Fine tuning of the *Bacillus cereus* stress response: role of transcriptional regulators

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Content

Abstract

The Gram-positive bacterium *Bacillus cereus* frequently causes foodborne illnesses and food spoilage. *B. cereus* can cause illnesses as it is able to produce a variety of virulence factors, including toxins. Food spoilage is caused by *B. cereus* because of its ability to survive and grow in a high diversity of environments, including foods, such as milk and pasta. The ability to display these features is encoded by the genes present in *B. cereus*. For the *B. cereus* type strain ~5300 genes were identified. Not all these genes can be expressed at the same time, as energy costs are too high and functions encoded by these genes can not all work simultaneously. To express the correct genes in the encountered environment the expression of the genes in *B. cereus* is tightly regulated. This thesis focuses on transcriptional regulation of *B. cereus* ATCC 14579 in response to changing environments. A comparison between anaerobic and aerobic growth of *B. cereus* revealed large differences in gene expression, including genes involved in metabolism and genes encoding enterotoxins and hemolytic enzymes. The enterotoxin and haemolytic genes are highest expressed under oxygen limiting conditions. The catabolite control protein CcpA in *B. cereus* was shown to be responsible for an optimal use of glucose as an energy source. In addition, CcpA was revealed to repress the gene expression of the enterotoxins Hbl and Nhe, indicating that CcpA represses the expression of *hbl* and *nhe* genes when glucose is present. These enterotoxin genes are functionally expressed in the gastro-intestinal tract, and it is suggested that the expression is at least partly regulated by CcpA-mediated glucose sensing. The genes regulated by three additional transcriptional regulators, so called alternative σ factors, have been assessed. Alternative σ factors function as co-factors of RNA polymerase and allow for modulation of gene expression by recognizing specific DNA sequences. The σ factor σ^B was previously shown to be involved in gene regulation in response to mild heat treatments. The genes identified in this thesis to be regulated by σ^B included genes involved in heat stress and with a function in formation of the cell wall of spores. Comparison of the σ^B regulon with that of other Gram-positive bacteria revealed the *B. cereus* σ^B regulon to be rather small, indicating a relatively recent introduction of σ^B on the *B. cereus* genome. In addition, the Extracytoplasmic Function (ECF) σ factors σ^M and σ^Z were studied. σ^M was activated by ethanol stress and its regulon included genes encoding functions involved in the Activated Methyl Cycle. This cycle includes the production of the precursor of the signalling molecule AI-2 by LuxS, and leads to the production of L-cysteine. This thiol-group containing amino acid has been shown in eukaryotic cells to counteract ethanol-induced oxidative stress, and a similar function is suggested in *B. cereus* and other bacteria. Finally, overexpression studies of the ECF σ factor σ^Z, which expression is increased under alkaline pH and ethanol stress conditions, revealed the σ^Z regulon to include genes encoding transporters and genes with functions in cell surface modification that may lead to alteration of the interaction of *B. cereus* with its environment. The research described in this thesis for *B. cereus* expands the understanding of the role of regulators in fine tuning of transcriptional regulation in response to changing environment.

1

Introduction and outline of the thesis

Abstract

The Gram-positive endospore-forming organism *Bacillus cereus* is a significant cause of food poisoning and food spoilage events. *B. cereus* is able to cause two different types of food poisoning, a diarrhoeal type and an emetic type. *B. cereus*-caused food poisoning occurs in a vast array of food products, while food spoilage by *B. cereus* is mainly a problem in the dairy industry. In this chapter, an overview is given of *B. cereus* as the cause of food poisoning and food spoilage. *B. cereus* has been shown to be part of a group of highly related bacteria, the so-called *B. cereus* group, which comprises amongst others *Bacillus thuringiensis* and *Bacillus anthracis*. A genomic overview of the species of the *B. cereus* group and their characteristics is given, with specific attention for the regulators of gene expression present in these species. At the end of this chapter, the outline of the thesis is presented that describes the research on the response of *B. cereus* ATCC 14579 to changing environments and the regulators involved in these responses.

Bacillus cereus, a foodborne pathogen and food spoilage microorganism

Bacillus cereus is a Gram-positive, endospore-forming, facultative anaerobic, rod-shaped bacterium, of which the spores and vegetative cells are ubiquitously found (Granum and Lund, 1997; Kotiranta *et al.*, 2000). *B. cereus* was first described in 1887, as it was isolated from a cow shed (Frankland and Frankland, 1887), and this isolate *B. cereus* ATCC 14579 is recognized as type-strain (Fig. 1).

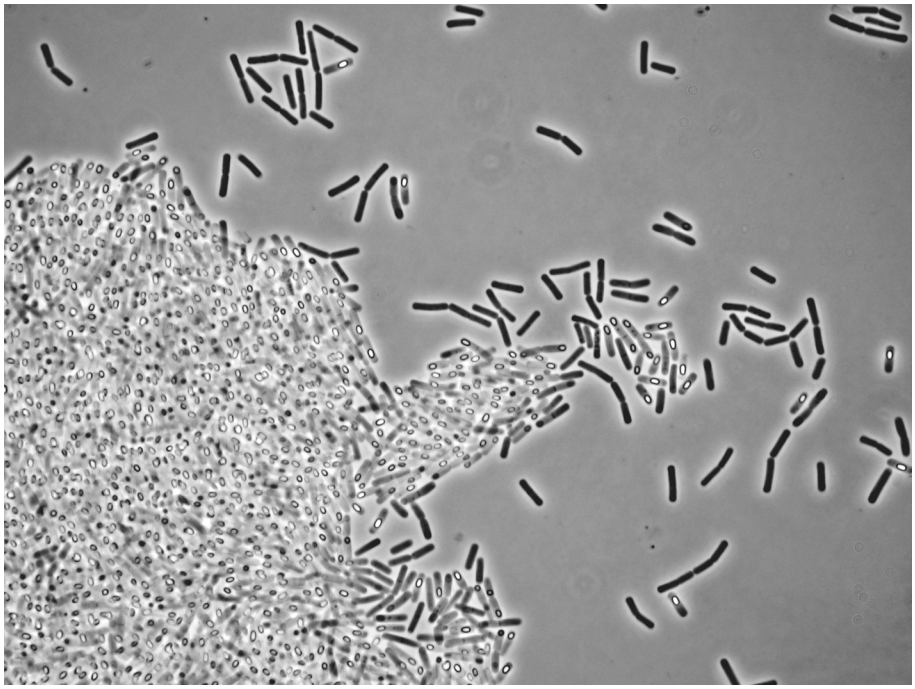

Fig. 1. Phase contrast microscopy picture of vegetative and sporulating cells of *B. cereus* ATCC 14579. The cells were grown on a sporulation agar plate.

In general, *B. cereus* is capable of growth in a temperature range between 10 and 50 °C, with a growth optimum between 28 and 35 °C. However, *B. cereus* variants have been isolated that are capable of growth at temperatures as low as 5 °C. Furthermore, *B. cereus* can grow in a pH range from 4.9 to 9.3 and at a minimum water activity of 0.91-0.95 (Johnson, 1984). One of the most interesting features of *B. cereus* is its capability to form endospores. Spores are survival vehicles that are metabolically inactive and extremely resistant to conditions such as freezing, heating, drying, radiation and nutrient shortage. Spores are formed in adverse conditions to enable survival in these conditions. Spores are able to germinate and grow out when more favourable conditions are encountered (Nicholson *et al.*, 2002).

The importance of *B. cereus* research emerged when the Norwegian doctor Steinar Hauge recognized and proved that *B. cereus* could be the cause of food poisoning (Hauge,

1955). After culturing *B. cereus* isolated from an outbreak of foodborne disease, Hauge artificially contaminated sterile vanilla sauce and ate the contaminated sauce. The symptoms of diarrhoeal disease that developed after 16 hours after ingestion could thus be attributed to *B. cereus*. Furthermore, in 1974 it was shown that *B. cereus* can also be the cause of an emetic type of food poisoning (Mortimer and McCann, 1974). Next to food poisoning, *B. cereus* is of major economic concern to the food industry being the cause of food spoilage events (te Giffel, 2001) and causing equipment failure by biofilm formation (Kumar and Prasad, 2006).

B. cereus food poisoning

One of the reasons why research is focusing on *B. cereus*, is that *B. cereus* can cause food poisoning. The importance of *B. cereus* in food poisoning is difficult to determine. The symptoms caused by *B. cereus* are generally mild and are therefore believed to be underreported in comparison to other foodborne diseases such as listeriosis and salmonellosis. Generally, *B. cereus* outbreaks are only reported when a large number of people is affected or when the symptoms are more severe than usual. A surveillance study of foodborne disease in Europe has shown great differences in causes of disease between different countries (Schmidt and Gervelmeyer, 2003). This surveillance showed that in some countries, such as the Netherlands and Norway, *B. cereus* is an important food pathogen, whereas in other countries, such as France and Belgium, the number of *B. cereus* outbreaks was marginal. In the Netherlands, *B. cereus* was even the most important cause of food poisoning outbreaks for cases in which a causative agent could be determined in 1999 and 2000, causing 26% and 25% of the outbreaks, respectively. Comparable numbers were found in Taiwan, where 18% of foodborne diseases between 1986 and 1995 with bacteria as the causative agent were caused by *B. cereus* (Pan *et al.*, 1997). In contrast, in France only 0.7% and 5% of the outbreaks of foodborne diseases in 1999 and 2000, respectively, were ascribed to *B. cereus*. For Belgium 2 of 156 outbreaks in 1999 were shown to be caused by *B. cereus* and 0 of 74 in 2000 (Schmidt and Gervelmeyer, 2003). These numbers were comparable to what was found in an epidemiological study of foodborne disease in the United States, where 0.2% of the cases was found to be caused by *B. cereus* (Mead *et al.*, 1999).

The high prevalence in countries as the Netherlands, Norway and Taiwan, may be due to local awareness of the occurrence of *B. cereus* infections. The recent lethal cases of *B. cereus*-caused gastro-intestinal infections in France (Lund *et al.*, 2000) and Belgium (Dierick *et al.*, 2005) show the relevance of determining *B. cereus*-caused infections. It will be of importance to see if guidelines for analysis of gastro-intestinal infections in these countries will change and how this will affect the reported occurrence of *B. cereus*-caused foodborne disease.

Diseases caused by *B. cereus*

It has been shown that *B. cereus* can be the causative agent of two different types of gastro-intestinal diseases, a diarrhoeal type or an emetic type of illness. The diarrhoeal type of illness can be associated with the consumption of foods such as meat products, soups, vegetables, sauces and dairy products (Schoeni and Wong, 2005). The symptoms of this type

of food poisoning occur between 8 to 16 h after ingestion of contaminated food and last no longer than 24 h. The symptoms are caused by enterotoxins produced in the gastro-intestinal tract, of which hemolysin BL, non-hemolytic enterotoxin and cytotoxin K are the three best studied (McKillip, 2000).

The first enterotoxin that was widely studied was hemolysin BL (Hbl), which is a three-component enterotoxin consisting of a B component and two L components, L1 and L2. Hbl has been named a hemolysin as it not only possesses enterotoxic properties, but also hemolytic properties. Hbl causes cells to lyse via a colloid osmotic mechanism through the formation of transmembrane pores (Beecher *et al.*, 2000). The three components that form Hbl are encoded by three genes, namely *hblC* encoding the L2 component, *hblD* encoding the L1 component and *hblA* encoding the B component (Heinrichs *et al.*, 1993; Ryan *et al.*, 1997). When present on the genome these three genes form one operon (Ivanova *et al.*, 2003).

The non-hemolytic enterotoxin (Nhe) has been shown to consist of three components as well, encoded by *nheA*, *nheB* and *nheC* (Granum *et al.*, 1999). NheA and NheB show similarity to the L2 and L1 components of Hbl, respectively. No similarity between NheC and the B component of Hbl exists. The Nhe encoding genes form one operon when present on the genome, as shown for Hbl (Ivanova *et al.*, 2003). In contrast to Hbl, no hemolytic activity was shown for Nhe (Lindback *et al.*, 2004).

In addition to Hbl and Nhe, cytotoxin K (CytK) has been well studied. CytK consists only of one component and was first isolated from a *B. cereus* strain that was the cause of a severe outbreak of gastro-enteritis in France in 1998. The extremely rare necrotic enteritis caused by *B. cereus* was found to be lethal for people (Lund *et al.*, 2000). CytK acts by the formation of pores in lipid bilayers that may infer cytotoxic effects on intestinal epithelial cells resulting in loss of their barrier function (Hardy *et al.*, 2001). The CytK encoding gene, *cytK*, can be found on the genome of many *B. cereus* strains which are associated with food poisoning, but also on the genome of the type-strain *B. cereus* ATCC 14579 (Fagerlund *et al.*, 2004; Guinebretiere *et al.*, 2002). The effectiveness of CytK in pathogenesis is affected by amino acid substitutions, and modifications in membrane-spanning regions have been reported (Fagerlund *et al.*, 2004). In addition, expression of *cytK* may differ between strains (Brillard and Lereclus, 2004).

The production of the three enterotoxins Hbl, Nhe and CytK is influenced by many different factors such as medium composition, pH, aeration and concentration of certain carbohydrates (reviewed by (McKillip, 2000)). Furthermore, it has been shown that the pleiotropic transcriptional regulator PlcR is involved in the expression of the genes encoding the Nhe, Hbl and CytK enterotoxins (Lund *et al.*, 2000; Okstad *et al.*, 1999). In general, enterotoxins are heat-labile and sensitive to proteolysis by gastric enzymes. Therefore, it is believed that the diarrhoeal type of illness is caused by production of enterotoxins after outgrowth of spores in the intestine (McKillip, 2000). This is in agreement with the fact that *B. cereus* mainly enters the human gastro-intestinal tract as spores (Granum and Lund, 1997), although more recently, evidence has been supplied that also a significant portion of vegetative cells may survive gastric passage (Wijnands *et al.*, 2006).

The emetic type of food poisoning caused by *B. cereus* has been attributed to the production of only one toxin, the so-called emetic toxin or cereulide. Foods associated with this type of disease are mainly rice and pasta (Schoeni and Wong, 2005), but recently, cereulide producing strains have been detected in milk powder and infant formula as well (Shaheen *et al.*, 2006). The emetic type of food poisoning was first described in 1974, when *B. cereus* could be linked to an outbreak of food poisoning in the United Kingdom of which vomiting was the major symptom (Mortimer and McCann, 1974). Symptoms of the emetic type of food poisoning occur 0.5 to 6 h after consumption of the contaminated food and last no longer than 24 h. Cereulide is a dodecadepsipeptide, with the chemical formula (D-*O*-Leu-D-Ala-*O*-Val-L-Val)$_3$~, that is the product of a single non-ribosomal peptide synthetase. This peptide synthetase is unique to cereulide forming strains (Ehling-Schulz *et al.*, 2004). Recently, it has been shown that the genetic determinants of the emetic toxin are plasmid-borne. This plasmid is not present in the type strain *B. cereus* ATCC 14579 (Hoton *et al.*, 2005). The mode of action of cereulide is possibly similar to the closely related ionophore valinomycin, as both compounds act as potassium ionophores on mitochondria (Mikkola *et al.*, 1999). Cereulide is resistant to heat, low pH and to proteolytic enzymes of the gastro-intestinal tract (Ehling-Schulz *et al.*, 2004). In contrast to enterotoxins, cereulide causes food poisoning when formed by *B. cereus* in food before consumption. This feature can lead to problems when food is prepared in advance for instance at large venues, where reheating is required before consumption. In the time span between preparation and reheating, *B. cereus* spores that survived the preparation steps can grow out and produce cereulide. Reheating will inactivate the bacteria, while the cereulide remains active, subsequently causing emetic food poisoning. This scenario has proven to occur in several occasions in the past years (1994; Asaeda *et al.*, 2005; Dierick *et al.*, 2005). Recent studies have focused on detecting and quantifying cereulide activity (Andersson *et al.*, 2007; Rajkovic *et al.*, 2006). In coming years, the research on cereulide and cereulide producing *B. cereus* strains is expected to provide new insights in occurrence of the emetic type of disease.

Additionally, *B. cereus* can occasionally cause severe non-gastro-intestinal infections such as periodontitis, fulminant endophthalmitis, and meningitis in immunocompromised patients (Kotiranta *et al.*, 2000). Interest in non-gastro-intestinal infections is growing as *B. cereus*-caused infections are emerging in clinical settings. Treatment of clinical *B. cereus* infections is often difficult, as *B. cereus* is usually resistant to frequently-used antibiotics like penicillins and cephalosporins (Gaur and Shenep, 2001).

B. cereus food spoilage

Growth of unwanted bacteria can cause enormous expenses for food industry, as this may lead to food spoilage (Gram *et al.*, 2002). Food spoilage caused by *B. cereus* occurs mainly in dairy industry, thereby for instance shortening the shelf-life of milk. *B. cereus* is present in soil, on cattle feed and in cattle faeces and is thus ubiquitously present in the dairy farm environment. From these sources raw milk can be easily contaminated with *B. cereus*, as its spores germinate more easily in milk than spores from other *Bacilli* (Wilkinson and Davies, 1973).

Spores and vegetative *B. cereus* cells present in food products can attach to processing equipment and form biofilms. Biofilms are multicellular complexes embedded in a matrix of exopolysaccharides that grow attached to a surface. Cells embedded in a biofilm are more resistant to cleaning agents and other anti-microbial substances, making them difficult to eradicate from processing equipment (Peng *et al.*, 2002; Stoodley *et al.*, 2002). Biofilms in processing equipment are a continuous source of contamination for food products by detachment of cells and spores from the biofilm. Next to this, biofilm formation may cause economic losses by causing equipment failure (Kumar and Prasad, 2006).

B. cereus strains isolated from food spoilage incidents generally do not produce cereulide. In contrast, enterotoxin producing strains are commonly isolated from food. Recently, however two psychrotrophic strains were also shown to produce cereulide (Thorsen *et al.*, 2006). Therefore, advanced knowledge is needed about *B. cereus* diversity, behaviour and pathogenic capacity in order to allow for better control of this pathogen in foods and in food production environments.

The *B. cereus* group of microorganisms

B. cereus is a member of the genus *Bacillus*, which is very heterogeneous. Species with a large variety in phenotypes and physiological and metabolic characteristics are found in this group. A phylogenetic study of the 3'end sequences of the 16S rRNA gene and the 16S-23S internal transcribed spacer (ITS) regions of 46 Bacillaceae showed that the genera *Bacillus*, *Geobacilllus*, *Paenibacillus* and *Brevibacillus* can be divided in 10 different clusters (Xu and Côté, 2003). The study showed that within these genera *B. cereus* is not closely related to *Bacillus subtilis*. This signifies the importance of studying *B. cereus* and related organisms separately, as it will not be possible to extrapolate all data gained on the Gram-positive model organism *B. subtilis* to *B. cereus* and related species. Phylogenetic studies of the Bacillaceae also showed that *B. cereus*, *Bacillus thuringiensis*, *Bacillus anthracis*, *Bacillus mycoides* and *Bacillus weihenstephanensis* are very closely related. Therefore, these bacteria are referred to as the *B. cereus* group (Lechner *et al.*, 1998) (Fig. 2). Although these Bacilli are closely related, they each possess specific phenotypic features, which are used to distinguish the different species of the *B. cereus* group. Notably, these pathogenic determinants are generally located on plasmids (Table 1).

Table 1: Basic characteristics of three species of the *B. cereus* group.

Characteristic*	*B. anthracis*	*B. cereus*	*B. thuringiensis*
Tripartite lethal toxin	Present	Absent	Absent
Enterotoxins production	Absent	Present	Present
Crystalline parasporal	Absent	Absent	Present
Hemolytic activity	Absent	Present	Present
Capsule synthesis	Present	Absent	Absent
Motility	Absent	Present	Present
Host range	Vertebrates	Unknown	Invertebrates
Distribution	Worldwide	Worldwide	Worldwide

* Characteristics were based on those reported by Han *et al.* (2006) and Jensen *et al.* (2003)

B. anthracis is the causative agent of anthrax. Research interest in *B. anthracis* grew after it was used for bioterrorist attacks in the USA in 2001 (Jernigan *et al.*, 2001). Three forms of anthrax are known, a cutaneous, gastro-intestinal and inhalation form. The most common one is the cutaneous form, which can be easily treated with antibiotics. The lethal forms are the gastro-intestinal and inhalation form, with the initial symptoms being not specific for anthrax. These initial mild symptoms resemble that of a mild flu, after which the disease suddenly develops into a systemic form that can not be treated and is rapidly fatal. Factors that are responsible for causing this systemic form of disease are encoded by *pagA*, *lef* and *cya*, which encode the anthrax toxins. Next to this, the presence of a capsule allows *B. anthracis* to circumvent the host immune response. The genes encoding the anthrax toxins are located on the plasmid pXO1, with the genes encoding the capsule located on pXO2 (Mock and Fouet, 2001).

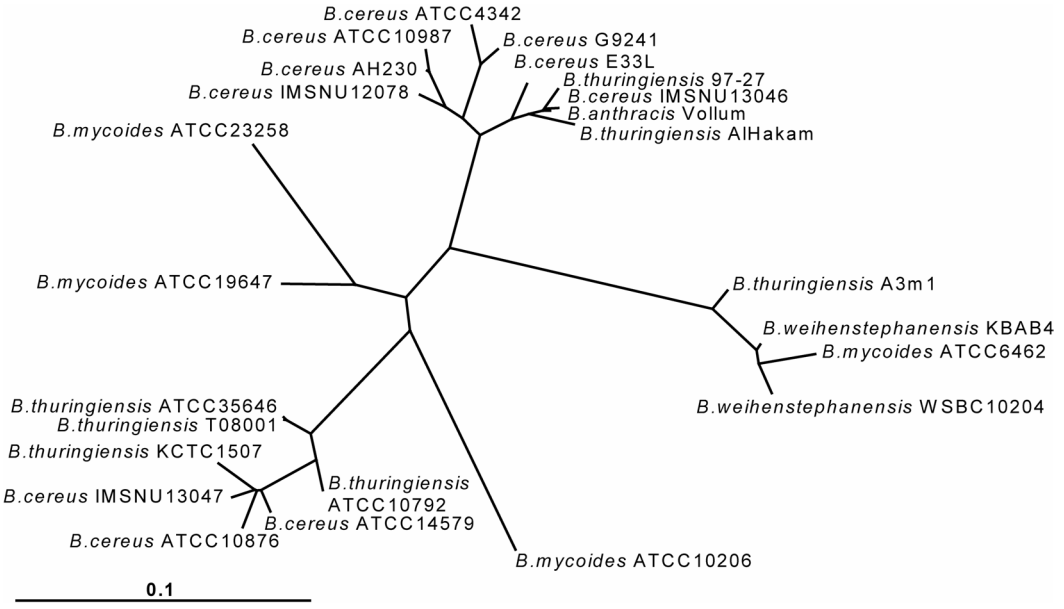

Fig. 2. Unrooted phylogenetic representation of the *B. cereus* group, obtained by multi-locus sequence typing (MLST) using SuperCAT (Tourasse and Kolsto, 2007). A selected number of species for which MLST is available are represented. Species were picked mainly on the basis of relevance for this thesis. *B. anthracis* isolates are represented as a single point on the tree, *B anthracis* Vollum, as the *B. anthracis* species cluster together and are in a dense part of the tree. *B. cereus* subsp. cytotoxis was not included in the representation, as the distance to the other species was too large.

In contrast to *B. anthracis* and *B. cereus*, *B. thuringiensis* is not considered to be a human pathogen, but an insect pathogen. This phenotypic trait is associated with its ability to produce insect-toxins that appear in the form of parasporal crystals. *B. thuringiensis* is widely used against insect pests, with the parasporal crystals acting as biocontrol agent. The genes encoding the parasporal crystals are situated on large plasmids (Berry *et al.*, 2002; Schnepf *et al.*, 1998).

B. mycoides can potentially be used as a biocontrol agent as well, as it triggers the resistance of sugar beets to pathogenic fungi (Bargabus *et al.*, 2003). *B. mycoides* is probably the least studied member of the *B. cereus* group. It can be easily distinguished from the other members by the appearance of its mucoid colonies, which have asymmetric 'hairy'-like extensions made of bundles of filaments that are curving either clockwise or counter clockwise (Di Franco *et al.*, 2002).

The psychrotrophic nature of *B. weihenstephanensis* was responsible for marking it as a distinctive member of the *B. cereus* group. Isolates of *B. weihenstephanensis* are able to grow at temperatures as low as 4 °C, but not above 43 °C (Lechner *et al.*, 1998). However, strains of *B. cereus* have been identified that grow at low temperature as well, showing that not all psychrotrophic strains of the *B. cereus* group belong to the species *B. weihenstephanensis* (Stenfors and Granum, 2001).

Attempts to find a genetics-based approach to distinguish between the different members of the *B. cereus* group have only been partially successful. The genomes of the different species actually are too similar to distinguish between the species (Priest *et al.*, 2004). The phenotypic differences between the strains of the *B. cereus* group are often caused by the presence of large plasmids, as stated above. These genetic elements are interchangeable making phenotypic traits interchangeable as well. This is illustrated by a *B. cereus* strain causing a severe inhalation anthrax-like illness. For *B. cereus* G9241 the presence of a plasmid with 99.6% similarity to pXO1 of *B. anthracis* was shown (Hoffmaster *et al.*, 2004). The interchangeability of these species-specific genotypic and phenotypic features further complicates speciation within the *B. cereus* group. However, it would be impractical for a group of species with such an amount of diverse phenotypes to all have the same species name. Besides this, it would now be practically impossible to rename all species of the *B. cereus* group to for instance *B. cereus* (Priest *et al.*, 2004). So for practical reasons it is desirable to keep the distinction between different species within the *B. cereus* group. The problem remains, however, for strains that show overlapping phenotypes such as *B. cereus* G9241, for which standard tests fail to show the pathogenic potential of the strain.

Members of the *B. cereus* group are of high economic and medical importance. Therefore, the genome sequence of many species of the *B. cereus* group was elucidated, leading to a high number of sequenced genomes (Table 2). Nine of these genomes are completely finished, two strains of *B. thuringiensis*, four strains of *B. cereus* and three strains of *B. anthracis*. In addition, sequencing of 26 genomes is incomplete or in progress, of which ten belong to the *B. cereus* group sequencing program of TIGR. A total number of 18 genomes is accessible via ERGO (Overbeek *et al.*, 2003) and 30 via NCBI (Benson *et al.*, 2007). Comparative genomic studies have been performed and show that indeed many of the differences between the members of the *B. cereus* group are encoded on large plasmids

(Rasko *et al.*, 2005). Next to this, it became apparent that subtle changes in regulation or regulators have major influences on species-specific phenotypic traits such as virulence (Brillard and Lereclus, 2004). Furthermore, the genomes of the different species are shown to be highly similar and to have a conserved gene order. Differences observed between the genomes of the different species seem to be the result of evolutionary adaptations to their specific niches.

Historically, *B. cereus* was thought to be a soil organism, like *B. subtilis* and *Streptomyces* spp.. However, analysis of both *B. cereus* and *B. anthracis* genomes give indications to suggest that the most recent ancestor of the *B. cereus* group was able to exist in animals and/or insects. This assumption is based on the presence of an expanded capacity for amino acid and peptide utilization (Read *et al.*, 2003). Besides this, it has been shown for soil bacteria, such as *B. subtilis,* that they contain a high variety of carbohydrate catabolic pathways. These enable consumption of the high diversity of carbon sources coming from degraded plant material, present in soil. For *B. cereus* ATCC 14579, the number of encoded enzymes involved in polysaccharide degradation was determined to be 14, whereas this number for *B. subtilis* was 41 (Ivanova *et al.*, 2003). This indicates that *B. cereus* is less adapted to a lifestyle in soil. Nevertheless, *B. cereus* was recently shown to be able to survive and grow in soil matter (Vilain *et al.*, 2006). The large capacity to grow on carbohydrates and on proteinaceous substrates shows that species of the *B. cereus* group can dwell in a variety of niches, including soil (Vilain *et al.*, 2006), the insect gut (Jensen *et al.*, 2003), the human gastro-intestinal tract and a large diversity of foods (Granum and Lund, 1997; Schoeni and Wong, 2005).

Table 2. Publicly available genome sequences of species of the *B. cereus* group.

Species*	Genome size (Mb)	Nr. of ORFs	Nr. of plasmids	GC %	Publication status
Ba 31-101	5.0	-	-	-	Incomplete
Ba 500	5.0	-	-	-	Incomplete
Ba A1055	5.3	-	-	35.3	Draft
Ba A2012	5.1	5544	-	35	Draft
Ba Ames	5.2	5311	-	35.4	1-5-2003
Ba Ames Ancestor A2084	5.2	5309	2	35.4	20-5-2004
Ba Australia 94	5.5	-	-	35.2	Draft
Ba CNEVA-9066	5.5	-	-	35.2	Draft
Ba Kruger B	5.5	-	-	35	Draft
Ba Sterne	5.2	5287	1	35.4	24-6-2004
Ba Tsiankovskii-I	5.5	-	-	-	Draft
Ba Vollum	5.5	-	-	35.2	Draft
Ba Western NA USA6153	5.5	-	-	35	Draft
Bc 03BB108	5.9	-	-	-	Draft
Bc AH1134	5.7	-	-	-	Draft
Bc AH187	5.5	-	-	35.5	Draft
Bc AH820	5.6	-	-	35.4	Draft
Bc ATCC 10987	5.2	5603	1	38	11-2-2004
Bc ATCC 14579	5.4	5234	1	35.3	1-5-2003
Bc B4264	5.3	-	-	-	Draft
Bc cytotoxis NVH 391-98	4.1	3833	1	-	17-7-2007
Bc E33L (ZK)	5.3	5134	5	35.4	16-9-2004
Bc F0837/76	-	-	-	-	Draft
Bc G9241	5.9	6147	-	35.2	Draft
Bc G9842	5.7	-	-	-	Draft
Bc H3081.97	5.6	-	-	-	Draft
Bc NVH0597-99	5.4	-	-	-	Draft
Bc Q1	-	-	-	-	Incomplete
Bc W	5.3	-	-	35.6	Draft
Bt Al Hakam	5.3	4736	1	35.4	27-11-2004
Bt ATCC35646	5.9	6132	-	35	Draft
Bt konkukian 97-27	5.2	5117	1	35.4	30-6-2004
Bt kurstaki	-	-	-	-	Incomplete
Bt NBT97	-	-	-	-	Incomplete
Bw KBAB4	5.6	5532	-	35.4	Draft

* *Bc*: *B. cereus*, *Bt*: *B. thuringiensis*, *Bw*: *B. weihenstephanensis*, *Ba*: *B. anthracis*. Data were obtained from the Genome OnLine Database (GOLD) v2.0.

Regulation of *B. cereus* gene expression

Bacteria continuously encounter changes in their environment, such as temperature shifts, nutrient limitation and the accumulation of toxic components. To be able to survive these environmental changes, an appropriate and fast reaction is required. The best known method for a bacterial cell to control a response to environmental changes is by the regulation of transcription of DNA to RNA by RNA polymerases (RNAP). Subsequently, the RNA is translated into proteins which can counteract the environmental changes (Hecker and Volker, 2001). Regulation of gene transcription occurs at several levels. First of all, RNAP is a complex enzyme, consisting of multiple subunits. One of these subunits is a dissociable σ factor, which is responsible for recognition of the DNA binding site for RNAP. In general, bacterial genomes encode one housekeeping σ factor, which is involved in transcription of

genes necessary for rapid growth. Upon an environmental change, it is possible to replace the housekeeping σ factor by an alternative σ factor, which subsequently initiates the expression of genes needed in the response to the environmental change (Hecker and Volker, 2001). A general rule seems to apply to the number of σ factors present on the genome of a bacterium: the number of σ factors is a reflection of the complexity of the lifestyle of the organism (Konstantinidis and Tiedje, 2004). This is illustrated by the fact that gene transcription in organisms encountering fast changing environments such as *Streptomyces coelicolor* and *Bacteroides thetaiotaomicron* is regulated by over 50 annotated σ factors and in obligate endocellular parasites such as *Mycoplasma* species by only one σ factor (Table 3). The fact that for species of the *B. cereus* group a reasonably high number of 20-30 σ factors are annotated (Table 4) is a reflection of the fairly complex lifestyle of these species. No general rules have been adapted to the nomenclature of σ factors, which causes a lot of confusion. Several orthologous σ factors carry different names, whereas the same name is given to σ factors with completely different roles in regulation. As long as general rules for nomenclature are absent, it is important to take extreme care in interpretation of σ factor functions.

Table 3. Overview of genomes and σ factors of different species.

Species	Genome size (Mb)	Nr. of ORFs	Nr. of σ factors	Nr. of ECF σ factors
Streptomyces coelicolor A3(2)	8.8	7825	65	50
Bacillus anthracis Ames	5.2	5311	28	16
Pseudomonas aeruginosa PAO1	6.3	5570	24	19
Bacillus cereus ATCC 14579	5.4	5366	20	10
Caulobacter crescentus	4	3767	17	13
Bacillus subtilis 168	4.2	4100	17	7
Escherichia coli K-12	4.7	4288	9	2
Listeria monocytogenes EGD-e	2.9	2844	5	1
Staphylococcus aureus Mu50	2.8	2593	4	1
Campylobacter jejuni NCTC11168	1.6	1654	3	0
Lactobacillus plantarum WCFS1	3.3	3053	3	0
Lactococcus lactis IL1403	2.4	2425	2	1
Lactococcus lactis MG 1363	2.5	2434	1	0
Mycoplasma genitalium G-37	0.6	470	1	0

A second mode to influence gene transcription is by activating or repressing binding of the RNAP to its promoter sequence. This occurs by binding to and release from DNA by transcriptional regulators, this either allows (activators) or deprives (repressors) binding of RNAP. In response to environmental changes, activators or repressors can bind or release the DNA, thereby facilitating transcription of specific genes (Seshasayee *et al.*, 2006). Next to transcriptional regulation, regulation of protein and enzyme levels occurs by translational regulation (Sierra and Zapata, 1994) and by the metabolic flux-control of reactions (Shimizu,

2004). For this thesis, the focus will be on transcriptional regulation of *Bacillus cereus* in response to changing environments.

Alternative σ factors

Alternative σ factors are known to regulate specific regulons that are activated in response to changes such as transitions in growth, morphological changes or when stress is encountered. The first alternative σ factors were discovered by biochemical studies of transcription selectivity (Haldenwang, 1995; Kroos *et al.*, 1999). However, as DNA sequence information is extending, more and more alternative σ factors are identified by sequence analysis. As the complete genome of a species becomes available, it is possible to make an overview of all alternative σ factors present in this species. This gives a good indication of the organism's possible lifestyles and abilities to survive different stresses. The σ factors can be divided into two families. Most of the alternative σ factors are related in sequence to σ^{70}, the identified housekeeping σ factor for *Escherichia coli* (Lonetto *et al.*, 1994). These σ factors comprise the σ^{70} family of σ factors. The other family is the σ^{54} family of σ factors, which seems unrelated in both sequence and function to the σ^{70} family of σ factors. Species of the *B. cereus* group contain 20-30 σ factors of which one belongs to the σ^{54} family and the others to the σ^{70} family of σ factors (Table 4).

The σ^{54} family of σ factors

The σ factor σ^{54} is also named σ^{L} or RpoN. As a high degree of similarity exists, the specific binding site of the σ^{54}-RNAP complex is similar in different organisms. σ^{54}-RNAP is shown to bind to the consensus DNA sequence YTGGCACGrNNNTTGCW (Barrios *et al.*, 1999). This consensus sequence can be used to identify putative targets of transcriptional control by σ^{54}, e.g. in species of which the genome is sequenced (Studholme *et al.*, 2000). However, binding of σ^{54}-RNAP to the DNA is not sufficient for initiation of transcription. In addition, a specific σ^{54} transcriptional activator is necessary to make DNA available for transcription (Buck *et al.*, 2000; Wedel *et al.*, 1990; Xu and Hoover, 2001). To initiate transcription the σ^{54}-RNAP complex must participate in an interaction with the transcriptional activator. This transcriptional activator is usually bound at least 100-bp up-stream of the promoter site, and DNA looping is required for the activator to contact the σ^{54}-RNAP complex to start transcription (Morett and Segovia, 1993; Studholme and Dixon, 2003). The diversity in genes regulated by σ^{54} factors makes it impossible to state a general function for this σ factor. Sequence analysis of the available genomes of the *B. cereus* group shows *sigL*, encoding σ^{L}, to be present on the genome of each of the species. For all 18 analyzed genomes, seven orthologous transcriptional activators were identified, and four genomes contained an additional eighth σ^{54} transcriptional activator.

The σ^{70} family of σ factors

The σ^{70} family of σ factors comprises the housekeeping σ factors. Therefore for each bacterium at least one member of the σ^{70} family is present on its genome. The σ^{70} family σ factors bind promoter binding sites consisting of a -10 and a -35 element, indicating their

position from the transcriptional start. Four conserved sequence regions have been identified for the σ^{70} family σ factors, of which regions 2 and 4 are involved in binding the -10 and -35 elements respectively, region 3 is known to assist in binding (extended) -10 elements, whereas region 1 appears to function in antagonizing the DNA-binding activity of the σ factor (Paget and Helmann, 2003)(Fig. 3). The σ^{70} family of σ factors can be divided into 5 groups (Helmann, 2002).

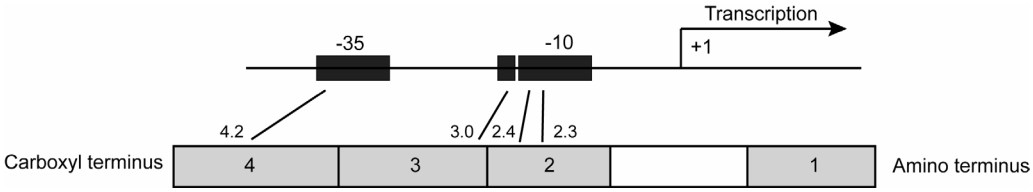

Fig. 3. Schematic representation of the binding of a σ factor to its promoter binding site. Transcription is induced by binding of the region 2 and region 4 of the σ factor to the -35 and -10 sequences. The numbers indicate the different regions of the σ factor. Region 1 and 3 are absent in group 3 and ECF σ factors. The figure is based on a figure presented by Paget and Helmann (2003).

The first group consists of the primary σ factors. Members of this group contain all four conserved sequence regions and are orthologous to σ^{70} of *E. coli*, which itself is a member of this group (Lonetto *et al.*, 1992). The primary σ factors are mainly responsible for transcription in vegetative growing cells, a single copy is present per organism and they are indispensable for growth. Therefore, they are often referred to as the housekeeping σ factors. The primary σ factors of different species show high similarity and consequently recognize similar binding sites: a -35 binding element of TTGaca and a -10 binding element of TAtaaT (uppercase nucleotides being the most conserved bases) (Gross *et al.*, 1998; Helmann and Chamberlin, 1988). The genomes of the species of the *B. cereus* group contain *sigA*, encoding their housekeeping σ factor σ^{A}.

Group 2 of the σ^{70} σ factors is the group of nonessential proteins which are highly similar to primary σ factors. Nevertheless, they are dispensable for growth (Helmann, 2002). Not all bacteria contain group 2 σ factors, as is shown for the species of the *B. cereus* group, for which no group 2 σ factors are annotated.

The σ factors belonging to group 3 are significantly smaller than σ factors belonging to group 1 and 2, which can be explained by the absence of conserved sequence regions 1 and 3 (Helmann, 2002). Group 3 σ factors are recognized as σ factors by the presence of conserved amino acid sequence regions 2 and 4. Together with group 4 and 5 σ factors, group 3 σ factors are regarded as secondary σ factors. The intracellular level of secondary σ factors will increase in the bacterial cell as a response to environmental changes, such as stress or developmental processes, whereas their level is low in normal vegetative growth (Hecker and Volker, 2001; Price, 2002). By the increase of the fraction secondary σ factors, the fraction RNAP bound with this secondary σ factor fraction increases as well, enabling the activation of the regulon of the induced σ factor. The level of secondary σ factors has to be tightly regulated and an increase should only occur when the correct trigger presents itself in the environment. Regulation of secondary σ factors occurs in three ways: at the synthesis level,

by proteolysis and by interaction of the σ factor with an anti-σ factor (Helmann, 1999; Hughes and Mathee, 1998). Group 3 σ factors can be subdivided in different classes by regarding their function (Lonetto *et al.*, 1992). Examples of group 3 σ factors present in species of the *B. cereus* group are the general stress σ factor σ^B and sporulation σ factors σ^E, σ^F, σ^G, σ^H and σ^K. Remarkably, no apparent σ factor involved in flagellar biosynthesis was found, as no orthologue of the *B. subtilis* σ^D could be identified for any of the species of the *B. cereus* group. Possibly, this function is performed by one of the σ factors of group 3 with an unknown function (Table 4).

The general stress σ factors σ^B is present in all members of the *B. cereus* group. Recently, σ^B of *B. cereus* ATCC 14579 has been studied extensively, revealing activation of σ^B in response to several stresses, including heat. In addition, it was shown that deletion of *sigB*, encoding σ^B, lead to an impaired heat resistance (van Schaik *et al.*, 2004a). Furthermore, the σ^B regulon was determined by proteome and in vitro transcription analysis (van Schaik *et al.*, 2004b), and more recently by comparative transcriptome analysis (van Schaik *et al.*, 2007). Besides the role of σ^B in heat adaptation, σ^B was shown to be involved in hydrogen peroxide resistance (van Schaik *et al.*, 2005b) and in determining spore properties (de Vries *et al.*, 2005). Moreover, the role of RsbV, RsbW, and RsbY in regulation of σ^B activity in *B. cereus* ATCC 14579 was established (van Schaik *et al.*, 2005a). Deletion of *sigB* in *B. anthracis* led to a changed morphology and slightly impaired virulence. This suggests σ^B to be a minor virulence factor in *B. anthracis* (Fouet *et al.*, 2000).

Sporulation σ factors σ^E, σ^F, σ^G, σ^H and σ^K of the *B. cereus* group and *B. subtilis* are homologous (Ivanova *et al.*, 2003; Kunst *et al.*, 1997). Moreover, expression patterns of the sporulation σ factors were shown to be similar in species of the *B. cereus* group and *B. subtilis* (de Vries *et al.*, 2004; Liu *et al.*, 2004). Therefore, the event of sporulation in species of the *B. cereus* group and *B. subtilis* is believed to be similar. Notably, sporulation σ factors of species of the *B. cereus* group have shown to be involved in the production of toxins. *B. anthracis* σ^H was shown to be essential for toxin gene expression (Hadjifrangiskou *et al.*, 2007). Several of the insecticidal crystal protein genes of *Bacillus thuringiensis* were also shown to be regulated by sporulation σ factors, amongst which σ^E, σ^H and σ^K (Komano *et al.*, 2000). Regulation of these toxin genes in *B. anthracis* and *B. thuringiensis* suggests that expression of enterotoxin genes in *B. cereus* could also be regulated by sporulation σ factors.

The ExtraCytoplasmic Function (ECF) σ factors constitute group 4 of the σ^{70} family as suggested by Helmann (Helmann, 2002). The first σ factor with a proposed regulon involved in processes that occur in extracytoplasmic compartments was the *Escherichia coli* σ^E (Mecsas *et al.*, 1993). Subsequently, it was noted that this *E. coli* σ^E, the *Streptomyces coelicolor* σ^E, and several other known activators formed a distinct group of σ factors within the σ^{70} family (Lonetto *et al.*, 1994). As a group, these σ factors posses certain common features. In most cases the ECF σ factor is co-transcribed with its anti-σ factor. This anti-σ factor generally is a transmembrane protein with an extracytoplasmic sensory domain and an intracellular σ factor binding domain. Next to this, the -35 element of the promoter binding site recognized by ECF σ factors often contain the base order 'AAC', for which the structural basis was recently clarified (Lane and Darst, 2006). Furthermore, the regulon of ECF σ

factors often regulate functions concerning the extracytoplasm, such as periplasmic stress, pathogenesis or transport (Manganelli *et al.*, 2004; Raivio and Silhavy, 2001). Hence, this group was called the extracytoplasmic function (or ECF) σ factor group (Helmann, 2002). The ever growing number of available genome sequences show that often a high number of σ factors present on the genome can be grouped within the ECF σ factor group (Table 3). For species of the *B. cereus* group, 10 to 18 ECF σ factors were identified (Table 4) (Ivanova *et al.*, 2003). In total 23 different orthologous clusters of ECF σ factors have been identified within the *B. cereus* group of which 8 were present in all species. For *B. anthracis* in each species the same 16 ECF σ factors were identified, of which one seems specific for *B. anthracis* species.

Table 4. The σ factors identified for species of the *B. cereus* group.

	σ^{54}	σ^{70}							Total
		Group 1	Group 2	Group 3			Group 4	Group 5	
***Species*#**		General		Stress	Spore	Other*	ECF		
Bc ATCC 14579	1	1	0	1	5	2	10	0	20
Bc ATCC 10987	1	1	0	1	5	2	14	0	24
Bc E33L	1	1	0	1	5	3	16	0	27
Bt ALH	1	1	0	1	5	2	15	0	25
Bt konkukian	1	1	0	1	5	2	16	0	26
Bw KBAB4	1	1	0	1	5	4	18	0	30
Ba species	1	1	0	1	5	4	16	0	28

* Other means with other or unknown function. # *Bc*: *B. cereus*, *Bt*: *B. thuringiensis*, *Bw*: *B. weihenstephanensis*, *Ba*: *B. anthracis*. The general σ factor is σ^A, the stress σ factor is σ^B, and the sporulation σ factors are σ^E, σ^F, σ^G, σ^H, and σ^K.

The number of ECF σ factors found in *B. cereus* species was quite diverse, as 10 were found for *B. cereus* ATCC 14579 and 16 for *B. cereus* E33L. Of the 12 ECF σ factors annotated for *B. cereus* ATCC 10987 there were 2 that were specific for this species. For *B. weihenstephanensis* KBAB4 18 ECF σ factors could be identified, which was the highest number within the *B. cereus* group. Of these 18 ECF σ factors, there were 2 that were specific for *B. weihenstephanensis* KBAB4. Two ECF σ factors of the *B. cereus* group have been studied in more detail. For *B. thuringiensis* it was shown that the ECF σ factor σ^W is involved in a pathway controlling β-exotoxin I production (Espinasse *et al.*, 2004). BA2502 of *B. anthracis* was observed to control β-lactamase gene expression (Ross *et al.*, 2005). These two ECF σ factors are also present in the other species of the *B. cereus* group. None of the other ECF σ factors of the *B. cereus* group were studied, leaving a great spectrum of functions and pathways to be discovered.

The last group of σ factors as proposed by Helmann (Helmann, 2002), is the TxeR group of σ factors, which up to know have only been identified for *Clostridium* species (Marvaud *et al.*, 1998) and were shown to be involved in toxin expression (Mani and Dupuy, 2001). Notably, this group of σ factors only show weak structural similarity with other members of the σ^{70} family and it is therefore very well possible that a larger number of proteins with an up to now unknown function may be identified as σ factors in the future.

Transcriptional regulators

In addition to σ factors, gene expression can also be regulated by transcriptional regulators. These transcriptional regulators are often two-domain proteins, in which one domain receives the signal and the second, DNA binding domain transduces this signal (Seshasayee *et al.*, 2006). Binding of the transcriptional regulator can either lead to activation or repression of transcription. For prokaryotic DNA binding proteins, the helix-turn-helix (HTH) motif is the most commonly observed motif for DNA binding. For almost 95% of all described transcription factors an HTH motif could be identified (Ramos *et al.*, 2005). Like σ factors, transcriptional regulators are divided into families on the basis of similarity, structural and functional criteria. Over 300 transcriptional regulators are annotated on each genome of the species of the *B. cereus* group (Ivanova *et al.*, 2003). Approximately 200 of these regulators could be assigned to a high diversity of families (Table 5). However, the others have not yet been assigned. Many of the transcriptional regulators studied have been shown to have a function in regulation of the metabolism, one of the best studied topics in microbiology. The best studied Gram-positive transcriptional regulator is the catabolite control protein CcpA, which is a member of the LacI-family of transcriptional regulators. This family of transcriptional regulators is involved in optimizing the utilization of carbon sources. Recently, a role for CcpA was also identified in regulation of other features of the bacterial cell, such as virulence in *Staphylococcus aureus* (Seidl *et al.*, 2006), and *Clostridium perfringens* (Varga *et al.*, 2004), biofilm formation in *B. subtilis* (Chagneau and Saier, 2004; Stanley *et al.*, 2003) and sporulation in *C. perfringens* (Varga *et al.*, 2004). As we further broaden our knowledge on other features of the bacterial cell, such as sporulation, stress response or biofilm formation, the role of transcriptional regulators in these processes will be elucidated in more depth.

The mechanism of sensing and DNA binding can also be performed by two separate proteins, as shown in a two-component system. In this system, one protein is generally a membrane bound kinase, which phosphorylates a DNA binding protein upon receiving the correct signal. Subsequently, the phosphorylated DNA binding protein is able to regulate transcription by recognition of a specific nucleotide sequence (Igo *et al.*, 1990). Two-component systems present in species of the *B. cereus* group have been analyzed in detail by the use of *in silico* techniques and putative biological functions were predicted (de Been *et al.*, 2006).

Table 5. Transcriptional regulators and possible functions identified for the type strain *B. cereus* ATCC 14579

Family	Action	Regulated functions	DBD motif*	Nr. identified
LysR	Activator/repressor	Carbon and nitrogen metabolism	HTH	15
AraC	Activator	Carbon metabolism, stress response and pathogenesis	HTH	13
TetR	Repressor	Biosynthesis of antibiotics, efflux pumps, osmotic stress, etc	HTH	28
LuxR	Activator	Quorum sensing, biosynthesis and metabolism, etc.	HTH	1
LacI	Repressor	Carbon source utilization	HTH	9
ArsR	Repressor	Metal resistance	HTH	14
IclR	Repressor/activator	Carbon metabolism, efflux pumps	HTH	1
MerR	Repressor	Resistance and detoxification	HTH	16
AsnC	Activator/repressor	Amino acid biosynthesis	HTH	5
MarR	Activator/repressor	Multiple antibiotic resistance	HTH	28
DeoR	Repressor	Sugar metabolism	HTH	11
CS	Activator	Low-temperature resistance	CSD	7
GntR	Repressor	General metabolism	HTH	23
Crp	Activator/repressor	Global responses, catabolite repression and anaerobiosis	HTH	2
PadR	Repressor	Phenolic acid metabolism	HTH	8
LytR	Repressor	Cell envelope	HTH	6
Xre	Repressor	Quinone oxidoreductase regulation	HTH	18
RpiR	Repressor	Ribose catabolism	HTH	2

* Family, Action, Regulated functions, and DNA binding domains (DBD) were based on data presented by Ramos *et al.* (2005). HTH: Helix turn helix, CSD: Cold shock domain (RNA binding).

Outline of this thesis

Regulation of transcription has been shown to be of great importance for the behaviour and features of the food-related bacterium *B. cereus*. This thesis focuses on diverse aspects of transcriptional regulation of *B. cereus* response to stresses related to food processing.

The first chapter of this thesis gives an overview of the general features of *B. cereus*, and its close relatives. Furthermore, the complexity of transcriptional regulation is described with specific attention for *B. cereus*. In Chapter 2, the transcriptional regulation of metabolic and toxigenic features during aerobic and anaerobic growth of *B. cereus* ATCC 14579 was addressed by microarray analysis. Microarray analyses were also used to determine the role of one of the major regulators of metabolism, the catabolite control protein CcpA. During different growth phases, the transcriptomes of both the ATCC 14579 wild-type and its *ccpA* deletion strain were analyzed to assess the genes under control of CcpA (Chapter 3). Furthermore, the regulon of the alternative stress σ factor σ^B was determined by microarray analysis and compared to the σ^B regulons of the Gram-positive bacteria *Bacillus subtilis*, *Listeria monocytogenes*, and *Staphylococcus aureus* (Chapter 4). Next to the alternative σ factor σ^B, also the role and function of the ExtraCytoplasmic Function σ factors σ^M (Chapter 5) and σ^Z (Chapter 6) in transcriptional regulation were analyzed. For both σ factors a putative regulon could be determined and a specific role in the response to different stresses was

established. The last chapter (Chapter 7) provides an overview of the results obtained and perspectives of the research are discussed.

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2

Anaerobic induction of metabolic pathways, alternative respiration capacity, nitrosative stress response and virulence factors in *Bacillus cereus* ATCC 14579

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Abstract

A comparative transcriptome approach was used to assess genes involved in the metabolism and pathogenesis that are specifically activated during anaerobic growth of the spore-forming foodborne human pathogen *Bacillus cereus* ATCC 14579. Growth under anaerobic conditions in Brain Heart Infusion broth revealed a reduced growth rate and a lower yield as compared to that under aerobic conditions. Comparative transcriptome analysis of cells harvested at early- and mid-exponential growth phase, transition phase and stationary phase, subsequently showed hundreds of genes to be induced under anaerobic condition. These included novel genes identified for anaerobic growth of *B. cereus*, encoding metabolic pathways, such as the arginine deiminase pathway (ArcABDC), a formate dehydrogenase (FdhF) and a pyruvate formate lyase (Pfl), and alternative respiratory proteins, such as arsenate reductases. Furthermore, the nitrosative stress response was induced in the anaerobic transition phase of growth, conceivably due to the production of nitric oxide as a by-product of nitrite and nitrate respiration. Notably, both hemolytic enzyme and enterotoxin encoding genes were activated in different oxygen limiting conditions, i.e. hemolytic enzyme encoding genes were induced during anaerobic growth, whereas enterotoxin encoding genes were induced in the transition and stationary phase of aerobic cultures reaching a high cell density. These data point to metabolic rearrangements, stress adaptation and activation of the virulent status of *B. cereus* under anaerobic conditions, such as encountered in the human GI-tract.

Introduction

Bacillus cereus is a Gram-positive, facultative anaerobe, which is frequently isolated from spoiled foods and from foods associated with poisoning outbreaks (Granum and Lund, 1997; Kotiranta *et al.*, 2000). *B. cereus* can cause two types of food poisoning, the diarrhoeal and emetic type. The diarrhoeal type of illness is caused by the production of enterotoxins in the gastro-intestinal (GI) tract after consumption of contaminated food. There are several enterotoxins that have been shown to cause the diarrhoeal type of illness, with the hemolytic enterotoxin (Hbl), the non-hemolytic enterotoxin (Nhe) and cytotoxin K (CytK) studied most extensively (Granum and Lund, 1997; McKillip, 2000; Schoeni and Wong, 2005). The emetic type of illness is caused by the production of the emetic toxin (cereulide) by *B. cereus* in foods before consumption. This toxin can cause nausea and vomiting (Ehling-Schulz *et al.*, 2004). Generally, symptoms caused by *B. cereus* food poisoning are regarded as mild. However recently, lethal *B. cereus* intoxications have been reported (Dierick *et al.*, 2005; Lund *et al.*, 2000). Next to food poisoning, a growing number of *B. cereus* infections, such as periodontitis, fulminant endophthalmitis, and meningitis, are reported (Beecher *et al.*, 2000; Gaur *et al.*, 2001; Gaur and Shenep, 2001). Furthermore, *B. cereus* growth can be a food spoilage factor, which may cause substantial economic losses (te Giffel, 2001). Anaerobic growth of *B. cereus* is supported by fermentation and/or anaerobic respiration (Rosenfeld *et al.*, 2005) and may enhance its performance in oxygen-deprived environments, such as soil (Vilain *et al.*, 2006), vacuum-packed foods (Tham *et al.*, 2000), and the human GI tract (Laohachai *et al.*, 2003). Fermentation products formed by *B. cereus* F4430/73 were shown to be mainly acetate, L-lactate, formate and ethanol, with only traces of succinate, pyruvate and 2,3-butanediol (Rosenfeld *et al.*, 2005). The expression of a limited number of *B. cereus* F4430/73 genes associated with this anaerobic metabolism were previously shown to be up-regulated using real-time PCR (Zigha *et al.*, 2006). Notably, also the gene expression of *hblC* and *nheA* was shown to be enhanced under anaerobic conditions (Zigha *et al.*, 2006), which are both part of a three gene operon encoding a hemolysin and a non-hemolytic toxin, respectively. Furthermore, a role for the transcriptional regulators Fnr and ResE was established in regulation of the transcription of these enterotoxin genes (Duport *et al.*, 2006; Zigha *et al.*, 2007).

In this study we report on the anaerobic transcriptome of *B. cereus* ATCC 14579, revealing activation of metabolic pathways not yet reported for *B. cereus* and a range of virulence factors, including enterotoxin genes.

Materials and methods

Strains and culture methods

B. cereus ATCC 14579 was cultured in Brain Heart Infusion (BHI) medium at 30 °C. Aerobically grown cells were obtained by culturing in 50 ml of BHI in a 250 ml Erlenmeyer flask with shaking at 200 rpm. Anaerobically grown cells, as assessed with the redox indicator resazurin, were obtained by culturing in 50 ml of BHI in a 220 ml flask, closed by rubber stoppers and after flushing for 30 minutes with N_2. Notably, no resazurin was added to cultures used for total RNA isolation to prevent interference with optical density measurements at OD_{600} used to monitor growth performance.

Microarray analysis

Samples for microarray analyses were taken at early-exponential and mid-exponential growth and in transition phase and stationary phase of both aerobically and anaerobically growth cultures. For aerobically grown cultures samples were thus taken at OD_{600} of 0.2, 0.8, 4.0 and 8.0 and for anaerobically grown cultures samples were taken at OD_{600} of 0.2, 0.4, 1.0 and 1.1 (Fig. 1). Sampling, RNA extraction and cDNA synthesis and labelling were performed as described previously (van Schaik *et al.*, 2007), and microarray hybridisation and transcriptome data analysis were as described for the microarray platform with GEO accession number GPL5161. The performed transcriptome analyses are listed in Table 1. For gene annotation and metabolic routes the ERGO (Overbeek *et al.*, 2003), KEGG databases, and the Simpheny software package (Genomatica) were used. Furthermore, Clusters of Orthologous Groups (COGs) were analyzed by the use of FIVA (Blom *et al.*, 2007)(Fig. 2). As there are genes that have not been annotated to be part of a COG, a small discrepancy exists between the number of genes represented to be differentially regulated in Table 1 and Fig. 2. The microarray data are deposited in the GEO database with accession number: GSE9846. Supplementary files can be found at www.fhm.wur.nl/UK/thesismenno.

Results and discussion

Aerobic and anaerobic growth

Assessment of growth performance revealed specific growth rates (μ) of 0.022 and 0.011 (h^{-1}) for aerobic and anaerobic cultures, respectively. Moreover, the OD_{600} at which the stationary phase was reached was 1.1 and 12.0 for anaerobic and aerobic cultures, respectively (Fig. 1). Furthermore, microscopic analysis of aerobically grown exponential phase cells revealed them to be highly motile and only single cells or cells in pairs could be observed. In contrast, anaerobically grown exponential phase cells were non-motile and mainly growing in chains (data not shown).

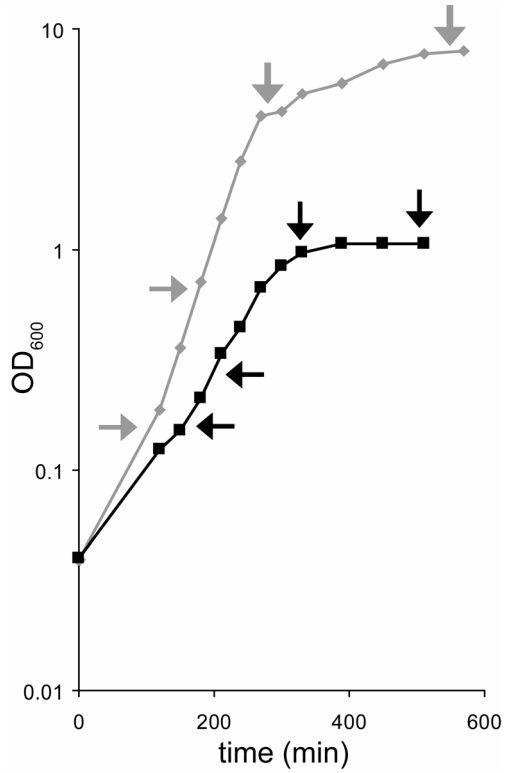


Fig. 1. Aerobic and anaerobic growth of *B. cereus* ATCC 14579 in BHI. Aerobic (grey) and anaerobic (black) growth as measured by optical density at OD_{600}. Arrows indicate at which point of growth samples were taken for microarray analysis (Table 1).

To identify genes that are specifically involved in anaerobic growth whole genome transcriptomes of the mid-exponential phases of both anaerobic and aerobic growth were compared directly (Table1). Furthermore, for both anaerobic and aerobic grown cultures, transcriptomes of cells harvested at the mid-exponential phase were compared to that of the early-exponential phase, the transition and the stationary phase of growth (Table 1).

Table 1. Compared transcriptomes and numbers of differentially regulated genes

Transcriptome analyses	**Phase compared to mid-exponential phase**	**Up-regulated**	**Down-regulated**
Anaerobic (OD_{600})			
0.2 compared to 0.4	Early-exponential	19	57
1.0 compared to 0.4	Transition	505	399
1.1 compared to 0.4	Stationary	50	4407
Aerobic (OD_{600})			
0.2 compared to 0.8	Early-exponential	6	34
4.0 compared to 0.8	Transition	491	538
8.0 compared to 0.8	Stationary	560	831
Aerobic to anaerobic (OD_{600})			
Anaerobic 0.6 to aerobic 0.6		164	80

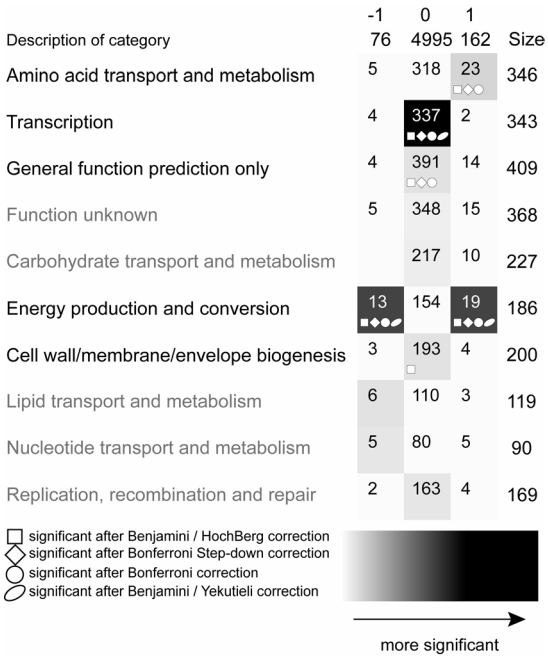
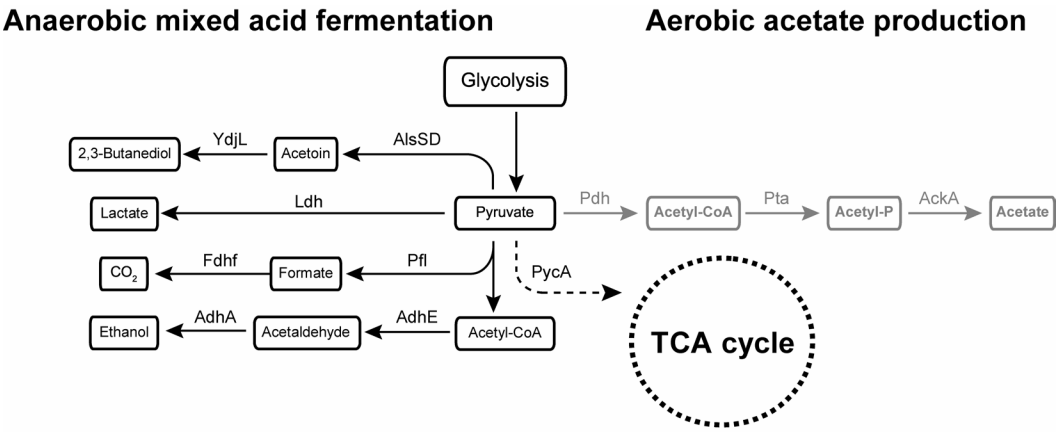


Fig. 2. COG functional classes comparing the anaerobic to aerobic mid-exponential phase of growth. Indicated are the number of genes in general and per functional class not-regulated (0), up-regulated (1) and down-regulated (-1) in anaerobic growth compared to aerobic growth. Significance of the representation of the regulated genes in the different COG functional classes is as determined by FIVA (Blom *et al.*, 2007).

Metabolic gene expression

Comparative transcriptome analysis revealed 164 genes to be up-regulated and 80 genes down-regulated in anaerobically grown cells harvested at mid-exponential phase compared to the aerobically grown mid-exponential phase cells. Interestingly, for both the anaerobic and aerobic mid-exponential phase analysis of the Clusters of Orthologous Groups (COGs) showed significant regulation of genes involved in energy production and conversion, respectively (Fig. 2). Glycolytic genes for example, were observed to be approximately 2-fold up-regulated during anaerobic growth (Table S1). No difference was observed in the expression of TCA-cycle genes between anaerobically and aerobically grown cells. However, a clear difference could be observed in expression of genes involved in the alternative catabolism of pyruvate (Fig. 3). Activation of pathways during the exponential phase of anaerobic growth revealed pyruvate to be catabolised to lactate, carbon dioxide (via formate), 2,3-butanediol (via acetoin), and ethanol, reflecting a so-called mixed acid fermentation. Pathways active during the exponential phase of aerobic growth showed pyruvate to be mainly catabolised to acetate. The high number of genes involved in energy production and conversion is in agreement with reported differences in metabolism between anaerobic and aerobic growth for *B. cereus* (Rosenfeld *et al.*, 2005; Zigha *et al.*, 2006).



Pyruvate to 2,3-Butanediol	Gene nr.	Ratio an/aer	Ratio aer/an
alsS	BC0883	13.8	
alsD	BC0884	19.5	
ydjL	BC0668	10.23	
Pyruvate to Lactate			
ldh[#]	BC4996	2.4	
Pyruvate to CO_2			
Pfl	BC0491	4.17	
fdhF	BC0589	2.34	
Pyruvate to Ethanol			
adhE	BC4365	28.18	
adhA	BC2220	7.94	
Pyruvate to Acetate			
pdhA	BC3973		3.72
pdhB	BC3972		2.88
pdhC	BC3971		4.27
pdhD	BC3970		1.45
pta	BC5387		0.93
ackA	BC4637		3.16
Pyruvate to TCA-cycle			
pycA	BC3947	1.11	0.9

Fig. 3. Anaerobic and aerobic pyruvate metabolism. A. Pathways induced in anaerobic (Black) and aerobic (light grey) exponential growth or similarly expressed in both (dashed). B. Gene expression of genes involved in pathways of the pyruvate metabolism. [#]One of the microarray probe values for *ldh* was not obtained in the mid-exponential comparison of anaerobic and aerobic growth, therefore the presented expression ratio for *ldh* is that of the comparison of anaerobic and aerobic at early-exponential growth.

Further analysis of the COGs indicated only small, non significant differences between the early- and the mid-exponential phases of both anaerobic and aerobic growth (Fig. S1). Clear differences were observed comparing the anaerobic and aerobic transcriptomes of mid-exponential phase cells with that of the respective transition or stationary phase cells. For anaerobic growth this comparison showed down-regulation in the transition phase of genes involved in translation, extracytoplasmic proteins and nucleotide metabolism and up-regulation of transcription (Fig. S1). In the aerobic transition phase also down-regulation of genes involved in translation and encoding extracytoplasmic proteins was observed. Furthermore, in this aerobic transition phase a change in metabolism could be observed by

up-regulation of the clusters involved in energy production and conversion, and in the metabolism of coenzymes, carbohydrates and amino acids (Fig. S1). In the stationary phase of anaerobic growth most genes were down-regulated (Table 1), however, a small amount of genes involved in amino acid and in carbohydrate metabolism were observed to be up-regulated (Fig. S1). In the aerobic stationary phase the change in metabolism observed for the transition phase continued, and also the down-regulated clusters were similar as in the transition phase. In addition, genes involved in cell motility were down-regulated in the aerobic stationary phase (Fig. S1). Notably, one of the changes in the energy production comparing the aerobic transition phase to the mid-exponential phase is up-regulation in the transition phase of genes involved in mixed-acid fermentation and the down-regulation of genes involved in generation of acetate. This change in energy conversion is similar to the difference observed between the anaerobic and aerobic exponential phases of growth.

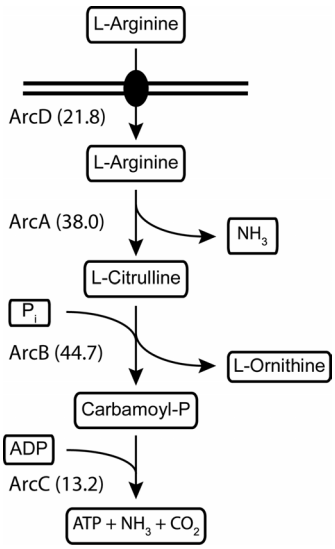


Fig. 4. Anaerobic mid-exponential growth induced ADI-pathway. L-Arginine is transported into the cell and catabolised to ATP and ammonia by enzymes encoded by genes constituting the *arc*-operon. Expression ratios of the genes encoding the ADI-pathway comparing the anaerobic to the aerobic mid-exponential phase of growth are indicated between brackets.

The *arc*-operon comprised the highest up-regulated genes (~40-fold) in anaerobic growth conditions compared to the aerobic growth conditions (Fig. 4). The *arc*-operon encodes the arginine deiminase pathway and consists of 4 genes, *arcABDC*, coding for arginine deiminase, ornithine carbamoyltransferase, arginine/ornithine antiporter and carbamate kinase (BC0406-BC0409) (Maghnouj *et al.*, 2000). Notably, expression of the *arc*-operon was also up-regulated (~10-fold) in the stationary phase of aerobic growth. The arginine deiminase pathway catabolises arginine, leading to the production of 1 ATP (Maghnouj *et al.*, 1998). An additional role of the arginine deiminase pathway may involve

the production of ammonia to counteract acidification of the medium by fermenting cells. In this way, anaerobic activation of the *arc*-operon in *B. cereus* may contribute to survival by generating ATP and by counteracting acidification. Notably, the gene encoding nitric oxide synthase (BC5444) was activated in the transition phase of anaerobic growth, which could lead to the conversion of arginine to citruline with concomitant production of nitric oxide (NO). This reaction is, however, dependent on the availability of oxygen, and it is therefore unlikely to proceed under anaerobic conditions (Liaudet *et al.*, 2000). NO synthases have been identified also in other bacteria including *B. subtilis* (Kunst *et al.*, 1997; Wang *et al.*, 2007), but their function remains elusive.

Remarkably, a 4.9-fold up-regulation of *luxS* was observed in the anaerobic mid-exponential phase as compared to the aerobic mid-exponential phase. The *luxS* gene encodes LuxS, which activity mediates the production of the signal molecule autoinducer-2 (AI-2) (Vendeville *et al.*, 2005). LuxS is one of the components of a metabolic pathway called the Activated Methyl Cycle, which is linked to methionine metabolism (Lombardia *et al.*, 2006). Other genes involved in methionine metabolism were also up-regulated as shown by the 4.3-fold induction of *yrhA* and *yrhB*, which are involved in cysteine metabolism, and a 3.2-fold up-regulation of *metA*, encoding homoserine O-succinyltransferase. Notably, LuxS of St*aphylococcus aureus* was shown to be involved in metabolism and not in the production of AI-2 (Doherty *et al.*, 2006). In anaerobic growth, but not in aerobic growth, the expression of *luxS*, appears to increase with increasing cell numbers, as has been reported before for other microorganisms (Kunst *et al.*, 1997). Addition of in vitro synthesized AI-2 showed an inhibitory effect on *B. cereus* biofilm formation (Auger *et al.*, 2006). The inhibitory effect of AI-2 on *B. cereus* biofilm formation suggests LuxS of *B. cereus* to be involved in quorum sensing. However, the exact role of *B. cereus* LuxS in anaerobic metabolism including its effect on biofilm formation remains to be elucidated.

Genes up-regulated in the anaerobic stationary phase were annotated to be involved in metabolism of fructose, trehalose and cellobiose, as reflected by the ~10-fold up-regulation of *fruRKA*-operon, the 12.6-fold up-regulation of *treP*, and the ~10-fold up-regulation of an operon consisting of 5 genes possibly involved in cellobiose metabolism (BC5212-BC5208), respectively (Fig. 5). In contrast, for the aerobic stationary phase, several operons involved in the catabolism of amino acids and lipids were observed to be induced (Table S1). This signifies glucose, which functions as the main carbon- and energy source for growth, to be catabolised in the exponential phase of growth, after which alternative metabolic pathways are activated. The alternative carbon sources used in energy generation in the anaerobic stationary phase were mainly sugars, whereas for aerobic growth, first alternative sugars are metabolized in the transition phase (Fig. 5), and in addition proteins and amino acids are metabolized in the aerobic stationary phase.

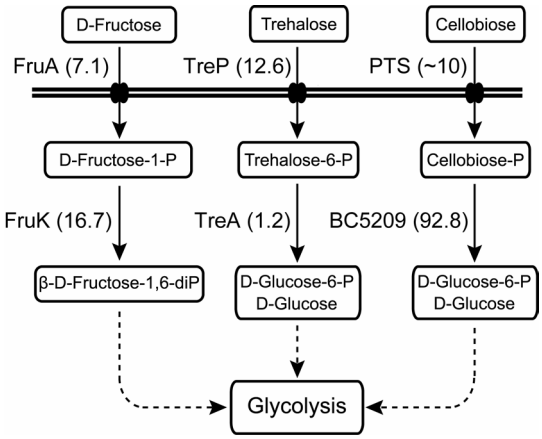


Fig. 5. Anaerobic stationary phase and aerobic transition phase induced pathways. The different substrates are transported by a PTS-system into the cells and catabolised into substrates for glycolysis. The expression ratios comparing the anaerobic stationary phase to the anaerobic mid-exponential phase of the genes encoding the responsible proteins are indicated between brackets.

Interestingly, two co-factors were shown to be differentially expressed. Genes of the biotin (vitamin H) synthesis pathway were clearly up-regulated in the stationary phase of aerobic growth and the genes of the riboflavin (vitamin B_2) synthesis pathway were clearly up-regulated in the transition phase of anaerobic growth. Biotin is a co-factor incorporated into various biotin dependent carboxylases, including acetyl-CoA, propionyl-CoA and pyruvate-carboxylase (Streit and Entcheva, 2003). In agreement with this, the propionyl-CoA carboxylase encoding gene (BC2484) is also highly up-regulated in the stationary phase of aerobic growth. Riboflavin is the universal precursor of flavocoenzymes, which are essential co-factors for a wide variety of redox reactions (Fischer and Bacher, 2005), which have been observed to occur in the transition phase of anaerobic growth (see below).

Transcriptional regulation in anaerobic growth

Comparison of the transcriptomes of the anaerobic and aerobic mid-exponential growth phase showed that in the anaerobic exponential phase 11 genes were up-regulated that encode proteins involved in signal transduction and none in the aerobic exponential phase. For instance, a clear 3-fold up-regulation was observed for *arcR*, encoding a transcriptional regulator of the Crp-family (BC0410), which positively regulates the *arc*-operon (Maghnouj *et al.*, 1998). Moreover, a 2.4-fold up-regulation was shown for BC0291 encoding the anaerobic transcriptional regulator Rex (Gyan *et al.*, 2006). In the anaerobic mid-exponential phase an up-regulation of ~2-fold was observed for the operon containing *ctsR*, encoding a general stress regulator. In addition, expression of the *ctsR*-operon was further elevated in the anaerobic transition phase by 6.1-fold. Next to this, *sigB*, encoding the stress related σ factor σ^B, was also shown to be up-regulated by 6.8-fold in the anaerobic transition phase. Both for *ctsR* and *sigB*, only a modest up-regulation of 2.3–fold was observed in the aerobic transition phase.

Strikingly, the gene *perR,* encoding a key regulator of oxidative stress (PerR), was up-regulated 4.3-fold in the transition phase of anaerobic growth. Genes known to be under control of PerR in *B. subtilis* (Helmann *et al.*, 2003), were also up-regulated in *B. cereus* including *katX*, encoding a catalase, *ahpCF*, encoding alkylhydroperoxide reductase, *hemA*, which is involved in heme biosynthesis and *fur*, encoding the ferric uptake regulator Fur (BC4091). Moreover, 5 out of 9 thioredoxin encoding genes and 2 out of 4 thioredoxin reductase encoding genes were up-regulated in the anaerobic transition phase. Up-regulation of these genes is conceivably caused by NO, a possible by-product of nitrate metabolism in *B. cereus* (Corker and Poole, 2003; Kalkowski and Conrad, 1991). NO is a lipophilic, freely diffusible radical that can inhibit enzymes and damage DNA. The NO stimulon in *Bacillus subtilis* was recently shown to contain Fur, PerR and σ^B and their regulons (Moore *et al.*, 2004). The induction of the genes encoding these proteins in *B. cereus* indicates these genes also to be part of the NO stimulon in *B. cereus*. The exact *B. cereus* NO stimulon remains to be elucidated.

Alternative respiration

Expression of operons encoding cytochromes was generally similar to results obtained for *B. subtilis* (Santana *et al.*, 1992; Saraste *et al.*, 1991; Winstedt *et al.*, 1998). However, the genome of *B. cereus* ATCC 14579 contains three distinct operons all encoding cytochrome *bd*, of which the BC4792-BC4793-operon was up-regulated ~5-fold in the anaerobic mid-exponential growth phase compared to the aerobic mid-exponential growth phase.

The respiratory nitrate (*narGHJI*) and nitrite (*nasDEF*) reductase operons, which were previously shown to be involved in alternative respiration in *B. cereus* (Richardson *et al.*, 2001; Zigha *et al.*, 2007), were ~5-fold up-regulated in the anaerobic mid-exponential growth phase as compared to the aerobic mid-exponential growth phase. Differential expression of *fnr* (BC2122), encoding the positive regulator of these operons (Spiro, 1994), was not observed. Oxygen limitation in the transition and stationary phase of aerobic growth described above was reflected in a 25-fold up-regulation of the *nar*- and the *nas*-operons in the transition phase compared to the aerobic mid-exponential growth phase (Table S1). In addition, it was observed that in the anaerobic transition phase 3 genes encoding arsenate reductase family proteins were up-regulated (Table S1), indicating arsenate to be a possible additional electron acceptor for anaerobic respiration. Amongst these genes BC3402 was identified to encode a putative arsenate reductase which shows high similarity to ArsC of *Shewanella* sp. strain ANA-3, which was recently shown to be involved in arsenate respiration (Saltikov *et al.*, 2005). Moreover, approximately 30 additional reductases were specifically up-regulated in the anaerobic transition phase (Table S1). This points to the use of other electron acceptors for anaerobic respiration by *B. cereus*, with the exact functions of the additional induced reductase genes remaining to be elucidated. Besides the anaerobic induction of alternative electron transfer components, a concomitant induction of transcriptional units encoding alternative metabolic pathways was observed (Fig. 2), and these may be partially interconnected. Notably, the up-regulation of both respiratory and

fermentative pathways in the aerobic transition and stationary phase may point to oxygen limitation in these high cell density cultures, reflected in a mixed aerobic/anaerobic performance.

Virulence factors gene expression

The *B. cereus* ATCC 14579 genome contains a large variety of virulence factors, including enterotoxins and hemolytic enzymes. For both enterotoxins and hemolytic enzymes an up-regulation of their production has been shown in anaerobic environments in members of the *B. cereus* group (Duport *et al.*, 2004; Duport *et al.*, 2006; Klichko *et al.*, 2003; Rosenfeld *et al.*, 2005; Zigha *et al.*, 2007). This comprises with the fact that in human food poisoning events *B. cereus* enterotoxin and hemolytic enzyme production occurs *in situ* in the gastro-intestinal tract, an environment where oxygen limitation can occur (Zigha *et al.*, 2006). Interestingly, in our study three of the five hemolytic enzyme encoding genes induced in anaerobic growth of *B. anthracis* were also induced in anaerobically grown *B. cereus*. In addition, a fourth gene encoding a hemolytic enzyme (BC5449) was observed to be induced under anaerobic conditions (Fig. 6). Elevated expression of three of these four genes was also observed in the oxygen limited transition phase of aerobic growth (Fig. S1).

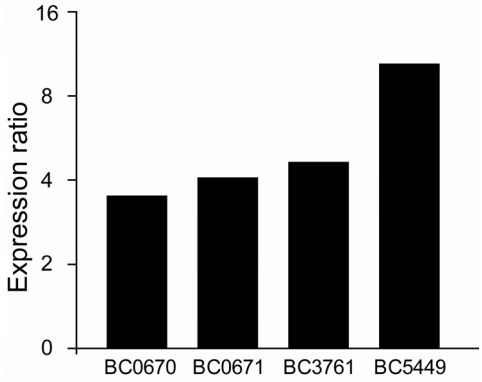


Fig. 6. The four genes encoding hemolytic enzymes induced during anaerobic growth. Expression of the genes is obtained by comparing the anaerobic and the aerobic mid-exponential growth. The ratios on the y-axis are on a 10log scale.

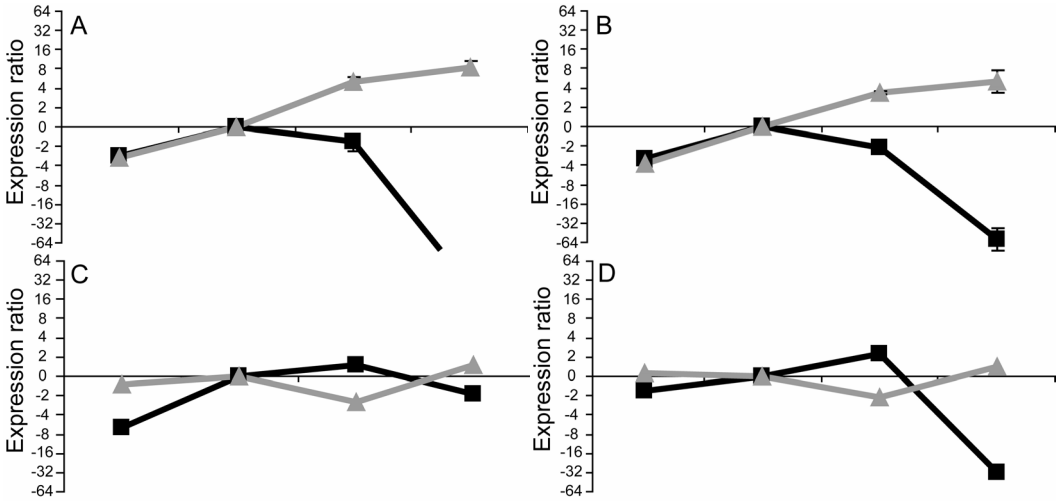


Fig. 7. Enterotoxin enzyme gene expression during anaerobic growth (black) and aerobic growth (grey). A. average expression of the *nhe*-operon, B. average expression of the *hbl*-operon, C. *cytK* and D. *plcR*. Anaerobic and aerobic expression are linked by expression at the mid-exponential phase, as no difference was observed at this point, for both anaerobic and aerobic growth the mid-exponential expression was set to zero. The four time point presented from left to right are early-, mid-exponential, transition and stationary phase. The ratios on the y-axis are on a log-value scale and are presented as absolute values.

The observed elevation in expression of the two enterotoxin encoding operons in the oxygen limited aerobic transition and stationary phase, indicate oxygen and/or substrate limitation and/or cell density to play a role in gene expression of the enterotoxin operons in these growth phases (Fig. 7A and B and Chapter 3). Besides this, for both *cytK*, encoding the enterotoxin cytotoxin K, and *plcR*, encoding the positive regulator PlcR of the enterotoxin gene clusters (Agaisse *et al.*, 1999), no or only minor changes in expression were observed (Fig. 7C and D). Next to PlcR, that is essential for enterotoxin gene expression (Agaisse *et al.*, 1999) a range of transcriptional regulators have been shown to play a role in the production of *B. cereus* enterotoxins. Amongst these regulators is CcpA, which represses enterotoxin gene expression in case of glucose availability (Chapter 3) and ResD (Duport *et al.*, 2006) and Fnr (Zigha *et al.*, 2007), which are redox-sensors (Nakano and Zuber, 1998), probably triggering enterotoxin gene expression upon oxygen limiting conditions. Notably, recent transcriptome analysis of anaerobically grown *Staphylococcus aureus* cells also showed expression of virulence-associated genes to be affected by oxygen concentrations, with highest toxin production levels reached under oxygen limiting conditions (Fuchs *et al.*, 2007). This indicates a similar sensing and transcriptional regulation system applies for *S. aureus*.

In conclusion, *B. cereus* ATCC 14579 activates a large number of alternative metabolic pathways and alternative respiratory chains that contribute to its performance under oxygen limiting conditions. Most remarkably, the concomitant increase in expression of enterotoxins and hemolytic enzymes indicates the virulent status of *B. cereus* to be induced under anaerobic conditions, such as encountered in the human GI-tract.

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3

Assessment of CcpA-mediated catabolite control of gene expression in *Bacillus cereus* ATCC 14579

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Abstract

CcpA is a transcriptional regulator conserved in many Gram-positives, controlling the efficiency of glucose metabolism. In this study we identified the role of *Bacillus cereus* ATCC 14579 CcpA in regulation of metabolic pathways and expression of enterotoxin genes by comparative transcriptome analysis of the wild-type and a *ccpA* deletion strain.

In *B. cereus* the catabolite control protein CcpA was shown to be involved in optimizing the efficiency of glucose catabolism by activating genes encoding glycolytic enzymes, including a non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase that mediates conversion of D-glyceraldehyde 3-phosphate to 3-phospho-D-glycerate in one single step, and by repressing genes encoding the citric acid cycle and gluconeogenic enzymes. Two *B. cereus*-specific CcpA-regulated operons were identified, encoding enzymes involved in the catabolism of fucose/ arabinose and aspartate. Notably, catabolite repression of the genes encoding non-hemolytic enterotoxin (Nhe) and hemolytic (Hbl) enterotoxin appeared CcpA-dependent, and for the corresponding enterotoxin operons, putative CRE-sites were identified.

First of all, it was shown that CcpA in *B. cereus* ATCC 14579 is involved in optimizing the catabolism of glucose. Furthermore, the results point to metabolic control of enterotoxin gene expression and suggest that CcpA-mediated glucose sensing provides an additional mode of control to PlcR activated expression of the *nhe*- and *hbl*-operons in *B. cereus* ATCC 14579.

Introduction

Bacillus cereus is an important Gram-positive, spore-forming foodborne pathogen. Many strains cause either an emetic or a diarrhoeal type of disease. The production of an emetic toxin, also referred to as cereulide, causes nausea and vomiting. In addition, several enterotoxins can be produced including Nhe, Hbl and CytK that can cause diarrhoea (Granum and Lund, 1997; Kotiranta *et al.*, 2000; Schoeni and Wong, 2005). Foodborne disease caused by *B. cereus* is generally characterized by mild symptoms. However, recently more severe cases with a lethal outcome have been described (Dierick *et al.*, 2005; Lund *et al.*, 2000). *B. cereus* can also be the causative agent of other diseases, such as periodontitis, fulminant endophthalmitis, and meningitis in immunocompromised patients (Drobniewski, 1993; Gaur *et al.*, 2001; Hilliard *et al.*, 2003; Kotiranta *et al.*, 2000). *B. cereus* is ubiquitously found in the environment, including in soil. Therefore, the transfer to food is not surprising and causes many problems (Kotiranta *et al.*, 2000). In nutrient-rich environments, such as food, *B. cereus* shows low generation times putatively gaining advantage from its capacity to use various carbohydrates and proteinaceous substrates (Ivanova *et al.*, 2003). The regulation of gene expression plays an important role in the efficient selection of the preferred carbon and energy source for growth. Annotation of the genome of *B. cereus* ATCC 14579 predicted the regulation of gene expression to be highly complex involving over two hundred transcriptional regulators managing its 5370 open reading frames (ORFs) (Ivanova *et al.*, 2003; Overbeek *et al.*, 2003). One of these is the catabolite control protein CcpA, which is a member of the LacI-family of transcriptional regulators. CcpA and the regulatory mechanism of the catabolite repression are highly conserved in low-GC Gram-positives (Warner and Lolkema, 2003). *B. cereus* ATCC 14579 CcpA shows 77% identity with *B. subtilis* CcpA. Furthermore, CcpA in *B. subtilis* has been shown to have a role in optimizing glucose metabolism and the underlying regulatory mechanisms have recently been reviewed (Bruckner and Titgemeyer, 2002; Stulke and Hillen, 2000; Titgemeyer and Hillen, 2002). Regulation of gene expression by CcpA is mediated by its binding to DNA at a specific *cis*-binding sequence, the Catabolite Responsive Element (CRE) (Deutsher *et al.*, 2002; Miwa *et al.*, 2000; Stulke and Hillen, 2000).

In recent years the regulon of *B. subtilis* CcpA has been studied extensively by transcriptome analyses, revealing genes and operons under direct and indirect control of CcpA (Blencke *et al.*, 2003; Lorca *et al.*, 2005; Lulko *et al.*, 2007; Yoshida *et al.*, 2001). Furthermore, Moreno *et al.* (2001) showed a clear correlation between the glucose-repressed genes and the presence of predicted CRE-sites. Moreover, they showed CcpA-mediated glucose-independent regulation of expression (Moreno *et al.*, 2001). Other organisms for which the role of CcpA in carbon metabolism was established are *Lactobacillus acidophilus* (Barrangou *et al.*, 2006) and *Lactococcus lactis* (Zomer *et al.*, 2007). Recently, a role for CcpA in the control of virulence of *Staphylococcus aureus* (Seidl *et al.*, 2006), *Streptococcus pneumoniae* (Iyer *et al.*, 2005), and *Clostridium perfringens* was reported (Varga *et al.*, 2004) and reviewed (Deutscher *et al.*, 2005).

Notably, comparative genomics of the different species of the *B. cereus* group revealed reduced capacity to metabolize carbohydrates and increased potential for protein

metabolism as compared to *B. subtilis* (Kunst *et al.*, 1997; Rasko *et al.*, 2005). Here we report on the role of CcpA in regulation of metabolism and virulence in *B. cereus* ATCC 14579.

Materials and methods

Bacterial strains, culture media, growth conditions, and genetic methods

B. cereus ATCC 14579 and its *ccpA* deletion strain FM1403 were cultured in Brain Heart Infusion broth (BHI, Becton and Dickinson, The Netherlands) medium at 30 °C, with shaking at 200 rpm. The growth of the culture was monitored by measurement of the optical density at OD_{600}. D-glucose concentrations were measured by use of a D-glucose measuring kit (Boehringer). Growth experiments and glucose measurements were performed in three fold. Plasmid DNAs were purified from *E. coli* with a Qiaprep Spin Miniprep kit (Westburg, Leusden, The Netherlands). Pwo polymerase (Roche Diagnostics, Almere, The Netherlands) was used for PCR generated fragments that were used in cloning and Taq polymerase (Fermentas, Amersfoort, The Netherlands) was used in control PCRs. *E. coli* HB101/pRK24 (Trieu-Cuot *et al.*, 1991) was used as the donor host in conjugation experiments. The antibiotics used were ampicillin (Sigma, Zwijndrecht, The Netherlands) at a concentration of 50 µg/ml, kanamycin (Sigma) at a concentration of 70 µg/ml, erythromycin (Sigma) at a concentration of 150 µg/ml (for *E. coli*) or 5 µg/ml (for *B. cereus*), spectinomycin (Sigma) at a concentration of 100 µg/ml, and polymyxin B (VWR, Amsterdam, The Netherlands) at a concentration of 50 µg/ml for counter-selection against *E. coli* upon conjugation.

Construction of *ccpA* deletion strain

To construct a double cross-over deletion strain of *ccpA*, an ~3.5-kb PCR product, comprising *ccpA* and 1-kb flanking regions was obtained by use of forward primer ccpAKOsacIforw (TCgagctcAGATTACGTTGATGTTATTC) and reverse primer ccpAKOxbaIrev (TGtctagaAGAAGAAGAAAAAGAGGAAGAAAT). This PCR product was cloned into pGemT-easy (Promega, Leiden, The Netherlands) according to the manufacturer's protocol resulting in pGemTccpA. Subsequently, an erythromycin-resistance cassette amplified from pUC18ERY (van Kranenburg *et al.*, 1997) with forward primer ErycasFBsrGI (TCtgtacaGTCCGCAAAAGAAAAACG) and reverse primer ErycasRClaI (TCatcgatCATACCTAATAATTTATCTAC) was cloned into pGemTccpA after digesting both with *Bsp*1407I (*Bsr*GI) and *Bsu*15I (*Cla*I) (Fermentas). The insert of the resulting plasmid, comprising the 1-kb flanking regions and the erythromycin-resistance cassette was cloned into the conjugal vector pATΔS28 (Trieu-Cuot *et al.*, 1990) by digestion of the insert and the vector with *Xba*I and *Sac*I (Fermentas). The resulting plasmid pATΔCcpAery was isolated from DH5α and transformed into *E. coli* HB101/pRK24. The resulting strain was used in a conjugation experiment with *B. cereus* ATCC 14579 following established procedures (Bron, 1990). Transconjugants were obtained by selection for spectinomycin sensitivity and erythromycin resistance and one was analyzed in comparison with the wild-type strain. PCR and Southern Blot analysis confirmed the deletion of *ccpA* by double homologous recombination (data not shown). The *B. cereus ccpA* deletion strain was designated *B. cereus* FM1403.

RNA isolation

RNA was extracted from both the *ccpA* deletion strain and the wild-type at four time points in the growth curve at OD_{600} of 0.2, 0.8, 4 and 8 which corresponds to early-exponential, mid-exponential, transition and stationary phase of growth from two independent cultures per phase by using RNAwiz (Ambion, Huntingdon, United Kingdom) according to the manufacturers protocol. Residual DNA from the RNA preparations was enzymatically removed by using TURBO DNA-*free* (Ambion). Extracted RNA samples were stored in 70% EtOH, 0.3 M sodium acetate buffer (pH 5.2) at -80 °C.

Microarray construction and transcriptome analysis

Amplicon based DNA-microarrays were constructed for *B. cereus* ATCC 14579 as described for *L. lactis* IL1403 (Kuipers *et al.*, 2002; van Hijum *et al.*, 2005) with modifications as detailed below. Amplicons were designed on 5199 genes selected from the 5311 annotated genes (ORFs smaller than 80-bp were omitted) on the genome of *B. cereus* ATCC 14579 (Ivanova *et al.*, 2003). To reduce cross-hybridization between probe and target DNA sequences the amplicons had sizes of 70 – 700-bp (depending on gene sizes) and comprised the most unique part of a gene. The amplicons were synthesized by EuroGentec (Seraing, Belgium) in two amplification steps. In the first amplification step, primers were used with a unique tag-sequence for *B. cereus* ATCC 14579 (forward primers were extended with the sequence: 5'-TCGGGCAGCTGCTCC-3'; and reverse primers with the sequence: 5'-TGGCGCCCCTAGATG -3'). Two copies of each amplicon were present per array, resulting in microarrays comprising 10398 spots.

Normalized expression data (Feature Extract, Agilent) for each spot was used in a statistical analysis. The biological replicate experiments were merged with the web-supported VAMPIRE microarray suite, based on a Bayesian frame work. Furthermore, VAMPIRE calculated p-values for individual spots and subsequently used this p-value to identify statistically differentially expressed spots between compared growth conditions by use of a false discovery rate (FDR) of 0.05 as a threshold (Hsiao *et al.*, 2004; Hsiao *et al.*, 2005). In addition, only ORFs of which both individual spots passed the FDR based threshold were considered to be putatively differentially regulated. Expression ratios per ORF were established by calculating the average of the log-values of individual spots. This value (R) was then used to calculate the average expression ratio (10^R) per ORF. Finally, only ORFs that showed a change in expression of at least 2-fold (up/down) were considered to be differentially expressed. Microarray data are submitted to the GEO database with accession number: GSE7843. Supplementary files can be found at www.fhm.wur.nl/UK/thesismenno.

To determine gene similarity, homology and gene context NCBI BLAST and the ERGO database were used (Overbeek *et al.*, 2003), while KEGG (Kanehisa *et al.*, 2006) was used for assessment of metabolic functions and pathways. Whether succeeding genes were part of one operon was determined according to operon prediction as performed by (Price *et al.*, 2005).

Definition and identification of *B. cereus* CRE-site

The 350-bp up-stream and 150-bp down-stream sequences of the translation start of genes identified by the array experiments to be significantly higher expressed in the *ccpA* deletion strain compared to the wild-type strain for early- and mid-exponential growth were analyzed with AlignACE 3.1 (Roth *et al.*, 1998), which searches the input sequences for stretches of nucleotides which align between the different input sequences. The 13-bp CRE-sites identified by AlignACE were aligned with MUSCLE 3.6 (Edgar, 2004) and a Hidden Markov Model (HMM) was constructed with the HMMER package (Durbin *et al.*, 1998). The HMM was used to search CRE-sites in the complete genome sequence of *B. cereus* ATCC 14579 (Ivanova *et al.*, 2003), and not only the 350-bp up-stream and 150-bp down-stream sequences of significantly higher expressed genes in the *ccpA* deletion strain. Subsequently, to analyse whether the *B. cereus* consensus was longer than 13-bp, the obtained sites after the HMM search were extended to 18-bp, as has been the reported length for *B. subtilis* (Miwa *et al.*, 2000). The resulting extended CRE-sites were again aligned using MUSCLE 3.6 (Edgar, 2004). This alignment was subsequently visualized with WebLogo (Crooks *et al.*, 2004) and a iteration HMM search was performed on the *B. cereus* ATCC 14579 sequence (Ivanova *et al.*, 2003) to identify all putative CRE-sites confirming to the new alignment in the *B. cereus* ATCC 14579 genome.

Results and Discussion

Growth and glucose utilization of the *ccpA* deletion strain compared to the wild-type

Growth of the wild-type and the *ccpA* deletion strain was assessed under aerobic conditions in BHI containing 2g/L D-glucose, and revealed specific growth rates (μ) of 0.024 and 0.022 (h^{-1}), respectively (Fig. 1). Statistical analysis showed the growth rates of the wild-type and deletion strain to be significantly different. Assessment of glucose concentrations at different time points during growth revealed a reduction in the glucose consumption rate for the *ccpA* deletion strain compared to that of the wild-type (Fig. 1). The glucose concentration at early-exponential growth for both the wild-type and the *ccpA* deletion strain was around 1.2 g/L, and glucose was still available in the mid-exponential growth phase. Notably, glucose was depleted in the wild-type culture upon entry into the transition phase, whereas glucose depletion in the *ccpA* deletion strain culture was only observed in the stationary phase of growth.

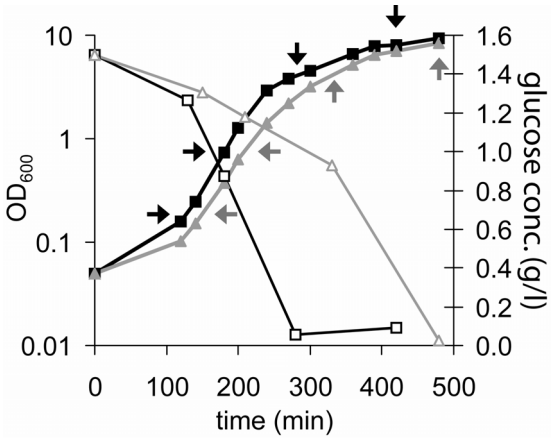

Fig.1. Growth and glucose consumption. Growth (closed symbols) and glucose consumption (open symbols) of the wild-type (black) and the *ccpA* deletion strain (grey) in BHI. Arrows indicate sampling points at OD_{600} of 0.2 (early-exponential), 0.8 (mid-exponential), 4.0 (transition) and 8.0 (stationary), for both glucose determination and transcriptional analysis. Experiments shown are representative for all performed experiments.

Overview of the transcriptome data

Analyses of the transcriptome data of the *ccpA* deletion strain compared to the wild-type in samples taken at the four time points indicated in figure 1, showed expression of a large number of ORFs to be affected upon *ccpA* deletion. Remarkably, the number of genes differentially expressed increased from 147 at early exponential phase to over 700 genes in the stationary phase (Fig. 2). The large differences in gene expression in transition and stationary phase cells of the *ccpA* deletion strain compared to that of the wild-type are conceivably affected by the respective presence and absence of glucose. Furthermore, these differences may point to the initiation of secondary effects of the *ccpA* deletion in these growth phases on gene expression and consequently cellular performance. Therefore, transcriptome analysis was focused on the early- and mid-exponential phase samples where glucose is still present at high levels in the cultures of both the *ccpA* deletion strain and the wild-type. In early-exponential phase, 103 genes showed higher expression and 44 genes showed a lower expression in the *ccpA* deletion strain compared to the wild-type. For mid-exponential phase cells these numbers were 127 and 54, respectively. When corrected for overlap between regulated genes in the early- and mid-exponential phase, a total of 173 genes expression in the exponential phase was shown to be higher in the *ccpA* deletion strain compared to the wild-type and for 80 genes expression in the *ccpA* deletion strain was observed to be lower than in the wild-type (Fig. 2). Consequently, genes that show a higher expression in the *ccpA* deletion strain are possibly repressed by CcpA, whereas genes that show a lower expression in the *ccpA* deletion strain may be activated by CcpA in the wild-type. The numbers of genes regulated are similar to those described for CcpA-regulated genes in *B. subtilis* (Blencke *et al.*, 2003; Lorca *et al.*, 2005; Moreno *et al.*, 2001; Yoshida *et al.*, 2001). Expression ratios of six randomly chosen genes with significantly altered expression in

the *ccpA* deletion strain (*cggR*, *acoR*, *gapB*, *ymfC*, *fruR* and *odhA*) were quantified using qPCR. Expression ratios obtained by microarray analysis were validated by ratios obtained by qPCR, showing the microarray platform to be suited for gene expression analysis (Table S1).

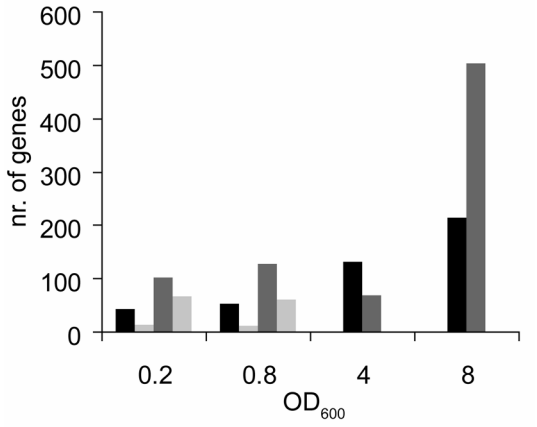

Fig. 2. Differential gene expression during growth. The number of genes differently expressed in the ccpA deletion strain compared to the wild-type in four different growth phases (OD_{600} 0.2 = early-exponential, OD_{600} 0.8 = mid-exponential, OD_{600} 4 = transition, OD_{600} 8 = stationary). Both, the genes with a higher (grey bars) and a lower (black bars) expression in the *ccpA* deletion strain are shown. The light grey bars indicate the number of genes putatively regulated by a CRE-site, these are not shown for the transition and stationary phase.

Identification of the *B. cereus* CRE-site consensus and *in silico* analysis

Direct regulation exerted by CcpA is indicated by the presence of a CRE-site. By a series of subsequent alignments searches, the *B. cereus* ATCC 14579 consensus CRE-site was identified and visualized in Figure 3 (for details see Methods). This showed the consensus sequence to consist of only 16-bp for *B. cereus* ATCC 14579 ,WWGWAARCGWWWWCAW, whereas an 18-bp consensus sequence was reported for *B. subtilis*, WWTGNAARCGNWWWCAWW, (Miwa *et al.*, 2000). Nevertheless, a high similarity between these consensus sequences exists. The *B. cereus* ATCC 14579 genome was scanned with the obtained CRE-site consensus (Fig. 3), identifying the putative CRE-sites, which are presented in Table S2. A number of 76 CRE-sites were identified for 83 out of 173 genes that show a higher expression in the *ccpA* deletion strain compared to the wild-type in exponential phase cells, and these 83 genes are part of 49 operons (Ivanova *et al.*, 2003; Price *et al.*, 2005). Furthermore, 21 CRE-sites were identified in the promoter region of 18 out of 80 genes with a lower expression in the *ccpA* deletion strain (Table S2) in exponential phase cells, these 18 genes are part of 17 operons (Ivanova *et al.*, 2003; Price *et al.*, 2005). These results are in agreement with the general observation that direct activation by CcpA occurs less frequently than direct repression (Moreno *et al.*, 2001). Recently, for *L. lactis* the position of the CRE-site in relation to the σ factor binding site was shown to be important in determining whether CcpA is able to repress or activate gene expression (Zomer *et al.*, 2007). For *B. cereus* ATCC 14579 a prediction of σ factor binding sites is complicated by the fact

that 20 σ factors are annotated on the genome. Furthermore, the -35 sequence of the σ^A promoter binding site of *B. cereus* showed a high divergence, complicating the prediction of σ^A promoter binding sites (data not shown). Therefore, CRE-sites could not be coupled to promoter binding sites in *B. cereus*.

Fig. 3. Visualization of the *B. cereus* ATCC 14579 CRE-site consensus. The consensus sequence was obtained by aligning putative CRE-sites identified on the genome of *B. cereus* ATCC 14579 in or in front of differently expressed genes between the *ccpA* deletion strain and the wild-type.

Analysis of the CcpA regulated genes

The COG annotation reveals that the differentially expressed genes are mainly involved in metabolic processes (Fig. 4), including glycolytic and gluconeogenic genes and genes encoding the citric acid cycle (Table 1). For approximately 35 CcpA regulated genes identified in our study a homologous gene which was also CcpA regulated was identified in *B. subtilis*. Of these genes a total number of 17 genes were annotated to have a function in the glucose metabolism (Table 1). Genes that were first identified in this study to be putatively regulated by CcpA with an apparent and interesting function are discussed below.

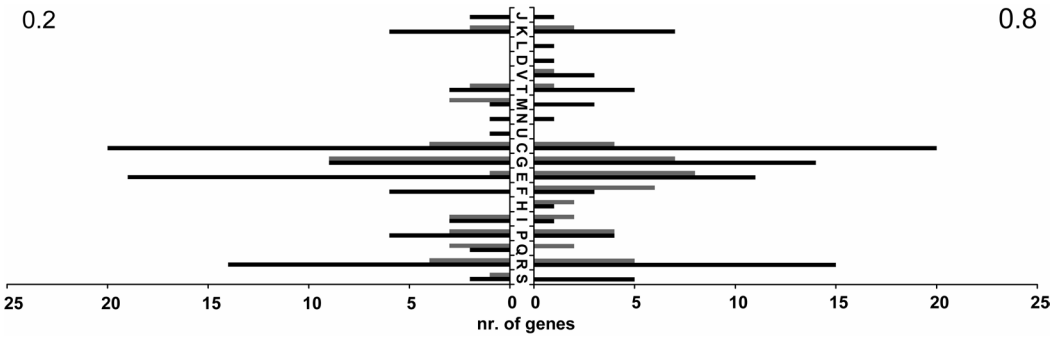

Fig. 4. Functional classes of differentially expressed genes. Functional classes according to COG to which the differently expressed genes in the *ccpA* deletion strain compared to the wild-type belong, for both the early-exponential (0.2) and mid-exponential (0.8) growth phase. Both, genes with a higher (black) and a lower (grey) expression in the *ccpA* deletion strain are shown. COG categories indicated are J: Translation, K: Transcription, L: Replication, recombination and repair, D: Cell cycle control, mitosis and meiosis, V: Defence mechanisms, T: Signal transduction mechanism, M: Cell wall/ membrane biogenesis, N: Cell motility, U, Intracellular trafficking and secretion, C: Energy production and conversion, G: Carbohydrate TM, E: Amino acid TM, F: Nucleotide TM, H: Coenzyme TM, I: Lipid TM, P: Inorganic ion TM, Q: Secondary metabolites TM, R: General function prediction only, S: Function unknown. TM: Transport and metabolism.

One of the glycolytic genes found to be expressed lower in the *ccpA* deletion strain than in the wild-type was *yfmT*. This gene encodes a non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase (GAPN), for which 2 putative CRE-sites could be identified. We propose to rename *yfmT* as *gapN*, since this gene shows similarity with *gapN* of the other members of the *B. cereus* group, and with *gapN* of *B. halodurans*, *Streptococci* and *Clostridia* (Iddar *et al.*, 2005). Notably, the gene is lacking in *B. subtilis* (Iddar *et al.*, 2005; Kunst *et al.*, 1997). Next to the non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase, two phosphorylating glyceraldehyde-3-phosphate dehydrogenases encoding genes (*gapA* and *gapB*) are present on the *B. cereus* ATCC 14579 genome. The function of GAPN in microbial metabolism is yet unclear, although it may play a role in accelerating glycolysis as it can produce 3-phospho-D-glycerate from D-glyceraldehyde 3-phosphate in a one-step reaction, instead of two steps. This may offer a competitive advantage in the use of glucose in mixed bacterial populations (Ahmed *et al.*, 2005). As a putative drawback, ATP is not produced in this one-step reaction. Recently, Asanuma and Hino (2006) showed a role for CcpA in expression control of *gapN* in *Streptococcus bovis* and proposed that NADPH is provided by GAPN activity for NADPH-dependent biosynthetic reactions, thereby maintaining an optimal redox balance at the same time. The exact role of GAPN in *B. cereus* metabolism remains to be elucidated.

The gluconeogenic genes expressed higher in the *ccpA* deletion strain included *ywjI* and *gapB*. The *ywjI* gene is part of the *murA2-ywjI*-operon and encodes a fructose-1,6-bisphosphatase. The *ywjI* gene of *B. subtilis*, that shows similarity to *ywjI* of *B. cereus*, is annotated to be a fructose-1,6-bisphosphatase, as a member of the *glpX*-family. The other annotated fructose-1,6-bisphosphatase gene for *B. subtilis*, *fbp*, which is no member of the *glpX*-family, is not regulated by CcpA and deletion of this gene seems to have no effect on gluconeogenesis (Fujita *et al.*, 1998). A corresponding *fbp* homologue appears to be absent in *B. cereus*. However, on the *B. cereus* genome two members of the *glpX*-family of fructose-1,6-bisphosphatases are present, of which *ywjI* was found to be affected by *ccpA* deletion, accordingly a CRE-site was identified in the promoter region of its operon. Therefore we suggest that *ywjI* encodes a fructose-1,6-bisphosphatase in *B. cereus* and also in *B. subtilis*. The *gapB* gene encodes a NAD(P)-dependent glyceraldehyde-3-phosphate dehydrogenase. Three obvious CRE-sites could be identified for *gapB* (Table 1) in *B. cereus*. The regulation of *ywjI* and also *gapB* by CcpA indicates that gluconeogenesis in *B. cereus* is regulated by CcpA. In contrast, gluconeogenesis in *B. subtilis* was shown to be regulated by the transcriptional regulator CcpN (Licht and Brantl, 2006; Servant *et al.*, 2005). CcpN is present on the genome of *B. cereus* ATCC 14579 as well and putative CcpN binding sites can be found in front of the genes encoding GapB and PckA (Servant *et al.*, 2005). This suggests a role for CcpN in the regulation of gluconeogenesis in *B. cereus* in addition to CcpA.

Table 1. Genes involved in *B. cereus* ATCC14579 glucose metabolism and their putative CRE-sites

Gene[§, ¶]		Annotation	BC nr.	RZC nr.	Δ*ccpA*/WT [†]		CRE[‡]
					early	mid	
Glycolysis							
ptsG[#]	1	PTS system, glucose-specific IIABC component	BC4050	RZC06596	0.52	1.12	2
ptsH[#]	2	Phosphocarrier protein HPr	BC4049	RZC00081	0.56	1.10	+
ptsI[#]	3	Phosphoenolpyruvate-protein phosphotransferase	BC4048	RZC06595	0.66	1.16	1
pgi		Glucose-6-phosphate isomerase	BC4898	RZC03793	0.31	0.20	1
pfkA	1	6-phosphofructokinase	BC4600	RZC02283	0.45	0.47	-
pykA	2	Pyruvate kinase	BC4599	RZC01414	0.29	0.33	-
fbaA		Fructose-bisphosphate aldolase	BC5335	RZC03557	0.39	0.52	2
cggR[#]	1	Central glycolytic genes regulator	BC5141	RZC08032	0.49	0.73	-
gapA[#]	2	Glyceraldehyde-3-phosphate dehydrogenase	BC5140	RZC00210	0.36	0.59	-
pgk*[#]	3	Phosphoglycerate kinase	BC5139	RZC00211	0.49	0.41	-
pgk*[#]	3	Phosphoglycerate kinase	BC5138	RZC08031	0.53	0.46	-
tpiA[#]	4	Triose-phosphate isomerase	BC5137	RZC08030	0.62	0.51	-
pgmA[#]	5	Phosphoglycerate mutase	BC5136	RZC05843	0.48	0.38	-
eno[#]	6	Phosphopyruvate hydratase	BC5135	RZC02971	0.42	0.51	-
Pyruvate dehydrogenase							
pdhA[#]	1	Pyruvate dehydrogenase (acetyl-transferring)	BC3973	RZC01920	1.24	0.77	2
pdhB[#]	2	Pyruvate dehydrogenase (acetyl-transferring)	BC3972	RZC01921	1.47	0.79	+
pdhC[#]	3	Dihydrolipoyllysine-residue acetyltransferase	BC3971	RZC06754	1.52	0.95	+
pdhD[#]	4	Dihydrolipoyl dehydrogenase	BC3970	RZC07919	1.28	1.01	+
Overflow metabolism							
alsS	1	Acetolactate synthase large subunit	BC0883	RZC01898	1.04	0.54	2
alsD	2	Alpha-acetolactate decarboxylase	BC0884	RZC01896	0.92	0.58	+
ldh		L-lactate dehydrogenase	BC1924	RZC05932	0.44	1.05	-
lctP		L-lactate permease	BC0612	RZC01585	0.34	1.21	-
pta		Phosphate acetyltransferase	BC5387	RZC05214	0.61	0.70	1
ackA		Acetate kinase	BC4637	RZC02436	0.93	1.29	1
Citric acid cycle							
citZ	1	Citrate synthase	BC4594	RZC01265	3.76	2.23	1
citC	2	Isocitrate dehydrogenase [NADP]	BC4593	RZC01263	5.56	4.38	1
mdh	3	Malate dehydrogenase	BC4592	RZC01264	5.81	4.32	+
citB		Aconitate hydratase	BC3616	RZC04993	1.62	1.26	1
odhA	1	Oxoglutarate dehydrogenase	BC1252	RZC06639	2.07	3.80	4
odhB	2	Dihydrolipoyllysine-residue succinyltransferase	BC1251	RZC03695	2.36	3.97	+
sucC	1	Succinate-CoA ligase (ADP-forming)	BC3834	RZC02282	8.23	6.32	1
sucD	2	Succinate-CoA ligase (ADP-forming)	BC3833	RZC02281	7.74	7.57	+
sdhC	1	Succinate dehydrogenase	BC4518	RZC05750	4.02	4.02	1
sdhA	2	Succinate dehydrogenase	BC4517	RZC07054	3.70	3.70	1
sdhB	3	Succinate dehydrogenase	BC4516	RZC07053	3.76	5.64	+
citG		Fumarate hydratase	BC1712	RZC03022	1.29	1.49	2
Gluconeogenensis							
fbpA		Fructose-bisphosphatase	BC4962	RZC03080	0.75	0.74	2
gapB		Glyceraldehyde-3-P dehydrogenase	BC4583	RZC07229	1.74	2.79	3
pckA		Phosphoenolpyruvate carboxykinase (ATP)	BC4762	RZC07251	1.37	1.09	1
ywjI		Fructose-bisphosphatase	BC5333	RZC00125	3.73	3.34	+

§ Genes of which expression of their homologous gene in *B. subtilis* was also CcpA dependent are in grey
# Genes of which effects of *ccpA* deletion on expression where shown to be indirect in *B. subtilis*
* *pgk* is represented on the microarray by two different amplicons, as Pgk is annotated in the ERGO database to be encoded by two genes.
¶ Numbers indicate the order of genes within one operon.
† Ratios are presented as expression in the *ccpA* deletion strain compared to the wild-type (WT), expression in the wild-type is set to 1. Early- and mid-exponential phase data are shown.
‡ In this column it is indicated whether a putative CRE-site was identified for the gene. Numbers indicate the number of putative CRE-sites identified, a + indicates that a gene is in the same operon as a gene for which a putative CRE-site was identified, and a – indicates no putative CRE-site could be identified.

The expression of genes with functions in routing metabolism towards the citric acid cycle appeared to be higher in the *ccpA* deletion strain compared to the wild-type. For a total number of 15 operons involved in amino acid catabolism the expression was shown to be higher. This included the *bkd*-operon (BC4163 to BC4157) that is common among the *Bacilli*, and that is involved in the catabolism of Valine and Leucine (Debarbouille *et al.*, 1999). One putative CRE-site in front of the *bkd*-operon and two within the operon were identified in *B. cereus* ATCC 14579. Notably, CcpA was previously shown to play no role in the regulation of expression of this operon in *B. subtilis* (Tam le *et al.*, 2007). In contrast, the *ilv-leu*-operon of *B. subtilis* coding for biosynthesis of branched chain amino acids has been shown to be activated by CcpA (Tojo *et al.*, 2005), whereas no involvement of CcpA was found in regulation of this operon in *B. cereus*. The fact that no regulation of the *ilv-leu*-operon was observed, could be due to a complex regulation of this operon under the tested circumstances involving CodY and TnrA (Shivers and Sonenshein, 2005; Tojo *et al.*, 2005). Expression of several operons encoding enzymes involved in catabolism of nucleosides was higher in the *B. cereus ccpA* deletion strain, this included the *yuf*-operon (BC3791 to BC3788) coding for nucleoside transport under the apparent control of the transcriptional regulator encoded by the also higher expressed *ymfC* (BC3792), for both the regulator and the *yuf*-operon CRE-sites could be identified (Table S2). Interestingly, expression of two operons with an apparent function in catabolism was higher in the *ccpA* deletion strain, and their putative functions were assessed using *in silico* analysis. The first operon consists of three genes and this operon is not found in *B. subtilis*. The genes are annotated as a 5-methylthioribose kinase (BC0378), a methylthioribose salvage protein (BC0379) and an L-fuculose phosphate aldolase (BC0380). It was shown for 5-methylthioribose kinases that they have high similarity with L-fuculose phosphate aldolases (Samuel *et al.*, 2001). Next to this, the *in silico* analysis of the methylthioribose salvage protein (BC0379) revealed similarity with an isomerase. This suggests that the first two genes may encode enzymes with specificity for fucose and/or arabinose. Together with the L-fuculose phosphate aldolase this operon could have a function in the catabolism of fucose and/or arabinose. Consequently, names and functions that we suggest are *fucI* for the L-fucose isomerase (BC0379), *fucK* for the L-fuculokinase (BC0378) and *fucA* for the L-fuculose phosphate aldolase (BC0380), as used for this gene cluster in *E. coli* (Elsinghorst and Mortlock, 1994) (Fig. 5). A clear CRE-site could be identified in front of this operon. The second operon consists of three genes and *in silico* analysis of this operon (BC1739-1741) revealed the transporter protein (BC1739) may function as a proton/sodium-

aspartate symporter (Lorca *et al.*, 2003), as an alternative to *gltT*. Subsequently, aspartate can be converted into ammonia and fumarate by the aspartate ammonia lyase (BC1740), after which fumarate can be metabolized to malate by one of the two fumarate hydratases encoded on the genome of *B. cereus*. The gene BC1741 encodes a malic enzyme, MalS, which is responsible for converting fumarate into pyruvate. This suggests that the operon encodes enzymes involved in the catabolism of aspartate to pyruvate. This operon is unique for the species of the *B. cereus* group, as the genes are not found as an operon in genomes of other bacteria. A CRE-site was identified for the first gene of the operon (Fig. 5). The two-component system (BC1742-BC1743) next to this operon shows high similarity with the two-component system GlnK-GlnL of *B. subtilis*, which is involved in glutamine utilization (Satomura *et al.*, 2005). Whether BC1742-BC1743 functions as a two-component system, involved in aspartate sensing and triggering expression of enzymes involved in its metabolism remains to be elucidated.

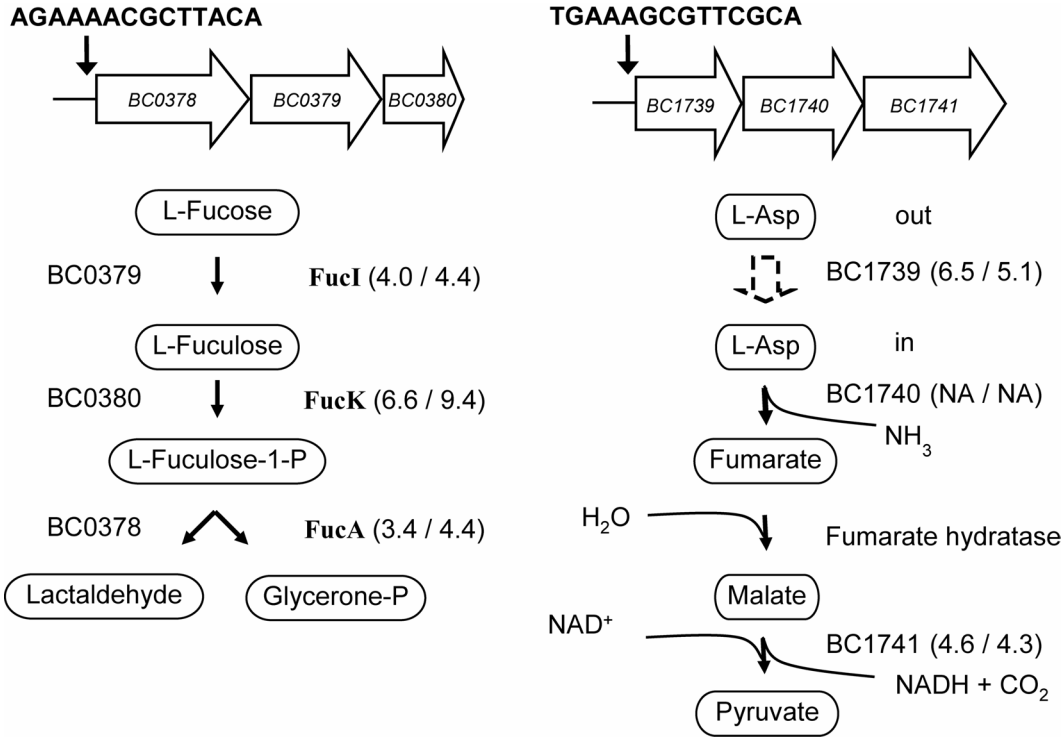

Fig. 5. CcpA-controlled metabolic pathways in *B. cereus*. Two operons observed to be higher expressed in the *ccpA* deletion strain compared to the wild-type with their proposed functions and between brackets the expression in the *ccpA* deletion strain compared to the wild-type in the early- and mid-exponential growth phase, respectively. Putative CRE-sites are indicated in front of the operon, the open arrow in the flow scheme stands for transport into the cell, whereas the closed arrows stand for reactions. L-Asp stands for L-Aspartate and P stands for phosphate.

Glutamate is a product of protein and amino acid catabolism and acts as link between nitrogen and carbon metabolism, mediated by glutamate synthase and glutamate dehydrogenase as described for *B. subtilis* (Belitsky and Sonenshein, 1998; Wacker *et al.*, 2003). The *B. subtilis* genome contains a glutamate synthase (*gltAB*) and two glutamate dehydrogenases (*rocG* and *gudB*) of which the GudB dehydrogenase appears to be inactive (Belitsky and Sonenshein, 1998). The microbial glutamate synthases generally belong to the NADPH-GltS family of glutamate synthases and are encoded by two genes (Vanoni and Curti, 1999). In the glutamate synthase reaction L-glutamate is produced from L-glutamine and 2-oxoglutarate, with the latter compound derived from the citric acid cycle (Vanoni and Curti, 1999). The *gltAB*-operon of *B. subtilis* is suggested to be regulated by CcpA (Faires *et al.*, 1999; Wacker *et al.*, 2003). On the genome of *B. cereus* ATCC 14579 only one glutamate synthase gene (*gltA*) is present and its expression at various phases of growth is similar in the wild-type and its *ccpA* deletion strain, indicating it not to be regulated by CcpA. Moreover, it is unclear to which family of glutamate synthases GltA of *B. cereus* belongs. Interestingly, *gudB* was the only gene present on the genome of *B. cereus* encoding a glutamate dehydrogenase, and its expression was observed to be clearly higher in the *ccpA* deletion strain. Glutamate dehydrogenase is responsible for the reversed reaction from L-glutamate to L-glutamine and 2-oxoglutarate supplying the latter to the citric acid cycle (Struck and Sizer, 1960). Higher expression of *gudB* in the *ccpA* deletion strain compared to the wild-type, together with the fact that it is the only glutamate dehydrogenase annotated on the genome of *B. cereus* suggests that GudB is the active glutamate dehydrogenase for *B. cereus*. This is supported by the fact that the 9-bp sequence, encoding the 3 amino acids causing the inactivity of GudB in *B. subtilis* (Belitsky and Sonenshein, 1998), are absent in the *gudB* sequence of *B. cereus* ATCC 14579. Two putative CRE-sites in front of and one within *gudB* were identified pointing to CcpA-controlled expression in *B. cereus*.

B. subtilis contains a large number of carbohydrate catabolic pathways (Kunst *et al.*, 1997), whereas the number of these pathways is limited in *B. cereus*. The observed deficiency of the *B. subtilis ccpA* deletion strain in growth with ammonium as the sole nitrogen-source, has been attributed to the regulation of the *gltAB* genes by CcpA (Faires *et al.*, 1999; Wacker *et al.*, 2003) and the read-through transcription of the *rocG* gene (Belitsky and Sonenshein, 1998). The lack of regulation of *gltA* in *B. cereus* by CcpA, together with its low similarity to the *B. subtilis gltA*, offers an explanation for the observation that the *ccpA* deletion strain of *B. cereus* is able to grow with ammonium as the sole nitrogen source (data not shown), a possible indirect role in this growth is proposed for GudB, as being the active glutamate dehydrogenase in *B. cereus*.

The role of CcpA in optimisation of glucose metabolism is apparent, since glycolytic enzymes were expressed lower and expression of genes encoding citric acid cycle enzymes was shown to be higher in the *ccpA* deletion strain compared to the wild-type. Direct repression by CcpA in *B. cereus* was found for a large number of genes encoding enzymes involved in protein, peptide and amino acid metabolism. This is seemingly in contrast with the proposed preferred use of proteinaceous substrates for growth of *B. cereus*, a hypothesis put forward by Ivanova *et al.* (Ivanova *et al.*, 2003). This hypothesis was supported by the

annotation of a large number of genes encoding proteolytic enzymes, a multiplicity of peptide and amino acid transporters, and a large variety of amino acid degradation pathways. Based on our results it is concluded however, that under the conditions tested, glucose is the preferred carbon and energy source for growth of *B. cereus* ATCC 14579.

CcpA-mediated catabolite control of enterotoxin gene expression in *B. cereus*

In early- and mid-exponential phase cells the expression of the *nhe*-operon (BC1809-BC1811), coding for the non-hemolytic toxin Nhe, was shown to be higher in the *ccpA* deletion strain compared to the wild-type. This higher expression was even more apparent in the stationary phase where an almost 20-fold stimulation was observed (Fig. 6). Furthermore, a putative CRE-site was identified for this operon. For the *hbl*-operon (BC3104-BC3102), coding for the Hbl enterotoxin, an 8-fold increase in the *ccpA* deletion strain was seen in the stationary phase and a putative CRE-site could be identified (Fig. 6). Differential expression of the *cytK* gene encoding CytK was not observed under the conditions tested, and no putative CRE-site could be identified. These results clearly show involvement of CcpA in the catabolite control of the expression of at least two major enterotoxins in *B. cereus* ATCC 14579. Many bacteria use glucose as their preferred carbon and energy source as it can be obtained from plant-derived polysaccharides, which can be found in many environments including foods. We hypothesize that glucose functions as a signalling molecule enabling CRE-site-mediated, CcpA-dependent repression of gene expression for microorganisms such as *Lactobacillus plantarum* (Bron *et al.*, 2004) and *B. cereus* that occur in several environments, including soil, plant and the human GI tract. In the GI tract, in the absence of glucose, CcpA-dependent repression is thus lifted, and this allows for induction of pathways involved in catabolism of other substrates present in the GI tract such as fucose, a major degradation product of mucus related fucosylated glycans (Hooper and Gordon, 2001). Notably, this study revealed the putative fucose utilisation operon of *B. cereus* to be repressed by CcpA in the presence of glucose, and this pathway may thus be activated in the GI-tract under glucose limiting conditions. A similar mode of regulation seems applicable for the *nhe* and *hbl* enterotoxin operons, for which the combination of transcriptome data and the identification of putative CRE-sites, indicates these to be CcpA controlled as well. Our results offer an explanation for the observations made by Ouhib *et al.* (Ouhib *et al.*, 2006), who showed that final enterotoxin levels reached, depended on the carbon and energy sources used for growth, with the lowest levels reached with cells grown on glucose.

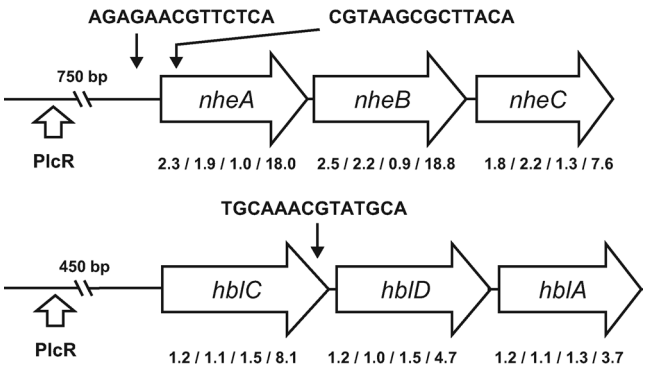

Fig. 6. CcpA regulated enterotoxin operons. Positioning of CRE-sites and PlcR binding sites in two major enterotoxin operons encoding Nhe and Hbl of *B. cereus* ATCC 14579. Ratios of gene regulation are given as found in the *ccpA* deletion strain compared to the wild-type, for the four different growth phases, early- and mid-exponential, transition and stationary phase, respectively. Closed arrows indicate the approximate position of the identified CRE-site, open arrows indicate binding sites for the pleiotropic regulator PlcR (Agaisse *et al.*, 1999; Gohar *et al.*, 2002).

These results suggests that action of the pleiotropic regulator PlcR (Agaisse *et al.*, 1999; Gohar *et al.*, 2002) can be overruled by CcpA-mediated catabolite repression. The anaerobic growth transcriptional regulators FNR and ResD have recently been found to play a role in enterotoxin production also (Duport *et al.*, 2006; Zigha *et al.*, 2007), and combined with our data, this points to an elaborate control of enterotoxin production in *B. cereus* at the transcriptional level based on a variety of input signals mediated by a range of transcriptional regulators. Notably, CcpA has been reported also to play a role in toxin formation in *C. perfringens* (Varga *et al.*, 2004), and in *S. aureus* deletion of *ccpA* was shown to affect the expression of the virulence gene *hla*, encoding α-hemolysin (Seidl *et al.*, 2006).

In conclusion, our study revealed glucose to be the preferred carbon source for growth of *B. cereus* ATCC 14579, with CcpA as the main regulator of gene expression leading to efficient glucose utilization and control of enterotoxin production.

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4

Identification of the σ^B regulon of *Bacillus cereus* and conservation of σ^B-regulated genes in low-GC Gram-positive bacteria

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Abstract

The alternative σ factor σ^B has an important role in the acquisition of stress-resistance in many Gram-positive bacteria including the foodborne pathogen *Bacillus cereus*. Here we describe the identification of the set of σ^B-regulated genes in *B. cereus* by DNA microarray analysis of the transcriptome upon a mild heat shock. Twenty-four genes could be identified as being σ^B-dependent as witnessed by (i) significantly lower expression-levels of these genes in mutants deleted for *sigB* and *rsbY* (which encode the alternative σ factor σ^B and a crucial positive regulator of σ^B-activity, respectively) than in the parental strain *B. cereus* ATCC 14579, and (ii) increased expression of these genes upon a heat shock. Newly identified σ^B-dependent genes in *B. cereus* include a histidine kinase and two genes that have predicted functions in spore germination. This study shows that the σ^B regulon of *B. cereus* is considerably smaller than that of other Gram-positive bacteria. This appears to be in line with phylogenetic analyses where σ^B of the *B. cereus* group was placed close to the ancestral form of σ^B in Gram-positives. The data described in this study and earlier studies in which the complete σ^B regulon of the Gram-positive bacteria *Bacillus subtilis*, *Listeria monocytogenes* and *Staphylococcus aureus* were determined, enabled a comparison of the sets of σ^B-regulated genes in the different Gram-positives. This showed that only three genes (*rsbV*, *rsbW* and *sigB*) are conserved in their σ^B-dependency in all four bacteria, suggesting that the σ^B regulon of the different Gram-positives has evolved to perform niche-specific functions.

Introduction

The ability of bacteria to respond rapidly to changing environmental conditions is a prerequisite for survival in their habitats. This bacterial stress response is triggered by a change in the microorganism's growth conditions. Such a change triggers a cascade of events that will lead to an increased stress-resistance of the bacterial cell; most often not only against the stress to which it was exposed, but also to other stresses, thereby ensuring its survival under a variety of conditions. A common strategy that bacteria use to counter stressful conditions, is to activate a specific alternative σ factor, which leads to the transcription of a set of genes (a so-called regulon) of which the products protect the cell against the adverse conditions (Kazmierczak *et al.*, 2005). In several Gram-positive bacteria the alternative σ factor σ^B is the key σ factor controlling the stress response (Kazmierczak *et al.*, 2005; Price, 2002; van Schaik and Abee, 2005).

In the Gram-positive model organism *Bacillus subtilis* and the human pathogens *Listeria monocytogenes* and *Staphylococcus aureus* numerous studies have shown that the activation of σ^B leads to an increased resistance to stress and that in pathogenic *Listeria* and *Staphylococcus* sp. σ^B has a role in virulence (Kazmierczak *et al.*, 2005; van Schaik and Abee, 2005). In the bacteria that comprise the physiologically diverse *Bacillus cereus* group, the role of σ^B has also been studied. The most prominent members of the *B. cereus* group are *B. anthracis* (a mammalian pathogen which causes the disease anthrax), *B. thuringiensis* (an insect pathogen that is widely used as a biopesticide), and *B. cereus*, which is a common cause of foodborne infections (Jensen *et al.*, 2003). Recent genomics studies have revealed that these bacteria share a common core-set of genes and that their phenotypic characteristics are largely determined by the presence or absence of large plasmids which may encode different virulence factors (Rasko *et al.*, 2005). Throughout the *B. cereus* group the genes encoding σ^B and the regulators of its activity are conserved. In *B. cereus*, σ^B was shown to be activated under a number of stress conditions, but most strongly upon a mild heat shock from 30 °C to 42 °C. Furthermore, the deletion of the *sigB* gene affected the heat adaptive response of vegetative cells and some spore properties, most notably the germination characteristics (de Vries *et al.*, 2005; van Schaik *et al.*, 2004a). Also in *B. anthracis*, σ^B was activated upon a heat shock and the *B. anthracis sigB* deletion mutant was shown to have slightly lower virulence than the parental strain (Fouet *et al.*, 2000).

Regulation of σ^B activity in Gram-positive bacteria occurs via a so-called partner switching mechanism. Under non-stress conditions, σ^B is held in an inactive state by sequestration to an anti-σ factor (RsbW). Under stress, an anti-σ factor antagonist, RsbV binds to RsbW and this leads to the release of σ^B from its complex with RsbW. Subsequently, σ^B can bind to core RNA polymerase, leading to the transcription of σ^B-dependent genes. RsbW is not only the anti-σ factor for σ^B, but it also acts as a kinase on RsbV. In its phosphorylated form, RsbV is unable to bind to RsbW and activate σ^B. However, under stress conditions, the phosphorylated form of RsbV can be dephosphorylated by the action of a PP2C-type phosphatase, which then leads to the formation of the RsbV-RsbW complex and the activation of σ^B (reviewed in (Kazmierczak *et al.*, 2005; Price, 2002; van Schaik and Abee, 2005). In the *B. cereus* group the PP2C phosphatase RsbY appears to be the only phosphatase responsible

for the activation of σ^B under stress, as deletion of the *rsbY* gene in *B. cereus* leads to an abolished σ^B-response under several stress conditions, including a heat shock (van Schaik *et al.*, 2005).

The identification of the complete σ^B regulons in *B. subtilis* (Helmann *et al.*, 2001; Petersohn *et al.*, 2001; Price *et al.*, 2001), *L. monocytogenes* (Kazmierczak *et al.*, 2003) and *S. aureus* (Bischoff *et al.*, 2004; Pane-Farre *et al.*, 2006) by DNA-microarray technology has revealed that a surprisingly low number of genes has a directly obvious role in the stress-response of these organisms. Instead, σ^B-dependent specialized metabolic adaptations may contribute to stress resistance. Furthermore, in both *L. monocytogenes* and *S. aureus* pathogenic traits seem to be governed by σ^B. These findings suggest that σ^B has evolved in response to the specific niches that are populated by the different bacteria.

Here we describe the σ^B regulon of *B. cereus* as determined by comparing the transcriptome of the parental *B. cereus* strain ATCC 14579 and its *sigB*- and *rsbY*-deletion mutant upon a mild heat shock. It appears that the σ^B regulon of *B. cereus* is considerably smaller than that of other Gram-positives, which may reflect the previously proposed close-to-ancestral state of σ^B in the *B. cereus* group (Ferreira *et al.*, 2004). In other studies the set of σ^B-regulated genes in *B. subtilis* (Helmann *et al.*, 2001; Petersohn *et al.*, 2001; Price *et al.*, 2001), *L. monocytogenes* (Kazmierczak et al., 2003) and *S. aureus* (Bischoff *et al.*, 2004; Pane-Farre *et al.*, 2006), was determined by DNA microarray technology. This allowed us to compare the σ^B regulons of *B. cereus*, *B. subtilis*, *L. monocytogenes* and *S. aureus* with each other to determine to what extent σ^B-regulated genes are conserved in the different bacteria and to assess in how far the σ^B-dependency of these genes is conserved.

Materials and methods

Strains, growth conditions and RNA isolation

The *B. cereus* strains ATCC 14579, and its isogenic derivatives the *sigB* deletion mutant FM1400 (van Schaik *et al.*, 2004a) and the *rsbY* deletion mutant FM1401 (van Schaik *et al.*, 2005) were used in this study. Strains were cultured overnight in 100 ml Erlenmeyer flasks containing 20 ml BHI broth at 30 °C with aeration by rotary shaking at 150 rpm. Subsequently, from a single overnight culture, pre-warmed BHI (20 ml in 100 ml Erlenmeyer flasks) was inoculated with 100 µl of the overnight culture. These cultures were then incubated at 30 °C as above. One of the three cultures was used to measure the absorbance at 600 nm (A_{600}). When the A_{600} of this culture reached 0.4 (mid-exponential growth phase), one of the other two cultures was transferred to a shaking waterbath at 42 °C and incubated for a further 10 min followed by RNA-isolation. We previously found that at 10 min after the start of heat shock the levels of σ^B protein reached maximum levels (van Schaik *et al.*, 2005), which leads to high-level expression of σ^B-dependent genes at this time-point. From the culture in the mid-exponential growth phase RNA was isolated immediately. RNA isolation was performed by transferring the cultures to a 50-ml Falcon tube and spun down at 13000 *g* for 20 sec. After decanting the supernatant, the cell pellets were snap frozen in liquid nitrogen. The time between removal from incubator and freezing of the cell pellets was approximately 60 seconds. Within 20 min after freezing the cell pellets, RNAwiz (Ambion,

Huntingdon, United Kingdom) was added to the pellets and RNA was extracted as described previously (van Schaik *et al.*, 2004a). Residual chromosomal DNA was removed by treating samples with DNA-*free* (Ambion). Extracted RNA samples were stored in 70% EtOH, 83 mM sodium acetate buffer (pH 5.2) at -80 °C.

cDNA synthesis and microarray design and hybridization

Before further analysis, the RNA samples were spun down at 13000 rpm in a microtube centrifuge pre-chilled at 4 °C. The RNA pellet was then washed with 70% EtOH (pre-chilled at -20 °C) and resuspended in RNAse-free water. Prior to cDNA synthesis, the quality of the extracted RNA was determined by analysis with an Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA), according to the manufacturer's instructions. cDNA with amino-allyl labelled dUTP (Ambion) was prepared in reverse transcription reactions using Superscript III (Invitrogen, Breda, The Netherlands). Cy3- and Cy5-labelling of the cDNAs was performed with the CyScribe Post-Labelling kit (GE Healthcare, Diegem, Belgium) as previously described (den Hengst *et al.*, 2005). Labeled cDNAs were purified using the CyScribe GFX Purification kit (GE Healthcare). Final cDNA yields and incorporation of the Cy-labels was determined by Nanodrop analysis (Isogen Life Science, IJsselstein, The Netherlands). Custom-made *B. cereus* microarrays (produced by Agilent, see below for details) were hybridized with 200 – 300 ng labeled cDNA. The microarray experiments for the comparison of the transcriptomes of the wild-type strain and the *sigB* and *rsbY*-mutant were performed with three and two independent biological replicates, respectively. Microarray experiments (using two biological duplicates), were also performed to identify the set of genes which were differentially expressed in *B. cereus* ATCC 14579 after heat shock from 30 °C to 42 °C for 10 min.

The microarrays used in this study were custom-made *B. cereus* ATCC 14579 microarrays using the 11K platform developed by Agilent Technologies. A total of 10263 spots represented 5058 *B. cereus* open reading frames, meaning that 96.6% of the predicted chromosomal *B. cereus* ATCC 14579 open reading frames (NCBI accession nr. NC_004722) are represented on the microarray. 95% of the open reading frames for which probes could be designed were represented by two or three non-overlapping probes on the array. The remaining 5% of the open reading frames was represented by a single oligo-nucleotide. After hybridization at 60 °C for 17 hours, the microarrays were washed according to the manufacturers with 6x SSC, 0.005% Triton X-102 at room temperature for 10 min and with prechilled (at 4 °C) 0.1x SSC, 0.005% Triton X-102 for 5 min. Slides were then scanned in an Agilent microarray scanner (G2565BA) and data was extracted from the scanned microarrays with Feature Extraction Software, which includes a Lowess normalization step of the raw data.

Analysis of microarray data

After removal of the data for the different controls printed on the microarray slides, the normalized data for each spot from the microarrays was analyzed for statistical significance using the web-based VAMPIRE microarray suite (Hsiao *et al.*, 2004; Hsiao *et*

al., 2005). A spot was found to be differentially expressed between two samples using the threshold of a false discovery rate smaller than 0.05. Subsequently, the data of the single spots were integrated to obtain expression ratios for an open reading frame. An open reading frame was found to be differentially expressed when all spots representing the open reading frame were significantly differentially expressed between samples. The average expression ratio was determined by calculating the average of the log-values of the expression ratios over all experiments. This value (R) was then used to calculate the average expression ratio (10^R). Finally, a fold-change of 1.5 (for up-regulated genes in the parental strain) and 0.67 (for down-regulated genes in the parental strain) was also introduced as significance limit. Microarray data was submitted to the GEO database with accession number GSE6005.

Validation of microarray data

The expression ratio of six genes was determined using quantitative real time PCR (qPCR) on the RNA samples isolated from the strains ATCC 14579 and FM1400. cDNA synthesis and qPCR were performed as described previously with *tufA* as reference gene (van Schaik *et al.*, 2005). All qPCR reactions were performed in triplicate. Expression ratios between the strains were determined by the REST-tool (Pfaffl *et al.*, 2002).

Table 1. Oligonucleotides used in this study

Name	Sequence (5'- 3')
tufAF	GCCCAGGTCACGCTGACTAT
tufAR	TCACGTGTTTGAGGCATTGG
BC0376F	GTAAACCGCCCTGGTGAAGA
BC0376R	GGAGCGCGTCCACTTACTTG
BC1008F	AAAGAGATTCACCCACTCATTGC
BC1008R	TTGCTCCTCAGTGTTACGGAAGT
BC1550F	CTACGGTCCGGCAATTGAAA
BC1550R	TCACCTTTGTAACTCTTTGTTGCATT
BC4641F	TGGTTGGAAATGGCATACACA
BC4641R	CGACAACTCCAGCAGCCATA
BC5149F	CCGCTCATACAATGGCAACA
BC5149R	CGGCTCCAGCGAAATCAAT
BC5391F	ACGTGGCAGTTCCCTTGGTA
BC5391R	GTTTTCCTGATTGCGGTTCA

Prediction of σ^B-dependent promoter sites

Prediction of σ^B-dependent promoter sites in *B. cereus* was performed by using the 500bp up-stream regions of the 26 genes identified in the σ^B regulon. Subsequently the up-stream regions were analyzed with AlignACE 3.1 (Roth *et al.*, 1998) and DBTBS 4.1 (Makita *et al.*, 2004) with a threshold of 5%. A total of twelve putative σ^B-dependent promoter sites were identified by these methods. These promoter sites were aligned with MUSCLE 3.6 (Edgar, 2004) and a Hidden Markov Model (HMM) was constructed with the HMMER package (Durbin *et al.*, 1998). The HMM was used for a first screening for σ^B-dependent promoters in the genome sequence of *B. cereus* ATCC 14579 (Ivanova *et al.*, 2003). Hits that were more than 300 nucleotides up-stream of the start codon of an open reading frame or had

an E-score of higher than 1 x 10^{-3} were discarded. Two of the putative promoter sites identified in the first round of the HMMER-search did not meet these standards. Therefore an iteration of the alignment, the HMM and the genome search was performed with the ten remaining promoters. Predicted sites that were more than 300 nucleotides up-stream of the start codon of an open reading frame or had an E-score of higher than 1 x 10^{-3} were again discarded. The ten σ^B-dependent promoter sites up-stream of genes belonging to the σ^B regulon that were identified by this approach were aligned and this alignment was visualized with WebLogo (Crooks *et al.*, 2004).

Comparison of the σ^B regulons of Gram-positive bacteria

To determine the conservation of σ^B-dependent genes in Gram-positive bacteria, the set of genes that is directly regulated by σ^B, was compiled for *B. cereus* (this study), *B. subtilis* (Helmann et al., 2001; Petersohn et al., 2001; Price et al., 2001), *L. monocytogenes* (Kazmierczak et al., 2003) and *S. aureus* (Bischoff et al., 2004). Direct regulation was defined as genes of which σ^B-dependency was determined by experimental confirmation (for example by promoter mapping through primer extension analysis) or by the prediction of a σ^B-dependent promoter (for example by the application of a HMM to identify σ^B-dependent promoters) up-stream of the gene. To obtain the set of genes directly regulated by σ^B in *B. subtilis*, three different papers in which the σ^B regulon was determined were compared and only genes that were found to be σ^B-dependent in at least two of the three studies were used in this comparative analysis. For the definition of the genes that were directly regulated by σ^B in *S. aureus,* the study of Bischoff *et al*. (2004) was used. Only genes directly regulated by σ^B and which had a homolog in *S. aureus* N315 (Kuroda *et al.*, 2001) were included in the analysis. The more limited study of the σ^B regulon performed by Pane-Farre *et al*. (2006) is not further discussed here, but the comparison of the results of this σ^B regulon with that of the other bacteria is included in the Supplementary Materials (Table S4) and is in line with the data from Bischoff *et al*. (2004).

TIGR's Multi-Genome Homology Comparison tool (http://cmr.tigr.org/tigr-scripts/CMR/shared/MakeFrontPages.cgi?page=circular_display), applying a P-value ≤ 1.0 x 10^{-5} for significance, was used for comparing the set of σ^B-dependent genes of each organism.

Results and discussion

Validation of transcriptome data by quantitative real-time PCR

Because this is the first report on the use of *B. cereus* whole genome microarrays were used, it was deemed essential to first validate the expression ratios generated by microarray analysis by quantitative real-time PCR. We used microarray-data that were obtained in the comparison of the *sigB*-mutant and its parental strain during mid-exponential growth at 30 °C and upon a heat shock to 42 °C for 10 min. We chose six genes with significantly different expression ratios under at least one growth condition (Fig. 1) and the validation by qPCR showed that all genes that were identified as significantly differentially expressed in the microarray-experiments, were also significant in the qPCR experiments.

Indeed the expression ratios found by microarray experiments exhibited a good correlation with the qPCR data.

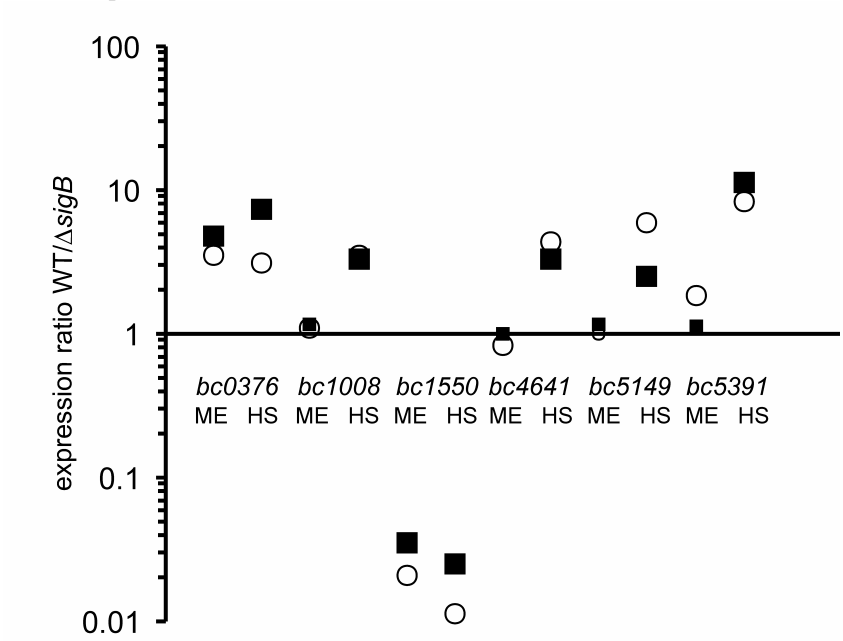

Fig. 1. Validation of microarray results by qPCR. Three independent RNA samples, isolated during mid-exponential growth at 30 °C and after a heat shock to 42 °C for 10 min were isolated from cultures of *B. cereus* strains ATCC 14579 (WT) and the *sigB* deletion mutant. The expression ratios between both strains of the indicated genes were measured using microarrays (closed squares) and qPCR (open circles). Small symbols indicate that no significant difference was measured for the expression-levels of the target gene between the two strains.

Identification of σ^B-dependent genes of *B. cereus* upon mild heat shock

To identify the set of σ^B-regulated genes in *B. cereus*, the transcriptomes of the *sigB* deletion mutant FM1400 and the *rsbY* deletion mutant FM1401 were compared with the transcriptome of the parental strain *B. cereus* ATCC 14579 after a heat shock to 42 °C for 10 min. This mild heat shock strongly activates σ^B-activity in the parental strain, while this response does not occur in both mutant strains (van Schaik *et al.*, 2004a; van Schaik *et al.*, 2005). Both the *sigB* and *rsbY* deletion mutants were used in these experiments to minimize the influence of mutant-specific, pleiotropic effects, which could give rise to false positives in the definition of the *B. cereus* σ^B regulon under this condition.

We observed that upon a heat shock to 42 °C for 10 min, 45 genes were significantly up-regulated in the parental strain in comparison with the *sigB*-mutant (Fig. 2). Similarly, 38 genes were found to be up-regulated after an identical heat shock in the wild-type strain as compared to the *rsbY* mutant. Expression data for these experiments can be found in the supplementary data (Table S1). A total of 28 genes from these two data-sets were found to be overlapping, which suggests that these genes are candidates for being dependent on σ^B for

their expression. No genes with expression ratios ≤0.67 (WT/mutant) were found in both the *sigB*- and *rsbY*-mutant, so neither σ^B nor σ^B-dependent proteins appear not to have a role in the down-regulation of genes in *B. cereus* upon a heat shock.

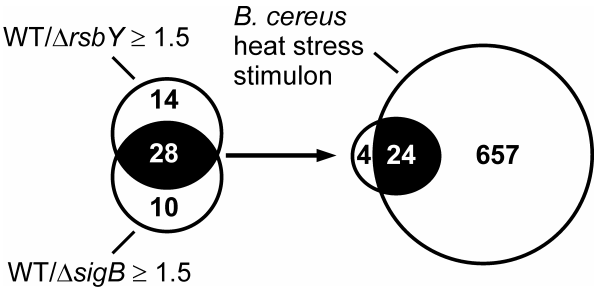

Fig. 2. Comparison of differentially expressed genes between *B. cereus* ATCC 14579 (WT) and the *sigB* and *rsbY* deletion mutants. The number of genes that are differentially expressed between the parental strain (WT) and the *rsbY*- and *sigB*-mutant upon a heat shock to 42 °C for 10 min. are indicated in the circles. The black overlapping area indicates that the same genes were identified as being differentially expressed in both the *sigB* and the *rsbY* deletion mutant under the indicated condition. Twenty-four of this group of genes was also up-regulated upon a heat shock and these form the σ^B regulon of *B. cereus*.

A further defining feature of a σ^B-dependent gene is that the gene exhibits up-regulated expression upon stress. Therefore, we subsequently performed microarray-experiments to define the set of genes that are up-regulated upon a heat shock from 30 °C to 42 °C for 10 min in the parental strain *B. cereus* ATCC 14579 and compared this data-set (Table S2 in the supplementary data) with the above described set of putative σ^B-dependent genes (Fig. 2). In total, 681 genes were up-regulated upon a heat shock from 30 °C to 42 °C for 10 min, which corresponds to 12.4% of the total number of genes in *B. cereus*. This substantial transcriptional reprogramming corresponds to data of the heat shock response in *B. subtilis* where over 10% of the total number of genes was induced upon a heat shock (Helmann *et al.*, 2001). The genes that were up-regulated upon a heat shock in *B. cereus* include *dnaK*, *groEL* and *groES* and the different *clp* genes, which is in line with a previous proteome-based study of the heat shock response of *B. cereus* (Periago et al., 2002). Twenty-four of the twenty-eight previously identified genes are also part of the *B. cereus* heat stress-stimulon and these genes were thus assigned to the σ^B regulon of *B. cereus* (Table 2). Indeed, the expression ratios between mid-exponentially growing and heat shocked *B. cereus* ATCC 14579 (4^{th} column in Table 2), is practically identical to the expression ratios when the mutant and parental strains were compared with one another upon a heat shock (2^{nd} and 3^{rd} column in Table 2). This reflects both the inactivity of σ^B under exponential growth conditions and the absence of a σ^B response in the mutants. Four genes (*bc1436*, *bc5148, bc5149*, and *bc5150*), which exhibited higher expression in *B. cereus* ATCC 14579 than in the *sigB*- and *rsbY*-mutant, were not assigned to the σ^B regulon as these were not up-regulated in their expression upon a heat shock in the parental strain.

Function of σ^B-dependent genes in *B. cereus*

The set of σ^B-dependent genes, which were identified by microarray analysis, includes genes, which were previously identified in a study using a combined proteomics- and *in vitro*-transcription-based approach (van Schaik *et al.*, 2004b), but a number of additional genes can now also be assigned to the σ^B regulon of *B. cereus* (Table 2). Note that two genes *(bc0999* and *bc1012*) which were not represented on the DNA-microarray are included in Table 2 as they were previously experimentally proven to be σ^B-dependent (van Schaik *et al.*, 2004b). The newly identified genes include *bc1007*, which encodes a predicted CheR-type methyl transferase and *bc1008* a histidine kinase fused to a Chase3-like N-terminal domain (Zhulin *et al.*, 2003) and a C-terminal CheY-type response regulator domain, which could be involved in a complex regulatory route in the *B. cereus* group. A large proportion of genes in the *B. cereus* σ^B regulon appears to have no known function. However, three of these genes have homologs in other bacteria, where they have a role in the stress response. These are *bc0998*, which has a homolog in *B. subtilis* (*yflT*), where it has a role in resistance to ethanol and hyper-osmotic conditions (Hoper *et al.*, 2005); *bc0999*, which is homologous to *csbD* in *B. subtilis* and which has a role in hyper-osmotic and cold stress (Hoper *et al.*, 2005) and *bc1000*, which is homologous to the GlsB-protein of *Enterococcus faecalis*, where it has a role in resistance to bile (Teng *et al.*, 2005). It is interesting to note that in the region delimited by *bc0995* and *bc1012* (a 12.4 kb fragment), σ^B is involved in the expression of 16 of the 18 open reading frames in this region. This region also includes the genes encoding all known regulators of σ^B-activity of *B. cereus*. Possibly, clustering of both regulators and important σ^B-dependent genes can be advantageous or it may be a remainder of an early event in the evolution of σ^B in Gram-positive bacteria.

Another newly identified member of the σ^B regulon of *B. cereus* is *bc2108*, which encodes the ECF-type σ factor, σ^Z. This apparent regulation of the transcription of *bc2108* by σ^B may very well be indirect as no candidate σ^B promoter site could be identified up-stream of *bc2108*. Two other genes (*bc2638* and *bc5391*), which also appear to be indirectly dependent on σ^B for their expression, have predicted roles in the processes of germination and sporulation. The *bc2638* gene is predicted to encode the C-subunit of a germination receptor. These receptors are important for sensing nutrients and triggering germination of the bacterial spore (Moir *et al.*, 2002). The *bc2638* gene is not part of one of the seven germination receptor operons of *B. cereus* (Hornstra *et al.*, 2006) and conceivably the encoded protein can act together with A- and B-subunits of other germination receptor complexes. The gene *bc5391* also has a proposed role in germination. The *B. subtilis* homolog of this gene (named *gerQ* or *ywdL*) is essential for the presence of the cortex-lytic enzyme CwlJ in the spore coat (Ragkousi *et al.*, 2003). In *B. subtilis* GerQ is cross-linked into high-molecular-mass forms by a transglutaminase. This may be important for building a spore coat that contributes to the resistance to environmental insults of the spore (Ragkousi and Setlow, 2004). Consequently, the germination defect and decreased heat resistance of the spores of the *sigB* deletion mutant of *B. cereus* (de Vries *et al.*, 2005) may be explained by lower expression levels of *bc2638* and *bc5391* during sporulation.

Table 2. Identification of σ^B-regulated genes in *B. cereus* ATCC 14579 upon mild heat shock.

Gene name	Exp. ratio[a] WT/Δ*sigB*	Exp. ratio WT/Δ*rsbY*	Exp. ratio 42°C/30°C	Annotation	Alias[b]	Experimentally defined and/or predicted promoter sequences[c]	E-score HMMER search
Bc0862	63.1	84.0	72.9	Protease I	*yfkM*	In operon with *bc0863*	
Bc0863	72.1	31.5	34.0	Catalase	*katE*	**ATGTTTAC -13bp- GGGTATC-N_{47}-ATG**	4.7 x 10^{-5}
Bc0995	1.9	2.0	2.2	Hypothetical protein		None identified	
Bc0996	2.1	1.8	2.0	Hypothetical protein		None identified	
Bc0998	123.6	86.0	70.9	General stress protein	*yflT*	**ATGTTTAA -14bp- GTGTACT-N_{45}-ATG**	6.4 x 10^{-5}
Bc0999[d]	ND	ND	ND	Hypothetical protein		In operon with *bc0998*	
Bc1000	98.1	42.4	36.3	Hypothetical protein		In operon with *bc0998*	
Bc1001	106.4	29.8	35.8	Hypothetical protein		**ATGTTTAA -13bp- GTGTATG-N_{37}–ATG**[f]	1.3 x 10^{-5}
Bc1002	42.6	31.7	38.3	Anti-σ^B factor antagonist	*rsbV*	**ATGTTTAA -13bp- GGGTAAT-N_{33}-ATG**	1.4 x 10^{-5}
Bc1003	51.1	38.3	52.4	Anti-σ^B factor	*rsbW*	In operon with *bc1002*	
Bc1004	-[e]	24.4	28.1	RNA polymerase σ factor σ^B	*sigB*	In operon with *bc1002*	
Bc1005	10.6	47.4	56.0	Putative bacterioferritin	*orf4*	In operon with *bc1002* and under control of own promoter: **ATGTTTAA -13bp- GGGTACT-N_{20}-ATG**	6.3 x 10^{-6}
Bc1006	4.0	-[e]	5.1	PP2C phosphatase, regulator of σ^B-activity	*rsbY*	In operon with *bc1002* and *bc1005*	
Bc1007	2.4	2.3	3.6	Chemotaxis protein methyltransferase		In operon with *bc1009*	
Bc1008	3.3	3.1	4.4	Two component system histidine kinase		In operon with *bc1009*	
Bc1009	188.5	110.2	87.5	Hypothetical protein		**ATGTTTAA -13bp- GGGTATG-N_{40}-ATG**	1.1 x 10^{-5}
Bc1010	8.2	7.0	5.5	Hypothetical protein		ACGTTTAG -13bp- GGGTATA-N_{285}-ATG	1.9 x 10^{-4}
Bc1011	33.9	25.8	23.7	Hypothetical protein		In operon with *bc1010*	
Bc1012[d]	ND	ND	ND	Hypothetical protein	*ybjQ*	In operon with *bc1010*	
Bc2108	2.9	2.8	3.8	RNA polymerase ECF-type σ factor	*sigZ*	None identified	
Bc2638	4.7	4.4	4.3	Spore germination protein LC		None identified	
Bc3130	20.7	29.3	10.3	Hypothetical protein		In operon with *bc3132*	
Bc3131	29.5	37.3	42.6	Hypothetical protein		In operon with *bc3132*	
Bc3132	7.6	2.0	2.0	General stress protein 17M		**AGGTTTAA -14bp- GTGTATT-N_{48}-ATG**	4.3 x 10^{-5}
Bc4641	3.3	3.9	3.8	Hypothetical protein		ATGAATAA -13bp- GGGTACG-N_{10}-ATG	9.3 x 10^{-5}
Bc5391	11.2	11.0	6.1	Spore coat protein	*gerQ*	None identified	

Genes were identified as being σ^B-dependent by a significant difference in expression between the parental strain and both the *sigB*- and *rsbY*-deletion mutant upon a heat shock from 30 °C to 42 °C for 10 min. ND: not determined.

[a] Exp. ratio stands for expression ratio.

[b] The aliases for the identified genes were based on the gene names of homologs in *B. subtilis* and/or *B. anthracis*.

[c] Experimentally confirmed promoter sites (van Schaik *et al.*, 2004a; van Schaik *et al.*, 2004b) are indicated in bold. Other promoters were identified by a Hidden Markov Model as outlined in the Materials and Methods section.

[d] These genes were not represented on the microarray, but were previously identified as being σ^B-dependent (van Schaik *et al.*, 2004b) and are therefore included in this table.

[e] These genes were disrupted by an erythromycin resistance cassette. Therefore, the expression data of these genes are omitted.

[f] A second σ^B-dependent promoter site was predicted in the HMMER-search up-stream of this gene. The E-score for this predicted site is higher (3.0 x 10^{-5}, Table S3) than for the experimentally confirmed promoter indicated in the table.

Prediction of σ^B-dependent promoters

A HMMER search was performed using the genes of the σ^B regulon of *B. cereus* to identify σ^B-dependent promoters in the *B. cereus* ATCC14579 genome (Ivanova *et al.*, 2003). The HMMER search identified promoters regulating 21 of the 26 genes belonging to the σ^B regulon of *B. cereus*, suggesting these are under the direct control of σ^B (Table 2). Promoters were identified in front of operons coding for 15 open reading frames of the island that are part of the σ^B regulon and in front of the *bc0863-bc0862* and *bc3132-bc3130* operons. Seven out of the ten predicted promoter sites have previously been experimentally defined (van Schaik *et al.*, 2004a; van Schaik *et al.*, 2004b).

Fig. 3. Consensus sequence of the σ^B-dependent promoter sites in *B. cereus*. The promoter sequences indicated in Table 2 were visualized with WebLogo (Crooks *et al.*, 2004). The height of the nucleotide is indicative for its frequency at that position in the promoter sites. Variable spacing between the -35 and -10 binding site for σ^B is signified by the absence of a nucleotide at position 22.

By aligning the predicted and experimentally verified σ^B-dependent promoter sites in *B. cereus*, a promoter consensus sequence for σ^B in *B. cereus* could be generated (Fig. 3). When the genome of *B. cereus* ATCC 14579 was searched with this consensus sequence, we found that the promoters of genes identified in this study were all found to have a low E-score, so a significant fit to the HMM (Table S3). Indeed the ten promoters with the lowest E-scores (correlating with optimal fit to the HMM) were all identified in this study. Generally, the promoters with the best fit to the HMM have the highest fold-change values in the microarray-analysis, suggesting that differences between expression levels of the genes of the σ^B regulon is primarily determined by the recognition of the promoter sequence by σ^B. The

other promoters are in all likelihood weakly (if at all) dependent on σ^B for their expression and/or may be expressed under other growth- or stress-conditions than used in this study. Even though we cannot completely rule out that under other stress conditions more σ^B-dependent genes may be identified, the fact that we identified the ten promoters with best fit to the HMM by microarray-analysis, implies that if additional σ^B-dependent genes can be identified under other conditions that their dependency on σ^B will probably be weak. Additionally, it is noteworthy that also in our previous proteomics-based study only a relatively small number of σ^B-dependent genes could be identified, even though we used an inducible overexpression system in *B. cereus* to obtain artificially high levels of σ^B and concomitant strong activation of σ^B-dependent genes (van Schaik *et al.*, 2004b). Both findings implicate that the σ^B regulon of *B. cereus* is not much larger than approximately 25 to 30 genes.

Evolutionary implications of the small σ^B regulon of *B. cereus*

When the findings of this study are compared to the results in other Gram-positive bacteria in which transcriptome profiling was used to determine the set of σ^B-regulated genes, it is striking that the σ^B regulon of *B. cereus* appears to be considerably smaller than that of other Gram-positives. In *L. monocytogenes* 54 genes were up-regulated in a σ^B-dependent fashion upon stress exposure. However, the total number of σ^B-dependent genes in *L. monocytogenes* may be higher as a microarray with limited genome coverage was used in that study (Kazmierczak *et al.*, 2003). In both *S. aureus* and *B. subtilis*, the number of genes in the σ^B regulon is considerably larger and appears to be around 100 (Bischoff *et al.*, 2004; Helmann *et al.*, 2001; Pane-Farre *et al.*, 2006; Petersohn *et al.*, 2001; Price, 2002).

Possibly the relatively small size of the σ^B regulon of *B. cereus*, is a reflection of the way σ^B has evolved throughout Gram-positive bacteria. The similarities between the operon structures for *sigB* and *sigF* (encoding σ^F, the σ factor which is active in the prespore), has been used to suggest that both operons have evolved from a common ancestor. Phylogenetic analysis indicates that the *sigB* operon of the *B. cereus* group most closely resembles the ancestral operon structure, whereas that of other Gram-positives seems to have evolved further (Ferreira *et al.*, 2004). Our finding of the relatively small size of the σ^B regulon of *B. cereus* seems to fit this hypothesis. While σ^B of *B. cereus* has a role in the development of stress resistance and is activated upon a number of different stress conditions (van Schaik *et al.*, 2004a), it does not seem to be very important for the regulation of metabolic pathways or the expression of virulence determinants as in the Gram-positive pathogens *L. monocytogenes* and *S. aureus* (Kazmierczak et al., 2005). Possibly, the evolution of σ^B has progressed in a modular fashion, in which functional groups of genes were added to the σ^B regulon in the different Gram-positive bacteria based on selective pressures encountered in the different environments. In *B. cereus* regulatory pathways other than σ^B may have evolved to regulate metabolic processes and the expression of virulence determinants, leaving *B. cereus* σ^B solely with the role of countering acute stresses and with a minor role in the process of sporulation.

Conservation of σ^B-dependent genes and σ^B-dependent gene expression in Gram-positive bacteria

In this study the σ^B regulon of *B. cereus* was determined by DNA microarray analysis. This allowed us to compare the set of σ^B-regulated genes in *B. cereus* with the previously published σ^B regulons of *B. subtilis*, *L. monocytogenes* and *S. aureus*, which encompasses the phylogenetic diversity of Gram-positive bacteria that have a bona fide *sigB* gene (Ferreira *et al.*, 2004). We performed this analysis to determine to what extent σ^B-regulated genes are conserved between Gram-positive bacteria and to assess in how far the σ^B-dependency of these genes is conserved (Fig. 4).

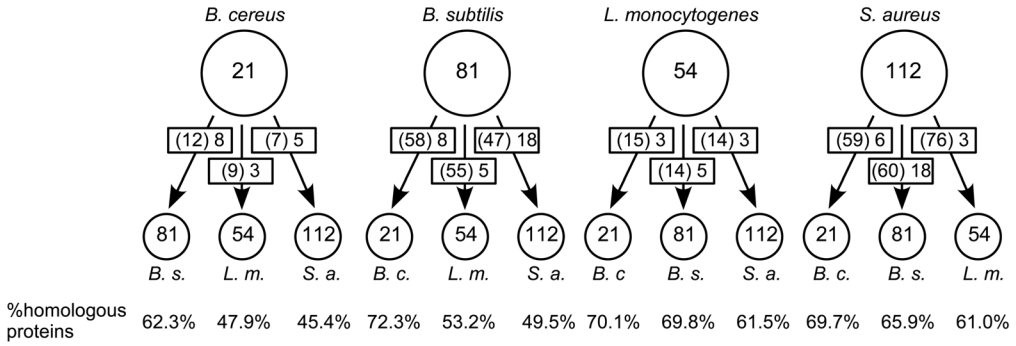

Fig. 4. Conservation of genes from the σ^B regulon in *B. cereus*, *B. subtilis*, *L. monocytogenes* and *S. aureus*. The number of genes that are directly regulated by σ^B in a given organism is shown in the large circle. The conservation of σ^B-dependent genes from this organism in three other Gram-positive bacteria (abbreviations used: *B. c.*: *B. cereus*, *B. s.*: *Bacillus subtilis*, *L. m.*: *L. monocytogenes* and *S. a.*: *S. aureus*) was determined with TIGR's Multi-Genome Homology Comparison tool (http://cmr.tigr.org/tigr-scripts/CMR/shared/MakeFrontPages.cgi?page=circular_display) with a cut-off for significance of a P-value $\leq 1.0 \times 10^{-5}$. The number of conserved genes of the σ^B regulon is indicated in the arrows to the small circles. The number in parentheses is the number of genes from the σ^B regulon of the query organism which have a homolog in the target organism. The second number in the arrow indicates the number of genes from this group, which are dependent on σ^B for their expression in both organisms. For comparative purposes the conservation of all protein-coding genes between the genomes is indicated at the bottom of the figure.

This comparison showed that genes from the σ^B regulon are generally conserved between the four Gram-positives to the same extent as all the genes of the genome. For example, of the 81 σ^B-dependent genes of *B. subtilis* 58 genes (71.6%) have a homolog in *B. cereus*. When all 4100 genes of *B. subtilis* are considered, 72.3% have a homolog in *B. cereus*. It should be noted, however, that σ^B-dependent genes that are strongly induced upon stress in *B. subtilis* such as *ctc*, *gsiB* and *gspA* have no homologs in *B. cereus*. This already suggests that σ^B-dependent genes which are important for the stress response in one organism are not necessarily conserved in other organisms, even when these organisms are relatively closely related like *B. subtilis* and *B. cereus*. It is also noteworthy that the genes of the σ^B regulon of *L. monocytogenes* are relatively poorly conserved in other bacteria. The peculiar lifestyle of *L. monocytogenes* as a gastro-intestinal intracellular pathogen may have led to the divergent evolution of the σ^B regulon in this organism.

The most important conclusion that can be drawn from Fig. 4 is that even though σ^B-dependent genes from one organism are generally well conserved in other organisms, their σ^B-dependency generally is not. This is clearly illustrated by comparing the σ^B regulons of the phylogenetically relatively closely related bacteria *B. subtilis* and *B. cereus*. Of the 81 σ^B-dependent genes of *B. subtilis*, 58 have a homolog in *B. cereus*. However, only 8 of these σ^B-dependent *B. subtilis* genes are also σ^B-dependent in *B. cereus*. The complete data-set of the genes which have conserved their σ^B-dependent expression in the compared bacteria discussed here can be found in Table S4.

Only three genes are conserved in their σ^B-dependency in all bacteria discussed here. These are the *sigB* gene itself and the genes encoding RsbV and RsbW, the anti-anti σ factor and anti-σ factor of σ^B, respectively. The gene *bc0999* from *B. cereus* also has σ^B-dependent homologs in *B. subtilis* (*ywmG* or *csbD*) and *S. aureus* (*sa0772*). It is also present in *L. monocytogenes* (*lmo2518*), but the σ^B-dependency of this gene has not been described, possibly because this gene was not represented on the microarray used in the determination of the *L. monocytogenes* σ^B regulon (Kazmierczak *et al.*, 2003). However, a putative σ^B-dependent promoter up-stream of *lmo2518* can be identified (data not shown), which strongly suggests that *lmo2518* is dependent on σ^B for its expression. In that case, it would be the fourth gene which is σ^B-dependent in all four different Gram-positives and this would be an indication that *csbD*-like genes may have an important role in the σ^B-mediated stress response. Interestingly, *csbD*-like genes are widely distributed in eubacteria, strongly suggesting that they perform an important, but hitherto unrecognized, function in eubacteria.

The poorly conserved control of stress response genes by σ^B in the different Gram-positives is in line with a recent article by Lozada-Chávez *et al*. (2006). This study predicted that bacterial regulatory networks are extremely flexible during evolution and that transcription factors appear to be primarily responsible for the plasticity of transcriptional regulatory networks. The evolution of σ^B and its regulon in Gram-positives appears to be an experimentally determined proof of this prediction. Even though σ^B's main function appears to be to control the stress response in the different organisms, it appears that it does so using a unique strategy in each Gram-positive through controlling the expression of different output genes.

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5

The regulon of the *Bacillus cereus* ATCC 14579 ECF σ factor σ^M is induced by ethanol stress and comprises transporter and oxidative stress genes

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Abstract

The *B. cereus* ATCC 14579 genome encodes ten ExtraCytoplasmic Function σ factors with nine of these being unique to the *B. cereus* group, and with only σ^M sharing 55% similarity with σ^M of *Bacillus subtilis*. Here, the role of σ^M and its regulon in stress response and survival of *B. cereus* ATCC 14579 were assessed by comparative transcriptome and phenotypic analysis of this strain and its *sigM* deletion strain. Exposure of *B. cereus* ATCC 14579 to a wide range of stresses revealed expression of *sigM*, encoding σ^M, to be up-regulated mainly in the presence of ethanol and after alkaline pH-shock. Next to this, disc diffusion tests showed the *sigM* deletion strain to be more sensitive to oxidizing agents and to be more resistant to cell-wall targeting antibiotics than the wild-type strain. The σ^M regulon was subsequently determined by comparative transcriptional analyses of the wild-type and its *sigM*-deletion strain after exposure to ethanol. The putative σ^M regulon was shown to consist of 29 genes, with several of these genes predicted to be involved in counteracting oxidative stress, such as an NADH oxidase, a ferredoxin, and a lysine decarboxylase and genes, including *luxS*, encoding the activated methyl cycle and L-cysteine production. Screening of promoter up-stream regions allowed for the assessment of a *B. cereus* consensus promoter binding site for σ^M. Since the consensus promoter binding site for *B. cereus* ATCC 14579 σ^M, its regulon and the predicted functionalities are different from the corresponding features in *B. subtilis*, it can be concluded that σ^M plays a unique role in *B. cereus* stress response and survival.

Introduction

Bacillus cereus is a food poisoning and food spoilage organism that can be found in a large variety of foods (Schoeni and Wong, 2005). In response to the different stresses encountered in food processing, *B. cereus* is capable of inducing an array of stress responses enhancing its resistance and survival capacity (van Schaik and Abee, 2005). Fine tuning of cellular responses is amongst others regulated by the activation of specific σ factors (Paget and Helmann, 2003). Next to the housekeeping σ factor (σ^A), the *B. cereus* strain ATCC 14579 was found to contain 19 alternative σ factors (Ivanova *et al.*, 2003; Overbeek *et al.*, 2003), including σ factors with roles in stress response (σ^B) and sporulation (σ^H, σ^E, σ^F, σ^G, σ^K) (de Vries *et al.*, 2004; Piggot and Hilbert, 2004; van Schaik *et al.*, 2004). A distinct class of alternative σ factors are the so-called ExtraCytoplasmic Function (ECF) σ factors (Helmann, 2002). The ECF σ factors are often involved in the regulation of functions related to cell surface modulation and repair and/or membrane transport (Helmann, 2002), and more recently evidence has been obtained revealing the involvement of ECF σ factors in stress responses associated with virulence (Bashyam and Hasnain, 2004; Kazmierczak *et al.*, 2005). As the cell surface is the first line of defence of the bacterial cell, ECF σ factors may have important roles in stress response and survival of bacteria during exposure to food-processing (Boor, 2006). The first ECF σ factor studied was σ^E in *Streptomyces coelicolor*, and it was shown to be involved in cell wall repair (Lonetto *et al.*, 1994). For the model Gram-positive organism *Bacillus subtilis*, the seven ECF σ factors σ^M, σ^V, σ^W, σ^X, σ^Y, σ^Z and σ^{YlaC} are extensively studied, and reviewed (Helmann, 2002, 2006). Only recently, σ^W of *Bacillus thuringiensis*, a member of the *B. cereus* group (Rasko *et al.*, 2005), was studied and shown to be involved in a pathway controlling β-exotoxin I production (Espinasse *et al.*, 2004).

Several reports have described the organisation of ECF σ factor operons and in general the gene encoding the ECF σ factor was found to precede a gene encoding its anti-σ factor. The anti-σ-factor is able to bind the ECF σ factor, making the ECF σ factor unavailable for transcription initiation. Furthermore, the anti-σ factors of ECF σ factors are all membrane bound proteins, thereby enabling sensing and responding to changes in the extracellular environment affecting cell surface characteristics (Yoshimura *et al.*, 2004). Not much is known yet about signals activating the release of ECF σ factors from their anti-σ factors. Stress-induced conformational changes (Hughes and Mathee, 1998) and intramembrane proteolysis (RIP) (Ades, 2004) have been implicated in the loss of anti-σ factor activity. Subsequent release of the σ factor from its anti-σ factor allows for complexation with the RNA polymerase and expression of the ECF σ factor regulon (Helmann, 2002). It has been suggested that the expression of ECF σ factor genes is autoregulated, as was shown for all *B. subtilis* ECF σ factors (Asai *et al.*, 2003).

B. cereus ATCC 14579 genome analysis predicts the presence of ten ECF σ factors, of which none has been studied in detail. This study describes the role of the ECF σ factor σ^M and its regulon in stress response and survival of *B. cereus* ATCC 14579.

Materials and methods

Bacterial strains and culture conditions

B. cereus ATCC 14579, its *sigM* deletion strain (FM1402), and the *B. cereus* ATCC 14579 strains carrying plasmids were cultured in Brain Heart Infusion broth (BHI, Difco) at 30 °C, with shaking at 200 rpm. *E. coli* HB101/pRK24 was used as the donor host in conjugation experiments (van Kranenburg *et al.*, 1997). Antibiotics used were ampicillin at a concentration of 50 µg/ml, kanamycin at a concentration of 70 µg/ml, chloramphenicol at a concentration of 5 µg/ml, erythromycin at a concentration of 150 µg/ml for *E. coli* or 5 µg/ml for *B. cereus*, spectinomycin at a concentration of 100 µg/ml, and polymyxin B at a concentration of 50 µg/ml for counter selection against *E. coli* upon conjugation.

Different stresses were applied for 10 min to 20 ml of mid-exponential cells growing in BHI broth in a 100 ml Erlenmeyer flask to an OD_{600} of 0.4. To apply a heat-shock Erlenmeyer flasks were transferred to a 42 °C waterbath, salt stress was applied by adding NaCl to a concentration of 2.5% directly to the culture, for acid stress HCl was added at a final concentration of 20 mM (~pH 5.2) directly to the culture, for alkaline exposure NaOH was added directly to the culture to a final concentration of 30 mM (~pH 8.5), oxidative stress was induced by adding H_2O_2 directly to the culture to a final concentration of 50 µM and ethanol was added to a final concentration of 4%.

Disc diffusion tests were performed by placing 6.35 mm (1/4 inch) paper discs (Becton Dickinson) on Nutrient Agar (NA, Difco) plates. Before pouring plates 5% of an overnight culture of the indicated *B. cereus* strain was added to NA. Subsequently, 10 µl was added to the paper disc of one of the following substances: 8.8 M H_2O_2 (Sigma), 1 M diamide (Sigma), 4 mg/ml plumbagin (Sigma), 400 mg/ml pyrogallol (Sigma), 0.5 M EDTA (Sigma), 10 mg/ml tellurite (Sigma), 10 mg/ml bacitracin (Sigma), 25 mg/ml kanamycin (Sigma), 100 mg/ml vancomycin (Sigma), 200 mg/ml fosfomycin (Sigma), 100 mg/ml streptomycin, 100 mg/ml neomycin and 100 mg/ml gentamycin.

For microarray analyses, ethanol was added to 50 ml mid-exponentially growing cultures at an OD_{600} of ~0.6 of wild-type and the *sigM* deletion strain to an end-volume of 4% v/v. Samples for RNA isolation were taken right before addition of the ethanol and after 10, 30 and 60 min. RNA isolations were performed as described below.

Genetic methods and construction of a *sigM* deletion strain

Plasmid DNA was purified from *E. coli* with a Qiaprep Spin Miniprep kit (Westburg, Leusden, The Netherlands). Pwo polymerase (Roche Diagnostics, Almere, The Netherlands) was used for PCRs that were used in cloning and Taq polymerase (Fermentas) for control amplifications. DNA digestions were performed as indicated by manufactures. For a double crossover event deleting part of the *sigM* two PCR products of *sigM* flanking regions were linked to both sites of an erythromycin resistance box. The up-stream homologous region was obtained by PCR with the forward primer 7902KOforw (CAGtctagaGGTTCGCGAATCCACTTTAC), introducing an *Xba*I-restriction site (small case) at the up-stream end and the reverse primer 7902rev (TCACTACATTTCCCTCCCTTCAT), the down-stream homologous region with the

forward primer 7902forw (ATAAGGAAATAGCAGAAATGACT) and the reverse primer 7902KOrev (TCgagctCTTATTGATCCGCCGTTACTTC), introducing a *Sac*I-restriction site (small case) at the down-stream end. The obtained PCR products were ligated separately into the pGemTeasy vector system according the manufacturers protocol (Invitrogen). Subsequently, the up-stream (*Xba*I-*Not*I) and down-stream (*Not*I-*Sac*I) flanking regions were digested from pGemTeasy and ligated together into the conjugal vector pATdeltaS28, *Xba*I-*Sac*I digested (Trieu-Cuot *et al.*, 1990). The erythromycin resistance box was amplified from pUC18ERY as described previously (Chapter 3) and ligated into the pGemTeasy vector system to attain a *Not*I restriction site on each site of the erythromycin resistance box. After digestion with *Not*I, the erythromycin resistance gene was cloned into the *Not*I-site created between the two flanking regions situated in pATdeltaS28 resulting in pATdeltaSigMery. After isolation of pATdeltaSigMery from DH5α and transformation into *E. coli* HB101/pRK24 it was used in a conjugation experiment with *B. cereus* ATCC 14579 (Bron, 1990). PCR and Southern Blot analysis confirmed the double homologous recombination event (data not shown). The *B. cereus sigM* deletion strain was designated *B. cereus* FM1402.

5'RACE and RT-qPCR

RT-qPCR experiments were performed as described previously (van Schaik *et al.*, 2005), with RNA isolated as described below. Primers used in qPCR experiments for *sigM* were sigMforwQpcr (CCGCTCGAAAGACGAAATGT) and sigMrevQpcr (CCGTCCCCTGTGTAATTCAA) and for *yhdL* were anti-sigM_forw_qPCR (GGAACCGTTGCAGAAGTAGCA) and anti-sigM_rev_qPCR (GGGAGTTCTTGTGCCTCGAA). Relative expression values were obtained by comparing expression with *rpoA* expression with the REST program (Pfaffl *et al.*, 2002). Primers used for *rpoA* were rpoAforw (ACCGCTTGAGCGTGGATATG) and rpoArev (TAGCAGTAACAGCGGCACCA). The expression of *sigM* after applying different stresses is presented as compared to the non-stress situation. The expression of *sigM* and *yhdL* after over-expression of *sigM* are presented as compared to the non-induced situation.

The 5' end of the *sigM* transcript was mapped using RNA isolated (as described below) from mid-exponential cultures of *B. cereus* ATCC 14579 with the 5' RACE System for Rapid Amplification of cDNA Ends (Invitrogen) using the GSP *sigM* primers (GSPsigM1: AATTGGTTCTGTTTTTGTTA and GSPsigM2: CGTTCCATTTTCATACTTCTTC) according to the manufacturer's instructions.

RNA isolation, cDNA synthesis and labelling, and microarray analysis

RNA isolation was performed by transferring the cultures to a 50ml Falcon tube and spun down at 13,000 *g* for 20 sec. After decanting the supernatant, the cell pellets were snap frozen in liquid nitrogen. The time between removal from incubator and freezing of the cell pellets was approximately 30 seconds. RNAwiz (Ambion, Huntingdon, United Kingdom) was added to the pellets and RNA was extracted as described previously (van Schaik *et al.*, 2007). Residual chromosomal DNA was removed by treating samples with DNA-*free* (Ambion).

Extracted RNA samples were stored in 70% EtOH, 0.3 M sodium acetate buffer (pH 5.2) at -80 °C.

Prior to cDNA synthesis, the quality of the extracted RNA was determined by analysis with an Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA), according to the manufacturer's instructions. cDNA synthesis and Cy3/Cy5 labelling were performed as previously described (van Schaik *et al.*, 2007). Final cDNA yields and incorporation of the Cy-labels was determined by Nanodrop analysis (Isogen Life Science, IJsselstein, The Netherlands). Custom-made *B. cereus* 60-mer oligo microarrays (produced by Agilent) were hybridized with 200 – 300 ng labeled cDNA. The microarray experiments were performed with two independent biological replicates. The used microarray platform and the subsequent hybridisation, washing, scanning and data normalization were performed as described before (van Schaik *et al.*, 2007). The resulting microarray data were analyzed for statistical significance using the web-based VAMPIRE microarray suite (Hsiao *et al.*, 2005). A spot was found to be differentially expressed between two samples using the threshold of a false discovery rate smaller than 0.05. Subsequently, the data of the single spots were integrated to obtain expression ratios per ORF. An ORF was found to be differentially expressed when all spots representing the ORF were significantly differentially expressed between samples. The average expression ratio per ORF was determined by calculating the average of the log-values of the expression ratios of all spots representing this ORF. This value (R) was then used to calculate the average expression ratio (10^R). Finally, a fold-change limit of expression of 1.5 was introduced. Microarray data were submitted to the GEO database with accession numbers: GSE9856 and GSE9855.

***In silico* analysis**

The binding site for σ^M, which was determined by 5'RACE , was used to screen 300-bp up-stream promoter regions of regulated genes for a putative σ^M binding site. In this search one modification from the determined -35 binding site was allowed, whereas for the -10 binding site a C was regarded to be the first nucleotide.

The amino acid sequences of the 17 σ-factor encoding genes of *B. subtilis* (Kunst *et al.*, 1997) and the 20 σ-factor encoding genes of *B. cereus* ATCC 14579 (Ivanova *et al.*, 2003) were aligned using Muscle 3.6 (Edgar, 2004). Subsequently, this alignment was visualized with the online available phylogenetic tree printer, Phylodendron. Colours were adjusted using Adobe Illustrator (Fig. 1).

Results

Comparison of *B. cereus* ATCC 14579 ECF σ factors with ECF σ factors from other microorganisms

An *in silico* alignment was performed of predicted σ factors of *B. cereus* ATCC 14579 with those from other microorganisms to search for homologies. Notably, high similarity was found for a number of alternative σ factors of *B. cereus* ATCC 14579 and *Bacillus subtilis* (Fig. 1), such as σ^B, σ^H and σ^L, but only one of the ECF σ factors of *B. subtilis* i.e. σ^M, showed similarity to the amino acid coding sequence of a *B. cereus* ECF σ

factor BC1114 (RZC07902), which was consequently named σ^M as well (Fig. 1). σ^M of *B. cereus* ATCC 14579 showed 36% identity and 55% similarity with σ^M of *B. subtilis*. Furthermore, *yhdL*, the second gene and putative anti-σ factor in the *B. cereus sigM*-operon, showed 27% identity and 48% similarity with the second gene of the *B. subtilis sigM*-operon (Fig. 2). The *B. subtilis sigM*-operon contains an additional third gene of unknown function that is lacking in the *B. cereus sigM*-operon. Moreover, detailed analysis of the *B. cereus* ATCC 14579 genome (Ivanova *et al.*, 2003) did not reveal the presence of homologues of this specific gene.

Because of its homology with *B. subtilis* σ^M, subsequent studies focussed on assessment of the role of *B. cereus* σ^M and its regulon in stress response and survival.

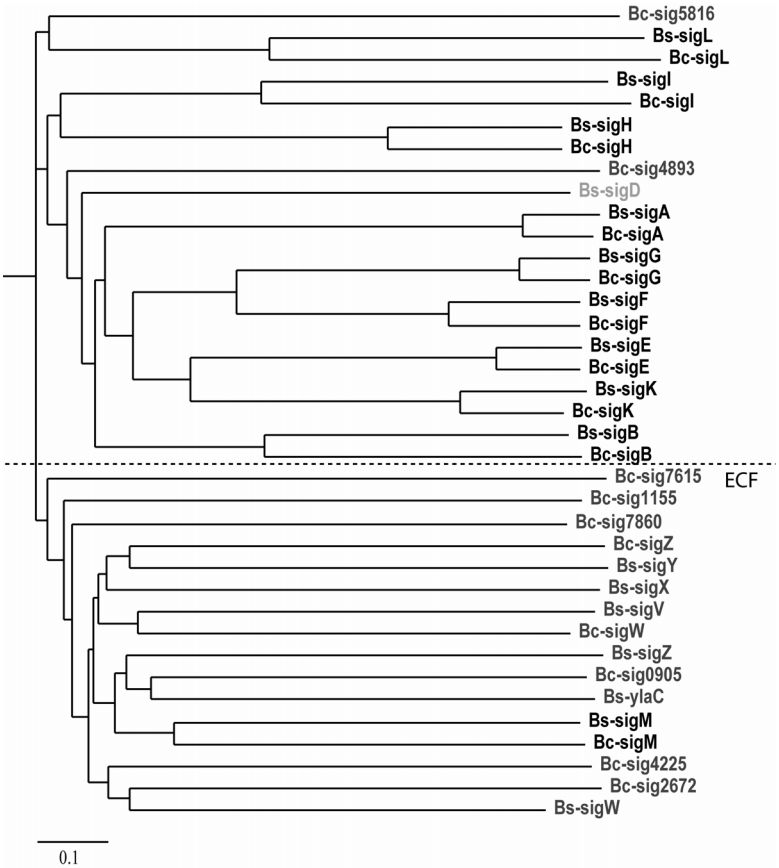
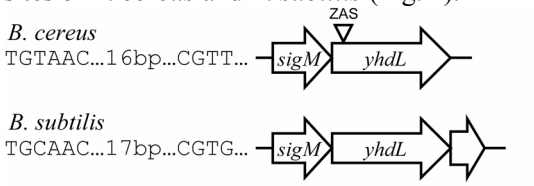


Fig. 1. Phylogenetic tree of all σ factors of *B. subtilis* (Kunst *et al.*, 1997) and *B. cereus* ATCC 14579 (Ivanova *et al.*, 2003) based on amino acid sequences. Names for *B. cereus* σ factors start with Bc and for *B. subtilis* with Bs. The last four numbers of the σ factors of *B. cereus* are the last four numbers of the RZC number of the σ factor they represent. ECF σ factors cluster at the lower half of the tree, except for Bc-sig5816. Putative homologous σ factors of *B. subtilis* and *B. cereus* are indicated in black, genes without a homolog in either species are presented in grey, and *sigD* of *B. subtilis* is colored light grey.

The *B. cereus* ATCC 14579 *sigM* operon

The *B. cereus* ATCC 14579 *sigM* operon consists of *sigM*, encoding the ECF σ factor σ^M, and *yhdL*, putatively encoding the anti-σ factor of σ^M. RT-PCR showed that *sigM* was expressed in mid-exponential growth in BHI broth (data not shown). An RNA sample of mid-exponentially grown cells was used in a 5' RACE experiment, revealing a single transcription start for the *sigM*-operon (Fig. 2). From the transcription start the putative -10 and -35 promoter sequences for σ factor binding could be established and subsequently aligned with known ECF σ factors of *B. subtilis*, revealing similarity between the σ^M promoter binding sites of *B. cereus* and *B. subtilis* (Fig. 2).



similar *B. cereus* ECF σ factor promoter binding sites:

TGGAAC…15bp…CTTG	Sig0905
CGTAAC…17bp…CGTT	Sig5816
TCAAAC…17bp…CGTC	σ^W
TGAACC…16bp…CGTC	Sig2672

similar *B. subtilis* ECF σ factor promoter binding sites:

TGTAAC…15bp…CGAC…	σ^X
TGAAAC…16bp…CGTA…	σ^W
TGAAAC…17bp…CGTcTa…	σ^V

Fig. 2. The *sigM*-operon of *B. cereus* compared to the *sigM*-operon of *B. subtilis*. Consensus promoter binding sites in *B. cereus* and *B. subtilis* are indicated and compared with other ECF σ factor promoter binding sites. The last four numbers of the *B. cereus* σ factors in the comparison of the promoter binding sites, are the last four numbers of the RZC nr. they represent. The ZAS domain is indicated with the open triangle, ZAS stands for Zinc-containing anti-σ factor.

Analyses of the putative anti-σ factor YhdL showed the presence of an N-terminal ZAS-domain (Fig. 2.), indicating the possible involvement of σ^M in cellular defence against disulfide or other oxidative stresses (Zdanowski *et al.*, 2006). YhdL was found to be the only putative anti-σ factor on the *B. cereus* ATCC 14579 genome that contains a ZAS-domain. Furthermore, it was shown that YhdL contains a membrane domain, predicting YhdL to be membrane bound (Hirokawa *et al.*, 1998). Remarkably, comparing the amino acid sequences of both YhdL and σ^M to those of other members of the *B. cereus* group showed that a higher identity was observed for σ^M than for YhdL within the *B. cereus* group (Table 1).

Table 1. Percentage identity and similarity for σ^M and YhdL compared to orthologous in *B. subtilis* and species of the *B. cereus* group.

	Bw KBAB4		*Bc* G9241		*Bc* ATCC1098		*Bt* konkukian		*Ba* ames		*Bc* E33L		*Bs* 168	
	% i	% s	% i	% s	% i	% s	% i	% s	% i	% s	% i	% s	% i	% s
σ^M	94	96	91	95	93	97	93	96	92	96	92	97	36	55
YhdL	91	95	85	93	70	83	69	82	70	83	69	81	27	48
ZAS*	+		+		+		+		+		+		-	

* Presence (+) or absence (-) of a ZAS-domain in YhdL. *Bw*: *B. weihenstephanensis*, *Bc*: *B. cereus*, *Bt*: *B. thuringiensis*, *Ba*: *B. anthracis*, *Bs*: *B. subtilis*. Identity (%i) and similarity (%s) are indicated.

sigM expression analysis and phenotypic assessment of *B. cereus* ATCC 14579 and its *sigM* deletion strain

To study the involvement of σ^M in the stress response of *B. cereus* ATCC 14579, mid-exponential cells were exposed to a large array of stresses. The expression of *sigM* after ten min was compared to that in the non-stressed control situation by use of RT-qPCR (Fig. 3). Exposure to ethanol or alkaline stress (~ pH 9) revealed marked induction of *sigM* by 7.5-fold and 5.9-fold, respectively. The 2.8-fold induction after a heat shock was less apparent, but still significant. No significant up-regulation was shown after salt or acid exposure, or with H_2O_2 generated oxidative stress (Fig 3).

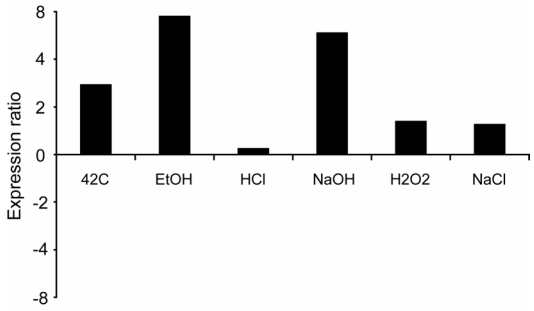


Fig. 3. Induction of *sigM* after exposure to different stresses for 10 min (42 °C,4% Ethanol, 20mM HCl (~pH 5.2), 30 mM NaOH (~pH 9.0), 50 µM H_2O_2 and 2.5% NaCl). Ratio values are presented relative to the expression of the household gene *rpoA* on the y-axis. Ratio values are presented in 10log.

A *sigM* deletion strain was constructed and its genotypic and phenotypic features were compared to that of the *B. cereus* ATCC 14579 wild-type strain. Using qPCR experiments loss of *sigM* expression was confirmed for the *sigM* deletion strain. Phenotypic analysis revealed the *sigM* deletion strain to be more sensitive to agents causing oxidative stress, such as diamide and plumbagin, whereas it appeared to be more resistant to cell wall targeting antibiotics, such as EDTA, kanamycin and streptomycin (Table 2). Notably, growth performance under stress conditions and stress survival capacity of wild-type and the *sigM* deletion strain appeared to be similar, even under conditions that evoked increased

transcription of *sigM* in the wild-type strain such as exposure to high concentrations of ethanol (data not shown).

Table 2. Growth inhibition zones for the wild-type and the *sigM* deletion strain exposed to antimicrobials in a disc diffusion test.

	WT (mm)*	Δ*sigM* (mm)*
Cell wall#		
Bacitracin	7	7
Fosfomycin	21	21
Gentamycin	25	23
Kanamycin	29	28
Neomycin	14	15.5
Streptomycin	29	27
Vancomycin	19	18
EDTA	22.5	21
Oxidative#		
H_2O_2	20	20
Diamide	17.5	19.5
Plumbagin	18.5	20
Pyrogallol	23	23.5
Tellurite	27.5	28

* The wild-type strain is indicated by WT and the *sigM* deletion strain by Δ*sigM*. The radius of the inhibition zone caused by the different agents is given in millimetres. # Concentrations of the tested agents are presented in Materials and Methods section.

Assessment of the σ^M regulon

The ethanol stress induction of *sigM* allowed for comparative transcriptome analysis of the wild-type and the *sigM* deletion strain and was used to identify genes under the control of σ^M. Analysis of the wild-type transcriptome after ethanol stress induction showed that *sigM* up-regulation was highest after 30 min by 3.1-fold, compared to 2.5 and 2.0-fold after 10 and 60 min, respectively. By use of qPCR a 7.5-fold *sigM* induction was observed after 10 min of ethanol stress exposure (Fig. 3). Genes that were induced significantly in the wild-type strain and not in the *sigM* deletion strain after 30 min of ethanol stress were assigned to be members of the σ^M regulon. A total number of 29 genes were found to be part of the putative σ^M regulon (Table 3). As observed for ECF σ factors in other organisms, it was shown that the *sigM*-operon was part of its own regulon, i.e. expression of *sigM* appears to be autoregulated. Other genes determined to be part of the σ^M regulon were annotated to have a role in the response to oxidative stress and to be involved in transport (Table 3).

Table 3. Genes putatively belonging to the σ^M regulon.

Gene	WT/ Δ*sigM*	Annotation	Alias	Putative σ^M binding site
BC0243	1.80	Oligopeptide transport system permease protein oppC	*appC*	
BC0244	3.95	Oligopeptide transport ATP-binding protein oppD	*appD*	
BC0245	3.26	Oligopeptide transport ATP-binding protein oppF	*appF*	
BC0294	1.58	10 kDa chaperonin GROES		TGTAAC…16bp…CAAC
BC0907	2.84	Oligopeptide-binding protein oppA	*appA*	TGTAAA…14bp…CGAA
BC1000	1.54	Hypothetical Membrane Spanning Protein		
BC1009	1.51	Hypothetical protein		
BC1047	1.57	Protease production regulatory protein hpr		TGTAAG…15bp…CACG
BC1113	NA*	Putative anti-σ factor σ^M	*yhdL*	
BC1114	21.85	RNA polymerase σ factor M	*sigM*	TGTAAC…16bp…CGTT
BC1714	5.52	Hypothetical protein		TGGAAC…16bp…CAAA
BC2329	1.78	Zinc uptake transporter		TCTAAC…15bp…CTAA
BC2355	2.25	Hypothetical protein		TGTAAA…13bp…CATT
BC2606	11.09	Hypothetical Membrane Spanning Protein		TGTATC…14bp…CTTT
BC2656	1.76	Cobalt transport protein cbiQ		
BC2795	4.03	Ferredoxin		TTTAAC…13bp…CAAT
BC3038	1.61	Transporter, Drug/Metabolite Exporter family	*yyaM*	TGTCAC…16bp…CATA
BC3987	5.01	NRDH-redoxin		
BC4174	1.51	Arginine repressor, argR		TGTAAA…19bp…CGAA
BC4259	1.66	Hypothetical Membrane Spanning Protein		TGTAAC…14bp…CATG
BC4366	2.69	Homocysteine gamma-lyase	*mccB*	TGTAAC…15bp…CAGT
BC4367	2.12	O-acetylserine thiol-lyase	*mccA*	TGTAAC…16bp…CATA
BC4368	1.92	S-Adenosylhomocysteine nucleosidase	*pfs*	TGTAAC…16bp…CATT
BC4369	1.90	Dimethyladenosine transferase	*yrrT*	TGTAAC…19bp…CATG
BC4516	1.58	Succinate dehydrogenase iron-sulfur protein	*sdhB*	AGTAAC…16bp…CACG
BC4789	2.46	AI-2 production protein/ Ribosylhomocysteinase	*luxS*	TATAAC…16bp…CGGG
BC4938	1.64	NADH dehydrogenase	*yutJ*	AGTAAC…13bp…CAGT
BC5046	1.75	Lysine decarboxylase family	*yvdD*	TGCAAC…17bp…CATC
BC5217	1.57	PTS system IIB component	*licB*	TGTAAC…19bp…CACC
BC5356	1.52	Methionine aminopeptidase	yflG	
		σ^M consensus		TGTAAC…16bp…CAtN

* Expression of *yhdL* in the *sigM* deletion strain is switched on due to read-through of the ery-resistance gene.

The genes encoding NrdH-redoxin (BC3987), NADH dehydrogenase (BC4938) and lysine decarboxylase (BC5046) have previously been shown to play a role in oxidative stress response. Next to this, 3 out of 5 genes encoding enzymes of the activated methyl cycle (AMC) were found to be part of the σ^M regulon. Two of these enzymes are encoded by the first two genes of a four gene operon that was identified to be part of the σ^M regulon. The first gene encodes a SAM-dependent methylase (BC4369), which is responsible for the conversion of S-adenosyl-L-methionine to S-adenosyl-L-homocysteine and the second gene encodes an S-adenosylhomocysteine nucleosidase (BC4368, Pfs) which converts S-adenosyl-L-homocysteine to adenosine and S-ribosyl-homocysteine. The last two genes of the operon *mccA* (BC4367) and *mccB* (BC4366) were previously revealed to encode the enzymes

responsible for the conversion of homocysteine to cysteine in *B. subtilis* (Hullo *et al.*, 2007). Notably, *luxS* was also identified to be part of the σ^M regulon. LuxS, the enzyme encoded by *luxS* is a component of the AMC and is responsible for the conversion of S-ribosyl-homocysteine to homocysteine and 4,5-dihydroxy-2,3-pentanedione (DPD), with the latter being the precursor of autoinducer-2 (AI-2) (De Keersmaecker *et al.*, 2006). Furthermore, a gene encoding a methionine aminopeptidase (BC5356), involved in the release of methionine from peptides, was identified to be part of the σ^M regulon as well.

In addition, several genes encoding transporters were found to be differentially regulated, including two oligopeptide transport systems (BC0241-BC0245 and BC0907-BC0911), a zinc uptake regulator (BC2329), the cobalt transport protein CbiQ (BC2656), a ferredoxin (BC2795), a transporter of the drug/metabolite exporter family (BC3038) and *licB*, encoding a PTS system component.

The promoter regions of the 29 genes of the putative σ^M regulon were analyzed for presence of a putative σ^M binding site. For 21 transcriptional units a putative σ^M promoter binding site could be identified, which included 22 of the 29 genes (Table 3).

Discussion

This study shows that extracytoplasmic function (ECF) σ factor σ^M, one of 10 ECF σ factors present on the genome of *Bacillus cereus* ATCC 14579, has a unique role in the stress response and survival of the foodborne human pathogen *B. cereus*. In total 29 genes were putatively identified to be part of the σ^M regulon by use of comparative transcriptome analysis of the wild-type and the *sigM* deletion strain in response to ethanol exposure. With both qPCR and microarray experiments *sigM* was observed to be induced in ethanol stress response 7.5-fold and 2.5-fold after 10 min, respectively. The higher induction observed by qPCR could be caused by a higher efficiency of the qPCR compared to the microarray experiment. The functional annotation of the 29 genes revealed these genes to encode proteins involved in transport and with a role in oxidative stress. The fact that 15 genes were predicted to be involved in transport, and 5 of the 6 genes have been annotated as hypothetical proteins containing a trans-membrane helix, strongly suggests σ^M to be involved in the regulation of proteins situated in the extracytoplasm of the cell, which fits with the predicted function in gene regulation of ECF σ factors (Helmann, 2002). By performing an *in silico* search for σ^M promoter binding sites, five to ten additional putative binding sites can be identified (data not shown). As the network of transcriptional regulation is different for every stress response, comparative analysis of the response of the wild-type and the *sigM* deletion strain to other stresses than ethanol may reveal additional genes to be part of the σ^M regulon.

Notably, oxidative stress is suggested to be a secondary effect of ethanol-induced stress in *B. subtilis* (Hoper *et al.*, 2005). It is very well possible that *B. cereus sigM* is up-regulated in response to the ethanol-induced oxidative stress, which would be in agreement with the presence of the ZAS-domain in the σ^M anti-σ factor. Only a minor 1.6-fold induction of *sigM* expression was observed after application of oxidative stress by H_2O_2, as monitored by qPCR, which may point to the fact that either the concentration of H_2O_2 used in this assay was not sufficient to fully induce *sigM* expression or that the stress response generated by

H_2O_2 is not mediated by σ^M. However, disc diffusion tests in which the resistance of the wild-type was compared to that of the *sigM* deletion strain revealed the *sigM* deletion strain to be more sensitive to several oxidative stress agents. This suggests σ^M to have a role in regulation of specific oxidative stress responses.

Indeed, many of the *B. cereus* genes putatively regulated by σ^M have a possible role in oxidative stress response. Amongst these is the cytoplasmic protein NrdH-redoxin (BC3987), a small redox protein, which was shown to be reduced by thioredoxin reductase in *E. coli* (Jordan *et al.*, 1997). In addition, both NADH dehydrogenase (BC4938) and ferredoxin (BC2795) have been assigned anti-oxidant functions in bacteria (Arner and Holmgren, 2000; Holmgren, 2000). Putative σ^M-controlled expression of the gene encoding lysine decarboxylase may also be associated with *B. cereus* oxidative stress response, since this gene was found to be up-regulated in *Vibrio vulnificus* under oxidative stress conditions (Kim *et al.*, 2006). The cadaverine formed in the decarboxylase reaction functions as a scavenger of superoxide radicals (Kim *et al.*, 2006). The cobalt and zinc transporter, putatively regulated by σ^M, could also have a role in oxidative stress as it is suggested that increased intracellular Zn(II) levels may protect thiols from oxidation. (Gaballa and Helmann, 2002; Valko *et al.*, 2005).

Remarkably, the activated methyl cycle (AMC) was revealed to be up-regulated after 30 min of ethanol stress induction. For three of the five genes encoding enzymes of the AMC transcriptome analysis showed their expression to be putatively σ^M dependent. Furthermore, *in silico* analysis revealed that a putative σ^M promoter binding site was present for the genes encoding the enzymes of the AMC that are part of the σ^M regulon based on our transcriptional data. This also includes *luxS*, encoding LuxS, the enzyme responsible for autoinducer-2 (AI-2) production. In addition, the homocysteine generated by the AMC is most likely converted into cysteine by MccA and MccB (Hullo *et al.*, 2007)(Fig. 2), and also for the genes encoding these enzymes a putative σ^M binding site was identified. Cysteine is a known antioxidant, which is formed in response to oxidative stress caused by ethanol in the human body (Vasdev *et al.*, 2006). It is therefore highly conceivable that increased production of cysteine can be supportive in coping with ethanol-induced oxidative stress in *Bacillus cereus*. As LuxS is the producer of DPD the precursor of the quorum sensing molecule AI-2, *luxS* up-regulation upon ethanol exposure could also be linked to induction of quorum sensing (De Keersmaecker *et al.*, 2006; Vendeville *et al.*, 2005). LuxS has been shown to affect biofilm formation in bacteria (Lombardia *et al.*, 2006), but both the wild-type and its *sigM* deletion strain showed similar biofilm forming capacity (data not shown).

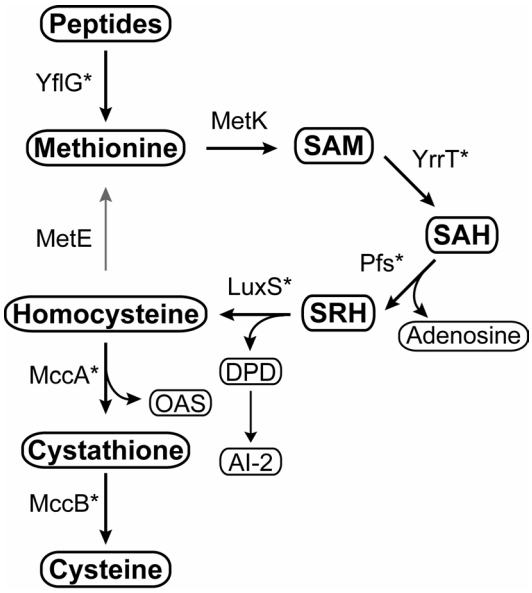


Fig. 4. *B. cereus* σ^M regulated cysteine and methionine metabolism pathways, including the Activated Methyl Cycle. Enzymes are underlined and putatively σ^M regulated genes are indicated with an asterisk (*). SAM, S-adenosyl-L-methionine, SAH, S-adenosyl-L-homocysteine, SRH, S-ribosyl-homocysteine, DPD, 4,5-dihydroxy-2,3-pentanedione, AI-2, autoinducer-2, OAS, *O*-acetylserine.

Comparison of the *B. cereus* and *B. subtilis* σ^M regulons showed no overlap between these regulons, indicating the role of the *B. cereus* σ^M to deviate from that of *B. subtilis*. This was already implied by the absence of the ZAS-domain from the anti-σ factor YhdL of *B. subtilis*. Genome comparisons between the different species within the *B. cereus* group revealed the *sigM*-operon to be present in all species. Next to this, the putative anti-σ factor YhdL showed less similarity between the different species of the *B. cereus* group than σ^M itself. Sensing of the extracellular environment occurs via the anti-σ factor, which can lead to activation of σ^M (Helmann, 2002). The differences in amino acid sequences of YhdL of species of the *B. cereus* group may indicate that stress sensing and signalling with activation of σ^M as a result may differ between representatives of the *B. cereus* group. It is noteworthy, however, that the ZAS-domain in YhdL is conserved in all species of the *B. cereus* group, which may indicate that YhdL is involved in mediating a σ^M-dependent oxidative stress response in the *B. cereus* group members.

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6

The ECF σ factor σ^Z of *Bacillus cereus* ATCC 14579 responds to different stresses and regulates cell surface modifications

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Abstract

The *Bacillus cereus* ATCC 14579 alternative σ factor σ^Z and its putative regulon have been characterized. σ^Z shows overall similarity with ECF σ factors and *sigZ* constitutes an operon together with *asfZ* encoding its putative anti-σ factor. Expression analysis revealed *sigZ* to be induced by an array of stresses, including exposure to ethanol, alkaline pH and heat shock, and a typical promoter binding site for the *sigZ*-operon was identified by 5'RACE. Phenotypic characterization of *B. cereus* ATCC 14579 and its *sigZ*-deletion strain revealed diminished growth performance and sporulation capacity. The σ^Z regulon was successfully established by transcriptome analysis of a nisin inducible *sigZ*-overexpression strain. Overexpression of *sigZ* was shown to affect expression of 50 genes, including 41 genes encoding proteins located in the extracytoplasm involved in signalling, transport and cell surface modifications. The role of σ^Z and its regulon in *B. cereus* stress response and survival is discussed.

Introduction

Bacteria have to cope with rapid changes in their environment bacteria in order to survive. Therefore, the bacterial cell comprises a diversity of mechanisms that can be activated under specific conditions. As a reaction to changing environments, the housekeeping σ factor can be replaced by an alternative σ factor to interact with the RNA polymerase (RNAP) allowing for modulation of gene expression, and consequently cellular performance (Hecker and Volker, 2001). The genome of the Gram-positive, endospore forming, food poisoning *B. cereus* ATCC 14579 contains 19 annotated alternative σ factors (Ivanova *et al.*, 2003; Overbeek *et al.*, 2003), including those involved in sporulation (σ^H, σ^E, σ^F, σ^G en σ^K) (de Vries *et al.*, 2004; Piggot and Hilbert, 2004) and general stress response (σ^B) (van Schaik *et al.*, 2004a). Furthermore, 10 of the alternative σ factors are part of a distinct family of σ factors, the so-called ExtraCytoplasmic Function (ECF) σ factors which mainly act to modulate extracytoplasmic functions (Helmann, 2002). This includes transport systems and enzymes involved in cell wall modification. More recently, ECF σ factor-controlled functions have also been linked to virulence (Bashyam and Hasnain, 2004; Kazmierczak *et al.*, 2005).

Genomic analysis revealed genes encoding ECF σ factors and anti-σ factors to be organized in an operon (Helmann, 2002). Notably, at least one trans-membrane helix was found to be conserved in the amino acid sequences of the ECF anti-σ factors, and these membrane-associated proteins have been suggested to be involved in sensing environmental changes. Furthermore, the activity of the ECF σ factors is controlled by binding to their respective anti-σ factors such that interaction with the RNAP is prevented (Yoshimura *et al.*, 2004). Until now, not much is known about triggers releasing the ECF σ factor from the anti-σ factor, after which the σ factor can bind to RNA polymerase, forming a RNAP holoenzyme that initiates transcription of the ECF σ factor regulon. As a general feature, expression of ECF σ factor genes is auto-regulated and they are thus part of their own regulon. Although ECF σ factors of *Bacillus subtilis* have been studied quite extensively (Helmann, 2002, 2006), no data are available about the functions of ECF σ factors in the foodborne human pathogen *B. cereus*. Notably, the ECF σ factor σ^W of *B. thuringiensis*, a member of the *B. cereus* group (Jensen *et al.*, 2003; Rasko *et al.*, 2005), was recently shown to have a role in regulating β-exotoxin I production (Espinasse *et al.*, 2004) and BA2502 encoding an ECF σ factor of *B. anthracis*, which is a member of the *B. cereus* group as well, is putatively involved in β-lactamase expression (Ross *et al.*, 2005). For neither of these ECF σ factors the corresponding regulon has been determined.

Here we performed a comparative phenotypic and transcriptomic analysis of *B. cereus* ATCC 14579, its *sigZ* deletion strain and a *sigZ* overexpression strain to assess the role of this ECF σ factor in the performance of this foodborne human pathogen**.**

Materials and Methods

Bacterial strains, culture conditions and sporulation

B. cereus ATCC 14579, its *sigZ* deletion strain (FM1500), and the *B. cereus* ATCC 14579 strains carrying plasmids were cultured as a standard in Brain Heart Infusion broth (BHI, Difco) at 30 °C, with shaking at 200 rpm. *E. coli* HB101/pRK24 (van Kranenburg *et*

al., 1997) was used as the donor host in conjugation experiments. Antibiotics used were ampicillin at a concentration of 50 µg/ml, kanamycin (70 µg/ml), chloramphenicol (5 µg/ml), erythromycin (150 µg/ml for *E. coli* or 5 µg/ml for *B. cereus*), spectinomycin (100 µg/ml), and polymyxin B (50 µg/ml) for counter selection against *E. coli* upon conjugation.

Different stresses were applied for 10 min to 20 ml of mid-exponential cells growing in BHI broth at an OD_{600} of 0.4. Stress conditions were 42 °C, 2.5% NaCl 20 mM HCl (~pH 5.2), 30 mM NaOH (~pH 9.0), 50 µM H_2O_2, and 4% of ethanol.

For microarray analyses, ethanol was added to 50 ml mid-exponentially growing wild-type cultures at an OD_{600} of ~0.6 to an end-volume of 4% v/v. Samples for RNA isolation were taken right before addition of the ethanol and 10, 30 and 60 min after its addition.

Sporulation of the wild-type and the *sigZ* deletion strain was induced by resuspending exponential cells grown in BHI (OD_{600} 0.8) in minimal medium without C- and N-source (Mols *et al.*, 2007). Subsequent overnight sporulation efficiency was tested by plating the spore suspension on BHI plates before and after killing non-sporulated cells by incubation for 15 min at 70 °C.

Genetic methods and construction of a *sigZ* deletion strain

Plasmid DNA was purified from *E. coli* with a Qiaprep Spin Miniprep kit (Westburg, Leusden, The Netherlands). Pwo polymerase (Roche Diagnostics, Almere, The Netherlands) was used for PCRs that were used in cloning and Taq polymerase (Fermentas) for control amplifications. DNA digestions were performed as indicated by manufacturers. A deletion strain of the *sigZ* designated gene (RZC03920, BC2108, (Ivanova *et al.*, 2003),) was obtained by a double crossover event. Two flanking regions of *sigZ* were coupled to an erythromycin resistance box by long flanking homology (LFH) PCR (Wach, 1996). The erythromycin resistance box was obtained by PCR as described previously (Chapter 3). Primers used for LFH-PCR were 3920KOforw, 3920revbinnen2, 3920forwbinnen and 3920KOrev2. The obtained PCR product was digested and subsequently cloned into the *Sac*I-*Bam*HI digested conjugal vector pATdeltaS28 (Trieu-Cuot *et al.*, 1990), resulting in pATdelta3920KO. After isolation of pATdelta3920KO from DH5α and transformation into *E. coli* HB101/pRK24, the resulting strain was used in a conjugation experiment with *B. cereus* ATCC 14579 (Bron, 1990). Conjugants were screened for erythromycin resistance and spectinomycin sensitivity. Subsequently, one conjugant was taken for further research and PCR and Southern Blot analysis confirmed the double homologous recombination event for this strain (data not shown). The *B. cereus sigZ* deletion strain was designated *B. cereus* FM1500.

5'RACE and RT-qPCR

RT-qPCR experiments were performed as described previously (van Schaik and Abee, 2005). Relative expression values were obtained by comparing expression with *rpoA* expression with the REST program (Pfaffl *et al.*, 2002). Primers used in qPCR experiments were for *sigZ* sigZforwQpcr and sigZrevQpcr, for *asfZ* asfZ_forw_qPCR and asfZ_rev_qPCR and for *rpoA* rpoAforw and rpoArev.

The 5' end of the *sigZ* transcript was mapped using RNA isolated (as described below) from mid-exponential cultures of *B. cereus* ATCC 14579 with the 5' RACE System for Rapid Amplification of cDNA Ends (Invitrogen). The used GSP *sigZ* primers were GSPsigZ1 and GSPsigZ2.

Inducible overproduction of *sigZ* in *B. cereus* ATCC 14579

The *sigZ* gene was amplified by use of the primers 3920over_forw and 3920over_rev. The PCR product was *Pag*I-*Sac*I digested and cloned into the *Nco*I-*Sac*I digested pNZ8048 (Eichenbaum *et al.*, 1998). The resulting vector, pOver3920, was transformed into *E. coli* MC1061. The purified pOver3920 or empty pNZ8048 were transformed into *B. cereus* ATCC 14579, which already carried pNZ9530 (Kleerebezem *et al.*, 1997), resulting in the *sigZ* overexpression strain and the empty-vector strain, respectively. For both the *sigZ* overexpression strain and the empty-vector strain, nisin (Sigma) was added to a mid-exponential-phase culture (OD_{600} of 0.4 to 0.5) to a final concentration of 0.2 ng/ml. For this concentration no inhibition of growth was observed. The cells were then cultured for another 90 min before being harvested for either total RNA or protein isolation.

Table 1. Oligonucleotides used in this study.

Oligonucleotides	Sequence 5'to 3'*
3920KOforw	TCgagctcGTCACGCAAATAACGGAAAAC
3920KOrev2	GTggatccTCAAAACTAGAAAACGGAGAAC
3920revbinnen2	cgaaccgtcttatctcccattatatcTGAACGACATCATCTACATCC
3920forwbinnen	gtagataaattattaggtatgAAAGAGGTGGATGCGACTACG
sigZforwQpcr	TGATAGCGAGAAGCCATTTC
sigZrevQpcr	TCGCATCCACCTCTTTCTTC
asfZ_forw_qPCR	AAACATGCCGGAGCAATGAC
asfZ_rev_qPCR	TCCTTCGCACCCATTTCATC
rpoAforw	ACCGCTTGAGCGTGGATATG
rpoArev	TAGCAGTAACAGCGGCACCA
GSPsigZ1	GATTTCACTGTACCGATTGG
GSPsigZ2	GCACTGGCTTTCTTTGCTCTTCTG
3920over_forw	CTGtcatgaAGGGTGAAATAGATTAC
3920over_rev	CTGgagctcTTTGCTTCTTCTTGTATAG
3920overHis_rev	CTGctcgagTTTGCTTCTTCTTGTATAG

* Introduced restrictions sites and gene aspecific sequences are in small caps

Overexpression and purification of σ^Z in *E. coli* and generation of polyclonal antibodies, protein extraction and immunoblotting techniques

sigZ was amplified from *B. cereus* chromosomal DNA by PCR by using primers 3920over_forw and 3920overHis_rev, which introduced an *Pag*I and an *Xho*I site, respectively. PCR product was cloned into pET28-b (Novagen, Madison, Wisconsin), *Nco*I-*Xho*I digested, and the resulting vector (pMTsigZ) was transformed into *E. coli* BL21-

Codonplus-(DE3)-RIL. σ^Z was produced and purified as described before (van Schaik *et al.*, 2004b). Protein concentration was measured by use of the bicinchoninic acid assay. Rabbit serum containing anti-σ^Z antibodies was prepared by Eurogentec S.A. (Herstal, Belgium) according to the company's standard protocol.

Total cellular protein was extracted by bead beating as described previously (Periago *et al.*, 2002). Protein concentrations were determined by use of the bicinchoninic acid assay. Samples containing 40 µg of protein were loaded on two SDS-PAGE gels. One of the gels was used in Western blotting experiments, and the other gel was stained with Coomassie blue and visually inspected to confirm that equal amounts of protein were loaded. Proteins were separated by SDS-PAGE on 15% polyacrylamide gels by use of a Criterion II vertical electrophoresis system (Bio-Rad, Richmond, California). Bio-Rad's broad-range prestained SDS-PAGE standards were used as molecular weight markers. Proteins were electroblotted and blocked as described previously (van Schaik *et al.*, 2004b). After blocking the blots were incubated with TBS (20 mM Tris-HCl [pH 7.5], 500 mM NaCl) with 0.05% Tween 20 and supplemented with 1,000-fold-diluted rabbit immune serum containing the polyclonal anti-σ^Z antibodies. Immunocomplexes were incubated with goat anti-rabbit peroxidase (Bio-Rad) and were visualized with 3,3'-diaminobenzidine tetrahydrochloride.

RNA isolation, cDNA synthesis and labelling, and microarray analysis

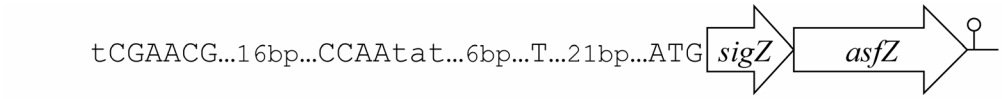
RNA isolation, cDNA synthesis and labelling, and custom-made *B. cereus* ATCC 14579 60-mer oligo microarrays (produced by Agilent) hybridization and analysis were performed as described previously (van Schaik *et al.*, 2007). For *sigZ* overexpression array analysis, the statistical test for significant up-regulation indicated genes to be significantly regulated with an up-or down-regulation, which was subsequently used as the cut-off value for these microarray experiments. For ethanol stress this cut-of was set to 1.5-fold. Microarray data were submitted to the GEO database with accession numbers: GSE9855, GSE9858 and GSE9860. The 300bp up-stream promoter sequences of up-regulated genes after σ^Z overexpression were screened for the presence of a putative -35 promoter binding site with similarity to the -35 binding site deduced from the transition start of the *sigZ*-operon. Consequently, a -10 binding site was linked to the identified -35 binding sites (Table 2).

Results

The *sigZ*-operon

The *sigZ*-operon consists of two genes, the first gene is *sigZ,* encoding σ^Z, and the second gene encodes the putative anti-σ factor, AsfZ. Similarity of σ^Z with the sub-family of ECF σ factors was assessed by BLAST (Altschul *et al.*, 1990). Furthermore, by use of the web-based tool SOSUI (Hirokawa *et al.*, 1998), a trans-membrane helix was identified in the predicted amino acid sequence of AsfZ. Phylogenetic analysis showed no homologues of σ^Z and AsfZ to be present outside the *B. cereus* group (data not shown). The transcription start for the *sigZ*-operon was determined by means of 5'RACE. A single transcription start site could be identified in the promoter region of *sigZ* (Fig.1). Alignment of the up-stream regions of orthologous genes within the *B. cereus* group showed the deduced -35 and -10 binding sites

to be highly conserved (Fig. 1). Expression of *sigZ* was monitored by RT-qPCR and induction of the *sigZ*-operon was tested during exposure to six different stress situations (Fig. 2). No induction of *sigZ* could be shown during exposure to low pH and high salt concentrations, and only 2-fold and 3-fold inductions were observed respectively after exposure to H_2O_2 and a 42 °C heat shock. Notably, a clear 6-6.5 fold induction of *sigZ* was observed after exposure to alkaline pH and ethanol. Induction of *asfZ* was comparable to *sigZ* induction after exposure to alkaline pH, however, after exposure to ethanol *asfZ* was found to be up-regulated only 3.3-fold.

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Bc ATCC14579 --AAAAAACTTTTTCGGAAAAATCGAACGAAATATGAATTACCTGCCAATATATAGTGTAAGACAAAGGAGGGCTGGAATATG
Bt 97-27     --TATAAAATTTTCTAGAAAAATCGAACGAAATATAAATTACTTACCAATATATTGTGTAAGACAAAGGAGGGCTGGAATATG
Bc E33L      --TATAAAATTTTCTAGAAAAATCGAACGAAATATAAATTACTTGCCAATATATTGTGTAAGACAAAGGAGGGCTGGAATATG
Ba A2012     --TATAAAATTTTCTAGAAAAATCGAACGAAATATAAATTACTTGCCAATATATTGTGTAAGACAAAGGAGGGCTGGTATATG
Bc ATCC10987 TATAAAAACTTTT--GAAAAAATCGAACGAAATATGGATTACTTGCCAATATAAAGTGTAAGACAAAGGAGGGATGGTACATG
               * *** ****    ******************  ***** * ********  ****************** *** * ***
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Fig. 1. *sigZ*-operon structure with promoter region. The *sigZ*-operon consists of 2 genes, *sigZ* and *asfZ*. In the promoter region are indicated. From left to right, the translation start (ATG), the transcription start (T), and the -10 promoter binding site and the -35 promoter binding site. The loop at the end of the operon indicates the end of transcription, as indicated in the Microbial Genome Viewer (Kerkhoven *et al.*, 2004). The alignment of the promoter regions of *sigZ* in different species of the *B. cereus* group, show high conservation of the identified -10 and -35 promoter binding sites.

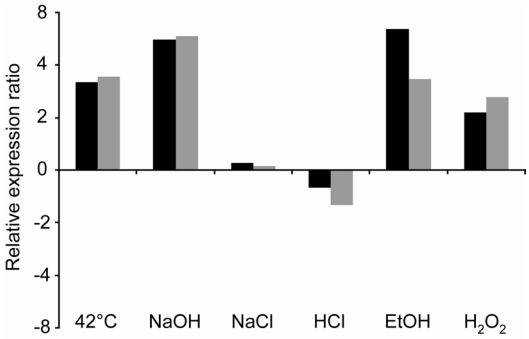

Fig. 2. Expression of both *sigZ* (black bars) and *asfZ* (grey bars) in the wild-type after exposure to different stresses for 10 min (42 °C, 2.5% NaCl, 20mM HCl (~pH 5.2), 30 mM NaOH (~pH 9.0) and 50 µM H_2O_2). Ratio values are presented relative to the expression of the household gene *rpoA* (the y-axes is on a 10log scale).

Phenotypic analysis of the *sigZ* deletion strain

A *sigZ* deletion strain was constructed to assess the role of σ^Z in *B. cereus* performance. Growth of the *sigZ* deletion strain appeared to be slightly impaired as reflected in the specific growth rates of 0.017 h^{-1} and 0.021 h^{-1}, determined for the *sigZ* deletion and the wild-type strain, respectively. Moreover, the sporulation capacity of the *sigZ* deletion strain appeared to be severely impaired (Fig. 3A), with the wild-type and the *sigZ* deletion strain showing 59% and 0.1% of the cells forming spores, respectively (Fig. 3B).

Comparative transcriptome analysis of *B. cereus* ATCC 14579 and its *sigZ* deletion strain did not allow for assessment of σ^Z-controlled genes, since expression of a large number of genes was found to be affected (data not shown). Therefore, the putative σ^Z regulon was assessed by transcriptome analysis of a nisin-inducible *sigZ* overexpression strain.

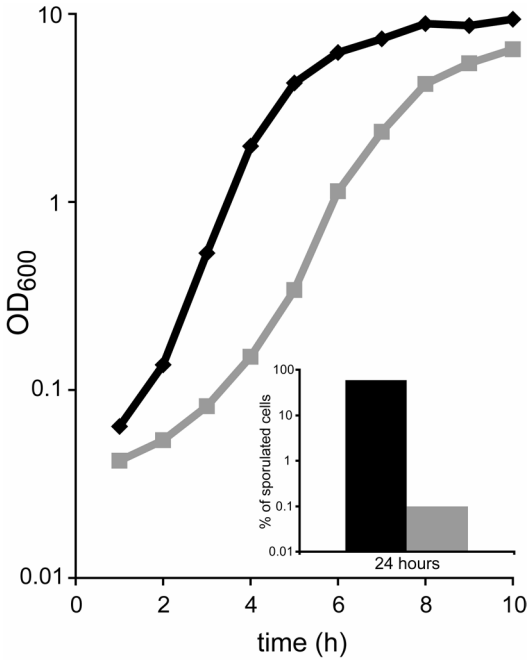

Fig. 3. Growth and sporulation (insert) of the wild-type (black bar and symbols) and the *sigZ* deletion strain (grey bar and symbols).

Establishing the putative σ^Z regulon

Nisin-induced overexpression of *sigZ* was confirmed by qPCR, and translation of overexpressed *sigZ* to σ^Z was confirmed by western-blotting (Fig. 4). Overexpression of σ^Z was used to determine the putative σ^Z regulon by comparative DNA microarray analysis of the overexpression strain and the empty-vector strain. A total number of 50 genes was up-regulated 3-fold or more after overexpression of σ^Z, including *sigZ* and *asfZ* (Table 2). The 50 genes included genes encoding proteins with functions in transport, cell-surface modification and transcriptional regulation. 23 genes are located in two single gene transcriptional units and six operons encoding a putative ABC-type transporter. In addition, BC5238 encodes a single protein that has been annotated as a glycine betaine transporter. Among the genes encoding cell-surface modification proteins are two genes (BC1161 and BC2272) encoding a peptidyl-prolyl isomerase (PPIase), one gene (BC3146) encoding a peptidoglycan N-acetylglucosamine deacetylase, and an operon consisting of genes encoding a cell surface protein BasI (BC4812), a sortase SrtC (*srtC*, BC4811) and a two-component system SctR-SctS (BC4810-BC4809, Table 2)(Marraffini and Schneewind, 2006). In addition, up-regulation after σ^Z overexpression of the gene encoding a hypothetical protein (BC4813)

suggests this gene to be part of this operon (BC4813-BC4809) as well. Moreover, an AraC-like transcriptional regulator (BC2507) was also found to be part of the σ^Z regulon. For all 11 genes up-regulated encoding a hypothetical protein at least one trans-membrane helix could be identified by use of SOSUI (Hirokawa *et al.*, 1998) pointing to their localization in, or association with, the membrane.

Quantitative PCR experiments revealed *sigZ* to be induced upon exposure to ethanol, and thus it is expected that the σ^Z regulon is also induced in response to ethanol stress. Therefore, the genes found to be induced in ethanol stress response were compared with the up-regulated genes after *sigZ* overexpression. Transcriptome assessment of ethanol-stressed cells revealed *sigZ* expression to be up-regulated 2.2–fold, 3.3-fold and 1.6-fold after 10 min, 30 min and 60 min, respectively. As shown by qPCR experiments, the induction of *asfZ* was approximately half of the up-regulation observed for *sigZ*. Comparing the gene expression profiles of ethanol- and nisin-induced *sigZ* revealed 17 genes to be up-regulated at least 1.5-fold in both conditions (Table 2).

Up-stream regions of genes suggested to be part of the σ^Z regulon were scanned for a putative σ^Z promoter binding site. For 23 of the 30 putative σ^Z regulated operons a possible binding site was identified. The consensus of these possible binding sites showed the -35 sequence to be more conserved than the -10 binding site (Table 2).

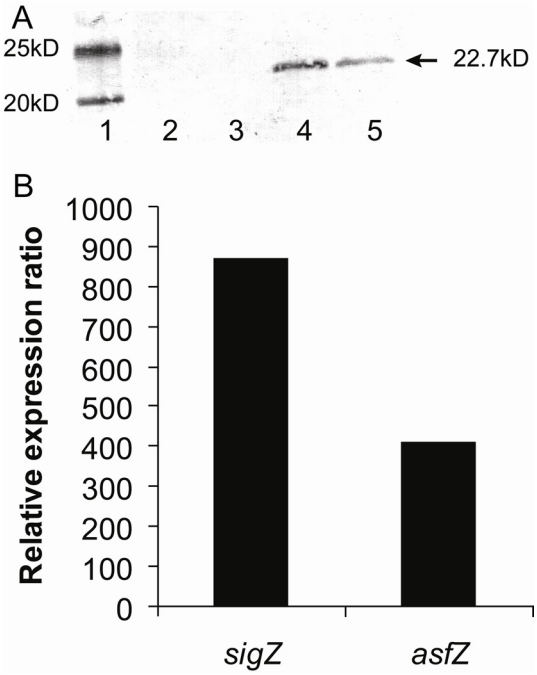

Fig. 4. Western-blot and qPCR of *sigZ* overexpression. A. Western blot, Lanes 2 and 3: σ^Z levels in the empty vector strain 90 min after addition of nisin. Lanes 4 and 5: σ^Z levels in the overexpression construct 90 min after addition of nisin. Lane 1: marker. B. qPCR: Expression ratios are presented of the overexpression strain after addition of nisin for 90 min, as compared to the empty vector strain after addition of nisin for 90 min, and relative to the control gene *rpoA*.

Table 2. Genes with a 3-fold higher expression after *sigZ* overexpression.

Gene ID	Ratio O.E.*	Ratio E.S.*	Alias	Annotation	Putative promoter binding site
Transport					
BC0207	3.71	0.79		Oligopeptide transport system permease protein oppB	TTGAACC-13bp-GCAA
BC0208	3.03	0.93		Oligopeptide transport system permease protein oppC	
BC0209	2.90	0.97		Oligopeptide transport ATP-binding protein oppD	
BC0210	2.66	1.28		Oligopeptide transport ATP-binding protein oppF	
BC0211	2.51	1.33		Oligopeptide-binding protein oppA	
BC0346	4.50	0.58		ABC transporter ATP-binding protein	TCGAAAG-16bp-CAGC
BC0347	6.50	0.94		ABC transporter permease protein	
BC0348	3.41	0.96	*yhcJ*	ABC transporter substrate-binding protein	
BC0814	3.42	3.54		ABC transporter permease protein	
BC0815	4.23	3.23	*yknY*	ABC transporter ATP-binding protein	
BC0816	4.93	3.60		HlyD family secretion protein	ACGAACG-17bp-GATG
BC2323	5.59	2.31		ABC transporter ATP-binding protein	GGGAACG-14bp-GAGA
BC2324	5.39	2.46		ABC transporter permease protein	
BC2372	3.18	0.87	*yfiC*	Multidrug/protein/lipid ABC transporter family	ATGAACA-16bp-GAGG
BC2909	2.74	0.73	*ssuD*	Alkanesulfonate monooxygenase	
BC2910	3.12	0.69	*ssuC*	Alkanesulfonates transport system permease protein	
BC2911	2.85	0.80	*ssuA*	Alkanesulfonates-binding protein	
BC2912	2.30	0.79	*ssuB*	Alkanesulfonates transport ATP-binding protein	AGAAACC-16bp-CAGA
BC4742	3.05	1.87		ABC transporter permease protein	
BC4984	3.25	0.62		ABC transporter substrate-binding protein	
BC4985	2.76	0.55	*metN*	ABC transporter substrate-binding protein	
BC4986	3.05	0.52	*metP*	ABC transporter permease protein	
BC4987	2.98	0.60	*metQ*	ABC transporter ATP-binding protein	TCAAACG-16bp-GAGC
BC5238	3.93	0.23	*opuD*	Glycine betaine transporter	CAAAACG-16bp-GAAC
Cell surface modification					
BC1161	3.01	3.02		Peptidyl-prolyl cis-trans isomerase	TACAACG-15bp-GATA
BC2272	5.26	3.45		Peptidyl-prolyl cis-trans isomerase	
BC3146	5.20	0.52		Peptidoglycan N-acetylglucosamine deacetylase	
BC4811#	11.27	0.53	*srtC*	Sortase C	
BC4812#	4.33	0.41	*basI*	Cell surface protein	
Transcriptional regulation					
BC2108	769.03	3.27	*sigZ*	RNA polymerase ECF-type σ factor	TCGAACG-15bp-GCCA
BC2109	715.46	1.04	*asfZ*	ECF-type σ factor negative effector	
BC2507	3.93	2.18		Transcriptional regulator, AraC family	GTGAACG-15bp-GGCC
BC4809#	3.22	1.02	*sctS*	Two component system histidine kinase	
BC4810#	4.90	1.01	*sctR*	Two-component response regulator	
Other or unknown function					
BC1041	5.94	0.41	*yvgS*	ATP-dependent DNA helicase rep	AGGAACG-16bp-AATT
BC1435	4.73	0.92		Hypothetical protein, with TMH	AAAAACG-17bp-GGAA
BC1436	4.48	0.93		Phage shock protein A	
BC1461	4.76	2.49		DNA integration/recombination/invertion protein	
BC1618	3.66	1.65		Hypothetical Membrane Spanning Protein	TAGAATG-15bp-GAAG
BC2438	3.28	1.04		Hypothetical protein, with TMH	
BC2554	5.31	0.74		Hypothetical protein, with TMH	
BC2603	3.72	1.76		Hypothetical protein, with TMH	
BC2953	3.69	0.85		Hypothetical protein, with TMH	ATGTACG-16bp-TTAA
BC3181	4.69	2.75		Hypothetical protein, with TMH	
BC3182	4.65	1.82		Hypothetical protein, with TMH	AGGAAAG-15bp-GACT
BC4036	3.11	0.56	*ykrW*	Ribulose bisphosphate carboxylase large chain	AGGGACG-15bp-GGCA
BC4482	5.80	2.32		Hypothetical protein, with TMH	ATTAACG-15bp-GTGA
BC4744	5.96	1.28		Hypothetical Membrane Spanning Protein	ATGAACT-16bp-GTAA
BC4813#	5.91	3.81		Hypothetical protein, with TMH	GATAACG-15bp-GATA
BC5406	4.25	0.95		Cysteine synthase	AAGAAAG-15bp-GAGA
				putative σ^Z consensus promoter	aNgAACG15/16-GaNa

* OE: Overexpression, ES: Ethanol Stress. Ratio is presented as overexpression strain compared to the empty vector strain after induction by nisin. Genes in italics show a not significant differential gene expression, and are present in the same operon as significantly differentially expressed genes. # Genes suggested to make up one operon. TMH: transmembrane helix.

Discussion

The *Bacillus cereus* ATCC 14579 ExtraCytoplasmic Function (ECF) σ factor σ^Z and its regulon have been characterized. The promoter binding site of the *sigZ*-operon was deduced from a 5'RACE determined transcription start and the -35 and -10 sequences of the promoter were found to be conserved in all species of the *B. cereus* group. Clear up-regulation of *asfZ* after σ^Z overexpression showed that σ^Z autoregulates the expression of its operon, and thus the promoter binding site identified by 5'RACE to be the σ^Z binding site. Notably, SDS-page and subsequent Western-blotting revealed σ^Z to be produced only moderately in overexpression experiments at the protein level, despite the large increase in RNA levels of *sigZ* indicating low translational efficiency. The moderate levels of σ^Z observed in the *sigZ* overexpression strain produced in overexpression fit with the defined impact on gene expression. The *sigZ*-operon was the only ECF σ factor operon to be up-regulated after σ^Z overexpression, which indicates that σ^Z can not activate other ECF σ factor operons. In total 50 genes were up-regulated after σ^Z overexpression, of which 17 genes, including the *sigZ*-operon, were also induced after exposure to ethanol. Genes found to be up-regulated after overexpression and not after ethanol exposure can be part of the σ^Z regulon in response to other stresses than ethanol, as the interplay of transcriptional regulators may be varying in different stresses. The putative -35 sequences for σ^Z binding were identified in front of 21 genes of the σ^Z regulon and these sites were subsequently linked to possible -10 sequences. The -35 sequence of the determined σ^Z consensus binding site was more conserved than the -10 sequence, which is in agreement with what was found previously for ECF σ factors of *B. subtilis* (Jervis *et al.*, 2007). The functionality of the putative promoter binding sites remains to be elucidated. Phenotypic analysis of the *sigZ* overexpression strain in response to a diversity of antibiotics and other stress agents, such as hydrogen peroxide did not show any clear differences in comparison with the empty vector strain (data not shown).

The genes constituting the σ^Z regulon function in the extracytoplasm, being involved in cell surface modification and transport, which is in line with previously described functions for ECF σ factors of other species (Helmann, 2002). The σ^Z regulon contains 3 genes that were identified to encode proteins involved in transcriptional regulation. One of these 3 genes encoded a transcriptional regulator of the AraC-family. Moreover, the putative σ^Z regulon comprised two genes encoding a two component system (BC4809 and BC4810). Stimulation of these transcriptional regulators may govern indirect regulation in response to σ^Z induction. A total number of 23 genes of the σ^Z regulon constitute two single gene transcriptional units and six operons encoding ABC-type transporters. ABC-type transporters have also been linked to stress resistance either by transporting substances, such as osmolytes, into the cell or by transporting substances, such as antibiotics, out of the cell (Poelarends *et al.*, 2002; Wood, 2006). For only two of these ABC-type transporters a putative function could be identified. The ABC-type transporter encoded by *ssuBACD* was shown to be required in the utilization of aliphatic sulphonates as sulphur sources in *B. subtilis* (van der Ploeg *et al.*, 1998). The ABC-type transporter *metNPQ* was recently studied in *B. subtilis*, and was shown to transport methionine sulfoxide, and D- and L-methionine (Hullo *et al.*, 2004; Merlin *et al.*, 2002). For both transport systems similar functions are presumed in *B. cereus*. Another ABC transporter

in the σ^Z regulon encodes an oligopeptide permease (BC0207-BC0211), which is required for the import of small peptides into the cell. A similar oligopeptide permease in *B. thuringiensis* was shown to be involved in regulation of hemolytic gene expression (Gominet *et al.*, 2001) and a role of this transporter in extracellular signal sensing has been suggested (Lazazzera, 2001). For the remaining five transcriptional units encoding ABC-type transporters no clear functions could be predicted. In addition, one gene (*opuD*) encodes a glycine betaine transporter, which may play a role in the response to osmotic stress (Wood, 2006).

In total five genes of the σ^Z regulon were annotated to be involved in cell surface modification. Two of these genes (BC1161 and BC2272) encode a peptidyl-prolyl isomerase (PPIase). On the genome of *B. cereus* ATCC 14579 four genes have been annotated to encode PPIases. In *B. subtilis* the PPIase PrsA was shown to be a membrane bound lipoprotein located at the membrane-cell wall interface, with a function in the folding of exported proteins, including extracytoplasmic domains of integral membrane proteins involved in the synthesis of cell wall matrix (Sarvas *et al.*, 2004; Vitikainen *et al.*, 2004). The PPiases of *B. cereus* ATCC 14579 show high similarity to PrsA and it is therefore conceivable that they have a function in the folding of secreted proteins involved in the synthesis of cell wall matrix. In addition, the peptidoglycan N-acetylglucosamine deacetylase identified to be part of the σ^Z regulon (BC3146) is one of the six genes annotated as such present on the genome of *B. cereus* ATCC 14579. Two of these peptidoglycan N-acetylglucosamine deacetylases, BC1960 and BC3618, were shown to have a role in deacetylation of cell wall peptidoglycan of *B. cereus*, which resulted in increased resistance to the action of lysozyme (Psylinakis *et al.*, 2005). A similar function may be assigned to the peptidoglycan N-acetylglucosamine deacetylase encoded by BC3146. Notably, the *basI-srtC-sctR-sctS*-operon (BC4812-BC4809) was identified to be part of the σ^Z regulon. For *B. anthracis* the sortase encoded by *srtC* was shown to process the LPNTA-motif present in the protein encoded by *basI*, and subsequently SrtC anchors BasI to the cell wall (Marraffini and Schneewind, 2007). This operon was shown to be positively regulated by the two component regulator encoded by *sctR* and *sctS* (Marraffini and Schneewind, 2006). In addition, the identified role of the *basI-srtC-sctR-sctS*-operon in sporulation of *B. anthracis* may offer an explanation for the impaired sporulation phenotype of the *sigZ* deletion strain. Notably, such a severely affected phenotype could not be identified for *B. subtilis* after deletion of a single ECF σ factor, only for a triple ECF σ factor deletion strain (*sigM*, *sigW* and *sigX*) in an undomesticated strain complete loss of multicellular differentiation was observed (Mascher *et al.*, 2007).

In conclusion, the *B. cereus* σ^Z regulon comprises a number of genes encoding proteins with putative functions in signalling, transmembrane transport and cell surface modifications. Whether the induction of the σ^Z regulon will lead to alteration of the cell surface, and subsequently to alteration of the interaction of *B. cereus* with its environment, including survival and/or pathogenic properties remains to be elucidated.

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7

Summary, discussion and perspectives

Introduction

Food industries have seen their challenges increased with respect to food safety issues because of consumer demands for higher quality, fresher and healthier foods. In certain foods this is achieved through implementation of mild processing steps. A combination of mild preservation techniques is generally referred to as the "hurdle concept" (Leistner, 2000). Several researchers have indicated that mild processing conditions may allow survival of subpopulations of bacteria that activated a so-called stress response, which provides (cross)protection to subsequent exposure to other otherwise lethal or growth inhibiting conditions (Abee and Wouters, 1999). This may increase the risks for consumers with respect to exposure to foodborne pathogens. Therefore, in this thesis the foodborne pathogen *Bacillus cereus* was studied to increase the understanding about the response of *B. cereus* to stress and to determine the role of transcriptional regulators in this process.

The research described in this thesis was part of an IOP genomics project (IGE01018) in which four food-relevant Gram-positive bacteria (*Bacillus subtilis*, *Lactococcus lactis*, *Lactobacillus plantarum* and *B. cereus*) were studied. By comparing the transcriptional responses of these four Gram-positive bacteria to different stresses, including metabolic stress, a general overview of stress responses within food-relevant Gram-positive bacteria could be obtained. A general objective of IOP genomics projects is to implement and maintain high quality of genomics research in the participating laboratories in the Netherlands. Moreover, the research on *B. cereus* was part of the Food Preservation and Safety project of the Wageningen Centre for Food Sciences. A summary of the work performed on *B. cereus* is presented and possible perspectives of the research will be discussed.

Transcriptional regulators

As a facultative anaerobe, *B. cereus* is capable of anaerobic growth. This anaerobic growth is supported by fermentation and/or anaerobic respiration (Rosenfeld *et al.*, 2005) and may enhance the performance of *B. cereus* in oxygen-deprived environments, such as soil (Vilain *et al.*, 2006), vacuum-packed foods (Tham *et al.*, 2000), and the human GI tract (Laohachai *et al.*, 2003). Since *B. cereus* can encounter anaerobic environments in food, it is important to understand the growth and transcriptional regulation of *B. cereus* in anaerobic conditions, in order to be able to prevent this growth. In Chapter 2, the anaerobic growth of *B. cereus* ATCC 14579 in a rich medium has been analyzed in comparison to aerobic growth. It was shown that both the growth rate and the yield were significantly lower under anaerobic conditions than under aerobic conditions. However, under anaerobic conditions *B. cereus* growth still yielded high numbers of cells. Transcriptome analysis of cells harvested in different growth phases during both anaerobic and aerobic growth, showed major differences between the expression of genes encoding metabolic pathways. Novel genes activated during anaerobic growth of *B. cereus* that encode metabolic pathways were identified, such as the arginine deiminase pathway (ArcABDC), a formate dehydrogenase (FdhF), a pyruvate fomate lyase (Pfl), and alternative respiratory proteins, such as arsenate reductases. Furthermore, the nitrosative stress response was induced in the anaerobic transition phase, conceivably due to

the production of nitric oxide as a by-product of nitrite and nitrate respiration. Moreover, expression of the genes encoding the transcriptional regulators ArcR, LuxS and Rex was higher in anaerobic cells than in aerobic cells. Gene deletion strains of these regulators can be used to further study the function of these regulators in anaerobic growth. Most interestingly, genes encoding the hemolytic enzymes and enterotoxins showed an up-regulation in oxygen limited conditions (Fig. 1). The hemolytic enzyme encoding genes were up-regulated in anaerobic growth compared to aerobic growth, whereas the enterotoxin operons were up-regulated in the oxygen limited, high cell density transition and stationary phase of aerobic growth. This is in agreement with previous studies that reported an enhanced production of the hemolytic enzymes and enterotoxins in anaerobic environments by members of the *B. cereus* group (Duport *et al.*, 2004; Duport *et al.*, 2006; Klichko *et al.*, 2003; Rosenfeld *et al.*, 2005; Zigha *et al.*, 2007). Both hemolytic enzymes and enterotoxins play a role in illnesses caused by *B. cereus* and these virulence factors are presumed to be produced in the human gastro-intestinal (GI)-tract, an environment where oxygen limitation may occur (Zigha *et al.*, 2006). To fully understand and reveal the transcriptional regulation of enterotoxin gene expression and their activity, research should focus on conditions mimicking the situation in the GI-tract, including the interaction of *B. cereus* with selected human cell lines, such as Caco-2 cells.

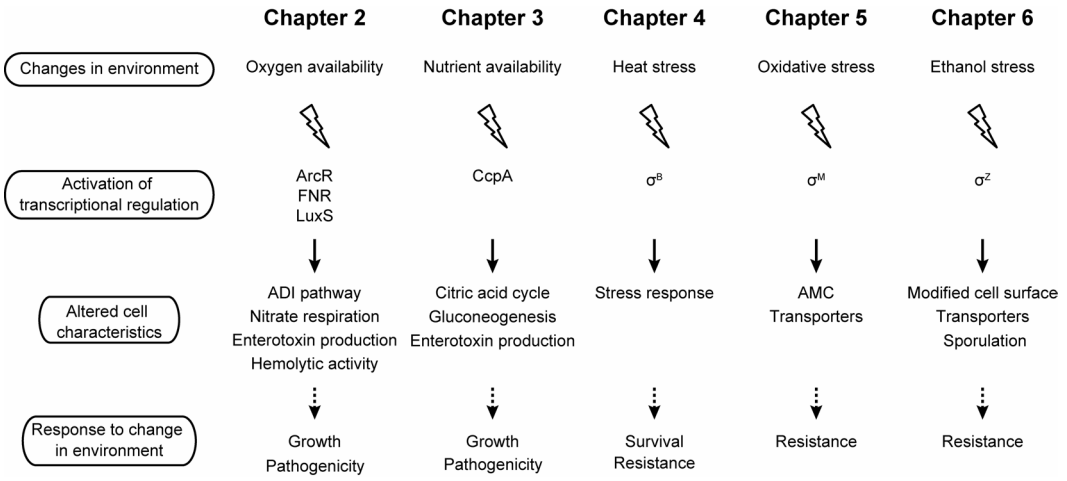

Fig. 1. Overview of the various stresses, environmental changes and subsequent responses of *B. cereus* ATCC 14579 addressed in this thesis.

Transcriptional regulation plays an important role in differential gene expression in the diverse growth phases and growth conditions. One of the best studied transcriptional regulators, conserved in many Gram-positive bacteria, is the catabolite control protein CcpA (Warner and Lolkema, 2003). In Chapter 3, the regulatory role of CcpA in *B. cereus* is studied (Fig. 1), further expanding the possibilities of comparing CcpA regulation in different Gram-positive bacteria. The genes regulated by CcpA were determined by transcriptional analysis

comparing the wild-type and a *ccpA* deletion strain. Consequently, the genes regulated by CcpA were used to determine the *B. cereus* Catabolite Responsive Element (CRE), the DNA binding site for this regulator. The consensus CRE-site proved to be highly similar to those found in other low GC-content Gram-positives. Furthermore, CcpA was shown to be involved in optimizing the efficiency of glucose catabolism by activating genes encoding glycolytic enzymes and by repressing genes encoding the citric acid cycle and gluconeogenic enzymes. Notably, transcription of the genes encoding non-hemolytic enterotoxin (Nhe) and hemolytic (Hbl) enterotoxin appeared to be repressed by CcpA, and putative CRE-sites were identified for the corresponding enterotoxin operons. This points towards metabolic control of enterotoxin gene expression and suggests that, besides activation by the major virulence transcriptional regulator PlcR, glucose sensing by CcpA is an additional mode of control of Nhe and Hbl expression in *B. cereus* (Granum *et al.*, 1999; Okstad *et al.*, 1999). In other microorganisms, CcpA has been shown to play roles in various processes, such as biofilm formation in *B. subtilis* (Stanley *et al.*, 2003), sporulation and production of enterotoxin in *C. perfringens* (Varga *et al.*, 2004) and production of virulence factors in *Staphylococcus aureus* (Seidl *et al.*, 2006). For *B. cereus*, it has also been shown that CcpA is involved in processes such as biofilm formation and sporulation (Wijman *et al.*, in preparation). Moreover, an *in silico* search of the genome using the CRE binding site for CcpA showed a number of possible targets for CcpA regulation that were not identified to be regulated under the conditions tested. Comparison of the genes regulated by CcpA in the different Gram-positives will identify the preferred metabolic pathways of these species, helping to recognize general and specific responses of different bacteria to metabolic and environmental stress.

The *B. cereus* genome includes over 300 annotated transcriptional regulators, indicating its large capacity to modulate gene expression (Ivanova *et al.*, 2003; Overbeek *et al.*, 2003). A number of these transcriptional regulators is commonly found amongst Gram-positives, such as CodY (Sonenshein, 2005) and CtsR (Chastanet *et al.*, 2003), whereas others are only present in the *B. cereus* group of species, such as PlcR, which has been shown to be involved in enterotoxin gene expression (Slamti and Lereclus, 2005) (Fig. 2). Only a limited number of regulators can be found in all prokaryotes (Fig. 2). Studying these regulators at different levels of speciation will help to identify general and species specific responses to a range of environmental conditions.

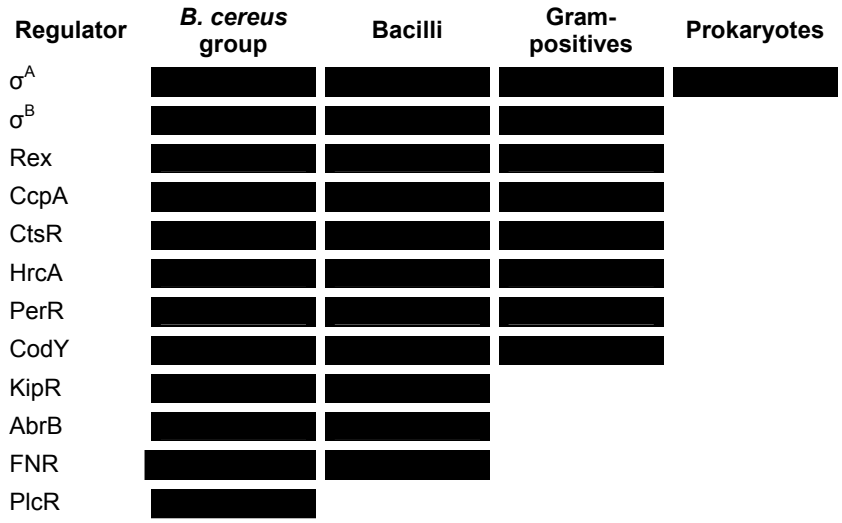

Fig. 2. Simplified overview of presence of transcriptional regulators in different levels of speciation.

Alternative σ factors

Alternative σ factors can interact with RNA polymerase (RNAP), replacing the housekeeping σ factor and initiating expression of the regulons of these alternative σ factors. Interactions of alternative σ factors with RNAP may be activated upon encountering changes in the environment, such as high temperature or nutritional shortage, which require a response of the bacterial cell.

The most studied alternative σ factor in Gram-positive bacteria is σ^B, which was shown to be involved in a range of stress responses of a highly diverse group of Gram-positives (van Schaik *et al.*, 2004a). The *B. cereus* σ^B regulon was initially characterized by proteome and in vitro transcription analyses (van Schaik *et al.*, 2004b). In Chapter 4 comparative transcriptome analysis of the wild-type versus the *sigB* deletion strain revealed the σ^B regulon in the response to heat stress (Fig. 1). New members of the σ^B regulon that could be identified included a histidine kinase and two genes that have predicted functions in spore germination. For *Bacillus anthracis*, it was shown that a large number of proteins present within the spore are already formed during growth, i.e. before the onset of the sporulation process (Bergman *et al.*, 2006), which add further evidence that properties of spores are determined by the history of the vegetative cells (Cortezzo and Setlow, 2005; de Vries *et al.*, 2004). This shows that studying vegetative cells is not only important to gain knowledge on stress response, growth and toxin production, but also to gain knowledge on spore properties, such as the ability to germinate and the resistance to food processing steps. Moreover, comparison of the *B. cereus* σ^B regulon with those of *B. subtilis*, *Listeria monocytogenes* and *Staphylococcus aureus* showed the different σ^B regulons to have only four genes in common, including *sigB*, and the σ^B regulatory protein encoding genes *rsbV* and *rsbW*. Interestingly, the fourth gene encodes a CsbD resembling protein (Chapter 4). Until now, the function of this small protein CsbD (62 and 66 amino acids in *B. subtilis* and *B.*

cereus, respectively) is unknown, and assessment of its function may clarify its role in stress response. An explanation for the relatively small size of the σ^B regulon of *B. cereus* could be that σ^B has been introduced in the *B. cereus* genome rather recently. This fits with the role of σ^B in *B. cereus* in the development of stress resistance and its activation upon a number of different stress conditions. However, σ^B does not seem to be very important for the regulation of metabolic pathways or the expression of virulence determinants in *B. cereus*, whereas the opposite has been shown in the Gram-positive pathogens *L. monocytogenes* and *S. aureus* (Kazmierczak *et al.*, 2003). It is believed that the different σ^B regulons have developed through evolution to the needs and niches occupied by the different species, in which transcription factors appear to be primarily responsible for the plasticity of the transcriptional regulatory networks (Lozada-Chavez *et al.*, 2006).

Although a number of studies addressed the function of other alternative σ factors of the *B. cereus* group, no studies had been performed on ECF σ factor regulons. Only two ECF σ factors of the *B. cereus* group had been studied previously. σ^W of *B. thuringiensis* was shown to have a role in regulating β-exotoxin I production (Espinasse *et al.*, 2004) and BA2502 of *B. anthracis* is putatively involved in β-lactamase expression (Ross *et al.*, 2005). Comparative genomic studies described in Chapter 1 of this thesis showed that for these two ECF σ factors, orthologous genes can be found on the genome of all *B. cereus* species sequenced until now (Ivanova *et al.*, 2003). The genomes of the individual species of the *B. cereus* group contain 10 to 18 ECF σ factors, which cluster into 23 orthologous groups. Eight of these twenty-three ECF σ factors were shown to be present in all species of the *B. cereus* group (Chapter 1). For *B. cereus* ATCC 14579, 10 ECF σ factors are annotated and one of these ten ECF σ factors (BC2794) was shown to divert phylogenetically from the other nine ECF σ factors (Bc-sig5861 in Chapter 4, Fig. 1). In contrast, the putative promoter binding site for this σ factor, BC2794, did show similarity with other ECF σ factor binding sites, and also overall similarity searches indicate BC2794 to be an ECF σ factor. Interestingly, the gene following BC2794 is not encoding a typical putative ECF anti- σ factor, as is the case for the other nine ECF σ factors annotated for *B. cereus* ATCC 14579. The gene preceding BC2794 encodes a Clp protease, and possibly has a role in activation of BC2794 by degradation of its σ factor. Clp proteases have been shown to be involved in specific degradation of ECF anti- σ factors in *B. subtilis* previously (Zellmeier *et al.*, 2006). This indicates this Clp protease might also be involved in activating ECF σ factors other than BC2794. This hypothesis is strengthened by the presence of this ECF σ factor and the Clp protease in all sequenced members of the *B. cereus* group.

The comparison of *B. subtilis* and *B. cereus* σ factors only revealed one ECF σ factor with reasonable similarity with an ECF σ factor, σ^M, of *B. subtilis* and this σ factor was studied in more detail (Chapter 5 and see Fig. 1). Exposure of *B. cereus* ATCC 14579 to a wide range of stresses revealed expression of *sigM*, encoding σ^M, to be up-regulated mainly in the presence of ethanol and after alkaline pH-shock. Next to this, disc diffusion tests showed the *sigM* deletion mutant to be more sensitive to oxidizing agents and to be more resistant to cell wall targeting antibiotics than its wild-type. The σ^M regulon was determined by comparative transcriptional analyses of the wild-type and the *sigM*-deletion strain after exposure to ethanol. The putative σ^M regulon was shown to consist of 29 genes with several

of these genes having putative roles in counteracting oxidative stress, such as an NADH oxidase, a ferredoxin, a lysine decarboxylase and several genes encoding enzymes involved in methionine metabolism leading toward L-cysteine production, including *luxS*. Next to this, the promoter binding site for the *sigM*-operon was determined by 5'RACE and a subsequent promoter binding site search was performed to identify σ^M binding sites for the putative σ^M regulated genes. Since the consensus promoter binding site for *B. cereus* ATCC 14579 σ^M, its regulon and corresponding functionalities are different from corresponding features in *B. subtilis*, it can be concluded that σ^M plays a unique role in *B. cereus* stress response and survival.

A second *B. cereus* ATCC 14579 ECF σ factor, σ^Z, was studied as well (see Chapter 6 and see Fig. 1). The protein sequence of σ^Z shows overall similarity with ECF σ factors. Furthermore, the gene encoding σ^Z, *sigZ*, constitutes an operon with *asfZ*, encoding the putative anti-σ factor of σ^Z. Expression analysis revealed *sigZ* to be induced by an array of stresses, including exposure to ethanol, alkaline pH and heat shock. Moreover, the promoter binding site for the *sigZ*-operon was identified by 5'RACE. Phenotypic characterization of *B. cereus* ATCC 14579 and its *sigZ*-deletion strain revealed diminished growth performance and sporulation capacity. Comparative transcriptome analysis of these strains did not allow for assessment of σ^Z-controlled genes, since expression of a large number of genes was found to be affected pointing to pleiotropic effects. However, the putative σ^Z regulon was successfully established by analyzing the transcriptome of a *sigZ*-overexpression strain. Overexpression of *sigZ* was shown to affect expression of 42 genes, including 33 genes encoding extracytoplasmic proteins with functions in transport and cell wall modification. One of the operons identified encodes an oligopeptide permease, involved in the transport of small peptides. In *B. thuringiensis,* a similar oligopeptide permease has been linked to activation of hemolytic activity (Gominet *et al.*, 2001). Moreover, the σ^Z regulon was shown to contain genes encoding cell wall modification proteins. Induction of σ^Z upon stress exposure may therefore alter the cell surface, consequently modulating the interaction of *B. cereus* with its environment. The human intestine is an environment where interactions of the cell's surface with for instance intestinal cells are of great importance, and where *B. cereus* displays hemolytic activities. A role for σ^Z and its regulon in regulation of the virulence status of *B. cereus* remains to be elucidated.

In comparison to σ^B, the activation of ECF σ factors upon stress exposure is generally low, as shown by both western-blotting for σ^Z and qPCR for both *sigM* and *sigZ*. Furthermore, several aspects complicate the determination of ECF σ factor regulons, due to the fact that different ECF σ factors may recognize the same promoter binding site, as is the case for σ^W and σ^X of *B. subtilis* (Helmann, 2002). Consequently, overexpression of a specific ECF σ factor may result in the induction of the regulons of other ECF σ factors. In addition, for the promoter binding site of the *B. subtilis* ECF σ factor σ^M it has been shown that the -35 sequence is more conserved than the -10 sequence (Jervis *et al.*, 2007), complicating the identification of ECF σ factor promoter binding sites. Another aspect influencing the determination of an ECF σ factor regulon is that often more than one σ factor binding sites are present in front of an ECF σ factor regulated gene, indicating the expression of such a gene is not solely dependent on one (ECF) σ factor. These complicating aspects were clearly shown

for the ECF σ factors of an undomesticated strain of *B. subtilis*, for which an approach of multiple deletions in one case showed additional phenotypes affected (triple deletion of *sigM*, *sigW* and *sigX*), whereas for other ECF σ factors the multiple deletions (quadruple deletion of *sigV*, *sigY*, *sigZ* and *ylaC*) did not affect additional phenotypic traits (Mascher *et al.*, 2007). Consequently, to study an ECF σ factor, a diversity of experimental approaches should be used to be able to define its role in transcriptional regulation. In general, for the three σ factors of *B. cereus* studied in his thesis, σ^B, σ^M and σ^Z, only a small regulon was defined, showing these σ factors to have a role in fine tuning of the response of *B. cereus* to environmental changes (Chapter 5 and 6). Moreover, fine tuning of gene expression becomes more important when the lifestyle of the bacterium is more complicated, and thus the number of annotated (ECF) σ factors for these bacteria is high, as in *Bacilli* and *Streptomyces* (see Chapter 1, Table 2).

Future perspectives

An overview of the different environmental changes encountered, and subsequent responses of *B. cereus* that were studied in this thesis, show that *B. cereus* is very versatile in its reaction to different stresses (Fig. 1). This makes it challenging to determine the role of specific transcriptional regulators in the stress response of this bacterium, as these regulators are often not responsible for the complete response, but are only involved in fine tuning of the response. Studying transcriptional regulators by use of transcriptome analysis, as shown for CcpA, σ^B, σ^M and σ^Z in this thesis (Chapter 3 to 6), can lead to the identification of novel responses of the bacterial cell to environmental changes that may occur in food processing. The research described in this thesis indicates that monitoring the behaviour of microorganisms by the use of omics techniques, including transcriptomics, proteomics and/or metabolomics in for instance food processing steps generates valuable data, but additional studies, including virulence assessment, are required. Therefore, in future research the integration of so-called omics data with phenotypic data should be further expanded, and for this purpose the knowledge and tools of bio-informatics will be of great use.

Another important aspect relates to the species and the selected strains studied, since most studies concentrate on only a limited number of species and corresponding strains. Such strains have often been studied in a laboratory environment for decades and have possibly lost essential traits in comparison to undomesticated strains. For instance, for domesticated strains of *B. subtilis* it has been shown that it is lacking multicellular differentiation, and consequently undomesticated strains of *B. subtilis* are now included in the research of complex phenotypes such as pellicle and biofilm formation (Aguilar *et al.*, 2007). More attention should also be given to the impact of strain diversity on the performance range of studied species. Notably, Wijman *et al.* (2007) recently showed large differences in biofilm forming capacity to exist between environmental, food and clinical isolates of *B. cereus*.

In conclusion, the combination of genomic, phenotypic and transcriptome data presented in this thesis showed the transcriptional regulation of *B. cereus* to be multicomponent and complex, making *B. cereus* highly versatile in coping with environmental changes. Further unravelling of the transcriptional regulation of the versatile

food pathogen *B. cereus* by a combination of techniques is challenging and may provide tools for enhanced control of this pathogen in food and clinical settings.

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Samenvatting

De alom voorkomende bacterie *Bacillus cereus* is in staat voedselvergiftiging te veroorzaken en voedsel te bederven. De symptomen van door *B. cereus* veroorzaakte voedselvergiftiging zijn over het algemeen mild en zijn geassocieerd met diarree of overgeven, welke worden veroorzaakt door respectievelijk enterotoxines en cereulide. Recentelijk zijn er een aantal gevallen van *B. cereus* voedselvergiftiging beschreven waarbij de symptomen zo ernstig waren dat deze uiteindelijk de dood tot gevolg hadden. Onderzoek naar deze bacterie is van belang om meer te weten te komen over de groei van *B. cereus* in voedsel en in welke situaties *B. cereus* in staat is het voedsel te bederven en toxines te vormen. Daarnaast is het belangrijk te weten hoe *B. cereus* reageert op verschillende stappen van de voedselverwerking en conservering. De moderne voedselverwerking en conservering is zo minimaal mogelijk om voedsel zo gezond en vers mogelijk te laten zijn, maar moet uiteindelijk wel een veilig product opleveren.

B. cereus is niet alleen in staat te groeien onder condities met zuurstof, maar ook onder condities zonder zuurstof. Deze zogenaamde anaerobe groei vindt plaats door middel van fermentatie en anaerobe respiratie, en maakt de groei van *B. cereus* mogelijk in omgevingen waar zuurstof schaars is, zoals vacuüm verpakt voedsel en het humane spijsverteringsstelsel. Om meer te weten te komen over de groei van *B. cereus* in anaerobe condities is de anaerobe groei in een rijk medium gevolgd en vergeleken met aerobe groei (Hoofdstuk 2). Uit deze vergelijking bleek allereerst dat *B. cereus* onder anaerobe condities minder snel groeit dan onder aerobe condities, daarnaast is gebleken dat *B. cereus* anaeroob niet in staat is om de hoge aantallen te bereiken die wel onder aerobe condities bereikt worden. De redenen hiervoor zijn onderzocht door te kijken naar de expressie van de genen van *B. cereus* met microarrays. Het vergelijk in genexpressie tussen anaerobe en aerobe groei liet zien dat er vooral verschillen waren in het metabolisme van de cel, waarbij nog niet eerder voor *B. cereus* omschreven metabole routes en reacties betrokken waren. Erg interessant was dat voor hemolytische enzymen en enterotoxines de genexpressie omhoog ging in situaties waar zuurstof gelimiteerd was. Voor de enterotoxines was dit aan het eind van de aerobe groei, waar de zuurstof schaars is door het grote aantal cellen dat zuurstof behoeft, en voor de hemolytische enzymen was dit verschil er in groeiende anaerobe cellen vergeleken met groeiende aerobe cellen. In het spijsverteringsstelsel kan zuurstoflimitatie voorkomen en dit is ook de plek waar *B. cereus* de diarree-veroorzakende toxines produceert. De activatie van de virulentie genen in zuurstof gelimiteerde condities, kan hieraan gekoppeld zijn.

Als het leefmilieu van de bacterie verandert, door bijvoorbeeld blootstelling aan stress (zoals in voedsel verwerking), zal deze zich moeten aanpassen. Om deze aanpassingen te reguleren heeft een bacterie verschillende transcriptionele regulatoren. In Hoofdstuk 3 wordt het onderzoek naar een specifieke transcriptonele regulator van het metabolisme gerapporteerd. Dit cataboliet controle eiwit (CcpA) is geconserveerd in een groot aantal bacteriën en is betrokken bij het zo efficiënt mogelijk gebruiken van suikers. Door middel van microarrays en het vergelijken van een wild-type stam met een *ccpA* deletie stam kon de rol

van CcpA in *B. cereus* worden aangetoond. CcpA in *B. cereus* controleert de genexpressie op een zodanige manier dat glucose op een efficiënte en voordelige manier wordt gebruikt. Opmerkelijk was het feit dat aangetoond kon worden dat ook de expressie van genen die enterotoxines coderen gereguleerd wordt door CcpA. Dit duidt er op dat deze enterotoxines alleen tot expressie komen als er geen glucose meer aanwezig is in het medium. In Hoofdstuk 2 was al aangetoond dat deze enterotoxines tot expressie komen aan het einde van de groei, het moment wanneer de glucose geconsumeerd is.

Een andere groep van transcriptionele regulatoren zijn de σ factoren, die fungeren als co-factoren van het RNA polymerase (RNAP). Elke σ factor herkent een bepaalde DNA sequentie en binding van het σ factor/RNAP-complex aan DNA kan de expressie van het verderop liggende gen activeren. Eén van de meest onderzochte σ factoren van Gram-positieve bacteriën is σ^B, waarvoor is aangetoond dat deze geactiveerd wordt in reactie op verschillende stressen. Ook voor *B. cereus* is het aangetoond dat σ^B een rol speelt bij het reguleren van genexpressie in reactie op verschillende stressen. Welke genen er in *B. cereus* door σ^B aangezet worden in reactie op hitte stress is in Hoofdstuk 4 onderzocht door middel van microarrays waarbij de genexpressie van de wild-type stam is vergeleken met die van een *sigB* deletie stam. Genen onder controle van σ^B codeerden voor eiwitten met functies gerelateerd aan stress, ontkieming van sporen en de regulatie van expressie van *sigB* zelf. Daarnaast liet dit onderzoek zien dat vergeleken met andere species zoals *Bacillus subtilis*, *Listeria monocytogenes* en *Staphylococcus aureus* het aantal door σ^B gereguleerde genen in *B. cereus* relatief klein is. Ook liet dit vergelijk zien dat er maar vier genen waren die in alle vier vergeleken species gereguleerd worden door σ^B. Aangenomen wordt dat de groep van σ^B gereguleerde genen zich door evolutie heeft aangepast aan de specifieke behoeftes van de verschillende species.

Een aparte groep binnen de σ factoren zijn de ExtraCytoplasmatische Functie (ECF) σ factoren. Deze ECF σ factoren reguleren de expressie van genen die coderen voor eiwitten met een functie buiten het cytoplasma en worden vooral gevonden in de celmembraan en de celwand. Twee van deze ECF σ factoren van *B. cereus* zijn in meer detail onderzocht. Hieruit bleek dat onderzoek aan deze ECF σ factoren bemoeilijkt wordt door een aantal zaken, zoals het feit dat verschillende ECF σ factoren dezelfde DNA sequentie herkennen en het feit dat veel genen die gereguleerd worden door een ECF σ factor ook nog gereguleerd worden door een andere (ECF) σ factor. In Hoofdstuk 5 is de rol van de ECF σ factor σ^M in *B. cereus* onderzocht door middel van microarrays en het vergelijken van de reactie van de wild-type stam en een *sigM* deletie stam op ethanol stress. Uit dit onderzoek blijkt dat σ^M genen reguleert die eiwitten coderen met een functie in het reageren op oxidatieve stress, zoals veroorzaakt kan worden bij blootstelling aan ethanol. De door σ^M gereguleerde genen omvatten een route die leidt tot de productie van L-cysteine. Voor eukaryote cellen is beschreven dat L-cysteine een rol speelt bij het weerstaan van oxidatieve stress. De tweede ECF σ factor die in meer detail is bestudeerd is σ^Z. Om de rol van deze ECF σ factor te bepalen is een stam gecreëerd die σ^Z tot overexpressie brengt, en de genexpressie van deze stam is vervolgens vergeleken met een controle stam. Uit dit onderzoek bleek dat σ^Z genen reguleert die eiwitten coderen met een functie in het transport van moleculen over de

celmembraan en in het veranderen van het cel oppervlak, hetgeen de interactie van de bacteriële cel met zijn omgeving kan beïnvloeden. Op deze manier kan de activatie van σ^Z en de daarop volgende productie van specifieke eiwitten, in reactie op verschillende types stress, ervoor zorgen dat de stress geen schade toebrengt aan *B. cereus*.

Het onderzoek naar de fine tuning van transcriptionele regulatie in *B. cereus*, zoals beschreven in dit proefschrift heeft geleid tot de identificatie van nieuwe responsen van *B. cereus* tegen stress. De verkregen resultaten kunnen gebruikt worden bij het optimaliseren van voedselverwerking, om een zo veilig en gezond mogelijk product te verkrijgen.

List of Publications

Y.P. de Vries, **M. van der Voort**, J. Wijman, W. van Schaik, L.M. Hornstra, Willem M. de Vos and T. Abee, 2004, Progress in food-related research focussing on *Bacillus cereus*, Microbes and Environments, Vol. 19, No. 4, 265-269.

W. van Schaik, **M. van der Voort**, D. Molenaar, R. Moezelaar, W.M. de Vos and T. Abee, 2007, Identification of the σ^B regulon of *Bacillus cereus* and conservation of σ^B-regulated genes in low-GC-content Gram-positive bacteria, Journal of Bacteriology, Vol. 189, No. 12, 4384-4390.

M. van der Voort, O.P. Kuipers, G. Buist, W.M. de Vos and T. Abee, Assessment of CcpA-mediated catabolite control of gene expression in *Bacillus cereus* ATCC 14579, submitted for publication.

M. van der Voort, T. Abee, Anaerobic induction of metabolic pathways, alternative respiration capacity, nitrosative stress response and virulence factors in *Bacillus cereus* ATCC 14579, submitted for publication

M. van der Voort, G. Nikitas, W.M. de Vos and T. Abee, The regulon of the *Bacillus cereus* ATCC 14579 ECF σ factor σ^M is induced by ethanol stress and comprises transporter and oxidative stress genes, submitted for publication.

M. van der Voort, W.M. de Vos and T. Abee, The ECF σ factor σ^Z of *Bacillus cereus* ATCC 14579 responds to different stresses and regulates cell surface modifications, submitted for publication.

Nawoord

Na zes jaar hard werken ligt er uiteindelijk dit boekje. Om op dit punt te komen heb ik me behoorlijk ingespannen, maar je doet het natuurlijk nooit alleen. Daarom wil ik de mensen bedanken die hebben geholpen om dit boekje voor elkaar te krijgen. Ik wil beginnen bij iedereen met wie ik op de zelfde afdeling of in het zelfde huis heb gewoond. Het is altijd prettig om met iemand te kunnen praten als je thuis komt van een dag op het lab. Daarnaast moest er natuurlijk gebasketbald worden. De mensen met wie ik de afgelopen zes jaar heb mogen spelen, hebben er niet alleen voor gezorgd dat ik prettig kon basketballen, maar ook voor een hoop gezelligheid. De ontspannig heeft de afgelopen zes jaar niet alleen plaatsgevonden in Wageningen, maar ook in andere delen van het land. Nog niet zo lang geleden is daar Valkenswaard bijgekomen, en ik wil dan ook mijn schoonfamilie bedanken voor het warme welkom in hun familie. Ook mijn aangetrouwde vrienden zorgen tegenwoordig voor leuke en lekkere etentjes. Maar de meeste tijd buiten Wageningen heb ik toch doorgebracht in Noordwijk. Een essentieel onderdeel daarvan was het bezoeken van de kroeg met de Hufters. We zien elkaar een stuk minder dan vroeger, maar het is wel altijd gezellig. Ook de bezoekjes aan mijn ouders en de familie van Leeuwen zorgen altijd voor de nodige ontspanning.

Naast alle gezelligheid moest er ook gewerkt worden om uiteindelijk tot dit boekje te komen. Willem heeft me daarbij in het begin op weg geholpen en was ook daarna bereid zijn mening en ideeën te delen. Met Marcel T. heb ik ook altijd prettig samengewerkt, jouw ervaring op het lab komt nog elke dag van pas. Daarnaast heb ik altijd een hoop lol gehad met de collega's met wie ik op de zelfde kamer heb gezeten, Wout, Mariël, Aarieke, Maarten en Diego, maar we hebben ook serieus over werk kunnen praten. Gerda, jij hebt alle zaken verzorgd die niets met wetenschap te maken hebben, maar die wel belangrijk zijn om wetenschappelijk onderzoek te kunnen doen. Bedankt alle collega's die de afgelopen zes jaar de revue zijn gepasseerd, het was erg gezellig om met jullie koffie te drinken, te lunchen, in de kroeg te zitten en alle andere activiteiten te ondernemen. Bij de MolGen groep uit Groningen heb ik de fijne kneepjes van het microarray werk geleerd. Het was erg prettig samenwerken. Naast de collega's zijn er ook nog de bazen. Marcel Z., als groot opperhoofd van de leerstoelgroep heb je de omstandigheden verzorgd waarin het onderzoek plaats kon hebben. Roy, jij hebt het C-009 project op rolletjes laten verlopen. Willem de V. met je altijd scherpe feed-back heb je de kwaliteit van dit boekje omhoog gekregen. Tjakko, ook voor jou waren de microarray analyses nieuw, ik vind dat we er uiteindelijk wat moois van hebben weten te maken. Jouw enthousiasme over mijn resultaten heeft me vaak over de dode punten heen geholpen.

Soms komen vrienden, geliefden, kamergenoten en collega's samen in één. Lieve Janneke, jij hebt het maken van dit boekje zoveel makkelijker gemaakt voor mij.

Bedankt!

Curriculum vitae

Menno van der Voort werd op 2 maart 1979 geboren te Noordwijk. In 1997 behaalde hij zijn VWO-diploma aan het Leeuwenhorst College te Noordwijkerhout. In september 1997 begon hij aan een studie Biologie aan de Universiteit Leiden, waarbij hij de specialisatie moleculaire biologie koos. Tijdens zijn studie heeft Menno onderzoek gedaan aan de promoter regio van het rijst vasculaire ontwikkelingsgen Oshox1 bij Moleculaire en Ontwikkelingsgenetica aan de Universiteit Leiden, onder begeleiding van Prof. Dr. J.H.C. Hoge. Tevens heeft hij gewerkt aan de transformatie efficiëntie van *Fusarium oxysporum* bij Fythopathologie aan de Universiteit van Amsterdam, onder begeleiding van Prof. Dr. B.J. Cornelissen. Nadat Menno in oktober 2001 was afgestudeerd, is hij in februari 2002 begonnen aan zijn promotieonderzoek bij de leerstoelgroep Levensmiddelenmicrobiologie van Wageningen Universiteit met als onderwerp de fine tuning van de stress response van *Bacillus cereus*. Dit onderzoek maakte deel uit van een IOP-Genomics project (IGE01018) en van het "Food Preservation and Safety" project van het Top Institute Food and Nutrition (voorheen Wageningen Centre for Food Sciences) en werd uitgevoerd onder begeleiding van Prof. Dr. Tjakko Abee en Prof. Dr. Willem M. de Vos. De resultaten van dit onderzoek staan in dit proefschrift beschreven. Vanaf 1 januari 2007 is Menno werkzaam als post-doctoraal onderzoeker bij de leerstoelgroep Levensmiddelenmicrobiologie van Wageningen Universiteit.

VLAG graduate school activities

Discipline specific activities
Courses
Bio-informatics course (Wageningen UR, 2002)
Microarray workshop (RU Groningen, 2003)

Meetings
WCFS Food summit (2003)
Gram positive genomics (Baveno, Italy, 2003)
Genomics momentum (2003)
Work visit microarray facility RUG (2003, 2004)
Current contents Experimental Evolution (Wageningen, 2004)
Geneyous general meeting (2004)
Gram positive genomics (San Diego, USA, 2005)
FEMS general meeting (Madrid, Spain, 2006)

General courses
Patent Day IOP genomics (2003)
Course "Leren presenteren" (WCFS, 2004)
Workshop "Mediatraining" (WCFS, 2007)

Optionals
WCFS WE-days (2002, 2003, 2004, 2005, 2006)
WCFS colloquia (2002, 2003, 2004, 2005)
IOP genomics meetings (2003, 2004, 2005, 2006)
Preparation of PhD research proposal
VLAG PhD trip Food Chemistry and Food Microbiology (USA, 2002)
VLAG PhD trip Microbiology (Japan, 2004)

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