Bacterial Stress Management

Novel Roles of SigB in the *Bacillus* General Stress Response

Kah Yen Claire Yeak

Propositions

1. Sigma B, a general stress-responsive sigma factor, has only a very minor role in the stress defense of *Bacillus cereus*.
   (This thesis)

2. The production of lichenysin by *Bacillus licheniformis* is an underestimated risk.
   (This thesis)

3. Storytelling is a prerequisite skill for scientists while disseminating research findings.

4. Newspaper articles covering breakthrough discoveries in basic science should refrain from overhyping foreseen applications.

5. To practice “Simplicity is the ultimate sophistication.” (attributed to Leonardo da Vinci) requires a lot of effort.

6. Emotional first aid is important to prevent psychological damage.

Propositions belonging to the thesis, entitled

Bacterial stress management – novel roles of Sigma B in the *Bacillus* general stress response

Kah Yen Claire Yeak

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Novel Roles of SigB in the *Bacillus* General Stress Response

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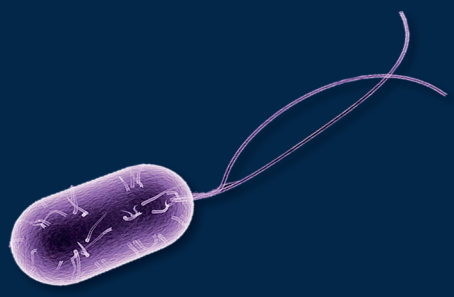
" Everything you know and
others worth knowing "

CHAPTER 1



General Introduction

Kah Yen Claire Yeak

1.1 Bacteria and natural habitat

The world we live in nurtures many visible and invisible life forms. Those invisible include microorganisms belonging to the three life domains: Bacteria, archaea, and eukarya. Bacteria are single-cell microbes, diverse in appearance, size, and function. They are one of the oldest life forms that inhabit all corners of the earth, like soil and seawater, but they are also intricately linked with higher forms of life, for instance, plants and animals. Archaea are also unicellular organisms and share similar characteristics as bacteria and eukarya, while eukarya are bigger, multicellular organisms, including plants, fungus, and animals.

Some bacteria exist in extreme habitats such as volcanoes, hot springs, the Antarctic region, deep ocean sediments, or salterns. Others that live in more "standard environments" like soil or water still face many challenges such as temperature change, lack of nutrients, change in alkalinity, acidity, salinity, etc. (Merino et al., 2019). Over millions of years of evolution, many bacteria have acquired robust stress management strategies to cope with the changes in their surroundings and thrive under extreme conditions (Merino et al., 2019).

The total number of bacteria on earth is enormous, but only a tiny fraction of this titanic amount has been discovered, explored, and studied by scientists since the first discovery of bacteria ~350 years ago, by the father of microbiology, Antonie v. Leeuwenhoek (Lane, 2015). Within the human body alone, the total number of bacterial cells is almost identical to the total number of human cells (~ 3.8×10^{13}) (Sender et al., 2016).

1.2 Beneficial and pathogenic bacteria

Out of those discovered and studied, some have beneficial roles for humans, but some are harmful to humans. Bacteria that have beneficial roles are essential, for instance, in maintaining skin and gut health in humans to prevent skin infection and the development of other chronic diseases such as colorectal cancer, gastrointestinal disorder, etc. (Hills et al., 2019). The human intestine hosts a vast number of bacterial species (500-1000) that serve to help in digestion, provide essential nutrients, maintain cells growth, regulate immune systems and produce neuroactive substances that act on the brain-gut axis (Bermúdez-Humarán et al., 2019; Dimidi et al., 2019). Some typical intestine bacteria are lactic acid bacteria (De Filippis et al., 2020) or bifidobacteria that are well-known to produce health-promoting metabolites, e.g., fatty acids (Arboleya et al., 2016).

Many bacteria residing on human skin also exert beneficial roles, e.g., suppressing inflammation, activating immune responses, inducing the expression of antimicrobial proteins on the skin, or producing antimicrobial peptides that give protection from pathogenic bacteria (Schommer and Gallo, 2013). Some well-known species are *Corynebacterium* spp. and

Propionibacterium spp. but there are at least 19 phyla in the bacterial skin microbiome (Schommer and Gallo, 2013).

On the contrary, some bacteria are pathogenic and harmful and can cause many human diseases. Some of the widely known deadly bacterial diseases are tuberculosis (caused by *Mycobacterium tuberculosis),* cholera (caused by *Vibrio cholera*), methicillin-resistant *Staphylococcus aureus* infection, pneumonia (caused by *Streptococcus pneumoniae*), meningitis (commonly caused by *S. pneumoniae, Haemophilus influenza*, *Listeria monocytogenes,* and *Escherichia coli*), etc. Most fatal cases are related to bacteria that cause systemic infections, especially ESKAPE (***E****nterococcus faecium,* ***S****. aureus,* ***K****lebsiella pneumoniae,* ***A****cinetobacter baumannii,* ***P****seudomonas* ***a****eruginosa*, and ***E****nterobacter* species) pathogens, that are resistant to antibiotic treatments (De Oliveira et al., 2020). Other than that, foodborne pathogens like *Salmonella, Campylobacter,* toxigenic *E. coli, L. monocytogenes, Bacillus cereus,* and *Clostridium botulinum* are a few culprits of foodborne outbreaks that cause different diseases that impact human health (Bintsis, 2017).

All the species mentioned above belong to different bacterial genera and are mainly vegetative microbes. Some bacteria genera can also form endospores (turning into a dormant state) as an ultimate survival mechanism, making them more challenging to deal with as spores are more resistant to external stresses, e.g., pH and temperatures. To date, there are 25 genera known to be endospore-forming, including the low GC bacteria *Bacillus, Clostridium, Thermoactinomyces*, *Sporolactobacillus*, and *Sporosarcina* (Wohlgemuth and Kämpfer, 2014). The largest and the most well-studied is the genus of *Bacillus,* comprising both beneficial and pathogenic species.

1.3 Three well-known *Bacillus* species

The genus *Bacillus* is Gram-positive endospore-forming rod-shaped bacteria. The taxonomic development of this genus changed over time and is now classified based on the DNA sequence of the gene encoding for the 16S ribosomal RNA, which is conserved within species (Cohn, 1872; Soule, 1932; Fritze, 2004). Of all *Bacillus* spp., the *Bacillus subtilis* and *Bacillus cereus* groups have gained the most attention in the scientific literature.

B. subtilis group members are typically non-pathogenic and include *Bacillus licheniformis, Bacillus pumilus, Bacillus amyloliquefaciens, Bacillus mojavensis, Bacillus vallismortis, Bacillus sonorensis*, *Bacillus atrophaeus,* the two sub-species *B. subtilis* subsp. *subtilis* and *B. subtilis* subsp. *spizizenii* (Fritze, 2004) and *B. subtilis* itself. Many of these have practical industrial applications.

The thoroughly investigated Gram-positive spore-forming *B. subtilis* has served as a model organism for decades. It is considered mainly harmless to humans and is broadly used in food fermentation (e.g., Natto, Soybean) (Kimura and Yokoyama, 2019) and for the production of

enzymes (amylases, xylanases, lichenase, β-galactosidase, cellulases, alkaline serine proteases) that are applied in different industries: food and feed, detergent, textile, leather, paper and pharmaceutical, as recently reviewed in Su et al. (2020). Nonetheless, it is also a major food spoiler (André et al., 2017; Moschonas et al., 2021).

B. cereus group (also known as *B. cereus sensu lato* group) includes *Bacillus anthracis, Bacillus thuringiensis, Bacillus mycoides, Bacillus pseudomycoides, Bacillus weihenstephanensis, Bacillus cytotoxicus*, *Bacillus wiedmannii*, *Bacillus toyonensis,* and *B. cereus* itself (Fritze, 2004; Liu et al., 2015; Ehling-Schulz et al., 2019). They are all pathogenic and exert varying degrees of pathogenic capacity. Since 2018, *B. weihenstephanensis* has been regarded the same as *B. mycoides* because they can hardly be distinguished based on the nucleotide identity of their whole genome sequences (Liu et al., 2018). Additionally, nine new species have been added to the *B. cereus* group but can only be differentiated from each other via whole-genome sequencing. These are *Bacillus paranthracis, Bacillus pacificus, Bacillus tropicus, Bacillus albus, Bacillus mobilis, Bacillus luti, Bacillus proteolyticus, Bacillus nitratireducens* and *Bacillus paramycoides* (Liu et al., 2017).

B. cereus has been excessively studied. It is a human pathogen that can cause foodborne diarrhea and intoxication when contaminated foods are ingested, or under certain circumstances, it can cause severe local infections such as endophthalmitis and septicemia (Ehling-Schulz et al., 2006; Guinebretière et al., 2010). Some *B. cereus* strains (also called *B. cereus sensu stricto*) are more toxigenic than others, depending on the presence of different virulence genes in isolates (Rossi et al., 2018).

Within the *B. cereus* group*, B. cereus, B. thuringiensis* (used as biopesticide), and *B. anthracis* (causative agent for anthrax) are the most studied and are renowned for their distinct phenotypic traits (Okinaka and Keim, 2016). In the *B. subtilis* group, *B. subtilis* (a Gram-positive spore-forming model organism) and *B. licheniformis* (an enzyme production workhorse) have gained the most attention from scientists, making them the most well-studied species in the genus of *Bacillus*.

1.3.1 *Bacillus subtilis* – a Gram-positive spore-forming model organism

Bacillus subtilis is a soil bacterium that can be isolated from the upper layer of soils, sediments, plants, or water. The organism is motile and aerobic, but can also grow in the absence of oxygen by using nitrate or nitrite as a terminal electron acceptor (Nakano and Zuber, 1998). The type strain (168) originated from Marburg and is very well characterized to date. It is one of the first bacteria from which the entire genome was sequenced (Kunst et al., 1997). The genome size of strain 168 is around 4.2 Mb and encodes 4244 proteins (Wipat and Harwood, 1999; Barbe et al., 2009). Knowledge of its physiology, morphology, nutritional requirements for growth, and methods for genome engineering is also well-developed. Detailed information on gene and protein functions, metabolic pathways, and regulatory networks are easily

accessible on the Subtiwiki platform, developed by the Stuelke research group at the University of Goettingen (www.subtiwiki.uni-goettingen.de) (Borriss et al., 2018; Pedreira et al., 2021).

As this species is generally safe, many well-established tools are exploited to modify the genome of *B. subtilis* for various purposes. Due to its natural competence and easy modification, it has gained broad interest among scientists in fundamental and applied sciences (Barák, 2021). In fundamental research, *B. subtilis* is used to study different cellular mechanisms and sporulation processes, and it is used as a heterologous expression model to understand important pathogens like *S. aureus* and *L. monocytogenes* (Barák, 2021).

To further decipher the cardinal demand of cellular life, the genome has been successfully reduced by 40% in the Minibacillus project (Reuß et al., 2016; Michalik et al., 2021), and more than 100 *B. subtilis* strains with sequentially reduced genomes are made available for investigations in basic science or industrial applications (Michalik et al., 2021; Pedreira et al., 2021). In the applied research field, *B. subtilis* has been employed as a cell factory to produce vaccines, vitamin B6, riboflavin, antibiotics, and valuable enzymes with particular functions, like lipases, proteases, etc. Some strains are also widely used in food fermentation or as probiotics; their applications were recently reviewed (Su et al., 2020). In short, *B. subtilis* is pivotal for microbiological researchers in academia and industry.

1.3.2 *Bacillus cereus* – a foodborne pathogen

B. cereus is a motile facultative anaerobe found in soil and water and can be isolated from various food products. The best-defined strain is the type strain ATCC (American Type Culture Collection) 14579, which has a genome size of ~ 5.4 Mb and encodes 5255 proteins (Ivanova et al., 2003). It also harbors a ~ 15 kb linear plasmid, pBClin15, that can lead to prophage activity (Stabell et al., 2009; Madeira et al., 2016).

B. cereus is a foodborne pathogen that can cause emetic disease and diarrhea. These symptoms are usually mild and are resolved within 1-2 days (Ehling-Schulz et al., 2019). As its endospores are highly resistant to a wide range of treatments like heat, UV light, drying, and cleaning chemicals, contamination of foods by *B. cereus* and the outgrowth in foods remains a concern for the food industry (André et al., 2017; Gauvry et al., 2017). Foods are either contaminated via ingredients or if the organism is present in food processing installations as vegetative cells or biofilms may release spores.

B. cereus strains can harbor various toxin genes (Ehling-Schulz et al., 2006; Guinebretière et al., 2010; Rossi et al., 2018). Strains harbor the *ces* gene cluster on a mega-plasmid can produce the emetic toxin cereulide. Such strains are regarded as emetic strains. Strains containing genes that encode enterotoxins like hemolysin BL (Hbl), non-hemolytic enterotoxin (Nhe), and cytotoxin K (CytK) are referred to as the diarrheal strains. These virulence genes belong to the

virulence regulon and are controlled by the pleiotropic transcriptional regulator PlcR (Gohar et al., 2008). Different combinations of toxin genes may be present in different strains (Ehling-Schulz et al., 2006; Guinebretière et al., 2010).

The cereulide toxin can be produced in food products under favorable conditions, and when ingested, this will incur vomiting after 1 h to 6 h. The emetic disease rarely leads to severe complications in adults, but in rare cases, it has led to acute liver failure and can be lethal for young children or immunocompromised adults (EFSA Panel on Biological Hazards (BIOHAZ), 2016; Ehling-Schulz et al., 2019). When high numbers of vegetative cells or spores of *B. cereus* are ingested, the organism can multiply in the human intestines and produce enterotoxins leading to diarrhea typically 8 h to 16 h after ingestion of contaminated food (EFSA Panel on Biological Hazards (BIOHAZ), 2016; Ehling-Schulz et al., 2019).

As the emetic or diarrhea diseases are usually non-lethal and resolve within 1-2 days, most of the foodborne illnesses caused by *B. cereus* are not reported to the local authorities, making the actual foodborne burden caused by this organism hard to estimate.

1.3.3 *Bacillus licheniformis* – the industrial workhorse

Bacillus licheniformis belongs to the *B. subtilis* group, is frequently isolated from soil, and is ubiquitously found in the environment in oil wells, compost, and plant materials (Rey et al., 2004). The organism grows preferably aerobically but is a facultative anaerobe in the presence of nitrogen sources. It can grow over a wide range of temperatures from 10°C to 58°C and optimally at 50°C (Warth, 1978; Baranyi and Tamplin, 2004). It is widely described as a mesophile but behaves more like a facultative thermophile as it can grow at a temperature of 10°C. The genome of the *B. licheniformis* type strain ATCC 14580 (or DSM13) was sequenced and published in 2004. The genome is ~ 4.2 Mb and encodes 4179 proteins (Rey et al., 2004).

Alternative to the cell factory *B. subtilis, B. licheniformis* is also frequently engineered to produce many valuable compounds with multifaceted properties. For instance, 1) biocontrol agents to remove biofilms (Ruiz et al., 2017; Galié et al., 2018; Sarwar et al., 2018); 2) detergent for household cleaning (Shaligram and Singhal, 2010); 3) skin penetration agent or foaming agent for cosmetics (Varvaresou and Iakovou, 2015); 4) drug coating agents to deliver drugs (Sen, 2010; Santos et al., 2018); or 5) bioactive compounds to treat cancers (Wu et al., 2017). Additionally, the organism is also applied directly in the agricultural industry to eliminate plant pathogens (Sachdev and Cameotra, 2013), for soil bioremediation or petroleum recovery (Golyshin et al., 1999; Mulligan, 2005; Alvarez et al., 2020), as animal feed additives, or as a probiotic (Huang et al., 2011; Wang et al., 2016; Elshaghabee et al., 2017; Nitschke and Silva, 2018; Bampidis et al., 2019; Lee et al., 2019).

In sporadic cases, *B. licheniformis* has been reported to cause illness, e.g., septicemia (Blue et al., 1995; Haydushka et al., 2012), peritonitis (Park et al., 2006), or endocarditis (Santini et al.,

1995). In addition, the bacterium has occasionally led to foodborne illness (Salkinoja-Salonen et al., 1999; Mikkola et al., 2003). The causative agent for foodborne illness has been linked to the production of a secondary metabolite-biosurfactant, lichenysin, a lipopeptide that exerts toxicity to mammalian cells (Madslien et al., 2013).

1.4 Transcriptional and translational regulation

As introduced above, *Bacillus* spp. can grow and survive in diverse environments, ranging from root nodules to composting materials, from sediments to oil wells, from soil and water to gastrointestinal tracts of animals, air and many other extreme surroundings (Fira et al., 2018; Merino et al., 2019). To live in these diverse habitats, *Bacillus* spp. require distinct repertoires of genes under different environmental conditions to grow and adapt to environmental stresses.

Bacillus spp. (also other bacteria) can adapt to environmental changes by altering their gene expression. These adaptations are governed by distinct transcription initiation proteins, namely, the sigma (σ) factors (Souza et al., 2014). They are prerequisites to guide the specific binding of RNA polymerase core enzyme to the promoter (a specific nucleotide consensus sequence on the DNA) to initiate transcription (Figure 1). The promoter is located upstream of the start codon (ATG) of genes that they regulate and ranges from 12 to 19 base pairs (Madigan et al., 2021). Although promoter sequences are not entirely identical, they contain conserved nucleotides at the regions of -35 and -10 upstream of the ATG start codon (Madigan et al., 2021). The -35 and -10 regions are recognized by specific Sigma factors and are also known as the promoter binding motif (PBM) (Figure 1). This is called an operon when a group of genes shares the same PBM and is recognized and regulated by the same transcription factor. When the same transcription factor regulates several operons (e.g., a specific sigma factor), these are known as a regulon (Madigan et al., 2021).

Each sigma factor has designated functions to regulate specific cell responses in *Bacillus* cells, but some have overlapping roles in managing multiple stresses. To date, there are 15 sigma factors described for the model organism for *Bacillus* spp., *B. subtilis*. Each of them recognizes a specific PBM and serves to either control the expression of housekeeping/reference genes or is involved in the regulation of other cellular responses (see Table 1). SigA is the housekeeping sigma factor and is constitutively active under the growth at optimal physiological conditions, whereas the alternative sigma factors are responsible for adaptive responses like morphological development and/or the management of stress (Haldenwang, 1995; Paget, 2015). These alternative Sigma-factor-mediated stress responses are important for bacterial transcriptional regulation and grant cells the ability of quick and reversible adaptations in various ecological niches.

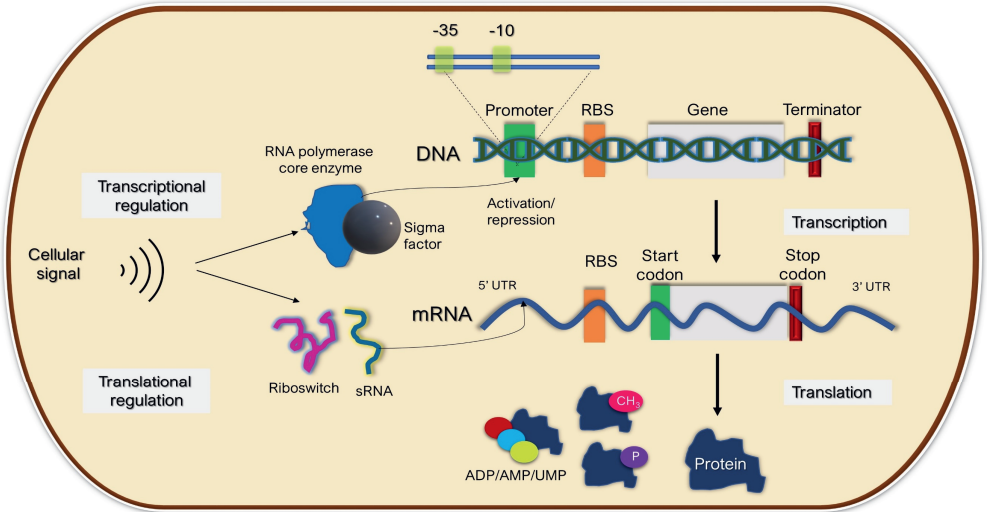


Figure 1- Bacterial transcriptional and translational regulation.

However, the quick adaptational stress responses do not solely rely on transcriptional regulations but also at the level of protein synthesis (translational regulation), which results in the production of proteins that calibrate responses to stresses (Tollerson and Ibba, 2020). Translational regulation is an intricated process, which involves regulation at initiation (control the number of available ribosomes, sequester ribosome binding site, ensure efficient mRNA folding, multiply ribosomes), elongation (programmed translational arrest and regulate translational rate), and termination (ensure immediate termination of ribosomes to maintain cellular homeostasis), reviewed in Tollerson and Ibba, (2020). Other than that, cells are also subjected to post-transcriptional regulation by small RNAs, RNA binding proteins, or riboswitches (Mandin and Guillier, 2013; Madigan et al., 2021); post-translational control (e.g., methylation, phosphorylation, attachment/removal of small molecules like adenosine monophosphate (AMP), adenosine diphosphate (ADP), and uridine monophosphate (UMP) (Madigan et al., 2021); or the coupling of transcriptional and translational control. All these regulation mechanisms are sophisticated, with the ultimate goal to provide cells with adequate resources to manage stresses with different survival strategies and ensure cell survival in diverse conditions.

Table 1: Sigma factors and functions in *Bacillus subtilis*

7	Promoter Sequence				Reference
	-35	spacer	-10	Function	
SigA	TTGACA	14	TATAAT	Housekeeping/ early sporulation	(Moran et al., 1982)
SigB	GTTTWW	12-15	GGGWAW	General stress response, fungal biocontrol, regulation of biofilm aging, and dispersal	(Petersohn et al., 1999a; Rodriguez Ayala et al., 2020; Vohradsky et al., 2021)
SigC	AAATC	15	TAXTGYTTZTA	Postexponential gene expression	(Johnson et al., 1983; Haldenwang, 1995)
SigD	TAAA	15	GCCGATAT	Regulation of flagella, motility, chemotaxis, and autolysis	(Helmann, 1991; Haldenwang, 1995)
SigE	KMATATT	13-14	CATACAMT	Transcription of sporulation genes (early mother cell)	(Haldenwang, 1995; Sierro et al., 2008)
SigF	YGYWTA	15	GGMAWAMTA	Transcription of sporulation genes (early forespore)	(Sun et al., 1991; Haldenwang, 1995; Sierro et al., 2008)
SigG	GHATR	18	GGCATXHTA	Transcription of sporulation genes (late forespore)	(Nicholson et al., 1989; Haldenwang, 1995; Sierro et al., 2008)
SigH	AGGTATT	14-17	GAATT	Transcription of early stationary phase genes (sporulation, competence)	(Predich et al., 1992; Haldenwang, 1995; Sierro et al., 2008)
SigK	MACM	16-17	CATANNTA	Transcription of sporulation genes (late mother cell)	(Zheng et al., 1992; Haldenwang, 1995; Sierro et al., 2008)
SigL	TGGCACN	5	TTGCA	utilization of arginine, acetoin, and fructose, required for cold adaptation	(Débarbouillé et al., 1991; Haldenwang, 1995; Sierro et al., 2008)
SigM	n.d.	n.d.	n.d.	Maintenance of cell wall integrity in response to environmental and antibiotic stress	(Luo and Helmann, 2012; Diehl et al., 2020)
SigN	ATTTACGT	13	GATATAA	Not yet determined	(Burton et al., 2019)
SigV	CATAACTGTGAAAC	17	CGTCTATAAATAGG	Resistance to lytic enzymes	(Zellmeier et al., 2005)
SigW	TGAAACN	16	CGTA	Adaptation to cell envelope stress, e.g., membrane-active compounds, antimicrobial peptides, and alkaline pH	(Cao et al., 2002; Sierro et al., 2008; Nicholson, 2012)
SigX	TGTAACN	17	CGAC	Resistance to cationic antimicrobial peptides. Control biofilm architecture	(Sierro et al., 2008; Ogura, 2022; Tzipilevich et al., 2022)
SigY	n.d.	n.d.	n.d.	Maintenance of the Sp prophage that contains genes necessary to produce and resist killing by the antibiotic sublancin	(Mendez et al., 2012)
SigZ	n.d.	n.d.	n.d.	Not yet determined	(Sorokin et al., 1997; Luo et al., 2010)

1.5 Survival strategies and stress responses of *Bacillus* spp.

Bacillus spp. can cope with stress by applying different strategies, depending on the type of stressors present. These stress responses can generally be grouped into five categories**: 1) switching metabolic state** to survive harsh conditions; Cells can switch to extreme slow-growth states to endure profound nutrient deprivation or highly unfavorable living condition like high salt, or become dormant, or shift into cannibalism mode to kill sister cells to initiate sporulation (González-Pastor, 2011; Ikryannikova et al., 2019); **2) evolve to survive** in harsh environments via gene mutations; e.g to low/high atmospheric pressure, high ultraviolet radiation, and unfavorable growth temperatures (Wassmann et al., 2010; Zeigler and Nicholson, 2017); **3) activate temporary stress adaptation mechanisms** to combat stress and allow for survival, which on certain occasions can involve the **4) production of toxic compounds** that inhibit other organisms and thereby promote survival (Petersohn et al., 1999b; Mendez et al., 2012; Bartolini et al., 2019a) and 5) **forming endospores** as the ultimate survival mechanisms to survive adverse conditions in a dormant state. The endospores are highly stress-resistant and can survive for an extended period without nutrients. Once the surrounding environments become hospitable, spores will germinate and return to their vegetative state. The entire sporulation process has been studied for > 180 years and has recently been reviewed by Riley et al. (2021).

Some common examples of stress adaptation mechanisms are temporal adaptations to oxidative stress (Zuber, 2009; Schäfer and Turgay, 2019; Tran and Bonilla, 2021), antibiotic stress (Butcher and Helmann, 2006; Shen et al., 2020), cell envelope stress (Guariglia-Oropeza and Helmann, 2011; Helmann, 2016), glucose, phosphate, and oxygen starvation, or other general stresses like heat, acid, alkaline or osmotic stresses (Antelmann et al., 2000; Bernhardt et al., 2003; Méndez et al., 2004; Voigt et al., 2007; Rodriguez Ayala et al., 2020; Gao et al., 2021). These adaptations are governed by the master regulator of general stress, the alternative Sigma factor B (SigB). SigB is one of the most prominent and well-characterized alternative Sigma factors in *Bacillus* spp., which regulates the General Stress Response (GSR), and the SigB-mediated GSR is deemed to be an essential component of the survival strategy for *Bacillus* spp.

1.6 The master regulator of general stress - Sigma factor B (SigB)

SigB was discovered > 40 years ago by William Hadenwang and Richard Losick (Haldenwang and Losick, 1979) and not much later shown to be induced by general stress (Moran et al., 1981). It was found that upon SigB activation, the transcription of a group of genes is elicited, followed by the translation of proteins that can protect cells in the presence of specific stress. Many studies following the discovery of SigB have since been conducted in *B. subtilis*, revealing

different stress conditions that can activate SigB, and a large group of genes (> 300) that are under the control of SigB have been identified (Richter and Hecker, 1986; Hecker et al., 1988, 2007; Benson and Haldenwang, 1993; Boylan et al., 1993; Völker et al., 1994; Hecker and Völker, 1998, 2001; Petersohn et al., 2001; Price et al., 2001a; Brigulla et al., 2003; Nannapaneni et al., 2012). These proteins have a non-specific but critical protective role for bacteria under stress conditions and are referred to as the general stress protein (GSP).

SigB-GSR has been extensively studied in *B. subtilis* and has also been well-characterized in other gram-positive species like *B. licheniformis* (Hoi et al., 2006; Voigt et al., 2007, 2013, 2014), *B. cereus* (Periago et al., 2002; van Schaik et al., 2004; van Schaik, 2005; Schaik et al., 2007; de Been et al., 2010; Scott and Dyer, 2012), *L. monocytogenes* (Wemekamp-Kamphuis et al., 2004; Abram et al., 2008; Hain et al., 2008; Shin et al., 2010; Ondrusch and Kreft, 2011; Dorey et al., 2019b, 2019a; Crespo Tapia et al., 2020; Guerreiro et al., 2020a, 2020b) and *S. aureus* (Gertz et al., 2000; Pané-Farré et al., 2006, 2009; Pförtner et al., 2014; Michalik et al., 2017). SigB-GSR and the different transduction routes in activating SigB in these species were recently reviewed in Pané-Farré et al. (2017) and Rodriguez Ayala et al. (2020). Additionally, SigB operon with the same structure as in *B. subtilis* has also been reported to be present in *Bacillus halodurans, Bacillus clausii, Oceanobacillus iheyensis, Clostridium difficile, Listeria innocua, and Listeria welshimeri* (Hecker et al., 2007).

Other than the well-known bacterial species described above, SigB has been found and investigated in other more distantly related bacterial species. This includes the high GC-gram-positive bacteria *Streptomyces coelicolor,* which harbors 9 homologs of SigB (Cho et al., 2001; Viollier et al., 2003; Fernández Martínez et al., 2009; Sevcikova et al., 2021), the pathogenic bacteria, *Mycobacterium tuberculosis,* that cause tuberculosis in humans (Hu and Coates, 1999; Lee et al., 2008; Fontán et al., 2009; Pisu et al., 2017; Hurst-Hess et al., 2019), and the gram-negative *Ralstonia eutropha,* and the Cyanobacteria *Synechocystis* species.

The wide distribution of SigB across species confirms the unique role of SigB in assuring bacterial cell survival in a natural environment, marking the importance of obtaining the complete picture of the SigB-dependent survival mechanisms in bacteria.

1.7 The SigB-mediated General Stress Response (GSR)

The SigB-mediated GSR is well-characterized in *B. subtilis.* It has been found that SigB GSR is induced when cells are exposed to two groups of stressors: either 1) physical (also refer as environmental) stressors when cells face challenging external conditions or 2) metabolic (also refer as energy or nutritional) stressors when cells are deprived or starved of nutrients.

Physical stressors include temperature: (heat or cold shock), pH (acid or alkaline shock) (Gaidenko and Price, 1998), high salt (osmotic shock), ethanol, certain antibiotics (e.g.,

vancomycin and bacitracin) (Mascher et al., 2003), oxidative or nitrosative stress (e.g., hydrogen peroxide, sodium nitropruside) (Tran et al., 2019), blue light, an elevated level of manganese (Guedon et al., 2003).

Metabolic stressors include the deprivation of glucose, phosphate, oxygen, red light and compounds that deplete energy-carrying molecules in cells (adenosine triphosphate ATP and/or guanosine triphosphate GTP). For instance, azide, carbonyl cyanide m-chlorophenylhydrazone (CCCP), nitric oxide (NO), mycophenolic acid, and decoyinine (Voelker et al., 1995; Zhang and Haldenwang, 2005). Moreover, unlike vancomycin and bacitracin, the antibiotic rifampin (also known as rifampicin), which blocks the β subunit of prokaryotic RNA polymerase (inhibit RNA synthesis), is classified as a metabolic stressor. This is because rifampin induces SigB via the same pathway as all other metabolic stressors in *B. subtilis* (Bandow et al., 2002).

1.7.1 SigB signaling cascades

Two major and well-known stress sensing signaling cascades mediated by SigB in *B. subtilis* are 1) the physical/environment signaling cascade monitored by a large protein complex called the stressosome, which is assembled by three SigB regulatory proteins RsbR, RsbS, and RsbT (where Rsb refers to the Regulator of SigB); and 2) the metabolic/energy/nutritional signaling cascade that is controlled by two other SigB regulatory proteins RsbQ and RsbP. Therefore, the physical/environment signaling cascade is often referred to as the RsbRST pathway, whereas the metabolic/energy/nutritional signaling cascade is referred to as the RsbQP pathway.

The ~1.8 MDa stressosome (RsbRST protein complex) and the RsbQP complex are activated in the presence of physical (environmental) and metabolic (energy/nutritional) stressors mentioned above, respectively. All components involved in these two signaling cascades are illustrated in Figure 2. Other than the two distinct RsbRST and RsbQP pathways, an Rsb-independent pathway is likely to exist as both low temperature (15°C) and high temperature (54°C) have been reported to induce SigB independently from the regulators that mediate SigB activation, i.e., RsbP/RsbU (Brigulla et al., 2003; Holtmann et al., 2004). In addition, SigB-dependent cross-protection for cells against nitrosative stress only requires SigB but not RsbP/RsbT regulators (Tran et al., 2019).

Interestingly, *B. cereus* does not use the above-mentioned SigB signaling cascades. Instead, *B. cereus* has evolved to exploit a completely different GSR sensing module, namely the RsbKMY two-component system, which was first discovered by de Been et al. (2010), and an additional methyltransferase (RsbM) was later found to be an essential regulator of the RsbKMY system (Chen et al., 2012, 2015, 2017) (see Figure 3 for SigB gene cluster arrangement in *B. subtilis* vs. *B. cereus*). RsbK is a transmembrane protein that consists of signal-receiving domains and is responsible for sensing the physical cues and transferring this signal

to its cognate response regulator, RsbY (Figure 2). It has been reported that both physical and metabolic stressors can induce SigB via the RsbKYM system in *B. cereus* (van Schaik et al., 2004).

1.7.1.1 RsbRST (stressosome)-mediated cascade

In unstressed conditions (e.g., during exponential growth with sufficient nutrients and without external stressors), SigB is inactive as a transcription factor due to the binding of the anti-sigma factor protein – RsbW serine kinase - and thus cannot bind to the core RNA polymerase. On top of that, RsbW phosphorylates the anti-anti sigma factor RsbV and keeps it in its inactive state. The structural insight of SigB regulation by RsbV and RsbW has recently been updated (Pathak et al., 2020). Together, the RsbV, RsbW, and SigB are key downstream elements of both stressosome-mediated or RsbQP-mediated signaling cascades, and these are the components shared between the two pathways (RsbRST, RsbQP) in *Bacillus* spp.

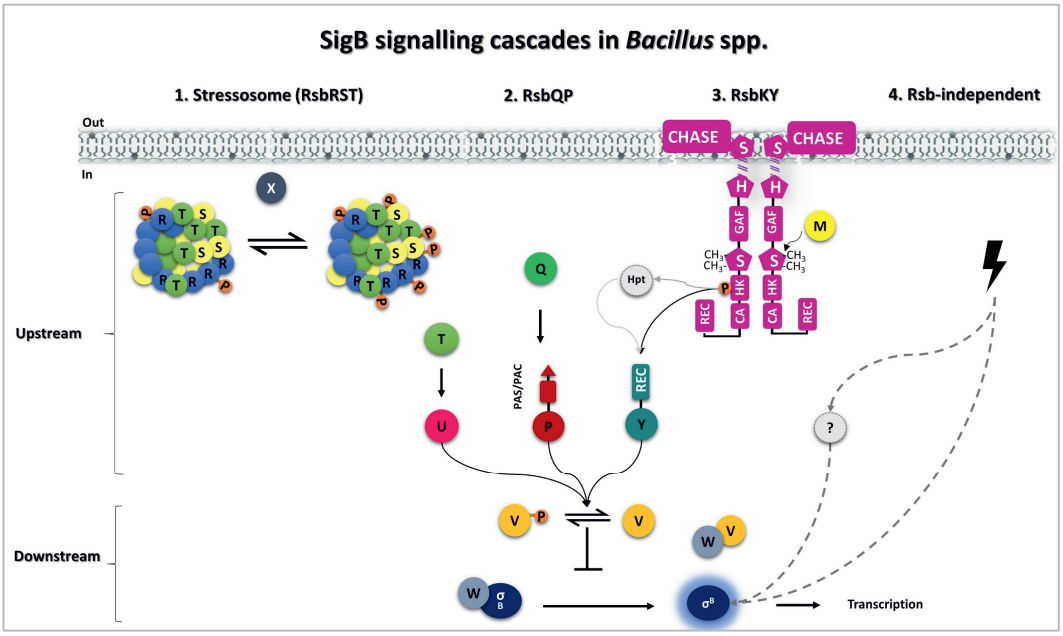


Figure 2- SigB signaling cascades in *Bacillus* species

The activation of RsbV by dephosphorylation leads to its binding to RsbW, which is subsequently released from SigB and renders SigB in its active form. This can be mediated by different protein phosphatases 2C (PP2C). In the presence of physical stressors, RsbV is dephosphorylated by the PP2C phosphatase RsbU, and in the presence of metabolic stressors, this is mediated by the PP2C phosphatase RsbP, as reviewed by Pané-Farré et al. (2017) and Rodriguez Ayala et al. (2020). The dephosphorylation of RsbV by either RsbU or RsbP increases its affinity to RsbW, thus uncoupling RsbW from the RsbW-SigB complex, thereby

leading to the transcription of SigB and genes under the control of SigB. Different upstream signaling protein components regulate the activities of these two PP2C phosphatases. RsbU is activated by the stressosome component RsbT kinase, whereas RsbP is regulated by the α/β hydrolase RsbQ, as reviewed by Pané-Farré et al. (2017) and Rodriguez Ayala et al. (2020).

Without physical stress, RsbT forms a complex with the two scaffold proteins RsbR and RsbS, in which RsbS contains a domain that acts as a sulfate transporter and anti-sigma factor antagonist (STAS) (Sharma et al., 2011; Pané-Farré et al., 2017) and RsbR contains a conserved C-terminal STAS domain and a diversified N-terminal domain (Chen et al., 2003; Pané-Farré et al., 2017), forming the stressosome complex. The stressosome complex can be heterogeneous because different RsbR paralogs (RsbRA, RsbRB, RsbRC, and RsbRD) can assemble with RsbS and RsbT into a fully functional stressosome. The heterogeneous stressosome acts as a protein signaling hub to sense the presence of physical stressors in the environment. Upon sensing physical stress signals, RsbT phosphorylates the STAS domains of RsbR (at position T171), its paralogs, and RsbS (at position S59) (see reviews of Pané-Farré et al. (2017) and Rodriguez Ayala et al. (2020)). The phosphorylation of RsbR and RsbS thus triggers a conformational change and releases RsbT from the stressosome. The free RsbT then switches its binding partner to the PP2C phosphatase RsbU and forms the RsbT: RsbU complex, leading to the dephosphorylation of RsbV and activation of SigB. Following the transient activation of SigB, the level of the stressosome resetter RsbX protein (also a PP2C phosphatase) also increases. RsbX possesses antagonistic activity toward RsbT and plays a critical role in resetting the RsbR-P and RsbS-P back to their resting states via dephosphorylation, and also blocks SigB activation in unstressed conditions (see reviews of Pané-Farré et al. (2017) and Rodriguez Ayala et al. (2020)).

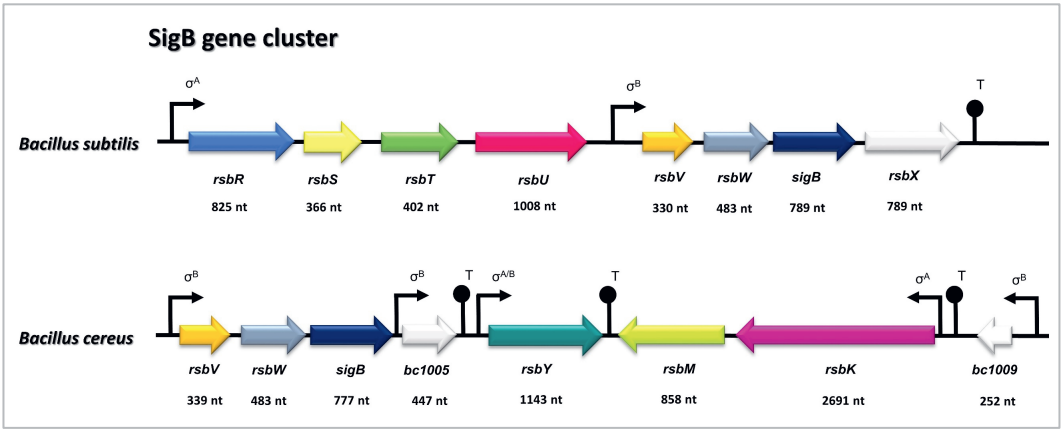


Figure 3- SigB gene cluster in *Bacillus subtilis* and *Bacillus cereus*

As the N-terminal domain of RsbR is diverse, it has long been hypothesized that the different RsbR paralogs hold a distinct role in sensing a specific physical stressor via its N-terminal domain. So far, there is no solid evidence that specific RsbR paralogs are more important than the other, but it appears that RsbRB, RsbRC, and RsbRD are redundant, and a functional stressosome can be formed solely with RsbRA. Vice versa, RsbRB, RsbRC, and RsbRD can substitute the role of RsbRA (Cabeen et al., 2017). To date, the function of each RsbR paralog and the exact mechanism in perceiving stress signals is still unclear. Only the YtvA paralog, a flavin mononucleotide that contains a light-oxygen-voltage domain, is known to be sensing blue light (Akbar et al., 2001; Losi et al., 2003; Jurk et al., 2013). It is also the only stressosome component with a confirmed specific activation signal (Gaidenko et al., 2006; Pané-Farré et al., 2017).

1.7.1.2 RsbQP-mediated cascade

When cells encounter metabolic stress, or when the ATP: ADP ratio falls, the α/β hydrolase RsbQ acts as an activator and transfers the metabolic stress signal to the Per-Arndt-Sim (PAS) domain of the PP2C phosphatase, RsbP, activating its C-terminal phosphatase domain. The activated RsbP then interacts with RsbU and leads to the sequential activation of the downstream elements: RsbV, RsbW, and SigB itself. Although it is found that RsbQP is indispensable in sensing metabolic stress in *B. subtilis,* the exact signal (or ligands) that is received either directly by RsbQ or via a so far unknown molecule remains elusive (Nadezhdin et al., 2011).

1.7.1.3 Rsb-independent cascade

Another uncharacterized SigB signaling cascade may exist based on experimental observations. As mentioned above, it has been shown that low temperature at 15°C can trigger SigB activation and SigB-dependent gene transcription independently from RsbV (Brigulla et al., 2003), and exposure to high temperatures (54°C for *B. subtilis*) is proposed to induce SigB directly (Holtmann et al., 2004). Recently, SigB was found to be the prerequisite for cross-protection against nitrosative stress, but the regulators RsbP or RsbU are not needed (Tran et al., 2019; Tran and Bonilla, 2021). These observations point to the possibility that SigB can be activated via so far undetermined regulators or direct activation by particular stressors.

1.7.1.4 RsbKMY- mediated cascade

Without stress signals, the S-helix of RsbK is methylated by the negative regulator RsbM, preventing the phosphate transfer from RsbK to RsbY (Chen et al., 2015). The unphosphorylated RsbY keeps the level of phosphorylated RsbV high, and as a consequence, RsbW remains bound to SigB, and SigB stays inactive (Figure 2). In contrast, in the presence of stress signals, the histidine residue auto-phosphorylates, and the phosphate group is transferred to the C-terminal receiver (REC) domain of RsbY directly (Chen et al., 2015) or via a putative phosphotransferase protein (de Been et al., 2010; Chen et al., 2012). RsbY is also a

PP2C phosphatase and becomes activated to dephosphorylate RsbV as described for the RsbRST cascade in *B. subtilis*. The dephosphorylated RsbV causes the release of RsbW from SigB, thereby leading to the transcription of SigB and subsequent transcription of genes belonging to this regulon in *B. cereus*.

However, the SigB GSR in *B. cereus* is not entirely analogous to *B. subtilis*. The SigB expression following stress imposition is transient in *B. subtilis* but lasts longer when growing cultures of *B. cereus* are exposed, namely from the exponential growth phase to the beginning of the stationary phase (Chen et al., 2017). In *B. subtilis,* the RsbX phosphatase is responsible for resetting the SigB level to its baseline after stress exposure by dephosphorylating the stressosome proteins (RsbR and RsbS). The reversion of the phosphorylated stressosome back to its ground state will recapture RsbT and cause the SigB-mediated transcription to cease. In *B. cereus,* it is unknown how cells control the level of SigB after its activation. Although RsbM negatively regulates SigB, it only prohibits SigB activation before stress (Chen et al., 2017). Thus, the extended activation of SigB may be attributed to the higher translation efficiency of RsbV than RsbW in *B. cereus* (Chen et al., 2017). As more RsbV proteins are produced, the formation of RsbV: RsbW complexes is promoted. Accordingly, the newly synthesized SigB is then "free" from its anti-sigma factor and fosters the continuum of SigB transcription.

Nonetheless, the SigB, RsbV, and RsbW expression levels gradually decrease until the early stationary phase, but decline rapidly upon entry into the stationary phase due to 1) down-regulated transcription and 2) possible enhanced proteolysis of components involved in the SigB signaling cascade by so far unidentified proteases (Chen et al., 2012, 2017).

1.7.1.5 SigB signaling in other *Bacillus* species, firmicutes, and bacteria

As set out in the previous paragraphs, there are at least four known SigB signaling cascades for *Bacillus* spp., the RsbRST, RsbQP, and Rsb-independent pathways are present in *B. subtilis,* and the RsbKMY is uniquely found in *B. cereus*. Therefore, the RsbRST or RsbQP pathways are referred to as the *B. subtilis* SigB signal sensing module and the RsbKMY as the *B. cereus* SigB signal sensing module in this thesis. These modules have also been found in other *Bacillus* spp. and firmicutes and explored to different extents in different species.

Other than *B. subtilis,* closely related species like *B. licheniformis* and *B. halodurans* and other firmicutes, including *O. iheyensis, L. innocua, L. monocytogenes, Moorella thermoacetica,* and *Thermoanaerobacter tengcongensis* are known to contain the *B. subtilis* RsbRST SigB sensing module (Pané-Farré et al., 2005). The RsbRST stressosome pathway was conserved in these species and has been extensively investigated in *B. licheniformis* and *L. monocytogenes,* revealing a highly similar SigB GSR to *B. subtilis* in these two species. However, the RsbQP metabolic stress sensing module is considered unique for *B. subtilis*. It has not been investigated in other species hitherto, even though a homologous system has been reported in *Geobacillus* and *Paenibacillus* spp. and in other more distant bacteria like *Streptomyces,*

Rhizobium, and *Pseudomonas* (Nadezhdin et al., 2011). The functionality of the predicted RsbQP-like systems in these species is yet to be explored. Thus far, there are no reports of direct SigB activation by either physical or metabolic stress in other species than *B. subtilis.*

Of the species known to have the SigB-mediated GSR, this stress response has been investigated extensively in the pathogenic organism *L. monocytogenes* that causes severe listeriosis in humans, including the role of SigB in pathogenicity and virulence. In *L. monocytogenes,* SigB is induced strongly by acidic stress but also responsive to ethanol, osmotic, heat, oxidative stress, blue light, and metabolic stresses (upon entering into the stationary phase) (Ondrusch and Kreft, 2011; Dorey et al., 2019b, 2019a, 2019a; Guerreiro et al., 2020b). The SigB-GSR and its activation via the sequential RsbRST (stressosome) signaling cascade in this species is almost identical to *B. subtilis* (Oliveira et al., 2021). However, it uses the RsbRST (stressosome)-mediated pathway instead of the RsbQP module for sensing metabolic (energy/nutritional) stress. Moreover, *L. monocytogenes* also employs SigB to control the production of a subset of virulence genes that are coregulated by the positive regulatory factor A (PrfA) (Ollinger et al., 2009) and uses the stressosome to uniquely configure stress within host cells post-infection (Dessaux et al., 2021).

On the other hand, the unique RsbKMY module of *B. cereus* has also been identified in other *B. cereus* group members (*B. thuringiensis, B. weihenstephanensis, B. anthracis,* and the high GC firmicutes, *Geobacillus* Y412MC10 and *Paenibacillus* JDR-2), and orthologs of the RsbK sensor kinase have been predicted for more distantly related bacteria: Actinobacteria, Proteobacteria, Cyanobacteria and Bacteroidetes (de Been et al., 2011). However, no detailed studies of the RsbKMY module in response to stress have been conducted in species other than *B. cereus.* Moreover, unlike *B. subtilis,* ATP depletion causes only minor SigB induction in *B. cereus* and is likely not the main trigger for SigB-GSR in species that employ the *B. cereus* SigB sensing module. The SigB signaling modules and stressors that trigger these modules in *Bacillus* spp., firmicutes, and other bacteria are summarized in Table 2.

1.7.2 SigB regulon

As mentioned above, the activation of SigB leads to the transcription of genes encoding specific or general stress proteins that can subsequently protect cells from the stressors. This includes genes directly and indirectly regulated by SigB, also known as the SigB regulon members. Direct SigB regulon members are genes that have a SigB PBM (with the consensus sequence of GTTTAA- $N_{13\text{-}15}$- GGGTAT), which allows for the direct binding of SigB to the upstream regions of genes for regulation. In contrast, indirect SigB regulon members are genes that do not have a SigB PBM. However, genes that do not contain a SigB PBM can still be regulated by SigB indirectly, for instance, via a regulator that is a SigB regulon member. Therefore, in this thesis, SigB regulon members with a SigB PBM are defined as the SigB direct regulon members, and those that do not have a SigB PBM as SigB indirect regulon members.

1.7.2.1 SigB regulon in *B. subtilis*

In the pre-genome sequencing era, SigB regulon members were unraveled through transposon mutagenesis and proteomics analysis of wild-type (wt) strain versus the *sigB* null mutant in response to various physical or metabolic triggers. Following the publication of the whole-genome sequence for the *B. subtilis* 168 type strain, the list of SigB regulon members was further expanded via transcriptomics and advanced proteomics analyses (Petersohn et al., 1999b, 1999a, 2001; Price et al., 2001b; Waters et al., 2014; Arrieta-Ortiz et al., 2015; Schumann, 2016), and via the combination of omics and predictive models (Nannapaneni et al., 2012). Over the years, the number of genes known to be regulated by SigB in *B. subtilis* has increased markedly. To date, more than 300 genes are thought to be SigB-dependent in *B. subtilis* (Nannapaneni et al., 2012; Rodriguez Ayala et al., 2020). These genes are activated or negatively regulated by SigB upon its activation via either RsbRST or RsbQP or the Rsb-independent pathway directly under stressful conditions.

Table 2: SigB signaling cascades found in *Bacillus* species, firmicutes, and other bacteria. *- indicates supported with experimental evidence.

Stressor /species	RsbRST	RsbQP	RsbKMY	Rsb-independent
Stressor	▪ Heat ▪ Acid ▪ Alkaline ▪ Osmotic (Salt) ▪ Antibiotics (Vancomycin, bacitracin) ▪ Oxidative (hydrogen peroxide) ▪ Nitrosative (sodium nitropruside) ▪ Blue light ▪ High manganese level	▪ Entry into stationary phase ▪ Glucose starvation ▪ Phosphate starvation ▪ ATP depletion (Azide, CCCP, Nitric oxide, Mycophenolic acid, Decoyinine, Rifampin) ▪ Red light	▪ Heat ▪ Ethanol ▪ Osmotic ▪ ATP depletion (minor induction)	▪ Low temperature (15°C) ▪ High temperature (54°C) ▪ Nitrosative
Species	***B. subtilis,*** ***B. licheniformis,*** *B. halodurans,* *B. amyloliquafaciens* *B. pumilus* *B. coagulans* *B. clausii* *O. iheyensis* ***L. monocytogenes*** ***L. innocua*** *L. welshimeri* *M. theroacetica* *T. tengcongensis*	***B. subtilis*** *Geobacillus* spp. *Paenibacillus* spp. *Streptomyces* *Rhizobium* *Pseudomonas*	***B. cereus*** *B. thuringiensis* *B.weihenstephanensis B. anthracis* *Geobacillus* Y412MC10 *Paenibacillus* JDR-2 Actinobacteria Proteobacteria Cyanobacteria Bacteroidetes	***B. subtilis***

The explicit function of each regulon member has not been designated fully. However, it is known that some of these genes (encoding for general stress proteins) respond universally to common physical stressors (i.e., heat, ethanol, salt, acid) and serve a generic role in protecting cells from damage, but not directed against a specific stimulus. Some characteristic examples of *B. subtilis* include DNA protection enzymes, chaperones (aid in degrading misfolded proteins), proteins that repair the cell envelope, transporters, exporters, osmoprotectants, etc. (reviewed by Rodriguez Ayala et al. (2020), see Appendix 1A for complete SigB regulon list for *B. subtilis*).

As the SigB regulon is relatively large, the expression of the entire regulon concomitantly would impose a tremendous energy burden on the cells. If SigB is continuously expressed, cells might cease to grow or even die. Therefore, the SigB-GSR is a tightly regulated process, and a subset of selected SigB-dependent genes are induced based on the type of stressors present. The sturdy governance of SigB-GSR in cells ensures that the activation of SigB does not reduce the robustness of cells, and cells generate just enough proteins to protect them from more severe stress.

1.7.2.2 SigB regulon in *B. licheniformis*

Within the *Bacillus* genus, the SigB regulon has also been elucidated in *B. licheniformis* and *B. cereus,* but to a somewhat lesser extent than in *B. subtilis.* Of the two, the SigB regulon in *B. licheniformis* is comparable to the SigB regulon of *B. subtilis.* Many SigB-dependent genes in *B. subtilis* were also proven to be induced or negatively regulated by SigB in *B. licheniformis* in response to physical stresses like heat, ethanol, salt, and hydrogen peroxide (Schroeter et al., 2011, 2013; Voigt et al., 2013). Both organisms thus appear to have a highly similar general stress management system. Additionally, the SigB regulon in *B. licheniformis* contains members not present in *B. subtilis,* e.g., some endonuclease and solute symporter genes (see Table S5 in Voigt et al. (2013)).

Albeit similar to *B. subtilis*, the size of the SigB regulon known to date for *B. licheniformis* is smaller than that of *B. subtilis,* likely due to two reasons: 1) fewer stressors have been used to study the SigB-GSR in *B. licheniformis,* e.g., it is not known whether acid, blue light, red light, and acid induce SigB GSR; and 2) *B. licheniformis* has evolved to employ a different system to deal with starvation (i.e., glucose, phosphate, and nitrogen starvation) because SigB GSR is not induced by these metabolic stresses in this organism (Hoi et al., 2006; Voigt et al., 2007, 2014). Moreover, the RsbQP SigB sensing module responsible for sensing metabolic stress in *B. subtilis* is missing in *B. licheniformis.* Thus far, the number of SigB regulon members revealed in *B. licheniformis* is around 200 (supplementary tables in Voigt et al. (2013) (Appendix 1B).

1.7.2.3 SigB regulon in *B. cereus*

The phylogenetically more distant *B. cereus* has a much smaller SigB regulon than *B. subtilis* and *B. licheniformis.* The last updated SigB regulon comprises only 32 members (de Been et al.,

2010), which is substantially smaller than that of *B. licheniformis* and *B. subtilis* (see Appendix 1C). This may be attributed to biological and technical factors.

Biologically, *B. cereus* has evolved to use a completely different SigB upstream sensing module (RsbKMY) than *B. licheniformis* (RsbRST) and *B. subtilis* (RsbRST and RsbQP). Thus, the SigB responses to general stress may also differ from the other species. Technically, fewer studies on SigB GSR have been conducted in *B. cereus* than in *B. subtilis.* It is known that the physical stressors, heat, ethanol, and salt all induce SigB GSR, with heat being the most potent SigB-GSR trigger in *B. cereus* (Periago et al., 2002; van Schaik et al., 2004; Schaik et al., 2007; de Been et al., 2010). However, ATP depletion in cells (e.g., by the addition of CCCP) only induces SigB slightly and hence has been concluded not to be the crucial stressor that leads to induction of SigB in *B. cereus* (van Schaik et al., 2004). The SigB-GSR has not been investigated in *B. cereus* using other stressors than heat, ethanol, salt, and CCCP. Although SigB has recently been reported to regulate glucose starvation and oxidative stress in *B. cereus* as in *B. subtilis* (Gao et al., 2021), no global transcriptomics or proteomics studies have been performed to elucidate the SigB regulon in *B. cereus* further.

Moreover, *B. cereus* has a divergent SigB regulon compared to other species in the *B. cereus sensu lato* group, in which the expression of a standard set of genes was reprogrammed to adapt to niche-specific survival strategies or to support different pathogenic roles (Scott and Dyer, 2012). It has been reported that the SigB regulons of 20 *B. cereus sensu lato* group members (*B. cereus, B. anthracis, B.mycoides, B. pseudomycoides, B. thuringiensis, B. weihenstephanensis and B. cytotoxicus*) show differences in the SigB PBM, and each organism shares a core SigB regulon which consists of only around 20 genes (Scott and Dyer, 2012).

The most recent SigB regulon members of *B. subtilis, B. licheniformis,* and *B. cereus* are listed in Appendixes 1A, 1B, and 1C, respectively, and the SigB consensus sequences (PBM) are listed in Table 3. In *B. subtilis,* the majority but not all SigB-dependent genes contain a SigB PBM (Petersohn et al., 1999a; Nannapaneni et al., 2012). Some direct or indirect regulon members are under dual or multiple control by SigB and other regulators. However, no SigB PBM search has been performed for SigB regulon members in *B. licheniformis,* and in *B. cereus,* only 23 out of 32 SigB regulon members were shown to contain a SigB PBM (de Been et al., 2010).

Table 3: SigB promoter binding motif (PBM) in *B. subtilis, B. licheniformis* and *B. cereus*

Species	Promoter Sequence			Reference
	-35	spacer	-10	
B. subtilis	GTTTWW	12-15	GGGWAW	(Petersohn et al., 1999a; Rodriguez Ayala et al., 2020; Vohradsky et al., 2021)
B. licheniformis	AGGTTTAA	14	GGGTAA	(Brody and Price, 1998)
B. cereus	AKGKTTA	13	GKGTA	(van Schaik et al., 2004; Scott and Dyer, 2012; Rodriguez Ayala et al., 2020)

1.7.3 SigB alternative functions in *Bacillus* species

1.7.3.1 Sporulation initiation and spore properties control

The relation between SigB and sporulation is sophisticated. At first, SigB was thought to be in charge of the switch from vegetative growth to spore formation in *B. subtilis* (Haldenwang and Losick, 1979; Moran et al., 1981). However, its role in sporulation was excluded when it was found that the deletion of the *sigB* gene did not affect sporulation and was not directly involved in the sporulation process (Binnie et al., 1986; Duncan et al., 1987). A potential link between SigB and the sporulation process was not confirmed until techniques used to decipher gene, and protein function became more mature and advanced. Findings at the beginning of 2000 indicated that SigB GSR and sporulation are not independent of each other (Méndez et al., 2004; van Schaik and Abee, 2005). In *B. subtilis,* Mandez et al. (2004) showed that SigB activity is needed for spore production at a low temperature, and in *B. cereus* de Vries et al. (2005) demonstrated that the deletion of *sigB* delays sporulation initiation and affects spore maturation. Moreover, further extended studies have been conducted in *B. subtilis,* revealing the molecular mechanism by which SigB is involved in the sporulation process. The ectopic expression of SigB (defect in regulation by RsbW) is shown to induce the expression of Spo0E phosphatase, which subsequently renders the sporulation master regulator (Spo0A) in its inactive state and thus prohibiting sporulation initiation (Reder et al., 2012b). In addition to this negative regulation, the SigB promoter preceding the *spo0E* gene is also repressed by the Rok protein (repressor of *comK*), forming a positive feedback loop in sporulation regulation through SigB (Reder et al., 2012a). Therefore, the influence of SigB on sporulation is now well-recognized.

1.7.3.2 Biofilm formation and motility

Another survival strategy, biofilm formation, is also linked with the SigB GSR. Cells lacking SigB form weaker biofilms than wt cells and are less motile; as a result, cells in such biofilms are less resistant to stresses and fail to disperse from the biofilm (Bartolini et al., 2019b). This regulatory circuit is established through SigB interaction with the master regulator of biofilm formation, SinR, via RsbP (metabolic SigB signaling module, see Figure 4). SigB downregulates SinR and provides an adaptation mechanism for the cells to either activate SigB or trigger sporulation within the biofilm (Nadezhdin et al., 2020). Although SigB is shown to modulate the distribution of sporulated cells via RsbQP, this control is deemed indirect because there is no SigB promoter found preceding the *sinR* gene, as reviewed by Rodriguez Ayala et al. (2020) and Arnaouteli et al. (2021).

Moreover, SinR is crucial for the swimming and swarming motility properties of *B. subtilis,* and the absence of SinR reduces cell motility. Bartolini et al. (2019b) also demonstrated that SigB regulates flagellum-dependent motility in *B. subtilis* cells, with cell motility being reduced in mutants lacking *sigB* or *sinR*. Therefore, the loss of motility within the biofilm indirectly impacts the dispersal of motile cells from the biofilm (Bartolini et al., 2019b).

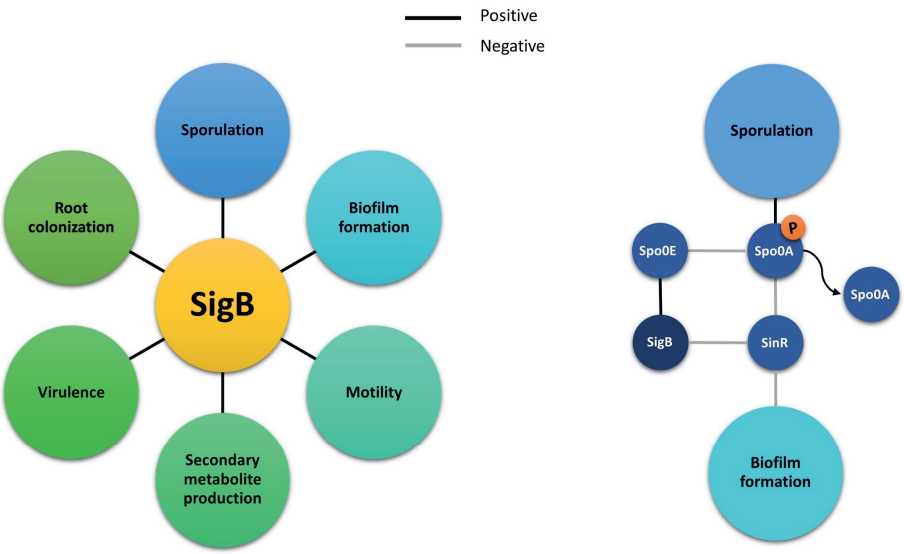


Figure 4- Influence of SigB in other processes in *Bacillus* species. (Black)- positive; (Grey)- negative

Similar to *B. subtilis,* it has only recently been discovered that the deletion of the *sigB* gene also impacts the ability of *B. cereus* cells to form biofilm under stress conditions, namely, low temperature and acidic pH (Gao et al., 2021). Wild-type cultures formed 2-3 fold more biofilms than the Δ*sigB* mutant at 16°C or 20°C. This apparent difference was not observed at optimal culturing temperatures (25°C, 30°C, or 37°C). When the pH of culturing medium was slightly acidic (pH 6.5), Δ*sigB* formed 30% less biofilm than wt cells, and at pH 5.5, Δ*sigB* formed 60% less biofilm than wt cells. Neither pH values close to the minimum pH growth boundary (pH 4.5) nor at a slightly alkaline pH of 7.5 affect biofilm formation (Gao et al., 2021).

Another *Bacillus* species well-known for its biofilm-forming capacity is *B. licheniformis* (Gopal et al., 2015). This may also occur in milk powder processing environments, such as evaporators, which operate at temperatures ranging from 50°C to 70°C (Wang et al., 2021). However, it is yet to be explored whether SigB also plays a role in biofilm development in this species.

1.7.3.3 Role in root colonization

In the natural environment, bacteria are exposed to stressful conditions such as changes in pH or temperature, the presence of reactive oxygen species, antibiotics, toxic compounds, disinfectants, conditions of nutrient deprivation, and changes between light and dark. Therefore, ecologically, *Bacillus* spp. form biofilm to protect cells from these environmental stresses (Jefferson, 2004; Caro-Astorga et al., 2020).

When starvation occurs in soil, predominant soil bacteria like *Bacillus* spp. can colonize plant roots and access nutrients available in the plant rhizosphere (Jefferson, 2004). As SigB is

known to be triggered under nutrient-deprived conditions, it is not surprising that SigB is also indirectly involved in root colonization. Although this has not been reported for *B. subtilis*, it has been recently uncovered that SigB in *B. cereus* controls such plant colonization, e.g., on wheat roots (Gao et al., 2021). The absence of SigB in *B. cereus* has been reported to affect colonization efficacy, which directly impacts cell survival (Gao et al., 2021). Therefore, this recent discovery further demonstrated the diverse roles of SigB.

1.7.3.4 Virulence

Moreover, a role of SigB in virulence has been reported for some *Bacillus* spp. and other related firmicutes like *L. monocytogenes* and *S. aureus*. Firstly, *B. thurigiensis,* which produces insecticidal toxins, is widely used for insect pest control. SigB is active during the infectious cycle of *B. thurigiensis,* which is critical for its pathogenicity towards insects and is required to adapt to the gut environment of the insect (Henry et al., 2020). Secondly, SigB appeared to play a role in the virulence of *B. anthracis,* which causes anthrax. When $10^4 – 10^8$ spores from *B. anthracis* were fed to a group of mice, spores of the wt strain caused a higher number of deaths in mice than spores of the Δ*sigB* strain (1 $\log_{10}$ unit higher in wt than in Δ*sigB,* but the exact LD_{50} value is not known) (Fouet et al., 2000).

Of the pathogenic *Bacillus* species, the role of SigB has been investigated the most in *B. cereus*. However, there is no evidence that SigB contributes to the virulence of this bacterium. The production of virulence factors and the nonhemolytic enterotoxin Nhe did not differ between the wt cells and Δ*sigB* mutant (van Schaik et al., 2004). Other pathogenic *Bacillus* spp. belonging to the *B. cereus sensu lato* group (i.e., *B. mycoides, B. pseudomycoides, B. weihenstephanensis,* and *B. cytotoxicus*) also contain a SigB ortholog and a distinctive SigB regulon (Scott and Dyer, 2012), but the exact role of SigB in these species has not been studied.

The role of SigB in virulence is evident for the firmicutes *L. monocytogenes* and *S. aureus* (O'Byrne and Karatzas, 2008; Liu et al., 2019). In *L. monocytogenes,* SigB influences adhesion to and invasion of host cells, survival within host cells, replication and survival in the gastric environment (resistance to bile), and resistance to host-cell phagosomes and antibiotics (nisin, lacticin, ampicillin, penicillin G and V, and tetracycline (Zhou et al., 2012)). In total, around 20% of the genes (51/304) that belong to the SigB regulon play a role in the virulence of *L. monocytogenes*, many of which are regulated by the virulence regulator PrfA but co-regulated by SigB. Some SigB regulon genes involved in glycolysis and glycerol metabolism also contribute to the pathogenicity in *L. monocytogenes* via interaction with PrfA, highlighting that SigB regulates specific metabolic functions that can contribute to infection (reviewed by Liu et al., 2019).

In *S. aureus,* SigB is involved in the production of virulence factors that, for instance, affect host cell invasion/internalization, antibiotic resistance (oxacillin, vancomycin, methicillin, beta-lactams antibiotics), persistence, and also biofilm formation and membrane transport

(reviewed by Guldimann et al., 2016; Jenul and Horswill, 2019). SigB moreover positively regulates the main virulence factor in *S. aureus,* called SarA. In addition, the expression of virulence factors like adhesins, exoproteins, toxins, and small-colony variants (important for chronic infections) are reported to be SigB-dependent (Bischoff et al., 2004; Moisan et al., 2006).

As this thesis focussed on *Bacillus* spp., the detailed role of SigB in virulence control of *L. monocytogenes* and *S. aureus* are not further discussed. Various comprehensive reviews on the role of SigB in these two pathogenic firmicutes are available (O'Byrne and Karatzas, 2008; Guldimann et al., 2016; Jenul and Horswill, 2019; Liu et al., 2019).

1.7.3.5 Influence on secondary metabolite production

It is clear that the SigB is critical for stress response and survival of *Bacillus* spp. and that the various defense mechanisms are closely intertwined. In addition, SigB can play a role in obtaining competitive advances over other microbes in the environment. For instance, there are some indications that SigB can influence the production of metabolites with antibacterial or antifungal effects that allow for better access to substrates, e.g., by increasing swarming (Bartolini et al., 2019a).

Secondary metabolites are biochemical products produced by plants, fungi, or bacteria that are derived from primary metabolism but may not be directly required for growth, development, or reproduction (Zähner, 1979). For example, amino acids crucial for growth are synthesized in primary metabolisms and later become the building blocks of complex secondary metabolites via a condensation step. Secondary metabolites have different ecological roles closely associated with the habitat of microorganisms or protecting them in harsh environments, e.g., protein complexes to transport nutrients and facilitators to compete for substrates or agents that may allow for interactions between organisms. To date, several secondary metabolite groups are well-known, like terpenoids and steroids, alkaloids, enzyme cofactors, fatty acids polyketides, and non-ribosomal peptides (NRP). Most of them are synthesized by plants, but many fungi and soil bacteria, including *Bacillus* species, produce a myriad of these compounds too. Among these, polyketides and NRPs possess versatile bioactive properties like antibacterial, antifungal, anticancer, or immunosuppressing activity (Martínez-Núñez and López, 2016; de Frias et al., 2018).

Polyketides and NRPs can either be linear or cyclic. They are synthesized and assembled by large enzymatic domains via a thiotemplate mechanism, which includes the initiation, elongation, and termination of peptides (Marahiel et al., 1997; Rajendran and Marahiel, 1999). These enzymatic domains are widely distributed across proteobacteria, cyanobacteria, actinobacteria, and firmicutes (Wang et al., 2014). For example, as much as 31 % of firmicutes harbor either the polyketide or the NRP synthetase gene clusters, and 70% are NRP synthetases, mainly present in the genus of *Paenibacillus* and *Bacillus* of this phylum (Aleti et al., 2015).

Renowned NRPs include the lipopeptides (surfactants) such as surfactin, iturin, fengycin, locillomycin, and lichenysin, produced by *Bacillus* spp (Luo et al., 2015). These lipopeptides are amphiphilic and contain a hydrophobic fatty acid chain linked to a hydrophilic cyclic peptide composed of several amino acids. For example, the well-known lipopeptide surfactin, produced by *B. subtilis,* contained a heptapeptide amino sequence of Glu-Leu-Leu-Val-Asp-Leu-Leu (Zhao et al., 2017) and was the first discovered by Arima et al. (1968).

It has been reported that the surfactin production in *B. subtilis* is influenced by SigB (Bartolini et al., 2019a). Upon deletion of the *sigB* gene in *B. subtilis,* cells produced lower amounts of surfactin and had a reduced efficacy in fungal control (Bartolini et al., 2019a). This report is another example of the involvement of SigB in physiological and ecological processes. No other similar studies on the relation between SigB and the production of NRP compounds are available for other *Bacillus* spp. such as *B. cereus* or *B. licheniformis*.

1.8 Aim of this thesis and outline

The master regulator of stress SigB plays a vital role in the adaptive stress response repertoire of *Bacillus* spp. This relates to various regulatory aspects, including 1) stressors that induce SigB; 2) signaling cascades that activate SigB; 3) genes/proteins regulated by SigB; and 4) alternative roles of SigB. The SigB general stress response (GSR) has been best characterized in the model organism *B. subtilis*, but to a lesser extent in the foodborne pathogen *B. cereus* and the industrial workhorse *B. licheniformis* and has not been examined for other Bacillales members. The function of SigB is not restricted to solely regulating the adaptive stress response in *B. subtilis*, but also other cellular functions or secondary process, as presented above (Figure 4). Similarly, this has not been explored in other *Bacillus* species.

Further understanding of the mechanisms underlying SigB GSR, SigB regulon functions, and alternative functions of SigB in *Bacillus* spp. may reveal novel specific triggers and adaptive responses to particular stresses that govern the persistence of the different species in particular niches.

This thesis aimed to dissect the SigB GSR of *B. subtilis* further and explore the roles of SigB in *B. cereus* and *B. licheniformis*. In addition to these three species, an *in silico* approach was used to characterize the composition and key players in SigB GSR in other Bacillales members.

The description of research topics investigated in this thesis are outlined below:

Chapter 1 introduces the genus of *Bacillus* and focuses on three prominent species: *B. subtilis*, *B. cereus,* and *B. licheniformis*. Their characteristics and beneficial and pathogenic potentials are presented. Comprehensive knowledge known to date concerning the master regulator of stress – SigB, its activation pathways, its regulon members, and its alternative function in addition to the adaptive stress response is described.

Chapter 2 extends the study on the SigB regulon in *B. subtilis* using a SigB promoter binding motif derived from all promoter sequences of the up-to-date knowledge on SigB regulated genes. Novel SigB regulon members with putative promoters were predicted, and the regulation of selected promoters was validated in *B. subtilis* wild-type versus Δ*sigB* mutants by use of a translational fusion of the selected promoters to the *lacZ* reporter gene. Novel SigB-regulated genes under the dual control of SigB and other regulators are described. Moreover, the presence and absence of SigB signaling cascades and SigB regulon members in other Bacillales members were predicted via comparative genomics.

Chapter 3 deciphers the core function of SigB and investigated the role of a SigB-dependent putative phosphocarrier protein (Bc1009) in *B. cereus* ATCC14579 via gene expression and omics studies in wild-type, Δ*sigB* and Δ*bc1009* mutants. Clean *sigB* and *bc1009* knockout mutants were constructed, and changes in proteome/genome profiles upon exposure to heat

stress at 42°C were compared to the wild-type cells. Novel SigB-dependent differentially expressed genes/proteins were identified. Comparison of expression data of the SigB and Bc1009 mutants with the wild type identified a subgroup of SigB-dependent genes/proteins regulated via Bc1009, with putative functions in stress response, cell motility, signal transduction, transcription, amino acid transport and metabolism. Phenotypic assays confirmed roles in heat resistance and motility. Notably, differential expression of SigB and Bc1009 dependent genes/proteins was also observed at 30°C non-stressed condition in both knockout mutants compared to wt, and functionality was confirmed in motility assays. Finally, the role of *B. cereus* SigB in regulating other cellular functions directly or via Bc1009, in non-stressed and stressed conditions is discussed.

Chapter 4 examines the potential involvement of SigB in the production of the biosurfactant (lichenysin) in *B. licheniformis*, and conversely, studies the role of lichenysin as a SigB stressor in *B. licheniformis* wild type and Δ*sigB* mutant. The total lichenysin yield in wild-type and Δ*sigB* mutant was quantified, showing that Δ*sigB* mutant produced less lichenysin than wild-type cells, but the lower production was shown to be not regulated by SigB directly in gene expression studies. However, the presence of lichenysin induced *sigB* and *sigB*- regulated genes mildly, contributing to slightly higher cell survival when exposed to lethal ethanol conditions. This modest protection was also seen for *B. licheniformis* when exposed to a lichenysin-like compound (surfactin) produced by *B. subtilis*.

Chapter 5 focuses on growth and lichenysin production by different food isolates of *B. licheniformis*. This bacterium showed robust growth under conditions such as high salt concentrations and high temperatures. Lichenysin production was investigated in a laboratory medium (Luria-Bertani) and a food matrix (milk) at optimal temperature (37°C) and high temperature (55°C). The amount produced per isolate, temperature, and condition was quantified using RP-HPLC-ESI-MS. Lichenysin toxicity toward human Caco-2 cells and pig ileum organoids was assessed and compared to the toxicity of the similar compound – surfactin - produced by *B. subtilis*. The relevance of *B. licheniformis* in foods in relation to potential food safety hazards is discussed.

Finally, **Chapter 6** discusses the fundamental and novel role(s) of SigB and SigB GSR in *Bacillus* species with respect to the results obtained in this thesis and provides outlooks and broader perspectives on persistence in environmental niches and their impact on food quality and safety.

Appendix 1A SigB regulon - *B. subtilis*

Supplementary Table S1: SigB regulon genes in *B. subtilis* (Pedreira et al., 2021). The expression of all genes/proteins that are SigB-dependent, with or without a SigB promoter binding motif.

Locus Tag	Gene name	Description	Function
BSU00870	*radA*	branch migration transferase	negative effector of activity
BSU00880	*disA*	DNA integrity scanning protein	the DisA-dependent checkpoint arrests spore outgrowth until the germinating spores genome is free of damage
BSU00850	*mcsB*	protein arginine kinase	modulator of protein dependent repression
BSU00860	*clpC*	AAA unfoldase	unknown
BSU00830	*ctsR*	transcription repressor of class III heat shock genes	unknown
BSU00840	*mcsA*	modulator of protein dependent repression	control of proteinactivity
BSU02940	*yceH*	similar to toxic anion resistance protein	unknown
BSU02890	*yceC*	similar to tellurium resistance protein	unknown
BSU02910	*yceE*	general stress protein	required for survival of ethanol stress and at low temperatures
BSU02900	*yceD*	general stress protein	required for survival of ethanol stress
BSU02920	*yceF*	general stress protein	resistance to Mn2+ intoxication
BSU02930	*yceG*	general stress protein	survival of ethanol stress
BSU04700	*rsbU*	protein serine phosphatase	unknown
BSU04730	*sigB*	RNA polymerase factor	biocontrol of fungal growth
BSU04720	*rsbW*	anti sigma factor	unknown
BSU04670	*rsbR*	stressosome sensor protein	control of protein activity
BSU04680	*rsbS*	part of the stressosome	control of protein activity
BSU04740	*rsbX*	protein serine phosphatase	unknown
BSU04710	*rsbV*	antagonist of protein	control of SigB activity
BSU04690	*rsbT*	PP2C activator	part of the stressosome
BSU39050	*katE*	catalase	detoxification (degradation) of hydrogen peroxide
BSU39040	*yxiS*	general stress protein	unknown
BSU04220	*ydaG*	putative pyridoxamine 5'-phosphate oxidase	unknown
BSU04190	*ydaD*	general stress protein	unknown
BSU04210	*ydaF*	similar to acetyltransferase	unknown
BSU04200	*ydaE*	lyxose isomerase	unknown
BSU16640	*ylxP*	general stress protein	protection against paraquat stress
BSU16630	*infB*	translation initiation factor IF-2	unknown
BSU16620	*rplGA*	similar to ribosomal protein	unknown
BSU16600	*nusA*	transcription termination factor of RNA polymerase	transcription termination factor
BSU16590	*ylxS*	similar to ribosome maturation protein	unknown
BSU16650	*rbfA*	ribosome-binding factor A	ribosome-binding factor A
BSU16610	*ylxR*	nucleoid-associated protein	unknown
BSU02840	*ycdG*	general stress protein	unknown
BSU02830	*ycdF*	general stress protein	unknown
BSU13170	*guaD*	guanine deaminase	unknown
BSU07900	*yfkH*	general stress protein	survival of stress conditions
BSU07880	*yfkJ*	general stress protein	unknown
BSU07890	*yfkI*	general stress protein	protection against paraquat stress
BSU30790	*menE*	o-succinylbenzoate-CoA ligase	o-succinylbenzoate-CoA ligase
BSU30780	*menC*	O-succinylbenzoate synthase	O-succinylbenzoate synthase
BSU05490	*mhqO*	hydroquinone-specific dioxygenase	resistence to methyl-hydroxyquinone
BSU05500	*mhqP*	may be involved in protection against methyl-hydroquinone	unknown
BSU13640	*spo0E*	Spo0A-P phosphatase	initiation of sporulation
BSU30650	*dps*	iron storage protein	unknown
BSU09140	*yhcM*	general stress protein	unknown
BSU40570	*yybO*	similar to permease	unknown
BSU38620	*aag*	hypoxanthine-DNA glycosylase	unknown
BSU38610	*yxzF*	general stress protein	unknown
BSU35670	*gtaB*	major vegetative UTP-glucose-1-phosphate uridylyltransferase	biosynthesis of teichoic acid
BSU09690	*nhaX*	general stress protein	unknown
BSU03910	*gabD*	succinate-semialdehyde dehydrogenase (NADP)	utilization of gamma-amino butyric acid
BSU06590	*yerD*	general stress protein	unknown
BSU02110	*ybyB*	general stress protein	survival of ethanol stress
BSU33610	*rnr*	RNase R	nonspecific degradation of rRNA
BSUmiscRNA55	*ssrA*	tmRNA	tmRNA
BSU33620	*yvaK*	general stress protein	unknown
BSU33600	*smpB*	tmRNA-binding protein	tmRNA-binding protein
BSU33630	*secG*	preprotein translocase subunit	preprotein translocase subunit
BSU04430	*ydbD*	general stress protein	survival of ethanol stress
BSU04440	*dctB*	C4-dicarboxylate binding protein	sensing of C4-dicarboxylates

Locus Tag	Gene name	Description	Function
BSU12170	*forE1*	partner subunit of formate:menadione oxidoreductase protein	unknown
BSU12160	*forC1*	formate:menadione oxidoreductase	unknown
BSU11130	*ipi*	intracellular proteinase inhibitor	intracellular proteinase inhibitor
BSU11500	*spx*	transcriptional regulator Spx	required for protection against paraquat stress
BSU02100	*cypC*	long chain-fatty acid beta-hydroxylating cytochrome P450	required for protection against paraquat stress
BSU12150	*yjgB*	general stress protein	survival of ethanol stress
BSU08600	*csbB*	lipoteichoic acid glycosyltransferase	required for protection against paraquate stress
BSU08610	*yfhO*	required for lipoteichoic acid glycosylation	glycosyltransferase
BSU07550	*yflT*	general stress protein	survival of ethanol stress
BSU04220	*ydaG*	putative pyridoxamine 5'-phosphate oxidase	unknown
BSU28340	*ysnF*	general stress protein	survival of ethanol stress
BSU33200	*yvrE*	glucoco-lactonase	unknown
BSU40450	*yycD*	general stress protein	survival of ethanol stress
BSU11120	*yitT*	general stress protein	protection against paraquat stress
BSU11130	*ipi*	intracellular proteinase inhibitor	intracellular proteinase inhibitor
BSU00160	*yaaH*	general stress protein	spore cortex lytic protein
BSU27020	*yraA*	general stress protein	unknown
BSU09530	*yhdN*	general stress protein	detoxification of methylglyoxal
BSU09540	*plsC*	acyl-ACP:1-acylglycerolphosphate acyltransferase	acyl-ACP:1-acylglycerolphosphate acyltransferase
BSU38940	*yxjI*	unknown	unknown
BSU24770	*mgsR*	transcriptional regulator of a subset of the general stress regulon	controls a subset of general stress genes
BSU39840	*yxbG*	general stress protein	unknown
BSU04370	*ydaS*	general stress protein	unknown
BSU29780	*ytxG*	general stress protein	unknown
BSU29770	*ytxH*	general stress protein	unknown
BSU29760	*brxC*	monothiol bacilliredoxin	removal of bacillithiol from oxidized cytosolic proteins
BSU34540	*clpP*	ATP-dependent Clp protease proteolytic subunit (class III heat-shock protein)	ATP-dependent Clp protease proteolytic subunit
BSU07760	*yfkT*	general stress protein	germination
BSU07790	*yfkQ*	part of the germinant receptor of unknown specificity	part of the germinant receptor
BSU07770	*yfkS*	D protein for the germinant receptor of unknown specificity	unknown
BSU07780	*yfkR*	part of the germinant receptor of unknown specificity	part of the germinant receptor
BSU40040	*yxaA*	putative glycerate kinase	putative glycerate kinase
BSU40030	*yxaB*	general stress protein	biofilm formation
BSU30930	*ytaB*	general stress protein	survival of ethanol and salt stresses
BSU17250	*ymaE*	unknown	unknown
BSU17240	*ymzB*	general stress protein	survival of ethanol and salt stresses
BSU09450	*yhdF*	similar to glucose 1-dehydrogenase	unknown
BSU38940	*yxjI*	unknown	unknown
BSU38930	*yxjJ*	general stress protein	unknown
BSU40000	*yxnA*	general stress protein	unknown
BSU04340	*ydaP*	general stress protein	putative pyruvate oxidase
BSU04290	*ydaL*	general stress protein	synthesis of extracellular polysaccharide
BSU04270	*ydaJ*	lipoprotein	unknown
BSU04280	*ydaK*	general stress protein	unknown
BSU04300	*ydaM*	general stress protein	synthesis of extracellular polysaccharide
BSU04310	*ydaN*	general stress protein	synthesis of extracellular polysaccharide
BSU23830	*yqjL*	general stress protein	unknown
BSU38430	*gspA*	general stress protein	unknown
BSU38630	*katX*	catalase	detoxification (degradation) of hydrogen peroxide
BSU38180	*ywzA*	general stress protein	unknown
BSU23329	*ypuC/1*	part of the ypuC pseudogene	unknown
BSU23340	*ypuB*	unknown	unknown
BSU06660	*opuE*	proline transporter	proline transporter
BSU29110	*phoP*	two-component response regulator (OmpR family)	regulation of phosphate metabolism
BSU29100	*phoR*	two-component sensor kinase	regulation of phosphate metabolism
BSU24020	*bmrR*	transcriptional activator operon	regulation of multidrug resistance
BSU24010	*bmr*	general stress protein	multidrug resistance
BSU27760	*csbX*	general stress protein	unknown
BSU27750	*bofC*	general stress protein	control of protein processing
BSU33410	*yvgO*	general stress protein	survival of ethanol stress
BSU29420	*ytkK*	similar to 3-oxoacyl- acyl-carrier protein reductase	unknown
BSU29410	*ytkL*	general stress protein	unknown
BSU35310	*hpf*	general stress protein	unknown
BSU13160	*ohrB*	general stress protein	unknown

Locus Tag	Gene name	Description	Function
BSU10430	*yhxD*	general stress protein	survival of salt and ethanol stresses
BSU07930	*yfkD*	unknown	unknown
BSU07920	*chaA*	Ca2+ exchanger	calcium export via proton antiporter
BSU36960	*ywlB*	general stress protein	unknown
BSU05790	*ydhK*	general stress protein	survival of stress conditions
BSU23300	*ypuD*	putative intramembrane metalloprotease	putative intramembrane metalloprotease
BSU08490	*yfhD*	general stress protein	survival of ethanol stress and low temperatures
BSU08510	*yfhF*	general stress protein	survival of ethonol stress and at low temperatures
BSU08500	*yfhE*	general stress protein	unknown
BSU28500	*trxA*	thioredoxin	unknown
BSU35970	*ywsB*	general stress protein	survival of ethanol and salt stresses
BSU08580	*yfhL*	general stress protein	survival of ethanol and salt stresses
BSU08570	*yfhK*	general stress protein	survival of ethonol stress and at low and high temperatures
BSU08590	*yfhM*	general stress protein	survival of ethanol stress
BSU28830	*ysdB*	control of SigW activity	survival of heat stress
BSU03130	*nadE*	NH3-dependent NAD+ synthetase	NH3-dependent NAD+ synthetase
BSU04370	*ydaS*	general stress protein	unknown
BSU04380	*ydaT*	general stress protein	survival of ethanol stress and low temperatures
BSU35180	*csbA*	general stress protein	protection against paraquat stress
BSU36670	*csbD*	general stress protein	unknown
BSU38830	*aldY*	aldehyde dehydrogenase (NAD)	unknown
BSU37210	*ywjC*	general stress protein	unknown
BSU24760	*rsbRD*	probably part of the stressosome	control of protein activity
BSU24000	*bmrU*	general stress protein	unknown
BSU24020	*bmrR*	transcriptional activator	regulation of multidrug resistance
BSU24010	*bmr*	general stress protein	multidrug resistance
BSU24740	*corA*	general stress protein	unknown
BSU25300	*cdd*	cytidine deaminase	cytidine deaminase
BSU25289	*yqzL*	unknown	unknown
BSU25280	*recO*	mediator of protein binding to ssDNA	unknown
BSU25290	*era*	GTP-binding protein	ribosome assembly
BSU24500	*yqhP*	unknown	unknown
BSU24490	*yqhQ*	general stress protein	survival of stress conditions
BSU24750	*yqhB*	similar to magnesium exporter	unknown
BSU07850	*yfkM*	glyoxalase III-like enzyme	paraquat and ethanol stresses
BSU04400	*gsiB*	general stress protein	response to water deficits
BSU30700	*rpmEB*	general stress protein	unknown
BSU38720	*yxkO*	ADP/ATP-dependent NAD(P)H-hydrate dehydratase	unknown
BSU07670	*yflI*	unknown	unknown
BSU07690	*yflG*	methionine aminopeptidase	methionine aminopeptidase
BSU07680	*yflH*	general stress protein	unknown
BSU00890	*yacL*	general stress protein	survival of salt and ethanol stresses
BSU00910	*ispF*	2-C-methyl-D-erythrol-2	unknown
BSU00900	*ispD*	2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase	MEP pathway of isoprenoid biosynthesis
BSU11500	*spx*	transcriptional regulator Spx	required for protection against paraquat stress
BSU11490	*yjbC*	general stress protein	survival of paraquat stress
BSU36670	*csbD*	general stress protein	unknown
BSU36680	*ywmF*	unknown	unknown
BSU18530	*yoaA*	similar to spermidine/spermine N-acetyltransferase	unknown
BSU18520	*yoxB*	general stress protein	unknown
BSU18510	*yoxC*	general stress protein	survival of ethanol stress
BSU18350	*dacC*	Class C penicillin-binding protein 4A	carboxypeptidase
BSU18360	*galM*	aldose-1-epimerase	aldose-1-epimerase (mutarotase)
BSU13010	*ykgB*	6-phosphogluconolactonase	pentose phosphate pathway
BSU13020	*ykgA*	similar to arginine-guanidine removing enzyme	unknown
BSU19240	*yocK*	general stress protein	general stress protein
BSU31380	*yuzA*	general stress protein	unknown
BSU00520	*ctc*	general stress protein	translation under stress conditions
BSU00540	*fin*	RNA polymerase-binding protein	control of sporulation
BSU00530	*spoVC*	general stress protein	spore coat formation
BSU00520	*ctc*	general stress protein	translation under stress conditions
BSU39810	*csbC*	general stress protein	unknown
BSU33530	*iolW*	scyllo-inositol dehydrogenase	utilization of scyllo-inosose
BSU30070	*opuD*	glycine betaine and arsenobetaine transporter	glycine betaine transporter
BSU30060	*ytfP*	putative NAD(FAD)-utilizing dehydrogenase	unknown
BSU25020	*sodA*	superoxide dismutase	unknown
BSU02590	*ycbP*	general stress protein	unknown
BSU04039	*yczO*	unknown	unknown
BSU04180	*ydaC*	similar to N-methyltransferase	unknown
BSU07750	*yflA*	general stress protein	unknown

Locus Tag	Gene name	Description	Function
BSU09390	*ygxB*	general stress protein	unknown
BSU10810	*yisP*	farnesyl diphosphate phosphatase	control of protein activity
BSU11839	*yjzE*	general stress protein	unknown
BSU14210	*ykuT*	mechanosensitive channel	unknown
BSU14660	*ykzI*	general stress protein	unknown
BSU19150	*yocB*	general stress protein	survival of stress conditions
BSU30640	*ytkC*	similar to autolytic amidase	unknown
BSU33850	*yvbG*	unknown	unknown
BSU33400	*yvgN*	glyoxal reductase	unknown
BSU35699	*yvzE*	putative UTP-glucose-1-phosphate uridylyltransferase	unknown
BSU35830	*ywtG*	general stress protein	unknown
new30363103036529c	*SR7P*	antisense RNA to RNase Y interaction	control of protein RNase Y activity
new30354753036308c	*S1134*	antisense RNA	antisense RNA
BSU00160	*yaaH*	general stress protein	spore cortex lytic protein
BSU00170	*yaaI*	general stress protein	survival of ethanol stress
BSU05150	*ydeC*	similar to transcription factor (AraC family)	unknown
BSU05140	*ydzE*	unknown	unknown
BSU07735	*yflB*	stress protein	unknown
BSU07750	*yflA*	general stress protein	unknown
BSU07720	*yflD*	stress protein	unknown
BSU37240	*ywiE*	minor cardiolipin synthetase	unknown
BSU37220	*ywjB*	unknown	unknown
BSU37230	*ywjA*	similar to ABC transporter (ATP-binding protein)	ABC transporter (ATP-binding protein)
BSU31279	*yuzH*	unknown	unknown
BSU31280	*yugU*	general stress protein	survival of stress conditions
BSU30690	*ytiB*	similar to carbonic anhydrase	unknown
BSU30670	*luxS*	S-ribosylhomocysteine lyase	unknown
BSU23329	*ypuC/1*	part of the ypuC pseudogene	unknown
BSU23340	*ypuB*	unknown	unknown
BSU23330	*ypuC/2*	part of the ypuC pseudogene	unknown
BSU13169	*ykzN*	unknown	unknown
BSU13160	*ohrB*	general stress protein	unknown
BSU14680	*ykzC*	unknown	unknown
BSU14670	*yktC*	inositol monophosphatase and 5'-nucleotidase with preference for GMP and IMP	nucleotidase
BSU40529	*yyzH*	unknown	unknown
BSU40259	*yyzG*	unknown	unknown
BSU40210	*yydC*	unknown	unknown
BSU36720	*ywmE*	general stress protein	survival of ethanol stress
BSU06400	*yebE*	unknown	unknown
BSU06410	*yebG*	unknown	unknown
new37081333708785	*S1384*	new RNA feature	unknown
BSU35980	*ywsA*	unknown	unknown
BSU12220	*yjiC*	UDP-dependent glycosyltransferase	synthesis of bacillaene
BSU12210	*yjiB*	cytochrome P450	oxidation of fatty acids
BSU04190	*ydaD*	general stress protein	unknown
BSU04200	*ydaE*	lyxose isomerase	unknown
BSU02890	*yceC*	similar to tellurium resistance protein	unknown
BSU02910	*yceE*	general stress protein	unknown
BSU02900	*yceD*	general stress protein	required for survival of ethanol stress
BSU02920	*yceF*	general stress protein	resistance to Mn2+ intoxication
new_3460206_3462957	*S1290*	antisense RNA	antisense RNA

Appendix 1B SigB regulon - *B. licheniformis*

Supplementary Table S2: SigB regulon members in *B. licheniformis* (Voigt et al., 2013). The expression of all genes/proteins that are SigB-dependent, with or without a SigB promoter binding motif.

Locus Tag	Gene	Description/Function
BLi00560	*sigB*	RNA polymerase sigma factor SigB
BLi00512	*ydaG*	BLAST putative general stress protein [Bacillus subtilis]
BLi02651	*yqgZ*	(mgsR) BLAST transcriptional regulator, controlls a subset of stress genes; thioredoxin-like-superfamily; ArsC-Spx-Hit
BLi04064	*gspA*	general stress protein A
BLi03887	*yqhB*	similar to hemolysin; BLAST TlyC domain
BLi04197	*katE2*	sigB dep. Catalase 3
BLi00779	*yflT*	hypothetical protein; BLAST heat stress induced protein [Bacillus subtilis subsp. subtilis str. 168 100%], YflT superfamily (heat induced protein)
BLi03885	*none*	BLAST CBS domain-containing protein [Bacillus licheniformis ATCC 14580 100%], CBS_pair superfamily
BLi02579	*bmrU*	multidrug resistance protein cotranscribed with bmr; BLAST DAGK_cat superfamily
BLi04196	*katE1*	sigB dep. Catalase 2
BLi03883	*ydbD*	similar to manganese-containing catalase; BLAST general stress protein,putative manganese-containing catalase, Mn-catalase domain
BLi01012	*yhdF*	Short-chain dehydrogenase/reductase SDR YhdF
BLi00747	*none*	putative 5-methylcytosine-specific restriction enzyme
BLi02212	*none*	putative symporter YidK
BLi01416	*ykzA*	organic hydroperoxide resistance protein OhrB, sigmaB regulon
BLi00815	*yfkM*	BLAST general stress protein YfkM [Bacillus licheniformis ATCC 14580 100%], GATase1_PfpI_like domain, GAT_1 superfamily
BLi02649	*yqhA*	similar to positive regulator of sigma-B activity; BLAST putative sulphate transporter YqhA [Bacillus licheniformis ATCC 14580 100%]
BLi01046	*nhaX*	putative regulatory gene for nhaC; BLAST stress response protein, NhaX [Bacillus licheniformis ATCC 14580 100%], AANH_like superfamily
BLi02221	*yjgD*	hypothetical protein
BLi03886	*yqxL*	BLAST Mg2+ transporter protein, CorA-like [Bacillus licheniformis ATCC 14580 100%], CorA domain
BLi00520	*ydaP*	pyruvate oxidase
BLi02834	*yrhK*	hypothetical protein
BLi01417	*none*	hypothetical protein
BLi02222	*yjgC*	similar to formate dehydrogenase; BLAST iron-sulfur binding domain-containing protein [Bacillus subtilis] 97%; Molybdopterin-binding superfamily
BLi00509	*ydaD*	short chain dehydrogenase
BLi00576	*none*	close homolog to Des membrane phospholipid desaturase; BLAST fatty acid desaturase
BLi02246	*yocK*	similar to general stress protein
BLi00510	*ydaE*	hypothetical protein
BLi01401	*ykgA*	BLAST transferase [Bacillus licheniformis ATCC 14580] 100%
BLi01684	*ykzI*	hypothetical protein
BLi04360	*yybO*	Major facilitator superfamily
BLi02648	*comGA*	late competence protein ComGA
BLi03884	*yqjF*	hypothetical protein
BLi00805	*none*	Putative uncharacterized protein
BLi00534	*gsiB*	general stress protein
BLi01145	*cotJA*	CotJA
BLi00231	*ybyB*	hypothetical protein
BLi02908	*none*	Putative uncharacterized protein
BLi02352	*none*	Putative uncharacterized protein
BLi01974	*cwlC*	CwlC
BLi03201	*ytlA*	Putative sulfonate transport system substrate-binding protein YtlA
BLi02966	*none*	Putative uncharacterized protein
BLi01146	*cotJB*	CotJB
BLi04356	*none*	spore coat protein F
BLi00341	*ycdF*	similar to glucose 1-dehydrogenase
BLi01491	*ykzB*	unknown Blast
BLi02120	*dctB*	possible C4-dicarboxylate binding protein
BLi00530	*ydaT*	hypothetical protein
BLi00719	*none*	hypothetical protein
BLi03203	*ytlD*	YtlD
BLi03651	*opuCA*	glycine betaine/carnitine/choline ABC transporter (ATP-binding protein)
BLi00804	*ytcB*	NAD-dependent epimerase/dehydratase
BLi01950	*yraD*	YraD
BLi04028	*ywcE*	Spore morphogenesis and germination protein ywcE
BLi00802	*none*	Spore coat biosynthesis protein
BLi01776	*pyrE*	orotate phosphoribosyltransferase
BLi02647	*comGB*	late competence protein ComGB / DNA transport protein ComGB
BLi03099	*none*	Small acid-soluble spore protein (Beta-type SASP)
BLi03888	*nrgA*	ammonium transporter
BLi00720	*none*	hypothetical protein
BLi01775	*pyrF*	Orotidine 5'-phosphate decarboxylase

Locus Tag	Gene	Description/Function
BLi00674	*none*	Putative uncharacterized protein
BLi04093	*none*	Putative transcriptional regulator
BLi02272	*none*	hypothetical protein
BLi03202	*ytlC*	Putative sulfonate transport system ATP-binding protein YtlC
BLi03835	*ywtD*	Gamma-DL-glutamyl hydrolase
BLi00527	*ydaS*	hypothetical protein
BLi00198	*none*	Arginase
BLi02026	*none*	BLAST Trp repressor binding protein, putative [Bacillus licheniformis ATCC 14580] 100%; FMN_red superfamily
BLi00978	*yhcN*	hypothetical protein
BLi02691	*yqfT*	YqfT
BLi02270	*yozR*	(yozR/gerT) Spore germination protein gerT
BLi02188	*yvmB*	YvmB; BLAST transcriptional regulator 98%
BLi03735	*hisB*	imidazoleglycerol-phosphate dehydratase
BLi02963	*none*	Putative uncharacterized protein
BLi04167	*none*	putative transcriptional regulator of anaerobic genes
BLi00879	*sspK*	acid-soluble spore protein K
BLi03644	*none*	close homolog to Abh transcriptional regulator of transition state genes (AbrB-like)
BLi03911	*ywnB*	BLAST putative oxidoreductase [Bacillus subtilis subsp. subtilis str. 168 100%], NADB_Rossmann superfamily
BLi03653	*yvbG*	YvbG
BLi03167	*ytdP*	similar to transcriptional regulator (AraC/XylS family)
BLi01050	*sspB*	Small acid-soluble spore protein (Beta-type)
BLi03207	*dps*	stress- and starvation-induced gene controlled by SigB
BLi04314	*none*	Putative uncharacterized protein
BLi01661	*ykpC*	hypothetical protein
BLi04094	*mmgD*	citrate synthase 3
BLi01622	*none*	hypothetical protein
BLi03468	*none*	Putative uncharacterized protein
BLi03737	*hisG*	ATP phosphoribosyltransferase catalytic subunit
BLi01774	*pyrD*	dihydroorotate dehydrogenase 1B
BLi01269	*cotY*	CotY
BLi01985	*none*	hypothetical protein
BLi03128	*ytxJ*	similar to general stress protein
BLi03516	*uxaC*	glucuronate isomerase
BLi04317	*none*	Putative uncharacterized protein
BLi01220	*yjzB*	Putative uncharacterized protein yjzB
BLi04313	*none*	Putative uncharacterized protein
BLi02482	*ypuA*	YpuA
BLi00528	*ansB*	aspartate ammonia-lyase
BLi00803	*none*	Putative uncharacterized protein
BLi00349	*none*	ABC transporter, ATP-binding protein; ABC_DR_subfamily_A; P-loop NTPase superfamily; CcmA-Domain
BLi00709	*none*	putative transposase; BLAST DUF772 superfamily
BLi00446	*ybfA*	Putative DNA binding protein YbfA
BLi00786	*yvaZ*	BLAST membrane protein SdpI [Bacillus licheniformis ATCC 14580 100%], DUF1648 superfamily
BLi03765	*yvjB*	Peptidase S41A, C-terminal protease
BLi02494	*spoVAA*	SpoVAA
BLi01951	*none*	YraE
BLi02124	*ydeT*	similar to transcriptional regulator (ArsR family)
BLi00243	*none*	putative nucleoside-diphosphate-sugar epimerases; BLAST NADB_Rossmann superfamily, Epimerase domain
BLi00824	*yfkD*	Conserved protein YfkD
BLi04246	*iolF*	inositol transport protein
BLi01490	*tnrA*	TnrA
BLi04318	*none*	Putative Type I restriction-modification system M subunit
BLi02093	*none*	SAM dependent methyltranferase
BLi01488	*none*	Putative uncharacterized protein
BLi00725	*none*	Putative alanine racemase
BLi00980	*yhcQ*	Putative uncharacterized protein yhcQ
BLi03832	*ywtG*	similar to metabolite transport protein
BLi00026	*yaaI*	similar to isochorismatase; putative isochorismatase hydrolase YaaI
BLi00025	*ydhD*	BLAST glycoside hydrolase family protein [Bacillus licheniformis ATCC 14580] 100%; spore germination protein [Bacillus pumilus] 97%
BLi03129	*ytxH*	similar to general stress protein
BLi00451	*yclG*	Pectin lyase-like protein
BLi04316	*none*	HsdS
BLi01047	*yheI*	ABC transporter YheI
BLi00433	*none*	hypothetical protein
BLi03474	*yfiZ*	ABC transport system permease protein
BLi04121	*none*	hypothetical protein
BLi00950	*none*	Putative metallopeptidase
BLi01828	*ylqH*	Putative uncharacterized protein ylqH
BLi00550	*ydcC*	Conserved membrane protein YdcC
BLi04299	*yvfS1*	YvfS1
BLi03311	*ydfR*	Putative uncharacterized protein ydfR
BLi04122	*none*	putative transcriptional regulator
BLi00464	*yclO*	similar to ferrichrome ABC transporter (permease); BLAST putative iron-siderophore ABC transporter (permease) [Bacillus subtilis 168 100%]; TM_ABC_iron-siderophores_like domain

Locus Tag	Gene	Description/Function
BLi01631	*ykuP*	similar to flavodoxin
BLi01739	*murG*	N-acetylglucosaminyl transferase
BLi04119	*none*	BLAST lantibiotic immunity protein, MFS type transporter [Bacillus licheniformis ATCC 14580] 100%
BLi00270	*none*	hypothetical protein, (possible membrane protein [Bacillus sp.]; Score 274 / 54%; Duf_421-superfamily)
BLi00985	*yhcU*	Putative uncharacterized protein yhcU
BLi03229	*glgC*	glucose-1-phosphate adenylyltransferase
BLi02663	*none*	SpoVAFA
BLi01292	*none*	Hypothetical DNA-binding protein,putative transcriptional regulator
BLi02424	*yphA*	BLAST putative integral inner membrane protein [Bacillus subtilis] 100%
BLi00539	*ydbL*	YdbL
BLi00594	*none*	hypothetical protein
BLi03712	*yvdC*	YvdC
BLi01051	*none*	hypothetical protein
BLi04033	*none*	Putative uncharacterized protein
BLi01112	*yfmD*	Putative transport system permease protein
BLi04300	*yvfS2*	YvfS2
BLi00171	*cwlD*	N-acetylmuramoyl-L-alanine amidase (germination)
BLi02422	*none*	Putative uncharacterized protein
BLi02423	*seaA*	involved in spore envelope assembly
BLi04034	*none*	Putative uncharacterized protein
BLi03713	*ydhJ*	Putative Metal-dependent phosphohydrolase, HD region

Appendix 1C SigB regulon - *B. cereus*

Supplementary Table S3: SigB regulon members in *B. cereus* (de Been et al., 2010). The expression of all genes/proteins that are SigB-dependent, with a SigB promoter binding motif.

Locus Tag	Gene	Description/Function
BC0861	*bc0861*	Hypothetical
BC0862	*yfkM*	Protease I
BC0863	*katE*	Catalase
BC0995	*n.i.*	Hypothetical
BC0996	*n.i.*	Hypothetical
BC0998	*yflT*	General stress protein
BC0999	*bc0999*	Hypothetical
BC1000	*bc1000*	Hypothetical
BC1001	*bc1001*	Hypothetical
BC1002	*rsbV*	Anti-sigma factor antagonist
BC1003	*rsbW*	Anti-sigma B factor
BC1004	*sigB*	Sigma factor B
BC1005	*bc1005*	bacterioferratin
BC1006	*rsbY*	PP2C phosphatase
BC1007	*rsbM*	Methyltransferase
BC1008	*rsbK*	Histidine sensor kinase
BC1009	*bc1009*	Hpt like phosphptransferase protein
BC1010	*bc1010*	Hypothetical
BC1011	*bc1011*	Hypothetical
BC1012	*bc1012*	Hypothetical
BC1154	*hemH-2*	Ferrochelatase
BC1155	*katA*	Catalase
BC2108	*sigZ*	Sigma factor Z
BC2638	*bc2638*	Spore germination protein
BC3129	*corA*	Mg2+ and Co2+ transporter
BC3130	*bc3130*	Hypothetical
BC3131	*bc3131*	Hypothetical
BC3132	*bc3132*	General stress protein 17M
BC4640	*ytfJ*	Hypothetical
BC4641	*bc4641*	Hypothetical
BC5390	*cwlJ*	Cell wall hydrolase cwlJ
BC5391	*gerQ*	spore coat protein

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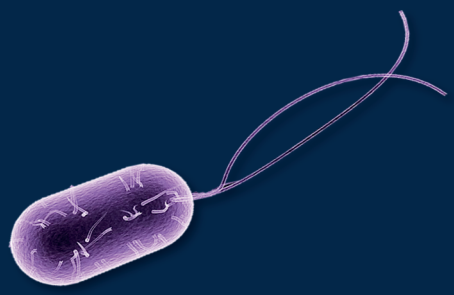
“ It is a well-known mechanism, but always more to explore ”

CHAPTER 2



Prediction and validation of novel SigB regulon members in *Bacillus subtilis* and regulon structure comparison to Bacillales members

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Abstract

Sigma factor B (SigB) is the central regulator of the general stress response in *Bacillus subtilis* and regulates a group of genes in response to various stressors, known as the SigB regulon members. Genes that are directly regulated by SigB contain a promotor binding motif (PBM) with a previously identified consensus sequence.

In this study, refined SigB PBMs were derived and take different spacer compositions and lengths (N_{12}-N_{17}) into account. These were used to identify putative SigB-regulated genes in the *B. subtilis* genome, revealing 255 genes: 99 had been described in the literature and 156 genes were newly identified, increasing the number of SigB putative regulon members (with and without a SigB PBM) to > 500 in *B. subtilis*. The 255 genes were assigned to five categories (I-V) based on their similarity to the original SigB consensus sequences. The functionalities of selected representatives per category were assessed using promoter-reporter fusions in wt and Δ*sigB* mutants upon exposure to heat, ethanol, and salt stress. The activity of the P_{rsbV} (I) positive control was induced upon exposure to all three stressors. P_{ytoQ} (II) showed SigB-dependent activity only upon exposure to ethanol, whereas P_{pucI} (II) with a N_{17} spacer and P_{ylaL} (III) with a N_{16} spacer showed mild induction regardless of heat/ethanol/salt stress. P_{ywzA} (III) and P_{yaaI} (IV) displayed ethanol-specific SigB-dependent activities despite a lower-level conserved -10 binding motif. P_{gtaB} (V) was SigB-induced under ethanol and salt stress while lacking a conserved -10 binding region. The activities of P_{ygaO} and P_{ykaA} (III) did not show evident changes under the conditions tested despite having a SigB PBM that highly resembled the consensus. The identified extended SigB regulon candidates in *B. subtilis* are mainly involved in coping with stress but are also engaged in other cellular processes. Orthologs of SigB regulon candidates with SigB PBMs were identified in other Bacillales genomes, but not all showed a SigB PBM. Additionally, genes involved in the integration of stress signals to activate SigB were predicted in these genomes, indicating that SigB signaling and regulon genes are species-specific.

2.1 Introduction

The general stress response (GSR) in bacteria constitutes a vital trait for cells to adapt to and survive conditions such as temperature change, nutrient depletion, or result of the exposure to reactive oxygen species in natural niches.

The GSR in *Bacillus subtilis* and many Bacillales members is under the transcriptional control of the alternative sigma factor B (σ^B or SigB) (Moran et al., 1981). Different environmental or nutritional signals, such as heat, ethanol, salt, and glucose starvation, can induce the SigB-mediated GSR, resulting in the expression of SigB-dependent genes and the production of proteins to protect cells from injuries (Petersohn et al., 1999a, 2001; Price et al., 2001a). The production of these proteins provides general protection to the cells and confers resistance to multiple stresses, allowing for rapid adaption to changing environments, thereby enhancing the survival of vegetative cells in extreme habitats (Nannapaneni et al., 2012).

The genes/proteins with SigB-dependent expression are defined as the SigB regulon members. Those that SigB directly regulates contain a promoter binding motif (PBM) consensus sequence GTTTAA-$N_{15\ (\pm\ 2\ bp)}$–GGGTAT (Sierro et al., 2008; Coelho et al., 2018; Vohradsky et al., 2021). Those that SigB indirectly controls do not have a SigB PBM and can either be controlled via SigB-dependent genes/proteins or regulated by other transcriptional regulators (Vohradsky et al., 2021). To date, 224 genes have been listed as members of the SigB regulon on Subtiwiki for *B. subtilis* (Petersohn et al., 1999b, 1999a, 2001; Nannapaneni et al., 2012; Waters et al., 2014; Arrieta-Ortiz et al., 2015; Schumann, 2016; Zhu and Stülke, 2018). A recent study by Vohradsky et al. (2021) further expanded the number to 411, with around 30 % of the SigB regulon genes reported to lack a SigB PBM and around 60 - 95% predicted to contain only a partial SigB PBM (i.e., only the -35 or the -10 promoter region, upstream of the AUG start codon).

Over time, more and more SigB regulon genes have been identified. This indicates that SigB regulatory networks are sophisticated and heavily interlinked with various other cellular mechanisms that are regulated by other transcriptional regulators. For example, SigB is indirectly involved in cellular responses such as sporulation and biofilm formation in *B. subtilis* (Reder et al., 2012; Bartolini et al., 2018). SigB-dependent genes may be expressed selectively to serve particular functions under certain conditions, e.g., 36 % of the 411 experimentally confirmed and predicted genes are expressed during spore germination and outgrowth in *B. subtilis* (Vohradsky et al., 2021), and roughly half are needed to cope with physical stresses such as ethanol, butanol, salt, high/low temperature (Nannapaneni et al., 2012). In *B. subtilis,* distinct sets of SigB-dependent genes are expressed under different environmental conditions in nature. This may also hold true for other *Bacillus* species like *Bacillus cereus* and *Bacillus licheniformis*, or other Bacillales members containing SigB. In members of the *B. cereus sensu*

lato group – which contain a common set of genes - it was found that the presence of varying SigB promoters gave rise to a unique SigB regulon structure per species, influencing pathogenesis via different strategies (Scott and Dyer, 2012). It is anticipated that Bacillales members that inhabit different niches have SigB regulon structures that are different from *B. subtilis* and may have developed to be mediated by different SigB activation routes.

Currently, four SigB activation routes are known for Bacillales members; namely, I) the stressosome RsbRST (Rsb= Regulator of SigB); II) the bipartite RsbQP, III) the two-component RsbKY system, and IV) direct activation (here refer to as Rsb-independent), as reviewed in Pané-Farré et al. (2017) and Rodriguez Ayala et al. (2020). The stressosome signaling complex is formed by RsbR and its paralogs (RsbRB, RsbRC, RsbRD, and YtvA), RsbS serine phosphatase, and RsbT serine kinase (Kim et al., 2004; Marles-Wright and Lewis, 2008; Pané-Farré et al., 2017). The stressosome becomes phosphorylated upon exposure to environmental stressors, and RsbT is then released to dephosphorylate the RsbU phosphatase, leading to further dephosphorylation of the RsbV anti-sigma factor antagonist (Yang et al., 1996). The dephosphorylated RsbV uncouples the binding between the anti-sigma factor RsbW and SigB, promoting the transcriptional activation of SigB and subsequently the expression of SigB regulon genes (Benson and Haldenwang, 1993; Alper et al., 1996; Kim et al., 2004). Activation of SigB via RsbQP involves the signal transfer from the α- hydrolase activator RsbQ to the RsbP phosphatase under nutritional stress (e.g., decrease in ATP, glucose starvation). RsbP then dephosphorylates RsbV, resulting in the same sequential SigB activation as activation via the stressosome (Kaneko et al., 2005; Nadezhdin et al., 2011). Thirdly, the RsbKY two-component system includes the histidine sensor kinase RsbK and its cognate response regulator, RsbY (de Been et al., 2010). By default, the methyltransferase (RsbM) methylates RsbK and negatively regulates SigB. Upon exposure to environmental/nutritional stressors, RsbK autophosphorylates and activates the RsbY phosphatase (Chen et al., 2012, 2015), and subsequent SigB activation takes place in the same way as for the stressosome and the RsbQP module (de Been et al., 2010; Chen et al., 2015, 2017). Lastly, in low-temperature or nitrosative stress adaptation, SigB is activated independently from its regulators, RsbU, RsbP, and RsbV (Brigulla et al., 2003; Tran et al., 2019).

To better understand the SigB regulon structures and functions in *B. subtilis*, this study employed *B. subtilis* 168 as a model, and novel SigB direct regulon members were identified using a newly derived SigB PBM. The functionality of several predicted PBMs was verified using translational fusions to a reporter in a wild type (wt) and Δ*sigB* background. The SigB regulons in 18 different *B. subtilis* strains and 106 Bacillales genomes were also assessed and a Bacillales SigB PBM consensus sequence was obtained. Lastly, the absence and presence of the four SigB activation routes in the 106 Bacillales genomes were predicted.

2.2 Materials and Methods

2.2.1 Sigma B (SigB) promoter binding motif (PBM) reconstruction

To identify novel SigB direct regulon members in *B. subtilis* 168, the SigB PBM was reconstructed as described by Wels et al. (2006, 2011) with slight modifications. In short, the respective operons of SigB regulon genes known to date in *B. subtilis* 168 were grouped according to their predicted operons (Supplementary Table S1). In total, 224 genes belonging to the SigB regulon were obtained for *B. subtilis* 168 on Subtiwiki (Zhu and Stülke, 2018), resulting in a compiled list with a total of 222 genes after removing duplicates. The operon structures of these genes were subsequently assessed, and genes were allocated to the same operon when: 1) adjacent genes were on the same coding strand, 2) the intergenic region between adjacent genes was <50 bp, and 3) no terminator was found between adjacent genes using Transterm (a tool to predict Rho-independent terminator) (Jacobs et al., 2006). Regions 300 bp upstream of each operon and the full intergenic region (if < 300 bp) were then inspected to identify the SigB PBM, and used to derive a standard SigB PBM in MEME Suite (Bailey et al., 2009, 2015). MEME was run using standard settings, with the following exceptions; -mod zoops (zero or one occurrence per sequence), –minw 10 (minimum width of 10), -maxw 50 (maximum width of 50), -dna (DNA molecule). The derived SigB PBM was used to repeat a search on the genome of *B. subtilis* 168 to obtain a new list of positive hit genes with a putative SigB PBM using the MAST search option in the MEME suite. The respective operons for these positive hit genes were predicted as aforementioned, and then a refined SigB PBM was built with MEME Suite. This refined SigB PBM with increased plasticity was used to search the genome of the 168 strain repeatedly, each with a different promoter spacer length, from N_{12} to N_{17}. Promoter space length was increased/decreased by deleting or copying the least informative position in the position-specific scoring matrix (PSSM). The p-value indicating the confidence of predicted PBMs was set at 10^{-5} (illustrated in Figure 1). Each promoter hit sequence was manually curated (Supplementary Table S2). Different spacers were screened in this study as Vohradky et al. (2021) indicated that genes controlled by SigB must contain both -35 and -10 binding motifs in the SigB PBM, and have a spacer length of 15 to 17 bp. Additionally, the similarity of the SigB PBM to the SigB consensus (indicated by the p-value) also took the nucleotide composition of spacers into account in this study. This is because the spacer compositions influence the promoter binding strength and gene expression, and may promote co-recognition by different transcriptional regulators (Liebeton et al., 2014; Gaballa et al., 2018; Han et al., 2019).

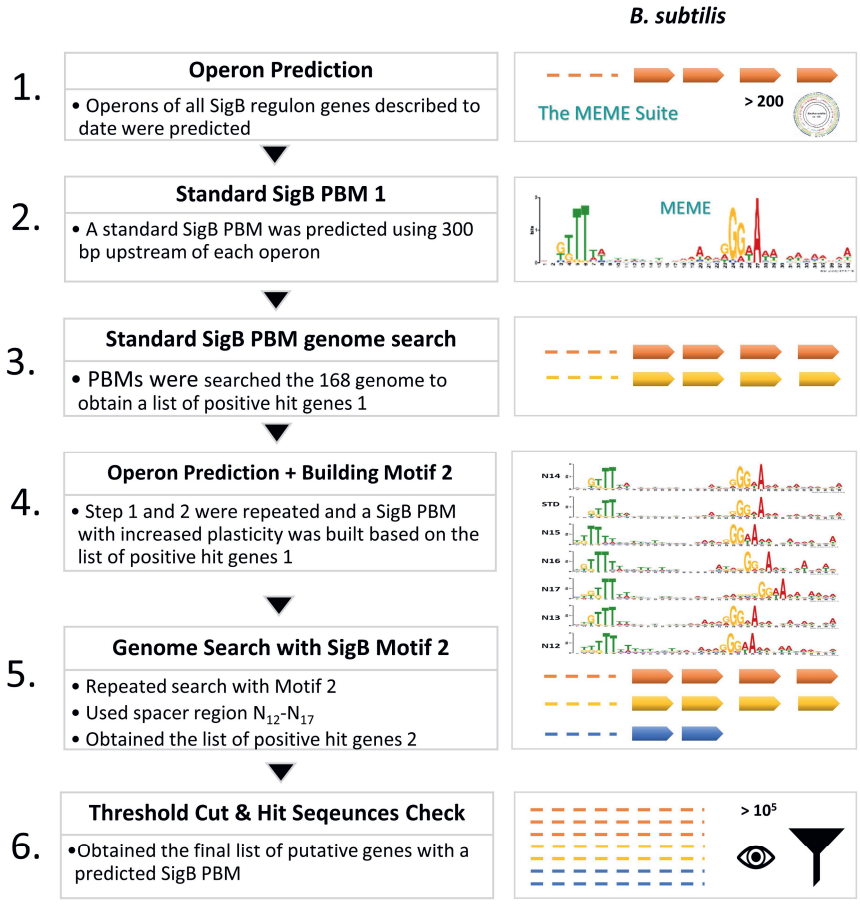


Figure 1- Flowchart of SigB promoter binding motif (PBM) reconstruction for *Bacillus subtilis*. All SigB PBMs were predicted using the MEME Suite version 5.0.5 (Bailey et al., 2009, 2015), and MAST was used to screen the genome for a potential SigB PBM, as described by Wels et al. (2006, 2011). The letter N indicates the number of base pairs in the spacer region. STD stands for the standard motif, built based on all the listed SigB regulon genes on Subtiwiki up to October 2020 (Zhu and Stülke, 2018). The p-value cut off threshold, indicating the confidence level of the predicted motif, was set as 10^5. All final sequences predicted for *B. subtilis* 168 were manually curated and listed in Supplementary Table S2.

2.2.2 Bacteria culture conditions, media, chemicals, and DNA manipulation

All strains of *B. subtilis* and *Escherichia coli* used in this study were cultured in Luria Bertani (LB) medium (Tritium Microbiologie, Eindhoven, The Netherlands) and propagated on LB agar plates unless stated otherwise. All incubations were performed at 37°C, and all liquid cultures were incubated using shaking at 220 rpm. For standard DNA cloning, plasmids were prepared and isolated from TOP10 *E. coli* cells (Thermo Fischer Scientific, Bleiswijk, The Netherlands). Chemically competent *E. coli* cells were transformed via heat shock (Froger and Hall, 2007). *B. subtilis* cells were transformed via natural competence in 1X MC competence medium

(containing 200 µl of 10X MC plus 6.7 µl of 1M $MgSO_4$, 10 µl of 1% tryptophan, and 1.8 ml of sterile water). A stock solution of 10X MC was prepared with 14.036 g $K_2HPO_4.3H_2O$, 5.239 g KH_2PO_4, 20 g glucose, 10 ml of 300 mM $Na_3C_6H_5O_7$, 1 ml of $C_6H_8FeNO_7$, 1 g of casein hydrolysate, and 2 g of $KC_5H_8NO_4$ to a total volume of 100 ml H_2O. The amylase activity of *B. subtilis* transformants was tested on 1% starch plates and stained with iodine. For the β- galactosidase assay, *B. subtilis* was cultured in a C-minimal medium supplemented with 1 g/L glucose and 8 g/L potassium glutamate (CE) as described by Commichau et al. (2008). Oligonucleotides and KOD hot-start DNA polymerase were purchased from Merck (Zwijndrecht, The Netherlands). *B. subtilis* chromosomal DNA was isolated using the GenElute Bacterial Genomic DNA Kit (Merck). Plasmids were isolated using the GeneJET Plasmid Miniprep Kit (Thermo Fischer Scientific). PCR products were purified using the PCR Purification Kit (Qiagen, Hilden, Germany). All FastDigest restriction enzymes and T4 DNA ligase were purchased (Thermo Fischer Scientific) and used according to the manufacturer's instructions. Bacterial culturing media were purchased (Tritium Microbiologie). DNA sequencing was performed by BaseClear B.V. (Leiden, The Netherlands).

2.2.3 Plasmids and reporter strains construction

The plasmids and strains constructed and used in this study are listed in Supplementary Table S3. First, the *B. subtilis* Δ*sigB* mutant was constructed using the long flanking homology recombination method described in Kunst and Rapoport (1995) with the selective marker chloramphenicol amplified from pNZ5319 (Lambert et al., 2007). Next, the *cre*-recombinase plasmid pDR244 (purchased from the *Bacillus* Genetic Stock Centre, Columbus, USA) was used to excise the chloramphenicol cassette at the *sigB* locus (Koo et al., 2017) to obtain a Δ*sigB* clean deletion mutant (BY47).

Plasmids pCY22, pCY23, pCY26, pCY27, pCY31, pCY32, and pCY33 were constructed, containing translational reporter fusions of P_{yaaI}, P_{ywzA}, P_{pucI}, P_{gtaB}, P_{ylaL}, P_{ykaA}, and P_{ygaO}, respectively (~ 150 bp upstream fragment), to *lacZ*. These SigB PBMs were selected as representative sequences as predicted in section 2.2.1 and were grouped into five categories irrespective of the spacer lengths: Category I (P_{rsbV}): exact match at -35 and -10 regions; Category II (P_{ytoQ}, P_{pucI}): exact match either at -35 or -10 region; Category III (P_{ylaL}, P_{ygaO}, P_{ykaA}): conserved GTTT at -35 and NGG at -10 region; Category IV (P_{yaaI}): less conserved motif with high p-values; and Category V (P_{gtaB}): with a duplicated -35 or -10 region. The predicted SigB PBM (~ 150 bp in length) upstream of each mentioned gene, including the native ribosomal binding site, was amplified by Polymerase Chain Reaction (PCR) using the corresponding oligonucleotides as listed in Supplementary Table S4. Each amplified fragment was ligated into either pDG1728 (purchased from BGSC) or pAC7 *amyE* integration plasmid (courtesy of Dr. J. Stülke, University of Goettingen, Germany) with the restriction enzymes *EcoRI* and *BamHI* (Weinrauch et al., 1991). Additionally, the plasmid pBP638 (courtesy of Dr. F. Commichau,

Cottbus-Senftenberg University, Germany) was used to study the activity of P_{ytoQ} and the plasmid pNW2205 (P_{rsbV}-*lacZ*) (courtesy of Dr. N. Stanley Wall, University of Dundee, UK) was used as a positive control in the β-galactosidase-assay.

The constructed plasmids were transferred into chemically competent TOP10 *E. coli* cells using heat shock, and transformants were selected on LB agar supplemented with 100 µg/ml carbenicillin. Plasmids were isolated from the positive *E. coli* colonies, sequenced, and subsequently introduced into *B. subtilis* 168 wt and Δ*sigB* mutant (BY47) cells via natural competence. *B. subtilis* transformants were selected on Brain Heart Infusion (BHI) agar supplemented with antibiotics (either 250 µg/ml spectinomycin or 10 µg/ml kanamycin). Activities of the promoters in response to stresses known to trigger SigB activation were investigated.

2.2.4 β-galactosidase reporter assay

The activity of a promoter *in vivo* was determined using the β-galactosidase assay. *B. subtilis* strains carrying promoter-*lacZ* reporter fusions were grown in C-Glc medium (Commichau et al., 2008), supplemented with 250 µg/ml spectinomycin or 10 µg/ml kanamycin. Overnight cultures in C-Glc were used to inoculate fresh C-Glc medium to an optical density of 0.05 at 600 nm (OD_{600}), and allowed to grow to OD_{600} ~ 0.35. Cells were then divided into different portions and subjected to either ethanol stress (4% v/v), NaCl (6% v/v), or heat (upshift from 37°C to 48°C) for 10 min. Cell pellets were collected before and after stress treatment and stored at -20°C. Quantitative studies of *lacZ* expression in *B. subtilis* were performed as described previously by Stannek et al. (2015). Briefly, cells were thawed and lysed with 400 µl Z-buffer (0.48 mM Na_2HPO_4. $2H_2O$; 0.32 mM NaH_2PO_4; 0.08 mM KCl; 8 µM $MgSO_4$; 0.4 mM ß-mercaptoethanol; 200 µg lysozyme and 200 µg DNAse I) for 1 hour (h) at 37°C. Lyzed cultures were centrifuged at 17,000 g to remove cell debris, and 100 µl of cell-free crude extract per sample was transferred into a new Eppendorf tube. 700 µl of Z-buffer without ß- mercaptoethanol was mixed with the 100 µl of crude extracts and incubated for 5 min at 28°C. 800 µl of Z-buffer without ß-mercaptoethanol was used as a reference. Subsequently, 200 µl of 4 mg/ml of ortho-nitrophenyl-β-galactoside (ONPG) was added to all the crude extracts and the reference and allowed to react at 28°C. When the cell extract turned visibly yellow, 500 µl of 62.5 mM Na_2CO_3 was added to stop the reaction. The absorption of samples at λ = 420 nm (absorption of O-nitrophenol) was measured. The protein concentration was determined via the Bradford assay (Bradford, 1976; Kruger, 2009) at λ = 595 nm using the commercial Roti®-Quant Bradford solution (Carl Roth, Karlsruhe, Germany) according to the manufacturer's protocol. The absorbance at A_{595nm} and A_{420nm} is corrected with the blank without cells. The specific ß- galactosidase activity (indicating the SigB activity) in Miller Units (MU)/mg protein was calculated using the formula:

$$\frac{\text{Units}}{\text{mg protein}} = \frac{2005.3475 \text{ x } A420nm}{\Delta T \text{ x } V \text{ x } A595}$$

One unit of β – galactosidase = the amount of enzyme produced to hydrolyze the chromogenic substrates ONPG to one nmol of o-nitrophenol (absorbs light at λ = 420nm) per minute at 28°C. V= 0.1 ml.

2.2.5 Bacillales core genome phylogenetic tree reconstruction, species-specific SigB PBM, and regulon structure prediction

To better understand the SigB regulon structure and function in *B. subtilis* and Bacillales members, a core genome phylogenetic tree of 18 *B. subtilis* strains (other than strain 168) and 106 Bacillales genomes was reconstructed (Supplementary Table S5). Species-specific SigB PBMs and SigB regulons were predicted and a Bacillales SigB consensus was derived as described below.

2.2.5.1 *B. subtilis* wild isolate strains and Bacillales members selection

The Bacillales members were selected when SigB operon genes have been described (Pané-Farré et al., 2017) and when they have been characterized for other properties (not necessarily related to SigB), such as producing high heat-resistance spores (Berendsen et al., 2016) or acid-tolerance (Patel et al., 2006). The genomes of strains analyzed in this study included strains of *B. subtilis* of different origins (18 plus reference strain 168), *Bacillus amyloliquefaciens* (4), *Bacillus vallismortis* (4), *Bacillus licheniformis* (11), *Bacillus velenzensis* (1), *Bacillus cereus* (33), *Bacillus coagulans* (5), *Bacillus thermoamylovorans* (5), *Bacillus pumilus* (6), *Anoxybacillus* (6), *Bacillus sporothermodurans* (1), *Geobacillus* (14), *Parageobacillus* (7), *Caldibacillus* (2), *Paenibacillus* (2), *Listeria* (3) and *Staphylococcus* (3) which were extracted from the NCBI bacterial genome database, available at (https://www.ncbi.nlm.nih.gov/genome/microbes/).

2.2.5.2 Core phylogenetic tree reconstruction and absence/presence of *B. subtilis* SigB regulon members in other Bacillales members

Orthologous groups were constructed using OrthAgogue (PMID24115168) (Ekseth et al., 2014). Orthologous protein sequences with exactly one copy in all 125 Bacillales genomes were aligned with Muscle (Edgar, R.C. Nucleic Acids Res 32(5), 1792-97) and a core genome phylogenetic tree was constructed from the concatenated alignments via PhyML (Guindon and Gascuel, 2003; Guindon et al., 2010). Subsequently, all genes belonging to the SigB regulon in *B. subtilis* 168 (Zhu and Stülke, 2018) were selected as genes of interest (GOI) (Supplementary Table S1). The absence or presence of these GOI in other 124 genome sequences was predicted via genome mining (Supplementary Table S5). Locus tags or gene symbols were used to identify the corresponding orthologous group of each GOI. Then, all GOI were clustered using the 1-(Spearman rank correlation of the gene copy number in each genome) as a distance measure, and neighbor-joining as the clustering algorithm (Phylip

package, (http://evolution.genetics.washington.edu/phylip.html). Lastly, the absence or presence of gene functions in a genome was predicted based on the absence or presence of one or more orthologs in that genome. The phylogenetic tree ("MLST trees") heat map was visualized using iTOL (PMID27095192) (Letunic and Bork, 2016).

2.2.5.3 Species-specific SigB PBMs and SigB regulon structures prediction for other Bacillales genomes

The predicted orthologs of the *B. subtilis* 168 SigB regulon members in 18 other *B. subtilis* strains and 106 Bacillales genomes (identified as described in section 2.2.5.2) were established and used to derive a species-specific SigB PBM via procedures reported in section 2.1.1 (for an example illustrated for constructing the *B. cereus* SigB PBM see Figure 2, step ii and iii). Subsequently, the species-specific SigB PBM was employed to screen for genes with potential SigB PBMs in the respective species. First, operons for the new positive hit genes were predicted, and the promoter regions of these genes were used to build the species-specific SigB PBM 2 (step v, Figure 2). Then, the genome of each species was screened repeatedly with the improved species-specific SigB PBMs with different promoter spacer lengths (N_{12} to N_{17}) as described in section 2.2.2.1, respectively. The screening thus resulted in a list of genes with putative SigB PBMs per species, forming the predicted SigB regulon for each inspected Bacillales genome. The overall presence/absence of the predicted species-specific SigB regulon members was cross-checked between all 125 genomes that were analyzed (Supplementary Table S6). The genome tree heat map for 125 Bacillales members (including *B. subtilis* 168) was generated using GENESIS 1.7.7 (Sturn et al., 2003) (Supplementary Figure S1).

2.2.6 Bacillales Sigma B (SigB) sensing modules prediction

To evaluate the ability of various Bacillales to employ different SigB signaling modules, the occurrence of the three known SigB sensing modules (RsbRST, RsbQP, and RsbKY) was evaluated in 125 Bacillales genomes as described in section 2.2.5.2. Briefly, genes encoding the proteins involved in these three known SigB signal transduction pathways for Bacillales (Petersohn et al., 1999a, 1999b, 2001; Price et al., 2001b; Schaik et al., 2007; de Been et al., 2010, 2011; Nannapaneni et al., 2012; Scott and Dyer, 2012; Waters et al., 2014; Schumann, 2016; Pané-Farré et al., 2017), and the SigB regulators *rsbX* and *rsbM* were set as the GOI. The absence/presence of these GOI in 125 genomes was predicted via genome mining to check for the absence or presence of an orthologous protein (section 2.2.5.2) (Supplementary Table S7) and a heat map was generated as mentioned in section 2.2.5.2 and visualized with iTOL.

Figure 2- Flowchart of the reconstruction of species-specific SigB promoter binding motif (PBM) for other species belonging to the order of Bacillales. All SigB PBMs were predicted using the MEME Suite version 5.0.5 (Bailey et al., 2009, 2015), and MAST was used to screen the genome for a potential SigB PBM, as described by Wels et al. (2006, 2011). The letter N indicates the number of base pairs in the spacer region. STD stands for the standard motif, built based on all the listed SigB regulon genes on Subtiwiki up to October 2020 (Zhu and Stülke, 2018). The Bacillales SigB promoter binding motif consensus was acquired from the predicted species-specific SigB PBM from the 125 Bacillales genomes, including the *B. subtilis* 168 strain.

2.3 Results and discussion

2.3.1 Two-step SigB promoter binding motif (PBM) derivation in *B. subtilis* 168

Based on the analysis of the *B. subtilis* genome 168 using the refined SigB PBM (see section 2.2.1), 255 genes (some belonging to the same operon) with a putative SigB PBM were predicted, indicating that they may be directly regulated by SigB (Supplementary Table S2). Of these, 99 overlap with SigB regulon members described in the literature: 74 were listed as SigB regulon members on Subtiwiki (Zhu and Stülke, 2018) (Figure 3), and an additional 25 were recently reported by Vohradsky et al. (2021). Thus, a total of 156 of the 255 genes with a predicted SigB PBM are novel putative SigB regulated genes that were not identified in earlier studies (Petersohn et al., 1999b, 1999a, 2001; Price et al., 2001a; Nannapaneni et al.,

2012; Waters et al., 2014; Arrieta-Ortiz et al., 2015; Schumann, 2016; Vohradsky et al., 2021). Each of the predicted sequences was manually checked to avoid possible false positives in this prediction (Supplementary Table S2).

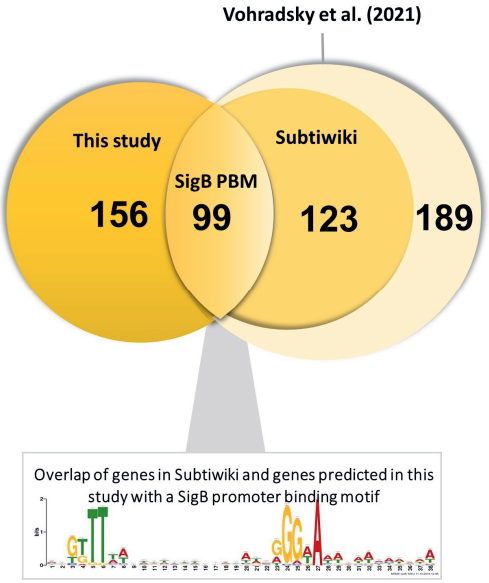


Figure 3- Novel SigB regulon genes detected for *Bacillus subtilis*. 255 SigB regulon genes with SigB PBMs were predicted, 99 overlapped with regulon genes already reported in the literature, and 156 were newly identified in this study.

The 255 genes predicted to have a SigB PBM in this study were grouped into five categories (Supplementary Table S2). **Category I (green)** contains 6 out of the 255 predicted genes with SigB PBMs that have an exact matching sequence to the previously reported SigB consensus motif (GTTTAA- $N_{15\ (\pm\ 2\ bp)}$ -GGGTAT) (Sierro et al., 2008; Coelho et al., 2018; Vohradsky et al., 2021), demonstrating that a very small number of the predicted genes have Category I SigB PBM. These are the well-known SigB-regulated genes, i.e., *sigB* itself, the anti-SigB antagonist *rsbV,* the serine protein kinase *rsbW,* the phosphoserine phosphatase *rsbX,* the general stress gene *ctc,* and the acetyltransferase *yjbC*. These genes with the category I SigB PBM and spacer length of N_{14} (except *yjbC* with N_{13}) have been shown to have extensive differential regulation under various conditions that lead to the induction of SigB (Petersohn et al., 1999b, 1999a, 2001; Price et al., 2001a).

Category II (**orange**) contains 27 out of 255 genes with a -35 binding motif that is identical to the consensus motif GTTTAA and only 1 to 2 base pairs variations in the -10 binding motif, or a -10 binding motif that is identical to the consensus motif GGGTAT and only 1 to 2 base pairs variations in the -35 binding motif. This category of SigB PBMs deviates the least from the SigB consensus and is likely easily recognized by SigB. Many general stress genes, such as the glucose starvation gene *gsiB* have the category II SigB PBMs (Supplementary Table S2). 11 newly identified genes in this category were not listed in Subtiwiki. Of these, *yjlB* was also

recently discovered as SigB dependent by Vohradsky et al. (2021), who identified SigB PBMs with -35 and -10 binding sites with spacers of 13-15 bp (which they referred to as Class I promoters). *pgcA, phoH,* and *yqhY* reported by Vohradsky et al. (2021) were not identified in our study, likely due to the use of different settings for the promoter searches. Moreover, the spacer length was up to 50 bp in the study of Vohradsky et al. (2021).

Most of the genes (137 out of 255) detected in this study contain **Category III** (**yellow**) SigB PBMs, which have the conserved GTTT bases at the -35 binding site and GG at the -10 binding site in the promoter region. Many of these SigB regulon genes (~ 50%) were identified in earlier studies (Petersohn et al., 1999b, 1999a, 2001; Price et al., 2001a; Nannapaneni et al., 2012), listed as SigB regulon genes in Subtiwiki, or reported by Vohradsky et al. (2021). These findings imply that SigB can recognize the binding motif well as long as the bases at the -35 and -10 binding sites are conserved, and the length of the spacer is between N_{12} and N_{17}.

Category IV (grey) contains 76 genes with low-level conserved SigB PBMs, which have p-values close to the cut-off threshold (section 2.2.1), likely indicating the presence of binding sequences for SigB and other transcriptional regulators. Several genes that are known to be coregulated by SigB and at least one other regulator were found in this group. These include, for instance, *secG* (preprotein translocase subunit), *yaaI* (general stress protein), and *plsC* (acylglycerolphosphate acyltransferase, biosynthesis of phospholipids). The *plsC* gene is known to be regulated by both SigB and FapR (a fatty acid synthetic gene repressor) to maintain membrane homeostasis (Albanesi and de Mendoza, 2016). Moreover, part of the SigB regulon overlaps with other regulons; it has been reported that SigB regulon members can be controlled by SigB and other regulators, of which 36 have been reported (SigB and/or different regulators at different cellular states) (Zhu and Stülke, 2018; Vohradsky et al., 2021). Therefore, genes that have less conserved SigB PBM or do not have a SigB PBM but show SigB dependency suggested that cells may fine-tune response to integrate multiple signals in various conditions.

Category V (blue) shows 9 out of 255 genes containing SigB PBM that deviated the most from the SigB consensus motif but were shortlisted likely due to the presence of either a duplicated -35 or -10 binding site or an occurrence of a single perfect -35 or -10 binding site (Supplementary Table S2). A previously described SigB regulon member, *gtaB* (Petersohn et al., 1999a, 2001), encoding the UTP-glucose-1-phosphate uridylyltransferase (a general stress protein), was found in this category. The duplicated GTTT region in the SigB PBM of *gtaB* might be the reason why the SigB PBM of *gtaB* had a low p-value (indicating higher similarity to the SigB consensus), suggesting that *gtaB* may be regulated by multiple regulators, with a more versatile promoter sequence. To confirm this hypothesis, the predicted *gtaB* SigB PBM sequence was submitted to the database of transcriptional regulation in *Bacillus subtilis* (DBTBS), and binding sequences of two other regulators SigA and DegU were found (Table 1).

In the study of Vohradsky et al. (2021), this gene was described to have a Class I promoter (i.e., contained both -35 and -10 binding sites), and the SigB PBM was indicated at 96 bp upstream of ATG. However, a manual check on the genome of strain 168 did not find a -35 binding site.

2.3.2 Experimental validation of predicted SigB PBMs

To further validate these *in silico* results, promoters of genes belonging to the five different assigned categories were selected, and their SigB-dependent activities were studied using a promoter-reporter approach in a wild type (wt) and Δ*sigB* background.

Eight predicted SigB PBMs (P_{ytoQ}, P_{pucI}, P_{ylaL}, P_{ygaO}, P_{ykaA}, P_{ywzA}, P_{yaaI}, P_{gtaB}) and the well-known P_{rsbV} were selected as representatives from different promoter categories (indicated in bold in Supplementary Table S2). The (putative) functions of these genes are presented in Table 1. SigB-dependent activities of translational promoter-*lacZ* fusions were determined in wt and *ΔsigB* mutants by measuring β- galactosidase activity upon temperature upshift from 37°C to 48°C, exposure to 4% (v/v) ethanol, and exposure to 6% (v/v) NaCl (in three independent experiments) (Figure 4).

2.3.2.1 Category I: P_{rsbV} as the positive control

The *rsbV* gene is a well-known SigB regulon gene. Thus, P_{rsbV}, which encompasses the sequence (GTTTAA-N_{14}-GGGTAT) that exactly matches the SigB PBM consensus, was used as the positive control and the Category I representative in the experimental validation. Exposure of wt cells containing P_{rsbV}-*lacZ* to heat, ethanol, and salt resulted in 8-fold, 15-fold, and 6-fold induction of β-galactosidase activity compared to unstressed cells, respectively (Figure 4A). In the Δ*sigB* mutant, no P_{rsbV}-dependent β-galactosidase activity was observed in either stressed or unstressed conditions, showing that the observed promoter activity could be attributed to SigB induction, as expected for this positive control.

2.3.2.2 Category II: P_{ytoQ} shows SigB-dependent induction under ethanol stress, P_{pucI} with longer spacer showed very mild SigB-dependent activity

Both *ytoQ* and *pucI* are newly identified putative SigB regulon genes in this study. P_{ytoQ} and P_{pucI}, with predicted SigB PBM of (GTTTAA-N_{14}-GGGTGA) and (GTTTAA-N_{17}-GGGAAA), respectively, were used as the Category II representatives. These sequences match the consensus -35 binding site and both contained three conserved GGG bases at the -10 binding site but have spacer lengths of 14 and 17 nucleotides, respectively.

Cells containing the P_{ytoQ}-*lacZ* (GTTTAA-N_{14}-GGGTGA) showed SigB-dependent LacZ induction only upon exposure to 4% (v/v) ethanol, but not upon temperature upshift from 37°C to 48°C nor upon 6% (v/v) NaCl shock (Figure 4B). This gene is likely also regulated by at least one other regulator because the baseline activity of P_{ytoQ} at T0 before stress exposure was already ~ 100 MU/mg protein. The average P_{ytoQ}-dependent β-galactosidase activity after

ethanol treatment was around 230 MU/mg protein for the wt carrying P_{ytoQ}-*lacZ*, which was about 130 MU/mg protein more than the activity at T0. The increase was lower (around 40 MU) in the Δ*sigB* mutant carrying P_{ytoQ}-*lacZ*. This difference between the wt and the Δ*sigB* mutant may imply that the product of the *ytoQ* gene is specific in response to stress caused by ethanol. Although the function of the *ytoQ* gene has not been fully elucidated, it was shown to be important under vitamin B6 starvation in *B. subtilis* (Rosenberg et al., 2018). Using the transcription regulator database DBTBS (Sierro et al., 2008), we found that the predicted SigB PBM for *ytoQ* also contained two alternative PBMs: the first was for *xre*, which is the repressor of a phage-like bacteriocin, and the second was for *codY,* the repressor involved in response to branched-chain amino acid limitation (see Table 1). These findings indicate that *ytoQ* may be regulated by SigB, CodY, and Xre under different conditions.

Table 1- Nine selected promoters with predicted SigB promoter binding motifs.

Locus Tag	Description[a]	Gene	Hit sequence	N	Other regulators[b]
BSU 04710	Anti-Sigma factor antagonist	*rsbV*	G**GTTTAA**CGTCTGTCAGACGA**GGGTAT**AAAGC AACTA	14	*ccpA, tnrA*
BSU 36470	Allantoin permease	*pucI*	TT**GTTTAA**GCTGTTCAAATACAAAC**GGGAAA**AT TGTA	17	*pucR,glnR, tnrA*
BSU 29850	Hypothetical protein	*ytoQ*	TC**GTTTAA**CATCGTGTACATGA**GGGTGA**AATT TACAG	14	*codY, xre*
BSU 38180	general stress protein	*ywzA*	T**GTTTAT**CTTATACAAAAAAG**AGGAAT**GATAT AAGCA	14	*codY, araR*
BSU 12850	unknown	*ykaA*	CCT**GTTTTT**TTTCGACAAAA**GGCTAT**ATAAT AATTT	12	*ctsR, rocR, sigA*
BSU 14820	unknown	*ylaL*	CGT**GTTTAT**CAGCTTTTGCCAAGGC**GGGAAT**AT AGCT	16	*sigG*
BSU 08890	unknown lipoprotein	*ygaO*	T**GTTTAT**TTATTTTTCGTCAA**GGGAAT**ATAAA AATTT	14	*sigH*
BSU 35670	Glucose uridylyltransferase	*gtaB*	A**GTTTTA**ATATTAAGGATAAA**GCTTGTTTAA**A AATGG	14	*degU*
BSU 00170	similar to isochorismatase	*yaaI*	**GTTTTT**TCATTGCCTAAAAA**GGCTAC**ATATTA ACTA	14	*codY*

[a] *description based on Subtiwiki* (Zhu and Stülke, 2018)
[b] *predicted regulators on the DBTBS database* (Sierro et al., 2008)

tnrA	Global nitrogen regulator. Negatively regulates glnR.
ccpA	Catabolite repressor or a positive regulator of genes involved in excretion of excess carbon
glnR	Repressor of glutamine synthesis
xre	Repressor of a phage-like bacteriocin, PBSX (phibacin damaged-prophage)
araR	Repressor of the L-arabinose metabolic operon
rocR	Activator of arginine utilization operons
ctsR	Repressor of class III heat shock genes
sigG	Transcription of sporulation genes
sigH	Transcription of early stationary phase genes (sporulation/competence). Not active in lab strains due to a mutation (V117A)
degU	Two-component response regulator, regulation of degradative enzyme and other adaptive responses
codY	Repressor in response to branched-chain amino acid limitation

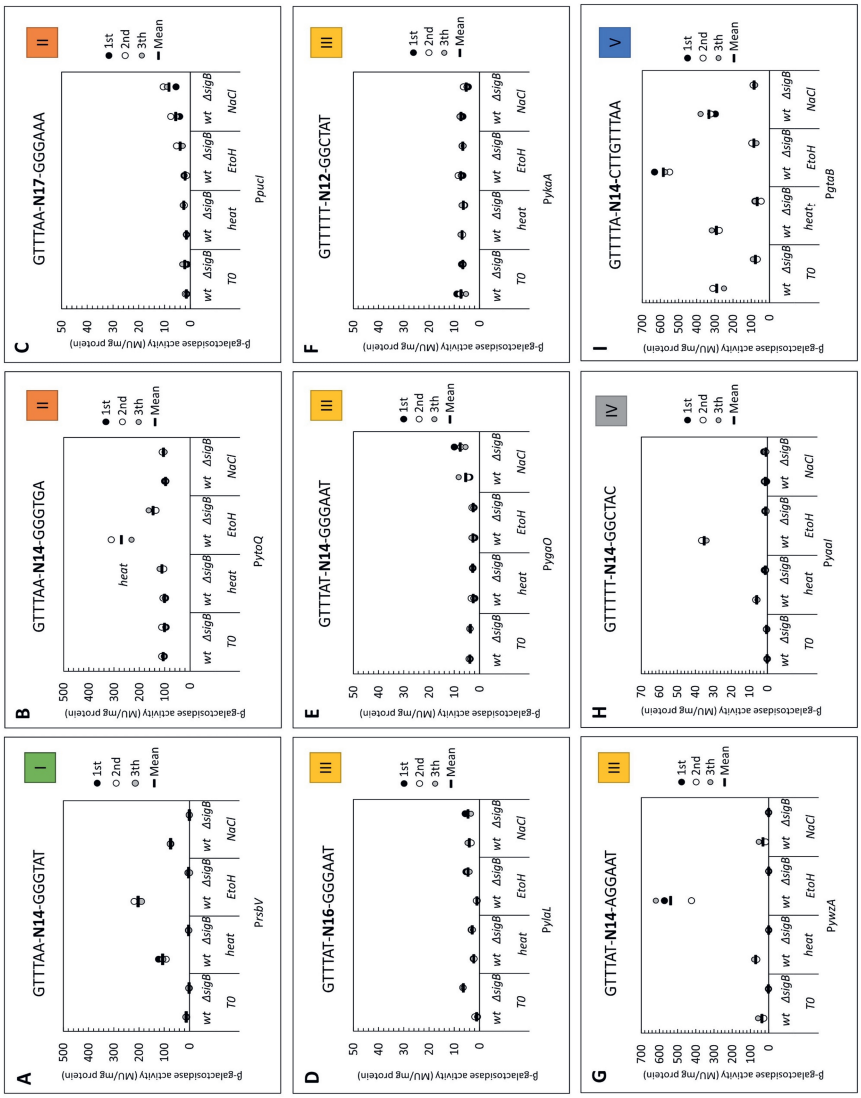


Figure 4- β-galactosidase activities of Category I-V predicted SigB promoters translationally fused to *lacZ* upon exposure to heat (37°C > 48°C), 4% (v/v) ethanol, and 6% (v/v) NaCl in wt cells and Δ*sigB* mutant in *B. subtilis*. Each data point represents a biologically independent replicate, and the bar indicates the average value for the three independent experiments. β-Galactosidase (LacZ) activities are presented in Miller units per milligram protein. The color-coded box at the right of each graph indicates the different categories to which the promoters belonged, based on the confidence level of the predicted PBM. Category I (green): the predicted PBM has the exact match at both -35 and -10 regions. Category II (orange): the predicted PBM has either an exact match at the -35 region, with 1- 2 bp variations at -10, or vice versa. Category III (yellow): the predicted PBM has conserved GTTT bases at the -35 region and the GG bases at the -10 region. Category IV (grey): low-level homology compared with the conserved motif with borderline p-values; and Category V (Blue): with a duplicated -35 or -10 region. **4A-** P_{rsbV}-*lacZ* activities; **4B-** P_{ytoQ} -*lacZ* activities; **4C-** P_{pucI} -*lacZ* activities; **4D-** P_{ylaL}-*lacZ* activities; **4E-** P_{ygaO}-*lacZ* activities; **4F-** P_{ykaA}-*lacZ* activities. 4G-P_{ywzA}-*lacZ* activities; **4H-** P_{yaaI} -*lacZ* activities; **4I-** P_{gtaB} -*lacZ* activities.

A mild yet notable SigB-dependent response was observed for *pucI* (encoding allantoin permease) upon exposure to ethanol and NaCl stresses (Figure 4C). After cells were exposed to 6% (v/v) NaCl, β- galactosidase activities of wt P_{pucI}-*lacZ* cultures increased around 4-fold compared to the control at T0. However, in the Δ*sigB* P_{pucI}-*lacZ* cultures, this increase was notably higher (~ 6-fold), suggesting that SigB may partially involve in the negative regulation of *pucI*. Upon exposure to ethanol stress, β- galactosidase activities of wt P_{pucI}-*lacZ* cultures did not change compared to the activity at T0, but the Δ*sigB* P_{pucI}-*lacZ* mutant showed ~ 2- fold higher β-galactosidase activities than the wt P_{pucI}-*lacZ* (see Figure 4C). This result indicated that the mild increase in β-galactosidase activities observed for the Δ*sigB* P_{pucI}-*lacZ* mutant did not result from the exposure to ethanol but the deletion of the *sigB* gene.

The promoter of *pucI* was induced by NaCl in wt and further induced in the Δ*sigB* mutant, implying that *pucI* may be co-regulated by other regulators/sigma factors, as found in Table 1. *pucI* may also have roles in other stress conditions triggered by other undiscovered stressors, or stressors other than heat, ethanol, and salt. It is noteworthy that the predicted SigB PBM of P_{pucI} has an extended spacer (17 nucleotides) between the -35 and -10 binding motifs compared with the consensus spacer. This longer spacer region may affect the promoter strength by influencing the binding of the RNA polymerase, thereby affecting transcription. In transcription initiation, the bacterial RNA polymerase first locates the promoter, and its largest subunit (β-zipper) will interact with the spacer between the -35 and -10 elements to form a holoenzyme complex (Yuzenkova et al., 2011; Lee and Borukhov, 2016).

2.3.2.3 Category III: P_{ylaL} with longer spacer showed very mild SigB-dependent activity, P_{ygaO} and P_{ykaA} did not show evident changes under tested conditions and P_{ywzA} showed ethanol-specific SigB-dependent induction

For category III, P_{ylaL} (GTTTAT-N_{16}-GGGAAT), P_{ygaO} (GTTTAT-N_{14}-GGGAAT), P_{ykaA} (GTTTTT-N_{12}-GGCTAT), and P_{ywzA} (GTTTAT-N_{14}-AGGAAT) were selected based on the conserved GTTT at the -35 binding site and the GG at the -10 binding sites and different spacer lengths. *ylaL, ygaO,* and *ykaA* are newly identified in this study as potential SigB regulon candidates, in which *ywzA* is a known SigB regulon gene, used as a control of Category III.

Mild β-galactosidase activities were observed for P_{ylaL} but were unrelated to the stressors (Figure 4D). In the wt P_{ylaL}-*lacZ* culture at T0 (before the exposure to heat, ethanol, or salt), no β-galactosidase activity was measured, whereas the Δ*sigB* P_{ylaL}-lacZ culture showed higher levels. The same results were observed in wt and the Δ*sigB* mutant after exposure to all three stressors, indicating that SigB may negatively affect *ylaL*. Based on the NCBI BlastP results, YlaL is 99.9% similar to the peptidyl-prolyl cis-trans isomerase and located next to the spore germination gene *ylaJ*. The predicted SigB PBM sequence for *ylaL* was also found to have a positive hit to the SigG binding motif with the consensus TGCATAT-N16-GATACTTA (DBTBS) (see Table 1), implying that YlaL may be coregulated by SigB and SigG, and may have a role

in sporulation. The role of isomerase in sporulation was described in *B. subtilis* subs. *spizizenii* (Berendsen et al., 2016), and the involvement of SigB in sporulation control was also reported before. SigB is known to induce the expression of *spo0E,* a suppressor of *spo0A* and *spoIIE* genes required for sporulation initiation (Reder et al., 2012; Rothstein et al., 2017). Similarly, as indicated above for P_{pucI}, the P_{ylaL} activities were relatively low, which may be attributed to the long spacer length of 16 nucleotides. Further experiments are thus needed to elucidate the potential regulation of PucI and YlaL by SigB.

P_{ygaO} and P_{ykaA} did not show evident changes in transcriptional activation in the three tested conditions despite having a relatively conserved SigB PBM (Figures 4E and 4F). Baseline β- galactosidase activities for both promoters were seen before stress imposition at T0, and the P_{ygaO} and P_{ykaA} activities in Δ*sigB* mutant did not differ from the wt upon exposure to heat, ethanol, or salt stress. Results suggested that *ygaO* and *ykaA* are likely controlled by regulators other than *sigB* (Table 1). Nonetheless, as only the three most commonly used SigB stressors were tested in this study, P_{ygaO} and P_{ywzA} may respond to other environmental or nutritional stressors.

In addition, the promoter activities of *ywzA* (a known SigB regulon gene predicted with a Category III SigB PBM) were also verified via experiments. The β-galactosidase activity in wt P_{ywzA}-*lacZ* cultures increased 2-fold after heat treatment compared to cultures without treatment, but the induction after ethanol treatment stood out (20-fold higher) (Figure 4G). No β- galactosidase activities were seen in the Δ*sigB* P_{ywzA}-*lacZ* cultures upon heat and ethanol stress, indicating that SigB was responsible for the increased expression in the wt cultures. Exposure to osmotic stress did not lead to changes in β- galactosidase activities in wt and Δ*sigB* P_{ywzA}-*lacZ* cultures. As the deletion of *sigB* abolished the activity of P_{ywzA} completely, *ywzA* is likely not co-regulated by other regulators under the conditions tested. However, alternative binding sites for *codY* (repressor in response to branched-chain amino acid limitation) and *araR* (repressor of the L-arabinose metabolic operon) were found for the predicted SigB PBM of *ywzA* (Table 1).

Of the newly predicted SigB regulon genes described so far (*ytoQ, pucI, ylaL, ygaO,* and *ykaA*), none of these five were identified as SigB regulated in previous studies, either via transposon mutagenesis (Boylan et al., 1991, 1992, 1993), gel-based proteomics (Antelmann et al., 2000; Brigulla et al., 2003; Höper et al., 2006; Wolff et al., 2006; Hahne et al., 2010), consensus promoter search (Petersohn et al., 1999b), transcriptional profiling (Petersohn et al., 1999a, 2001), the combination of microarray and machine learning algorithm in defining the SigB regulon structure (Nannapaneni et al., 2012) or SigB modeling (Vohradsky et al., 2021). Our data show that three out of the five have SigB-dependent promoter activity, indicating that these genes might have been overlooked in earlier studies. The predicted P_{ygaO} and P_{ykaA} SigB

PBM with high confidence did not show an apparent SigB-dependent activation, suggesting that they might be induced by other stressors than heat, ethanol, or NaCl or that the control by SigB is affected by the consensus and the spacer of the promoter.

2.3.3.4 Category IV and V: P_{yaaI} and P_{gtaB} showed SigB-dependent activities despite deviating considerably from the consensus

Lastly, P_{yaaI} (GTTTTT-N_{14}-GGCTAC) and P_{gtaB} (GTTTTA-N_{14}-CTTGTTTAA) were included as a representative from category IV and category V, respectively. Both *yaaI* and *gtaB* are known SigB regulon genes but were selected for verification in this study because the predicted SigB PBMs deviate considerably from the original SigB consensus (see section 2.2.3).

The P_{yaaI}-LacZ activity in wt was induced the most upon exposure to ethanol stress, with a 35-fold increase, and heat stress resulted in a 4-fold increase compared with untreated wt P_{yaaI}-*lacZ* cultures, whereas no difference of wt P_{yaaI}-LacZ activity was observed under salt stress (Figure 4H). This result suggested that the general stress gene *yaaI* plays a vital role in protecting cells from damage caused by ethanol. The deletion of the *sigB* gene diminished the activity of P_{yaaI} under all three tested conditions, suggesting that the expression of *yaaI* may be solely-dependent on SigB. However, an alternative binding site for *codY* was also found for the predicted SigB PBM of *yaaI* (Table 1), but the interaction of SigB, CodY, and YaaI is yet to be explored.

The β-galactosidase activities of P_{gtaB} were also investigated in wt and Δ*sigB* mutant (Figure 4I). Petersohn et al. (2001) reported that the putative SigB PBM for *gtaB* is located inside the gene coding region, but this study identified that the SigB PBM for *gtaB* is located upstream of the AUG start codon, containing the sequence GTTTTA-N_{14}-GCTTGTTTAA. This SigB PBM met the selection criteria (section 2.2.1) only because of the duplicated GTTT sequence at both -35 and -10 binding sites, and it was chosen as a target to verify if SigB could bind to this predicted SigB PBM.

Despite the poor binding motif, the wt P_{gtaB}-*lacZ* culture displayed SigB-dependent induced β- galactosidase activity upon exposure to ethanol and salt stress (Figure 4I). At T0 before stress exposure, the baseline P_{gtaB} activity in wt cells was already around 290 MU/mg protein, indicating that other transcriptional regulators may co-regulate this gene. A promoter sequence search using the DBTBS database revealed binding sites for two other alternative regulators, SigA and DegU (Table 1). Despite the high baseline activity, an increase in P_{gtaB}-dependent β-galactosidase activity was seen after ethanol (584 MU) and salt shock (333 MU), but no significant increase was observed upon heat treatment. Notably, the P_{gtaB}-*lacZ* activity in the Δ*sigB* mutant at T0 was also ~3-fold lower than in the wt, indicating that SigB might play a role in regulating *gtaB* even under the control (presumably unstressed) condition. No induction was seen in the Δ*sigB* P_{gtaB}-*lacZ* mutant in response to ethanol, salt, and heat stress.

These results align with available transcriptomics data for *B. subtilis* 168 wt and Δ*sigB* mutant, showing more profound expression of *gtaB* upon ethanol and salt shocks than upon heat shock (Petersohn et al., 2001). This example demonstrated that SigB recognizes the predicted binding sequence, at least weakly, despite the large deviation from the SigB consensus sequence (GGGTAT) at the -10 binding site.

The identification of putative SigB regulon members (see section 2.3.1), of which nine predicted SigB PBMs of Category I-V were validated in section 2.3.2, suggests that the SigB regulon in *B. subtilis* 168 may be even more extensive than currently thought. The number of theoretical SigB regulon genes was recently estimated to be 411 (Vohradsky et al., 2021), and taken together with the predicted genes in this study, the total number may exceed 500 (Figure 3). This large number of SigB regulon genes aligned with the notion that many SigB-regulated genes are also co-regulated by other transcriptional regulators, interlinking SigB regulation with other cellular processes (Vohradsky et al., 2021). Category III representatives that did not show SigB dependence in this study may respond to other so far unknown stressors or their promoter activities may be affected by the spacer length and/or compositions of the promoter, which requires further confirmation.

2.3.3 Functional distribution of known and predicted SigB regulon genes

The functions of the 156 predicted SigB regulon candidates in this study and all genes listed in Subtiwiki are presented in a functional distribution map (see Figure 5). The list of genes with known functions (data extracted from Subtiwiki) is presented in Supplementary Table S2b. The sunburst map illustrates genes with and without SigB PBM (with shaded regions indicating genes with a predicted SigB PBM in this study, section 2.3.1), and indicates that 30% of the genes encode for proteins involved in lifestyles (e.g., coping with stress, sporulation), 21% in information processing (e.g., protein synthesis and modification, transcription or translational regulation), 17% in metabolism regulation (e.g., biosynthesis of amino acid, lipids, utilization of carbon sources), 8% in cellular processing (e.g., transporter, exporter, homeostasis), 6 % in phage-related function, and 18% constitute proteins with unknown functions.

Many SigB regulon members involved in lifestyle management have generic functions in general stress protection. Some genes are likely regulated by SigB directly as SigB PBMs were predicted; some encode proteins with a role in resistance to toxins or antibiotics, and others are linked to sporulation. Among the ~21% of members involved in information processing, many are well-known SigB-dependent genes such as *ctc, rsbV, rsbW,* and *sigB* itself that are involved in the regulation of gene expression, and many play a role in protein synthesis, modification, and degradation as well as DNA repair and recombination. Interestingly, most genes involved in cellular processes encompassed a putative SigB PBM, such as ABC transporters, ions transporters, and amino acids transporters. Other metabolic genes

responsible for the biosynthesis of lipid synthesis, acquisition of amino acids, and utilization of different carbon sources also have predicted SigB PBMs (Figure 5). The remaining genes were either prophages and mobile genetic elements, or genes encoding membrane proteins with undefined functions. Further functional investigation of these genes may help to understand better their involvement in stress response regulation in *B. subtilis*.

This study identified an additional 156 SigB regulon candidates with a putative SigB PBM in *B. subtilis* based on a computational approach using a more plastic SigB PBM. Multiple factors may have limited the detection of such genes in previous studies: **1)** The use of a more restricted SigB PBM in earlier studies (Sierro et al., 2008; Coelho et al., 2018); **2)** Utilization of the same stressors in global SigB- mediated GSR studies**.** Ethanol, heat, and salt were used primarily because of their potent SigB triggering response. Although other investigators studied the induction of SigB by acid, cold, antibiotics, reduced ATP, GTP, low oxygen, glucose limitation, blue light, red light, carbonyl cyanide m-chlorophenylhydrazone (CCCP), butanol, pH, low pressure, high-level iron, and oxidative stress, global transcriptomic or proteomic analyses were not performed in all studies (Völker et al., 1999; Petersohn et al., 2001; Bernhardt et al., 2003; Brigulla et al., 2003; Helmann et al., 2003; Mascher et al., 2003; Holtmann et al., 2004; Zhang and Haldenwang, 2005; Gaidenko et al., 2006; Avila-Pérez et al., 2010; Ondrusch and Kreft, 2011; Nannapaneni et al., 2012; Reder et al., 2012; Jurk et al., 2013; Waters et al., 2014; Yu and Ye, 2016; Tran et al., 2019). Therefore, SigB-regulated genes that are specific for other stressors may have not been detected. Moreover, stimuli or stressors that play a role in unexplored ecological niches may trigger SigB as well; **3)** Analysis restricted to the SigB induction response. The standard setting in many studies of the global transcriptomics or proteomics SigB stress response focused on the induction pattern of SigB, and thus often checked for the "loss-of-gene-function" in the Δ*sigB* mutant. Such approaches may overlook genes that may be negatively regulated by SigB; **4)** SigB is likely active without "triggers" in the control condition and co-regulates other cellular mechanisms than the SigB GSR. Several known or predicted SigB candidate genes with SigB PBMs are involved in miscellaneous functions in *B. subtilis*, for instance, biofilm formation, sporulation, utilization of sugars, biosynthesis of amino acids, and homeostasis. Reder et al. (2012) and Rothstein et al. (2017) reported the negative regulation of SigB in sporulation initiation. Bartolini et al., (2018) demonstrated the role of SigB in regulating biofilm growth rate via the interaction with the SinR transcriptional regulator. SigB was also shown to indirectly affect the expression of surfactin, a cyclic lipopeptide (biosurfactant) (Bartolini et al., 2019).

Other than the functions reported in *B. subtilis,* SigB can influence motility, virulence, and invasiveness in other Bacillales members, e.g., *L. monocytogenes* and *S. aureus* (Kim et al., 2005; Ondrusch and Kreft, 2011; Mitchell et al., 2013), indicating that the structure of SigB regulons between species may diverge due to differences in physiological responses upon exposure to a broad range of stressors. Thus, to obtain a global outlook of the SigB general stress regulon

in other species in the Bacillales order, putative SigB regulons of 18 other *B. subtilis* strains and 106 Bacillales genomes were predicted as described in section 2.2.5.

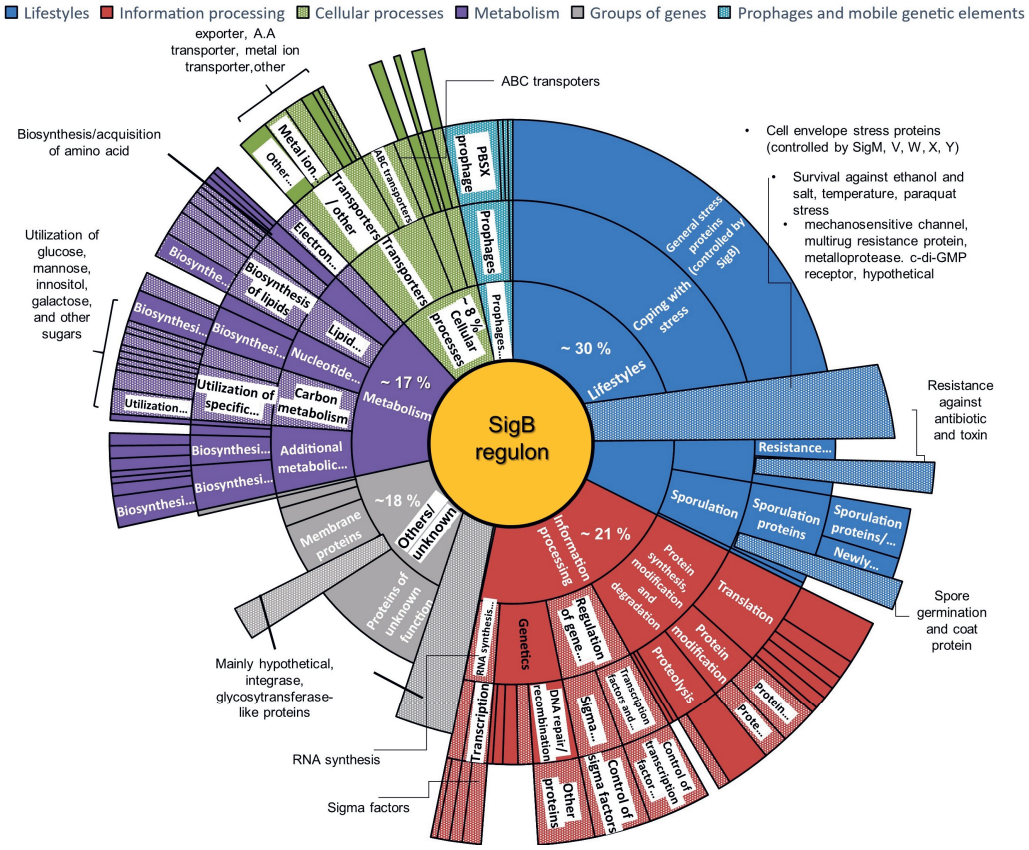


Figure 5- Functional distribution map for the predicted and existing SigB regulon members. The sunburst map shows five known functional groups and a group with unknown functions, labeled with different colors of the predicted and existing SigB regulon members. The dotted regions in the map refer to genes with either a known SigB PBM or a predicted SigB PBM in each functional category. **Blue-** genes involved in lifestyles (e.g., coping with stress, sporulation) (~30%); **Red-** genes for information processing (e.g., protein synthesis and modification, transcription, or translational regulation) (~ 21%); **Purple-** genes for metabolism regulation (e.g., biosynthesis of amino acid, lipids, utilization of carbon sources) (~17%); **Green-** genes for cellular processing (e.g., transporter, exporter, homeostasis) (~8%); **Turquoise-** genes for phage-related function (~6%); and **Grey-** genes with unknown functions (~18%). Underlying background data are shown in Supplementary Table S6.

2.3.4 SigB regulon prediction for *B. subtilis* wild isolates and Bacillales genomes

Genomes of 18 *B. subtilis* wild isolates and 106 other Bacillales genomes, including different *Bacillus* species, *Listeria* spp., and *Staphylococcus* spp. (Supplementary Table S5, were mined for the presence of SigB regulon members that had been identified in *B. subtilis* 168 (Supplementary Table S1). Based on the conserved protein sequences, the reconstructed phylogenetic tree heat map in Figure 6 showed that nearly all genes that belong to the SigB regulon in *B. subtilis* 168 had orthologs in 18 other wild *B. subtilis* isolates, except for a small cluster of germination genes (*yfkR, yfkS, yfkT*) and a group of genes with unknown function (*ykzN, ypuB, yydC*) (details in Supplementary Table S5). More prominent differences were seen between SigB regulons of *B. subtilis* and other *Bacillus* species and Bacillales genomes, such as *B. licheniformis, B. cereus,* and other further related species like *Geobacillus, Listeria,* and *Staphylococcus.* Around 25% of the *B. subtilis* 168 SigB regulon genes were absent in *B. licheniformis,* around 50% were missing in *B. cereus,* and three quarters were lacking in *Geobacillus, Listeria,* and *Staphylococcus* (Figure 6, Supplementary Table S5).

The prediction results showed that SigB regulates different sets of genes in different species, e.g., in *B. cereus, L. monocytogenes,* or *S. aureus.* Therefore, the SigB PBMs are likely species-specific and deviate from the SigB consensus of *B. subtilis* (GTTTAA-$N_{15\ (\pm\ 2\ bp)}$-GGGTAT), or have the same PBM but is/are not present in front of the same genes. This assumption was further investigated by reconstructing species-specific SigB PBMs per inspected species (described in section 2.2.5.3), illustrated in Figure 2, in a step-wise approach; 1) orthologous genes of the *B. subtilis* 168 SigB regulon members in other analyzed species were first predicted, 2) the predicted orthologous genes were grouped into operons, 3) promoters of these operons were used to reconstruct a species-specific SigB PBM, 4) the respective genome of a species was screened for the presence of the constructed species-specific PBM, resulting in a new list of genes with putative SigB PBMs, 5) operons for these genes with putative SigB PBMs were again predicted, and species-specific SigB PBM 2 was constructed, and lastly, 6) the respective genome of a species was repeatedly screened for the presence of the species-specific SigB PBM 2 with different spacers N_{12} – N_{17} (Figure 2). The same procedures were performed for other species included in this study, and each species-specific SigB PBM was used to derive a Bacillales consensus (KTTTW- N_{12}-N_{17}- GGGWAW). The Bacillales consensus is less conserved at the first guanine nucleotide in the -35 region when compared with the *B. subtilis* SigB consensus and contains more thymine than adenine nucleotides.

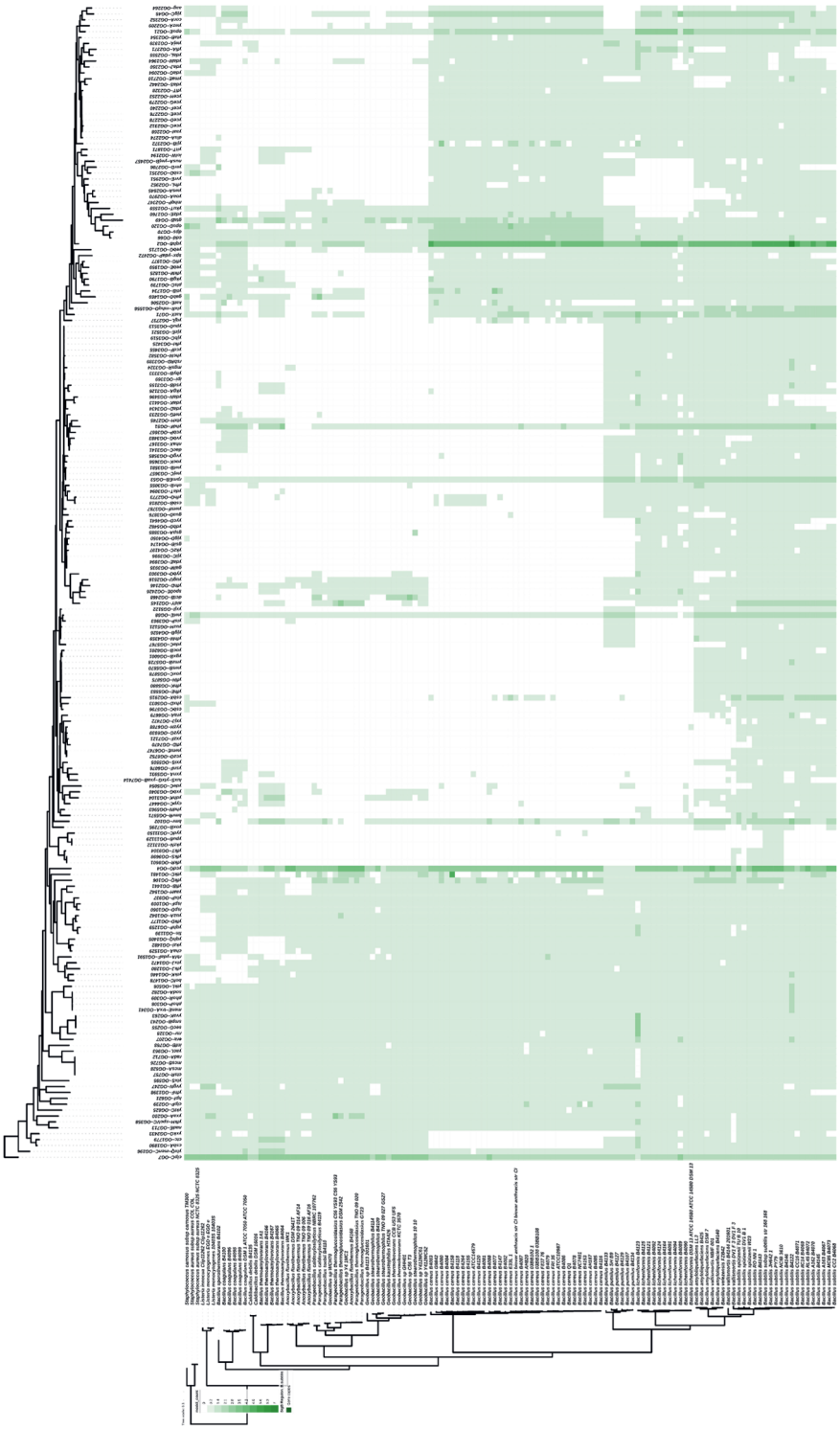


Figure 6- Genome tree heat map of 18 other *B. subtilis* strains and 106 Bacilalles genomes and the prediction of the presence of orthologs of *B. subtilis* 168 SigB regulon genes. The phylogenetic genome tree heat map for 19 *Bacillus subtilis* genomes (including *B. subtilis* 168) and 106 Bacillales members was generated using iTOL (PMID27095192) (Letunic and Bork, 2016). The vertical genome tree shows the phylogenetic relationships of all genomes based on the core conserved protein sequences in each genome. The horizontal genome tree shows the clustering of *B. subtilis* 168 SigB regulon genes listed in Subtiwiki up to October 2020 (Supplementary Table S1). A green square indicates the presence of a target gene, the intensity of the green color indicates the gene copy number, and white indicates the absence of a gene. Underlying background data are shown in Supplementary Table S5. The original resolution of the heat map is available upon request (tjakko.abee@wur.nl or kahyen.yeak@wur.nl).

The predicted species-specific SigB regulon members with/without SigB PBM are presented in a heat map (Supplementary Figure S1), showing the 1) absence/presence of predicted genes that are orthologous to the *B. subtilis* 168 SigB regulon members in 124 Bacillales genomes, and 2) the putative species-specific SigB regulon genes with or without a predicted SigB PBM. The complete list of these genes is shown in Supplementary Table S6. Four major observations can be made based on the heat map: 1) many other Bacillales genomes contain genes that are orthologous to the SigB regulon members of *B. subtilis* 168, but they do not necessarily have a SigB PBM; 2) groups of predicted regulon genes with/without SigB PBM are species-specific; 3) A group of genes that are orthologous to the SigB regulon members of *B. subtilis* 168 or the predicted species-specific regulon genes do not have a SigB PBM, and lastly 4) A group of genes with/without SigB PBM that are specific for *Bacillus* species but is absent in other Bacillales genomes.

Overall, the results obtained confirm that SigB plays a role in adaptive stress response in many species, but that the actual cellular responses and genes involved are different for different species. Species-specific SigB regulons may correspond with distinct physiological responses of species when dealing with a broad range of stressors in their environments. Orthologs of SigB-regulated genes with a SigB PBM as found in *B. subtilis* were mainly found in other *Bacillus* species but did not necessarily contain an upstream SigB PBM, and the majority of the *B. subtilis* SigB regulon genes were absent in *Listeria* spp. and *Staphylococcus* spp.

These results are in line with the studies of Scott et al. (2012), who reported on the divergence of the SigB GSR regulons within the *B. cereus sensu lato* group (containing species that are not included in this study: *B. anthracis, B. mycoides, B. pseudomycoides, B. thuringiensis, B. weihenstephanensis and B. cytotoxicus*). Four lineages of the SigB regulon were described in their study, and each lineage has arisen from the selection of a set of genes from the common gene pool, with the "reassignment" of a SigB promoter to these genes to support pathogenesis for different *sensu lato* members. The extra members in addition to the SigB core regulon (consisting of ~20 members) was suggested to serve a distinct function in different habitats and support the phenotype of a specific member, such as enhancing pathogenic potential or increasing competence against other microorganisms in the soil (Scott and Dyer, 2012). Moreover, the SigB PBM predicted for *B. cereus* in this study (shown in Figure 2) is highly similar to the one described in the study of Scott et al. (2012) despite using different species of *B. cereus* group members (in this study, only *B. cereus* genomes were used, Supplementary Table S6).

2.3.5 The Occurrence of SigB sensing modules in other *B. subtilis* strains and Bacillales genomes

Our analysis showed differences between the predicted SigB regulons for various Bacillales genomes. We furthermore examined the presence of the three well-known SigB signaling modules in Bacillales, i.e., RsbRST, RsbQP, and RsbKY (as described in section 2.2.6).

The absence/presence of genes involved in sensing stressors, SigB transduction, activation, and regulation in 19 *B. subtilis* genomes and 106 Bacillales members is presented in Figure 7. The complete datasets relating to the presence of the sensing modules are presented in Supplementary Table S7. The majority of the inspected genomes carried the *sigB* gene. However, it was absent in several species like *B. thermoamylovorans, Parageobacillus thermoglucosidasius,* and *Anoxybacillus*. These species likely evolved to utilize other stress sensing systems and were therefore excluded from further analyses.

All species that contained the *sigB* gene also carried the *rsbV* and *rsbW* genes. These three genes are highly conserved in Bacillales, including the *Geobacillus* and *Paenibacillus* species with high GC content. In general, the complete stressosome system (*rsbRST* and *rsbU*) and its feedback regulator (*rsbX*) were found in *B. subtilis, B. vallismortis, B. licheniformis, B. amyloliquefaciens, B. coagulans, B. pumilus, B. valezensis, B. sporothermodurans, L. monocytogenes,* and *L. innocua* (Figure 7). In addition, the RsbQP module was found in *B. subtilis, B. vallismortis,* and *Paenibacillus* spp. and the two-component system (*rsbKY*) and its regulator *rsbM* were identified in *B. cereus, Paenibacillus* spp. and *B. coagulans* (Figure 7).

2.3.5.1 The stressosome RsbRST SigB activation pathway

The RsbRST stressosome system was detected in many species, but only the RsbRA ortholog is conserved (Figure 7). Many species that contain the stressosome genes lacked one or more of either the RsbRB, RsbRC, RsbRD, or the YtvA ortholog. *B. licheniformis, B. pumilus,* and *B. sporothermodurans* did not have the RsbRB and RsbRC orthologs, whereas *B. coagulans* did not have the RsbRC and RsbRD orthologs. Only *B. subtilis, B. vallismortis, B. amyloliquafaciens, B. pumilus, L. monocytogenes,* and *L. innocua* contained the fifth RsbR ortholog, YtvA, which is involved in the sensing of blue light. Even in *B. subtilis*, not every strain contained the genes encoding the same RsbR orthologs. Some of the *B. subtilis* food isolate strains (B4068 and B4073) lost the gene encoding the RsbRC ortholog, and *B. subtilis* B4122 had two gene copies encoding the RsbRD ortholog (Figure 7, Supplementary Table S7).

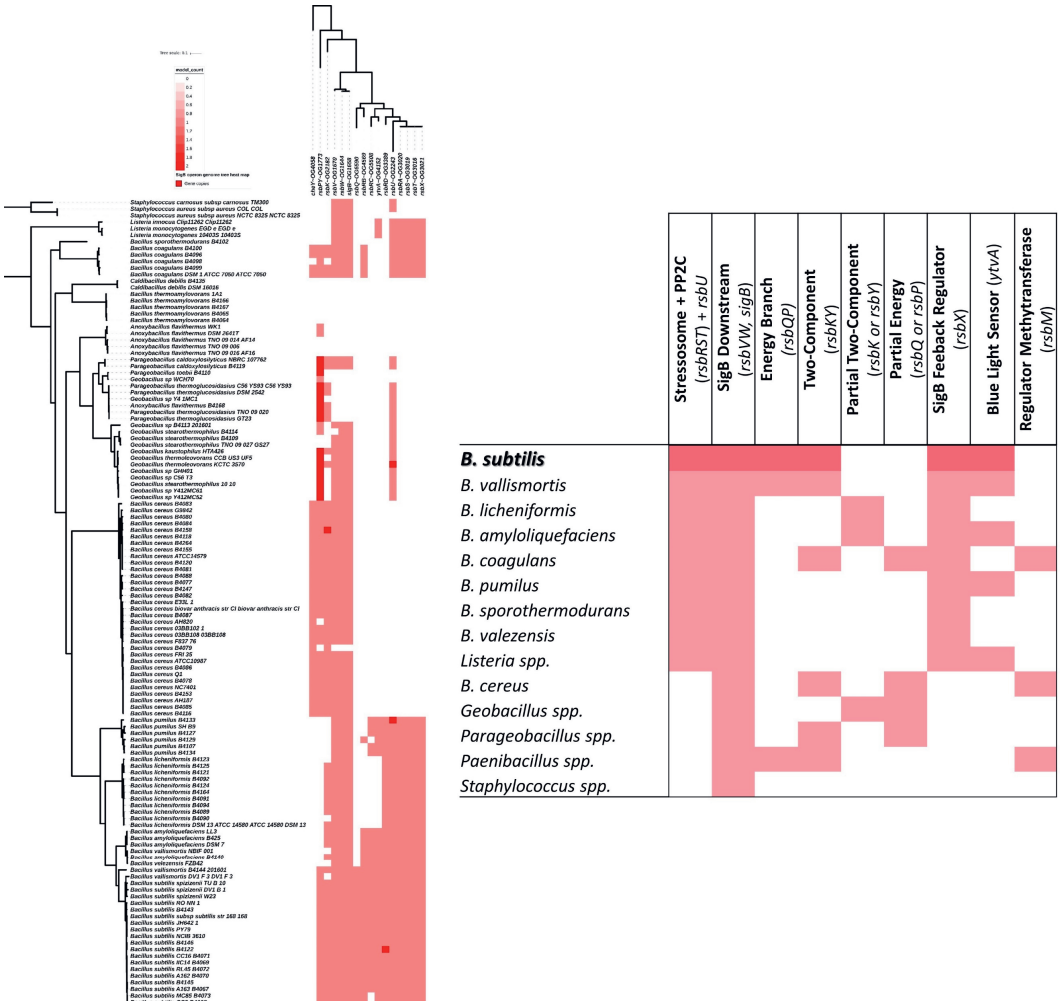


Figure 7- Genome tree heat map of sensing modules of the SigB general stress for 19 *Bacillus subtilis* genomes and 106 Bacillales members. The heat map of the core genome tree of sensing modules of the SigB general stress for 19 *Bacillus subtilis* genomes and 106 Bacillales members was generated using iTOL (PMID27095192) (Letunic and Bork, 2016). The tree on the left shows the phylogenetic relationships of all genomes based on the core conserved protein sequences in each genome. The tree on the top shows the clustering of genes involved in SigB signal sensing. The red square indicates the presence of a target gene, and the intensity of the red color indicates the gene copy number. White indicates the absence of a gene. Underlying background data are shown in Supplementary Table S7. The insert shows the summary of the general distribution of the three sensing modules for each species belonging to the Bacillales order. Stressosome refers to the *rsbRST* stressosome genes and stressosome downstream elements to *rsbV, rsbW* and *sigB*. The energy branch refers to the *rsbQP* genes and the two-component to the *rsbKY* genes. Inspected strains that contain only a single gene of a signaling module, e.g., the presence of an orphan *rsbK* gene without its cognate response regulator gene *rsbY,* or the presence of an *rsbP* phosphatase gene without its partner *rsbQ*, are referred to as having a partial two-component, or a partial energy system, respectively. Other SigB sensors and regulators included the blue light sensor (YtvA), the regulator of SigB methyltransferase Bc1007 (renamed to RsbM by Chen et al., 2012), and the feedback regulator RsbX phosphoserine phosphatase. Underlying background data are shown in Supplementary Table S7.

The occurrence of different types and numbers of RsbR orthologs results in the formation of heterogeneous stressosome complexes (Delumeau et al., 2006), thereby affecting specific stress sensing via the turrets (referring to the protein structure of the RsbR and its paralogs) with different ligands (Marles-Wright and Lewis, 2008; Pané-Farré et al., 2017). Moreover, from an evolutionary point of view, different species may be exposed to specific stress conditions in particular ecological niches, putting selective pressures on retaining certain RsbR orthologs for sensing specific stress. Different RsbR orthologs can have distinct functions in mediating stress; RsbRC has for instance been shown to be responsible for a slow progressive stress response upon ethanol stress, whereas RsbRA mediates a fast transient response (Cabeen et al., 2017). However, RsbRC was absent in most genomes carrying genes encoding stressosome members (Figure 7), which implies that its role may not be essential, or that its function is redundant in the presence of RsbRA. In *B. subtilis*, it was shown that RsbRA and RsbRB orthologs are responsible for light sensing, and the role of RsbR can be complemented by RsbRC or RsbRD (van der Steen et al., 2012).

Additionally, the gene *rsbX* was found only in strains that contained the stressosome genes, confirming the reported function of the RsbX in forming a negative feedback loop by dephosphorylating the RsbR and RsbS, resetting the activated stressosome back to its original state (Chen et al., 2004). Therefore, the absence of *rsbX* in other strains that do not contain the stressosome genes was not surprising as this feedback loop was probably not required.

2.3.5.2 The bipartite RsbQP SigB activation pathway

The entire RsbQP module was detected in *B. subtilis, B. vallismortis,* and *Paenibacillus* spp. (Figure 7), indicating that the nutritional stress sensing branch is not restricted to just *B. subtilis*. The *rsbQ* gene was missing in most genomes, but the *rsbP* gene, encoding for a PP2C phosphatase, was distributed more broadly. As *rsbP* and *rsbY* belong to the same OG1773 group, an "*rsbP*" ortholog was also found in the genomes of *B. cereus, Geobacillus* spp, *Paenibacillus* spp, and *Parageobacillus* spp. (see Figure 7).

The finding was in line with the publication of Nadezhdin et al. (2011), who suggested that the predicted RsbQP could be functional in other species, or react with other stressors, but not the same as described for *B. subtilis* (Zhang and Haldenwang, 2005; Avila-Pérez et al., 2010). The observation that RsbQ is generally absent while RsbP is generally present in Bacillales (Supplementary Table S7) suggests RsbP may have other so far unidentified functions, and its interaction partner may not be limited to RsbQ. This speculation can be supported by reports on alternative functions of RsbP in earlier studies, which showed that RsbP is also involved in sensing red light (Avila-Pérez et al., 2010) and that this protein interacted with the stressosome to mediate resilience toward oxidative and nitrosative stress in *B. subtilis* (Guldimann et al., 2016).

2.3.5.3 The two-component RsbKY SigB activation pathway

The two-component system encoded by *rsbKY* and *rsbM* was found in *B. cereus, Paenibacillus* spp., and *B. coagulans*. This sensing module was well-known to be specific to *B. cereus* and its group members (de Been et al., 2011), while an entire RsbKYM system in *B. coagulans* has not been described previously. In addition, the *rsbK* (*bc1008*) gene in *B. cereus* was found to have an ortholog in *B. subtilis* and other group members like *B. licheniformis, B. amyloliquefaciens, B. pumilus,* and *B. vallismortis* (Figure 7, Supplementary Table S7), but no cognate response regulator was detected adjacent to the predicted *rsbK* gene in these species. The functionality of an RsbK ortholog in *B. subtilis* and its potential role in the SigB activation pathway remains to be confirmed.

The suggestion of an alternative SigB activation pathway in *B. subtilis* is not new, as extreme heat and chill conditions have been reported to induce SigB either directly, or independently from RsbV (Brigulla et al., 2003; Holtmann et al., 2004), and nitrosative stress triggered SigB in the absence of RsbT or RsbP (Tran et al., 2019). Moreover, the RsbW (the anti-sigma factor) exhibited high cross-phosphorylation activity by other kinases (Shi et al., 2014), which may cause unexpected SigB activation in *B. subtilis*.

2.4 Conclusion

This study generated a SigB PBM that took spacer composition into account and has higher plasticity than the previously reported consensus sequence (Sierro et al., 2008; Coelho et al., 2018; Vohradsky et al., 2021). This was used to identify potential novel candidates that belong to the SigB regulon of *B. subtilis*. Of the 255 genes with predicted SigB PBMs identified in this study, 99 genes have previously been reported in the literature, indicating the identification of 156 new putative SigB regulon members. The functionality of nine predicted SigB PBMs (including a positive control P_{rsbV}) was further validated via experiments, and results obtained showed that 1) some promoters containing the predicted SigB PBMs are stressor-specific; 2) spacer length likely influences the promoter activity with a spacer length of 14 bp appearing to be optimal; 3) less conserved SigB PBMs are still recognized by SigB (e.g., P_{gtaB}) but may be co-regulated by other transcriptional regulators.

Furthermore, this study demonstrated the diversity of SigB regulons in different species of the Bacillales order. The various SigB regulons are likely linked with distinct strategies required for survival in ecological niches of species. While all Bacillales members can be present in soils, some inhabit salt lakes, hot springs, guts of invertebrates, etc. Thus, the strategies used to cope with stressors found in the soil environments likely overlap between these species but have diverse stress management strategies in other niches. A Bacillales SigB consensus was predicted, with the sequence of KTT at the -35 and the GG at the -10 binding site, respectively.

The SigB stress sensing modules were also species-specific and may even vary between different strains of the same species, likely due to the evolution of Bacillales members in specific habitats, demanding different needs to sense unique stressors.

Overall, the entire SigB regulatory network is sophisticated and not yet fully understood, even for the well-characterized organism *B. subtilis* 168. Knowledge and information gained in this study can be used in further SigB GSR studies to uncover a complete picture of the role of SigB in *B. subtilis* and other species.

2.5 Supplementary Materials

Supplementary Table S1: List of SigB regulon genes described to date October 2020 for *Bacillus subtilis* 168

No	Locus Tag	Gene	No	Locus Tag	Gene	No	Locus Tag	Gene
1	BSU_misc_RNA_55	*ssrA*	**41**	BSU04370	*ydaS*	**81**	BSU08500	*yfhE*
2	BSU00160	*yaaH*	**42**	BSU04380	*ydaT*	**82**	BSU08510	*yfhF*
3	BSU00170	*yaaI*	**43**	BSU04400	*gsiB*	**83**	BSU08570	*yfhK*
4	BSU00520	*ctc*	**44**	BSU04430	*ydbD*	**84**	BSU08580	*yfhL*
5	BSU00530	*spoVC*	**45**	BSU04440	*dctB*	**85**	BSU08590	*yfhM*
6	BSU00540	*fin*	**46**	BSU04670	*rsbR*	**86**	BSU08600	*csbB*
7	BSU00830	*ctsR*	**47**	BSU04680	*rsbS*	**87**	BSU08610	*yfhO*
8	BSU00840	*mcsA*	**48**	BSU04690	*rsbT*	**88**	BSU09140	*yhcM*
9	BSU00850	*mcsB*	**49**	BSU04700	*rsbU*	**89**	BSU09390	*ygxB*
10	BSU00860	*clpC*	**50**	BSU04710	*rsbV*	**90**	BSU09450	*yhdF*
11	BSU00870	*radA*	**51**	BSU04720	*rsbW*	**91**	BSU09530	*yhdN*
12	BSU00880	*disA*	**52**	BSU04730	*sigB*	**92**	BSU09540	*plsC*
13	BSU00890	*yacL*	**53**	BSU04740	*rsbX*	**93**	BSU09690	*nhaX*
14	BSU00900	*ispD*	**54**	BSU05140	*ydzE*	**94**	BSU10430	*yhxD*
15	BSU00910	*ispF*	**55**	BSU05150	*ydeC*	**95**	BSU10810	*yisP*
16	BSU02100	*cypC*	**56**	BSU05490	*mhqO*	**96**	BSU11120	*yitT*
17	BSU02110	*ybyB*	**57**	BSU05500	*mhqP*	**97**	BSU11130	*ipi*
18	BSU02590	*ycbP*	**58**	BSU05790	*ydhK*	**98**	BSU11490	*yjbC*
19	BSU02830	*ycdF*	**59**	BSU06400	*yebE*	**99**	BSU11500	*spx*
20	BSU02840	*ycdG*	**60**	BSU06410	*yebG*	**100**	BSU11839	*yjzE*
21	BSU02890	*yceC*	**61**	BSU06590	*yerD*	**101**	BSU12150	*yjgB*
22	BSU02900	*yceD*	**62**	BSU06660	*opuE*	**102**	BSU12160	*yjgC*
23	BSU02910	*yceE*	**63**	BSU07550	*yflT*	**103**	BSU12170	*yjgD*
24	BSU02920	*yceF*	**64**	BSU07670	*yflI*	**104**	BSU12210	*yjiB*
25	BSU02930	*yceG*	**65**	BSU07680	*yflH*	**105**	BSU12220	*yjiC*
26	BSU02940	*yceH*	**66**	BSU07690	*yflG*	**106**	BSU13010	*ykgB*
27	BSU03130	*nadE*	**67**	BSU07720	*yflD*	**107**	BSU13020	*ykgA*
28	BSU03910	*gabD*	**68**	BSU07735	*yflB*	**108**	BSU13160	*ohrB*
29	BSU04039	*yczO*	**69**	BSU07750	*yflA*	**109**	BSU13169	*ykzN*
30	BSU04180	*ydaC*	**70**	BSU07760	*yfkT*	**110**	BSU13170	*guaD*
31	BSU04190	*ydaD*	**71**	BSU07770	*yfkS*	**111**	BSU13640	*spo0E*
32	BSU04200	*ydaE*	**72**	BSU07780	*yfkR*	**112**	BSU14210	*ykuT*
33	BSU04210	*ydaF*	**73**	BSU07790	*yfkQ*	**113**	BSU14660	*ykzI*
34	BSU04220	*ydaG*	**74**	BSU07850	*yfkM*	**114**	BSU14670	*yktC*
35	BSU04270	*ydaJ*	**75**	BSU07880	*yfkJ*	**115**	BSU14680	*ykzC*
36	BSU04280	*ydaK*	**76**	BSU07890	*yfkI*	**116**	BSU16590	*ylxS*
37	BSU04290	*ydaL*	**77**	BSU07900	*yfkH*	**117**	BSU16600	*nusA*
38	BSU04300	*ydaM*	**78**	BSU07920	*chaA*	**118**	BSU16610	*ylxR*
39	BSU04310	*ydaN*	**79**	BSU07930	*yfkD*	**119**	BSU16620	*ylxQ*
40	BSU04340	*ydaP*	**80**	BSU08490	*yfhD*	**120**	BSU16630	*infB*

No	Locus Tag	Gene	No	Locus Tag	Gene	No	Locus Tag	Gene
121	BSU16650	*rbfA*	**162**	BSU30640	*ytkC*	**203**	BSU38720	*yxkO*
122	BSU17240	*ymzB*	**163**	BSU30650	*dps*	**204**	BSU38830	*aldY*
123	BSU17250	*ymaE*	**164**	BSU30670	*luxS*	**205**	BSU38930	*yxjJ*
124	BSU18350	*dacC*	**165**	BSU30690	*ytiB*	**206**	BSU38940	*yxjI*
125	BSU18360	*galM*	**166**	BSU30700	*rpmEB*	**207**	BSU39040	*yxiS*
126	BSU18510	*yoxC*	**167**	BSU30780	*menC*	**208**	BSU39050	*katE*
127	BSU18520	*yoxB*	**168**	BSU30790	*menE*	**209**	BSU39810	*csbC*
128	BSU18530	*yoaA*	**169**	BSU30930	*ytaB*	**210**	BSU39840	*yxbG*
129	BSU19150	*yocB*	**170**	BSU31279	*yuzH*	**211**	BSU40000	*yxnA*
130	BSU19240	*yocK*	**171**	BSU31280	*yugU*	**212**	BSU40030	*yxaB*
131	BSU23300	*ypuD*	**172**	BSU31380	*yuzA*	**213**	BSU40040	*yxaA*
132	BSU23329	*ypuC/1*	**173**	BSU33200	*yvrE*	**214**	BSU40210	*yydC*
133	BSU23330	*ypuC/2*	**174**	BSU33400	*yvgN*	**215**	BSU40259	*yyzG*
134	BSU23340	*ypuB*	**175**	BSU33410	*yvgO*	**216**	BSU40450	*yycD*
135	BSU23830	*yqjL*	**176**	BSU33530	*iolW*	**217**	BSU40529	*yyzH*
136	BSU24000	*bmrU*	**177**	BSU33600	*smpB*	**218**	BSU40570	*yybO*
137	BSU24010	*bmr*	**178**	BSU33610	*rnr*	**219**	BSU16640	*ylxP*
138	BSU24020	*bmrR*	**179**	BSU33620	*yvaK*	**220**	new_3035475_3036308_c	*S1134*
139	BSU24490	*yqhQ*	**180**	BSU33630	*secG*	**221**	new_3036310_3036529_c	*S1136*
140	BSU24500	*yqhP*	**181**	BSU33850	*yvbG*	**222**	new_3708133_3708785	*S1384*
141	BSU24740	*corA*	**182**	BSU34540	*clpP*			
142	BSU24750	*yqhB*	**183**	BSU35180	*csbA*			
143	BSU24760	*rsbRD*	**184**	BSU35310	*hpf*			
144	BSU24770	*mgsR*	**185**	BSU35670	*gtaB*			
145	BSU25020	*sodA*	**186**	BSU35699	*yvzE*			
146	BSU25290	*era*	**187**	BSU35830	*ywtG*			
147	BSU25300	*cdd*	**188**	BSU35970	*ywsB*			
148	BSU27020	*yraA*	**189**	BSU35980	*ywsA*			
149	BSU27750	*bofC*	**190**	BSU36670	*csbD*			
150	BSU27760	*csbX*	**191**	BSU36680	*ywmF*			
151	BSU28340	*ysnF*	**192**	BSU36720	*ywmE*			
152	BSU28500	*trxA*	**193**	BSU36960	*ywlB*			
153	BSU28830	*ysdB*	**194**	BSU37210	*ywjC*			
154	BSU29100	*phoR*	**195**	BSU37220	*ywjB*			
155	BSU29110	*phoP*	**196**	BSU37230	*ywjA*			
156	BSU29410	*ytkL*	**197**	BSU37240	*ywiE*			
157	BSU29420	*ytkK*	**198**	BSU38180	*ywzA*			
158	BSU29760	*ytxJ*	**199**	BSU38430	*gspA*			
159	BSU29770	*ytxH*	**200**	BSU38610	*yxzF*			
160	BSU29780	*ytxG*	**201**	BSU38620	*aag*			
161	BSU30070	*opuD*	**202**	BSU38630	*katX*			

Supplementary Table S2: The list of newly predicted SigB regulon genes with a SigB PBM in this study
a- bold locus tag indicates genes found in this study that are also listed in the Subtiwiki SigB regulon

Locus Tag[a]	Operon	Annotation/Description/Function	Gene	SigB PBM	C
BSU04720	operon_2365	anti- sigma factor B; serine-protein kinase RsbW	*rsbW*	G**GTTTAA**CGTCTGTCAGACGA**GGGTAT**AAAGCAACTA	I
BSU04730	operon_2365	RNA polymerase sigma-B	*sigB*	G**GTTTAA**CGTCTGTCAGACGA**GGGTAT**AAAGCAACTA	I
BSU04710	operon_2365	anti-sigma-B factor antagonist	***rsbV***	G**GTTTAA**CGTCTGTCAGACGA**GGGTAT**AAAGCAACTA	I
BSU04740	operon_2365	protein serine phosphatase, feedback PP2C, dephosphorylates RsbS and RsbR	*rsbX*	G**GTTTAA**CGTCTGTCAGACGA**GGGTAT**AAAGCAACTA	I
BSU11490	operon_1888	acetyltransferase YjbC, general stress protein, required for survival of salt and paraquat stresses	*yjbC*	CT**GTTTAA**ACAAGAAGAAATG**GGGTAT**ATCTAAAAGT	I
BSU00520	operon_2634	general stress protein, similar to ribosomal protein L25	*ctc*	AG**GTTTAA**ATCCTTATCGTTAT**GGGTAT**TGTTTGTAA	I
BSU09530	operon_2033	general stress protein, broad specificity aldo-keto reductase that converts MG to acetol	*yhdN*	G**GTTTAA**CATTTTTTCCAGAG**GGGAAA**AGATAGTGAA	II
BSU04220	operon_2402	putative pyridoxamine 5'-phosphate oxidase , general stress protein, required for protection against paraquat stress	*ydaG*	T**GTTTAA**ATCTTCCCCGGATGT**GGAAAA**GTAACAGCG	II
BSU04400	operon_2388	glucose starvation-inducible protein; general stress protein, prevents enzyme inactivation upon freeze-thaw treatments	***gsiB***	TT**GTTTAA**AAGAATTGTGAGC**GGGAAT**ACAACAACCA	II
BSU13020	operon_1790	general stress protein, survival of salt and ethanol stresses	*ykgA*	TG**GTTTAA**TGATTTTCATGATGA**GGGAAT**AATAAATG	II
BSU00890	operon_2615	PIN and TRAM-domain containing protein; general stress protein, survival of salt and ethanol stresses	*yacL*	CG**GTTAAA**ACCTTATGAATAC**GGGTAT**ATTAATGTT	II
BSU00900	operon_2615	2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase, third step in the MEP pathway of isoprenoid biosynthesis	*ispD*	CG**GTTAAA**ACCTTATGAATAC**GGGTAT**ATTAATGTT	II
BSU00910	operon_2615	2-C-methyl-D-erythrol-2,4-cyclodiphosphate synthase, 5th step in the MEP pathway of isoprenoid biosynthesis	*ispF*	CG**GTTAAA**ACCTTATGAATAC**GGGTAT**ATTAATGTT	II
BSU38430	operon_176	general stress protein	*gspA*	GT**GTTTAT**TTTTTTGAAAAA**GGGTAT**GTAACTTGTA	II
BSU38930	operon_144	general stress protein, survival of ethanol, paraquat and salt stresses	*yxjJ*	TTGT**GTTCAA**TTCCGCAAAATA**GGGTAT**AAACCAAACA	II
BSU02590	operon_2519	general stress protein	*ycbP*	AG**GTTTAA**CTTTTTACATTTGA**GGAATT**ATACATAAC	II
BSU36720	operon_283	general stress protein, survival of ethanol stress	*ywmE*	TG**GTTTAA**AAACAGTTTGGGC**GGGAAT**GATACCCTAA	II
BSU02830	operon_2499	glucose 1-dehydrogenase 2; general stress protein, similar to glucose 1-dehydrogenase, survival of ethanol stress and low temperatures	*ycdF*	CT**GTTTTC**AACTCGGAAAAACA**GGGTAT**TTTCCACTGC	II
BSU02840	operon_2499	oligo-1,6-glucosidase 2 ; general stress protein, similar to oligo-1,6-glucosidase	*ycdG*	CT**GTTTTC**AACTCGGAAAAACA**GGGTAT**TTTCCACTGC	II
BSU14210	operon_1691	MscS family protein ; mechanosensitive channel, similar to MscS, general stress protein	*ykuT*	AC**GTTTAA**CATGGTCATGTACA**GGGTAA**CTAGTCTCAA	II
BSU13160	operon_1779	organic hydroperoxide resistance OhrB; general stress protein	*ohrB*	AT**GTTTAA**AAAGATCAGAAA**GGGAAA**TATAACAACTA	II
BSU13169	operon_1779	hypothetical protein; unknown	*ykzN*	AT**GTTTAA**AAAGATCAGAAA**GGGAAA**TATAACAACTA	II
BSU36470	operon_302	allantoin permease	***pucI***	TT**GTTTAA**GCTGTTCAAATACAAAC**GGGAAA**ATTGTA	II
BSU13920	operon_1720	transcriptional regulator SplA ; transcriptional repressor of the spore photoproduct lyase splA-splB operon	*splA*	CTG**GTTTTT**TTCATAAGTAA**GGGTAT**AGAAGGACAC	II
BSU23040	operon_1137	ferredoxin	*fer*	AC**GTTTAA**AGTCATCATTAAGG**GGGATG**TTATGTAGA	II
BSU29850	operon_720	hypothetical protein	***ytoQ***	TC**GTTTAA**CATCGTGTACATGA**GGGTGA**AATTTACAG	II
BSU27630	operon_852	hypothetical protein	*yrvD*	T**GTTTAA**AACCAGTCTTGATT**GGGAAA**GTTACCTCAA	II
BSU23900	operon_1082	hypothetical protein	*yqjF*	TC**GTTTAA**AAAGAAGGAAATC**GGGAAT**ACCTTTACTC	II
BSU03390	operon_2457	hypothetical protein	*yckC*	GT**GTTTAA**AAGCGTCTATGAA**GGGAAA**GGTGATGAA	II
BSU12270	operon_1825	hypothetical protein	*yjlB*	CT**GTTTAA**TATAAAGGTAACG**GCGAAA**AAGAGTGAA	II
BSU33510	operon_473	copper chaperone CopZ; copper transport protein, metallochaperone	*copZ*	T**TTTTT**ATTGTAATACCCTACGG**GGGTAT**GGTAGGAT	II
BSU34640	operon_408	putative lysine decarboxylase;	*yvdD*	AT**GTTTAA**CACGATATATAGATGC**GGAGAT**GACAAGA	II
BSU18370	operon_1439	probable multidrug resistance protein	*yoeA*	AT**GTTTAA**AAACGATTTCAAATAAA**GGAGTC**CTTTAA	II
BSU29410	operon_751	general stress protein; metal-dependent hydrolase	*ytkL*	**GTTTTT**CAGCTTTTTAAAAA**GGGAAA**ATAAAAAAAA	III
BSU02110	operon_2553	GSP, survival of ethanol stress	*ybyB*	AG**GTTTAG**CAATTTCCAAAAC**GGGAAT**GATACAGGAA	III

Locus Tag[a]	Operon	Annotation/Description/Function	Gene	SigB PBM	C
BSU38620	operon_164	hypoxanthine-DNA glycosylas, GSR protect against paraquat stress	*aag*	C**GTTTTT**TTTGATCTGCTTC**GGGAAT**GGTACAATGT	III
BSU38610	operon_164	general stress protein	*yxzF*	C**GTTTTT**TTTGATCTGCTTC**GGGAAT**GGTACAATGT	III
BSU39840	operon_91	general stress protein, similar to glucose 1-dehydrogenase; oxidoreductase	*yxbG*	AT**GTTTAT**CACTGCACATAGC**GGGAAG**ACAAATAGAA	III
BSU38180	operon_193	general stress protein	***ywzA***	T**GTTTAT**CTTATACAAAAAAG**AGGAAT**GATATAAGCA	III
BSU23300	operon_1122	putative intramembrane metalloprotease	*ypuD*	CG**GTTTTT**TATTCATGAAAAAA**AGGAAT**AACTCATATG	III
BSU19150	operon_1366	general stress protein, survival of stress conditions	*yocB*	AG**GTTTGA**TCGTTTTTAAGAG**AGGAAA**AAGAAAACTA	III
BSU10430	operon_1959	general stress protein, similar to alcohol dehydrogenase, oxidoreductase	*yhxD*	T**GTTTTT**CTGCTTATGCTCAG**GGGTAC**ACATACGAAT	III
BSU39810	operon_94	general stress protein, similar to sugar symporter, required for protection against paraquat stress metabolite transport protein CsbC	*csbC*	AT**GTTTCA**AATGAGATAGGAAAT**GGGTAC**TAATCTAT	III
BSU08570	operon_2104	general stress protein, similar to cell division inhibitor	*yfhK*	**GTTTCA**CCAGCCTGTCAATCA**GGGAAT**ACCACTTATA	III
BSU05790	operon_2282	general stress protein, survival of ethanol and paraquat stresses	*ydhK*	T**GTTTGG**CTTTGCAAACAAAG**GGGAAT**AGGACAAACT	III
BSU40529	operon_42	hypothetical protein	*yyzH*	AT**GTTTTG**AAGTTTTTAAAAA**GGGAAA**TTCACCTTAT	III
BSU29780	operon_724	general stress protein	*ytxG*	AT**GTTTAT**GATTGAAGAAAAC**GGGTAA**ACAGCAGTAT	III
BSU29770	operon_724	general stress protein	*ytxH*	AT**GTTTAT**GATTGAAGAAAAC**GGGTAA**ACAGCAGTAT	III
BSU29760	operon_724	general stress protein, putative bacillredoxin	*ytxJ*	AT**GTTTAT**GATTGAAGAAAAC**GGGTAA**ACAGCAGTAT	III
BSU04340	operon_2394	general stress protein, required for survival of ethanol, cold (4°C) and oxidative stress (superoxide/paraquat), putative pyruvate oxidase; thiamine pyrophosphate-containing protein YdaP	*ydaP*	G**GTTTTA**AAGCCTTTCTCCTG**TGGTAT**TGAAAAAAGG	III
BSU04430	operon_2386	general stress protein, manganese catalase	*ydbD*	TC**GTTTAT**CTTTCTATCGAT**CGGAAAT**ATAAAAAGAG	III
BSU38630	operon_163	Catalase X, general stress protein	*katX*	**GTTTTA**AAATCTTTCCATTCA**GGGAAT**ATTGTTACCG	III
BSU09140	operon_2063	general stress protein	*yhcM*	ACG**GTTAAT**TTGTCTAACGAGG**GGGAAA**ATATGAATAC	III
BSU24000	operon_1075	general stress protein, multidrug resistance protein, similar to diacylglycerol kinase; lipid kinase BmrU	*bmrU*	TC**GTTTAC**TGCTTACAGAAAAAG**GGGATT**ATATAACCA	III
BSU30700	operon_665	general stress protein, binds in the stationary phase to the ribosome, replaces RpmE under conditions of zinc limitation;	*rpmE*	AG**GTTTAC**GATGTGAAACAGA**GGGAAG**GATATAAGAA	III
BSU30070	operon_703	glycine betaine and arsenobetaine transporter	*opuD*	T**GTTTTG**TTTCACCTGATTGCG**GGGTAA**TTTTAAGAG	III
BSU04380	operon_2390	general stress protein, survival of ethanol stress and low temperatures	*ydaT*	C**GTTTTT**ATTTTTCACCTGC**GGGTAC**CATTTTTATA	III
BSU11120	operon_1912	general stress protein, required for protection against paraquat stress	*yitT*	GT**GTTCAA**TATTTGTTTTAA**GGGAAA**ACATAATAAA	III
BSU19240	operon_1358	general stress protein	*yocK*	AT**GTTTGA**CAGAAGGCAAAAC**GGGAAA**CAGGATAGAA	III
BSU34540	operon_412	ATP-dependent Clp protease proteolytic subunit (class III heat-shock protein)	*clpP*	**GTTTGA**ACCCCTGTATTTTTG**GGGAAA**ATGGGAAAAA	III
BSU40450	operon_48	general stress protein, survival of ethanol stress	*yycD*	C**GTTTCG**GACAGTAACAAGGC**GGGAAA**AATGCAATAA	III
BSU00870	operon_2616	DNA repair protein RadA; branch migration transferase, 6-O-methylguanine-DNA methyltransferase, negative effector of DisA activity, participates in the stabilization and/or processing of holliday junction intermediates	*radA*	C**GTTTAT**CTGAAGAACTCCTCAG**AGGAAA**TATTCATA	III
BSU00880	operon_2616	DNA integrity scanning protein, diadenylate cyclase, delays [SW|sporulation] in the case of chromosome damage, the DisA-dependent checkpoint arrests [SW|DNA replication] during B. subtilis spore outgrowth until the germinating spores genome is free of damage	*disA*	C**GTTTAT**CTGAAGAACTCCTCAG**AGGAAA**TATTCATA	III
BSU00850	operon_2617	ATP:guanido phosphotransferase, tags proteins for degradation by ClpC and	*mcsB*	G**GTTTTG**TGGACCGGGAAAAT**GGAAAT**AATGAAGGA	III

Locus Tag[a]	Operon	Annotation/Description/Function	Gene	SigB PBM	C
		ClpP protein, modulator of CtsR - dependent repression			
BSU00840	operon_2617	modulator of CtsR-dependent repression, McsA activates kinase activity of McsB	*mcsA*	G**GTTTTG**TGGACCGGGAAAAT**GGAAAT**AATGAAGGA	III
BSU00860	operon_2617	negative regulator of genetic competenceClpC/MecB	*clpC*	G**GTTTTG**TGGACCGGGAAAAT**GGAAAT**AATGAAGGA	III
BSU00830	operon_2617	transcriptional regulator CtsR; transcription repressor of class III heat shock genes	*ctsR*	G**GTTTTG**TGGACCGGGAAAAT**GGAAAT**AATGAAGGA	III
BSU36680	operon_286	hypothetical protein	*ywmF*	AC**GTTTAT**TTCCGGGGAGAAC**AGGAAA**AATCGCTACA	III
BSU36670	operon_287	general stress protein	*csbD*	AT**GTTTAT**TGCCTCTCAGATC**GGGAAG**TTAACAAGTA	III
BSU08510	operon_2109	general stress protein, similar to nucleoside-diphosphate sugar epimerase	*yfhF*	C**GTTTTC**TTTTATTACAATG**AGGTAA**AGTATATTTA	III
BSU11839	operon_1863	general stress protein	*yjzE*	C**CTTTTA**TTCCCTCTTCAAAG**AGGAAT**TGATAATGTT	III
BSU18360	operon_1440	aldose-1-epimerase	*galM*	GGT**GTTTTT**TTATTTGATAG**GGGAAAA**TATAAAATG	III
BSU18350	operon_1440	penicillin-binding protein 4A, D-alanyl-D-alanine carboxypeptidase	*dacC*	GGT**GTTTTT**TTATTTGATAG**GGGAAAA**TATAAAATG	III
BSU04300	operon_2397	glycosyltransferase YdaM; general stress protein, synthesis of extracellular polysaccharide	*ydaM*	CT**GTTTTC**TTAATGTTCAAAAA**GGGAAA**AAAAAGCTA	III
BSU04270	operon_2397	lipoprotein, may modify the extracellular polysaccharide synthesized by YdaLYdaM and YdaN	*ydaJ*	CT**GTTTTC**TTAATGTTCAAAAA**GGGAAA**AAAAAGCTA	III
BSU04280	operon_2397	general stress protein, may act as c-di-GMP receptor, required for extracellular polysaccharide synthesis by [[protein|YdaL]]-[[protein|YdaM]]-[[protein|YdaN]]	*ydaK*	CT**GTTTTC**TTAATGTTCAAAAA**GGGAAA**AAAAAGCTA	III
BSU04290	operon_2397	general stress protein, synthesis of extracellular polysaccharide	*ydaL*	CT**GTTTTC**TTAATGTTCAAAAA**GGGAAA**AAAAAGCTA	III
BSU04310	operon_2397	general stress protein, synthesis of extracellular polysaccharide	*ydaN*	CT**GTTTTC**TTAATGTTCAAAAA**GGGAAA**AAAAAGCTA	III
BSU33610	operon_465	RNase R, required for protection against paraquat stress	*rnr*	AC**GTTTTT**TTCTGATTAAACTG**TGGAAA**ACTAAAATGA	III
BSU33620	operon_465	general stress protein, carboxylesterase	*yvaK*	AC**GTTTTT**TTCTGATTAAACTG**TGGAAA**ACTAAAATGA	III
BSU25490	operon_989	transcriptional repressor of class I heat-shock genes	*hrcA*	CC**GTTTAT**TTGACTCATCAA**GGGAAA**TTATTAGGCA	III
BSU18690	operon_1410	hypothetical protein	*yoaP*	G**GTTTTA**CAGTTAATTGGCAAAA**TGGAAA**AGAAAAAG	III
BSU23230	operon_1125	hypothetical protein	*ypuF*	**CTTTTT**TCCTTTTATCCCTTCTA**TGGTAC**ACTAAAAG	III
BSU18400	operon_1436	hypothetical protein	*yoeD*	G**GTTTTT**TAGCTTCCGCGTGAAAA**GGGACA**CTTCCAG	III
BSU14820	operon_1646	hypothetical protein	*ylaL*	CGT**GTTTAT**CAGCTTTTGCCAAGGC**GGGAAT**ATAGCT	III
BSU15650	operon_1595	calcium-transporting ATPase	*yloB*	TTG**GTTTTT**TCCCTTGGCACGGATG**TGGAAA**ATCACA	III
BSU12840	operon_1799	low-affinity inorganic phosphate transporter, proton symporter	***pit***	CCT**GTTTT**TTTTCGACAAAA**GGCTAT**ATAATAATTT	III
BSU12850	operon_1799	ypothetical protein	***ykaA***	CCT**GTTTT**TTTTCGACAAAA**GGCTAT**ATAATAATTT	III
BSU35160	operon_384	excinuclease ABC (subunit A), required for transcription-dependent asymmetry in mutation rates of genes in the two orientations	*uvrA*	G**GTTTTT**TATACACAAAAACAGC**TGGAAA**TAAAAAAC	III
BSU35170	operon_384	excinuclease ABC (subunit B), required for transcription-dependent asymmetry in mutation rates of genes in the two orientations	*uvrB*	G**GTTTTT**TATACACAAAAACAGC**TGGAAA**TAAAAAAC	III
BSU08890	operon_2075	hypothetical protein	***ygaO***	T**GTTTAT**TTATTTTTCGTCAA**GGGAAT**ATAAAAATTT	III
BSU08899	operon_2075	similar to transcription regulator Xre family	*ygzD*	T**GTTTAT**TTATTTTTCGTCAA**GGGAAT**ATAAAAATTT	III
BSU32380	operon_547	hypothetical protein	*yunE*	**GTTTTT**AAAATCTCATCAACG**TGGTAT**CTTTTTTTA	III
BSU32390	operon_547	hypothetical protein	*yunF*	**GTTTTT**AAAATCTCATCAACG**TGGTAT**CTTTTTTTA	III
BSU32370	operon_547	hypothetical protein	*yunD*	**GTTTTT**AAAATCTCATCAACG**TGGTAT**CTTTTTTTA	III
BSU32400	operon_547	hypothetical protein	*yunG*	**GTTTTT**AAAATCTCATCAACG**TGGTAT**CTTTTTTTA	III
BSU14030	operon_1708	similar to macrolide-efflux protein, MFS transporter	*ykuC*	CG**GTTTAT**AATATGATAAAGT**GGGACC**TTTAGGGTA	III
BSU15350	operon_1609	N-formyl-4-amino-5-aminomethyl-2-methylpyrimidine deformylase, thiamine salvage pathway	*ylmB*	C**GTTTCA**TCACATTTGAAAC**GGCTTT**TTTGATTTTA	III
BSU08160	operon_2132	hypothetical protein	*yfjB*	AGT**GTTTTT**TCCCATAGAGC**GGGAAA**TTTCTCATTT	III
BSU08150	operon_2132	hypothetical protein	*yfjC*	AGT**GTTTTT**TCCCATAGAGC**GGGAAA**TTTCTCATTT	III
BSU08170	operon_2132	hypothetical protein	*yfjA*	AGT**GTTTTT**TCCCATAGAGC**GGGAAA**TTTCTCATTT	III
BSU08140	operon_2132	lipoprotein	*yfjD*	AGT**GTTTTT**TCCCATAGAGC**GGGAAA**TTTCTCATTT	III
BSU25210	operon_1001	DNA primase, part of the [SW|replisome]	*dnaG*	GG**GTTTTT**GGCTGTGCCAAAA**GGGAAT**AATGAAAAAC	III
BSU25230	operon_1001	hypothetical protein	*yqxD*	GG**GTTTTT**GGCTGTGCCAAAA**GGGAAT**AATGAAAAAC	III
BSU23510	operon_1109	tyrosine recombinase XerD, site-specific integrase/recombinase,	*ripX*	**GTTTAT**ATAACTGGAACCATA**GGGAAA**GGATCGATTA	III

Locus Tag[a]	Operon	Annotation/Description/Function	Gene	SigB PBM	C
		partitioning of the terminus region after replication,			
BSU23519	operon_1109	hypothetical protein	*yqzK*	**GTTTAT**ATAACTGGAACCATA**GGGAAA**GGATCGATTA	III
BSU19220	operon_1360	ATP-dependent DNA helicase RecQ, acts together with RecJ	*yocI*	A**GTTTTA**ACCTTTAAGCTGTAATA**GGGAGT**TTTATAT	III
BSU12500	operon_1809	PBSX prophage, phage-like element PBSX XkdA	*xkdA*	GT**GTTTAT**TTTTTAAAGAAAA**GGGAAA**GATTTCTACA	III
BSU10900	operon_1926	spore coat protein, similar to chloride peroxidase, AB hydrolase superfamily protein YisY	*yisY*	CGG**GTTCAA**TTTAATAGATCGTTA**AGGAAA**TTGATAC	III
BSU12440	operon_1815	response regulator aspartate phosphatase RapA inhibitor, control of the [SW|phosphorelay]	*phrA*	CG**GTTTTC**ACTAAAAGAAAGCAC**GGGTGT**TTGAAAAA	III
BSU12430	operon_1815	response regulator aspartate phosphatase A, dephosphorylates Spo0F-P, control of the phosphorelay	*rapA*	CG**GTTTTC**ACTAAAAGAAAGCAC**GGGTGT**TTGAAAAA	III
BSU06150	operon_2266	D-sorbitol dehydrogenase	*gutB*	GT**GTTAAA**AAGCAGATAAAATG**GGGGCAGTA**ACAGAG	III
BSU12240	operon_1827	similar to amino acid ABC transporter (membrane protein)	*yjkA*	CG**GTTTGT**TCATTATAATAGCG**GGGATG**ATGTTACAT	III
BSU12250	operon_1827	similar to amino acid ABC transporter ATP-binding protein	*yjkB*	CG**GTTTGT**TCATTATAATAGCG**GGGATG**ATGTTACAT	III
BSU27610	operon_853	adenine phosphoribosyltransferase, universally conserved protein	*apt*	A**TTTTT**CAGGTAATGAAATTGAAGC**GGGAAA**TAAAAA	III
BSU27620	operon_853	single-strand DNA-specific exonuclease RecJ	*recJ*	A**TTTTT**CAGGTAATGAAATTGAAGC**GGGAAA**TAAAAA	III
BSU37790	operon_213	catabolic NAD-specific glutamate dehydrogenase RocG, trigger enzyme and effector protein for GltC	*rocG*	AT**GTTTTC**AGTCTCTTTTTTTG**TGGATT**CAAAAGCTG	III
BSU12860	operon_1798	serine/ threonine exchanger transporter SteT	*steT*	TC**GTTTAT**AGACCATTAAAAC**AGGTAT**TTCTACAGTT	III
BSU24290	operon_1061	similar to exodeoxyribonuclease VII (small subunit)	*yqiC*	G**GTTTTC**TTTAGTTATGGTGC**AGGAAT**GCTTAGGAGA	III
BSU24300	operon_1061	similar to exodeoxyribonuclease VII (large subunit)	*yqiB*	G**GTTTTC**TTTAGTTATGGTGC**AGGAAT**GCTTAGGAGA	III
BSU24280	operon_1061	farnesyl diphosphate synthase	*yqiD*	G**GTTTTC**TTTAGTTATGGTGC**AGGAAT**GCTTAGGAGA	III
BSU30470	operon_684	hypothetical protein	*ytzC*	GC**GTTTTT**CCGTATTTGAATA**AGGAAA**AGGATTGTTC	III
BSU40700	operon_28	hypothetical protein	*yybB*	CCG**GTTTTA**CTTCAGAGGAAA**AGGAAA**CCATTTCTGCA	III
BSU27150	operon_884	hypothetical protein	*yrhK*	**GTTTAC**ATGCCGTTTACGTAA**GGGTAA**GCCAAAATTG	III
BSU29700	operon_728	acetoin utilization protein AcuB	*acuB*	AT**GTTGAA**AACGCTTTATAATT**TGGTAT**TCTTAAAGA	III
BSU29710	operon_728	acetoin utilization protein AcuC	*acuC*	AT**GTTGAA**AACGCTTTATAATT**TGGTAT**TCTTAAAGA	III
BSU29690	operon_728	acetoin utilization protein AcuA	*acuA*	AT**GTTGAA**AACGCTTTATAATT**TGGTAT**TCTTAAAGA	III
BSU29190	operon_762	6-phosphofructokinase	*pfkA*	CG**GTTGAA**GATCAATATATCG**GGGTAA**ACTAAATAA	III
BSU29180	operon_762	pyruvate kinase, glycolytic enzyme	*pyk*	CG**GTTGAA**GATCAATATATCG**GGGTAA**ACTAAATAA	III
BSU01010	operon_2610	transcription termination/antiterminationprotein NusG	*nusG*	**CTTTTT**GCCCTCCTTGACAC**AGGAAT**TTCTCAATTA	III
BSU19420	operon_1343	UDP-glucosyltransferase YojK, similar to macrolide glycosyltransferase	*yojK*	TG**CTTTTT**GTCTTGAAGACA**GGGAAT**ATAAACTGGT	III
BSU33760	operon_456	sporulation-delaying SdpB membrane protein, required for SdpC toxin maturation	*sdpB*	AT**GATTAT**ATTAGCTTAGAGG**AGGTAA**TCTACATCAA	III
BSU33770	operon_456	killing factor SdpC, toxin, collapses the proton motive force and induces autolysis, kills non-sporulating cells, induces activity of SigW	*sdpC*	AT**GATTAT**ATTAGCTTAGAGG**AGGTAA**TCTACATCAA	III
BSU33750	operon_456	sporulation-delaying protein SdpA, required for SdpC toxin maturation	*sdpA*	AT**GATTAT**ATTAGCTTAGAGG**AGGTAA**TCTACATCAA	III
BSU18830	operon_1396	phosphoenolpyruvate synthase, similar to rifampicin phosphotransferase	*pps*	**GTTTTT**TAATGCGACTAGCTA**TGGAAA**TTAAGAAAAG	III
BSU30380	operon_687	bacitracin export ATP-binding protein BceA, or the export of bacitracin, plectasin, mersacidin and actagardine	*bceA*	**CTTTTT**TGTTCGCCGTATCGA**AGGAAA**AGCCCGGCAT	III
BSU30370	operon_687	bacitracin export permease protein BceB, ABC transporter (permease) for the export of bacitracin, plectasin, mersacidin and actagardine, sensory component of the Bce regulatory & detoxification system	*bceB*	**CTTTTT**TGTTCGCCGTATCGA**AGGAAA**AGCCCGGCAT	III
BSU06220	operon_2261	hypothetical protein	*ydjJ*	TT**GTTTCA**GACTCAGGATAAA**AGGAAA**TAGAGAAGAA	III
BSU29805	operon_722	DNA translocase SftA,, translocation of non-segregated chromosomes prior to septum closure	*sftA*	T**CTTTTC**TGGGCCGACCAGCAG**TGGTAT**ACTAAAAACC	III
BSU17550	operon_1501	hypothetical protein	*ynaG*	T**TTTTTT**AAAAACGATTTATC**AGGAAA**GGCAGAATTA	III
BSU17540	operon_1501	hypothetical protein	*ynaF*	T**TTTTTT**AAAAACGATTTATC**AGGAAA**GGCAGAATTA	III
BSU25910	operon_961	hypothetical protein, holin, skin element	*yqxH*	AC**GTTTGC**CACTAGCACAAA**AGGAAA**ATCAGAAGCTT	III

Locus Tag[a]	Operon	Annotation/Description/Function	Gene	SigB PBM	C
BSU16799	operon_1552	germination protein, required for TepA activity	*ylzJ*	T**CTTTAT**TTTTTGTCCTTCAAGCC**GGGATT**GAATGAA	III
BSU16790	operon_1552	translocation-enhancing protein TepA, orphan ClpP- like germination protease, contributes to SASP degradation	*tepA*	T**CTTTAT**TTTTTGTCCTTCAAGCC**GGGATT**GAATGAA	III
BSU21090	operon_1250	hypothetical protein	*yonH*	GGT**GTTTTG**AACCCTTTAAAATCAC**TGGTAT**ATTTTT	III
BSU19290	operon_1353	hypothetical protein	*yozO*	CG**GTTTTTT**TTGTGGTTAAAAC**AGGAAA**TATAAAACAA	III
BSU16010	operon_1579	hypothetical protein	*ylqD*	C**CTTTTT**TTACACGCAAAAAAA**GGGTAA**ACTGATAGAA	III
BSU16020	operon_1579	ribosome maturation factor, 16S rRNA processing protein, RNase	*rimM*	C**CTTTTT**TTACACGCAAAAAAA**GGGTAA**ACTGATAGAA	III
BSU16030	operon_1579	tRNA methyltransferase, methylates G37 residue at the N1 position	*trmD*	C**CTTTTT**TTACACGCAAAAAAA**GGGTAA**ACTGATAGAA	III
BSU38190	operon_192	galactose-1-phosphate uridylyltransferase	*galT*	CC**GTTTTC**TATGAAGAAATAAG**GGGAAT**AAAAAATAAA	III
BSU38200	operon_192	galactokinase	*galK*	CC**GTTTTC**TATGAAGAAATAAG**GGGAAT**AAAAAATAAA	III
BSU24200	operon_1067	extracellular lipoprotein	*yqiH*	CTC**GTTATC**TTTCTAACAAC**AGGAAA**TGATCGGGTC	III
BSU24190	operon_1067	secreted N-acetylmuramoyl-L-alanine amidase	*yqiI*	CTC**GTTATC**TTTCTAACAAC**AGGAAA**TGATCGGGTC	III
BSU14640	operon_1660	hypothetical protein	*yktA*	**CTTTTT**TATGGCCGGGAATG**TGGAAT**TGAACAGGCT	III
BSU02030	operon_2560	serine/threonine protein kinase D	*prkD*	**CTTTTT**TATTCTCCGTCTAGA**AGGTAA**ACTATAGTCA	III
BSU01650	operon_2580	hypothetical protein	*ybbC*	CG**GTTTTT**TATTGACCTATGCATT**CGGATAT**AAAGAT	III
BSU01660	operon_2580	N-acetylglucosaminidase, lipoprotein	*nagZ*	CG**GTTTTT**TATTGACCTATGCATT**CGGATAT**AAAGAT	III
BSU01670	operon_2580	N-acetylmuramyl-L-alanine amidase	*amiE*	CG**GTTTTT**TATTGACCTATGCATT**CGGATAT**AAAGAT	III
BSU40980	operon_6	hypothetical protein	*yyaB*	AT**GTTTTT**TTGACTTAAAAA**AGGAAT**TTCTTAAAGA	III
BSU36730	operon_282	hypothetical protein	*ywmD*	CC**GTTTTT**CTGTCATAATCAA**GGCGAA**AAAAGAATGAA	III
BSU08620	operon_2100	hypothetical protein	*yfhP*	CT**GTTTTT**TGTTGTACACTAA**GGGCAC**AGTACGATAA	III
BSU29140	operon_766	citrate synthase 2	*citZ*	**GTTTTT**ACAACACTCTTAAAG**GGGAAA**TTTATTGAAA	III
BSU25700	operon_976	lipoprotein	*yqeF*	T**GTTTTT**CAGATTGGAAAGAA**GGGAAT**TTTAAAAAAT	III
BSU18390	operon_1437	integrase/recombinase YoeC	*yoeC*	AG**GTTTTT**TGATGATTAGTAGG**GGGATA**AGTATCCAA	III
BSU18800	operon_1399	beta-lactamase	*penP*	G**GTTTGT**TACACTAACTGTCAT**GGGAAA**CATTTCAAA	III
BSU11040	operon_1916	hypothetical protein	*yitM*	T**GTTTTT**CCTTTTAACAAAAA**TGGAAT**TTCTCATACT	III
BSU11070	operon_1916	hypothetical protein	*yitP*	T**GTTTTT**CCTTTTAACAAAAA**TGGAAT**TTCTCATACT	III
BSU11055	operon_1916	hypothetical protein	*yitO*	T**GTTTTT**CCTTTTAACAAAAA**TGGAAT**TTCTCATACT	III
BSU02310	operon_2538	hydrolase YbfO, erythromycin esterase	*ybfO*	G**GTTTTA**CTTTCATCAGGTGATGA**GAGAAT**CCAGCTA	III
BSU33490	operon_475	copper(I)-transporting ATPase, cadmium transporting ATPase, resistance to cadmium	*cadA*	T**GTTTTT**CATTGACACTTTCT**TGGAAA**ACAACATATA	III
BSU33630	operon_464	protein-export membrane protein SecG, preprotein translocase subunit	*secG*	C**CTTTGA**CCTTTTCATATGAATA**GGGTAA**CCAAGATA	IV
BSU09540	operon_2032	acyl-ACP:1-acylglycerolphosphate acyltransferase	*plsC*	GA**GTTTAT**GGCCCCGCCGACCAGA**GAGGAA**ATATAAC	IV
BSU00170	operon_2655	general stress protein, similar to isochorismatase	***yaaI***	**GTTTTT**TCATTGCCTAAAA**AGGCTAC**ATATTAACTA	IV
BSU32130	operon_566	GMP reductase	*guaC*	AT**GTTTGA**CATCCTAAATAAAACAGAGTACATTATAT	IV
BSU01640	operon_2581	AraC family transcriptional regulator, AraC family DNA-binding domain fused to protein FeuA	*btr*	TA**CTTTTA**CACATAAGTAA**GGGTTTT**TTTCATGCTA	IV
BSU02450	operon_2528	two-component response regulator GlnL, regulation of the glsA-glnT operon	*glnL*	GC**GTTAAA**CGCTTTCATAATAG**CGGAAG**GTATAATTA	IV
BSU02440	operon_2528	two-component sensor kinase, regulation of the [[gene|glsA]]-[[gene|glnT]] operon	*glnK*	GC**GTTAAA**CGCTTTCATAATAG**CGGAAG**GTATAATTA	IV
BSU34200	operon_429	RNA polymerase sigma-54 factor, Sigma-54, Sigma L	*sigL*	T**GTTTTA**AATTCTCAATAAAATAA**GGGACG**GAAAAAA	IV
BSU25430	operon_991	threonylcarbamoyladenosine tRNAmethylthiotransferase MtaB	*mtaB*	**TTTTTT**CTTGCCCGGGATAAA**AGGAAT**TGAAAAATCA	IV
BSU25440	operon_991	ribosomal RNA small methyltransferaseE	*yqeU*	**TTTTTT**CTTGCCCGGGATAAA**AGGAAT**TGAAAAATCA	IV
BSU25460	operon_991	chaperone protein DnaJ, heat-shock activator protein, DnaK	*dnaJ*	**TTTTTT**CTTGCCCGGGATAAA**AGGAAT**TGAAAAATCA	IV
BSU25450	operon_991	ribosomal protein L11methyltransferase,	*yqeT*	**TTTTTT**CTTGCCCGGGATAAA**AGGAAT**TGAAAAATCA	IV
BSU31510	operon_606	hypothetical protein	*yufK*	T**GTTTTA**AGTCCTCCATAATAAAT**GAGGTA**GATTATA	IV
BSU17500	operon_1505	hypothetical protein	*ynaB*	GG**GTTTCC**TTATGGCTCAACAGATA**GGGTAG**TAATTA	IV
BSU30220	operon_696	8-amino-7-oxononanoate synthase	*bioF*	AT**TGTTAA**CCTTTGAATATAATT**GGTTAA**CAATTTAG	IV
BSU30230	operon_696	BioA aminotransferase	*bioA*	AT**TGTTAA**CCTTTGAATATAATT**GGTTAA**CAATTTAG	IV
BSU30200	operon_696	biotin synthase	*bioB*	AT**TGTTAA**CCTTTGAATATAATT**GGTTAA**CAATTTAG	IV
BSU30210	operon_696	ATP-dependent dethiobiotin synthetase BioD	*bioD*	AT**TGTTAA**CCTTTGAATATAATT**GGTTAA**CAATTTAG	IV
BSU30240	operon_696	pimeloyl-CoA synthase (6-carboxyhexanoate-CoA ligase)	*bioW*	AT**TGTTAA**CCTTTGAATATAATT**GGTTAA**CAATTTAG	IV
BSU19430	operon_1342	sporulation-specific diadenylate cyclase, synthesis of c-di-AMP	*cdaS*	CG**GTTTCG**TTTTTCTAAACATTT**GGGCAT**AAATAAAA	IV

Locus Tag[a]	Operon	Annotation/Description/Function	Gene	SigB PBM	C
BSU33680	operon_460	HTH-type transcriptional regulator, transcription repressor of *cat D* & E	*catR*	CC**GATTTTC**AATAGTCAAAAAT**GGGAAC**ATCTCTCAAA	IV
BSU37610	operon_224	hypothetical protein	*ywzC*	**TTTTTT**GAAACGTGATTTT**AGGTTA**TTCACATAGAT	IV
BSU13490	operon_1754	stress-responsive membrane protease	***htpX***	T**GTTTTA**TAATTTAAATATAC**GGGTTC**TTTTGAACTT	IV
BSU01530	operon_2589	N-acetylmuramoyl-L-alanine amidase, spore cortex peptidoglycan synthesis	*cwlD*	**GTTTTTT**GATTATCTCTATAAT**TGGATA**CACGAGCAA	IV
BSU09510	operon_2034	anti-sigma-M factor YhdL	*yhdL*	AA**CTTTAA**ACCTTTCTTATGC**GTGTAT**AACATAGAG	IV
BSU09500	operon_2034	anti-sigma-M factor YhdK	*yhdK*	AA**CTTTAA**ACCTTTCTTATGC**GTGTAT**AACATAGAG	IV
BSU09520	operon_2034	RNA polymerase sigma factor SigM, adapt inhibitors of peptidoglycan synthesis	*sigM*	AA**CTTTAA**ACCTTTCTTATGC**GTGTAT**AACATAGAG	IV
BSU06510	operon_2242	phosphoribosylglycinamide formyltransferase	*purN*	C**CTTTCT**CTTTCTGCCCTCGTTCGGGG**AGATAT**TTTG	IV
BSU06530	operon_2242	phosphoribosylglycinamide synthetase	*purD*	C**CTTTCT**CTTTCTGCCCTCGTTCGGGG**AGATAT**TTTG	IV
BSU06520	operon_2242	phosphoribosylaminoimidazole carboxamide formyltransferase,	*purH*	C**CTTTCT**CTTTCTGCCCTCGTTCGGGG**AGATAT**TTTG	IV
BSU06500	operon_2242	phosphoribosylaminoimidazole synthetase	*purM*	C**CTTTCT**CTTTCTGCCCTCGTTCGGGG**AGATAT**TTTG	IV
BSU35680	operon_356	galactosamine-containing minor teichoic acid biosynthesis, membrane protein	*ggaB*	A**TTTTT**AAAAGTTTGCTTACAA**GGGTTT**TTAAAAAAA	IV
BSU34810	operon_398	TPR repeat-containing protein YvcD	*yvcD*	G**CTTTTG**TTTGTTGAAAAAG**GGGAAA**TTACCGGTCG	IV
BSU12890	operon_1796	glycosyltransferase YkcC	*ykcC*	TGGCTTAAAACC**CTTTGC**AAATATGGGTGAT**TGGTAT**	IV
BSU12880	operon_1796	mannosyltransferase YkcB	*ykcB*	TGGCTTAAAACC**CTTTGC**AAATATGGGTGAT**TGGTAT**	IV
BSU35660	operon_358	UDP-N-acetylglucosamine 2-epimerase	*mnaA*	T**GTTTTT**ACACATTTTTATGATAT**CGGATA**AACCATT	IV
BSU29220	operon_760	NAD-dependent malic enzyme	*ytsJ*	GC**GTTTAT**CTCTATTATGAA**AGGCAA**AAACAGACGAT	IV
BSU38030	operon_203	hypothetical protein	*ywdA*	T**GTTTCT**CGACGCGTCATCTG**TGGAAA**TCTTTATCAA	IV
BSU00930	operon_2613	serine O-acetyltransferase	*cysE*	**CTTTTT**TTATTGGGTAGA**GGAAAT**CAGATAGAGAAAC	IV
BSU00950	operon_2613	RNase Mini-III, mini-ribonuclease 3	*mrnC*	**CTTTTT**TTATTGGGTAGA**GGAAAT**CAGATAGAGAAAC	IV
BSU00960	operon_2613	23S rRNA(guanosine(2251)-2'-O)-methyltransferase RlmB	*yacO*	**CTTTTT**TTATTGGGTAGA**GGAAAT**CAGATAGAGAAAC	IV
BSU00970	operon_2613	ribosome -associated endoribonuclease	*yacP*	**CTTTTT**TTATTGGGTAGA**GGAAAT**CAGATAGAGAAAC	IV
BSU00940	operon_2613	cysteine-tRNA synthetase	*cysS*	**CTTTTT**TTATTGGGTAGA**GGAAAT**CAGATAGAGAAAC	IV
BSU33550	operon_469	hypothetical protein	*yvaC*	C**GTTTTT**GGGCATACTAAAGCG**AAGAAA**CTGGAAAAG	IV
BSU29600	operon_737	branched-chain amino acid transporter	*braB*	**TTTTTT**ACTCCTTTATGAAAG**CGGTAC**AAGGTATGAT	IV
BSU22150	operon_1189	ATP-dependent helicase YpvA, similar to ATP-dependent helicase	*ypvA*	A**GTTTTT**TATTGTATTCATCAG**CGGAAA**ATGATATTA	IV
BSU09900	operon_2004	similar to ABC transporter membrane protein	*yhaP*	GT**CTTTCT**TCATTTATATTCTAAAA**AGGAAA**TCAAAA	IV
BSU09890	operon_2004	similar to ABC transporter membrane protein	*yhaQ*	GT**CTTTCT**TCATTTATATTCTAAAA**AGGAAA**TCAAAA	IV
BSU15300	operon_1614	bacillopeptidase F	*bpr*	**TTTTT**ATCATTTTCATATATA**TGGAAA**TTGAATGACA	IV
BSU04990	operon_2356	hypothetical protein	*yddJ*	CAG**GTTAAG**GGAAATGGATAAAAGG**TGGAAA**AGATCA	IV
BSU12030	operon_1848	hypothetical protein	*yjdF*	T**GTTACA**CTCTTTTCTGTATA**TGGTAA**AGAATGAAAA	IV
BSU04250	operon_2399	AsnC family transcriptional regulator protein-DNA complexes	*lrpC*	A**TTTTT**ACATGCCTTTTCTA**GGGAAC**TGTACTTGTC	IV
BSU10030	operon_1991	Hit-like protein involved in cell-cycle regulation	*hit*	CT**GTTTTC**TATTTTCTGCATTTCTG**TGGTAC**GATGAA	IV
BSU07400	operon_2186	similar to multidrug-efflux transporter	*yfmO*	TT**GTTAAA**CGCTATTTTTGTA**GCGTTT**CTTTTAATGG	IV
BSU32719	operon_528	hypothetical protein	*yuzK*	**GTTTTT**GCGGCATCCAACGCAGCAC**TGGTAC**AATAAA	IV
BSU26920	operon_899	hypothetical protein	*yraJ*	TAT**GTTCTA**TCGTTTCTTAAAATGA**AGGAAT**TGAAGA	IV
BSU38229	operon_190	transcriptional regulator SlrA, negative regulator of the fla-che operon, anti-repressor, antagonist SinR	*slrA*	**CTTTTA**TTGCCCGCATGTTGC**AGGAAA**ACATTTTATA	IV
BSU06270	operon_2255	hypothetical protein	*ydjO*	A**TTTTTC**TCTACAGCAGCTG**GGGAAC**ACTTGCGCAG	IV
BSU35790	operon_348	mannose-6-phosphate isomerase YvyI	*pmi*	GC**GTTTGT**AAGAGGGGCGCACC**GGGAAA**ATGATGATC	IV
BSU25720	operon_974	hypothetical protein	*yqeD*	CTG**GTTTTC**AGTTGAACAGCTGAAA**TGGCAC**CAGTT	IV
BSU12630	operon_1804	phage-like element PBSX protein XkdI	*xkdI*	CC**TGTTAA**CGGACAGAGAAA**AGGAAA**TGTTTTTGCT	IV
BSU12620	operon_1804	phage-like element PBSX protein XkdH	*xkdH*	CC**TGTTAA**CGGACAGAGAAA**AGGAAA**TGTTTTTGCT	IV
BSU12570	operon_1804	PBSX phage terminase small subunit	*xtmA*	CC**TGTTAA**CGGACAGAGAAA**AGGAAA**TGTTTTTGCT	IV
BSU12619	operon_1804	unknown PBSX prophage protein	*ykzL*	CC**TGTTAA**CGGACAGAGAAA**AGGAAA**TGTTTTTGCT	IV
BSU12660	operon_1804	phage-like element PBSX protein XkdM,	*xkdM*	CC**TGTTAA**CGGACAGAGAAA**AGGAAA**TGTTTTTGCT	IV
BSU12640	operon_1804	phage-like element PBSX protein XkdJ	*xkdJ*	CC**TGTTAA**CGGACAGAGAAA**AGGAAA**TGTTTTTGCT	IV
BSU12650	operon_1804	phage-like element PBSX protein XkdK	*xkdK*	CC**TGTTAA**CGGACAGAGAAA**AGGAAA**TGTTTTTGCT	IV
BSU12649	operon_1804	hypothetical protein	*ykzM*	CC**TGTTAA**CGGACAGAGAAA**AGGAAA**TGTTTTTGCT	IV
BSU12600	operon_1804	phage-like element PBSX protein XkdF	*xkdF*	CC**TGTTAA**CGGACAGAGAAA**AGGAAA**TGTTTTTGCT	IV
BSU12610	operon_1804	phage-like element PBSX protein XkdG	*xkdG*	CC**TGTTAA**CGGACAGAGAAA**AGGAAA**TGTTTTTGCT	IV
BSU12590	operon_1804	phage-like element PBSX protein XkdE	*xkdE*	CC**TGTTAA**CGGACAGAGAAA**AGGAAA**TGTTTTTGCT	IV
BSU12580	operon_1804	PBSX phage terminase large subunit	*xtmB*	CC**TGTTAA**CGGACAGAGAAA**AGGAAA**TGTTTTTGCT	IV
BSU14630	operon_1661	arginine decarboxylase for biofilm	*speA*	CG**GTTGTC**CAATCTTCATTCATC**GGATAT**TGATACTC	IV
BSU15230	operon_1617	UDPacetylenolpyruvoylglucosamine reductase	*murB*	TAT**GTTATA**GAGAAACTTAGACTGG**GGGAAA**AAAGAA	IV
BSU15220	operon_1617	N-acetylglucosamine transferase	*murG*	TAT**GTTATA**GAGAAACTTAGACTGG**GGGAAA**AAAGAA	IV
BSU32120	operon_567	hypothetical protein	*yuzG*	**CTTTCT**TTATGGCATGGACAT**GGGAAA**GGAGCGAATA	IV

Locus Tag[a]	Operon	Annotation/Description/Function	Gene	SigB PBM	C
BSU35670	operon_357	UTP-glucose-1-phosphate uridylyltransferase, GSP	***gtaB***	A**GTTTTA**ATATTAAGGATAAAGCTT**GTTTAA**AAATGG	V
BSU40000	operon_79	Oxidoreductase, GSP	*yxnA*	AG**GGGTAA**GACCCTTCCGGATG**GGGTAA**TGTACAAAA	V
BSU28190	operon_820	GTP-binding protein	*ysxC*	**CTTTTT**CATATCAGAAAGAAA**GGGTAT**ACTACGAGGA	V
BSU28200	operon_820	class III heat-shock ATP-dependent protease	*lonA*	**CTTTTT**CATATCAGAAAGAAA**GGGTAT**ACTACGAGGA	V
BSU19120	operon_1369	Repressor of *cadA* and *czcD*	*czrA*	C**CTTTTT**TATTATGACATAAGA**GGGTAT**ATCAGACCAA	V
BSU34110	operon_435	PP2C phosphatase	*rsbP*	CACATGAGCACCTCCTGATC**GTTTAC**ATTGATTGTA	V
BSU34100	operon_435	alpha/beta hydrolase for RsbP activation	*rsbQ*	CACATGAGCACCTCCTGATC**GTTTAC**ATTGATTGTA	V
BSU29210	operon_761	acetyl-CoA carboxylase (beta subunit)	*accD*	GG**GTTTAA**CATTATTTTTGAATGCGCTGTCAACCTTG	V
BSU29200	operon_761	acetyl-CoA carboxylase (alpha subunit)	*accA*	GG**GTTTAA**CATTATTTTTGAATGCGCTGTCAACCTTG	V

Supplementary Table S3: The list of newly predicted SigB regulon genes with a SigB PBM in this study

Bacillus subtilis	**Genotype**	**References**
168	*TrpC2*	Kunst et al., 1997; Zeigler et al., 2008; Barbe et al., 2009
BY47	168 *ΔsigB*	This study
BY62	168 *trpC2 amyE::* P*ylaL-lacZ aphA*	This study
BY63	168 *trpC2 amyE::* P*ykaA-lacZ aphA*	This study
BY64	168 *trpC2 amyE::* P*ygaO-lacZ aphA*	This study
BY65	168 *trpC2 amyE::* P*ywzA-lacZ spc*	This study
BY66	168 *trpC2 amyE::* P*yaaI-lacZ spc*	This study
BY67	168 *trpC2 amyE::* P*rsbV-lacZ spc*	This study
BY69	168 *trpC2 amyE::* P*ytoQ-lacZ aphA*	This study
BY70	168 *trpC2 amyE::* P*pucI-lacZ aphA*	This study
BY71	168 *trpC2 amyE::* P*gtaB-lacZ aphA*	This study
BY72	168 Δ*sigB amyE::* P*rsbV-lacZ spc*	This study
BY82	168 Δ*sigB amyE::* P*ylaL-lacZ aph*	This study
BY83	168 Δ*sigB amyE::* P*ykaA-lacZ aph*	This study
BY84	168 Δ*sigB amyE::* P*ygaO-lacZ aph*	This study
BY85	168 Δ*sigB amyE::* P*ywzA-lacZ spc*	This study
BY86	168 Δ*sigB amyE::* P*yaaI-lacZ spc*	This study
BY89	168 Δ*sigB amyE::* P*ytoQ-lacZ aphA*	This study
BY90	168 Δ*sigB amyE::* P*pucI-lacZ aphA*	This study
BY91	168 Δ*sigB amyE::* P*gtaB-lacZ aphA*	This study

Escherichia coli	**Genotype**	**References**
TOP10	*F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(Str^R) endA1 λ-*	Commercially available from Invitrogen

Plasmid	**Construction and description**	**References**
pDR244	Cre- recombinase plasmid with spectinomycin antibiotic cassette	Koo et al., 2017
pNZ5319	Cre-lox plasmid with chloramphenicol and erythromycin antibiotic cassettes	Lambert et al., 2006
pAC7	Translational fusion to *lacZ aph,* ingetration at the *amyE* locus	Weinrauch et al. 1991
pDG1728	Transriptional fusion to *lacZ spc* ingetration at the *amyE* locus	Guérout-Fleury et al., 1996
pBP638	P*ytoQ-lacZ*-PAC7	Rosenberg et al., 2017
pNW2205	P*rsbV-lacZ*-pDG1728	Constructed by Manisha Pandey, courtesy Dr. N. Stanley Wall, University Dundee, UK
pCY22	P*yaaI*-pDG1728	This study
pCY23	P*ywzA*-pDG1728	This study
pCY26	P*pucI*-lacZ-PAC7	This study
pCY27	P*gtaB*-lacZ-PAC7	This study
pCY31	P*ylaL- lacZ-PAC7*	This study
pCY32	P*ykaA- lacZ-PAC7*	This study
pCY33	P*ygaO- lacZ-PAC7*	This study

Supplementary Table S4: Oligonucleotides used in this study

Oligonucleotides	Description	Sequence 5' to 3'
CY51	pNZ5319 *cat* gene check reverse	TAGGATCCAAGTACAGTCGG
CY316	*sigB* left flank FWD	TTCTTGGAGCGTCCTGATCTG
CY317	*sigB* left flank REV *cat* overhang	TTTAAACGTGCTCCAGTGGCGATCAACTCGCTCCCCATTTAAATA
CY318	pNZ5319 cat check FWD overhang	AATGGGGAGCGAGTTGATCGCCACTGGAGCACGTTTAAAC
CY319	pNZ5319 cat check REV overhang	TTTTCTTCAACCTGGATCATCGTCTTCTCGTAGCGATCGG
CY320	*sigB* right flank FWD *cat* overhang	CCGATCGCTACGAGAAGACGATGATCCAGGTTGAAGAAAACGAG
CY321	*sigB* right flank REV	TGCTTCAGCGCCTTCTAATACA
CY322	*sigB* genomic DNA check FWD	AGGACTCGTTCTCGGCATCT
CY323	*sigB* genomic DNA check REV	TCTGGATATGCGTCTCTCGGA
CY414	PrsbV-pNW2205 check forward	CAATTCGATCAGCATCTGGAAAAGG
CY415	PrsbV-pNW2205 check reverse	TCAAATTTTCCTTCAAATCACTAGTTGC
CY328	*PywzA EcoRI* overhang	TAAGCAGAATTCCCTCGTCTAGCATCTTTTCACAC
CY329	*PywzA BamHI* overhang	CCGCCGGATCCCTCATCATGCAACACATCCTAT
CY334	*PyaaI EcoRI* overhang	TATCGAGGATCCAATCGACAGTCTCCTTTCCGT
CY335	*PyaaI BamHI* overhang	TCTGTGAATTCGCACGCGCTATAATTTCACCA
CY384	*amyE* genomic integration check FWD	CTGGCTTACAGAAGAGCGGT
CY385	*amyE* genomic integration check REV	CCCGCTCCGATTAAAGCTACT
CY414	*PrsbV*-pNW2205 check FWD	CAATTCGATCAGCATCTGGAAAAGG
CY415	*PrsbV*-pNW2205 check REV	TCAAATTTTCCTTCAAATCACTAGTTGC
CY419	*PpucI BamHI* overhang	TTTGGATCCATAGCCGGCTCCCCTTTCC
CY420	*PpucI EcoRI* overhang	TTTGAATTCAAGAAAGAGACGGGCTCTGAG
CY421	*PgtaB EcoRI* overhang	TTTGAATTCGGTCCCGAAAACGGTCATCA
CY422	*PgtaB BamHI* overhang	TTTGGATCCATTTAAAAGGCACCTTCCTTATGATCGAAAAA
CY423	*PylaL BamHI* overhang	AAAGGATCCATGATATGACCCCCTTCTCTCAAAAT
CY424	*PylaL EcoRI* overhang	AAAGAATTCGCAAGGGAAAGGACTTTCGATATACT
CY425	*PykaA BamHI* overhang	AAAGGATCCATTAAAAACCCTCCATAGTTTGAATGAAC
CY426	*PykaA EcoRI* overhang	AAAGAATTCTGAAACAGAAGATAAAAGTTGACAGGAAA
CY427	*PygaO BamHI* overhang	TTTGGATCCATGACGTCTGTCCCCTTCAC
CY428	*PygaO EcoRI* overhang	AAAGAATTCTTGGAAGGCTGAGGTTTTACCA

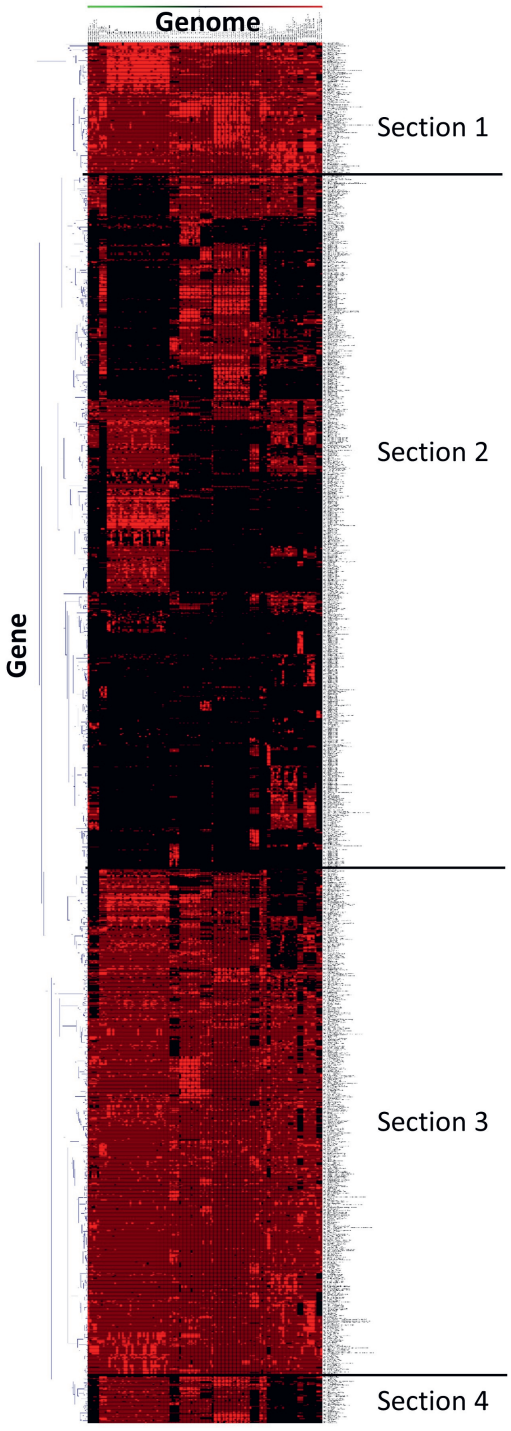
Following supplementary materials contained large confidential data files: available upon request (tjakko.abee@wur.nl) or (kahyen.yeak@wur.nl)

Supplementary Table S2b: functional distribution of SigB regulon candidates

Supplementary Table S5: Presence and absence of *B. subtilis* 168 SigB regulon genes described to date October 2020 in other Bacillales

Supplementary Table S6: Predicted putative species-specific SigB regulon genes in 19 *Bacillus subtilis* genomes and 106 Bacillales members

Supplementary Table S7: Presence and absence of genes involved in SigB sensing modules in Bacillales described to date October 2020



Supplementary Figure S1- Heat map of SigB regulon members with SigB promoter binding motifs in 125 Bacillales.*original resolution picture is available upon request[1]. **Section 1** of the heat map shows that predicted SigB regulon genes with/without a putative SigB PBM were found in nearly every inspected species. This includes orthologs of genes with SigB PBMs in *B. subtilis* such as *rsbV, rsbW,* and *sigB* itself. However, in other species, not all genes harbor a SigB PBM (bright red = ortholog present with a predicted SigB PBM, dark red = ortholog present without SigB PBM, black = absence of ortholog). For example, some *B. subtilis* SigB regulon genes had orthologs in *B. cereus, Listeria* spp., and *Staphylococcus* spp., but only the orthologs in *B. cereus* had a predicted SigB PBM (genes indicated in bright red for *B. cereus*, and dark red for other species) (Supplementary Table S6). **Section 2** presents the predicted species-specific SigB regulon genes, with or without a SigB PBM. Some of these predicted SigB regulon genes were the orthologs of the *B. subtilis* 168 SigB regulon members, and some are uniquely identified for the different species (Supplementary Table S6). This section of the heat map showed that a group of putative SigB regulon genes with SigB PBMs are unique to *B. subtilis* and group members (including *B. licheniformis, B. pumilus, B. vallismortis, B. valezensis,* and *B. amyloliquafaciens*) but not *B. cereus, Geobacillus, L. monocytogenes,* and *S. aureus* (illustrated in black), and vice versa. **Section 3** of the heat map presents *B. subtilis* SigB regulon members contained orthologs in nearly all of the inspected genomes. However, these genes did not have a predicted SigB PBM (dark red), implying that they are either not regulated by SigB or indirectly regulated by SigB. Lastly, **Section 4** shows a group of genes that had orthologs in the *Bacillus* species, but these were utterly absent in more distantly related species such as *Geobacillus* spp., *Listeria* spp., and *Staphylococcus* spp.

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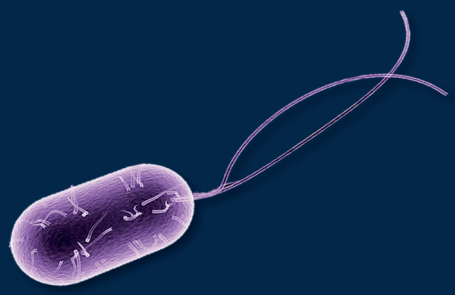
“The well-defined function is not the only observed crucial function”

CHAPTER 3



SigB modulates expression of novel SigB regulon members via Bc1009 in non-stressed and heat-stressed cells revealing its alternative roles in *Bacillus cereus*

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Abstract

The *Bacillus cereus* Sigma B (SigB) dependent general stress response is activated via the two-component RsbKY system, which involves a phosphate transfer from RsbK to RsbY. It has been hypothesized that the Hpr-like phosphocarrier protein (Bc1009) encoded by *bc1009* in the SigB gene cluster may play a role in this transfer, thereby acting as a regulator of SigB activation. Alternatively, Bc1009 may be involved in activation of a subset of SigB regulon members. We first investigated the potential role of *bc1009* to act as a SigB regulator but ruled out this possibility as the deletion of *bc1009* did not affect the expression of *sigB* and other SigB gene cluster members. The SigB-dependent functions of Bc1009 were further examined in *B. cereus* ATCC14579 via comparative proteome profiling (backed up by transcriptomics) of wt as well as Δ*bc1009* and Δ*sigB* deletion mutants under heat stress at 42°C. This revealed 284 proteins displaying SigB-dependent alterations in protein expression levels in heat-stressed cells, including a subgroup of 138 proteins for which alterations were also Bc1009-dependent. Next to proteins with roles in stress defense, newly identified SigB and Bc1009-dependent proteins have roles in cell motility, signal transduction, transcription, cell wall biogenesis, and amino acid transport and metabolism. Analysis of lethal stress survival at 50°C after pre-adaptation at 42°C showed intermediate survival efficacy of *Δbc1009* cells, highest survival of wt, and lowest survival of *ΔsigB* cells, respectively. Additional comparative proteome analysis of non-stressed wt and mutant cells at 30°C revealed 96 proteins with SigB and Bc1009-dependent differences in levels, 51 were also identified under heat stress, and 45 showed significant differential expression at 30°C, including proteins with roles in carbohydrate/ion transport and metabolism. Overlapping functions at 30°C and 42°C included proteins involved in motility, and Δ*sigB* and Δ*bc1009* cells showed reduced motility compared to wt cells in swimming assays at both temperatures.

In conclusion, our results extend the *B. cereus* SigB regulon to >300 members, with a novel role of SigB-dependent Bc1009 in the activation of a subregulon of >180 members, conceivably via phosphotransfer-based interactions with other transcriptional regulatory networks. Many of these SigB regulon and subregulon members have unknown functions, which requires further exploration.

3.1 Introduction

Bacillus cereus is a Gram-positive endospore-forming facultative anaerobe bacterium found in soil, invertebrates, plants, and in fresh and stored foods (Ehling-Schulz et al., 2019). It is a foodborne pathogen that can cause emetic and diarrheal disease due to the production of the emetic toxin cereulide in foods (Lund and Granum, 1997; Ehling-Schulz et al., 2019), and the production of diarrhoeal toxins in the human intestine once foods contaminated with *B. cereus* are ingested. The diarrhoeal symptoms can involve non-hemolytic and hemolytic enterotoxin (Nhe and Hbl), and cytotoxin K (Lund et al., 2000; Ceuppens et al., 2012; Ehling-Schulz et al., 2019).

Environmental transmission of *B. cereus* is strongly supported by the production of highly stress-resistant spores (Ehling-Schulz et al., 2019), while the resistance of vegetative cells to various stress conditions is enhanced by the activation of the so-called general stress response (GSR) (van Schaik et al., 2004a). This includes stresses encountered during food processing and preservation (Desriac et al., 2013; Warda et al., 2016). In Gram-positive bacteria, including *B. cereus*, the GSR is governed by the master regulator of stress, i.e., the alternative sigma factor B (SigB) (van Schaik et al., 2004a; Rodriguez Ayala et al., 2020). In the presence of environmental/nutritional stressors (e.g., temperature shifts and starvation), SigB is triggered to reprogram the transcriptional and translational machinery in the cells, resulting in the production of defense proteins that mediate increased survival (Höper et al., 2005; van Schaik et al., 2007; Pané-Farré et al., 2017; Rodriguez Ayala et al., 2020).

In *B. cereus,* SigB is activated via a two-component system, comprising the signal-receiving sensor kinase RsbK and its cognate response regulator, RsbY phosphatase (de Been et al., 2010a; Abee et al., 2011). In the presence of stressors, RsbK autophosphorylates its histidine residue and initiates the transfer of a phosphate group to the C-terminal receiver (REC) domain of the RsbY protein (de Been et al., 2010b; Chen et al., 2015). The phosphorylated RsbY then dephosphorylates the anti-sigma factor antagonist RsbV, increasing its affinity to the anti-sigma factor RsbW and their subsequent association. The formation of RsbVW complexes releases RsbW from SigB, leading to SigB activation (de Been et al., 2010b; Chen et al., 2015, 2017). Without stressors, the methyltransferase RsbM methylates the S-helix of RsbK and prohibits its phosphate transfer to RsbY (Chen et al., 2015). The unphosphorylated RsbY does not dephosphorylate RsbV; thus, RsbW remains bound to SigB to keep SigB inactive.

In *B. subtilis*, SigB activity is controlled by two pathways, which independently sense energy and environmental stresses. Both pathways converge to the anti-anti-sigma factor RsbV (Haldenwang and Losick, 1979; Haldenwang, 1995; Price et al., 2001; Price, 2010). Sensing energy stress, such as low adenosine triphosphate (ATP) levels, requires the activity of the hydrolase RsbQ and phosphatase RsbP, although the specific signal is unknown (Brody et al.,

2001; Price et al., 2001; Nadezhdin et al., 2011). Environmental stress uses the stressosome complex consisting of the RsbR, RsbS, and RsbT sensor proteins. Once environmental stress is sensed, RsbT is activated and released from the stressosome, activating the phosphatase RsbU. Active RsbU dephosphorylates RsbV, in analogy to *B. cereus* RsbY, promoting the partner switching of RsbW bound to SigB to the anti-anti-sigma factor RsbV, resulting in SigB activation (Marles-Wright and Lewis, 2010; Pané-Farré et al., 2017; Rodriguez Ayala et al., 2020).

B. cereus uses the RsbKY system to sense environmental and nutritional cues (van Schaik et al., 2004a). Temperature upshift induces the strongest SigB GSR in *B. cereus*. Ethanol exposure and osmotic upshock activate SigB moderately, while energy stress (i.e., ATP depletion) activates SigB only mildly (van Schaik et al., 2004a). Upon SigB activation, around 30 genes have been reported to be upregulated by SigB in *B. cereus* (van Schaik et al., 2004a, 2007; de Been et al., 2010b), and these genes are referred to as the SigB regulon members. The reported RsbKY-controlled SigB regulon in *B. cereus* and its group members (Scott and Dyer, 2012) is relatively small in comparison to the stressosome-controlled SigB regulons in *B. subtilis* (Nannapaneni et al., 2012; Rodriguez Ayala et al., 2020) and *Listeria monocytogenes* (Liu et al., 2017, 2019), which both have at least > 300 SigB regulon members described to date. Alternative functions of SigB other than its role in the GSR have also been reported for *B. subtilis,* i.e., biofilm formation, sporulation, and fungal control (Reder et al., 2012a, 2012b; Bartolini et al., 2018, 2019), and for *L. monocytogenes*, i.e., virulence, antibiotic resistance and carbon metabolism (Nadon et al., 2002; Gaballa et al., 2018; Liu et al., 2019).

It has previously been suggested that a Hpr-like phosphocarrier protein encoded by *bc1009*, located in the SigB gene cluster, may affect the efficacy of the RsbKY stress sensor and SigB activation (de Been et al., 2010a). However, so far, no evidence has been provided to substantiate this suggestion, and alternative roles of *B. cereus* SigB remain to be identified. Therefore, in this study, we used comparative proteome profiling, backed up by transcriptomics, to determine the *B. cereus* SigB regulon following quantification of differentially expressed genes/proteins in *B. cereus* ATCC14579 wt versus *sigB* (Δ*sigB*) and *bc1009* mutants (Δ*bc1009*) in non-heat-stressed and heat-stressed cells at 30°C and 42°C, respectively. The approach combined with selected phenotyping experiments provides further insight into the *B. cereus* SigB regulon composition and the possible roles of Bc1009 in RsbKY-induced SigB activation and the control of SigB regulon members.

3.2 Materials and Methods

3.2.1 Strains, media, and growth conditions

Bacillus cereus ATCC14579 wild-type (wt) strain (laboratory stock) and its isogenic mutant strains Δ*sigB* and Δ*bc1009* were used in this study (Table 1). The ATCC14579 strain contains virulence genes encoding Nhe, Hbl and CytK, but not the *ces* gene cluster for cereulide production. The two mutants were constructed as described by Warda et al. (2016). Bacteria were routinely cultured in Brain Heart Infusion (BHI) medium (Becton, Dickinson Difco, Breda, The Netherlands) with incubation at 30°C and 200 rpm, unless otherwise stated. Overnight (ON) cultures were prepared by inoculating strains from -80°C stocks to 50 ml Falcon tubes (Greiner BioOne, Alphen aan den Rijn, The Netherlands) containing 5 ml of BHI and incubating the cultures for 16 -18 hours (h). Optical density at 600 nm (OD_{600}) was measured with the spectrophotometer (Amersham Bioscience, Twente, The Netherlands) and used as a measure for cell biomass.

Table 1: Strains and plasmids used in this study

Strain	**Description**	**References**
ATCC14579	*B. cereus* wild type strain	Laboratory collection
FM1004	*B. cereus* Δ*sigB* clean KO mutant	This study
FM1009	*B. cereus* Δ*bc1009* clean KO mutant	This study
FM1642	*B. cereus* Δ*flgG* clean KO mutant	Laboratory collection
Plasmid	**Description**	**References**
pAUL-A	Heat-inducable suicide vector for *B. cereus* group, Erm^R	Chakraborty et al., 1992

3.2.2 Gene expression study

The differential SigB gene cluster expressions in wt, Δ*sigB,* and Δ*bc1009* cells before and after heat shock (30°C versus an upshift from 30°C to 42°C) were compared via Reverse Transcription Quantification Polymerase Chain Reaction PCR (RT-qPCR) as described below.

3.2.2.1 Cell culture and sample collection

ON cultures were used to inoculate 60 ml of fresh BHI to an OD_{600} of 0.01 and grown to the mid-exponential phase (OD_{600} ~ 0.4; time point zero, T0). A volume of 8 ml of the cell culture was collected as the control (T0), and the tube containing the remaining cultures was transferred to a 42°C water bath. Then, 8 ml of heat-treated cultures were collected after 20 minutes (min). The collected cells were centrifuged at 8000 rpm for 5 min, resuspended in 1 ml of Tri-reagent (Merck, Zwihndrecht, The Netherlands), transferred to a lysing matrix B tube (MP Biomedicals, Eschwege, Germany), and stored at -80°C. Four biological replicates were collected and analyzed as described below.

3.2.2.2 RNA isolation

Cell samples in lysing matrix B tubes were homogenized using a Fastprep-24 beat beater (MP Biomedicals) for 30 seconds (s) at 6.0 m/s. The procedure was repeated 6 times with resting intervals of 1 min. After 10 min settling at room temperature (RT), 200 µl of chloroform was added to the tubes, mixed, incubated for 15 min at RT, then centrifuged at 13,000 rpm for 15 min. The liquid phase (top layer) was transferred to a new Eppendorf tube, adjusted to 1 ml with RNAse-free water, mixed with 1 ml of ice-cold isopropanol, incubated at RT for 10 min, and centrifuged at 13,000 rpm for 15 min. The cell pellet was washed with 75% ice-cold ethanol, air-dried, and resuspended in 90 µl of RNAse-free water. The extracted RNA was treated with DNaseI to remove DNA, as described in the manufacturer's protocol (Thermo Fischer Scientific, Bleiswijk, The Netherlands) and stored with 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of 100% ethanol (v/v).

3.2.2.3 Reverse Transcription Quantification Polymerase Chain Reaction (RT-qPCR)

All RT-primers were designed using the Primer3 and DNASTAR software to amplify genes in the SigB gene cluster*: bc1002* (*rsbV*), *bc1003* (*rsbW*), *bc1004* (*sigB*), *bc1005*, *bc1006* (*rsbY*), *bc1008* (*rsbK*) and *bc1009* (Figure 1A), and four reference genes *nifU, gatB, rpsU* and *tufA*. All primers are listed in Supplementary Table S1. As *bc1007* (*rsbM*) is co-regulated with *bc1008* (*rsbK*) and does not have a SigB promoter binding motif (Figure 1A), it was excluded from the gene expression studies.

The purified RNA samples were precipitated at 13,000 rpm for 15 min, washed with 75% ethanol, air-dried, and resuspended in 200 µl RNAse-free water. For each reaction, 600 ng of total RNA was mixed with 2 µl reverse primer (5 µM), 2 µl dNTP mix (10 mM), and added to 29 µl with RNAse-free water. The reaction mixture was incubated at 65°C for 5 min for primer annealing and then cooled on ice for 1 min. 11 µl of reverse transcriptase master mix containing 8 µl 5x first strand buffer, 2 µl 0.1 M DTT, and 1 µl SuperScript™ III Reverse Transcriptase (Thermo Fischer Scientific) was added to each reaction. cDNA was synthesized at 55°C for 1 h, followed by enzyme inactivation at 70°C for 15 min. The synthesized cDNAs were diluted 10fold, and qPCRs were performed using primers listed in Supplementary Table S1.

For qPCR, each reaction contained: 12.5 µl Power SYBR Green PCR Master Mix (Thermo Fisher Scientific), 1 µl forward primer (5 µM), 1 µl reverse primer (5 µM), 5 µl of cDNA template, and 5.5 µl of RNAse free water. DNA denaturation was done at 95°C for 10 min, and PCRs were performed for 40 cycles (Denaturation: 95°C, 15 secs; Annealing and elongation: 61°C, 1 min). The cycles of quantification (Cq) values were recorded to compare differential gene expressions. Efficiencies of primers were checked with serial dilutions of the tested sample and calculated via the REST2 qPCR data analysis tool as described (Pfaffl, 2001; Pfaffl et al.,

2002). Cq values of all tested genes were uploaded to the COTTON EST DATABASE to look for stable reference genes. *tufA, gatB,* and *rpsU* were selected (Vandesompele et al., 2002; Pfaffl et al., 2004) and used to normalize the gene expression data with the REST2 qPCR program.

All recorded values were first normalized with the expression ratio of the three reference genes *gatB, rpsU,* and *tufA,* then normalized with the expression values recorded for wt in the control condition at 30°C. The changes in gene expression of Δ*sigB* mutant vs. wt and Δ*bc1009* mutant vs. wt were expressed in $\log_2$ ratios.

3.2.3 Transcriptome analysis

3.2.3.1 Cell culture and sample collection

Wt, Δ*sigB,* and Δ*bc1009* cells were cultured as described in section 3.2.2.1, and RNAs were isolated and purified as mentioned in section 3.2.2.2.

3.2.3.2 cDNA synthesis and labeling

The cDNA synthesis and labeling were performed as reported in van Schaik et al. (2007) and Mols et al. (2013) with slight modification. Briefly, the purified RNA was reversely transcribed using the SuperScript™ IV Reverse Transcriptase kit (Invitrogen, Groningen, the Netherlands) according to the manufacturer's instruction. mRNAs within the synthesized cDNA were treated with 3 µl of 2.5 M NaOH, spun down, incubated at 37°C for 15 min, and neutralized with 15 µl 2 M HEPES free acid. Then, the amino-allyl-labeled dUTP cDNA was synthesized, purified with the CyScribe GFX purification kit, and labeled with the Amersham CyDye Reactive Dye (Cy3 and Cy5) (Sigma).

3.2.3.3 Microarray design, hybridization, and scanning

Microarray design, hybridization, and scanning were performed as described in Mols et al. (2013) with slight modification. Briefly, ~100 ng of Cy3 and Cy5-labelled cDNA (in 1:1 ratio) were combined in a total volume of 18 µl, heated to 98°C for 3 min, mixed with 36 µl of 2x Hi-RPM Hybridization Buffer (Agilent Technologies, CA, USA) and hybridized onto the DNA MicroArray slides. The array slides were scanned with an Agilent G2505C scanner (Agilent Technologies).

3.2.3.4 Microarray data acquisition and analysis

Images were analyzed and processed with the Agilent Feature Extraction software (version 10.7.3.1). The obtained extracted data files were analyzed using the limma software package (Ritchie et al., 2015) in R (R Core Team, 2014). For Agilent-based arrays, global loess normalization was used when the bulk of genes is not differentially expressed. The analysis was based on a 2-color experimental design using a linear modeling approach by lmfit and empirical Bayes statistics (Phipson et al., 2016).

DNA arrays generated gene expression data for 5283 annotated coding sequences (CDSs) and 69 RNA features (sRNAs). Genes were considered as significantly differentially expressed

when the transcript level ratio between the two conditions (i.e., 30°C vs. 30°C > 42°C) was ≥ 2 folds (= upregulation of 1 $\log_2$ expression unit) or ≤ 2 folds (downregulation of 1 $\log_2$ expression unit) and the false discovery rate was ≤ 0.05.

3.2.4 Proteome analysis

3.2.4.1 Cell culture and sample collection

The proteomes of wt, Δ*sigB,* and Δ*bc1009* cells grown before (30°C) and after heat shock (42°C) were analyzed. ON cultures of each strain were used to inoculate 80 ml of fresh BHI to an OD_{600} of 0.01, and cultures were grown to an OD_{600} ~ 0.4 at 30°C and 200 rpm, respectively. Then, 20 ml aliquots of each culture were transferred to 50 ml Falcon tubes, and cells were spun down at 8000 rpm for 5 min at RT. After centrifugation, the cell pellet of one of the four tubes was frozen immediately in liquid nitrogen and used as the T0 control. For the remaining three tubes, cells were resuspended in 20 ml of 42°C-preheated BHI medium and further incubated for another 40 min. Samples were harvested after 20 min (T20) and 40 min (T40), respectively, and stored at -20°C. Four independent biological replicate experiments were performed. T20 was selected for proteome sample collection because SigB expression was shown to be the most abundant at 20 min after heat shock (data not shown), whereas T40 was selected to ensure proteins that required extended expression time were also analyzed and covered.

3.2.4.2 Protein extraction and preparation for Mass-spectrometry analysis

The pellets were each resuspended in 100 µl Tris-HCl (5 mM pH 7.4 containing 5% SDS) and immediately disrupted using a Dismembrator (Retsch, Haan, Germany) at 2600 rpm for 3 min (in a 4,8 ml Teflon vessel precooled in liquid nitrogen with an 8 mm diameter steel ball). Next, the cell powder was resuspended with 400 µl of preheated (95°C) Tris-HCl buffer (5 mM pH 7.4) and the viscous lysate was transferred into a fresh 1.5 ml low bind pre-lubricated Eppendorf tube and shaken for 1 min at 95°C and 1400 rpm. The lysate was cooled to RT and 2 µl of a 1 M $MgCl_2$ stock solution (final 4 mM $MgCl_2$) was added. Next, 1 µl of a 1:100 diluted benzonase (Pierce Universal Nuclease No#88702) (Pierce, Rockford, IL, USA) stock solution (final 0.005 U/µl) was added and mixed by short vortexing. The samples were incubated in an ultrasonic bath at RT for 5 min until the viscous lysate was liquefied by complete degradation of DNA and RNA. The raw lysates were centrifuged at 17000 g at RT for 30 min. After centrifugation, the protein lysate was transferred into a fresh 1.5 ml low bind pre-lubricated Eppendorf tube, and the pelleted cell debris was discarded. The protein concentration of the samples was determined using the Micro BCA Protein Assay Kit following the manufacturer's protocol (Pierce, Prod. No. 23235) using a FLUOstar Omega Plate Reader (BMG Labtech, Ortenberg, Germany). Samples were stored at -80°C. Sample preparation for mass spectrometry measurements was performed using the SP3 protocol as described in Blankenburg et al. (2019).

3.2.4.3 Liquid chromatography-mass spectrometry (LC-MS) measurements

Protein samples were measured using the LC-MS/MS platform containing reversed-phase nano liquid chromatography (nano Acquity M-class UPLC) (Waters Corporation, Massachusetts, USA), which was coupled to nanospray ionization tandem mass spectrometry with traveling wave ion mobility using high-definition data-independent (HD-MS^E) acquisition and enabled with hybrid quadrupole orthogonal acceleration time of flight mass spectrometer (Synapt G2Si, Waters Corporation).

The peptide mixture was separated on ACQUITY UPLC® M-Class HSS T3 1.8 um, 75 um x 200 mm column (Waters Corporation) using a mixture of Buffers A (0.1% v/v Acetic acid in water) and B (0.1% v/v acetic acid in acetonitrile) through a linear concentration gradient of Buffer B in 170 min, with a flow rate of 300 nl/min from 5-26% (v/v). The eluents were sprayed at a voltage of 1.85-1.90 kV using PicoTip emitters (Waters Corporation), and other source parameters were set (sampling cone = 40V, source offset = 0 V, source temperature = 80°C, gas flows, cone gas = 50 l/h, nano gas flow = 0.4 bar, purge gas = unchanged, IMS = optimized for wave velocity with a start velocity of 870 m/s to end at 564 m/s that corresponds to the separation of GluFib fragments in the drift time range of 0-200 bins).

The data was acquired using the MassLynx™ software program version V4.1 (Waters Corporation) that automatically switches between MS and MS/MS (HDMS^E) scans in resolution mode at 20000 with 1 second (sec) scan time and the scan range of 50-2000 m/z. Calibration was done by injecting GluFib at an interval of 1 min. The acquired data were analyzed for protein identifications against the Uniprot fasta database of *B. cereus* ATCC14579 strain that contained 5240 sequences via the PLGS v3.3 program (Water Corporation). Spectra were processed using low and high-energy thresholds of 135, 20 counts, and lock mass calibration 785.8456 m/z for GluFib. The workflow search parameters were set as: (protease = trypsin, one missed cleavage, carbamidomethyl for cysteine as fixed, variable modifier = oxidation of methionine). The protein was quantified when 1) the top 3 peptides had no modifications; 2) pass one match had peptide fragment one; and 3) ranked the first three highest peptides. The PLGS independent identification output was imported to ISOQuant 1.8 (Distler et al., 2014) for sample analyses.

3.2.4.4 Proteome data analysis

The quantitative protein data were obtained based on identifying a minimum of two peptides, the protein areas were used, and the data analysis was performed using the tidyverse package (version 1.3.0) in R version 4.0.2 (Wickham et al., 2019). Briefly, the IsoQuant protein intensities were median normalized using the global median as reference. The PCA analysis was carried out using the FactoMineR package (version 2.3) (Lê et al., 2008) with normalized $\log_2$ protein intensities scaled to unit variance. The sample correlation was calculated using Kendall's methodology (Kendall, 1938) and displayed using the ggcorrplot package (version

0.1.3) (Kassambara, 2019). The statistical analysis was carried out using the PECA package (version 1.24.0) (Suomi et al., 2021) by applying a modified T-test for the pairwise comparisons to proteins having valid protein quantity values in at least two replicates, by calculating empirical Bayes moderated T-statistics using the linear modeling approach implemented in the limma package (version 3.44.3) (Ritchie et al., 2015). The raw p-values (p) were multiple tests adjusted (p.fdr) using the Benjamini-Hochberg method (Benjamini and Hochberg, 1995). Finally, volcano plots were generated using the ggplot2 package (version 3.3.2) (Wickham, 2016) using an absolute fold-change cut-off of 1.5 and 0.05 as q-value (adjusted p-value) cut-off.

Mass spectrometric analyses detected between ~1100 to ~1800 proteins, depending on the strain and condition. Proteins were considered to be significantly differentially expressed when the absolute fold change (FC) was ≥ 1.5 folds ($\log_2$ FC ≥ 0.585) (induction/upregulation) or ≤ 1.5 folds ($\log_2$ FC ≤ 0.585) (downregulation), and the false discovery rate was ≤ 0.05.

3.2.5 Cell survival study

ON cultures were inoculated into 60 ml of fresh BHI to an OD_{600} of 0.01 and grown to the mid-exponential phase (OD_{600} ~ 0.4, T0) at 30°C. A control sample ($T0_{\text{non-adapted}}$) was collected, and the remaining cultures were divided into two portions. One portion was heated at 50°C without preadaptation, and samples were collected after 10, 20, and 30 min. Another portion was preadapted at 42°C for 45 min, a control sample ($T0_{\text{adapted}}$) was collected, and the tube with the remaining culture was heated at 50°C. Samples were taken every 20 min intervals up to 120 min. Preadaptation for 45 min at 42°C was chosen to allow an adequate time for the culture medium to reach 42°C, as a 15 - 60 min preheating treatment showed similar effects upon exposure to this lethal temperature of 50°C (van Schaik et al., 2004a).

At every time point, cultures were 10-fold serially diluted and plated on BHI agar plates. Colony-forming units per ml (CFU)/ml were calculated, and the survival of the heat-adapted and non-heat-adapted cells was expressed in $\log_{10}$ CFU/ml reduction ($\log_{10}$ CFU/ml T_0 - $\log_{10}$ CFU/ml T_x). The experiment was done with biological and technical duplicates, respectively.

3.2.6 Cell motility assay of wt, Δ*sigB*, and Δ*bc1009* cells

A cell motility assay was performed to check the phenotype for the wt, Δ*sigB,* and Δ*bc1009* cells at the control temperature (30°C) and under the condition with heat stress (30°C → 42°C). A flagella deficient strain Δ*flgG* (laboratory collection) was used as the negative control. The motility of each strain was tested on a BHI medium with 0.25% agar. Plates were prepared by pouring 50 ml in 120 x18 mm square Petri dishes (Greiner Bio-one). The BHI plates were dried for 15 min in a flow cabinet before use.

All strains were cultured ON at 30°C, 200 rpm. An aliquot of 250 µl of an ON culture was used to inoculate 20 ml of fresh BHI medium, and the culture was grown to an OD_{600} of ~ 0.4. Of

these cultures, cells were collected from 5 ml via centrifugation at 5500 g for 5 min. The supernatant was discarded, and the cell pellet was resuspended in 5 ml Phosphate-buffered saline (PBS). The motility under heat stress was assessed by first subjecting 5 ml of the cultures to 42°C in a water bath for 30 min, followed by centrifugation and resuspension in PBS. 5 µl of the resuspended cells were pipetted in the middle of the BHI agar plates, dried in the flow cabinet, and incubated either at 30°C or 42°C. The closed agar Petri dishes were covered with wet tissues to prevent dehydration. After ~ 20 h, all plates were photographed, and the colony diameter was measured using the ImageJ software (Schneider et al., 2012). The colony diameter and maximum plate size in pixels were measured in ImageJ with the 'Rectangle' setting. The colony diameter (in pixels) was divided by the maximum plate size (in pixels) to obtain a colony/plate ratio. This ratio was subsequently multiplied by the known plate size (150 mm) to obtain the colony diameter in mm.

3.3 Results

The *bc1009* gene in the SigB gene cluster (Figure 1A) encodes a putative Hpr-like phosphocarrier protein (Bc1009) in *B. cereus*. The potential role of *bc1009* acting as a SigB regulator was first examined via gene expression studies, and its SigB-dependent functions were investigated via proteome profiling (complemented with transcriptomic data). The global protein/gene expressions of the Δ*bc1009* mutant before and after heat shock were analyzed and compared with the protein/gene expressions of the *B. cereus* wt cells and a marker-free Δ*sigB* mutant under the same conditions.

3.3.1 The activity of *bc1009* is dependent on *sigB,* but *bc1009* does not regulate SigB

Changes in expression within the SigB gene cluster*: bc1002* (*rsbV*), *bc1003* (*rsbW*), *bc1004* (*sigB*), *bc1005*, *bc1006* (*rsbY*), *bc1008* (*rsbK*) and *bc1009* of *B. cereus* wt, Δ*sigB* and Δ*bc1009* cells upon heat shock (30°C → 42°C) were determined via RT-qPCR. Results were first compared for wt versus the Δ*sigB* mutant (Figure 1B), and then for wt versus the Δ*bc1009* mutant (Figure1C).

Upon heat shock of wt cells, the expression of the three known SigB-dependent genes *rsbV* (*bc1002*), *bc1005*, *bc1009,* and *sigB* itself increased > 200-fold (~ $\log_2$ 8) compared to wt cells without heat shock. The *rsbY* (*bc1006*) and *rsbK* (*bc1008*) genes that encode the two-component system were also expressed ~ 4- fold (~ $\log_2$ 2) higher in wt cells after heat shock than before heat shock (Figures 1B and 1C). Upon heat shock of the Δ*sigB* mutant, *rsbV* and *bc1005* were mildly expressed (< 4- fold) (~ $\log_2$ 2), whereas *bc1009* was expressed at a lower level after heat shock in the Δ*sigB* mutant (Figure 1B), showing that its heat induction was strictly dependent on SigB. The expression of the histidine kinase encoding gene *rsbK* is controlled by *sigA*. The observation that its expression was similar for the Δ*sigB* mutant and the wt cultures after heat shock is in line with this. Its cognate regulator partner *rsbY* was expressed at a lower

level in the Δ*sigB* mutant than in wt cultures, indicating partial dependency of its expression on SigB (Figure 1B). Notably, when the *bc1009* gene was deleted, the expression of all other genes in the SigB cluster was unaffected and similar to that in wt cultures (Figure 1C). This implied that *sigB* expression did not rely on *bc1009*, ruling out a possible role of *bc1009* as an additional phosphocarrier in the activation of SigB via the RsbKY system.

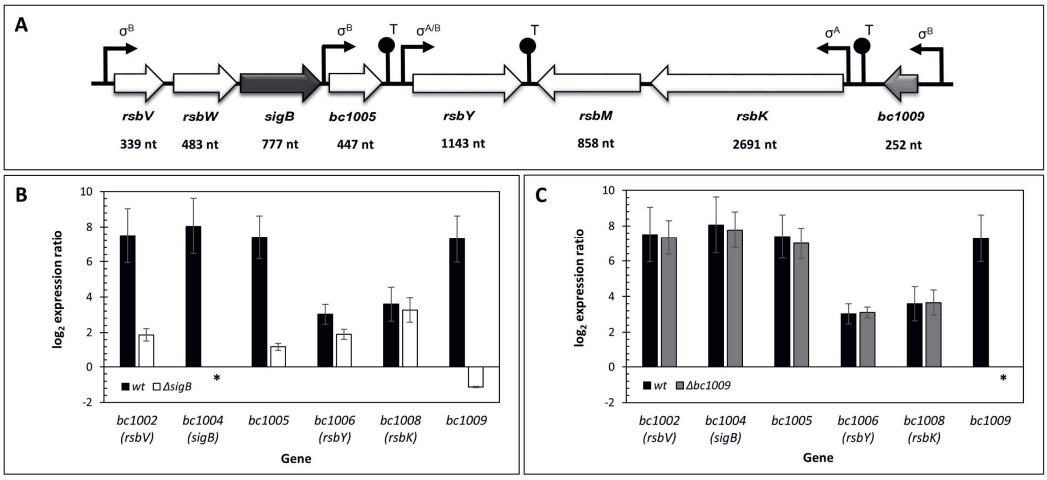


Figure 1- SigB gene cluster in *Bacillus cereus* and differential gene expression after heat shock. **1A-** SigB gene cluster arrangement in *B. cereus* ATCC14579. **1B –** Change in expression of genes belonging to the *sigB* gene cluster for wt and Δ*sigB* mutant after 20 min of heat shock at 42°C compared to the non-heat-stressed condition at 30°C. N=4, $p < 0.001$. **1C-** Change in expression of genes belonging to the SigB gene cluster expression for wt and Δ*bc1009* mutant after heat shock at 42°C compared to the non-heat-stressed condition at 30°C; **Black** bar- wt; **white** bar- Δ*sigB* mutant; **grey** bar- Δ*bc1009* mutant. * indicates the absence of gene activity. N=4, $p < 0.001$.

3.3.2 Comparison of proteomic profiles of Δ*sigB* and Δ*bc1009* mutants to wt upon heat shock reveals novel SigB-dependent proteins mediated via Bc1009 in *B. cereus*

To further explore the role of Bc1009 and SigB in *B. cereus,* we compared the proteome profiles of *B. cereus* cultures of wt, Δ*sigB,* and Δ*bc1009* mutants grown at 30°C with cultures that were heat-shocked at 42°C after growth at 30°C. The proteomics results of wt, Δ*sigB,* and Δ*bc1009* cells obtained in this study are presented below and complemented by transcriptomics results (Supplementary files).

3.3.2.1 Heat-induced changes of proteome profiles in wt cells

After heat shock treatment, ~ 1500 proteins were detected for wt cells. Among these, 429 proteins displayed significantly increased (≥ 1.5 folds, $\log_2 FC \geq 0.585$), and 435 significantly decreased (≤ 1.5 folds, $\log_2 FC \leq 0.585$) levels in wt cells after heat shock compared to control conditions, respectively (Figure 2). The complete list of proteins displaying significantly

different levels is presented in Supplementary Table S2A, and the clusters of orthologous group (COG) functions are presented in Supplementary Figure S1.

As expected, next to previously described SigB regulon members (see below), well-known protein chaperones (GroEL, DnaJ, DnaK), transcription repressors (HrcA, CtsR) that control Clp proteases (ClpC, ClpP, ClpB, ClpY, ClpQ), DNA repair proteins (RadA, MutS) and other well-known heat shock proteins like GrpE, YflT, and FtsH were all present at ≥ 1.5 folds higher levels in wt cultures after heat shock at 42°C compared to control conditions at 30°C. These heat shock proteins were also identified in an earlier study on *B. cereus* heat stress response (Periago et al., 2002), confirming the congruency of the results obtained in this study. Extended information on the *B. cereus* heat shock regulon was acquired (Supplementary Table S2A), and ~ 20% of the proteins with temperature-dependent changes in level found in wt cultures upon heat shock were also influenced at the gene expression level (Supplementary Table S2B).

3.3.2.2 Impact of SigB and Bc1009 on *B. cereus* protein profiles after heat shock

SigB-dependent and SigB/Bc1009-dependent heat shock induction of proteins

The protein profiles of Δ*sigB* and Δ*bc1009* mutants were further compared with wt cells. Out of 429 proteins displaying increased levels after heat shock in wt cells, 175 showed lower levels in the Δ*sigB* mutant, indicating that the expression of the encoding genes was inducible via SigB in wt cells (Figure 2). Additionally, for 98 of these 175 proteins, this effect was not only dependent on SigB but also on Bc1009 because they also exhibited lower levels in the Δ*bc1009* mutant than in the wt (Figure 2), hereby referred to as SigB (and Bc1009)-dependent proteins. The complete list of the SigB-induced and SigB (and Bc1009)-induced proteins with their functional annotation is listed in Table 2. The $\log_2$ fold changes of the protein levels are presented in the volcano plot in Figure 3A, and their COG functions in Figure 3B. The top 30 most significant SigB-induced proteins are indicated with numbers in Figure 3A on the right (marked in white), and for half of these, the increase in level was also dependent on Bc1009 (marked in Grey in Figure 3A) (see details in Table 2).

The 175 proteins displaying SigB-dependent increases in level after heat shock include the general stress defense proteins previously described by van Schaik et al. (2007) and De Been et al. (2010), i.e., Bc0861, Bc0862 (YfkM), Bc0863 (KatE), Bc0998 (YflT), Bc0999 (CsbD), Bc1002 (RsbV), Bc1003 (RsbW), Bc1004 (SigB), Bc1005 (bacterioferritin), Bc1010 (hypothetical), Bc1012 (hypothetical), Bc1154 (ferrochelatase), and Bc3132 (general stress protein) (Table 2). In line with these findings, significantly induced transcription was seen for genes that encode these general stress proteins (Supplementary Table S3B). Although the Bc1009 protein was not detected, the complementary transcriptional results showed that the *bc1009* gene was induced (Supplementary Table S3B).

Other newly identified members of the SigB-regulon mainly belong to the COG groups of cell motility, signal transduction mechanisms, transcription, amino acid transport, and metabolisms, or a group without assigned function (Figure 3B). Representatives of the cell motility COG group included many flagella or chemotaxis proteins (FliM, FliN, FliG, YvzB, Bc1637, FlgE, PhnB, McpBH, YoaH, CheV, CheA, Bc0404, MotA, Bc0678, TlpA), and the signal transduction and transcription COG groups contained proteins that are related to sporulation (SigK, YndF, Spo0A, Bc4463). Other strongly induced proteins included Bc0409 (carbamate kinase), Bc0566 (endonuclease/exonuclease phosphatase family protein), and Bc0666 (Immune inhibitor A precursor). Strikingly, a large number of transcriptional regulators were shown to be SigB-dependent, including SigK (sporulation sigma factor), Spo0A (sporulation initiation protein), CggR (central glycolytic regulator), ArsR family transcriptional regulator Bc0613, GntR family transcriptional regulator YvfI, PadR, YdgH, and the MerR family transcriptional regulator Bc3356 (Table 2). The most significantly induced protein - Bc0107 (YacN), a 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase, has a putative role in lipid transport and metabolism. Together with the strongly induced protein Bc4345 that encodes a lipase, this suggests an additional role of SigB in the modulation of membrane lipid composition. Remarkably, a cluster of proteins (Bc5117 - Bc5125) that is likely involved in the transport of nutrients, and Bc0423 (a non-ribosomal peptide synthetase that is involved in secondary metabolite production) also showed significant induction in wt cells and impaired production in Δ*sigB* cells after heat shock compared to before heat shock (Figure 3B, Table 2).

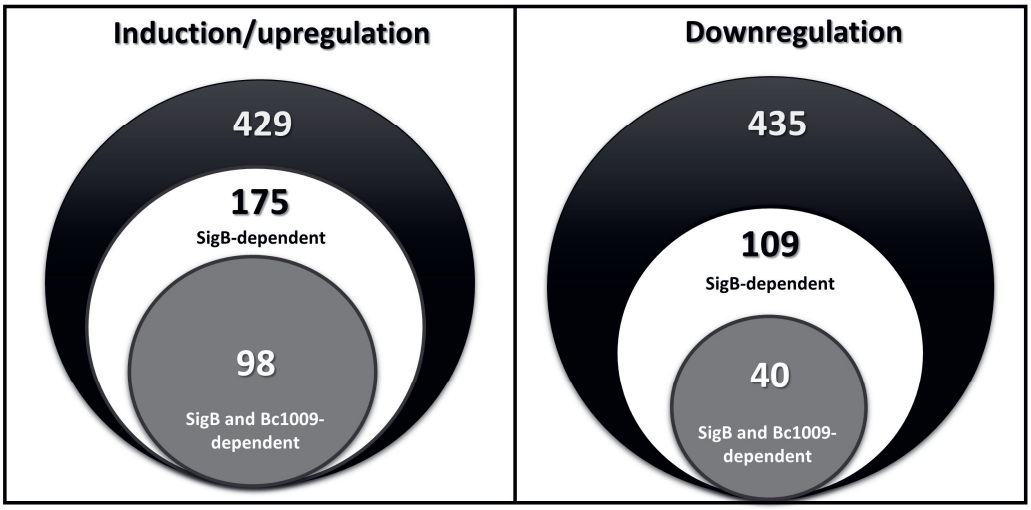


Figure 2- Proteomics analyses of *B. cereus* ATCC14579 wt, Δ*sigB,* and Δ*bc1009* mutants in upon heat shock. **Left:** Venn diagram showing 429 significantly induced (upregulated) proteins in *B. cereus* heat-stressed wt cells (**black circle**) compared to the non-heat-stressed wt cells at 30°C (see Supplementary Table S2A). **White circle:** 175 heat-induced proteins in wt that are SigB-dependent, i.e., proteins that show upregulation of > 0.6 $\log_2$ fold change in wt/Δ*sigB* cells upon heat shock compared to the non-heat-stressed condition at 30°C. **Grey circle:** For 98 proteins, the heat-mediated increase in level in the wt is dependent on SigB and Bc1009, i.e., proteins that show upregulation of > 0.6 $\log_2$ fold change in wt/Δ*sigB* and wt/Δ*bc1009* cells upon heat shock compared to the non-heat-stressed condition at 30°C (see Table 2 for the complete list of proteins). **Right:** Venn diagram showing 435 significantly downregulated proteins in *B. cereus* heat-stressed wt cells (**black circle**) compared to the non-heat-stressed wt cells at 30°C (Supplementary Table S2A). **White circle:** 109 downregulated proteins in heat-stressed wt cells vs. non-heat-stressed wt cells are SigB-dependent, i.e., proteins that show downregulation of > 0.6 $\log_2$ fold change in wt/Δ*sigB* cells upon heat shock compared to the non-heat-stressed condition at 30°C. **Grey circle:** For 40 proteins, reduction in level in wt cells upon heat shock was dependent on SigB and Bc1009, i.e., proteins that show downregulation of > 0.6 $\log_2$ fold change in wt/Δ*sigB* and wt/Δ*bc1009* cells upon heat shock vs. non-heat-stressed condition (see Table 3). The underlying transcriptome data supporting this figure are presented in Supplementary Table S2B.

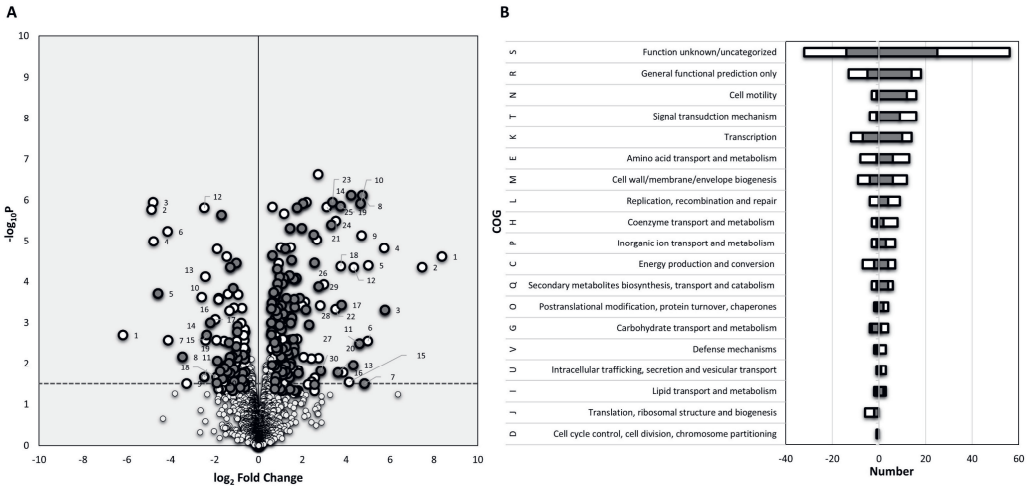


Figure 3- SigB and SigB (and Bc1009)-dependent induced and downregulated proteins in wt cells after heat shock (30°C to 42°C) versus before heat shock and their cluster of orthologous group (COG). **3A-** $\log_2$ fold change in protein expression. Positive and negative fold change values indicate induced and downregulated proteins, respectively. Numbers indicate the top 30 induced (Table 2) or top 20 downregulated (Table 3) proteins, respectively. The p-value threshold for each protein was < 0.05, N = 4. **Grey symbols:** induced/downregulated proteins that are SigB (and Bc1009)-dependent; i.e., proteins that show up(or down) regulation of > 0.6 $\log_2$ fold change in wt/Δ*sigB* and wt/Δ*bc1009* cells upon heat shock vs. non-heat-stressed condition**. White symbols:** induced/downregulated proteins that are SigB-dependent; i.e., proteins that show up(or down) regulation of > 0.6 $\log_2$ fold change in wt/Δ*sigB* cells upon heat shock vs. non-heat-stressed condition**. 3B-** cluster of orthologous group (COG) function for both SigB-dependent induced **(Right)** and downregulated proteins **(Left)** in wt cells upon heat shock. The number on the x-axis indicates the total number of induced/downregulated proteins, and the negative sign indicates downregulation. **Grey bar:** induced/downregulated proteins after heat shock that are SigB (and Bc1009)-dependent; i.e., proteins that show up(or down) regulation of > 0.6 $\log_2$ fold change in wt/*ΔsigB* and wt/Δ*bc1009* cells; **(White bar);** induced/downregulated proteins that are SigB-dependent; i.e., proteins that show up(or down) regulation of > 0.6 $\log_2$ fold change only in wt/Δ*sigB* cells respectively, compared to the expression in the non-heat-stressed condition at 30°C. The underlying transcriptome data supporting this figure are presented in Supplementary Table S3B.

Table 2: SigB-dependent induced proteins upon heat shock in *B. cereus* ATCC14579
Bold: SigB and Bc1009-dependent-induced proteins upon heat shock in *B. cereus*
Not Bold: SigB-dependent-induced proteins upon heat shock in *B. cereus*
log_2 FC: log_2 protein fold change values at either T20 or T40 timepoint
COG: Cluster of orthologous groups, see description in Figure 3.
Only SigB or SigB and Bc1009-dependent proteins are listed here

No.	Locus Tag	Protein	Annotation	wt 42°C/ Δ*sigB* 42°C log2 FC	wt 42°C/ Δ*bc1009* 42°C log2 FC	COG
1	BC0107	YacN	2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase	8.4		I
2	BC0998	YflT	General stress protein 17M	7.5		S
3	**BC4345**	**BC4345**	**Lipase; Pimeloyl-ACP methyl ester carboxylesterase (Coenzyme transport & metabolism)**	**5.8**	**5.5**	**R**
4	BC1004	SigB	RNA polymerase sigma-B factor	5.7		K
5	BC1005	BC1005	Bacterioferritin	5.0		P
6	BC4336	SigK	RNA polymerase sigma-K factor, sporulation sigma factor	5.0		K
7	**BC0409**	**BC0409**	**Carbamate kinase**	**4.8**	**1.5**	**E**
8	**BC5066**	**BC5066**	**Endonuclease/Exonuclease/phosphatase family protein**	**4.7**	**4.2**	**S**
9	BC0863	KatE	Catalase	4.7		P
10	**BC0666**	**BC0666**	**Immune inhibitor A precursor**	**4.7**	**3.7**	**S**
11	**BC0862**	**YfkM**	**Protease I**	**4.6**	**1.1**	**R**
12	BC4153	BC4153	Phosphohydrolase (MutT/nudix family protein)	4.3		L
13	**BC3935**	**BC3935**	**hypothetical Cytosolic Protein**	**4.3**	**4.1**	**S**
14	**BC1010**	**BC1010**	**YflT heat induced stress protein family**	**4.2**	**0.7**	**S**
15	BC0422	BC0422	Methyl-accepting chemotaxis protein	4.1		NT
16	BC0612	BC0612	L-lactate permease	3.9		C
17	**BC1663**	**FliN**	**Flagellar motor switch protein fliN**	**3.8**	**3.6**	**NU**
18	BC0613	BC0613	Transcriptional regulator, ArsR family	3.8		K
19	**BC5077**	**BC5077**	**Protein with unknown function**	**3.7**	**3.8**	**S**
20	**BC3110**	**YndF**	**Spore germination protein BC**	**3.6**	**1.2**	**S**
21	BC3229	YxkD	hypothetical Membrane Spanning Protein	3.5		S
22	BC0773	YdjE	Fructokinase	3.5		G
23	**BC3576**	**BC3576**	**Spore germination protein SC**	**3.4**	**3.2**	**S**
24	**BC5410**	**YocJ**	**FMN-dependent NADH-azoreductase**	**3.3**	**5.2**	**I**
25	BC0999	BC0999	General stress protein (hyperosmotic & cold)	3.1		S
26	BC3292	BC3292	hypothetical protein	3.0		S
27	**BC1662**	**FliM**	**Flagellar motor switch protein fliM**	**2.8**	**2.9**	**N**
28	BC1661	BC1661	Flagellar motor switch protein fliN	2.8		NU
29	**BC3406**	**BC3406**	**Oxidoreductase**	**2.8**	**3.5**	**R**
30	BC1127	BC1127	Malate synthase	2.7		C
31	BC1002	RsbV	Anti-sigma B factor antagonist	2.7		T
32	BC1003	RsbW	Anti-sigma B factor	2.7		T
33	BC1025	BC1025	Glyoxalase family protein	2.6		E
34	BC3132	BC3132	General stress protein 17M	2.6		S
35	**BC0423**	**SrfAAH**	**Non-ribosomal peptide synthetase (adenylation domain)**	**2.6**	**2.6**	**Q**
36	**BC2006**	**TlpA**	**Methyl-accepting chemotaxis protein, pH sensor**	**2.6**	**2.6**	**NT**
37	**BC5422**	**BC5243**	**O-Antigen ligase - like protein**	**2.5**	**2.9**	**S**
38	BC1226	YjcH	Acetyl esterase	2.4		P
39	BC0655	BC0655	Universal stress protein family	2.4		T
40	**BC5436**	**BC5436**	**Peptide methionine sulfoxide reductase**	**2.3**	**2.4**	**O**
41	BC1237	TrpB	Tryptophan synthase beta chain	2.3		E
42	BC1333	BC1333	CBS domain containing protein	2.2		R
43	**BC1349**	**BC1349**	**Acetyltransferase**	**2.2**	**0.8**	**KR**
44	BC1369	DltD	Protein dltD precursor	2.1		M
45	BC1403	LeuD	3-isopropylmalate dehydratase small subunit	2.1		E
46	**BC1303**	**YvfV**	**(S)-2-hydroxy-acid oxidase, iron-sulfur chain**	**2.0**	**2.1**	**C**
47	BC4463	BC4463	Stage II sporulation protein B	2.0		S

No.	Locus Tag	Protein	Annotation	wt 42°C/ Δ*sigB* 42°C log2 FC	wt 42°C/ Δ*bc1009* 42°C log2 FC	COG
48	**BC3980**	**YkuR**	**putative N-acetyldiaminopimelate deacetylase, peptidoglycan synthesis**	**2.0**	**1.9**	**R**
49	**BC4786**	**BC4786**	**hypothetical Cytosolic Protein**	**1.9**	**4.8**	**S**
50	**BC1410**	**HisF**	**HisF protein**	**1.8**	**1.7**	**E**
51	BC5223	YdaJ	hypothetical protein	1.8		S
52	BC1431	BC1431	Cell wall endopeptidase, family M23/M37	1.8		MS
53	BC4820	BC4820	hypothetical protein	1.8		S
54	**BC5141**	**CggR**	**Central glycolytic genes regulator**	**1.8**	**1.7**	**K**
55	**BC1370**	**DltC**	**D-alanyl carrier protein**	**1.7**	**2.9**	**IQ**
56	**BC0678**	**BC0678**	**Methyl-accepting chemotaxis protein, signaling domain**	**1.7**	**1.4**	**NT**
57	**BC5123**	**BC5123**	**homolog of lantibiotic biosynthesis dehydratase C-term**	**1.7**	**1.7**	**S**
58	**BC0597**	**YueK**	**Nicotinate phosphoribosyltransferase**	**1.7**	**1.7**	**H**
59	**BC5118**	**BC5118**	**ABC transporter ATP-binding protein**	**1.6**	**1.8**	**E**
60	**BC1275**	**BC1275**	**Methyltransferase**	**1.6**	**1.6**	**QR**
61	**BC5155**	**YvcK**	**hypothetical Cytosolic Protein**	**1.6**	**2.2**	**S**
62	**BC5103**	**YclP**	**Ferric anguibactin transport ATP-binding protein**	**1.6**	**2.0**	**P**
63	**BC3203**	**BC3203**	**hypothetical protein**	**1.6**	**1.5**	**S**
64	BC2303	DhbC	Isochorismate synthase	1.6		HQ
65	**BC3257**	**BC3257**	**N-acetylmuramoyl-L-alanine amidase, Cell wall or membrane biogenesis**	**1.5**	**1.6**	**TM**
66	**BC5122**	**BC5122**	**YcaO cyclodehydratase, ATP-ad Mg2+-binding**	**1.5**	**1.8**	**S**
67	BC0571	BC0571	Serine/threonine protein phosphatase	1.5		T
68	**BC0675**	**BC0675**	**hypothetical protein**	**1.5**	**1.7**	**S**
69	BC1586	TuaA	Undecaprenyl-phosphate galactosephosphotransferase	1.5		M
70	BC3350	BC3350	TPR-repeat-containing protein	1.5		R
71	**BC2850**	**YkfB**	**Mandelate racemase/muconate lactonizing enzyme family protein**	**1.5**	**1.1**	**MR**
72	**BC3165**	**PucA**	**Xanthine dehydrogenase subunit**	**1.5**	**1.0**	**O**
73	BC3287	BC3287	hypothetical protein	1.4		S
74	**BC5255**	**YknX**	**periplasmic component of efflux system**	**1.4**	**1.6**	**M**
75	**BC1857**	**BC1857**	**Exonuclease SbcD**	**1.4**	**1.8**	**L**
76	BC3278	BC3278	hypothetical protein	1.4		S
77	**BC1658**	**YvzB**	**Flagellin**	**1.4**	**1.3**	**N**
78	**BC2757**	**BC2757**	**Tryptophan 2,3-dioxygenase**	**1.4**	**2.4**	**E**
79	BC5125	BC5125	Peptidase family M50	1.4		M
80	BC0419	ThiM	Hydroxyethylthiazole kinase	1.4		H
81	BC1725	DsdA	D-serine dehydratase	1.4		E
82	**BC1756**	**PadR**	**Transcriptional repressor PadR**	**1.4**	**1.0**	**K**
83	**BC5168**	**UvrB**	**Excinuclease ABC subunit B**	**1.3**	**1.4**	**L**
84	**BC5243**	**BC5243**	**hypothetical protein**	**1.3**	**1.6**	**S**
85	**BC5119**	**BC5119**	**In operon with BC5120, which is a nitroreductase family protein**	**1.3**	**1.6**	**S**
86	**BC0244**	**AppD**	**Oligopeptide transport ATP-binding protein oppD**	**1.3**	**0.7**	**EP**
87	**BC2147**	**RapG**	**Response regulator aspartate phosphatase**	**1.3**	**1.0**	**R**
88	**BC1637**	**BC1637**	**Flagellar hook-associated protein 3**	**1.2**	**1.0**	**N**
89	**BC4300**	**YqfG**	**hypothetical Metal-Binding Protein**	**1.2**	**1.3**	**R**
90	**BC2285**	**MmgD**	**2-methylcitrate synthase**	**1.2**	**1.5**	**C**
91	BC2431	BC2431	PhnB protein	1.2		S
92	**BC1651**	**FlgE**	**Flagellar hook protein flgE**	**1.2**	**1.1**	**N**
93	BC2425	BC2425	hypothetical protein	1.2		S
94	BC2464	BC2464	S-layer protein / Peptidoglycan endo-beta-N-acetylglucosaminidase	1.2		G
95	BC2750	BC2750	Protein with unknown function	1.2		L
96	**BC0792**	**YrkD**	**hypothetical Cytosolic Protein**	**1.2**	**1.4**	**S**
97	**BC4170**	**SpoOA**	**Stage 0 sporulation protein A**	**1.1**	**1.3**	**TK**
98	**BC3356**	**BC3356**	**Transcriptional regulator, MerR family**	**1.1**	**0.9**	**K**
99	BC3565	BC3565	hypothetical protein	1.1		H
100	**BC2760**	**BC2760**	**Transcriptional regulator, TetR family**	**1.1**	**1.1**	**K**

No.	Locus Tag	Protein	Annotation	wt 42°C/ Δ*sigB* 42°C log2 FC	wt 42°C/ Δ*bc1009* 42°C log2 FC	COG
101	**BC3852**	**YlpC**	**PaaI family protein, possible transcriptional regulator**	**1.1**	**0.7**	**Q**
102	**BC4791**	**YtiB**	**Carbonic anhydrase**	**1.1**	**1.0**	**P**
103	BC2862	PrsA	Protein export protein prsA precursor	1.1		O
104	**BC4513**	**MotA**	**Chemotaxis motA protein**	**1.1**	**1.0**	**N**
105	**BC3894**	**BC3894**	**DnaK suppressor protein**	**1.1**	**1.3**	**T**
106	**BC4371**	**BC4371**	**hypothetical protein**	**1.1**	**0.9**	**S**
107	BC2919	YokD	Aminoglycoside N3'-acetyltransferase	1.1		V
108	**BC5121**	**BC5121**	**hypothetical protein**	**1.1**	**1.6**	**S**
109	**BC5117**	**BC5117**	**ABC transporter permease protein**	**1.1**	**1.2**	**S**
110	BC2936	YdgH	Transcriptional repressor Bm3R1	1.0		K
111	**BC3433**	**BC3433**	**hypothetical protein**	**1.0**	**1.8**	**S**
112	BC3070	BC3070	Signal peptidase I	1.0		U
113	BC0887	BC0887	Collagen adhesion protein	1.0		M
114	**BC3192**	**BC3192**	**precursor of the glucomannan utilization protein ydhR**	**1.0**	**2.8**	**S**
115	**BC0191**	**BC0191**	**hypothetical Membrane Spanning Protein**	**1.0**	**1.2**	**S**
116	BC1645	FliG	Flagellar motor switch protein fliG	1.0		N
117	**BC5241**	**YvbJ**	**IG hypothetical 16680**	**1.0**	**1.1**	**S**
118	BC4938	YutJ	NADH dehydrogenase	1.0		C
119	**BC0954**	**BC0954**	**tcdA-E operon negative regulator**	**1.0**	**1.0**	**S**
120	BC4723	BC4723	Molybdopterin biosynthesis MoeB protein	0.9		H
121	BC3264	BC3264	hypothetical protein	0.9		L
122	**BC2150**	**YpgQ**	**metal-dependent phosphohydrolase**	**0.9**	**1.0**	**R**
123	**BC2846**	**BC2846**	**Protein dltD precursor**	**0.9**	**1.2**	**M**
124	**BC3281**	**BC3281**	**hypothetical protein**	**0.9**	**2.0**	**S**
125	**BC0404**	**TarH**	**Methyl-accepting chemotaxis protein**	**0.9**	**0.7**	**NT**
126	**BC1657**	**BC1657**	**Flagellin**	**0.9**	**1.1**	**S**
127	BC1882	BC1882	Phage protein	0.9		S
128	**BC1247**	**BC1247**	**hypothetical protein**	**0.9**	**1.1**	**S**
129	BC0295	GroEL	60 kDa chaperonin GROEL	0.9		O
130	**BC5120**	**BC5120**	**hypothetical Cytosolic Protein**	**0.9**	**1.7**	**S**
131	**BC3286**	**BC3286**	**hypothetical protein**	**0.9**	**0.8**	**C**
132	BC1384	YubB	Bacitracin resistance protein (Putative undecaprenol kinase)	0.9		V
133	BC0040	YabB	Methyltransferase	0.9		R
134	BC3550	BC3550	Argininosuccinate lyase	0.9		E
135	**BC1302**	**YvfI**	**Transcriptional regulator, GntR family**	**0.9**	**0.8**	**K**
136	**BC3601**	**YclJ**	**Two-component response regulator**	**0.9**	**0.9**	**TK**
137	**BC3699**	**BC3699**	**Antigen/ lysozyme like protein**	**0.8**	**3.1**	**M**
138	**BC0061**	**YabN**	**MazG protein**	**0.8**	**0.9**	**R**
139	BC3802	YmxH	hypothetical protein	0.8		S
140	**BC1924**	**BC1924**	**L-lactate dehydrogenase**	**0.8**	**1.0**	**C**
141	BC4178	BC4178	Exodeoxyribonuclease VII small subunit	0.8		E
142	BC1736	YfiN	Export ABC transporter permease protein	0.8		V
143	**BC4224**	**BC4224**	**Glycine dehydrogenase [decarboxylating]**	**0.8**	**1.8**	**E**
144	BC5445	BC5445	Superoxide dismutase [Mn]	0.8		P
145	**BC4696**	**YtmQ**	**SAM-dependent methyltransferase**	**0.8**	**1.7**	**R**
146	BC4246	BC4246	hypothetical protein	0.8		S
147	**BC4469**	**HemB**	**Delta-aminolevulinic acid dehydratase**	**0.8**	**0.7**	**H**
148	BC1628	CheA	Chemotaxis protein cheA	0.8		NT
149	BC4702	YtjP	Xaa-His dipeptidase	0.8		E
150	BC4261	BC4261	hypothetical Cytosolic Protein	0.8		S
151	BC1479	YvqK	hypothetical Cytosolic Protein	0.8		S
152	**BC0888**	**CwlH**	**N-acetylmuramoyl-L-alanine amidase,Cell wall or membrane biogenesis**	**0.8**	**0.7**	**M**
153	**BC0576**	**McpBH**	**Methyl-accepting chemotaxis protein**	**0.8**	**1.9**	**NT**
154	BC4381	YrrK	hypothetical Cytosolic Protein	0.7		L
155	**BC3921**	**YlbP**	**Acetyltransferase**	**0.7**	**0.9**	**KR**
156	**BC1654**	**CheV**	**Chemotaxis protein cheV**	**0.7**	**0.8**	**NT**

No.	Locus Tag	Protein	Annotation	wt 42°C/ Δ*sigB* 42°C log2 FC	wt 42°C/ Δ*bc1009* 42°C log2 FC	COG
157	**BC4579**	**DnaI**	**Primosomal protein dnaI, DNA replication**	**0.7**	**2.2**	**L**
158	**BC4644**	**BC4644**	**PhnB protein**	**0.7**	**1.0**	**S**
159	BC3976	BC3976	putative transcriptional regulator	0.7		S
160	BC4668	BC4668	Virulence factor mviM	0.7		R
161	**BC4741**	**BC4741**	**DNA integration/recombination/invertion protein**	**0.7**	**0.6**	**L**
162	BC3857	YloS	Thiamin pyrophosphokinase	0.7		H
163	**BC4832**	**YdeE**	**Transcriptional regulator, AraC family**	**0.6**	**1.3**	**KS**
164	BC4847	BC4847	D-alanyl-D-alanine carboxypeptidase	0.6		MS
165	**BC1659**	**BC1659**	**hypothetical protein**	**0.6**	**0.6**	**S**
166	**BC3713**	**YmaH**	**Hfq protein**	**0.6**	**0.6**	**R**
167	BC4961	YutE	hypothetical Cytosolic Protein	0.6		S
168	BC4856	MenF	Isochorismate synthase	0.6		HQ
169	**BC3372**	**YqeC**	**6-phosphogluconate dehydrogenase**	**0.6**	**0.6**	**G**
170	BC0570	BC0570	Glycerol-3-phosphate-binding protein	0.6		G
171	**BC0203**	**BC0203**	**hypothetical protein**	**0.6**	**0.7**	**S**
172	BC1313	BC1313	PhaP protein	0.6		S
173	BC4179	YqiB	Exodeoxyribonuclease VII large subunit	0.6		L
174	**BC5034**	**YoaH**	**Methyl-accepting chemotaxis protein**	**0.6**	**0.7**	**NT**
175	**BC5124**	**BC5124**	**Protein with unknown function**	**0.6**	**1.9**	**S**

A total of 98 of the newly identified SigB-dependent proteins are also Bc1009-dependent, including flagella/chemotaxis proteins, transcriptional activator/repressors, ABC transporters, proteins involved in amino acid transport and metabolism, and cell wall/membrane/envelope biogenesis (Figure 3B, and bold-highlighted in Table 2). Similarly, as reported above, the Bc5117- Bc5125 cluster and Bc0423 (non-ribosomal peptide synthetase) also showed reduced levels in Δ*bc1009* culture compared to wt cells. Bc0423 was also downregulated at the gene level in both mutants (complementing transcriptomic data are presented in Supplementary Table S3B). Several other proteins that showed significantly different levels in Δ*sigB* and Δ*bc1009* cultures versus wt do not belong to the COG groups mentioned above. For instance, YocJ (FMN-dependent NADH-azoreductase) was induced > 50-fold ($\log_2 \sim 5.6$) stronger in wt cultures compared to Δ*sigB* mutant, and > 10-fold ($\log_2 \sim 3.3$) stronger in wt cultures compared to Δ*bc1009* mutant. Similar observations were made for other proteins with unknown functions (Bc4786, Bc5066, Bc3935 cytosolic protein, Bc5077, Bc0666 immune inhibitor A precursor) (see Table 2).

These results were supported by the transcriptomics data (Supplementary Table S3B), which showed differential transcription of genes encoding the reported motility proteins in Δ*sigB* and Δ*bc1009* cultures, pointing to a role of SigB and Bc1009 in the control of cell motility. Notably, despite the SigB-induced transcription of a group of phage genes, the corresponding encoded proteins were either not detected or not differentially expressed in heat-stressed cells.

SigB-dependent and SigB/Bc1009- dependent downregulated proteins

On the other hand, 109 of 435 proteins that showed lower levels in wt cells after heat shock than before heat shock showed higher levels in the Δ*sigB* mutant, indicating that the expression of the encoding genes is likely indirectly regulated by SigB in wt cells (Figure 2). 40 of these 109 proteins also showed higher levels in the Δ*bc1009* mutant than in wt, suggesting that the

encoding genes are regulated by SigB and Bc1009 (Figure 2). The complete list of the SigB-dependent and SigB (and Bc1009)-dependent proteins that displayed lower levels after heat shock with their annotated functions is listed in Table 3. The $\log_2$ fold changes of the levels of these proteins are presented in the volcano plot (Figure 3A), and their COG functions are shown in Figure 3B. The top 20 SigB-dependent proteins with lower reductions of levels after heat shock in Δ*sigB* and Δ*bc1009* cells compared to wt are indicated by numbers in Figure 3A on the left (marked in white), with five of these being SigB and Bc1009- dependent (marked in Grey in Figure 3A) (see details in Table 3).

Many of the 109 proteins that revealed SigB-dependent reductions in levels in wt cells after heat shock compared to control samples have undefined functions or fall into the COG group of proteins with transcription, cell wall biogenesis, and energy production and conversion functions (Figure 3B, Table 3). For instance, the most prominently downregulated proteins in wt cells (~25 to 75 fold; $\log_2$ FC = ~ 4.6 - 6.2) after heat shock vs. before heat shock include RpsT (SSU ribosomal protein, involved in translation, ribosomal structure, and biogenesis), Bc2026 (oligopeptide-binding protein OppA involved in amino acid transport and mechanism), Bc1699 (ECF-type sigma factor negative effector with unknown function), YkfJ (protein tyrosine phosphatase in signal transduction), and Bc3442 (hypothetical protein). Remarkably, several other proteins involved in transcription were significantly downregulated in wt cells but less so in Δ*sigB* and Δ*bc1009* cells, such as the ArgR arginine repressor, Sigma-54-dependent transcriptional activator GlcR regulator, TetR family regulator Bc3592, and ArsR family transcriptional regulator Bc4256 (Table 3).

For 40 of these proteins, the reduction in level after heat shock was not only dependent on SigB but also on Bc1009, and for many of them the function has not been defined yet. Those showing significant differential expression with known functions are mainly engaged in transcription, including YitH acetyltransferase, ArgR arginine repressor, Bc2298 transcriptional repressor, Bc4652 IcaR transcriptional regulator, Bc4256 ArsR family transcriptional regulator, BkdR sigma 54-dependent transcriptional activator, and Bc2369 acetyltransferase.

Transcriptional analysis generally supported proteomics data, except for a group of *nar* genes involved in anaerobic respiration; these showed SigB dependency in wt cells upon heat shock (Supplementary Table S3B), while no differential expression of corresponding proteins was observed (Table 3). Moreover, comparative proteomics and transcriptomics data of wt vs. Δ*bc1009* also showed an additional group of Bc1009-induced/downregulated proteins/genes that are not dependent on SigB. As this study focussed on the SigB-mediated responses, these data are not further discussed here, but details are listed in Supplementary Table S4A and S4B, respectively.

The results presented in section 3.3.2 show more than 300 newly identified SigB-dependent proteins, and more than 100 of these require both SigB and Bc1009 for changes in their level in heat-stressed cells, indicating a significant extension of the *B. cereus* SigB regulon and a subregulon additionally requiring the Hpr-like phosphocarrier protein Bc1009. Most of these SigB and Bc1009-dependent proteins are involved in cell motility, signal transduction mechanisms, transcription, amino acid transport and metabolism, and cell wall biogenesis. Other proteins are responsible for DNA replication and repair, protein quality maintenance, and cell wall remodeling, suggesting a role of Bc1009 in adaptive heat stress response in *B. cereus* as well.

Table 3: SigB-dependent downregulated proteins upon heat shock in *B. cereus* ATCC14579
Bold: SigB and Bc1009-dependent-downregulated proteins under heat shock in *B. cereus*
Not Bold: SigB-dependent-induced proteins upon heat shock in *B. cereus*
$\log_2$ FC: $\log_2$ protein fold change values at either T20 or T40 timepoint
COG: Cluster of orthologous groups, see description in Figure 3.
Only SigB or SigB and Bc1009-dependent proteins are listed here

No.	Locus Tag	Protein	Annotation	wt 42°C/ Δ*sigB* 42°C log2 FC	wt 42°C/ Δ*bc1009* 42°C log2 FC	COG
1	BC4320	RpsT	SSU ribosomal protein, translation	-6.2		J
2	BC1699	BC1699	ECF-type sigma factor negative effector	-4.9		S
3	BC2026	BC2026	Oligopeptide-binding protein oppA	-4.8		E
4	BC0450	YfkJ	Protein tyrosine phosphatase	-4.8		T
5	**BC3442**	**BC3442**	**hypothetical protein**	**-4.6**	**-1.2**	**R**
6	BC2675	BC2675	Acetyltransferase	-4.1		K
7	BC4168	BC4168	hypothetical protein	-4.1		S
8	**BC0066**	**YabR**	**S1-type RNA-binding domain**	**-3.5**	**-0.7**	**J**
9	BC3321	YfkO	NAD(P)H-dependent flavin reductase	-3.3		C
10	BC4935	YutM	Fe-S carrier protein, assembly of Fe-S clusters, DNA repair	-2.6		S
11	BC0544	BC0544	iron-sulfur cluster-binding protein	-2.5		C
12	BC4742	BC4742	ABC transporter permease protein	-2.5		V
13	BC0575	BC0575	hypothetical protein	-2.4		S
14	BC4619	BC4619	Protein with unknown function	-2.4		S
15	**BC2272**	**BC2272**	**Protein export protein prsA precursor**	**-2.4**	**-0.6**	**O**
16	**BC1650**	**FlgD**	**Basal-body rod modification protein flgD**	**-2.2**	**-1.7**	**N**
17	BC1970	BC1970	Protein with unknown function	-2.0		H
18	BC5166	BC5166	hypothetical protein	-1.9		S
19	BC5232	BC5232	Phosphoglycerol transferase	-1.9		M
20	**BC1593**	**YitH**	**Acetyltransferase**	**-1.9**	**-1.3**	**KR**
21	BC4102	YbaC	Alpha/beta hydrolase	-1.9		S
22	**BC2421**	**BC2421**	**hypothetical protein**	**-1.9**	**-0.7**	**S**
23	BC1137	AddB	ATP-dependent nuclease subunit B	-1.8		L
24	BC2084	BC2084	hypothetical Cytosolic Protein	-1.8		S
25	BC1548	BC1548	Endonuclease III	-1.8		L
26	**BC0949**	**BC0949**	**hypothetical Membrane Spanning Protein**	**-1.7**	**-1.7**	**EH**
27	BC3592	BC3592	Transcriptional regulator, TetR family	-1.7		K
28	**BC1638**	**BC1638**	**Flagellar hook-associated protein 2**	**-1.7**	**-1.5**	**N**
29	**BC5271**	**BC5271**	**UDP-N-acetylglucosamine 4-epimerase**	**-1.6**	**-1.4**	**MG**
30	BC0848	BC0848	Transcriptional regulator, AsnC family	-1.6		K
31	BC4448	BC4448	Protein with unknown function	-1.6		S
32	**BC0875**	**BC0875**	**hypothetical protein**	**-1.6**	**-1.2**	**S**
33	BC1991	BC1991	putative murein endopeptidase	-1.4		D
34	BC1520	YpiB	hypothetical Cytosolic Protein	-1.4		S
35	**BC0673**	**BC0673**	**Flavin-dependent dehydrogenase**	**-1.4**	**-1.1**	**C**
36	BC0489	BC0489	Glycosyltransferase involved in cell wall biogenesis	-1.4		M
37	BC2508	BC2508	Collagen adhesion protein	-1.4		M
38	**BC4652**	**YttP**	**Transcriptional regulator IcaR**	**-1.3**	**-0.8**	**K**
39	**BC3958**	**YktC**	**Myo-inositol-1(or 4)-monophosphatase**	**-1.3**	**-1.2**	**G**

No.	Locus Tag	Protein	Annotation	wt 42°C/ Δ*sigB* 42°C log2 FC	wt 42°C/ Δ*bc1009* 42°C log2 FC	COG
40	BC3931	BC3931	hypothetical protein	-1.3		S
41	**BC5377**	**YwhC**	**Membrane metalloprotease**	**-1.3**	**-0.7**	**R**
42	**BC3609**	**BC3609**	**hypothetical protein**	**-1.3**	**-0.6**	**S**
43	**BC0984**	**BC0984**	**DNA-binding protein**	**-1.3**	**-1.2**	**S**
44	**BC2077**	**BC2077**	**YukE protein of unknown function**	**-1.3**	**-1.5**	**S**
45	**BC5274**	**YveM**	**UDP-N-acetylglucosamine 4,6-dehydratase**	**-1.3**	**-1.6**	**MG**
46	BC2060	BC2060	hydrolase (HAD superfamily)	-1.2		R
47	BC2926	BC2926	hypothetical protein	-1.2		L
48	**BC3437**	**BC3437**	**hypothetical Cytosolic Protein**	**-1.2**	**-1.6**	**S**
49	BC4150	YqiX	Arginine-binding protein	-1.2		ET
50	**BC0405**	**ArgR**	**Arginine repressor, argR**	**-1.2**	**-0.9**	**K**
51	**BC2298**	**BC2298**	**Transcriptional repressor**	**-1.1**	**-2.9**	**K**
52	BC0115	SecE	Protein translocase subunit SecE	-1.1		U
53	BC1545	YpmB	hypothetical protein	-1.1		S
54	**BC3953**	**YlaI**	**hypothetical protein**	**-1.1**	**-1.8**	**S**
55	**BC0235**	**YdaL**	**hypothetical protein**	**-1.1**	**-0.7**	**S**
56	BC4495	GerM	Germination protein germ	-1.1		R
57	BC4735	BC4735	hypothetical protein	-1.1		S
58	**BC4006**	**BC4006**	**hypothetical Cytosolic Protein**	**-1.1**	**-0.6**	**S**
59	BC5270	YvfC	Undecaprenyl-phosphate galactosephosphotransferase	-1.0		M
60	BC3791	YufN	Nucleoside-binding protein	-1.0		R
61	BC5126	BC5126	Transposase	-1.0		L
62	BC2248	YodQ	Acetylornithine deacetylase	-1.0		E
63	**BC0709**	**BC0709**	**Ferrous iron transport protein A**	**-1.0**	**-0.7**	**P**
64	**BC2578**	**BC2578**	**Phage protein**	**-1.0**	**-0.7**	**S**
65	**BC3398**	**BC3398**	**Serine transporter**	**-1.0**	**-0.8**	**E**
66	BC5401	YpmR	Lipase/Acylhydrolase with GDSL-like motif	-1.0		E
67	**BC4256**	**BC4256**	**Transcriptional regulator, ArsR family**	**-1.0**	**-1.0**	**K**
68	**BC5047**	**YvdC**	**IG hypothetical 16995**	**-0.9**	**-1.3**	**R**
69	**BC0493**	**UgtP**	**1,2-diacylglycerol 3-glucosyltransferase**	**-0.9**	**-0.8**	**M**
70	BC3595	YvaA	Oxidoreductase	-0.9		R
71	BC0062	YabO	Heat shock protein 15	-0.9		J
72	BC4963	BC4963	hypothetical Cytosolic Protein	-0.9		S
73	BC0163	TruA	tRNA pseudouridine synthase A	-0.9		J
74	BC2770	GlcR	Transcriptional regulator, DeoR family	-0.9		KG
75	BC1641	FlgB	Flagellar basal-body rod protein flgB	-0.8		N
76	BC0563	BC0563	Biotin carboxyl carrier protein	-0.8		C
77	**BC4436**	**RpmA**	**LSU ribosomal protein L27P**	**-0.8**	**-1.3**	**J**
78	BC5327	BC5327	Stage II sporulation protein R	-0.8		S
79	BC0383	FhuD	Ferrichrome-binding protein	-0.8		P
80	BC1804	YbfQ	Rhodanese-related sulfurtransferases	-0.8		R
81	BC1517	AroB	3-dehydroquinate synthase	-0.7		E
82	**BC4830**	**BC4830**	**ABC transporter permease protein**	**-0.7**	**-0.7**	**V**
83	BC1365	YrdC	Isochorismatase	-0.7		Q
84	BC0851	YraB	Mercuric resistance operon regulatory protein	-0.7		K
85	BC4952	YutI	NifU protein	-0.7		O
86	**BC1901**	**BC1901**	**Phage protein**	**-0.7**	**-1.9**	**S**
87	BC3093	BC3093	hypothetical protein	-0.7		S
88	BC0873	YckJ	Cystine transport system permease protein	-0.7		E
89	**BC3209**	**BC3209**	**hypothetical Cytosolic Protein**	**-0.7**	**-1.4**	**S**
90	BC4789	LuxS	Autoinducer-2 production protein luxS / Ribosylhomocysteinase	-0.7		T
91	BC2054	GatA	Glutamyl-tRNA(Gln) amidotransferase subunit A	-0.7		J
92	**BC1984**	**BC1984**	**hypothetical protein**	**-0.7**	**-1.0**	**S**
93	BC0304	BC0304	FrnE protein	-0.7		Q
94	BC1188	YjbD	Arsenate reductase family protein	-0.6		P
95	BC0353	YerQ	hypothetical protein	-0.6		IR
96	BC0371	BC0371	Mandelate racemase/muconate lactonizing enzyme family protein	-0.6		MR
97	BC1648	BC1648	hypothetical Cytosolic Protein	-0.6		S
98	**BC4165**	**BkdR**	**Sigma-54-dependent transcriptional activator**	**-0.6**	**-1.0**	**KT**
99	BC2940	BC2940	Histidinol-phosphate aminotransferase	-0.6		E

No.	Locus Tag	Protein	Annotation	wt 42°C/ Δ*sigB* 42°C log2 FC	wt 42°C/ Δ*bc1009* 42°C log2 FC	COG
100	BC1377	BC1377	hypothetical protein	-0.6		S
101	**BC2215**	**YfkC**	**Mechanosensitive ion channel**	**-0.6**	**-2.0**	**M**
102	**BC3380**	**BC3380**	**Quinone oxidoreductase**	**-0.6**	**-0.7**	**C**
103	BC4925	YumB	NADH dehydrogenase	-0.6		C
104	BC5312	AtpB	ATP synthase A chain	-0.6		C
105	**BC3548**	**YueD**	**Benzil reductase**	**-0.6**	**-2.9**	**IQR**
106	BC0449	BC0449	hypothetical protein	-0.6		S
107	BC4724	BC4724	Molybdenum cofactor biosynthesis protein A	-0.6		H
108	**BC2369**	**BC2369**	**Acetyltransferase**	**-0.6**	**-0.8**	**K**
109	BC1195	YjbI	Globin Family Protein	-0.6		R

3.3.3 Bc1009 and SigB contribute to the survival of severe heat stress

To determine the impact of *bc1009* and *sigB* on survival of severe heat stress, the wt strain and isogenic mutants lacking Bc1009 or SigB were exposed to 50°C, with or without pre-adaptation at 42°C (Figure 4). Pre-adaption of wt, Δ*sigB,* and Δ*bc1009* cultures resulted in a significantly higher survival rate at 50°C than the non-pre-adapted control cells. The wt showed a 1.5 $\log_{10}$ reduction at 120 min. Although the adapted Δ*sigB*, Δ*bc1009,* and wt cultures showed similar survival during the first 40 min of exposure, the survival of the Δ*sigB* mutant then rapidly declined, resulting in approximately 2 $\log_{10}$ reductions after 120 min compared to the wt. The thermotolerance of adapted Δ*bc1009* cells was higher than that of Δ*sigB* cells. Survival rates were also similar to wt cells for the first 80 min, but a stronger decrease in survival was observed after 120 min at 50°C with an approximate 1.3 $\log_{10}$ reduction compared to the wt. This points to a modest role of Bc1009 in activating heat stress defense, in line with its role in controlling the expression of a subset of SigB-dependent genes/proteins.

Exposure to 50°C of cultures of wt, Δ*sigB* and Δ*bc1009* that were not pre-adapted at 42°C showed rapid killing, and a 3 $\log_{10}$ reduction was observed for all three strains already after 30 min (Figure 4).

3.3.4 Impact of SigB and Bc1009 on the *B. cereus* protein profile under control conditions at 30°C

The impact of SigB and Bc1009 on the level of motility proteins after heat shock (section 3.3.2), and previous studies on the regulation of *B. cereus* motility at 30°C by MogR and RpoN (Hayrapetyan et al., 2015; Smith et al., 2020), prompted us to perform an additional comparative omics analysis of non-stressed wt, Δ*sigB* and Δ*bc1009* cells at 30°C.

In total, 96 proteins showed significant differences in level in both Δ*sigB* and Δ*bc1009* mutants compared to wt cells already at 30°C, indicating that even their basal levels are dependent on SigB and Bc1009. This included 72 proteins with higher levels in the wt compared to the mutants and 24 with lower levels in the wt compared to the mutants (Figure 5A, Table 4). The proteins displaying the largest SigB and Bc1009-dependent differences in levels are indicated

with numbers in Figure 5A on the right and left, respectively. The COG functions of these proteins are shown in Figure 5B, and the complete list of differentially regulated proteins is presented in Table 4 (detailed in Supplementary Table S5A), with the complemented transcriptome data shown in Supplementary Table S5B. Interestingly, ~ 50% (41 of 72 induced and 12 of 24 downregulated) of the differentially expressed proteins that were detected at 30°C were also differentially regulated in heat-stressed cells (bold highlighted in Table 4) and described above (section 3.3.2).

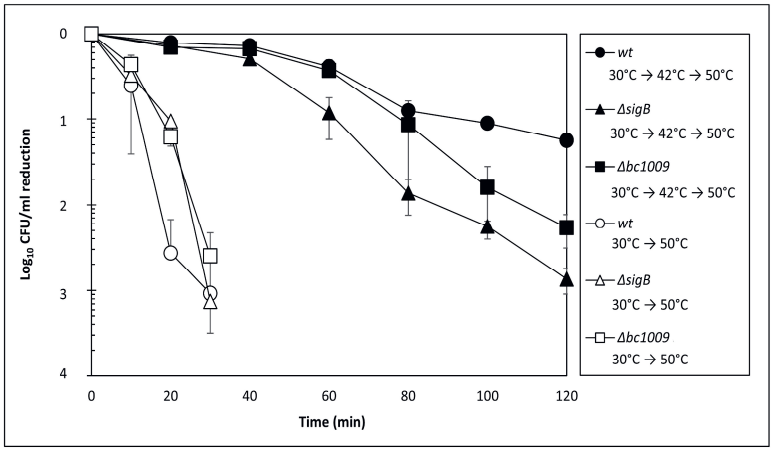


Figure 4- The relative survival of *B. cereus* wt, Δ*sigB,* and Δ*bc1009* mutants upon lethal heat exposure at 50°C. The relative survival at 50°C of heat-preadapted cells (30°C to 42°C for 45 min) of *B. cereus* wt (**filled circle**), Δ *sigB* (**filled triangle**), and Δ *bc1009* cells (**filled square**) compared to cells that were not preadapted to heat (42°C) (wt- **open circle**; Δ *sigB*- **open triangle**; Δ *bc1009*- **open square**) for 120 min. N=4.

SigB and Bc1009 dependently induced proteins at 30°C

SigB and Bc1009-dependent induced proteins at 30°C include flagella and chemotaxis proteins, transcriptional regulators like Spo0A and CggR, the Bc5117- Bc5125 cluster (conceivably involved in transporting nutrients), and Bc0423 (Non-ribosomal peptide synthetase) (Table 4). Next to proteins that were also differentially expressed under heat shock (described above in section 3.2.2), an additional group of proteins involved in carbohydrate/ion transport and metabolisms was present at lower levels in Δ*sigB* and Δ*bc1009* cells compared to wt cells at 30°C (Figure 5B, Table 4). These were YtzE, Bc2464, and Bc0896- Peptidoglycan endo-beta-N-acetylglucosaminidase, Bc1157- alpha-amylase, CutC- copper homeostasis protein, YclP- ferric transport ATP binding protein, Bc4984 ABC transporter, YtiB carbonic anhydrase, and YvgY copper chaperon associated protein. The remaining uniquely SigB and Bc1009-induced proteins at 30°C mainly have undefined functions or are additional proteins found in the same COG groups described above, including motility (Table 4). Proteomics results were supported by transcriptional data (Supplementary Table S5B).

SigB and Bc1009 dependently downregulated proteins at 30°C

An additional 24 proteins were present at lower levels in wt cells compared to Δ*sigB* and Δ*bc1009* mutants at 30°C, of which 12 also displayed lower levels in wt cells upon heat shock (section 3.3.2), and 12 were uniquely regulated at 30°C (Table 4). Four SigB and Bc1009-dependent downregulated proteins have putative roles in transcription regulation, including LytR family transcriptional regulator (Bc3587), leucine-responsive regulatory protein LrpC (Bc1363), ArsR family transcriptional regulator (Bc4256) and arginine repressor ArgR (Bc0405), of which the latter two were also upregulated in heat-stressed Δ*sigB* and Δ*bc1009* mutants compared to wt (section 3.2.2). Remarkably, the arginine repressor ArgR (Bc0405) and Bc0385 thioredoxin reductase were both present at 100-fold lower levels (log_2 fold change ~ 6) in wt cells compared to Δ*sigB* and Δ*bc1009* mutants (Figure 5, Table 4). However, genes that encode these proteins did not show differential expression (Supplementary Table S5B), suggesting regulation at the post-transcriptional level.

Taken together, this section provides evidence that SigB may be active already during control conditions at 30°C, showing its alternative role in cellular functions other than the SigB GSR, potentially via the putative Hpr-like protein, Bc1009.

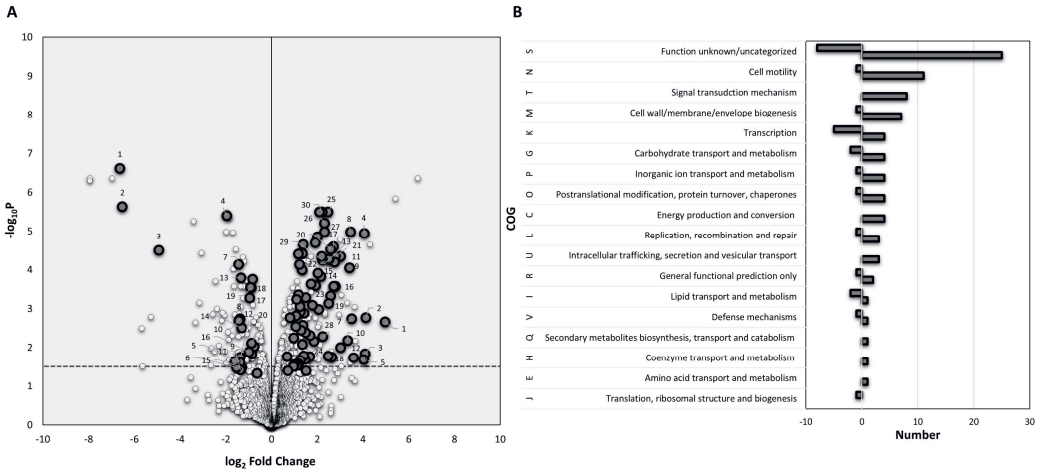


Figure 5- SigB-dependent induced and downregulated proteins for Δ*sigB* and Δ*bc1009* mutants compared to wt cells at 30°C and their cluster of orthologous group (COG). **5A-** log_2 fold change in protein expression. Positive and negative fold change values indicate induced and downregulated proteins, respectively. **Grey symbols:** induced/downregulated proteins that are SigB (and Bc1009)- dependent, i.e., differentially expressed proteins in Δ*sigB* and Δ*bc1009* mutants compared to wt cells at 30°C; the numbers indicate the top 30 induced proteins (positive x-axis) or top 20 downregulated proteins (negative x-axis), with details listed in Table 4. **White symbols:** induced/downregulated proteins at 30°C that are either only SigB-dependent or Bc1009-dependent, i.e., differentially expressed proteins in Δ*sigB* or Δ*bc1009* mutants compared to wt cells at 30°C (see details in Supplementary Table S5A). The threshold of the p-value for each protein was < 0.05. N=4. **5B-** cluster of orthologous group (COG) function for SigB (and Bc1009)-induced and downregulated proteins. The number on the x-axis indicates the number of induced/downregulated proteins in respective COG groups. The underlying transcriptome data supporting this figure are presented in Supplementary Table S5B.

Table 4: Differentially regulated SigB and Bc1009-dependent proteins at 30°C in *B. cereus* ATCC14579 wt cells versus Δ*sigB* and Δ*bc1009* mutants

Bold: SigB and Bc1009-dependent-proteins that were also differentially regulated upon heat shock in *B. cereus*
Not Bold: SigB and Bc1009-dependent proteins uniquely regulated at 30°C in wt cells versus Δ*sigB* and Δ*bc1009* mutants
log_2 FC: Protein fold change values at T0 time point
COG: Cluster of orthologous groups, see description in Figure 5.
Only SigB and Bc1009-dependent proteins are listed here

No.	Locus Tag	Protein	Annotation	SigB-dependent wt 30°C / Δ*sigB* 30°C log2 FC	Bc1009-dependent wt 30°C / Δ*bc1009* 30°C log2 FC	COG
1	BC5408	YfnB	2-haloalkanoic acid dehalogenase	5.0	3.9	R
2	**BC0423**	**SrfAAH**	**Non-ribosomal peptide synthetase (adenylation domain)**	**4.1**	**3.9**	**Q**
3	BC2294		hypothetical Cytosolic Protein	4.1	1.6	S
4	BC2724	OmdAH	LAAC/ Bacteriocin-protection, YdeI or OmpD-Associated	4.1	4.2	S
5	BC1718		DUF family protein of unknown function	4.0	4.9	S
6	BC3071	CutC	Copper homeostasis protein cutC	3.6	3.1	P
7	BC2752	YpeB	hypothetical Membrane Spanning Protein	3.5	3.5	S
8	**BC1303**	**YvfV**	**(S)-2-hydroxy-acid oxidase, iron-sulfur chain**	**3.5**	**3.0**	**C**
9	**BC5077**		**hypothetical protein**	**3.4**	**3.2**	**S**
10	BC4830		ABC transporter permease protein	3.3	1.0	V
11	BC0544		iron-sulfur cluster-binding protein	3.0	3.1	C
12	**BC1662**	**FliM**	**Flagellar motor switch protein fliM**	**3.0**	**2.9**	**N**
13	**BC5121**		**protein of unknown function**	**2.8**	**1.8**	**S**
14	**BC0576**	**McpBH**	**Methyl-accepting chemotaxis protein**	**2.8**	**1.4**	**NT**
15	**BC5243**		**protein with unknown function**	**2.8**	**2.6**	**S**
16	BC4496	RacE	Glutamate racemase	2.7	2.6	M
17	**BC3257**		**N-acetylmuramoyl-L-alanine amidase, Cell wall or membrane biogenesis**	**2.7**	**2.4**	**TM**
18	BC3698		Cell wall endopeptidase, family M23/M37	2.6	3.9	M
19	BC0232		hypothetical Membrane Spanning Protein	2.6	1.4	S
20	**BC0678**		**Methyl-accepting chemotaxis protein, signaling domain**	**2.6**	**2.0**	**NT**
21	**BC5123**		**homolog of lantibiotic biosynthesis dehydratase C-term**	**2.5**	**1.8**	**S**
22	BC4019		hypothetical protein	2.5	1.5	S
23	BC1202		Serine/threonine protein phosphatase	2.5	1.1	T
24	**BC1663**	**FliN**	**Flagellar motor switch protein fliN**	**2.5**	**2.8**	**NU**
25	**BC3406**		**Oxidoreductase**	**2.5**	**2.6**	**R**
26	**BC5118**		**ABC transporter ATP-binding protein**	**2.3**	**1.9**	**E**
27	BC1688	YmfQ	IG hypothetical 17894	2.3	1.6	S
28	**BC4170**	**SpoOA**	**Stage sporulation protein A**	**2.2**	**1.3**	**TK**
29	**BC5125**		**Peptidase family M50**	**2.2**	**2.3**	**M**
30	BC5198	YviA/degV	DegV family fatty acid binding protein, phosphorylation of fatty acids	2.2	2.0	S
31	**BC5122**		**YcaO cyclodehydratase, ATP-ad Mg2+-binding**	**2.2**	**1.9**	**S**
32	**BC5117**		**ABC transporter permease protein**	**2.2**	**1.7**	**S**
33	**BC5119**		**In operon with BC5120, which is a nitroreductase family protein**	**2.1**	**1.8**	**S**
34	**BC1857**	**SbcD**	**Exonuclease SbcD, DNA recombination and repair**	**2.1**	**2.8**	**L**
35	BC4512	MotB	Chemotaxis motB protein	2.0	0.7	N
36	**BC5103**	**YclP**	**Ferric anguibactin transport ATP-binding protein**	**2.0**	**1.7**	**P**
37	**BC1658**	**YvzB/ FliC**	**Flagellin**	**1.9**	**2.4**	**N**
38	**BC5141**	**CggR**	**Central glycolytic genes regulator**	**1.9**	**2.3**	**K**
39	BC1168	ClpB	ClpB protein	1.9	1.5	O
40	**BC1651**	**FlgE**	**Flagellar hook protein flgE**	**1.8**	**1.8**	**N**
41	BC5128	SmpB	SsrA-binding protein, required for rescue of stalled ribosomes	1.7	1.7	O
42	BC4703	YtzE	Transcriptional regulator, DeoR family	1.7	1.1	KG
43	**BC5034**	**YoaH**	**Methyl-accepting chemotaxis protein**	**1.7**	**1.1**	**NT**
44	BC4403	YrvE	Single-stranded-DNA-specific exonuclease recJ	1.5	1.3	LS

No.	Locus Tag	Protein	Annotation	SigB-dependent wt 30°C / Δ*sigB* 30°C log2 FC	Bc1009-dependent wt 30°C / Δ*bc1009* 30°C log2 FC	COG
45	**BC5410**	**YocJ**	**FMN-dependent NADH-azoreductase**	**1.5**	**1.1**	**I**
46	**BC5120**		**hypothetical Cytosolic Protein**	**1.5**	**1.3**	**S**
47	BC1850		Transcriptional regulator	1.5	1.0	K
48	BC1660	YjbJ	Soluble lytic murein transglycosylase	1.4	1.8	M
49	BC4984		ABC transporter substrate-binding protein	1.4	1.1	P
50	**BC5255**	**YknX**	**periplasmic component of efflux system**	**1.4**	**1.1**	**M**
51	**BC1654**	**CheV**	**Chemotaxis protein cheV**	**1.4**	**1.0**	**NT**
52	**BC2285**	**MmgD**	**2-methylcitrate synthase**	**1.4**	**1.3**	**C**
53	**BC2464**		**S-layer protein / Peptidoglycan endo-beta-N-acetylglucosaminidase**	**1.4**	**2.0**	**G**
54	BC1639	Flis	Flagellar protein fliS	1.3	1.0	NUO
55	**BC0888**	**CwlH**	**N-acetylmuramoyl-L-alanine amidase**	**1.3**	**1.2**	**M**
56	BC3947	PycA	Pyruvate carboxylase	1.3	1.0	C
57	BC3081	YhjR	hypothetical protein	1.3	1.2	S
58	BC0896		S-layer protein / Peptidoglycan endo-beta-N-acetylglucosaminidase	1.2	0.9	G
59	**BC1657**		**hypothetical protein**	**1.2**	**1.5**	**S**
60	**BC5436**		**Peptide methionine sulfoxide reductase**	**1.2**	**0.9**	**O**
61	**BC1659**		**hypothetical protein**	**1.2**	**1.0**	**S**
62	**BC0404**	**TarH**	**Methyl-accepting chemotaxis protein (motility, Signal transduction)**	**1.2**	**1.6**	**NT**
63	**BC2431**	**PhnB**	**PhnB protein**	**1.1**	**0.6**	**S**
64	**BC0597**	**YueK**	**Nicotinate phosphoribosyltransferase**	**1.1**	**1.5**	**H**
65	BC1157		Alpha-amylase	1.1	0.8	G
66	BC1653		hypothetical protein	1.1	0.7	S
67	**BC4178**		**Exodeoxyribonuclease VII small subunit**	**1.0**	**0.7**	**L**
68	BC1726		hypothetical Membrane Spanning Protein	1.0	1.8	S
69	**BC0675**		**hypothetical protein**	**1.0**	**2.0**	**S**
70	BC5189	SecA	Protein translocase subunit SecA	0.8	1.1	U
71	**BC5241**	**YvbJ**	**IG hypothetical 16680**	**0.7**	**1.3**	**S**
72	**BC4791**	**YtiB**	**Carbonic anhydrase**	**0.7**	**1.3**	**P**
1	BC0385		Thioredoxin reductase, posttranslational modification, protein turnover, chaperones	-6.6	-5.8	O
2	**BC0405**	**ArgR**	**Arginine repressor, argR**	**-6.5**	**-6.5**	**K**
3	BC2355		hypothetical protein	-4.9	-3.2	S
4	**BC2077**		**YukE protein of unknown function**	**-2.0**	**-2.0**	**S**
5	BC3587		Transcriptional regulator, LytR family	-1.6	-0.8	K
6	BC1363	LrpC	Leucine-responsive regulatory protein	-1.5	-0.9	K
7	BC3728		hypothetical protein	-1.4	-1.4	L
8	**BC1520**	**YpiB**	**hypothetical Cytosolic Protein**	**-1.4**	**-1.1**	**S**
9	BC5341	AcdA	Acyl-CoA dehydrogenase, short-chain specific	-1.4	-1.5	I
10	BC1359	YhcH	Bacitracin transport ATP-binding protein bcrA	-1.4	-0.6	V
11	BC5101		Perfringolysin O precursor	-1.4	-2.4	S
12	**BC3281**		**hypothetical protein**	**-1.4**	**-0.9**	**S**
13	**BC1638**	**Flg**	**Flagellar hook-associated protein 2**	**-1.3**	**-1.5**	**N**
14	BC5002	YusJ	Acyl-CoA dehydrogenase	-1.3	-0.9	I
15	BC1914		Phage protein	-1.3	-0.7	K
16	BC0366		hypothetical protein	-1.0	-0.7	S
17	**BC4256**		**Transcriptional regulator, ArsR family**	**-1.0**	**-0.8**	**K**
18	**BC5422**		**hypothetical protein**	**-0.9**	**-0.8**	**S**
19	**BC5274**	**YveM**	**UDP-N-acetylglucosamine 4,6-dehydratase**	**-0.9**	**-1.2**	**MG**
20	BC3731	YvgY	COP associated protein	-0.9	-0.7	P
21	BC0679		Cell wall-binding protein	-0.9	-1.3	S
22	BC0858		Modulator of drug activity B	-0.8	-0.7	R
23	BC0157	RpsK	SSU ribosomal protein S11P	-0.8	-0.8	J
24	BC5335	FbaA	Fructose-bisphosphate aldolase	-0.6	-0.7	G

3.3.5 Δ*bc1009* and Δ*sigB* mutants both show a defective motility phenotype

Based on the observation that many motility/chemotaxis proteins were present at higher levels in both non-stressed and heat-stressed wt cells compared to non-stressed and heat-stressed Δ*sigB* and Δ*bc1009* mutants, we compared the motility of Δ*sigB* and Δ*bc1009* mutants with that of wt cells on BHI agar plates with a low agar percentage (0.25%) under three conditions: 1) at 30°C (isothermal); 2) at 30°C, following a heat-shock at 42°C for 30 min; and 3) at 42°C (isothermal). A mutant unable to produce flagella (Δ*flgG*) was used as a negative control. Results that are presented in Figure 6 show that at 30°C, the colony diameter of Δ*flgG* mutant was lowest, followed by that of Δ*bc1009*, then Δ*sigB*, and with the wt showing the highest motility (Figure 6 left and right; observed phenotypes). Following a mild heat shock for 30 min at 42°C and subsequent incubation at 30°C, again, the colony diameter of Δ*flgG* mutant was lowest, with both Δ*sigB* and Δ*bc1009* mutants displaying intermediate levels of motility, with the wt displaying the highest motility. Similarly, incubation at isothermal 42°C showed the lowest and highest motility of Δ*flgG* mutant and wt cells, respectively, while Δ*sigB* and Δ*bc1009* cells showed comparable intermediate motility. These results show that SigB-induced motility in non-heat-stressed and heat-stressed cells depends on Bc1009.

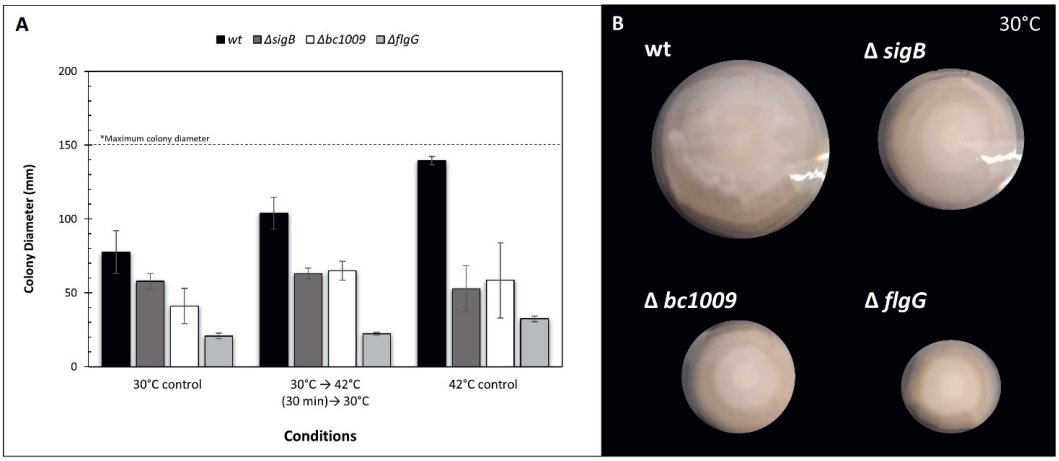


Figure 6- Motility phenotype of wt, Δ*sigB,* and Δ*bc1009* mutants. **6A-** the motility of wt, Δ*sigB,* and Δ*bc1009* was compared on Brain Heart Infusion (BHI) agar with 0.25% agar and indicated by the colony diameter (mm) formed on the agar after 24 h incubation. **Black bar:** wt; **Grey bar:** Δ*sigB* mutant; **white bar:** Δ*bc1009* mutant; **light grey bar:** Δ*flgG* mutant (negative control without flagella). The motility of all cells was tested under three different conditions, 1) at 30°C for 24 h; 2) upon heat shock from 30°C to 42°C for 30 min, and back to 30°C for 24 h; and 3) at 42°C for 24 h. The dotted line shows the maximum plate size. **6B-** Colony of wt, Δ*sigB,* Δ*bc1009,* and Δ*flgG* cells on 0.25% BHI agar at 30°C after 24 h.

3.4 Discussion

This study investigated the potential of *bc1009* that encodes the Hpr-like phosphocarrier protein Bc1009 in regulating SigB and/or modulating the expression of SigB regulon members. Comparative gene expression analysis of wt, *sigB*, and *bc1009* mutants ruled out a possible role of Bc1009 in regulating SigB expression, as its absence did not affect the expression of *sigB* and other SigB gene cluster members. Further proteomics studies of *B. cereus* wt, *sigB* and *bc1009* mutants provided novel insights into the SigB regulon and a role for Bc1009 in controlling the expression of a subset of genes/proteins in non-heat-stressed cells at 30°C and heat-stressed cells at 42°C.

Proteomics analyses backed up by transcriptomics data in this study revealed at least 284 SigB-controlled proteins upon heat shock in *B. cereus* (175 significantly induced/upregulated and 109 downregulated), including most SigB regulon members that were previously reported by De Been et al. (2010). In the current study, we excluded *rsbK* and *rsbM* from the SigB regulon list because the expression of *rsbK*, with *rsbM* in the same operon, was shown to be independent of SigB (Figure 1). Notably, a total of 96 SigB-dependent proteins (72 induced; 24 downregulated) were identified at 30°C for non-heat-stressed cells. Of these, 51/96 (53%) proteins overlapped with the results of SigB-dependent proteins that were identified upon heat shock, indicating that an additional 45 members were significantly differentially expressed at 30°C (i.e., differentially expressed proteins in Δ*sigB* mutant compared to wt), potentially expanding the SigB regulon members to more than 300 members in *B. cereus*. The presumed size of the SigB regulon in *B. cereus* is thereby comparable with the size of the SigB regulons of *B. subtilis* (Nannapaneni et al., 2012; Rodriguez Ayala et al., 2020; Vohradsky et al., 2021) and *L. monocytogenes* (Liu et al., 2017, 2019). At least 300 SigB regulon members have been described for the latter two species.

The *B. subtilis* SigB regulon contained SigB-dependent members (i.e., genes/proteins) that are either directly or indirectly controlled by SigB, based on the presence or absence of SigB promoter binding motif (PBM), respectively (Haldenwang, 1995; Petersohn et al., 1999; Nicolas et al., 2012). SigB regulon members indirectly controlled by SigB may occur via a cascade effect through a direct SigB-controlled regulator/protein. In this study, we provided evidence that ~ 70% of the newly identified SigB regulon members in *B. cereus* were regulated via the direct SigB-controlled member, Hpr-like phosphocarrier protein Bc1009. In line with this, all genes/gene clusters encoding newly identified proteins do not contain SigB PBMs (Chapter 2, data not shown). Several of these proteins appeared to contribute to the heat stress response, as proteins with putative functions in heat stress resistance (e.g., DNA recombination and repair, cell wall remodeling, and protein quality maintenance) were present at lower levels in Δ*bc1009* and Δ*sigB* mutants compared with wt upon heat shock. In line with these findings,

mild heat-induced stress resistance of the Δ*bc1009* mutant was lower than that of wt cells but higher than the Δ*sigB* mutant when lethal heat stress was imposed.

A group of sporulation-related proteins (with COG functions of transcription/signal transduction) was also found to be inducible via SigB after heat shock (SigK sporulation sigma factor, Bc4463, Spo0A, YndF, Bc3576). The expression of the latter three were dependent on Bc1009, providing further support for the previously reported role of SigB in affecting spore properties in *B. cereus* (de Vries et al., 2005). In addition, studies in *B. subtilis* have also shown that SigB impairs sporulation initiation by inactivating the sporulation master regulator Spo0A via the Spo0E phosphatase (Reder et al., 2012b, 2012a).

Other SigB and Bc1009-dependent proteins have putative functions in cell motility, signal transduction mechanism, transcription, and cell wall/membrane/envelope biogenesis. Approximately ~40% of these proteins were differentially regulated in non-heat-stressed control cells, suggesting that the baseline level of SigB at 30°C without further induction already contributes to the regulation of cellular functions (Figure 7). The significant induction of a large group of motility/chemotaxis proteins and the observed defect in motility phenotypes in Δ*sigB* and Δ*bc1009* mutants strongly suggests that SigB positively regulates motility in *B. cereus*. Based on this evidence, SigB can be added to the list of regulators, including MogR (Smith et al., 2020) and RpoN (Hayrapetyan et al., 2015), that play a role in the control of flagella synthesis and motility/chemotaxis in *B. cereus*. As *B. cereus* wt, Δ*sigB,* and Δ*bc1009* mutants contained flagella (Supplementary Figure S2), higher motility of wt than the two mutants may be linked to the increased levels of motor switch proteins (FliN, FliM, and FliG) and/or chemotaxis proteins (Bc0422, TlpA, Bc0678, MotA, MotB, TarH, CheA, CheV, McpB/H, and YoaH). In *B. subtilis*, indirect roles of SigB in surfactin production and swimming and swarming activity have been reported (Bartolini et al. (2018; Bartolini et al., 2019). Interestingly, Bc0423, a homolog of surfactin synthetase, was substantially downregulated in SigB and Bc1009 mutants compared to wt in non-heat-stressed cells at 30°C and heat-stressed cells at 42°C. However, further studies are required to confirm the role of Bc0423 in *B. cereus* motility in the tested conditions.

Our data also showed that proteins with putative functions in the modulation of membrane composition were under the control of SigB. This is in line with an earlier report on the putative functions of augmented SigB regulon members predicted for clade A *B. cereus sensu lato* group, including *B. cereus* ATCC14579 (Scott and Dyer, 2012). In addition, the expression of the *sinI* gene (antagonist of *sinR,* a regulator in biofilm formation) was significantly reduced in the *sigB* mutant but not in the *bc1009* mutant, implying that SigB may play a role in biofilm formation in *B. cereus,* in line with observations showing that the Δ*sigB* mutant of *B. cereus* 905 forms a weaker biofilm than the wt (Gao et al., 2021). Moreover, SigB has been reported to regulate biofilm aging and cell dispersal in *B. subtilis* (reviewed in Rodriguez Ayala et al. (2020).

In non-heat-stressed cells of *B. cereus,* proteins that showed SigB-dependent differential expression were involved in carbohydrate/ion transport and metabolisms, while upon a heat shock from 30°C to 42°C, a switch was observed to the expression of proteins involved in amino acid transport and metabolisms (Figure 7). This may indicate a rearrangement of cell resources to cope with heat stress in *B. cereus*. It is conceivable that amino acids are needed to maintain protein quality homeostasis under heat stress (e.g., as building blocks to aid protein repair/degradation). To the best of our knowledge, no known amino acid metabolism regulator has been reported to be SigB-controlled in *B. cereus* (de Been et al., 2011; Scott and Dyer, 2012) or *B. subtilis* (Subtiwiki, (Zhu and Stülke, 2018). However, the global regulator of branched-chain amino acid limitation in *Bacillus* species, CodY, is found to be SigB-dependent upon heat shock in *B. licheniformis* (Voigt et al., 2013). Nonetheless, based on information obtained in this study, SigB does not regulate CodY in *B. cereus,* or *B. subtilis* (Subtiwiki).

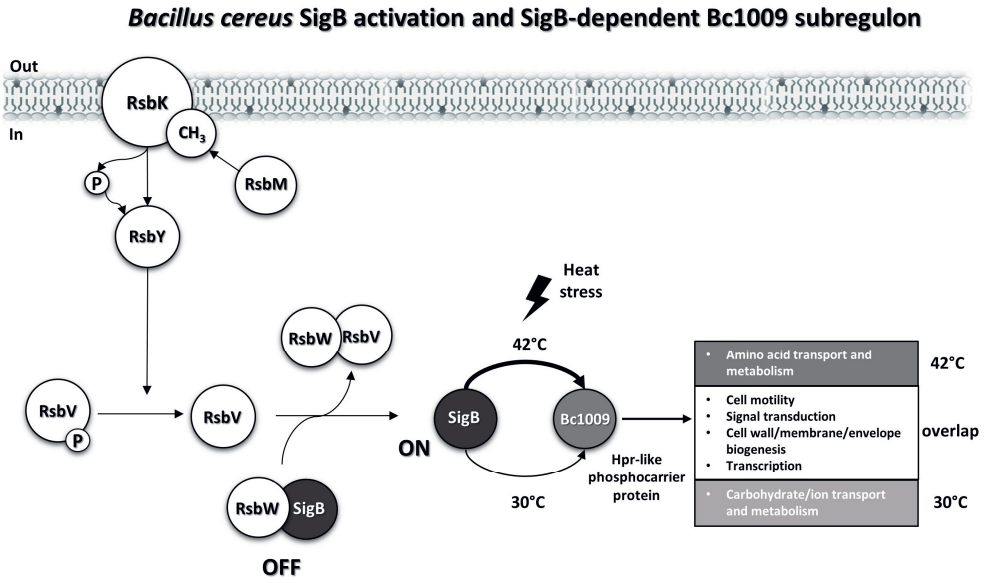


Figure 7- Activation model of *Bacillus cereus* SigB and the SigB-dependent Bc1009 subregulon. The RsbKY two-component system mediates the SigB general stress response in *B. cereus*. Under heat shock (temperature shift from 30°C to 42°C), SigB is induced (**bold arrow**) via the RsbKY signaling cascade and directly regulates the Hpr-like phosphocarrier protein Bc1009 to mediate amino acid transport and metabolism, cell motility, signal transduction, cell wall/membrane/envelope biogenesis and transcription. At 30°C, the baseline level of SigB also appears to regulate Bc1009 (**non-bold arrow**) to mediate similar functions as described for the heat shock condition, except for amino acid transport and metabolism (which switch to carbohydrate/ion transport and metabolism at 30°C instead, indicated in **dark grey** and **light grey box**, respectively). Upon SigB activation, other SigB regulon members not controlled by Bc1009 are also activated (see main text, Tables 2 and 3, and Supplementary Tables S5A and S5B).

B. cereus SigB (and Bc1009) dependent transcriptional regulators such as ArgR (arginine biosynthesis transcriptional regulator) may also play a role in metabolic shifts. Van Schaik et al. (2005) previously showed that *B. cereus* failed to utilize a variety of nitrogen sources,

including L-arginine, in the absence of SigB, suggesting that SigB may be linked to the control of nitrogen metabolism. Furthermore, SigB interaction with ArgR has also been described in *L. monocytogenes,* in which SigB is repressed by ArgR when arginine is absent and de-repressed by ArgR when arginine is present, thus forming feedback regulation (Cheng et al., 2017).

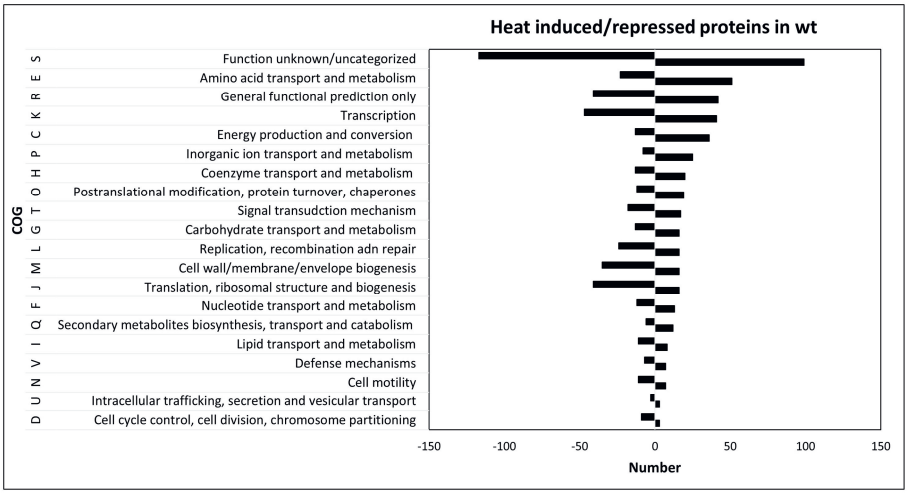
SigB may also be indirectly engaged in controlling virulence factors in *B. cereus*. The expression of two genes (*bc3103* and *bc3104*) that encode Hemolysin BL lytic component L1 (Hbl-L1) and L2 (Hbl-L2), and two genes (*bc1809* and *bc1810*) that encode non-hemolytic enterotoxin component NheA and NheB, was de-repressed in the Δ*sigB* mutant after heat shock, and the expression of the *cytK* gene that encodes the cytotoxin CytK was induced in both Δ*sigB* and Δ*bc1009* mutants under the non-heat-stressed condition in this study. Notably, in *B. anthracis,* a Δ*sigB* mutant was found to be less virulent in the mouse model (Fouet et al., 2000; Lereclus et al., 2000), whereas in *B. thurigiensis,* SigB was required for its pathogenicity towards insect larvae and is required for adaptation to the insect gut environment (Henry et al., 2020). We also found that Bc0385 (thioredoxin reductase) was dependent on SigB (and Bc1009). This protein is crucial for resistance against various disinfectant treatments in *B. cereus* (Ceragioli et al., 2010), suggesting further involvement of SigB in controlling other stresses. However, the impact of SigB on emetic and diarrheal toxin production and virulence in *B. cereus* remains to be elucidated.

It is known that the phosphotransferase system (PTS) components in *Bacillus* regulate carbon utilization and carbohydrate uptake/transport in response to metabolic/environmental challenges (Neira et al., 2021). This system links with the control of chemotaxis and cell motility, which supports the ability to scavenge additional carbon and/or nitrogen sources in the environment (Galinier and Deutscher, 2017). Such regulation is controlled via phosphorylation of target proteins, including transcriptional regulators, signal transduction proteins, transporters, and catabolic enzymes (Galinier and Deutscher, 2017; Neira et al., 2021). Our results provide evidence for an additional level of control exerted by Hpr-like phosphocarrier protein Bc1009, following the stress-induced RsbKY-dependent activation of SigB in *B. cereus*. Such indirect control of SigB via Bc1009 may be exerted via the phosphogroup transfer that leads to the modulation of other two-component systems or transcriptional regulators, e.g., those involved in chemotaxis/motility and PTS-type carbohydrate transport and utilization. Despite the significant increase in putative SigB regulon members, the number of genes/proteins that SigB directly controls and that contain a SigB PBM is low, i.e., around 30 of 300, an approximate 10 %. The stark contrast between the current and previously reported size of the *B. cereus* SigB regulon can be explained by the advanced proteomics approach used in the current study and the heat shock conditions used, which constituted a prolonged exposure time of 20-40 min to mild heat versus 10 min in the previous studies (van Schaik et al., 2004b, 2007; de Been et al., 2010b). The established relatively small SigB direct regulon may be related to the use of different stress sensing systems,

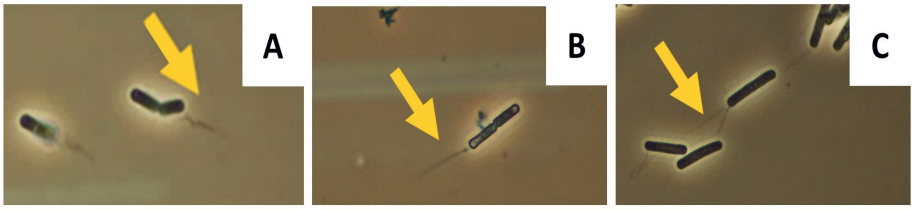
i.e., RsbKY in *B. cereus* versus RsbRST and/or RsbQP in *B. subtilis* and *L. monocytogenes*. The latter two species have at least 100 SigB direct regulon members with a SigB PBM (Price, 2010; Nannapaneni et al., 2012; Liu et al., 2019).

In conclusion, this study revealed novel SigB regulon members for *B. cereus* and provided evidence that expression of a SigB subregulon is controlled by Hpr-like phosphocarrier protein Bc1009. These subregulon members contribute to heat stress resistance and cell motility and have putative functions in signal transduction, cell wall/membrane/envelope biogenesis, transcription, amino acid/carbohydrate/ion transport and metabolism (Figure 7). Further exploration is required to investigate the possible roles of RsbKY in SigB-dependent or independent activation of Bc1009 upon exposure to other stresses and its impact on fitness and survival efficacy. In addition, identifying Bc1009 phosphotransfer-based interaction partners may shed light on other cellular regulatory networks.

3.5 Supplementary Materials



Supplementary Figure S1: Heat regulon genes/proteins in *Bacillus cereus* ATCC14579



Supplementary Figure S2: Flagella quick staining of wt cells (A), Δ*sigB* cells (B) and Δ*bc1009* cells (C). Flagella indicated with yellow arrow.

Supplementary Table S1: Oligonucleotides used in this study

qPCR amplification	**Target genes**	**Sequence 5' to 3'**
QPCR_sigB_F	*sigB (bc1004)*	CAATGTGATGAAGCGCAGGA
QPCR_sigB_R	*sigB (bc1004)*	CGGTCCGCCTTTTGAATAGC
QPCR_tufA_F	*tufA (bc0129)*	GCCCAGGTCACGCTGACTAT
QPCR_tufA_R	*tufA (bc0129)*	TCACGTGTTTGAGGCATTGG
QPCR_BC4306_F	*gatB/Yqey (bc4306)*	AGCTGGTCGTGAAGACCTTG
QPCR_BC4306_R	*gatB/Yqey (bc4306)*	CGGCATAACAGCAGTCATCA
QPCR_BC4307_F	*rpsU (bc4307)*	AAGATCGGTTTCTAAAACTGGTACA
QPCR_BC4307_R	*rpsU (bc4307)*	TTTCTTGCCGCTTCAGATTTC
QPCR_BC4952_F	*nifU (bc4952)*	TGGAAAACCCACATATGCAA
QPCR_BC4952_R	*nifU (bc4952)*	GCGCTCGATACCAGCTTTT
QPCR_BC1002_F	*rsbV (bc1002)*	TGCCTATTGCAAGCGAAAAA
QPCR_BC1002_R	*rsbV (bc1002)*	CACCTAAACCGGTGCTATCCA
QPCR_BC1003_F	*rsbW (bc1003)*	TGCGGATAATGGGGTTAGCTT
QPCR_BC1003_R	*rsbW (bc1003)*	TTCCGGCAAATGTTCTACTGG
QPCR_BC1005_F	*orf4 (bc1005)*	CCCACTACGATCCCTTTACCAG
QPCR_BC1005_R	*orf4 (bc1005)*	TTTCGCTGCCTGTTCTTTTCTC
QPCR_BC1006_F	*rsbY (bc1006)*	TGCCTGAAATTGATGGACTTGA
QPCR_BC1006_R	*rsbY (bc1006)*	CGGCCAATTTATTTGCATCC
QPCR_BC1008_F	*rsbK (bc1008)*	TGGATTGTTTTCCAACATCAGC
QPCR_BC1008_R	*rsbK (bc1008)*	CAAACTTCTTGCGGTGCATTT
QPCR_BC1009_F	*hpt (bc1009)*	GAACGCCATTCACTCTACTACTATTTC
QPCR_BC1009_R	*hpt (bc1009)*	TGTTGTCATTAAAGCATAAAAATGATG
Mutant construction	**Target genes**	**Sequence 5' to 3'**
KO BC1009_Up_BamHI_F	*hpt (bc1009)*	CACCGGATCCCTTTTAAATAGCATGATAGAACTTGA
KO BC1009_Up_NotI_R	*hpt (bc1009)*	TGAAGCGGCCGCATCGTAATATTCCTTTCCATATAGTAA
KO_BC1009_DOWN_NotI_F	*hpt (bc1009)*	CACTGCGGCCGCCTTAGAATGTTTGTTATCCTCT
KO_BC1009_DOWN_SalI_R	*hpt (bc1009)*	ACTAGTCGACTTACTGCAATCACTTCATCTTCA
KO_BC1004_UP_BamHI_F	*sigB (bc1004)*	TCGAGGATCCTTATTCTTACGACTTGCCTTGGTTC
KO_BC1004_UP_NotI_R	*sigB (bc1004)*	ATCTGCGGCCGCCATCATTCTCCACCTGCTCTC
KO_BC1004_DOWN_NotI_F	*sigB (bc1004)*	TGCAGCGGCCGCATTTTTAGATACATAA
KO_BC1004_DOWN_HindIII_R	*sigB (bc1004)*	CAGTAAGCTTATTTAATTCCGATTTCAAG
Genomic integration check primers	**Target genes**	**Sequence 5' to 3'**
KO BC1004_UPFlank_F	*sigB (bc1004)*	CCCCCACTCATTAACATTACACTTTCT
KO BC1004_DOWNFlank_R	*sigB (bc1004)*	TATATGATCTTCTCTTAATGGGCTACTT
KO BC1009_UPFlank_F	*hpt (bc1009)*	GTTTACGAAACCATTTCTTCACATACT
KO BC1009_DOWNFlank_R	*hpt (bc1009)*	GAATTCGCATACGTCCCATAATACTA

Supplementary Table S2A: Induced and downregulated proteins upon heat shock (30°C to 42°C) in *Bacillus cereus* ATCC14579 wt

				wt 42°C / wt 30°C		
No	**Locus Tag**	**Protein**	**Annotation**	**log2 FC_T20**	**log2 FC_T40**	**COG**
1	BC0075	CysK	Cysteine synthase	1.0	1.2	E
2	BC0099	CtsR	Transcriptional regulator ctsR	3.6	3.3	K
3	BC0100	McsA	ClpC ATPase	5.2	5.2	S
4	BC0101	McsB	Arginine kinase	4.7	4.7	E
5	BC0102	ClpC	Negative regulator of genetic competence clpC/mecB	2.4	2.5	O
6	BC0215	Pbp2_oppA	substrate-binding component of an ABC-type oligopetide import system	1.3	1.0	E
7	BC0218		Pyrroline-5-carboxylate reductase	0.7	1.1	E
8	BC0291	YdiH	AT-rich DNA-binding protein	0.8	0.9	R
9	BC0294	GroES	10 kDa chaperonin GROES	0.9	0.7	O
10	BC0295	GroEL	60 kDa chaperonin GROEL	2.3	2.2	O
11	BC0361	YxkH	Polysaccharide deacetylase	0.9	1.2	G
12	BC0371		Mandelate racemase/muconate lactonizing enzyme family protein	2.1	2.8	MR
13	BC0385	TrxBH	Thioredoxin reductase, posttranslational modification, protein turnover, chaperones	4.2	4.0	O
14	BC0405		Arginine repressor, argR	5.9	5.4	K
15	BC0442	YceC	Tellurium resistance protein terD	0.9	1.3	T
16	BC0491		Formate acetyltransferase, key step in anaerobic glycolysis	0.7	1.4	C
17	BC0492		Pyruvate formate-lyase activating enzyme, key step in anaerobic glycolysis	0.9	1.2	O
18	BC0538		protein with unknown function	1.3	1.5	S
19	BC0558	YuaG	Flottilin	1.0	1.4	S
20	BC0616	YfiY	Iron(III) dicitrate-binding protein	1.0	0.8	P
21	BC0668	YdjL	(R,R)-butanediol dehydrogenase	0.9	1.6	ER

22	BC0679		Cell wall-binding protein	0.7	0.7	S, S
23	BC0707		Ferrous iron transport protein B	0.9	2.2	P
24	BC0805		outer surface protein	1.0	2.2	S
25	BC0824		Lactoylglutathione lyase	1.8	3.6	E
26	BC0862	YfkM	Protease I	2.9	2.2	R
27	BC0863	KatE	Catalase	3.6	2.3	P
28	BC0883	AlsS	Acetolactate synthase large subunit	1.2	2.7	EH
29	BC0884	AlsD	Alpha-acetolactate decarboxylase	1.6	3.5	Q
30	BC0998	YflT	General stress protein 17M	7.3	6.8	S
31	BC0999	CsbDH	General stress protein (hyperosmotic & cold)	8.6	8.0	S
32	BC1002	RsbV	Anti-sigma B factor antagonist		2.7	U
33	BC1003	RsbW	Anti-sigma B factor	4.3	3.5	T
34	BC1004	SigB	RNA polymerase sigma-B factor	6.0	5.3	K
35	BC1005		Bacterioferritin	4.4	3.4	P
36	BC1010		hypothetical protein	3.9	2.9	S
37	BC1012		unknown	2.7	2.9	S
38	BC1069	HemH	Ferrochelatase	1.2	1.6	H
39	BC1115		Transcriptional regulator, AraC family	0.6	1.0	K, S
40	BC1125		S-layer homology domain	2.0	2.0	S
41	BC1126		S-layer homology domain	2.3	3.2	S
42	BC1127		Malate synthase	2.7	2.0	C
43	BC1168	ClpB	ClpB protein	1.3	1.5	O
44	BC1181	OppC	Oligopeptide transport system permease protein oppC	3.8	3.7	EP
45	BC1185		Oligopeptide-binding protein oppA	1.1	1.1	E
46	BC1225	YjcG	2'-5' RNA ligase	1.4	2.3	J
47	BC1246		NADH dehydrogenase	0.9	1.3	R
48	BC1251	OdhB	Dihydrolipoamide succinyltransferase component (E2) of 2-oxoglutarate dehydrogenase complex	1.4	1.4	C
49	BC1252	OdhA	2-oxoglutarate dehydrogenase E1 component	1.6	1.2	C
50	BC1259		hypothetical protein	1.3	1.2	S
51	BC1285	YwdH	Aldehyde dehydrogenase (NAD(P)+)	0.9	0.9	C
52	BC1305	YvbY	hypothetical protein	0.6	1.2	S
53	BC1308	YwcJ	Nitrite transporter	2.8	3.9	P
54	BC1316	PhaR	PhaR protein	1.5	1.4	S
55	BC1415		Glyoxylate reductase (NADP+)	0.8	1.3	CHR
56	BC1448		Nitric oxide dioxygenase	1.1	3.2	C, C
57	BC1498	YpfD	SSU ribosomal protein S1P	1.0	1.3	J
58	BC1515		Nucleoside diphosphate kinase	1.2	2.7	F
59	BC1528	YugP	hypothetical Membrane Spanning Protein	0.6	0.8	R
60	BC1540	PanB	3-methyl-2-oxobutanoate hydroxymethyltransferase	2.0	3.1	H
61	BC1542	PanD	Aspartate 1-decarboxylase	2.1	2.7	H
62	BC1566	YpwA	Thermostable carboxypeptidase 1	0.7	1.3	E
63	BC1576		Thiosulfate sulfurtransferase	0.9	1.7	P
64	BC1603		0	0.6	0.7	K
65	BC1652		hypothetical protein	1.9	2.1	S
66	BC1726		hypothetical Membrane Spanning Protein	2.0	2.1	S
67	BC1814		Transcriptional regulator, TetR family	1.4	2.4	K
68	BC1952		Nitroreductase family protein	2.7	3.8	R
69	BC1964		Homoserine dehydrogenase	1.8	1.8	E
70	BC1994	NadE	NH(3)-dependent NAD(+) synthetase	0.7	0.8	H
71	BC2011	Dps1	Non-specific DNA-binding protein Dps / Iron-binding ferritin-like antioxidant protein / Ferroxidase	1.4	2.0	P
72	BC2035		Magnesium and cobalt transport protein corA	1.6	1.4	P
73	BC2051		Ribosomal-protein-alanine acetyltransferase	2.2	2.6	KR
74	BC2118	NarG	Respiratory nitrate reductase alpha chain	1.2	2.7	C
75	BC2119	NarH	Respiratory nitrate reductase beta chain	1.0	1.8	C
76	BC2136	NasD	Nitrite reductase [NAD(P)H] large subunit	1.9	4.4	C
77	BC2147	RapG	Response regulator aspartate phosphatase	1.1	0.8	R
78	BC2213	YueJ	Pyrazinamidase	0.8	1.0	Q
79	BC2214		Small heat shock protein	1.7	1.7	O
80	BC2220	AdhA	Alcohol dehydrogenase	0.8	1.8	R
81	BC2241		Succinate-semialdehyde dehydrogenase [NADP+]	0.9	0.9	C
82	BC2295	SbcD	Exonuclease SbcD	1.0	1.4	L
83	BC2299		hypothetical protein	2.3	3.8	S
84	BC2313		unknown	1.3	2.3	L
85	BC2355		hypothetical protein	5.0	5.6	S
86	BC2752	YpeB	hypothetical Membrane Spanning Protein	2.1	2.0	S
87	BC2753	SleB	Cell wall hydrolase cwlJ	1.5	1.1	M, M
88	BC2850	YkfB	Mandelate racemase/muconate lactonizing enzyme family protein	3.1	2.6	MR
89	BC2892		hypothetical protein	1.7	1.6	S
90	BC2939	TyrA	Arogenate dehydrogenase	1.9	2.2	E
91	BC2940		Histidinol-phosphate aminotransferase	3.0	3.0	E
92	BC2942	AroA	Chorismate mutase	1.3	1.3	E, E
93	BC3028		Phosphohydrolase (MutT/nudix family protein)	1.3	1.7	LR
94	BC3065		Permease	1.2	1.2	S
95	BC3110	YndF	Spore germination protein BC	5.9	5.7	S

96	BC3132		General stress protein 17M	2.7	2.9	S
97	BC3201	YkoH	Two component system histidine kinase	0.7	1.6	T
98	BC3272		hypothetical Cytosolic Protein	0.8	1.6	S
99	BC3298		hypothetical Cytosolic Protein	1.5	1.7	S
100	BC3329		ABC transporter ATP-binding protein	3.3	3.0	V
101	BC3353		hypothetical protein	1.4	1.9	S
102	BC3600		Protease HhoA	0.7	1.6	O
103	BC3601	YclJ	Two-component response regulator	0.8	1.4	TK
104	BC3603		Anaerobic ribonucleoside-triphosphate reductase	3.0	5.0	F
105	BC3676		hypothetical protein	0.9	1.5	S
106	BC3760	YckE	6-phospho-beta-glucosidase	0.7	1.2	G
107	BC3772	YmcB	tRNA 2-methylthioadenosine synthase	1.0	1.5	J
108	BC3798	DapG	Aspartokinase	0.8	1.0	E
109	BC3828	ClpQ	ATP-dependent protease hslV	0.9	1.4	O
110	BC3831	TopA	DNA topoisomerase I	1.9	2.1	L, L
111	BC3856	RpmB	LSU ribosomal protein L28P	6.0	6.3	J
112	BC3870	YlzA	hypothetical protein	0.7	1.0	S
113	BC3891	PyrR	Uracil phosphoribosyltransferase	0.7	0.8	F
114	BC3908		Cell division protein ftsQ	2.2	2.5	M
115	BC3994		Exopolyphosphatase	2.1	2.5	FP
116	BC4054	NagB	Glucosamine-6-phosphate isomerase	1.5	2.2	G
117	BC4055	NagA	N-acetylgalactosamine-6-phosphate deacetylase	2.6	2.9	G
118	BC4093		Ribosomal-protein-serine acetyltransferase	1.1	1.8	J
119	BC4099		hypothetical protein	1.2	2.0	S
120	BC4121	NhaC	Tyrosine transporter	1.2	0.9	C
121	BC4124		Transcriptional regulator, MarR family	1.2	1.1	K
122	BC4179	YqiB	Exodeoxyribonuclease VII large subunit	0.6	0.6	L
123	BC4240		Transcriptional regulator	1.8	2.3	K
124	BC4250		5-methyltetrahydrofolate--homocysteine methyltransferase	1.0	3.1	E, E
125	BC4311	DnaJ	Chaperone protein dnaJ	1.0	1.0	O
126	BC4312	DnaK	Chaperone protein dnaK	0.7	0.7	O
127	BC4313	GrpE	GrpE protein	1.0	1.0	O
128	BC4315	HemN	Coproporphyrinogen oxidase, anaerobic	4.3	4.2	H
129	BC4329	YqeJ	Nicotinate-nucleotide adenylyltransferase	1.0	0.9	H
130	BC4354	YfiE	Glyoxalase family protein	5.2	3.4	R
131	BC4369	YrrT	Dimethyladenosine transferase	1.2	1.2	QR
132	BC4392	YrvO	Cysteine desulfhydrase	0.9	0.8	E
133	BC4394	YrvN	ATPase, AAA family	1.0	1.8	L
134	BC4427	PheA	Prephenate dehydratase	0.9	1.0	E
135	BC4449		hypothetical Membrane Spanning Protein	1.5	1.2	L
136	BC4454		hypothetical protein	1.4	1.0	S
137	BC4459		Type I restriction-modification system methylation subunit	1.7	1.2	V
138	BC4510	YhaQ	Sodium export ATP-binding protein	0.8	0.6	R
139	BC4525	YsiA	Transcriptional regulator, TetR family	1.5	1.7	K
140	BC4571	YsdC	Deblocking aminopeptidase	1.0	1.3	G
141	BC4584	YtaG	Dephospho-CoA kinase	1.7	1.8	H
142	BC4607	YtrI	hypothetical protein	1.4	3.4	S
143	BC4613	YtkL	Metal-dependent hydrolase	1.3	1.4	R
144	BC4623		Alanine dehydrogenase	0.8	1.1	E
145	BC4625		Universal stress protein family	1.1	1.6	T
146	BC4650		Transcriptional regulators, LysR family	2.9	2.3	K
147	BC4653	YtsP	GAF domain-containing proteins	1.0	1.0	T
148	BC4678		Aminopeptidase	0.7	0.9	E
149	BC4693	YtoP	Deblocking aminopeptidase	1.1	1.4	G
150	BC4702	YtjP	Xaa-His dipeptidase	1.2	1.0	E
151	BC4792		Cytochrome d ubiquinol oxidase subunit I	1.6	3.7	C
152	BC4816	BmrU	hypothetical protein	0.8	0.9	IR
153	BC4860		hypothetical protein	1.0	0.7	S
154	BC4870		L-lactate dehydrogenase	1.1	1.5	C
155	BC4900	YugI	S1-type RNA-binding domain	0.8	0.7	J
156	BC4915		ComA operon protein 2	1.1	0.8	Q
157	BC4926	YumC	Thioredoxin reductase	1.9	2.2	O
158	BC4931	YbbK	hypothetical Cytosolic Protein	0.8	1.4	S
159	BC4936	DapF	Diaminopimelate epimerase	1.1	1.4	E
160	BC4954	YutH	CotS-related protein	0.7	1.2	S
161	BC4958		NAD(P)H dehydrogenase [quinone]	0.7	0.9	R
162	BC4961	YutE	hypothetical Cytosolic Protein	1.0	1.4	S
163	BC4996	Ldh	L-lactate dehydrogenase	0.6	1.0	C
164	BC5008	YqgV	hypothetical Cytosolic Protein	1.1	1.6	S
165	BC5028		Two-component response regulator vanRB	1.7	1.9	TK
166	BC5035	YitW	Phenylacetic acid degradation protein paaD	1.0	0.8	R
167	BC5059		unknown	1.6	2.6	K
168	BC5065		Methyl-accepting chemotaxis protein	2.3	1.4	NT
169	BC5080		Methyltransferase	3.9	3.8	QR
170	BC5152	ClpP	ATP-dependent Clp protease proteolytic subunit	1.1	1.2	OU
171	BC5177	YwqN	Trp repressor binding protein	4.3	4.8	R

172	BC5190	YvyD	Probable Sigma (54) modulation protein / SSU ribosomal protein S30P	1.2	1.4	J
173	BC5199	YvyE	Xaa-Pro dipeptidase	1.2	1.1	S
174	BC5234	LytE	N-acetylmuramoyl-L-alanine amidase	1.1	1.9	M, T
175	BC5279		Tyrosine-protein kinase (capsular polysaccharide biosynthesis)	1.3	2.1	D
176	BC5322	YwrF	Nitrilotriacetate monooxygenase component B	1.4	2.2	R
177	BC5327		Stage II sporulation protein R	1.2	1.1	S
178	BC5337	YwjG	hypothetical protein	0.8	1.0	KE
179	BC5376	YwhD	hypothetical Cytosolic Protein	0.7	0.6	S
180	BC5388	YwfI	IG hypothetical 16794	0.9	1.5	S
181	BC5435	YerP	Acriflavin resistance plasma membrane protein	1.6	1.4	V
182	BC5478		hypothetical Cytosolic Protein	0.9	1.0	S
183	BC1182	OppD	Oligopeptide transport ATP-binding protein oppD	0.9		EP
184	BC1195	YjbI	Globin Family Protein	1.0		R
185	BC1304	YvfW	iron-sulfur cluster-binding protein	1.0		C
186	BC1317		Acetoacetyl-CoA reductase	1.3		IQR
187	BC1581		Mg(2+) P-type ATPase-like protein	0.6		P
188	BC1712	FumC	Fumarate hydratase	1.8		C
189	BC1715		Transcriptional regulator	2.3		K, S
190	BC1970		hypothetical protein	3.7		H
191	BC2087		hypothetical protein	0.6		S
192	BC2121	NarI	Respiratory nitrate reductase gamma chain	0.8		C
193	BC2526	YcxD	Transcriptional regulator, GntR family	1.1		KE
194	BC3163	YdeS	Transcriptional regulator, TetR family	0.9		K
195	BC3194		Transcriptional regulator, MarR family	3.2		K
196	BC3438		Transcriptional regulator, PadR family	1.4		K
197	BC3681	YneE	IG hypothetical 18106	1.8		S
198	BC3808	TruB	tRNA pseudouridine synthase B	1.3		J
199	BC3852	YlpC	PaaI family protein, possible transcriptional regulator	1.1		Q
200	BC4000		Multidrug resistance protein B	1.2		GEPR
201	BC4140		Mg(2+) transport ATPase, P-type	2.8		P
202	BC4199	YqhS	3-dehydroquinate dehydratase	0.8		E
203	BC4309	YqeU	surface protein	0.9		S
204	BC4314	HrcA	Heat-inducible transcription repressor hrcA	0.8		K
205	BC4336	SigK	RNA polymerase sigma-K factor, sporulation sigma factor	3.6		K
206	BC4345	MhpCH	Lipase; Pimeloyl-ACP methyl ester carboxylesterase (Coenzyme transport & metabolism)	3.8		R
207	BC4384		hypothetical protein	1.3		S
208	BC4416		Ferrichrome-binding protein	1.3		P
209	BC4458		Type I restriction-modification system specificity subunit	1.8		V
210	BC4463		Stage II sporulation protein B	2.7		S
211	BC4469	HemB	Delta-aminolevulinic acid dehydratase	0.9		H
212	BC4516	SdhB	Succinate dehydrogenase iron-sulfur protein	0.7		C
213	BC4517	SdhA	Succinate dehydrogenase flavoprotein subunit	0.6		C
214	BC4592		Malate dehydrogenase	0.7		C
215	BC4649	EzrA	Septation ring formation regulator	1.6		D
216	BC4674		Chorismate mutase	2.0		E, E
217	BC4679		hypothetical protein	1.0		S
218	BC4723		Molybdopterin biosynthesis MoeB protein	1.4		H
219	BC4820		hypothetical protein	1.2		S
220	BC5031		Methionyl-tRNA synthetase	1.2		J
221	BC5155	YvcK	hypothetical Cytosolic Protein	1.3		S
222	BC5205	LevR	Transcriptional regulatory protein levR	1.5		KT, K
223	BC5237	YdaM	N-acetylglucosaminyltransferase	1.0		M
224	BC5333	YwjI	Fructose-1,6-bisphosphatase	0.9		G
225	BC5422		O-Antigen ligase - like protein	1.6		S
226	BC0061	YabN	MazG protein	0.6		R
227	BC0622		L-threonine 3-dehydrogenase	0.8		MG
228	BC0666		Immune inhibitor A precursor	4.6		S
229	BC0786	YfhC	Nitroreductase family	1.4		C
230	BC0792	YrkD	hypothetical Cytosolic Protein	1.4		S
231	BC1323		2-aminoethylphosphonate-binding protein	1.0		P
232	BC1410	HisF	HisF protein	0.6		E
233	BC0016	YaaE	pyridoxine biosynthesis amidotransferase		0.6	H
234	BC0041	YabC	Corrin/porphyrin methyltransferase		0.8	R
235	BC0103	RadA	DNA repair protein RadA		3.4	O
236	BC0107	YacN	2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase		1.5	I
237	BC0186	YbbP	hypothetical Membrane Spanning Protein		0.9	S
238	BC0265	YdcD	hypothetical Cytosolic Protein		1.0	K
239	BC0464	YfkA	Thioredoxin-like oxidoreductases		2.2	R
240	BC0465		unknown		1.0	S
241	BC0466		Fumarate hydratase		0.8	C, C
242	BC0482	NagP	PTS system, N-acetylglucosamine-specific IIBC component		1.1	G, G
243	BC0551	YhbH	Glycosyltransferase		0.8	S
244	BC0580		NAD-dependent malic enzyme		2.5	C
245	BC0589		Formate dehydrogenase alpha chain		1.1	R

246	BC0604		hypothetical protein		0.6	S
247	BC0612		L-lactate permease		1.6	C
248	BC0708		Ferrous iron transport protein B		1.5	P
249	BC0709		Ferrous iron transport protein A		1.3	P
250	BC0740		Cell wall endopeptidase, family M23/M37		0.7	M, T
251	BC0902		S-layer protein / N-acetylmuramoyl-L-alanine amidase		1.7	M
252	BC0958		Transcriptional regulator		6.0	K
253	BC0991		S-layer homology domain / putative murein endopeptidase		0.9	D
254	BC1049		Bis(5'-nucleosyl)-tetraphosphatase (asymmetrical)		0.9	FGR
255	BC1060		Collagen adhesion protein		1.1	M
256	BC1154		Ferrochelatase		1.8	H
257	BC1202		Serine/threonine protein phosphatase		0.9	T
258	BC1226	YjcH	Acetyl esterase		1.5	P
259	BC1247		hypothetical protein		1.2	S
260	BC1271		hypothetical protein		1.7	S
261	BC1277		D-alanyl-D-alanine carboxypeptidase		2.3	M
262	BC1333		CBS domain containing protein		1.3	R
263	BC1345	YkvS	hypothetical protein		1.0	S
264	BC1350		hypothetical protein		1.0	S
265	BC1353	YosM	NrdI protein		0.8	F
266	BC1403	LeuD	3-isopropylmalate dehydratase small subunit		1.8	E
267	BC1408	HisH	Amidotransferase hisH		1.0	E
268	BC1422	Sat	Sulfate adenylyltransferase		2.0	P
269	BC1431		Cell wall endopeptidase, family M23/M37		1.4	M, S
270	BC1490	YpbH	genetic competence negative regulator mecA		1.8	OTN
271	BC1496	YccC	L-asparaginase		0.6	EJ
272	BC1514	HepT	Farnesyltransferase		1.4	H
273	BC1619	NfrA	Oxygen-insensitive NADPH nitroreductase		1.5	C
274	BC1623	Hfq	Hfq protein		4.6	R
275	BC1625	MotA	Chemotaxis motA protein		1.6	N
276	BC1626	YtxE/ motS	Chemotaxis motB protein		0.7	N
277	BC1647		Flagellum-specific ATP synthase		1.0	NU
278	BC1651	FlgE	Flagellar hook protein flgE		1.8	N
279	BC1653		hypothetical protein		2.0	S
280	BC1660	YjbJ	Soluble lytic murein transglycosylase		0.9	M
281	BC1725	DsdA	D-serine dehydratase		1.8	E
282	BC1746		Aspartate--ammonia ligase		0.9	E
283	BC1793		Chlorohydrolase/deaminase family protein		0.7	FR
284	BC1818		Transcriptional regulator, TetR family		1.8	K
285	BC1827		hypothetical Cytosolic Protein		1.8	S
286	BC1828		Xaa-Pro aminopeptidase		0.9	E
287	BC1867		Phage protein		2.3	S
288	BC1938	CydA	Cytochrome d ubiquinol oxidase subunit I		1.0	C
289	BC1962		Phosphohydrolase (MutT/nudix family protein)		1.2	LR
290	BC2006	TlpA	Methyl-accepting chemotaxis protein, pH sensor		1.2	NT
291	BC2013		Alpha/beta hydrolase		6.5	R
292	BC2032	YjhB	Phosphohydrolase (MutT/nudix family protein)		1.2	F
293	BC2074		Acetyltransferase		2.9	KR
294	BC2077		YukE protein of unknown function		0.8	S
295	BC2101		Formate--tetrahydrofolate ligase		0.6	F
296	BC2105		Lactoylglutathione lyase		1.8	E
297	BC2120	NarJ	Respiratory nitrate reductase delta chain		4.7	C
298	BC2124	MoeBH	thiamine/molybdopterin biosynthesis MoeB-like protein		3.5	H
299	BC2125	MoeAH	Molybdopterin biosynthesis MoeA protein, nitrate respiration		2.2	H
300	BC2163		TPR-repeat-containing protein		1.2	R
301	BC2164		Isoleucyl-tRNA synthetase, mupirocin resistant		1.8	J
302	BC2195		1-acyl-sn-glycerol-3-phosphate acyltransferase		1.3	I
303	BC2202	YpmQ	Cytochrome c oxidase Cu(A) center assembly protein		0.6	R
304	BC2254	YodN	IG hypothetical 18022		1.0	S
305	BC2298		Transcriptional repressor		1.2	K
306	BC2353		unknown		0.6	S
307	BC2424		hypothetical protein		0.8	S
308	BC2431		PhnB protein		0.7	S
309	BC2435		Phosphoglycerate mutase		1.7	G
310	BC2439		Aminoacyl-histidine dipeptidase		0.6	E
311	BC2448		D-alanyl-D-alanine carboxypeptidase		0.6	M
312	BC2456		Peptide synthetase		0.8	Q, Q
313	BC2567		Phage protein		0.6	S
314	BC2579		hypothetical Cytosolic Protein		1.6	J
315	BC2723	YurR	D-amino acid dehydrogenase small subunit		0.7	E
316	BC2772		Methyltransferase		1.6	QR
317	BC2919	YokD	Aminoglycoside N3'-acetyltransferase		1.5	V
318	BC2926		hypothetical protein		1.8	L
319	BC2938	AroE	3-phosphoshikimate 1-carboxyvinyltransferase		2.1	E
320	BC3021		hypothetical Cytosolic Protein		1.1	S

321	BC3078		Acetyltransferase		1.3	R
322	BC3121		5'-nucleotidase		1.1	F
323	BC3192		precursor of the glucomannan utilization protein ydhR		6.5	S
324	BC3264		hypothetical protein		1.1	L
325	BC3281		hypothetical protein		2.1	S
326	BC3303		hypothetical protein		0.8	J
327	BC3334		2-haloalkanoic acid dehalogenase		0.8	R
328	BC3355	YrkA	Magnesium and cobalt efflux protein corC		2.1	R
329	BC3368	YkgB	6-phosphogluconolactonase		5.1	G
330	BC3392		unknown		3.6	S
331	BC3437		hypothetical Cytosolic Protein		1.0	S
332	BC3439		Hydroxylamine reductase		5.0	C
333	BC3460	YwfD	Short chain dehydrogenase		0.7	IQR
334	BC3497	YhjG	Transcriptional regulator, ArsR family		1.0	K
335	BC3533	YoaR	Vancomycin B-type resistance protein vanW		1.6	V
336	BC3535	YetN	IG hypothetical 17116		1.1	S
337	BC3548	YueD	Benzil reductase		1.7	IQR
338	BC3550		Argininosuccinate lyase		2.6	E
339	BC3571	YrhF	hypothetical Cytosolic Protein		1.3	S
340	BC3597	ParE	Topoisomerase IV subunit B		1.2	L
341	BC3599	YneT	Succinyl-CoA synthetase, alpha subunit-related enzymes		0.9	R
342	BC3731	YvgY	COP associated protein		0.8	P
343	BC3743	PepT	Peptidase T		0.8	E
344	BC3769	MutS	DNA mismatch repair protein mutS		1.4	L
345	BC3790	YufP	Nucleoside transport ATP-binding protein		1.8	R
346	BC3797	DapA	Dihydrodipicolinate synthase		0.7	EM
347	BC3799		Aspartate-semialdehyde dehydrogenase		0.7	E
348	BC3802	YmxH	hypothetical protein		1.0	S
349	BC3827	ClpY	ATP-dependent hsl protease ATP-binding subunit hslU		0.9	O
350	BC3900	YlmE	hypothetical Cytosolic Protein		0.7	R
351	BC3952		GTPase		1.5	J
352	BC3960	YktB	hypothetical protein		0.8	S
353	BC3974	YkrB	Polypeptide deformylase		0.7	J
354	BC3987		NRDH-redoxin		1.3	O
355	BC3995	YkyB	hypothetical Cytosolic Protein		1.9	S
356	BC4041		hypothetical protein		1.5	S
357	BC4052		hydrolase (HAD superfamily)		0.6	R
358	BC4084	YfjQ	Magnesium and cobalt transport protein corA		1.5	P
359	BC4085		Pyrimidine-nucleoside phosphorylase		0.6	F
360	BC4094	NudF	ADP-ribose pyrophosphatase		0.9	LR
361	BC4123		Lipase/Acylhydrolase with GDSL-like motif		1.4	E
362	BC4143	YqjE	Peptidase T		0.7	E
363	BC4170	SpoOA	Stage 0 sporulation protein A		0.7	TK,
364	BC4174	ArgR	Arginine repressor, argR		1.1	K
365	BC4180	FolD	Methylenetetrahydrofolate dehydrogenase (NADP+)		0.8	H
366	BC4203	YhcW	Phosphoglycolate phosphatase		1.7	R
367	BC4206		Transcriptional regulator, PadR family		0.9	K
368	BC4225		Glycine dehydrogenase [decarboxylating]		0.9	E
369	BC4226	GcvT	Aminomethyltransferase		0.9	E
370	BC4256		Transcriptional regulator, ArsR family		0.7	K
371	BC4258	YqgX	Hydroxyacylglutathione hydrolase		0.6	R
372	BC4277		Zinc-specific metalloregulatory protein		1.6	P
373	BC4323		ComE operon protein 2		0.8	F
374	BC4340	YdfN	NAD(P)H nitroreductase		0.6	C
375	BC4365		Alcohol dehydrogenase		0.7	C, C
376	BC4382	YrzL	hypothetical Cytosolic Protein		1.4	S
377	BC4393	YrzC	RRF2 family protein		0.8	K
378	BC4468	HemL	Glutamate-1-semialdehyde 2,1-aminomutase		0.7	H
379	BC4481	YsoA	TPR repeat protein		0.7	R
380	BC4483	YvbH	hypothetical protein		0.7	S
381	BC4492		putative phosphoesterase		0.7	R
382	BC4509	YhaP	Sodium export permease protein		1.0	CP
383	BC4555		hypothetical protein		2.9	S
384	BC4579	DnaI	Primosomal protein dnaI		2.5	L
385	BC4593		Isocitrate dehydrogenase [NADP]		0.8	C
386	BC4614	YkvY	Xaa-Pro dipeptidase		0.8	E
387	BC4642	YtdI	ATP-NAD kinase		1.0	G
388	BC4644		PhnB protein		1.1	S
389	BC4692		hypothetical protein		4.6	S
390	BC4703	YtzE	Transcriptional regulator, DeoR family		1.3	KG
391	BC4743		unknown		1.4	V
392	BC4762	PckA	Phosphoenolpyruvate carboxykinase [ATP]		1.9	C
393	BC4832	YdeE	Transcriptional regulator, AraC family		1.2	K, S
394	BC4847		D-alanyl-D-alanine carboxypeptidase		4.0	M
395	BC4909	KapB	Kinase-associated protein B		0.7	S
396	BC4913		hypothetical protein		1.0	L

397	BC4959	YutF	4-nitrophenylphosphatase		0.9	G
398	BC4992	YusI	Arsenate reductase family protein		2.1	P
399	BC5000	YobS	Transcriptional regulator, TetR family		0.6	K
400	BC5076		Short chain dehydrogenase		2.1	R
401	BC5180		unknown		1.3	S
402	BC5187		Cytochrome c551		0.7	C
403	BC5251		RNA polymerase sigma factor		4.2	K
404	BC5317	YwlG	hypothetical protein		1.1	S
405	BC5325	YwlC	SUA5 protein		1.0	J
406	BC5379	YwfO	dGTP triphosphohydrolase		0.6	R
407	BC5413	ThiD	Phosphomethylpyramidine kinase		0.9	H
408	BC5436		Peptide methionine sulfoxide reductase		1.0	O, O
409	BC5341	AcdA	Acyl-CoA dehydrogenase, short-chain specific	1.7		I
410	BC0044	YabD	Sec-independent secretion protein tatD	0.7		L
411	BC0191		hypothetical Membrane Spanning Protein	0.7		S
412	BC0245	AppF	Oligopeptide transport ATP-binding protein oppF	4.1		E
413	BC0268	YdcK	Zinc metalloprotease	3.7		S
414	BC0344	YcgN	Delta-1-pyrroline-5-carboxylate dehydrogenase	0.9		C
415	BC0387		hypothetical protein	1.5		S
416	BC0446	YceG	hypothetical tellurium resistance protein	0.9		S
417	BC0457		Tetratricopeptide repeat family protein	1.0		S
418	BC0657	YusO	Transcriptional regulator, MarR family	0.8		K
419	BC1275		Methyltransferase	1.1		Q
420	BC1491	GudB	NAD-specific glutamate dehydrogenase	0.6		E
421	BC1796		hypothetical protein	2.1		S
422	BC2649		Transcriptional regulator, MerR family		0.8	K
423	BC3406		Oxidoreductase	1.2		R
424	BC3980	YkuR	putative N-acetyldiaminopimelate deacetylase	2.0		R
425	BC4097		2,5-diketo-D-gluconic acid reductase		1.0	R
426	BC4150	YqiX	Arginine-binding protein	0.9		ET
427	BC4802		hypothetical protein		0.7	S
428	BC5077		hypothetical protein	0.9		S
429	BC5410	YocJ	FMN-dependent NADH-azoreductase	1.9		I
1	BC1190	MecA	genetic competence negative regulator mecA	-0.6		OTN
2	BC1517	AroB	3-dehydroquinate synthase			E
3	BC1531	YpjD	hypothetical Transcriptional Regulatory Protein			R
4	BC0004	RecF	DNA replication and repair protein recF	-1.1	-1.2	L
5	BC0003	YaaA	hypothetical protein	-0.6		S
6	BC0021		Deoxyguanosine kinase		-1.0	F
7	BC0039	YabA	hypothetical protein	-0.7	-1.1	S
8	BC0045	RnmV	Ribonuclease M5		-1.1	L
9	BC0056		Peptidyl-tRNA hydrolase	-0.9	-1.7	J
10	BC0058		Transcription-repair coupling factor		-0.9	LK
11	BC0065		Cell division protein DIVIC		-1.4	D
12	BC0066	YabR	S1-type RNA-binding domain		-3.4	J
13	BC0069		Stage II sporulation protein E	-0.6	-0.8	TK
14	BC0072	FtsH	Cell division protein ftsH	-1.0	-1.1	O
15	BC0078	PabC	4-amino-4-deoxychorismate lyase		-0.9	EH
16	BC0081	FolK	2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase		-0.8	H
17	BC0082	YazB	Transcriptional regulator, Xre family		-1.7	K
18	BC0106	YacM	2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase		-0.8	I
19	BC0112	YacO	23S rRNA methyltransferase	-0.6	-0.6	J
20	BC0117	RplK	LSU ribosomal protein L11P		-0.7	J
21	BC0119	RplJ	LSU ribosomal protein L10P		-0.7	J
22	BC0122		DNA-directed RNA polymerase beta chain		-0.6	K
23	BC0131	RplC	LSU ribosomal protein L3P		-0.7	J
24	BC0132	RplD	LSU ribosomal protein L1E/L4P		-0.7	J
25	BC0133	RplW	LSU ribosomal protein L23P		-1.0	J
26	BC0135	RpsS	SSU ribosomal protein S19P		-0.9	J
27	BC0138	RplP	LSU ribosomal protein L16P		-0.7	J
28	BC0144	RpsN	SSU ribosomal protein S14P		-0.8	J
29	BC0147	RplR	LSU ribosomal protein L18P		-0.7	J
30	BC0159	RplQ	LSU ribosomal protein L17P		-1.2	J
31	BC0161	YbaE	Cobalt transport ATP-binding protein cbiO		-0.8	P
32	BC0163	TruA	tRNA pseudouridine synthase A	-1.9	-2.8	J
33	BC0167	CwlD	Spore-specific N-acetylmuramoyl-L-alanine amidase		-0.8	M
34	BC0198		ABC transporter substrate-binding protein		-0.6	P
35	BC0232		hypothetical Membrane Spanning Protein		-4.6	S
36	BC0235	YdaL	hypothetical protein		-0.7	S
37	BC0236		Transglycosylase	-1.1	-1.0	M
38	BC0241		Oligopeptide-binding protein oppA	-0.9	-1.2	E
39	BC0258	MurF	UDP-N-acetylmuramoylalanyl-D-glutamyl-2,6-diaminopimelate--D-alanyl-D- alanyl ligase	-0.7	-0.9	M
40	BC0259	YdbR	ATP-dependent RNA helicase		-0.9	LKJ
41	BC0325	PurB	Adenylosuccinate lyase		-0.7	F
42	BC0331	PurM	Phosphoribosylformylglycinamidine cyclo-ligase		-1.0	F

43	BC0340	PcrA	DNA helicase II		-0.8	L
44	BC0353	YerQ	hypothetical protein		-0.8	IR
45	BC0364	YefA	tRNA (Uracil-5-) -methyltransferase	-0.7	-0.9	J
46	BC0365	YfjN	Nitrogen regulation protein NIFR3	-0.6	-0.7	J
47	BC0368		hypothetical Cytosolic Protein	-0.8	-1.4	S
48	BC0376	AhpF	Alkyl hydroperoxide reductase subunit F		-0.7	O
49	BC0388	YoeD	hypothetical protein	-2.1	-2.4	S
50	BC0400		Fatty acid desaturase	-1.2	-1.3	I
51	BC0407		Ornithine carbamoyltransferase	-1.6	-1.8	E
52	BC0409		Carbamate kinase	-1.4	-3.0	E
53	BC0416		Phage infection protein		-0.9	S
54	BC0417	TopB	DNA topoisomerase I		-1.2	L, L
55	BC0419	ThiM	Hydroxyethylthiazole kinase		-1.8	H
56	BC0422		Methyl-accepting chemotaxis protein, signaling domain		-1.3	NT
57	BC0423	SrfAAH	Non-ribosomal peptide synthetase (adenylation domain)		-2.9	Q, Q
58	BC0439		Prolyl-tRNA synthetase	-1.2	-1.2	J
59	BC0445	YceF	TerC-like protein		-0.7	P
60	BC0453	YkvW	Zinc uptake P-type ATPase		-1.1	P
61	BC0471		tRNA pseudouridine synthase A	-0.8	-1.5	J
62	BC0545	YhbB	hypothetical Cytosolic Protein		-1.4	S
63	BC0563		Biotin carboxyl carrier protein	-0.7		C
64	BC0576	McpBH	Methyl-accepting chemotaxis protein	-0.7	-1.4	NT
65	BC0631	TreP	PTS system, trehalose-specific IIBC component	-1.4	-1.9	G, G
66	BC0644		OsmC-like protein		-2.1	O
67	BC0673		Flavin-dependent dehydrogenase		-1.1	C
68	BC0675		hypothetical protein		-0.7	S
69	BC0682	SrtC	Sortase		-0.7	M
70	BC0773	YdjE	Fructokinase		-1.8	G
71	BC0774	SacA	Sucrose-6-phosphate hydrolase		-0.9	G
72	BC0780	YhdB	hypothetical protein		-0.7	S
73	BC0801		Transcriptional regulator, LytR family	-0.6	-0.8	K
74	BC0812		PBS lyase HEAT-like repeat		-1.6	C
75	BC0841	YbbI	Glucokinase regulatory protein		-1.5	R
76	BC0858		Modulator of drug activity B	-0.6	-1.6	R
77	BC0875		0		-1.5	S
78	BC0892		hypothetical protein	-1.9	-2.9	S
79	BC0899		protein with unknown function	-1.2	-1.8	S
80	BC0903		Methyltransferase		-1.2	L
81	BC0939	YdiS	Type II restriction-modification system restriction subunit		-0.9	V
82	BC0954		tcdA-E operon negative regulator	-0.9	-1.7	S
83	BC0981		Dihydroxyacetone kinase		-0.9	G
84	BC0984		DNA-binding protein		-1.4	S
85	BC0992		hypothetical protein		-1.0	S
86	BC0997		hypothetical protein		-1.4	S
87	BC1018		unknown		-1.6	S
88	BC1025		Glyoxalase family protein		-1.1	E
89	BC1037		Transcriptional regulator, PadR family		-1.0	K
90	BC1064	YdbS	hypothetical Membrane Spanning Protein		-2.8	S
91	BC1086	YhfJ	Lipoate-protein ligase A		-0.8	H
92	BC1101		Internalin G		-0.9	S
93	BC1124		Methyl-accepting chemotaxis protein		-1.5	NT
94	BC1129		Trifolitoxin immunity protein	-1.1	-2.3	M, R
95	BC1131		Protein of unknown function	-0.9	-1.3	K
96	BC1157		Alpha-amylase		-1.5	G
97	BC1160	YitL	S1 RNA binding domain	-0.7	-1.1	S
98	BC1172		ComZ protein		-1.9	G
99	BC1198	YjbM	GTP pyrophosphokinase		-0.6	S
100	BC1237	TrpB	Tryptophan synthase beta chain	-1.4		E
101	BC1244		IG hypothetical 16680		-0.7	S
102	BC1286		Spermidine/putrescine transport ATP-binding protein potA		-1.1	E
103	BC1288		Spermidine/putrescine transport system permease protein potC	-1.3	-1.6	E
104	BC1302	YvfI	Transcriptional regulator, GntR family		-0.7	K
105	BC1327		(2-aminoethyl)phosphonate--pyruvate transaminase		-1.6	E
106	BC1349		Acetyltransferase		-1.1	KR
107	BC1357	YhcG	ABC transporter ATP-binding protein	-0.7	-0.7	V
108	BC1366		SSEB protein	-0.7		S
109	BC1371	DltB	Protein dltB	-0.6	-0.9	M
110	BC1398		Acetolactate synthase small subunit	-0.6	-1.6	E
111	BC1436		Phage shock protein A	-2.6	-2.5	KT
112	BC1443		SAM-dependent methyltransferase	-0.7	-0.7	QR
113	BC1472	RluB	Ribosomal large subunit pseudouridine synthase B	-2.2	-1.9	J
114	BC1479	YvqK	hypothetical Cytosolic Protein		-1.0	S
115	BC1497		Cytidylate kinase		-0.7	F
116	BC1504	YphC	GTP-binding protein	-0.8	-0.9	R
117	BC1512	HepS	Heptaprenyl diphosphate synthase component I		-0.7	S
118	BC1545	YpmB	hypothetical protein		-1.1	S

119	BC1550		Multimodular transpeptidase-transglycosylase PBP 1A		-1.4	M
120	BC1557		hypothetical protein		-4.3	S
121	BC1565	YpvA	ATP-dependent helicase, DinG family	-0.7		KL
122	BC1568		Methionine aminopeptidase		-0.6	J
123	BC1582	YpcP	5'-3' exonuclease		-0.7	L
124	BC1586	TuaA	Undecaprenyl-phosphate galactosephosphotransferase	-0.8	-1.2	M
125	BC1593	YitH	Acetyltransferase	-1.0		KR
126	BC1632	CheR	Chemotaxis protein methyltransferase		-1.6	NT
127	BC1633		hypothetical protein	-0.9		S
128	BC1641	FlgB	Flagellar basal-body rod protein flgB		-1.8	N
129	BC1642	FlgC	Flagellar basal-body rod protein flgC	-1.3	-1.2	N
130	BC1643	FliE	Flagellar hook-basal body complex protein fliE	-2.2		NU
131	BC1650	FlgD	Basal-body rod modification protein flgD		-1.2	N
132	BC1699		ECF-type sigma factor negative effector		-3.7	S
133	BC1718		DUF family protein of unknown function		-0.8	S
134	BC1737		Cardiolipin synthetase		-1.5	I
135	BC1740		unknown	-0.6	-1.5	S
136	BC1741	MalS	NAD-dependent malic enzyme	-0.8	-1.2	C
137	BC1756	PadR	Transcriptional repressor PadR	-0.9	-0.7	K
138	BC1788		Lysophospholipase L2		-1.3	I
139	BC1794		unknown		-0.8	E
140	BC1804	YbfQ	Rhodanese-related sulfurtransferases	-1.3	-2.3	R
141	BC1893		Scaffold protein	-2.7		S
142	BC1914		Phage protein		-1.3	K
143	BC1935		hypothetical protein	-0.9		S
144	BC1936		Transcriptional regulator, MarR family	-2.1	-2.0	K
145	BC1947	YybP	hypothetical protein	-3.0		S
146	BC1956		Two-component response regulator	-0.7		TK
147	BC1968		hypothetical protein		-5.7	H
148	BC1984		unknown		-1.3	S
149	BC1998	YczG	Transcriptional regulator, ArsR family		-0.9	K
150	BC2017		Ribosomal-protein-serine acetyltransferase	-0.6	-0.8	J
151	BC2037	YcgQ	hypothetical Membrane Spanning Protein		-1.2	S
152	BC2060		hydrolase (HAD superfamily)		-1.6	R
153	BC2084		hypothetical Cytosolic Protein		-2.7	S
154	BC2094		Acetyltransferase	-1.3		R
155	BC2102		hypothetical protein	-0.6	-0.7	S
156	BC2123	MoeAH	Molybdenum cofactor biosynthesis protein A	-1.1		H
157	BC2134	NasF	Uroporphyrin-III C-methyltransferase	-1.3	-0.8	H, H
158	BC2137	DnrN	Nitric oxide-dependent regulator DnrN		-1.1	D
159	BC2157	YphP	hypothetical protein		-0.7	J
160	BC2172		IG hypothetical 1888 Protein of unknown function	-1.1	-1.9	S
161	BC2183		Acetyltransferase		-1.0	KR
162	BC2187		Phosphoglycolate phosphatase		-1.8	R
163	BC2190		Penicillin-binding protein	-1.1		V
164	BC2194	YvaB	FMN-dependent NADH-azoreductase	-1.1		I
165	BC2204	AsnB	Asparagine synthetase [glutamine-hydrolyzing]	-0.6		E
166	BC2208		Iron(III) dicitrate-binding protein	-0.9	-0.7	P
167	BC2225		6-phosphogluconate dehydrogenase	-0.7		G
168	BC2242	YueI	hypothetical Cytosolic Protein	-0.6		S
169	BC2285	MmgD	2-methylcitrate synthase	-1.0		C
170	BC2286	MmgE	2-methylisocitrate dehydratase	-2.3		R
171	BC2318		hypothetical protein		-0.7	S
172	BC2321		tRNA pseudouridine synthase A	-1.6	-2.5	J
173	BC2335		Catabolite gene activator	-1.0	-1.1	T
174	BC2357		Cold shock protein	-1.6		S
175	BC2358		hypothetical protein	-1.5	-2.2	K
176	BC2379		Transcriptional regulator		-0.8	K
177	BC2390		hypothetical protein		-1.4	S
178	BC2421		unknown			S
179	BC2464		S-layer protein / Peptidoglycan endo-beta-N-acetylglucosaminidase	-0.8		G
180	BC2498		Two-component response regulator vanR	-0.9	-2.1	TK
181	BC2513		Excinuclease ABC subunit A		-0.9	L
182	BC2550		D-alanine--D-alanine ligase	-0.7	-1.1	M
183	BC2578		Phage protein		-1.3	S
184	BC2580		Phage protein		-1.2	S
185	BC2601		hypothetical protein		-1.3	S
186	BC2607		hypothetical protein		-1.2	S
187	BC2644		hypothetical protein	-0.9	-0.8	S
188	BC2662		Division specific D,D-transpeptidase / Cell division protein ftsI			M
189	BC2671	YoaZ	ThiJ/PfpI family		-3.5	R
190	BC2705	YjqA	hypothetical Cytosolic Protein	-0.7	-0.8	S
191	BC2713	YurQ	UvrC-like protein	-1.9	-3.1	L
192	BC2724	OmdAH	LAAC/ Bacteriocin-protection, YdeI or OmpD-Associated	-4.4	-4.9	S
193	BC2731		IG hypothetical 18565		-2.3	S

194	BC2749		Acetyltransferase	-1.2		KR
195	BC2770	GlcR	Transcriptional regulator, DeoR family	-1.2		KG
196	BC2773		RRF2 family protein		-1.1	K
197	BC2779	AcoA	Acetoin dehydrogenase E1 component alpha-subunit		-1.4	C
198	BC2809	YwnH	Phosphinothricin N-acetyltransferase	-3.1	-3.5	M
199	BC2821		unknown		-4.9	L
200	BC2824	YfjM	hypothetical Cytosolic Protein		-2.2	S
201	BC2848		unknown	-1.3	-5.5	E
202	BC2862	PrsA	Protein export protein prsA precursor		-0.8	O
203	BC2871	YitS	DegV family protein		-0.7	S
204	BC2882	YhbE	hypothetical Cytosolic Protein	-1.7	-1.6	M
205	BC2923		Acetyltransferase	-2.5	-1.6	KR
206	BC2933	YkpA	ABC transporter ATP-binding protein	-0.7	-0.8	R
207	BC2936	YdgH	Transcriptional repressor Bm3R1	-1.6	-0.9	K
208	BC2937		Acetyltransferase		-1.1	R
209	BC2977		Pyrroline-5-carboxylate reductase		-0.7	E
210	BC3058		Phosphohydrolase (MutT/nudix family protein)		-1.3	LR
211	BC3062	YhdZ	SIR2 family protein	-1.0		K
212	BC3081	YhjR	hypothetical protein		-0.9	S
213	BC3093		unknown	-0.8	-1.1	S
214	BC3135		Carboxymethylenebutenolidase-related protein	-2.3		R
215	BC3174		Short chain type dehydrogenase/reductase	-3.4	-1.2	IQR
216	BC3209		hypothetical Cytosolic Protein	-0.9		S
217	BC3216		1-aminocyclopropane-1-carboxylate deaminase	-0.6		E
218	BC3240		Thermitase	-0.7	-1.2	O
219	BC3257		N-acetylmuramoyl-L-alanine amidase, Cell wall or membrane biogenesis	-1.2	-1.2	T, M
220	BC3286		hypothetical protein	-1.4	-3.4	S
221	BC3308	CapA	Capsule biosynthesis protein capA	-1.2	-2.2	M
222	BC3394		4-oxalocrotonate tautomerase		-2.7	R
223	BC3484		Oligoendopeptidase F		-0.8	E
224	BC3501	RapC	Response regulator aspartate phosphatase	-0.6	-0.9	R
225	BC3520	TarH	Methyl-accepting chemotaxis protein (motility, Signal transduction)	-1.6	-1.5	NT
226	BC3587		Transcriptional regulator, LytR family	-1.2		K
227	BC3592		Transcriptional regulator, TetR family		-2.3	K
228	BC3606	YneP	Esterase	-1.0	-1.7	R
229	BC3608		Thioredoxin related protein	-1.5	-1.4	S
230	BC3659		hypothetical protein	-0.6	-1.0	S
231	BC3661	YneJ	CcdC protein		-0.7	O
232	BC3667b		unknown	-2.0	-2.1	S
233	BC3682		Transketolase		-0.6	G
234	BC3693		Transcriptional regulator, PadR family	-0.8		K
235	BC3696	XhlAH	cell-surface associated haemolysin		-1.0	S
236	BC3698		Cell wall endopeptidase, family M23/M37	-3.2	-2.9	M
237	BC3699		Antigen/ lysozyme like protein	-1.8	-3.9	M
238	BC3706	GlnA	Transcriptional regulator, MerR family		-1.7	K
239	BC3708	YnbA	GTP-binding protein hflX		-1.0	R
240	BC3714	MiaA	tRNA delta(2)-isopentenylpyrophosphate transferase		-0.9	J
241	BC3777	YmdB	IG hypothetical 15594	-1.1	-1.6	S
242	BC3785	YmfI	3-oxoacyl-[acyl-carrier protein] reductase		-1.3	IQR
243	BC3792	YmfC	Transcriptional regulator, GntR family		-1.4	K
244	BC3793		Cell division protein ftsK	-1.3		D
245	BC3796		Zn-dependent hydrolase	-0.6	-0.9	R
246	BC3803	MlpA	Zinc protease		-0.6	R
247	BC3805	PnpA	Polyribonucleotide nucleotidyltransferase		-0.6	J
248	BC3811	InfB	Bacterial Protein Translation Initiation Factor 2 (IF-2)		-0.7	J
249	BC3815	YlxS	hypothetical Cytosolic Protein	-0.7	-1.1	S
250	BC3830		Glucose inhibited division protein A	-0.9	-1.1	J
251	BC3835	RnhB	Ribonuclease HII	-0.8	-1.4	L
252	BC3837		Signal peptidase I	-1.1	-0.8	U
253	BC3846		Chromosome partition protein smc		-1.1	D
254	BC3847		Ribonuclease III	-0.6		K
255	BC3849	FabG	3-oxoacyl-[acyl-carrier protein] reductase	-0.7	-1.0	IQR
256	BC3859	YloQ	GTPase	-0.9	-0.9	R
257	BC3862	YloN	Radical SAM family enzyme		-1.4	R
258	BC3863	YloM	16S rRNA m(5)C 967 methyltransferase	-1.0	-0.9	J
259	BC3869		Guanylate kinase		-0.7	F
260	BC3874	LprIH	lysozyme inhibitor, binds to and inhibits macrophage lysozyme, aid bacterial survival	-3.8	-4.3	S
261	BC3892	YlyB	Ribosomal large subunit pseudouridine synthase D	-0.8	-0.7	J
262	BC3916	PbpB	Division specific D,D-transpeptidase / Cell division protein ftsI		-0.6	M
263	BC3931		hypothetical protein		-1.6	S
264	BC3934	YhfR	Phosphoglycerate mutase	-4.4	-3.0	G
265	BC3935		hypothetical Cytosolic Protein			S
266	BC3938	YlbA	hypothetical Cytosolic Protein	-1.9	-1.7	S
267	BC3945	CtaB	Protoheme IX farnesyltransferase		-1.8	O

268	BC3950	YlaL	hypothetical protein		-0.8	S
269	BC3956	YlaG	GTP-binding protein TypA/BipA		-0.6	T
270	BC3961	YktA	putative transcriptional regulator	-1.2	-2.1	S
271	BC3962	SpeA	Arginine decarboxylase		-1.0	E
272	BC3989	YkuI	Diguanylate cyclase/phosphodiesterase domain 2 (EAL)		-1.5	T
273	BC4051	GlcT	Transcription antiterminator, BglG family		-1.5	K
274	BC4063		hydrolase (HAD superfamily)		-1.0	R
275	BC4082		hypothetical protein	-2.9	-3.1	S
276	BC4112	RibH	6,7-dimethyl-8-ribityllumazine synthase		-1.4	H
277	BC4146	YqjB	Protein erfK/srfK precursor		-1.6	S
278	BC4151	YqiW	hypothetical protein		-1.1	S
279	BC4155	RodA	Rod shape-determining protein rodA		-0.9	D
280	BC4158		2-oxoisovalerate dehydrogenase beta subunit	-0.6		C
281	BC4165	BkdR	Sigma-54-dependent transcriptional activator	-0.9	-2.1	KT
282	BC4178		Exodeoxyribonuclease VII small subunit		-1.0	L
283	BC4181	NusB	N utilization substance protein B		-0.7	K
284	BC4186		Stage III sporulation protein AH	-1.1	-0.7	S
285	BC4204	MntR	Manganese transport transcription regulator	-1.1	-1.6	K
286	BC4208		hypothetical protein		-0.7	S
287	BC4221		ABC transporter ATP-binding protein			V
288	BC4224	GcvPBH	Glycine dehydrogenase [decarboxylating]	-1.3	-1.0	E
289	BC4239		ComG operon protein 1		-1.7	NU
290	BC4243		hypothetical protein	-1.0	-5.4	S
291	BC4249		UDP- N-acetylglucosamine transferase	-1.0		M
292	BC4262	YqgN	5-formyltetrahydrofolate cyclo-ligase		-1.0	H
293	BC4280	YqfU	hypothetical Membrane Spanning Protein	-2.7	-2.5	S
294	BC4283	YqfR	ATP-dependent RNA helicase	-0.7	-1.0	LKJ
295	BC4293	YqfL	hypothetical Cytosolic Protein		-0.8	S
296	BC4297		GTP-binding protein		-1.5	R
297	BC4320	RpsT	SSU ribosomal protein, translation	-4.9		J
298	BC4326	YqeM	Methyltransferase	-0.9	-1.0	QR
299	BC4328	YqeK	hydrolase (HAD superfamily)		-0.7	H
300	BC4332	YqeH	GTP-binding protein		-0.9	R
301	BC4371		hypothetical protein		-1.0	S
302	BC4375		Uridine kinase	-0.8	-1.3	F
303	BC4379	YrrL	hypothetical protein		-1.2	R
304	BC4381	YrrK	hypothetical Cytosolic Protein		-2.4	L
305	BC4396	YrvM	Molybdopterin biosynthesis MoeB protein		-0.7	H
306	BC4401	RelA	GTP pyrophosphokinase		-0.6	TK
307	BC4402		Adenine phosphoribosyltransferase		-0.8	F
308	BC4406		hypothetical protein		-2.5	S
309	BC4411		Queuine tRNA-ribosyltransferase	-0.8	-1.0	J
310	BC4415	RuvA	Holliday junction DNA helicase ruvA		-0.7	L
311	BC4434		GTP-binding protein	-0.9	-0.9	R
312	BC4436	RpmA	LSU ribosomal protein L27P		-0.8	J
313	BC4437	YsxB	hypothetical ribosome-associated protein	-0.7	-1.0	J
314	BC4443	MinC	Cell division inhibitor MinC		-0.8	D
315	BC4448		Protein with unknown function	-1.7		S
316	BC4453		Phage protein		-3.2	S
317	BC4477	LonA	ATP-dependent protease La	-0.6	-0.9	O
318	BC4484		hypothetical Cytosolic Protein		-1.6	S
319	BC4487		Superfamily I DNA and RNA helicases		-1.0	L
320	BC4489		hypothetical Cytosolic Protein	-2.6	-3.2	S
321	BC4493	YsnA	Nucleoside-triphosphatase		-1.3	F
322	BC4496	RacE	Glutamate racemase		-2.3	M
323	BC4505		Transcriptional regulator		-1.9	K
324	BC4512	MotB	Flagella motor protein	-0.6		N
325	BC4519	YslB	hypothetical Cytosolic Protein		-0.6	S
326	BC4523	EtfA	Electron transfer flavoprotein beta-subunit	-1.1	-1.3	C
327	BC4541	YwbD	Methyltransferase	-0.9	-1.2	R
328	BC4574	RpmI	LSU ribosomal protein L35P		-0.6	J
329	BC4580	DnaB	Replication initiation and membrane attachment protein	-0.8	-1.2	L
330	BC4583		NAD(P)-dependent glyceraldehyde-3-phosphate dehydrogenase	-1.3		G
331	BC4587	PolA	DNA polymerase I		-0.7	L, L
332	BC4594	CitZ	Citrate synthase		-0.8	C
333	BC4619		hypothetical protein	-2.2	-1.8	S
334	BC4647	YtbJ	Thiamine biosynthesis protein thiI		-0.9	H
335	BC4648	NifZ	Cysteine desulfhydrase	-0.8	-1.3	E
336	BC4658		Maltose O-acetyltransferase		-1.4	R
337	BC4667		Ankyrin		-1.0	R
338	BC4668		Virulence factor mviM		-0.6	R
339	BC4704	YtzF	Ribosomal small subunit pseudouridine synthase A	-0.9	-1.2	J
340	BC4746	YtqA	Fe-S oxidoreductase		-1.1	R
341	BC4795		hypothetical Membrane Spanning Protein	-1.2		S
342	BC4799		hypothetical protein	-1.1	-1.3	S

343	BC4818		hypothetical protein		-1.2	S
344	BC4830		ABC transporter permease protein		-1.2	V
345	BC4836		Two component system histidine kinase		-0.7	T
346	BC4846		Dihydroorotase	-1.4	-2.2	R
347	BC4911		Arsenical pump membrane protein	-0.6	-0.9	P
348	BC4925	YumB	NADH dehydrogenase		-1.3	C
349	BC4927		Cell surface protein		-1.0	L
350	BC4934		protein of unknown function	-0.9		S
351	BC4935	YutM	Fe-S carrier protein, assembly of Fe-S clusters, DNA repair		-2.4	S
352	BC4940	YjkB	ABC transporter ATP-binding protein		-0.9	P
353	BC4951	YuzD	hypothetical protein	-2.0	-1.6	S
354	BC4952	YutI	NifU protein	-0.8	-0.7	O
355	BC4956		Ribosomal-protein-alanine acetyltransferase	-0.9		KR
356	BC4966	YutD	hypothetical Cytosolic Protein	-1.7	-2.7	S
357	BC4983	YurY	ABC transporter ATP-binding protein		-0.6	O
358	BC5007	YusU	IG hypothetical 17Protein of unknown function29	-1.9		S
359	BC5010		Transcriptional regulator	-0.7	-1.0	K
360	BC5015		hypothetical exported repetitive protein	-1.6	-1.0	S
361	BC5018		hypothetical Membrane Spanning Protein		-1.3	S
362	BC5047	YvdC	IG hypothetical 16995	-1.2	-1.4	R
363	BC5051	AlsT	Sodium/proton-dependent alanine carrier protein		-1.0	E
364	BC5056		Collagen adhesion protein		-0.8	S
365	BC5064		protein with unknown function	-0.7	-1.1	R
366	BC5086		putative lantibiotic biosynthesis protein	-3.5	-2.4	S
367	BC5106		Ferric anguibactin-binding protein	-0.6	-0.7	P
368	BC5111		Transposase	-1.7	-1.7	L
369	BC5117		ABC transporter permease protein	-0.9	-1.4	S
370	BC5118		ABC transporter ATP-binding protein		-1.3	E
371	BC5119		In operon with BC5120, which is a nitroreductase family protein	-0.7	-1.1	S
372	BC5120		hypothetical Cytosolic Protein	-0.6	-0.9	S
373	BC5121		protein of unknown function	-0.8	-1.1	S
374	BC5122		YcaO cyclodehydratase, ATP-ad Mg2+-binding	-0.6	-1.3	S
375	BC5123		homolog of lantibiotic biosynthesis dehydratase C-term		-0.7	S
376	BC5124		Protein with unknown function		-1.8	S
377	BC5125		Peptidase family M50		-1.4	M
378	BC5128	SmpB	SsrA-binding protein, required for rescue of stalled ribosomes	-1.6	-0.6	O
379	BC5168	UvrB	Excinuclease ABC subunit B		-2.1	L
380	BC5194		unknown		-1.0	S
381	BC5198	YviA/degV	DegV family fatty acid binding protein, phosphorylation of fatty acids	-2.3		S
382	BC5222	YybE	Transcriptional regulators, LysR family	-3.6	-1.6	K
383	BC5232		Phosphoglycerol transferase		-1.1	M
384	BC5235	YutK	Nucleoside permease nupC		-1.3	F
385	BC5239		0		-3.8	T, S
386	BC5241	YvbJ	IG hypothetical 16680		-0.8	S
387	BC5242		Membrane protein with C2C2 zinc finger	-4.4		J
388	BC5243		hypothetical protein		-0.7	S
389	BC5252		hypothetical Membrane Spanning Protein	-0.6	-1.1	S
390	BC5253	YknZ	ABC transporter permease protein	-1.0	-0.9	V
391	BC5254	YvrO	ABC transporter ATP-binding protein	-1.5	-1.7	V
392	BC5255	YknX	periplasmic component of efflux system		-2.0	M
393	BC5263		UDP-glucose 4-epimerase	-1.8	-1.9	M
394	BC5264		EPSX protein	-0.6	-1.1	E
395	BC5265	LytR	Transcriptional regulator, LytR family		-2.1	K
396	BC5267	YveT	Glycosyltransferase	-0.7	-1.4	M
397	BC5269		Amylovoran biosynthesis AmsK	-0.9	-1.2	M
398	BC5271		UDP-N-acetylglucosamine 4-epimerase	-1.2		MG
399	BC5272		Carbamoyl-phosphate synthase small chain	-0.7	-1.5	F, I
400	BC5273	SpsC	UDP-bacillosamine synthetase	-0.7	-0.8	M
401	BC5277	YwqD	Tyrosine-protein kinase (capsular polysaccharide biosynthesis)	-0.8	-1.4	D
402	BC5278	YwqC	Chain length regulator (capsular polysaccharide biosynthesis)	-0.8	-1.1	M
403	BC5288		UDP-N-acetylglucosamine 1-carboxyvinyltransferase	-0.6	-1.1	M
404	BC5303		Integral membrane protein		-0.6	S
405	BC5312	AtpB	ATP synthase A chain		-0.8	C
406	BC5328	YwkE	Methyltransferase	-1.0	-1.2	J
407	BC5330		Thymidine kinase		-0.7	F
408	BC5332		Transcription termination factor rho		-0.6	K
409	BC5338	PyrG	CTP synthase		-0.7	F
410	BC5339	RpoE	DNA-directed RNA polymerase delta chain		-0.7	K
411	BC5365	YwiB	hypothetical protein	-0.8	-0.9	S
412	BC5367		hypothetical protein		-1.4	S
413	BC5374	YqiK	Glycerophosphoryl diester phosphodiesterase	-0.6		C
414	BC5377	YwhC	Membrane metalloprotease		-0.7	R
415	BC5386	YwfL	Lipoate-protein ligase A		-1.2	H
416	BC5387		Phosphate acetyltransferase		-0.6	C
417	BC5389		D-alanyl-D-alanine carboxypeptidase	-1.7	-1.8	M

418	BC5393		hypothetical protein		-1.5	S
419	BC5408	YfnB	2-haloalkanoic acid dehalogenase/ putative phosphatase	-6.2	-7.2	R
420	BC5419	TagA	N-acetylglucosaminyldiphosphoundecaprenol N-acetyl-beta-D-mannosaminyltransferase		-1.2	M
421	BC5420		Glycosyltransferase	-0.6		M
422	BC5426	YkhA	Acyl-CoA hydrolase		-0.8	I
423	BC5427		Glycosyltransferase involved in cell wall biogenesis		-0.7	M
424	BC5429		Beta-1,3-N-acetylglucosaminyltransferase		-1.0	M
425	BC5430		Oligosaccharide translocase (flippase)		-0.7	R
426	BC5433	YfmM	ABC transporter ATP-binding protein uup		-1.0	R
427	BC5454		Molybdenum cofactor biosynthesis enzyme and related Fe-S oxidoreductases		-1.5	R
428	BC5461	YycH	hypothetical membrane protein yycH	-0.8	-1.2	S
429	BC5462	YycG	Two-component sensor kinase yycG		-0.7	T
430	BC5474	RpsR	SSU ribosomal protein S18P		-0.7	J
431	BC5479	YkuT	Mechanosensitive ion channel		-0.6	M
432	BC5482		Sporulation initiation inhibitor protein soj		-0.6	D
433	BC5485	GidA	Glucose inhibited division protein A		-0.8	D
434	BC5487		Jag protein		-1.1	R
435	BC5489	RnpA	Ribonuclease P protein component	-0.7		J

Supplementary Table S2B: Induced and downregulated genes upon heat shock (30°C to 42°C) in *Bacillus cereus* ATCC14579 wt

No	Locus Tag	Gene	Annotation	log2 expression	COG
1	BC1000	*bc1000*	hypothetical Membrane Spanning Protein	6.8	S
2	BC0863	*katE*	Catalase	6.6	P
3	BC0998	*yflT*	General stress protein 17M	6.6	S
4	BC0862	*yfkM*	Protease I	6.4	R
5	BC1005	*rsbM*	Bacterioferritin	5.6	P
6	BC1003	*rsbW*	Anti-sigma B factor	5.4	T
7	BC1004	*sigB*	RNA polymerase sigma-B factor	5.1	K
8	BC1168	*clpB*	ClpB protein	5.1	O
9	BC1011	*bc1011*	hypothetical protein	5.0	S
10	BC1002	*rsbV*	Anti-sigma B factor antagonist	4.9	T
11	BC0999	*#N/A*	CsbD family protein	4.6	S
12	BC2214	*bc2214*	Small heat shock protein	4.6	O
13	BC0295	*groEL*	60 kDa chaperonin GROEL	4.5	O
14	BC0101	*mcsB*	Arginine kinase	4.4	E
15	BC0294	*groES*	10 kDa chaperonin GROES	4.3	O
16	BC2134	*nasF*	Uroporphyrin-III C-methyltransferase	4.3	H
17	BC0100	*mcsA*	ClpC ATPase	4.3	S
18	BC0102	*clpC*	Negative regulator of genetic competence clpC/mecB	4.2	O
19	BC4365	*bc4365*	Alcohol dehydrogenase	4.1	C
20	BC2119	*narH*	Respiratory nitrate reductase beta chain	4.1	C
21	BC2135	*nasE*	Nitrite reductase [NAD(P)H] small subunit	3.8	PR
22	BC0099	*ctsR*	Transcriptional regulator ctsR	3.7	K
23	BC3131	*bc3131*	hypothetical protein	3.7	S
24	BC2133	*bc2133*	CbiX protein	3.7	S
25	BC4454	*bc4454*	hypothetical protein	3.6	S
26	BC4455	*bc4455*	IG hypothetical 17788	3.6	S
27	BC4456	*bc4456*	Type I restriction-modification system restriction subunit	3.6	V
28	BC2120	*narJ*	Respiratory nitrate reductase delta chain	3.6	C
29	BC4457	*bc4457*	Type I restriction-modification system restriction subunit	3.6	V
30	BC2136	*nasD*	Nitrite reductase [NAD(P)H] large subunit	3.5	C
31	BC3130	*bc3130*	hypothetical protein	3.5	S
32	BC2128	*narK*	Nitrite extrusion protein	3.3	P
33	BC4458	*bc4458*	Type I restriction-modification system specificity subunit	3.2	V
34	BC3603	*bc3603*	Anaerobic ribonucleoside-triphosphate reductase	3.2	F
35	BC4793	*bc4793*	Cytochrome d ubiquinol oxidase subunit II	3.2	C
36	BC1941	*cydD*	Transport ATP-binding protein cydC	3.2	CO
37	BC1694	*bc1694*	hypothetical protein	3.2	S
38	BC4459	*bc4459*	Type I restriction-modification system methylation subunit	3.1	V
39	BC1010	*bc1010*	hypothetical protein	3.1	S
40	BC5211	*bc5211*	PTS system, lichenan oligosaccharide-specific IIC component	3.0	G
41	BC5152	*clpP*	ATP-dependent Clp protease proteolytic subunit	2.9	OU
42	BC1693	*bc1693*	putative hydrolase	2.9	R
43	BC0492	*bc0492*	Pyruvate formate-lyase activating enzyme	2.8	O
44	BC0884	*alsD*	Alpha-acetolactate decarboxylase	2.7	Q
45	BC2121	*narI*	Respiratory nitrate reductase gamma chain	2.7	C
46	BC2118	*narG*	Respiratory nitrate reductase alpha chain	2.7	C

47	BC1009	*bc1009*	putative phosphocarrier protein	2.7	S
48	BC1695	*bc1695*	Transcriptional regulator, MarR family	2.7	K
49	BC5212	*bc5212*	PTS sugar transporter subunit IIB	2.6	S
50	BC0883	*alsS*	Acetolactate synthase large subunit	2.6	EH
51	BC3883	*pyrF*	Orotidine 5'-phosphate decarboxylase	2.6	F
52	BC1940	*cydC*	Transport ATP-binding protein cydD	2.6	CO
53	BC3884	*pyrD*	Dihydroorotate dehydrogenase, catalytic subunit	2.5	F
54	BC0707	*bc0707*	Ferrous iron transport protein B	2.5	P
55	BC4312	*dnaK*	Chaperone protein dnaK	2.5	O
56	BC4460	*bc4460*	Type I restriction-modification system specificity subunit	2.5	V
57	BC4792	*bc4792*	Cytochrome d ubiquinol oxidase subunit I	2.5	C
58	BC0491	*bc0491*	Formate acetyltransferase	2.5	C
59	BC3882	*pyrE*	Orotate phosphoribosyltransferase	2.5	F
60	BC2125	*bc2125*	Molybdopterin biosynthesis MoeA protein	2.4	H
61	BC4313	*grpE*	GrpE protein	2.4	O
62	BC2220	*adhA*	Alcohol dehydrogenase	2.4	R
63	BC2123	*bc2123*	Molybdenum cofactor biosynthesis protein A	2.3	H
64	BC2023	*yqjM*	NADH oxidase	2.3	C
65	BC1305	*yvbY*	hypothetical protein	2.2	S
66	BC1923	*ydbI*	hypothetical Membrane Spanning Protein	2.2	R
67	BC2011	*dps1*	Non-specific DNA-binding protein Dps / Iron-binding ferritin-like antioxidant protein / Ferroxidase	2.2	P
68	BC1126	*bc1126*	S-layer homology domain	2.2	S
69	BC3886	*bc3886*	Carbamoyl-phosphate synthase large chain	2.2	EF
70	BC0708	*bc0708*	Ferrous iron transport protein B	2.2	P
71	BC2165	*bc2165*	hypothetical Membrane Spanning Protein	2.2	S
72	BC2941	*aroF*	Chorismate synthase	2.1	E
73	BC1304	*yvfW*	iron-sulfur cluster-binding protein	2.1	C
74	BC1411	*hisI*	Phosphoribosyl-AMP cyclohydrolase	2.1	E
75	BC2942	*aroA*	Chorismate mutase	2.1	E
76	BC1317	*bc1317*	Acetoacetyl-CoA reductase	2.1	IQR
77	sRNABC050			2.1	S
78	BC2939	*tyrA*	Arogenate dehydrogenase	2.1	E
79	BC0482	*nagP*	PTS system, N-acetylglucosamine-specific IIBC component	2.1	G
80	BC2940	*bc2940*	Histidinol-phosphate aminotransferase	2.1	E
81	BC1939	*cydB*	Cytochrome d ubiquinol oxidase subunit II	2.0	C
82	BC4469	*hemB*	Delta-aminolevulinic acid dehydratase	2.0	H
83	BC4241	*yqgY*	hypothetical protein	2.0	S
84	BC3439	*bc3439*	Hydroxylamine reductase	2.0	C
85	BC0668	*ydjL*	(R,R)-butanediol dehydrogenase	2.0	ER
86	BC3885	*pyrK*	Dihydroorotate dehydrogenase electron transfer subunit	2.0	HC
87	BC5012	*ydhL*	Chloramphenicol resistance protein	2.0	G
88	BC0371	*bc0371*	Mandelate racemase/muconate lactonizing enzyme family protein	1.9	MR
89	BC1999	*iolS*	IolS protein	1.9	C
90	BC2166	*bc2166*	Transcriptional regulator, TetR family	1.9	K
91	BC5018	*bc5018*	hypothetical Membrane Spanning Protein	1.9	S
92	BC3602	*bc3602*	Anaerobic ribonucleoside-triphosphate reductase activating protein	1.9	O
93	BC4268	*bc4268*	Phosphate transport system permease protein pstC	1.9	P
94	BC1717	*ygaJ*	Peptidase E	1.8	E
95	BC1965	*thrC*	Threonine synthase	1.8	E
96	BC2341	*#N/A*	MMPL family transporter	1.8	S
97	BC4971	*bc4971*	Phosphoglycerate mutase	1.8	G
98	BC3437	*bc3437*	hypothetical Cytosolic Protein	1.8	S
99	BC0709	*bc0709*	Ferrous iron transport protein A	1.8	P
100	BC3759	*bc3759*	6-phospho-beta-glucosidase	1.8	G
101	BC0817	*ywaF*	hypothetical Membrane Spanning Protein	1.8	S
102	BC2935	*#N/A*	MMPL family transporter	1.8	S
103	BC1006	*rsbY/ rsbP*	Sigma factor sigB regulation protein rsbU	1.8	TK
104	BC2758	*bc2758*	Metal-dependent hydrolase	1.8	R
105	BC0805	*bc0805*	outer surface protein	1.8	S
106	BC1523	*bc1523*	Menaquinol-cytochrome c reductase cytochrome b subunit	1.8	C
107	BC5055	*bc5055*	Wall-associated protein precursor	1.8	S
108	BC4468	*hemL*	Glutamate-1-semialdehyde 2,1-aminomutase	1.8	H
109	BC4055	*nagA*	N-acetylgalactosamine-6-phosphate deacetylase	1.8	G
110	BC3356	*bc3356*	Transcriptional regulator, MerR family	1.8	K
111	BC1780	*ilvD*	Dihydroxy-acid dehydratase	1.7	EG
112	BC3435	*bc3435*	Daunorubicin resistance transmembrane protein	1.7	V
113	BC1966	*thrB*	Homoserine kinase	1.7	E
114	BC4870	*bc4870*	L-lactate dehydrogenase	1.7	C
115	BC2978	*yqjN*	Peptidase family M20	1.7	E
116	BC1407	*hisB*	Imidazoleglycerol-phosphate dehydratase	1.7	E
117	BC1778	*bc1778*	ACT domain-containing protein	1.7	S
118	BC0370	*bc0370*	hypothetical protein	1.7	S
119	BC1251	*odhB*	Dihydrolipoamide succinyltransferase component (E2) of 2-oxoglutarate dehydrogenase complex	1.7	C

120	BC4140	*bc4140*	Mg(2+) transport ATPase, P-type	1.7	P
121	BC5219	*bc5219*	Integral membrane protein	1.7	S
122	BC1413	*bc1413*	Histidinol-phosphatase	1.7	ER
123	BC1922	*bc1922*	Glutamyl-tRNA(Gln) amidotransferase subunit A	1.7	J
124	BC0612	*bc0612*	L-lactate permease	1.7	C
125	BC1303	*yvfV*	(S)-2-hydroxy-acid oxidase, iron-sulfur chain	1.7	C
126	BC3616	*citB*	Aconitate hydratase	1.7	C
127	BC4471	*hemC*	Porphobilinogen deaminase	1.7	H
128	BC1308	*ywcJ*	Nitrite transporter	1.7	P
129	BC0444	*terD*	Tellurium resistance protein terD	1.7	T
130	BC4470	*hemD*	Uroporphyrinogen-III synthase	1.7	H
131	sRNABC051			1.7	S
132	BC1316	*bc1316*	PhaR protein	1.6	S
133	BC4926	*yumC*	Thioredoxin reductase	1.6	O
134	BC5322	*ywrF*	Nitrilotriacetate monooxygenase component B	1.6	R
135	BC5207	*bc5207*	Chromate transport protein	1.6	P
136	BC0442	*yceC*	Tellurium resistance protein terD	1.6	T
137	BC1524	*qcrC*	Menaquinol-cytochrome c reductase cytochrome c subunit	1.6	C
138	BC2757	*bc2757*	Tryptophan 2,3-dioxygenase	1.6	E
139	BC1007	*bc1007*	Chemotaxis protein methyltransferase	1.6	NT
140	BC4972	*bc4972*	DNA-binding protein	1.6	S
141	BC4311	*dnaJ*	Chaperone protein dnaJ	1.6	O
142	BC4121	*nhaC*	Tyrosine transporter	1.6	C
143	BC5388	*ywfI*	IG hypothetical 16794	1.6	S
144	BC1522	*qcrA*	Menaquinol-cytochrome c reductase iron-sulfur subunit	1.6	C
145	BC5003	*yusK*	3-ketoacyl-CoA thiolase	1.6	I
146	BC1025	*bc1025*	Glyoxalase family protein	1.6	E
147	BC0103	*radA*	DNA repair protein RadA	1.6	O
148	BC4927	*bc4927*	Cell surface protein	1.6	L
149	BC0104	*yacK*	DNA-binding protein	1.6	R
150	BC3555	*dhaS*	Aldehyde dehydrogenase	1.6	C
151	BC3355	*yrkA*	Magnesium and cobalt efflux protein corC	1.6	R
152	BC3887	*bc3887*	Carbamoyl-phosphate synthase small chain	1.5	EF
153	BC3760	*yckE*	6-phospho-beta-glucosidase	1.5	G
154	BC0870	*yheI*	Multidrug resistance ABC transporter ATP-binding and permease protein	1.5	V
155	BC3467	*bc3467*	Ferrichrome transport system permease protein fhuG	1.5	P
156	BC4776	*bc4776*	hypothetical protein	1.5	S
157	BC0443	*yceE*	Tellurium resistance protein terD	1.5	T
158	BC3758	*bc3758*	Transcriptional regulator, GntR family	1.5	K
159	BC4472	*hemX*	HEMX protein	1.5	O
160	BC1070	*hemY*	Protoporphyrinogen oxidase	1.5	H
161	BC5391	*ywdL*	Spore coat protein gerQ	1.5	S
162	BC1008	*rsbK*	Two component system histidine kinase	1.5	T
163	BC4584	*ytaG*	Dephospho-CoA kinase	1.5	H
164	BC2759	*bc2759*	L-kynurenine hydrolase	1.4	E
165	BC0802	*yhfP*	Alcohol dehydrogenase	1.4	CR
166	BC0663	*rbsC*	Ribose transport system permease protein rbsC	1.4	G
167	BC4314	*hrcA*	Heat-inducible transcription repressor hrcA	1.4	K
168	BC0408	*bc0408*	Arginine/ornithine antiporter	1.4	E
169	BC4902	*alaR*	Transcriptional regulator, AsnC family	1.4	K
170	BC4995	*bc4995*	regulatory protein (pfoS/R)	1.4	R
171	BC4901	*alaT*	Aromatic amino acid aminotransferase	1.4	E
172	BC4703	*ytzE*	Transcriptional regulator, DeoR family	1.4	KG
173	BC2351	*bc2351*	Transcriptional regulator, MerR family	1.4	K
174	BC4898	*bc4898*	Glucose-6-phosphate isomerase	1.4	G
175	BC0937	*citT*	Two-component response regulator citT	1.4	KT
176	BC4198	*yqhT*	Xaa-Pro dipeptidase	1.4	E
177	BC1225	*yjcG*	2'-5' RNA ligase	1.3	J
178	BC3123	*bc3123*	hydrolase	1.3	Q
179	BC5022	*bc5022*	Cytolysin immunity CylI	1.3	S
180	BC0806	*licR*	Transcription antiterminator, BglG family	1.3	GTK
181	BC4481	*ysoA*	TPR repeat protein	1.3	R
182	BC1498	*ypfD*	SSU ribosomal protein S1P	1.3	J
183	BC2130	*bc2130*	Transporter	1.3	R
184	BC2171	*bc2171*	Proline iminopeptidase	1.3	R
185	BC4199	*yqhS*	3-dehydroquinate dehydratase	1.3	E
186	BC1128	*bc1128*	Isocitrate lyase	1.3	C
187	BC1964	*bc1964*	Homoserine dehydrogenase	1.3	E
188	BC2479	*bc2479*	ABC transporter permease protein	1.3	V
189	BC4614	*ykvY*	Xaa-Pro dipeptidase	1.3	E
190	BC1588	*bc1588*	Secreted polysaccharide polymerase	1.3	M
191	BC3787	*ymfG*	Zinc protease	1.3	R
192	BC5035	*yitW*	Phenylacetic acid degradation protein paaD	1.3	R
193	BC0195	*yjdA*	3-oxoacyl-[acyl-carrier protein] reductase	1.3	IQR
194	BC3372	*yqeC*	6-phosphogluconate dehydrogenase	1.3	G
195	BC0632	*treA*	Trehalose-6-phosphate hydrolase	1.3	G

196	BC1069	*hemH*	Ferrochelatase	1.3	H
197	BC3438	*bc3438*	Transcriptional regulator, PadR family	1.3	K
198	BC5400	*bc5400*	Bacitracin transport ATP-binding protein bcrA	1.3	V
199	BC0631	*treP*	PTS system, trehalose-specific IIBC component	1.3	G
200	BC5220	*bc5220*	Lactoylglutathione lyase	1.3	E
201	BC1252	*odhA*	2-oxoglutarate dehydrogenase E1 component	1.3	C
202	BC0618	*yfiZ*	Iron(III) dicitrate transport system permease protein fecD	1.3	P
203	BC3000	*bc3000*	Proline/betaine transporter	1.2	GEPR
204	BC0237	*bc0237*	Galactose-1-phosphate uridylyltransferase	1.2	C
205	BC1937	*bc1937*	Luciferase-like monooxygenase	1.2	C
206	BC5062	*bc5062*	Tyrosyl-tRNA synthetase	1.2	J
207	BC0759	*bc0759*	Serine protease	1.2	R
208	BC4844	*bc4844*	4-Hydroxy-2-oxoglutarate aldolase	1.2	V
209	sRNABC052			1.2	S
210	BC1224	*yjcF*	Acetyltransferase	1.2	KR
211	BC1814	*bc1814*	Transcriptional regulator, TetR family	1.2	K
212	BC0662	*rbsA*	Ribose transport ATP-binding protein rbsA	1.2	G
213	BC0372	*bc0372*	hypothetical protein	1.2	S
214	BC3947	*pycA*	Pyruvate carboxylase	1.2	C
215	BC0555	*bc0555*	Glycine betaine transporter	1.2	M
216	BC4866	*glgD*	Glucose-1-phosphate adenylyltransferase	1.2	G
217	BC3993	*bc3993*	Polyphosphate kinase	1.2	P
218	BC5047	*yvdC*	IG hypothetical 16995	1.2	R
219	BC0439	*bc0439*	Prolyl-tRNA synthetase	1.2	J
220	BC2938	*aroE*	3-phosphoshikimate 1-carboxyvinyltransferase	1.2	E
221	BC3743	*pepT*	Peptidase T	1.2	E
222	BC4693	*ytoP*	Deblocking aminopeptidase	1.2	G
223	BC2170	*yocR*	Sodium-dependent leucine transporter	1.2	R
224	BC4996	*ldh*	L-lactate dehydrogenase	1.2	C
225	BC1246	*bc1246*	NADH dehydrogenase	1.2	R
226	BC1412	*bc1412*	Phosphoribosyl-ATP pyrophosphatase	1.1	E
227	BC4218	*bc4218*	ABC transporter permease protein	1.1	R
228	BC4219	*bc4219*	ABC transporter permease protein	1.1	R
229	BC2354	*bc2354*	Protoporphyrinogen oxidase	1.1	H
230	BC5136	*pgm*	Phosphoglycerate mutase	1.1	G
231	BC4592	*bc4592*	Malate dehydrogenase	1.1	C
232	BC4084	*yfjQ*	Magnesium and cobalt transport protein corA	1.1	P
233	BC4310	*yqeT*	Ribosomal protein L11 methyltransferase	1.1	J
234	BC1619	*nfrA*	Oxygen-insensitive NADPH nitroreductase	1.1	C
235	BC4220	*bc4220*	ABC transporter permease protein	1.1	R
236	BC5312	*atpB*	ATP synthase A chain	1.1	C
237	BC1065	*bc1065*	hypothetical Membrane Spanning Protein	1.1	S
238	BC1415	*bc1415*	Glyoxylate reductase (NADP+)	1.1	CHR
239	BC5399	*bc5399*	Bacitracin transport permease protein BCRB	1.1	R
240	BC4900	*yugI*	S1-type RNA-binding domain	1.1	J
241	BC3668	*alsR*	Transcriptional regulators, LysR family	1.1	K
242	BC0539	*yeeI*	hypothetical Cytosolic Protein	1.1	S
243	BC0696	*qoxC*	Cytochrome aa3 quinol oxidase polypeptide III	1.1	C
244	BC0695	*qoxD*	Cytochrome aa3 quinol oxidase polypeptide IV	1.1	C
245	BC4240	*bc4240*	Transcriptional regulator	1.1	K
246	BC1587	*bc1587*	Oligosaccharide translocase (flippase)	1.1	R
247	BC2370	*bc2370*	hypothetical protein	1.1	S
248	BC3719	*fruK*	1-phosphofructokinase	1.1	G
249	BC5335	*fbaA*	Fructose-bisphosphate aldolase	1.0	G
250	BC3662	*ccdA*	Cytochrome c-type biogenesis protein ccdA	1.0	O
251	BC5140	*gapA*	Glyceraldehyde 3-phosphate dehydrogenase	1.0	G
252	BC1815	*bc1815*	Acetyltransferase	1.0	KR
253	BC3960	*yktB*	hypothetical protein	1.0	S
254	BC1833	*bc1833*	hypothetical protein	1.0	S
255	BC0617	*bc0617*	Iron ABC transporter permease	1.0	S
1	BC0123	*bc0123*	DNA-directed RNA polymerase beta' chain	-1.0	K
2	BC5427	*bc5427*	Glycosyltransferase involved in cell wall biogenesis	-1.0	M
3	BC3811	infB	Bacterial Protein Translation Initiation Factor 2 (IF-2)	-1.0	J
4	BC0244	appD	Oligopeptide transport ATP-binding protein oppD	-1.0	EP
5	BC3506	*bc3506*	Ribosomal-protein-alanine acetyltransferase	-1.0	J
6	BC3819	*bc3819*	1-deoxy-D-xylulose 5-phosphate reductoisomerase	-1.0	I
7	BC1713	*bc1713*	hypothetical Membrane Spanning Protein	-1.1	S
8	BC5050	*bc5050*	Nucleoside permease nupC	-1.1	F
9	BC3836	ylqF	GTP-binding protein	-1.1	R
10	BC5456	yydA	hypothetical Cytosolic Protein	-1.1	S
11	BC3814	nusA	N utilization substance protein A	-1.1	K
12	sRNABC079			-1.1	S
13	BC3845	ftsY	Cell division protein ftsY	-1.1	U
14	BC4208	*bc4208*	hypothetical protein	-1.1	S
15	BC5278	ywqC	Chain length regulator (capsular polysaccharide biosynthesis)	-1.1	M
16	BC5358	*bc5358*	Collagen adhesion protein	-1.1	M

17	BC0122	*bc0122*	DNA-directed RNA polymerase beta chain	-1.1	K
18	sRNABC055			-1.1	S
19	BC3815	ylxS	hypothetical Cytosolic Protein	-1.1	S
20	BC5452	guaC	GMP reductase	-1.1	F
21	BC3820	cdsA	Phosphatidate cytidylyltransferase	-1.1	I
22	BC5006	yusM	Prolyne dehydrogenase	-1.1	E
23	BC1384	yubB	Bacitracin resistance protein (Putative undecaprenol kinase)	-1.1	V
24	BC1383	yojI	Na+ driven multidrug efflux pump	-1.1	V
25	BC0201	yitY	L-gulonolactone oxidase	-1.1	C
26	BC3699	*bc3699*	Antigen	-1.1	M
27	BC5034	yoaH	Methyl-accepting chemotaxis protein	-1.1	NT
28	BC5277	ywqD	Tyrosine-protein kinase (capsular polysaccharide biosynthesis)	-1.1	D
29	BC0190	glmS	Glucosamine--fructose-6-phosphate aminotransferase [isomerizing]	-1.1	M
30	BC3846	*bc3846*	Chromosome partition protein smc	-1.1	D
31	BC3830	*bc3830*	Glucose inhibited division protein A	-1.1	J
32	BC4335	*bc4335*	Phosphatidylserine decarboxylase	-1.2	I
33	BC2006	tlpA	Methyl-accepting chemotaxis protein	-1.2	NT
34	BC3679	*bc3679*	Multidrug resistance ABC transporter ATP-binding and permease protein	-1.2	V
35	BC5255	yknX	periplasmic component of efflux system	-1.2	M
36	sRNABC002			-1.2	S
37	BC3837	*bc3837*	Signal peptidase I	-1.2	U
38	BC1355	nrdF	Ribonucleoside-diphosphate reductase beta chain	-1.2	F
39	BC1615	*bc1615*	Na+ driven multidrug efflux pump	-1.2	V
40	BC4136	*bc4136*	L-serine dehydratase	-1.2	E
41	BC5264	*bc5264*	EPSX protein	-1.2	E
42	BC3812	ylxQ	LSU ribosomal protein L7AE	-1.2	J
43	BC5370	speB	Agmatinase	-1.2	E
44	BC0469	rlmD	23S rRNA (uracil(1939)-C(5))-methyltransferase RlmD	-1.2	S
45	sRNABC005			-1.2	S
46	BC0202	yitZ	Multidrug resistance protein B	-1.2	GEPR
47	BC0874	yckI	Cystine transport ATP-binding protein	-1.2	E
48	BC0654	ybaR	Sulfate transporter	-1.2	P
49	BC3868	yloH	DNA-directed RNA polymerase omega chain	-1.2	K
50	BC2153	*bc2153*	hypothetical protein	-1.2	S
51	BC1277	*bc1277*	D-alanyl-D-alanine carboxypeptidase	-1.2	M
52	BC5338	pyrG	CTP synthase	-1.2	F
53	BC4295	recO	DNA repair protein recO	-1.2	L
54	BC3817	proS	Prolyl-tRNA synthetase	-1.2	J
55	BC4744	*bc4744*	hypothetical Membrane Spanning Protein	-1.2	S
56	BC3810	ylxP	hypothetical Cytosolic Protein	-1.2	S
57	BC5365	ywiB	hypothetical protein	-1.2	S
58	BC5106	*bc5106*	Ferric anguibactin-binding protein	-1.2	P
59	BC1742	*bc1742*	Two component system histidine kinase	-1.2	T
60	BC1110	*bc1110*	Cytotoxin K	-1.2	S
61	BC5231	*bc5231*	hypothetical protein	-1.2	S
62	BC0576	*bc0576*	Methyl-accepting chemotaxis protein	-1.2	NT
63	BC0873	yckJ	Cystine transport system permease protein	-1.2	E
64	BC1729	*bc1729*	Multi-TM2 domain protein	-1.2	S
65	BC0907	appA	Oligopeptide-binding protein oppA	-1.2	E
66	BC5364	argS	Arginyl-tRNA synthetase	-1.3	J
67	BC3257	*bc3257*	N-acetylmuramoyl-L-alanine amidase	-1.3	TM
68	BC4174	*bc4174*	Arginine repressor, argR	-1.3	K
69	BC0909	*bc0909*	Oligopeptide transport system permease protein oppC	-1.3	EP
70	BC1564	*bc1564*	hypothetical protein	-1.3	S
71	BC3840	rimM	16S rRNA processing protein rimM	-1.3	J
72	BC3962	speA	Arginine decarboxylase	-1.3	E
73	BC1290	*bc1290*	Phosphatidylglycerophosphatase B	-1.3	I
74	BC4630	argG	Argininosuccinate synthase	-1.3	E
75	BCp0001		hypothetical protein	-1.3	S
76	BC4137	*bc4137*	hypothetical protein	-1.3	S
77	BC0468	yfjP	DNA-3-methyladenine glycosylase II	-1.3	L
78	BC3808	truB	tRNA pseudouridine synthase B	-1.3	J
79	BC5242	*bc5242*	Membrane protein with C2C2 zinc finger	-1.3	J
80	BC5331	ytiA	LSU ribosomal protein L31P	-1.3	J
81	BC3809	rbfA	Ribosome-binding factor A	-1.3	J
82	BC5339	rpoE	DNA-directed RNA polymerase delta chain	-1.3	K
83	BC0115	*bc0115*	Protein translocase subunit SecE	-1.3	U
84	BC1131	cspA	RNA chaperone/antiterminator CspA	-1.3	K
85	BC5489	rnpA	Ribonuclease P protein component	-1.3	J
86	BC3821	uppS	Undecaprenyl pyrophosphate synthetase	-1.3	I
87	sRNABC029			-1.3	S
88	BC5490	*bc5490*	LSU ribosomal protein L34P	-1.3	J
89	BC3678	*bc3678*	Multidrug resistance ABC transporter ATP-binding and permease protein	-1.3	V
90	BC3839	trmD	tRNA (Guanine-N1) -methyltransferase	-1.3	J
91	BC5243	*bc5243*	hypothetical protein	-1.3	S
92	BC5230	*bc5230*	hypothetical Membrane Spanning Protein	-1.3	R

93	BC0162	ybaF	Cobalt transport protein cbiQ	-1.4	P
94	BC5241	yvbJ	IG hypothetical 16680	-1.4	S
95	BC0422	*bc0422*	Methyl-accepting chemotaxis protein	-1.4	NT
96	BC1497	*bc1497*	Cytidylate kinase	-1.4	F
97	BC3680	yneF	hypothetical protein	-1.4	S
98	BC1223	yjcD	ATP-dependent DNA helicase	-1.4	L
99	BC5487	*bc5487*	Jag protein	-1.4	R
100	BC1730	*bc1730*	hypothetical protein	-1.4	S
101	BC2933	ykpA	ABC transporter ATP-binding protein	-1.4	R
102	BC3681	yneE	IG hypothetical 18106	-1.4	S
103	BC1709	mleN	malate-2H+/lactate-NA+ antiporter	-1.4	C
104	BC0769	ytiP	Guanine-hypoxanthine permease	-1.4	R
105	BC4337	*bc4337*	hypothetical Membrane Spanning Protein	-1.4	S
106	BC0872	yckK	Cystine-binding protein	-1.4	ET
107	BC0160	ybxA	Cobalt transport ATP-binding protein cbiO	-1.4	P
108	BC3911	*bc3911*	Cell division protein ftsW	-1.4	D
109	BC4859	*bc4859*	cold-shock protein	-1.4	K
110	BC1563	ypsC	Methyltransferase	-1.4	L
111	BC2358	*bc2358*	cold-shock protein	-1.4	K
112	BC1767	*bc1767*	Sodium-dependent serine transporter	-1.4	R
113	BC1809	*bc1809*	Non-hemolytic enterotoxin lytic component L2	-1.4	S
114	BC0658	yusP	Multidrug resistance protein B	-1.4	GEPR
115	BC5389	*bc5389*	D-alanyl-D-alanine carboxypeptidase	-1.4	M
116	BCp0002		hypothetical protein	-1.5	S
117	BC1821	nupC	Nucleoside permease nupC	-1.5	F
118	BC3805	pnpA	Polyribonucleotide nucleotidyltransferase	-1.5	J
119	BC0163	truA	tRNA pseudouridine synthase A	-1.5	J
120	BC0967	*bc0967*	hypothetical protein	-1.5	S
121	BC5397	ydzA	hypothetical Membrane Spanning Protein	-1.5	S
122	BC0677	*bc0677*	Bacitracin resistance protein (Putative undecaprenol kinase)	-1.5	V
123	BC0161	ybaE	Cobalt transport ATP-binding protein cbiO	-1.5	P
124	BC5065	*bc5065*	Methyl-accepting chemotaxis protein	-1.5	NT
125	BC3104	*bc3104*	Hemolysin BL lytic component L2	-1.5	S
126	BC0908	appB	Oligopeptide transport system permease protein oppB	-1.5	EP
127	BC4435	*bc4435*	Sporulation initiation phosphotransferase B	-1.5	T
128	BC3694	*bc3694*	N-acetylmuramoyl-L-alanine amidase	-1.5	M
129	BC0021	*bc0021*	Deoxyguanosine kinase	-1.5	F
130	BC3697	*bc3697*	hypothetical protein	-1.5	S
131	BC3986	ykuJ	hypothetical Cytosolic Protein	-1.5	S
132	BC4379	yrrL	hypothetical protein	-1.6	R
133	BCp0006		LexA repressor	-1.6	KT
134	BC5329	prfA	Bacterial Peptide Chain Release Factor 1 (RF-1)	-1.6	J
135	BC0981	*bc0981*	Dihydroxyacetone kinase	-1.6	G
136	BC0902	*bc0902*	S-layer protein / N-acetylmuramoyl-L-alanine amidase	-1.6	M
137	BC5235	yutK	Nucleoside permease nupC	-1.6	F
138	sRNABC007			-1.6	S
139	BC2621	*bc2621*	Signal peptidase I	-1.6	U
140	BC5408	yfnB	2-haloalkanoic acid dehalogenase	-1.6	R
141	BC3103	*bc3103*	Hemolysin BL lytic component L1	-1.6	S
142	BC1434	*bc1434*	Na+/H+ antiporter NhaC	-1.6	C
143	BC0020	#N/A	deoxynucleoside kinase	-1.6	S
144	BC0323	*bc0323*	Phosphoribosylaminoimidazole carboxylase catalytic subunit	-1.6	F
145	BC4396	yrvM	Molybdopterin biosynthesis MoeB protein	-1.6	H
146	BC1565	ypvA	ATP-dependent helicase, DinG family	-1.7	KL
147	BCp0003		hypothetical cytosolic protein	-1.7	S
148	BC2620	pbpA	Penicillin-binding protein transpeptidase	-1.7	M
149	BC0670	*bc0670*	Phospholipase C	-1.7	S
150	BC3702	*bc3702*	Antirepressor	-1.7	S
151	BC5328	ywkE	Methyltransferase	-1.7	J
152	BC4434	*bc4434*	GTP-binding protein	-1.7	R
153	sRNABC008			-1.7	S
154	BC2103	yfmL	ATP-dependent RNA helicase	-1.7	LKJ
155	BC2357	*bc2357*	Cold shock protein	-1.7	S
156	BC0423	*bc0423*	Peptide synthetase	-1.7	Q
157	BC3520	*bc3520*	Methyl-accepting chemotaxis protein	-1.7	NT
158	BC4704	ytzF	Ribosomal small subunit pseudouridine synthase A	-1.7	J
159	BC0607	ywtF	Transcriptional regulator, LytR family	-1.7	K
160	BC1739	*bc1739*	Proton/sodium-glutamate symport protein	-1.7	C
161	BC0297	pbuG	Guanine-hypoxanthine permease	-1.7	R
162	BC0243	appC	Oligopeptide transport system permease protein oppC	-1.7	EP
163	BC2021	ycdH	High-affinity zinc uptake system protein znuA precursor	-1.7	P
164	BC4489	*bc4489*	hypothetical Cytosolic Protein	-1.8	S
165	BCp0005		type B DNA polymerase	-1.8	S
166	BC0259	ydbR	ATP-dependent RNA helicase	-1.8	LKJ
167	BC2713	yurQ	UvrC-like protein	-1.8	L
168	BC2067	*bc2067*	hypothetical Membrane Spanning Protein	-1.8	S

169	BC2102	*bc2102*	hypothetical protein	-1.8	S
170	BC0363	*bc0363*	Nucleoside permease nupC	-1.8	F
171	BC3804	ylxY	Chitooligosaccharide deacetylase	-1.9	G
172	BC3695	*bc3695*	holin	-1.9	S
173	BC3776	*bc3776*	Stage V sporulation protein S	-1.9	S
174	BC3696	#N/A	hypothetical protein	-1.9	S
175	BC4925	yumB	NADH dehydrogenase	-1.9	C
176	BC0564	*bc0564*	hypothetical lipoprotein	-2.0	C
177	BC3777	ymdB	IG hypothetical 15594	-2.0	S
178	BC4999	*bc4999*	CAAX amino terminal protease family	-2.0	R
179	BC3177	*bc3177*	DNA helicase	-2.0	L
180	BC0656	glpT	Glycerol-3-phosphate transporter	-2.0	G
181	BC4283	yqfR	ATP-dependent RNA helicase	-2.0	LKJ
182	BC4795	*bc4795*	hypothetical Membrane Spanning Protein	-2.0	S
183	BC2300	*bc2300*	Oxalate/formate antiporter	-2.0	GEPR
184	BC1810	*bc1810*	Non-hemolytic enterotoxin lytic component L1	-2.0	S
185	BC2234	*bc2234*	Arginine/ornithine antiporter	-2.1	E
186	BC5330	*bc5330*	Thymidine kinase	-2.1	F
187	BC0954	*bc0954*	tcdA-E operon negative regulator	-2.1	S
188	BC3835	rnhB	Ribonuclease HII	-2.1	L
189	BC0580	*bc0580*	NAD-dependent malic enzyme	-2.1	C
190	BC5064	*bc5064*	hypothetical protein	-2.2	R
191	BC0121	ybxB	16S rRNA m(2)G 1207 methyltransferase	-2.2	J
192	BC5438	ysbB	Murein hydrolase export regulator	-2.2	M
193	BC0965	*bc0965*	hypothetical protein	-2.2	S
194	sRNABC010			-2.2	S
195	BC0241	*bc0241*	Oligopeptide-binding protein oppA	-2.3	E
196	BC5439	ysbA	Murein hydrolase exporter	-2.3	R
197	BC3183	*bc3183*	hypothetical Membrane Spanning Protein	-2.4	S
198	BC0966	*bc0966*	Fimbria associated protein	-2.4	M
199	BC3542	*bc3542*	Transcriptional regulator	-2.5	S
200	BC0242	*bc0242*	Oligopeptide transport system permease protein oppB	-2.5	EP
201	BC1599	*bc1599*	hypothetical Membrane Spanning Protein	-2.5	S
202	BC4507	*bc4507*	Aquaporin	-2.6	G
203	BC3703	*bc3703*	Phage protein	-2.6	S
204	BCp0017		hypothetical membrane spanning protein	-2.6	S
205	BC2465	*bc2465*	hypothetical protein	-2.6	S
206	BCp0020		hypothetical protein	-2.7	S
207	BCp0021		N-acetylmuramoyl-L-alanine amidase	-2.7	M
208	BC5443	*bc5443*	Glycine betaine transporter	-2.7	M
209	BC0865	yvsH	Arginine/ornithine antiporter	-2.8	E
210	BC3922	*bc3922*	Prespore specific transcriptional activator rsfA	-2.8	S
211	BCp0015		hypothetical membrane spanning protein	-3.0	S
212	sRNABC020			-3.0	S
213	BCp0019		hypothetical protein	-3.1	S
214	BCp0008		hypothetical cytosolic protein	-3.1	S
215	BCp0018		hypothetical cytosolic protein	-3.4	S
216	BC5218	*bc5218*	Proton/sodium-glutamate symport protein	-3.5	C
217	BCp0016		N-acetylmuramoyl-L-alanine amidase	-3.6	NU
218	BCp0007		hypothetical membrane spanning protein	-3.9	S
219	BCp0014		hypothetical cytosolic protein	-3.9	S
220	BC0579	maeN	Malate-sodium symport	-4.0	C
221	BC4242	yhcL	Proton/sodium-glutamate symport protein	-4.2	R
222	BCp0013		hypothetical cytosolic protein	-4.3	S
223	BCp0012		hypothetical protein	-4.3	S
224	BCp0010		hypothetical cytosolic protein	-4.4	S
225	BCp0011		hypothetical membrane spanning protein	-4.5	S
226	BCp0009		hypothetical cytosolic protein	-5.1	S

Supplementary Table S3B: SigB-dependent induced and downregulated genes upon heat shock (30°C to 42°C) in *Bacillus cereus* wt cells versus Δ*sigB* and Δ*bc1009* mutants

Bold: SigB and Bc1009-dependent-induced and downregulated genes under heat shock in B. cereus
Non-bold: SigB-dependent-induced genes upon heat shock in B. cereus
COG: Cluster of orthologous groups

				wt 42°C/ Δ*sigB* 42°C	**wt 42°C/ Δ*bc1009* 42°C**	
No	**Locus Tag**	**Gene**	**Annotation**	**Log2 expression**	**Log2 expression**	**COG**
1	BC1000	*bc1000*	hypothetical Membrane Spanning Protein	7.3		S
2	BC0998	*yflT*	General stress protein 17M	7.1		S
3	BC1004	*sigB*	RNA polymerase sigma-B factor	6.6		K
4	BC0863	*katE*	Catalase	6.6		P
5	BC0862	*yfkM*	Protease I	6.4		R
6	BC0999	*#N/A*	CsbD family protein	5.6		S
7	BC1003	*rsbW*	Anti-sigma B factor	5.5		T
8	BC1005	*bc1005*	Bacterioferritin	5.4		P
9	BC1002	*rsbV*	Anti-sigma B factor antagonist	5.3		T
10	**BC1011**	***bc1011***	**hypothetical protein**	**5.2**	**1.2**	**S**
11	**BC1852**	***bc1852***	**Exonuclease SbcC**	**4.5**	**3.7**	**L**
12	BC3131	*bc3131*	hypothetical protein	4.4		S
13	**BC1009**	***bc1009***	**hypothetical protein**	**4.3**	**4.6**	**S**
14	BC3130	*bc3130*	hypothetical protein	4.2		S
15	**BC1853**	***bc1853***	**hypothetical protein**	**4.1**	**3.7**	**S**
16	**BC2300**	***bc2300***	**Oxalate/formate antiporter**	**3.6**	**3.3**	**GEPR**
17	BC5391	*ywdL*	Spore coat protein gerQ	3.6		S
18	**BC1010**	***bc1010***	**hypothetical protein**	**3.5**	**1.1**	**S**
19	**BC1855**	***bc1855***	**Chromosome segregation ATPases**	**3.4**	**3.0**	**L**
20	**BC1857**	***bc1857***	**Exonuclease SbcD**	**3.3**	**3.6**	**L**
21	**BC1860**	***bc1860***	**Phage protein**	**3.2**	**3.1**	**S**
22	**BC1856**	***bc1856***	**Phage protein**	**3.1**	**3.3**	**S**
23	**BC3699**	***bc3699***	**Antigen**	**3.0**	**3.2**	**M**
24	**BC1862**	***bc1862***	**Phage protein**	**3.0**	**2.6**	**S**
25	**BC3698**	***bc3698***	**Cell wall endopeptidase, family M23/M37**	**2.8**	**3.0**	**M**
26	**BC1858**	***bc1858***	**Phage protein**	**2.6**	**2.4**	**S**
27	**BC1660**	***yjbJ***	**Soluble lytic murein transglycosylase**	**2.5**	**2.4**	**M**
28	**BC1001**	***bc1001***	**hypothetical protein**	**2.4**	**1.3**	**S**
29	**BC1671**	***flgG***	**Flagellar basal-body rod protein flgG**	**2.4**	**2.1**	**N**
30	**BC1861**	***bc1861***	**DNA/RNA helicase (DEAD/DEAH box family)**	**2.3**	**2.3**	**KL**
31	**BC3695**	***bc3695***	**holin**	**2.3**	**2.8**	**S**
32	BC5390	*cwlJ*	Cell wall hydrolase cwlJ	2.3		M
33	**BC1669**	***flhA***	**Flagellar biosynthesis protein flhA**	**2.2**	**2.1**	**S**
34	**BC3696**		**hypothetical protein**	**2.2**	**2.7**	**S**
35	**BC1670**	***flhF***	**Flagellar biosynthesis protein flhF**	**2.2**	**1.9**	**S**
36	BC3132	*bc3132*	General stress protein 17M	2.2		S
37	BC3129	*yqxL*	Magnesium and cobalt transport protein corA	2.2		P
38	**BC1661**	***fliN***	**Flagellar motor switch protein fliN**	**2.2**	**2.3**	**NU**
39	**BC1666**	***fliQ***	**Flagellar biosynthetic protein fliQ**	**2.1**	**2.3**	**NU**
40	**BC1668**	***flhB***	**Flagellar biosynthetic protein flhB**	**2.1**	**1.9**	**NU**
41	BC5449	*bc5449*	Conserved membrane protein (hemolysin III homolog)	2.1		R
42	BC3648	*ydeB*	CarD-like transcriptional regulator	2.0		K
43	**BC1663**	***fliN***	**Flagellar motor switch protein fliN**	**2.0**	**2.1**	**NU**
44	**BC1665**	***fliP***	**Flagellar biosynthetic protein fliP**	**2.0**	**2.0**	**NU**
45	**BC1667**	***fliR***	**Flagellar biosynthetic protein fliR**	**2.0**	**1.9**	**NU**
46	**BC3694**	***bc3694***	**N-acetylmuramoyl-L-alanine amidase**	**2.0**	**2.3**	**M**
47	**BC1664**	***fliN***	**Flagellar motor switch protein fliN**	**2.0**	**2.2**	**S**
48	**BC1854**	***bc1854***	**hypothetical Cytosolic Protein**	**2.0**	**2.2**	**S**
49	**BC1870**	***bc1870***	**Phage protein**	**1.9**	**1.5**	**S**
50	**BC2130**	***bc2130***	**Transporter**	**1.9**	**1.2**	**R**
51	**BC1863**	***bc1863***	**MCM domain family protein**	**1.9**	**2.0**	**L**
52	**BC1662**	***fliM***	**Flagellar motor switch protein fliM**	**1.9**	**1.9**	**N**
53	BC2131	*bc2131*	hypothetical protein	1.9		S
54	**BC1851**	***bc1851***	**Transcriptional regulator**	**1.8**	**1.8**	**K**
55	**BC1866**	***bc1866***	**Phage protein**	**1.8**	**1.4**	**S**
56	**BC0852**	***bc0852***	**Quaternary ammonium compound-resistance protein**	**1.8**	**1.5**	**P**
57	**BC1864**	***bc1864***	**DNA polymerase I**	**1.7**	**2.4**	**L**
58	**BC3697**	***bc3697***	**hypothetical protein**	**1.7**	**1.7**	**S**
59	BC1006	*rsbP/Y*	Sigma factor sigB regulation protein rsbU	1.7		TK
60	**BC1869**	***bc1869***	**Phage protein**	**1.7**	**1.8**	**S**
61	**BC0853**	***bc0853***	**Quaternary ammonium compound-resistance protein**	**1.6**	**1.5**	**P**
62	**BC5438**	***ysbB***	**Murein hydrolase export regulator**	**1.6**	**1.4**	**M**

63	**BC1873**	***bc1873***	**Phage protein**	**1.6**	**1.3**	**S**
64	BC4482	*bc4482*	hypothetical protein	1.6		S
65	BC1245	*bc1245*	hypothetical protein	1.6		S
66	**BC4267**	***bc4267***	**Phosphate transport system permease protein pstA**	**1.6**	**1.1**	**P**
67	BC1264	*yonV*	hypothetical protein	1.6		S
68	**BC1612**	***bc1612***	**Na+/H+ antiporter NapA (inosine-dependent germination)**	**1.5**	**1.2**	**P**
69	BC4644	*bc4644*	PhnB protein	1.5		S
70	BC5006	*yusM*	Prolyne dehydrogenase	1.5		E
71	**BC1880**	***bc1880***	**Phage protein**	**1.5**	**1.2**	**S**
72	BC1443	*bc1443*	SAM-dependent methyltransferase	1.5		QR
73	**BC1436**	***bc1436***	**Phage shock protein A**	**1.5**	**1.2**	**KT**
74	**BC4268**	***bc4268***	**Phosphate transport system permease protein pstC**	**1.5**	**1.4**	**P**
75	BC4714	*bc4714*	CarD-like transcriptional regulator	1.5		K
76	**BC1876**	***bc1876***	**Phage protein**	**1.5**	**1.3**	**S**
77	**BC1872**	***bc1872***	**Phage protein**	**1.5**	**1.6**	**C**
78	BC1187	*bc1187*	hypothetical protein	1.4		S
79	**BC0251**	***#N/A***	**DUF3948 family protein**	**1.4**	**1.8**	**S**
80	BC1265	*bc1265*	hypothetical Cytosolic Protein	1.4		S
81	BC4813	*bc4813*	hypothetical protein	1.4		S
82	**BC3882**	***pyrE***	**Orotate phosphoribosyltransferase**	**1.4**	**2.2**	**F**
83	**BC3458**	***bc3458***	**XpaF1 protein**	**1.4**	**1.1**	**S**
84	**BC1874**	***bc1874***	**Phage protein**	**1.3**	**1.0**	**S**
85	BC1848	*bc1848*	Prophage helix-turn-helix protein	1.3		S
86	**BC3884**	***pyrD***	**Dihydroorotate dehydrogenase, catalytic subunit**	**1.3**	**2.2**	**F**
87	BC3723	*bc3723*	hypothetical protein	1.3		S
88	**BC4046**	***bc4046***	**hypothetical protein**	**1.3**	**1.1**	**S**
89	BC0404	*bc0404*	Methyl-accepting chemotaxis protein	1.3		NT
90	BC3459	*bc3459*	hypothetical protein	1.3		S
91	**BC1493**	***bc1493***	**hypothetical protein**	**1.3**	**1.1**	**S**
92	BC4075	*dacF*	D-alanyl-D-alanine carboxypeptidase	1.3		M
93	BC2107	*bc2107*	Quinone oxidoreductase	1.3		CR
94	BC1878	*bc1878*	Phage protein	1.3		S
95	BC0555	*bc0555*	Glycine betaine transporter	1.3		M
96	**BC4269**	***bc4269***	**Phosphate-binding protein**	**1.3**	**1.2**	**P**
97	BC5020	*bc5020*	hypothetical protein	1.2		S
98	BC5009	*mcpC*	Methyl-accepting chemotaxis protein	1.2		NT
99	**BC5435**	***yerP***	**Acriflavin resistance plasma membrane protein**	**1.2**	**2.9**	**V**
100	**BC3883**	***pyrF***	**Orotidine 5'-phosphate decarboxylase**	**1.2**	**1.8**	**F**
101	BC2842	*bc2842*	hypothetical protein	1.2		S
102	BC0557	*bc0557*	hypothetical protein	1.2		S
103	BC2358	*bc2358*	cold-shock protein	1.2		K
104	**BC1435**	***bc1435***	**hypothetical protein**	**1.2**	**1.1**	**S**
105	**BC1887**	***bc1887***	**Phage protein**	**1.2**	**1.2**	**S**
106	BC0683	*bc0683*	hypothetical protein	1.1		S
107	BC1625	*bc1625*	Chemotaxis motA protein	1.1		N
108	BC4266	*bc4266*	Phosphate transport ATP-binding protein pstB	1.1		P
109	BC1494	*bc1494*	N-acetylmuramoyl-L-alanine amidase	1.1		M
110	BC2357	*bc2357*	Cold shock protein	1.1		S
111	BC4047	*bc4047*	hypothetical protein	1.1		S
112	**BC3886**	***bc3886***	**Carbamoyl-phosphate synthase large chain**	**1.1**	**1.4**	**EF**
113	BC2841	*bc2841*	hypothetical protein	1.1		S
114	**BC1886**	***bc1886***	**Phage protein**	**1.1**	**1.2**	**S**
115	BC0423	*bc0423*	Peptide synthetase	1.1		Q
116	**BC5033**	***#N/A***	**HAMP domain-containing protein**	**1.1**	**1.1**	**S**
117	**BC3885**	***pyrK***	**Dihydroorotate dehydrogenase electron transfer subunit**	**1.0**	**1.5**	**HC**
118	**BC1882**	***bc1882***	**Phage protein**	**1.0**	**1.2**	**S**
119	BC3075	*ydbM*	EpiH/GdmH-related protein	1.0		S
120	**BC1658**	***yvzB***	**Flagellin**	**1.0**	**1.2**	**N**
121	BC1550	*bc1550*	Multimodular transpeptidase-transglycosylase PBP 1A	1.0		M
122	BC1888	*bc1888*	Phage protein	1.0		EP
123	BC3653	*hutP*	Hut operon positive regulatory protein	1.0		S
124	BC3000	*bc3000*	Proline/betaine transporter	1.0		GEPR
1	BC3590	*bc3590*	ABC transporter permease protein	-1.0		R
2	BC0865	*yvsH*	Arginine/ornithine antiporter	-1.0		E
3	BC0763	*bc0763*	ABC transporter permease protein	-1.0		V
4	BC0363	*bc0363*	Nucleoside permease nupC	-1.0		F
5	sRNABC020	*sRNABC020*		-1.1		S
6	BC0991	*bc0991*	S-layer homology domain / putative murein endopeptidase	-1.1		D
7	BC3245	*yecA*	Amino acid permease	-1.1		E
8	BC0746	*tenI*	Regulatory protein TENI	-1.1		H
9	BC2973	*yxjA*	Nucleoside permease nupC	-1.1		F
10	BC5219	*bc5219*	Integral membrane protein	-1.1		S
11	BC0189	*bc0189*	hypothetical protein	-1.1		S
12	BC3103	*bc3103*	Hemolysin BL lytic component L1	-1.1		S
13	**BCp0005**	***bcp0005***	**type B DNA polymerase**	**-1.2**	**-1.3**	**S**

14	BC0674	*bc0674*	Permease	-1.2		V
15	sRNABC063	*sRNABC063*		-1.2		S
16	BC1589	*tuaG*	putative N-acetylgalactosaminyl-diphosphoundecaprenol glucuronosyltransferase	-1.3		M
17	**BCp0016**	***bcp0016***	**N-acetylmuramoyl-L-alanine amidase**	**-1.3**	**-1.1**	**NU**
18	BC1725	*dsdA*	D-serine dehydratase	-1.3		E
19	BC0556	*bc0556*	Microbial collagenase	-1.3		R
20	BC4795	*bc4795*	hypothetical Membrane Spanning Protein	-1.3		S
21	sRNABC002	*sRNABC002*		-1.4		S
22	sRNABC010	*sRNABC010*		-1.4		S
23	BC5174	*bc5174*	hypothetical Membrane Spanning Protein	-1.4		S
24	BC1809	*bc1809*	Non-hemolytic enterotoxin lytic component L2	-1.4		S
25	BC1540	*panB*	3-methyl-2-oxobutanoate hydroxymethyltransferase	-1.4		H
26	BC3104	*bc3104*	Hemolysin BL lytic component L2	-1.4		S
27	BCp0015	*bcp0015*	hypothetical membrane spanning protein	-1.5		S
28	BC2129	*bc2129*	hypothetical protein	-1.5		S
29	**BCp0019**	***bcp0019***	**hypothetical protein**	**-1.5**	**-1.7**	**S**
30	BC1541	*panC*	Pantoate--beta-alanine ligase	-1.5		H
31	BC1542	*panD*	Aspartate 1-decarboxylase	-1.6		H
32	**BCp0020**	***bcp0020***	**hypothetical protein**	**-1.6**	**-1.7**	**S**
33	BC5351	*bc5351*	Bacillolysin	-1.7		E
34	BC1448	*bc1448*	Nitric oxide dioxygenase	-1.7		C
35	BC4140	*bc4140*	Mg(2+) transport ATPase, P-type	-1.7		P
36	BC4141	*bc4141*	Mg(2+) transport ATPase protein C	-1.7		S
37	BCp0014	*bcp0014*	hypothetical cytosolic protein	-1.7		S
38	BC4109	*ribD*	Diaminohydroxyphosphoribosylaminopyrimidine deaminase	-1.8		H
39	**BCp0012**	***bcp0012***	**hypothetical protein**	**-1.8**	**-1.2**	**S**
40	BC4110	*ribE*	Riboflavin synthase alpha chain	-1.8		H
41	BCp0017	*bcp0017*	hypothetical membrane spanning protein	-1.8		S
42	BC1810	*bc1810*	Non-hemolytic enterotoxin lytic component L1	-1.8		S
43	BC4112	*ribH*	6,7-dimethyl-8-ribityllumazine synthase	-1.8		H
44	**BCp0011**	***bcp0011***	**hypothetical membrane spanning protein**	**-1.9**	**-1.6**	**S**
45	**BC4111**	***ribA***	**GTP cyclohydrolase II**	**-1.9**	**-1.1**	**H**
46	BC3439	*bc3439*	Hydroxylamine reductase	-2.0		C
47	**BCp0013**	***bcp0013***	**hypothetical cytosolic protein**	**-2.0**	**-1.4**	**S**
48	BCp0009	*bcp0009*	hypothetical cytosolic protein	-2.0		S
49	**BCp0021**	***bcp0021***	**N-acetylmuramoyl-L-alanine amidase**	**-2.0**	**-1.5**	**M**
50	**BCp0010**	***bcp0010***	**hypothetical cytosolic protein**	**-2.1**	**-1.4**	**S**
51	**BCp0018**	***bcp0018***	**hypothetical cytosolic protein**	**-2.2**	**-1.7**	**S**
52	**BC2136**	***nasD***	**Nitrite reductase [NAD(P)H] large subunit**	**-2.5**	**-1.5**	**C**
53	**BC2124**	***moeB***	**thiamine/molybdopterin biosynthesis protein MoeB**	**-2.5**	**-1.3**	**H**
54	**BC2118**	***narG***	**Respiratory nitrate reductase alpha chain**	**-2.5**	**-1.3**	**C**
55	**BC2132**	***bc2132***	**Ferrochelatase**	**-2.6**	**-1.8**	**H**
56	**BC2135**	***nasE***	**Nitrite reductase [NAD(P)H] small subunit**	**-2.6**	**-1.7**	**PR**
57	**BC2123**	***bc2123***	**Molybdenum cofactor biosynthesis protein A**	**-2.7**	**-1.3**	**H**
58	**BC5101**	***bc5101***	**Perfringolysin O precursor**	**-2.7**	**-1.7**	**S**
59	**BC2125**	***moeA***	**Molybdopterin biosynthesis MoeA protein**	**-2.7**	**-1.3**	**H**
60	**BC2134**	***nasF***	**Uroporphyrin-III C-methyltransferase**	**-2.8**	**-2.0**	**H**
61	**BC2133**	***bc2133***	**CbiX protein**	**-2.9**	**-2.1**	**S**
62	**BC5026**	***bc5026***	**hypothetical protein**	**-3.0**	**-2.2**	**S**
63	**BC2126**	***moaE***	**molybdopterin synthase catalytic subunit MoaE**	**-3.0**	**-1.3**	**H**
64	BC2127	*bc2127*	Molybdopterin (MPT) converting factor, subunit 1	-3.1		H
65	**BC5027**	***bc5027***	**Protein erfK/srfK precursor**	**-3.2**	**-2.9**	**S**
66	**BC2119**	***narH***	**Respiratory nitrate reductase beta chain**	**-3.5**	**-2.5**	**C**
67	**BC2121**	***narI***	**Respiratory nitrate reductase gamma chain**	**-3.5**	**-2.3**	**C**
68	**BC2120**	***narJ***	**Respiratory nitrate reductase delta chain**	**-3.7**	**-2.6**	**C**
69	**BC2128**	***narK***	**Nitrite extrusion protein**	**-4.1**	**-2.9**	**P**

Supplementary Table S4A: Bc1009-dependent proteins in *Bacillus cereus* upon heat shock (30°C to 42°C) in wt cells versus Δ*bc1009* mutant

				wt 42°C/ Δ*bc1009* 42°C		
No	**Locus Tag**	**Protein**	**Annotation**	**log2 FC_T20**	**log2 FC_T40**	**COG**
1	BC1113	YhdL	Sigma-M negative effector	7.0		S
2	BC5205	LevR	Transcriptional regulatory protein levR	6.2	0.7	TK
3	BC1837		Methylmalonyl CoA epimerase	5.5		E
4	BC0236		Transglycosylase	5.2	5.5	M
5	BC5239		protein with unknown function	4.8		T
6	BC0232		hypothetical Membrane Spanning Protein	4.8		S
7	BC0961		Transcriptional regulator, TetR family	3.6		K
8	BC2770	GlcR	Transcriptional regulator, DeoR family	3.6		KG
9	BC1064	YdbS	hypothetical Membrane Spanning Protein	3.2		S
10	BC5177	YwqN	Trp repressor binding protein	2.9	1.1	R
11	BC4150	YqiX	Arginine-binding protein	2.9		TE
12	BC5056		Collagen adhesion protein	2.8		S
13	BC4496	RacE	Glutamate racemase	2.8		M
14	BC3812	YlxQ	LSU ribosomal protein L7AE	2.7	3.1	J
15	BC0185	RocF	Arginase	2.6	2.7	E
16	BC4453		Phage protein	2.3		S
17	BC3251		hypothetical protein	2.2		S
18	BC5163		Prolipoprotein diacylglyceryl transferase	2.2	3.6	M
19	BC0622		L-threonine 3-dehydrogenase	2.1		MG
20	BC1439	YvqC	Two-component response regulator yvqC	2.1		TK
21	BC1712	FumC	Fumarate hydratase	2.0	2.6	C
22	BC1796		hypothetical protein	1.9		H
23	BC2085		hypothetical protein	1.9		S
24	BC3159		NAD-dependent epimerase/dehydratase / Alpha/beta hydrolase	1.7		S
25	BC1548		Endonuclease III	1.6		L
26	BC4270		Penicillin-binding protein	1.6		M
27	BC4249		UDP-N-acetylglucosamine--N-acetylmuramyl-(Pentapeptide) pyrophosphoryl-undecaprenol N-acetylglucosamine transferase	1.5		M
28	BC1970		hypothetical protein	1.5		H
29	BC2753	SleB	Cell wall hydrolase cwlJ	1.5	1.0	M
30	BC2121	NarI	Respiratory nitrate reductase gamma chain	1.4		C
31	BC0644		OsmC-like protein	1.4		O
32	BC4679		hypothetical protein	1.3		S
33	BC0889	HemZ	Coproporphyrinogen oxidase, anaerobic	1.3		H
34	BC3107		UvrC-like protein	1.3		L
35	BC0903		Methyltransferase	1.2		L
36	BC1905		Phage protein	1.2		S
37	BC1568		Methionine aminopeptidase	1.2		J
38	BC0780	YhdB	hypothetical protein	1.2		S
39	BC5189	SecA	Protein translocase subunit SecA	1.2	1.2	U
40	BC0651		Two component system histidine kinase	1.2		T
41	BC0895		putative pyruvyl-transferase	1.2		S
42	BC3520	TarH	Methyl-accepting chemotaxis protein (motility, Signal transduction)	1.1		NT
43	BC2498	VanR	Two-component response regulator vanR	1.1		TK
44	BC5322	YwrF	Nitrilotriacetate monooxygenase component B	1.1		R
45	BC4102	YbaC	Alpha/beta hydrolase	1.0		S
46	BC1640		hypothetical protein	1.0		S
47	BC4302	PhoH	PhoH protein	1.0	0.7	T
48	BC4487		Superfamily I DNA and RNA helicases	1.0	0.7	L
49	BC3312		3-Oxoadipate enol-lactonase	1.0		R
50	BC3586		Oligopeptide-binding protein oppA	1.0		E
51	BC1862		Phage protein	1.0		S
52	BC2192	DfrA	Dihydrofolate reductase	0.9		H
53	BC4562	YsgA	23S rRNA methyltransferase	0.9		J
54	BC0814		ABC transporter permease protein	0.9		V
55	BC0896		S-layer protein / Peptidoglycan endo-beta-N-acetylglucosaminidase	0.9		G
56	BC0755		Potassium-transporting ATPase C chain	0.9		P
57	BC3058		Phosphohydrolase (MutT/nudix family protein)	0.9		L
58	BC3596	ParC	Topoisomerase IV subunit A	0.9		L
59	BC2254	YodN	IG hypothetical 1822	0.8		S
60	BC5031		Methionyl-tRNA synthetase	0.8		J
61	BC1048		IG hypothetical 1777	0.8		R
62	BC1641	FlgB	Flagellar basal-body rod protein flgB	0.8		N
63	BC4594	CitZ	Citrate synthase	0.8		C
64	BC1542	PanD	Aspartate 1-decarboxylase	0.8		H
65	BC0447	YceH	Tellurite resistance protein	0.8		P
66	BC5046	YvdD	Lysine decarboxylase family	0.8		R

67	BC3874		lysozyme inhibitor, binds to and inhibits macrophage lysozyme, aid bacterial survival	0.8		S
68	BC1129		Trifolitoxin immunity protein	0.8		M
69	BC0426		Hydroxymethylpyrimidine-binding protein	0.7		P
70	BC2087		hypothetical protein	0.7		S
71	BC1365	YrdC	Isochorismatase	0.7		Q
72	BC4859			0.7		0
73	BC5236		Diguanylate cyclase/phosphodiesterase domain 1 (GGDEF)	0.7		S
74	BC1124		Methyl-accepting chemotaxis protein	0.7		NT
75	BC5482	Soj	Sporulation initiation inhibitor protein soj	0.7		D
76	BC4454		hypothetical protein	0.7		S
77	BC3391		Glycosyl hydrolase	0.6		S
78	BC0453	YkvW	Zinc uptake P-type ATPase	0.6		P
79	BC1387		Transcriptional regulator, MarR family	0.6		K
80	BC1629	FliY	Chemotaxis protein cheC	0.6		NTU
81	BC4762	PckA	Phosphoenolpyruvate carboxykinase [ATP]		3.0	C
82	BC4250		5-methyltetrahydrofolate--homocysteine methyltransferase		2.9	E
83	BC4514	YodJ	D-alanyl-D-alanine carboxypeptidase		2.6	M
84	BC1718		DUF family protein of unknown function		2.6	S
85	BC3355	YrkA	Magnesium and cobalt efflux protein corC		2.5	R
86	BC3995	YkyB	hypothetical Cytosolic Protein		1.8	S
87	BC1818		Transcriptional regulator, TetR family		1.6	K
88	BC5059				1.5	K
89	BC3439		Hydroxylamine reductase		1.3	C
90	BC3769	MutS	DNA mismatch repair protein mutS		1.0	L
91	BC4509	YhaP	Sodium export permease protein		1.0	CP
92	BC3597	ParE	Topoisomerase IV subunit B		0.9	L
93	BC1799	YoaA	Ribosomal-protein-alanine acetyltransferase		0.8	J
94	BC3790	YufP	Nucleoside transport ATP-binding protein		0.8	R
95	BC0144	RpsN	SSU ribosomal protein S14P		0.7	J
96	BC1202		Serine/threonine protein phosphatase		0.7	T
97	BC4792		Cytochrome d ubiquinol oxidase subunit I		0.7	C
98	BC4512	MotB	Chemotaxis motB protein		0.6	N
99	BC4799		hypothetical protein		0.6	S
100	BC2102		protein with unknown function		0.6	S
1	BC4006		hypothetical Cytosolic Protein	-0.6		S
2	BC3690	LexA	LexA repressor	-0.6		KT
3	BC4586	MutM	Formamidopyrimidine-DNA glycosylase	-0.6		L
4	BC1408	HisH	Amidotransferase hisH	-0.7		E
5	BC0164	RplM	LSU ribosomal protein L13P	-0.6		J
6	BC5106		Ferric anguibactin-binding protein	-0.6		P
7	BC0824		Lactoylglutathione lyase	-0.6		E
8	BC0470		S-layer protein / N-acetylmuramoyl-L-alanine amidase	-0.6		S
9	BC4280	YqfU	hypothetical Membrane Spanning Protein	-0.6	-1.7	S
10	BC4539		0	-0.6		S
11	BC2004		hypothetical protein	-0.6		S
12	BC3609		hypothetical protein	-0.6		S
13	BC1033	GlpP	Glycerol uptake operon antiterminator regulatory protein	-0.6		K
14	BC3021		hypothetical Cytosolic Protein	-0.6		S
15	BC2272		Protein export protein prsA precursor	-0.6		O
16	BC5408	YfnB	2-haloalkanoic acid dehalogenase/ putative phosphatase	-0.6	-1.2	R
17	BC2133		CbiX protein	-0.7		S
18	BC4327	YqeL	iojap protein family	-0.7		S
19	BC1867		Phage protein	-0.7		S
20	BC3728		hypothetical protein	-0.7	-1.7	L
21	BC4049	PtsH	Phosphocarrier protein HPr	-0.7		G
22	BC0235	YdaL	hypothetical protein	-0.7		S
23	BC2580		Phage protein	-0.7	-1.3	S
24	BC2883	YhbD	hypothetical Cytosolic Protein	-0.7		S
25	BC5377	YwhC	Membrane metalloprotease	-0.7		R
26	BC4097		2,5-diketo-D-gluconic acid reductase	-0.8		R
27	BC5381	FhuC	Ferrichrome transport ATP-binding protein fhuC	-0.7		PH
28	BC5019		Ribosomal-protein-serine acetyltransferase	-0.7		J
29	BC1830		Copper resistance protein A	-0.7		Q
30	BC0142	RplX	LSU ribosomal protein L24P	-0.7		J
31	BC0365	YfjN	Nitrogen regulation protein NIFR3	-0.7		J
32	BC2882	YhbE	hypothetical Cytosolic Protein	-0.7		M
33	BC3380		Quinone oxidoreductase	-0.7	-0.6	C
34	BC3478		Phosphoglycerate mutase	-0.7		G
35	BC5476	RpsF	SSU ribosomal protein S6P	-0.7		J
36	BC3711		DNA integration/recombination/invertion protein	-0.8		S
37	BC3398		Serine transporter	-0.8		E
38	BC1308	YwcJ	Nitrite transporter	-0.9	-0.6	P
39	BC1983		hypothetical Cytosolic Protein	-0.9		S
40	BC4652	YttP	Transcriptional regulator IcaR	-0.8	-0.7	K
41	BC3934	YhfR	Phosphoglycerate mutase	-0.9		G

42	BC1852		Exonuclease SbcC	-0.8		S
43	BC0493	UgtP	1,2-diacylglycerol 3-glucosyltransferase	-0.8	-0.7	M
44	BC2369		Acetyltransferase	-0.8		KR
45	BC4174	ArgR	Arginine repressor, argR	-0.8		K
46	BC1625	MotA	Chemotaxis motA protein	-0.9		N
47	BC0405	ArgR	Arginine repressor, argR	-0.9		K
48	BC4795		hypothetical Membrane Spanning Protein	-1.0		S
49	BC4247		hypothetical protein	-0.9		S
50	BC2449		Lipase/Acylhydrolase with GDSL-like motif	-1.0		E
51	BC4256		Transcriptional regulator, ArsR family	-1.0	-0.7	K
52	BC4934		hypothetical protein	-1.6		S
53	BC2368	YflK	hypothetical Cytosolic Protein	-1.2		S
54	BC3618		Peptidoglycan N-acetylglucosamine deacetylase	-1.1	-0.8	G
55	BC3667b		protein with unknown function	-1.2		S
56	BC4607	YtrI	hypothetical protein	-1.3		S
57	BC2135	NasE	Nitrite reductase [NAD(P)H] small subunit	-1.4		PR
58	BC0139	RpmC	LSU ribosomal protein L29P	-1.5	-0.7	J
59	BC2210		NAD(P)H nitroreductase	-1.1		C
60	BC1650	FlgD	Basal-body rod modification protein flgD	-1.1	-1.7	N
61	BC1969		Transcriptional regulator, LytR family	-1.2		K
62	BC2094		Acetyltransferase	-1.2		R
63	BC3718	FruA	PTS system, fructose-specific IIABC component	-1.2	-0.6	GT
64	BC3958	YktC	Myo-inositol-1(or 4)-monophosphatase	-1.2		G
65	BC2456		Peptide synthetase	-1.2		Q
66	BC2125		Molybdopterin biosynthesis MoeA protein, nitrate respiration	-1.2		H
67	BC3442		hypothetical protein	-1.2		R
68	BC5000	YobS	Transcriptional regulator, TetR family	-1.2	-1.0	K
69	BC3023	YcnE	hypothetical Cytosolic Protein	-1.3		S
70	BC1593	YitH	Acetyltransferase	-1.3		K
71	BC5047	YvdC	IG hypothetical 16995	-1.3		R
72	BC0596	YvgW	Zinc-transporting ATPase	-1.3		P
73	BC2797	YybD	Acetyltransferase	-1.3	-1.9	K
74	BC3062	YhdZ	SIR2 family protein	-1.4		K
75	BC1514	HepT	Farnesyltransferase	-1.4		H
76	BC5271		UDP-N-acetylglucosamine 4-epimerase	-1.4		MG
77	BC0778		Thioredoxin	-1.4		OC
78	BC3209		hypothetical Cytosolic Protein	-1.4		S
79	BC1638		Flagellar hook-associated protein 2	-1.4	-1.5	N
80	BC2077		YukE protein of unknown function	-1.5	-0.8	S
81	BC4175	YqxC	putative cell adhesion protein (contact hemolysin TlyA-related)	-1.5		J
82	BC0103	RadA	DNA repair protein RadA	-1.6		O
83	BC5274	YveM	UDP-N-acetylglucosamine 4,6-dehydratase	-1.7	-1.4	MG
84	BC0143	RplE	LSU ribosomal protein L5P	-1.7		J
85	BC4232		Shikimate kinase	-1.7		E
86	BC0949		hypothetical Membrane Spanning Protein	-1.7		S
87	BC3953	YlaI	hypothetical protein	-1.8		S
88	BC1422	Sat	Sulfate adenylyltransferase	-1.9		P
89	BC1814		Transcriptional regulator, TetR family	-1.9		K
90	BC1901		Phage protein	-1.9		S
91	BC4298		Cytidine deaminase	-2.2		F
92	BC5478		hypothetical Cytosolic Protein	-2.4		S
93	BC0069		Stage II sporulation protein E	-2.5		TK
94	BC3793		Cell division protein ftsK	-2.5		D
95	BC2749		Acetyltransferase	-2.6		K
96	BC2649		Transcriptional regulator, MerR family	-2.7		K
97	BC2298		Transcriptional repressor	-2.9	-1.4	K
98	BC2587		Phage Prohead Protease	-2.9		R
99	BC3548	YueD	Benzil reductase	-3.0		IQ
100	BC1623	Hfq	Hfq protein	-3.2		R
101	BC2051		Ribosomal-protein-alanine acetyltransferase	-3.1		K
102	BC4373	YxiP	hypothetical protein	-3.1		S
103	BC3047		Serine/threonine protein phosphatase	-3.1		T
104	BC3813	YlxR	hypothetical Cytosolic Protein	-3.2		K
105	BC3952		GTPase	-5.5		J
106	BC1126		S-layer homology domain		-0.6	S
107	BC2335		Catabolite gene activator		-0.6	T
108	BC4954	YutH	CotS-related protein		-0.6	S
109	BC2353				-0.6	S
110	BC2526	YcxD	Transcriptional regulator, GntR family		-0.6	KE
111	BC4830		ABC transporter permease protein		-0.7	V
112	BC3394		4-oxalocrotonate tautomerase		-0.7	R
113	BC2379		Transcriptional regulator		-0.7	K
114	BC0709		Ferrous iron transport protein A		-0.7	P
115	BC2421				-0.7	S
116	BC0066	YabR	S1-type RNA-binding domain		-0.7	J
117	BC5393		hypothetical protein		-0.7	S

118	BC2578		Phage protein		-0.7	S
119	BC1788		Lysophospholipase L2		-0.9	I
120	BC5035	YitW	Phenylacetic acid degradation protein paaD		-0.8	R
121	BC3682		Transketolase		-0.8	G
122	BC0159	RplQ	LSU ribosomal protein L17P		-0.8	J
123	BC1284				-0.8	S
124	BC4484		hypothetical Cytosolic Protein		-0.9	S
125	BC3308	CapA	Capsule biosynthesis protein capA		-0.9	M
126	BC2318		hypothetical protein		-0.9	S
127	BC3317		Histidyl-tRNA synthetase		-1.0	J
128	BC1984				-1.0	S
129	BC4314	HrcA	Heat-inducible transcription repressor hrcA		-1.0	K
130	BC4165	BkdR	Sigma-54-dependent transcriptional activator		-1.0	TK
131	BC2163		TPR-repeat-containing protein		-1.0	R
132	BC0387		hypothetical protein		-1.6	S
133	BC4221		ABC transporter ATP-binding protein		-1.1	V
134	BC5386	YwfL	Lipoate-protein ligase A		-1.1	H
135	BC0673		Flavin-dependent dehydrogenase		-1.1	C
136	BC2321		tRNA pseudouridine synthase A		-1.2	J
137	BC1968		hypothetical protein		-1.2	H
138	BC0875				-1.2	S
139	BC3606	YneP	Esterase		-1.3	R
140	BC0984		DNA-binding protein		-1.2	S
141	BC3961	YktA	putative transcriptional regulator		-1.2	S
142	BC0331	PurM	Phosphoribosylformylglycinamidine cyclo-ligase		-1.3	F
143	BC4257		hypothetical Cytosolic Protein		-1.3	S
144	BC0437		Phosphoglyceromutase/phosphopentomutase		-1.3	R
145	BC1537	BirA	Biotin operon repressor / Biotin--[acetyl-CoA-carboxylase] synthetase		-1.3	HK
146	BC4436	RpmA	LSU ribosomal protein L27P		-1.3	J
147	BC2848		protein with unknown function		-1.4	E
148	BC2172		IG hypothetical 1888		-1.5	S
149	BC3437		hypothetical Cytosolic Protein		-1.6	S
150	BC2358				-1.7	K
151	BC0416		Phage infection protein		-1.9	S
152	BC1822		Pyrimidine-nucleoside phosphorylase		-1.9	F
153	BC0631	TreP	PTS system, trehalose-specific IIBC component		-1.9	G
154	BC2215	YfkC	Mechanosensitive ion channel		-2.0	M
155	BC4718	MoaD	Molybdopterin (MPT) converting factor, subunit 1		-2.2	H
156	BC4323		ComE operon protein 2		-2.5	F
157	BC4692		hypothetical protein		-2.7	S
158	BC4613	YtkL	Metal-dependent hydrolase		-2.9	R
159	BC4458		Type I restriction-modification system specificity subunit		-3.2	V
160	BC4580	DnaB	Replication initiation and membrane attachment protein		-4.6	L
161	BC1512	HepS	Heptaprenyl diphosphate synthase component I		-4.7	S

Supplementary Table S4B: Bc1009-dependent genes in *Bacillus cereus* upon heat shock (30°C to 42°C) in wt cells versus Δ*bc1009* mutant

				wt 42°C/ Δ*bc1009* 42°C	
No	**Locus Tag**	**Gene**	**Annotation**	**Log2 expression**	**COG**
1	BC1859		Phage protein	2.3	S
2	BC0579	*maeN*	Malate-sodium symport	1.6	C
3	BC5241	*yvbJ*	IG hypothetical 16680	1.5	S
4	BC5242		Membrane protein with C2C2 zinc finger	1.5	J
5	BC1877		Phage protein	1.5	S
6	BC0161	*ybaE*	Cobalt transport ATP-binding protein cbiO	1.4	P
7	BC1883		Phage protein	1.4	S
8	BC2149		hypothetical protein	1.4	S
9	BC1867		Phage protein	1.4	S
10	BC5243		hypothetical protein	1.4	S
11	BC5226	*ywrK*	Arsenical pump membrane protein	1.4	P
12	BC0160	*ybxA*	Cobalt transport ATP-binding protein cbiO	1.3	P
13	BC3856	*rpmB*	LSU ribosomal protein L28P	1.3	J
14	BC3986	*ykuJ*	hypothetical Cytosolic Protein	1.3	S
15	BC4637	*ackA*	Acetate kinase	1.3	C
16	BC0564		hypothetical lipoprotein	1.3	C
17	BC4916		hypothetical Cytosolic Protein	1.2	S
18	BC2752	*ypeB*	hypothetical Membrane Spanning Protein	1.2	S
19	BC1868		Phage protein	1.2	S
20	BC5134		Inosine-uridine preferring nucleoside hydrolase	1.2	F
21	BC5117		ABC transporter permease protein	1.2	S
22	BC2854		Oxidoreductase	1.2	C

23	BC5034	*yoaH*	Methyl-accepting chemotaxis protein	1.2	NT
24	BC5266		Heteropolysaccharide repeat unit export protein	1.2	S
25	BC3702		Antirepressor	1.1	S
26	BC0162	*ybaF*	Cobalt transport protein cbiQ	1.1	P
27	BC5268		Secreted polysaccharide polymerase	1.1	S
28	BC3887		Carbamoyl-phosphate synthase small chain	1.1	EF
29	BC5269		Amylovoran biosynthesis AmsK	1.1	M
30	BC5439	*ysbA*	Murein hydrolase exporter	1.1	R
31	BC3703		Phage protein	1.1	S
32	BC5265	*lytR*	Transcriptional regulator, LytR family	1.1	K
33	BC1879		Transglycosylase	1.1	S
34	BC1885		Phage protein	1.1	S
35	BC5118		ABC transporter ATP-binding protein	1.1	E
36	BC5267	*yveT*	Glycosyltransferase	1.0	M
37	BC3626		hypothetical Cytosolic Protein	1.0	S
38	BC5125		hypothetical protein	1.0	M
39	BC1884		Transcription state regulatory protein abrB	1.0	K
40	BC5120		hypothetical Cytosolic Protein	1.0	S
1	BC0185	*rocF*	Arginase	-1.1	E
2	BC0208	*dppC*	Oligopeptide transport system permease protein oppC	-1.9	EP
3	BC0209		Oligopeptide transport ATP-binding protein oppD	-1.7	EP
4	BC0210		Oligopeptide transport ATP-binding protein oppF	-1.6	E
5	BC0237		Galactose-1-phosphate uridylyltransferase	-1.0	C
6	BC0347		ABC transporter permease protein	-1.8	P
7	BC0348	*yhcJ*	ABC transporter substrate-binding protein	-1.7	P
8	BC0743		Hydroxymethylpyrimidine transport ATP-binding protein	-1.4	P
9	BC0813		enterotoxin / cell-wall binding protein	-1.0	TS
10	BC0855		Multidrug resistance protein B	-1.2	GEPR
11	BC1180	*oppB*	Oligopeptide transport system permease protein oppB	-1.1	EP
12	BC1181	*oppC*	Oligopeptide transport system permease protein oppC	-1.2	EP
13	BC1182	*oppD*	Oligopeptide transport ATP-binding protein oppD	-1.3	EP
14	BC1183	*oppF*	Oligopeptide transport ATP-binding protein oppF	-1.2	E
15	BC1296	*ysfD*	(S)-2-hydroxy-acid oxidase subunit GlcF	-1.0	C
16	BC1316		PhaR protein	-1.6	S
17	BC1317		Acetoacetyl-CoA reductase	-1.5	IQR
18	BC1318		Poly-beta-hydroxybutyrate polymerase	-1.5	I
19	BC1569		Xanthine phosphoribosyltransferase	-1.0	F
20	BC1717	*ygaJ*	Peptidase E	-1.1	E
21	BC1746		Aspartate--ammonia ligase	-1.2	E
22	BC2190		Penicillin-binding protein	-1.7	V
23	BC2292		3-hydroxyisobutyryl-coenzyme A hydrolase	-1.1	I
24	BC2629		Methionyl-tRNA formyltransferase	-1.3	J
25	BC2939	*tyrA*	Arogenate dehydrogenase	-1.1	E
26	BC3880		hypothetical Membrane Spanning Protein	-1.1	S
27	BC4036	*ykrW*	Ribulose bisphosphate carboxylase large chain	-1.0	G
28	BC4073		Anti-sigma F factor	-1.1	T
29	BC4158		2-oxoisovalerate dehydrogenase beta subunit	-1.1	C
30	BC4212		Transcriptional regulator, TetR family	-1.0	K
31	BC4213		Quaternary ammonium compound-resistance protein	-1.2	P
32	BC4214	*ykkD*	Quaternary ammonium compound-resistance protein	-1.3	P
33	BC4365		Alcohol dehydrogenase	-1.0	C
34	BC4986	*yusB*	ABC transporter permease protein	-1.1	P
35	BC5233		D-alanine aminotransferase	-1.2	EH
36	BC5239		SH3 domain-containing protein	-1.5	TS
37	BCp0001		hypothetical protein	-1.5	S
38	BCp0002		hypothetical protein	-1.2	S
39	sRNABC051			-1.3	S
40	sRNABC052			-1.0	S

Supplementary Table S5A: Differentially regulated SigB- dependent or SigB and Bc1009-dependent proteins in non-heat-stressed condition at 30°C in *B. cereus* ATCC14579 wt cells versus Δ*sigB* and Δ*bc1009* mutants

Bold: indicate SigB and Bc1009-induced or downregulated proteins in wt cells versus Δ*sigB* and Δ*bc1009* mutants at 30°C
Not Bold: indicate SigB-induced or downregulated proteins in wt cells versus Δ*sigB* mutant at 30°C
log2 FC: log 2 protein fold change values at T0
COG: Cluster of orthologous groups

Induction				SigB-dependent wt 30°C / Δ*sigB* 30°C	Bc1009-dependent wt 30°C / Δ*bc1009* 30°C	
No.	**Locus Tag**	**Protein**	**Annotation**	**log2 FC**	**log2 FC**	**COG**
1	BC3699		Antigen/ lysozyme like protein	6.4		M
2	BC0944		hypothetical protein	5.4		S
3	BC5408	YfnB	2-haloalkanoic acid dehalogenase/ putative phosphatase	5.0		R
4	BC5205	LevR	Transcriptional regulatory protein levR	4.3		KTK
5	**BC0423**	**SrfAAH**	**Non-ribosomal peptide synthetase (adenylation domain)**	**4.1**	**3.9**	**Q**
6	**BC2294**		**hypothetical Cytosolic Protein**	**4.1**	**1.6**	**S**
7	**BC2724**	**OmdAH**	**LAAC/ Bacteriocin-protection, YdeI or OmpD-Associated**	**4.1**	**4.2**	**S**
8	**BC1718**		**DUF family protein of unknown function**	**4.0**	**4.9**	**S**
9	BC3874	LprIH	lysozyme inhibitor, binds to and inhibits macrophage lysozyme, aid bacterial survival	3.7		S
10	BC4667		Ankyrin	3.6		R
11	**BC3071**	**CutC**	**Copper homeostasis protein cutC**	**3.6**	**3.1**	**P**
12	**BC2752**	**YpeB**	**hypothetical Membrane Spanning Protein**	**3.5**	**3.5**	**S**
13	**BC1303**	**YvfV**	**(S)-2-hydroxy-acid oxidase, iron-sulfur chain**	**3.5**	**3.0**	**C**
14	BC4453		Phage protein	3.5		S
15	**BC5077**		**hypothetical protein**	**3.4**	**3.2**	**S**
16	**BC4830**		**ABC transporter permease protein**	**3.3**	**1.0**	**V**
17	BC2512		hypothetical protein	3.3		S
18	BC0198		ABC transporter substrate-binding protein	3.1		P
19	BC0580		NAD-dependent malic enzyme	3.0		C
20	**BC0544**		**iron-sulfur cluster-binding protein**	**3.0**	**3.1**	**C**
21	**BC1662**	**FliM**	**Flagellar motor switch protein fliM**	**3.0**	**2.9**	**N**
22	BC4165	BkdR	Sigma-54-dependent transcriptional activator	3.0		KT
23	BC2296	YirY	Exonuclease SbcC	3.0		L
24	BC4124		Transcriptional regulator, MarR family	3.0		K
25	BC1640		hypothetical protein	2.9		S
26	**BC5121**		**protein of unknown function**	**2.8**	**1.8**	**S**
27	**BC0576**	**McpBH**	**Methyl-accepting chemotaxis protein**	**2.8**	**1.4**	**NT**
28	**BC4236**		**ComG operon protein 4**	**2.8**	**3.0**	**NU**
29	**BC5243**		**protein with unknown function**	**2.8**	**2.6**	**S**
30	**BC4496**	**RacE**	**Glutamate racemase**	**2.7**	**2.6**	**M**
31	**BC3257**		**N-acetylmuramoyl-L-alanine amidase, Cell wall or membrane biogenesis**	**2.7**	**2.4**	**TM**
32	BC5124		Protein with unknown function	2.6		S
33	**BC3698**		**Cell wall endopeptidase, family M23/M37**	**2.6**	**3.9**	**M**
34	BC2210		NAD(P)H nitroreductase	2.6		C
35	BC2941	AroF	Chorismate synthase	2.6		E
36	**BC0232**		**hypothetical Membrane Spanning Protein**	**2.6**	**1.4**	**S**
37	**BC0678**		**Methyl-accepting chemotaxis protein, signaling domain**	**2.6**	**2.0**	**NT**
38	**BC5123**		**homolog of lantibiotic biosynthesis dehydratase C-term**	**2.5**	**1.8**	**S**
39	**BC4019**		**hypothetical protein**	**2.5**	**1.5**	**S**
40	**BC1202**		**Serine/threonine protein phosphatase**	**2.5**	**1.1**	**T**
41	BC4518	SdhC	Succinate dehydrogenase cytochrome b558 subunit	2.5		C
42	**BC1663**	**FliN**	**Flagellar motor switch protein fliN**	**2.5**	**2.8**	**NU**
43	**BC3406**		**Oxidoreductase**	**2.5**	**2.6**	**R**
44	BC3107		UvrC-like protein	2.5		L
45	BC5280	YwpB	(3R)-hydroxymyristoyl-[acyl carrier protein] dehydratase	2.4		I
46	BC2846		Protein dltD precursor	2.4		M
47	BC0453	YkvW	Zinc uptake P-type ATPase	2.3		P
48	BC4547		Cell surface protein	2.3		M
49	**BC5118**		**ABC transporter ATP-binding protein**	**2.3**	**1.9**	**E**
50	**BC1688**	**YmfQ**	**IG hypothetical 17894**	**2.3**	**1.6**	**S**
51	**BC4170**	**SpoOA**	**Stage sporulation protein A**	**2.2**	**1.3**	**TK**
52	**BC5125**		**Peptidase family M50**	**2.2**	**2.3**	**M**
53	**BC5198**	**YviA/degV**	**DegV family fatty acid binding protein, phosphorylation of fatty acids**	**2.2**	**2.0**	**S**
54	**BC5122**		**YcaO cyclodehydratase, ATP-ad Mg2+-binding**	**2.2**	**1.9**	**S**
55	**BC5117**		**ABC transporter permease protein**	**2.2**	**1.7**	**S**
56	BC4341	YwaC	GTP pyrophosphokinase	2.2		S
57	BC3520	TarH	Methyl-accepting chemotaxis protein (motility, Signal transduction)	2.1		NT

58	**BC5119**		**In operon with BC5120, which is a nitroreductase family protein**	**2.1**	**1.8**	**S**
59	BC4239		ComG operon protein 1	2.1		NU
60	**BC1857**	**SbcD**	**Exonuclease SbcD, DNA recombination and repair**	**2.1**	**2.8**	**L**
61	BC1472	RluB	Ribosomal large subunit pseudouridine synthase B	2.1		J
62	**BC4512**	**MotB**	**Chemotaxis motB protein**	**2.0**	**0.7**	**N**
63	BC2842		hypothetical protein	2.0		S
64	**BC5103**	**YclP**	**Ferric anguibactin transport ATP-binding protein**	**2.0**	**1.7**	**P**
65	BC2357		Cold shock protein	2.0		S
66	**BC1658**	**YvzB**	**Flagellin**	**1.9**	**2.4**	**N**
67	**BC5141**	**CggR**	**Central glycolytic genes regulator**	**1.9**	**2.3**	**K**
68	BC4530		hypothetical protein	1.9		S
69	BC3995	YkyB	hypothetical Cytosolic Protein	1.9		S
70	BC0185	RocF	Arginase	1.9		E
71	BC5000	YobS	Transcriptional regulator, TetR family	1.9		K
72	**BC1168**	**ClpB**	**ClpB protein**	**1.9**	**1.5**	**O**
73	BC0336		Somatin-like protein	1.8		O
74	**BC1651**	**FlgE**	**Flagellar hook protein flgE**	**1.8**	**1.8**	**N**
75	**BC5128**	**SmpB**	**SsrA-binding protein, required for rescue of stalled ribosomes**	**1.7**	**1.7**	**O**
76	BC0756		Sensor protein kdpD	1.7		T
77	BC0903		Methyltransferase	1.7		L
78	**BC4703**	**YtzE**	**Transcriptional regulator, DeoR family**	**1.7**	**1.1**	**KG**
79	**BC5034**	**YoaH**	**Methyl-accepting chemotaxis protein**	**1.7**	**1.1**	**NT**
80	BC3355	YrkA	Magnesium and cobalt efflux protein corC	1.7		R
81	BC1422	Sat	Sulfate adenylyltransferase	1.6		P
82	BC0073	YacB	Bvg accessory factor	1.6		K
83	BC0026	RecR	Recombination protein recR	1.6		L
84	BC1398		Acetolactate synthase small subunit	1.6		E
85	BC3812	YlxQ	LSU ribosomal protein L7AE	1.6		J
86	BC1277		D-alanyl-D-alanine carboxypeptidase	1.6		M
87	BC0422		Methyl-accepting chemotaxis protein, signaling domain	1.5		NT
88	**BC4403**	**YrvE**	**Single-stranded-DNA-specific exonuclease recJ**	**1.5**	**1.3**	**L, S**
89	BC5410	YocJ	FMN-dependent NADH-azoreductase	1.5		I
90	**BC5120**		**hypothetical Cytosolic Protein**	**1.5**	**1.3**	**S**
91	**BC1850**		**Transcriptional regulator**	**1.5**	**1.0**	**K**
92	BC4449		hypothetical Membrane Spanning Protein	1.5		L
93	BC4619		hypothetical protein	1.5		S
94	BC1632	CheR	Chemotaxis protein methyltransferase	1.5		NT
95	BC1282	SinR	SinR protein	1.4		K
96	**BC4984**		**ABC transporter substrate-binding protein**	**1.4**	**1.1**	**P**
97	BC5263		UDP-glucose 4-epimerase	1.4		M
98	BC5428		hypothetical protein	1.4		S
99	**BC5255**	**YknX**	**periplasmic component of efflux system**	**1.4**	**1.1**	**M**
100	BC5018		hypothetical Membrane Spanning Protein	1.4		S
101	BC5200	YvhJ	Transcriptional regulator, LytR family	1.4		K
102	BC2740	SecY	Protein translocase subunit SecY	1.4		U
103	**BC1654**	**CheV**	**Chemotaxis protein cheV**	**1.4**	**1.0**	**TN**
104	**BC2285**	**MmgD**	**2-methylcitrate synthase**	**1.4**	**1.3**	**C**
105	BC1771		Fibronectin-binding protein	1.4		S
106	**BC2464**		**S-layer protein / Peptidoglycan endo-beta-N-acetylglucosaminidase**	**1.4**	**2.0**	**G**
107	**BC1639**	**FliS**	**Flagellar protein fliS**	**1.3**	**1.0**	**NUO**
108	BC0267	YdcI	Transcription accessory protein (S1 RNA binding domain)	1.3		K
109	BC0001	DnaA	Chromosomal replication initiator protein dnaA	1.3		L
110	BC1760		3-oxoacyl-[acyl-carrier-protein] synthase III	1.3		I
111	**BC0888**	**CwlH**	**N-acetylmuramoyl-L-alanine amidase**	**1.3**	**1.2**	**M**
112	BC1725	DsdA	D-serine dehydratase	1.3		E
113	BC5404		Homoserine dehydrogenase	1.3		E
114	BC1624		hydrolase (HAD superfamily)	1.3		R
115	**BC3947**	**PycA**	**Pyruvate carboxylase**	**1.3**	**1.0**	**C**
116	BC4262	YqgN	5-formyltetrahydrofolate cyclo-ligase	1.3		H
117	**BC3081**	**YhjR**	**hypothetical protein**	**1.3**	**1.2**	**S**
118	**BC0896**		**S-layer protein / Peptidoglycan endo-beta-N-acetylglucosaminidase**	**1.2**	**0.9**	**G**
119	BC1060		Collagen adhesion protein	1.2		M
120	BC0604		hypothetical protein	1.2		S
121	BC4209	YqhM	Lipoate-protein ligase A	1.2		H
122	**BC1657**	**#N/A**	**#N/A**	**1.2**	**1.5**	**S**
123	BC4579	DnaI	Primosomal protein dnaI	1.2		L
124	BC4741		DNA integration/recombination/invertion protein	1.2		L
125	BC5436		Peptide methionine sulfoxide reductase	1.2		O
126	**BC1659**	**#N/A**	**#N/A**	**1.2**	**1.0**	**S**
127	**BC0404**	**TarH**	**Methyl-accepting chemotaxis protein (motility, Signal transduction)**	**1.2**	**1.6**	**NT**
128	BC0053		Stage V sporulation protein G	1.2		M
129	BC3687		2',3'-cyclic-nucleotide 2'-phosphodiesterase	1.1		F

130	BC1794	#N/A	#N/A	1.1		E
131	**BC2431**	**PhnB**	**PhnB protein**	**1.1**	**0.6**	**S**
132	BC4970		hypothetical protein	1.1		S
133	BC3919	YllA	hypothetical Membrane Spanning Protein	1.1		S
134	**BC0597**	**YueK**	**Nicotinate phosphoribosyltransferase**	**1.1**	**1.5**	**H**
135	BC1581		Mg(2+) P-type ATPase-like protein	1.1		P
136	BC4492		putative phosphoesterase	1.1		R
137	BC3394		4-oxalocrotonate tautomerase	1.1		R
138	BC2435		Phosphoglycerate mutase	1.1		G
139	**BC1157**		**Alpha-amylase**	**1.1**	**0.8**	**G**
140	**BC1653**		**hypothetical protein**	**1.1**	**0.7**	**S**
141	BC0331	PurM	Phosphoribosylformylglycinamidine cyclo-ligase	1.1		F
142	BC1962		Phosphohydrolase (MutT/nudix family protein)	1.1		LR
143	BC5157	YvcI	Mutator mutT protein (7,8-dihydro-8-oxoguanine-triphosphatase)	1.0		LR
144	BC3571	YrhF	hypothetical Cytosolic Protein	1.0		S
145	BC1593	YitH	Acetyltransferase	1.0		KR
146	BC5374	YqiK	Glycerophosphoryl diester phosphodiesterase	1.0		C
147	BC4286		NIF3-related protein	1.0		S
148	BC3209		hypothetical Cytosolic Protein	1.0		S
149	BC2862	PrsA	Protein export protein prsA precursor	1.0		O
150	**BC4178**		**Exodeoxyribonuclease VII small subunit**	**1.0**	**0.7**	**L**
151	BC4437	YsxB	hypothetical ribosome-associated protein	1.0		J
152	BC2579		hypothetical Cytosolic Protein	1.0		J
153	**BC1726**		**hypothetical Membrane Spanning Protein**	**1.0**	**1.8**	**S**
154	**BC0675**		**hypothetical protein**	**1.0**	**2.0**	**S**
155	BC0499	YfhH	Transcriptional regulator	1.0		S
156	BC2087		hypothetical protein	1.0		S
157	BC0449		hypothetical protein	0.9		S
158	BC3976	YkzG	putative transcriptional regulator	0.9		S
159	BC1936		Transcriptional regulator, MarR family	0.9		K
160	BC0038	YaaT	Tpl protein	0.9		S
161	BC5422		O-Antigen ligase - like protein	0.9		S
162	BC1131		Protein of unknown function	0.9		K
163	BC1338		Oligoendopeptidase F	0.9		E
164	BC0303		Alpha/beta hydrolase	0.9		R
165	BC2896		Aspartate aminotransferase	0.9		E
166	BC3996		hypothetical protein	0.9		S
167	BC4155	RodA	Rod shape-determining protein rodA	0.9		D
168	BC5222	YybE	Transcriptional regulators, LysR family	0.8		K
169	BC4469	HemB	Delta-aminolevulinic acid dehydratase	0.8		H
170	BC1454	YkaA	putative pit accessory protein	0.8		P
171	**BC5189**	**SecA**	**Protein translocase subunit SecA**	**0.8**	**1.1**	**U**
172	BC0056		Peptidyl-tRNA hydrolase	0.8		J
173	BC1288		Spermidine/putrescine transport system permease protein potC	0.8		E
174	BC4955	YutG	Low temperature requirement C protein	0.8		I
175	BC0364	YefA	tRNA (Uracil-5-) -methyltransferase	0.8		J
176	BC2021	YcdH	High-affinity zinc uptake system protein znuA precursor	0.8		P
177	BC4958		NAD(P)H dehydrogenase [quinone]	0.8		R
178	BC0044	YabD	Sec-independent secretion protein tatD	0.8		L
179	BC5252		hypothetical Membrane Spanning Protein	0.8		S
180	BC0954		tcdA-E operon negative regulator	0.7		S
181	BC4694		hypothetical protein	0.7		S
182	**BC5241**	**YvbJ**	**IG hypothetical 16680**	**0.7**	**1.3**	**S**
183	BC1195	YjbI	Globin Family Protein	0.7		R
184	BC3831	TopA	DNA topoisomerase I	0.7		L
185	BC3058		Phosphohydrolase (MutT/nudix family protein)	0.7		LR
186	BC1532	DapB	Dihydrodipicolinate reductase	0.7		E
187	BC3865		Polypeptide deformylase	0.7		J
188	BC0883	AlsS	Acetolactate synthase large subunit	0.7		EH
189	BC3807	RibC	Riboflavin kinase	0.7		H
190	BC3929	YlbI	Phosphopantetheine adenylyltransferase	0.7		H
191	BC1637		Flagellar hook-associated protein 3	0.7		N
192	BC3659		hypothetical protein	0.7		S
193	BC4198	YqhT	Xaa-Pro dipeptidase	0.7		E
194	**BC4791**	**YtiB**	**Carbonic anhydrase**	**0.7**	**1.3**	**P**
195	BC0437		Phosphoglyceromutase/phosphopentomutase	0.7		R
196	BC0383	FhuD	Ferrichrome-binding protein	0.7		P
197	BC1477	ResD	Transcriptional regulatory protein resD	0.7		TK
198	BC1261		ATP/GTP-binding protein	0.6		LR
199	BC3774		Pyruvate synthase alpha chain	0.6		C
200	BC0368		hypothetical Cytosolic Protein	0.6		S
201	BC5380		Ferrichrome-binding protein	0.6		P
202	BC4909	KapB	Kinase-associated protein B	0.6		S
203	BC5454		Molybdenum cofactor biosynthesis enzyme and related Fe-S oxidoreductases	0.6		R
204	BC4900	YugI	S1-type RNA-binding domain	0.6		J

205	BC5063		UDP-N-acetylenolpyruvoylglucosamine reductase	0.6		M
206	BC2103	YfmL	ATP-dependent RNA helicase	0.6		LKJ
207	BC2321		tRNA pseudouridine synthase A	0.6		J
208	BC4801	YvqH	Phage shock protein A	0.6		KT
209	BC0546	CspR	23S rRNA methyltransferase	0.6		J
210	BC2019		Low-affinity zinc transport protein	0.6		R
211	BC1066	YhgC	Signal transduction protein TRAP	0.6		R
212	BC1497		Cytidylate kinase	0.6		F
213	BC0426		Hydroxymethylpyrimidine-binding protein		0.8	P
214	BC0815	yknY	ABC transporter ATP-binding protein		0.6	V
215	BC1165	yitU	hydrolase (HAD superfamily)		0.8	R
216	BC1172		ComZ protein		2.7	G
217	BC1359	yhcH	Bacitracin transport ATP-binding protein bcrA		0.6	V
218	BC1370	dltC	D-alanyl carrier protein		3.6	IQ
219	BC1603		0		0.7	K
220	BC1628	cheA	Chemotaxis protein cheA		1.1	NT
221	BC1629	fliY	Chemotaxis protein cheC		0.7	NTU
222	BC1642	flgC	Flagellar basal-body rod protein flgC		0.9	N
223	BC1652		hypothetical protein		0.6	S
224	BC1661		Flagellar motor switch protein fliN		2.4	NU
225	BC1800		Two-component response regulator vanR		1.1	TK
226	BC1914		Phage protein		0.7	K
227	BC1991		putative murein endopeptidase		1.0	D
228	BC2057		Stomatin like protein		1.3	O
229	BC2102		hypothetical protein		0.6	S
230	BC2299		hypothetical protein		0.9	S
231	BC2607		hypothetical protein		0.8	S
232	BC3135		Carboxymethylenebutenolidase-related protein		1.6	R
233	BC3194		Transcriptional regulator, MarR family		1.0	K
234	BC3286		hypothetical protein		0.9	S
235	BC3587		Transcriptional regulator, LytR family		0.8	K
236	BC3595	yvaA	Oxidoreductase		1.4	R
237	BC3886		Carbamoyl-phosphate synthase large chain		1.4	EF
238	BC4224		Glycine dehydrogenase [decarboxylating]		3.2	E
239	BC4302	phoH	PhoH protein		1.4	T
240	BC4371		hypothetical protein		1.3	S
241	BC4746	ytqA	Fe-S oxidoreductase		0.9	R
242	BC4934		hypothetical protein		2.0	S
243	BC5168	uvrB	Excinuclease ABC subunit B		1.7	L
244	BC5242		Membrane protein with C2C2 zinc finger		2.5	J

Downregulation				**SigB-dependent wt 30°C / Δ*sigB* 30°C**	**Bc1009-dependent wt 30°C / Δ*bc1009* 30°C**	
No.	**Locus Tag**	**Protein**	**Annotation**	**log2 FC**	**log2 FC**	**COG**
1	BC3923		LSU ribosomal protein L32P	-7.9		J
2	BC2051		Ribosomal-protein-alanine acetyltransferase	-7.9		KR
3	BC2353		hypothetical protein	-7.0		S
4	**BC0385**		**Thioredoxin reductase, posttranslational modification, protein turnover, chaperones**	**-6.6**	**-5.8**	**O**
5	**BC0405**	**ArgR**	**Arginine repressor, argR**	**-6.5**	**-6.5**	**K**
6	BC3856	RpmB	LSU ribosomal protein L28P	-5.7		J
7	BC2371	YfiB	Multidrug resistance ABC transporter ATP-binding and permease protein	-5.6		V
8	BC1970		hypothetical protein	-5.3		H
9	**BC2355**		**hypothetical protein**	**-4.9**	**-3.2**	**S**
10	BC3550		Argininosuccinate lyase	-3.4		E
11	BC1647		Flagellum-specific ATP synthase	-3.3		NU
12	BC1403	LeuD	3-isopropylmalate dehydratase small subunit	-3.2		E
13	BC1619	NfrA	Oxygen-insensitive NADPH nitroreductase	-3.1		C
14	BC4463		Stage II sporulation protein B	-2.6		S
15	BC4692		hypothetical protein	-2.6		S
16	BC0288	YdiD	Ribosomal-protein-alanine acetyltransferase	-2.6		R
17	BC4373	YxiP	hypothetical protein	-2.5		D
18	BC3982	CcpC	Transcriptional regulators, LysR family	-2.4		K
19	BC1011		hypothetical protein	-2.4		S
20	BC3110	YndF	Spore germination protein BC	-2.3		S
21	BC3132		General stress protein 17M	-2.2		S
22	BC4058	YpuG	hypothetical Cytosolic Protein	-2.1		S
23	BC4250		5-methyltetrahydrofolate--homocysteine methyltransferase	-2.1		E
24	BC4221		ABC transporter ATP-binding protein	-2.0		V
25	BC3821	UppS	Undecaprenyl pyrophosphate synthetase	-2.0		I
26	BC3298		hypothetical Cytosolic Protein	-2.0		S
27	BC2424		hypothetical protein	-2.0		S
28	**BC2077**		**YukE protein of unknown function**	**-2.0**	**-2.0**	**S**

29	BC3938	YlbA	hypothetical Cytosolic Protein	-1.9		S
30	BC0857		hypothetical protein	-1.9		R
31	BC0142	RplX	LSU ribosomal protein L24P	-1.8		J
32	BC0041	YabC	Corrin/porphyrin methyltransferase	-1.8		R
33	BC3125		hypothetical protein	-1.7		S
34	BC0365	YfjN	Nitrogen regulation protein NIFR3	-1.7		J
35	BC3322		hypothetical protein	-1.7		S
36	BC4151	YqiW	hypothetical protein	-1.6		S
37	BC4203	YhcW	Phosphoglycolate phosphatase	-1.6		R
38	BC1316		PhaR protein	-1.6		S
39	BC3587		Transcriptional regulator, LytR family	-1.6		K
40	BC3005		Glycosyltransferase involved in cell wall biogenesis	-1.6		M
41	**BC1363**	**LrpC**	**Leucine-responsive regulatory protein**	**-1.5**	**-0.9**	**K**
42	BC2984		Immune inhibitor A precursor	-1.5		S
43	BC3718	FruA	PTS system, fructose-specific IIABC component	-1.5		GT
44	BC2590		Phage protein	-1.5		S
45	**BC3728**		**hypothetical protein**	**-1.4**	**-1.4**	**L**
46	BC1848		Prophage helix-turn-helix protein	-1.4		S
47	**BC3480**		**DNA mismatch repair protein mutS**	**-1.4**	**-1.4**	**L**
48	BC2753	SleB	Cell wall hydrolase cwlJ	-1.4		M
49	BC2379		Transcriptional regulator	-1.4		K
50	**BC1520**	**YpiB**	**hypothetical Cytosolic Protein**	**-1.4**	**-1.1**	**S**
51	**BC1660**	**YjbJ**	**Soluble lytic murein transglycosylase**	**-1.4**	**-1.8**	**M**
52	**BC5341**	**AcdA**	**Acyl-CoA dehydrogenase, short-chain specific**	**-1.4**	**-1.5**	**I**
53	BC1359	YhcH	Bacitracin transport ATP-binding protein bcrA	-1.4		V
54	**BC5101**		**Perfringolysin O precursor**	**-1.4**	**-2.4**	**S**
55	**BC3281**		**hypothetical protein**	**-1.4**	**-0.9**	**S**
56	BC0416		Phage infection protein	-1.4		S
57	BC3341		1-deoxy-D-xylulose 5-phosphate reductoisomerase	-1.4		I
58	**BC1638**		**Flagellar hook-associated protein 2**	**-1.3**	**-1.5**	**N**
59	BC2251	KamA	Lysine 2,3-aminomutase	-1.3		E
60	**BC5002**	**YusJ**	**Acyl-CoA dehydrogenase**	**-1.3**	**-0.9**	**I**
61	BC3693		Transcriptional regulator, PadR family	-1.3		K
62	BC1914		Phage protein	-1.3		K
63	BC4039	YkrZ	2-hydroxy-3-oxo-5-methylthiopent-2-enoate oxidase	-1.3		S
64	BC1535	YpjH	Glycosyltransferase	-1.2		M
65	BC4460		Type I restriction-modification system specificity subunit	-1.2		V
66	BC4927		Cell surface protein	-1.2		L
67	BC1898		Phage protein	-1.2		S
68	BC3159		NAD-dependent epimerase/dehydratase / Alpha/beta hydrolase	-1.2		MGRQ
69	BC5048		Ferritin	-1.2		P
70	BC2126	MoeEH	Molybdopterin synthase catalytic subunit	-1.1		H
71	BC0972		hypothetical protein	-1.1		R
72	BC2448		D-alanyl-D-alanine carboxypeptidase	-1.1		M
73	BC2037	YcgQ	hypothetical Membrane Spanning Protein	-1.1		S
74	BC4634		Protein ecsC	-1.1		S
75	BC5325	YwlC	SUA5 protein	-1.1		J
76	BC1353	YosM	NrdI protein	-1.0		F
77	**BC0366**		**hypothetical protein**	**-1.0**	**-0.7**	**S**
78	BC0361	YxkH	Polysaccharide deacetylase	-1.0		G
79	BC1017		hypothetical protein	-1.0		S
80	BC3121		5'-nucleotidase	-1.0		F
81	**BC4256**		**Transcriptional regulator, ArsR family**	**-1.0**	**-0.8**	**K**
82	BC0076	PabB	Para-aminobenzoate synthase component I	-1.0		EH
83	BC1540	PanB	3-methyl-2-oxobutanoate hydroxymethyltransferase	-0.9		H
84	**BC5274**	**YveM**	**UDP-N-acetylglucosamine 4,6-dehydratase**	**-0.9**	**-1.2**	**MG**
85	**BC3731**	**YvgY**	**COP associated protein**	**-0.9**	**-0.7**	**P**
86	BC3007		Acetylornithine aminotransferase	-0.9		E
87	**BC0679**		**Cell wall-binding protein**	**-0.9**	**-1.3**	**S**
88	BC0808		PTS system, diacetylchitobiose-specific IIB component	-0.8		G
89	BC2147	RapG	Response regulator aspartate phosphatase	-0.8		R
90	BC0135	RpsS	SSU ribosomal protein S19P	-0.8		J
91	**BC0858**		**Modulator of drug activity B**	**-0.8**	**-0.7**	**R**
92	BC2032	YjhB	Phosphohydrolase (MutT/nudix family protein)	-0.8		F
93	BC0042	AbrB	Transcription state regulatory protein abrB	-0.8		K
94	BC2655	YkoD	Cobalt transport ATP-binding protein cbiO	-0.8		P
95	BC0021		Deoxyguanosine kinase	-0.8		F
96	**BC0157**	**RpsK**	**SSU ribosomal protein S11P**	**-0.8**	**-0.8**	**J**
97	BC0052	YabJ	Translation initiation inhibitor	-0.7		J
98	BC1341	YkvJ	Aluminum resistance protein	-0.7		R
99	BC3303		hypothetical protein	-0.7		J
100	BC2773		RRF2 family protein	-0.7		K
101	BC1246		NADH dehydrogenase	-0.7		R
102	BC4374	GreA	Transcription elongation factor greA	-0.7		K
103	BC0981		Dihydroxyacetone kinase	-0.7		G
104	BC0824		Lactoylglutathione lyase	-0.7		E

105	**BC5335**	**FbaA**	**Fructose-bisphosphate aldolase**	**-0.6**	**-0.7**	**G**
106	BC4415	RuvA	Holliday junction DNA helicase ruvA	-0.6		L
107	BC3680	YneF	hypothetical protein	-0.6		S
108	BC0083	yacF	Nif3 family protein		-2.0	J
109	BC0100	mcsA	ClpC ATPase		-1.1	S
110	BC0101	mcsB	Arginine kinase		-0.7	E
111	BC0164	rplM	LSU ribosomal protein L13P		-0.8	J
112	BC0489		Glycosyltransferase involved in cell wall biogenesis		-2.2	M
113	BC0504	yfhQ	A/G-specific adenine DNA glycosylase		-1.3	L
114	BC0707		Ferrous iron transport protein B		-1.3	P
115	BC0821		CotJC protein		-0.7	P
116	BC0875		hypothetical protein		-1.2	S
117	BC0999	#N/A	hypothetical protein		-0.9	S
118	BC1024	ykcA	Glyoxalase family protein		-0.7	E
119	BC1050	ecsA	ABC-type transporter ATP-binding protein ecsA		-0.7	V
120	BC1179		Oligopeptide-binding protein oppA		-1.7	E
121	BC1690		D-alanyl-D-alanine carboxypeptidase		-1.6	V
122	BC1715		Transcriptional regulator		-1.2	KS
123	BC1719		Transcriptional regulator, MecI family		-1.7	K
124	BC1746		Aspartate--ammonia ligase		-3.5	E
125	BC1861		DNA/RNA helicase (DEAD/DEAH box family)		-1.8	KL
126	BC2437		hypothetical protein		-1.9	S
127	BC2923		Acetyltransferase		-1.2	KR
128	BC3095	ansR	Ans operon repressor protein		-2.0	K
129	BC3270		hypothetical Membrane Spanning Protein		-1.9	S
130	BC3321	yfkO	NAD(P)H-dependent flavin reductase		-2.7	C
131	BC3690	lexA	LexA repressor		-0.6	KT
132	BC3790	yufP	Nucleoside transport ATP-binding protein		-1.9	R
133	BC3952		GTPase		-0.6	J
134	BC4205	splB	Spore photoproduct lyase		-0.9	L
135	BC4384		hypothetical protein		-0.6	S
136	BC4462		Septum formation protein Maf		-7.8	D
137	BC4508	ywnA	RRF2 family protein		-1.9	K
138	BC4525	ysiA	Transcriptional regulator, TetR family		-0.8	K
139	BC4649	ezrA	Septation ring formation regulator		-2.2	D
140	BC4839	#N/A	hypothetical protein		-0.8	V
141	BC5221		Phage infection protein		-2.8	S
142	BC5237	ydaM	N-acetylglucosaminyltransferase		-1.2	M
143	BC5239		hypothetical protein		-0.9	TS
144	BC5309	atpH	ATP synthase delta chain		-0.6	C
145	BC5377	ywhC	Membrane metalloprotease		-0.8	R

Supplementary Table S5B: Differentially regulated SigB- dependent or SigB and Bc1009-dependent genes in non-heat-stressed condition at 30°C in *B. cereus* ATCC14579 wt cells versus Δ*sigB* and Δ*bc1009* mutants

Bold: indicate SigB and Bc1009-induced or downregulated genes in wt cells versus Δ*sigB* and Δ*bc1009* mutants at 30°C
Not Bold: indicate SigB-induced or downregulated genes in wt cells versus ΔsigB mutant at 30°C
log2 FC: log 2 protein fold change values at T0
COG: Cluster of orthologous groups

No	Locus Tag	Gene	Annotation	wt 30°C/ Δ*sigB* 30°C log2 expression	wt 30°C/ Δ*bc1009* 30°C log2 expression	COG
1	BC0423	*srfAAH*	Non-ribosomal peptide synthetase (adenylation domain)	1.8	1.8	Q
2	BC0576	*mcpBH*	Methyl-accepting chemotaxis protein	1.4	1.8	NT
3	BC0675		hypothetical protein	1.4	1.5	S
4	BC0678		Methyl-accepting chemotaxis protein	1.4	1.3	NT
5	BC0888	*cwlH*	N-acetylmuramoyl-L-alanine amidase	1.1	1.3	M
6	BC1653		LysR transcriptional regulator, can function as activator/repressor	1.0	1.1	S
7	BC1658	*yvzB/fliC*	Flagellin	1.6	1.6	N
8	BC1660	*yjbJ*	Soluble lytic murein transglycosylase	2.2	2.3	M
9	BC1662	*fliM*	Flagellar motor switch protein fliM	2.5	2.6	N
10	BC1663	*fliN*	Flagellar motor switch protein fliN	2.3	2.3	NU
11	BC1857	*sbcD*	Exonuclease SbcD	3.4	3.3	L
12	BC3257		N-acetylmuramoyl-L-alanine amidase	1.4	1.4	TM
13	BC3698		Cell wall endopeptidase, family M23/M37	3.7	3.6	M
14	BC5117		ABC transporter permease protein	1.2	1.3	S
15	BC5118		ABC transporter ATP-binding protein	1.2	1.5	E
16	BC5119		hypothetical protein	1.3	1.5	S
17	BC5120		hypothetical Cytosolic Protein	1.0	1.2	S
18	BC5121		hypothetical protein	1.2	1.3	S
19	BC5122		hypothetical Cytosolic Protein	1.2	1.3	S
20	BC5123		hypothetical protein	1.1	1.3	S
21	BC5241	*yvbJ*	IG hypothetical 16680	1.3	1.4	S
22	BC5243		hypothetical protein	1.4	1.6	S
23	BC0215		peptide ABC transporter substrate-binding protein	2.9	1.7	E
24	BC0492		Pyruvate formate-lyase activating enzyme	3.8	1.0	O
25	BC0559		Methyl-accepting chemotaxis protein	1.2	1.2	NT
26	BC0965		hypothetical protein	1.7	2.3	S
27	BC0966		Fimbria associated protein	1.5	1.5	M
28	BC1110	*cytK*	Cytotoxin K	1.8	2.1	S
29	BC1625		Chemotaxis motA protein	1.5	1.7	N
30	BC1626	*ytxE*	Chemotaxis motB protein	1.3	1.5	N
31	BC1656		Flagellin	1.2	1.3	N
32	BC1661	*fliN*	Flagellar motor switch protein fliN	2.5	2.4	NU
33	BC1664		Flagellar motor switch protein fliN	2.3	2.4	S
34	BC1665	*fliP*	Flagellar biosynthetic protein fliP	2.3	2.6	NU
35	BC1666	*fliQ*	Flagellar biosynthetic protein fliQ	2.3	2.4	NU
36	BC1667	*fliR*	Flagellar biosynthetic protein fliR	2.5	2.5	NU
37	BC1668	*flhB*	Flagellar biosynthetic protein flhB	2.3	2.4	NU
38	BC1669	*flhA*	Flagellar biosynthesis protein flhA	2.4	2.4	S
39	BC1670	*flhF*	Flagellar biosynthesis protein flhF	2.3	2.5	S
40	BC1671	*flhA*	Flagellar basal-body rod protein flgG	2.3	2.5	N
41	BC1672		Metal-dependent hydrolase related to alanyl-tRNA synthetase	1.4	1.1	R
42	BC1852		Exonuclease SbcC	3.2	2.3	L
43	BC1853		hypothetical protein	3.3	2.5	S
44	BC1855		Chromosome segregation ATPases	3.6	2.0	L
45	BC1856		Phage protein	3.0	2.0	S
46	BC1858		Phage protein	1.9	1.6	S
47	BC1860		Phage protein	2.9	1.5	S
48	BC1862		Phage protein	2.4	1.8	S
49	BC1873		Phage protein	1.7	1.0	S
50	BC1884	*abrB*	Transcriptional regulator during the transition from growth to stationary phase	1.8	1.3	K
51	BC1885		Phage protein	1.4	1.2	S
52	BC1887		Phage protein	1.2	1.3	S
53	BC2006	*tlpA*	Methyl-accepting chemotaxis protein	1.4	1.5	NT
54	BC2008		hypothetical protein	1.2	1.0	S
55	BC2056		hypothetical protein	1.7	1.1	OU
56	BC2057		Stomatin like protein	1.7	1.4	O
57	BC2465		hypothetical protein	1.2	1.1	S
58	BC3458		XpaF1 protein	1.5	1.3	S
59	BC3520		Methyl-accepting chemotaxis protein	1.8	2.1	NT

60	BC3694		N-acetylmuramoyl-L-alanine amidase	3.4	3.0	M
61	BC3695		holin	3.4	3.3	S
62	BC3696	*#N/A*	hypothetical protein	3.5	3.5	S
63	BC3697		hypothetical protein	2.4	1.6	S
64	BC3699		Antigen	4.0	3.7	M
65	BC3703		Phage protein	1.5	1.8	S
66	BC3763	*#N/A*	LysM peptidoglycan-binding domain-containing protein	1.6	1.7	S
67	BC4337		hypothetical Membrane Spanning Protein	1.4	1.5	S
68	BC4340	*ydfN*	NAD(P)H nitroreductase	1.4	1.2	C
69	BC4792		Cytochrome d ubiquinol oxidase subunit I	3.2	1.5	C
70	BC4793		Cytochrome d ubiquinol oxidase subunit II	3.5	1.9	C
71	BC4933		Methyltransferase	1.4	1.3	R
72	BC5033	*#N/A*	HAMP domain-containing protein	2.9	1.8	S
73	BC5116		hypothetical protein	1.5	1.7	S
74	BC5126		Transposase	1.3	1.4	L
75	BC5242		Membrane protein with C2C2 zinc finger	1.8	2.0	J
1	BC1060		Collagen adhesion protein	-1.7	-1.9	M
2	BC1283	*sinI*	SinI protein	-2.1	-1.0	S
3	BC2078		hypothetical Membrane Spanning Protein	-1.9	-1.5	S
4	BC2080	*yukC*	hypothetical Membrane Associated Protein	-2.4	-1.4	S
5	BC2081	*yukA*	DNA segregation ATPase and related proteins (FtsK/SpoIIIE family)	-1.7	-1.3	D
6	BC3186	*yrrS*	hypothetical protein	-1.9	-1.2	S
7	BC5026		hypothetical protein	-4.7	-2.9	S
8	BC5027		Protein erfK/srfK precursor	-4.1	-3.2	S
9	BCp0001		hypothetical protein	-1.4	-1.1	S
10	BCp0002		hypothetical protein	-1.3	-1.1	S
11	BCp0003		hypothetical cytosolic protein	-1.1	-1.0	S
12	BCp0005		type B DNA polymerase	-1.5	-1.2	S
13	BCp0006		LexA repressor	-1.6	-1.4	KT
14	BCp0007		hypothetical membrane spanning protein	-2.0	-2.1	S
15	BCp0008		hypothetical cytosolic protein	-1.7	-1.9	S
16	BCp0009		hypothetical cytosolic protein	-2.0	-2.2	S
17	BCp0010		hypothetical cytosolic protein	-1.7	-2.0	S
18	BCp0011		hypothetical membrane spanning protein	-1.6	-2.2	S
19	BCp0012		hypothetical protein	-1.8	-2.2	S
20	BCp0013		hypothetical cytosolic protein	-1.7	-2.0	S
21	BCp0014		hypothetical cytosolic protein	-1.8	-1.9	S
22	BCp0015		hypothetical membrane spanning protein	-1.5	-1.7	S
23	BCp0016		N-acetylmuramoyl-L-alanine amidase	-1.7	-1.9	NU
24	BCp0017		hypothetical membrane spanning protein	-1.8	-1.8	S
25	BCp0018		hypothetical cytosolic protein	-1.7	-1.7	S
26	BCp0019		hypothetical protein	-1.6	-1.9	S
27	BCp0020		hypothetical protein	-1.5	-1.8	S
28	BCp0021		N-acetylmuramoyl-L-alanine amidase	-1.5	-1.8	M

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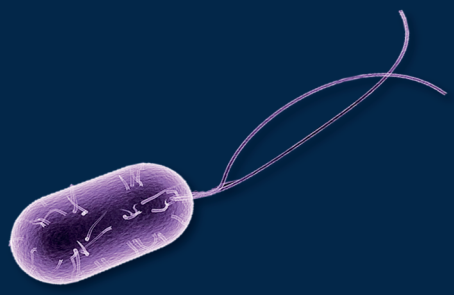
“A mildly stressful condition is stressful, a mild response is still a response.

CHAPTER 4



The role of SigB in biosurfactant synthesis and the effect of biosurfactant on SigB general stress response in *Bacillus licheniformis*

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Abstract

In this study, the role of SigB - the master regulator of the General Stress Response (GSR) in the production of lichenysin (a biosurfactant and lipopeptide) in *Bacillus licheniformis* wild type and its SigB mutant was investigated. Additionally, the effect of lichenysin and a highly similar lipopeptide (surfactin) on the induction of the SigB GSR and lethal stress resistance was studied.

B. licheniformis MW3 wt cells produced more lichenysin than Δ*sigB* cells, i.e., 3.3 versus 2.5 µg per g of biomass, respectively, but lichenysin production was shown to be not directly regulated by SigB: No SigB promoter binding motif (PBM) was found preceding the *lch* (*lic*) operon, and the expression of lichenysin-producing enzymes (*lchAA /licA and lchTE /licTE*) was not affected upon SigB activation in exponential or stationary phase cultures. When cells of *B. licheniformis* were exposed to lichenysin, a ~2-fold induction of the expression of *sigB* and the SigB-dependent gene *rsbW* was observed. Similar ~2-fold induction of SigB-dependent *rsbV* by lichenysin and surfactin was shown in a *B. subtilis* wt strain containing a P_{rsbV}-*lacZ* reporter. The mild surfactant-induced SigB GSR provided modest protection to *B. licheniformis* in the first 120 min when cells were exposed to harsh stress (12% (v/v) ethanol).

In conclusion, this study showed modest roles of SigB in *B. licheniformis* surfactant production and surfactant-induced lethal stress resistance. The exact environmental conditions and efficacy of surfactants in triggering SigB GSR, and their impact on the competitive fitness of *B. licheniformis* remain to be elucidated.

4.1 Introduction

Bacillus licheniformis is a mesophilic endospore former, commonly found in soil, rhizospheres of plants, plant material, compost, aquatic environments, and animal intestinal tracts. As a result, this bacterium and its spores are also inevitably present in food products (Vos et al., 2009; Connor et al., 2010; López et al., 2021). The endospores survive harsh conditions, such as wet/dry heat, exposure to ultraviolet light, ionizing radiation, microwave treatment (Sella et al., 2014), chemical sporicides, high hydrostatic pressure, high voltage electric fields, and many others, as reviewed in (Cho and Chung, 2020). Spores of strains that contain the mobile genetic element *Tn1546* are extremely heat resistant and can even survive boiling for ~ 10 h (Berendsen et al., 2016).

Bacterial spores can withstand many environmental insults, but cells are more sensitive once the spores germinate and the bacterium is in its vegetative state. For instance, conditions of nutrient depletion or exposure to osmotic, acid, oxidative stress, or antibacterial compounds may occur, or cells may be exposed to fluctuations in the growth conditions, such as temperature shifts (Voigt et al., 2013; Wells-Bennik et al., 2016; Bartolini et al., 2019; Cho and Chung, 2020). Yet, vegetative cells of *B. licheniformis* are also relatively resilient during growth compared with many other non-spore-forming bacteria. It is a facultative anaerobe, can grow at high salt concentrations (Gekas et al., 1998; Yeak et al., 2022), and is facultative thermophilic with an ability to grow at temperatures up to ~ 58°C (Warth, 1978; Baranyi and Tamplin, 2004). It is also well known for producing xylanase and cellulase, enzymes that enable this bacterium to break down plant cell walls made up of hemicellulose (Seo et al., 2013; López et al., 2021). These properties allow *B. licheniformis* to thrive on plant sources in the soil, other natural niches, extreme habitats (e.g., salt lakes), or even oil reservoirs (Yakimov et al., 1997; Hashemi et al., 2014; Singh and Jha, 2016; Nikolova and Gutierrez, 2020).

Moreover, *B. licheniformis* synthesizes the lichenysin lipopeptide, which has antimicrobial properties (Nerurkar, 2010; Coronel-León et al., 2016). It is synthesized by the lichenysin synthetase cluster *lchAA-AB-AC-TE* (also annotated as *licA – licTE*) (Konz et al., 1999), and is a biosurfactant that is structurally very similar to the biosurfactant produced by *B. subtilis* (surfactin). Surfactin is synthesized via proteins encoded by the *srf* operon: *srfAA-AB-AC-TE* (Nakano et al., 1991). Both surfactants are commonly used as household cleaning agents (Shaligram and Singhal, 2010), in soil bioremediation and oil recovery (Golyshin et al., 1999; Mulligan, 2005; Alvarez et al., 2020), but also exert antimicrobial properties, e.g., inhibiting the growth of *Staphylococcus aureus, Escherichia coli, Enterobacter* spp., and *Pseudomonas* spp. (Yakimov et al., 1995; Coronel-León et al., 2016). However, both compounds have also been previously reported to be associated with foodborne illness (Salkinoja-Salonen et al., 1999; Mikkola et al., 2003; Pavić et al., 2005).

B. licheniformis and *B. subtilis* are relatively stress-resistant due to the presence of stress-adaptive mechanisms, such as the renowned general stress response (GSR), which is mediated by the alternative Sigma factor B (SigB). They utilize SigB-GSR to adapt to abrupt changes or stresses in the environments, e.g., temperature shifts, osmotic shock, and ethanol (Petersohn et al., 1999, 2001; Price et al., 2001; Voigt et al., 2013). In addition, the antimicrobial action of lichenysin and surfactin conceivably provides producer cells with a competitive advantage over other coexisting microorganisms, thereby supporting their survival. Moreover, biosurfactants can also reduce water surface tension, promoting cellular swarming on surfaces and pellicle biofilm formation, which may allow for enhanced scavenging of nutrients and contribute to the colonization of new habitats (Raaijmakers et al., 2010; Ke et al., 2015).

To date, SigB GSR has been well characterized in *B. subtilis*. In the presence of physical stressors (extreme temperature, pH, salt, ethanol, antibiotics, oxidative or nitrosative stress, blue light, elevated manganese), SigB is triggered via the stressosome signaling cascade, which is made up of the Regulator of SigB proteins R, S, and T (encoded by *rsbR, rsbS*, and *rsbT*, respectively) (Marles-Wright and Lewis, 2010; Pané-Farré et al., 2017). In response to nutritional stressors (e.g., glucose, nitrogen, or phosphate), SigB is activated via the RsbQP energy signaling module, recently reviewed in Rodriguez Ayala et al. (2020). In *B. licheniformis,* physical signals including high temperature, salt, and ethanol also trigger the SigB GSR (Schroeter et al., 2013; Voigt et al., 2013), while nutritional stress caused by glucose, nitrogen, and/or phosphate starvation does not trigger SigB GSR (Hoi et al., 2006; Voigt et al., 2007). Whether this latter phenomenon reflects differences in roles of SigB in *B. subtilis* and *B. licheniformis* remains to be studied.

Recently, Bartolini et al. (2019) reported that a comparative analysis of *B. subtilis* wild type and its SigB mutant showed significantly decreased surfactin-dependent antifungal activity in the latter strain. The absence of SigB led to an apparent reduction in surfactin production in *B. subtilis*, conceivably via indirect effects on the expression of the *srf* operon, since no SigB promoter binding site was detected in the promoter region (Bartolini et al., 2019). So far, it was not known whether SigB has an effect on lichenysin production in *B. licheniformis*. Moreover, lichenysin/surfactin can interact with microbial membranes and cause growth inhibition, as previously reported for ethanol (a well-known inducer of the SigB-mediated GSR in *B. subtilis* and *B. licheniformis*). The question is whether these surfactants induce the SigB GSR in *B. licheniformis*.

In this study, firstly, the effect of SigB on lichenysin production was assessed. Secondly, the effect of lichenysin and surfactin on SigB GSR and subsequent lethal stress survival of *B. licheniformis* was investigated.

4.2 Materials and Methods

4.2.1 Strains, media, growth conditions, and chemicals used

All strains used in this study are listed in Table 1. *B. licheniformis* strains used were MW3 (parent strain DSM13 Δ*hsdR1* and Δ*hsdR2*) and MW3 Δ*sigB* (DSM13 Δ*hsdR1*, Δ*hsdR2*, ΔrsbWsigBrsbX) (Courtesy Dr. J. Panne Farre, University of Greifswald, Germany). The wild-type (wt) MW3 strain was selected instead of the DSM13 type strain because this strain lacked two restriction enzyme genes Δ*hsdR1* and Δ*hsdR,* allowing for increased transformation efficiency (Waschkau et al., 2008; Hoffmann et al., 2010) needed to generate an isogenic Δ*sigB* mutant (Voigt et al., 2013). The *B. subtilis* strain used was the 168 wt strain of Marburg origin (Zeigler et al., 2008; Barbe et al., 2009; Misirli et al., 2014).

All strains were cultured in Luria Bertani (LB) medium or propagated on LB agar plates (Tritium Microbiologie, Eindhoven, The Netherlands), and the MW3 Δ*sigB* mutant was grown in LB medium or agar plates supplemented with 200 µg/ml spectinomycin unless otherwise stated. The chemically defined medium used was the C-minimal medium described by Commichau et al. (2008), supplemented with 1 g/L glucose (C-Glc). The *Bacillus* transformation medium used was the 1X MC competence medium (containing 6.7 µl of 1M $MgSO_4$, 10 µl of 1% (v/v) tryptophan, 200 µl of 10X MC, and 1.8 ml of sterile water). The 10 X MC medium contained 140.36 g/L $K_2HPO_4.3H_2O$, 52.39 g/L KH_2PO_4, 200 g/L glucose, 300 mM $Na_3C_6H_5O_7$, 0.22 g/L of $C_6H_8FeNO_7$, 10 g/L of casein hydrolysate, and 20 g/L of $KC_5H_8NO_4$. All incubations were done at 37°C, and all liquid cultures were incubated in a shaking incubator at 200 rpm. Growth was monitored by determining the optical density at 600 nm (OD_{600}).

All chemicals, oligonucleotides, KOD polymerases, and genomic DNA isolation kit (GenElute) were purchased from Merck (Zwijndrecht, The Netherlands). Plasmids were isolated with the GeneJET Miniprep kit (Thermo Fischer Scientific, Bleiswijk, The Netherlands), and the amplified PCR products were purified with the QIAquick PCR purification kit (Qiagen, Hilden, Germany). The DNA of received plasmids and transformants were sequenced via Sanger sequencing (BaseClear B.V., Leiden, The Netherlands).

4.2.2 Lichenysin synthetase cluster and *sigB* gene deletion verification in *B. licheniformis* MW3

To perform a qualitative check of the *B. licheniformis* MW3 wt and MW3 Δ*sigB* strains (Courtesy Dr. J. Panne Farre, University of Greifswald, Germany), PCR was carried out to check for 1) the presence of each of the genes in the lichenysin synthetase gene cluster (*lchAA, lchAB, lchAC, lchTE)* (or *licA, licB, licC, licTE*) and 2) the absence of *sigB* in the MW3 Δ*sigB* mutant. All of the corresponding primers used are listed in Table 2. The amplicons were

visualized on 1% (w/v) agarose gel stained with ethidium bromide. The molecular marker used was the 1 Kb Plus DNA ladder (Thermo Fischer Scientific).

4.2.3 Lichenysin extraction from biomass and quantification

To achieve optimal lichenysin production, *B. licheniformis* wt and Δ*sigB* mutant were cultured on LB agar (2 % w/v) at 37°C for 10 days, and lichenysin was extracted from collected biomass as described by Madslien et al., (2013) and Rønning et al., (2015), with slight modifications as described in detail in Yeak et al. (2022). The Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC) with high-resolution accurate mass spectroscopic (MS) detection was used to quantify the amount of lichenysin produced by the *B. licheniformis* wt strain and its isogenic Δ*sigB* mutant. All chemicals and reagents, lichenysin calibration standards, and instruments used were the same as described in Yeak et al. (2022). The occurrence of lichenysin with the molecular masses listed in Table 3 was assessed, and the total amount was quantified in the positive detection mode. Data analysis was performed as described in Yeak et al. (2022).

4.2.4 Screening of the SigB promoter binding consensus

The complete FASTA sequence of *B. licheniformis* DSM13 was uploaded to the bacterial gene regulation database, PRODORIC2 (Eckweiler et al., 2018), available at http://www.prodoric2.de. The presence or absence of the SigB transcription factor binding consensus (GTTTAA-N_{14}-GGGTAT) upstream of the lichenysin synthetase operon in the DSM13 genome was assessed using PRODORIC2. A manual check of the SigB binding site was also performed using the Benchling Biology Software, 2020, retrieved from https://benchling.com.

4.2.5 Quantification of changes in gene expression for *B. licheniformis* upon ethanol shock or lichenysin treatment

To determine whether the induction of SigB influences the expression of the lichenysin gene cluster directly, the changes in the expression of SigB-dependent genes (*rsbW, yfkM,* and *sigB* itself) and two lichenysin synthetase genes (*lchAA, lchTE*) were quantified upon 4% (v/v) ethanol shock of cultures via Reverse-Transcription quantification polymerase chain reaction (RT-qPCR). The changes in the expression of *rsbW, yfkM,* and *sigB* upon treatment with 10, 20, or 30 µM of lichenysin (Lipofabrik, Villeneuve-d'Ascq, France) were also determined via RT-qPCR to assess whether the exposure of cells to lichenysin mediates a SigB GSR.

4.2.5.1 Cultivation conditions and RNA isolation

For the gene expression studies, *B. licheniformis* was cultivated in the chemically defined medium (C-Glc) to ensure that the changes in gene expression were not affected by the slight difference in nutrient compositions/concentrations in different batches of LB. Briefly, *B. licheniformis* wt strain and Δ*sigB* mutant were cultured overnight (ON) in C-Glc medium and

C- Glc supplemented with 200 µl spectinomycin, respectively. ON cultures of wt and *ΔsigB* mutant were inoculated into pre-warmed C- Glc without spectinomycin to an OD_{600} of 0.05, and the cultures were allowed to grow to the exponential phase (an OD_{600} of ~ 0.35). Subsequently, 10 ml of the culture was collected as a no-stress control, and another 10 ml was treated with either 4 % ethanol (v/v), 10, 20, or 30 µM of commercially available lichenysin (Lipofabrik) for 10 min. Lichenysin stock (5 mg/ml) was dissolved in water (pH 11.5 to obtain maximum solubility) instead of other solvents (such as ethanol or DMSO) to avoid SigB induction in cells upon addition of the concentrated lichenysin stock solution. Cells pellet were collected via centrifugation at 8000 g for 5 min at room temperature and resuspended in 400 µl ice-cold Tris Ethylene-diamine-tetra-acetic acid (TE) (pH 7.5) buffer. The resuspended cells were transferred to a sterile bead beater tube containing 0.5 g zirconia beads (Merck). Subsequently, 250 µl phenol (pH 4.3, 99% v/v), 250 µl chloroform (99% v/v), 30 µl sodium acetate (3M pH 5.5), and 30 µl 10% (v/v) sodium dodecyl sulfate (pH 7.2) were added to the tube, and the content was frozen with liquid nitrogen, followed by storage at -80°C.
RNA was isolated using the High Pure RNA Isolation kit (Roche, Almere, The Netherlands). The RNA concentration in the samples was measured with the NanoDrop Spectrophotometer (Thermo Fischer Scientific), and 3 µg was taken and digested with DNAseI (Thermo Fischer Scientific). A test PCR was performed using the CY9 and CY10 primers targeting *lchAA* (*licA*) to ensure the digested RNA was free of DNA contamination (see Table 2).

4.2.5.2 Reverse-Transcription quantification polymerase chain reaction (RT-qPCR)

To achieve the best qPCR efficiency as described in the protocol of the iScript™ One-Step RT-PCR Kit With SYBR® Green (Biorad, Lunteren, The Netherlands), an exact amplicon size of 145-150 bp for each targeted gene was designed using the Primer 3 plus program (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) (Untergasser et al., 2007, 2012). All RT primers used to amplify the corresponding PCR products using MW3 wt chromosomal DNA as a template are listed in Table 2. PCR products were purified, and standard curves were generated as Mulder et al. (2017) described. The *ccpA* (encoding the carbon catabolite protein) and *rpoB* (encoding RNA polymerase beta subunit) genes were used as the RT-qPCR reference genes for *B. licheniformis*.

The changes in gene expression of three SigB-dependent genes (*sigB, rsbW, yfkM*), two lichenysin synthetase genes (*lchAA, lchTE*), and two reference genes (*ccpA, rpoB*) in MW3 wt and MW3 Δ*sigB* mutant were quantified using the iScript™ One-Step RT-PCR Kit With SYBR® Green (Biorad). The instrument used was the CFX384 Touch Real-Time PCR system (Biorad). All samples were prepared, and procedures were performed according to the manufacturer's protocol. 100 ng of total RNA was used as the template, and the standard reaction protocol was followed. Briefly, complementary deoxyribonucleic acid (cDNA) synthesis was performed

at 50°C for 10 min, and then the reverse transcriptase was inactivated at 95°C for 1 min. Following cDNA synthesis, qPCR was performed in a cycle of two steps 1) DNA denaturation step: 95 °C for 10 s and 2) annealing and elongating step: 55°C for 30 s, for a total of 39 PCR cycles. To determine the PCR product specificity, the melting curve of the reaction products was analyzed over a temperature range from 55°C to 95°C, at 0.5°C increments every 3 s.

The cycle threshold (CT) values were recorded, indicating the cycle number upon which the fluorescent signal exceeded the background level. Next, the relative transcript level of each tested gene in MW3 wt and the MW3 Δ*sigB* mutant was normalized using the average transcript levels of the two reference genes *rpoB* and *ccpA* in both strains, respectively. Subsequently, the fold changes in gene expression of MW3 wt versus the MW3 Δ*sigB* mutant under the control condition (no treatment) and upon ethanol shock or lichenysin treatment were calculated using the Livak method formula (Livak and Schmittgen, 2001).

$$\text{Fold changes} = 2^{-\text{ddCT}}$$

$$\text{ddCT} = \text{RNA2}\ (\text{CT} - \text{CT}_{\text{constant}}) - \text{RNA1}\ (\text{CT} - \text{CT}_{\text{constant}})$$

CT: Transcript amount of the respective gene/cycles until the threshold value is reached;

$\text{CT}_{\text{constant}}$: Median of the CT values of the reference genes *ccpA* and *rpoB* in the respective strain;

RNA1: RNA of wt or Δ*sigB* under the control condition (no treatment);

RNA2: RNA of wt or Δ*sigB* under the induced condition (4% ethanol v/v or 10, 20, 30 µM lichenysin).

4.2.6 Construction of the *Bacillus subtilis* Δ*sigB* mutant

The *B. subtilis* 168 *sigB* knockout mutant was constructed using the long flanking homology recombination method Kunst and Rapoport, (1995), and all primers used were designed using the Benchling Biology Software, 2020, retrieved from https://benchling.com.

The purified chromosomal DNA of *B. subtilis* wt was used as a template. First, 1 kb DNA fragments that flanked the upstream and the downstream regions of the *sigB* gene (BSU04730) were amplified with the primer pairs CY316, CY317, and CY320, CY321 (Table 2), respectively, via PCR. Next, the chloramphenicol cassette was amplified from the pNZ5319 plasmid using primers CY318 and CY319, and each end contained a 20 bp complementary (overhang) sequence which matched the upstream and downstream DNA flanking fragment, respectively. The three PCR fragments were purified, then 100 ng of the left flanking fragment, 100 ng of the right flanking fragment, and 150 ng of the chloramphenicol cassette were added into a PCR reaction tube to generate one 3.2 kb linear DNA fragment via a two-steps sewing PCR as described by Wach (1996). The 3.2 kb linear DNA fragment was introduced into *B. subtilis* wt

competent cells via natural competence in 1X MC competence medium (see section 4.2.1 for composition), and the transformed cells were selected on LB agar supplemented with 5 µg/ml chloramphenicol. Positive colonies were isolated, chromosomal DNA of colonies was purified using the genomic DNA isolation kit (section 4.2.1) and sequenced (outsourced to BaseClear) with primers CY322 and CY323 (Table 2) to check for the complete integration of the 3.2 kb linear DNA fragment into the *sigB* locus. The sequenced positive transformants with complete integration of the 3.2 kb linear DNA fragment into the *sigB* locus were propagated on LB with 5 µg/ml chloramphenicol.

To further construct the marker-free Δ*sigB* mutant, transformation procedures described above were repeated to introduce the pDR244 *cre*-recombination plasmid (Koo et al., 2017) that was used to excise the chloramphenicol cassette (Table 2). The absence of a marker in the Δ*sigB* mutant BY47 (Table 1) was confirmed by sequencing, and this strain was further used to introduce translational promoter-*lacZ* constructs, as described in the next section.

4.2.7 Construction of *Bacillus subtilis lacZ*-reporter strains

B. subtilis lacZ-reporter strains were constructed by translationally fusing the promoter of *rsbV* (P_{rsbV}) to the *lacZ*-reporter, and inserting this fragment into the *amyE* locus of *B. subtilis* 168 wt and Δ*sigB* mutant (BY47). Briefly, the plasmid pNW2205 (courtesy of Dr. N. Stanley Wall, University of Dundee, UK) containing the translational reporter fusions of P_{rsbV} to *lacZ*, was inserted into the *amyE* locus via the same transformation procedures as described in section 4.2.6. Positive colonies were selected on LB agar containing 200 µg/ml spectinomycin, and the loss of amylase activity was evaluated using 1% (w/v) starch plates. Positive colonies were isolated, and chromosomal DNA was purified and sequenced (BaseClear) with primers CY414 and CY415 to verify the genomic integration (Table 2). *B. subtilis* wt with P_{rsbV} -*lacZ* integrated into the *amyE* locus was named BY67 (168 *trpC2 amyE::*P_{rsbV}*-lacZ::spc*), and the Δ*sigB* mutant was named BY72 (168 *trpC2* Δ*sigB amyE::*P_{rsbV}*-lacZ::spc*). All strains constructed for this study are shown in (Table 1) and the primers used are listed in Table 2.

Table 1: Strains and plasmids used in this study

Strain	Description and Genotype	Reference
168	*B. subtilis* 168 type strain *trpC2*	(Kunst et al., 1997; Zeigler et al., 2008; Barbe et al., 2009)
MW3	*B. licheniformis* MW3 Δ*hsDR1* Δ*hsdR2*	(Hoffmann et al., 2010; Voigt et al., 2013)
MW3 Δ*sigB*	*B. licheniformis* MW3 Δ*hsDR1* Δ*hsdR2* Δ*rsbWsigBrsbX::spec*	(Voigt et al., 2013)
BY44	*B. subtilis* 168 *trpC2* Δ*sigB::cat*	This study
BY47	*B. subtilis* 168 *trpC2* Δ*sigB*	This study
BY67	*B. subtilis* 168 *trpC2 amyE::PrsbV-lacZ::spec*	This study
BY72	*B. subtilis* 168 *trpC2* Δ*sigB amyE::PrsbV-lacZ::spec*	This study
pNZ5319	Cre-lox plasmid with *cat* and *erm* antibiotic cassettes	(Lambert et al., 2007)
pDR244	Cre- recombinase plasmid with *spec* antibiotic cassette	(Koo et al., 2017)
pNW2205	pDG1728 P*rsbV-lacZ::spc*	Courtesy Dr. N. Stanley Wall, University of Dundee, UK

4.2.8 β-galactosidase (LacZ) reporter assay

BY67 and BY72 carrying the P_{rsbV}-*lacZ* reporter fusion were grown in the C-Glc medium supplemented with 200 µg/ml spectinomycin. ON cultures in C-Glc were used to inoculate fresh C-Glc medium to an OD_{600} of 0.05 and incubation was continued until an OD_{600} of ~ 0.35 was reached. Half of the culture was then used as the control, and the other half was exposed to a selected compound at a concentration of choice. This included 4% (v/v) ethanol, 10-100 µM of surfactin (Merck), or 30 µM of lichenysin (Lipofabrik) for 10 min. Stock solutions of surfactin (10 mg/ml) and lichenysin (5 mg/ml) were made in water (pH 11.5) instead of solvents such as ethanol or DMSO to avoid induction of the SigB GSR by solvents present in stock solutions. Upon exposure to ethanol and the surfactants for 10 min, cell pellets were collected immediately via centrifugation at 8000 g for 2 min at room temperature, supernatants were discarded, and pellets were stored at - 20°C.

The *in vivo* SigB activity of BY67 and BY72 was determined using the quantitative β- galactosidase (LacZ) assay described by Stannek et al. (2015). (see Chapter 2). Briefly, cells were thawed and lysed with 400 µl Z-buffer (0.48 mM $Na_2HPO_4.2H_2O$; 0.32 mM NaH_2PO_4; 0.08 mM KCl; 8.0 µM $MgSO_4$; 0.4 mM ß- mercaptoethanol; 200 µg lysozyme and 200 µg DNAse I) for 1 h at 37°C. Lyzed cells were centrifuged at 17,000 g to remove cell debris, and 100 µl of cell-free crude extracts per sample were collected into a new Eppendorf tube. 700 µl of Z- buffer without ß- mercaptoethanol was mixed with 100 µl of the crude extracts and incubated for 5 min at 28°C. 800 µl of Z-buffer without ß- mercaptoethanol was used as a reference. Subsequently, 200 µl of 4 mg/ml of ortho-nitrophenyl-β-galactoside (ONPG) was added to all the crude extracts and the reference and allowed to react at 28°C. When a yellow color became visible, 500 µl of 62.5 mM Na_2CO_3 was added to stop the reaction. The absorption of samples at λ = 420 nm was measured. Protein concentration was determined at λ = 595 nm using the commercial Roti®-Quant Bradford solution (Carl Roth, Karlsruhe, Germany) according to the manufacturer's protocol. The absorbance at A_{595nm} and A_{420nm} is corrected with the blank without cells. The specific ß- galactosidase (LacZ) activity (indicating the SigB activity) in Miller Units/mg protein was calculated using the formula:

$$\frac{\text{Units}}{\text{mg protein}} = \frac{2005.3475 \text{ x } A420nm}{\Delta T \text{ x } V \text{ x } A595}$$

One unit of β-galactosidase = the amount of enzyme produced to hydrolyze the chromogenic substrates ONPG to one nmol of o-nitrophenol (absorbs light at λ = 420nm) per min at 28°C. V= 0.1 ml.

4.2.9 Stress adaptation and cell survival assay

The survival of MW3 wt and its isogenic Δ*sigB* mutant was established upon exposure to 12% (v/v) ethanol, with or without pre-exposure to 10 µM lichenysin, 10 µM surfactin, or 4% (v/v) ethanol. Wt cells were cultured ON in C- Glc medium, and the Δ*sigB* mutant was cultured in C- Glc supplemented with 200 µg/ml spectinomycin. ON cultures of the wt strain and the Δ*sigB* mutant were diluted (to OD_{600} 0.05) in pre-warmed C-Glc without spectinomycin, and grown to an OD_{600} of ~ 0.35, at which point the viable counts in the culture were determined. For each strain, three different cultures of OD_{600} 0.35 were exposed to lichenysin (final concentration 10 µM), surfactin (final concentration 10 µM), or ethanol (final concentration 4% (v/v) for 30 min before subjecting to lethal ethanol stress (12% v/v). Another culture was immediately exposed to 12% (v/v) ethanol. The viable counts of each culture were determined just before exposure to the lethal ethanol stress. Following exposure to 12% (v/v) ethanol, viable counts were determined every hour. In all cases, viable counts were determined by preparing 10-fold serial dilutions and plating these on C- Glc agar plates that were subsequently incubated at 37°C ON and counted.

Table 2: Primers used in this study. Underscore (_) indicates the complementary overhang sequences; *Bsu* (*B. subtilis*); *Bli* (*B. licheniformis*); RT: Reverse transcription

Primer	Name	Sequence 5'- 3'
CY51	p*NZ5319 cat* gene check	TAGGATCCAAGTACAGTCGG
CY316	*Bsu sigB* upstream flanking forward	TTCTTGGAGCGTCCTGATCTG
CY317	*Bsu sigB* upstream flanking reverse with *cat* overhang	TTTAAACGTGCTCCAGTGGCGA TCAACTCGCTCCCCATTTAAATA
CY318	*sigB cat* gene forward overhang	AATGGGGAGCGAGTTGATC GCCACTGGAGCACGTTTAAAC
CY319	*sigB cat* gene reverse overhang	TTTTCTTCAACCTGGATCAT CGTCTTCTCGTAGCGATCGG
CY320	*Bsu sigB* downstream flanking forward with *cat* overhang	CCGATCGCTACGAGAAGACGATGAT CCAGGTTGAAGAAAACGAG
CY321	*Bsu sigB* downstream flanking reverse	TGCTTCAGCGCCTTCTAATACA
CY322	*Bsu sigB* genomic DNA check forward	AGGACTCGTTCTCGGCATCT
CY323	*Bsu sigB* genomic DNA check reverse	TCTGGATATGCGTCTCTCGGA
CY414	*PrsbV-pNW2205 check forward*	CAATTCGATCAGCATCTGGAAAAGG
CY415	*PrsbV-pNW2205 check reverse*	TCAAATTTTCCTTCAAATCACTAGTTGC
CY21	*Bli lchAA* forward	CGATTCCGGGTTATTGACTG
CY22	*Bli lchAA* reverse	CGCTTCATATTGTGCGTTCC
CY23	*Bli lchAB* forward	ATTTGAGGATCTTGCGGACTTG
CY24	*Bli lchAB* reverse	TATAGCGGGAGATAGTCGTACC
CY25	*Bli lchAC* forward	GGCTGCGTATATCATGCTTG
CY26	*Bli lchAC* reverse	CTTGTTGGCTTCCATCAAGG
CY27	*Bli lchTE* forward	GATATTCGGCATCATTCAGACC
CY28	*Bli lchTE* reverse	GCTACTTGTTCCGTTTCTGATA
CY29	*Bli sigB* forward	ATCAGGAGAACCAGGATGAAG
CY30	*Bli sigB* forward	GATCTTCAGCCAATGCTTCC
CY1	*Bli rpoB*_RT forward	GCTCGCAGCTTTCTCAGTTT
CY2	*Bli rpoB*_RT reverse	CCGGCCGTAGTGTGAATAGT
CY3	*Bli ccpA*_RT forward	CCTCGCCAGCTCTGAATAAA
CY4	*Bli ccpA*_RT reverse	CGACGACGAGAAAGAAGGTG
CY9	*Bli lchAA*_RT forward	ACATCGTCGGCTATATCGAA
CY10	*Bli lchAA*_RT reverse	CTCCATCCAGCTCTAAGCAA
CY15	*Bli lchTE*_RT forward	GATCGCCTGTGCATATTCTG
CY16	*Bli lchTE*_RT reverse	CGGCTACTTGTTCCGTTTCT
CY17	*Bli sigB*_RT reverse	ATCTATGCCAGGTTGGGATG
CY18	*Bli sigB*_RT forward	TGGACGCTCCAGGTCTTATC

4.3 Results

4.3.1 Lichenysin production differs between *B. licheniformis* MW3 wt and Δ*sigB* mutant

The total lichenysin yield of wt *B. licheniformis* was higher than the isogenic Δ*sigB* mutant after growth on LB agar. Based on analyses of three independent biological replicates, wt and Δ*sigB* mutant cells grown on LB agar produced a total of 3.3 ± 0.2 and 2.5 ± 0.5 µg lichenysin /g of wet biomass, respectively (Figure 1). The molar masses of the various isoforms and their respective concentrations produced by the wt and Δ*sigB* mutant are listed in Table 3. The acyl chain lengths of the detected isoforms varied from C12 to C17, in line with previously reported results (Yeak et al., 2022).

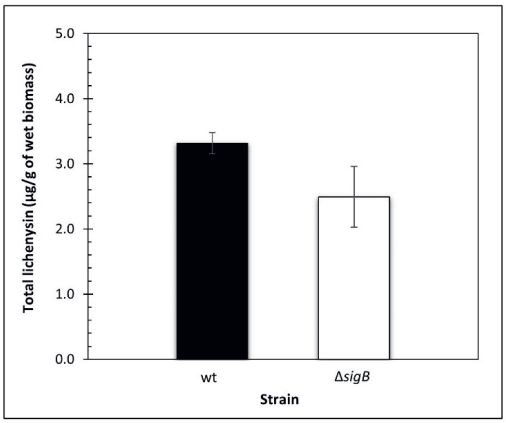


Figure 1- Total lichenysin yield from *Bacillus licheniformis* MW3 wt and Δ*sigB* mutant. The production of lichenysin was quantified in three independent biological replicates using Reversed-RP-HPLC-MS. The total lichenysin yield consisted of C12 to C17 isoforms. Total production in wt cells was 3.3 ± 0.2 µg lichenysin /g of wet biomass and in Δ*sigB* cells was 2.5 ± 0.5 µg lichenysin /g of wet biomass.

Table 3: Lichenysin isoforms, molar masses, and mean concentrations of three independent biological replicates

Isoform	Molecular Formula	$[M + H]^+$ m/z	Concentration (µg/g wet biomass) in wt	Concentration (µg/g wet biomass) in Δ*sigB*
C12-Lichenysin	$C_{50}H_{88}N_8O_{12}$	993.6595	0.013	0.010
C13-Lichenysin	$C_{51}H_{90}N_8O_{12}$	1007.6751	0.255	0.188
C14-Lichenysin	$C_{52}H_{92}N_8O_{12}$	1021.6908	0.640	0.482
C15-Lichenysin	$C_{53}H_{94}N_8O_{12}$	1035.7064	2.232	1.683
C16-Lichenysin	$C_{54}H_{96}N_8O_{12}$	1049.7221	0.138	0.102
C17-Lichenysin	$C_{55}H_{98}N_8O_{12}$	1063.7377	0.039	0.028
Total concentration			3.317	2.493

4.3.2 The *lch (lic)* operon lacks a SigB promoter binding motif and expression is not influenced by SigB activation

No SigB promoter binding motif (PBM) was identified preceding the lichenysin synthetase operon (*lch/lic*), indicating that SigB does not directly regulate lichenysin production. The search of whole-genome sequences of *B. licheniformis* type strain (DSM13) using the Prodoric2 bacterial gene regulation database resulted in 46 potential promoter sequences with a SigB PBM (Supplementary Table S1), but none of these were found upstream of the lichenysin operon. Therefore, an additional manual check was performed by screening 500 nucleotides upstream of the ATG start codon preceding the *lch/lic* operon. Two potential hit sequences were shortlisted, but neither displayed a clear SigB PBM. One sequence, namely, GTTTCC-N_{14}-TTGGAAAT was located 104 bp upstream of the ATG start codon, but in the anti-sense direction, eliminating it as a SigB PBM involved in the regulation of the *lch/lic* operon. The second sequence GTTTTC-N_{14}-GTCAATTT was found at 402 bp upstream of the ATG in the sense direction of the DNA but showed limited similarity to the SigB consensus sequence in *B. licheniformis,* which is GTTTAA-N_{14}-GGGTAT. Despite the presence of the GTTT in the –35 region, the well-known guanine bases at the -10 region were not conserved (Figure 2A).

While direct binding of SigB to this predicted motif is unlikely, the expression of the lichenysin gene cluster upon SigB activation was investigated. Cultures of wt and Δ*sigB* strains of *B. licheniformis* were exposed to 4% (v/v) ethanol stress, which is known to induce the SigB-mediated GSR (Voigt et al., 2013). For wt cells at the exponential phase, ethanol stress resulted in strong induction of the three SigB-dependent genes, i.e., *sigB* itself, *rsbW,* and *yfkM*. Expression of *sigB* was increased 81-fold while *rsbW* (encoding the anti-SigB protein RsbW) and *yfkM* (encoding the general stress protein YfkM) were induced 99-fold and 51-fold, respectively. However, *lchAA* (also annotated as *licA*; encoding the lichenysin synthetase A LchAA/LicA protein) was not differentially expressed ($2^{-\,ddCT}$ fold change value =1). Expression of *lchTE* (also annotated as *licTE*; encoding the LchTE/LicTE thioesterase protein) was mildly increased ~ 2- fold, which was significantly lower than the three SigB-dependent genes. These results suggest that SigB does not directly regulate the expression of the *lch (lic)* operon (Figure 2B). Ethanol exposure resulted in a ~ 2-fold increase in *rsbW* and *yfkM* expression in Δ*sigB* cells compared to non-exposed Δ*sigB* cells, while *lchAA* (*licA*) and *lchTE* (*licTE*) were not differentially expressed (2^{-ddCT} fold change value =1) (Figure 2B).

The effect of SigB induction by 4% (v/v) ethanol stress on the expression of *sigB, rsbW, yfkM*, *lchAA,* and *lchTE* was also examined when cells enter the early stationary phase of growth (see insert in Figure 2C). For wt cells in the stationary phase, ethanol strongly induced *sigB, rsbW,* and *yfkM* (241, 118, or 56 fold, respectively). The induction of these genes was not observed in the Δ*sigB* mutant. Although *lchAA* (*licA*) and *lchTE* (*licTE*) were mildly induced ~ 2 fold in the wt cultures in the stationary phase, no apparent dependence on SigB was observed (Figure

2C). Overall, these results showed that the lichenysin gene cluster is not regulated by SigB at the gene transcriptional level, neither in the exponential phase nor the stationary phase.

4.3.3 Lichenysin induces a mild SigB general stress response in *B. licheniformis*

Surfactants are known to affect biological membranes. Therefore, we determined whether exposure of cells to lichenysin mediates a SigB GSR.

Figure 3 shows the changes in expression of the *sigB, rsbW,* and *yfkM* genes in wt and Δ*sigB* mutants after exposure to 10, 20, and 30 µM of lichenysin. In wt cells, *sigB* and *rsbW* gene expression was induced ~ 2-fold upon lichenysin exposure, but no changes were measured for the *yfkM* gene (2^{-ddCT} fold change value =1). The mild induction of both *sigB* and *rsbW* genes in the tested conditions contrasts with the 80 to 100-fold increase of both genes in wt cells after exposure to 4% (v/v) ethanol (Figure 2). On the contrary, neither the *rsbW* nor the *yfkM* gene was differentially expressed (2^{-ddCT} fold change value =1) in the Δ*sigB* mutant after lichenysin exposure (Figure 3). These results show that exposure to 10, 20, and 30 µM of lichenysin results in the induction of SigB GSR genes, reflecting mild activation of adaptive stress response in *B. licheniformis.*

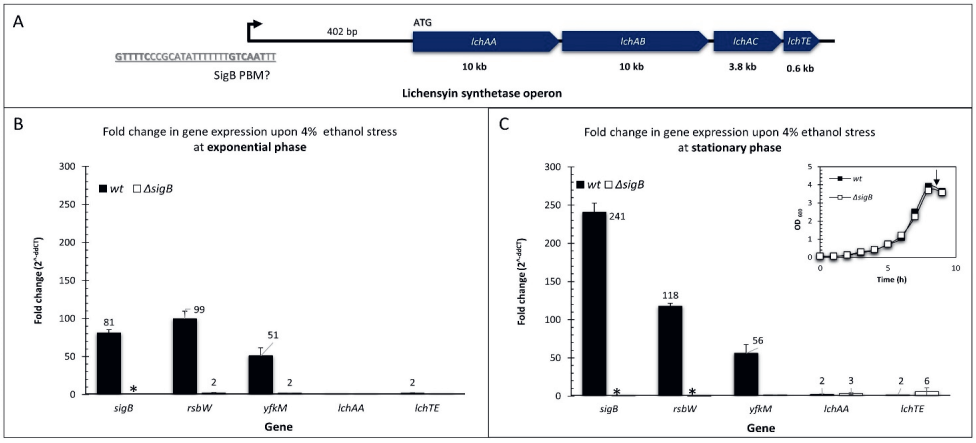


Figure 2- The lichenysin synthetase operon and ethanol-induced changes in gene expressions. **2A-** The lichenysin synthetase operon, also known as the *lch* or *lic* operon in *B. licheniformis*. **2B-** Fold changes (2^{-ddCT}) in the expression of three SigB-dependent genes (*sigB* itself, *rsbW, yfkM*) and two lichenysin operon genes (*lchAA, lchTE*) in *B. licheniformis* MW3 wt (**black bar**) and Δ*sigB* (**white bar**) cells upon exposure to 4% (v/v) ethanol at exponential phase (OD_{600nm} of ~ 0.35); **2C insert-** shows the growth of *B. licheniformis* MW3 wt (**black square**) and Δ*sigB* (**white square**). The arrow indicates the time point at which stationary phase cells (OD_{600nm} of ~ 4) were collected. **2C-**Fold changes (2^{-ddCT}) in the expression of *sigB, rsbW, yfkM, lchAA*, and *lchTE* in *B. licheniformis* MW3 wt (**black bar**) and Δ*sigB* (**white bar**) cells upon exposure to 4% (v/v) ethanol at stationary phase. Fold changes ($2^{\Lambda-ddCT}$) indicate the change of transcript level for each tested gene in wt and Δ*sigB* mutant cells upon exposure to 4% (v/v) ethanol or lichenysin compared to the control condition (no treatment) and were calculated with the Livak method (Livak and Schmittgen, 2001) (section 4.2.5). Fold change = 1 indicates no differential expression; fold change > 1 = induction in gene expression; N= 3.

4.3.4 Lichenysin and surfactin induce a mild SigB general stress response in *B. subtilis*

As lichenysin appeared to induce SigB GSR in *B. licheniformis,* we next tested whether exposure to lichenysin or surfactin induces SigB GSR in *B. subtilis* using the wt and the Δ*sigB* P_{rsbV}-*lacZ* reporter strains.

The effect of a range of surfactin concentrations (from 5 µM to 100 µM) on induction of P_{rsbV} activity in *B. subtilis* was tested, showing a slight average induction at 5 µM and higher compared to the unexposed control (Supplementary Figure S1). A concentration of 30 µM of surfactin and lichenysin was selected as the concentration to expose the *B. subtilis* reporter strains.

Figure 4A shows that the P_{rsbV} LacZ activity in wt cells under control condition (no treatment) was ~10 MU/mg protein, and a significant increase to ~ 70 MU/mg protein was measured after exposure to 4% (v/v) ethanol. When wt cells were treated with 30 µM of lichenysin, the P_{rsbV} LacZ activity was ~ 20 MU/mg protein, which was lower than cells exposed to ethanol stress but ~ 2-fold higher than the control cells without any treatment. This increment was subtle, but it can be concluded that this was SigB-dependent as no P_{rsbV} LacZ activity was detected in the *B. subtilis* Δ*sigB* mutant (Figure 4A). This mild SigB-dependent increased gene expression in *B. subtilis* caused by lichenysin exposure of cells is in line with the results in *B. licheniformis* (Figure 3).

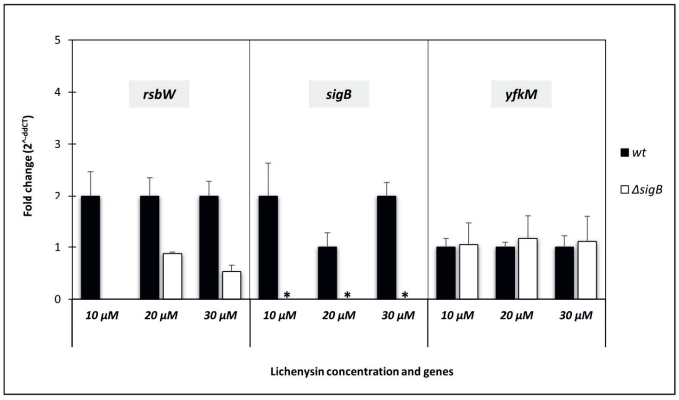


Figure 3 - Changes in expression of SigB-dependent genes in *Bacillus licheniformis* MW3 wt and Δ*sigB* mutant upon exposure to lichenysin. Fold changes (2^{-ddCT}) in the expression of three SigB-dependent genes (*sigB* itself, *rsbW, yfkM*) upon exposure to 10, 20, and 30 µM of lichenysin in *B. licheniformis* MW3 wt (**black bar**) and Δ*sigB* (**white bar**) cells, respectively. Fold changes ($2^{\wedge\text{-}ddCT}$) indicate the change of transcript level for each tested gene in wt and Δ*sigB* mutant cells upon exposure to lichenysin compared to the control condition (no treatment) and were calculated with the Livak method (Livak and Schmittgen, 2001) (section 4.2.5). Fold change = 1 indicates no differential expression; fold change > 1 = induction in gene expression; N= 3. Asterisk indicates the absence of gene activity.

Surfactin also induced a mild SigB GSR in *B. subtilis*: the P_{rsbV} LacZ activity of wt cells after exposure to 30 µM surfactin was ~100 MU/mg protein, which was twice the level seen without treatment (~50 MU/mg protein) (Figure 4B). Exposure to 4% (v/v) ethanol resulted in even higher levels of P_{rsbV} LacZ activity, which was ~ 250 MU/mg protein (Figure 4B). Similar to the lichenysin-treated cells, the P_{rsbV} LacZ activity recorded for wt cells after surfactin treatment was ~ 2-fold higher than the P_{rsbV} LacZ activity recorded for wt cells without treatment but ~ 5-fold lower than wt cells treated with ethanol, indicating that surfactin also induced SigB GSR mildly. For the Δ*sigB* cells that were tested under the same conditions, no P_{rsbV} LacZ activity was detected, indicating that the changes in P_{rsbV} LacZ activities measured after exposure to surfactin were SigB-dependent (Figure 4B).

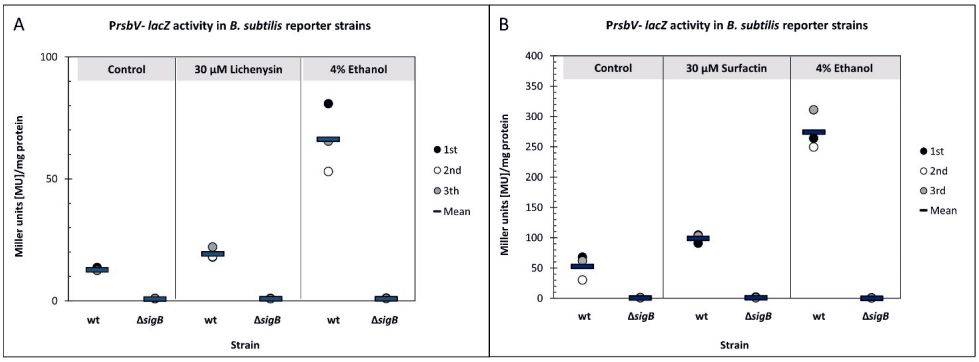


Figure 4- Assessment of SigB induction in *Bacillus subtilis* by lichenysin and surfactin using P_{rsbV} LacZ-reporter strains. **4A-** P_{rsbV}-*lacZ* activities in response to 30 µM of lichenysin (Lipofabrik) and 4% (v/v) of ethanol in *B. subtilis* wt and Δ*sigB* mutant. **4B-** P_{rsbV}-*lacZ* promoter activities in response to 30 µM of surfactin (Merck) and 4% (v/v) of ethanol in *B. subtilis* wt and Δ*sigB* mutant. The results of three independent biological replicates are shown (N=3). The **circles** depict values from individual experiments, and the **bar** shows the mean value of the replicates.

4.3.5 Pre-exposure to lichenysin and surfactin shows a modest protective effect for *B. licheniformis* when subjected to 12% (v/v) ethanol

It is conceivable that the mild induction of selected SigB GSR genes upon lichenysin and surfactin exposure activates a stress defense. Therefore, we determined the survival of *B. licheniformis* cells upon exposure to 12% (v/v) ethanol (lethal stress) without and with pre-exposure to lichenysin and surfactin (10, 20, and 30 µM).

As the cell survival of *B. licheniformis* upon exposure to 10-30 µM of lichenysin and surfactin was similar over this concentration range (data not shown), biological triplicates were performed using only the lowest concentration (10 µM), and the results are presented in Figure 5. Without pre-exposure to lichenysin, surfactin, and 4% (v/v) ethanol, wt and Δ*sigB* cells died quickly upon exposure to 12% (v/v) ethanol (Figures 5A, C, and E). In the case of pre-exposure

to 10 µM of lichenysin or surfactin, wt cells were slightly more robust than the Δ*sigB* mutant when exposed to 12% (v/v) ethanol, showing ~ 0.5 and 1- 2 log CFU/ml higher CFUs in the first 120 min, respectively (Figure 5B and D). Prolonged exposure up to 3 h resulted in similar viable counts for wt and Δ*sigB* mutant cells. Pre-exposure of wt and Δ*sigB* mutant cells to 4% (v/v) of ethanol before being subjected to 12% (v/v) ethanol, resulted in ~ 2 logs CFU/ml higher viable counts for the wt than for the Δ*sigB* cells (Figure 5F). These results provided evidence for 4% (v/v) ethanol-induced SigB dependent adaptive GSR in *B. licheniformis* that protects against lethal 12 % (v/v) ethanol stress, while putative mild surfactant-induced SigB GSR only showed a modest protective effect against lethal ethanol stress for the first 120 min in *B. licheniformis*.

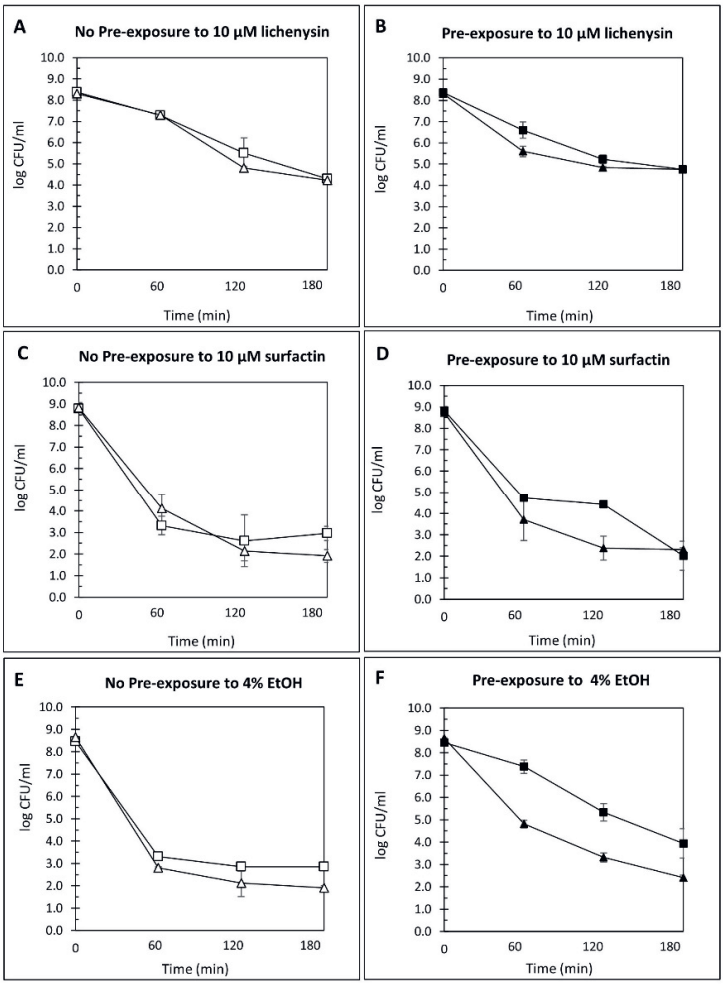


Figure 5- Viable counts of *Bacillus licheniformis* strain MW3 upon exposure to 12% (v/v) ethanol with and without pre-exposure to 10 µM lichenysin, 10 µM surfactin, or 4% (v/v) ethanol. Viable counts of wt and Δ*sigB* cultures without pre-exposure to 10 µM lichenysin (**5A**); 10 µM surfactin (**5C**); 4% (v/v) ethanol (**5E**); Viable counts of wt and Δ*sigB*

cultures with pre-exposure to 10 µM lichenysin (**5B**); 10 µM surfactin (**5D**); 4% (v/v) ethanol (**5F**); **Square**- wt; **triangle**- Δ*sigB* mutant; **open symbol** – without pre-exposure; **closed symbol** – with pre-exposure. N = 3

4.4 Discussion

This study demonstrated that the deletion of SigB affected lichenysin production negatively in *B. licheniformis*, but the expression of lichenysin-producing enzymes is not under the direct control of SigB. The exposure of *B. licheniformis* and its close relative *B. subtilis* to lichenysin and surfactin led to mild induction of SigB-dependent genes that mediate the adaptive GSR, and exposure to these compounds provided a modest protective effect against lethal ethanol stress (12% v/v) for the first 120 min *B. licheniformis*.

In this study, the *B. licheniformis* MW3 wt (with the deletion of two restriction endonucleases *hsDR1* and *hsdR2*) was used instead of its isogenic parental strain DSM13, which has naturally poor genetic competence (Hoffmann et al., 2010; Voigt et al., 2013). *B. licheniformis* MW3 produces ~ 3.3 µg of lichenysin per g of wet biomass under the optimal condition (LB agar at 37°C) (Figure 1). This amount is ~ 8 fold lower than DSM13, which was previously shown to produce ~ 25 µg of lichenysin per g of wet biomass on LB agar at 37°C (Yeak et al., 2022). The deletion of *sigB* in the MW3 wt strain further reduced the potential of MW3 to synthesize lichenysin (Figure 1), suggesting that SigB plays a role in lichenysin production.

However, SigB does not regulate lichenysin production directly at the gene level as 1) no SigB PBM was found preceding the *lch* (*lic)* gene cluster and 2) activation of SigB at the exponential or stationary phase did not influence the expression of *lchAA* (*licA*) or *lchTE* (*licTE*) gene. These findings are in line with the role of SigB that was reported for surfactin production by *B. subtilis* (Bartolini et al., 2019). In *B. subtilis,* SigB is believed to indirectly increase the production of surfactin (for which the enzymes encoded by *srfA* are required) via an unknown pathway (Bartolini et al. 2019) because 1) no SigB PBM was detected preceding the *srfA* operon, and 2) the two well-known *srfAA* regulators (ComA two-component system and the RapC phosphatase) (Core and Perego, 2003) are not SigB dependent (Price et al., 2001; Hecker et al., 2007). Nevertheless, PhoP – the regulator of phosphate metabolism (Salzberg et al., 2015), and Spx - the regulator of disulfide stress (Nakano et al., 2003), which are involved in *srfAA* regulation (Zhu and Stülke, 2018) are SigB-dependent. This implies that SigB may indirectly affect *srfAA* expression via PhoP or Spx. Moreover, the *srfA* operon is required for competent cell development in *B. subtilis* because the *comS* gene (encodes the regulatory component in the competence signal transduction pathway) resides within the *srfA* operon. Thus, all cells that produce surfactin also produce ComS, facilitating a partial subpopulation of cells to develop competence (Rahman et al., 2021). This suggests that SigB may be further involved in the indirect control of other regulatory pathways that are connected, such as competence. In *B. licheniformis,* the expression of the *lch/lic* operon is regulated by ComA (regulator of

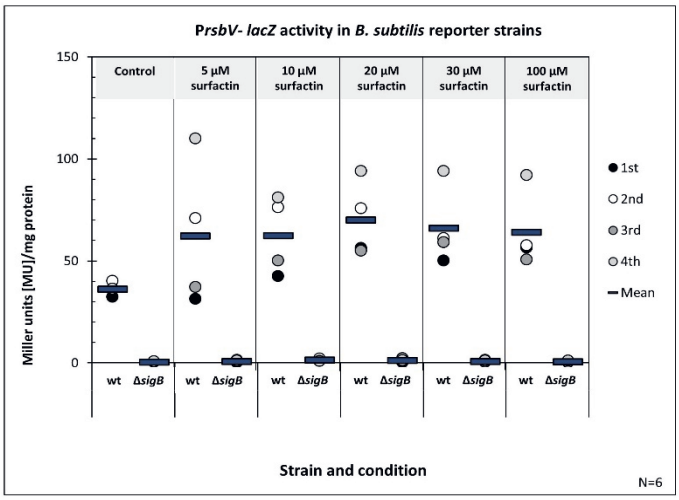
competence) as the *srfA* operon in *B. subtilis* (Yakimov and Golyshin, 1997). However, no competence-related genes reside within the *B. licheniformis lch/lic* operon. The *B. licheniformis lch/lic* operon is also regulated by CodY (regulates response to limitation of branched-chain amino acid) (Zhu et al., 2017), which in turn is SigB-dependent (Voigt et al., 2013). In Δ*codY* cells, the transcription of *lchAA* (*licA*) and its regulators (ComA, DegQ, and DegU) was upregulated to generate more lichenysin precursor amino acids (Gln, Ile, Leu, and Val), implying that SigB may have an indirect effect on the synthesis of lichenysin precursors, thereby affecting the assembly of lichenysin.

Indirect regulation of gene expression by SigB via regulation of other transcription regulators is not uncommon, and the regulatory pathways of various adaptive processes are known to be highly intertwined (Hamoen et al., 2003). While a role of SigB has been established in sporulation, competence, biofilm formation, and cellular motility in *B. subtilis,* such roles are not known in *B. licheniformis*. In this study, we observed that the overnight cultures of *B. licheniformis* MW3 wt and Δ*sigB* mutant were both able to form pellicle biofilms, initiate sporulation, and showed similar motility on low percentage agar plates (Supplementary Figure S2), additionally pointing to a limited role for SigB in *B. licheniformis* adaptive processes.

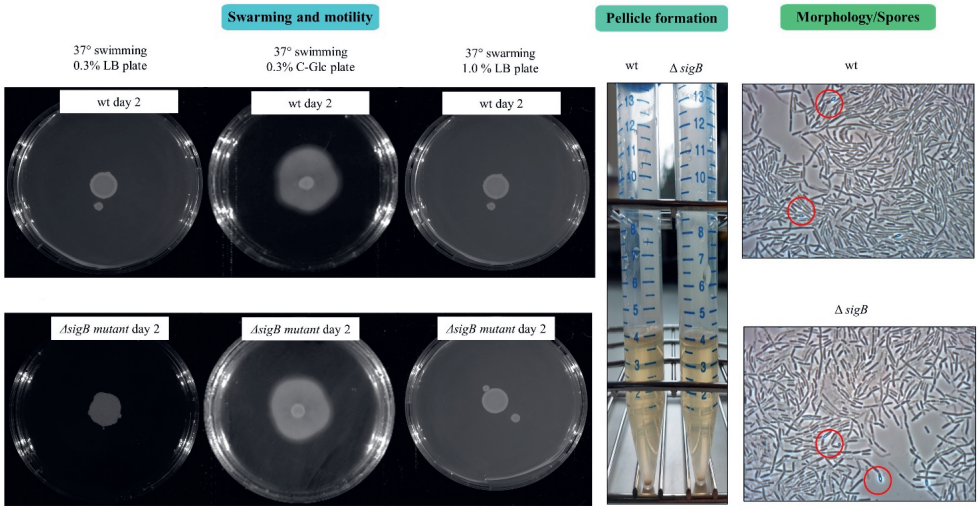
Exposure of *B. licheniformis* and *B. subtilis* to lichenysin and surfactin resulted in an approximate 2-fold induction of SigB-dependent genes, and the presumed GSR only had a modest effect on survival following exposure to 12% (v/v) ethanol. The exact environmental conditions and efficacy of surfactants in triggering SigB GSR remain to be elucidated. However, it is noteworthy that the production of surfactin triggers the differentiation of cells into competence development, matrix production, and sporulation, resulting in different subpopulations (Rahman et al., 2021). Lichenysin and surfactin are moreover good ion chelators that form complexes with ions (Grangemard et al., 2001; Zamboulis et al., 2011), and may scavenge beneficial ions as micronutrients to the proximity of the cells, conceivably supported by enhanced swarming motility to promote colonization of new habitats (Raaijmakers et al., 2010).

In conclusion, this study showed a modest impact of *B. licheniformis* SigB on lichenysin production and a minor effect of surfactant-induced SigB GSR on lethal stress resistance in the conditions tested. Further studies are required to confirm additional roles of lichenysin in *B. licheniformis* ecology and physiology, and its impact on its competitive fitness and environmental transmission.

4.5 Supplementary Materials



Supplementary Figure S1: A- Assessment of SigB induction by 5- 100 µM of surfactin in *Bacillus subtilis* wt and Δ*sigB* mutant P_{rsbV}-*lacZ*-reporter strains.



Supplementary Figure S2: Motility, pellicle formation, and spore formation of *B. licheniformis* MW3 wild type and Δ*sigB* mutant cells.

Supplementary Table S1: Prediction of operons with SigB promoter binding motif (PBM) in *Bacillus licheniformis* DSM13 using the Prodoric 2 database

SigB PBM	strand	positive hit sequence	locus_tag	gene
MX000072	-	ATCGTTTTTCTGATTTTTTCCCCCGGGAAATCG		
MX000072	+	TTTGTTTGATCATATATTGATACCGGGTAATGT	BL01687	*ycdF*
MX000072	-	GTCGTTTCCAATAAAACATTATATTGGAAATAA		
MX000072	+	CAGGTTTTGCAAAAAACGTTTGAAAGGCAAATG		
MX000072	+	TAAGTTTGACATTGTTAGGCACATCGGAATAAA		
MX000072	+	CTAGTTTAGTATAAGATGGATCTAAGGTAAGAG		
MX000072	+	GGAGTTTTTTCATTTTTATCAAAAGGGAAAGGA	BL01489	*BL01489*
MX000072	-	GATGTTTTTCACGGTTGATTGATTGGGAATAGT		
MX000072	-	AAGGTTTATATTTTCCCTTTTCAAGGGAAATCA		
MX000072	+	GACGTTTTACCTTGTCCGGTTTGGTGGAAAAAG		
MX000072	+	AGTGTTTTTTCTTTCTGAAAAGTGGGGAAAGAA	BL03081	*yfkJ*
MX000072	+	GGCGTTTTTTAGCGGAAACAAAAGGGGGATACA	BL03092	*yfjM*
MX000072	-	TCTGTTTGAAAGTGGCCTTTCGCCGGGAATGAT		
MX000072	-	TTCGTTTTCAAAAAAAGTTTTTAAGGGTAGATA		
MX000072	-	TTTGTTTTATTAAGAATGATTAAGGGGGAAAAC	BL01306	*yitK*
MX000072	+	GACGTTTTCAAAATATTGTTTTAAGGGTAAACC	BL05104	*yitT*
MX000072	-	GTCGTTTTTAACGTCTTCCCGATAGGGAAAATA		
MX000072	+	CGAGTTTTCGATAGTCGGGGGACACGGAAATAT		
MX000072	+	GGTGTTTTTCAAAGAACAGCAGACGGAAAAATG	BL00557	*moeB*
MX000072	-	TCTGTTTTTCGGATTTCGTTAAACGGGAAAAAC	BL01621	*ykoA*
MX000072	+	TTTGTTTTAGAAAAGATGTTTTGGAGGAATGGT	BL02267	*ileS*
MX000072	+	TAGGTTTTGCCCAAGTTCGTTAAAGGGAAATGT		
MX000072	+	TATGTTTTATGAAGGTGCAAGTCCGGGTAATAG	BL00155	*BL00155*
MX000072	-	GGGGTTTTTTTTTATGTCTAAAAAAGGGGAAAGC	BL05172	*BL05172*
MX000072	+	GGCGTTTTGGAAATGACAAGATCCTGGTAAGCG		
MX000072	+	CAGGTTTACGGAACGGGCAGCCCGGGGAAAAAT	BL01511	*bmrU*
MX000072	+	TGTGTTTTCAAGAATTGTTTTGTGCGGAAACAG		
MX000072	-	TGGGTTTTGGTTTAATTTTAAAAGGGGAAATTT		
MX000072	+	AGAGTTTTGTTTCACCTGATTGTGGGGTAATAG	BL00066	*opuD*
MX000072	-	GACGTTTTCCTCAATGGTTTTCCAGGGAAATAA		
MX000072	+	TTCGTTTTATTATTGAATTTAAAAGGGAAAACG		
MX000072	+	CATGTTTTTCGATCGCTTTCGCATGGGAATGCG		
MX000072	-	GCTGTTTCTTTTTTATAGTATAATGGGAAAAAA	BL05321	*yueG*
MX000072	+	AACGTTTTTGAAGCGCCTTGACCTGGGAACCGG		
MX000072	+	AGCGTTTTAGAGTTGAATCCTCCAAGGTATTGG		
MX000072	+	TGAGTTTGTAATTGTAATTTTTCGGGGTATATC	BL02431	*gtaB*
MX000072	-	GCTGTTTTGCATATGGGGATTCTCTGGAAATTC		
MX000072	+	CGGGTTTGTCGTGACGCCGCCAGCGGGAAATGA		
MX000072	+	CTCGTTTTTGATTTAGCCGAAAAGGGAAATCAT	BL02454	*yqjF*
MX000072	-	ACTGTTTTTTAGCTCATCAAAGAGGGGAAGGGA	BL04018	*feuC*
MX000072	-	TCTGTTTTAAAACGCTTTTTTCATGGGAAAACG	BL03895	*ung*
MX000072	-	TTAGTTTAAAACCGGTTGTCAGCAGGGAAAAGG	BL03870	*galE*
MX000072	+	TTGGTTTTTTTAGTCAGTTATTTAGGGAATAGG	BL05363	*BL05363*
MX000072	-	AATGTTTTTAAAAAATGATGAAAAAGGAAAAAA		
MX000072	+	TCGGTTTTATAGGAAGGCACAGGGGGGCAAGCG		
MX000072	-	GACGTTTTAGACCGTTTTGGAAAGGGGAAGTCA		

4.6 References

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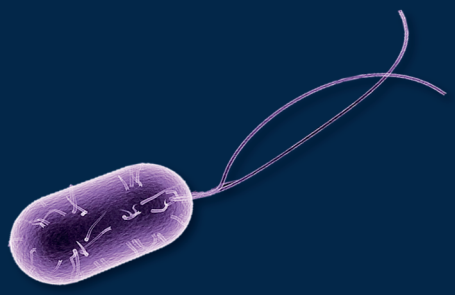
“ Unknown toxicity is not non-toxic,
the toxin toxicity is cumulative ”

CHAPTER 5



Lichenysin production by *Bacillus licheniformis* food isolates and toxicity to human cells

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FIELD CONTRIBUTION

Bacterial foodborne outbreaks related to *Bacillus* species in foods are commonly due to *Bacillus cereus*, but cases of intoxications caused by *Bacillus licheniformis* have been reported due to production of lichenysin. Information is lacking on levels produced in foods and on the stage of growth during which this surfactant is synthesized. This is needed for risk assessments. We investigated the production of lichenysin by different *Bacillus licheniformis* food isolates. The amount of lichenysin produced strongly depended on the strain and on the cultivation medium and incubation temperature. The organism did not produce detectable amounts of lichenysin up to cell densities of 5 $\log_{10}$/ml in milk and LB. At higher cell densities, appreciable amounts of lichenysin were measured. We also demonstrated that lichenysin is toxic to human epithelial cells at levels that can be produced in foods. This study stresses that control of outgrowth of *B. licheniformis* in foods is important to prevent foodborne illness due to lichenysin production. In addition, active introduction of specific *B. licheniformis* strains into the food chain, either indirectly as crop bioprotectant or feed additive, or directly as food additives (probiotic), requires thorough assessment of surfactant production capacity

Abstract

Bacillus licheniformis can cause foodborne intoxication due to the production of the surfactant lichenysin. The aim of this study was to measure the production of lichenysin by food isolates of *B. licheniformis* in LB medium and skimmed milk and its cytotoxicity for intestinal cells. Out of 11 *B. licheniformis* isolates tested, most showed robust growth in high salt (1M NaCl), 4% ethanol, at 37°C or 55°C, and aerobic and anaerobic conditions. All strains produced lichenysin (in varying amounts), but not all strains were hemolytic. Production of this stable compound by selected strains (high producers B4094 and B4123, and type strain DSM13^{T}) was subsequently determined using LB medium and milk, at 37°C and 55°C. Lichenysin production in LB broth and milk was not detected at cell densities < 5 $\log_{10}$ CFU/ml. The highest concentrations were found in the stationary phase of growth. Total production of lichenysin was 4-20 times lower in milk than in LB broth (maximum 36 µg/ml), and ~10 times lower in the biomass obtained from milk agar than LB agar. Under all conditions tested, strain B4094 consistently yielded the highest amounts. Besides strain variation and medium composition, temperature also had an effect on lichenysin production, with two-fold lower amounts of lichenysin produced at 55°C than at 37°C. All three strains produced lichenysin A with varying acyl chain lengths (C11 to C18). The relative abundance of the C14 variant was highest in milk and the C15 variant highest in LB. The concentration of lichenysin needed to reduce cell viability by 50% (IC_{50}) was 16.6 µg/ml for Caco-2 human intestinal epithelial cells and 16.8 µg/ml for pig ileum organoids. Taken together, the presence of low levels (< 5 $\log_{10}$ CFU/ml) of *B. licheniformis* in foods is unlikely to pose a foodborne hazard related to lichenysin production. However, depending on the strain present, the composition, and storage condition of the food, a risk of foodborne intoxication may arise if growth to high levels is supported and such product is ingested.

5.1 Introduction

Bacillus licheniformis is a facultative anaerobic endospore-producing bacterium that is ubiquitously found in the environment, plant material, and soil. The organism belongs to the *Bacillus subtilis* group and is known for its use in the production of enzymes or antibiotics (e.g., bacitracin), while spore preparations of selected strains are used as crop bioprotectants and feed additives (Wang et al., 2016; Elshaghabee et al., 2017; Radhakrishnan et al., 2017).

B. licheniformis is not considered to be a pathogen and has not been shown to be able to invade the outer barriers of the body without previous lesions (e.g., mucosal tissue or skin), but sporadic cases of human infection related to *B. licheniformis* have been reported (Sugar and McCloskey, 1977; Park et al., 2006; Haydushka et al., 2012; Padhi et al., 2012). In cows, the organism has been linked to rare cases of abortion (Johnson et al., 1994; Agerholm et al., 1995), and in a study by Nieminen et al. (2007), high numbers were found in some samples of mastitic milk.

While generally considered safe, *B. licheniformis* has occasionally been reported as a causative agent of foodborne intoxication after the consumption of cooked meat, vegetables, milk powders, and dairy products (Salkinoja-Salonen et al., 1999; Pavić et al., 2005; Logan, 2012; Rønning et al., 2015). Reported cases were characterized by a relatively short incubation time (2–14 h) and high infective dose (> 5 $\log_{10}$ CFU/g) followed by mild gastrointestinal symptoms (e.g., nausea, stomach cramps, vomiting, abdominal pain, and sometimes diarrhea) lasting for 6–24 h (Kramer and Gilbert, 1989; Salkinoja-Salonen et al., 1999). The agent causing foodborne illness was identified as the surfactant lichenysin (Salkinoja-Salonen et al., 1999; Mikkola et al., 2003), with one fatal case linked to the consumption of infant formula containing lichenysin (Salkinoja-Salonen et al., 1999).

Spores of *B. licheniformis* may be present in food ingredients and survive commonly applied heating processes such as pasteurization. The heat resistance of spores can vary significantly between isolates: some carry genetic elements that give rise to the production of high-level heat-resistant spores (Berendsen et al., 2016a). Surviving spores can germinate, return to their vegetative state, and then grow in the finished food, depending on the composition of the product and its storage condition. The organism tolerates a relatively low water activity (A_w) of ~ 0.9 and a broad temperature range (~ 10°C - 58°C) for growth (Warth, 1978; Baranyi and Tamplin, 2004). This includes food processing conditions at relatively high temperatures, for instance, in evaporators that are operated at temperatures around 55°C. *B. licheniformis* can be the predominant spore former in milk powders made from pasteurized milk that is evaporated and spray-dried (Miller et al., 2015; Eijlander et al., 2019), even though the raw milk used to make powder contains a broad range of spore-forming species (Coorevits et al., 2008; Miller et al., 2015). High temperatures in evaporators (operated under vacuum) in

combination with a low Aw in the concentrated product stream put selective pressures that favor outgrowth and subsequent spore formation by *B. licheniformis* (Burgess et al., 2010; Eijlander et al., 2019; Delaunay et al., 2021). Besides dairy products, the organism is also frequently found in spices, dry herbs (e.g., pepper and turmeric) (Mathot et al., 2021), and flours (Rosenkvist and Hansen, 1995; Iurlina et al., 2006). As a common contaminant in food ingredients, the processing conditions will determine whether viable spores are present in the finished product (Postollec et al., 2012). If the product and storage conditions subsequently support the growth of *B. licheniformis*, a possibility of lichenysin production in the food exists (Warth, 1978; Postollec et al., 2012).

Lichenysin is a secondary metabolite. It is synthesized by the proteins encoded by the *lchAA-AB-AC-TE* gene cluster, also annotated as *licA-TE* (Konz et al., 1999). The biosynthesis of the peptide fraction of lichenysin is catalyzed by nonribosomal peptide synthetases (NRPS). The presence of *lch* genes in *B. licheniformis* appears to be very common: the *lchAA* gene was detected by PCR in all 53 isolates studied by Madslien et al. (2013). The whole-genome sequences of these isolates are not available, but lichenysin production was confirmed for all strains in their study. The presence of the *lch* cluster in a particular strain does not necessarily mean that the compound is produced. This depends on the level of transcription, translation, and enzyme (lichenysin synthetase) activity level (Harwood et al., 2018). Environmental conditions, such as the type of carbon, nitrogen, or phosphate sources present in the environment strongly influence production (Coronel-León et al., 2015b).

Lichenysin is an amphiphilic lipopeptide. Its structure is highly similar to surfactin, a well-known surfactant produced by *B. subtilis* (Arima et al., 1968) (Figure 1). Both compounds can be produced aerobically or anaerobically (Javaheri et al., 1985; Willenbacher et al., 2015; Coronel-León et al., 2016a; Hoffmann et al., 2020). They consist of a hydrophilic peptide ring of seven amino acids, connected to a hydrophobic β-hydroxy fatty acid chain (Joshi et al., 2008; Coronel-León et al., 2016a, 2016b). The β-hydroxy fatty acids can vary in length between 12–17 carbons and can be normal or branched in *iso* or *anteiso* forms (Yakimov et al., 1995). Lichenysin A contains glutamine (Gln) and isoleucine (Ile) in the 1st and 7th position of the cyclic peptide, respectively, whereas surfactin has glutamate (Glu) and leucine (Leu) in these positions (Figure 1). Other lichenysin isoforms are more similar to surfactin, e.g., lichenysin B only differs in the fatty acid chain, and lichenysin C has one amino acid difference in the peptide ring (Leu > Ile) (Nerurkar, 2010). Lichenysin D and G isoforms are almost identical to lichenysin A, with just one amino acid difference at the 7th position; lichenysin D has either Leu or Valine (Val), whereas lichenysin G has Val (Nerurkar, 2010).

The various isoforms of these surfactants share similar features, such as good solubilizing, foaming, emulsifying, and detergent activity (Rajendran and Marahiel, 1999; Zhao et al., 2017; Santos et al., 2018; Thakur et al., 2020), but they can have different physicochemical and

bioactive properties, depending on the amino acids in the peptide ring and the length and type of the fatty acid chain (Santos et al., 2018). Lichenysin A, for instance, has higher surfactant power than surfactin, likely due to the two different amino acids in the peptide ring, and it has 2 to 10-fold lower critical micelle concentrations (CMC) than surfactin (Grangemard et al., 2001; Nerurkar, 2010). The multifaceted properties of these amphiphilic lipopeptides account for their broad range of applications, such as biocontrol in the agricultural industry (Henry et al., 2011; Sachdev and Cameotra, 2013), emulsifiers in the oil industry (Yao et al., 2021), foaming agents in cosmetics (Varvaresou and Iakovou, 2015), and detergents in household cleaning products (Shaligram and Singhal, 2010).

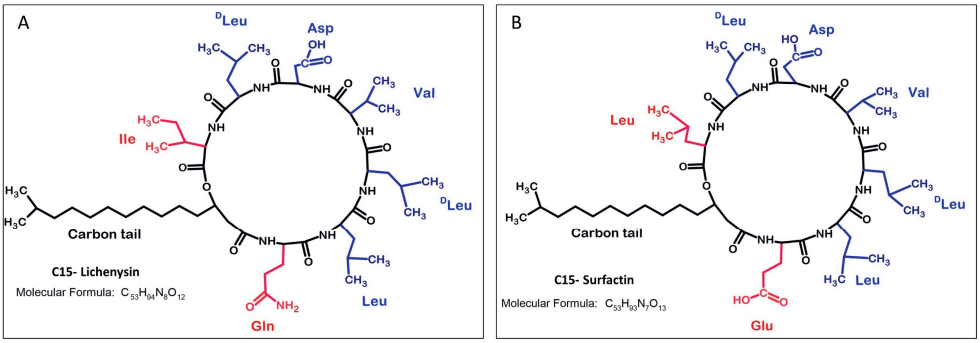


Figure 1- Chemical structures of C15-lichenysin and C15-surfactin. **(A)** C15 Lichenysin; **(B)** C15- Surfactin; The lipopeptide lichenysin A (cyclo-[Gln1-Leu2-Leu3-Val4-Asp5-Leu6-Ile7 β-OH fatty acid]) produced by *B. licheniformis* (Yakimov et al., 1995) is structurally similar to surfactin (cyclo-[Glu1-Leu2-Leu3-Val4-Asp5-Leu6-Leu7 β-OH fatty acid]) produced by *B. subtilis* (Zhao et al., 2017), which was first discovered by Arima et al. (1968). The 1st and 7th position of the peptide ring differs between surfactin and lichenysin A.

Once produced, lichenysin is highly stable under extreme conditions (Coronel-León et al., 2015a, 2015b). Its surfactant properties are unaffected over a wide pH range (pH 6 – 11), in the presence of high salt concentration (~ up to 20 % NaCl), and upon exposure to temperatures as high as 121 °C (Coronel-León et al., 2015b; Purwasena et al., 2019). Next to its action as a surfactant, lichenysin is also a good ion chelator, exerts antibiotic activity, interacts with phospholipids, and induces the formation of ion channels in artificial membranes (Grangemard et al., 2001; Coronel-León et al., 2017).

Production of lichenysin in foods may pose a food safety risk. It has hemolytic activity and toxic effects on boar spermatozoa cells at concentrations above 10 µg/ml and Vero cells at concentrations above 33 µg/ml (Madslien et al., 2013), whereas toxicity data for human-derived cell lines have not been reported. This activity is tightly linked to the capacity of lichenysin to interact with cell membranes and disrupt membrane barrier functions (Coronel-León et al., 2017). Although lichenysin A production by *B. licheniformis* in food has been connected with cases of food poisoning (Salkinoja-Salonen et al., 1999; Mikkola et al., 2003; Nieminen et al., 2007; Logan, 2012; Madslien et al., 2013; Rønning et al., 2015), data on

amounts and types of lichenysin synthesized during growth in food matrices, levels produced at different stages of growth, and dependency on culturing conditions are not available.

In this study, we determined the types and amounts of lichenysin produced by selected *B. licheniformis* food isolates in nutrient-rich Luria Bertani (LB) medium and skimmed milk at different temperatures. Additionally, we assessed the cytotoxicity of lichenysin with surfactin as a reference.

5.2 Materials and Methods

5.2.1 Bacterial strains and culture conditions

The following foodborne isolates of *B. licheniformis* from the NIZO culture collection were used: B4089, B4090, B4091, B4092, B4094, B4121, B4123, B4124, B4125, and B4164. Details related to their isolation sources and whole-genome accession numbers were reported (Berendsen et al., 2016a). Genomes were checked for the presence of the lichenysin gene cluster (*licA, licB, licC,* and *licTE*) using the Benchling biology software, available at https://benchling.com. *B. licheniformis* DSM13^{T} type strain was obtained from the German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany (DSMZ). Bacteria were routinely cultured using LB medium (Bertani, 2004) (Tritium Microbiologie BV, Eindhoven, The Netherlands) and incubated aerobically at 37°C, using shaking at 220 rpm, unless stated otherwise.

5.2.2 Screening and selection of *B. licheniformis* food isolates

5.2.2.1 β – hemolytic activity on Columbia Blood Agar

To screen strains for hemolytic activity, the Columbia blood agar method (Mulligans et al., 1984) was used as described by Madslien et al. (2013) with minor adjustments. Briefly, the optical densities (OD_{600}) of the overnight (ON) cultures in LB were measured and adjusted to OD_{600} 2.0. Ten µl of each culture was spotted on Columbia agar base supplemented with 5% sheep blood (Tritium Microbiologie BV) and incubated at 37°C. The hemolytic activity of each strain was inspected after 24 and 48 hours (h) of incubation. Strains that caused lysis of the red blood cells exhibited a transparent clearing zone around the colonies, indicating the presence of the biosurfactant lichenysin.

5.2.2.2 Oil displacement test

To assess the ability of *B. licheniformis* strains to produce biosurfactants, the oil displacement method as described by Walter et al. (2013) was used with modifications. Briefly, the oil used in this study was a mixture of linseed: wheat germ: and sesame oils in the ratio of 3: 0.5: 0.05 (Mitterzumleben, Oberthal, Germany). 1 ml of the oil mixture was evenly placed on top of 3.5 ml of distilled water on a 6-wells plate (Thermo Fischer Scientific, Bleiswijk, The Netherlands).

An aliquot of 10 µl of an ON culture of *B. licheniformis* was dropped on the oil layer, and the diameter of displaced oil was measured.

5.2.2.3 Aerobic and anaerobic growth in 96-wells plate

An ON culture of *B. licheniformis* in LB was adjusted to OD_{600} 0.05 in fresh LB medium and grown to OD_{600} ~ 0.5 at 37°C. Then 2 µl was added to either 198 µl of LB medium, or LB medium containing 1 M NaCl, or LB containing 4% ethanol (v/v) in 96 well plates (Greiner Bio-One B.V, Alphen aan den Rijn, The Netherlands). Two different plates were incubated at 37°C or 55°C in the Epoch2 microplate reader (Biotek, Hellevoetsluis, The Netherlands) in dual orbital shaking mode, and growth was followed for ~20 h by measuring the OD_{600}.

For anaerobic growth, all lab materials and cultivation media were transferred into an anaerobic chamber (model #830 -ABE/OTA) (Plas-Labs, Michigan, United States), 24 h before the experiment. The gas mixture used was 5% H_2, 5% CO_2, 90 % N_2. All strains were first cultured to an OD_{600} ~ 0.5 aerobically, then 2 µl of cultures were added to 198 µl of anaerobic LB medium containing 0.2% of potassium nitrate (KNO_3). Growth was followed by monitoring the OD_{600} for ~ 20 h using the Epoch2 microplate reader (BioTek) inside the anaerobic chamber. 2 µl of the blue fluorogenic dye Resazurin (1 g/l in distilled water) (Merck, Zwijndrecht, Netherlands) was added to an additional well that contained (198 µl) cell culture and was used as a redox indicator. The color change from blue to pink and then to colorless indicated the absence of oxygen.

5.2.3 Growth conditions and sample collection for lichenysin extraction

5.2.3.1 Growth curves in LB broth and skimmed milk

Two selected *B. licheniformis* food isolates B4123, B4094, and the type strain DSM13^T were cultured either in LB or skimmed milk (Tritium Microbiologie BV) ON at 37°C, 220 rpm. The OD_{600} of ON cultures was measured and adjusted to OD_{600} 0.05, diluted 6-fold, resulting in around 10^2 colony-forming units (CFU) ml^{-1} in 100 ml of fresh prewarmed medium. Samples were collected at time point 0 (T0) and subsequently, cultures were incubated at 37°C, with continuous shaking (220 rpm) for 72 h. During the growth of these cultures, samples were collected to determine the CFUs in time and to determine the concentrations of lichenysin in the culture.

In LB, samples were collected every 2 h for the first 24 h and for the first 28 h in skimmed milk to determine the CFUs in time. Lichenysin produced in the culture was determined at every 4 h in LB and skimmed milk, with additional selected time points in between the 4 h interval after cells reached the early stationary phase. Aliquots of 5 ml cell cultures were collected and frozen immediately at -20°C until used for lichenysin extraction.

At each mentioned time point, 1 ml of the culture was 10-fold serially diluted using sterile phosphate-buffered saline (PBS), and appropriate dilutions were plated out either on LB agar

or Plate count skimmed milk agar (PCMA) (Tritium Microbiologie BV) followed by incubation at 37°C ON and counting of the colonies. The CFU/ml at each time point for each strain was determined using the formula (Number of colonies x dilution factor) / volume spread on the plate. In addition, lichenysin concentrations in the cultures at each time point were analyzed and quantified (section 5.2.4).

To measure the OD_{600} of ON cultures in skimmed milk, the milk was cleared by mixing 1 ml of ON culture with 9 ml of citrate milk clearing solution (2%) (Tritium Microbiologie BV). For growth in skimmed milk, two additional samples were collected at time points 26 and 28 h as all three strains grew slower in skimmed milk than in LB, and cultures reached the stationary phase later than in LB. Incubation was performed at 37°C.

5.2.3.2 Estimation of lag time, lambda (λ), and specific growth rate, mu (μ)

The average $\log_{10}$ CFU/ml for the strain B4094, B4123, and DSM13 were calculated in LB and skimmed milk from two independent experiments. Their respective specific growth rate (mu/μ) and lag time (lambda/λ) for each strain/medium were estimated using the Baranyi primary growth model (Baranyi and Roberts, 1994; Buchanan et al., 1997), performed using the Github version of the Biogrowth R package. The Biogrowth R package is available at the Comprehensive R Archive Network (CRAN) (https://CRAN.R-project.org/package=biogrowth) and the Github page at https://github.com/albgarre/biogrowth_web. The Baranyi model was selected in this study because it has the lowest variability for estimating growth rates compared to Gompertz, Richards, and the logistic model (Pla et al., 2015).

5.2.3.3 Surface growth on LB and skimmed milk agar and sample collection for endpoint lichenysin quantification

To compare lichenysin produced in diverse environments (liquid broth versus solid surface), B4094, B4123, and $DSM13^T$ were cultured on LB and skimmed milk agar. ON cultures in LB or skimmed milk of the three selected strains were used to inoculate fresh LB broth or skimmed milk (15 ml) to an OD_{600} of 0.05, and cultures were grown to an OD_{600} of 0.5 by incubation at 37°C with agitation (200 rpm). Aliquots of 500 µl of the cultures grown in LB or skimmed milk were then spread evenly on the LB and skimmed milk agar plates containing 2% (w/v) agar, respectively, and allowed to dry under the laminar flow hood. All plates were sealed with parafilm and incubated at 37 °C for 10 days, after which biomass was collected from the agar surface using a sterile scraper. The collected biomass (150 mg) was weighed inside an empty soda-lime glass tube (DWK Life Sciences, Staffordshire, England), and stored at -20°C until used for lichenysin extraction.

5.2.4 Lichenysin extraction

5.2.4.1 Extraction from liquid culture samples during growth in LB and skimmed milk

Lichenysin was extracted as described by Madslien et al. (2013) and Rønning et al. (2015) with slight modifications. Briefly, a 5 ml aliquot of the collected liquid cultures (see section 5.2.4.1) was mixed with 5 mL of pure methanol (Merck) in a soda-lime glass tube (DWK Life Sciences), and vortexed for 5 min until a homogenous mixture was obtained. The mixture was heated for 30 min at 80°C in a water bath. At 10 min intervals, each tube was vortexed for 20 s. Subsequently, the mixture of methanol and cells was cooled down for 20 min on the bench, and centrifuged for 10 min at 4000 g at room temperature. The liquid phase was transferred to a new glass tube. The cell pellet was subsequently resuspended in 2 ml of methanol, and the extraction process was repeated. Per sample, the liquid phases collected from the first and second extraction procedures were mixed and evaporated under N_2 flow at 40°C in a heating block. The dry residue was resuspended in 500 µl of methanol and lichenysin was quantified in these samples.

5.2.4.2 Extraction of lichenysin from biomass collected from surface growth on LB agar and skimmed milk agar

Extraction of lichenysin from biomass was performed as described above for the liquid culture extractions. 3 ml of pure methanol was added to 150 mg of cell biomass weighted in a soda-lime glass tube (DWK Life Sciences) and vortexed for 5 min until a homogenous mixture was obtained. The second extraction was performed using 1 ml of methanol. After evaporation, the dry lichenysin extract was resuspended in 150 µl of methanol before quantification.

5.2.4.3 Quantification of lichenysin by Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC) – Electro Spray Ionization Mass Spectrometry (ESI-MS)

Qualitative and quantitative analyses of lichenysin A variants as extracted from either liquid cultures or biomass were performed using Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC) with high-resolution accurate mass spectroscopic (MS) detection. All chemicals and reagents used were HPLC grade (Merck). Surfactin (≥ 98.0% purity with >99% consisting of the C15 variant, HPLC grade) (Merck) and lichenysin ≥ 90.0% purity (Lipofabrik, Villeneuve-d'Ascq, France) were used as standards in the quantification.

The instrumentation used for RP-HPLC was the Agilent 1260 SL system (Agilent Technologies, Middelburg, The Netherlands) consisting of a degasser, binary pump, thermostatted autosampler, and column compartment. Aliquots of 5 µl of lichenysin samples were injected onto a Kinetex C8 Minibore column (2.6 µm, 100 Å, 100 x 2.1 mm, p/n: 00D-4497-AN) (Phenomenex, Utrecht, The Netherlands), and separation was performed at 50°C. The temperature of the autosampler was 10°C. Lichenysin A was eluted at a flow rate of 0.2 ml/min

with a linear gradient of 0.10 % formic acid in 40 % water + 55 % acetonitrile + 5 % tetrahydrofuran (mobile phase A) to 0.1 % formic acid in 75 % acetonitrile + 25 % tetrahydrofuran (mobile phase B) in 20 min.

The mass spectrophotometer (Agilent Technologies) comprised a high-resolution accurate mass quadrupole time of flight G6530B, equipped with a dual Electrospray Ionisation (ESI) source, operated in 2 GHz, in extended dynamic range mode. The ionization was performed in positive mode with reference mass correction. The fragmentor voltage was 150 V, gas temperature 350°C, drying gas flow 10 l min^{-1}, nebulizer pressure 25 psi, capillary voltage 3500 V, and collision energies 25 psi. A calibration curve of lichenysin was made for external calibration based on lichenysin standards in acetonitrile (14 different concentrations in the range of 10 – 40,000 µg/l) from a stock concentration of 5 mg/ml (Lipofabrik).

The LCMS was operated in a full-scan mode to scan the parent ions $(M + xH)^{x+}$ from the range of 100 m/z to 2500 m/z. The most abundant ion representing lichenysin A in the standard was [M + H]+ 1021.6908 (m/z), similar to the description by Madslien et al. (2013) and Rønning et al. (2015). Lichenysin A and its variants in the samples were identified according to the masses listed in supplementary Table S1. Quantitative data were acquired in the positive detection mode, and data analysis was done with MassHunter Qualitative and Quantitative Analysis software version B.07.00 (Agilent Technologies). The Limit of detection LOD (= Limit of quantification) was 0.005 µg/ml.

5.2.5 Cytotoxicity assay

5.2.5.1 Preparation of concentrated lichenysin extract

To perform cytotoxicity assay, two batches of concentrated lichenysin were prepared from biomass grown on LB agar plates as described in section 5.2.4.2. Instead of using 150 mg biomass, lichenysin was extracted from 6.5 g of biomass from strain B4123, and dissolved in a final volume of 300 µl methanol. A small aliquot of 1 µl was taken to determine the concentration of the concentrated lichenysin extract via RP-HPLC-ESI-MS. The remaining concentrated extract was entirely evaporated under a flow of nitrogen at 40°C in a heating block. The dry residue was resuspended in either 99% Dimethyl Sulfoxide (DMSO) or Dulbecco's Modified Eagle Medium (DMEM; Gibco, Thermo Fischer Scientific, Bleiswijk, The Netherlands) without phenol red serum or antibiotics. Surfactin (purchased from Merck) was also used in cytotoxicity assays by dissolving 10 mg in 1 ml of distilled water (10 mM) (with an adjusted pH of ~11.7 to obtain full solubility). Both lichenysin and surfactin were diluted to the desired concentration in DMEM medium without phenol red, serum and antibiotics before adding to the cells.

5.2.5.2 Propagation and culturing of human cell

Human Embryonic Kidney (HEK293) cells were obtained from Invivogen (San Diego, California, United States), and Caco-2 human intestinal epithelial cells (ATCC HTB-37) were purchased from the American Type Culture Collection (Manassas, Virginia, United States). Both cell lines were maintained in DMEM medium containing 10 % Fetal Bovine Serum (FBS) (Thermo Fischer Scientific) and 1 % penicillin/streptomycin (Merck) to attain a confluent growth. The grown HEK293 and Caco- 2 cells were seeded and suspended at a final concentration of 2 x 10^5 cells/ml (in 200 µl) in 96 wells-plates (Greiner). The 96 wells-plates were then incubated for 24 h at 37°C, under a gas atmosphere of 5 % CO_2 for cell adherence, and washed with PBS to remove unbound cells. The attached cells were exposed to different concentrations (0 – 200 µM) of surfactin (diluted with distilled water (pH 11.7) from a 10 mM stock) or lichenysin (dissolved in DMEM, prepared as described in 2.4.2) for a period of 72 h.

Generation of 3D pig ileum organoids

Pig ileum organoids were generated by the method described by Sato et al. (2011) using modifications described by van der Hee et al. (2018, 2020). Briefly, 3D organoids were dissociated into single cells by TrypLE digestion, seeded in a 96-well plate, and incubated until reaching full confluency. Subsequently, cells were exposed to a range of 0 – 200 µM of surfactin diluted with distilled water (pH ~11.7) from a 10 mM stock or lichenysin (dissolved in DMSO, prepared as described in 2.4.2) for a period of 72 h.

AlamarBlue assay

The alamarBlue (Thermo Fischer Scientific) fluorometric assay was used to examine the cytotoxicity of lichenysin and surfactin toward two human cell lineages (HEK293, Caco-2) and ileum organoids from pigs. Culturing medium DMEM with Alamar blue (i.e., a no-cells control) and cells exposed to 10 % DMSO were used to correct background fluorescence and cell death, respectively. To assess cell viability or death upon exposure to surfactin or lichenysin, cells without treatment were used as a negative control. In the cytotoxicity assessment of surfactin and lichenysin toward pig ileum cells, cells treated with 1 % DMSO instead of cells without treatment were used as a negative control because lichenysin was dissolved in DMSO instead of the DMEM medium.

The alamarBlue assay was performed as described in Burczynski et al. (2000); Hamid et al. (2004); Rampersad (2012). Briefly, the Alamar Blue solution was added to a final concentration of 10 % (v/v) in each well. The plates were incubated for 4 h and exposed to an excitation wavelength of 530 nm. The fluorescence at the emission wavelength of 590 nm was measured with a SpectraMax M5 (Molecular Devices, San Jose, CA, United States) and expressed as relative fluorescence units. The average fluorescence of the no-cells control was used to normalize all recorded fluorescence emission values.

The cell viability (%) was calculated by using the formula:

$$\left(\frac{Fs - Favg\ no\ cells\ control}{Favg\ untreated\ cells\ OR\ Favg\ cells\ treated\ with\ 1\%\ DMSO}\right) \times 100$$

F_s = the recorded fluorescence for cells in each well after the exposure to surfactin or lichenysin
F_{avg} no cells control = the average fluorescence recorded for the no-cells control;
F_{avg} untreated cells = the average fluorescence recorded for the untreated cells
F_{avg} 1% DMSO = the average fluorescence recorded for the cells treated with 1% DMSO

5.3 Results

5.3.1 Initial screening experiments for the growth of eleven *B. licheniformis* food isolates and lichenysin production in different conditions

The growth of 10 food isolates of *B. licheniformis* (B4089, B4090, B4091, B4092, B4094, B4121, B4123, B4124, B4125, B4164) and type strain DSM13^T under various conditions are presented in Table 1. The underlying growth data are presented in Supplementary Figures S1-S4. In addition, all strains were screened for hemolytic activity (Supplementary Figure S3), and the ability to produce lichenysin via RP-HPLC-MS (Table 1, Supplementary Table S2).

All 11 isolates showed comparable growth at 37°C in LB broth, except strain B4089 that grew more slowly. All isolates grew at 55°C in LB broth, a temperature close to their reported maximum growth limit of 58°C (Warth, 1978; Berendsen et al., 2016b) (Table 1, Supplementary Figure S1). Strains B4094, B4090, and B4091 showed growth at an even higher temperature, at 60°C, with B4094 growing the best with the highest cell count (see Table 1 and Supplementary Figure S2). The presence of high salt concentrations of 1M NaCl in LB broth (resulting in an A_w of ~0.927 - 0.935 (Gekas et al., 1998)) did not have a substantial impact on the final OD_{600} that was reached by the cultures of all 11 tested isolates at 37°C, while at 55°C the additional salt stress resulted in lower cell densities (Supplementary Figure S1). Growth of all isolates in the presence of 4% (v/v) ethanol in LB was observed except for B4089. Under anaerobic conditions with 0.2% KNO_3 at 37°C, three isolates (B4089, B4092, B4164) of 11 isolates failed to grow, and B4094 showed the most growth.

The hemolytic activity of all isolates on the Columbia blood agar varied substantially (Table 1 and Supplementary Figure S3). Out of the 11 tested strains, three food isolates (B4090, B4091, B4164) and the DSM13^T strain did not show hemolytic activity, while obvious clearing zones were formed on blood agar plates by the other eight strains. Strains B4089, B4092, B4094, B4124 showed weak hemolytic activity, strains B4125 and B4121 showed medium and strong hemolytic activity, respectively, and strain B4123 showed the strongest hemolytic activity (Table 1 and Supplementary Figure S3). All strains showed the ability to produce lichenysin as

detected by RP-HPLC-MS in biomass obtained from LB agar at 37 °C (Supplementary Table S2), with reference strain DSM13 producing the lowest amounts. The complete lichenysin gene cluster (*lchAA-TE*, also annotated as *licA-licTE*) was found in 10 of 11 strains, based on the analysis of the published genomes (Berendsen et al., 2016b). The available genome sequences of strain B4089 showed the presence of *lchAC* (*licC*) and *lchTE* (*licTE*) but not *lchAA (licA)* and *lchAB (licB)*. Both genes were detected in this strain based on PCR analysis (using *lchAA* and *lchAB* specific primers and B4089 chromosomal DNA as a template; data not shown) (Supplementary Table S3). Based on lichenysin production and the positive PCR results, the available genome sequence of B4089 may be incomplete.

5.3.1.1 Growth and lichenysin production in strains B4094, B4123, and DSM13^T

The growth and lichenysin production under different conditions was further assessed for three selected strains, namely: B4094, which showed robust growth in the presence of ethanol and NaCl, growth at 60°C, and produced heat-resistant spores (Berendsen et al., 2016b); B4123 which showed strong hemolytic activity and intermediate growth in all tested conditions; and DSM13^T which was used as a reference strain.

A direct comparison of the hemolytic activity of the strains and the growth under different conditions is presented in Figure 2, with B4123 showing strong hemolytic activity (large clearing zone), B4094 forming a small clearing zone, while DSM13^T was not hemolytic. DSM13^T did not displace oil from water, but both B4094 and B4123 showed clear displacement activity (Figure 2). Given the potential of DSM13^T to produce lichenysin (see the previous section), these results indicate that B4094 and B4123 produce higher amounts of lichenysin than DSM13^T, resulting in the observed hemolytic activity of erythrocytes and oil displacement.

When grown in LB at 37°C, food isolates B4094 and B4123 grew better than DSM13^T (Figure 2A), whereas, at 55°C, strains B4094 and DSM13^T reached a higher cell density than B4123 (Figure 2B). Under high salt concentration (1M NaCl), both food isolates showed a growth advantage over DSM13^T but did not differ evidently from one another (Figure 2C) and all three strains showed growth under anaerobic conditions in the presence of nitrate (Figure 2D).

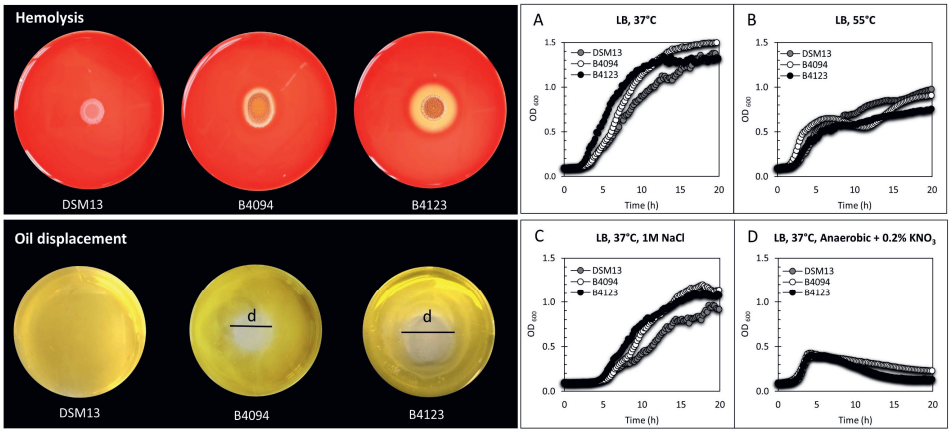


Figure 2- Hemolysis, oil displacement, and representative growth data for the three selected strains, B4094, B4123, and DSM13^T. (**Top left**) DSM13^T, B4094, and B4123 growth on Columbia blood agar showing hemolytic activity. The clearing zone on the blood agar indicates results from lysis of erythrocytes, indicative of the presence of the biosurfactant lichenysin. (**Bottom left**) The oil displacement activity of DSM13^T, B4094, and B4123. The displacement zone (d) shows the oil-repelling movement away from water, indicative of the presence of a surfactant. (**Right**) Aerobic and anaerobic growth data for DSM13T (**grey**), B4094 (**white**), and B4123 (**black**) in LB medium. (**A**) Growth in LB at 37°C; (**B**) Growth in LB at 55°C; (**C**) Growth in LB + 1M NaCl at 37°C; (**D**) Anaerobic growth in LB + 0.2% KNO3 at 37°C.

Table 1: Properties of *Bacillus licheniformis* food isolates and the type strain DSM13^T

Medium	**Blood agar**	**Growth in LB broth[c]**						**Growth on LB agar[d]**	**LB agar**
Strain[a]	**Hemolysis[b]**	**37°C**	**55°C**	**1M NaCl, 37°C**	**1M NaCl, 55°C**	**4% EtOH, 37°C**	**0.2% KNO_3 37°C**	**60°C**	**Lichenysin production[e]**
B4089	Weak	+	+ + +	+ +	+	-	-	NT	+
B4090*	None	+ + + +	+ + +	+ + + +	+ +	+ + +	+	+ + +	+
B4091	None	+ + + +	+ + +	+ +	+ +	+ + + +	+	+ + +	+
B4092*	Weak	+ + + +	+ + +	+ +	+ +	+ + +	-	+ +	+
B4094*	**Weak**	+ + + +	**+ + +**	**+ + + +**	**+ +**	**+ + + +**	**++**	**+ + + +**	+
B4121	Strong	+ +	+ +	+ + +	+	+ + + +	+	+	+
B4123	**Super strong**	**+ + + +**	**+ + +**	**+ + +**	**+**	**+ +**	**+**	**+**	+
B4124	Weak	+ + + +	+ + +	+ +	+ +	+ +	+	+ +	+
B4125	Medium	+ + +	+	+ + +	+	+ +	+	+	+
B4164	None	+ + +	+	+ +	+ +	+ +	-	+	+
DSM13^T	**None**	**+ +**	**+ +**	**+ +**	**+ +**	**+ +**	**+**	**+ + +**	+

[a]Food isolates sources as indicated in Berendsen et al., 2016(b); * indicates isolates that are heat resistant with Tn1546 transposons encompassing the *spoVA*^{2mob} operons. DSM13^T type strain was purchased from the German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany (DSMZ). [b]Hemolysis test carried out on Columbia blood agar plates; None - no clearing zone was observed; Weak - very small clearing zone; medium- medium size clearing zone; strong - big clearing zone; Super strong - very big clearing zone (Supplementary Figure S3)

[c]Growth data measured in OD_{600nm}; Minus (-) = no growth; + sign indicates growth robustness based on OD_{600} measurement (Supplementary Figures S1 and S4).

[d]Growth data at 60°C was checked on LB agar via spot platting of 3 µl of OD_{600} 1.0 day culture. NT= not tested; ++++ = growth observed in 10^{-9} dilution; +++ = growth observed > 10^{-7} but < 10^{-9} dilution; ++ = growth observed in dilution > 10^{0} but <10^{-7}; + = growth only observed at 10^{0} dilution (Supplementary Figure S2).

[e]Lichenysin production screening test via RP-HPLC ESI-MS; no quantification data; positive = lichenysin peak was detected (Supplementary Figure S2).

5.3.2 Growth and lichenysin production by selected food isolates B4094, B4123, and DSM13^T in LB broth and skimmed milk

5.3.2.1 Growth in LB broth and skimmed milk

The growth of strains B4094, B4123, and DSM13^T in LB and skimmed milk is presented in Figures 3A and 3B. The specific growth rate (μ, h^{-1}), lag phase (λ), and the doubling time (Td) were calculated for each strain under different conditions (section 5.2.3.2) and the results are shown in Table 2. Starting from an initial level of 1 to 2 $\log_{10}$ CFU/ml, each of the strains reached similar cell densities in a stationary phase of around 9 $\log_{10}$ CFU/ml within 12 to 14 h in LB (Figures 3A), with B4123 having the shortest lag time, and DSM13 having the highest growth rate (μ= 2.75 h^{-1}; Td = ~15 min) (Table 2). Lag times and growth rates in skimmed milk were similar for the three strains. All had shorter lag times and lower growth rates (μ ~ 0.7 h^{-1}) than in LB and entered the stationary phase approximately 10 h later. Final cell densities of around 9 $\log_{10}$ CFU/mL were reached after 22 to 24 h incubation (Figure 3B). At this moment, the milk showed spoilage.

Table 2: Estimated growth rate mu (μ) and lag time (λ) for DSM13^T, B4094 and B4123 in LB and skimmed milk using the Baranyi primary growth model

Medium	Strain	Parameter	$\log N_0$ ($\log_{10}$CFU /ml)	$\log N_{max}$ ($\log_{10}$CFU /ml)	μ (h^{-1})	λ (h)	Td (min)
LB	DSM13^T	Estimate	1.8	8.9	2.75	5.88	15.1
		Std. Error	0.2	0.1	0.12	0.39	
	B4094	Estimate	1.4	8.8	2.05	4.51	20.3
		Std. Error	0.1	0.1	0.05	0.32	
	B4123	Estimate	0.7	8.9	1.93	1.70	21.5
		Std. Error	0.2	0.1	0.04	0.38	
Skimmed milk	DSM13^T	Estimate	0.8	9.0	0.74	0.24	56.4
		Std. Error	0.4	0.2	0.02	1.66	
	B4094	Estimate	1.1	9.0	0.69	0.66	60.2
		Std. Error	0.3	0.2	0.01	1.32	
	B4123	Estimate	1.3	8.8	0.74	0.67	56.4
		Std. Error	0.2	0.1	0.01	1.00	

5.3.2.2 Lichenysin production in LB broth and skimmed milk

The total lichenysin concentrations during the growth of strains B4094, B4123, and DSM13^T at 37 °C were determined in LB and skimmed milk. Lichenysin was not found in cultures of DSM13^T in both media, except for a very low level (0.1 μg/ml) in LB at 20 h (Figures 3C and 3D). For the two food isolates, lichenysin was not detected in LB and skimmed milk at cell densities < 5 $\log_{10}$ CFU/ml.

In LB, lichenysin was first detected at time point 10 h, namely, 0.2 μg/ml at a cell density of 7 $\log_{10}$ CFU/ml (strain B4094) and 5.4 μg/ml at a cell density of 8 $\log_{10}$ CFU/ml (strain B4123) (Figure 3C). Upon entering into stationary phase, lichenysin concentrations increased to

35.9 µg/ml and 19.1 µg/ml for strain B4094 and B4123, respectively, with cell densities of around 8-9 $\log_{10}$ CFU/ml (Figure 3C).

The production of lichenysin was significantly lower in skimmed milk than in LB for both strains, with B4094 producing more lichenysin than strain B4123 in both cases. In the milk, low lichenysin levels were first detected after 22 h for strain B4094, namely 0.2 µg/ml at a cell density of ~ 7.9 $\log_{10}$ CFU/ml, with levels increasing to 8.1 µg/ml in the stationary stage (8.8 $\log_{10}$ CFU/ml) after 28 h of incubation (Figure 3D). For strain B4123, low levels of lichenysin (0.1 µg/ml) were first detected at 16 h, and levels reached only 0.8 µg/ml during the stationary phase at cell densities of ~ 8.5 $\log_{10}$ CFU/ml (Figure 3D). Lichenysin production and total viable counts were furthermore determined at two later time points (48 and 72 h), showing that cell concentrations in LB and skimmed milk did not change in cultures of B4094, B4123, and DSM13^T, and the lichenysin concentrations did not increase (Supplementary Tables S4 & S5).

It can be concluded from Figure 3 that 1) lichenysin was not detected in LB and skimmed milk at cell densities < 5 $\log_{10}$ CFU/ml; 2) lichenysin production started when cells reached the late exponential phase in LB and skimmed milk; 3) lichenysin production was strain and medium-dependent at similar cell densities.

5.3.2.3 Identification and quantification of lichenysin A variants in LB broth and skimmed milk

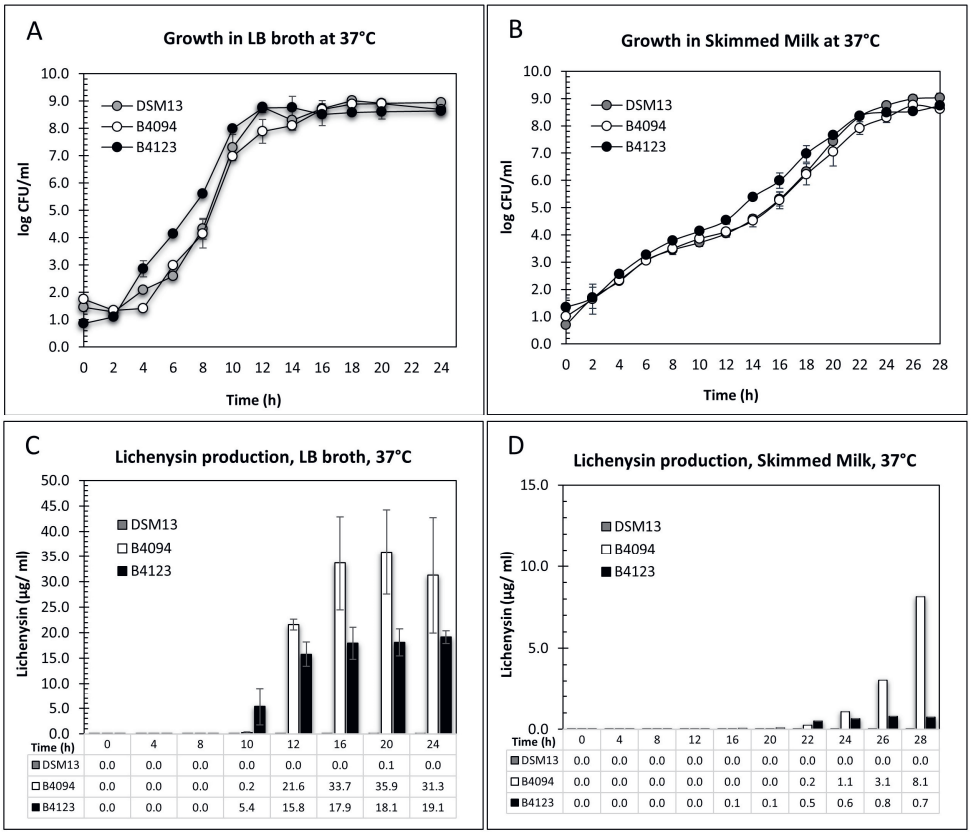
The concentrations of lichenysin reported in the previous section consisted of the sum of lichenysin variants, all corresponding with lichenysin A variants. The molar masses of the detected variants are listed in Supplementary Table S1. The percentages of individual variants based on the total amount found are presented in Figures 4A and 4B for strains B4094, B4123, and DSM13^T upon growth in LB for 24 h and skimmed milk for 28 h. An example of chromatograms with mass spectra from the extracts of the B4094 cultured cells in skimmed milk as retrieved at 28 h is shown in Supplementary Figure S5.

The five main lichenysin A variants found were C12, C13, C14, C15, and C16 (molar mass of 993.7 Da, 1007.7 Da, 1021.7 Da, 1035.7 Da, and 1049.7 Da, respectively). In addition, strains B4094, B4123, and DSM13^T synthesized two other variants, namely, C11-lichenysin (979.6 Da) and C17-lichenysin (1063.7 Da). Lichenysin with the shortest hydrophobic tail (C11) was only detected in the case of the two food isolates (B4094 and B4123) grown in skimmed milk but not in LB. The relative abundance of lichenysin with the longest hydrophobic tail (C17) was higher in LB than in skimmed milk for all three strains (Figure 4 and Supplementary Table S4). The 14 Da mass shift between the molecular masses detected could arise from amino acid substitutions in the peptide moiety (e.g., valine vs. isoleucine) or different fatty acid chain lengths. Given the ~1 min shorter elution time of the variant with the 14 Da lower mass than C12 (Supplementary Figure S5), this mass difference could be assigned to the acyl chain length.

As a comparison, C15-surfactin and C15-lichenysin (differing in peptide ring but not chain length) had very similar retention times (~13 min).

Approximately half of the main variants produced in LB consisted of C15-lichenysin, followed by C14 and smaller amounts of C13 > C16 > C12 (Figure 4A). In skimmed milk, on the other hand, C14-lichenysin had the highest relative abundance (around 55%), followed by C15 and smaller amounts of C13 > C16 > C12. This was observed for each of the three strains, indicating that the composition of the medium has a strong influence on the distribution of the chain length.

To further test whether the medium composition and culture conditions affect the levels of lichenysin produced and the synthesis of different isoforms, the total lichenysin production of B4094, B4123, and DSM13^T on agar surfaces was also quantified.



Time (h)	0	4	8	10	12	16	20	24
DSM13	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0
B4094	0.0	0.0	0.0	0.2	21.6	33.7	35.9	31.3
B4123	0.0	0.0	0.0	5.4	15.8	17.9	18.1	19.1

Time (h)	0	4	8	12	16	20	22	24	26	28
DSM13	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
B4094	0.0	0.0	0.0	0.0	0.0	0.0	0.2	1.1	3.1	8.1
B4123	0.0	0.0	0.0	0.0	0.1	0.1	0.5	0.6	0.8	0.7

Figure 3- Growth and lichenysin production for DSM13^T, B4094, and B4123 in LB and skimmed milk at 37°C. (**A**) Growth of three selected strains, DSM13^T, B4094 and B4123 in LB medium for 24 h. (**B**) Growth of three selected strains, DSM13^T, B4094 and B4123 in skimmed milk for 28 h. (**C**) Total lichenysin production (mg/ml) in LB broth for DSM13^T, B4094, and B4123. (**D**) Total lichenysin production (mg/ml) in skimmed milk for DSM13^T, B4094, and B4123. The amount of lichenysin produced by all three strains in LB at selected time points as quantified with RP-HPLC-QTOF-ESI/MS. **Grey circle or bar-** DSM13^T, **white circle or bar**- B4094, **black circle or bar**- B4123.

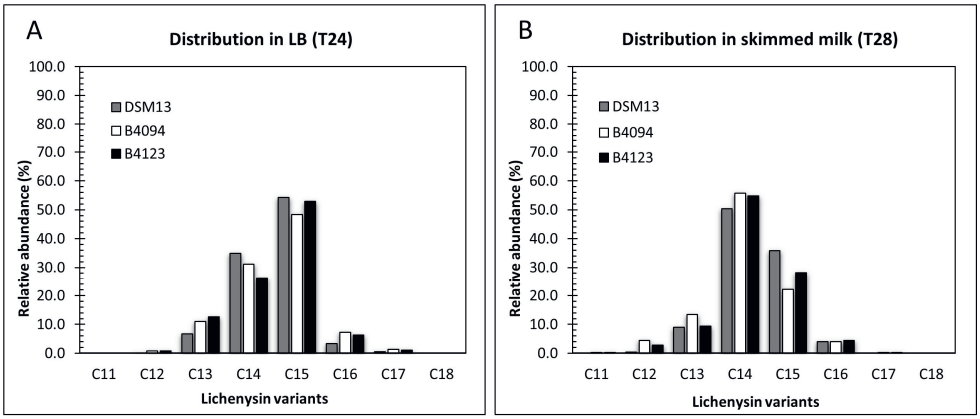


Figure 4- Distribution of lichenysin A variants, produced in LB broth and skimmed milk at 37°C. Relative abundance of individual lichenysin variants as part of the total amount in (**A**)- LB broth at 24 h; (**B**) skimmed milk at 28 h. **Grey bar-** DSM13^T , **white bar-** B4094, **black bar-** B4123.

5.3.3 Surface growth on LB agar and skimmed milk agar and production of lichenysin and its variants at optimal temperature (37°C) and high temperature (55°C)

5.3.3.1 Lichenysin production on LB agar and skimmed milk agar at 37°C and 55°C

The amounts and variants of lichenysin produced by strains B4094, B4123, and DSM13^T upon growth on LB agar and skimmed milk agar at 37°C and 55°C, are presented in Figure 5. The amount of lichenysin produced by all three strains was measured at a late time point (after 10 days of incubation of the agar plates).

On LB agar at 37°C, DSM13^T, B4094, and B4123 yielded 25 µg lichenysin per g of wet biomass (µg /g), 267 µg/g, and 218 µg/g, respectively (Figure 5A). All three strains also produced lichenysin at 55°C, but at lower concentrations than at 37°C, namely, ~ 60 % less for DSM13^T, ~50 % less (138 µg/g) for B4094, and ~ 30 % less (168 µg/g) for B4123 (Figure 5B).

Similar trends were observed upon growth on skimmed milk agar, albeit the total concentrations produced on milk agar were around ten-fold lower than on LB agar. At 37 °C, strains DSM13^T, B4094, and B4123 produced 10 µg/g, 23 µg/g, and 25 µg/g of lichenysin per g of wet biomass, respectively (Figure 5C), and at 55°C, the total lichenysin concentrations were between ~ 50 and 90 % lower (Figure 5D).

Similar to the results reported in section 5.3.2, B4094 and B4123 produced significantly higher amounts of lichenysin than the type strain DSM13 under all conditions tested.

5.3.3.2 Identification and quantification of lichenysin A variants on LB agar and skimmed milk agar at 37°C and 55°C

The distribution of lichenysin A variants upon production on LB agar and skimmed milk agar at 37°C and 55°C is presented in Figure 6. Upon growth on LB agar at 37°C and 55°C, all three strains produced C15 as the dominant variant (in most cases > 60%), followed by the variants C14 > C13 > C16 > C12-lichenysin. The relative abundance of the C17 variant was low but similar for the three strains (Figures 6A and 6B).

On skimmed milk agar, C14- lichenysin had the highest relative abundance at 37°C for all three strains, followed by C15 > C13 > C16-lichenysin (Figure 6C). At 55°C, the C14 and C15 variants made up 70 to 90% of the total lichenysin, with similar ratios for strains B4094 and B4123, but two times higher levels of C15 than C14 for DSM13^T. At this high temperature, C13 and C16- lichenysin each constituted around 10% of the total amount, with only very low levels of C17 and the C12-variant (Figure 6D).

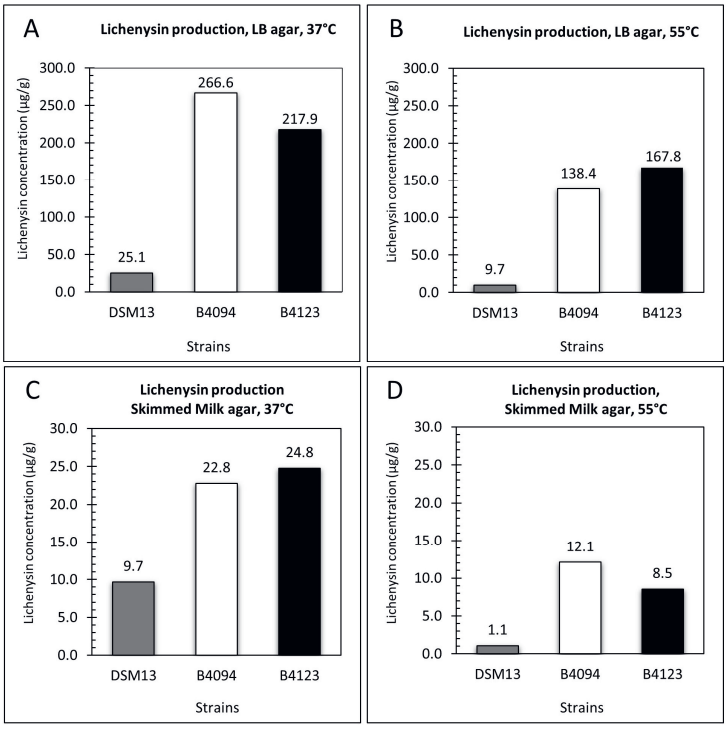


Figure 5- Lichenysin production upon surface growth on LB agar and skimmed milk at 37 and 55°C. Total lichenysin produced by DSM13^T, B4094, and B4123 per g of wet biomass after 10 days of incubations on (**A**) LB agar at 37°C; (**B**) LB agar at 55°C; (**C**) skimmed milk agar at 37°C; (**D**) skimmed milk agar at 55°C. **Grey bar**- DSM13^T, **white bar-** B4094, **black bar-** B4123.

A variant with the longest hydrophobic tail (C18) was found to be produced at very low levels by all three strains at 37°C on LB agar but not on skimmed milk agar (Figure 6 and Supplementary Table S6). The C18 variant (with ~ 1 min higher elution time) was detected in biomass of B4094 obtained after growth at 55°C on LB agar, but not for the other two strains growth at 55°C on LB. In contrast, the shortest C11 variant was detected only in skimmed milk or skimmed milk agar, but not in LB broth or on LB agar at 37°C and 55°C (Supplementary Table S6). However, one exception was observed, as the B4123 strain produced the C11 variant on LB agar at 55°C. Together, these results indicate that the tail lengths of the lichenysin variants and their relative abundances depend on the strain, cultivation medium, and temperature.

Considering the above outcomes that culturing conditions can strongly influence the amount and type of lichenysin produced, lichenysin production was also evaluated upon culturing DSM13^T, B4094, and B4123 in liquid broth using stagnant conditions. Results presented in Supplementray Figure S6 show that lichenysin concentrations were significantly higher in the fractions that contained cells (pellicle and cell pellet) than in the supernatant, with the highest amount observed in the pellicle that was formed at the liquid-air interface.

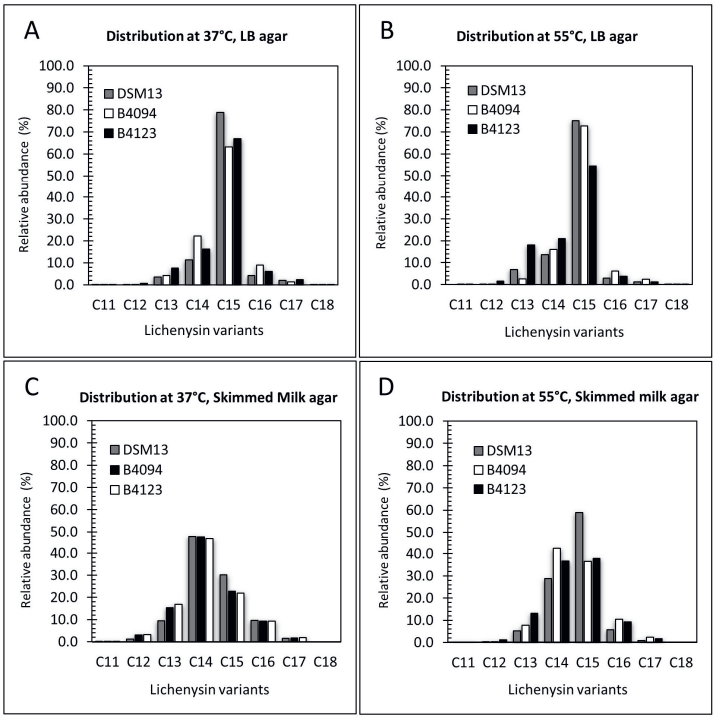


Figure 6- Distribution of lichenysin A variants upon surface growth on LB agar and skimmed milk agar at 37 and 55°C. Relative abundance of individual lichenysin variants as part of the total amount extracted from biomass of DSM13^T, B4094, and B4123 after 10 days of incubations on (**A**) LB agar at 37°C; (**B**) LB agar at 55°C; (**C**) skimmed milk agar at 37°C; (**D**) skimmed milk agar at 55°C. **Grey bar-** DSM13T , **white bar**- B4094, **black bar**- B4123.

5.3.4 Lichenysin is slightly more toxic to Caco-2 cells than surfactin

The toxicity of lichenysin toward human cells and pig organoids was investigated, using *B. subtilis*-derived surfactin as a control. The concentration of lichenysin and surfactin needed to reduce cell viability by 50% (IC_{50}) is presented for different cell lines and organoids in Table 3. Lichenysin and surfactin were not toxic to HEK293 embryonic kidney cells at the highest tested concentration of 200 µM (~200 µg/ml), even after prolonged exposure for 72 h. Lichenysin and surfactin led to a reduction of the viability of Caco-2 cells after 72 h incubation with IC_{50} values of 16.6 µg/ml and 23.4 µg/ml, respectively (Figure 7). Cells exposed to lichenysin or surfactin at 100 µM (~100 µg/ml) or 200 µM (~200 µg/ml) showed different degrees of cell impairment indicated by reduced fluorescence in the Alamar Blue assay and cells incubated with the solvent DMSO alone at 10% w/v showed severe impairment (Figure 7). The viability of the pig organoids upon exposure to lichenysin and surfactin was also determined, showing IC_{50} for lichenysin of 16.8 µg/ml, while surfactin at the highest tested concentration of 200 µM did not show toxicity, indicating an IC_{50} > 200 µg/ml.

Table 3: Cytotoxic level of lichenysin and surfactin (IC_{50}) to human and mammalian cells

Cell line	Surfactin IC_{50} (µg/g)	Lichenysin IC_{50} (µg/g)
HEK293	>200	>200
Caco-2	23,5	16,6
Pig ileum organoids	>200	16,8

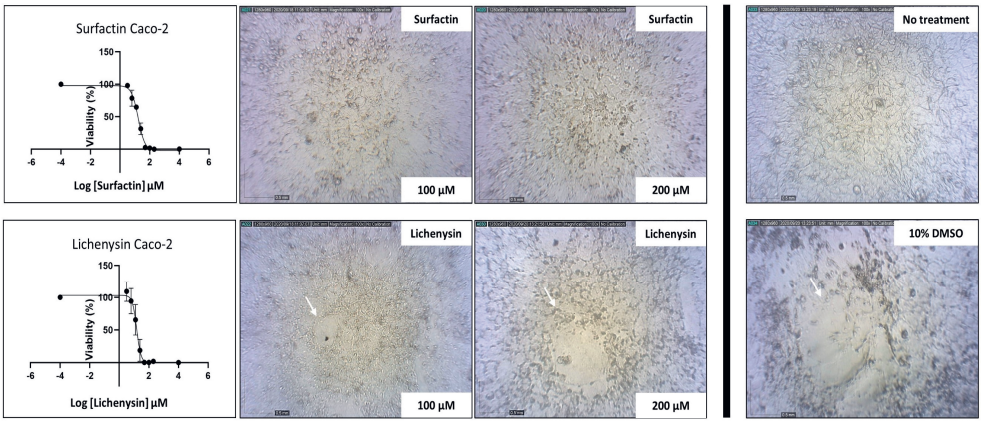


Figure 7- Toxicity of lichenysin and surfactin toward human Caco-2 cells. Caco-2 cells were seeded ($2x10^5$ cells/ml) in 96 wells-plates and exposed to different lichenysin and surfactin concentrations (only 100 µM and 200 µM are shown; in log_{10} scale = 2 and 2.3 µM, respectively). No-treatment cells were the negative control, and 10% DMSO was used as a control for cell death. Cell viability was monitored for 72 h, and microscopic pictures of cell disruption for lichenysin after 24 and 72 h are shown. The IC50 for lichenysin is 16,6 µM (= ~ 16.6 µg/ml), in log_{10} scale = 1.22 µM; and for surfactin is 23,5 µM (= ~ 25.5 µg/ml), in log_{10} scale = 1.37 µM.

5.4 Discussion

The results presented in this study showed that nine out of ten *B. licheniformis* food isolates have robust growth properties. The amount of lichenysin produced by the isolates depended on the strain, cultivation medium, and incubation temperature. Lichenysin showed toxic effects in pig ileum organoids and human epithelial Caco-2 cells.

Strain B4094 consistently produced more lichenysin than strain B4123 under all conditions tested, and both high producers synthesized much more lichenysin than DSM13^T. The observed variation between strains is in line with an earlier report by Madslien et al. (2013), who demonstrated that the amounts produced by 53 *B. licheniformis* strains varied more than two orders of magnitude (ranging from <0.013 µg to >3.3 µg per mg biomass) between strains. Some high producers in their study were associated with foodborne intoxications, and they classified strain ATCC14580 (=DSM13^T) as a weak producer.

The analysis of lichenysin production in liquid LB or skimmed milk (as a food matrix) revealed that lichenysin was not detected at cell densities < 5 $\log_{10}$ CFU/ml. Production did not reach maximum levels until the stationary stage in both media. It is known that lichenysin production can be influenced by environmental factors, e.g., medium composition (Yakimov et al., 1996), oxygen availability, temperature, and pH (Yakimov et al., 1995; Joshi et al., 2008; Li et al., 2008; Gogotov and Miroshnikov, 2009), and the type of carbon source (important for energy generation) or nitrogen sources (for amino acids and protein synthesis). Reported yields of lichenysin were for instance, higher in cultivation media containing glucose than in media with other carbon sources like sucrose, maltose, glycerol, sodium citrate, sodium acetate, lactose, corn starch, and starch (Yakimov et al., 1995; Qiu et al., 2014). In addition, the yield increased in the presence of specific amino acids like L-glutamic acid and L-asparagine in the medium (Yakimov et al., 1996) and when inorganic nitrogen sources [such as KNO_3, $NaNO_3$, NH_4Cl, $(NH_4)_2SO_4$, and NH_4NO_3] were present instead of organic nitrogen sources (peptone, yeast extract, etc.) (Qiu et al., 2014). LB contains glucose and readily available nitrogen sources (yeast extract and peptone), whereas skimmed milk contains the sugar lactose and casein as a nitrogen source, requiring proteolytic cleavage prior to uptake by cells. These differences in composition likely account for the significantly lower levels of lichenysin produced in milk than in LB, as found in this study.

We furthermore showed that lichenysin yields were temperature-dependent, with lower yields at 55°C than 37°C in biomass obtained from agar plates (LB and milk agar) (Figure 5). This result is consistent with the study of Yakimov et al. (1995), who showed that lichenysin production by *B. licheniformis* is optimal at 35°C to 45°C. Similarly, surfactin yield from *B. subtilis* was also influenced by temperature, with lower production at 45°C than at 35°C (Park et al., 2019) and higher yields at 37°C than at 25°C (Abdel-Mawgoud et al., 2008; Rahman

and Ano, 2009). In another study, the production of surfactin by *Bacillus amyloliquefaciens* was, however, lower at 25°C or 30°C than at 15°C (Monteiro et al., 2016).

In our study, cultures contained lichenysin A variants with different acyl chain lengths (C11 to C18). The length of the fatty acid chain lengths may influence the toxicity of the compound; Previous studies on surfactin showed that C15 acyl chains had more efficient penetration strength into the cell membrane than its shorter counterparts, C13 and C14-surfactin (Liu et al., 2010; Wu et al., 2017), and exhibited enhanced antifungal and antibacterial activity (Meena and Kanwar, 2015). This is thought to result from more efficient interactions of a longer fatty acid chain of surfactin with the acyl chains of phospholipids in the cell membrane. Cultures containing lichenysin with a skew toward long-chain variants (which is medium and culture condition dependent) may therefore be more toxic to different cells than cultures with a higher proportion of short-chain variants.

The concentration of lichenysin needed to reduce cell viability by 50% (IC_{50}) was 16.6 µg/ml for Caco- 2 human intestinal epithelial cells and 16.8 µg/ml for pig ileum organoids. These levels correspond with concentrations resulting in toxic effects on boar spermatozoa cells (i.e.,> 10 µg/ml) (Madslien et al., 2013). For surfactin, the IC_{50} value was 23.5 µg/ml for Caco-2 cells, while no toxicity was seen for the ileum organoids at the highest levels tested (> 200 µg/ml). This indicates that lichenysin is more toxic to these cell types than surfactin. In the case of surfactin, the purchased compound largely contained C15 acyl chains (> 99%). The lichenysin extracts used in the toxicity assays contained a mix of lichenysin variants, with C15-lichenysin being the most abundant (> 65 %, produced by B4094 on LB agar 37°C, see Figure 6). In addition to differences in the peptide ring between surfactin and lichenysin, the presence of longer acyl chains (~10% C16) may have contributed to the observed differences in toxicity toward the organoids. A further detailed mechanistic study with pure lichenysin variants having different carbon chain lengths would be required to elucidate this; however, such compounds are not commercially available.

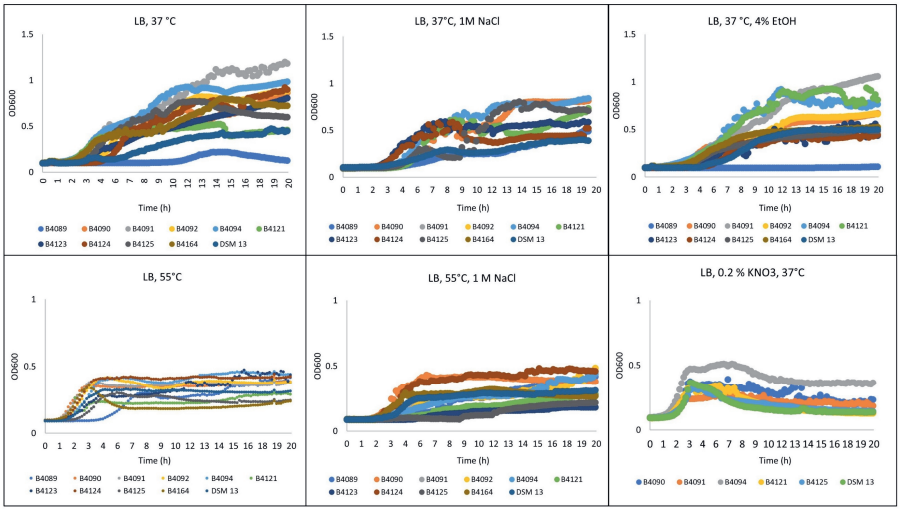
Overall, levels of *B. licheniformis* up to 5 $\log_{10}$ viable cells/ml in liquid foods are unlikely to pose a foodborne hazard related to the presence of lichenysin. However, at higher cell densities, a risk of foodborne intoxication may arise due to the production of the compound. Lichenysin levels produced in milk and LB were in the same range as the IC_{50} values of lichenysin for epithelial cells. The actual amounts to cause illness *in vivo* upon ingestion of foods will depend on various factors, most notably the amount of food ingested and the concentration of the lichenysin in the product. High producers are commonly found in foods: in the study by Madslien et al. (2013), 28 out of 53 isolates produced > 3.3 µg/mg biomass on tryptic soy agar after 10 days at 37°C. The highest level found in our study was 0.27 µg/mg biomass for B4094 on LB agar. This 10-fold difference indicates that production of higher levels than reported in this study is conceivable in food products with high levels of *B. licheniformis*, depending on the

strain present, the composition of the food and conditions during processing and storage in the chain. In solid foods, the distribution of the organism in the product may be inhomogeneous with 'hot spots' of high cell concentrations and lichenysin production. In confirmed foodborne intoxication cases due to consumption of solid product contaminated with *B. licheniformis*, high levels of the organism were observed (i.e., 2 x 10^6 CFU/g in curried chicken and mayonnaise sandwich; 1 x 10^8 CFU/g in minced beef pies; 1.1 x 10^8 CFU/g in pancakes) (Salkoninen et al., 1999).

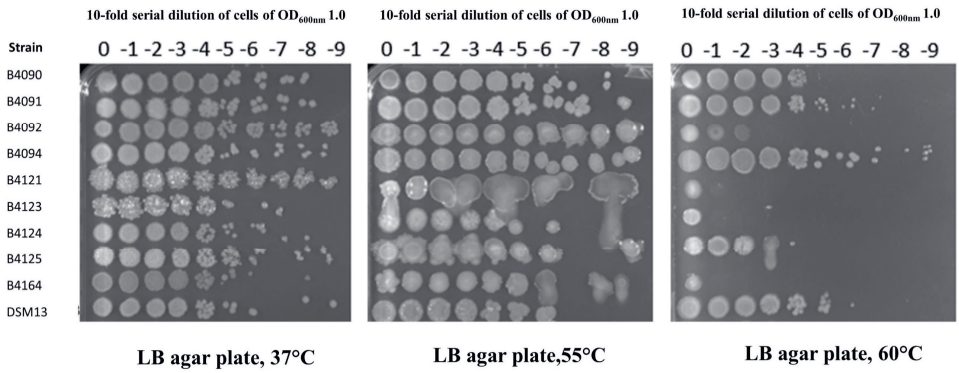
In addition to natural contamination of foods, *B. licheniformis* can be actively introduced into the food chain (e.g., as crop bioprotectants or feed/food additives). This is usually done in the form of spores. Therefore, from a food processing point of view, it is crucial that the selected strains do not possess genetic elements that enable the production of highly heat-resistant spores (Berendsen et al., 2016a, b), resulting in potential non-sterility issues in finished products.

From a food safety point of view, the production of lichenysin must be prevented, requiring a thorough assessment of surfactant production capacity. Once lichenysin is produced in food upon the growth of *B. licheniformis*, the compound is stable even when cells (or even spores) are inactivated. This stresses the importance of controlling this organism throughout the entire production chain by inactivating viable cells and spores in ingredients and preventing outgrowth using appropriate preservation systems and/or low-temperature control.

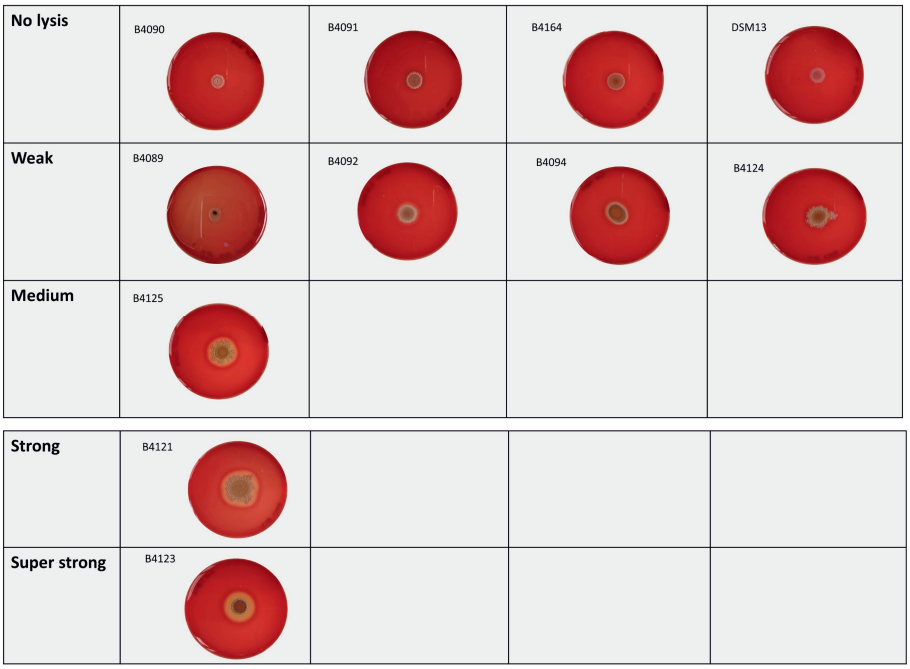
5.5 Supplementary Materials



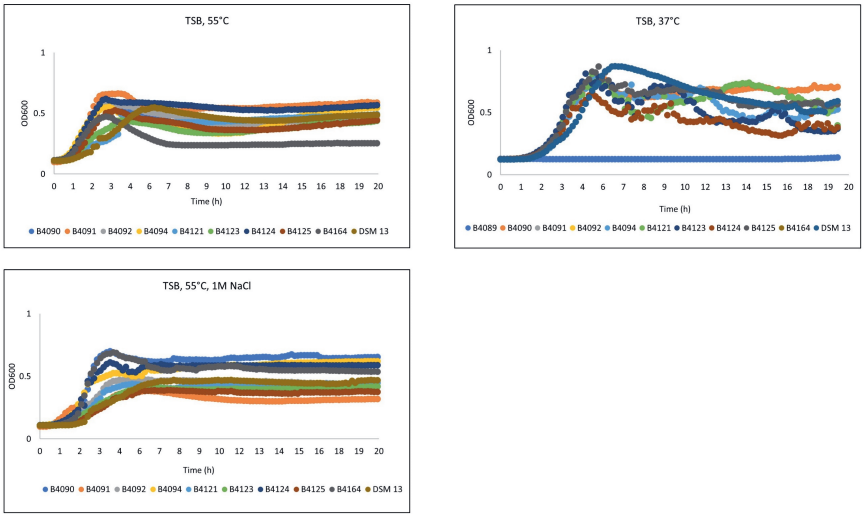
Supplementary Figure S1- Growth of different *Bacillus licheniformis* strains in LB medium under different conditions



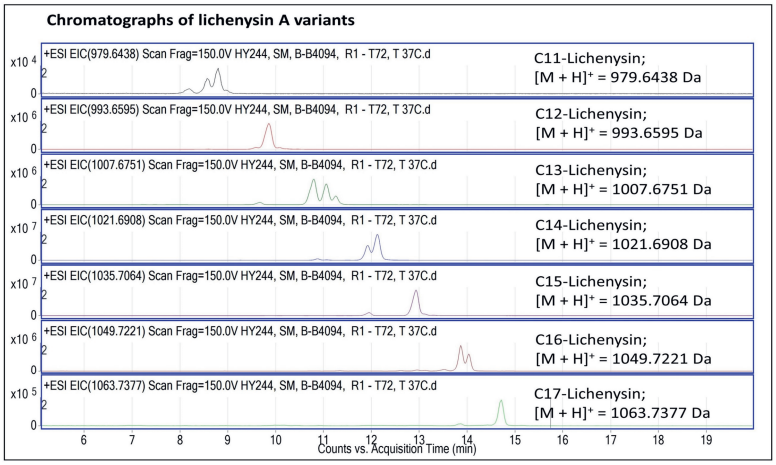
Supplementary Figure S2- Growth of different *Bacillus licheniformis* strains on agar plates at 37°C, 55°C, and 60°C. The OD_{600nm} of the ON cultures in LB media was measured and used to inoculate 5 ml of fresh LB medium to the OD_{600nm} 0.05. Cultures were then grown at 37 °C, 200 rpm until OD_{600nm} 0.5. This step was performed to get endospores-free culture. 1 ml of cells from the day culture in LB medium were collected by centrifugation at 10000 x g for 2 min (5430 R Eppendorf centrifuge) and resuspended in 1 ml sterile 0.9 % NaCl solution. The OD_{600nm} of the cells was measured and adjusted to 1.0 with the 0.9 % NaCl solution. A 10-fold dilution series in the NaCl solution was prepared up to 10^{-9}. 3 µl of cells from each dilution tube was spotted on LB agar plates and dried under the flow cabinet. The plates were incubated at 37 °C, 55 °C, and 60 °C.



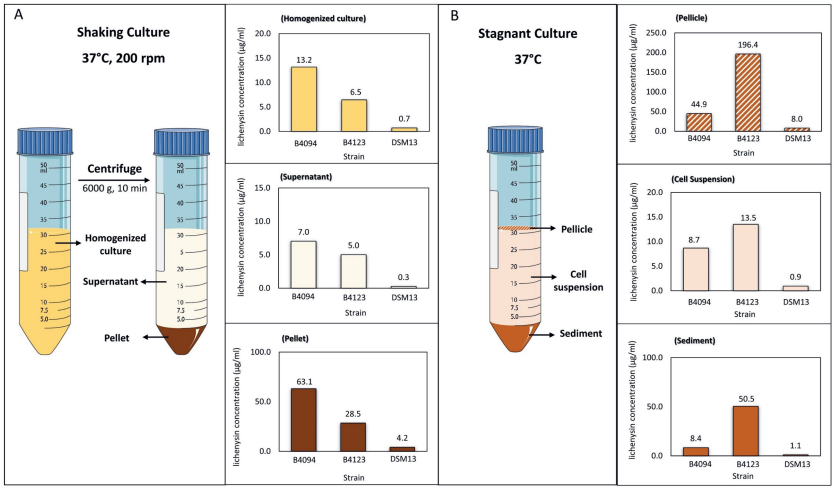
Supplementary Figure S3- Hemeolysis Test for different *Bacillus licheniformis* strains. The growth of all 10 *B. licheniformis* food isolates and the type strain DSM13T on Columbia blood agar show hemolytic activity. The clearing zone on the blood agar indicates the lysis of erythrocytes, indicative of the presence of the biosurfactant lichenysin. A small clearing zone indicated weak hemolytic activity and a big clearing zone indicated strong hemolytic activity.



Supplementary Figure S4- Growth of different *Bacillus licheniformis* strains in TSB medium under different conditions



Supplementary Figure S5- Chromatographs of lichenysin A variants. Chromatographs of C11 to C17- lichenysin A variants detected for B4094 via RP-HPLC-QTOF-ESI/MS. 5 µL sample was injected on a C8 analytical column (Phenomenex) thermostated at 50°C. Lichenysin A was eluted at a flow rate of 0.2 mL/min with a linear gradient of 0.10% formic acid in 40% water + 55% acetonitrile + 5% tetrahydrofuran to 0.1% formic acid in 75 % acetonitrile + 25% tetrahydrofuran in 20 min. Lichenysin A variants were screened according to the masses in Supplementary Table S1. Quantitative analyses were carried out using lichenysin A standard (range 10 – 40 000 µg/L) (Lipofabrik).



Supplementary Figure S6- *Bacillus licheniformis* growth and lichenysin production in different matrices

Supplementary Table S1: Molar masses of lichenysin A variants

No	Variant	Molecular Formula	M [da]	$[M + H]^+$
	^{L}C11-Lichenysin*	$C_{49}H_{86}N_8O_{12}$	978.6365	979.6438
	^{L}C12-Lichenysin	$C_{50}H_{88}N_8O_{12}$	992.6522	993.6595
	^{M}C13-Lichenysin	$C_{51}H_{90}N_8O_{12}$	1006.6678	1007.6751
Lichenysin A	^{H}C14-Lichenysin	$C_{52}H_{92}N_8O_{12}$	1020.6835	1021.6908
	^{H}C15-Lichenysin	$C_{53}H_{94}N_8O_{12}$	1034.6991	1035.7064
	^{M}C16-Lichenysin	$C_{54}H_{96}N_8O_{12}$	1048.7148	1049.7221
	^{M}C17-Lichenysin*	$C_{55}H_{98}N_8O_{12}$	1062.7304	1063.7377
	^{M}C18-Lichenysin*	$C_{56}H_{100}N_8O_{12}$	1076.7534	1077.7534
*identified in this study, L- low, M- medium, H- high				

Supplementary Table S2: Lichenysin production screening in biomass obtained from LB agar at 37°C

	Rt = 12.7 - 13.0 min	**Rt = 9.8 min**	**Rt = 10.8 min**	**Rt = 12.0 - 12.2 min**	**Rt = 13.0 min**	**Rt = 13.6 + 13.9 min**	**Rt = 14.8 min**	**Rt = 15.3 min**
	C15-Surfactin	C12-Lichenysin*	C13-Lichenysin*	C14-Lichenysin*	C15-Lichenysin*	C16-Lichenysin*	C17-Lichenysin*	C18-Lichenysin*
		$C_{50}H_{88}N_8O_{12}$	$C_{51}H_{90}N_8O_{12}$	$C_{52}H_{92}N_8O_{12}$	$C_{53}H_{94}N_8O_{12}$	$C_{54}H_{96}N_8O_{12}$	$C_{55}H_{98}N_8O_{12}$	$C_{56}H_{100}N_8O_{12}$
	$[M + H]^+$	$[M + H]^+$	$[M + H]^+$	$[M + H]^+$	$[M + H]^+$	$[M + H]^+$	$[M + H]^+$	$[M + H]^+$
	Conc (µg/kg)	Conc (µg/kg)	Conc (µg/kg)	Conc (µg/kg)	Conc (µg/kg)	Conc (µg/kg)	Conc (µg/kg)	Conc (µg/kg)
strain no.	**1036.690**	**993.659**	**1007.675**	**1021.690**	**1035.706**	**1049.722**	**1063.737**	**1077.753**
B4089	42855841	66400	8790159	11999823	74615281	97665105	35531669	1114756
B4090	47158178	851787	49332070	20573729	81912012	10316775	28541879	677605
B4091	75910640	108704	11622944	32236428	12939537	43191399	20808074	6856817
B4092	43824269	80315	3455921	95918454	76118297	10993278	22410919	496102
B4094	64747932	425735	55469001	33570524	10861941	24775599	94752748	2543627
B4121	65258378	10583906	21377135	46903198	10773611	22490580	76602476	1766166
B4123	58876876	520364	52015840	27770900	10087992	19885884	74148616	2014192
B4124	10274490	85037665	73973754	11443050	17473608	87987855	34270586	33499914
B4125	54810004	8904784	17756391	33025520	93745718	12453498	40078496	588291
B4164	81673484	2814232	18361653	66246781	14209978	48338781	22711756	5394420
DSM 13	11487866	237825	7134384	23338008	20527716	5983426	2955461	13876

Supplementary Table S3: The presence/absence of the lichenysin gene cluster in strains used in this study

STRAIN	***licA / lchAA***	***licB / lchAB***
B4089	positive PCR on genomic DNA*	positive PCR on genomic DNA**
B4090	B4090_RS06670 B4090_RS07745 B4090_RS05175	B4090_RS06670 B4090_RS07745 B4090_RS05175
B4091	B4091_RS01540 B4091_RS07175 B4091_RS13370	B4091_RS01540 B4091_RS07175 B4091_RS13370
B4092	B4092_RS01230 B4092_RS05480 B4092_RS11525	B4092_RS01230 B4092_RS05480 B4092_RS11525
B4094	B4094_RS09780 B4094_RS04415 B4094_RS20155 B4121_RS17190 B4121_RS17195 B4121_RS07900	B4094_RS09780 B4094_RS04415 B4094_RS20155
B4121	B4121_RS21390 B4123_RS02770 B4123_RS10570 B4123_RS13065	B4121_RS17190 B4121_RS17195 B4121_RS07900 B4121_RS21390
B4123	B4123_RS07720	B4123_RS02770 B4123_RS10570 B4123_RS13065 B4123_RS07720
B4124	B4124_RS11270 B4124_RS05120 B4124_RS04610 B4125_RS10415 B4125_RS14280 B4125_RS08345	B4124_RS11270 B4124_RS05120 B4124_RS04610
B4125	B4125_RS19185	B4125_RS10415 B4125_RS14280 B4125_RS08345 B4125_RS19185
B4164	B4164_RS04965 B4164_RS16945 B4164_RS08070	B4164_RS04965 B4164_RS16945 B4164_RS08070
DSM13	TRNA_RS23485	TRNA_RS23490
STRAIN	***licC / lchAC***	***licTE / lchTE***
B4089	B4089_RS00540	B4089_RS00535
B4090	B4090_RS10120	B4090_RS10125
B4091	B4091_RS20640	B4091_RS20645
B4092	B4092_RS19250	B4092_RS19245
B4094	B4094_RS08740 B4121_RS21395 B4121_RS22340 B4121_RS17200	B4094_RS08735
B4121	B4121_RS09725	B4121_RS17205 B4121_RS14020 B4121_RS22335
B4123	B4123_RS13590	B4123_RS08525
B4124	B4124_RS07445 B4125_RS08340 B4125_RS19175 B4125_RS10420	B4124_RS07450
B4125	B4125_RS10425	B4125_RS10430 B4125_RS19170
B4164	B4164_RS14050	B4164_RS14045
DSM13	TRNA_RS23495	TRNA_RS23495
*	primers used: CY21 and CY22	
**	primers used: CY23 and CY24	

Pri-mer	**Description**	**5'-3' sequence**
CY21	Bli lchAA forward	CGATTCCGGGTTATTGACTG
CY22	Bli lchAA reverse	CGCTTCATATTGTGCGTTCC
CY23	Bli lchAB forward	ATTTGAGGATCTTGCGGACTTG
CY24	Bli lchAB reverse	TATAGCGGGAGATAGTCGTACC

Supplementary Table S4: Lichenysin production and variant distribution in LB broth and skimmed milk at 37°C.

*recovery percentage for standards were 60-72%

Sample name	Medium	Standard/strain	Replicate timepoint	Injected volume [µL]	Dilution factor	**Rt = 8.7 min** C11-Lichenysin $C_{58}H_{96}N_8O_{12}$ [M + H]$^+$ 979.6438 Final Conc. [ug/L]	**Rt = 9.7 min** C12-Lichenysin $C_{59}H_{98}N_8O_{12}$ [M + H]$^+$ 993.6595 Final Conc [ug/L]	**Rt = 10.6 min** C13-Lichenysin $C_{51}H_{96}N_8O_{12}$ [M + H]$^+$ 1007.6751 Final Conc. [ug/L]	**Rt = 11.8 min** C14-Lichenysin $C_{52}H_{92}N_8O_{12}$ [M + H]$^+$ 1021.6908 Final Conc. [ug/L]	**Rt = 12.8 min** C15-Lichenysin $C_{53}H_{94}N_8O_{12}$ [M + H]$^+$ 1035.7064 Final Conc. [ug/L]	**Rt = 13.8 min** C16-Lichenysin $C_{54}H_{96}N_8O_{12}$ [M + H]$^+$ 1049.7221 Final Conc. [ug/L]	**Rt = 14.6 min** C17-Lichenysin $C_{55}H_{98}N_8O_{12}$ [M + H]$^+$ 1063.7377 Final Conc. [ug/L]	**Rt = 15.2 min** C18-Lichenysin $C_{56}H_{100}N_8O_{12}$ [M + H]$^+$ 1077.7534 Final Conc. [ug/L]
HY135	LB	STD R1		5	0.1	0.0	0.2	1.7	3.5	4.8	0.6	0.0	0.0
HY136	LB	STD R2		5	0.1	0.0	1.0	6.7	12.6	16.1	1.3	0.2	0.0
HY137	LB	STD R3		5	0.1	0.0	113.9	582.8	943.0	602.5	44.3	6.7	0.0
HY138	SM	STD R1		5	0.1	0.0	5.2	29.5	47.4	36.3	2.2	0.3	0.0
HY139	SM	STD R2		5	0.1	0.0	25.1	143.6	228.1	166.9	9.1	1.4	0.0
HY140	SM	STD R3		5	0.1	0.0	78.7	602.2	841.5	590.6	27.0	3.4	0.0
HY141	LB	DSM13	R1-T0	5	0.1	0.0	0.0	0.0	0.7	0.5	0.0	0.0	0.0
HY142	LB	B4094	R1-T0	5	0.1	0.0	0.0	0.0	0.9	0.6	0.0	0.0	0.0
HY143	LB	B4123	R1-T0	5	0.1	0.0	0.0	0.0	0.2	0.4	0.0	0.0	0.0
HY144	LB	DSM13	R1-T4	5	0.1	0.0	0.0	0.0	0.3	0.4	0.0	0.0	0.0
HY145	LB	B4094	R1-T4	5	0.1	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0
HY146	LB	B4123	R1-T4	5	0.1	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0
HY147	LB	DSM13	R1-T6	5	0.1		0.0	0.0	0.0	0.3	0.0	0.0	0.0
HY148	LB	B4094	R1-T6	5	0.1	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0
HY149	LB	B4123	R1-T6	5	0.1		0.0	0.0	0.0	0.2	0.0	0.0	0.0
HY150	LB	DSM13	R1-T8	5	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
HY151	LB	B4094	R1-T8	5	0.1	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0
HY152	LB	B4123	R1-T8	5	0.1	0.0	0.0	0.2	1.5	2.7	0.0	0.0	0.0
HY153	LB	DSM13	R1-T10	5	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
HY154	LB	B4094	R1-T10	5	0.1	0.0	1.5	14.6	154.2	155.0	22.1	2.0	0.0
HY155	LB	B4123	R1-T10	5	0.1	0.0	9.2	130.3	817.2	723.7	92.5	7.4	0.0
HY156	LB	DSM13	R1-T12	5	0.1	0.0	0.0	0.0	0.0	0.6		0.0	0.0
HY157	LB	B4094	R1-T12	1	0.5	0.0	247.0	2545.9	7875.0	10106.7	1671.6	218.0	0.0
HY158	LB	B4123	R1-T12	1	0.5	0.0	116.8	1847.7	3541.3	6973.2	785.6	126.9	0.0
HY159	LB	DSM13	R1-T16	5	0.1	0.0	0.1	2.0	9.3	11.6	0.6	0.1	0.0
HY160	LB	B4094	R1-T16	1	0.5	0.0	284.4	2740.8	8432.9	10949.0	1816.5	241.5	0.0
HY161	LB	B4123	R1-T16	1	0.5	0.0	130.6	1895.4	4173.7	7559.9	887.3	141.0	0.0
HY162	LB	DSM13	R1-T20	5	0.1	0.0	0.1	2.1	10.1	11.2	0.0	0.1	0.0
HY163	LB	B4094	R1-T20	1	0.5	0.0	273.8	3048.5	9117.0	12774.1	2032.5	300.5	0.0
HY164	LB	B4123	R1-T20	1	0.5	0.0	147.2	2037.2	4324.5	7833.2	928.1	148.0	0.0
HY165	LB	DSM13	R1-T24	5	0.1	0.0	0.2	1.6	8.6	10.2	0.7	0.1	0.0
HY166	LB	B4094	R1-T24	1	0.5	0.0	208.1	2345.0	6737.7	9057.2	1337.4	241.0	0.0
HY167	LB	B4123	R1-T24	1	0.5	0.0	170.3	2301.3	4984.7	9143.3	1131.2	182.4	0.0
HY168	LB	DSM13	R1-T48	5	0.1	0.0	0.0	1.3	8.4	14.7	1.1	0.2	0.0
HY169	LB	B4094	R1-T48	1	0.5	0.0	60.4	1106.4	4857.4	7470.2	1057.5	199.7	0.0
HY170	LB	B4123	R1-T48	1	0.5	0.0	46.7	1173.0	4235.1	8790.1	1124.0	204.3	0.0
HY171	LB	DSM13	R1-T72	5	0.1	0.0	0.0	1.3	9.7	16.8	1.2	0.3	0.0
HY172	LB	B4094	R1-T72	1	0.5	0.0	20.6	624.4	5455.3	10909.8	1745.6	305.6	0.0
HY173	LB	B4123	R1-T72	1	0.5	0.0	0.9	74.4	882.3	3187.7	345.6	82.3	0.0
HY174	LB	DSM13	R2-T0	5	0.1	0.0	0.0	0.0	0.4	0.5	0.0	0.0	0.0
HY175	LB	B4094	R2-T0	5	0.1	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0
HY176	LB	B4123	R2-T0	5	0.1	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0
HY177	LB	DSM13	R2-T4	5	0.1		0.0	0.0	0.0	0.2	0.0	0.0	0.0
HY178	LB	B4094	R2-T4	5	0.1	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0
HY179	LB	B4123	R2-T4	5	0.1	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0
HY180	LB	DSM13	R2-T6	5	0.1		0.6	10.2	66.8	109.1	8.9	1.9	0.0
HY181	LB	B4094	R2-T6	5	0.1		0.0	0.0	0.0	0.0	0.0	0.0	0.0
HY182	LB	B4123	R2-T6	5	0.1	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0
HY183	LB	DSM13	R2-T8	5	0.1		0.0	0.0	0.0	0.1	0.0	0.6	0.0
HY184	LB	B4094	R2-T8	5	0.1	0.0	0.0	0.2	1.5	2.9	0.3	0.0	0.0
HY185	LB	B4123	R2-T8	5	0.1	0.0		0.2	4.2	6.8	0.9	0.0	0.0
HY186	LB	DSM13	R2-T10	5	0.1	0.0				0.1	0.0		0.0

HY187	LB	B4094	R2-T10	5	0.1	0.0	0.0	1.6	22.3	28.7	4.9	0.5	0.0
HY188	LB	B4123	R2-T10	5	0.1	0.0	74.7	900.3	4045.4	3394.9	532.2	76.6	0.0
HY189	LB	DSM13	R2-T12	5	0.1	0.0	0.0	0.3	1.9	2.7	0.0	0.0	0.0
HY190	LB	B4094	R2-T12	1	0.5	0.0	222.6	2035.5	7580.6	8830.0	1658.9	187.1	0.0
HY191	LB	B4123	R2-T12	1	0.5	0.0	176.8	2447.4	4821.0	9362.5	1152.3	184.0	0.0
HY192	LB	DSM13	R2-T16	5	0.1		0.0	0.9	4.7	7.8	0.3	0.1	0.0
HY193	LB	B4094	R2-T16	1	0.5	0.0	492.0	5328.7	12728.2	20692.6	3062.9	562.8	0.0
HY194	LB	B4123	R2-T16	1	0.5	0.0	214.0	2972.7	5279.2	11208.8	1212.6	195.7	0.0
HY195	LB	DSM13	R2-T20	5	0.1	0.0	0.3	5.9	27.0	42.3	2.8	0.3	0.0
HY196	LB	B4094	R2-T20	1	0.5	0.0	441.0	5353.7	13137.4	21417.8	3298.8	596.0	0.0
HY197	LB	B4123	R2-T20	1	0.5	0.0	179.2	2741.6	5144.8	11232.4	1272.1	207.0	0.0
HY196	LB	B4094	R2-T20	5	0.1	0.0	407.2	3692.2	17651.7	8004.4	2362.3	444.0	0.0
HY198	LB	DSM13	R2-T24	5	0.1	0.0	0.0	3.9	20.0	34.2	2.1	0.3	0.0
HY199	LB	B4094	R2-T24	1	0.5	0.0	309.4	4533.1	12798.7	21247.5	3215.5	587.2	0.0
HY200	LB	B4123	R2-T24	1	0.5	0.0	146.2	2504.3	5114.0	11096.0	1289.9	215.6	0.0
HY199	LB	B4094	R2-T24	5	0.1	0.0	296.9	3238.9	17141.4	8143.3	2366.3	453.8	0.0
HY201	LB	DSM13	R2-T48	5	0.1	0.0	0.0	3.0	15.2	23.0	1.3	0.3	0.0
HY202	LB	B4094	R2-T48	1	0.5	0.0	93.1	2651.1	10646.8	19985.9	3127.4	628.5	0.0
HY203	LB	B4123	R2-T48	1	0.5	0.0	14.7	617.7	2157.3	6231.0	634.8	123.9	0.0
HY204	LB	DSM13	R2-T72	5	0.1	0.0	0.0	0.5	4.1	7.5	0.3	0.1	0.0
HY205	LB	B4094	R2-T72	1	0.5	0.0	56.4	2103.9	9402.5	18828.9	2892.0	587.5	0.0
HY206	LB	B4123	R2-T72	1	0.5	0.0	13.9	735.6	3047.1	9293.4	1028.5	205.0	0.0
HY207	SM	DSM13	R1-T0	5	0.1	0.0	0.0	0.3	0.8	0.7	0.0	0.0	0.0
HY208	SM	B4094	R1-T0	5	0.1	0.0		0.0	0.0	0.3	0.0		0.0
HY209	SM	B4123	R1-T0	5	0.1	0.0	0.0	0.1	0.5	0.4			0.0
HY210	SM	DSM13	R1-T4	5	0.1	0.0	0.0	0.1	0.0	0.6			0.0
HY211	SM	B4094	R1-T4	5	0.1	0.0	0.0	0.1	0.0	0.3			0.0
HY212	SM	B4123	R1-T4	5	0.1	0.0	0.0	0.4	0.5	0.7	0.0		0.0
HY213	SM	DSM13	R1-T8	5	0.1	0.0	0.0	0.0	0.0	0.3	0.0		0.0
HY214	SM	B4094	R1-T8	5	0.1	0.0	0.0	0.3	0.6	1.2			0.0
HY215	SM	B4123	R1-T8	5	0.1	0.0	0.0	0.2	0.3	0.6	0.0	0.0	0.0
HY216	SM	DSM13	R1-T12	5	0.1	0.0	0.0	0.1	0.0	0.3	0.0		0.0
HY217	SM	B4094	R1-T12	5	0.1	0.0	0.0	0.2	0.4	0.9	0.0	0.0	0.0
HY218	SM	B4123	R1-T12	5	0.1	0.0	0.0	0.4	1.0	2.0	0.0	0.0	0.0
HY219	SM	DSM13	R1-T16	5	0.1	0.0	0.0	0.1	0.0	0.2		0.0	0.0
HY220	SM	B4094	R1-T16	5	0.1	0.0	0.0	0.1	0.2	0.4			0.0
HY221	SM	B4123	R1-T16	5	0.1	0.0	0.0	0.0	0.0	0.3	0.0		0.0
HY222	SM	DSM13	R1-T18	5	0.1	0.0	0.0	0.0	0.0	0.3			0.0
HY223	SM	B4094	R1-T18	5	0.1	0.0	0.0	0.0	0.0	0.2	0.0		0.0
HY224	SM	B4123	R1-T18	5	0.1	0.0	0.0	0.1	0.2	0.5		0.0	0.0
HY225	SM	DSM13	R1-T20	5	0.1	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0
HY226	SM	B4094	R1-T20	5	0.1	0.0	0.0	0.0	0.0	0.5			0.0
HY227	SM	B4123	R1-T20	5	0.1	0.0	0.0	0.3	6.8	1.9	0.0		0.0
HY228	SM	DSM13	R1-T22	5	0.1	0.0	0.0	0.0	0.3	0.4		0.0	0.0
HY229	SM	B4094	R1-T22	5	0.1	0.0	0.9	3.4	19.2	7.3	1.3	0.0	0.0
HY230	SM	B4123	R1-T22	5	0.1	0.0	3.1	18.6	182.4	126.3	30.5	2.1	0.0
HY231	SM	DSM13	R1-T24	5	0.1	0.0	0.0	0.1	1.2	0.9	0.0	0.0	0.0
HY232	SM	B4094	R1-T24	5	0.1	0.0	15.9	76.7	698.4	200.3	70.3	2.2	0.0
HY233	SM	B4123	R1-T24	5	0.1	0.0	9.9	51.0	289.7	192.6	46.4	3.8	0.0
HY234	SM	DSM13	R1-T26	5	0.1	0.0	0.0	0.8	4.1	6.0	0.2	0.0	0.0
HY235	SM	B4094	R1-T26	5	0.1	0.0	109.7	411.7	1769.0	653.8	139.1	5.3	0.0
HY236	SM	B4123	R1-T26	5	0.1	0.0	17.0	73.6	380.0	231.2	46.8	3.4	0.0
HY237	SM	DSM13	R1-T28	5	0.1	0.0	0.0	0.9	4.9	2.6	0.4		0.0
HY238	SM	B4094	R1-T28	5	0.1	0.0	1.1	4.2	22.1	10.2	1.4	0.0	0.0
HY239	SM	B4123	R1-T28	5	0.1	0.0	16.5	68.0	375.7	223.7	39.3	2.8	0.0
HY240	SM	DSM13	R1-T48	5	0.1	0.0	0.0	1.0	7.1	3.9	0.0	0.0	0.0
HY241	SM	B4094	R1-T48	1	0.5	0.0	602.9	2011.1	6983.7	4178.1	554.8	34.4	0.0
HY242	SM	B4123	R1-T48	5	0.1	0.0	40.2	114.2	682.8	302.7	50.5	3.2	0.0
HY243	SM	DSM13	R1-T72	5	0.1	0.0	0.0	0.3	2.3	1.8	0.0	0.0	0.0
HY244	SM	B4094	R1-T72	5	0.1	15.0	338.0	1057.0	4222.5	1908.8	328.7	21.7	0.0
HY245	SM	B4123	R1-T72	5	0.1	3.0	31.1	92.1	544.4	258.2	36.5	2.2	0.0
HY246	SM	DSM13	R2-T0	5	0.1	0.0	0.0	0.2	0.0	0.2			0.0
HY247	SM	B4094	R2-T0	5	0.1	0.0	0.1	0.3	0.0	0.5	0.0		0.0
HY248	SM	B4123	R2-T0	5	0.1	0.0	0.0	0.3	0.6	1.0	0.0	0.0	0.0
HY249	SM	DSM13	R2-T4	5	0.1	0.0	0.0	0.6	1.3	1.8	0.0		0.0
HY250	SM	B4094	R2-T4	5	0.1	0.0	0.0	0.4	0.7	1.0	0.0	0.0	0.0
HY251	SM	B4123	R2-T4	5	0.1	0.0	0.0	0.6	0.9	1.3			0.0
HY252	SM	DSM13	R2-T8	5	0.1	0.0	0.0	0.6	1.2	1.5	0.0		0.0
HY253	SM	B4094	R2-T8	5	0.1	0.0	0.0	0.4	1.2	1.2	0.0		0.0
HY254	SM	B4123	R2-T8	5	0.1	0.0	0.0	0.4	0.7	1.1	0.0		0.0
HY255	SM	DSM13	R2-T12	5	0.1	0.0	0.0	0.3	0.0	0.7		0.0	0.0
HY256	SM	B4094	R2-T12	5	0.1	0.0	0.0	0.6	1.4	3.0	0.0	0.0	0.0
HY257	SM	B4123	R2-T12	5	0.1	0.0	0.0	0.4	0.7	1.0	0.0	0.0	0.0
HY258	SM	DSM13	R2-T16	5	0.1	0.0	0.0	0.4	0.6	1.1		0.0	0.0
HY259	SM	B4094	R2-T16	5	0.1	0.0	0.0	0.0	0.2	0.3		0.0	0.0
HY260	SM	B4123	R2-T16	5	0.1	0.0	2.2	9.9	61.4	44.9	8.6	0.8	0.0
HY261	SM	DSM13	R2-T18	5	0.1	0.0	0.0	0.1	0.0	0.3	0.0		0.0

HY262	SM	B4094	R2-T18	5	0.1	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0
HY263	SM	B4123	R2-T18	5	0.1	0.0		0.0	0.0	0.3		0.0	0.0
HY264	SM	DSM13	R2-T20	5	0.1	0.0	0.0	0.1	0.4	0.7	0.0	0.0	0.0
HY265	SM	B4094	R2-T20	5	0.1	0.0	0.0	1.8	14.9	4.1	0.7	0.0	0.0
HY266	SM	B4123	R2-T20	5	0.1	0.0	1.1	6.8	81.5	36.0	6.5	0.2	0.0
HY267	SM	DSM13	R2-T22	5	0.1	0.0	0.0	0.0	0.4	0.4	0.0	0.0	0.0
HY268	SM	B4094	R2-T22	5	0.1	0.0	4.6	27.3	241.5	136.8	47.2	2.1	0.0
HY269	SM	B4123	R2-T22	5	0.1	0.0	7.4	47.2	293.1	241.6	59.3	4.5	0.0
HY270	SM	DSM13	R2-T24	5	0.1	0.0	0.0	0.4	2.5	1.5	0.0	0.0	0.0
HY271	SM	B4094	R2-T24	5	0.1	0.0	19.6	109.6	619.2	253.9	65.6	2.6	0.0
HY272	SM	B4123	R2-T24	5	0.1	0.0	12.2	61.4	330.2	229.3	44.7	3.1	0.0
HY273	SM	DSM13	R2-T26	5	0.1	0.0	0.0	0.8	6.7	4.0	0.4	0.0	0.0
HY274	SM	B4094	R2-T26	5	0.1	14.8	100.6	359.0	1730.2	690.8	115.0	5.0	0.0
HY275	SM	B4123	R2-T26	5	0.1	0.0	19.0	80.6	409.5	260.1	41.9	2.7	0.0
HY276	SM	DSM13	R2-T28	5	0.1	0.0	0.1	0.8	4.3	3.9	0.3	0.0	0.0
HY277	SM	B4094	R2-T28	5	0.1	15.5	358.0	1086.0	4527.6	1793.6	329.4	15.9	0.0
HY278	SM	B4123	R2-T28	5	0.1	0.0	25.1	70.8	434.9	194.2	26.8	1.8	0.0
HY279	SM	DSM13	R2-T48	5	0.1	0.0	0.0	0.5	4.0	2.0	0.0	0.0	0.0
HY280	SM	B4094	R2-T48	5	0.1	23.2	169.7	537.8	2582.6	1039.2	158.9	8.7	0.0
HY281	SM	B4123	R2-T48	5	0.1	0.0	17.6	67.5	377.2	228.4	39.2	2.7	0.0
HY282	SM	DSM13	R2-T72	5	0.1	0.0	0.0	1.1	7.0	6.3	0.7	0.1	0.0
HY283	SM	B4094	R2-T72	5	0.1	10.4	85.6	300.9	1569.7	688.2	105.2	5.9	0.0
HY284	SM	B4123	R2-T72	5	0.1	0.4	34.6	107.2	705.2	362.9	53.5	3.6	0.0

Supplementary Table S5: Cell concentration (log10 CFU/ ml) and total lichenysin production (µg/ml) at T48 and T72 h in LB medium and skimmed milk

Timepoint/ concentration		**48 h**		**72 h**	
Medium	**Strain**	Cell concentration (log CFU/ml)	Lichenysin (µg/ml)	Cell concentration (log CFU/ml)	Lichenysin (µg/ml)
LB	DSM13	8.7	0.0	8.7	0.0
	B4094	8.9	25.9	8.9	26.5
	B4123	8.9	12.7	8.7	9.5
Skimmed	DSM13	8.6	0.0	9	0.0
milk	B4094	8.8	9.4	9.1	5.3
	B4123	8.7	1.0	9.1	1.1

Supplementary Table S6: Lichenysin production and variant distribution in biomass obtained from LB agar and skimmed milk agar at 37°C

Strain	Medium	injection vol (µL)	Rt = 9.4 min **C11-Lichenysin** $C_{49}H_{86}N_8O_{12}$ $[M+H]^+$ 979.6438 Final Conc. [µg/kg]	Rt = 10.3 min **C12-Lichenysin** $C_{50}H_{88}N_8O_{12}$ $[M+H]^+$ 993.6595 Final Conc. [µg/kg]	Rt = 11.2 min **C13-Lichenysin** $C_{51}H_{90}N_8O_{12}$ $[M+H]^+$ 1007.6751 Final Conc. [µg/kg]	Rt = 12.4 min **C14-Lichenysin** $C_{52}H_{92}N_8O_{12}$ $[M+H]^+$ 1021.6908 Final Conc. [µg/kg]	Rt = 13.3 min **C15-Lichenysin** $C_{53}H_{94}N_8O_{12}$ $[M+H]^+$ 1035.7064 Final Conc. [µg/kg]	Rt = 14.3 min **C16-Lichenysin** $C_{54}H_{96}N_8O_{12}$ $[M+H]^+$ 1049.7221 Final Conc. [µg/kg]	Rt = 15.1 min **C17-Lichenysin** $C_{55}H_{98}N_8O_{12}$ $[M+H]^+$ 1063.7377 Final Conc. [µg/kg]	Rt = 15.4 min **C18-Lichenysin** $C_{56}H_{100}N_8O_{12}$ $[M+H]^+$ 1077.7534 Final Conc. [µg/kg]	**Total-Lichenysin** Final Conc. [µg/kg]
B4094	LB agar	1	< 5	252.4	11084.0	59083.3	168680.3	23845.1	3687.2	77.3	266709
B4123	LB agar	1	< 5	1328.2	16753.1	35310.9	145947.4	13329.9	5270.4	42.8	217983
DSM13	LB agar	1	< 5	46.4	893.9	2849.0	19781.1	1046.4	484.4	4.2	25105
B4094	LB agar	1	< 5	93.2	3562.4	22246.2	100760.1	8534.5	3245.6	43.9	138486
B4123	LB agar	1	21.1	2555.8	30212.8	35076.2	91533.5	6206.5	2207.2	< 5	167813
DSM13	LB agar	1	n.d.	20.4	659.1	1315.6	7268.3	289.3	124.7	< 5	9677
			Rt = 8.7 min	Rt = 9.7 min	Rt = 10.6 min	Rt = 11.8 min	Rt = 12.8 min	Rt = 13.8 min	Rt = 14.6 min	Rt = 15.2 min	

Standard	Medium	injection vol (µL)	**C11-Lichenysin** $C_{49}H_{86}N_8O_{12}$ $[M+H]^+$ 979.6438 Final Conc. [µg/kg]	**C12-Lichenysin** $C_{50}H_{88}N_8O_{12}$ $[M+H]^+$ 993.6595 Final Conc. [µg/kg]	**C13-Lichenysin** $C_{51}H_{90}N_8O_{12}$ $[M+H]^+$ 1007.6751 Final Conc. [µg/kg]	**C14-Lichenysin** $C_{52}H_{92}N_8O_{12}$ $[M+H]^+$ 1021.6908 Final Conc. [µg/kg]	**C15-Lichenysin** $C_{53}H_{94}N_8O_{12}$ $[M+H]^+$ 1035.7064 Final Conc. [µg/kg]	**C16-Lichenysin** $C_{54}H_{96}N_8O_{12}$ $[M+H]^+$ 1049.7221 Final Conc. [µg/kg]	**C17-Lichenysin** $C_{55}H_{98}N_8O_{12}$ $[M+H]^+$ 1063.7377 Final Conc. [µg/kg]	**C18-Lichenysin** $C_{56}H_{100}N_8O_{12}$ $[M+H]^+$ 1077.7534 Final Conc. [µg/kg]	**Total-Lichenysin** Final Conc. [µg/kg]
STD R1	CanolaPro TM + cal st 10	1	n.d.	33.6	219.4	378.8	291.0	15.0	1.8	n.d.	939.4
STD R2	CanolaPro TM + cal st 12	1	n.d.	154.5	1025.6	1697.4	1369.9	67.7	12.8	n.d.	4327.8
STD R3	CanolaPro TM + cal st 14	1	n.d.	667.9	4341.9	7657.7	5360.1	282.0	63.9	n.d.	18373.4
B4094	Skimmed milk agar skimmed	1	29.1	676.1	3483.9	10873.3	5189.5	2128.0	392.3	n.d.	22772.2
B4123	milk agar skimmed	1	37.3	796.9	4171.5	11515.8	5451.6	2312.4	477.3	n.d.	24762.7
DSM13	milk agar skimmed	1	0.3	117.1	921.5	4639.2	2925.5	924.7	148.2	n.d.	9676.6
B4094	milk agar skimmed	1	0.3	47.5	937.4	5135.9	4428.8	1270.6	282.2	n.d.	12102.6
B4123	milk agar skimmed	1	0.9	102.7	1120.1	3138.0	3242.2	797.5	146.7	n.d.	8548.1
DSM13	milk agar	1	0.0	3.6	56.6	310.0	635.9	62.6	9.3	n.d.	1077.9

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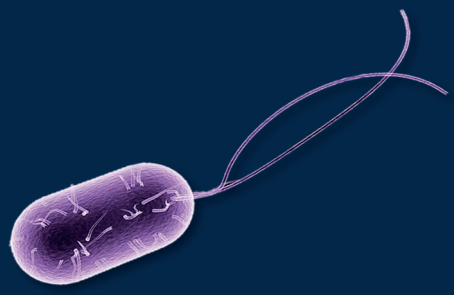
“ Discovery is worth discussing in perspectives based on evidence ”

CHAPTER 6



General Discussion

Kah Yen Claire Yeak

This thesis describes the investigation of the general stress response (GSR) mediated by the master regulator of adaptive stress - Sigma factor B (SigB), in three well-known *Bacillus* species: *Bacillus subtilis* (the model organism for spore-forming bacteria), *Bacillus cereus* (a foodborne pathogen), and *Bacillus licheniformis* (an industrial 'workhorse'). They are Gram-positive, spore-forming, and facultative anaerobic bacteria found ubiquitously in the environment. The SigB-mediated GSR plays a vital role in conferring resistance to cells to cope with physical or nutritional stresses, and recently, the roles of SigB in regulating various other cellular functions have become evident (**Chapter 1**). Novel SigB regulon members in *B. subtilis* were predicted in **Chapter 2,** and a number of these members were validated via promoter studies, extending the putative SigB regulon in *B. subtilis* (containing ~ 500 members). These members mainly cope with general stress and other cellular functions. Additionally, SigB signaling cascades and SigB regulon members orthologous to the *B. subtilis* 168 regulon members were found to be species-specific in other Bacillales members. Subsequently, in **Chapter 3,** novel genes and proteins (~ 300) that are SigB-dependent were revealed in *B. cereus* upon exposure to heat shock and under the control condition at 30°C, indicating that SigB controls a set of cellular functions unrelated to heat stress. A subgroup of the newly identified SigB-dependent genes/proteins was co-regulated by the putative phosphocarrier protein Bc1009. This protein was demonstrated to play a role in heat resistance and motility. In **Chapter 4,** a mild effect of SigB on the production of the secondary metabolite lichenysin (a surfactant) was demonstrated in *B. licheniformis*. This compound mildly induced GSR and provided modest protection to cells in lethal ethanol stress conditions. In **Chapter 5,** it was concluded that the production of lichenysin by different *B. licheniformis* food isolates strongly depend on the strain, medium composition, and incubation conditions. *In vitro* studies showed that lichenysin is toxic to human cells at ID_{50} of ~16 µg/ml. Therefore, when levels of *B. licheniformis* cells in foods reach high levels and such foods are ingested, this can cause foodborne intoxication.

Following the introduction in **Chapter 1**, the observations and results of **Chapters 2-5** provided a better understanding of bacterial stress adaptation. In this general discussion, three major topics are discussed. **Firstly,** the new insights on SigB mechanisms in *Bacillus*. **Secondly**, the ecological aspects of SigB and SigB-GSR, and **thirdly,** implications of the knowledge gained on SigB and SigB GSR for the food sector and the study of other foodborne pathogens.

6.1 New insights on SigB mechanisms in *Bacillus*

New information on the four main aspects of SigB GSR was acquired; namely, **i)** SigB regulon: genes/proteins that are SigB-dependent, regulated either directly or indirectly, **ii)** alternative roles of SigB in *Bacillus,* **iii)** SigB signal sensing cascades in *Bacillus*, and **iv)** known stressors that induce SigB in *Bacillus*.

6.1.1 Genes and proteins regulated by SigB: the SigB regulon

The activation of SigB leads to the transcription of a group of genes regulated by SigB, and thereby the expression of proteins (**Chapter 1).** These genes/proteins regulated by SigB either directly (have an upstream SigB **P**romoter **B**inding **M**otif) or indirectly (without a SigB PBM) are known as the SigB regulon members. In this thesis, those genes with a SigB PBM are referred to as the SigB direct regulon members; SigB can bind to the promoter of these genes and regulate their transcription directly. Those that do not have a SigB PBM are the SigB indirect regulon members and are likely regulated by SigB via an intermediate (e.g., a SigB-dependent member).

This section provides up-to-date information on the SigB regulon members of *B. subtilis* and *B. cereus* based on the results of **Chapters 2** and **3**, respectively, and the reported SigB regulon members in the literature **(**Chapter 1 appendixes**).** SigB regulon members for these two species are grouped into either direct or indirect members based on the presence/absence of either a known/predicted SigB PBM (Figure 1). No update is available for *B. licheniformis* as the locus tags used by Voigt et al. (2013) differ from those used for *B. licheniformis* type strain DSM13 in **Chapter 2;** therefore, no direct comparison was made.

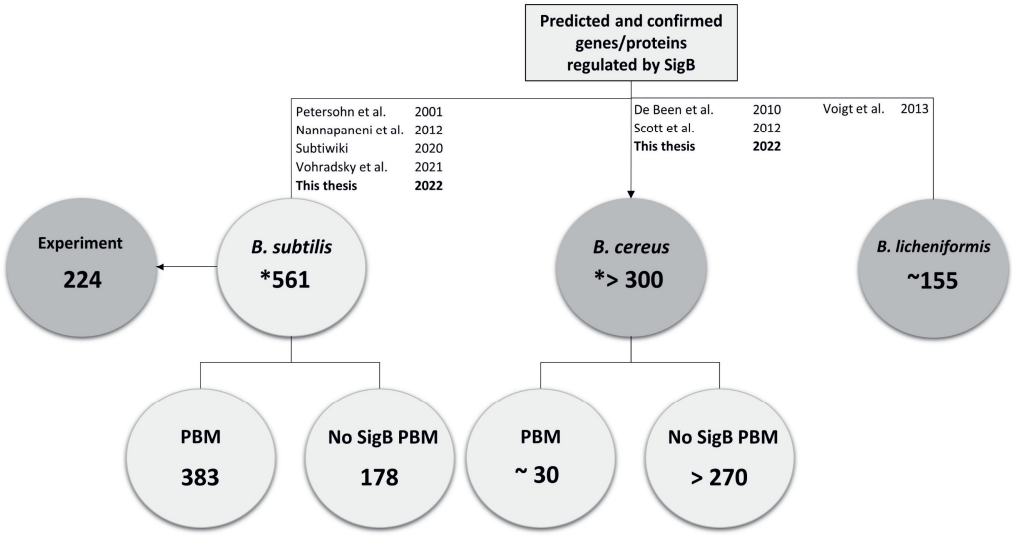


Figure 1- Up-to-date knowledge on SigB regulon members in *B. subtilis* 168, *B. cereus* ATCC14579 and *B. licheniformis* DSM13. Total genes or proteins that are identified via experiments or predicted to be regulated by SigB in the literature and this thesis. **Light grey circle-** SigB-dependent genes/proteins identified via predictions and experiments. **Dark grey circle-** SigB-dependent genes/proteins identified via experiments.

6.1.1.1 SigB regulon in *B. subtilis*

The updates on *B. subtilis* SigB regulon members compared to the list of SigB regulon genes listed on Subtiwiki are mainly based on the study of Vohradsky et al. (2021) and the results obtained in **Chapter 2**. Firstly, 411 SigB regulon members were reported by Vohradsky et al. (2021), including all the up-to-date SigB regulon members extracted from Subtiwiki and the literature. Secondly, 255 genes were predicted to have a SigB PBM of (Category I-V) in **Chapter 2.** Of these, 99 overlapped with the list published by Vohradsky et al. (2021), resulting in 156 newly predicted genes with a SigB PBM. The SigB regulon members were identified via experiments or predicted based on machine learning expression kinetics, kinetic modeling, or the presence of a SigB PBM in the promoter region of these genes (Nannapaneni et al., 2012; Vohradsky et al., 2021), including the 156 newly predicted members reported in **Chapter 2** of this thesis. Taking together all the genes/proteins identified via experiments or predicted in the literature and this thesis, *B. subtilis* carries a total of 567 potential SigB regulon candidates. By removing five genes that do not have a locus tag (BSU number) in the list of Vohradsky et al. (2021), 562 candidates remained (Appendix 1A Chapter 1, Tables S1 and S2 Chapter 2).

Out of these 562 members, 383 were predicted to have a SigB PBM based on information available in the literature (Petersohn et al., 2001; Nannapaneni et al., 2012; Vohradsky et al., 2021) and this thesis **(Chapter 2),** indicating ~ 68% are SigB direct regulon members in *B. subtilis*. The remaining 178 (~ 22%) did not have a SigB PBM and are SigB indirect regulon members. A total of 224 out of 562 showed significant differential regulation in proteomics approaches/DNA array-based expression studies in at least one of the following conditions tested: exposure to ethanol, butanol, osmotic, oxidative stress, growth at low temperature, and heat shock (Nannapaneni et al., 2012). For the remaining predicted members, their SigB-controlled activity remains to be confirmed via experimental work. Nonetheless, the vast set of SigB regulon candidates in *B. subtilis* demonstrated that the SigB regulon might be more extensive than previously thought. Many candidates are known to be regulated by additional transcriptional regulators. These may thus be involved in other regulatory networks than GSR and do not have a SigB PBM that resembles the consensus (**Chapter 2,** (Nannapaneni et al., 2012; Vohradsky et al., 2021)), nor display standard SigB-dependent GSR upon exposure to standard stressors (discussed in section 6.1.4).

6.1.1.2 SigB regulon in *B. cereus*

In comparison to *B. subtilis*, not many large-scale DNA array-based or proteomics-based studies have been performed to investigate the SigB GSR in *B. cereus*. Only ~30 SigB regulon members (both direct and indirect) were initially described in the literature by de Been et al. (2010) and Scott and Dyer (2012). Thus, it is generally recognized that the SigB regulon in *B. cereus* is smaller than in *B. subtilis* (Rodriguez Ayala et al., 2020).

In this thesis, the core function of SigB in *B. cereus* was deciphered by analyzing the changes in proteome/transcriptome profiles of the clean Δ*sigB* mutant versus wt cells under heat stress (30°C > 42°C) and at 30°C, and novel SigB-dependent genes/proteins under both conditions

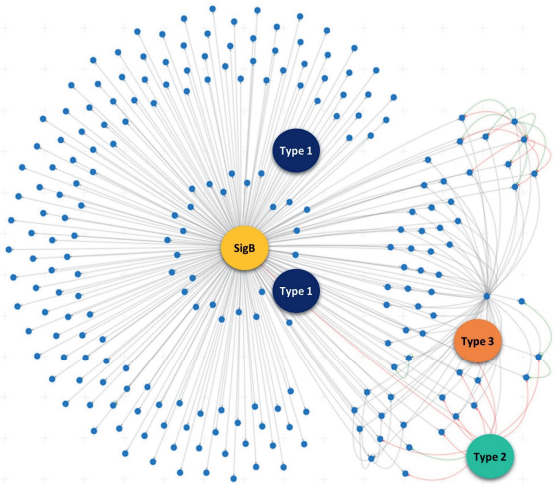
were identified (**Chapter 3**). While differentially regulated proteins were highlighted in **Chapter 3**, differential expression of specific gene clusters (but not detected in proteomes) was also observed, suggesting that the SigB regulon in *B. cereus* may exceed 300 members (see Supplementary Tables Chapter 3). Nevertheless, SigB regulon members containing a SigB PBM remained low, approximately 10%. (discussed in Chapter 3).

Using the same definition described for *B. subtilis,* ~10% of the candidates (~ 300) are SigB direct regulon members, and the remaining candidates appeared (so far) to be SigB indirect regulon members in *B. cereus*. Whether more of these SigB regulon members contain a functional SigB PBM is yet to be elucidated. This finding showed that the size of the SigB direct regulon in *B. cereus* is still much smaller than in *B. subtilis,* which may be attributed to more pleiotropic regulators that are SigB-dependent in *B. cereus*. Moreover, as discussed in **Chapter 3,** most newly identified SigB-dependent genes and proteins formed a SigB subregulon controlled by the putative phosphocarrier protein Bc1009, signifying that these are indirectly regulated by SigB via Bc1009.

The SigB subregulon members and other newly identified SigB regulon members (**Chapter 3**) have putative functions in cell motility, signal transduction, transcription, amino acid/carbohydrate/ion transport, and metabolism. Of which, roles in heat resistance and motility were confirmed in phenotypic assays. Remarkably, many of these are transcriptional regulators, indicating that SigB may also be involved in other cellular responses as reported for *B. subtilis* (e.g., sporulation, biofilm formation, etc.) discussed in section 6.1.2.

6.1.1.3 SigB direct and indirect regulation in *Bacillus*

Based on the results of **Chapters 2 and 3**, Figure 2 illustrates the direct and indirect regulatory relationships between SigB and genes/proteins that it controls. Genes with a SigB PBM allow the direct binding of SigB to execute direct regulation (Type 1). Genes without a SigB PBM but show SigB-dependent expressions may be regulated by SigB direct regulon members (Type 2) or their expression may be affected by SigB indirectly via a cascade effect (Type 3). Both direct and indirect regulation can be either positively controlled (induction/upregulation) or negatively controlled (repression/inhibition) (Green line: positive; red line: negative in Figure 2). The role of SigB as a transcriptional activator resulting in gene induction response is typically better known than the negative gene response (Rodriguez Ayala et al., 2020). However, SigB inhibition activity is not new, as SigB has been reported to negatively impact sporulation by prohibiting the onset of spore formation in *B. subtilis* (Reder et al., 2012a, 2012b; Rothstein et al., 2017). Taking an example from **Chapter 2,** the deletion of the *sigB* gene in *B. subtilis* also resulted in slightly higher promoter activity of the *ylaL* gene under the control condition without further stress imposition, indicating putative SigB-dependent repression activity. Therefore, SigB may be a repressor, either indirectly or directly. More evidence of the SigB-dependent repression/inhibition was gathered for *B. cereus,* in which the expression of regulators such as ArgR or a group of genes involved in anaerobic respiration was higher in the absence of SigB than in its presence (discussed in Chapter 3).



SigB regulon members	Genes/proteins
Type 1	Directly regulated by SigB, have a SigB promoter binding motif
Type 2	Indirectly regulated by SigB via SigB-direct regulon members, does not have a SigB promoter binding motif
Type 3	Indirectly regulated by SigB via cascade effects

Figure 2- SigB direct and indirect regulatory relationships in *Bacillus* cells

SigB-dependent repression is rarely mentioned in the literature, despite evidence of genes/proteins being upregulated when SigB is absent in *B. subtilis* (Rodriguez Ayala et al., 2020). This is because studies of SigB binding to the upstream regions of these genes/operons were not done to confirm the repression effect of SigB further. Additionally, most studies compared genes/proteins expression patterns in a setting of the wt expression levels versus Δ*sigB* mutants. The upregulation observed in the Δ*sigB* mutant can result from perturbations in gene expressions caused by the deletion of SigB or attributed to the Sigma factor competition for shared promoters, contributing to the transcriptional repression of associated genes (Mauri and Klumpp, 2014). For instance, if gene A is co-regulated by SigB and a competitor $SigB_C$, both compete to bind to the promoter of gene A, causing repression of gene A. Thus, deletion of SigB leads to the "de-repression" effect observed for gene A, but in a wt situation, SigB does not necessarily "repress" gene A. Furthermore, temperature, pH, water activity, or the medium composition used in the experiment may affect bacteria integrating the signaling response, causing indirect "repression" effects.

On the other hand**,** different SigB threshold levels (i.e., amount) in *Bacillus* cells may regulate distinct sets of genes. It is known that elevated expression of SigB followed by its activation (uncoupling from RsbW) mediates the GSR (**Chapter 1**), but the threshold levels of SigB required to regulate each gene are unknown. If the threshold level of SigB varies in different physiological states (i.e., unstressed and stressed conditions), a low threshold level of SigB may limit the binding of SigB to all known SigB-regulated promoters. Thus, *Bacillus* cells may program their expression machinery to prioritize the expression of genes that are only required at a specific physiological state (see conceptual model, Figure 3). For instance, two of the tested candidates with SigB PBMs that highly resembled the consensus failed to show SigB-dependent activity under the tested conditions (**Chapter 2**). Other than the reasons described

in **Chapter 2** (i.e., low binding affinity due to extended spacer, triggered by unknown stressors), these genes may be regulated at a high SigB threshold level, and the induction of SigB does not necessarily increase the promoter activity (e.g., *ylaL, ygaO, ykaA,* Chapter 2 Figure 3). This is in line with the notion that SigB is not only responsible for the adaptive stress regulation in *Bacillus* spp., but also for indirect regulation of other cellular responses (discussed in **Chapters 2 and 3**), likely at different SigB threshold levels.

The extensive information gained on the SigB regulon in this thesis further elucidated SigB regulon structures for *B. subtilis,* especially for *B. cereus*. Such detailed data are useful for further studies to obtain a complete picture of the SigB-controlled regulatory functions in *Bacillus* spp.

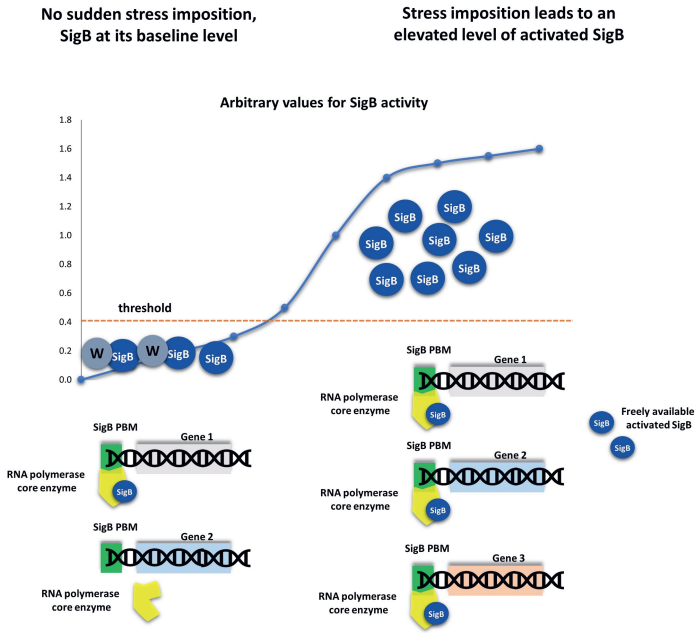


Figure 3- Conceptual model for SigB regulation at different threshold values in cells. SigB is expressed at its baseline level as controlled by SigA in the absence of stress, and remains bound to RsbW. Low amount of SigB may be free and may guide RNA polymerase to the SigB promoter binding motif (PBM), and regulate Gene 1 putatively. Gene 2 which also has a SigB PBM was not regulated by SigB at baseline due to the low threshold. Upon exposure to stressors, the expression of SigB is elevated, and uncoupled from RsbW, allowing Gene 1 and 2, and additionally Gene 3 to be regulated concomitantly by SigB at a high threshold.

6.1.2 SigB regulatory networks: alternative roles of SigB

More than 500 and 300 confirmed and predicted genes/proteins are potentially regulated by SigB in *B. subtilis* and *B. cereus*, respectively. Not all are directly controlled by SigB (Figure 1), but those indirectly controlled provide further insights into the SigB regulatory networks.

In *B. subtilis,* the functional distribution map of SigB regulon members (including newly predicted members) illustrated that, other than in the GSR, SigB also participates in controlling sporulation, biofilm formation, information processing such as protein synthesis/modification/ degradation, transcription of other regulators, DNA repair and recombination, carbon metabolisms, biosynthesis or acquisition of amino acids, and a group of phage genes with unknown function (**Chapter 2).** Hence, the SigB regulon may show overlap with regulons that other transcriptional regulators control. The results obtained in **Chapter 2** aligned with the report of Vohradsky et al., 2021, who provided evidence that 37 other regulators shared governing roles with SigB in regulating SigB regulon members (both direct and indirect). This includes other sigma factors, i.e., SigA for housekeeping; SigM and SigW for dealing with cell surface stress; SigG, SigF, SigH, and SigE in sporulation for the transcription of early stationary phase genes, early forespores, mother cells, and sporulation specific genes, respectively; SigX for resistance against cationic antimicrobial peptides and temporary phage resistance; SigD for the regulation of flagella, motility, chemotaxis and autolysis; and SigI for control of heat shock genes (Zhu and Stülke, 2018; Vohradsky et al., 2021). SigB regulon members co-regulated by SigX in *B. subtilis* (Vohradsky et al., 2021) may provide cells with an adaptive phage tolerance to resist temporary phage attacks (Tzipilevich et al., 2022). Although no other studies have investigated the role of SigB in phage tolerance, 15 genes encoding for phage elements were predicted to have a SigB PBM in *B. subtilis* (**Chapter 2**), and the expression of a large group of phage genes was found inducible via SigB in *B. cereus* (**Chapter 3**). However, the functions of these phage genes are unknown, and SigB and SigX co-regulatory mechanisms are yet to be explored.

Additionally, SigB has been reported to indirectly influence the production of the antimicrobial peptide (surfactin) that has antifungal activities in *B. subtilis* (Bartolini et al., 2019a), and it was also shown in **Chapter 4** that SigB influenced the production of a similar compound, namely lichenysin in *B. licheniformis.* The production of these biosurfactants likely increases the motility of cells by reducing the surface tension in their environment, which may promote swimming toward nutrients or resources, especially in water-containing habitats like upper layers of mud/soils, and stagnant lakes, or ponds.

While the alternative roles of SigB in other processes are described in *B. subtilis,* e.g., sporulation (Reder et al., 2012b, 2012a; Rothstein et al., 2017), biofilm development (Bartolini et al., 2019b; Nadezhdin et al., 2020; Arnaouteli et al., 2021), motility (Bartolini et al., 2018), surfactin production for antifungal control (Bartolini et al., 2019a), less information is available for *B. cereus*. Only one study reported that SigB affected spore properties (de Vries et al., 2005)

and one that biofilm formation was influenced (Gao et al., 2021). However, it was not studied how SigB interacts with genes/proteins or regulators in the sporulation or biofilm formation pathway.

Based on the evidence of differentially regulated genes/proteins as presented in **Chapter 3,** a SigB regulatory network is mapped out in Figure 4 to illustrate the potential integration of SigB activity in lifestyle decision-making and metabolic crosstalk in *B. cereus*. Major regulators that showed prominent differential regulation by SigB are listed in Table 1, genes/proteins identified in **Chapter 3** are colored, and their potential interaction partners are marked in grey. Pointed arrows indicate positive interaction, and thwart arrows indicate negative interaction (Figure 4).

Firstly, the central glycolytic genes regulator CggR (Bc5141) showed SigB-dependent induction. It is a transcriptional repressor of the glycolytic *gapA* operon genes which encode catabolic enzymes in glycolysis (Brantl and Licht, 2010), indicating that SigB influences glycolysis. The CggR-mediated pathway was previously shown to be affected by the deletion of RpoN (a regulator of growth, carbohydrate metabolism, motility, biofilm formation, and toxin production), negatively influencing pyruvate availability when *B. cereus* is switching to fermentative metabolism under anaerobic conditions (Hayrapetyan et al., 2015).

Secondly, SigB may be involved in the positive or negative feedback regulation of sporulation in *B. cereus*. The phosphorelay regulator Spo0A (Bc4170) - responsible for sporulation initiation, and SigK (Bc4336) - responsible for mother cell-specific gene expression during sporulation (Sun et al., 2021), were induced by SigB under heat stress. On the other hand, AbrB (the transcriptional regulator of transition state genes that repressed sporulation sigma factor SigH) (Schultz et al., 2009; Rodriguez Ayala et al., 2020) showed increased expression in *ΔsigB* cells compared to wt at 30°C without further stress imposition.

Table 1: Summary of differential regulation of transcriptional regulators by SigB in *B. cereus*

Regulator	Description	Regulation by SigB
CggR	Central glycolytic genes regulator	Positive
Spo0A	Phosphorelay regulator for sporulation initiation	Positive
SinI, IcaR	SinR biofilm repressor antagonist	Negative
ArgR	Regulator of arginine metabolic genes	Negative
SigK	Sporulation-specific sigma factor	Positive
AbrB	Regulator of transition state genes	Positive

This discussion proposes that in positive regulation, SigB induces Spo0A, and the phosphorylated Spo0A becomes active and positively influences sporulation. Spo0A also activates AbbA (the AbrB repressor) to inhibit the expression of AbrB. The reduced expression of AbrB may release the SigH repression, thereby partially allowing SigH to contribute to sporulation. In negative regulation, SigB induces AbrB, AbrB repressed SigH and may impact

the sporulation process negatively. Moreover, Spo0E is SigB-dependent and blocks the transcription of *spo0A* and *spoIIE* genes, leading to a sporulation-deficient phenotype (Reder et al., 2012b).

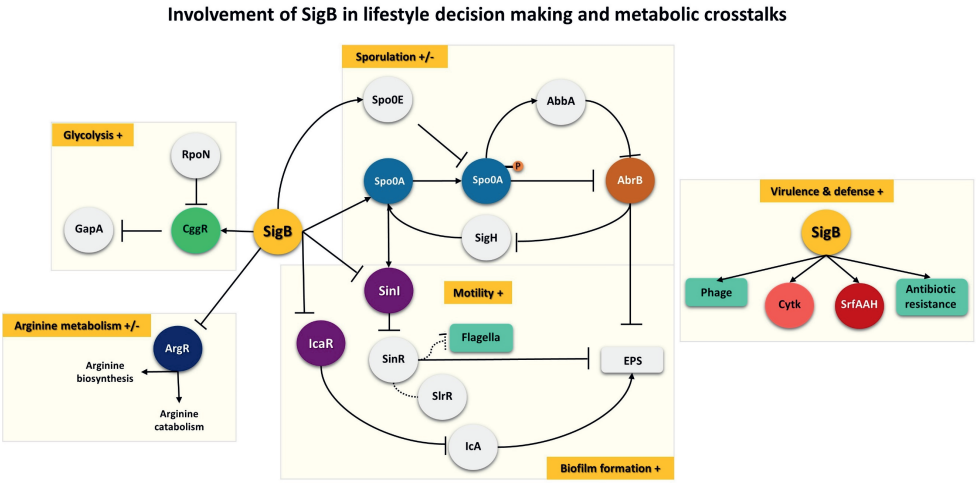


Figure 4 – Potential integration of SigB activity in lifestyle decision-making and metabolic crosstalk in *B. cereus*

Thirdly, SigB may regulate biofilm formation in a feedback loop. SigB was shown to induce Spo0A, and inhibit the expression of SinI (antagonist of SinR repressor for biofilm formation) and IcaR (Bc4652) (**Chapter 3**), a repressor that blocks IcA (a protein important for intracellular adhesion during biofilm formation) (Cue et al., 2009). The scenario depiction in positive regulation: 1) Spo0A is induced, becomes active upon phosphorylation, and in turn activates the expression of SinI, and SinI represses SinR to promote biofilm formation (Xu et al., 2017; Lin et al., 2022); 2) IcaR is inhibited, and thus IcA is derepressed, facilitating adhesion during biofilm formation. In negative regulation, SinI is repressed, the SinI-SinR interaction may be disrupted and SinR represses biofilm formation, forming a feedback loop.

Fourth, SigB positively regulates motility indirectly in *B. cereus,* which was confirmed via phenotypic assays in **Chapter 3.** This regulation may occur through the control of SinI. As SinI represses SinR, SlrR forms a complex with SinR and de-represses the inhibition of the expression of motility genes (Cozy et al., 2012).

Fifth, SigB is connected to nitrogen source utilization. It likely interacts with the ArgR feedback regulator (Pandey et al., 2020) depending on the absence or presence of arginine. In the presence of arginine, ArgR represses the expression of genes for arginine biosynthesis and activates the expression of genes for arginine catabolism, whereas, in the absence of arginine, ArgR activates the genes for biosynthesis (Cheng et al., 2017). In **Chapter 3,** it was shown that

SigB negatively impacted the level of ArgR, and Van Schaik et al. (2005) also reported that *B. cereus* cells failed to utilize many nitrogen sources, including L-arginine, in the absence of SigB.

Lastly, SigB may play a role in regulating genes/proteins related to virulence in *B. cereus* ATCC14579. For example, the *cytK (bc1110)* gene encoding for cytotoxin K, *bc0423*/Bc0423 (peptide synthase similar to *srfA* that is required to synthesize surfactin), a group of phage genes (with unknown function), and proteins related to antibiotic resistance were induced by SigB in *B. cereus* ATCC14579 (**Chapter 3)**. However, the interaction of SigB with these genes/proteins remains to be elucidated.

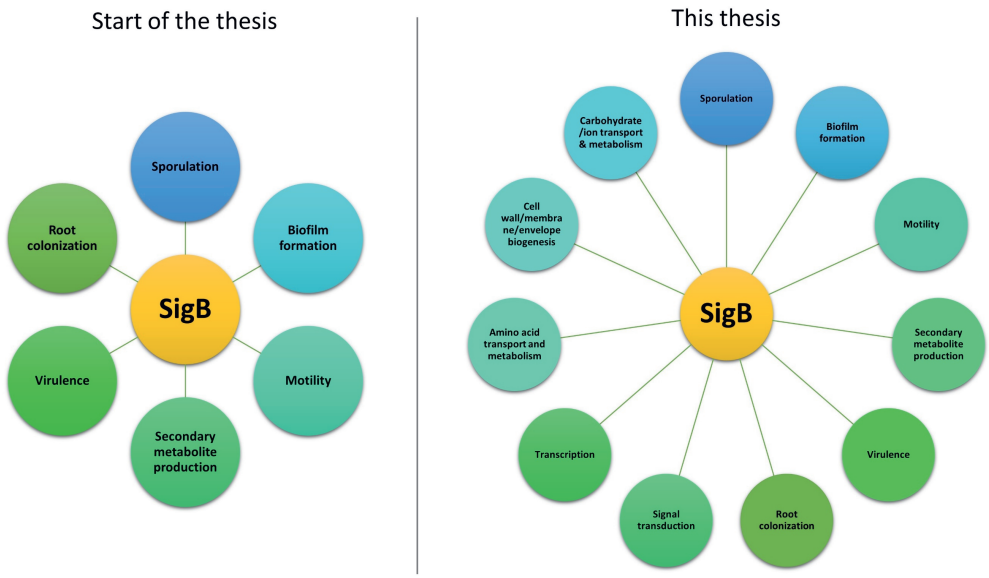


Figure 5- Influence of SigB in cellular responses and secondary processes in *Bacillus* species. Evidence was gathered via experiments performed with *B. cereus* (Chapter 3) and *B. licheniformis* (Chapter 4). *In silico* predictions for additional roles of SigB in other functions than the general stress response for *B. subtilis* are not shown in this figure but are described in Chapter 2.

The indications of SigB involvement in different cellular responses of *B. cereus* in **Chapter 3** and the indirect involvement of SigB in lichenysin production in **Chapter 4** provided additional evidence that SigB may influence more cellular functions/secondary processes than previously described **(Chapter 1),** at least in *Bacillus* species (Figure 5).

6.1.3 SigB signal sensing cascades

As introduced in **Chapter 1,** the stressosome is the signaling protein complex that detects the stress signals in *B. subtilis* group members and integrates the signals into the SigB signaling cascades (Delumeau et al., 2006). It comprises the RsbR, RsbS, and RsbT proteins, in which RsbR consists of N-terminal domains of RsbRA dimers, protruding from the protein as turrets and is thought to act as the sensory domain to integrate stress signals (Marles-Wright and Lewis, 2008). However, how the signals are being sensed by the stressosome and integrated into the SigB GSR mechanism is unknown.

Besides *B. subtilis* group members, the Bacillales member *L. monocytogenes* also employs a stressosome for stress signaling (Impens et al., 2017; Williams et al., 2019). The *Listeria* stressosome has been reported to interact with the mini membrane anchoring protein- Prli42 to sense environmental cues. Impens et al. (2017) described that Prli42 is conserved in firmicutes, able to interact with the *Bacillus* stressosome components, and is critical for stressosome activation in *L. monocytogenes*.

The amino acid sequence alignment of *L. monocytogenes* Prli42 and the *B. subtilis* homolog are presented in Figure 6A. The function of the Prli42 homolog in *B. subtilis* and its interaction with the *Bacillus* stressosome components were evaluated but not described in the previous chapters. No solid evidence was obtained to show that Prli42 is required to activate the SigB GSR nor that it interacts with any of the four RsbR paralogs in *B. subtilis*. A clean Δ*prli42* mutant was constructed to investigate its role in activating the stressosome-mediated GSR upon exposure to ethanol, heat, and oxidative stress via gene expression studies (Figure 6B). The gene expression results (measured via One-step RT- qPCR as described in Chapter 4) showed that the expression of *sigB* and two SigB-dependent genes (*ctc* and *rsbV*) in response to I) heat stress (upshift from 37°C to 48°C); II) 4% (v/v) ethanol stress and III) 0.05% (v/v) oxidative stress, did not differ between wt and Δ*prli42* cells (expression values for Δ*sigB* cells were lower than the cycle threshold, and not included in Figure 6B). Moreover, the deletion of *prli42* did not impact growth nor lead to decreased robustness of *Bacillus* cells when exposed to 10% (v/v) lethal ethanol conditions, with or without pre-adaptation to 4% (v/v) ethanol (Figure 6C).

Furthermore, the Prli42 homolog of *B. subtilis* was chemically synthesized with the TAMRA fluorophore conjugated to the carboxy-terminal end of the peptide to study the interaction of Prli42 with the N-terminal domains of four purified RsbR paralogs (RsbRA, RsbRB, RsbRD, RsbRD) via fluorescence polarization assays. This work was performed in collaboration with Sema Edjer, Rick Lewis, and John Marles-Wright (unpublished data reported in the PhD thesis entitled 'Structure-function relationships in stressosome complexes from *Listeria monocytogenes* and *Bacillus subtilis*', 2021, Newcastle University). No interaction was found between the cytosolic tail of the *B. subtilis* Prli42 homolog and the RsbR N-terminal domains *in vitro*. Based on these results, we concluded that the Prli42 homolog does not interact with the *Bacillus* stressome and is not critical for activating SigB GSR in *B. subtilis*. As no other potential ligands were found to interact with RsbR, the mechanisms of how the stressosome

senses and integrates the stress signal into the SigB signaling cascades remain to be established.

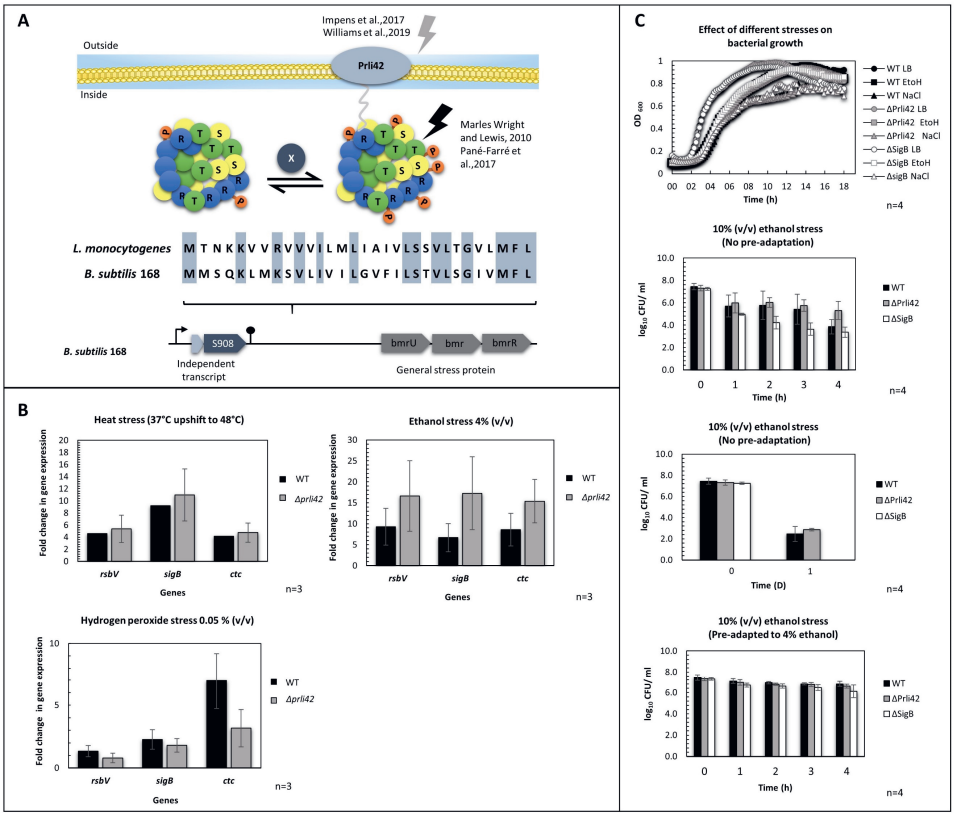


Figure 6- Prli42 homolog and functionality in *B. subtilis*. **6A-** Interaction model of Prli42 to the stressosome component as suggested by Impens et al. (2017) and Williams et al. (2019) and the multiple sequence alignment of the Prli42 homolog sequence in *B. subtilis* 168 to *L. monocytogenes*. Conserved amino acids higher than 55% are highlighted. The Prli42 homolog in *B. subtilis* is an independent transcript located upstream of the new RNA feature S908. **6B-** Fold changes in the expression of *sigB* and two SigB-dependent genes (*ctc* and *rsbW*) in *B. subtilis* wt (**black bar**) and Δ*prli42* (**grey bar**) cells upon exposure to heat stress (37°C upshifted to 48°C), 4% (v/v) ethanol and 0.05% (v/v) hydrogen peroxide at exponential phase (OD_{600nm} of ~ 0.35). **6C-** Growth and viable counts of wt (**black bar**), Δ*prli42* (**grey bar**), and Δ*sigB* (**white bar**) cells upon exposure to 10% (v/v) ethanol with and without preadaptation to 4% ethanol (v/v). **Circle-** growth in Luria Bertani (LB) medium; **square-** growth in LB with 4% ethanol; **triangle** – growth in LB with 1M NaCl, all at 37°C. N=3

Moreover, it is noteworthy that the presence of an ortholog to the *B. cereus* RsbK protein was predicted in *B. subtilis* **(Chapter 2),** but no cognate response regulator was detected adjacent to the predicted *rsbK* gene in the *B. subtilis* genome. This finding may imply that an additional SigB activation pathway exists in *B. subtilis*. This notion is not new. As introduced in **Chapter 1,** SigB is proposed to be activated directly at high/low temperatures (54°C/15°C) or in the presence of nitrosative stress via an Rsb-independent pathway (Brigulla et al., 2003; Holtmann et al., 2004; Tran et al., 2019a). These stressors may be able to interact with so far unidentified

membrane signaling proteins, such as possibly the RsbK ortholog, that sense specific stress. Nonetheless, the potential functionality of the RsbK ortholog in *B. subtilis* and its putative role in the SigB activation pathway remains to be confirmed.

In addition, the protein serine kinase RsbW (the anti-sigma factor) has been shown to exhibit high cross-phosphorylation activity with other kinases (Shi et al., 2014). It is generally recognized that the dephosphorylated RsbV sequesters RsbW to promote the transcriptional activation of SigB **(Chapter 1).** However, Shi and colleagues (2014) showed that PrkC and YabT kinases could efficiently cross-phosphorylate the RsbW, thereby influencing its phosphorylation capacity. Such cross-phosphorylation events may lead to either an unexpected SigB activation or the loss of the ability of RsbW to phosphorylate RsbV, leading to a potential constitutional activation of SigB (updated scheme of SigB activation routes for *Bacillus* species in Figure 7).

Lastly, a Hpt protein (Bc1009) in the SigB gene cluster of *B. cereus* has previously been proposed to be the intermediate protein that transfers the phosphate group from RsbK to RsbY (de Been et al. (2010). While the phosphate group can transfer from RsbK to RsbY directly (Chen et al., 2012, 2015), the transfer via a Hpt protein might be possible. Based on the evidence obtained in **Chapter 3,** it could however be concluded that Bc1009 is not a direct SigB regulator: its deletion did not influence the expression of SigB or the other SigB gene cluster members. Therefore, the possibility of Bc1009 being the Hpt intermediate that mediates the phosphoryl group transfer in *B. cereus* was ruled out.

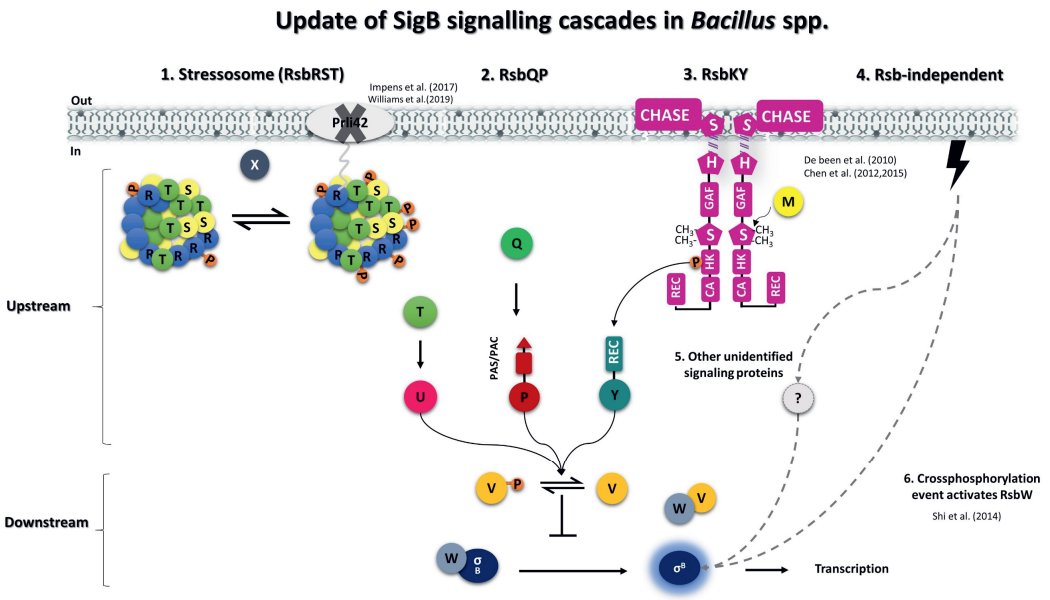


Figure 7- Updated scheme of SigB activation routes for *Bacillus* species

6.1.4 Stressors that induce SigB

In published reports of SigB GSR in *Bacillus* species and other Bacillales members such as *L. monocytogenes* and *S. aureus*, standard stressors (i.e., heat, ethanol, salt, and glucose starvation) are often employed to study the induction response of SigB upon exposure to the stressors. Although many other physical (pH, antibiotics, oxidative or nitrosative stress, blue light, red light, elevated level of manganese) and metabolic stressors (phosphate, oxygen, azide, carbonyl cyanide m-chlorophenylhydrazone (CCCP), nitric oxide (NO), mycophenolic acid, and decoyinine) are known (Voelker et al., 1995; Gaidenko and Price, 1998; Guedon et al., 2003; Mascher et al., 2003; Zhang and Haldenwang, 2005; Tran et al., 2019b) **(Chapter 1)**, most of the global gene and protein studies were performed using only the standard stressors. However, these are not the only stressful conditions bacteria encounter in their ecological niches. Moreover, as SigB is the master regulator of stress, a stressor is also generally recognized as the prerequisite to triggering the SigB GSR. In this thesis, it was shown in **Chapter 3** that SigB might not need an external stressor to exert active functions in *B. cereus* cells, and an alternative stressor that induces SigB moderately was identified in *B. licheniformis* as well as *B. subtilis* (**Chapter 4).**

SigB is known to be under the control of the housekeeping sigma factor, SigA, and is expressed at low levels during growth. The baseline expression level of SigB increases markedly when cells encounter stresses in their surroundings, providing cells with rapid stress adaptive response (Rodriguez Ayala et al., 2020). However, the "housekeeping" level of SigB may already involve in regulating other cellular responses in the absence of external stressors (illustrated in Figure 3), supported by the evidence obtained in **Chapter 3** for *B. cereus*. The proteomics analyses of the *B. cereus* wild-type (wt), Δ*sigB,* and Δ*bc1009* mutants upon exposure to heat stress revealed novel proteins differentially regulated by SigB, potentially via the putative phosphocarrier protein Bc1009. However, a subgroup of these newly identified proteins also showed differential regulation at constant temperature (30°C, without further stress imposition) in the Δ*sigB* cells compared to wt cells. These proteins were mainly involved in cell motility, transcription, signal transduction, cell wall/membrane biogenesis, carbohydrate/ion transport, and metabolism. Of these cellular functions, the motility phenotype was confirmed, and both SigB-deficient and Bc1009-deficient cells showed defects in swimming motility on low-percentage agar plates. These findings in **Chapter 3** imply that the SigB-mediated GSR is activated by a stressor and may be regulated by the elevated level of SigB, but other SigB-dependent cellular processes (either directly or indirectly) may not require an external stressor. These processes may be governed by SigB at its baseline expression level controlled by SigA.

The function of the baseline level of SigB has not been reported in the literature for *Bacillus* spp.. Based on the results of **Chapter 3,** two scenarios of SigB functions in *Bacillus* cells can be deduced (Figure 8). Without stress, baseline levels of SigB produced in the cells (as controlled by SigA) interact with other transcriptional regulators/proteins to control other

cellular responses, e.g., motility. With stress, the baseline levels of SigB are elevated in the cells as a result of the stressor-induced GSR, ***Scenario one****:* switching all cells to cope with stresses and enhance survival; or ***Scenario two****:* switching some cells to transiently cope with stresses while other cells keep regulating other cellular responses (Figure 8). As the proteomics analyses (supported by transcriptomics data) in **Chapter 3** showed that proteins involved in motility, signal transduction, transcription, and cell wall/membrane biogenesis were similarly regulated regardless of heat stress, *Scenario two* more likely depicts the SigB mechanisms in *Bacillus* cells, at least for *B. cereus*. Notably, as introduced in **Chapter 1,** SigB is deemed to be at its inactive state (bound by RsbW) in unstressed conditions, and in the presence of stress, the dephosphorylated RsbV sequester RsbW and leads to SigB activation and thus the transcription of SigB-controlled genes. If *Scenario two* would hold true, this indicates that *not all* SigB in unstressed conditions is inactive (see the conceptual model in Figure 3). This activation of SigB may occur due to a high cross-phosphorylation event exhibited by RsbW (discussed in section 6.1.3).

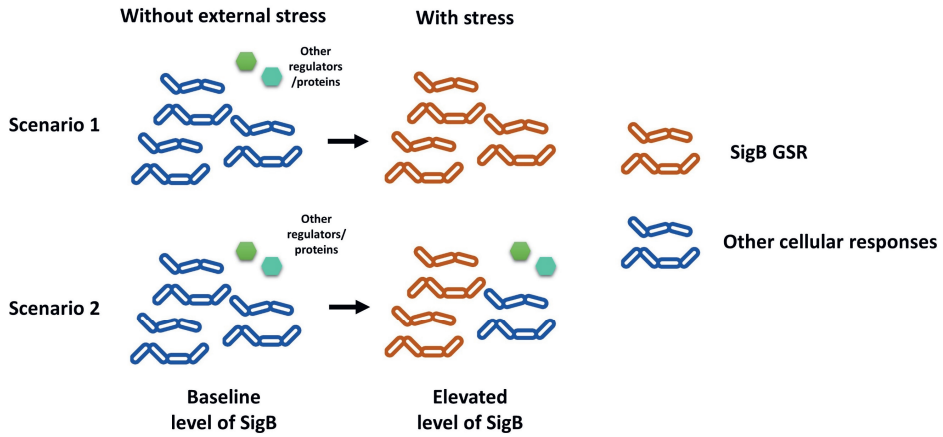


Figure 8- Illustration of the function of SigB with and without external stress in *Bacillus* cells.

However, the temperature used in the experimental works in **Chapter 3** (30°C) may still be stressful for *Bacillus* cells as the average temperature of soils in nature (niches for *Bacillus*) is likely below 30°C. Nevertheless, the continuous culture of cells at 30°C did not introduce a sudden switch of environments for the cells, which is deemed to be a crucial factor for SigB activation in *Bacillus* cells (Rodriguez Ayala et al., 2020). Therefore, the group of genes/proteins differentially regulated in Δ*sigB* cells compared to wt cells at 30°C **(Chapter 3)** supported the notion that SigB functions at its baseline level.

Besides external factors, compounds that *Bacillus* cells produce themselves may induce the SigB-GSR and lead to protection from other stresses. For example, the deletion of SigB was shown to negatively affect lichenysin production in *B. licheniformis* in **Chapter 4**, which has

antimicrobial/antifungal properties **(Chapter 1)** and is toxic to mammalian and human cells **(Chapter 5)**. Further experiments in **Chapter 4,** which exposed cells to lichenysin or surfactin (a similar compound to lichenysin), showed that lichenysin induces a mild SigB GSR, which confers cells with a slight advantage to survive subsequent exposure to lethal stress (12% ethanol v/v). Similarly, in *B. subtilis,* SigB has been reported to influence surfactin production indirectly (Bartolini et al., 2019a), and surfactin-producing cells are unaffected by surfactin itself (Rahman et al., 2021). This thesis presents evidence that surfactin and lichenysin are mild stressors that can induce the SigB GSR in *B. licheniformis* and *B. subtilis,* respectively, which may also be true for other *Bacillus* spp. Although the exact environmental conditions and efficacy of surfactants in triggering SigB GSR remain to be elucidated, it is noteworthy that surfactin has also been reported to act as a signaling molecule to further trigger the differentiation of cells into competence development, matrix production, and sporulation, resulting in different subpopulations within a cellular population (Rahman et al., 2021).

6.2 Ecological aspects of SigB and SigB-GSR

Ecological niches are specific habitats where a group of microorganisms populates an ecosystem (Polechová and Storch, 2019). In the following sections, the contribution of SigB and SigB-GSR to the persistence of *Bacillus* spp. in environmental niches and the roles of *Bacillus* spp. in environments are discussed.

6.2.1 Persistence of microorganisms in environmental niches

The core function of SigB in mediating GSR and its alternative functions in regulating other processes (section 6.1) shows that SigB plays a crucial role throughout the life cycle of *Bacillus* cells and assists the rapid adaptation in different niches. While the alternative functions of SigB discussed in the sections above were seemingly independent of each other, these functions are interrelated ecologically.

In natural habitats, *Bacillus* cells may be exposed to conditions of drought, temperature shift, change in salinity or pH, nutrient deprivation, absence of O_2, presence of harmful compounds, e.g., antimicrobials and cell wall disruptors, and the presence of other competitors. At each particular condition, SigB appears to play a role in managing the specific stresses alone or with other Sigma factors/regulators (depicted in Figure 9). When cells face harsh changing living conditions, SigB induces the GSR in *Bacillus* to prepare cells to cope with environmental/nutritional stressors or may form biofilm or sporulate as one of the mitigation strategies upon entering the stationary phase (Arnaouteli et al., 2021). This switch of bacterial lifestyle from the motile form into the sessile form is monitored by SigB via regulating genes involved in biofilm formation and sporulation (Shemesh and Chai, 2013). As reported in section 6.1.2, the regulation of biofilm formation and sporulation are closely linked to the regulation of cell motility, in which the motile cells within the mature biofilm will disperse and colonize new niches. Again, SigB is known to be involved in such control of biofilm aging and triggering cell

dispersal in *B. subtilis* (Bartolini et al., 2018). Moreover, *Bacillus* cells commonly colonize soil and are often nutrient-deprived; thus, controlling cell motility may allow sessile colonies at one nutrient-deprived location to move to another, e.g., different layers of soil surfaces. This movement can be further enhanced by surfactant-producing cells, which can lower the surface tension of a water-contained environment.

Other than the environmental/nutritional limiting conditions, *Bacillus* cells may face threats from other microbes that coinhabit the same niche and compete for nutrients with *Bacillus* (e.g., other bacteria/fungus) or attack them (e.g., viruses). Species like *Pseudomonas* can inhibit cell differentiation, e.g., biofilm formation and sporulation in *Bacillus* (Powers et al., 2015). Therefore, *Bacillus* cells are known to secrete metabolites that exert antibacterial, antifungal, or antiviral properties to gain competitive advantages. Such metabolites can be surfactants (e.g., surfactin/lichenysin). Surfactin production is indirectly influenced by SigB in *B. subtilis* (Bartolini et al., 2019a), and lichenysin production was also shown to be reduced in the absence of SigB in *B. licheniformis* **(Chapter 4).** Metabolites like surfactin conceivably contribute to competitive fitness and can act as signaling cues to trigger cell differentiation into different subpopulations, e.g., competence development, matrix production, and sporulation in *B. subtilis* (Rahman et al., 2021).

6.2.2 Contribution of SigB to the role of *Bacillus* spp. within the soil ecosystem

Bacillus spp. as rhizobacteria play active roles in maintaining the homeostasis of the soil ecosystem. *Bacillus* interacts with plants and establishes mutualistic relationships: on the one hand, plants fix carbon and secrete the carbohydrates via root exudates to provide carbon sources for *Bacillus.* On the other hand, *Bacillus* colonizes plant roots to promote the cycling of nutrients and organic matter, solubilize soil for nutrient access, enhance nitrogen fixation, stimulate plant growth and prevent plant drought (Radhakrishnan et al., 2017; Hashem et al., 2019). They confer stress tolerance to plants by inducing plants to produce phytohormones and other stress-related metabolites to fight pathogens, e.g., cell-wall degrading enzymes and antioxidants (Allard-Massicotte et al., 2016; Hashem et al., 2019; Saxena et al., 2020). Moreover, *Bacillus* spp. produce surfactants, and surfactants that are good ion chelators can play a role in the bioremediation of metal-contaminated soil and reduce the toxic effects of metal ions on plant growth (Radhakrishnan et al., 2017).

These ecological roles of *Bacillus* spp. are not directly related to the SigB GSR. However, SigB has recently been shown to regulate SodA2 (superoxide dismutase) positively (important in surviving ethanol and toxic chemical like herbicides) to contribute to the colonization of wheat roots in *B. cereus* (Gao et al., 2021). This report is in line with the results obtained in **Chapter 3,** as biofilms and motility-related genes/proteins were found to be influenced by SigB (section 6.1.2), implying that SigB of *Bacillus* spp. may involve triggering of biofilm formation for root colonization. Moreover, the role of SigB in the nitrogen cycle has been described by Van Schaik et al. (2005), who showed that SigB-deficient cells failed to utilize many nitrogen sources, which are the intermediates of the urea cycle, such as arginine, ornithine, citrulline, and aspartate.

Similarly, **Chapter 3** also indicated that SigB interacts with ArgR (the arginine metabolism regulator), and many genes encode nitrate reductase enzyme complex members (essential for anaerobic growth), nitrogenases (nitrogen fixation), and molybdopterin synthases (nitrate assimilation in plants and anaerobic respiration in bacteria) also showed SigB-dependent expression (Chapter 3 Supplementary Materials), stressing the functional role of SigB in the nitrogen cycle and anaerobic respiration.

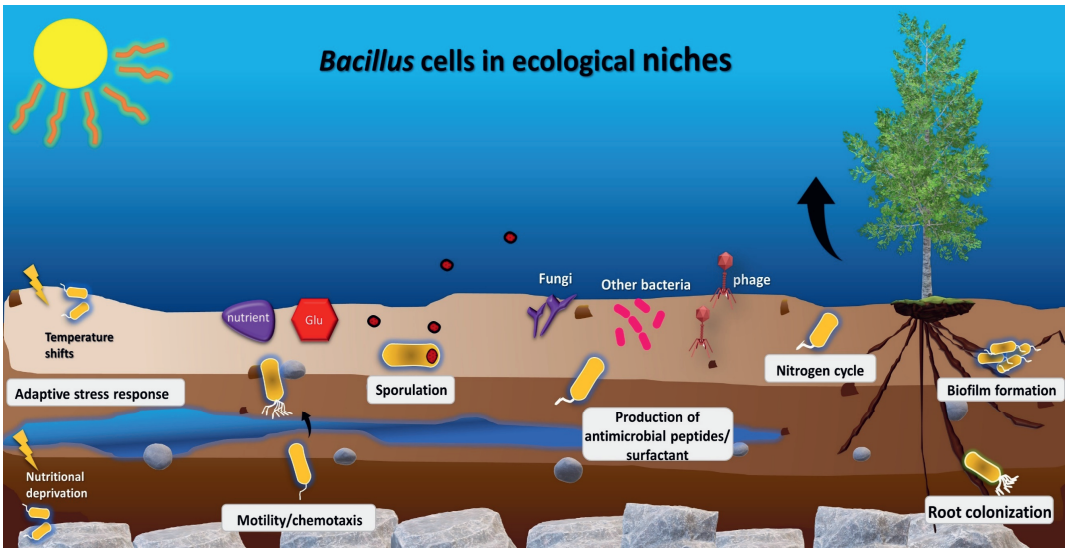


Figure 9- Depiction of direct and indirect roles of SigB in *Bacillus* cells in the environment

6.3 Implications in the study of other foodborne pathogens and the food sector

B. cereus, L. monocytogenes, and *S. aureus* are well-known foodborne hazards that can cause foodborne illnesses (European Food Safety Authority, 2021) (SigB GSR for *L. monocytogenes* and *S. aureus* described in **Chapter 1**). They have been identified in the list of top 32 microbiological hazards in Europe based on the major outbreaks, global public health impact, and food contamination data (WHO, 2015; ECDC, 2019; European Commission, 2021; European Food Safety Authority, 2021; Yeak et al., 2022). While *B. licheniformis* is generally considered non-pathogenic, it can cause rare foodborne intoxications (Salkinoja-Salonen et al., 1999; Mikkola et al., 2000). Knowledge gained on SigB-GSR in *Bacillus* is transferable to studying the above-mentioned foodborne pathogens and has practical implications for food quality and safety concerning food processing design and improving food safety prediction modeling tools.

6.3.1 Implications in the study of other foodborne pathogens

The studies of GSR in *L. monocytogenes* and *S. aureus* are discussed in other reviews (see Chapter 1), so this discussion only highlights the transferable knowledge gained on SigB and SigB-GSR to the development of new therapeutics in treating pathogens like *L. monocytogenes* and *S. aureus*. SigB enhances the tolerance of *L. monocytogenes* and *S. aureus* to different classes of antibiotics and supports cells to escape the host cell immune systems. Therefore, SigB inhibitors like fluoro-phenyl-styrene-sulfonamide (Ringus et al., 2013) or PrfA inhibitors in *L. monocytogenes* (methyl-benzothiazole, acetamide) (Tran et al., 2022) could offer a novel solution to reduce the resistance of such a pathogen and provide alternative therapeutic approaches in addition to antibiotics. Similarly, finding inhibiting ligands that bind to the stressosome proteins may provide SigB inhibition effects. The FragLites molecules (halogenated drug-like fragment compounds used in drug discovery studies) (Wood et al., 2019) were found to potentially bind to the RsbR ligand-binding pocket of the *L. monocytogenes* stressosome and may provide information to target-based drug discovery (Unpublished data, PhD thesis Sema Edjer, Newcastle University, 2021), opening up new avenues of research.

6.3.2 Implications for food quality and food safety

As *Bacillus* (and other pathogens) are highly persistent in the environment, they can contaminate raw food ingredients and enter the food chains. Their ability to form biofilms and spores (influenced by SigB) also contributed to their persistence in the food processing plant by contaminating surfaces like storage tanks, pipes, or conveyor belts (Lin et al., 2022). The impact of spore resistance on food safety and quality is well-known, which contributes to cell survival, emergence, and outgrowth in food, reviewed in Wells-Bennik et al. (2016); den Besten et al. (2018) and Setlow and Christie (2021). Moreover, microbes with robust stress adaptation systems may reside in the same processing environments for a long time.

This scenario is of particular importance when foods undergo mild processing, as SigB-GSR in bacteria can be induced by mild stressors and thereby provide cross-protection to the cells against lethal conditions. Mild processing techniques like high-pressure processing, pulsed electric fields, ultrasounds, UV light irradiation, and cold plasma are favored in the food industries as these techniques preserve nutrients and the organoleptic properties of foods (Barba et al., 2017). Moreover, changes in consumer behavior preference for non-processed/mildly-processed foods to date also give impulses to using mild processing techniques. When foods are mildly processed, pathogens in foods (or their toxins) may not be fully inactivated (e.g., *S. aureus* and its heat-resistant toxin) due to their stress-adaptation capability and may lead to increased chances of foodborne outbreak or intoxication. Such events must be avoided by careful analysis of the impact of food processing procedures and conditions on the survival of species of concern.

6.3.2.1 Improvement of food safety prediction modeling tools

Bacillus spp. produce spores that can survive many harsh treatments (Wells-Bennik et al., 2016; Cho and Chung, 2020), but the inactivation of *Bacillus* vegetative cells is nonetheless important, particularly upon germination and outgrowth. The activation of stress sensing mechanisms such as the SigB GSR potentially impacts the survival of vegetative cells, indicating that variance may exist in the prediction of bacterial inactivation kinetics. Figure 10 shows such differences using *B. subtilis* wt cells and the Δ*sigB* mutant under isothermal heat treatment at 52.5°C, representing the temperature for mild processing. As expected, with a preadaptation treatment at 48°C, wt persisted longer and survived much better (> 3 logs) than the Δ*sigB* mutant at 52.5°C. Without the preadaptation treatment, the difference between wt and Δ*sigB* cells was smaller, but wt cells still showed better fitness than the Δ*sigB* cells (higher logs). As SigB is described to be crucial for adaptive stress response but does not have much impact on growth or survival in long-term stress (Pané-Farré et al., 2017; Rodriguez Ayala et al., 2020), these data further emphasized that such stress adaptation mechanisms like SigB GSR may contribute to adaptation to persisting stress, resulting in robust phenotypes. Such physiological responses in bacteria can cause variance in the prediction of bacterial inactivation parameters, and data demonstrated here indicate that it is crucial to determine the dynamics of bacterial inactivation kinetics in specific food compositions under different processing conditions.

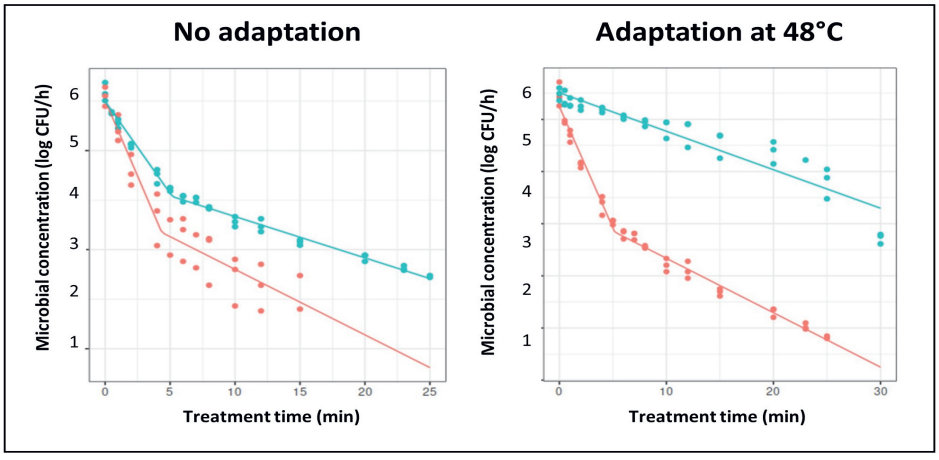


Figure 10- Inactivation kinetic model predictions and the microbial counts of *B. subtilis*. wild type (wt) cells versus Δ*sigB* mutant during isothermal inactivation at 52.5°C in LB medium without adaptation and with adaptation. wt (**blue**) and Δ*sigB* mutant (**red**).

Today, food safety management includes more risk-based than pure hazard-based assessment, leading to the development of many prediction tools, databases, and repositories for improved data analysis (Barlow et al., 2015; Possas et al., 2022). However, uncertainty and variability in risk-based prediction models hamper realistic predictions of microbial behaviors in foods. One such factor is the adaptation capability of organisms (e.g., as influenced by SigB-GSR).

Adaptational events have been shown to account for altered heat resistance traits of *L. monocytogenes* and *Escherichia coli*, leading to inaccurate prediction of inactivation model parameters, and it has been suggested that inactivation model parameters need to be calibrated using experimental data that represent the ability of an organism to adapt (Garre et al., 2018, 2019).

6.4 Concluding remarks and future perspectives

In conclusion, this thesis dissected the SigB-mediated GSR and the role of SigB in *B. subtilis, B. cereus,* and *B. licheniformis,* and provided new insights into the sophisticated SigB regulatory networks of *Bacillus* species, including the identification of SigB-regulated genes/proteins, identification of mild stressors that induce SigB and (putative) proteins involved in SigB signal sensing cascades, and impact on the production of lichenysin.

Notably, not all stressors that trigger the SigB GSR in *Bacillus* environments are known, and studying the response of wt and mutants in the laboratory may not fully reflect actual environmental conditions in the ecological niches. Further studies using strains isolated from natural sources may provide more insight into other stressors and/or SigB signal sensing components that can trigger SigB. Furthermore, the function of SigB may not be restricted to the protection of *Bacillus* cells from sudden exposure to physical or nutritional stressors, but it may play a role in ‘’baseline’’ functions in other conditions (e.g., unstressed or stresses caused by other factors or long-term stress). However, such baseline level functions need to be further verified, and phenotypes need to be confirmed.

This thesis also sheds more light on lichenysin production by *B. licheniformis*. SigB was found to play a minor role in the production of this secondary metabolite. Biosurfactants like surfactin and lichenysin are stressors that mildly induce SigB GSR, which conferred a slight advantage to cell survival under lethal stress. Such compounds were found to be produced in different amounts by different strains based on medium composition and culture conditions and are toxic to mammalian and human cells. Therefore, their presence in foods such as milk, or cooked meat and vegetables must be carefully examined via detection followed by quantification. Cell growth to high CFU levels (10^4-10^5) which facilitates lichenysin production must be prevented.

The fundamental knowledge gained on SigB GSR and the alternative roles of SigB in other cellular functions/secondary processes in this thesis provided a deeper understanding of stress adaptation mechanisms in *Bacillus* species. Such knowledge can be transferred to studying other related pathogens and have practical implications for the food sector.

6.5 References

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APPENDIX

Summary

The endospore formers *Bacillus subtilis, Bacillus cereus,* and *Bacillus licheniformis* can rapidly adapt to environmental stresses via the general stress response (GSR), which is mediated by the master regulator of stress: the alternative Sigma factor B (SigB). The SigB-GSR is one of the most important stress responses and is crucial to conferring multiple resistances to *Bacillus* cells and supporting their survival in changing environments. Additionally, SigB is involved in regulating several other cellular processes related to stress resistance, e.g., sporulation, biofilm formation, and the production of antimicrobial peptides. The various elements involved in the SigB GSR are well-characterized for a model strain of *B. subtilis*, i.e., the stress signals (stressors) that trigger SigB, the different SigB signaling cascades, the group of SigB-dependent genes regulated in response to stress (namely, the SigB regulon), and the alternative functions of SigB in other cellular processes **(Chapter 1)**. However, the entire SigB GSR mechanism is sophisticated and has not yet been fully elucidated to date. The complete structure of the SigB regulon and regulatory networks in *B. subtilis* remains incomplete. In addition, the SigB GSR mechanisms and functions are less understood in other *B. subtilis* group members and the foodborne pathogen *B. cereus*.

This thesis presents further insights into the SigB-mediated GSR in *B. subtilis, B. cereus,* and *B. licheniformis*, as well as other Bacillales members. **Chapter 2** of this thesis further dissected the SigB regulon of *B. subtilis* using a SigB promoter binding motif (PBM) derived from the promoter sequences of all so far known SigB regulated genes. Novel putative SigB regulon candidates with SigB PBMs were revealed (156), extending the SigB theoretical regulon to > 500 members in this organism. SigB-dependent regulation of selected newly identified promoters was confirmed experimentally. Additionally, species-specific SigB regulon structures and signaling cascades in 106 other Bacillales members were unraveled. Although many Bacillales members contain genes orthologous to the SigB regulon members of *B. subtilis* 168, not all have a SigB PBM. Some Bacillales members appeared to have reassigned SigB PBM to selected genes from the shared gene pool, likely related to survival in different ecological niches.

Notably, many SigB regulon members of *B. subtilis* 168 have orthologs in the closely-related species, *B. cereus*. The SigB regulon of *B. cereus* in response to heat stress was examined in-depth via a proteomics approach backed up with transcriptomics data in **Chapter 3**. The putative SigB regulon of *B. cereus* was expanded from ~ 30 to > 300 members, and a SigB subregulon (> 180) controlled by the SigB-dependent phosphotransferase protein (Bc1009) was identified. Additionally, the Bc1009-controlled SigB subregulon was also differentially regulated in non-heat-stressed Δ*sigB* and Δ*bc1009* mutants compared to wild-type cells at 30°C,

implying putative SigB functions at 30°C. Phenotypic assays confirmed the roles of SigB and Bc1009 in heat resistance and motility.

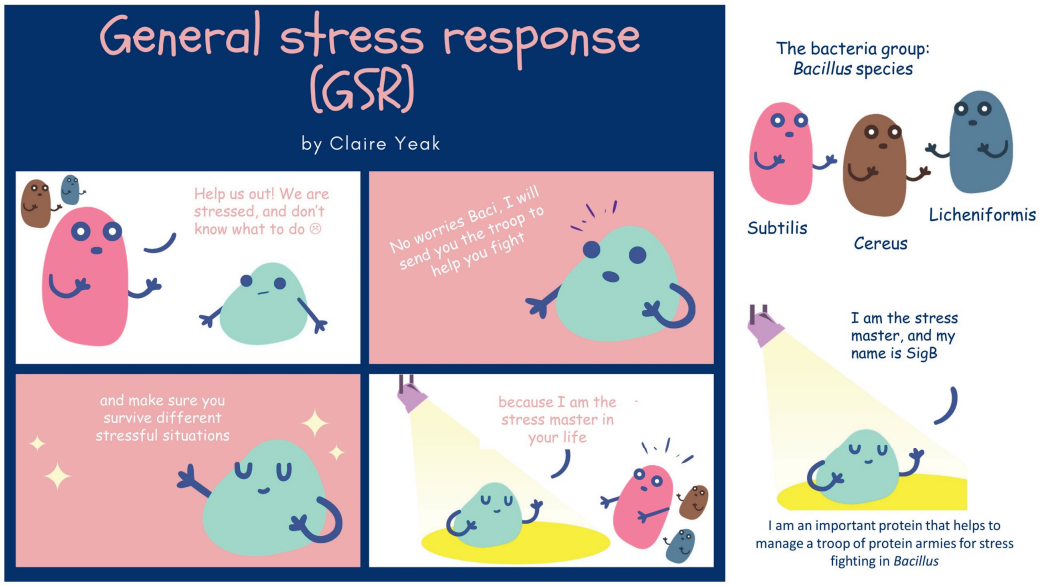
Subsequently, in **Chapter 4,** the influence of SigB on secondary metabolite (the surfactant and antimicrobial peptide-lichenysin) production was demonstrated in *B. licheniformis*, showing that SigB-deficient cells produced ~ 25% less lichenysin than the wild type cells under optimal growth conditions. However, lichenysin production was not directly regulated by SigB. A mild surfactant-induced SigB-GSR on lethal stress resistance was shown by exposing *B. licheniformis* cells to lichenysin or surfactin, and subsequently to lethal ethanol conditions. Likewise, the similar compound - surfactin produced by *B. subtilis* induced mild SigB GSR in *B. sub*tilis and provided modest protection to the cells under lethal ethanol conditions.

As lichenysin and surfactin are potential food poisoning agents**,** lichenysin production by *B. licheniformis* food isolates was investigated in **Chapter 5**. The amounts produced by three selected isolates under different conditions showed that lichenysin production is highly dependent on medium composition, strain, temperature, and culture condition. The toxicities of lichenysin and surfactin to human cells were also determined, implicating that risks of foodborne intoxication may arise if growth to high levels is supported in foods and the product is subsequently ingested by humans.

Chapter 6 discussed the experimental results obtained in the research chapters (2-5). Firstly the new insights gained on the SigB-mediated mechanisms in *Bacillus* species are discussed. These insights are linked with ecological aspects of SigB and SigB-GSR in relation to the persistence of microorganisms. Lastly, implications of the role of SigB in the study of other pathogenic species and practical implications relevant to the food sector are discussed.

Layman summary for general audiences (English)

The group of bacteria called *Bacillus* is one of the biggest and most well-known groups of bacteria living in soils. This thesis investigated the stress management strategies of this bacterial group in depth, specifically in three renowned members, namely *Bacillus subtilis, Bacillus cereus,* and *Bacillus licheniformis*. Two of them (Subtilis and Licheniformis) are often used as a cell factory to produce many valuable enzymes, like your detergents at home, but Cereus is the bad guy and can contaminate the food you eat and make you sick. So, when you experience diarrhea or vomiting, Cereus can be the culprit! These three bacteria are widespread in the environment and thus often exposed to stressful situations, like extreme heat or cold temperature or lack of nutrients. However, they manage such stressful situations very well, specifically through a stress management mechanism known as the general stress response (GSR). This GSR response is controlled by a crucial protein called Sigma B (SigB).



SigB is the master for stress management for this group of bacteria (*Bacillus*), just like humans' brains. When humans are stressed, our brain processes this information and activates responses to either prepare us to run away or fight back. Like humans, when *Bacillus* encounter stress (imagine a 42°C hot summer day), the SigB master gets activated and acts like a commander, to order the production of many proteins to help *Bacillus* to fight stress and survive. Besides managing stress, the SigB master controls several other processes in *Bacillus*, such as turning the cells into a dormant state or producing slimy compounds to protect cells.

The entire SigB-controlled mechanism is very complicated, including 1) different environmental stress signals that turn on this master regulator, 2) the different pathways used to activate the SigB master, 3) a troop of genes and proteins armies controlled by SigB to fight stress, and 4) the miscellaneous functions

of SigB other than dealing with stress. **Chapter 1** introduces **everything you know or others worth knowing** about *Bacillus,* its beneficial or pathogenic roles, and their stress management strategies. **Chapter 2** investigated whether the commands from SigB are delivered to the stress-fighting protein armies directly or indirectly through a secondary messenger, and found that it can occur either way. Furthermore, we revealed that the number of known stress-fighting army troops increased from about 200 members to > 500 in *Bacillus.* Therefore, **even though the SigB-controlled mechanism is well-known, there is always more to explore**. Besides, we also checked for the presence or absence of such SigB-controlled stress management strategies in the close and far relatives of *Bacillus,* and uncovered that it exists in many other bacteria groups.

Chapter 3 showed that the primary responsibility of the SigB master is to manage stress but still has other roles in *Bacillus,* such as controlling the bacterium swimming activity. Besides that, the SigB manager communicates with other *Bacillus* managers, like the manager for foods and nutrients, to select the carbohydrate or protein sources to take in. Results obtained implied that even though the stress management **function for the SigB master is well-defined, it is not the only observed crucial function.**

Subsequently, in **Chapter 4,** we studied the role of the SigB master in controlling the production of a detergent-like compound (called lichenysin) and examined whether the exposure to this compound caused stress in *Bacillus.* Our results showed that exposure to lichenysin activated the SigB master modestly. This activation provided small protection to *Bacillus* when exposed to other lethal stress, demonstrating that a **mildly stressful condition is stressful, and a mild response is still a response**.

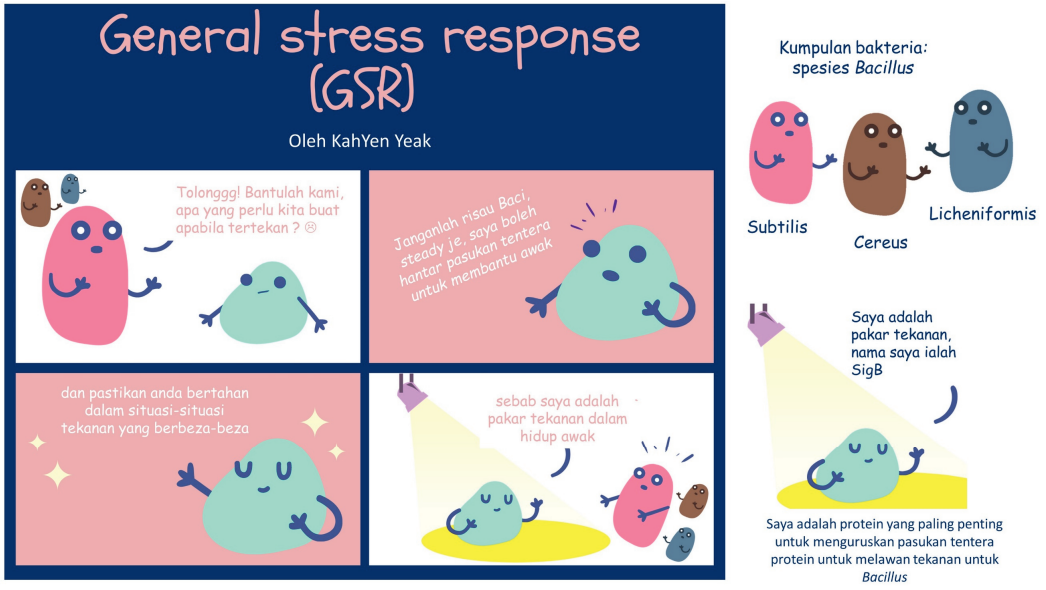
This detergent-like compound (lichenysin) can be a potential food poisoning agent and is produced by all Licheniformis members. Therefore, we also investigated the production of this compound by selected Licheniformis members in food and determined its toxicity to human cells in **Chapter 5.** Results showed that the amounts of produced lichenysin varied extensively between different Licheniformis members and depended on food compositions, temperatures, and cultivation conditions. We also showed that this compound is toxic to humans and mammalian cells. These results implied that there may be a risk of foodborne intoxication if the bacterium is allowed to grow in foods and such foods are subsequently ingested by humans. Hence, **unknown toxicity of a compound is not non-toxic, and the toxin (lichenysin) toxicity is cumulative**.

Lastly, **Chapter 6** discusses **the discovery in this thesis from different perspectives based on scientific evidence.** Firstly, the new insights obtained for the SigB master and other mechanisms that it controls. Secondly, how these stress management strategies contributed to the persistence of *Bacillus* in environments. Finally, the implications of such obtained knowledge in the study of other pathogenic bacteria and other practical implications relevant to the food sector.

Ringkasan khalayak Publik (Bahasa Malaysia)

PS: Thanks Theo and Sylviani for cross-checking the text

Kumpulan bakteria yang dipanggil *Bacillus* adalah salah satu kumpulan bakteria terbesar dan paling terkenal yang hidup di dalam tanah. Tesis ini menyiasat strategi pengurusan tekanan kumpulan bakteria ini secara mendalam, khususnya dalam tiga ahli yang terkenal, iaitu *Bacillus subtilis*, *Bacillus cereus*, dan *Bacillus licheniformis*. Dua daripadanya (Subtilis dan Licheniformis) sering digunakan sebagai kilang sel untuk menghasilkan banyak enzim berharga, seperti detergen di rumah anda, tetapi Cereus adalah patogenik, dan boleh menyebabkan penyakit keracunan makanan. Apabila anda mengalami cirit-birit atau muntah, Cereus boleh menjadi puncanya! Ketiga bakteria ini tersebar luas di alam sekitar, oleh itu sering terdedah kepada situasi tekanan, seperti haba melampau, suhu sejuk atau kekurangan nutrien. Walau bagaimanapun, mereka menguruskan situasi tekanan sedemikian dengan baik, khususnya melalui mekanisme pengurusan tekanan yang dikenali sebagai General Stress Response (GSR). Tindak balas GSR ini dikawal oleh protein penting yang dipanggil Sigma B (SigB).



SigB ialah pakar tekanan utama untuk kumpulan bakteria ini (*Bacillus*), sama seperti otak manusia. Apabila manusia tertekan, otak kita memproses maklumat ini dan mengaktifkan tindak balas untuk menyediakan kita melarikan diri atau melawan. Seperti manusia, apabila *Bacillus* menghadapi tekanan (bayangkan hari musim panas 42°C), SigB akan diaktifkan dan bertindak seperti komander, untuk memerintahkan pengeluaran banyak protein untuk membantu *Bacillus* melawan tekanan dan terus hidup. Selain daripada pengurusan tekanan, SigB juga mengawal beberapa proses yang lain dalam *Bacillus*, seperti mengubah sel ke keadaan yang tidak aktif atau menghasilkan sebatian berlendir untuk melindungi sel.

Keseluruhan mekanisme yang dikawalkan oleh SigB adalah sophistikated, termasuk 1) isyarat tekanan persekitaran berbeza yang mengaktifkan SigB, 2) laluan berbeza yang digunakan untuk mengaktifkan SigB, 3) sekumpulan tentera gen dan protein dikawalkan oleh SigB untuk melawan tekanan, dan 4) pelbagai fungsi SigB selain daripada menangani tekanan.

Bab 1 memperkenalkan segala-gala maklumat tentang *Bacillus* and lain-lain yang patut diketahui tentang *Bacillus*, iaitu peranannya yang bermanfaat atau patogenik, dan strategi pengurusan tekanan mereka. **Bab 2** menyiasat sama ada arahan daripada SigB dihantar kepada tentera protein yang melawan tekanan secara langsung, atau tidak langsung melalui pengawal selia lain, dan mendapati bahawa ia boleh berlaku sama ada cara. Selain itu, kami mendedahkan bahawa bilangan tentera-tentera protein yang melawan tekanan meningkat dari kira-kira 200 anggota menjadi > 500 di *Bacillus*. Oleh kerana itu, walaupun mekanisme dikawal oleh SigB terkenal, masih ada yang lain perlu diterokai. Sebagai tambahan, kami juga menyiasat kehadiran atau ketiadaan strategi pengurusan tekanan terkawal SigB dalam saudara bakteria terdekat atau pun jauh daripada *Bacillus*. Kami mendapati bahawa ia wujud dalam banyak kumpulan bakteria yang lain.

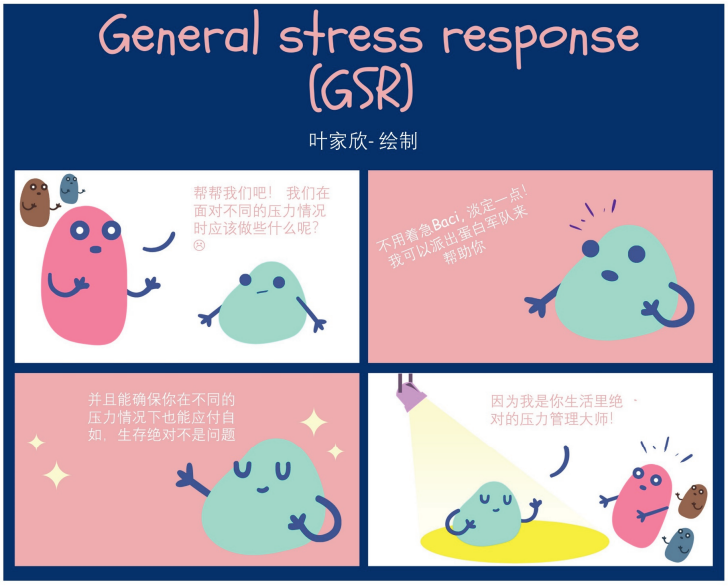
Bab 3 menunjukkan bahawa tanggungjawab utama SigB adalah untuk menguruskan tekanan, tetapi masih mempunyai peranan lain dalam *Bacillus*, seperti mengawal aktiviti bergerak bakteria. Selain itu, SigB berkomunikasi dengan pengurus lain di *Bacillus* juga, seperti pengurus untuk makanan dan nutrien, untuk memilih sumber karbohidrat atau protein. Keputusan yang diperoleh menunjukkan bahawa walaupun fungsi pengurusan tekanan untuk SigB ditafsirkan dengan baik, ia bukan satu-satunya fungsi penting yang diperhatikan. Selepas itu, dalam **Bab 4,** kami mengkaji peranan SigB dalam mengawal pengeluaran kompaun seperti detergen (dipanggil lichenysin) dan mengkaji sama ada pendedahan kepada sebatian ini menyebabkan tekanan dalam *Bacillus*. Keputusan kami menunjukkan bahawa pendedahan kepada lichenysin mengaktifkan SigB secara sederhana. Pengaktifan ini memberikan perlindungan kecil kepada *Bacillus* apabila terpapar kepada tekanan maut yang lain. Walaupun tindak balas terhadap tekanan boleh dikatakan ringan, tekanan yang berlaku mahupun tidak boleh diabaikan. Kompaun seperti detergen (lichenysin) ini boleh menjadi agen keracunan makanan yang berpotensi dan dihasilkan oleh semua ahli Licheniformis. Oleh itu, kami juga menyiasat pengeluaran kompaun ini oleh ahli Licheniformis yang terpilih dalam makanan dan menentukan ketoksikannya terhadap sel manusia dalam **Bab 5**. Keputusan menunjukkan bahawa jumlah lichenysin yang dihasilkan berbeda secara meluas antara ahli Licheniformis yang berbeza, dan bergantung kepada komposisi makanan, suhu, dan kondisi pertumbuhan. Kami juga menunjukkan bahawa kompaun ini adalah toksik untuk manusia dan sel mamalia. Keputusan ini mengimplikasikan potensi risiko keracunan makanan jika bakteria dibiarkan tumbuh dalam makanan, dan makanan tersebut kemudiannya dimakan oleh manusia. Oleh itu, ketoksikan yang tidak diketahui bukan bererti tidak toksik, dan ketoksikan (lichenysin) adalah kumulatif.

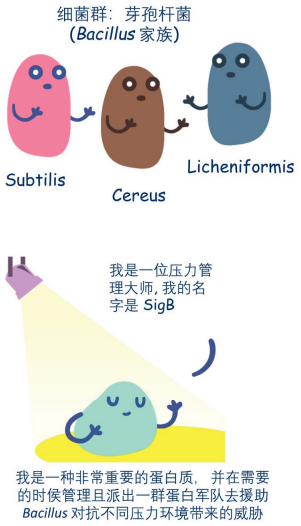
Terakhir, **Bab 6** membincangkan penemuan dalam tesis ini dari perspektif yang berbeza berdasarkan bukti saintifik. Pertama, cerapan baharu yang diperoleh untuk SigB dan mekanisme lain yang dikawalnya. Kedua, bagaimana strategi pengurusan tekanan ini menyumbang kepada ketahanan *Bacillus* dalam lingkungannya, dan akhirnya, implikasi pengetahuan yang diperoleh untuk mengkaji bakteria patogen yang lain dan implikasi praktikal lain yang berkaitan dengan sektor makanan.

论文简明摘要总结 (马来西亚中文)

PS: Thanks Yue and Jingya for cross-checking the text

称为芽孢杆菌的细菌群 （*Bacillus*）是生活在土壤中的最大和最知名的细菌群之一。本论文深入研究了该菌群的压力管理策略，特别是最著名的三个成员，*Bacillus subtilis*, *Bacillus cereus*, 和 *Bacillus licheniformis*。其中两个（Subtilis 和 Licheniformis）经常被用作细胞工厂来生产许多有价值的 enzymes（酶），就像在我们家中的洗涤剂一样，但 Cereus 是三个成员中不好的那一个，会污染食物并致病。因此，当我们出现腹泻或呕吐时，Cereus 可能就是罪魁祸首！这三种细菌广泛存在于环境中，因此经常会面对不同的压力环境，例如极热或极冷的温度又或是缺乏营养。然而，他们还是可以很好地处理这种压力情况，特别是通过一种称为 General Stress Response (GSR) 的压力管理机制。这种 GSR 反应由一种称为 Sigma B (SigB) 的关键蛋白质控制。





SigB 是这组细菌（*Bacillus*）的压力管理大师，就像人类的大脑一样。当人类受到压力时，我们的大脑会处理这些信息并激活反应，让我们准备逃跑或反击。与人类一样，当 *Bacillus* 遇到压力时（想象一个 42°C 的炎热夏天）, SigB 会被激活并充当指挥官，命令体内的管理系统去生产许多蛋白质来帮助 *Bacillus* 去对抗压力并生存。除了管理压力外，SigB 还可以控制 *Bacillus* 的其他机制，例如使细胞进入休眠状态或产生粘稠的化合物来保护细胞免受破坏。整个 SigB 控制的机制非常复杂，包括 1) 不同的环境压力信号可以激活 SigB，2) 不同的途径可以用于激活 SigB，3) SigB 可以控制一组基因和蛋白质军队去对抗不同的压力环境, 以及 4) SigB 除了处理压力外, 还有其他的主要功能。

第一章 介绍了有关于 *Bacillus* 的所有知识或其他值得了解的内容、包括了其有益或致病作用以及它们的压力管理策略。**第二章** 研究了来自 SigB 的命令是直接还是通过次要信使间接传递给抗压蛋白大军，并发现这两种方式都可能发生。此外，我们还发现，在 *Bacillus* 里的已知抗压蛋白大军数量已经从大

约 200 个增加到大约 500 个。因此，尽管我们已经掌控 SigB 控制的基本机制，当中还是有非常多值得探索的地方。此外，我们还检查了 *Bacillus* 以外的其他细菌群是否存在这种 SigB 控制的压力管理策略，并发现它存在于许多其他的细菌群中。

第三章 表明， 虽然 SigB 的主要功能是压力管理，但仍然具有其他功能，例如控制细菌的游泳活动。除此之外，SigB 像一个管理员，还能与在 *Bacillus* 里的其他管理员（如食物和营养管理员）进行沟通，并一起选择要摄入的碳水化合物或蛋白质来源。获得的结果证明，即使 SigB 的主要功能是压力管理，它并不是唯一观察到的关键功能。

随后，在**第四章**中，我们首先研究了 SigB 对类似于洗涤剂物质（称为 Lichenysin-表面活性剂）制造的控制管理，并检查了当将 *Bacillus* 暴露于这种物质时，是否会对它们造成一种压力环境。我们获得的结果表明，暴露于 Lichenysin 这种物质会导致细菌遭受轻度的压力，而这种轻度压力环境可以适度激活 *Bacillus* 的 SigB 功能，并在激活后可以更佳地保护细菌不受其他致命压力的损害。这章表明轻度的压力还是一种压力，并会激活连锁和轻度的压力反应。

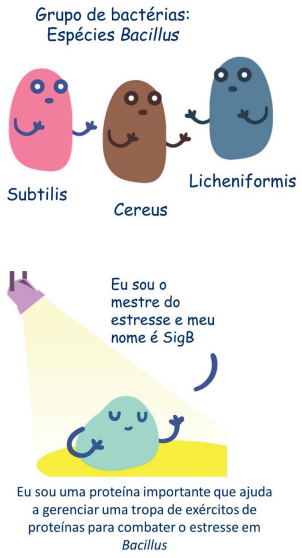
所有的 Licheniformis 细菌成员都可以在体内制造这种 Lichenysin 表面活性剂, 如果这些细菌刚好生存在食物中并制造这种物质，就有可能会导致食物中毒。因此，我们在**第五章**中研究了 Licheniformis 细菌成员在食物里制造 Lichenysin 的情况，并确定了其对人体细胞的毒性。研究结果表明，不同 Licheniformis 细菌成员之间制造的 Lichenysin 数量差异非常大，并且取决于食物成分、温度和生长条件。我们还发现这种物质对人类和哺乳动物细胞有毒。这些结果都表明，如果允许细菌在食物中生长并制造 Lichenysin，而这些食物随后被人类摄入的话，很大可能会导致食源性中毒。因此，未知毒性的物质并非都是无毒的，而 Lichenysin 毒素的毒性通常都是累积性的。

最后，**第六章里，**基于科学证据， 我们从不同的角度探讨了本论文的发现。首先，我们获得了关于 SigB 及其控制的其他机制的新见解。其次，我们了解了这些压力管理策略如何帮助 *Bacillus* 在不同的地理环境中持续生存。最后，我们提出了如何将获得的这些细菌抗压知识用于其他病原菌的研究，与其他用于提升相关食品安全的实际用途。

O resumo da tese para o público em geral (Português)

PS: Thanks Nayara for translating the text

O grupo de bactérias chamado Bacillus é um dos maiores e mais conhecidos grupos de bactérias que vivem em solos. Esta tese investigou aprofundadamente as estratégias de gestão de stress deste grupo bacteriano, especificamente três membros de renome, chamados de Bacillus subtilis, Bacillus cereus e Bacillus licheniformis. Dois deles (Subtilis e Licheniformis) são frequentemente usados como fábrica de células para produzir muitas enzimas valiosas e importantes, tais como os detergentes usados na limpeza de casa. No entanto, o Cereus é um vilão que pode contaminar os alimentos e causar doenças nos seres humanos. Dessa forma, quando você tiver diarréia ou vômito, Cereus pode ser o culpado disso! Essas três bactérias estão espalhadas no meio ambiente e, portanto, são frequentemente expostas a situações estressantes, tais como calor ou frio extremos ou falta de nutrientes. Nessas situações, elas driblam essas condições de estresse muito bem, especificamente por meio de um mecanismo de gerenciamento de estresse conhecido como resposta geral ao estresse (GSR). Esta resposta GSR é controlada por uma proteína crucial nesse processo, chamada de Sigma B (SigB).



A SigB é o mestre em gerenciamento de estresse para este grupo de bactérias (Bacillus), assim como os cérebros dos seres humanos. Quando os humanos estão estressados, nosso cérebro processa essas informações e ativa respostas para nos preparar para fugir ou revidar. Assim como os humanos, quando Bacillus encontra-se em estado de estresse (imagine um dia quente de verão de 42°C), o mestre SigB é ativado e age como um comandante, para ordenar a produção de muitas proteínas para ajudar o Bacillus a combater o estresse e sobreviver. Além de gerenciar o estresse, o mestre SigB controla vários outros processos em Bacillus, tais como transformar as células em estado dormente ou produzir compostos viscosos para proteger as células.

Todo o mecanismo controlado por SigB é muito complicado, incluindo 1) diferentes sinais de estresse ambiental que ativam esse regulador mestre, 2) as diferentes vias usadas para ativar o mestre SigB, 3) uma tropa de genes e exércitos de proteínas controlados por SigB para combater o estresse, e 4) as diversas funções do SigB além de lidar com o estresse. **O Capítulo 1** apresenta tudo o que você sabe ou que vale a pena saber sobre Bacillus, seus papéis benéficos ou patogênicos e suas estratégias de gerenciamento de estresse. O **Capítulo 2** investigou se os comandos do SigB são entregues direta ou indiretamente aos exércitos de proteínas de combate ao estresse por meio de um mensageiro secundário e descobriu que isso pode ocorrer de qualquer maneira. Além disso, revelamos que o número de tropas de combate ao estresse conhecidas aumentou de cerca de 200 membros para > 500 em Bacillus. Portanto, embora o mecanismo controlado por SigB seja bem conhecido, sempre há mais o que explorar. Além disso, também verificamos a presença ou ausência de tais estratégias de gerenciamento de estresse controladas por SigB em espécies de bactérias similares e distantes dos Bacillus e descobrimos que elas existem em muitos outros grupos de bactérias.

O **Capítulo 3** mostrou que a principal responsabilidade do mestre SigB é gerenciar o estresse, mas essa proteína ainda tem outros papéis no Bacillus, como controlar a "atividade de natação" da bactéria. Além disso, o manager do SigB se comunica com outros managers do Bacillus, como o manager dos alimentos e nutrientes, para selecionar as fontes de carboidratos ou proteínas a serem ingeridas. Os resultados obtidos indicaram que, embora a função de gerenciamento de estresse para o mestre SigB esteja bem definida, essa não é a única função crucial observada.

Posteriormente, no **Capítulo 4**, estudamos o papel do mestre SigB no controle da produção de um composto do tipo detergente (chamado liquenisina) e examinamos se a exposição a esse composto causava estresse no Bacillus. Nossos resultados mostraram que a exposição à liquenisina ativou modestamente o mestre SigB. Essa ativação forneceu pequena proteção ao Bacillus quando exposto a outro estresse letal, demonstrando que uma condição mesmo que levemente estressante é ainda estressante, e que uma resposta leve ainda é uma resposta.

Este composto (liquenisina) é um tipo de detergente que pode ser um potencial agente de intoxicação alimentar e é produzido por todos os membros do Licheniformis. Portanto, também investigamos a produção deste composto por membros selecionados de Licheniformis em alimentos e determinamos sua toxicidade para células humanas no **Capítulo 5**. Os resultados mostraram que as quantidades de liquenisina produzidas variaram amplamente entre diferentes membros de Licheniformis e dependem da composição dos alimentos, temperaturas e condições de cultivo. Também demostramos que este composto é tóxico para células humanas e de mamíferos. Esses resultados indicam que pode haver um risco de intoxicação alimentar se a bactéria crescer em alimentos e esses alimentos forem posteriormente ingeridos por humanos. Portanto, a toxicidade desconhecida de um composto não significa que ela seja não-tóxica, e a toxicidade da toxina (liquenisina) é acumulativa.

Por fim, o **Capítulo 6** discute todas as descobertas encontradas nesta tese a partir de diferentes perspectivas baseadas em evidências científicas. Em primeiro lugar, descobrimos os novos insights obtidos para o mestre SigB e outros mecanismos que ele controla. Em segundo lugar, descobrimos como essas estratégias de gerenciamento de estresse contribuíram para a persistência de Bacillus nos ambientes e, por fim, elucidamos as implicações desse conhecimento obtido no estudo de outras bactérias patogênicas e outras implicações práticas relevantes para o setor de alimentos.

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Being independent is an outstanding value that one can have, but one's success is not only the result of individual effort. Completing a PhD is never a journey alone, so this thesis is undoubtedly the result of collaborative teamwork. Also, I have finally come to realize what my Ex-supervisors from Germany meant when they told me that completing a PhD in the Netherlands is much more challenging! However, thanks to the training they provided me in my MSc., which prepared me well to complete this "odyssey" in the Netherlands today.

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I would like to extend my sincere gratitude to all the **co-authors** from other departments at Wageningen University or other organizations involved in this thesis, especially **Jos** and **Michiel,** for helping me with the bioinformatics analysis during your time at NIZO, **Marcel. T** for your brilliant idea and initiative on the Bc1009 project, for collecting and analyzing the transcriptomics data, for the co-supervision of students, and for the fruitful discussion on the *B. cereus* project (sometimes also some complaints together ;p). **Alex. R** and **Uwe** for running and analyzing the proteomics samples, and **Blanca** and **Jerry** for arranging the toxicity assays at HMI.

This endeavor would not have been possible without my colleagues and friends at FHM. **Andy**, thank you very much for your invites to all FHM's events, especially the most remarkable PhD trip to China. I was so glad to have joined in 2019; that was the first time I connected with all the PhDs in our department. Thank you also for all your life hack tips, lunches, and dinners you prepared, and all the nice encouraging words you have told me! **Alberto G,** for being the person who drags me to all the Vrijmibo events, for playing many different roles in my life, my officemate at FHM, my colleague who works late with me, my friend who listens to me, drinks 7 gin-tonic with me, and most importantly, my brother who truly cares

for me and help me to solve all the life-psychological related issues, haha, and finally, for helping me to organize the party and supporting me all along the way. **Rebecca**, thank you for your support, especially in this last year. I admire your personality and your courage to voice out and also appreciate that it is always so easy to talk to you. I can be myself with you, knowing that telling you any mean jokes will not hurt. Haha. Thank you for accepting and helping me to organize my PhD party, listening to all my naggings and complaints and comforting me, and giving me the Rebecca type of advice! **Nathalia B.,** we met at FHM with my "intrusive'' introduction, and soon we realized we shared a similar experience, mainly making fun of the mandatory German-speaking events. I regretted not joining your two solo trips to France because of the thesis writing! Promise for next year :D **Jeroen,** thank you for helping me with the volcano plots, for witnessing the epic ''hotpot'' night, for the leisure drinking days, and for meeting us during the pandemic ;p!!. **Pjotr** and **Alex**, you were the two I first met at the PhD weekend. Thank you for the fun time, especially in China, and Alex the genius in the department, thanks for the good talks in China and also sharing some nice project ideas together ;) **Nathalia C.,** thank you for recommending jobs to me and the limited time shared in China too, **Angela**, thank you for telling all the stories, and answering my 10 million questions about FHM in China. **Bernard**, **Maren, Jasper B., and Anneloes,** thanks for the unforgettable trip together. **Yue,** thank you for advising me on what to do at the last phase of PhD, writing the guide together with me, and being my loyal listener of all my presentations :), sorry to bother you with so many questions in the last few months ;p, and for your patience with me by answering all my texts and emails :D. **Georgios,** thank you for being more excited than me when I submitted the thesis reading version, haha :D, and all the dinners, tennis, and drinks together. **Alberto B.** thanks for the good time in Germany and Switzerland, for being my Tennis coach, some funny moments in the lab practical and the party (you know, haha, I am following your red light), and also the time together with other active Vrijmibo members, **Sylviani** (again ;p), **Domiziana**, **Tamara** and **Xuchuan,** on all Fridays on drinking, playing tennis, dinner out, the BDDD, the durian experience, and many others! A huge thanks to the PhD trip organizers of 2022 (**Linda, Xuchuan, Jasper B., Georgios, Domi, Frank)**. Together, we made the trip to Germany and Switzerland from impossible to possible! The crazy late-night climbing in the middle of nowhere to this creepy house was the highlight of the entire trip. Thank you also for being so considerate and taking more responsibility for the follow-up works when I finalize the thesis. Thank you to all other FHM colleagues, **Oscar** (for your home brew wine, home grew veges, and your persistency in forming a veluweloop run team haha)**, Jasper Z. Thelma, Wilma, Eddy, Judith, Dennis, Anne,** and **Ingrid** (for always making sure that my legs are not exposed in the lab :P)**,** for the coffee breaks, time in the practical and lunch together. **Gerda,** thank you for helping me to deal with all administrative issues, for reminding me to filling in the hours, and your kindness with me when I made you stressed out in your birthday :) **James,** thank you for constantly forcing me to go home, especially during the last few months of the thesis writing phase and sharing my struggles, sometimes just a few words with you comfort my agitated mind! Ps: I still insist that I am not a robot ;p

When home is far away, your friends are your family. I am very fortunate to have many and would love to take this chance to express my most profound appreciation to you who gave me unconditional support in different moments during my PhD. **Guille,** you witnessed a lot of ups and downs in my life. Thank you for being there, coming to see me wherever I am, making an effort to make me happy, and bringing me to your family events so that I am not left alone in Christmas. Those crazy stories we shared, and times in London, Spain, Malaysia, Italy.. we left many footprints together, and our most outstanding achievement is going from being poor to okay! Hahaha! Thank you also for putting an effort into

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PS: Please excuse me that I am not able to mention each of your names here, but a big thanks to all of you, other technicians, collaboration partners, coworkers from other companies and organizations, and friends who have given me help these years.

About the author

Thank you for your interest in reading about the author; this is the story of how life takes you on unexpected twists and turns, which ended relatively, well. =)

Kah Yen Claire Yeak was born on 31st January 1991 in Selangor, Malaysia. She completed up to A-level in Kuala Lumpur, with biology as her worst science subject compared to chemistry/physics. Yet, she decided to continue pursuing her BSc. in Applied Biology in Germany, merely to go abroad, but the subject of study was kinda secondary ;p. The reason was also easy because Biology was the only subject that was taught in English at that time. She then turned down the offer from UPM and left for Bonn, Germany, the beautiful town of Beethoven, but not to study music apparently. She still clearly remember the shocking facial expression of her biology teacher when the teacher found out that the rebellion Claire who never did all the homeworks and slept through all biology classes at school was enrolled in a biology BSc!

The journey in Germany began. Thanks to the strict and excellent education system in Germany, she failed one subject once and never dared to fail a second time; because 3 times failing means that one is not talented and will be kicked out from the study and never able to do a similar course in entire Germany. Guess which subject? Of course, it was biology-related: Microbiology. Nevertheless, also thanks to this failure, she finished her BSc. with the best grade in microbiology and graduated with high distinction. After that, she did BSc. internship at the University Hospital of Bonn and worked on stem cell research, assisted medical doctors and PhDs in characterizing human mesenchymal stem cells isolated from patients with osteoporosis. At graduation, she decided NOT to continue her MSc. study related to microbiology or genetic engineering, so she returned to Malaysia and worked as a product development intern in Kuala Lumpur.

She was later accepted to the University of Lübeck and the University of Göttingen, and very unexpectedly, decided to go to the latter and continued her MSc. in Microbiology and Molecular life sciences in Göttingen because it was closer to Bonn! During 2 years of her MSc. study, she received intensive training in genetic engineering techniques and other research methods used in microbiology and bacteriology studies. In her MSc. thesis and internship projects, she worked on the yeast and bacteria genetics research projects in the lab of AG Krebber, AG Commichau and AG Stülke, respectively. She investigated novel vitamin B6 metabolic and production pathways in bacteria and life in the absence of vitamin B6 and graduated MSc. with the higest distinction.

Upon finishing, she was determined NOT to continue a PhD, but look for a job. However, history is often strikingly similar! She was advised that a PhD is a prerequisite in Germany, so she applied for the Marie Skłodowska-Curie grant and (very fortunately), received the grant to continue her PhD in the Netherlands. Remembering the interview in Amsterdam, a beautiful hot summer day that (has set ;p) her up for a full commitment of another 3 years at NIZO, the contract-based research company in Ede. During her PhD, she studied bacterial stress management, and specifically investigated the novel roles of SigB in the

general stress response of *Bacillus* (which is the title of this book, and hopefully, you read :). At the end of 2020, she finished her experimental works, but history seems to repeat itself again. She started her 4th year of PhD and the meantime also her first year of Post-doc position in 2021 at the Food Microbiology department at the University of Wageningen. Not hard to guess; it was also an unexpected decision..which is ongoing....and going...

Now, she knows that being a professor IS NOT her next plan, and she is certained to switch her career to the corporate world for future work. However, no one can predict whether the history will once again, repeat itself. To continue discovering whether she will make again ''unexpected'' decisions in her future career, you can stay connected with her on LinkedIn or contact her via email.

Scan this QR code to connect with her on LinkedIn.

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Full CV available on LinkedIn.

A

List of publications

(This dissertation)

1. **Yeak, K.Y.C.,** Boekhorst, J., Wels, M., Abee, T., Wells-Bennik, M.H.J. Prediction and validation of novel SigB regulon members in *Bacillus subtilis* and regulon structure comparison to Bacillales members. (Submitted to BMC Microbiology).

2. **Yeak, K.Y.C.,** Templaars, M., Wu, J.L., Westerveld, W., Reder, A., Voelker, U., Panne-Farre, J., Wells-Bennik, M.H.J., Abee, T. SigB modulates novel SigB regulon members via Bc1009 in non-stressed and heat-stressed cells revealing its alternative roles in *Bacillus cereus*. (Submitted to BMC Microbiology).

3. **Yeak, K.Y.C.,** Wells-Bennik, M.H.J., Perko, M., Staring, G., Fernandez-Ciruelos, B., Wells, J., et al. (2022b). Lichenysin production by *Bacillus licheniformis* food isolates and toxicity to human cells. *Front. Microbiol.* 13. Available at: https://www.frontiersin.org/article/10.3389/fmicb.2022.831033 [Accessed January 24, 2022].

4. **Yeak, K.Y.C.,** Bijlaart, M., Abee, T., Wells-Bennik, M.H.J. The role of SigB in biosurfactant synthesis and the effect of biosurfactant on SigB general stress response in *Bacillus licheniformis*. (Manuscript in preparation).

5. **Yeak, K.Y.C.,** Ejder, S., Guerreiro, D., Dessaux, C., Lewis, R., Abee, T., Wells-Bennik, M.H.J., O'Byrne, C., Marles-Wright, J. The role of Prli42 mini-protein in SigB adaptive stress response of *Bacillus subtilis* and *Listeria Monocytogenes*. (Manuscript in preparation).

(Others)

1. **Yeak, K.Y.C.,** Palmont, P., Rivière, G., Bemrah, N., den Besten, H. M. W., and Zwietering, M. H. (2022a). Microbial and chemical hazard identification in infant food chains. *Glob. Pediatr.* 2, 100010. doi: 10.1016/j.gpeds.2022.100010.

2. **Yeak, K. C.,** Fanning, S., Bourdichon, F., Zwietering, M., Betts, R., and Forsythe, S. *Cronobacter* in the Spotlight: New Insights Into a Known Organism [White paper]. ILSI Europe. New Food Mag. Available at: https://www.newfoodmagazine.com/whitepaper/167276/cronobacter-in-the-spotlight-new-insights-into-a-known-organism/.

3. Georgalis L., **Yeak, K.Y.C.,** Tsimpou C., Wells-Bennik, M.H.J., Fernandez P., den Besten, H. M. W., Garre A. Analysis of wt and Δ*sigB* mutant adaptation behaviors in isothermal and dynamic heat treatment improved predictive model parameters for microbial inactivation. (Manuscript in preparation).

Overview of completed training

A. Discipline-specific activities

Name of the course/meeting	Organizing institute(s)	City	Year
Microbial Stress Kinsale, Co Cork Conference	FEM	Kinsale, Ireland	2018
ListMaps Symposium	FEM	Kinsale, Ireland	2018
The Power of Microscopy: Seeing is Believing	Cambridge University	Groningen, Netherlands	2018
Translating Postgenomic Microbiology to Advancing Food Science & Safety	Nestle, NIZO, Natac	Groningen, Netherlands	2018
Secondment at University of Greifswald, Germany	Greifswald University	Greifswald, Germany	2018
Elucidating Structure & Function in Biology	Newcastle University	Newcastle, UK	2019
Advanced Proteomics	University Greifswald	Greifswald, Germany	2019
20th Gram positive Meeting Washington	National Institutes of Health	Washington, USA	2019
Symposium "The Brave New World of Smart Data & Semantics in the Life Sciences"	WUR	Wageningen, Netherlands	2019
Microbial Stress Kinsale, Rome, Conference (Online)	PATHSENSE	Rome, Italy	2020
International Commision on Micriobiological Specifications for Foods (ICMSF)- Latin American Sub-commission	ICMSF	Online	2021
IAFP Europe Food protection	IAFP	Online	2021
10th Europaediatrics 2021	EPA/UNESPA	Zagreb, Croatia	2021
56th Congress of the Turkish Pediatric Association (TPA) 2021,	TPA	Istanbul, Turkey	2021

B. General courses

Name of the course	Organizing institute(s)	City	Year
Bench to Market: Starting up a Spin-Off Company	NUIG	Galway, Ireland	2018
Public Engagement and Outreach	NUIG	Galway, Ireland	2018
VLAG PhD week	VLAG	Baarlo, Netherlands	2018
Scientific Integrity & Ethics	RUG	Groningen, Netherlands	2018
Developing a Research Proposal: Grant Writing Skills	University Regensburg	Ede, Netherlands	2019

Introduction to R	WUR	Wageningen, Netherlands	2019
Career Planning for PhD Graduates	NIZO	Madrid, Spain	2020
Intensive writing course	Radbound University	Wageningen, Netherlands	2020
Project and Time management	WGS	Wageningen, Netherlands	2020
Reviewing a Scientific Manusript	WGS	Wageningen, Netherlands	2020
R-mark down	WGS	Wageningen, Netherlands	2020
Popular Scientific writing	WGS	Wageningen, Netherlands	2021

C. Other activities

Name of the course	**Organizing institute**	**Place**	**Year**
Preparation of research proposal	FHM	Wageningen	2018
FHM PhD knowledge exchange visit China	FHM	China	2019
Food Microbiology department seminars	FHM	Wageningen	2018-2022
FHM PhD knowledge exchange visit Germany and Switzerland (Including organization)	FHM	Germany, Switzerland	2022

Supervision

MSc. student	**Year**	**Course**	**Year**
Victoria Martinez Sanchez	2019	Food Microbiology practical	2021
Manca Perko	2019	Food Microbiology practical	2022
Eline van Ophem	2020		
Mounia Bijlaart	2020		
Wouter Westerfeld	2020		
Lize Fang	2021		
Cheyenne van Ee	2021		
Lois Hiltjesdam	2021		
Natisha Vatwani	2022		
Jingya Wang	2022		
Ioanna Vouga	2022		
Han Xiao Li	2022		
Wirasha Ghisai	2022		
Han Zhang	2022		

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