The alternative sigma factor σ^B and the stress response of *Bacillus cereus*

Willem van Schaik

Promotoren

Prof. Dr. T. Abee
Persoonlijk hoogleraar bij de leerstoelgroep Levensmiddelenmicrobiologie
Wageningen Universiteit

Prof. Dr. W. M. de Vos
Hoogleraar Microbiologie
Wageningen Universiteit

Prof. Dr. Ir. M. H. Zwietering
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Prof. Dr. O. P. Kuipers
Rijksuniversiteit Groningen

Prof. Dr. J. van der Oost
Wageningen Universiteit

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Proefschrift

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ABSTRACT

Bacillus cereus is a gram-positive pathogen that is a frequent cause of outbreaks of foodborne disease. It is closely related to the insect pathogen *Bacillus thuringiensis* and the mammalian pathogen *Bacillus anthracis*, which causes the disease anthrax. All these bacteria can form spores, which are resistant to many extreme conditions. Upon germination of the spores in a food product or in the host, vegetative cells may multiply leading to product spoilage, toxin production or infection. The mechanisms that *B. cereus* vegetative cells use to counter adverse environmental conditions, determine their capacity to grow in food products or to cause disease. This thesis describes the stress response of vegetative cells of *B. cereus* and the role of the alternative sigma factor σ^B in this process. Upon exposure to a mild stress, *B. cereus* will gain the ability to survive for a prolonged period of time under conditions that are rapidly lethal for non-stressed cells. Several proteins of *B. cereus* are induced upon exposure to stress. These "stress-proteins" include chaperones, but also metabolic enzymes and RsbV, the anti-anti-sigma factor of the alternative sigma factor σ^B. Sigma factors are dissociable subunits of the enzyme RNA polymerase, which coordinate the process of transcription. In *B. cereus*, σ^B is activated under several stress conditions including osmotic stress, exposure to ethanol and upon a mild heat shock. Furthermore, σ^B contributes to the adaptation to and survival at high and low temperatures. Remarkably, the *sigB* deletion mutant showed a defect in the use of a number of amino acids as nitrogen sources, thereby showing that stress response and cellular metabolism are interconnected pathways. Surprisingly, the *sigB* deletion mutant of *B. cereus* showed hyperresistance to hydrogen peroxide. The increased expression of the vegetative cell catalase in the *sigB* deletion mutant apparently contributes to this notable phenotypic trait. Several σ^B-dependent genes were identified by a combined proteome and *in vitro* transcription analysis. Some of the identified genes encode enzymes with roles in cellular metabolism, which shows that metabolic rearrangements are part of the stress response of *B. cereus*. The intracellular concentration and activity of the σ^B protein is regulated on at least two different levels: transcriptionally and post-translationally. The transcriptional regulation of *sigB* gene expression appears to be relatively simple: *sigB* is preceded by a σ^B-dependent promoter and so σ^B autoregulates the expression of its structural gene. Post-translational regulation of σ^B activity involves a partner-switching mechanism involving σ^B, the anti-sigma factor RsbW and the anti-anti-sigma factor RsbV. Furthermore, a unique regulator, RsbY, has a key role in the process of σ^B activation. This phosphatase is structurally different from the regulators of σ^B activity in other gram-positive bacteria as it has a characteristic N-terminal CheY response regulator domain. The research described in this thesis contributes to the understanding of the stress response of *B. cereus* and the role of σ^B in this process. It has revealed that in *B. cereus* σ^B not only has a role in stress response, but that it also contributes to metabolic flexibility, which can be a factor in the successful colonization of the different ecological niches that *B. cereus* inhabits. This knowledge may be applied for the development and optimization of techniques for the production of high-quality, microbiologically safe foods.

1 INTRODUCTION AND OUTLINE OF THE THESIS

ABSTRACT

Bacillus cereus is a gram-postive, rod-shaped, facultatively anaerobic, spore-forming bacterium. It is frequently isolated from foods and it can cause two types of foodborne disease, which are described as diarrheal type and emetic type, to reflect the main symptoms of the disease. Furthermore, it is an important food spoilage organism, especially in dairy products. In this chapter several topics will be discussed, including the significance of *B. cereus* as a foodborne pathogen, the virulence factors that are produced by *B. cereus*, and the phylogenetic relationship of *B. cereus* with other *Bacilli*, in particular with *Bacillus anthracis*. Furthermore, the knowledge gained from the complete genome sequences of *B. cereus* and its close relatives is appraised. Finally, the outline of this thesis is provided.

THE SIGNIFICANCE OF *B. CEREUS* AS A FOODBORNE PATHOGEN

B. cereus was first described in 1887, when it was isolated from the air in a cow-shed in the United Kingdom (31). This isolate is the type strain of *B. cereus*, *B. cereus* ATCC 14579. An electron micrograph of vegetative cells of *B. cereus* ATCC 14579 is shown in Fig. 1.

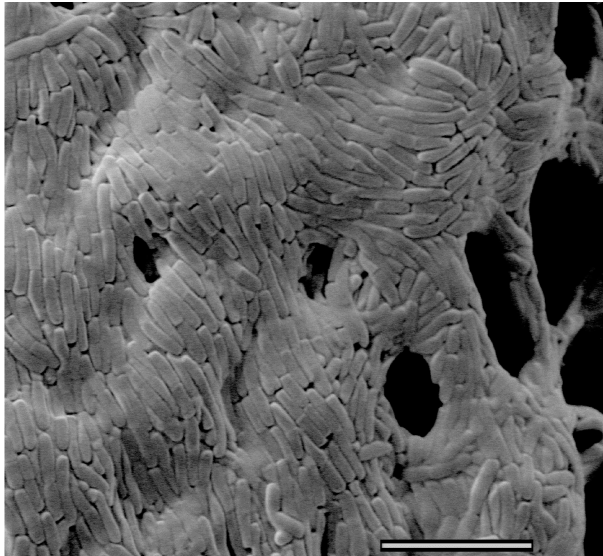

Fig. 1. Scanning electron micrograph of vegetative cells of *Bacillus cereus* ATCC 14579. The scale bar represents 10 µm. This figure was kindly provided by Jan Dijksterhuis.

In 1950 the Norwegian doctor Steinar Hauge, who investigated an outbreak of foodborne disease characterized by diarrhea, was the first to recognize that *B. cereus* could cause food poisoning. The symptoms in this particular case were caused by the consumption of a vanilla sauce that was heavily contaminated by *B. cereus*. To prove that this bacterium did indeed cause the disease, Hauge cultured the *B. cereus* strain, artificially contaminated sterile vanilla sauce with it and swallowed the sauce. After 16 hours, he developed symptoms such as abdominal pain, nausea and, finally, diarrhea, thereby proving that *B. cereus* is indeed able to cause diarrheal disease (38). The emetic variant of the disease was first described in 1974 when *B. cereus* was linked to outbreaks of foodborne disease in the United Kingdom, which had vomiting as the major symptom (64). The symptoms of the diarrheal type of *B. cereus* food poisoning occur between 8 – 16 h after ingestion of contaminated food. Symptoms of the emetic syndrome (most notably vomiting) occur much faster: between 0.5 – 6 h after consumption of contaminated food. In both types of disease symptoms generally do not last longer than 24 h (23, 51).

It is difficult to assess the current importance of *B. cereus* as an agent of foodborne disease. Because it is not a notifiable disease like other foodborne diseases such as listeriosis and salmonellosis, it is generally only reported when there are large outbreaks of *B. cereus* intoxications or when the symptoms of the disease are more severe than usual. These practices are thought to lead to a substantial underestimation of the number of cases of *B. cereus* foodborne disease. This may be especially true for the situation in the United States where, until recently, isolation of *B. cereus* in a clinical laboratory was frequently considered a contamination. This resulted in *B. cereus* plates being discarded rather than to follow up the initial isolation of *B. cereus* with further characterization and reporting (56). A comprehensive surveillance study in Europe has shown that in some countries, most notably in The Netherlands and Norway, *B. cereus* is an important foodborne pathogen (75). In The Netherlands, it was the single most important causative agent of outbreaks of food poisoning in 1999 and 2000, causing 26% and 25%, respectively, of the outbreaks in which a causative agent could be determined. Similar data were obtained in Norway where *B. cereus* accounts for 35% and 32% of the outbreaks investigated in 1999 and 2000, respectively. Comparable data come from Taiwan where between 1986 and 1995 *B. cereus* was responsible for 18% of outbreaks of foodborne disease in which a bacterial agent was responsible (66). Other countries report much lower incidences of outbreaks of *B. cereus* food poisoning. In France, for example, between 0.7% (1999) and 5.0% (2000) of outbreaks of foodborne disease were attributable to *B. cereus*. Other countries like England, Italy, and Germany report even lower numbers ranging between 0.5 and 1% (75). These last figures are in line with an epidemiological study of foodborne disease in the United States in which *B. cereus* was estimated to be responsible for 0.2% of cases of foodborne disease (61).

The differences in reported incidences between the different countries may be caused by different food consumption patterns or disparities in food production practices, but the most important factor appears to be that in many countries there is both underdiagnosis and underreporting of disease caused by *B. cereus*. This is not difficult to explain because the gastrointestinal symptoms during a *B. cereus* intoxication are generally mild and self-limiting and consequently doctors do not come into contact with patients that suffer from *B. cereus* foodborne disease. Furthermore, when gastrointestinal infections are studied in more detail, confirmation of the cause by culture often does not include *B. cereus* but is focused on pathogens like *Salmonella* and *Shigella*. The high prevalence of *B. cereus* foodborne disease in countries like the Netherlands, Norway or Taiwan, may thus be a reflection of local procedures, which more often lead to the detection of *B. cereus*. It will be interesting to see whether the recent severe cases of *B. cereus* intoxications in France (57) and Belgium (Koen De Schrijver, personal communication) that led to the deaths of three and one patient(s), respectively, will lead to altered guidelines in these countries and how these will affect the reported prevalence of *B. cereus* foodborne disease.

B. CEREUS VIRULENCE FACTORS

The full repertoire of virulence factors that *B. cereus* can produce and which contribute to its pathogenic properties are shown in Fig. 2. Their contributions to the

different symptoms of *B. cereus* disease are discussed in more detail in the next sections.

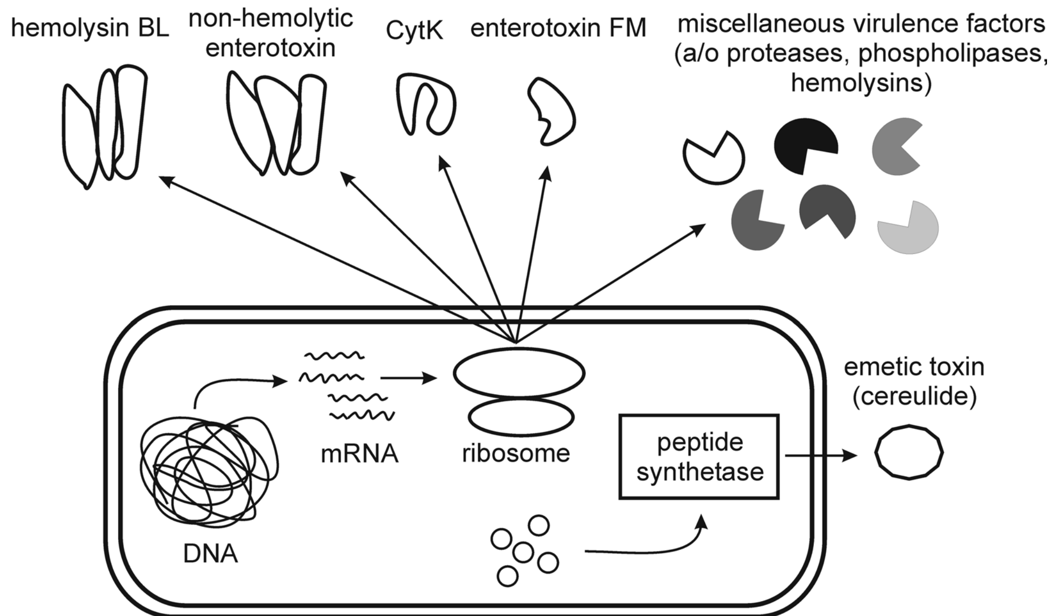

Fig. 2. *Bacillus cereus* virulence factors. The hemolytic enterotoxin BL and the non-hemolytic enterotoxin Nhe are three-component toxins. CytK and enterotoxin FM are single-component toxins. Other proteins (like phospholipases, proteases and hemolysins) can also play a role in the infectious process. All these virulence factors are ribosomally synthesized proteins. The emetic toxin (or cereulide) is a dodecadepsipeptide, which is assembled by a non-ribosomal peptide synthetase (25).

Diarrheal type of foodborne infection by *B. cereus*

The foods that are associated with the diarrheal type of *B. cereus* gastrointestinal disease include meat products, soups, vegetables, sauces and dairy products. Diarrheal disease is caused by at least two types of three-component enterotoxins. The first well characterized enterotoxin is termed hemolysin BL (Hbl), which consists of a B component and two L components, L_1 and L_2. Apart from its enterotoxic properties, it also causes hemolysis, hence its name. Separately, the three components of Hbl are nontoxic, but together they form a complex, which causes the lysis of cells by a colloid osmotic mechanism through the formation of transmembrane pores (10, 55).

A second enterotoxin that is involved in the diarrheal *B. cereus* syndrome is the non-hemolytic enterotoxin Nhe. This also is a three-component enterotoxin, consisting of the subunits NheA, NheB, and NheC. In contrast with Hbl, Nhe is not hemolytic and it only has enterotoxic properties (54).

A third toxin, CytK, was first isolated from a *B. cereus* strain, which was the causative agent in an especially severe outbreak of gastro-enteritis in France (in 1998).

Three people died in this outbreak due to necrotic enteritis, which is an extremely rare but very serious symptom in *B. cereus* foodborne disease (57). CytK forms pores in planar lipid bilayers and is cytotoxic to intestinal epithelial cells (37). CytK-type enterotoxins occur in many *B. cereus* strains that are associated with foodborne disease, but also in the type strain *B. cereus* ATCC 14579 which is generally thought to be non-pathogenic (28, 35). Factors that influence the importance of CytK in *B. cereus* pathogenesis can include amino acid substitutions, especially in important membrane-spanning regions of the toxin, or differences in *cytK* expression levels between strains (13, 28).

Two single-component enterotoxins have also been described in *B. cereus*: enterotoxin FM (5) and enterotoxin T (2). Considerably less information exists on these enterotoxins than on Hbl and Nhe. Recently, a deletion in the gene encoding enterotoxin FM was generated. This mutant had a reduced cytotoxicity and hemolytic activity, compared to the wild-type strain (58), which strongly suggests that enterotoxin FM can play a role in *B. cereus* intoxication. Enterotoxin T, first described by Agata *et al.* (2), is probably not an enterotoxin at all, as no biological activity was observed when the gene proposed to be encoding enterotoxin T (*bceT*) is overexpressed in *Escherichia coli* (15). Furthermore, the originally described *bceT* gene was unintentionally created by the accidental joining of four DNA fragments during ligation, resulting in a fusion protein with an open reading frame (ORF 101) that originated from the pathogenicity island of the *Bacillus anthracis* pXO1 virulence plasmid. This fusion protein may be responsible for the biological activity that was observed in the original description of enterotoxin T (36).

All enterotoxins are heat-labile and sensitive to proteolysis by gastric enzymes (60). This has led to the hypothesis that spores, which are very resistant to the acidity of the human stomach (17), can attach to epithelial cells in the small intestine upon stomach passage. Subsequent germination and vegetative growth will then result in enterotoxin formation, leading to diarrheal symptoms (4, 60).

Emetic type of foodborne infection by *B. cereus*

While there is a wide variety of toxins involved in the diarrheal syndrome of *B. cereus* foodborne poisoning, there is only one toxin responsible for the emetic syndrome. Foods that are associated with this type of disease are mainly rice and pasta, but it has also been reported in milk powder and infant milk formula (23). The emetic toxin, or cereulide, that is responsible for the emetic type of disease is a small dodecadepsipeptide with the chemical formula (D-*O*-Leu-D-Ala-L-*O*-Val-L-Val)$_3$. This means that it is chemically very closely related to the ionophore valinomycin. Indeed, the mode of action of emetic toxin resembles that of valinomycin, as it acts as a potassium ionophore on mitochondria (62). However emetic toxin seems to be more toxic than valinomycin in an animal model (the rodent *Suncus murinus* or house musk shrew) as between 100 – 1000 fold higher doses of valinomycin have to be administered to the animals to obtain the same incidence of emesis as for emetic toxin (3). The receptor of emetic toxin in *Suncus murinus* is the serotonin 5-HT$_3$ receptor (3). Also in humans this receptor has been linked to emesis (see for example (65)) and it therefore seems safe to assume that in humans too the serotonin 5-HT$_3$ receptor is the target of emetic toxin.

The emetic toxin is extremely resistant to heat, low pH and proteolytic enzymes in the gastrointestinal tract. This implies that the emetic syndrome is caused by the consumption of food in which the emetic toxin has been preformed by growth of *B. cereus*. An illustrative example is the practice of pre-cooking rice and subsequent storage at room-temperature to avoid the clumping of rice that can occur during low temperature storage. *B. cereus* spores, which may have survived the cooking process or which have contaminated the cooked rice during handling and storage, can germinate and grow, thereby producing emetic toxin. When the rice is reheated, *B. cereus* viable counts can be dramatically reduced, so the food may be safe to eat from a microbiological point-of-view. However, the food still contains the heat-stable emetic toxin, which may cause vomiting upon ingestion of the reheated rice. Indeed, these circumstances led to the first described cases of the emetic type of *B. cereus* foodborne disease (64) and remains an important exposure route to this date as several outbreaks of foodborne disease that follow this scenario have been described over the past years, including one in The Netherlands in 2000, in which 116 persons were affected (26, 49, 72).

The peculiar chemical composition of the emetic toxin already suggests that it is not a ribosomally synthesized peptide. Indeed, recently a number of studies have provided evidence that the emetic toxin is a product of a single non-ribosomal peptide synthetase, which is unique to emetic toxin producing strains. Consequently, this gene is not found in *B. cereus* ATCC 14579 (24, 25, 44, 80). It is not yet known if the non-ribosomal peptide synthetase gene is carried on the chromosome or on a plasmid in emetic toxin producing strains.

The role of other toxins in disease caused by *B. cereus*

The above-described toxins are the ones that are most important in foodborne disease caused by *B. cereus*, but it should be noted that *B. cereus* can produce a variety of other virulence factors, including phosphatidylcholine- and phosphatidylinositol-specific phospholipase, sphingomyelinase, collagenase, proteases, and several hemolytic proteins (9, 33, 69). A remarkable toxin, which has a poorly understood role in pathogenesis is the ADP-ribosyltransferase C3cer, which targets Rho GTPases in host target cells and thereby disrupts the actin skeleton of eukaryotic cells (81, 82).

With such a veritable arsenal of virulence factors, it is not surprising that *B. cereus* can also cause non-gastro-intestinal infections in which these factors, but also the enterotoxins or the emetic toxin described above, play important roles. *B. cereus* can be the cause of severe, even lethal infections such as sepsis, pneumonia, meningitis, endocarditis, or wound infections, especially in immunocompromised patients (reviewed in (51) and (21)). Especially neonates may be at considerable risk for a hospital-acquired *B. cereus* infection (42). Additionally, *B. cereus* can cause fulminant eye infections (endophthalmitis) usually upon trauma, and sometimes upon an operation on the eye (14, 18, 27). The treatment of clinical *B. cereus* infections may be difficult because *B. cereus* is usually resistant to often-used antibiotics like penicillins and cephalosporins (32).

B. CEREUS AND CLOSELY RELATED BACTERIA IN THE *B. CEREUS* GROUP

The genus *Bacillus* is a very heterogeneous group of bacteria, which consists of species with large variations in phenotypes, nutritional requirements and other physiological and metabolic characteristics (16, 79). Currently, ribosomal RNA sequences are considered the most useful marker to infer phylogenetic relationships because these sequences are present in all organisms and changes in the nucleotide sequences are supposed to occur in a clocklike manner (83). In a study of 46 *Bacillaceae* a phylogenetic tree was constructed on the basis of the sequence of the 3' end of the 16S rRNA gene and the 16S-23S internal transcribed spacer (ITS) region (84). This revealed that *Bacillus* and the closely related genera *Geobacillus*, *Paenibacillus* and *Brevibacillus* can be grouped in 10 different clusters (Fig. 3).

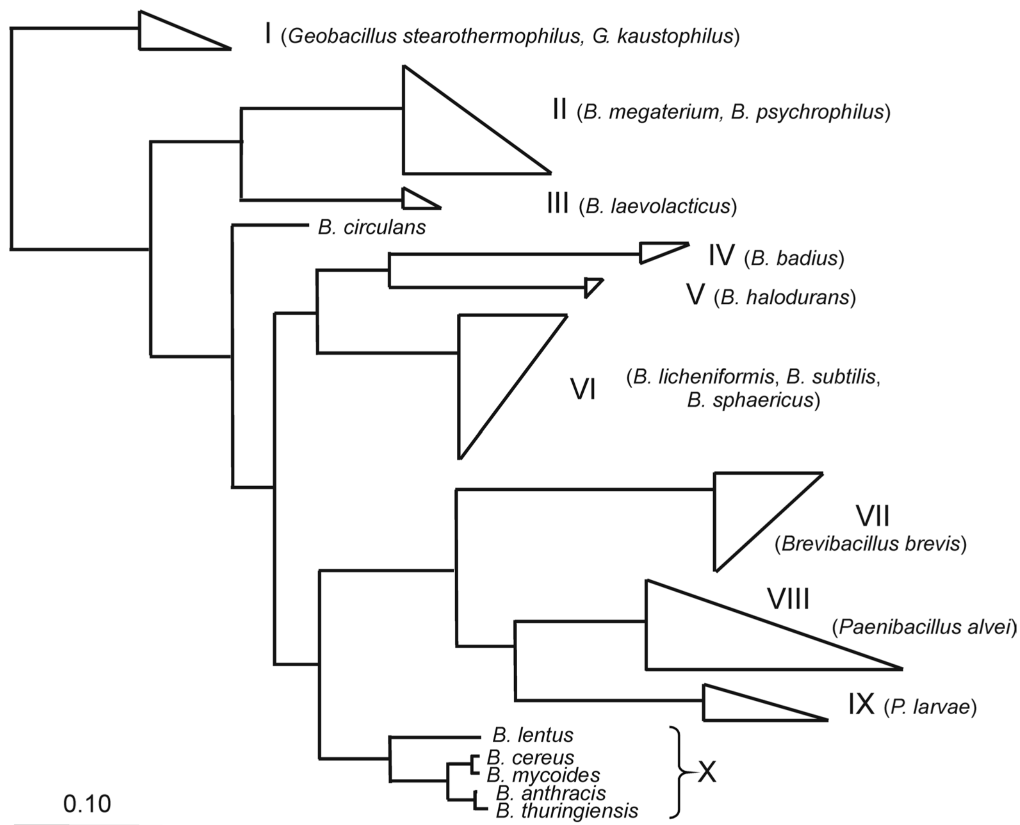

Fig 3. Phylogenetic relationships of the genus *Bacillus* and closely related genera. This figure is based on the alignment of the 150 bp 3' end 16S rDNA and the 70 bp 5' 16S–23S ITS region, adapted from Xu and Côté (84). Ten different groups can be assigned. These groups are indicated by Roman numerals. Some of the members of each group are indicated. *B. circulans* is the only species that cannot be assigned to a group. The bar represents the number of nucleotide substitutions per site.

With relation to *B. cereus*, two important conclusions can be drawn from Fig. 3. First of all it is clear that *B. cereus* is not particularly closely related to the gram-positive model organism *Bacillus subtilis*, indicating that many of the findings concerning metabolism, physiology or genetics of *B. subtilis* cannot simply be extrapolated to *B. cereus*. Furthermore, it is clear that *B. cereus* clusters very closely to other *Bacillus* species in particular *Bacillus mycoides*, *Bacillus anthracis* and *Bacillus thuringiensis.* These bacteria, together with *Bacillus weihenstephanensis*, are usually referred to as the *B. cereus* group. Historically, strains from the *B. cereus* have been assigned to one of the above species on the basis of their phenotypic characteristics, which are outlined below.

B. anthracis is the causative agent of anthrax, which has gained notoriety after having been used in bioterrorist attacks in the USA in the autumn of 2001 (48). Anthrax can occur in three different forms: cutaneous, gastrointestinal, and inhalational. The first form is the most common and can easily be treated with antibiotics. The gastrointestinal and inhalational forms are far more dangerous because the first symptoms appear relatively unremarkable (symptoms resemble a mild flu with slight fever and mild forms of gastroenteritis) but the disease abruptly develops into a systemic form that is resistant to treatment and rapidly fatal. Key factors in pathogenesis are the anthrax toxins, encoded by the genes *pagA*, *lef*, and *cya*, and the presence of a capsule, which allows the bacteria to escape the host immune response. The toxin genes are located on the plasmid pXO1 (181,654 nucleotides long) and the capsule genes are found on the plasmid pXO2 (96,231 nucleotides long) (63).

B. thuringiensis is generally not considered to be a human pathogen. *B. thuringiensis* strains can produce toxins in the form of parasporal crystal proteins that have been widely used for the biocontrol of insect pests of crops or vectors of human disease. The insecticidal toxins of *B. thuringiensis* are encoded on extrachromosomal elements (12, 76). *B. mycoides* is probably the least studied member of the *B. cereus* group. Its phenotypic characteristic that sets it apart from other *B. cereus* group members is its striking colony morphology. It has a typical asymmetric hairy shape, made by bundles of filaments curving clock- or counterclockwise (20). *B. mycoides* is also a potential biological control agent which can trigger resistance of sugar beets to pathogenic fungi (7). B. *weihenstephanensis* was first described in 1998 as a psychrotolerant species of the *B. cereus* group (53). Isolates of *B. weihenstephenensis* could grow at 4-7°C but not at temperatures above 43°C. This means that these bacteria can grow at refrigeration temperatures, which can have important consequences for the food industry. Psychrotrophic *B. cereus* strains are important spoilage organisms in the dairy industry because heat-resistant spores can survive pasteurization, which is used to preserve milk or milk-based products. Surviving spores can germinate during low temperature storage and upon vegetative cell growth the dairy product may spoil (30, 52). It should be noted however that not all *B. cereus* strains that are able to grow at 6°C are in fact *B. weihenstephanensis* and so it seems that intermediate forms between *B. cereus* and *B. weihenstephanensis* exist (77).

The above description of the members of the *B. cereus* group has focused on their phenotypic traits. Considerable effort has been made to separate these bacteria on a genetic level, so that specific markers for the different species can be identified and evolutionary

relationships may be recognized. The standard method of using ribosomal RNA sequences as markers has proven to be ineffective because these are practically identical in all members of the *B. cereus* group (6). These and similar findings of close genetic relationships between the members of the *B. cereus* group has even led to the claim that all bacteria in the *B. cereus* group should be considered a single species (39). Despite such claims, a separate species status for these bacteria has been maintained because of their distinctive pathogenic features. In recent years a large number of high-resolution studies have been performed, which have given extensive insights in the relationships in the *B. cereus* group (8, 40, 41, 50, 70).

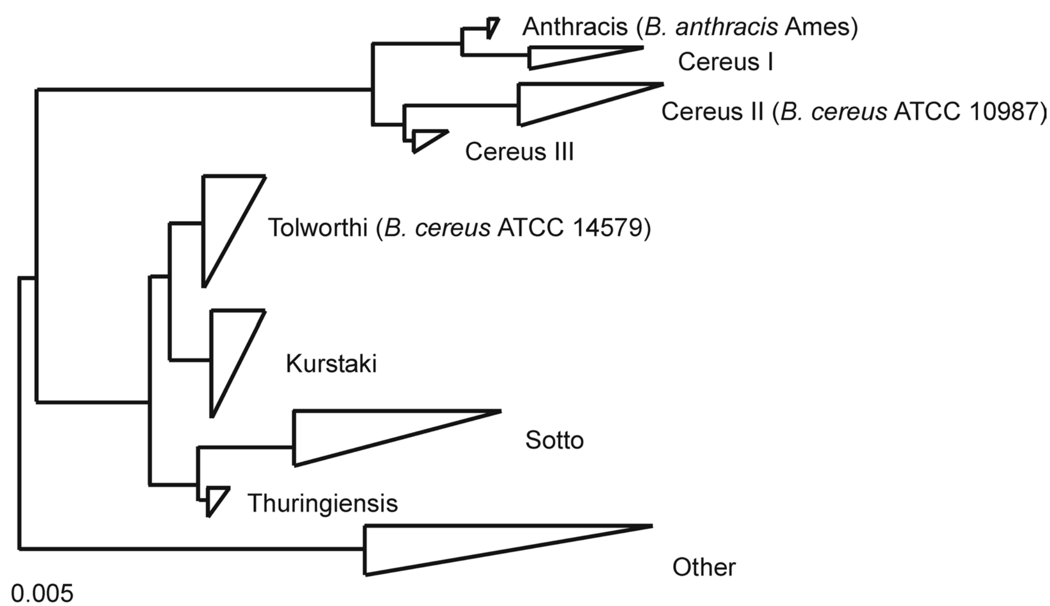

Fig 4. Schematic phylogenetic relationships in the *B. cereus* group. This tree is a schematic representation of the high-resolution phylogenetic tree of the *B. cereus* group as published by Priest *et al.* (70). The positions of *B. cereus* ATCC 14579, *B. cereus* ATCC 10987 and *B. anthracis* Ames (the complete genome sequences of these strains have been published) in this phylogenetic tree are indicated. The bar represents the number of nucleotide substitutions per site (note the large difference in scale with Fig. 3).

A schematic overview of a phylogenetic tree of the *B. cereus* group as recently constructed by Priest *et al.* (70) is shown in Fig 4. The results from the other studies mentioned above are consistent with this tree. The most important conclusion that can be drawn from these trees is that there is extensive diversity within *B. thuringiensis* and *B. cereus.* A large number of *B. cereus* strains and most *B. thuringiensis* strains fall into the lineages named Tolworthi and Kurstaki by Priest *et al.* (70). The lineages Sotto and Thuringiensis appear to be smaller and are predominantly formed by *B. thuringiensis*

strains. *B. weihenstephanensis* and *B. mycoides* strains are found in the remaining (Other) lineage. In contrast, *B. anthracis* is remarkably monomorphic and clearly forms a separate cluster from *B. cereus/thuringiensis*. It seems that the ancestral *B. anthracis* descended from a single *B. cereus*-like isolate that acquired the pXO1 and pXO2 plasmids by a gene transfer or genetic exchange event (70). *B. cereus* strains which are associated with foodborne disease generally cluster close to this *B. anthracis* branch in the phylogenetic tree (41). The strain *B. cereus* ATCC 10987 of which the complete genome sequence recently has become available (73), falls in the Cereus II lineage. This strain is a food-isolate (it was isolated from spoilt cheese) but, to our knowledge, has not been involved in foodborne disease. The type strain of *B. cereus*, ATCC 14579, which is used throughout this study and of which the genome sequence has been determined (45), falls into the Tolworthi lineage. The close proximity of this strain to *B. thuringiensis* isolates and the proposed role of *B. cereus* as an inhabitant of the insect gut (59) seem to suggest that the type strain of *B. cereus* may have adapted to a life-style as an insect symbiont (47).

The high-resolution mapping of the members of the *B. cereus* group has revealed the genetic relationships of the bacteria in the *B. cereus* group in incredible detail. However, these trees sometimes give the impression that the differences between the different *B. cereus* group members are relatively large, which may not necessarily be correct. The availability of complete genome sequences of *B. cereus*, *B. thuringiensis* and *B. anthracis* strains makes it possible to give a comprehensive overview of the differences and similarities between the different members of the *B. cereus* group.

COMPLETE GENOME SEQUENCES OF *B. CEREUS* GROUP MEMBERS

Three publications have described the full genome sequences of the *B. cereus* strains ATCC 14579 (45), ATCC 10987 (73), and G9241 (43). This last strain is an exceptional case as it is a clinical isolate which can cause an inhalational anthrax-like disease. The extreme pathogenicity of this strain can be explained by its ability to form a capsule (this trait is conferred by the presence of a 218-kb plasmid) and the presence of a second plasmid that is practically identical to pXO1 in *B. anthracis* and which carries the anthrax toxin genes. Also for *B. anthracis* strain Ames (74) a complete genome sequence has been published. In addition, the genome sequences of at least nine more *B. anthracis*, one *B. cereus*, and two *B. thuringiensis* strains are available in public databases like ERGO Light (http://www.ergo-light.com) and NCBI (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi) An overview of the genome characteristics of *B. cereus* ATCC 14579, *B. cereus* ATCC 10987, and *B. anthracis* Ames and selected differences in toxins and metabolic pathways are provided in Table 1.

Table 1. Comparison of the genome features and encoded toxins and pathways of *B. cereus* ATCC 14579, *B. cereus* ATCC 10987 and *B. anthracis* Ames[a]

	B. cereus ATCC 14579	*B. cereus* ATCC 10987	*B. anthracis* Ames
Genome features			
Size (bp)	5,426,909	5,224,283	5,227,293
No. genes	5,366[b]	5,642	5,508
Percentage coding	84	85	84.3
%G+C content	35.3	35.6	35.4
Toxins			
Anthrax toxins	-	-	+
Hemolytic enterotoxin BL	+	-	-
Non-hemolytic enterotoxin Nhe	+	+	+
Enterotoxin FM	+	+	+
CytK	+	+	-
Pathways			
Arginine deiminase	+	+	-
Urease	-	+	-
Nitrate reduction	+	-	+
Xylose utilization	-	+	-
Tagatose utilization	-	+	-

[a]Data on the genome features were adapted from Rasko *et al.* (73). The overview of the toxins and pathways that are present or absent in the different strains was adapted from Abee *et al.* (1).
[b]Note that the number of genes is somewhat higher than the total number of proteins mentioned in the text due to the presence of frameshift mutations.

An important reason for the intense genome sequencing efforts in the *B. cereus* group, is the identification of *B. anthracis*-specific genes which may contribute to the virulence of *B. anthracis*. Clearly the presence of plasmids carrying anthrax-virulence genes is of crucial importance, as is illustrated by *B. cereus* G9241 (43), but chromosomal differences between *B. cereus* and *B. anthracis* may also exist. The comparison of the genomes of *B. cereus* and *B. anthracis* may thus lead to the identification of additional factors that are needed for the virulent phenotype of *B. anthracis*. A full genome comparison between *B. cereus* G9241, ATCC 10987, and ATCC 14579 and *B. anthracis* Ames revealed that *B. cereus* G9241 is most closely related to *B. anthracis* followed by *B. cereus* ATCC 10987. *B. cereus* ATCC 14579 is most distant from *B. anthracis* (43, 73). Still, for 4364 proteins in *B. cereus* ATCC 14579 (of a predicted total of 5229) a homologue could be identified in *B. anthracis* (73), which serves to emphasize that the similarities between the *B. cereus* group members are larger than the differences.

A number of unique pathways or genes for *B. cereus* can be identified from the complete genome sequences. To what extent these unique genes can explain the differences between *B. cereus* and *B. anthracis* is still not known, but they give some tantalizing insights. This is in particular the case for the genes encoding enzymes from the arginine

deiminase pathway. These were identified in both *B. cereus* ATCC 10987 and ATCC 14579 but not in *B. anthracis*. As pointed out by Ivanova *et al.* (45), this pathway enables *Streptococcus pyogenes* to survive acidic conditions in the presence of arginine owing to release of ammonium (19). However, ammonium inhibits receptor-mediated internalization of the lethal toxin (34), and this may explain why ammonium production by arginine deiminase was counterselected for in *B. anthracis*. None of the chromomal genes that are unique for *B. anthracis* have any immediately obvious role in virulence. Less than 150 unique *B. anthracis* genes have an assigned function and these include phage genes or transposases (74). In addition, two studies in which suppressive subtractive hybridization was used to identify specific chromosomal sequences unique to *B. anthracis* have yielded additional insights into unique *B. anthracis* genes (22, 71). Here too, bacteriophage related genes were frequently found, but also cell-wall associated proteins, including the S-layer protein, of *B. anthracis* were identified.

In summary, it appears that although chromosomal differences can affect pathogenic properties of bacteria of the *B. cereus* group, it is evident that plasmid-encoded traits are most important in conferring virulence. This is dramatically illustrated by *B. cereus* G9241 in which the presence of two plasmids confers properties that can cause an anthrax-like illness. Bacteria in the *B. cereus* group appear to exchange genetic material quite readily as witnessed by the spread of genes from the *B. anthracis* virulence plasmids pXO1 and pXO2 throughout the *B. cereus* group (67, 68, 74) and this may thus be an important way by which bacteria in the *B. cereus* group can gain pathogenic traits.

The genome sequences have also provided meaningful insights into the lifestyle of the members of the *B. cereus* group. The historically held assumption that *B. cereus* is a soil bacterium, like *B. subtilis* or *Streptomyces* spp., seems to be wrong. Soil bacteria have a variety of carbohydrate catabolic pathways, which can be used to degrade the diverse carbohydrates, mainly from plant origin, in the soil. However, *B. cereus* ATCC 14579 has only 14 genes that code for polysaccharide degrading enzymes, while in *B. subtilis* this number stands at 41. The only polysaccharides that can be degraded by *B. cereus* ATCC 14579, as predicted by its genome sequence, are starch, glycogen, chitosan and chitin. The last two polysaccharides are important constituents of the exoskeletons of insects and this provides an important clue that *B. cereus* group bacteria are more adapted to life inside insect hosts than in soil (45). In contrast with their reduced polysaccharide metabolism, the members of the *B. cereus* group seem to have a larger capacity to metabolize proteins and amino acids than *B. subtilis*. For example a total of 51 protease-encoding genes were identified in *B. cereus*, compared to 30 in *B. subtilis* (45). Similarly, study of the *B. anthracis* genome has revealed that it has 17 ABC-type peptide binding proteins (four in *B. subtilis*), nine homologues of the BrnQ branched chain amino-acid transporter (two in *B. subtilis*), six LysE/Rht amino-acid efflux systems (two in *B. subtilis*). The LysE/Rht systems prevent the accumulation of amino acids to bacteriostatic concentrations during growth on peptides (11, 74). Both *B. cereus* and *B. anthracis* may thus be adapted for life in a protein-rich environment. For *B. cereus* this can mean the insect gut, but for *B. anthracis* this can also be decaying animal matter. Its large capacity for the metabolism of proteins and amino

acids may also explain why *B. cereus* can grow better than other *Bacilli* in protein-rich foods, such as milk (52).

An important feature of all *Bacilli* is their ability to form spores. In this regard the complete genome sequences of the *B. cereus* group have also shed light on important differences with *B. subtilis*, which is the model organism for the study of sporulation and germination. Spores have no metabolic activity and they are very resistant to extreme environmental conditions such as heating, freezing, drying and radiation. When spores find themselves in an environment that is favorable for growth, spores germinate and rapid vegetative growth can resume. In *B. cereus* the outgrowth of spores in a food product may cause spoilage or vegetative cells may reach such high levels that consumption of the food product may be hazardous to the health of the consumer (51). In addition, the germination of spores is a crucial step in anthrax pathogenesis (63). Even though the basic sporulation machinery of the *B. cereus* group is broadly similar to that of *B. subtilis* (46) there are some important differences. The proteins with the highest degree of sequence divergence between the species are spore coat constituents and spore polysaccharide biosynthesis components, which suggests that the composition of the outer surface of spores from the *B. cereus* group is different from *B. subtilis* spores (74). This may explain why *B. cereus* spores are hydrophobic and *B. subtilis* spores hydrophilic, which causes major differences in the attachment of *B. cereus* and *B. subtilis* spores to various materials (29). In addition, there is considerable variation in the sensing and signaling pathways leading to sporulation, which may reflect differences in which environmental triggers lead to the initiation of the process of sporulation in *Bacilli* (78). There also appear to be important differences in the repertoire of germination receptors of *B. cereus* and *B. subtilis* and the conditions under which the spores of *B. cereus* and *B. subtilis* can germinate (Luc Hornstra, personal communication).

CONCLUDING REMARKS

It is clear that the bacteria of the *B. cereus* group form an interesting and highly relevant field of study. Both fundamental science and applied research can be performed to answer the many questions that remain concerning the evolution of the members of the *B. cereus* group, their lifestyle, their pathogenic properties and how these bacteria can be dealt with, both in clinical and food-processing settings.

The complete genome sequences of the members of the *B. cereus* group have revealed important traits unique to this group of bacteria, as outline above. Furthermore, because of the increasing popularity of minimally processed, ready-to-eat food products and the increasing number of elderly and immunocompromised people, *B. cereus* will remain a concern for the food industry for years to come (23).

The study of *B. cereus* genetics and physiology may provide clues for strategies for the control of this bacterium. An important factor in determining the survival and growth of the bacteria is to what extent they can adapt to the continuously changing conditions in their environment. This process of adaptation is generally called the stress response. The stress response usually confers cross-protection against numerous other stresses to the cell. The fact that microbes can adapt to changing environments, which in turn can lead to their

increased resistance to various stresses including those encountered in the human gastrointestinal tract, can have serious implications for food safety (1). Whereas the stress response of other gram-positive bacteria, most notably *B. subtilis*, *Staphylococcus aureus* and *Listeria monocytogenes*, has been studied in great detail, data describing this response in *B. cereus* are lacking.

OUTLINE OF THIS THESIS

The research described in this thesis was initiated because there is a need for accurate data on the inactivation kinetics of *B. cereus* so that it can be efficiently controlled during food processing. The stress response of *B. cereus* may lead to the increased survival of vegetative cells of *B. cereus* under several mild food-processing conditions. In addition, the elucidation of the molecular mechanisms that are involved in the stress response of *B. cereus* can lead to the identification of targets for preservation strategies.

The alternative sigma factor σ^B is an important regulator of the stress response in several gram-positive bacteria. σ^B is also present in *B. cereus*, but, prior to this study, virtually nothing was known about its role in the stress response of this organism. A review on the role of σ^B in the stress response, virulence and cellular differentiation in gram-positive bacteria is provided in **Chapter 2**. Here too, the role of sigma factors in prokaryotic transcription and the mechanism of the regulation of the activity of σ^B are discussed. In **Chapter 3** the heat stress response of *B. cereus* is described. When vegetative cells are exposed to a mild heat shock or other stresses, they exhibit an increased survival at the lethal temperature of 50°C compared to non-stressed cells. Furthermore, a number of stress-induced proteins were identified by proteome analysis. The activation of σ^B upon stress in *B. cereus* is described in **Chapter 4**. The autoregulation of transcription of the *sigB* gene and the effect of the deletion of the *sigB* gene on the adaptive heat shock response was also examined. In **Chapter 5** genes that are regulated by σ^B in *B. cereus* are described. These genes were identified by a combination of proteome and *in vitro* transcription analysis with reconstituted *B. cereus* σ^B – RNA polymerase. The regulation of the activity of σ^B by the regulators RsbV, RsbW and RsbY is described in **Chapter 6**. Some important differences were shown to exist between the regulation of σ^B-activity in *B. cereus* and other gram-positive bacteria. In **Chapter 7** the phenotypic characterization of the *sigB* deletion mutant of *B. cereus* using Phenotype MicroArray technology is described. In addition, the growth and survival of the *sigB* deletion mutant under low temperature conditions was studied. In **Chapter 8** the remarkable hyperresistance to H_2O_2 of the *sigB* deletion mutant of *B. cereus* during exponential growth in rich medium is described. Subsequent study of the expression of the catalases of *B. cereus* revealed that in the *sigB* deletion mutant, the expression of the *katA* gene is upregulated, resulting in high catalase activity. **Chapter 9** includes the general discussion, the conclusions and future perspectives of the research described in this thesis.

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2 THE ROLE OF σ^B IN THE STRESS RESPONSE OF GRAM-POSITIVE BACTERIA - TARGETS FOR FOOD PRESERVATION AND SAFETY

Willem van Schaik and Tjakko Abee

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ABSTRACT

The alternative sigma factor σ^B modulates the stress response of several Gram-positive bacteria, including *Bacillus subtilis* and the foodborne human pathogens *Bacillus cereus*, *Listeria monocytogenes* and *Staphylococcus aureus*. In all these bacteria, σ^B is responsible for the transcription of genes that can confer stress resistance to the vegetative cell. Recent findings indicate that σ^B also plays an important role in antibiotic resistance, pathogenesis and cellular differentiation processes such as biofilm formation and sporulation. Although there are important differences in the regulation of σ^B and in the set of genes regulated by σ^B in *B. subtilis*, *B. cereus*, *L. monocytogenes* and *S. aureus*, there are also some conserved themes. A mechanistic understanding of the σ^B-activation processes and assessment of its regulons could provide tools for pathogen control and inactivation both in the food industry and clinical settings.

INTRODUCTION

Genome sequences of hundreds of microorganisms are now available and provide a wealth of information on the strategies that can be employed by bacteria to conquer a variety of niches in the environment and in the host. The information encoded in bacterial genomes allows cells to produce thousands of different proteins. Obviously, these are not synthesized all at the same time and tight control of the production of the optimal set of proteins under specific conditions is of competitive advantage. Sigma factors, which are dissociable subunits of RNA polymerase (RNAP), play a key role in this process. The core form of RNAP consists of five subunits ($\alpha_2\beta\beta'\omega$), but this form is unable to initiate transcription from promoters. For this process a sixth protein, a sigma factor (σ), is necessary. Sigma factors can bind to RNAP, forming RNAP holoenzyme and this complex can recognize promoters and melt DNA, thus initiating transcription. Most transcription in exponentially growing cells is mediated by RNAP holoenzyme, which carries a "housekeeping" sigma factor, that is similar to σ^{70} in *Escherichia coli* and σ^A in *B. subtilis*. However, in most bacteria other sigma factors can simultaneously be present in the cell. These so-called alternative sigma factors target specific recognition sequences and control specialized regulons that are activated under specific conditions including exposure to stresses and during cellular differentiation (21, 36).

Table 1. Selected gram-positive bacteria in which σ^B plays a role in the stress response

	Bacillus subtilis	*Bacillus cereus*	*Listeria monocytogenes*	*Staphylococcus aureus*
Cell morphology	Rod	Rod	Rod	Coccus
Spore-former	+	+	-	-
Pathogenic	-	+	+	+
Relevant characteristics	Used in industrial fermentations. Gram-positive model organism	Foodborne pathogen. Closely related to *Bacillus anthracis* (cause of anthrax).	Foodborne pathogen. Grows at refrigeration temperatures and resistant to low pH and high salt concentrations.	Foodborne pathogen. Leading cause of nosocomial infections. Increasingly resistant to antibiotics.
References	(42)	(26)	(28)	(33, 34)

One of the best-studied alternative sigma factors in gram-positive bacteria is σ^B. This sigma factor functions as a central regulator of the stress response in bacteria in the genera *Bacillus*, *Staphylococcus* and *Listeria*. Other Gram-positive bacteria with a low GC content (e.g. lactic acid bacteria and Clostridia) do not contain σ^B. Although σ^B-like proteins have been reported in high GC content Gram-positive bacteria such as *Mycobacterium* and

Streptomyces, these are in fact more closely related to the group of σ^F-like proteins (14) and these distant σ^B homologs are not further discussed in this review.

Not only does σ^B play a prominent role in the stress response of the gram-positive model organism, *B. subtilis*, but it also has a key role in the stress response of the human pathogens *B. cereus*, *L. monocytogenes* and *S. aureus* (Table 1). It is noteworthy that the role and regulation of σ^B in these bacteria is quite different from the *B. subtilis* paradigm. Earlier research on σ^B has been covered extensively, with a distinct focus on *B. subtilis*, in several excellent reviews (22, 39, 40). In this review, we will focus on recent developments in the study of σ^B in gram-positive bacteria, highlighting work in *B. cereus, L. monocytogenes* and *S. aureus*. We will also give pointers as to how the scientific knowledge on σ^B may be applied to ensure the microbial safety of industrially processed foods.

A KEY REGULATOR IN THE GENERAL STRESS RESPONSE

In all gram-positive bacteria the activation of σ^B confers protection to the cell against adverse conditions. A schematic overview of this process is depicted in Fig. 1A. Upon exposure to stress, the stress has to be sensed and signaled through a regulatory cascade, leading to the activation of σ^B and, subsequently, to the transcription of the set of σ^B-regulated genes (the σ^B regulon). The encoded proteins perform specific functions, which protect the cell against stress.

The question to what extent and under which conditions σ^B is responsible for survival during stress has been addressed by phenotypic characterization of *sigB* deletion mutants (see Fig. 1B and 1C for examples). These studies revealed that σ^B is involved in the resistance to a variety of stresses including heat, high osmolarity, high ethanol concentrations, high and low pH, and oxidizing agents (3, 15, 16, 19, 24, 25, 40, 46, 49). In *L. monocytogenes* and *B. subtilis* σ^B was shown to have a role in growth and survival under low temperatures (4, 6). This condition is of special interest for practical reasons as chilled storage is often a crucial factor in the preservation of minimally processed foods.

Remarkably, under low temperature conditions, the *sigB* deletion mutant of *B. subtilis* also has a distinct sporulation defect (35). Also in *B. cereus*, the deletion of *sigB* results in a delayed onset of sporulation and subsequent maturation of the spores. Furthermore the spores of the *sigB* deletion mutant germinate less efficiently than wild-type spores (De Vries *et al.*, unpublished data). These findings complement the data from Mendez *et al.* (35) and strongly suggest that stress response and sporulation are not two completely independent pathways for *Bacilli*, but that they are in fact intertwined, giving the cell the utmost flexibility to adjust its physiology to changes in the environment.

σ^B also has a function in the resistance against antibiotics. In *B. subtilis* the *sigB* mutant cannot resume growth after exposure to rifampin as quickly as the parental strain (2). Even more remarkable is the role that σ^B plays in the resistance of *S. aureus* to several important antibiotics, including methicillin and vancomycin (43, 44, 51). This is of special interest, as infections with antibiotic-resistant *S. aureus* strains are an increasing problem in hospitals around the world and these infections are exceedingly difficult to treat (34). In

***Staphylococcus epidermidis*, which is closely related to *S. aureus* and is a frequent cause of infections on implanted biomaterials, σ^B has a role in the regulation of biofilm formation. The colonization of biomaterials by biofilm formation is an important step in the pathogenesis of *S. epidermidis* and in this way σ^B is involved in the regulation of a key virulence factor in this organism (12, 31, 32).**

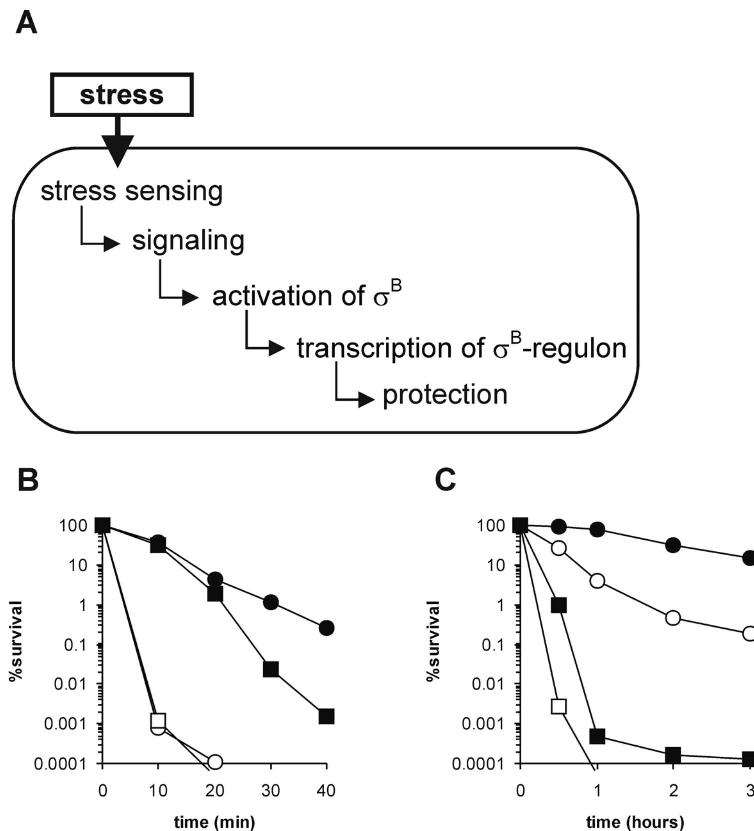

Fig. 1. The role of σ^B in the general stress response in gram-positive bacteria. (A) The pathway leading to the activation of σ^B. After a stress is sensed, a signal is relayed, which leads to the activation of σ^B. σ^B then associates with core RNA polymerase, and transcription of the σ^B regulon is initiated. The protein products encoded by the members of the σ^B regulon confer stress protection to the cell. (B) Survival at 50°C of *B. cereus* ATCC 14579 (circles) and its *sigB* deletion mutant (squares). Cells from the mid-exponential growth phase (open symbols) and cells that were pretreated with a mild heat shock (closed symbols) were exposed to 50°C (46). (C) Survival of *L. monocytogenes* EGD-e (circles) and its *sigB* deletion mutant (squares) during incubation at pH 2.5 with (closed symbols) and without (open symbols) pre-exposure to pH 4.5 for 1 h (49). Fig. 1B and 1C are reproduced (with slight modifications) with permission of the American Society for Microbiology.

For many pathogens, the ability to mount a stress response is a prerequisite for virulence as they have to withstand the many defenses that the host has available to battle invading micro-organisms (20). Because σ^B is such an important regulator of the microbial stress response, it is not surprising that many groups have attempted to identify a function for σ^B in the infection process. In *L. monocytogenes* the deletion of *sigB* had only very minor effects on virulence as tested in animals models (50), even though several virulence factors in these organisms are under control of σ^B (see below). Perhaps, this reflects redundancy in the pathways connecting stress response and virulence. Another explanation may be that current animal models for the infection of *L. monocytogenes* are too crude to pick up subtle differences in virulence caused by the deletion of *sigB*. In *S. aureus*, the deletion of *sigB* has no effect on virulence in a murine subcutaneous skin abscess model (25). Recently however, when a murine model for septic arthritis was used to compare the virulence of a *sigB* deletion mutant with a parent strain with functional σ^B, a marked decrease in the severity of arthritis, weight loss, and mortality was observed (27). Also in *Bacillus anthracis*, which causes the often deadly disease anthrax, there is a clear link between σ^B and virulence. Here, there is an approximately 1-log-unit difference in the 50% lethal dose (LD_{50}) between the *sigB* mutant and its parent strain (17).

THE REGULATION OF σ^B ACTIVITY

The exposure of cells to stress may lead to activation of σ^B and its regulon. A tight control of σ^B activity is achieved through a signal transduction network in which key protein interactions are regulated by serine and threonine phosphorylation (22, 39). Two regulators (RsbV and RsbW) are conserved in all gram-positive bacteria that have σ^B (14); their role in σ^B activation is described in detail in Fig. 2A.

The activation-state of σ^B is determined by the phosphorylation state of the anti-sigma factor antagonist RsbV. This phosphorylation state is determined by the kinase activity of RsbW and the phosphatase activity of a regulator that feeds into this regulatory system. All these phosphatases have a C-terminal PP2C phosphatase domain, but there is variation in the N-terminal part of the protein. The N-terminal domain can sense signals that are transferred through elements acting further upstream in the regulatory cascade. The differences in the N-terminal domains of the PP2C phosphatases appear to reflect divergence in the regulation of σ^B activity (Fig. 2B).

Although many aspects of the regulation of σ^B activity are well understood, some are still unclear. For example, the physiological stimulus that triggers σ^B-activation is, as yet, unknown. It has been proposed that ribosomes may act as the sensors for stress (52), but recently the existence of large protein complexes (up to 1 MDa) in the σ^B activation pathway has been revealed. Subunits of these complexes may take part in both sensing and relaying the stress signal (10, 30). These data have been obtained in *B. subtilis* however, and it seems unlikely that they can be extrapolated to other gram-positive bacteria, some of which lack the genes encoding the subunits of these complexes. There are also σ^B-activating pathways

present that remain to be discovered; for example RsbV- and RsbU-independent routes leading to σ^B^ activation have been described in *B. subtilis* (6), and in *S. aureus* (37), respectively.

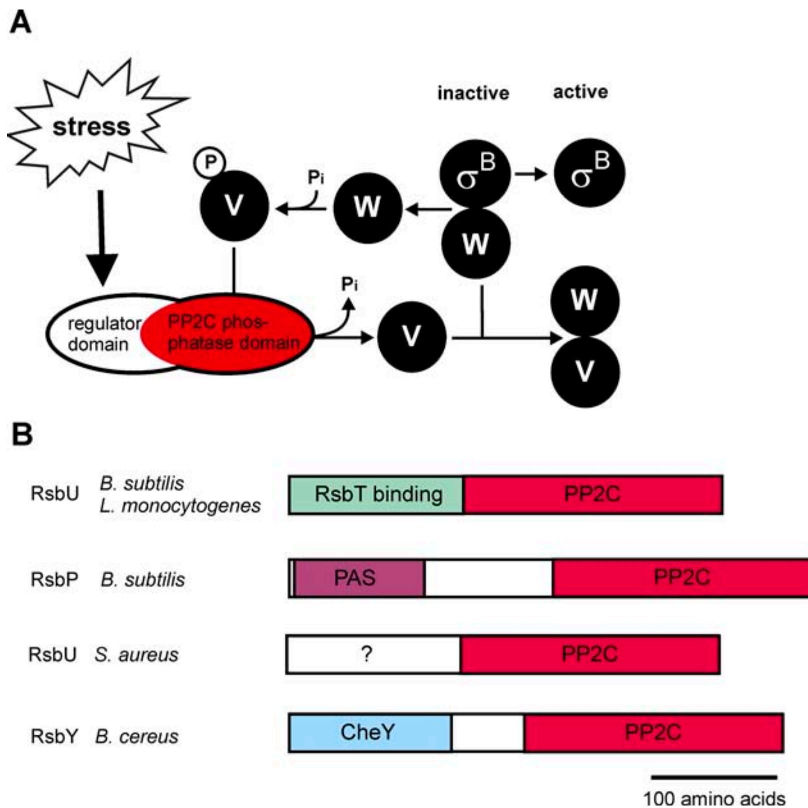

Fig. 2. Regulation of σ^B^ activity. (A) Regulation of σ^B^ by the association with an anti-sigma factor. Under non-stress condition σ^B^ is bound (and thus inactive) to its cognate anti-sigma factor, RsbW. This protein also functions as a kinase on the anti-sigma factor antagonist, RsbV. When RsbV is dephosphorylated by the action of a phosphatase with a PP2C-domain, RsbV can bind to RsbW. This leads to the release of σ^B^ from its complex with RsbW, and, upon association of σ^B^ to core RNA polymerase, to the transcription of the σ^B^ regulon (22, 39, 40). (B) Schematic overview of the PP2C-phosphatases that act on RsbV. Environmental stress is signaled through RsbU in *B. subtilis*, which has an N-terminal domain that can associate with another regulator, RsbT. RsbP is only present in *B. subtilis*, and is activated upon energy stress (e.g. entry into stationary phase and glucose limitation) and has a C-terminal PAS domain (14, 48). RsbU in *L. monocytogenes* is very similar to RsbU in *B. subtilis*, as it also has a C-terminal RsbT-binding domain, but it can relay both environmental and energy stress signals (9, 13). In *S. aureus*, the RsbU-homologue does not have an RsbT-binding domain (13, 37). How this phosphatase is activated is currently not known. In *B. cereus*, the PP2C phosphatase RsbY has a N-terminal domain, which is homologous to the CheY response regulator domain. This strongly suggests that an, as yet unidentified, kinase can phosphorylate this domain leading to the activation of the phosphatase (chapter 6 of this thesis).

THE σ^B REGULON

In *B. subtilis*, *B. cereus*, *L. monocytogenes*, and *S. aureus* σ^B-regulated genes have been identified by various approaches, including two-dimensional gel electrophoresis, genome-wide searches for σ^B-dependent promoter consensus sequences, and transcriptome profiling using DNA microarrays (5, 18, 23, 29, 38, 41, 47). Fig. 3 provides an overview of the genes that are under control of σ^B. A comparison of the set of σ^B-regulated genes reveals considerable overlap in functionality between the four bacteria. A surprisingly small number of the proteins that are encoded by σ^B-dependent genes have a direct role in stress response, like catalases and intracellular proteases that can turn-over misfolded proteins. In *L. monocytogenes* the glutamate decarboxylase system, which protects against acid stress, is under control of σ^B. A larger group of proteins seems to have a role in metabolic reprogramming of the cell. These proteins have diverse functions, including the synthesis of vitamins or cofactors, modulation of carbon metabolism and mediating the in- and efflux of amino acids, ions and osmolytes. Moreover, the function of a considerable portion of σ^B-dependent proteins is still unknown (5, 18, 23, 29, 38, 41, 47). The determination of the function of these proteins is a challenge for future research and may lead to the discovery of novel mechanisms that can contribute to stress resistance.

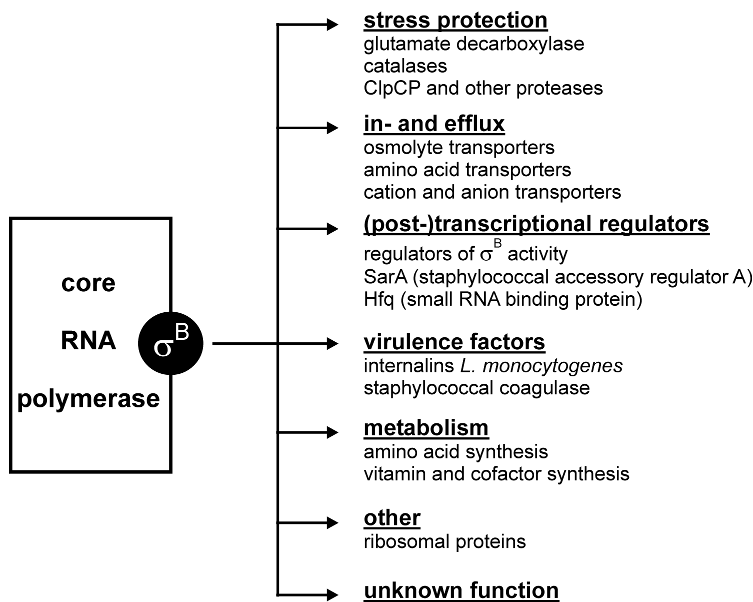

Fig. 3. Schematic overview of σ^B regulated genes in *B. subtilis*, *B. cereus*, *L. monoytogenes* and *S. aureus*. Note that not all genes presented are σ^B-dependent in all bacteria under review here, but that this figure is meant to convey the wide variety of protein functions in the various σ^B regulons. For more detailed information see the references mentioned in the text

Several virulence determinants are also under control of σ^B. In *L. monocytogenes*, *bsh* (encoding a conjugated bile salt hydrolase) and two genes from the internalin family, which contribute to host cell internalization, are partially σ^B-dependent (29, 45). In *S. aureus*, σ^B represses the expression of most of the exoenzymes and toxins, while it positively regulates the expression of many adhesins (5). Because the adhesins are important virulence determinants in the murine septic arthritis model, this could explain the attenuated virulence of an *S. aureus sigB* deletion mutant in this model (27). Another important virulence regulator that is under positive control of σ^B is staphylococcal accessory regulator A (SarA). SarA is a DNA-binding protein that binds to an A/T-rich recognition motif in the promoter regions of target genes, leading to induction or repression of the transcription of downstream genes (5, 7).

The central role of σ^B in the regulatory network of the microbial stress response is illustrated by the fact that σ^B can control other important regulatory proteins. A remarkable example is the σ^B-dependency of the RNA-binding protein Hfq in *L. monocytogenes*. Hfq is thought to play a crucial role in the post-transcriptional regulation of gene expression by small RNAs and the σ^B-dependency of this regulator adds another level of complexity to the function of σ^B in *L. monocytogenes* (11).

A TARGET FOR FOOD PRESERVATION AND SAFETY

It is obvious that σ^B is an important player in the stress response of several gram-positive bacteria. But how can this fundamental knowledge be applied in a practical sense? Currently, there is a trend in the food industry towards the use of mild preservation methods, allowing maintenance of flavor and texture of the natural products. At the same time, it remains of crucial importance that the final product is free of food pathogens. However, pathogens can enter the production chain with the raw materials used, and at various other stages; for example via handling or by the release of cells from microbial biofilms that might have developed in the production line. For an efficient control of foodborne pathogens there is a need for more accurate and precise data on the inactivation kinetics and growth inhibition of bacteria, based on the current genomics knowledge (1, 8). The activation of σ^B may lead to a considerable increase in stress resistance and so σ^B may serve as an important biomarker to assess the resistance of strains to preservation. In addition, the knowledge obtained on σ^B could be used to support the design of a process with sequential preservation steps in which the stress response is not activated or even repressed, thereby sensitizing the bacteria to subsequent preservatand inactivation treatments.

CONCLUSIONS

In recent years, it has become increasingly clear that σ^B, in addition to its role in the general stress response, can have a role in cellular differentiation processes, including biofilm formation and sporulation. This indicates that σ^B is a key player in pathogen eco-physiology and contributes to survival in industrial and clinical settings.

It will be a challenge for future research to incorporate the knowledge gathered on σ^B in concepts that can be used for food preservation on an industrial scale. In addition, its role in antibiotic resistance and virulence may provide tools for control of pathogens in a clinical setting. Furthermore, we expect that the knowledge and tools obtained and developed in the research on σ^B will stimulate and facilitate the research on other alternative sigma factors including those involved in the stress response, stability and performance of gram-positive lactic acid bacteria and bifidobacteria used as industrial starters and probiotics.

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3 IDENTIFICATION OF PROTEINS INVOLVED IN THE HEAT STRESS RESPONSE OF *BACILLUS CEREUS* ATCC 14579

Paula M. Periago, Willem van Schaik, Tjakko Abee, Jeroen A. Wouters

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ABSTRACT

To monitor the ability of the foodborne opportunistic pathogen *Bacillus cereus* to survive during minimal processing of food products, we determined its heat-adaptive response. During pre-exposure to 42°C, *B. cereus* ATCC 14579 adapts to heat exposure at the lethal temperature of 50°C (maximum protection occurs after 15 min to 1 h of pre-exposure to 42°C). For this heat-adaptive response, *de novo* protein synthesis is required. By using two-dimensional gel electrophoresis, we observed 31 heat-induced proteins, and we determined the N-terminal sequences of a subset of these proteins. This revealed induction of stress proteins (CspB, CspE, and SodA), proteins involved in sporulation (SpoVG and AldA), metabolic enzymes (FolD and Dra), identified heat-induced proteins in related organisms (DnaK, GroEL, ClpP, RsbV, HSP16.4, YflT, PpiB, and TrxA), and other proteins (MreB, YloH, and YbbT). The upregulation of several stress proteins was confirmed by using antibodies specific for well-characterized heat shock proteins (HSPs) of *Bacillus subtilis*. These observations indicate that heat adaptation of *B. cereus* involves proteins that function in a variety of cellular processes. Notably, a 30-min pre-exposure to 4% ethanol, pH 5, or 2.5% NaCl also results in increased thermotolerance. Also, for these adaptation processes, protein synthesis is required, and indeed, some HSPs are induced under these conditions. Collectively, these data show that during mild processing, cross-protection from heating occurs in pathogenic *B. cereus*, which may result in increased survival in foods.

INTRODUCTION

Bacillus cereus is one of the major foodborne pathogenic bacteria and a common contaminant of food and dairy products. It is a gram-positive, facultatively anaerobic, spore-forming bacterium that is the causative agent of two types of food poisoning, the emetic and diarrheal forms. The mechanism of the pathogenesis of *B. cereus* has not yet been fully clarified. The vegetative bacterial cells can produce a number of virulence factors, including enterotoxins, of which at least three have been characterized (13, 21). One of the reasons that *B. cereus* is an important cause of food poisoning in industrialized parts of the world (13) might be the increasing demand of consumers for fresher and more natural food products, which results in a reduction in the intrinsic preservation of foods (12). The microbial safety and stability of most minimally processed foods are based on the application of combined preservative factors, of which (mild) heating is the most common preservation technique used (22).

Bacteria have evolved adaptive networks to face the challenges of a changing environment and to survive under conditions of stress (1). An initial nonlethal heat dose can induce transient resistance to subsequent heat treatment, a phenomenon termed thermotolerance. Heat-induced thermotolerance has been studied in several food pathogens, such as *Listeria monocytogenes* (28) and *B. cereus* (23). For both psychrotrophic and mesophilic *B. cereus* variants, increased thermotolerance at 50°C is observed after incubation under mild heat conditions (37 or 40°C for several hours). Several proteins were induced during heat pre-exposure, but these were not identified (23). The mechanisms of heat adaptation and the production of heat-induced proteins have not been studied in *B. cereus*.

In a wide variety of bacteria, the heat shock response includes increased synthesis of a set of conserved heat shock proteins (HSPs) (41). The molecular genetics of the heat shock response has been most extensively studied in *Escherichia coli* and *B. subtilis* (14, 41). Classical HSPs are the molecular chaperones (e.g., DnaK, GroEL, and their cohorts) or ATP-dependent proteases (e.g., ClpP). These proteins play roles in protein folding, assembly, and repair and prevention of aggregation under stress and nonstress conditions. The chaperones and proteases act together to maintain quality control of cellular proteins (11). The *B. subtilis* heat-inducible genes are divided into four different classes based on their regulatory mechanisms (8, 14). Class I genes encode classical chaperones such as DnaK, DnaJ, GroES, and GroEL, the expression of which involves a highly conserved CIRCE (controlling inverted repeat of chaperone expression) operator sequence, which is the binding site for the HrcA repressor. Class II genes are induced by both heat and other stresses, such as salt or ethanol. Expression of these genes is regulated by the alternative sigma factor σ^B. Class III genes form part of the CtsR stress response regulon and include genes encoding the ClpP protease and two ATPases, ClpC and ClpE. Class IV genes are devoid of regulation by the CIRCE operator sequence and σ^B, as well as CtsR, and the regulation of these genes remains to be elucidated. This group includes, among others, the genes encoding the ClpX ATPase and FtsH (8, 14). Recent DNA microarray experiments with *B. subtilis* revealed that more than 100 genes are heat induced (15). In addition, array experiments on σ^B-regulated genes indicated that

approximately 100 genes belong to this regulon and that these genes are involved in a variety of cellular processes, including protective processes, (post)transcriptional regulation, solute influx and efflux, and carbon metabolism (29, 30).

For several bacteria, it has been observed that stress exposures other than heat, such as exposure to ethanol, acid, or oxidative stress or during macrophage survival, might result in increased thermotolerance. Several HSPs are also induced under these conditions (1, 2, 3, 41). Common regulatory pathways might be responsible for the production of HSPs under different stress conditions, and in *B. subtilis* and *Staphylococcus aureus*, for example, a central role in these processes is thought to be played by σ^B (7, 14). Understanding cross-adaptation to different stresses and the involvement of stress proteins during these exposures might be instrumental in optimizing processing conditions to guarantee the microbial safety of food products (5). Another important applicative aspect of the heat shock response is the observation that *B. subtilis* cells pre-exposed to mild heat produce spores that are more heat stable (26). Understanding the heat-adaptive response of vegetative cells might also shed light on the mechanism of heat resistance of spores.

In this report, we provide evidence for a heat-protective response in *B. cereus* ATCC 14579. By using a proteomics approach, we observed the production of heat shock proteins. A reference map of the protein components of *B. cereus* was generated, and a group of 31 HSPs was identified. The role of these proteins in heat adaptation is discussed.

MATERIALS AND METHODS

Bacterial strain and growth conditions

The mesophilic strain *B. cereus* ATCC 14579 was used throughout this study. Cells were grown at 30°C in brain heart infusion (BHI) medium (Difco, Le Pont de Claix, France) with shaking at 130 rpm. Growth of *B. cereus* was monitored at 30, 35, 40, and 45°C by plate counting and measurement of optical density at 600 nm.

Viability and thermotolerance of *B. cereus* exposed to different stresses

B. cereus cells, cultured at 30°C, were pre-exposed to mild heat treatment at 42°C for 0, 7.5, 15, 30, 60, 120, or 240 min, after which their thermotolerance at 50°C was determined. Cultures in the mid-exponential growth phase (optical density at 600 nm, 0.5), grown at 30°C, were harvested by centrifugation (3,000 x g, 10 min), resuspended in preheated BHI medium, and kept at 42°C for the above-mentioned time periods. Consecutively, cells were exposed to 50°C and viable counts were measured after 0, 5, 10, 15, 20, and 25 min of exposure. For all heat exposures, three independent experiments were performed and samples were plated in duplicate for each time point. The heat tolerance of cells pre-exposed to the following other stresses was also assessed: low temperature (7°C for 2 h), salt upshock (additional 2.5% [wt/vol] NaCl at 30°C for 30 min), low pH (pH 5 adjusted with lactic acid at 30°C for 30 min), and the presence of ethanol (4% [vol/vol] ethanol at 30°C for 30 min). The heat sensitivity of cells pre-exposed to these stresses was analyzed in BHI medium as described above. The pre-exposures to heat or other stresses were also

performed in the presence of chloramphenicol (100 μg ml^{-1}) to inhibit *de novo* protein synthesis.

Protein extraction from *B. cereus*

Total cellular protein extractions were performed essentially as described by Wouters *et al.* (38). For each sample, 10-ml cultures were concentrated and, consecutively, cells were disrupted by bead beating with an MSK cell homogenizer (B. Braun Biotech International, Melsungen, Germany) and zirconium beads (0.1-mm diameter; Biospec Products, Bartlesville, Okla.) six times for 1 min (with cooling on ice between treatments). Subsequently, proteins in the homogenate were analyzed by Western blotting and two-dimensional gel electrophoresis (2D-E). The protein concentration in cell extracts was determined by using the bicinchoninic acid assay (Sigma Chemical Co., St. Louis, Mo.).

Western blotting.

Protein extracts (40 μg) were separated by using sodium dodecyl sulfate-12.5% polyacrylamide gels in a Criterion II vertical electrophoresis system (Bio-Rad, Richmond, Calif.) with a molecular size standard containing proteins of 94, 67, 43, 30, 20, and 14 kDa. After electrophoresis, proteins were electroblotted at 100 V on nitrocellulose membranes (Bio-Rad) and blocked with 0.1% sodium caseinate. Blots were then incubated with either GroEL, GroES, DnaK, DnaJ, ClpC, ClpP, ClpX, or FtsH rabbit antibodies raised against these proteins of *B. subtilis*. The antibodies were generous gifts of W. Schumann, University of Bayreuth, Bayreuth, Germany (GroEL, DnaK, DnaJ, and FtsH); K. Turgay, Albert-Ludwigs-Universität, Freiburg, Germany (ClpC); and U. Gerth, Ernst-Moritz-Arndt-Universität, Greifswald, Germany (ClpP and ClpX). Immunocomplexes were incubated with goat anti-rabbit peroxidase and visualized with 3,3'-diaminobenzidine tetrahydrochloride.

Protein analysis by 2D-E

Protein analysis was performed with a Multiphor 2D-E system (Pharmacia Biotech, Uppsala, Sweden) as described previously (38). Equal amounts of protein (50 μg) were separated on isoelectric-point gels at pI 4 to pI 7 and subsequently on homogeneous sodium dodecyl sulfate-12 to 14% polyacrylamide gels (Pharmacia Biotech). The gels were silver stained as described by Blum *et al.* (4). The experiments were performed in duplicate or triplicate, and representative gels are shown. The gels were analyzed, integrated, and normalized by using PD-Quest software (Bio-Rad). Induction factors for each heat-induced protein were calculated as the ratio of the normalized spot values in a stress gel to those in the control gel.

Determination of N-terminal amino acid sequences

For determination of the N-terminal amino acid sequences of specific spots, protein samples (1.5 mg) were separated on the 2D-E gels under conditions identical to those used for the running of analytical gels. The proteins were blotted on a polyvinylidene difluoride membrane optimized for protein transfer (Amersham Life Science, Buckinghamshire, England) with a Trans-Blot unit in accordance with the instructions of the manufacturer

(Bio-Rad) and stained with Coomassie brilliant blue. Protein spots were cut from the blot and subjected to consecutive Edman degradation and subsequent analysis with the model 476A Protein Sequencing System (Applied Biosystems, Foster City, Calif.) at the Sequence Center, University Utrecht (Utrecht, The Netherlands). By using BlastP and the *B. cereus* ATCC 14579 genome sequence database (Integrated Genomics, Chicago, Ill.; www.integratedgenomics.com), the derived N termini were screened for sequence similarities.

RESULTS

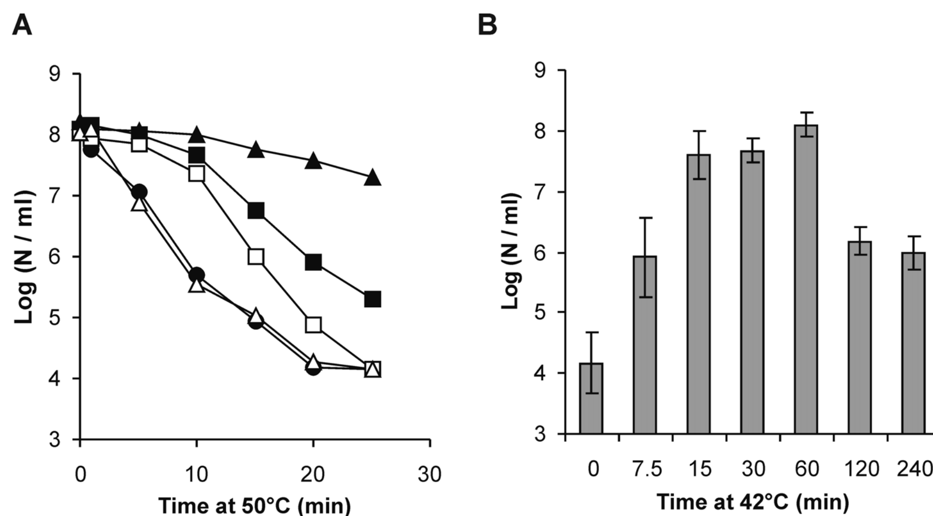

Fig. 1. Effect of mild heat treatment on the survival of *B. cereus* ATCC 14579 at 50°C. (A) Survival (log number of cells [N] per milliliter) of exponential-phase control cells grown at 30°C (circles) and that of cells pretreated for 7.5 (squares) and 15 (triangles) min at 42°C in the absence and presence of chloramphenicol (closed and open symbols, respectively). (B) Survival, after 20 min at 50°C, of mid-exponential-phase *B. cereus* ATCC 14579 cells pretreated at 42°C for 0, 7.5, 15, 30, 60, 120, and 240 min.

Heat sensitivity and thermotolerance of *B. cereus* pre-exposed to mild-heat stresses

In order to select optimal temperatures for the heat exposure experiments, the growth rates of *B. cereus* ATCC 14579 at different temperatures in BHI medium were defined. The maximum growth rates determined were 0.8, 1.1, 1.0, and 0.3 h^{-1} at 30, 35, 40, and 45°C, respectively. No growth was observed at 50°C within 24 h after inoculation. A temperature of 30°C was chosen as the standard growth temperature in order to expose *B. cereus* cells to a significant temperature upshift (from 30 to 42°C) to study their thermotolerance at the lethal temperature of 50°C. Upon exposure of mid-exponential-phase cells grown at 30 to 50°C, a 4-log reduction in viable counts was observed after 20 min (Fig.

1A). Cells pre-exposed to 42°C for 15 min showed a less-than-0.5-log reduction in viable cells after 20 min at 50°C (Fig. 1A).

Thus, pre-exposed cells showed strongly enhanced thermotolerance at 50°C compared to control cells. An increase in heat survival was also observed after pre-exposure at 42°C for 7.5 min, but to a lesser extent (approximately 100-fold greater survival). Longer pre-exposures of 30 min and 1 h at 42°C also produced a major increase in *B. cereus* thermotolerance compared to control cells (3- to 4-log increased survival after 20 min of heat treatment; Fig. 1B). After 2 and 4 h of pre-exposure, the thermotolerance decreased and was similar to that observed after 7.5 min of pre-exposure at 42°C (Fig. 1B). Interestingly, in the presence of chloramphenicol (an inhibitor of *de novo* protein synthesis) during pre-exposure at 42°C for 15 min, no increase in thermotolerance was observed compared to that of control cells. Exposure to heat in the presence of chloramphenicol for 7.5 min only partly blocked the development of thermotolerance, which is possibly explained by the time required to obtain a complete block of protein synthesis (Fig. 1A).

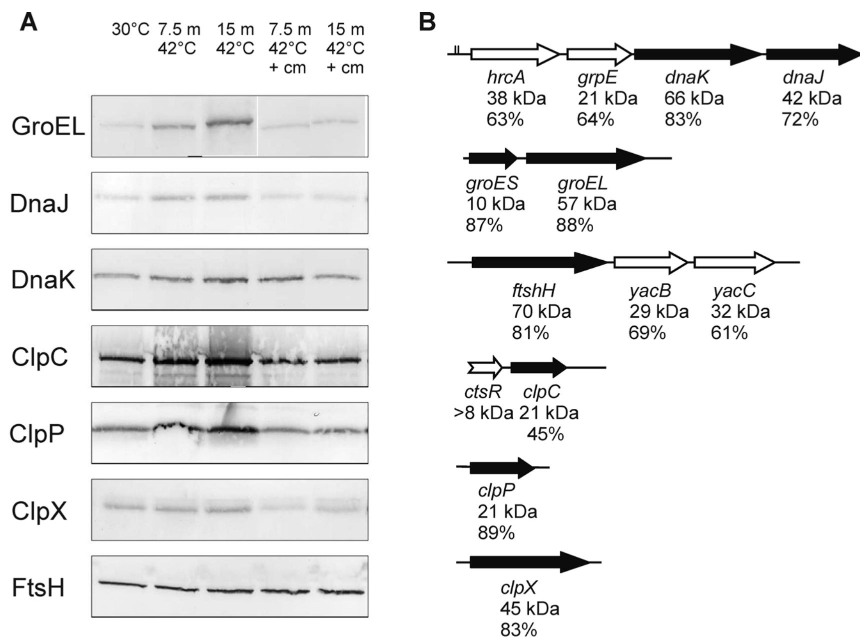

Fig. 2. Production of HSPs upon heat shock in *B. cereus.* (A) Western blot analysis of HSP (GroEL, DnaK, DnaJ, ClpC, ClpP, ClpX, and FtsH) production in *B. cereus*. Cells were grown to mid-exponential phase at 30°C (control) and exposed to mild heat treatment (42°C) for 7.5 and 15 min (m) in the absence and presence of chloramphenicol (cm). (B) Schematic representation of the gene clusters encoding the analyzed HSPs in *B. cereus* (black arrows) and proteins homologous to heat-induced proteins in *B. subtilis* (white arrows; HrcA, GrpE, the transcriptional regulator YacB, the redox regulated chaperone YacC, and CtsR), which were not found to be heat induced in *B. cereus* in our study. For each gene, the percent identity to the *B. subtilis* homologs and the molecular mass of the encoded protein are indicated. The CIRCE sequence preceding the gene encoding HrcA is indicated by two vertical lines.

Production of HSPs in *B. cereus* exposed to mild heat conditions

By using Western blotting with antibodies against the well-characterized *B. subtilis* HSPs GroEL, GroES, DnaK, DnaJ, ClpC, ClpP, ClpX, and FtsH, we analyzed the heat-induced production of these proteins in *B. cereus*. Except for GroES, all *B. subtilis* HSP antisera cross-reacted to their *B. cereus* counterparts (Fig. 2A).

We observed a significant (greater than threefold) induction of all HSPs after exposure of *B. cereus* to 42°C for 7.5 and 15 min. The only exception was FtsH, which was not heat induced. In the presence of chloramphenicol, no increased HSP levels were found (except for DnaK, which was slightly induced after 7.5 min), which indicates that the increased HSP levels are dependent on *de novo* protein synthesis. The genes encoding all of the above-described HSPs could be identified in the *B. cereus* genome sequence, including the *groES* gene. The gene clusters encoding these HSPs show orientations identical to those observed for their homologs in *B. subtilis*. Upstream of the gene encoding HrcA, a CIRCE element is found that exactly matches the conserved CIRCE sequence of *B. subtilis* (TTAGCACTC-N_9-GAGTCGTAA) (14; Fig. 2B). Because the upstream region of the *groESL* gene cluster is lacking in the *B. cereus* genome sequence, no CIRCE element could be identified upstream this operon. No other perfect CIRCE sequences were observed in the *B. cereus* genome sequence.

2D-E of extracts of *B. cereus* cells exposed to mild heat stress conditions

2D-E was used to gain an overview of the proteins induced by heat shock. On gels containing extracts of control and heat-shocked *B. cereus* cells, a total of approximately 250 proteins could be identified. Detailed analysis of the 2D-E gels revealed that 31 proteins were induced twofold or more in *B. cereus* by 15 min, 1 h, or 4 h of incubation at 42°C (Fig. 3; Table 1).

The HSPs could be grouped on the basis of the time points of maximal production. For the first group (proteins 2, 3, 4, 5, 10, and 17), the induction factors increased upon longer exposure to 42°C and were significantly higher after 4 h than after 15 min of incubation at 42°C. The majority of HSPs (proteins 6, 7, 8, 9, 11, 15, 16, 18, 20, 21, 22, 23, 24, 25, 29, 30, and 31) fall into a second category of proteins that were maximally produced after 15 min at 42°C but whose levels decreased again upon longer exposure, returning to (near) prestimulus levels. A third group (proteins 1, 12, 13, 14, 19, 26, 27, and 28) were induced after 15 min at 42°C and stayed approximately at this level during longer heat exposure (up to 4 h; Table 1). The N-terminal sequences of a subset of HSPs were determined (Table 2).

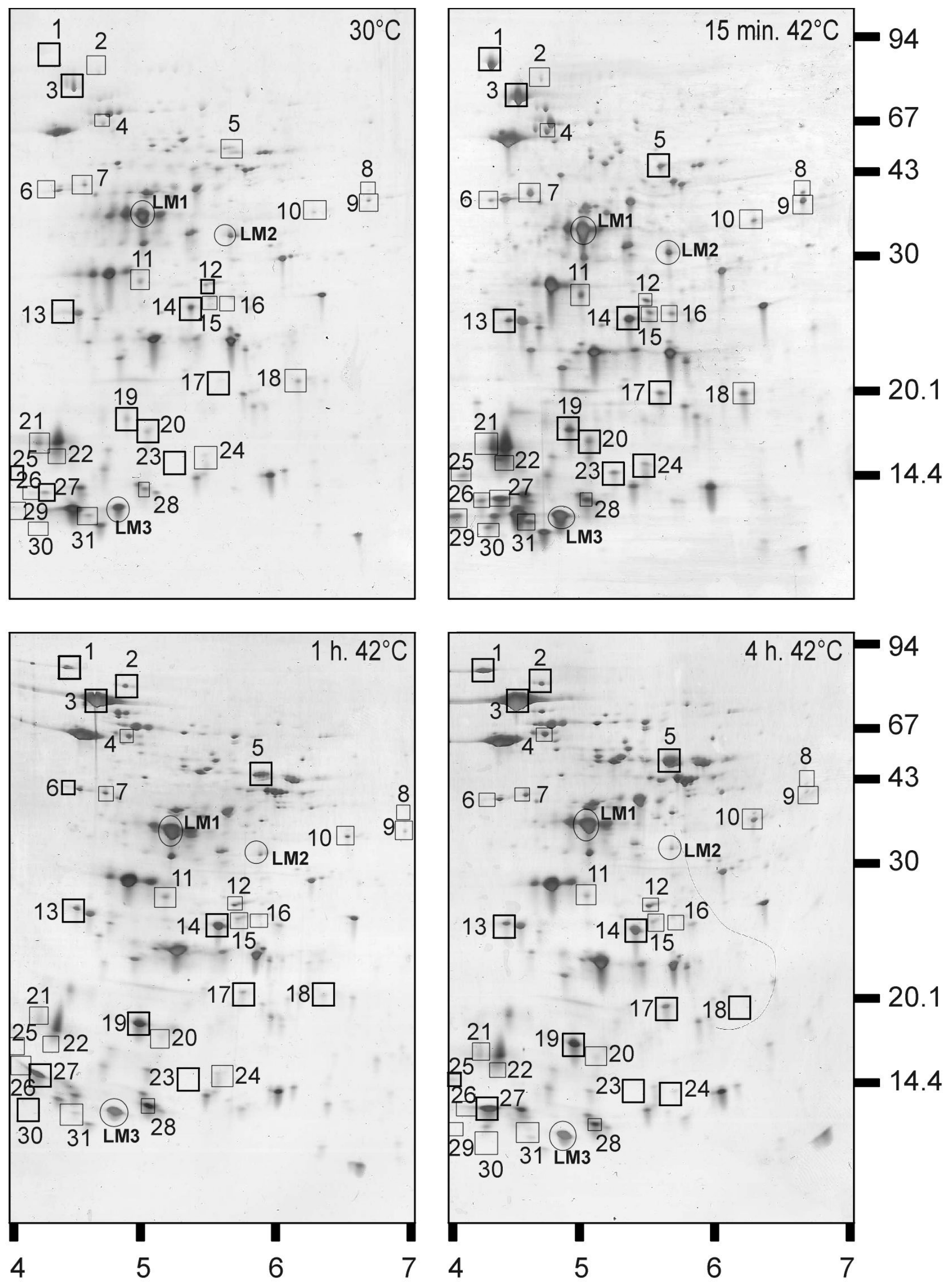

Fig. 3. 2D-E of extracts of exponential-phase *B. cereus* cells grown at 30°C and after mild heat treatment (42°C) for 15 min, 1 h, and 4 h. Molecular masses (in kilodaltons) of marker bands (right side) and pI ranges (bottom) are indicated. Heat-induced proteins are boxed and numbered (see also Tables 1 and 2).

Table 1. Induction factors[a] of heat-shock proteins of *B. cereus* ATCC14579

			Heat (42°C)			Stress conditions			
Spots	Protein[b]	Group[c]	15 min[d]	1 h	4h	7°C	NaCl	EtOH	pH 5
1	DnaK	I	>20	>20	>20	4.2	>20	3	2.5
2	n.d.	I	-	7.6	7.4	8.9	20	14.9	13.7
3	GroEL	I	3.5	5.5	7.5	-	2.1	-	-
4	YbbT	I	-	3.8	4.6	2.1	2.4	2.5	2.6
5	AldA	I	-	10.9	12.9	-	-	2.2	-
6	n.d.	II	2.6	-	-	4.8	8.6	-	3.7
7	MreB	II	2.3	-	-	-	-	2.5	2
8	n.d.	II	4	-	-	2.8	-	3.7	3.1
9	n.d.	II	2.9	-	-	-	-	3	3.1
10	FolD	I	-	2.8	6.3	-	-	3.1	-
11	Dra	II	2	-	-	-	-	4.5	-
12	SodA	III	2	2	2	2.3	-	4.8	-
13	n.d.	III	2	2	2.1	-	-	-	-
14	ClpP	III	3.5	3.9	4.1	-	-	3.6	-
15	n.d.	II	2.2	-	-	2.4	-	2.4	-
16	n.d.	II	2	-	-	-	-	-	-
17	HSP 16.4	I	8	4.4	16.2	-	-	-	-
18	PpiB	II	2	-	-	-	-	3	2.3
19	YflT	III	4.1	6.1	5.3	-	-	-	-
20	n.d.	II	2.4	-	-	-	-	-	-
21	n.d.	II	3.9	-	-	-	2.9	-	-
22	n.d.	II	7.1	-	-	-	-	-	-
23	RsbV	II	>20	3.7	4	-	-	-	-
24	SpoVG	II	2.5	-	-	-	-	3.8	-
25	TrxA	II	>20	-	-	-	>20	-	-
26	n.d.	III	4.9	2	2	-	5.2	-	3.5
27	YloH	III	8	7	10.4	-	-	-	-
28	n.d.	III	2.3	2.2	2	-	-	2.9	-
29	n.d.	II	17.6	-	-	-	9.9	-	2.2
30	CspE	II	4.2	-	-	-	3.5	-	-
31	CspB	II	2.9	-	-	-	-	2.2	-

[a] Normalized value in stress gel/normalized value in control gel.
[b] Identified heat-induced protein. n.d., N-terminal amino acid sequence not determined.
[c] Grouping is based on time point of induction (see text for details).
[d] Less-than-twofold induction.

Table 2. List of identified heat-shock proteins of *B. cereus* ATCC14579

HSP no.	Designation	N-terminal sequence[a]	Molecular mass[b] (kDa)	Description	% Identity (closest hit)
1[c]	DnaK	SKIIGIID |||||||| SKIIGIID	72	Heat shock chaperone	83 (*B. subtilis*)
3	GroEL	MAKDIKFSEEA ||||||||||| MAKDIKFSEEA	60	60-kDa chaperonin	89 (*B. subtilis*)
4	YbbT	XXKYFGTD |||||||| MGKYFGTD	48	Phosphoglucosamine mutase	78 (*B. subtilis*)
5	AldA	MRXGIPXE |||||||| MRIGIPTE	40	Alanine dehydrogenase	73 (*B. sphaericus*)
7	MreB	MFGFXGF ||||||| MFGFGGF	36	Rod shape-determining protein	84 (*B. subtilis*)
10	FolD	XVAVIIKG |||||||| MVAVIIKG	31	Methylene tetrahydrofolate dehydrogenase	68 (*B. subtilis*)
11	Dra	MNIAKLI ||||||| MNIAKLI	24	Deoxyribose phosphate aldolase	76 (*B.subtilis*)
12	SodA	XAKHELPN |||||||| MAKHELPN	23	Superoxide dismutase	79 (*G. stearo-thermophilus*)
14	ClpP	MNLIPTVIEQ |||||||||| MNLIPTVIEQ	21	ATP-dependent protease	90 (*B. subtilis*)
17	HSP 16.4	MRNLFPE ||||||| MRNLFPE	16	Heat shock chaperone	35 (*S.thermo-philus*)
18	PpiB	MKTLGYI ||||||| MKTLGYI	16	Peptidyl-prolyl *cis-trans* isomerase	74 (*B. halodurans*)

Table 2 (continued). List of identified heat-shock proteins of *B. cereus* ATCC14579

19	YflT	METXYRK ||||||| METKYRK	17	General stress protein 17M	47 (*B. subtilis*)
23	RsbV	MNLAIN |||||| MNLAIN	12	Anti-sigma factor B antagonist	95 (*B. anthracis*)
24	SpoVG	MEVTDVR ||||||| MEVTDVR	10	Stage V sporulation protein G	85 (*B. subtilis*)
25	TrxA	XAIVNAND |||||||| MAIVNAND	11	Thioredoxin	72 (*B. halodurans*)
27	YloH	MLXP |||| MLNP	7	RNA polymerase omega subunit	67 (*B. subtilis*)
30	CspE	MQGKVK |||||| MQGKVK	7	Cold shock protein E	92 (*B. cereus*)
31	CspB	MTLTGKV ||||||| MTLTGKV	71	Cold shock protein B	97 (*B. cereus*)

[a] The upper sequence is the derived N-terminal sequence, and the lower sequence represents the N-terminal sequence of the respective protein in the *B. cereus* ATCC 14579 genome. The letter X indicates an unknown residue.
[b] Calculated molecular mass based on the encoded gene sequence.
[c] The N-terminal sequences of proteins 2, 6, 8, 9, 13, 15, 16, 20, 21, 22, 26, 28, and 29 have not been determined.

Firstly, the identified proteins include HSPs reported for other organisms, such as DnaK, GroEL, ClpP, SodA, Hsp16.4, PpiB, RsbV, SpoVG, and TrxA. Next, a set of proteins were also identified that are induced in response to stresses other than heat in several bacteria but have been shown to be heat induced in *B. cereus*: YflT, CspB, and CspE. In addition, some *B. cereus* HSPs have not been reported to be heat induced before, such as YbbT, AldA, MreB, FolD, Dra, and YloH. The genes corresponding to the identified proteins were determined by using the *B. cereus* genome sequence, and percentages of homology to related proteins were calculated (Table 2).

Thermotolerance of *B. cereus* pre-exposed to different stress conditions.

To verify whether other stresses affect heat survival, *B. cereus* cells were exposed to 7°C, 2.5% NaCl, 4% ethanol, or pH 5 prior to exposure to heat treatment at 50°C. All of these pretreatments resulted in increased thermotolerance, although to different levels (Fig. 4). The greatest cross-protection was provided by salt stress; no cell number reduction was observed after 25 min of incubation at 50°C, compared to a 4-log reduction in control cells. No reduction in cell counts was noted during the first 10 min upon exposure to 50°C after ethanol or low-pH pretreatment. However, after 25 min at 50°C, 2- and 3.3-log reductions were observed after the low-pH and ethanol exposures, respectively. Pre-exposure to a low temperature had a marginal effect (a 10-fold increase) on *B. cereus* thermotolerance (Fig. 4). In the presence of chloramphenicol during the stress pre-exposures, some thermotolerance was acquired compared to that of control cells (a maximal 20-fold increase in salt-, pH-, and ethanol-stressed cells and no increase in low-temperature-stressed cells after 25 min at 50°C; data not shown). This indicates that the increase in thermotolerance obtained during these processes is not dependent solely on *de novo* protein synthesis.

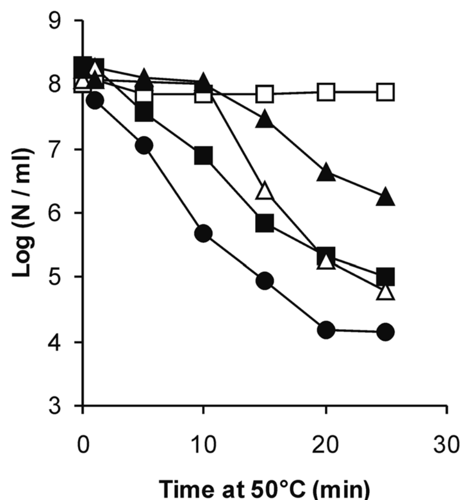

Fig. 4. Effect of pre-exposure to stress on survival of *B. cereus* cells at 50°C. Survival (log number of cells [N] per milliliter) of exponential-phase control cells grown at 30°C (circles) and that of cells pretreated for 30 min at 30°C with 2.5% NaCl (open squares), 4% ethanol (open triangles), and pH 5 (closed triangles) and cells preincubated at 7°C for 2 h (closed squares).

Production of HSPs in *B. cereus* exposed to different stress conditions

Next, we analyzed whether the identified *B. cereus* HSPs are induced by stresses other than heat by using Western blotting or 2D-E. By using Western blotting, we observed that the production of *B. cereus* HSPs GroEL, DnaK, DnaJ, ClpC, ClpP, ClpX, and FtsH increased after exposure to 4% ethanol. Upon exposure to NaCl, the production of DnaJ,

DnaK, ClpC, and ClpX increased. Under low-pH and low-temperature conditions, a weak induction of only DnaK and DnaJ was found (Fig. 5).

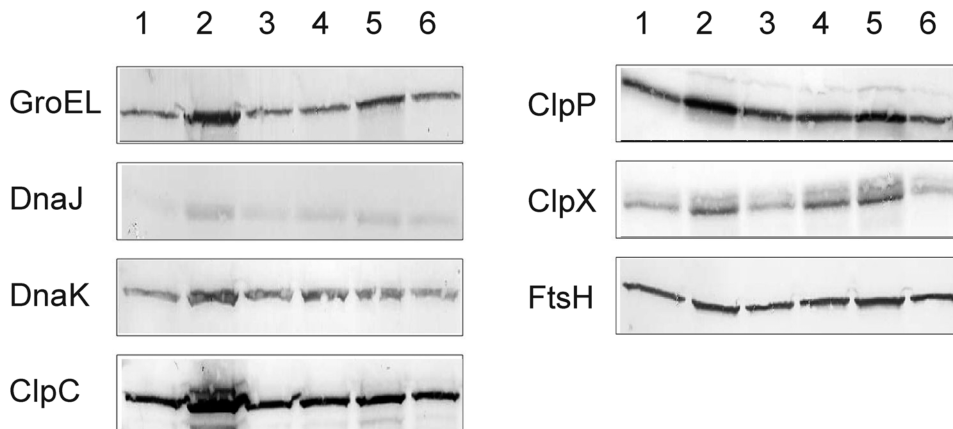

Fig. 5. Western blot analysis of HSP (GroEL, DnaK, DnaJ, ClpC, ClpP, ClpX, and FtsH) production in *B. cereus*. Cells were grown to mid-exponential phase at 30°C (column 1) and exposed to mild heat (42°C, 15 min) (column 2), a low temperature (7°C, 2 h) (column 3), 2.5% NaCl (30°C, 30 min) (column 4), 4% ethanol (30°C, 30 min) (column 5), or pH 5 (30°C, 30 min) (column 6).

Next, comparison of the 2D-E gels showed that 23 of the 31 HSPs identified were also induced during one of the stress exposures tested (Fig. 6; Table 2). Enhanced (greater than twofold) production levels were observed for 10, 16, 10, and 7 proteins after exposure to salt, ethanol, a low pH, and a low temperature, respectively. Strikingly, three HSPs (DnaK, protein 2, and YbbT) were upregulated under all of the stress conditions tested. The production of four proteins (GroEL, protein 21, TrxA, and CspE) increased only during the heat and salt stresses, while the enhanced production of seven proteins (AldA, FolD, Dra, ClpP, SpoVG, protein 28, and CspB) overlapped during heat and ethanol exposures (Table 2). Finally, we also observed increased production of non-heat-induced proteins after exposure to the different stress conditions (Fig. 6).

DISCUSSION

In the present study, we analyzed the heat stress response of *B. cereus* ATCC 14579. During a shift from 30 to 42°C, *B. cereus* cells develop an increased tolerance to lethal heat exposure at 50°C. This phenomenon of increased thermotolerance after mild pre-exposure has been shown in other bacteria (2, 3, 23, 28, 41) and is now also known in *B. cereus* ATCC 14579. Furthermore, it was shown that protein synthesis is required to obtain this increase in thermotolerance. By using specific antibodies against the HSPs DnaK, DnaJ, GroEL, ClpC,

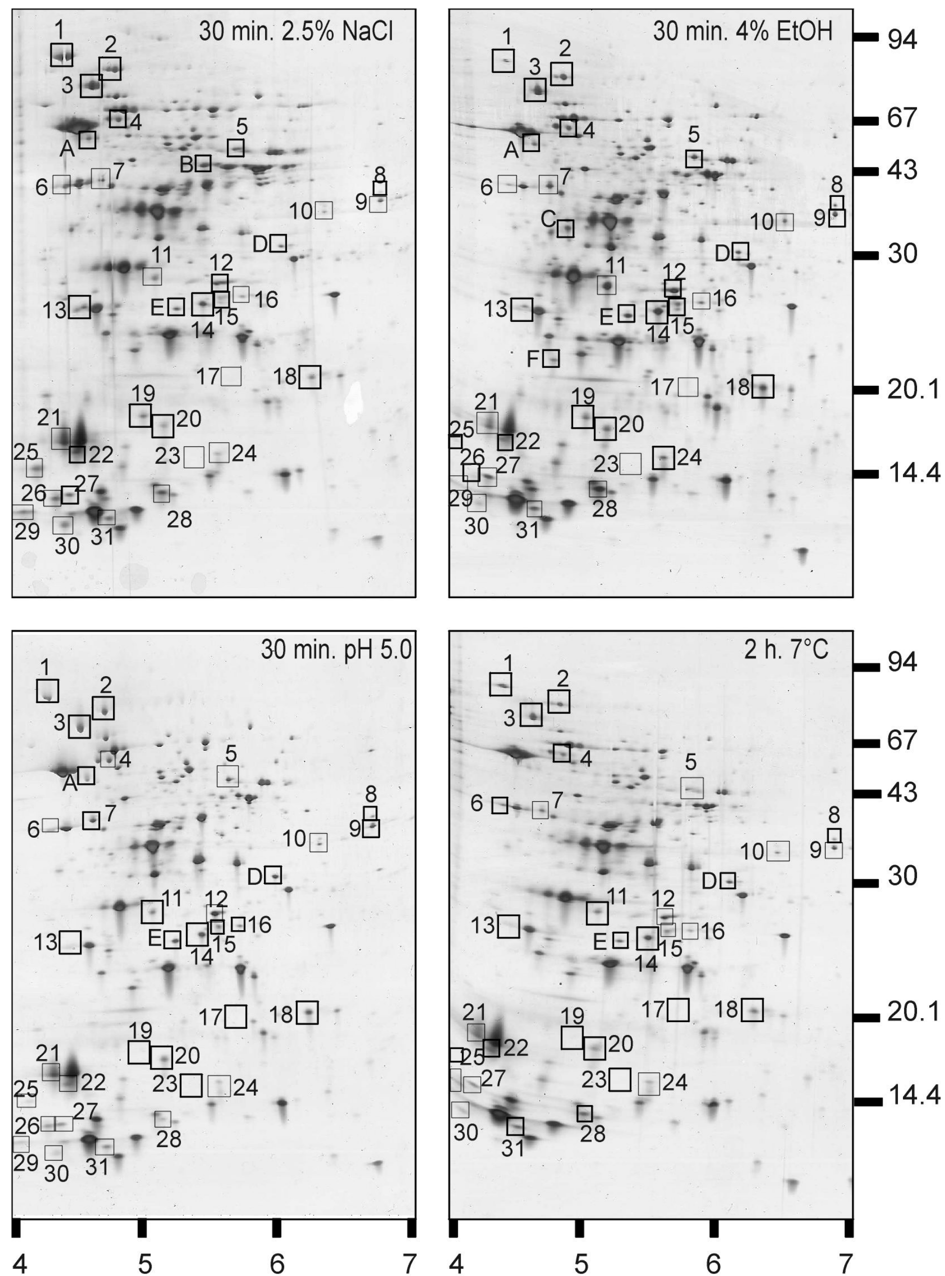

Fig. 6. 2D-E of extracts of *B. cereus* cells exposed to 2.5% NaCl, 4% ethanol, or pH 5 for 30 min at 30°C or to a low temperature (7°C) for 2 h. Molecular masses (in kilodaltons) of marker bands (right side) and pI ranges (bottom) are indicated. HSPs are boxed and numbered. Proteins typically induced by exposure to a stress other than heat are boxed and lettered in each panel for the respective stress conditions

ClpP, and ClpX, we showed that the production of these proteins increases during mild heat exposure and that this increase is absent in the presence of chloramphenicol. 2D-E revealed that 31 *B. cereus* proteins were induced during heat shock. Determination of the N-terminal sequences of a series of these proteins revealed that these proteins are involved in a variety of cellular processes. The majority of the proteins identified are homologous to stress proteins in other microorganisms but also to proteins involved in sporulation and in the biosynthesis of cellular compounds.

Several of the proteins identified belong to the group of chaperones (DnaK and GroEL) and proteases (ClpP) that have been described in great detail in other microorganisms (for reviews, see references 11 and 41). The induction of proteins of this type forms a highly conserved response to heat stress, and their induction by heat in *B. cereus* confirms their universal importance in stress adaptation. Other chaperones that are heat induced in *B. cereus* are HSP16.4, PpiB, YloH, CspB, and CspE. HSP16.4 is a member of the small heat shock protein (Hsp20) family and shows the greatest homology to a family of this type of proteins in the lactic acid bacterium *Streptococcus thermophilus* (34). PpiB is a peptidyl-prolyl *cis-trans* isomerase that can catalyze the refolding of proteins in *B. subtilis*. Although the disruption of *ppiB* in *B. subtilis* did not increase the sensitivity of the cells to heat stress, it is upregulated during heat shock (10). YloH is the omega subunit of RNA polymerase, and it has recently been shown to enhance the *in vitro* reconstitution of *E. coli* core RNA polymerase (9). YloH might act as a core RNA polymerase-specific chaperone that counters the core enzyme destabilization caused by elevated temperatures. To our knowledge, this is the first example of stress induction of the omega subunit of RNA polymerase, possibly suggesting a role for YloH in stress adaptation of the transcription machinery. Finally, in this chaperone family, two cold shock proteins, CspB and CspE, were induced upon heat stress. CspB and CspE have been described in *Bacillus weihenstephanensis* WSBC10201 and were only mildly induced upon cold shock (25), which is in agreement with our observations. Cold shock proteins are believed to act as RNA chaperones under low-temperature and other stress conditions (39). Here we show that CspB and CspE of *B. cereus* are induced during heat shock and osmotic or ethanol shock, respectively.

Within the group of *B. cereus* HSPs, we found several homologs to stress proteins. A protein with a possible role in protection of the cell against oxidative oxygen species is the thioredoxin TrxA. Thioredoxins are small, heat-stable proteins, and in *B. subtilis*, thioredoxin is induced under several stress conditions. Thioredoxin has multiple functions: it can serve as a hydrogen donor, it has been implicated in the formation of disulfide bonds in proteins, and it may be involved in the defense against oxidative stress (17, 32). Interestingly, the important regulatory protein RsbV is also induced by heat shock. RsbV is the anti-sigma factor antagonist of the alternative sigma factor σ^B, and its upregulation suggests the activation of σ^B under these conditions in *B. cereus*. Next, SodA is a superoxide dismutase that is highly homologous to superoxide dismutases from other members of the family *Bacillaceae*. This enzyme not only plays a role in protecting vegetative cells from reactive oxygen species but is also important in sporulation, as it is involved in the assembly

of the insoluble matrix of the spore (16, 18). Finally, the role of the general stress protein YflT is unclear. It is also upregulated under heat shock conditions in *B. subtilis* (37).

Apart from SodA, two other HSPs of *B. cereus* have a potential role in sporulation. Firstly, AldA is homologous to the Ald protein in *B. subtilis*, which catalyzes the deamination of alanine to pyruvate and ammonia and is required for normal sporulation. The generation of pyruvate by this mechanism may be a source of energy during the sporulation process (33). SpoVG is involved in sporulation as a negative regulator of the pathway leading to asymmetric septation. SpoVG is upregulated upon heat shock in *B. subtilis*, and this may function to block the sporulation process and favor the vegetative growth of cells (24).

The production of several proteins presumably involved in metabolic processes also increased at 42°C in *B. cereus*. FolD is a methylene-tetrahydrofolate dehydrogenase that is involved in the biosynthesis of essential cellular compounds such as purines, methionine, and histidine. Dra is the last enzyme in the cascade of the catabolism of deoxyribonucleosides and is thought to furnish the cells with an extra source of energy (31, 35). YbbT is homologous to a phosphoglucose mutase in *B. subtilis* that can be involved in the conversion of glucose 1-phosphate into glucose 6-phosphate in glycolysis. YbbT is also closely related (68% identity) to GlmM, a phosphoglucosamine mutase from *S. aureus* that is involved in peptidoglycan production and methicillin resistance (19, 40). Finally, the production of the rod shape-determining protein MreB increased in *B. cereus*. MreB was identified in *B. cereus* ATCC 10987 by Narahara *et al.* (27) and was recently characterized as the bacterial homologue of actin (20, 36). In *B. subtilis*, this protein is essential as its deletion causes inflated morphology and, ultimately, cell lysis (20). The upregulation of MreB in *B. cereus* may be required for retention of the rod-shaped cell form under stress conditions.

On the basis of the time after which heat-activated production occurred, the *B. cereus* HSPs can be divided into three groups: I, increasing induction within the exposure time (up to 4 h); II, maximum induction after 15 min with a decrease to basic levels after 1 or 4 h; III, constant overproduction during heat exposure (15 min to 4 h). By analogy to *B. subtilis*, the grouping of the *B. cereus* HSPs might correlate to common regulatory features. Transient induction of σ^B regulated genes and proteins in *B. subtilis* upon heat exposure has been observed (14, 15), which correlates to that of the *B. cereus* group II HSPs. Indeed, group II contains a regulator of σ^B, RsbV, which might point to σ^B-dependent expression of the genes in this group. In addition, two genes that are σ^B-dependent in *B. subtilis* (*trxA* and *ppiB*; 14, 32) are found in this group. However, group III also contains some genes that are σ^B regulated in *B. subtilis* (*yflT* and *sodA*; 14, 29). Continuous production of the classical heat shock chaperones and proteases, such as GroEL and DnaK, upon heat exposure has been observed (correlating to groups I and III of the *B. cereus* HSPs). This might relate to regulation by HrcA via CIRCE elements. Indeed, close to the transcriptional start of the *hrcA-grpE-dnaK-dnaJ* operon, which encodes, among other proteins, DnaK (group III), a CIRCE element is present. GroEL, which is HrcA regulated in *B. subtilis*, also falls into the cluster of proteins the production of which increases during heat exposure (group I).

In relation to these regulatory phenomena, it is important to note that *B. cereus* ATCC 14579 developed cross-protection from heat after exposure to salt, a low pH, and

ethanol, stresses in which common regulators may be involved. We also observed that the increase in the thermotolerance of *B. cereus* cells exposed to stresses other than heat is not solely protein synthesis dependent. An alternative or complementary mechanism can be the accumulation of compatible solutes or osmoprotectants that might function as thermoprotectants, as has been shown, for example, for glycine betaine in *E. coli* (6).

The increased use of mild heat preservation treatments and hurdle technology makes food products more susceptible to bacterial contamination than heavily processed foods. This development may have contributed to the reported rise in *B. cereus*-related food poisoning outbreaks in developed countries (21). Within the concept of hurdle technology, it is important to keep in mind that microorganisms can be more resistant to adverse conditions after a previous stress exposure and thus survive normally lethal conditions that occur during food processing. Here, we analyzed the initial responses of *B. cereus* to heat and other stresses. This will contribute to the further understanding of *B. cereus* adaptive mechanisms that may be applicable to food processing and cause increased survival of *B. cereus* during food processing. Even when more severe heat treatments or other preservation strategies are used, the understanding of the stress response of vegetative cells is of vital importance, as this process may lead to the generation of more resistant spores (26). In the development of cross-protection, key regulators, such as σ^B, are thought to play a central role. In future studies, we will characterize σ^B of *B. cereus* and study genes controlled by this sigma factor.

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4 THE ALTERNATIVE SIGMA FACTOR σ^B OF *BACILLUS CEREUS*: RESPONSE TO STRESS AND ROLE IN HEAT ADAPTATION

Willem van Schaik, Marcel H. Tempelaars, Jeroen A. Wouters, Willem M. de Vos, Tjakko Abee

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ABSTRACT

A gene cluster encoding the alternative sigma factor σ^B, three predicted regulators of σ^B (RsbV, RsbW, and RsbY), and one protein whose function is not known (Orf4) was identified in the genome sequence of the food pathogen *Bacillus cereus* ATCC 14579. Western blotting with polyclonal antibodies raised against σ^B revealed that there was 20.1-fold activation of σ^B after a heat shock from 30 to 42°C. Osmotic upshock and ethanol exposure also upregulated σ^B, albeit less than a heat shock. When the intracellular ATP concentration was decreased by exposure to carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), only limited increases in σ^B levels were observed, revealing that stress due to ATP depletion is not an important factor in σ^B activation in *B. cereus*. Analysis of transcription of the *sigB* operon by Northern blotting and primer extension revealed the presence of a σ^B-dependent promoter upstream of the first open reading frame (*rsbV*) of the *sigB* operon, indicating that transcription of *sigB* is autoregulated. A second σ^B-dependent promoter was identified upstream of the last open reading frame (*orf4*) of the *sigB* operon. Production of virulence factors and the nonhemolytic enterotoxin Nhe in a *sigB* null mutant was the same as in the parent strain. However, σ^B was found to play a role in the protective heat shock response of *B. cereus*. The *sigB* null mutant was less protected against the lethal temperature of 50°C by a preadaptation to 42°C than the parent strain was, resulting in a more-than-100-fold-reduced survival of the mutant after 40 min at 50°C.

INTRODUCTION

Bacillus cereus is a spore-forming gram-positive rod that is increasingly being recognized as a foodborne pathogen. It may cause illness through the production of a range of virulence factors. The most important virulence factors are a heat-stable emetic toxin, which causes vomiting, and several enterotoxins that cause diarrhea (14, 23). The symptoms of foodborne disease caused by *B. cereus* are generally mild and self-limiting, but in rare instances they can also be life-threatening, as was shown in 1998 when a food-poisoning outbreak in France, which was attributable to *B. cereus*, caused the deaths of three persons. The *B. cereus* strain that caused this outbreak produced a novel cytotoxin, CytK, which caused necrotic enteritis (30). *B. cereus* can also be the causative agent of other diseases, such as periodontitis, fulminant endophthalmitis, and meningitis in immunocompromised patients (2, 11, 12).

Because of the ubiquitous presence of *B. cereus* in the environment, it can easily contaminate food production or processing systems (23). *B. cereus* has the potential for multiple adaptive response pathways (20). These pathways may contribute to survival of the cells during food processing and storage and thus may contribute to the importance of *B. cereus* as a foodborne pathogen. Vegetative cells of *B. cereus* also play an important role in the pathogenesis of foodborne illness, because they produce the diarrheal enterotoxins in the host small intestine (31). In this situation, *B. cereus* has to deal with the stresses that it experiences in the gastrointestinal tract. Indeed, for some foodborne pathogens, the ability to mount a stress response is a prerequisite for virulence in the gastrointestinal tract (10).

Taxonomically, *B. cereus* is closely related to *Bacillus thuringiensis* and *Bacillus anthracis*. Together with *Bacillus weihenstephanensis* and *Bacillus mycoides*, these organisms form the *B. cereus* group. The members of this group are genotypically so similar that it has been proposed that the members of the *B. cereus* group should be considered members of the same species (17). However, the phenotypic differences among *B. cereus*, *B. anthracis*, and *B. thuringiensis* are substantial. While *B. cereus* causes generally mild cases of foodborne illness, *B. anthracis* is the etiological agent of the often lethal disease anthrax (22). *B. thuringiensis*, on the other hand, is generally considered a beneficial microorganism; it produces insecticidal toxins and is widely used as a biological control agent to counter insect pests in agriculture (41). Whole-genome sequencing of *B. cereus* ATCC 14579 (20) and *B. anthracis* Ames (39) and suppressive subtraction hybridization (38) have revealed some distinct genomic differences that distinguish *B. cereus* and *B. thuringiensis* from *B. anthracis*, but these differences do not seem to explain the phenotypic disparities in the *B. cereus* group mentioned above. The functional properties that differentiate these organisms are thought to be mostly caused by genes carried on plasmids or, possibly, by altered gene expression among strains (39).

Previously, a number of stress-induced proteins of *B. cereus* were identified by two-dimensional gel electrophoresis. These proteins included RsbV, the antagonist of the anti-sigma factor of σ^B, which was found to be upregulated during heat shock (33). This strongly suggested that a σ^B response is triggered during heat shock and potentially also under other stress conditions. σ^B has been studied extensively in several gram-positive bacteria. This

protein is a secondary subunit of RNA polymerase that is known to play an important role in regulating gene expression when there are major changes in the environment. The model organism for study of σ^B is *Bacillus subtilis* (see reference 36 for a recent review). *sigB* null mutants of *B. subtilis* have decreased resistance to heat, acid, ethanol, salt, and oxidative stress (35). Similar effects have also been described for *sigB* null mutants of the human pathogens *Listeria monocytogenes* and *Staphylococcus aureus* (1, 5, 7, 8).

The regulatory network leading to expression of σ^B in *B. subtilis* has been extensively studied for a number of years. Two differentially regulated pathways lead to activation of σ^B in *B. subtilis*. The first pathway is induced under environmental stress conditions (like ethanol exposure and osmotic shock), and the second pathway is induced by a decrease in the level of intracellular ATP (36, 48). The regulatory network leading to σ^B activation and repression functions by a so-called partner switching mechanism. In this system, interactions between the anti-sigma factor of σ^B (RsbW) and the anti-sigma factor antagonist (RsbV) and more regulators further upstream in the regulatory cascade are controlled by serine phosphorylation and dephosphorylation. This leads to the formation or dissociation of protein-protein complexes, which can finally lead to the release of σ^B from an RsbW-σ^B complex (35). More than 200 general stress response genes are under the control of σ^B in *B. subtilis*, and these genes encode proteins with a wide variety of cellular functions (19, 34, 37). In *B. anthracis* the alternative sigma factor σ^B was shown to be a minor virulence factor and to be activated during the stationary growth phase and after a heat shock (9).

In this paper, we describe the *sigB* operon of *B. cereus* ATCC 14579 and a predicted novel regulator of σ^B activity (RsbY), which is located directly downstream of the *sigB* operon. σ^B was activated under several stress conditions, particularly during heat shock but also during other stresses, such as osmotic upshock and ethanol exposure. No correlation between intracellular ATP levels and σ^B activation was found, indicating that σ^B activation is not triggered by energy depletion. We mapped two σ^B-dependent promoters in the *sigB* operon, which revealed the transcriptional organization of the σ^B operon in *B. cereus*. Finally, a *sigB* null mutant exhibited impaired survival at 50°C after preadaptation to 42°C compared to the survival of the parent strain. This indicates that σ^B plays a role in the adaptive response of *B. cereus* during heat stress.

MATERIALS AND METHODS

Bacterial strains, culture media, growth conditions, and plasmids

B. cereus ATCC 14579 was cultured in brain heart infusion (BHI) medium at 30°C with aeration at 200 rpm. All *Escherichia coli* strains were cultured in Luria broth at 37°C (40). *E. coli* DH5α (40) was used as a general-purpose cloning host. *E. coli* BL21-Codonplus-(DE3)-RIL (Stratagene, La Jolla, Calif.) was used as the host for SigB overproduction. *E. coli* HB101/pRK24 (44) was used as the donor host in conjugation experiments. The antibiotics used were ampicillin at a concentration of 50 µg/ml, kanamycin at a concentration of 70

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 counterselection against *E. coli* upon conjugation. The plasmids used in this study are listed in Table 1.

Table 1. Plasmids used in this study

Plasmid	Relevant properties	Reference
pGEM-T	PCR cloning vector, Amp^R	Promega
pET28-b	*E. coli* overexpression vector, Kan^R	Novagen
pMT01	pET28-b derivative containing sigB under control of the T7 RNA polymerase promoter	This study
pUC18ERY	Amp^R, Ery^R	(45)
pATΔS28	tra^+ conjugative suicide vector for *B. cereus* group, Spc^R	(32)
pATΔS28ery	pATΔS28 derivative containing erythromycin resistance cassette from pUC18ERY, Spc^R, Ery^R	This study
pATΔS28eryBY	pATΔS28ery derivative containing 1.1 kb downstream flanking region of *sigB*, Spc^R, Ery^R	This study
pATΔsigB	pATΔS28ery derivative containing 1.1 kb upstream and downstream flanking regions of *sigB*, Spc^R, Ery^R	This study

DNA manipulation and sequencing

Plasmid DNA was purified with a Qiaprep Spin Miniprep kit (Westburg, Leusden, The Netherlands). *B. cereus* chromosomal DNA was isolated by using a Wizard genomic DNA purification kit (Promega, Madison, Wis.) according to the manufacturer's instructions. DNA sequencing was performed with an ABI Prism 377 DNA sequencer (Applied Biosystems, Foster City, Calif.) and a DYEnamic ET terminator cycle sequencing kit (Amersham Biosciences, Roosendaal, The Netherlands). Restriction endonucleases and DNA ligase (MBI Fermentas GmbH, St. Leon-Rot, Germany) were used according to the manufacturer's instructions. PCR experiments for cloning DNA into vectors were performed with *Pwo* polymerase (Roche Diagnostics, Almere, The Netherlands). The oligonucleotides used are listed in Table 2.

The *B. cereus* ATCC 14579 genome sequence database was used throughout this study (http://www.integratedgenomics.com). Comparisons with the *B. anthracis* Ames genome and the unfinished *B. cereus* ATCC 10897 genome were made by using BLAST with microbial genomes at http://www.ncbi.nlm.nih.gov/cgi-bin/Entrez/genom_table_cgi. Comparisons with the incomplete *B. thuringiensis* subsp. *israelensis* ATCC 35646 genome were made at ERGO Light (http: //www.ergo-light.com). The free energy of stem-loop structures was calculated by using the Mfold server at http://mfold.burnet.edu.au.

Overexpression and purification of σ^B in *E. coli* and generation of polyclonal antibodies

sigB was amplified from *B. cereus* chromosomal DNA by PCR by using primers OEcSigBF and OEcSigBR, which introduced an NcoI site and an XhoI site, respectively. The

PCR product was cloned into pET28-b (Novagen, Madison, Wis.), and the resulting vector (pMT01) was transformed into *E. coli* BL21-Codonplus-(DE3)-RIL. To produce SigB, a 200-ml culture of this strain was grown at 37°C in Luria broth with kanamycin. When the cells reached the mid-exponential growth phase (optical density at 600 nm [OD_{600}], ~0.5), isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM, and incubation was continued for 2 h. Cells were then harvested, resuspended in lysis buffer (50 mM HEPES-NaOH [pH 7.5], 0.5 M NaCl, 1 mM dithiothreitol, 5 mM Pefabloc, 0.5 mg of lysozyme per ml), and incubated for 30 min at 4°C. Cells were then lysed by addition of Triton X-100 to a final concentration of 1% and subsequent sonication. The extract was then treated with DNase I (20 µg/ml) for 1 h at 37°C. The insoluble fraction containing the inclusion bodies was centrifuged (30,000 x *g*, 20 min, 4°C) and washed twice with phosphate-buffered saline with 1% Triton X-100. The washed pellet was resuspended in 20 ml of binding buffer (20 mM sodium phosphate buffer [pH 7.4], 8 M urea, 0.5 M NaCl, 10 mM imidazole, 1 mM dithiothreitol, 5 mM Pefabloc, 10% glycerol) and kept at 4°C for 1 h. After removal of debris by centrifugation, an aliquot of the supernatant, corresponding to 12 mg of protein, was loaded on a 1-ml Hi-Trap chelating HP Ni^{2+} column (Amersham Biosciences). The column was then washed with 10-ml portions of binding buffer containing decreasing concentrations of urea (8, 6, 4, 2, 1, and 0 M), which allowed on-column refolding of the protein. The protein was then eluted with an imidazole gradient (20 to 500 mM) and dialyzed against dialysis buffer (50 mM sodium phosphate buffer [pH 7.8], 0.3 M NaCl, 50% glycerol). Protein purity was assessed by Coomassie blue staining of a sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) gel. The protein concentration was measured by the bicinchoninic acid assay.

Table 2. Oligonucleotides used in this study

Oligonucleotides	Sequence (5' – 3')
OEcSigBF	GCAGCCATGGTGGAAATCCAATCTCAACCT[a]
OEcSigBR	GCAGCTCGAGTGTATCTAAAAATGCGGCTTG
EryCasF	GCGATCTAGAGTCCGCAAAAGAAAAACG
EryCasR	GCGAGGATCCCATACCTAATAATTTATCTAC
FlSigbupF	CACGTCTAGACTTACGACTTGCCTTGGTTC
FlSigbupR	CCCTTCTAGACCTGCGCTTCATCACATTGG
FlSigbdownF	GCGACCCGGGGGTTAGGTATTTCACAAATG
FlSigbdownR	GCGAGAATTCTTTAATTCCGATTTCAAGCG
PrRsbVF	AAATGATGGAGGTTATACG
PrRsbVR	TAATATTTCTGTTAACCCTG
PrOrf4F	TTTAGCAGGAGAATACTCAG
PrOrf4R	AACTCTGTCATATTTAATTTCG
PERsbV	AATCTACGTTATGAAAATCTA
PEOrf4	TGTCCTTGTTCATCACTAAT
SeqRsbV	GGAATGATTACGGGAAAAGACT
SeqOrf4	AATGAAAATTCCTGCAAAGG

[a] Introduced restriction sites are underlined

Rabbit serum containing anti-σ^B antibodies was prepared by Eurogentec S.A. (Herstal, Belgium) according to the company's standard protocol. Two rabbits were used to raise antibodies against σ^B. At the start of the protocol 100 µg of purified σ^B was mixed with Freund's adjuvant and injected intradermally. After 14, 28, and 56 days booster injections consisting of 100 µg of antigen mixed with incomplete Freund's adjuvant were administered. Finally, the animals were bled at day 66. The serum of the animal with the highest antibody titer was used for all experiments.

Protein extraction and immunoblotting techniques

Total cellular protein was extracted by bead beating as described previously (33). The protein concentration was determined by 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 by using 15% polyacrylamide gels and a Criterion II vertical electrophoresis system (Bio-Rad, Richmond, Calif.). Bio-Rad's broad-range prestained SDS-PAGE standards were used as molecular weight markers. After electrophoresis, proteins were electroblotted at 100 V onto nitrocellulose membranes. After the membranes were blocked by incubation with TBS (20 mM Tris-HCl [pH 7.5], 500 mM NaCl) with 0.1% sodium caseinate, the blots were incubated with TBS-0.05% Tween 20 supplemented with 2,000-fold-diluted rabbit immune serum containing the polyclonal anti-σ^B antibodies. Immunocomplexes were incubated with goat anti-rabbit peroxidase (Bio-Rad) and were visualized with 3,3'-diaminobenzidine tetrahydrochloride. The signal intensities of the Western blots were quantified by using the Gel-Pro Analyzer software package (Media Cybernetics, Silver Spring, Md.).

Determination of the intracellular ATP pool

ATP was measured by the firefly luciferase assay by using the the LuminATE luciferin-luciferase reagent (Celsis, Landgraaf, The Netherlands). Cells were lysed by adding 1 ml of culture to 2 ml of absolute ethanol that was prechilled to -20°C. The suspension was incubated for 10 min at -20°C before the luciferase reaction results were determined with a Lumac biocounter M2500. To correct for ATP present in the culture broth, 1-ml aliquots of the cultures were centrifuged (12,000 x *g*, 1 min), and each supernatant was analyzed to determine the ATP concentration as described above.

Construction of a *sigB* null mutant

First, an erythromycin resistance cassette was amplified from pUC18ERY with primers EryCasF and EryCasR. After digestion with XbaI and BamHI, the erythromycin resistance cassette was cloned into pATΔS28, resulting in pATΔS28ery. Subsequently, a 1.2-kb downstream flanking region of *sigB*, which contained 82 bp of the 3' end of *sigB*, was amplified by PCR with primers FlSigbdownF and FlSigbdownR and, after digestion with XmaI and EcoRI, inserted into pATΔS28ery, resulting in pATΔS28eryBY. Finally, a 1.2-kb

upstream flanking region of *sigB*, which contained 96 bp of the 5' end of *sigB*, was amplified by PCR with primers FlSigbupF and FlSigbupR and, after digestion with XbaI, inserted into pATΔS28eryBY, resulting in pATΔsigB. The orientation of the inserts in the vector was checked by restriction analysis and sequencing. The vector was then transformed into *E. coli* HB101/pRK24, and the resulting strain was used in conjugation experiments with *B. cereus*. Conjugation was carried out by using the standard protocol for conjugative plasmid transfer from *E. coli* to gram-positive bacteria (4). Transconjugants were selected for erythromycin resistance and screened for spectinomycin sensitivity. PCR and Southern analysis confirmed that the strains selected harbored the deleted allele of *sigB* and that the erythromycin resistance cassette had recombined into the chromosome through a double-crossover event rather than integration of the entire plasmid (data not shown). The *B. cereus sigB* null mutant was designated *B. cereus* FM1400.

Isolation of total RNA, Northern blotting, and primer extension

Total RNA was isolated from *B. cereus* by using Trizol (Invitrogen, Breda, The Netherlands). After precipitation of the nucleic acid, the residual DNA was removed with 10 U of RNase-free DNase I (Roche). After phenol-chloroform extraction and precipitation, the RNA was quantitated by measuring the OD_{260}. All RNA samples had an OD_{260}/OD_{280} ratio of ≥1.9.

RNA was separated on a 1.2% agarose-0.66 M formaldehyde-morpholine-propanesulfonic acid (MOPS) gel, which was electrophoresed at 40 V (constant voltage) and blotted onto Zeta-Probe membranes (Bio-Rad). Blots were hybridized and washed according to the manufacturer's instructions. Internal PCR fragments of *rsbV* (generated with primers PrRsbVF and PrRsbVR, resulting in a 270-bp product) and *orf4* (generated with primers PrOrf4F and PrOrf4R, resulting in a 302-bp product) were used as probes. Gel-purified probes were radiolabeled with [γ-^{32}P]dATP by nick translation. After hybridization and washing, the blots were exposed to a PhosphorImager screen. After exposure for 16 to 72 h, the screen was scanned with a Storm 840 system (Amersham Biosciences). A 0.24- to 9.5-kb RNA ladder (Invitrogen) was used to determine the transcript sizes.

Primer extension analysis was performed as described by Kuipers *et al.* (24). The oligonucleotides used were PERsbV and PEOrf4, which are complementary to *rsbV* and *orf4*, respectively. Four picomoles of primer was added to 50 μg of RNA in reaction buffer containing 10 nmol of dCTP, 10 nmol of dGTP, 10 nmol of dTTP, and 3.3 nmol of [α-^{32}P]dATP. cDNA was synthesized by addition of 200 U of Superscript II RNase H$^-$ reverse transcriptase (Invitrogen) and incubation for 10 min at 42°C, followed by addition of 10 nmol of cold dATP and incubation for 10 min at 42°C; the final volume of the reaction mixture was 20 μl. After the enzyme was inactivated by heating the preparation for 15 min at 70°C, 12 μl of formamide loading dye (95% formamide, 18 mM EDTA, 0.025% SDS, xylene cyanol, bromophenol blue) was added. After the samples were denatured by heating them at 80°C for 10 min, 5-μl aliquots were analyzed on a 7 M urea-8% PAGE sequencing gel prior to visualization by autoradiography. Sequence ladders were obtained by cloning template DNA (generated with PERsbV and SeqRsbV, resulting in a 941-bp product, and with PEOrf4 and SeqOrf4, resulting in a 1,429-bp product) into pGEM-T (Promega) and performing

radioactive sequencing with a T7 DNA polymerase sequencing kit (USB, Cleveland, Ohio) with the same primers that were used for the primer extension reaction.

Assay for virulence factors and heat resistance of *B. cereus* cells

Protease, lecithinase, and hemolytic activities were assayed on BHI agar plates supplemented with 5% milk, 5% egg yolk, and 5% sterile sheep blood (Biotrading, Mijdrecht, The Netherlands), respectively. Two microliters of an overnight culture of *B. cereus* was spotted on each plate. The plates were examined after 24 h of incubation at 30 and 37°C. The presence of enterotoxins in the supernatants of overnight cultures of *B. cereus* was determined with a Tecra BDE kit (Tecra Diagnostics, Frenchs Forest, Australia), which detects the NheA subunit of the nonhemolytic enterotoxin Nhe.

The heat resistance of vegetative *B. cereus* cells was assayed as described previously (33). Briefly, cells from a culture in the mid-exponential growth phase were exposed to a lethal temperature (50°C) with or without preexposure to 42°C for 30 min. Survival at 50°C was determined by plating appropriate dilutions on BHI agar plates, followed by overnight incubation at 30°C. For all heat exposures, three independent experiments were performed, and samples were plated in duplicate for each time point.

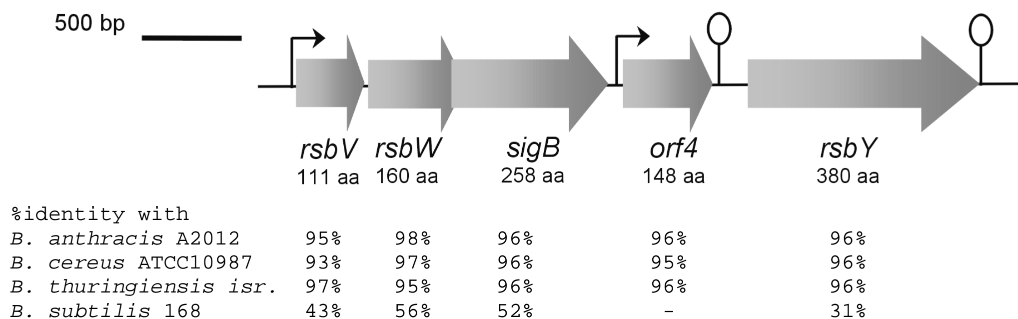
RESULTS

Sequence analysis of the *sigB* gene cluster in *B. cereus* ATCC 14579

A gene cluster encoding σ^B and its regulators was identified in the recently completed genome sequence of *B. cereus* ATCC 14579 (20) (Fig. 1). This gene cluster consisted of five open reading frames, which encode regulators of σ^B activity, the σ^B structural gene, and a protein with an unknown function. The products of all these open reading frames exhibit high amino acid identity with homologs encoded in the recently completed *B. anthracis* Ames genome (39), the unfinished *B. cereus* ATCC 10897 genome, and the unfinished *B. thuringiensis* subsp. *israelensis* ATCC 35646 genome, highlighting the close relationship among the different members of the *B. cereus* group. All of the open reading frames, except *orf4*, also have homologs in *B. subtilis* (26) with amino acid identities between 31 and 56% (Fig. 1).

The first open reading frame of the gene cluster is *rsbV*, which encodes a 111-amino-acid protein with the predicted function of an anti-sigma factor antagonist. The next open reading frame is *rsbW*, which is predicted to encode an 160-amino-acid protein that can function as an anti-sigma factor of σ^B. The *sigB* gene overlaps *rsbW* for 12 codons and codes for a protein consisting of 258 amino acids. The overlap between *rsbW* and *sigB* is conserved in *B. subtilis* (13 codons overlap), *L. monocytogenes* (13 codons overlap), and *S. aureus* (8 codons overlap) (1, 21, 25, 50). In *B. subtilis* these genes have been shown to be translationally coupled, ensuring that equimolar amounts of σ^B and its cognate anti-sigma factor are present (3). The fourth open reading frame was designated *orf4* and could code for a 148-amino-acid protein. The BLAST hit in the GenBank database with the highest significance for the gene encoding this protein is the *B. anthracis* homolog (96% identity),

which is also situated directly downstream of *sigB*. Orf4 from *B. cereus* is distantly related to bacterioferritins and Dps-like DNA binding proteins, as previously reported for Orf4 of *B. anthracis* (9). A homolog with an unknown function from the recently discovered and sequenced organism *Oceanobacillus iheyensis* strain HTE831 (29, 43) is also relatively closely related to Orf4, with a level of identity of 63%. This homolog is not part of the σ^B operon of *O. iheyensis*. Directly downstream of *orf4* a stem-loop structure was identified, which may function as a terminator. The free energy of formation of this structure is -9.4 kcal/mol.



%identity with					
B. anthracis A2012	95%	98%	96%	96%	96%
B. cereus ATCC10987	93%	97%	96%	95%	96%
B. thuringiensis isr.	97%	95%	96%	96%	96%
B. subtilis 168	43%	56%	52%	-	31%

Fig. 1. Diagram of the organization of the *sigB* gene cluster of *B. cereus* ATCC 14579. The large arrows represent open reading frames and indicate their orientations and sizes. The code numbers of these open reading frames in the *B. cereus* genome database (20) are RZC05131, RZC05126, RZC05124, RZC05127, and RZC05128. The predicted sizes of the encoded proteins (in amino acids [aa]) are also indicated. Predicted terminators downstream of *orf4* and *rsbY* are indicated by stem-loop structures. σ^B-dependent promoters identified in this study are indicated by small arrows. The levels of amino acid identity with homologs of the open reading frames in *B. anthracis* Ames (39), *B. cereus* ATCC 10987 (http://www.tigr.org), *B. thuringiensis* subsp. *israelensis* ATCC 35646 (http://www.ergo-light.com), and *B. subtilis* 168 (26) are indicated at the bottom. Note that in *B. subtilis* 168 the gene encoding the RsbY homolog (*rsbP*) is not located directly downstream of the *sigB* operon like *rsbY* in the *B. cereus* group.

The last open reading frame in the *sigB* gene cluster is *rsbY*. The 380-amino-acid protein that could be encoded by this open reading frame contains a C-terminal PP2C serine phosphatase domain and an N-terminal response regulator receiver domain homologous to CheY. The latter domain retained the highly conserved and functionally important residues equivalent to D12, D13, the phosphorylation site D57, T87, and K109 of CheY (49). Previously, *rsbY* was not identified in *B. anthracis* (9), presumably because an incomplete version of the *B. anthracis* genome sequence was used. A new search of the genomes available for the *B. cereus* group revealed that in all cases *rsbY* is present and located directly downstream of *orf4*. The closest well-described relative of RsbY is RsbP in *B. subtilis*. RsbP is also a two-domain protein; the N-terminal domain is a sensor PAS domain, while the C-terminal domain is a PP2C serine phosphatase domain. RsbP is activated upon energy stress and is then able to dephosphorylate RsbV, leading to σ^B activation (46). Directly downstream

of *rsbY* a strong stem-loop structure with a calculated free energy of formation of -20.6 kcal/mol was identified.

The genome of *B. cereus* ATCC 14579 was also searched for homologs of other regulators (RsbQ, -R, -S, -T, -U, and -X) of the environmental stress and energy stress pathway of σ^B activation in *B. subtilis*. RsbU exhibited some homology with the C-terminal part of RsbY and with stage II sporulation protein E, but the other σ^B regulators of *B. subtilis* have no apparent homologs in *B. cereus* ATCC 14579.

Overexpression of *sigB* in *E. coli*, purification of σ^B, and generation of anti-σ^B antibodies

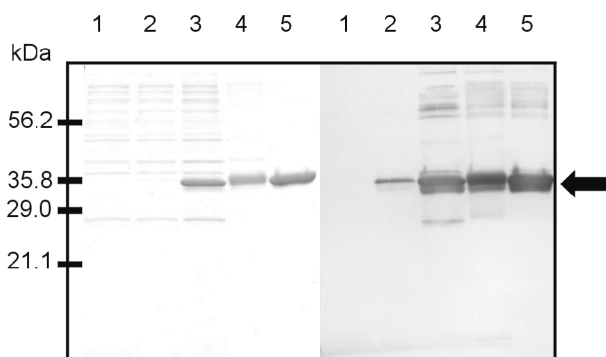


Fig. 2. Overproduction and purification of σ^B. (Left panel) SDS-PAGE of protein extracts from *E. coli* BL21-Codonplus-(DE3)-RIL carrying either pET28-b or pMT01. (Right panel) Immunodetection of σ^B with anti-σ^B antiserum. Ten micrograms of protein was loaded for each sample. Lane 1, crude protein extract from *E. coli* carrying pET28-b; lane 2, crude protein extract from *E. coli* carrying pMT01; lane 3, crude protein extract from *E. coli* carrying pMT01 after induction with 1 mM IPTG for 2 h; lane 4, inclusion bodies isolated from *E. coli* carrying pMT01 after induction with 1 mM IPTG for 2 h; lane 5, representative fraction of purified σ^B after elution from an Ni^{2+} affinity column. The arrow indicates the position of the overproduced σ^B protein.

To analyze the role of σ^B in the stress response of *B. cereus*, we raised polyclonal antibodies against σ^B to determine intracellular σ^B levels under a variety of stress conditions. To obtain sufficient amounts of antigen for the immunization protocol, σ^B was overexpressed in *E. coli*. The *sigB* gene was cloned into the overexpression vector pET28-b, creating a fusion with six C-terminal histidine residues. Subsequently, σ^B was purified by Ni^{2+} affinity chromatography. In the standard overexpression host *E. coli* strain BL21λDE3(pLysS), only very limited production of σ^B could be obtained (data not shown), presumably because of the differences in codon usage between *E. coli* and *B. cereus*. In the codon bias-adjusted BL21 derivative *E. coli* BL21-Codonplus-(DE3)-RIL, σ^B production was dramatically increased. Purification of σ^B resulted in a >95% pure protein as judged on a Coomassie blue-stained

SDS-PAGE gel. The purified protein was then used for generation of polyclonal antibodies against σ^B (Fig. 2).

Even when the histidine tag was taken into account, σ^B migrated as a slightly larger protein in the SDS-PAGE gel than predicted on the basis of its predicted molecular mass (29 kDa). This is a property of many sigma factors because of highly positive and negative charge clusters in the proteins (6, 13, 15, 42). The antiserum that was raised against σ^B reacted specifically with purified σ^B, although some cross-reaction was also seen with larger proteins in the purified σ^B sample (Fig. 2).

Activation of σ^B under stress conditions

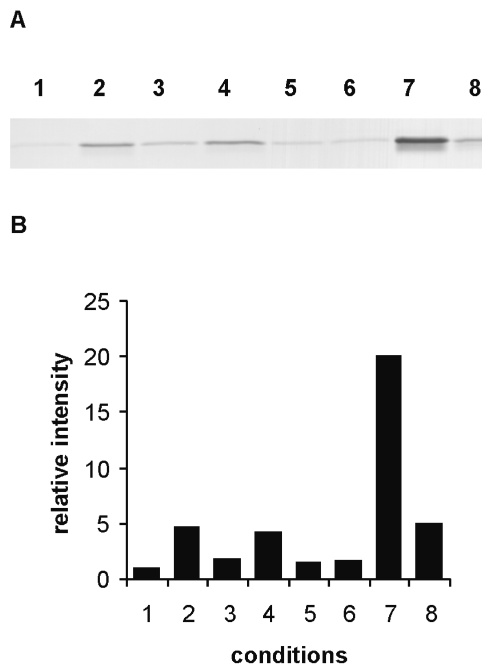


Fig. 3. Stress-induced activation of σ^B in *B. cereus*. (A) Cellular σ^B levels upon exposure to stress. Protein extracts from mid-exponential-phase *B. cereus* cells (lane 1) and stressed *B. cereus* cells were prepared as described in Materials and Methods. *B. cereus* cells in the mid-exponential growth phase were exposed to 4% (vol/vol) ethanol (lane 2), pH 5.2 (the pH was adjusted by addition of HCl) (lane 3), 2.5% (wt/vol) NaCl (lane 4), 50 μM H_2O_2 (lane 5), 1 mM diamide (lane 6), and 42°C for 30 min (lane 7). Proteins were also extracted from an overnight culture (lane 8). Forty micrograms of protein of each sample was loaded on the SDS-PAGE gel. Immunoblotting was performed with the sample by using the σ^B antiserum. (B) Relative amounts of σ^B. The signal intensities from the Western blot shown in panel A were quantified by using the Gel-Pro Analyzer software package (Media Cybernetics). The value for the mid-exponential-phase culture was defined as 1.

To characterize the σ^B response of *B. cereus* under stress conditions, σ^B levels in total protein extracts from stressed *B. cereus* cells were determined by Western blotting by using the anti-σ^B antiserum. The antiserum reacted strongly with a protein band at 34 kDa, which corresponded to the expected migration of native σ^B in *B. cereus.* This band was absent when protein extracts from the *B. cereus sigB* null mutant (see below) were studied, confirming that it was indeed σ^B.

We found that several types of stress can lead to activation of σ^B (Fig. 3A). In cells taken from a culture in the mid-exponential growth phase (OD_{600}, 0.4 to 0.5), low levels of

σ^B were present, but upon exposure to stress conditions, the σ^B levels rose rapidly. To quantify activation of σ^B under stress conditions, we determined the signal intensities of the σ^B bands (Fig. 3B). The greatest effect was observed after a heat shock at 42°C. Densitometric analysis of the σ^B band on the Western blot revealed that there was 20.1-fold activation of σ^B. In an overnight culture, which had been in the stationary phase for several hours, σ^B was found to be expressed at levels that were 5.0 fold higher than the levels in cells in the mid-exponential growth phase. Addition of 4% ethanol or 2.5% NaCl or an acid shock at pH 5.2 led to 4.6-, 4.2-, and 1.8-fold induction of σ^B, respectively. A limited effect on σ^B levels was observed after oxidative stress induced by addition of 50 μM H_2O_2 or the thiol-specific oxidizing agent diamide at a concentration of 1 mM (1.5- and 1.6-fold σ^B activation, respectively).

Energy stress is not an important trigger for activation of σ^B

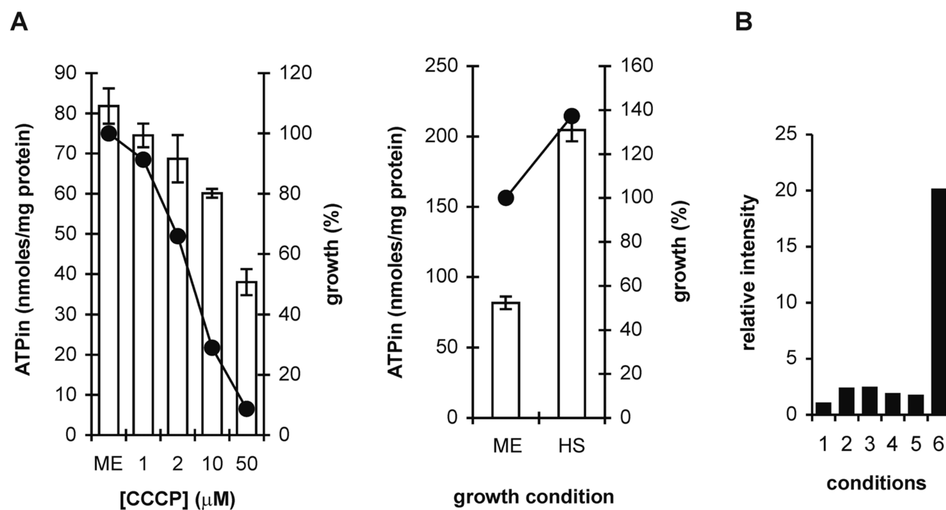


Fig. 4. Effects of CCCP on growth, the intracellular concentration of ATP, and σ^B expression of *B. cereus*. (A) Growth (circles) and intracellular ATP concentration (bars) in *B. cereus* cells from a mid-exponential-phase culture (ME) after exposure to 1, 2, 10, and 50 μM CCCP (left panel) and after a heat shock (HS) at 42°C for 30 min (right panel). Growth of the culture was monitored by determining the increase in the OD_{600} during the 30 min of exposure to CCCP or 42°C. The increase in the OD_{600} over 30 min for an untreated culture was defined as 100%, and growth in the CCCP-treated and heat-shocked cultures was related to this value. Intracellular ATP concentrations were determined by the firefly luciferase assay as described in Materials and Methods. (B) Relative levels of σ^B in CCCP-treated *B. cereus* cells (lanes 1 to 5 contained 0, 1, 2, 10, and 50 μM CCCP, respectively) and heat-shocked *B. cereus* cells (lane 6). The cellular levels of σ^B were estimated by immunoblotting with anti-σ^B antiserum and quantification of the signal by the Gel-Pro Analyzer software package as described in the text. Forty micrograms of protein from each sample was loaded on the SDS-PAGE gel used for Western blotting.

In *B. subtilis*, the energy stress pathway of σ^B activation is controlled by RsbP, which responds to a decrease in the size of the intracellular ATP pool (46, 48). Because of the presence of the RsbP homolog RsbY in the proteins encoded by the *B. cereus sigB* gene cluster, we decided to test if a reduction in the intracellular ATP level would also result in σ^B activation in *B. cereus*. The intracellular ATP pool was depleted by using increasing concentrations of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), which is an agent that uncouples oxidative phosphorylation. A 30-min exposure to CCCP resulted in a decrease in the intracellular ATP concentration and inhibition of growth (Fig. 4A). A limited σ^B-activating effect (≤2.5-fold induction on the protein level) was observed in the cultures exposed to CCCP (Fig. 4B). During a heat shock from 30 to 42°C for 30 min, the intracellular ATP concentration rose more than twofold and the σ^B level increased 20.1-fold. These results indicate that the σ^B response in *B. cereus* is not triggered by a drop in the intracellular ATP concentration, as in *B. subtilis* (48), but seems to occur solely under environmental stress conditions.

Transcriptional analysis of the *sigB* operon

To study transcriptional regulation of the *sigB* operon and the physiological role of σ^B in the resistance of *B. cereus* to stress, a *sigB* null mutant (*B. cereus* FM1400) was constructed, in which the *sigB* gene was disrupted by an erythromycin resistance cassette. PCR and Southern blotting analysis showed that the erythromycin resistance cassette had correctly integrated into the *sigB* gene (data not shown).

Transcription of the *sigB* operon in the *sigB* null mutant and its parent strain was studied. The activation of σ^B under stress conditions was confirmed by Northern blot experiments performed with RNA isolated from cultures of *B. cereus* FM1400 and the parent strain in the mid-exponential growth phase and after 10 min of exposure to 42°C (Fig. 5A). In the *sigB* null mutant, no transcription of the *sigB* operon was observed under both these conditions. In wild-type cells in the mid-exponential growth phase no transcription of the *sigB* operon was observed, which corresponded to the barely detectable σ^B levels in the Western blot experiments. In the RNA isolated from wild-type cells after a 10 min of exposure to 42°C, a 2.1-kb mRNA transcript hybridized with the *rsbV* probe, corresponding to a transcript covering the *rsbV-rsbW-sigB-orf4* region. This transcript could also be visualized after the blot was probed with DNA probes specific for *rsbW* and *sigB* (data not shown). These results demonstrate that the *sigB* operon is autoregulated by σ^B. When an *orf4*-specific probe was used, the 2.1-kb transcript was also identified after a heat shock, but in addition a strong band at 0.5 kb was also observed. This transcript could not be visualized in the RNA samples from *B. cereus* FM1400, which indicates that the transcript is σ^B-dependent.

On the basis of the results obtained in the Northern blot experiments, we performed primer extension analysis and mapped two σ^B-dependent promoters in the *sigB* operon (Fig. 5B and C). These promoters are located upstream of *rsbV* and *orf4*, and both of them are silent during mid-exponential growth but are activated upon a heat shock.

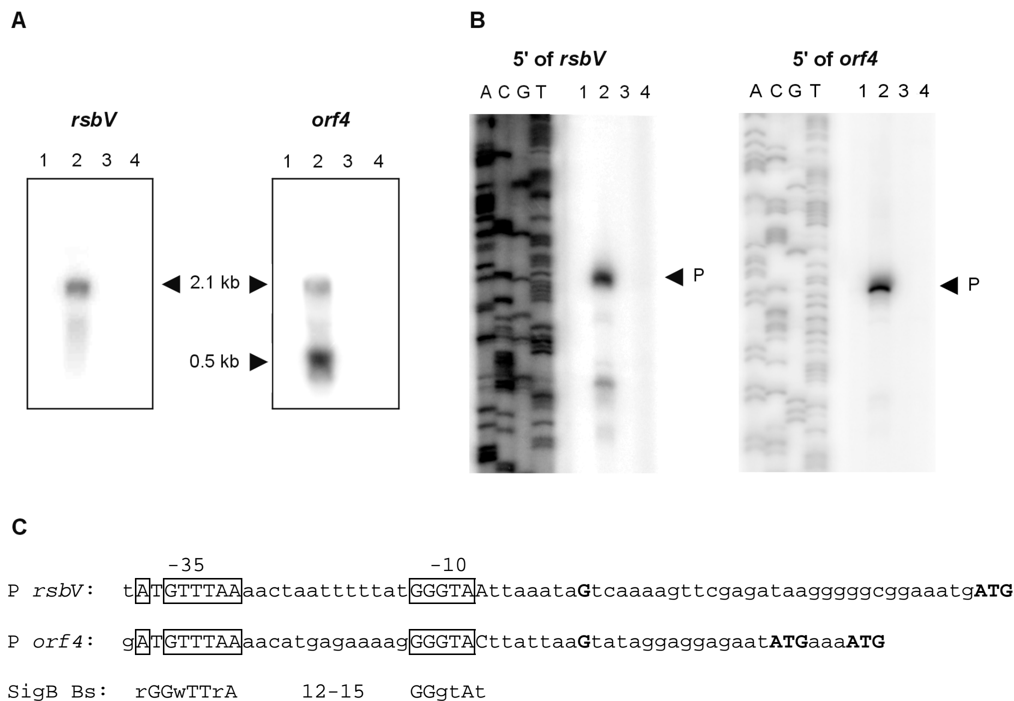


Fig. 5. Analysis of transcription of the *sigB* operon in *B. cereus*. (A) Northern blot analysis of transcription of the *sigB* operon. Total RNA was extracted from *B. cereus* ATCC 14579 and *B. cereus* FM1400 cells during exponential growth in BHI medium (lanes 1 and 3, respectively) and after a 10-min exposure to 42°C (lanes 2 and 4, respectively). ^{32}P-labeled internal PCR products of *rsbV* and *orf4* were used as probes. Hybridization of the probe with target RNA was visualized by exposure to a Phosphoscreen and scanning with a Storm scanner. Transcript sizes are indicated by arrowheads. (B) Primer extension analysis of promoters 5' of *rsbV* and *orf4.* For all reactions 50 µg of RNA was used. Total RNA was extracted from *B. cereus* ATCC 14579 and *B. cereus* FM1400 cells during exponential growth in BHI medium (lanes 1 and 3, respectively) and after a 10-min exposure to 42°C (lanes 2 and 4, respectively). Mapped transcriptional start sites are indicated by arrowheads. Lanes A, C, G, and T contained the corresponding sequencing ladders for localization of the transcripts. (C) Promoter sequence alignment for the σ^B-dependent promoters 5' of *rsbV* and *orf4.* The positions of identified -35 and -10 regions are indicated. The nucleotides in boxes in the -35 and -10 regions fit the σ^B promoter consensus sequence of *B. subtilis* (18). Uppercase letters in the *B. subtilis* σ^B promoter consensus sequence indicate highly conserved residues, and lowercase letters indicate less conserved residues (R = A or G, W = A or T). Transcriptional start sites and ATG start codons are indicated by boldface type. Putative Shine-Dalgarno sequences are underlined.

In *B. cereus* FM1400 these promoters are silent under both conditions, showing that they are σ^B-dependent. The promoter upstream of *rsbV* has -35 and -10 sequences of ATGTTTAA and GGGTAA, respectively, with a spacing of 13 nucleotides. This promoter sequence closely resembles the consensus sequence of σ^B promoters in *B. subtilis.* For this organism, the consensus sequences for the -35 and -10 regions have been described as rGGwTTrA and GGgtAt, respectively (uppercase letters indicate highly conserved residues,

and lowercase letters indicate less conserved residues; R = A or G, W = A or T), which are separated by 12 to 15 nucleotides (18). The promoter 5' of *orf4* was also shown to be activated only in the wild-type strain upon heat shock. The -35 and -10 regions of this promoter are ATGTTTAA and GGGTAC, respectively, which are separated by 13 nucleotides. This means that the -35 and -10 regions of this promoter are practically identical to the regions of the promoter 5' of *rsbV*; the only difference is the last residue of the -10 region (C instead of A).

Role of σ^B in production of virulence factors and heat resistance of vegetative cells

We did not observe any radical difference between the phenotypes of the *B. cereus sigB* null mutant and its parent strain. The growth rate at 30°C of the *sigB* null mutant in BHI broth did not differ from the growth rate of the parent strain, and cultures of both strains reached the same cell density in the stationary phase. We assayed protease, lecithinase, and hemolytic activities and the production of nonhemolytic enterotoxin by both wild-type and *sigB* null mutant cells and found no significant differences between the strains in any of the assays. Hence, σ^B does not play a role in the production of virulence factors in *B. cereus.*

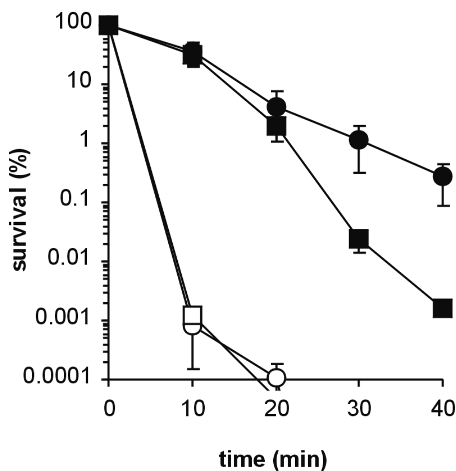


Fig. 6. The protective heat shock response of *B. cereus* ATCC 14579 and its *sigB* deletion mutant. Survival of of *B. cereus* ATCC 14579 cells (circles) and *B. cereus* FM1400 cells (squares) in the mid-exponential growth phase at 30°C (open symbols) and after pretreatment at 42°C for 30 min before exposure to 50°C (solid symbols). The averages of three independent experiments are shown. The error bars indicate standard deviations

To examine the role of σ^B in the adaptive response of *B. cereus*, we focused on the thermotolerance of vegetative cells. Previously, it has been shown that mid-exponential cells of *B. cereus* are very sensitive to a high temperature (50°C) but that they can be protected by preadaptation to a permissive temperature, 42°C (33). We showed that upon heat shock from 30 to 42°C the σ^B levels increased substantially (Fig. 3), suggesting that σ^B may play a role in survival of the cells at high temperatures. Therefore, we tested the thermotolerance of *B. cereus* FM1400 and its parent strain in the mid-exponential growth phase with and without preadaptation to 42°C (Fig. 6). In the nonadapted cultures there was no significant

difference between the levels of survival of the two strains; both died rapidly at 50°C, and the viable counts were around or below the detection limit after 20 min of incubation at 50°C. After preadaptation to 42°C, the wild-type cells showed dramatically increased survival at 50°C. The *sigB* null mutant was also protected by preadaptation at 42°C, but it was clearly protected less than the wild-type cells, which resulted in >100-fold-lower survival after 40 min at 50°C compared to the survival of the parent strain. These results demonstrate that in *B. cereus* σ^B is involved in the protective response of vegetative cells against high temperature.

DISCUSSION

In this paper we describe the transcriptional organization and expression of the *sigB* operon of *B. cereus* and provide data concerning activation of σ^B upon exposure to stress. In this study we also found that σ^B is involved in the protective heat stress response. Our results establish a starting point for further studies of the role of σ^B in the stress response of the food pathogen *B. cereus* and related members of the *B. cereus* group. We specifically studied regulation of σ^B activity, and hence we can compare and contrast the *B. cereus* σ^B response with the responses of other gram-positive bacteria.

Several stresses activate σ^B in *B. cereus*. A heat shock from 30 to 42°C leads to 20.1-fold activation of σ^B and is by far the most powerful trigger leading to σ^B activation in *B. cereus*. This is in agreement with a previous study, in which several stress-induced proteins of *B. cereus* were identified by two-dimensional gel electrophoresis (33). One of the proteins identified was the anti-sigma factor antagonist RsbV, which was upregulated >20-fold after a heat shock. The data obtained suggested that σ^B is activated upon heat shock. In this study, we obtained experimental evidence that σ^B is indeed activated upon heat shock. Furthermore, we found that σ^B is also activated under other stress conditions, albeit at a level that is an order of magnitude less than the level during a heat shock, which may explain why the effects were not observed in a previous proteomic study by our group (33).

In *B. subtilis*, activation of σ^B in response to heat stress has been well documented (16, 19, 36, 37) and seems to be signaled through the environmental stress pathway (48). It has been proposed that in *B. subtilis* structural changes in the ribosome during stress could lead to induction of this pathway (51). Conceivably, a similar mechanism could also be involved in triggering σ^B activation in *B. cereus*. Energy stress can be ruled out as an important factor in σ^B activation in *B. cereus*, because (i) decreasing the intracellular ATP concentration results in limited activation of σ^B (≤2.5-fold) and (ii) during a heat shock at 42°C the intracellular concentration of ATP increases but, nevertheless, σ^B is strongly activated.

Analysis of the transcriptional organization of the *sigB* operon revealed that this operon is transcribed as a 2.1-kb transcript encompassing *rsbV*, *rsbW*, *sigB*, and *orf4*. *orf4* is also under control of an additional σ^B-dependent promoter, and this makes *orf4* a member of the σ^B regulon in *B. cereus*. The exact role of Orf4 in the stress response of *B. cereus* and

other members of the *B. cereus* group is still unknown. This protein may function as a bacterioferritin, as predicted on the basis of its distant homologs, as proposed previously for Orf4 in *B. anthracis* (9), but experimental data are needed to verify this.

The *sigB* operon ends directly downstream of *orf4*, because the transcripts starting upstream of *rsbV* and *orf4* both end at this site, which suggests that the stem-loop structure downstream of *orf4* functions as a terminator. Downstream of this structure we identified an open reading frame, *rsbY*. The encoded protein is annotated as a σ^B regulatory protein. The *rsbY* gene was found to be present directly downstream of the σ^B operon in all members of the *B. cereus* group whose genome sequences are available. The position of RsbY in the *sigB* operon and its domain structure strongly suggest that it can function like the PP2C serine phosphatase proteins RsbU and RsbP in *B. subtilis*, which play crucial regulatory roles in activation of σ^B in that organism (36).

In other gram-positive bacteria, σ^B has not been studied as extensively as it has been in *B. subtilis*, but there are some intriguing similarities and differences between the σ^B responses of these organisms and the σ^B response of *B. cereus.* In *L. monocytogenes*, σ^B is also activated in response to different stresses, but in this organism osmotic shock is the most powerful trigger. Heat shock is also an important activating factor, and other stresses, like ethanol stress and acid shock, result in significantly less marked activation of σ^B (1). In *S. aureus*, a thorough examination of the stresses which activate σ^B has not been performed, but the available data suggest that heat shock is the most potent inducer in this organism, while ethanol shock has a limited effect (25). Of all the *sigB* operons in gram-positive bacteria, the pattern of activation in the *S. aureus* operon seems to be the most comparable to the pattern in the *B. cereus* operon. Interestingly, in *S. aureus*, σ^B seems to be regulated by three Rsb proteins, just as it is in *B. cereus*. An important difference between *S. aureus* and *B. cereus*, however, is the fact that the phosphatase containing the PP2C phosphatase domain is a single domain protein (RsbU) in *S. aureus* (25, 50). This again underlines the uniqueness of σ^B in the *B. cereus* group; both the stresses that activate σ^B and the organization of the operon are markedly different from the stresses that activate σ^B and the organization of the operon in other gram-positive bacteria studied thus far. In *B. cereus* σ^B also plays a role in protecting the cells against high temperature. In *B. subtilis* (47) and *S. aureus* (5), σ^B is also involved in the protective heat stress response, but an *L. monocytogenes sigB* null mutant was not more heat sensitive than its parent (7). This inconsistency in the phenotypes of *sigB* null mutants may be caused by differences in the set of σ^B-regulated genes in the various organisms.

The observation that σ^B does not play a role in the production of virulence factors and the nonhemolytic enterotoxin Nhe was not unexpected, since in the *B. cereus* group the production of these factors is governed by the pleiotropic regulator PlcR and σ^B seems not to be involved in transcription of the *plcR* gene (27, 28). However, a *sigB* null mutant may show a weakened stress response. This may indirectly decrease its virulence by impeding the growth or survival of the organism in food or the host, analogous to the role of the stress response in the virulence of the food pathogens *L. monocytogenes* and *Salmonella* spp. (10).

The fact that σ^B is activated when *B. cereus* is exposed to several different stress conditions and the observation that σ^B plays a role in survival during exposure to high temperature can have significant consequences for the control of *B. cereus. B. cereus* is a pathogen whose responses to different environmental situations can lead to cross-protection against normally lethal conditions (23, 33). The data presented here prove that σ^B is activated under several conditions and that σ^B has an important role in the adaptive response of *B. cereus*. Activation of σ^B may lead to increased survival of *B. cereus* during food processing and thus to increased risk of food poisoning outbreaks. In future research, the role of σ^B in the stress response of *B. cereus* will be elucidated further, and studies will focus on the σ^B regulon and the pathway leading to σ^B activation in *B. cereus*.

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5 IDENTIFICATION OF σ^B-DEPENDENT GENES IN *BACILLUS CEREUS* BY PROTEOME AND *IN VITRO* TRANSCRIPTION ANALYSIS

Willem van Schaik, Marcel H. Zwietering, Willem M. de Vos, and Tjakko Abee

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ABSTRACT

The alternative sigma factor σ^B of the food pathogen *Bacillus cereus* is activated upon stress exposure and plays a role in the adaptive response of vegetative cells. This study describes the identification of σ^B-dependent genes in *B. cereus*. Two-dimensional gel electrophoresis was performed with protein extracts from a σ^B-overproducing *B. cereus* strain. Nine protein spots, which were absent from the negative control, were identified by matrix-assisted laser desorption ionization-time of flight mass spectrometry or N-terminal sequencing. The σ^B-dependent expression of the corresponding genes was confirmed by Northern blot analysis with RNA isolated from *B. cereus* ATCC 14579 and its *sigB* null mutant. Northern blot analysis also revealed that six other genes were part of σ^B-dependent operons. The proteins that are predicted to be encoded by the σ^B-dependent genes include an intracellular protease, a Mg^{2+} transporter, and a thiamine biosynthesis protein (ThiG). Highly conserved promoter sites were found to precede all σ^B-dependent genes, with the exception of *thiG*. By searching the *B. cereus* genome for this conserved promoter sequence, five more candidate σ^B-dependent genes were identified. Northern blot analysis and *in vitro* transcription experiments with reconstituted *B. cereus* σ^B-RNA polymerase holoenzyme confirmed the σ^B-dependency of two of these genes and strongly suggested that two other genes, encoding an oligopeptide-binding OppA-like protein and subunit II of the cytochrome *d* ubiquinol oxidase, are also σ^B-dependent. In conclusion, σ^B of *B. cereus* not only regulates genes directly involved in the stress response but may also control specific metabolic rearrangements.

INTRODUCTION

Bacillus cereus is a common foodborne pathogen, which can cause outbreaks of foodborne disease. Symptoms are generally mild and self-limiting, ranging from diarrhea to vomiting (17, 31). Occasionally, however, the symptoms of *B. cereus* foodborne disease can be life-threatening. For instance, in a *B. cereus* food-poisoning outbreak in France in 1998, one of the symptoms was bloody diarrhea, which resulted in the deaths of three persons (35). *B. cereus* is also an emerging pathogen in clinical settings, where it can cause severe infections, especially in immunocompromised patients (8, 14, 20).

B. cereus is closely related to *Bacillus anthracis*, the causative agent of anthrax, and *Bacillus thuringiensis*, which is widely used as a biological pest control agent. These organisms, together with *Bacillus weihenstephanensis* and *Bacillus mycoides*, form the *B. cereus* group (for a recent review, see reference 24). Whole-genome sequencing of *B. cereus* and *B. anthracis* showed a remarkably conserved core set of genes. The presence of virulence-associated plasmids and subtle chromosomal differences may explain the phenotypic differences between the different members of the *B. cereus* group (23, 47, 48).

For both *B. cereus* and *B. anthracis*, the alternative sigma factor σ^B has been studied in considerable detail. For *B. anthracis*, σ^B was shown to be upregulated upon heat shock and during the stationary growth phase. Furthermore, a *sigB* null mutant had attenuated virulence in a mouse model (13, 34). In *B. cereus*, heat shock also had a strong σ^B-activating effect, but other stresses (such as ethanol shock, osmotic shock, and acid stress) were also found to lead to the activation of σ^B. There was no detectable effect of the *sigB* deletion on the production of *B. cereus* virulence factors such as hemolysins, lecithinases, and the nonhemolytic enterotoxin Nhe, but σ^B was shown to play a role in the adaptive heat stress response of *B. cereus* (52).

The role of σ^B in the stress response of vegetative cells has been studied in the human pathogens *Staphylococcus aureus* and *Listeria monocytogenes*, but most extensively in *Bacillus subtilis*. A *B. subtilis sigB* null mutant has an increased sensitivity to a wide variety of stresses, including acid, ethanol, heat, salt, and oxidative stress (9, 53). The set of σ^B-dependent genes (the σ^B regulon) has been identified by a number of techniques, including two-dimensional gel electrophoresis, σ^B-promoter consensus searching, and transcriptome profiling by DNA microarray analysis. This resulted in a set of approximately 200 σ^B-dependent genes. Relatively few of these genes seem to have a role in actively protecting the cell against environmental stress. The majority of σ^B-dependent genes code for proteins that seem to be involved in a metabolic rearrangement that can confer passive stress resistance (for a recent review, see reference 45). In the human pathogen *S. aureus*, σ^B plays a role in both stress resistance and the expression of virulence determinants (21). In a proteomics study, 23 σ^B-dependent genes were identified in *S. aureus*. Several of the encoded proteins were predicted to have a function in the generation of NADH or in membrane transport mechanisms (15). For the foodborne pathogen *L. monocytogenes*, σ^B has been shown to be involved in protection against osmotic stress and oxidative stress and in the acid tolerance response (2, 11, 12). The σ^B regulon of *L. monocytogenes* was recently

identified by DNA microarray analysis. Several stress response genes and genes involved in virulence were thus identified as being σ^B-dependent (29).

A comparison between the σ^B regulons of *B. subtilis*, *S. aureus*, and *L. monocytogenes* revealed a considerable overlap in the functions of the σ^B-dependent genes in these organisms. However, the divergence of the σ^B regulons suggests that the σ^B regulon has evolved to serve different roles among gram-positive bacteria (29). The natural niche of *B. cereus* has been proposed to be the nutrient-rich environment of the insect intestine (23, 24, 36), and as a consequence the σ^B regulon of *B. cereus* may have evolved to serve specific functions in this environment. Furthermore, the identification of the σ^B regulon of *B. cereus* may provide an explanation of the weakened heat stress response of the *sigB* null mutant of *B. cereus* (52) and give clues about further roles for σ^B in *B. cereus*.

In this paper, we describe the identification of a total of15 σ^B-dependent proteins by a two-dimensional gel electrophoresis (2D-E) approach upon σ^B overproduction in *B. cereus*, followed by a Northern blot analysis. By performing a σ^B promoter consensus search of the *B. cereus* genome, we identified five more candidate σ^B-dependent genes. Northern blot analysis and *in vitro* transcription experiments with a reconstituted *B. cereus* σ^B-RNA polymerase (RNAP) holoenzyme confirmed the σ^B-dependency of two of these genes and strongly suggested that two other genes are also σ^B-dependent. Several of the identified σ^B-dependent proteins do not have a clearly defined function, but others may have roles in the turnover of misfolded proteins or in influencing metabolic fluxes through the cell.

MATERIALS AND METHODS

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

B. cereus ATCC 14579 and its *sigB* null mutant FM1400 (52) were cultured in brain heart infusion (BHI) medium at 30°C, with aeration at 200 rpm. The growth of the culture was monitored by measurement of the optical density at 600 nm (OD_{600}). *Escherichia coli* MC1061 (7) was used as a host for the vectors of the nisin inducible controlled expression (NICE) system and was cultured in Luria broth at 37°C with aeration at 200 rpm (49). The antibiotics used were chloramphenicol at 10 µg/ml and erythromycin at 150 µg/ml (for *E. coli*) or 5 µg/ml (for *B. cereus*).

Plasmid DNAs were purified from *E. coli* with a Qiaprep Spin Miniprep kit (Westburg, Leusden, The Netherlands). *B. cereus* was transformed with plasmid DNA by electroporation, as described previously for *B. thuringiensis* (3). For the purification of plasmids from *B. cereus*, 5 ml of a culture in the mid-exponential growth phase was spun down, resuspended in 250 µl of THMS (30 mM Tris-HCl [pH 8.0], 3 mM $MgCl_2$, 25% sucrose) plus 2 mg of lysozyme/ml, and incubated for 1 h at 37°C before proceeding with the standard plasmid purification protocol. Pwo polymerase (Roche Diagnostics, Almere, The Netherlands) was used for all PCRs in this study. Radiochemicals were obtained from Hartmann Analytic GmbH, Braunschweig, Germany. Other genetic methods have been described previously (52).

Inducible overproduction of σ^B in *B. cereus*

sigB was amplified by a PCR employing the primers OBcSigBF (GCAGCCATGGT-GGAAATCCAATCTCAACCT) and OBcSigBR (GCAGCTGCAGTGTATCTAAAAATGCGG-CTTG), which introduced an NcoI and a PstI site (underlined), respectively. The PCR product was cloned into pNZ8048 (32), and the resulting vector, pFM100T, was transformed into *E. coli* MC1061. From this strain, the plasmid DNA was purified, and after sequencing to check for the absence of mutations in the insert, the vector was transformed into *B. cereus* ATCC 14579, which already carried pNZ9520 (30). The overproduction of σ^B was induced by the addition of nisin to a mid-exponential-phase culture (OD_{600} = 0.4 to 0.5) to a final concentration of 10 ng/ml. The cells were then cultured for a further 90 min before being harvested.

Total RNA isolation and Northern blotting techniques

RNA was isolated from *B. cereus* by the use of RNAwiz (Ambion, Huntingdon, United Kingdom). After precipitation of the nucleic acid, residual DNA was removed with 20 U of RNase-free DNase I (Ambion). After phenol-chloroform extraction and precipitation, the RNA was quantified by measuring the OD_{260}. All RNA samples had an OD_{260}/OD_{280} ratio of $\geq$1.9.

Five micrograms of total RNA was separated in a 1.2% agarose-0.66 M formaldehyde-morpholinepropanesulfonic acid (MOPS) gel which was run at a 40-V constant voltage and blotted onto a Zeta-Probe membrane (Bio-Rad, Richmond, Calif.). Internal PCR fragments of open reading frames were used as probes. The PCR fragments were radiolabeled with [α-^{32}P]dATP by nick translation. After hybridization with ULTRAhyb hybridization buffer (Ambion) and stringent washing according to the manufacturer's instructions, the blots were exposed to a phosphorimager screen. After an exposure time of 16 to 24 h, the screen was scanned on a Storm 840 system (Amersham Biosciences, Roosendaal, The Netherlands). ImageQuant TL software (Amersham Biosciences) was used for image analysis. A 0.24- to 9.5-kb RNA ladder (Invitrogen, Breda, The Netherlands) was used to determine the transcript sizes.

Protein extraction, Western blotting techniques, and 2D-E

Protein extraction and Western blotting with an anti-σ^B antiserum were performed as described previously (52). 2D-E was performed as described previously (43). In brief, equal amounts of protein (40 μg for analytical gels and 800 μg for preparative gels) were first separated in 11-cm-wide Immobiline DryStrip gels (Amersham Biosciences) at pHs 4 to 7 and subsequently separated in ExcelGel precast sodium dodecyl sulfate (SDS)-12 to 14% polyacrylamide gradient gels (Amersham Biosciences). The Precision Plus protein standard (Bio-Rad) was used as a molecular weight standard. The gels were silver stained and analyzed with PD-Quest software (version 7.1; Bio-Rad). Experiments were performed at least in triplicate, and representative gels are shown in the figures.

Identification of protein spots by MALDI-TOF and N-terminal sequencing

Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) analysis of manually excised spots was performed at the Maastricht Proteomics Center (Department of Human Biology, University of Maastricht, Maastricht, The Netherlands) by trypsin digestion in a MassPrep station (Micromass, Almere, The Netherlands) and subsequent analysis with a MALDI-TOF LR mass spectrometer (Micromass). Peptide mass fingerprints were analyzed with Mascot software (Matrix Science Ltd., London, United Kingdom).

For determination of the N-terminal sequences of specific spots, gels were blotted onto Hybond-P polyvinylidene difluoride membranes (Amersham Biosciences) in a Trans-Blot unit (Bio-Rad) with 10 mM CAPS buffer (pH 11.0) plus 15% methanol at 50 V for 30 min and were then stained with Coomassie blue. Protein spots were cut from the blot and analyzed by consecutive Edman degradation with the model 476A protein sequencing system (Applied Biosystems) at the Sequence Center, University Utrecht (Utrecht, The Netherlands).

Purification of *B. cereus* RNAP and *in vitro* transcription techniques

B. cereus cells (20 g of wet weight) from a culture grown in BHI medium at 30°C to an OD_{600} of 1 were homogenized in 25 ml of lysis buffer (0.05 M Tris-HCl [pH 8.0], 5% glycerol, 2 mM EDTA, 0.1 mM dithiothreitol, 1 mM ß-mercaptoethanol, 0.23 M NaCl, and 23 µg of phenylmethylsulfonyl fluoride/ml) and lysed by two passages through a French press at 10,000 lb/in^2. Subsequently, RNAP was purified by following established protocols for the purification of *E. coli* RNAP (5, 16), using Polymin-P fractionation, heparin-Sepharose affinity chromatography, A5M gel filtration, and phosphocellulose chromatography. The RNA core and holoenzyme were eluted from the phosphocellulose column with P50 buffer (40 mM potassium phosphate buffer [pH 8.0], 1 mM dithiothreitol, 0.1 mM EDTA, 50% glycerol) supplemented with 0.2 and 0.5 M KCl, respectively. Aliquots from eluted fractions were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) in a 15% polyacrylamide gel followed by silver staining. The peqGOLD protein marker (Peqlab Biotechnologie GmbH, Erlangen, Germany) was used as a molecular weight standard.

For *in vitro* transcription experiments, a reaction mixture containing 600 nM σ^B (52), 30 nM *B. cereus* core RNAP, and a 30 nM PCR-generated template DNA in transcription buffer (20 mM Tris-HCl [pH 8.0], 50 mM K-glutamate, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 20 µM EDTA, 5% glycerol) in a final volume of 10 µl was incubated on ice for 30 min. In control experiments, σ^B was replaced with an equal volume of σ^B dialysis buffer (50 mM sodium phosphate buffer [pH 7.8], 0.3 M NaCl, 50% glycerol). The mixture was subsequently incubated at 30°C for 5 min, followed by the addition of 10 µl of 1 mM (each) ATP, CTP, GTP, and [α-^{32}P]UTP (3,000 Ci/mmol) in transcription buffer supplemented with 0.1 M NaCl. After a 30-min incubation at 30°C, 5 µl of chase mix (10 mM [each] ATP, CTP, GTP, and UTP plus 400 µg of heparin/ml) was added, and the mix was incubated for a further 10 min to finish the transcription reaction and to prevent reinitiation. The transcription reaction was stopped by the addition of 25 µl of formamide loading buffer (95% formamide, 18 mM EDTA, 0.025% SDS, xylene cyanol, and bromophenol blue). After denaturation of the samples by heating at 95°C for 5 min, 5-µl aliquots were analyzed on a 7

M urea-8% polyacrylamide gel, which was run at 90 W. The transcripts were visualized by autoradiography using phosphorimager screens and a Storm 840 system. Size estimates of the runoff transcription products were made by using a ^{32}P-labeled low-range RNA ladder (MBI Fermentas GmbH, St. Leon-Rot, Germany).

RESULTS

Inducible overproduction of σ^B in *B. cereus*

The σ^B-regulated genes of *B. cereus* were initially identified by a proteomics-based approach. First, protein profiles of the *sigB* null mutant and its parent during exponential growth at 30°C and upon heat shock to 42°C for 30 min were determined (data not shown). Only two σ^B-dependent protein spots, corresponding to the previously identified stress-induced proteins YflT and RsbV (43), could be identified. To get a more complete overview of the σ^B regulon, the protein profile of a *B. cereus* strain in which overproduction of σ^B could be induced was determined by 2D-E. To confirm the σ^B-dependent expression of the genes corresponding to the proteins that were identified by 2D-E, we performed a Northern blot analysis, using RNA samples from *B. cereus* ATCC 14579 and the *sigB* null mutant FM1400.

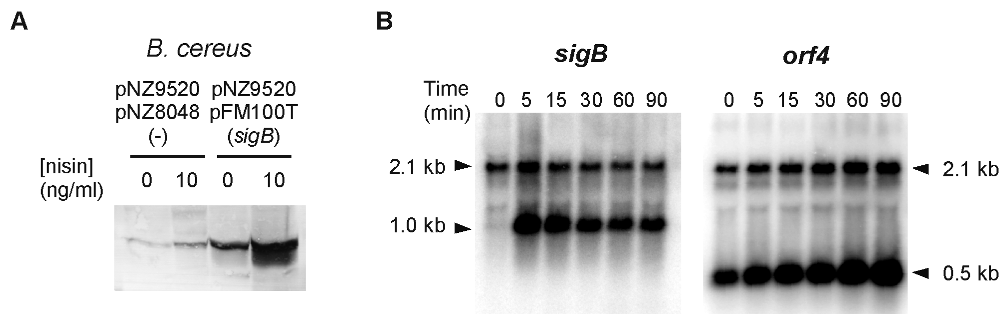

Fig. 1. Inducible overproduction of σ^B in *B. cereus*. (A) Intracellular σ^B levels in *B. cereus* during inducible overproduction of σ^B. Immunoblotting with an anti-σ^B antiserum was performed on protein extracts from *B. cereus* harboring pNZ9520 and pNZ8048 or pNZ9520 and pFM100T. Proteins were extracted 90 min after the addition of nisin (final concentration, 10 ng/ml) to a mid-exponential-phase culture. (B) Northern blot analysis of inducible σ^B overproduction. The total RNA was isolated from *B. cereus* harboring pNZ9520 and pFM100T at mid-exponential phase (t = 0) and 5, 15, 30, 60, and 90 min after the addition of 10 ng of nisin/ml. The blot was probed with a ^{32}P-labeled internal PCR product of *sigB* (left panel). After visualization of the hybridized probe, the blot was stripped and probed with a ^{32}P-labeled internal PCR product of *orf4* (right panel). The sizes of the transcripts (in kilobases) are indicated.

The NICE system was used to obtain inducible overproduction of σ^B in *B. cereus*. The NICE system consists of two vectors, one of which (pNZ9520) contains the genes for

NisR and NisK. NisK senses the presence of nisin in the medium and phosphorylates NisR, which in its turn activates the *nisA* promoter on the second vector (pNZ8048) (32). The *sigB* gene was cloned downstream of the *nisA* promoter on pNZ8048, resulting in pFM100T. The addition of subinhibitory amounts of nisin to a culture of *B. cereus* carrying pNZ9520 and pFM100T led to the overproduction of σ^B. This was assayed by Western blotting with an anti-σ^B antiserum. In cultures harboring pNZ9520 and the empty vector pNZ8048, σ^B was present at low levels. In noninduced cultures of *B. cereus* carrying pNZ9520 and pFM100T, elevated σ^B levels were present, which increased further when nisin was added to the culture to activate the overproduction system (Fig. 1A).

To analyze the kinetics of σ^B overproduction and the biological activity of the overproduced σ^B, we isolated total RNA at different time points during the induction of the σ^B overproduction system and performed Northern blotting with these RNA samples. The blots were probed with a *sigB*- and *orf4*-specific probe (Fig. 1B) and showed the transcription of the *sigB* overexpression vector and the *sigB* operon during σ^B overproduction and the activation of the σ^B-dependent promoter upstream of *orf4* (52). For the RNA isolated from a *B. cereus* strain carrying pNZ9520 and the empty vector pNZ8048, barely detectable levels of *sigB* and *orf4* expression were observed under the conditions tested (data not shown), corresponding with the Western blot data.

In the blot probed with the *sigB*-specific probe, a weak signal at 1.0 kb, corresponding to the mRNA of the *sigB* overexpression plasmid pFM100T, could be visualized before the addition of nisin to the culture. This indicates that the *nisA* promoter is somewhat leaky, which explains why the 2.1-kb transcript that was observed in both blots and which corresponds to the chromosomal *rsbV-rsbW-sigB-orf4* mRNA is already present before the induction of the NICE system. Even though the NICE system is not particularly leaky, even a low-level production of σ^B is presumably enough to switch on transcription of the *sigB* operon, because it is autoregulated by σ^B (52). However, after the induction of σ^B overproduction by the addition of nisin to the culture, the σ^B levels were highly increased (Fig. 1A), and a Northern blot analysis of the σ^B-dependent 0.5-kb *orf4* transcript under these conditions showed that the overproduced σ^B protein was biologically active. The transcript levels of the *orf4* mRNA increased steadily over time, presumably because some time is needed for the production of functional σ^B protein after the induction of σ^B overproduction.

These data show that chromosomal σ^B-dependent promoters of *B. cereus* are activated to high levels upon σ^B overproduction. Subsequently, 2D-E was used to map the protein profiles of *B. cereus* during σ^B overproduction.

2D-E of proteins extracted from *B. cereus* upon σ^B overproduction

Protein profiling was performed on protein extracts of *B. cereus* cells carrying pNZ9520 and pNZ8048 (the negative control) or pNZ9520 and pFM100T (the σ^B-overproducing strain) that were isolated 90 min after the addition of 10 ng of nisin/ml to a mid-exponential-phase culture. Clear differences between the protein profiles could be

observed (Fig. 2). Fourteen spots for the protein sample isolated upon σ^B overproduction could not be matched with spots in the control gel. These proteins were prime candidates for being σ^B-dependent. Nine of these 14 spots could be identified by MALDI-TOF analysis, or if no satisfactory peptide mass fingerprint was obtained, by N-terminal sequencing (Table 1). A subsequent Northern blot analysis showed that all nine of these proteins are indeed σ^B-dependent (see below).

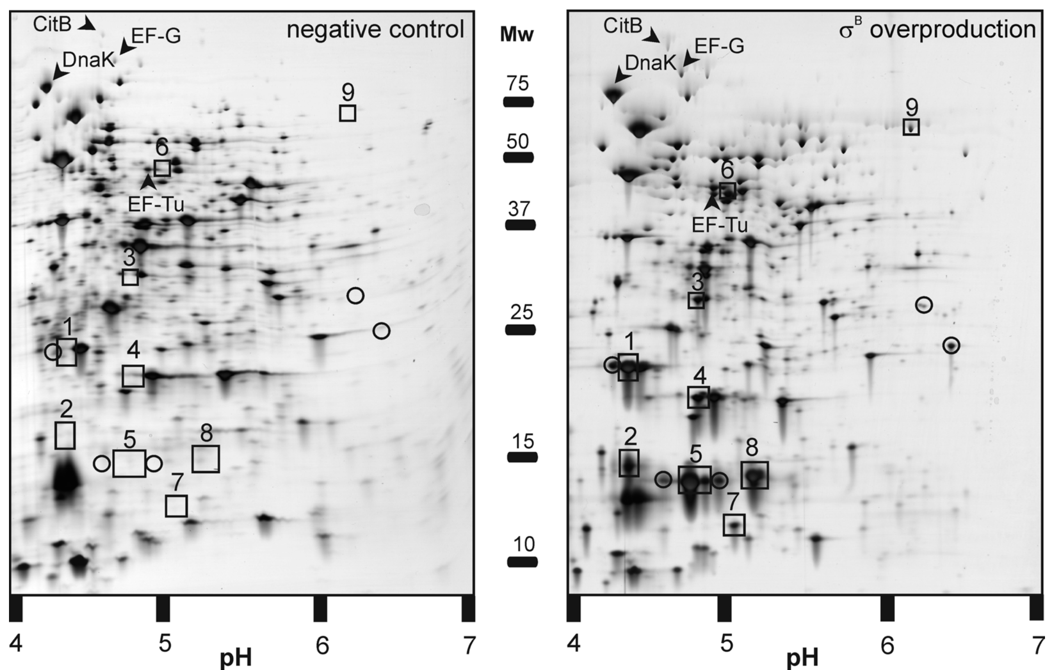

Fig. 2. 2D-E of extracts of *B. cereus* cells carrying pNZ9520 and pNZ8048 (left panel) or pNZ9520 and pFM100T (right panel). Proteins were extracted 90 min after the addition of nisin (final concentration, 10 ng/ml) to a mid-exponential-phase culture. The molecular masses (in kilodaltons) of the markers and the pH range are indicated. Proteins that were upregulated, but were not σ^B-dependent, as shown by a subsequent Northern blot analysis, are indicated by arrows and their respective identifications. Unidentified proteins are circled. Identified proteins in the σ^B overproduction sample which could not be matched with a protein in the control sample and which were confirmed to be σ^B-dependent by a subsequent Northern blot analysis are boxed and numbered, corresponding to the data in Table 1.

Four proteins that were moderately (two- to threefold) upregulated were also identified (Fig. 2, arrows). These proteins, the chaperone DnaK, the protein elongation factors EF-Tu and EF-G, and the aconitase CitB, were shown not to be σ^B-dependent by subsequent Northern blotting (data not shown), and their upregulation may be explained by the stress caused by the artificial overproduction of σ^B. Their upregulation reflects findings

for *E. coli*, in which the expression of many genes, including genes for central metabolism and heat shock response genes, is upregulated upon protein overproduction (39).

Table 1. Identification of σ^B-dependent proteins of *B. cereus* after 2D-E analysis

Spot	N-terminal sequence or Maldi-TOF analysis[a]	Identified protein[b]	Predicted function	Mw/pI[c]	Predicted localization[d]
1	44 (6)	YfkM (RZC01423)	Intracellular protease I	19/4.46	C
2	MSHDVKEL	Orf4 (RZC05127)	Putative bacterioferritin	17/4.53	C
3	MLNIGPF	ThiG (RZC02927)	Thiazole biosynthesis protein	27/4.76	C
4	68 (9)	RZC04880	Unknown	18/4.85	M
5	38 (7)	YflT (RZC05134)	General stress protein	15/4.69	C
6	65 (12)	RZC04727	Unknown	39/4.91	M
7	MNLAINIL	RsbV (RZC05131)	Anti-σ^B-antagonist	13/5.17	C
8	67 (10)	RZC04730	General stress protein	15/5.04	C
9	113 (18)	KatE (RZC01424)	Catalase	75/6.62	C

[a] For proteins identified by N-terminal sequencing, the derived sequence is shown. For proteins identified by Maldi-TOF analysis, the probability based MOWSE score and the number of peptides matched (in parentheses) is indicated. A score higher than 63 is considered significant. Identifications of proteins with scores lower than 63 were confirmed by matching predicted Mw and pI with the position of the spot on the 2D-E gel.
[b] Protein designations were based on homologous proteins from other bacteria. The *B. cereus* genome sequence codes are also specified.
[c] Molecular weight is indicated in kDa.
[d] The subcellular localization of the proteins was predicted with the PSORT server (http://psort.nibb.ac.jp). C; predicted cytoplasmic protein, M; predicted membrane protein.

Identification of σ^B-dependent proteins in *B. cereus* and their possible functions in the *B. cereus* stress response

The upregulation upon σ^B overproduction of the proteins listed in Table 1 strongly suggested that they are members of the σ^B regulon of *B. cereus*. This was confirmed by Northern blotting experiments in which blots containing total RNA from cultures of the *B. cereus sigB* deletion mutant and its parent strain, isolated during the mid-exponential growth phase and after a 10-min exposure to 42°C, were hybridized with probes specific for the structural genes of all nine identified proteins (Fig. 3A). The genes *orf4* and *rsbV* were previously shown to be σ^B-dependent (52) and were not included in this experiment. Northern blot analysis with probes corresponding to *yfkM* and *rzc04727* gave results that were identical to those of Northern blot analysis with probes corresponding to *katE* and *rzc04730*, respectively (data not shown). This was expected, because these open reading

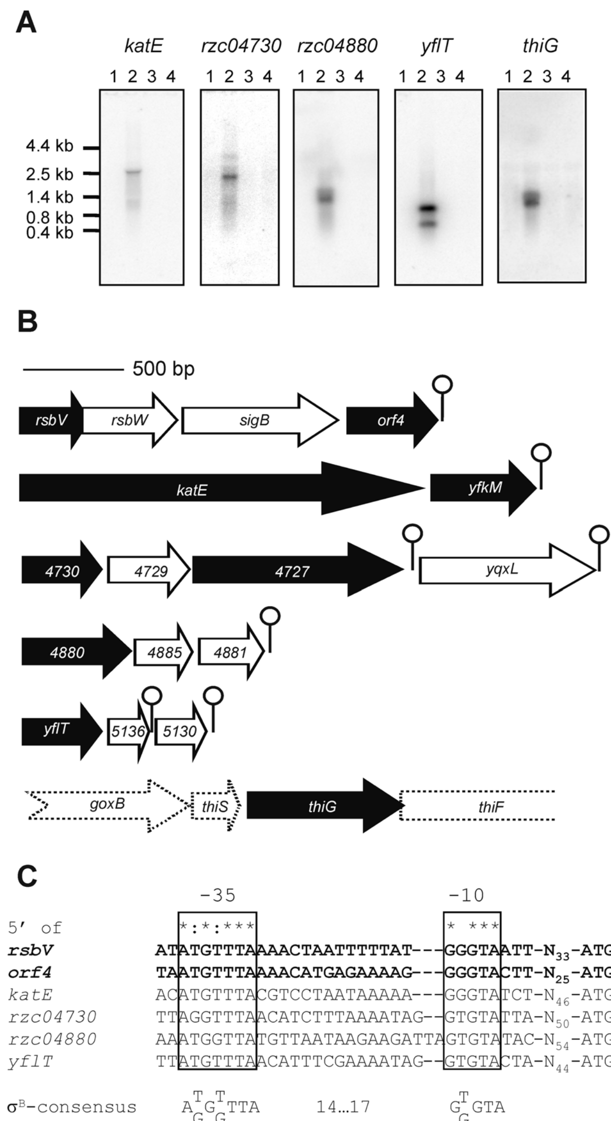

Fig. 3. Transcriptional analysis of σ^B-dependent genes in *B. cereus*. (A) Northern blot analysis of transcription of σ^B -dependent genes in *B. cereus*. Total RNAs were extracted from *B. cereus* ATCC 14579 and *B. cereus* FM1400 cells during mid-exponential growth in BHI medium (lanes 1 and 3, respectively) and after a 10-min exposure to 42°C (lanes 2 and 4, respectively). ^{32}P-labeled internal PCR products of the different genes were used as probes. Northern blot analyses with probes corresponding to *yfkM* and *rzc04727* gave identical results as the Northern blot analyses with probes corresponding to *katE* and *rzc04730*, respectively (data not shown). Marker sizes (in kilobases) are indicated. (B) Operon structure of σ^B-dependent genes in *B. cereus*. The arrows represent open reading frames and indicate their orientations and sizes. Black arrows correspond to genes that were identified on the basis of 2D-E experiments. White arrows denote genes that are cotranscribed. Predicted stem-loop structures are indicated as lollipop structures. The three-letter codes of the genes, or when no such code exists, the *B. cereus* genome sequence code is indicated. The common part (RZC0) of the *B. cereus* genome sequence designations was omitted because of lack of space. The dashed arrows of the genes surrounding *thiG* indicate that it is not clear if these genes are cotranscribed with *thiG*. (C) Alignment of predicted σ^B-dependent promoter sequences. The –35 and –10 regions are indicated. The spacing to the start codon is also specified. The promoters 5' of *rsbV* and *orf4* are shown in bold to indicate that these have been experimentally defined (52). Completely conserved residues are indicated with asterisks. Residues that are conserved in five of the six promoter sites are indicated with colons. The extracted σ^B promoter consensus sequence is also indicated.

frames are in the same operons. In all cases, transcripts were only visualized in RNA samples isolated from *B. cereus* ATCC 14579 upon a heat shock from 30 to 42°C, not in RNA samples from the *sigB* null mutant, indicating that all of the examined genes are σ^B-dependent

Northern blotting also revealed the operon structure of the different σ^B-dependent genes and resulted in the identification of six additional σ^B-dependent genes in *B. cereus* (Fig. 3B and Table 2). The operons were further examined for the presence of predicted stem-loop structures, which can function as terminators, and for the presence of predicted σ^B-dependent promoters upstream of the operon (Fig. 3C). These promoters were subsequently experimentally proven to be σ^B-dependent by *in vitro* transcription experiments (see below).

The functions of the 15 σ^B-dependent proteins, which were identified by 2D-E analysis upon σ^B overproduction are summarized in Tables 1 and 2. Eight of the σ^B-dependent proteins do not have a clearly defined function in *B. cereus*, and their role in the physiology, and more specifically, the stress response of *B. cereus* remains unclear. One of these is YflT, which was previously found to be a heat-shock-inducible protein of *B. cereus* (43). The *B. subtilis* homologue of YflT is also heat inducible and σ^B-dependent and responds strongly to σ^B activation (19, 46), suggesting that this protein has a significant role in the σ^B-regulated component of the stress response in *Bacilli*. RZC04881, whose structural gene is cotranscribed with *rzc04880*, is highly homologous (90% amino acid identity) with the protein encoded by the open reading frame *pX02-45* from the capsule plasmid of *B. anthracis*, reflecting the previously reported spread of *B. anthracis* virulence plasmid genes throughout the *B. cereus* group (40, 41). Finally, RZC04727 is a protein that is unique to the *B. cereus* group. Its C-terminal part, however, has a low-level homology (23% amino acid identity) with a predicted ATPase of the HSP70 class in *Clostridium acetobutylicum*, suggesting that RZC04727 may function as a protein with chaperone activity in *B. cereus*.

Table 2. New σ^B-dependent *B. cereus* genes that are part of σ^B-dependent operons

Co-transcribed gene[a]	Predicted protein function	Mw/pI[b]	Predicted localization[c]
rzc04729	unknown	15/10.14	M
yqxL (*rzc01861*)	CorA-like Mg^{2+} and Co^{2+} transporter protein	37/8.69	M
rzc04885	unknown	11/9.89	C
rzc04881	unknown	11/4.05	C
ywmG (*rzc05136*)	unknown	7/10.17	C
rzc05130	conserved membrane protein	9/9.67	M

[a] Gene designations were based on homologous genes in other bacteria. The *B. cereus* genome sequence codes are also specified.
[b] Molecular weight is indicated in kDa.
[c] The subcellular localization of the proteins was predicted with the PSORT server (http://psort.nibb.ac.jp). C; predicted cytoplasmic protein, M; predicted membrane protein.

The σ^B-dependent proteins that have predicted functions in *B. cereus* may serve a variety of roles in the stress response. YfkM is annotated as an intracellular protease and belongs to the Pfam (1) DJ-1/PfpI family. Proteins from this family are widespread throughout all kingdoms of life and have a wide range of functions, but most importantly they possess chaperone and proteolytic activities (33). YfkM may therefore also function as a chaperone during heat stress in *B. cereus* and may contribute to the correct folding of proteins or the breakdown of misfolded proteins under this condition. The catalase KatE is one of three predicted catalases in the *B. cereus* genome sequence. Its homologue in *B. subtilis* is also σ^B-dependent, but its role in stress resistance is thought to be insignificant, as the deletion of *katE* had no detectable effect on the resistance of *B. subtilis* against oxidative stress (9). *yqxL* is transcribed from the promoter 5' of *rzc04730*. Although there is a stem-loop structure present downstream of *rzc04727*, this structure is not strong enough to completely stop transcription, as shown by the weak readthrough transcript at approximately 3.2 kb visualized on the Northern blot that was probed with a *rzc04730*-specific DNA (Fig. 3A). A subsequent Northern blot analysis of the transcription of *yqxL* showed that its expression is completely σ^B-dependent (data not shown). YqxL is one of three predicted CorA-type transporters in *B. cereus*. CorA-type transporters have been suggested to be the major constitutive Mg^{2+} uptake system of both the *Bacteria* and the *Archaea* (37, 50). These findings suggest that the σ^B-dependent activation of YqxL influences the flux of Mg^{2+} ions over the cytoplasmic membrane during stress.

The mechanism of the σ^B-dependent transcriptional activation of *thiG* is not immediately apparent. We were unable to locate a candidate σ^B-dependent promoter or a clear downstream stem-loop structure which could match the approximately 1.3-kb transcript visualized on the Northern blot, and consequently we could not identify the genes that are cotranscribed with *thiG* with any certainty. Possibly a promoter is involved which is different from the other proposed σ^B-dependent promoters (Fig. 3C), or the expression of *thiG* may be indirectly regulated by σ^B. On the basis of its homology (80% amino acid identity) with ThiG of *B. subtilis*, we can assume that ThiG catalyzes the formation of the phosphate ring in the thiazole moiety of thiamine, which is one of the last steps in thiamine biosynthesis (42). Thiamine pyrophosphate (or vitamin B_1) is an essential cofactor for several enzymes in carbohydrate metabolism, and its mechanistic role is to stabilize the acyl carbanion (25). In *B. cereus*, the biosynthesis of thiamine is not always needed, because it can take up thiamine from the medium (51). This explains why *thiG* is not expressed during exponential growth in rich BHI broth (Fig. 3A). The stress-induced upregulation of *thiG* may be explained by a disturbance of the thiamine uptake system under stress conditions, after which the biosynthesis of thiamine is required for further cellular growth.

Identification of candidate σ^B-dependent promoters and promoter consensus search

In Fig. 3C, an alignment of the candidate σ^B-dependent promoters that could be identified upstream of the set of σ^B-dependent genes in *B. cereus* (with the exception of *thiG*) is shown. These sites were identified because they are practically identical to the

experimentally defined σ^B^-dependent promoters 5' of *rsbV* and *orf4* (52). The alignment of these promoters revealed a preliminary σ^B^ promoter consensus sequence with a –35 region sequence of AKGKTTA (K = T or G) and a –10 region sequence of GKGTA, with a spacing of 14 to 17 nucleotides. σ^B^ promoter consensus sequences have also been determined for *B. subtilis* and *L. monocytogenes*. For *B. subtilis*, the σ^B^ promoter consensus sequences for the –35 and –10 regions were defined as rGGwTTrA and GGgtAt, respectively (capital letters indicate highly conserved residues and lowercase letters indicate less conserved residues [R = A or G and W = A or T]), with a spacing of 12 to 15 nucleotides (18). In *L. monocytogenes*, the σ^B^-dependent promoter consensus is GTTT for the –35 region and GGGWAT for the –10 region, with a spacing of 13 to 17 nucleotides (29). This indicates that there may be some differences, both in the sequences of the –35 and –10 regions and in the spacing between these regions, in the promoter sequences that are recognized by σ^B^ in *B. cereus*, *B. subtilis*, and *L. monocytogenes*. However, more σ^B^-dependent promoters in *B. cereus* should be identified before definitive conclusions can be drawn.

The *B. cereus* σ^B^ promoter consensus, extracted from the alignment of the predicted promoter sites upstream of the four σ^B^-dependent operons identified in this study and of the *sigB* operon, was used to further search the *B. cereus* genome sequence to identify other candidate σ^B^-dependent genes. Five hits were found in an intergenic region with an open reading frame within 1 kb of the predicted promoter site (Fig. 4A). Northern blot analysis was subsequently performed to study if these open reading frames were transcribed in a σ^B^-dependent fashion.

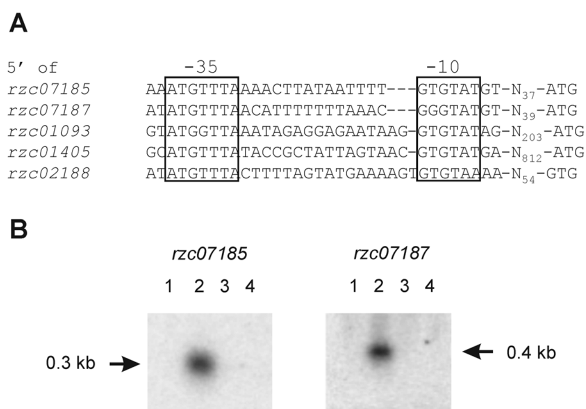

Fig. 4. Identification of σ^B^-dependent genes in *B. cereus* by σ^B^-dependent promoter consensus search. (A) Alignment of predicted σ^B^-dependent promoter sequences, identified by a σ^B^-dependent promoter consensus search. The –35 and –10 regions are indicated. The spacing to the start codon is also specified. (B) Northern blot analysis of transcription of *rzc07185* and *rzc07187*. Total RNA was extracted from *B. cereus* ATCC 14579 and *B. cereus* FM1400 cells during mid-exponential growth in BHI medium (lanes 1 and 3, respectively) and after a 10-min exposure to 42°C (lanes 2 and 4, respectively). ^{32}P-labeled internal PCR products of *rzc07185* and *rzc07187* were used as probes. The transcript sizes (in kilobases) are indicated.

Of these five hits, *rzc07185* and *rzc07187* were found to be dependent on σ^B for their expression (Fig. 4B). Both of these genes code for small (7.6 and 9.5 kDa, respectively, with pIs of 7.68 and 10.47) proteins that are monocistronically transcribed. RZC07185 and RZC07187 are predicted to be a membrane-associated and a cytoplasmic protein, respectively. Northern blot analysis of the other predicted σ^B-dependent genes (*rzc01093*, encoding one of 15 oligopeptide-binding OppA proteins of *B. cereus*; *rzc01405*, encoding a sporulation kinase; and *rzc02188*, encoding subunit II of the cytochrome *d* ubiquinol oxidase) showed weak transcripts under all conditions tested (data not shown). Therefore, these genes are expressed at low levels in a σ^B-independent fashion. However, a subsequent *in vitro* transcription analysis showed that the promoter sites upstream of *rzc01093* and *rzc02188* can be recognized by σ^B-RNAP (see below), indicating that the σ^B-dependent transcription of these genes may be relevant under conditions other than the ones used for the isolation of RNAs for Northern blot analysis in this study.

rzc07185 and *rzc07187* lie in an approximately 10-kb region of the *B. cereus* genome where a number of σ^B-regulated genes are clustered. This region starts with *yflT* (at position 982113), ends with *rzc04881* (at position 992843), and encompasses a total of 15 open reading frames, including the *sigB* operon, of which 12 have been experimentally proven to be σ^B-dependent. A clustering of σ^B-dependent transcriptional units has also been reported for *B. subtilis* (19), but the significance of this observation in both *B. cereus* and *B. subtilis* is still unclear.

Determination of σ^B dependency of promoters by *in vitro* transcription with reconstituted *B. cereus* σ^B-RNAP

There may be several reasons for the apparent inactivity of the promoters upstream of *rzc01093*, *rzc01405*, and *rzc02188*. Because their –35 and –10 sequences fit the σ^B promoter consensus that was defined previously, a σ^B-dependent effect on transcription was expected. However, there may be additional control mechanisms which cause the apparently σ^B-independent transcription under the conditions that we tested. Additional proof for the σ^B-dependency of these upstream promoter sites was obtained by *in vitro* transcription experiments with a reconstituted σ^B-RNAP holoenzyme. Furthermore, we obtained biochemical evidence of the σ^B-dependence of the candidate σ^B-dependent promoters that were defined previously (Fig. 3C). A similar methodology has also been used for *B. subtilis*, e.g., for the determination of σ^W-dependent promoter sites (6, 22). From 20 g of wet *B. cereus* cells, we were able to purify approximately 5.2 mg of RNAP. In the final phosphocellulose step, RNAP was eluted with buffers containing two concentrations (0.2 and 0.5 M) of KCl. This allowed us to separate fractions corresponding to approximately 3 mg of core RNAP (which eluted at 0.2 M KCl) and 2.2 mg of holoenzyme (which eluted at 0.5 M KCl). This distinction between the core and holoenzyme forms was first based on data resulting from an SDS-PAGE analysis of the purified fractions (Fig. 5).

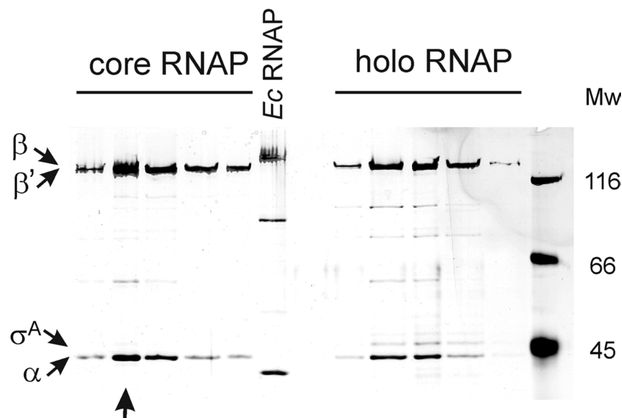

Fig. 5. Purification of *B. cereus* RNAP. An SDS-PAGE analysis of 10-µl aliquots of fractions after phosphocellulose chromatography with P50 buffer containing 0.2 M KCl (core RNAP) and 0.5 M KCl (holo-RNAP) is shown. The proteins were visualized by silver staining. Purified *E. coli* RNAP holoenzyme (Ec RNAP) was also loaded for comparison. The predicted sizes of the ß, ß', σ, and σ^A subunits of the *B. cereus* RNAP are indicated. The fraction used as the core RNAP in subsequent *in vitro* transcription experiments is indicated with an arrow. The sizes of the molecular weight markers (in kilodaltons) are specified.

In the fractions eluted with 0.2 M KCl, we could not visualize a protein corresponding to the size of σ^A, whereas such a protein was seen in the fractions that were eluted with 0.5 M KCl. This was confirmed when an aliquot of the first fraction was used for *in vitro* experiments, in which an extraneously added sigma factor was needed to start transcription (Fig. 6), while an aliquot from the latter fraction could start transcription by itself (data not shown). In all fractions, proteins with sizes corresponding to the α, β, and β' subunits of RNAP were present. The 21-kDa δ subunit of RNAP was not seen on SDS-PAGE gels and was thus presumably lost during our purification protocol. For *B. subtilis*, it was observed that the presence of this subunit in multiple-round *in vitro* transcription reactions can increase the amount of RNA synthesized but that it is not essential for the transcription process (27). A weak band corresponding to the size of the ω subunit was visible in both the core and holoenzyme preparations (data not shown).

For *in vitro* transcription experiments, the different proposed σ^B-dependent promoter regions were amplified by PCR. The primers were chosen in such a way that the σ^B-dependent transcripts were sized between 150 and 240 nucleotides. For *in vitro* transcription reactions with these templates, no transcripts were observed when σ^B was not added (Fig. 6), showing that the purified RNAP fraction used behaves as a core enzyme.

When σ^B was included in the reaction mixture, resulting in a reconstituted σ^B-RNAP holoenzyme, transcripts with the expected sizes could be visualized for the promoter sites upstream of *rsbV* and *orf4* (Fig. 6, samples 1 and 2), which were previously experimentally determined to be σ^B-dependent by primer extension analysis (52). When templates with the promoter regions upstream of the σ^B-dependent genes *katE*, *rzc04730*, *yflT*, *rzc07185*, and *rzc07187* were included in the *in vitro* transcription reaction mix, transcripts with sizes that

matched those expected for the predicted σ^B^-dependent promoter sites were also formed (Fig. 6, samples 3 to 7). A template with the candidate σ^B^-dependent promoter site upstream of *rzc04880* was also used in this experiment, but for this sample the result was that many strong nonspecific transcripts were formed in the reaction (data not shown).

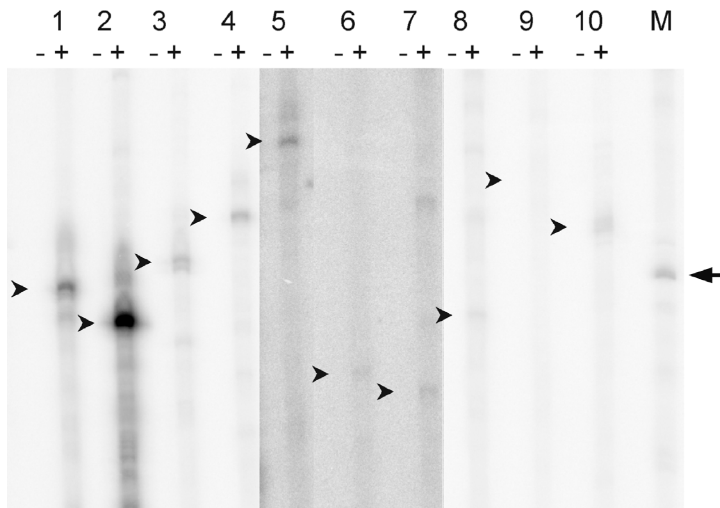

Fig. 6. *In vitro* transcription of predicted σ^B-dependent promoters. PCR products containing the promoter regions of *rsbV* (lane 1), *orf4* (lane 2), *katE* (lane 3), *rzc04730* (lane 4), *yflT* (lane 5), *rzc07185* (lane 6), *rzc07187* (lane 7), *rzc01093* (lane 8), *rzc01405* (lane 9), and *rzc02188* (lane 10) were incubated with *B. cereus* RNAP in the absence (–) or presence (+) of σ^B. After electrophoresis, runoff transcription products were visualized by exposure to a phosphorimager screen and scanning with a Storm scanner. The expected sizes of the σ^B-dependent transcription products are indicated with small arrows. The band in the marker lane (M), indicated with an arrow, corresponds to an RNA size of 200 nucleotides. All data are from a single, representative experiment. The signals for samples 5, 6, and 7 were weaker than the other samples and were enhanced with ImageQuant TL software for optimal clarity.

Interestingly, we could identify σ^B-dependent transcription of the promoter sites upstream of *rzc01093* and *rzc02188*, but not of that upstream of *rzc01405*. These sites were predicted to be σ^B-dependent in our promoter consensus search, but this could not be confirmed by Northern blot analysis. These data suggest that σ^B may play a role in the transcription of both *rzc01093* and *rzc02188* under different growth conditions than the ones used for this study.

DISCUSSION

For this study, we used two different approaches, 2D-E upon σ^B overproduction and *in vitro* transcription with reconstituted σ^B-RNAP holoenzyme, to identify σ^B-dependent

genes in *B. cereus*. We have introduced an inducible σ^B overproduction system in *B. cereus*, and have shown that upon induction of this system, a strong σ^B response was triggered, resulting in the *de novo* production of 14 proteins, as visualized by 2D-E. Nine of these 14 proteins were identified by MALDI-TOF or N-terminal sequencing. The transcription of the structural genes coding for these nine proteins was confirmed to be σ^B-dependent by subsequent Northern blot analyses.

Some of the σ^B-dependent proteins are predicted to have a role in degrading incorrectly folded proteins (YfkM, and possibly, RZC04727). These proteins may thus have a role in protecting vegetative cells of *B. cereus* against high temperatures. Their σ^B-dependency may explain the attenuated heat shock response of the *sigB* null mutant of *B. cereus*. Other proteins do not have such obvious roles in the stress response, but they may subtly tweak the cellular metabolism, which could lead to an increased passive stress resistance. An example of this metabolic rerouting is most apparent in the case of the σ^B-dependent upregulation of ThiG, which may serve to increase the thiamine pool in *B. cereus*. The fact that only ThiG and not the whole thiamine biosynthesis pathway is upregulated may be explained by the finding that for *Paenibacillus alvei* (originally named *Bacillus paraalvei*), which is closely related to the *B. cereus* group (26), a lack of thiamine can be overcome by the addition of specific amino acids (phenylalanine, alanine, valine, isoleucine, and cysteine) to the medium (28). Indeed, in *B. subtilis*, ThiG alone can catalyze the formation of the thiazole moiety of thiamine, thereby bypassing the rest of the thiamine biosynthesis pathway (42). The σ^B-dependent upregulation of the expression of *thiG* may be explained by a metabolic rearrangement, which shuttles amino acids into the thiamine biosynthesis pathway. By following this metabolic route, only ThiG, and not the entire thiamine biosynthesis pathway, would be needed for the generation of the thiazole moiety.

All of the σ^B-dependent genes that were identified on the basis of 2D-E experiments, with the exception of *thiG*, were preceded by a highly conserved promoter motif, which fits the experimentally determined σ^B-dependent promoter sequence in the *sigB* operon (52). An alignment of these promoter sites resulted in a σ^B promoter consensus sequence, and subsequently the *B. cereus* genome was searched with this sequence. Of the five genes that were directly preceded by a promoter site that matched the σ^B promoter consensus sequence, two (*rzc07185* and *rzc07187*) were found to be σ^B-dependent by Northern blot analysis. The fact that we did not detect their corresponding proteins in the 2D-E experiments can be explained by their small sizes and their pIs, which lie outside the range tested in our experiments. The three other open reading frames, even though they were preceded by a conserved σ^B-dependent promoter, were not dependent on σ^B for their expression under the tested conditions.

By performing *in vitro* transcription experiments with a reconstituted *B. cereus* σ^B-RNAP holoenzyme, we were able to identify the σ^B-dependency of genes in a system that was independent from the growth phase or other regulatory factors. Furthermore, these experiments provided further proof for the σ^B-dependency of the predicted promoter sites. Purification of both core and holoenzyme forms of the RNAP of *B. cereus* was performed and yielded an active enzyme. Two genes, encoding an oligopeptide permease subunit (*rzc01093*)

and subunit II of the cytochrome *d* ubiquinol oxidase (*rzc02188*), were preceded by promoters that could be recognized *in vitro* by σ^B-RNAP. Northern blot analysis showed that these genes were not transcribed in a σ^B-dependent fashion under our experimental stress conditions. This may, however, be explained by additional control mechanisms that operate under the conditions in which RNA was extracted from *B. cereus*. For *B. cereus*, cytochrome *d* is not expressed in a rich medium with a fermentable sugar substrate, but is only expressed when fermentable sugars are not present or under conditions of oxygen limitation or anaerobiosis (10). Because RNA was isolated from aerobically growing cells in the mid-exponential growth phase, there may not have been a trigger for the cell to produce cytochrome *d*, and therefore no σ^B-dependent transcription of *rzc02188* was observed. A similar mechanism may operate for the expression of *rzc01093*, which encodes one of the oligopeptide-binding OppA proteins of *B. cereus*. In other bacteria, OppA expression is tightly regulated, and this subunit of the oligopeptide permease system is generally expressed only under specific circumstances, which include changes in the intracellular amino acid pools (38). Further studies incorporating other growth conditions may reveal situations in which the σ^B-dependent expression of *rzc01093* and *rzc02188* is physiologically relevant.

A comparison of the σ^B-dependent genes in *B. cereus* that have been described in this study with the σ^B regulons of *B. subtilis* (19, 44, 46) and *L. monocytogenes* (29) reveals a considerable overlap in functionality among the three organisms, with the overlap being the largest between *B. cereus* and *B. subtilis*, as can be expected because of the relatively close phylogenetic relationship between the two. Of the 19 known σ^B-dependent genes of *B. cereus*, 8 have a homologue in *B. subtilis* and all of these are also σ^B-dependent in *B. subtilis*. There is considerably less homology with *L. monocytogenes*. Five σ^B-dependent genes from *B. cereus* have a homologue in *L. monocytogenes*. Of these five, only *rsbV*, *rsbW*, and *sigB* are σ^B-dependent in both organisms. However, this may be an underestimation, because a DNA microarray with a partial covering of the *L. monocytogenes* genome was used to define the σ^B regulon of this organism, so more σ^B-dependent genes in *L. monocytogenes* may be identified in the future. A comparison with the known σ^B-dependent genes of *S. aureus* (15) revealed that only YfkM is σ^B-dependent in both *B. cereus* and *S. aureus*. This indicates that the σ^B regulon of *B. cereus* has probably evolved to serve specific functions in the *B. cereus* group. This may reflect differences in the ecological niches of these organisms. The ecological niche of *B. cereus* is quite different from those of the other organisms discussed here, as it may be an important symbiont in the nutrient-rich environment of the insect gut (23, 24, 36). In other bacteria, σ^B is not directly involved in vitamin metabolism, so the σ^B-dependent upregulation of ThiG may be specifically coupled to the particular environments in which *B. cereus* lives. Furthermore, the possible σ^B-dependent proteins Rzc01093 (an oligopeptide-binding OppA protein) and Rzc02188 (subunit II of the cytochrome *d* ubiquinol oxidase) may also be important during growth in the nutrient-rich environment of the insect gut, where oxygen concentrations are less than atmospheric (4). More definitive conclusions about the role of σ^B in the lifestyle of *B. cereus* can, however, only be drawn

when more σ^B-dependent genes are identified. For the identification of more σ^B-dependent genes, transcriptome profiling of the *B. cereus sigB* null mutant and its parent strain may be necessary. In addition, further *in vitro* transcription analysis using the reconstituted σ^B-RNAP holoenzyme may also shed further light on the σ^B regulon of *B. cereus*. We have demonstrated, however, that in *B. cereus* the σ^B regulon can play a role in protecting the cell against stress by upregulating chaperone activity in the cell and by adjusting its metabolism.

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6 ANALYSIS OF THE ROLE OF RSBV, RSBW AND RSBY IN REGULATING σ^B ACTIVITY IN *BACILLUS CEREUS*

Willem van Schaik, Marcel H. Tempelaars, Marcel H. Zwietering, Willem M. de Vos and Tjakko Abee

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ABSTRACT

The alternative sigma factor σ^B is an important regulator of the stress response of *Bacillus cereus*. Here, the role of the regulatory proteins RsbV, RsbW, and RsbY in regulating σ^B activity in *B. cereus* is analyzed. By performing *in vitro* transcription experiments with reconstituted σ^B-*B. cereus* RNA polymerase holoenzyme, RsbV and RsbW were shown to act as an anti-sigma factor antagonist and an anti-sigma factor of σ^B, respectively. Furthermore, RsbW was found to be able to act as a kinase on RsbV *in vitro*. These data are in line with earlier functional characterizations of RsbV- and RsbW-homologues in *B. subtilis*. The *rsbY* gene, which is predicted to encode a protein with an N-terminal CheY-like response regulator domain and a C-terminal PP2C phosphatase domain, is unique to *B. cereus* and its closest relatives. Genetic evidence for a central role of RsbY was obtained when the *rsbY* gene was deleted, which resulted in an almost completely abolished σ^B response upon stress. The activation of σ^B upon stress exposure could be restored by *in trans* complementation with full length *rsbY*. Expression analysis of *rsbY* revealed that it is expressed during exponential growth at low-levels from a σ^A-dependent promoter. Upon stress exposure, *rsbY* transcript levels increase 4.6-fold, because then a σ^B-dependent promoter upstream of *rsbY* contributes to *rsbY*-expression. The central role of RsbY in regulating the activity of σ^B shows that in *B. cereus* the pathway leading to the activation of σ^B is markedly different from that in other gram-positive bacteria.

INTRODUCTION

The gram-positive rod-shaped bacterium *Bacillus cereus* is a frequent cause of foodborne disease, which has vomiting or diarrhea as its, relatively mild, main symptoms (9, 15, 23, 27). However, more serious complications, such as necrotic enteritis and fulminant liver failure, arising from the ingestion of food contaminated with *B. cereus* have also been described (25, 26). *B. cereus* can also cause dangerous non-gastrointestinal infections, including periodontitis, fulminant endophthalmitis and meningitis in immunocompromised patients (7, 13, 18).

B. cereus is part of a group of bacteria which is called the *B. cereus* group (reviewed in (21)). This group also includes *Bacillus thuringiensis*, which is an insect pathogen and therefore is widely used as a biopesticide, and *Bacillus anthracis*, which can cause the disease anthrax. The relationships between *B. cereus*, *B. thuringiensis*, and *B. anthracis* are complex. Strains were first named according to their phenotype, but studies using high-resolution DNA-based techniques have recently revealed that there is significant overlap between *B. cereus* and *B. thuringiensis* (1, 17, 22, 33). The *B. anthracis* lineage seems to be distinct from *B. cereus* and *B. thuringiensis*. However, based on the complete genome sequence of a number of *B. cereus* and *B. anthracis* strains it appears that a large number of proteins that serve important cellular functions are highly conserved throughout the *B. cereus* group (19, 20, 34, 35).

The alternative sigma factor σ^B and the regulators of its activity are proteins that are conserved throughout the *B. cereus* group (41). σ^B plays an important role in redirecting gene expression under stress conditions in several gram-positive bacteria, like *Bacillus subtilis*, *Staphylococcus aureus* and *Listeria monocytogenes* (reviewed in (43)). In *B. cereus* and *B. anthracis* σ^B is activated upon a heat shock and entry into stationary phase (5, 11, 24, 41). In addition, other stresses (exposure to ethanol or osmotic upshock), also lead to the activation of σ^B in *B. cereus* (41). The role of σ^B in the physiology of *B. cereus* and *B. anthracis* has been studied by analysis of the phenotype of *sigB* deletion mutants. In *B. anthracis,* σ^B was shown to be a minor virulence factor as the deletion of the *sigB* gene led to a decreased virulence in a mouse model (11). In *B. cereus* σ^B was shown to be involved in the adaptive heat stress response (41).

It is obvious that there is a need for *B. cereus* and other gram-positive bacteria to tightly control the activity of σ^B. Under non-stress conditions, the cell has no need for the production of stress proteins, but once a stress situation is encountered, σ^B should be activated rapidly. This allows the vegetative cell to respond quickly to the changes in its environment, either leading to a rapid resumption of its growth or increasing its chances of survival before the cell is irreversibly damaged. Studies in *B. subtilis* have shown how this bacterium solves these challenges (reviewed in (32)). In non-stressed cells, σ^B is present in an inactive form by complexation with the anti-sigma factor RsbW. In this form σ^B is unable to bind to RNA polymerase and thus cannot initiate the transcription of stress response genes. Under stress, however, an anti-sigma factor antagonist, RsbV, can bind to RsbW, thereby forming an RsbV-RsbW complex, which leads to the release of σ^B. σ^B can then bind

to RNA polymerase after which the transcription of σ^B-dependent genes can take place. RsbW not only acts as an anti-sigma factor for σ^B but it also is a kinase for RsbV in which it phosphorylates a serine residue. The phosphorylated form of RsbV is unable to complex with RsbW and thus cannot release σ^B from its complex with RsbW. However, under stress conditions a phosphatase can be activated which can dephoshorylate RsbV. Dephosphorylated RsbV can then form a complex with RsbW, leading to the release of σ^B.

There is considerable variation in the biochemical make-up of the phosphatases, which can dephosphorylate RsbV in the different bacteria (6, 43). However, a common theme is that they all have a C-terminal PP2C phosphatase domain, which is responsible for the dephosporylation of RsbV. In *B. subtilis*, there are two PP2C phosphatases that act on RsbV~P, which were termed RsbU and RsbP. RsbU has an N-terminal domain that can bind an upstream regulator (RsbT) (6). The second is RsbP, which is unique for *B. subtilis* and has an N-terminal PAS-domain (44). *L. monocytogenes* also has an RsbU homolog with an N-terminal RsbT-binding domain, but no RsbP-homologue (4). *S. aureus* also has a homolog of RsbU, but the role of the N-terminal part of this protein is unknown (14). The RsbU homologue in the *B. cereus* group has an N-terminal CheY-like domain. The CheY-domain is a widespread regulatory domain in prokaryotes. It is named after the single-domain CheY protein, which is involved in chemotaxis, but in many bacteria the CheY-domain, is coupled to a C-terminal effector domain, which can have a wide variety of functions (38, 45). We have earlier proposed the name RsbY for the RsbU-homologue of *B. cereus*, to reflect its structural differences with other PP2C phosphatases which perform the crucial role of dephosphorylating RsbV~P in the σ^B activation pathway in other bacteria (41).

In this article we provide data on the regulatory role of RsbV, RsbW and RsbY in the process of σ^B activation in *B. cereus*. RsbV acts as an RsbW-antagonist and RsbW is the anti-sigma factor of σ^B, which is in line with data from other gram-positive bacteria. The role of RsbY in the process of regulation of σ^B activity in *B. cereus* was studied by generating an *rsbY* deletion mutant. In this mutant the σ^B response of *B. cereus* is almost completely abolished and can be restored by complementation with a plasmid containing *rsbY*. The *rsbY* gene is transcribed at low levels during exponential growth conditions, but it is upregulated in a σ^B-dependent fashion upon a heat shock. The central role of RsbY in regulating σ^B activity is unique for *B. cereus* and indicates that there are important differences in the regulation of σ^B activity with other gram-positive bacteria.

MATERIALS AND METHODS

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

B. cereus was cultured in brain heart infusion (BHI) medium at 30°C with shaking at 200 rpm. All *E. coli* strains were cultured in Luria broth at 37°C (40). *E. coli* DH5α (36) was used as a general purpose cloning host. *E. coli* BL21-Codonplus-(DE3)-RIL (Stratagene, La Jolla, CA.) was used as the host for RsbV- and RsbW-overproduction. *E. coli* HB101/pRK24 (39) was used as the donor host in conjugation experiments. Antibiotics used were ampicillin at 50 μg/ml, kanamycin at 70 μg/ml, erythromycin at 150 μg/ml (for *E. coli*)

or 5 μg/ml (for *B. cereus*), chloramphenicol at 5 μg/ml, spectinomycin at 100 μg/ml and polymyxin B at 50 μg/ml for counterselection against *E. coli* upon conjugation. Oligonucleotides used in this study are listed in Table 1. Other genetic methods have been described previously (41).

Table 1. Oligonucleotides used in this study

Oligonucleotides	Sequence (5' – 3')[a]
OERsbV-PagI-F	GGGCGGAAATCATGAATTTGGCAATAAA
OERsbV-XhoI-R	CTCCCTCGAGCCTTCTTTCTACTTTTTCAA
OERsbW-NcoI-F	GGTGCCATGGAGAGATTTGAAAAGATAG
OE RsbW-XhoI-R	GTGGCTCGAGGTAAGATTCGTAGGTTGAGATTG
KORsbY-XbaI-F	GTTCTAGAGATTATGGATGCG
KORsbY-EcoRI-R	GGAGGAATTCCAATGCCAAATGATAAGGAAAAA
Erycas-SacI-F	CCCAGAGCTCGGTCCGCAAAAGAAAAAC
Erycas-EcoRI-R	CCACGAATTCCATACCTAATAATTTATCTAC
PEOrf4-R	TGTCCTTGTTCATCACTAAT
PrSigB-F	GAAATCGCAAATCATTTAGG
qPCRrsbY-F	TGCCTGAAATTGATGGACTTGA
qPCRrsbY-R	CGGCCAATTTATTTGCATCC
qPCRtufA-F	GCCCAGGTCACGCTGACTAT
qPCRtufA-R	TCACGTGTTTGAGGCATTGG
GSP1-rsbY	TGATCTTCTCTTAATGGGCTACTT
GSP2-rsbY	GATTTTCTTCTTGCTCTTTATGC
ComprsbY-HindIII-F	GGAGAAGCTTGCAGCGAAATTAAATATGACAGAG
ComprsbY-BamHI-R	CACCGGATCCACCCAATTTAATCCTAGTGAACAA

[a]Underlined nucleotides indicate introduced restriction sites.

Overexpression and purification of RsbV and RsbW

rsbV and _rsbW_ were amplified from *B. cereus* chromosomal DNA by PCR and digested with the appropriate restriction enzymes (see Table 1) and cloned into pET-28b (Novagen, Madison, WI), thereby generating fusions with six C-terminal histidine residues. The resulting vectors were named pETrsbV and pETrsbW. These vectors were transformed to *E. coli* BL21-Codonplus-(DE3)-RIL and further overexpression and purification of the proteins was performed as previously described for σ^B (41), with the exception that after elution from the Ni^{2+}-column, the proteins were dialyzed against a solution of 10 mM Tris-HCl (pH 8), 50 mM KCl, 10 mM $MgCl_2$, 0.4 mM dithiothreitol (DTT), and 20% glycerol (29). Purity of the proteins was assessed by Coomassie staining of an SDS-PAGE gel loaded with the final protein preparation.

In vitro transcription and phosphorylation assays

In vitro transcription assays with purified *B. cereus* core RNA polymerase (RNAP) were carried out essentially as described previously (42). The reaction mixtures contained purified proteins at the following concentrations, RNAP: 30 nM, σ^B: 60 nM, RsbW: 0.3 μM, RsbV: 1.5 μM (final volume 20 μl). As template for the *in vitro* transcription reaction a PCR product, generated with primers BcSigBF and PEOrf4, which contains the σ^B-dependent

promoter upstream of *orf4* was used at a concentration of 30 nM. For the reactions the template, nucleotides, σ^B and the regulators RsbV and RsbW were mixed and incubated at 30°C for 5 min before *B. cereus* core RNAP was added.

In vitro phosphorylation of RsbV by RsbW was performed based on previously described methodology (28, 46). The reaction mixture contained 1 μM RsbV, 1 μM RsbW, 40 μCi γ-^{32}P-ATP (3000 Ci/mmol) in kinase buffer (50 mM Tris-HCl pH 7.6, 50 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA). Non-radioactively labeled ATP was added at a concentrations of 20 μM. To check for autophosphorylation of RsbV control reactions in which RsbW was replaced with dialysis buffer were also performed. Phosphorylation reactions were incubated at 30°C for 30 min after which the reaction was terminated by the addition of 2x SDS-PAGE sample buffer. Samples were then heated at 85°C for 5 min. before electrophoresis on an 18% polyacrylamide Criterion gel (Bio-Rad, Richmond, CA).

Generation of an *rsbY* deletion mutant

The *rsbY* gene was deleted by allelic replacement with an erythromycin resistance cassette. First a 3.2-kb product was amplified by PCR with the primers KORsbY-XbaI-F and KORsbY-EcoRI-R (Table 1) from *B. cereus* chromosomal DNA, digested with the appropriate restriction enzymes and cloned into pATΔS28 (30), resulting in pATΔrsbY. Note that the XbaI site in this PCR product is a natural restriction site, which lies in the *sigB* gene. Restriction of this site did not generate mutations in the *sigB* gene as was confirmed by subsequent sequencing of the *sigB* gene in the *rsbY* deletion mutant. Subsequently the plasmid pATΔrsbY was digested with SacI and MunI. These enzymes cut in the *rsbY* gene at positions 110 and 980, respectively (the complete *rsbY* gene is 1143 bases long). Subsequently, the erythromycin-resistance cassette of pUC18ERY (40) was amplified with the primers Erycas-SacI-F and Erycas-EcoRI-R. The generated PCR-product was cut with SacI and EcoRI and subsequently cloned into the digested pATΔrsbY vector (note that digestion with MunI and EcoRI results in compatible sites), resulting in pATΔrsbYery. This plasmid was then transformed to *E. coli* HB101/pRK24 and the resulting strain was used in conjugation experiments with *B. cereus*. Conjugation and confirmation of the deletion of the *rsbY* allele was performed as described previously (41). The *rsbY* deletion mutant was termed *B. cereus* FM1402.

Effect of deletion of *rsbY* on the activation of σ^B under stress conditions

Cultures of *B. cereus* ATCC 14579 and FM1401 in the mid-exponential growth phase (OD_{600} = 0.4 – 0.5) were stressed by a heat shock from 30°C to 42, by the addition of ethanol or NaCl to final concentrations of 4% (vol/vol) and 2.5% (wt/vol), respectively. Proteins were extracted before and 2.5, 5, 10, and 30 min after the stress exposure. Proteins were extracted and Western blotting with anti-σ^B antiserum was performed as described previously (41), with the modification that the resulting immunocomplexes were visualized using the SuperSignal West Pico Chemiluminescent Substrate (Perbio, Etten-Leur, The Netherlands).

Complementation analysis of the *rsbY* deletion mutant

The *rsbY* deletion mutant of *B. cereus* was complemented by cloning the full-length *rsbY* and its upstream sequence, which was amplified from *B. cereus* with primers ComprsbY-HindIII-F and ComprsbY-BamHI-R, into the gram-positive shuttle vector pKSV7 (37), resulting in pKSV7-rsbY. *B. cereus* ATCC 14579 and FM1401 were transformed with the plasmids pKSV7 and pKSV7-rsbY by electroporation (2). Colonies resistant to chloramphenicol were picked and the resulting strains were cultured in the presence of chloramphenicol until mid-exponential growth phase, followed by heat shock from 30°C to 42°C. Proteins were extracted before and 10 and 30 min after a heat shock. Levels of σ^B under these conditions were determined using immunoblotting with anti-σ^B antiserum as described above.

Expression analysis of *rsbY* with real-time PCR and 5' RACE

The expression level of *rsbY* was determined in *B. cereus* ATCC 14579 and the *sigB* deletion mutant FM1400 (41) during exponential growth in BHI and upon a heat shock from 30°C to 42°C for 10 min using real-time PCR, based on previously described methodology (3) with slight modifications. RNA was extracted from two independent cultures of mid-exponential phase and heat shocked *B. cereus* ATCC 14579 and FM1400 cells. Aliquots (2 ml) were spun down in an eppendorf centrifuge at 13000 rpm for 15 sec. After decanting the supernatant, pellets were frozen in liquid nitrogen. RNA was then isolated using RNAwiz (Ambion, Huntingdon, United Kingdom) and residual DNA was enzymatically removed using TURBO DNA-*free* (Ambion). cDNA was synthesized from RNA by using Superscript III reverse transcriptase (Invitrogen, Breda, The Netherlands), 2 pmol of a *rsbY* gene-specific primer (qPCRrsbY-R), which was designed with the Primer 3 website (www.genome.wi.mit.edu), each deoxynucleoside triphosphate at a concentration of 0.5 mM, and 1 µg of total RNA. Reverse transcription was performed at 55°C for 30 min, and this was followed by inactivation of the reverse transcriptase by incubation at 70°C for 15 min. Quantitative PCR was performed with the synthesized cDNAs by using an ABI Prism 7700 with SYBR Green technology (PE Applied Biosystems, Nieuwekerk a/d IJssel, The Netherlands). Each 25-µl reaction mixture contained 1x SYBR Green master mixture (Applied Biosystems), each primer at a concentration of 400 nM and 10 ng of reverse-transcribed RNA. Amplification was initiated at 95°C for 10 min, and this was followed by 40 cycles of 95°C for 15 s and 55°C for 60 s. Specificity of the reaction products was confirmed by melting curve analysis. Relative transcript levels were calculated by using the relative expression software tool (REST) (31). The expression of the *tufA* gene was used as a reference for the determination of induction levels.

The 5' end of the *rsbY* transcript was mapped using RNA isolated from mid-exponential and heat-shocked cultures of *B. cereus* ATCC 14579 and the *sigB* deletion mutant *B. cereus* FM1400 with the 5' RACE System for Rapid Amplification of cDNA Ends (Invitrogen) using the GSP RsbY primers (Table 1) according to the manufacturer's instructions.

RESULTS

Functional characterization of RsbV and RsbW

RsbV and RsbW of *B. cereus* show only limited homology (43 and 56% at the amino acid level, respectively (41)) with their counterparts in *B. subtilis*. This means that there is a possibility that RsbV and RsbW of *B. cereus* may have properties or functions different from the *B. subtilis* homologs and so an *in vitro* examination of important predicted functional characteristics of RsbV and RsbW of *B. cereus* was performed.

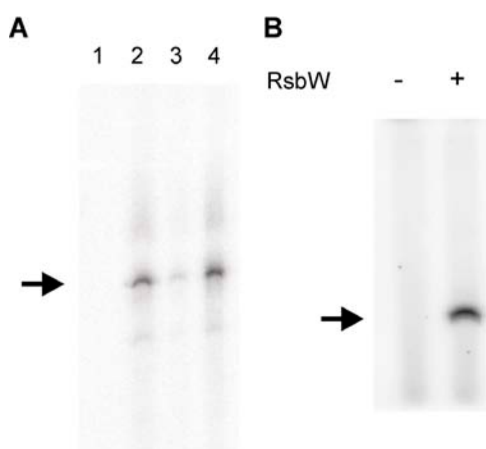

Fig. 1. Functional analysis of RsbV and RsbW of *B. cereus*. (A) *In vitro* transcription assays for the determination of the function of RsbV and RsbW. A PCR-template containing the σ^B-dependent promoter site 5' of *orf4* was used in the *in vitro* transcription reactions containing *B. cereus* core RNAP (lane 1), core RNAP and σ^B (lane 2), core RNAP, σ^B and RsbW (lane 3) and core RNAP, σ^B, RsbW and RsbV (lane 4). The concentrations of the different proteins in the *in vitro* transcription reactions were 30 nM for *B. cereus* core RNAP, 60 nM for σ^B, 0.3 μM for RsbW, and 1.5 μM for RsbV. After electrophoresis, run-off transcription products were visualized by autoradiography. The size of the σ^B-dependent transcription product is indicated with the arrow. (B) Phosphorylation of RsbV by RsbW. The phosphorylation reaction mixture contained 40 μCi γ-^{32}P-ATP (3000 Ci/mmol), 1 μM RsbV, and, where indicated, 1 μM RsbW. Non-radioactively labeled ATP was added at a concentration of 20 μM. Proteins were separated on an 18% polyacrylamide gel SDS-PAGE and, after Coomassie staining, phosphorylated proteins were visualized by autoradiography. The position of RsbV (determined by running a sample of purified RsbV in parallel to the phosphorylation reactions) is indicated by the arrow.

In vitro transcription experiments using purified *B. cereus* RNA polymerase with added σ^B, RsbW and RsbV confirmed the predicted functions of RsbW as an anti-sigma factor and RsbV as an anti-sigma factor antagonist (Fig. 1A). Using a template that contains the σ^B-dependent promoter upstream of *orf4* (41, 42), σ^B-dependent transcription is almost completely abolished when RsbW is added to the transcription reaction. However, addition

of RsbV, which presumably forms a complex with RsbW and thus allows σ^B to bind to core RNA polymerase, completely restores σ^B-dependent transcription.

In *B. subtilis* RsbW acts as a kinase on RsbV (8, 46). This property of RsbV and RsbW from *B. cereus* was also tested in an *in vitro* phosphorylation assay. This revealed that also in *B. cereus* RsbW can phosphorylate RsbV (Fig. 1B). In conclusion, the basic functions of RsbV and RsbW in *B. cereus* are essentially identical to the *B. subtilis* homologues and thus this part of the pathway leading to σ^B activation is in effect identical in both bacteria.

RsbY has a crucial role in regulating σ^B activity of *B. cereus*

Previously, we have identified the *rsbY* gene, which is directly downstream of the *sigB* operon (41). Its C-terminal PP2C-domain, its close proximity to the *sigB* gene, and the absence of other genes with obvious homology to the important regulatory protein RsbU in *B. subtilis*, *L. monocytogenes* and *S. aureus* already suggested that RsbY has a role in regulating σ^B activity of *B. cereus*. To check this hypothesis, a deletion mutant of *rsbY* was constructed by allelic replacement of the *rsbY* gene with an erythromycin-resistance cassette, resulting in *B. cereus* FM1401. Subsequently, the activation of σ^B under various stress conditions was studied by immunoblotting with σ^B-antiserum. Under all tested conditions (heat shock, ethanol exposure and osmotic upshock) an almost completely abolished σ^B-response was observed in the *rsbY* null mutant (Fig. 2A).

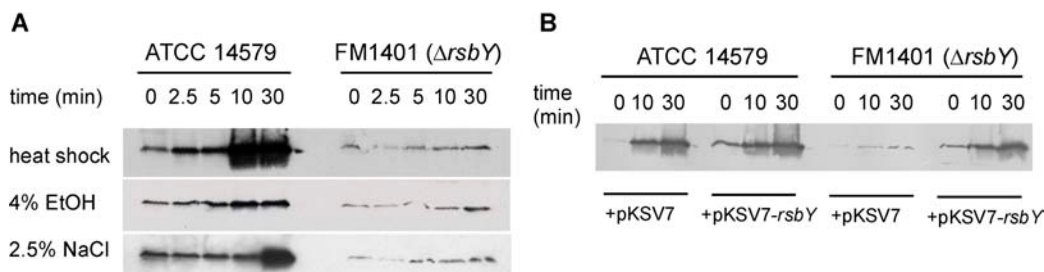

Fig. 2. The effect of deletion of *rsbY* on the stress induced activation of σ^B in *B. cereus*. (A) Cellular σ^B-levels in *B. cereus* ATCC 14579 and its *rsbY* deletion mutant FM1401 upon stress exposure. Bacterial proteins were extracted of cultures in the mid-exponential growth phase (time: 0 min) and upon exposure to the indicated stress for 2.5, 5, 10 and 30 min. 40 μg of protein of each sample was loaded on a 15% polyacrylamide gel SDS-PAGE gel. σ^B was detected by immunoblotting with anti-σ^B antiserum as described in the Materials and Methods section. (B) *In trans* complementation of the *rsbY* deletion mutant restores the activation of σ^B under stress. *B. cereus* ATCC 14579 and its *rsbY* deletion mutant FM1401 carrying the vector pKSV7 or pKSV7-*rsbY* (which contains full length *rsbY* under the control of its natural promoter) where grown until mid-exponential growth phase and heat shocked from 30°C to 42°C. Proteins were extracted at the indicated times. Electrophoresis and the detection of σ^B was performed as described above.

This makes it clear that RsbY is the key regulator of σ^B activity in *B. cereus*; the slight σ^B activating effect upon stress exposure that remains in *B. cereus* FM1401 may be due

to an as yet unidentified regulatory mechanism of minor importance. During stress exposure, proteins were isolated at regular intervals and this allowed us to study the time-course of σ^B activation. It is clear that in *B. cereus* this response can be extremely rapid: already after 2.5 min upon a heat shock, an increase in σ^B levels can be noted and after 10 min σ^B has reached maximal levels. Also during ethanol stress, there is a rapid increase of σ^B levels. The response of σ^B to osmotic stress is slower: after 10 min there is still no increase in σ^B levels, but after 30 min a strong activation of σ^B can be observed.

The important role of RsbY in the σ^B activation pathway of *B. cereus* was confirmed by complementation of the *rsbY* deletion mutant *in trans* with a wild-type copy of *rsbY* expressed under the control of its own σ^A-dependent promoter (see below for the description of the promoter of *rsbY*). Immunoblotting with anti-σ^B antiserum showed that the activation of σ^B upon a heat shock was restored in the *rsbY* deletion mutant upon complementation with the wild-type copy of *rsbY* (Fig. 2B). The basal levels of σ^B during exponential growth seem to be somewhat higher when extra copies of *rsbY* are present on the vector. The presence of multiple copies of *rsbY* may lead to higher RsbY protein levels, which may subtly shift the equilibrium of the σ^B-activation pathway to a state in which more free σ^B is present under non-stress conditions.

Expression analysis of *rsbY*

The expression of *rsbY* was determined in *B. cereus* ATCC 14579 and its *sigB* deletion mutant during the mid-exponential growth phase and upon a heat shock from 30°C to 42°C. Northern analysis was first used to asses the expression of *rsbY*, but no transcripts could be visualized (data not shown). The more sensitive technique of real-time PCR was then used to detect *rsbY* transcripts. The expression of *rsbY* is upregulated approximately 4.6-fold upon a heat shock in *B. cereus* ATCC 14579 but not in the *sigB* deletion mutant, which indicates that the expression of *rsbY* is upregulated under a heat shock in a σ^B-dependent fashion (Fig. 3A).

The 5' ends of the *rsbY* transcripts were mapped using 5' RACE. During exponential growth, *rsbY* is transcribed from a promoter that is situated directly upstream from *rsbY*. This promoter is probably σ^A-dependent even though its sequence is somewhat different from the σ^A promoter consensus sequence from *B. subtilis*. These apparent mismatches may result in the low level transcription of the *rsbY* gene under exponential growth conditons. Using RNA that was isolated upon a heat shock, a smear was observed in the 5' RACE reaction (data not shown), which may indicate degradation of the mRNA. Upon cloning and sequencing of an approximately 0.8-kb product, which appeared to be the largest fragment visible upon electrophoresis, a second promoter site was mapped. The 5' RACE reaction product mapped to a position in *orf4*, the open reading frame 5' of *rsbY*. However, the fact that no clear σ^B-dependent promoter site exists at this position leads to the conclusion that this is probably a degradation product of a transcript that originates from the σ^B-dependent promoter site upstream of *orf4*. The fact that transcripts originating from *orf4* contribute to *rsbY* expression is remarkable because directly downstream of *orf4* a stem-loop structure exists, with a calculated free energy of formation of -9.4 kcal/mol. Previous Northern

analysis of the transcription of *orf4* did not show transcription proceeding beyond this structure (41). However, using a more sensitive PCR-based method, it is shown that transcription through this stem-loop structure occurs and that it contributes significantly to the upregulation of *rsbY* transcription under stress conditions.

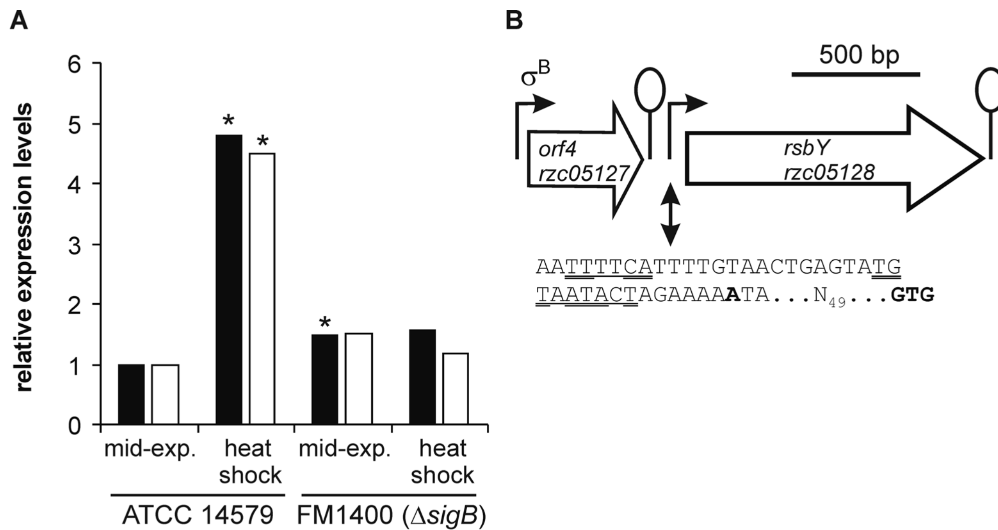

Fig. 3. Expression analysis of *rsbY*. (A) Real-time PCR quantification of *rsbY* transcript levels. Cultures of *B. cereus* ATCC 14579 and the *sigB* deletion mutant FM1400 were grown until the mid-exponential growth phase and exposed to a mild heat shock from 30°C to 42°C for 10 min. The results of RNA extraction from two independently treated cultures (black and white bars) are shown. The expression level of *rsbY* was set at 1 during the mid-exponential growth phase in *B. cereus* ATCC 14579 and the other conditions were compared to that condition using the REST software tool (31). Significantly different ($P<0.05$) expression levels of *rsbY*, compared to the expression of *rsbY* during mid-exponential growth phase in *B. cereus* ATCC 14579, are indicated with an asterisk. (B) Result of 5' RACE mapping of *rsbY* promoter sites. Identified -35 and -10 regions are underlined. Double underlined residues indicate matches with the *B. subtilis* σ^A promoter consensus sequence (16). Bold type indicates the mapped transcriptional start site. The spacing to the GTG start-codon of *rsbY* is also indicated. Upstream of *rsbY* the *orf4* reading frame with its σ^B-dependent promoter is indicated. Lollipops indicate stem-loop structures. The original *B. cereus* ATCC 14579 genome sequence codes for *orf4* and *rsbY* are also indicated.

Discussion

In the gram-positive bacteria where σ^B is an important regulator of the stress response, the activity of σ^B is tightly regulated by a process termed "partner switching" (46). In this mechanism, specific protein-protein interactions are determined by serine or threonine phosphorylation. The mechanism of "partner switching" is evident in RsbW. It can either be bound to σ^B, in which case it acts as an anti-sigma factor by preventing the

association of σ^B to core RNA polymerase, or RsbW can be bound to RsbV in which case σ^B is free to associate with core RNA polymerase. The phosphorylation state of RsbV determines to which protein RsbW binds. When RsbV is in its phosphorylated form, RsbW binds to σ^B, but when RsbV is not phosphorylated RsbW can bind to RsbV. The phosphorylation state of RsbV is thus the key factor in determining if σ^B is active or not. The phosphorylation state of RsbV is determined by at least two proteins, which function as an RsbV-specific kinase and phosphatase. RsbW acts as a kinase on RsbV. RsbV can be dephosphorylated by phosphatases, which have a C-terminal PP2C domain. These core components of the σ^B activation pathway are conserved in bacteria that have σ^B (10). However, there is considerable variation between *B. subtilis*, *B. cereus*, *S. aureus*, and *L. monocytogenes* in the PP2C phosphatase that dephosphorylates RsbV (6, 43) and components that are even further upstream in the σ^B activation pathway. This means that the mechanism of the regulation of σ^B activity in *B. subtilis*, cannot be assumed to be identical in other gram-positive bacteria.

In this study we set out to determine the roles of three regulators of σ^B activity in the foodborne pathogen *B. cereus*, resulting in the model of σ^B activation as depicted in Fig 4. This study reveals that the function of RsbV and RsbW are basically identical in *B. cereus* and *B. subtilis*, but that RsbY, which is only found in the *B. cereus* group, is a unique regulator of the stress response in gram-positive bacteria.

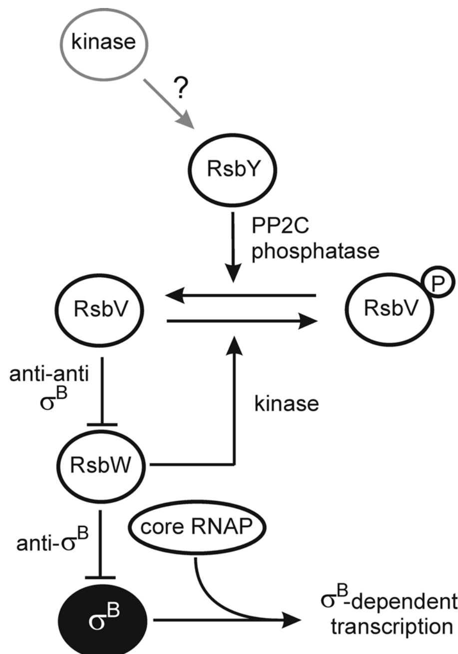

Fig. 4. Model for the regulation of σ^B activity in *B. cereus*. Under non-stress condition σ^B is kept in an inactive state by its anti-sigma factor, RsbW. This protein also functions as a kinase on the anti-sigma factor antagonist, RsbV. When RsbV is dephosphorylated by the action of the RsbY phosphatase, RsbV can bind to RsbW. This leads to the release of σ^B from its complex with RsbW, and, upon association of σ^B to core RNAP, to the transcription of σ^B-dependent genes. RsbY has a N-terminal CheY response regulator domain. This strongly suggests that an, as yet unidentified, kinase (shown in gray) can phosphorylate this domain leading to the activation of the phosphatase

On the basis of analysis of the *B. cereus* ATCC 14579 genome sequence (20) RsbY was already proposed to act as a crucial regulator of σ^B activity in *B. cereus*. Here, we have shown that RsbY truly has an important role in regulating σ^B activity. The deletion of the *rsbY* gene leads to an almost completely abolished σ^B response in *B. cereus*. The fact that there still seems to be slight upregulation of σ^B under stress conditions suggests that there may be additional factors present that can interfere with the phosphorylation state of RsbV, but clearly these factors are of minor importance compared to RsbY. The N-terminal CheY response regulator domain of RsbY suggests that RsbY is activated through a mechanism, which involves phosphorylation of a conserved aspartate residue in the CheY domain by an as-yet unidentified kinase. The coupling of a CheY-domain to a PP2C phosphatase domain in itself is not unique, but it is a rare occurence, as most CheY-domains are coupled to a C-terminal binding DNA-output domain that activates or represses transcription of specific target genes (12). If the activation of σ^B in *B. cereus* is directly coupled to a sensor kinase, as in a classical two-component signal transduction cascade, this would mean a major difference with the σ^B activation pathway of *B. subtilis* in which more partner-switching units and large protein complexes form important parts of the sensing and signaling cascade. So it appears that even though *B. cereus* and *B. subtilis* are relatively closely related bacteria, two different sensing and signaling pathways leading to the activation of the σ^B have evolved. In this respect it is also noteworthy that the *rsbY* gene is partially under transcriptional control of σ^B. In other gram-positive bacteria, the PP2C phosphatase that is responsible for the dephosphorylation of RsbV is constitutively transcribed. It remains to be determined if this positive feed-back effect of σ^B levels on the expression of *rsbY* contributes to the process of σ^B activation in *B. cereus*.

The identification of mechanisms that regulate the activity of RsbY is currently under way in our laboratory and may provide important mechanistic clues on how stress conditions are sensed and signaled leading to the activation of σ^B in *B. cereus*. This may provide indications on how the activation pathway of σ^B can be perturbed. A disruption of the stress response may lead to the sensitization of bacteria under stress conditions. This approach may then be used to counter the growth and survival of bacteria from the *B. cereus* group during food processing or pathogenesis.

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THE ROLE OF THE ALTERNATIVE SIGMA FACTOR σ^B OF *BACILLUS CEREUS* IN LOW TEMPERATURE ADAPTATION AND NITROGEN METABOLISM

Willem van Schaik, Peter A. Boleij, Marcel H. Zwietering, Willem M. de Vos and Tjakko Abee

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ABSTRACT

The alternative sigma factor σ^B is an important regulator in the stress response of many gram-positive bacteria including *Bacillus cereus*. Here, the effect of the deletion of the *sigB* gene in *B. cereus* on the growth and survival at low temperature is described. When *B. cereus* ATCC 14579 and its isogenic *sigB* deletion mutant FM1400 were grown in a defined medium at 15°C, a major growth defect was observed in the *sigB* deletion mutant. Whereas the wild-type strain resumes growth immediately after inoculation into pre-chilled defined medium, the *sigB* deletion mutant only resumes growth after more than two days at 15°C. Survival at 4°C, which is below the minimal growth temperature of *B. cereus*, was also affected by the deletion of *sigB*. After twelve days at 4°C the *sigB* deletion mutant showed a survival of 2%, while the parent strain was not negatively affected by low temperature storage. To further characterize the *sigB* deletion mutant, high-throughput characterization of the phenotype of the *sigB* deletion mutant was performed using Phenotype MicroArray analysis. Several phenotypic traits of *B. cereus* ATCC 14579 were negatively affected by the deletion of the *sigB* gene, most notably the growth on several nitrogen-sources, including the amino acids L-asparagine, L-aspartic acid, and L-glutamic acid. These data show that in *B. cereus* σ^B plays a role in low temperature adaptation and the control of important pathways involving nitrogen metabolism.

INTRODUCTION

Bacillus cereus is a gram-postive, rod-shaped, facultatively anaerobic, spore-forming bacterium. It is frequently isolated from foods and it can cause two types of foodborne disease, which are described as diarrheal type and emetic type, to reflect the main symptoms of the disease (11, 26, 28). *B. cereus* is of special concern to the dairy industry because *B. cereus* spores that can be present in raw milk can survive pasteurization. During storage, spores can germinate and cell growth can occur in milk or milk-based products (16). In addition, *B. cereus* can cause non-gastrointestinal infections such as sepsis, pneumonia, meningitis, endocarditis, or wound infections, especially in immunocompromised patients and neonates (10, 20). Often these infections are hospital-acquired and may be difficult to treat because *B. cereus* is usually resistant to widely-used antibiotics like penicillins and cephalosporins (17).

B. cereus is a member of the *B. cereus* group, which includes *B. cereus* itself and *Bacillus anthracis* and *Bacillus thuringiensis* (22). *B. anthracis* is a virulent pathogen which causes the often lethal disease anthrax (29). *B. thuringiensis* is generally not considered to be a human pathogen, but it can produce insecticidal toxins in the form of parasporal crystal proteins and therefore *B. thuringiensis* is widely used for the biocontrol of insect pests (35). Within the *B. cereus* group, there is considerable overlap between *B. cereus* and *B. thuringiensis*, whereas *B. anthracis* is a very monomorphic species (33). There is a large core group of genes (about 4100 on a total of approximately 5300) that can be found in all three species (34). Many of the conserved genes encode proteins that serve important cellular functions in the *B. cereus* group. These include the genes that code for the alternative sigma factor σ^B and its regulators (38). In *B. cereus*, σ^B is activated under several stress conditions and it was shown to have a role in the adaptive heat stress response (38). In *B. anthracis* σ^B was shown to be a minor virulence factor as the deletion of the *sigB* gene led to an approximately 1-log-unit higher 50% lethal dose (LD_{50}) in a mouse model (15).

The phenotypic characterization of *sigB* deletion mutants in the gram-positive bacteria *Bacillus subtilis*, *Listeria monocytogenes*, and *Staphylococcus aureus* has revealed that σ^B serves important roles in the stress response of these bacteria (reviewed in (40)). In *B. subtilis* the deletion of *sigB* leads to a decreased resistance of vegetative cells to heat, acid, ethanol, salt and oxidative stresses (12, 41). In *L. monocytogenes* the deletion of *sigB* negatively affects the acid tolerance response, the survival during oxidative stress and freeze-thaw cycles (13, 14, 42). In *S. aureus* σ^B has a role in oxidative stress resistance and the resistance to several important antibiotics, including methicillin and vancomycin (21, 36, 44).

In both *B. subtilis* and *L. monocytogenes* σ^B also has a role in the growth at low temperatures (2, 6, 30). This is an important phenotype because at present many food products are minimally processed and stored at refrigeration temperatures. Minimal processing may not completely inactivate robust bacteria like *B. subtilis*, *B. cereus* and *L. monocytogenes* and their survival and possible subsequent growth during low temperature storage may affect the microbiological safety of the food product (1).

Apart from effects on stress response or adaptation, the deletion of *sigB* may also have an effect on apparently unrelated metabolic processes. The identification of σ^B-dependent genes by global transcriptome analysis in *B. subtilis* (18, 31, 32), *L. monocytogenes* (25) and *S. aureus* (3) has revealed that the number of genes encoding obvious functions in stress response is surprisingly low. Instead a relatively large proportion of genes encode proteins with diverse functions in a wide variety of metabolic pathways. Also in *B. cereus* a number of σ^B-dependent genes seem to serve metabolic functions (39). However, so far no insight has been provided in the metabolic effects of the deletion of *sigB* in gram-positive bacteria. Recently, several high-throughput approaches have been developed to analyze metabolic phenotypes (7). Amongst these, Phenotype MicroArray-technology is especially suited for microbial systems and allows for the characterization of many aspects of cellular function, including the catabolism of carbon, nitrogen, phosphorus, and sulfur sources, and growth under stress conditions (4, 5, 45). Here, we show that σ^B contributes to the growth of *B. cereus* at the sub-optimal temperature of 15°C in a defined medium, but not in a rich medium. In addition, the deletion of *sigB* also negatively affects the survival of *B. cereus* at 4°C. Furthermore, Phenotype MicroArray analysis showed that several metabolic functions, most notably regarding the use of a number of nitrogen-sources is affected.

MATERIALS AND METHODS

Strains, growth conditions, and determination of low temperature survival

B. cereus ATCC 14579 and its isogenic *sigB* deletion mutant FM1400 (38) were cultured overnight at 30°C in brain heart infusion (BHI) broth (BD Biosciences, Alphen aan de Rijn, The Netherlands) with shaking at 200 rpm. Subsequently, 250 µl aliquots of these precultures were used to inoculate 250 ml Erlenmeyer flasks with 50 ml prechilled (at 15°C) BHI or defined medium (DM). The latter medium was previously used to follow the growth, sporulation and expression of key sigma factors of *B. cereus* ATCC 14579 (9). For the inoculation of DM 1 ml aliquots of the precultures were spun down in a microtube centrifuge (13000 rpm, 1 min) and washed twice with prechilled DM before inoculating (with 250 µl of the washed cell suspension), to minimize carry-over of BHI medium components. Viable counts of the inoculum were determined by plate counting and showed that equal numbers of live cells were added to the pre-chilled medium. After inoculation, cultures were incubated at 15°C with aeration at 200 rpm. Growth was assayed by turbidity measurements at 600 nm (OD_{600}).

The determination of the survival of *B. cereus* ATCC 14579 and its *sigB* deletion mutant at 4°C was performed as described by Kandror *et al.* (23) with some modifications. Cultures of the two strains were grown at 30°C in BHI until the mid-exponential phase (OD_{600} of 0.4), diluted 1,000- and 100,000- fold in peptone physiological salt solution (8.5 g NaCl, 1 g neutralized bacteriological peptone (Oxoid, Basingstoke, United Kingdom) per liter). 100 µl of the dilutions were plated on BHI agar plates, containing 15 g/l bacteriological agar (Oxoid). Plates were then sealed in plastic bags to prevent drying and

stored at 4°C. At three-day intervals, the number of survivors was determined by removing the plates from 4°C and incubating them for 24 h at 30°C. All growth and survival experiments were performed in triplicate.

Phenotype MicroArray analysis of *B. cereus* ATCC 14579 and FM1400

Phenotype MicroArray analysis (Biolog, Hayward, CA) was performed, essentially as described previously for *Escherichia coli* (45), with the modification that the Phenotype MicroArray plates were incubated at 30°C. Complete maps of the Phenotype MicroArray plates that were used in these experiments can be found at http://www.biolog.com/PM_Maps.html. The plates that were used for this study are coded PM1-PM10 and PM31-PM40.

The analysis of *B. cereus* ATCC 14579 and the *sigB* deletion mutant FM1400 was performed in duplicate. Growth was measured using the OMNILOG-PM automated incubation and monitoring system. The OMNILOG-PM software generates time course curves for respiration (by following the formation of a colored tetrazolium salt) and calculates the difference between the areas under the curves for *B. cereus* ATCC 14579 and the *sigB* deletion mutant FM1400. The differences reported are arithmetic differences between the average height for *B. cereus* ATCC 14579 and FM1400. The average height is defined as the area under the curve divided by the number of reads. Positive values indicate that *B. cereus* FM1400 has a greater rate of respiration than *B. cereus* ATCC 14579. Negative values indicate the opposite. Growth was followed for 24 h or, for the plates in which nitrogen, phosphorous and sulfur sources were studied, for 48 h.

RESULTS AND DISCUSSION

σ^B has a role in low temperature adaptation and survival of *B. cereus*

The low temperature growth of *B. cereus* ATCC 14579 and its isogenic *sigB* deletion mutant FM1400 was assessed at 15°C in both a rich, complex medium (BHI) and in a defined medium (DM) (Fig 1A). The deletion of *sigB* hardly affects growth in BHI at 15°C. However, in DM, there is a dramatic difference in the growth of *B. cereus* ATCC 14579 and its *sigB* deletion mutant. Whereas the wild-type strain resumes growth rapidly upon inoculation of cold DM, the *sigB* deletion mutant needs much more time to adapt to the low temperature conditions, resulting in an approximately 50 h delay in growth.

When we streaked pure cultures of *B. cereus* ATCC 14579 and FM1400 on BHI plates and stored these plates at 4°C, we noticed that the colonies of the *sigB* deletion mutant lost their viability much earlier than those of the wild-type strain. To quantify this effect, dilutions of mid-exponential cultures of the two strains were plated on BHI plates and stored at 4°C. The number of survivors was determined by removing the plates from 4°C and incubating them for 24 h at 30°C. *B. cereus* ATCC 14579 did not lose viability during storage at 4°C (Fig. 1B). However, the cells of the *sigB* deletion mutant started losing viability as soon as they were exposed to 4°C, with survival reaching approximately 2% after 12 days of low temperature storage.

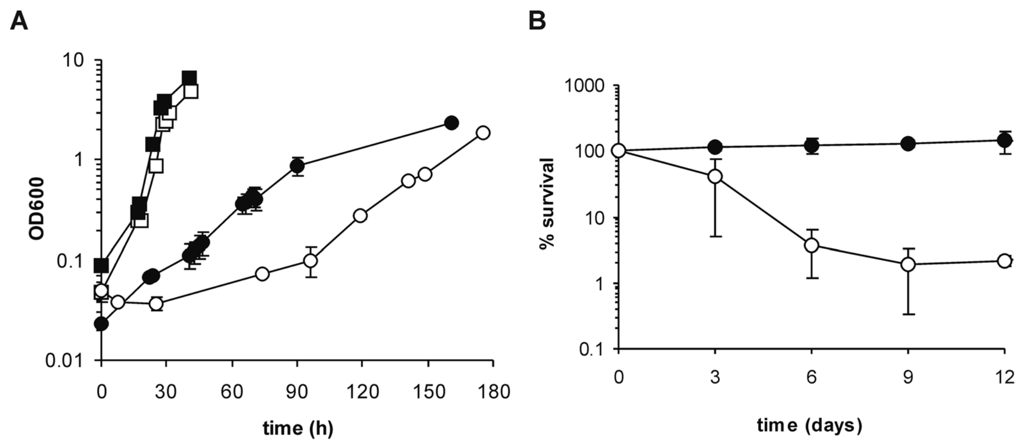

Fig. 1. Low temperature growth and survival of *B. cereus* ATCC 14579 and the *sigB* deletion mutant FM1400. (A) Growth (OD_{600}) of *B. cereus* ATCC 14579 (closed symbols) and FM1400 (open symbols) at 15°C in BHI (squares) and DM (circles). (B) Survival of *B. cereus* ATCC 14579 (closed symbols) and FM1400 (open symbols) at 4°C. Experiments were performed in triplicate and standard deviations are indicated by the error bars.

Phenotype MicroArray analysis reveals metabolic defects in the *sigB* deletion mutant of *B. cereus*

A number of the known σ^B-dependent genes in *B. cereus* encode proteins with metabolic functions (39). To analyze the effects of the deletion of *sigB* on a wide variety of traits, including many metabolic parameters, high-throughput phenotypic screening was performed using Phenotype MicroArray technology (4). This analysis revealed that the large majority of phenotypic traits of *B. cereus* ATCC 14579 was not affected by the deletion of *sigB* (Fig. 2). No significant differences were observed with regard to carbon source utilization, nutrient supplements and growth under osmotic- or pH-stress conditions between *B. cereus* ATCC 14579 and the *sigB* deletion mutant. The conditions, under which there is a significant difference between the growth of *B. cereus* ATCC 14579 and its *sigB* deletion mutant, are provided in Tables 1 and 2.

The erythromycin resistance cassette that was used to replace the *sigB* gene of *B. cereus* can explain most of the phenotypes that were gained in the *sigB* deletion mutant (Table 1). Not only does this cassette confer resistance to erythromycin in *B. cereus*, but also to the eythromycin analogues lincomycin, tylosin, josamycin, and oleandomycin. Lomefloxacin is a fluoroquinolone antibiotic (not related to erythromycin), which has DNA gyrase as its primary target thus affecting DNA replication (43). The reason for the apparent increased resistance to lomefloxacin of the *sigB* deletion mutant of *B. cereus* is not known. The increased resistance to sodium selenite of the *sigB* deletion mutant is discussed elsewhere (chapter 8 of this thesis).

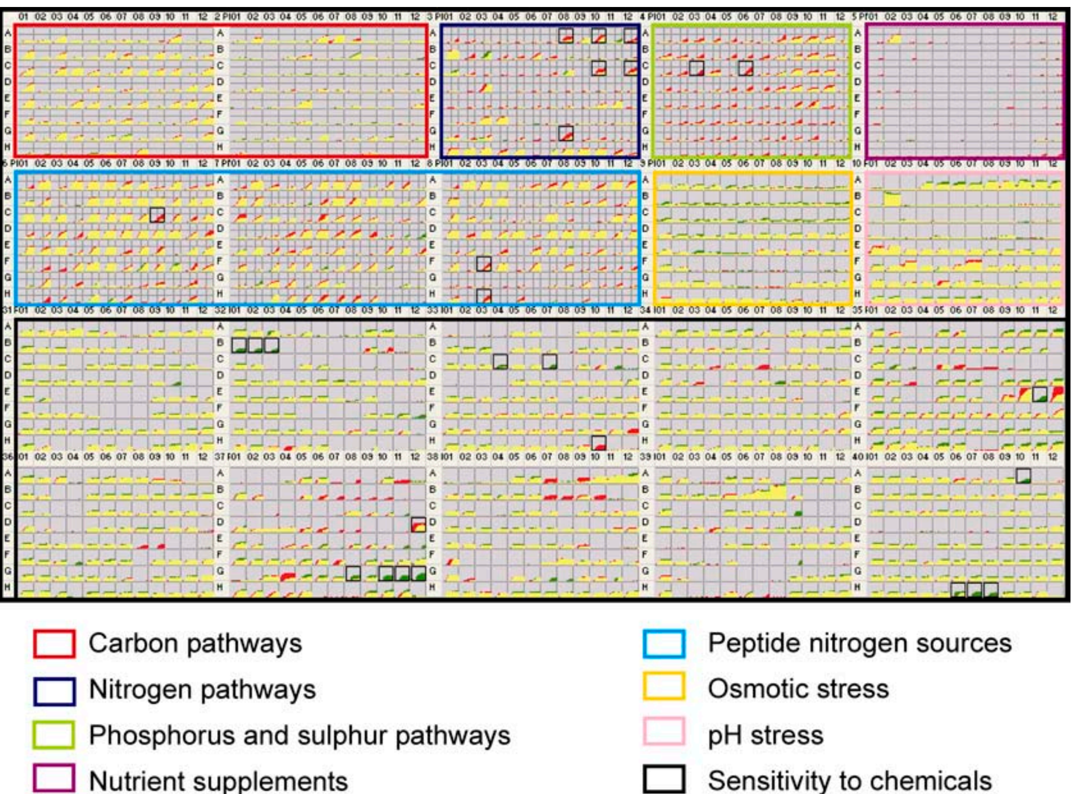

Fig. 2. Complete overview of the Phenotype MicroArray analysis of B. cereus ATCC 14579 and the sigB deletion mutant FM1400. Yellow indicates that growth of the wild type and growth of the sigB deletion mutant were similar. Red indicates increased growth of B. cereus ATCC 14579. Green indicates increased growth of the sigB deletion mutant. Significant changes are enclosed in boxes. The different classes of the tested phenotypes are indicated. Detailed maps of the Phenotype MicroArray plates can be found at http://www.biolog.com/PM_Maps.html The plates that were used are PM1-PM10 and PM31-PM40.

Table 1. Phenotypes gained by the deletion of *sigB* in *B. cereus* ATCC 14579

Trait	Plate-well	Difference[a]	Remarks
Erythromycin	PM40-H07	51	Macrolide antibiotic, targets protein synthesis
Erythromycin	PM40-H08	44	,,
Erythromycin	PM40-H06	35	,,
Lincomycin	PM32-B01	43	Erythromycin analogue
Lincomycin	PM32-B02	32	,,
Lincomycin	PM32-B03	25	,,
Tylosin	PM33-C07	30	,,
Josamycin	PM37-G12	74	,,
Josamycin	PM37-G11	54	,,
Josamycin	PM37-G10	51	,,
Oleandomycin	PM37-G08	33	,,
Oleandomycin	PM33-C04	30	,,
Sodium selenite	PM35-E11	44	Toxic anion
Lomefloxacin	PM40-A10	31	Fluoroquinolone, affects DNA unwinding, gyrase and topoisomerase

[a]The differences between *B. cereus* ATCC 14579 and FM1400 were determined using OMNILOG PM software as outlined in the Materials and Methods section.

Table 2. Phenotypes lost by the deletion of *sigB* in *B. cereus* ATCC 14579

Trait	Plate-Well	Difference[a]	Remarks
γ-amino-N-butyrate	PM03-G08	-83	N-source
L-citrulline	PM03-C10	-78	,,
L-glutamate	PM03-A12	-54	,,
L-arginine	PM03-A08	-74	,,
L-ornithine	PM03-C12	-74	,,
L-aspartate	PM03-A10	-70	,,
Gly-Gly-Gly	PM08-H03	-63	,,
Asp-Asp	PM06-C09	-75	,,
β-Ala-Gly	PM08-F03	-55	,,
D-glucosamine-6-phosphate	PM04-C06	-69	P-source
D-glucose-1-phosphate	PM04-C03	-51	,,
Piperacillin	PM33-H10	-52	β-lactam antibiotic
Ketoprofen	PM37-D12	-60	non-steroidal anti-inflammatory drug

[a]The differences between *B. cereus* ATCC 14579 and FM1400 were determined using OMNILOG PM software as outlined in the Materials and Methods section.

Analysis of the Phenotype MicroArray data shows that most of the phenotypes that were negatively affected by the deletion of the *sigB* gene in *B. cereus* concern nitrogen-metabolism (Table 2). Three amino acids (glutamate, aspartate and arginine), three peptides and three other nitrogen sources were less efficiently metabolized by the *sigB* deletion mutant. Therefore, a reconstruction of the amino acid metabolism of *B. cereus* ATCC 14579 was made using the KEGG database (24). It is remarkable that many of the nitrogen-sources that are less efficiently used by the *sigB* deletion mutant are intermediates in the urea cycle

or closely associated to the urea cycle (8; Fig. 3). This strongly suggests that the deletion of *sigB* affects this pathway for nitrogen metabolism, thereby leading to less efficient growth. Alternatively, the metabolic pathways depicted in Fig. 3 suggest that some of the nitrogen sources tested may contribute to the formation of succinate and fumarate, which have important roles in energy generation, since they are intermediates in the tricarboxylic acid cycle. Finally, *B. cereus* can directly generate ATP from L-citrulline by the deiminase pathway, which is catalyzed by ornithine carbamoyltransferase (EC 2.1.3.3) and carbamate kinase (EC 2.7.2.2) (8). This pathway could also be affected in the *B. cereus sigB* deletion mutant. Future determination of the complete σ^B regulon in *B. cereus* may lead to the identification of σ^B-dependent genes that encode enzymes in these pathways. This will provide a mechanistic explanation of the phenotypes concerning nitrogen metabolism that were observed by the Phenotype MicroArray analysis described here.

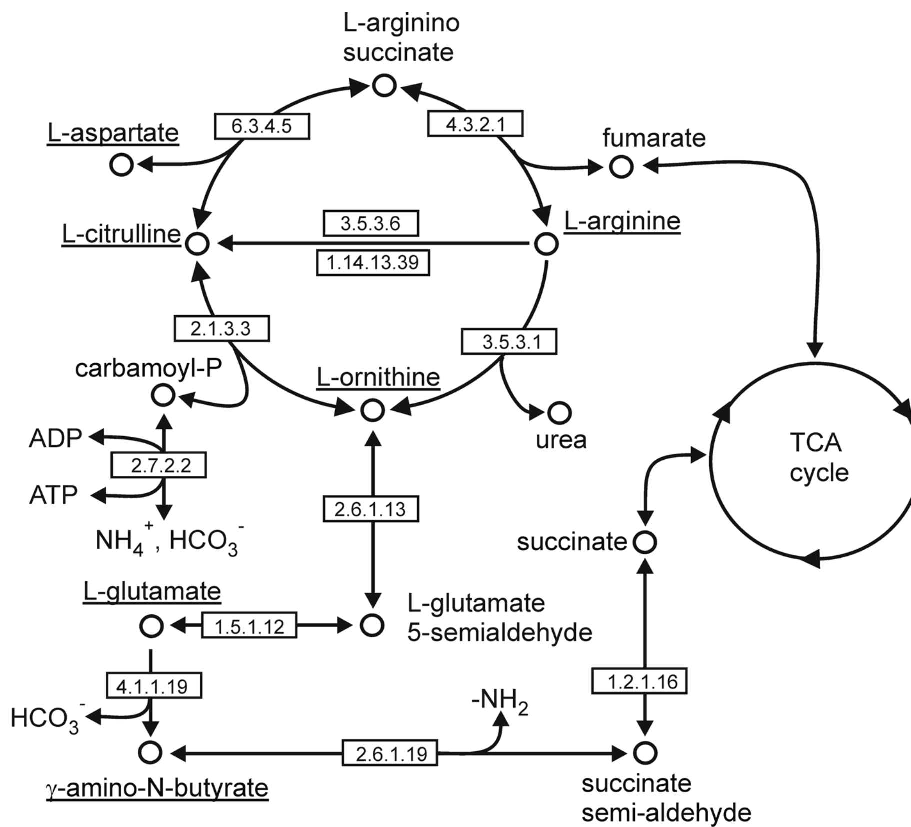

Fig. 3. The urea cycle of *B. cereus* ATCC 14579 and its connection to the tricarboxylic acid (TCA) cycle. The nitrogen sources, of which the metabolism was affected by the deletion of *sigB*, are underlined. The boxed numbers give the EC numbers of enzymes that are present in *B. cereus* and that can catalyze the indicated conversions. The pathways were constructed using the KEGG database (24).

In addition, deletion of *sigB* appears to sensitize *B. cereus* to the antibiotic piperacillin. This is a β-lactam antibiotic, which can be used to treat *B. cereus* infections (19). The development of additional antimicrobial strategies, which target σ^B may thus sensitize *B. cereus* to piperacillin, resulting in more effective killing of the pathogen.

CONCLUSIONS AND FUTURE PERSPECTIVES

In this study we focused on the phenotypic characterization of the *sigB* deletion mutant of *B. cereus* ATCC 14579. First the growth and survival of the *sigB* deletion mutant under low temperature conditions was studied. We found that the *sigB* deletion mutant showed a clear growth defect compared to the parent strain when the strains were grown in a defined medium at 15°C. This growth defect is not observed when *B. cereus* ATCC 14579 and its *sigB* deletion mutant are grown in BHI or DM at 30°C (data not shown), so the effect that is described here is specific for low temperature adaptation in DM.

To assess the metabolic implications of the deletion of *sigB* in *B. cereus*, an extensive analysis of the phenotype of the *sigB* deletion mutant was performed using Phenotype MicroArray technology (4). This approach allows the identification of differences in many traits between two strains, and is especially effective in identifying metabolic pathways that may be affected by the deletion of the *sigB* gene. Remarkably, the amino acid metabolism of the *sigB* deletion mutant appears to be affected, which shows that in *B. cereus* σ^B has a role in nitrogen metabolism. The members of the *B. cereus* group seem to have a larger capacity to metabolize proteins and amino acids than other gram-positive bacteria like *B. subtilis,* and so the careful adjustment of amino acid metabolism under stress conditions may be of crucial importance for the survival of *B. cereus* in the protein-rich niches that it can inhabit (22). In the defined medium that was used to monitor growth at 15°C, glutamate is the most abundant amino acid (9). To study if the defective glutamate metabolism of the *sigB* deletion mutant contributes to its growth deficiency at low temperature, growth experiments were performed in DM to which 4 g/l Cas Aminoacids (Difco, Detroit, MI) was added. This supplementation with a mixture of amino acids did not repair the growth defect of the *sigB* deletion mutant at 15°C (data not shown) suggesting that the defect in glutamate metabolism does not have role in the poor growth of the *sigB* deletion mutant at low temperatures.

The effect of σ^B on the growth and survival at low temperatures can have significant implications for the development of strategies to prevent the growth of *B. cereus* in chilled, ready-to-eat food products. If the σ^B response of *B. cereus* can be suppressed, it may inhibit the growth or the survival of *B. cereus* under low temperature conditions, thereby increasing the microbial safety of the product. In this respect, it should be noted that *B. cereus* ATCC 14579 is a mesophilic strain, which cannot grow at refrigeration temperatures. However, psychrotolerant *B. cereus* strains and the closely related bacterium *Bacillus weihenstephanensis* (27), can actually grow at low temperatures and can readily be isolated from chilled food products (37). The study of the role of σ^B on cold adaptation in psychrotolerant *B. cereus* strains may provide additional information on the role of σ^B in the

low temperature adaptation of the bacteria comprising the *B. cereus* group. Furthermore, it is obvious that the deletion of *sigB* can have important metabolic effects that can have a serious impact on the bacterium's fitness. By contributing to metabolic flexibility, σ^B may thus affect the growth and survival of *B. cereus* under adverse conditions.

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8 DELETION OF THE *SIGB* GENE IN *BACILLUS CEREUS* ATCC 14579 LEADS TO HYDROGEN PEROXIDE HYPERRESISTANCE

Willem van Schaik, Marcel H. Zwietering, Willem M. de Vos and Tjakko Abee

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ABSTRACT

In several gram-positive bacteria the alternative sigma factor σ^B, which is encoded by the *sigB* gene, is a key regulator of the general stress response. Consequently, the deletion of *sigB* generally leads to a stress-sensitive phenotype. The *sigB* deletion mutant of *B. cereus*, for example, displays a defective adaptive heat stress response [Van Schaik *et al.*, 2004. J. Bacteriol. 186:316-325]. However, the *sigB* deletion mutant of *B. cereus* ATCC 14579 also exhibits an extraordinary resistance to H_2O_2, resulting in an approximately 10,000-fold higher survival upon exposure to 10 mM H_2O_2 than the parent strain. Anaerobically grown *B. cereus* cells are more sensitive towards H_2O_2 than aerobically grown cells, but under anoxic conditions the *sigB* deletion mutant is again more resistant to H_2O_2 than the wild-type with an approximately 100-fold difference in survival upon exposure to 2 mM H_2O_2. The hyperresistant phenotype of the *sigB* null mutant under aerobic conditions coincides with elevated catalase activity. In the *B. cereus* ATCC 14579 genome sequence three catalase genes were annotated but only one, *katA*, is expressed during aerobic exponential growth. Expression of *katA* is upregulated in the *sigB* deletion mutant and primer extension analysis showed that *katA* is expressed from a σ^A-dependent promoter. The catalase activity of anaerobically grown cells is similar between the *sigB* deletion mutant and its parent strain. This suggests that the hyperresistance to H_2O_2 of the *sigB* deletion mutant is a complex phenotype, in which KatA is an important, but, at least for anaerobic conditions, not the only player.

INTRODUCTION

Bacillus cereus is a gram-positive bacterium that is a frequent cause of foodborne illnesses, with diarrheal and emetic symptoms (21, 31, 34). In clinical settings it is increasingly being recognized as a cause of life-threatening infections, especially in immunocompromised patients (12, 19, 24). Furthermore, *B. cereus* is an important spoilage organism, especially in the dairy industry where *B. cereus* spores that are present in raw milk can survive pasteurization. During storage, spores can germinate and cell growth can occur in milk or milk-based products (16).

Recently, the close genetic relationship between bacteria in the *B. cereus* group has received considerable attention. The *B. cereus* group includes *B. cereus* itself but also *Bacillus anthracis*, which is the causative agent of anthrax, and *Bacillus thuringiensis*, which is used as a biopesticide (30). The type strain of *B. cereus* (*B. cereus* ATCC 14579), which is used in this study, and *B. anthracis* strains are not considered to be particularly closely related, but it is nonetheless striking that more than 80% of the *B. cereus* ATCC 14579 coding sequences have homologues in *B. anthracis* with 80–100% identity on the amino acid level. This strongly supports the view that the identification of genes that are crucial for important cellular processes in *B. cereus* can have implications for understanding the biology of *B. anthracis* (29). This may be especially true for the alternative sigma factor σ^B, because the genes coding for σ^B and its regulators are highly conserved throughout the *B. cereus* group (41).

The alternative sigma factor σ^B, which is encoded by the *sigB* gene, has a central role in the stress response of several gram-positive bacteria, including *Bacillus subtilis*, *Listeria monocytogenes*, *Staphylococcus aureus*, and *B. cereus* (reviewed in reference 43). In *B. cereus* σ^B is activated upon stress exposure (including exposure to ethanol and osmotic upshock, but most profoundly by heat shock) and entry into stationary phase (11, 41). The phenotypic characterization of the *B. cereus sigB* deletion mutant revealed that σ^B plays a role in the adaptive heat stress response (41). The data available for the role of σ^B in *B. anthracis* suggest that it has a similar function in this organism. Here too, σ^B is activated upon heat shock and entry in the stationary phase. Furthermore, the deletion of *sigB* was found to lower the pathogenicity of the organism in an animal model (15, 33). In other gram-positive bacteria the deletion of *sigB* generally leads to a decreased resistance to reactive oxygen species (13, 14, 20, 25, 32). There is one report in which the increased resistance of a *sigB* deletion mutant of a *L. monocytogenes* strain towards the oxidative agent cumene hydroperoxide is described, but neither quantitative nor mechanistic details were provided in this study (35).

An important line of defense of bacteria against H_2O_2 is the enzyme catalase, which breaks down two molecules of H_2O_2 to water and oxygen. Most bacteria produce one or more catalases to cope with the generation of H_2O_2, which is a fortuitous by-product of aerobic growth of bacteria (8, 23, 28). It was reported in the 1960s that *B. cereus* has two catalases: a thermolabile catalase is present in vegetative cells and a thermostable catalase is present in spores (2). In the *B. cereus* ATCC 14579 genome sequence (29), three open reading frames

encoding catalases can be identified. One of these, *katE*, was previously found to be expressed upon a heat shock in a σ^B-dependent fashion (42).

Here we describe the remarkable hyperresistance to H_2O_2 of the *sigB* deletion mutant of *B. cereus* ATCC 14579. Furthermore, evidence is provided that upregulated transcription of the *katA* gene, which encodes the main vegetative cell catalase of *B. cereus*, plays an important role in the hyperresistance to H_2O_2 of the *sigB* deletion mutant.

MATERIALS AND METHODS

Bacterial strains, culture media, and growth conditions

B. cereus ATCC 14579 and its *sigB* null mutant FM1400 (41) were cultured in BHI medium at 30°C with aeration at 200 rpm. Growth of the culture was followed by measurement of the optical density at 600 nm (OD_{600}). Aerobically grown cultures were in the mid-exponential growth phase at an OD_{600} of 0.4. For experiments under anaerobic conditions, *B. cereus* was pre-cultured overnight (final OD_{600} of 1.0 – 1.2) and grown until mid-exponential phase (OD_{600} of 0.2) in BHI medium in tightly stoppered flasks, which were purged with N_2 gas for 45 min. before inoculation.

E. coli DH5α was used as a general purpose cloning host and was grown in Luria broth at 37°C (37). Other genetic methods have been described previously (41). *Pwo* polymerase (Roche Diagnostics, Almere, The Netherlands) was used for all PCR reactions in this study. Radiochemicals were obtained from Hartmann Analytic GmbH, Braunschweig, Germany. Oligonucleotides used in this study are listed in Table 1.

Table 1. Oligonucleotides used in this study

Oligonucleotides	Sequence (5' – 3')
PrKatAF	CGTATTCCGGAGCGTGTT
PrKatAR	CGATCTGGCGTTTGAATA
PrKatBF	AAGGATTTGGTGCGTTCG
PrKatBR	GGCCGTACCATTATTAGAGT
PrPerRF	GCGCTAGAAATGCTGAAAAA
PrPerRR	TACGCCTTATGACACTCTGGAC
PEKatA	AAGTGTGCAAGTTTTTCTAC
SeqKatA	AGCGCCGCATTATTCTA

Determination of H_2O_2-resistance of *B. cereus*

The H_2O_2-resistance of vegetative *B. cereus* cells was assayed by adding H_2O_2 from a 30% stock-solution directly to a culture in the mid-exponential growth phase to a final concentration of 10 mM H_2O_2. In a parallel experiment, aerobic cultures were heat-shocked from 30°C to 42°C for 30 min to activate σ^B, prior to the addition of H_2O_2. Anaerobic cultures in the mid-exponential growth phase were exposed to 2 mM H_2O_2. Survival was determined by plating appropriate dilutions on BHI plates, followed by overnight incubation at 30°C. For each time-point samples were plated in duplicate. All survival experiments were performed with three independent cultures.

Catalase activity assay

The catalase activity assay of whole cells was performed based on previously described methodology (3, 9) with slight modifications. Two ml (for aerobically grown cultures) or 10 ml (for anaerobically grown cultures) aliquots of cultures in the mid-exponential growth phase and cultures that were heat shocked from 30°C to 42°C for 30 min. were pelleted by centrifugation (13000 g, 1 min), washed once in ice-cold phosphate-buffered saline (PBS; 138 mM NaCl, 2.7 mM KCl, 140 mM Na_2HPO_4, 1.8 mM KH_2PO_4, adjusted to pH 7.4 with HCl) and resuspended in 1 ml ice-cold PBS. 100 µl of the cell suspension was then added to a quartz cuvet containing 900 µl PBS with 44.4 mM H_2O_2 (final concentration of H_2O_2 in the assay is 40 mM). After mixing, the cuvet was placed in a Shimadzu UV-1601 spectrophotometer and the decrease of the absorbance at 240 nm (A_{240}) was measured over time at a constant temperature of 30°C. One unit of catalase activity was defined as a decrease of 1 in A_{240} per minute. Catalase activity was corrected for the amount of cells added to the assay buffer (1/10th of the value of the OD_{600} of the cell suspension added to the assay). This assay was performed in triplicate for all tested conditions.

Visualization of catalase activity on polyacrylamide gels

Total proteins were isolated from *B. cereus* cultures as described previously (36). Crude cell-free protein extracts of *B. cereus* were obtained by pelleting insoluble material by centrifugation at 13,000 g for 30 min. The protein concentrations in the extract were determined using the bicinchoninic acid assay. 100 µg of cell-free protein extracts were separated on a native 10% polyacrylamide Criterion precast gel (Bio-Rad, Richmond, CA). Catalases were stained by the method described by Woodbury *et al.* (44), which results in yellow catalase bands against a dark-green background.

RNA isolation, Northern blotting and primer extension techniques

Total RNA isolation with RNAwiz (Ambion, Huntingdon, United Kingdom), Northern blotting and primer extension analysis was performed as described previously (41, 42). As probes for Northern analysis, internal PCR-fragments of *katA* (generated with primers PrKatAF and PrKatAR), *katB* (generated with primers PrKatBF and PrKatBR) and *perR* (generated with primers PrPerRF and PrPerRR) were used. The oligonucleotide used in the primer extension reaction was PEKatA, which is complimentary to *katA*. For the generation of a sequence ladder in the primer extension analysis the upstream region of *katA* was amplified by PCR with primers PEKatA and SeqKatA. This PCR product was cloned into pGEM-T (Promega, Madison, WI) and after plasmid isolation, radioactive sequencing of this template was performed with the T7 DNA Polymerase Sequencing kit of USB (Cleveland, OH).

Visualization of carbonylated proteins

Detection of carbonylated proteins was performed using the chemical and immunological reagents of the OxyBlot oxidized protein detection kit (Serologicals Corporation, Norcross, GA). Cell-free extracts were prepared as described previously (36), with the exception that 50 mM DTT was added to the cell lysis solution to prevent the

oxidation of proteins after cell lysis. 20 μg of protein from cell free extracts was used in the derivatization reaction. Proteins were separated on 12.5% Tris-HCl polyacrylamide gels and blotted as described previously (41). Protein bands were detected using the SuperSignal West Pico Chemiluminescent substrate (Pierce, Rockford, IL).

Computer methods

To determine the relatedness of *B. cereus* catalases to their homologues in other gram-positive bacteria, multiple sequence alignments and phylogenetic trees were generated with the ClustalX program (40) and the Phylodendron phylogenetic tree printer at http://iubio.bio.indiana.edu/treeapp. The *B. cereus* ATCC 14579 genome sequence (29) is used throughout this study and is freely available through http://www.ergo-light.com.

RESULTS

The *B. cereus sigB* deletion mutant is hyperresistant to H_2O_2

To get an insight into the resistance to stress conditions and the role that σ^B plays in this resistance, the growth of *B. cereus* ATCC 14579 and its *sigB* deletion mutant (FM1400) were assayed in BHI medium to which different concentrations of ethanol (up to 8% [vol/vol]), NaCl (up to 7.5% [wt/vol]), HCl (down to pH 4.5) or H_2O_2 (0.5 mM) were added. Growth at 30°C was then followed by OD_{600} measurements over an 8-hour period. No significant differences between the growth of the *sigB* deletion mutant and its parent strain were observed in BHI with added ethanol, NaCl or HCl. However, when 0.5 mM H_2O_2 was added to the medium, the lag-time for *B. cereus* FM1400 was shorter than for the wild-type strain (data not shown), suggesting that the *sigB* deletion mutant is more resistant to H_2O_2 than its parent. This was confirmed when the killing kinetics of a single exposure to 10 mM H_2O_2 was analyzed. The *sigB* deletion mutant survived between 6,000 and 10,000-fold better than the parent strain (Fig. 1).

In wild-type cells a heat shock from 30°C to 42°C has a slight cross-protecting effect on H_2O_2-resistance. Interestingly, the survival of heat-shocked *B. cereus* FM1400 cells after exposure to H_2O_2 is 2-fold lower than of cells of the mid-exponential growth phase. The resistance to H_2O_2 under anaerobic conditions was also tested. *B. cereus* can grow under anaerobic conditions in BHI until an OD_{600} of approximately 1.0 – 1.2. In the mid-exponential growth phase (OD_{600} of 0.2) during anaerobic growth in BHI, the resistance of *B. cereus* to H_2O_2 was determined. After an exposure to 10 mM H_2O_2 no survivors could be detected, which clearly shows that the anaerobically cultured cells are more sensitive to H_2O_2 than aerobically grown cells. When anaerobically grown wild-type cells were exposed to 2 mM H_2O_2 a killing efficiency was observed that was comparable to the exposure of aerobically grown wild-type cells to 10 mM H_2O_2. Under anaerobic conditions the *sigB* deletion mutant is also more resistant to H_2O_2 than the parent strain, but the difference in survival is now approximately 100-fold, which is markedly less than under aerobic conditions.

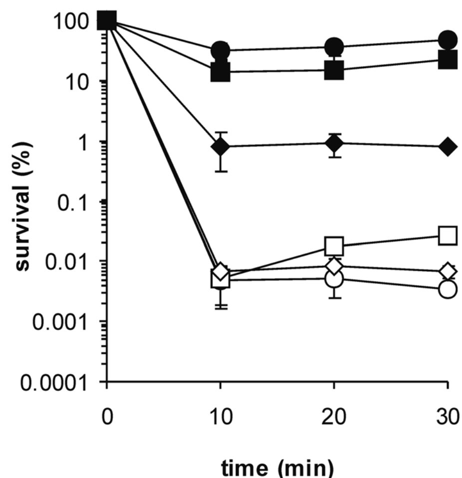

Fig. 1. Survival of *B. cereus* ATCC 14579 and *B. cereus* FM1400 during exposure to H_2O_2. The survival of *B. cereus* ATCC 14579 cells (open symbols) and the *sigB* deletion mutant *B. cereus* FM1400 (closed symbols) cells was determined upon exposure to H_2O_2. Cells were grown until the mid-exponential growth phase either aerobically or anaerobically. Aerobically grown cultures were exposed to 10 mM H_2O_2 in the mid-exponential growth phase (circles) or after a heat shock from 30°C to 42°C for 30 min (squares). Anaerobically grown cultures were exposed to 2 mM H_2O_2 (diamonds). The averages of three independent experiments are shown. The error bars indicate standard deviations.

In an independent experiment, cells were first washed and resuspended in prewarmed BHI medium with added H_2O_2, but no differences in killing kinetics or efficiency were observed compared to the data in Fig. 1 (data not shown). This indicates that extracellular enzymes do not play a role in the resistance towards H_2O_2 and that the H_2O_2-scavenging enzymes of *B. cereus* are cell-associated or located intracellularly.

Catalase activity is increased during aerobic growth in the *sigB* deletion mutant

The enzyme catalase forms an important line of defense against H_2O_2 and therefore the activity of this enzyme was assessed in *B. cereus* ATCC 14579 and *B. cereus* FM1400. *B. cereus* ATCC 14579 and its *sigB* deletion mutant were cultured until the mid-exponential growth phase and isolated before and after a heat shock from 30°C to 42°C, and during anaerobic growth (Fig. 2).

These are the same conditions under which the survival of the cells exposed to H_2O_2 was tested. The assay of the catalase activity of whole cells revealed that during the mid-exponential growth phase catalase activity is 3-fold higher in the *sigB* deletion mutant than in the parent strain. A heat shock from 30°C to 42°C has no effect on the cellular catalase activity of wild-type cells, which suggests that the σ^B-dependent catalase KatE, which is expressed under these conditions, has a minor role in H_2O_2-resistance of *B. cereus*. However, in the *sigB* deletion mutant a significant decrease is observed in catalase activity upon a heat shock and this may explain the somewhat lower survival of heat-shocked *B. cereus* FM1400 cells. The catalase activity of anaerobically grown cells is lower than that of cells growing in an aerobic culture, which may explain the increased H_2O_2-sensitivity of the anaerobically grown cells. Furthermore, under anaerobic conditions, there is no significant difference between the catalase activity of *B. cereus* ATCC 14579 and the *sigB* deletion mutant. This suggests that there are other, as yet unidentified, mechanisms than catalase

that are operating in the *sigB* deletion mutant which are responsible for the increased resistance to H_2O_2 under anaerobic conditions.

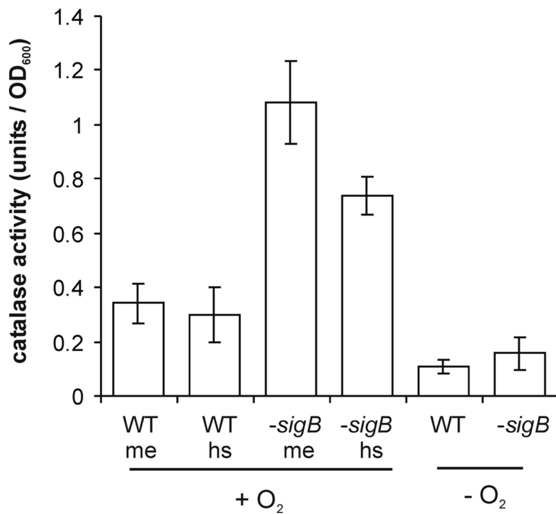

Fig. 2. Catalase activity in *B. cereus* ATCC 14579 and *B. cereus* FM1400. Catalase activity of whole cells was determined of *B. cereus* ATCC 14579 (WT) and *B. cereus* FM1400 (*-sigB*). Catalase activity was determined of aerobically (+O_2) grown cells in the mid-exponential growth phase in BHI (me) and after a 30 min heat shock from 30°C to 42°C (hs). Catalase activity was also determined of cells grown in anaerobic (-O_2) culture. Cells were pelleted by centrifugation, washed once in PBS and resuspended in PBS containing 40 mM H_2O_2. One unit of catalase activity was defined as a decrease in the absorbance at 240 nm of 1 per minute and adjusted for the OD_{600} of the cultures. The averages of three independent experiments are shown. The error bars indicate standard deviations.

The main vegetative cell catalase (KatA) is upregulated in the *sigB* deletion mutant

Because there is such a dramatic increase in catalase activity in the *sigB* deletion mutant under aerobic conditions, the expression of catalases under these conditions was studied in more detail. The catalases that are present in *B. cereus* during mid-exponential aerobic growth and upon a heat shock from 30°C to 42°C for 30 min were visualized by activity staining of catalases on a native polyacrylamide gel (Fig. 3). The results of this experiment are in line with the previously described catalase activity assays of whole cells. Highest activity staining is observed in the cell-free extract of the *B. cereus sigB* deletion mutant, while a heat shock leads to a decrease of catalase activity staining. A single catalase seems to be responsible for the staining, with the exception of the heat-shocked wild-type sample, in which a relatively weak second catalase band appears. This band is probably the σ^B-dependent catalase KatE (42), as no such band is present in the *sigB* deletion mutant after a heat shock.

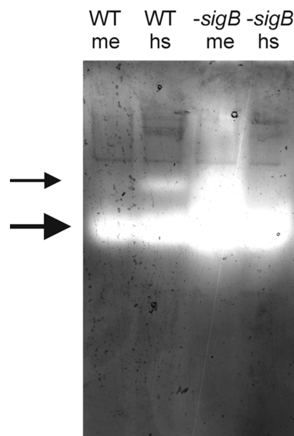

Fig. 3. Visualization of catalase activity on a native polyacrylamide gel. Cell-free protein extracts were isolated from *B. cereus* ATCC 14579 (WT) and *B. cereus* FM1400 (-*sigB*) cells in the mid-exponential growth phase in BHI (me) and after a 30 min exposure to 42°C (hs). Proteins (100 μg) were separated on a non-denaturing 10% polyacrylamide gel and stained for catalases as described by Woodbury *et al.* (44), resulting in light catalase bands against a dark background. The large arrow points to the main vegetative cell catalase. The small arrow points to the σ^B-dependent catalase KatE.

In the genome sequence of *B. cereus* ATCC 14579 three catalases can be identified. Fig. 4 shows a phylogenetic tree of the catalases of *B. cereus* and other gram-positive bacteria generated with the ClustalX program.

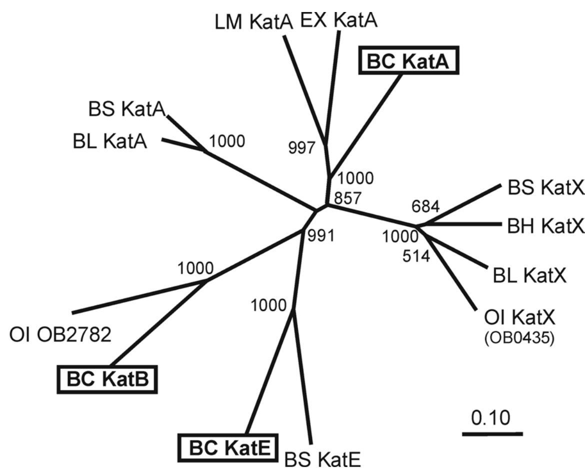

Fig. 4. Phylogenetic tree of *B. cereus* catalases with several of its homologues from other bacteria. Amino acid sequences were aligned by the ClustalX program and an unrooted phylogenetic tree was generated by the Phylodendron phylogenetic tree printer at http://iubio.bio.indiana.edu/treeapp. Bootstrap values (the relative probabilities for occurrence of internal nodes for the tree), based on 1000 random runs, are indicated. Catalase protein sequences were obtained from complete genome sequence projects available at Genbank. Abbreviations: BC: *Bacillus cereus* ATCC 14579; OI: *Oceanobacillus iheyensis* HTE831; LM: *Listeria monocytogenes* EGD-e; BS: *Bacillus subtilis* 168; BH: *Bacillus halodurans* C-125; BL: *Bacillus licheniformis* ATCC 14580; EX: *Exiguobacterium* strain 255-15. Because no nomenclature exists for the *O. iheyensis* catalases, the original genome sequence codes are indicated. The scale bar represents 0.1 substitutions per amino acid.

It is clear from Fig. 4 that the *B. cereus* catalases are quite distinct from those of *B. subtilis*. It is especially remarkable that *B. cereus* does not have a clear homologue of KatX, which in *B. subtilis* is the catalase that is present in spores (1). KatE (encoded by *rzc01424*) is the catalase of which the expression is σ^B-dependent. Our data strongly suggest that it only has a minor role in H_2O_2-resistance: after induction of *katE* by a heat shock there is no dramatic increase in H_2O_2-resistance and the assay of the catalases by native gel electrophoresis shows that KatA contributes to a far larger extent to the total catalase activity of the cell. KatB (encoded by *rzc07268*) is more distantly related to other catalases of gram-positive bacteria. The catalases from *B. cereus* ATCC 14579 that are described here have homologues in the other bacteria of the *B. cereus* group. These are very similar (with amino acid homologies between 85 and 98%), which means that they cluster in the same place as, or very close to, the *B. cereus* ATCC 14579 catalases.

The expression of *katA* and *katB* during mid-exponential growth and upon a heat shock from 30°C to 42°C was determined by Northern analysis in the *sigB* deletion mutant and the parent strain. The expression of *katE* under these growth conditions was determined previously and was found to be strictly σ^B-dependent (42). *katB* is not expressed under the tested conditions, which makes *katA* the only catalase that is expressed during aerobic exponential growth. Indeed, *katA* is clearly more expressed during exponential growth in the *sigB* deletion mutant than in the parent strain (Fig 5A). *katA* is mainly transcribed as a mono-cistronic transcript of approximately 1.6 kb. A second, very weak, transcript can be a read-through mRNA from the upstream gene *rzc05783.* Upon a heat shock, the expression of *katA* is upregulated in both *B. cereus* ATCC 14579 and the *sigB* deletion mutant. This is seemingly at odds with the enzyme activity data in Fig. 2, but it is not unlikely that a heat shock leads to the partial inactivation of the catalase as this enzyme has been previously reported to be thermolabile (2). Furthermore, σ^B-dependent proteins that are upregulated after a heat shock may act as chaperones, which can repair misfolded proteins in *B. cereus* ATCC 14579 (42). The absence of these σ^B-dependent rescue processes may explain the relatively large decrease in cellular catalase activity after a heat shock in the *sigB* deletion mutant.

Subsequently, the transcriptional start site of the *katA* mRNA was mapped by primer extension analysis to determine which promoter sites are responsible for the upregulated *katA* expression in the *sigB* deletion mutant (Fig. 5B and 5C). A single transcriptional start site upstream of *katA* could be determined. The promoter site that is predicted to be responsible for the transcription of *katA* is practically identical to the consensus *B. subtilis* σ^A-dependent promoter (22). No other promoter sites could be identified upstream from *katA*, which shows that the σ^A-dependent promoter is responsible for the transcription of *katA*. Overlapping the transcriptional start site of the *katA* mRNA is a sequence that is essentially identical to the recognition site of the regulator PerR in *B. subtilis*, which has a consensus sequence of TTATAATnATTATAA (23). A practically identical consensus PerR-binding sequence has been described in *S. aureus* (26). PerR is a metal-binding protein which functions as the central regulator of the peroxide stress response in gram-positive bacteria. In *B. subtilis* and *S. aureus* deletion of *perR* leads to the

upregulation of catalase activity and increased H_2O_2-resistance by abolishing the repression of *katA* transcription (6, 26). If in *B. cereus* expression of *perR* would be dependent on σ^B, this could serve as an explanation for the H_2O_2 hyperresistant phenotype of the *sigB* deletion mutant. However Northern analysis showed that transcription of *perR* is not dependent on σ^B. In fact, *perR* is transcribed at higher levels in the *sigB* mutant than in the parent strain (data not shown), which indicates that an abolished repression of *katA* expression by PerR is not a likely explanation of the H_2O_2-resistant phenotype.

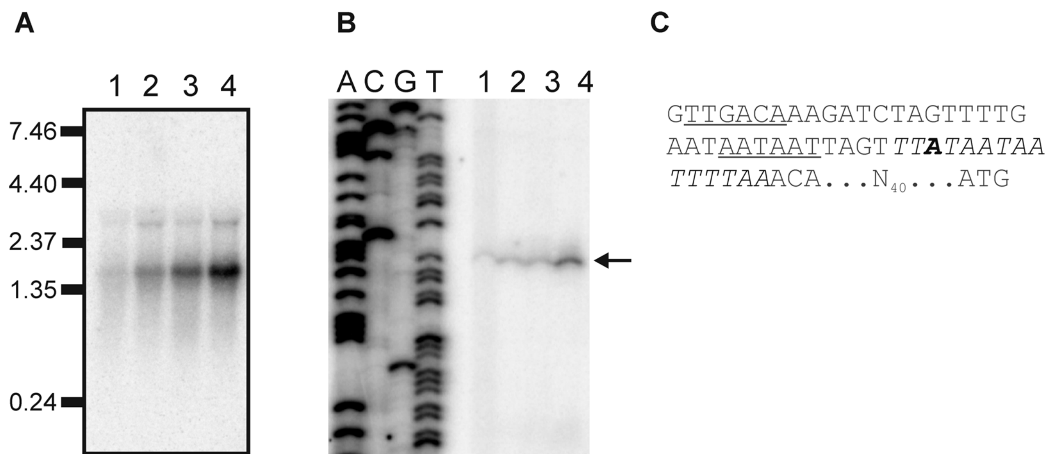

Fig. 5. Transcriptional analysis of *katA* of *B. cereus*. (A) Northern blot analysis of the transcription of *katA*. Total RNA was extracted from *B. cereus* ATCC 14579 and *B. cereus* FM1400 cells during mid-exponential growth in BHI (lane 1 and 3, respectively) and after a 10 min exposure to 42°C (lane 2 and 4, respectively). A ^{32}P-labeled internal PCR products of *katA* was used as probe. Hybridization of the probe with target RNA was visualized by exposure to a Phosphoscreen and scanning with a Storm scanner. Marker sizes (in kb) are indicated. (B) Primer extension analysis of the promoters 5' of *katA*. Total RNA was extracted from *B. cereus* ATCC 14579 and *B. cereus* FM1400 cells during mid-exponential growth in BHI (lane 1 and 3, respectively) and after a 10 min exposure to 42°C (lane 2 and 4, respectively). The mapped transcriptional start site is indicated with an arrow. The lanes labeled A, C, G and T are the corresponding sequencing ladder for the localization of the transcripts. (C) Sequence of the promoter 5' of *katA*. Identified -35 and -10 regions are underlined. Bold type indicates the mapped transcriptional start site. The putative PerR-binding site is indicated in italics. The spacing to the ATG start-codon of *katA* is also indicated.

DISCUSSION

The alternative sigma factor σ^B, encoded by the *sigB* gene, is a central regulator of the stress response in the genera *Bacillus*, *Listeria* and *Staphylococcus* (43). Here we show that in *B. cereus* the deletion of *sigB* leads to a remarkable hyperresistance to H_2O_2. This is in stark contrast with findings in other bacteria, where the deletion of *sigB* generally leads to a reduced resistance to reactive oxygen species. *B. subtilis* cells that are in the mid-

exponential growth phase become 10-fold more sensitive to 10 mM H_2O_2 after deletion of the *sigB* gene (13). In *S. aureus* deletion of *sigB* leads to a fourfold-greater minimum bactericidal concentration for H_2O_2, but the minimal inhibitory concentration of H_2O_2 was not affected by the deletion of the *sigB* gene (20, 25, 32). In an experiment where the survival of *S. aureus* was observed at regular intervals after exposure to H_2O_2, showed that cultures of the *sigB* deletion mutant that were either starved or in stationary growth phase were more sensitive towards H_2O_2 than the parent strain. In the exponential growth phase no differences in H_2O_2-resistance were observed (7). The viability of a *sigB* deletion mutant in *L. monocytogenes* strain 10403S (serotype 1/2a) was 100-fold lower upon exposure to the oxidizing agent cumene hydroperoxide than its parent (14). In another study the resistance of two other *L. monocytogenes* strains, L61 (serotype 1/2a, isolated from a meat product) and L99 (a clinical isolate with serotype 4c) to cumene hydroperoxide was tested. No significant difference in the reduction of viable counts was observed between L61 and its *sigB* deletion mutant. Surprisingly, the *sigB* deletion mutant of L99 was significantly more resistant to cumene hydroperoxide than the parent strain (35). However, neither quantitative details nor a mechanistic explanation of the cumene hydroperoxide hyperresistance of the *sigB* deletion mutant of *L. monocytogenes* L99 was provided.

The upregulation of *katA*-expression upon deletion of *sigB* in *B. cereus* is an important contributing factor in the H_2O_2-resistant phenotype of the *sigB* deletion mutant. However, resistance to H_2O_2 is multifactorial and bacteria have developed many systems to detoxify H_2O_2 and repair the damage caused by this molecule. Our data suggest that systems other than the main vegetative cell catalase may also be involved in the H_2O_2 hyperresistance of the *B. cereus sigB* deletion mutant. The primary scavengers of H_2O_2 are catalases, which exclusively degrade H_2O_2, and alkyl hydroperoxide reductases, which reduce organic hydroperoxides but also H_2O_2. Catalase appears to be the most important H_2O_2-scavenging enzyme when H_2O_2-concentrations are high (38).

Other cellular systems that protect against H_2O_2 in gram-positive bacteria include homologues to the *E. coli* Dps protein, which protects the DNA from oxidative damage and scavenges free iron (see reference (10) for a recent review). Iron scavenging is important because free ferrous iron can transfer an electron to H_2O_2 leading to the formation of the hydroxyl-radical (OH•), which can cause more damage to the cell than H_2O_2 itself. Generally speaking, metal homeostasis is of particular importance in H_2O_2-resistance. For example, the accumulation of manganese and zinc can protect against reactive oxygen (17, 27). All these mechanisms may also be partially responsible for the H_2O_2-resistance in the *sigB* deletion mutant. Recently, Phenotype MicroArray technology (5) was used for a complete characterization of the phenotype of the *sigB* deletion mutant of *B. cereus* (chapter 7 of this thesis). This revealed an increased resistance towards sodium selenite of the *sigB* deletion mutant compared to the parent strain. This toxic anion can be detoxified by the thioredoxin/thioredoxin reductase system (4, 18) and the increased resistance of the *sigB* deletion mutant towards selenite suggests that the thioredoxin system is upregulated in the *sigB* deletion mutant of *B. cereus*. Thioredoxin also has a role in the resistance towards reactive oxygen because it can reactivate proteins that are damaged by oxidative stress (39).

This makes thioredoxin a prime candidate for contributing to the H_2O_2 hyperresistance of the *sigB* deletion mutant of *B. cereus*.

In follow-up experiments complete transcriptome analysis of the *sigB* deletion mutant and the parent strain will be performed using DNA microarrays. This should not only reveal the genes of which the expression is dependent on σ^B, but also the full set of genes that are upregulated, like *katA* and *perR*, in the *sigB* deletion mutant. This may reveal important aspects of the regulatory network that leads to the H_2O_2 hyperresistance of the *sigB* deletion mutant of *B. cereus*. This study illustrates the fact that mutations in regulatory genes can have unexpected phenotypic consequences. Such pleiotropic effects should be taken into account when industrial processes or antibiotic therapies are developed which target the σ^B-response of gram-positive bacteria. The inactivation of σ^B may have important consequences as it may actually lead to an unexpected increased resistance to a subset of stress conditions. This study underlines the importance of thorough phenotypic investigations of *sigB* deletion mutants, as this can provide crucial knowledge on the precise role of σ^B in the parental strains.

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9 SUMMARY, CONCLUDING REMARKS AND FUTURE PERSPECTIVES

INTRODUCTION

Bacillus cereus and its close relatives *Bacillus anthracis* and *Bacillus thuringiensis* thrive in many different ecological niches (12). *B. cereus* is a common cause of foodborne disease and is increasingly being recognized as an infectious agent in clinical settings. *B. thuringiensis* is generally regarded as a beneficial organism because it is used as a biological control agent to counter insect pests. In recent years, *B. anthracis*, the causative agent of anthrax, has become one of the world's most notorious bacteria. The use of *B. anthracis* in bioterrorism in the autumn of 2001 has met with worldwide shock and revulsion, and the possibility of future bioterrorist attacks with *B. anthracis* remains a concern.

Considerable research efforts have been aimed at discerning the genetic relationships between *B. cereus*, *B. thuringiensis* and *B. anthracis* (the so-called *B. cereus* group), the mode-of-action of several toxins and the distribution of these toxins among the *B. cereus* group (chapter 1). However, there is considerably less information available on the basic biology of *B. cereus*. This is remarkable because the growth and survival mechanisms of *B. cereus* actually determine its success in any environment, including food and the human host. For the control of *B. cereus* it is especially relevant to understand how it can grow and survive in food products for human consumption. This knowledge may then be applied to optimize food production processes and storage conditions on an industrial scale, with the aim of minimizing the danger of foodborne *B. cereus* infections and intoxications.

An overview of the results described in this thesis is provided in Fig. 1. In the remainder of this chapter a more detailed summary and discussion of the results is provided.

THE STRESS RESPONSE OF *B. CEREUS*

The microbial stress response is an important factor in determining the survival of bacteria under adverse conditions. Upon triggering the stress response, bacteria gain an increased resistance towards conditions that are rapidly lethal for non-stressed cells. An important regulator of the microbial stress response in several gram-positive bacteria is the alternative sigma factor σ^B (chapter 2). In chapter 3 the stress response of *B. cereus* is described. Our results show that pre-exposure to stress leads to an increased survival at 50°C. The largest protecting effects were provided by a heat shock from 30°C to 42°C and by an osmotic upshock, prior to exposure to 50°C. The other stresses tested (exposure to ethanol, acid and cold shock) also led to an increased survival at 50°C, although to a lesser extent. These data unequivocally show that *B. cereus* is capable of displaying a stress response which can dramatically increase the resistance of the vegetative cells against adverse conditions.

Using Western blotting with antibodies raised against known stress proteins in *Bacillus subtilis* and two-dimensional (2D) gel electrophoresis, a number of proteins that were upregulated upon exposure to stress were identified. RsbV, which has the predicted function of an anti-anti-sigma factor of the alternative sigma factor σ^B could be visualized on a 2D-gel upon heat stress. This finding suggests a role for σ^B in the stress response of *B.*

cereus. In addition, a number of chaperones and proteases that are predicted to be involved in repair and turn-over of misfolded proteins under stress, are upregulated. Perhaps surprisingly, the levels of several metabolic proteins were also increased, which indicates that metabolic processes may have an important role in contributing to stress resistance, conceivably by contributing to the metabolic flexibility of the bacterial cell.

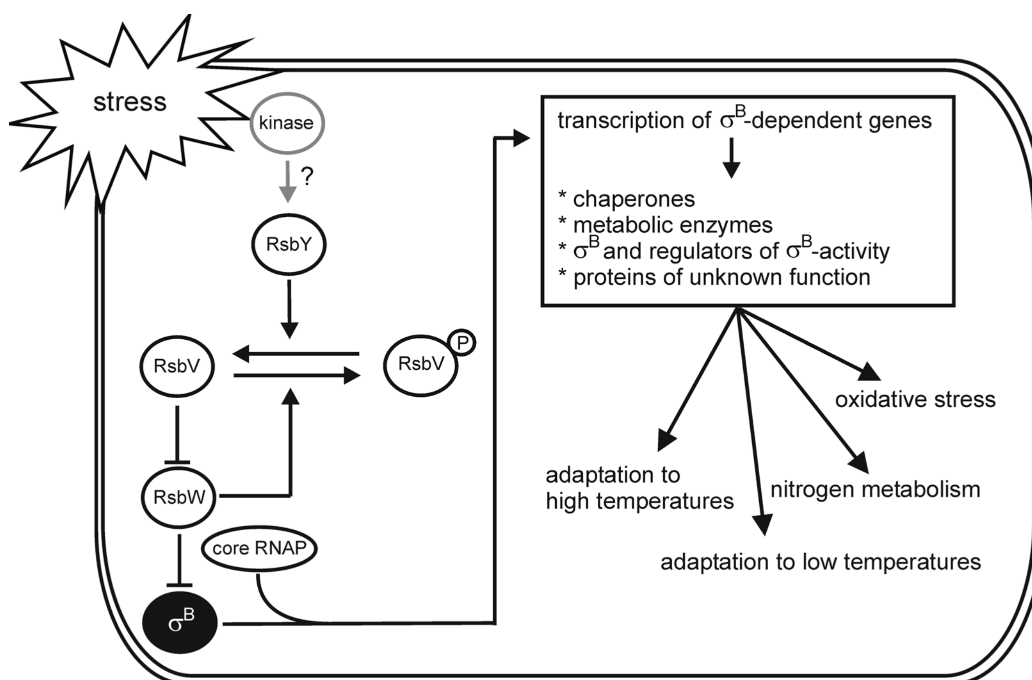

Fig. 1. The role of σ^B in *B. cereus*. Upon exposure to stress. σ^B is activated through a regulatory cascade, involving the anti-sigma factor RsbW, the anti-anti-sigma factor RsbV and the phosphatase RsbY and a putative kinase. . Active σ^B can bind to core RNA polymerase (RNAP), which leads to the transcription of the set of σ^B-regulated genes in *B. cereus.* The proteins encoded by these σ^B-regulated genes have important roles in stress adaptation and metabolic processes.

SIGMA FACTORS PLAY KEY ROLES IN REGULATING GENE EXPRESSION IN PROKARYOTES

Sigma factors are dissociable subunits of RNA polymerase (RNAP), which direct RNAP to specific promoter-sites, leading to the transcription of downstream genes. Bacteria have at least one major housekeeping sigma factor, but, in addition, they generally also have a number of alternative sigma factors, which are specifically activated under certain environmental conditions or during developmental processes. There is a general rule that the number of sigma factors in a bacterium reflects the need by that bacterium to be able to

adapt to changes in its environment (Table 1). Bacteria which have complex lifestyles, like the soil-dwelling, antibiotic-producing filamentous bacterium *Streptomyces coelicolor* and the human gut-symbiont *Bacteroides thetaiotaomicron*, have a need to extensively regulate their gene expression, which is reflected by the high number of sigma factors encoded in their genome sequences. Bacteria that do not have to cope with major changes in their environment, like obligate endocellular parasites, generally have small genomes, with little need for fine-tuning of transcription by sigma factors (14).

Table 1. Overview of the number of sigma factors in selected bacterial genomes[a]

Organism	Genome size (Mb)	Number of open reading frames	Total number of sigma factors
Streptomyces coelicolor A3(2)	8.77	7825	65
Bacteroides thetaiotaomicron ATCC 29148	6.3	4779	54
Pseudomonas aeruginosa PAO1	6.3	5570	24
Bacillus cereus ATCC 14579	5.4	5366	20
Caulobacter crescentus	4.0	3767	17
Bacillus subtilis 168	4.2	4100	17
Escherichia coli K-12	4.7	4288	9
Listeria monocytogenes EGD-e	2.9	2853	5
Staphylococcus aureus Mu50	2.8	2593	4
Campylobacter jejuni NCTC11168	1.64	1654	3
Lactococcus lactis IL1403	2.4	2266	2
Mycoplasma genitalium G-37	0.6	470	1

[a]This table is adapted from reference (9) with additional information from references (3, 8, 11, 22).

Table 2. Genes encoding sigma factors and their proposed functions in *B. cereus* ATCC 14579[a]

Housekeeping	Stress	Sporulation	ECF[b]	Other[c]
sigA	*sigB*	*sigE*	*rzc00905*	*rzc04893*
		sigF	*rzc01155*	*sigI*
		sigG	*rzc02672*	*sigL*
		sigH	*rzc03920*	
		sigK	*rzc04225*	
			rzc05816	
			rzc07615	
			rzc07816	
			sigM	
			sigW	

[a]Genes encoding sigma factors were identified in the *B. cereus* ATCC 14579 genome sequence at http://www.ergo-light.com. No nomenclature exists for a number of sigma factor encoding genes and here the original *B. cereus* ATCC 14579 genome sequence codes are indicated.
[b]ECF: extracytoplasmic function.
[c]The sigma factors in this column cannot be assigned to one of the other functional categories in this table.

The total number of sigma factors of *Bacillus cereus* is twenty. This is a moderately high number and may reflect its fairly complex lifestyle and the wide variety of ecological niches this bacterium can inhabit (12). The sigma factors of *B. cereus* can be categorized in different functional groups (Table 2).

In a number of gram-positive bacteria the alternative sigma factor σ^B, which is encoded by the *sigB* gene, plays an important role in redirecting gene expression upon stress exposure. The function of σ^B and the molecular mechanisms that lead to its activation in different gram-positive bacteria are reviewed in chapter 2. Although there are conserved themes in the role and regulation of σ^B in different gram-positive bacteria, it is becoming increasingly clear that there is considerable variation in the exact function of σ^B in different gram-positive bacteria. This may reflect the different niches that the different bacteria can inhabit. Because of the significance of *B. cereus* as a foodborne pathogen (15) and the characteristic ecological niches that this bacterium and its close relatives can inhabit (12), the study of the role of σ^B in *B. cereus* is a very relevant area of research.

ACTIVATION OF σ^B UPON STRESS EXPOSURE AND EFFECTS OF DELETION OF THE *SIGB* GENE

The effect of several stresses on the levels of the σ^B protein in *B. cereus* is described in chapter 4. Oxidative stress and the depletion of ATP-levels by adding the uncoupling agent CCCP barely led to an increase in the levels of σ^B. Other stresses, however, like ethanol exposure, osmotic upshock and acidification of the medium did lead to a clear increase in σ^B levels. However, by far the largest effect on σ^B levels was observed upon a heat shock from 30°C to 42°C. After the construction of a *sigB* deletion mutant of *B. cereus*, the effect of σ^B on the growth and survival of *B. cereus* under several conditions was assayed. We found that σ^B plays a role in the adaptive heat stress response and growth and survival at sub-optimal temperatures (chapters 4 and 7). In addition, the *sigB* deletion mutant was shown to have a defect in the use of several amino acids as nitrogen sources (chapter 7). These metabolic effects of the deletion of *sigB* show that the function of σ^B is not strictly confined to the stress response, but that it also has a role in important metabolic routes. The *sigB* deletion mutant also exhibited a remarkable hyperresistance to H_2O_2. The upregulated expression of *katA* (the gene that encodes the vegetative cell catalase) in the *sigB* deletion mutant appears to play an important role in this phenotypic trait (chapter 8).

A number of σ^B-dependent genes in *B. cereus* were identified by a combination of proteome and *in vitro* transcription analysis (chapter 5). A specific protease, which may be involved in turn-over of misfolded or damaged proteins was dependent on σ^B and this finding may explain the defective adaptive heat stress response of the *sigB* deletion mutant. This is the only protein that has a directly obvious function in the stress response. A number of proteins, which have a function in various metabolic pathways were found to be dependent on σ^B. Finally, no function could be assigned to a large number of the proteins that are encoded by σ^B-dependent genes in *B. cereus*. Determining the function of these

proteins is a challenge for future research and might lead to the discovery of novel mechanisms that can contribute to stress resistance in *B. cereus*.

TRANSCRIPTIONAL AND POST-TRANSLATIONAL REGULATION OF THE ACTIVITY OF σ^B

The regulation of the levels of the σ^B protein in *B. cereus* is accomplished at two different levels, transcriptionally and post-translationally. The transcriptional regulation is relatively simple. The *sigB* operon is preceded by a σ^B-dependent promoter, and so the transcription of the *sigB* gene is autoregulated. In principle, high levels of σ^B should thus lead to high levels of *sigB* expression (chapter 4). However, research in *B. subtilis* has shown that post-translational control of σ^B activity is also an important factor (18) and our data extend these findings to *B. cereus*. In *B. cereus*, the σ^B regulators RsbV and RsbW perform essentially identical functions as their homologs in *B. subtilis* (chapter 6). RsbW is an anti-sigma factor, which can bind to σ^B thereby preventing the association of σ^B to core RNAP. RsbV can sequester σ^B from this complex by binding to RsbW, thereby leading to the initiation of σ^B-dependent transcription. We have also shown that RsbW acts as a kinase on RsbV, leading to the phosphorylation of RsbV. In *B. subtilis* it has previously been shown that the phosphorylated form of RsbV cannot bind to RsbW and so, the equilibrium between phosphorylated and unphosphorylated RsbV determines the activation state of σ^B (7, 23). In gram-positive bacteria that carry σ^B, one or more phosphatases from the PP2C-family also influence the activation state of σ^B by catalyzing the dephosphorylation of RsbV~P (chapter 2). In *B. cereus*, the deletion of the *rsbY*, which encodes the PP2C phosphatase RsbY, virtually completely abolished the activation of σ^B under stress conditions. *In trans* complementation with full-length *rsbY* restored the activation of σ^B under stress. These data strongly suggest that RsbY is the key phosphatase that can dephosphorylate RsbV~P leading to the activation of σ^B in *B. cereus* (chapter 4).

PERSPECTIVES FOR FURTHER RESEARCH

Even though the results described in this thesis provide extensive insight in the role of σ^B in the stress response of *B. cereus*, there are many aspects of the exact function of σ^B in the physiology of *B. cereus* and the regulation of σ^B activity, that remain to be elucidated.

First of all, it will be of great importance to determine the complete set of genes that are regulated by σ^B (the σ^B regulon). Transcriptome profiling using DNA microarray has become the method of choice for defining stress-regulons in bacteria. Using a DNA microarray which only had a partial coverage of the *L. monocytogenes* genome, already 55 σ^B-dependent genes could be identified (13). When DNA micro- or macroarrays were used which complete genome coverage, more than 200 σ^B-dependent genes were identified in *B. subtilis* and *S. aureus* (4, 10, 17, 19). The comparison of the transcriptome of *B. cereus* ATCC 14579 and its *sigB* deletion mutant by DNA microarray technology will no doubt lead to the

identification of a large number of σ^B-dependent genes in *B. cereus*. With this knowledge it may be possible to explain many of the phenotypes of the *sigB* deletion mutant that have been described in this thesis (chapters 4, 7 and 8). On the other hand, as in other gram-positive bacteria, many genes that encode proteins with no known function, will probably be identified as being σ^B-dependent. This underscores the need for further functional characterization of proteins with no known function in the bacterial stress response. A prime candidate for such a functional characterization is the protein YflT. This protein is conserved in *Bacilli* and it is strongly upregulated upon stress exposure in a σ^B-dependent fashion in both *B. cereus* (chapter 5) and *B. subtilis* (10, 17, 19). However, nothing is known about its functional role in the stress response of *Bacillus*.

The process of σ^B activation in *B. cereus* differs in important aspects from other gram-positive bacteria, most notably in the role of the phosphatase RsbY in the regulation of σ^B activity as outlined above and in chapters 2 and 6. However, there are several questions, which remain to be addressed before the description of the σ^B activation pathway in *B. cereus* is complete. First of all, several of the functions of the regulatory proteins RsbV, RsbW and RsbY were inferred based on their homologs to proteins in *B. subtilis* of which the function has been experimentally defined. We have experimentally proven most of the predicted functions of the *B. cereus* homologs (i.e. the kinase activity of RsbW and the anti-sigma factor and anti-anti-sigma factor activities of RsbW and RsbV, respectively). However, more biochemical evidence may be needed to unequivocally prove the occurrence of other protein-protein interactions in the σ^B activation pathway of *B. cereus*. The need for such experiments is most obvious for determining the interaction of RsbY and RsbV~P. Although unlikely, it is in principle possible that RsbY is not involved in the dephosphorylation of RsbV~P, but in some other dephosphorylation reaction, which would then occur further upstream in the σ^B activation pathway. To directly determine protein-protein interactions between regulators of σ^B activity in *B. cereus*, techniques like yeast two hybrid screening, which has been applied successfully in validating predicted protein-protein interactions in the σ^B activation pathway of *B. subtilis* (21), would be a powerful method to prove the direct interactions between RsbV, RsbW and RsbY. Yeast two hybrid screening with the response regulator domain of RsbY as "bait" and a genomic library of *B. cereus* as "prey" may also lead to the identification of the kinase, which we have proposed to be involved in the phosphorylation of the N-terminal receiver domain of RsbY, leading to the activation of RsbY and, ultimately, of σ^B.

Finally, there may be additional phenotypes that are exhibited by the *sigB* deletion mutant, that we have so far missed in our analyses. Even though Phenotype MicroArray screening of the *sigB* deletion mutant revealed that growth under osmotic and acid stress conditions is not negatively affected by the deletion of *sigB*, we cannot rule out the possibility that there are important differences in the survival kinetics of the *sigB* deletion mutant and the parent strain under these and other stress conditions.

POTENTIAL PRACTICAL APPLICATION OF THE RESEARCH DESCRIBED IN THIS THESIS

Because of the ubiquitous occurrence of *B. cereus* in the environment it is practically unavoidable that raw materials used in food production will contain *B. cereus* spores and/or vegetative cells. Especially spores form a problem, because they can only be inactivated using fairly harsh treatments, which mainly rely on heat sterilization. In most cases, heat sterilization will lead to a significant deterioration of product quality. This is no longer acceptable for many consumers, who prefer foods that have a fresh and natural taste and texture. This has led to the development of novel preservation methods to control foodborne microorganism. Many of these technologies are not based on "classical" thermal inactivation of spores and vegetative cells, but instead rely on non-thermal technologies including acidification of the product, use of anti-microbial agents (such as bacteriocins), low temperature or modified atmosphere storage, and inactivation techniques like pulsed electric fields, ultrasonication, high hydrostatic pressure and irradiation (1, 2, 20). A combination of preservation techniques is commonly referred to as "hurdle technology", which should ensure a microbiologically safe food product (16).

The main drawback of these novel techniques is that spores, and sometimes even vegetative cells, may survive these relatively mild preservation conditions. The surviving spores and cells may subsequently germinate and grow in the final food product. For the application of novel preservation techniques it is therefore of crucial importance that there is adequate data on the growth and survival kinetics (and underlying mechanisms) of spoilage and pathogenic bacteria so that clear "safety boundaries" may be defined (5). The data described in this thesis can contribute to setting the limits under which *B. cereus* can still grow and survive. There is a major effect of the stress response on the survival of cells as they can survive for a prolonged period of time under conditions, which are lethal to non-stressed cells. Clearly, the possible impact of the stress response on the survival capacity of food spoilage and pathogenic bacteria should be taken into account during product development and accompanying risk assessment. Our data also show that σ^B plays a role in heat and cold adaptation. The disruption of the σ^B response in *B. cereus*, may thus lead to a stress-sensitive phenotype, making the bacteria easier to inactivate. However, the data in chapter 8 show that inactivation of σ^B can, unexpectedly, lead to resistance against oxidative stress. Whether this also affects the survival of *B. cereus* under food-relevant conditions remains to be determined. The rapid activation of σ^B during stress exposure (see chapters 4 and 6), means that σ^B could also be used as a biosensor for the stress state of the organism. The use of biosensors is a novel concept by which the physiological state of an organism can be determined in a much faster and more sensitive way than by using culture-based methods (6).

In conclusion, detailed knowledge of the ecology and cellular mechanisms of *B. cereus* is essential for the optimization of novel intervention strategies for the elimination or control of *B. cereus*.

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SAMENVATTING

Bacillus cereus is een ziekteverwekkende bacterie die een frequente veroorzaker is van uitbraken van voedselvergiftiging of -infectie. De symptomen van een door *B. cereus* veroorzaakte voedselvergifting zijn over het algemeen mild en worden gekarakteriseerd door diarree of overgeven. Recentelijk zijn er ook enkele gevallen van *B. cereus* voedselvergiftiging beschreven waarbij zich ernstige complicaties voordeden, die uiteindelijk zelfs de dood tot gevolg hadden.

B. cereus maakt deel uit van een grotere groep van bacteriën die gezamenlijk de *Bacillus cereus* groep wordt genoemd. Behalve *B. cereus* omvat deze groep ook de bacteriën *Bacillus thuringiensis* en *Bacillus anthracis*. *B. thuringiensis* maakt gifstoffen die dodelijk zijn voor verschillende insecten en deze bacterie wordt dan ook veelvuldig gebruikt als biopesticide bij insectenplagen. *B. anthracis* veroorzaakt de ziekte miltvuur en is berucht geworden door aanslagen die met deze bacterie in de herfst van 2001 in de Verenigde Staten zijn gepleegd. De mogelijkheid van toekomstige bioterreur-aanslagen met deze bacterie blijft een punt van zorg voor overheden over de gehele wereld. De verschillen in de pathogene eigenschappen van de bacteriën in de *B. cereus* groep zijn vooral te wijten aan de aanwezigheid van plasmiden waarop de verschillende gifstoffen en andere virulentie-determinanten gecodeerd zijn. Op het niveau van het chromosomaal DNA zijn de verschillen tussen de bacteriën van de *B. cereus* groep relatief gering, wat inhoudt dat belangrijke cellulaire processen, zoals de hieronder beschreven stress-respons, in al deze bacteriën grofweg volgens dezelfde lijnen verloopt.

De stress-respons is een belangrijk overlevingsmechanisme van bacteriën. Stress kan zich bij bacteriën voordoen als de omstandigheden in de omgeving van de cel veranderen. De cel moet snel reageren op deze veranderingen omdat de cel anders beschadigd kan worden en daardoor in de groei geremd of zelfs gedood kan worden. Omdat bacteriën in hun natuurlijke omgeving in feite continu te maken hebben met veranderingen in hun leefomgeving, zijn zij voorzien van een veelvoud aan systemen om te reageren op stress-situaties. In een aantal gram-positieve bacteriën is een belangrijke rol in het reguleren van de stress-respons weggelegd voor de alternatieve sigma factor σ^B. Sigma factoren zijn onderdelen van het enzym RNA polymerase, dat een centrale rol speelt in het proces van transcriptie (het overschrijven van DNA in RNA). Sigma factoren sturen het RNA polymerase naar specifieke promoter-sites waarna transcriptie van de daarachter gelegen genen plaatsvindt. Bacteriën hebben in het algemeen meerdere sigma factoren, waarvan er één (de zgn. "huishoud sigma factor") noodzakelijk is voor de transcriptie van genen die essentieel zijn voor de groei. De overige sigma factoren spelen alleen een rol tijdens specifieke condities of cellulaire ontwikkeling. Voor aanvang van deze studie was al bekend dat de sigma factor σ^B in verschillende bacteriën (met name in *Bacillus subtilis*, *Listeria monocytogenes* en *Staphylococcus aureus*) een belangrijke rol speelt in de stress-respons, maar over de stress-respons van *B. cereus* en de rol van σ^B hierin was nog vrijwel niets bekend. Dit proefschrift biedt een uitgebreid overzicht van de stress-respons van *B. cereus* met speciale aandacht voor de rol van σ^B in dit proces.

In hoofdstuk 3 van dit proefschrift wordt de hittestress-respons van *B. cereus* beschreven. Cellen in de mid-exponentiele groei-fase die rechtstreeks van 30°C naar 50°C worden overgebracht sterven snel af bij deze hoge temperatuur. Echter, als cellen eerst worden gestrest door ze over te brengen van 30°C naar 42°C of door zout, ethanol of zuur toe te voegen, kunnen ze aanmerkelijk langer overleven bij 50°C. Met de twee-dimensionale gel-electroforese techniek werden verschillende eiwitten die een rol hebben in de stress-respons geïdentificeerd. Eén van deze stress-eiwitten is RsbV (de anti-anti-sigma-factor van σ^B in *B. cereus*). Dit resultaat suggereert reeds dat σ^B ook in *B. cereus* een rol heeft in de stress-respons.

In hoofdstuk 4 wordt de activatie van σ^B gedurende blootstelling aan stress in detail beschreven. σ^B wordt geactiveerd onder verschillende stress-condities, in het bijzonder gedurende een hitteschok van 30°C naar 42°C, maar ook na toevoeging van ethanol, zout of zuur aan het groeimedium. Verder reguleert σ^B zijn eigen expressie d.w.z. het *sigB* operon wordt vooraf gegaan door een σ^B-afhankelijke promoter. In hoofdstuk 4 wordt ook een mutant beschreven waarin het *sigB*-gen is verwijderd. Deze mutant bleek een verzwakte hittestress-respons te hebben in vergelijking met een stam die nog wel σ^B kon produceren. Een andere opmerkelijke eigenschap van de *sigB*-mutant is dat deze stam een verminderde groei en overleving heeft bij lage temperaturen in vergelijking met de wild-type stam. Ook in het gebruik van verschillende aminozuren als stikstofbron speelt σ^B een aanzienlijke rol. Deze data staan beschreven in hoofdstuk 7. Verder vertoont de *sigB*-mutant een opmerkelijke hyperresistentie t.o.v. waterstofperoxide (H_2O_2). De verhoogde expressie van *katA* (het gen dat codeert voor het enzym catalase in de vegetatieve cel) lijkt een belangrijke rol te spelen in dit fenotype (hoofdstuk 8).

Een aantal genen waarvan de expressie σ^B-afhankelijk is, werd geïdentificeerd door een combinatie van proteome- en *in-vitro*-transcriptieanalyse (hoofdstuk 5). Het is opmerkelijk dat van de geïdentificeerde eiwitten er slechts één (een protease dat mogelijk beschadigde eiwitten kan afbreken of repareren) een voor de hand liggende rol lijkt te hebben in de stress-respons. Verrassend was ook dat een groter aantal σ^B-afhankelijke genen coderen voor eiwitten die een rol hebben in verschillende metabole processen. Dit suggereert dat modificatie van het cel-metabolisme een belangrijke rol heeft in de stress-respons van *B. cereus*. Tenslotte werden een aantal σ^B-afhankelijke genen geïdentificeerd die coderen voor eiwitten met een onbekende functie. Een eventuele toekomstige opheldering van de functie van deze eiwitten kan mogelijk leiden tot inzichten in nieuwe mechanismen die bijdragen aan de stress-respons van *B. cereus* en andere gram-positieve bacteriën.

Het is van evident belang voor de cel om de activiteit van σ^B zo nauwkeurig mogelijk te reguleren. Onder optimale groei-omstandigheden is het niet nodig om stress-eiwitten te produceren, maar zodra een stress-situatie optreedt moet er een snelle tegenreactie plaatsvinden om de cel te beschermen tegen de veranderende omstandigheden. Een onderdeel van dit proces is de hierboven beschreven autoregulatie van de transcriptie van het *sigB* gen. De regulatie van de activiteit van σ^B vindt echter voor een belangrijk deel ook post-translationeel plaats. Dit aspect van de regulatie van σ^B-activiteit staat beschreven in hoofdstuk 6. Ten eerste werd vastgesteld dat RsbV en RsbW respectievelijk als anti-anti-

sigma factor en als anti-sigma factor functioneren. Ook bleek RsbW in staat te zijn om RsbV te fosforyleren. In *B. subtilis* bepaalt de fosforylatie-staat van RsbV of σ^B al dan niet geactiveerd wordt. Als RsbV gefosforyleerd is, dan kan RsbV niet aan RsbW binden. RsbW vormt in dit geval een complex met σ^B en inactiveert zo σ^B. RsbV kan op zijn beurt gedefosforyleerd worden door een fosfatase. Dit is een cruciale stap in het proces van σ^B-activatie omdat ongefosforyleerd RsbV wel een complex met RsbW kan vormen. Dit geeft dan de mogelijkheid voor σ^B om te binden aan RNA polymerase, gevolgd door σ^B-afhankelijke transcriptie. De in dit proefschrift beschreven resultaten wijzen erop dat de essentiële functies van RsbV en RsbW in *B. cereus* identiek zijn aan die van RsbV en RsbW in *B. subtilis*. Er bestaan echter grote verschillen tussen de fosfatases die RsbV kunnen defosforyleren. In alle gram-positieve bacteriën waarin σ^B een rol speelt in de stress-respons, heeft dit fosfatase een C-terminaal PP2C-domein. Er is echter aanmerkelijke variatie in de N-terminale domeinen die de signalen opvangen van andere regulatoren in de σ^B-activatie cascade. In *B. cereus* (en de overige bacteriën in de *B. cereus* groep) heeft dit fosfatase (RsbY) een N-terminaal CheY domein, hetgeen uniek is voor regulatoren van σ^B-activiteit in gram-positieve bacteriën. Dit geeft aan dat de σ^B-activatie route in *B. cereus* verschilt van die in andere gram-positieve bacteriën. De cruciale rol van dit fosfatase in het proces van σ^B activatie werd aangetoond door een deletiemutant te maken in het *rsbY* gen. Deze mutant bleek niet meer in staat te zijn om σ^B te activeren na blootstelling aan stress.

Het onderzoek dat in dit proefschrift staat beschreven kan verschillende praktische toepassingen vinden. Deze studie toont aan dat de stress-respons de overleving van bacteriën onder verschillende condities dramatisch kan beïnvloeden. Omdat tegenwoordig veel voedingsmiddelen met betrekkelijk milde methoden worden geconserveerd (zgn. “minimal processing”), is het van bijzonder belang dat een processtap zo wordt afgesteld dat nog steeds alle ziekteverwekkende of bederf-veroorzakende bacteriën worden geïnactiveerd. Het is daarom van belang dat ook de stress-respons van *B. cereus* in acht wordt genomen bij het inregelen van industriële processen voor de conservering van voedingsmiddelen. Door het dusdanig optimaliseren van “minimal processing”-technieken kunnen de nutritionele en organoleptische eigenschappen van een natuurlijk product zo goed mogelijk behouden blijven, terwijl ook de microbiologische veiligheid van het eindproduct gegarandeerd blijft.

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NAWOORD

Toen ik in de loop van 20o0 besloot dat ik me wetenschappelijk verder wilde verdiepen, zag ik vrij snel een advertentie in de krant staan, waarin werd gevraagd naar een AIO die wilde werken aan de stress-respons van *Bacillus cereus*. Mijn sollicitatie-brief zat net voor een vakantie naar Toscane op de bus en nog tijdens die vakantie kreeg ik een uitnodiging voor een gesprek (Yvonne, bedankt voor het openmaken van de brief!). Vanaf de trappen van één van de vele kerken in Assisi heb ik nog geprobeerd om dat gesprek te verzetten, want eigenlijk stond er nog een bezoek aan Florence gepland. Helaas lukte het me niet om iemand aan de telefoon te krijgen, en dus moest de terugreis naar Nederland vroegtijdig ingezet worden. Gelukkig was het de moeite waard, want ik kijk met grote tevredenheid en plezier terug op de afgelopen vier jaar. Alleen Florence heb ik nog steeds niet gezien…

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In het eerste jaar van een promotie-onderzoek wordt de basis gelegd voor de rest van het onderzoek en hier hebben Patrick en Jeroen een belangrijke bijdrage geleverd. I would also like to thank Paula for the pleasant and fruitful cooperation during the first year of my PhD-research. Marcel T. was de juiste man op de juiste plaats en tijd: je bijzondere efficiëntie bij het zuiveren van σ^B hebben geleid tot een stroomversnelling in het onderzoek. Verder heeft iedereen, die samen met mij in lab 406/7 heeft gewerkt, me op hun eigen manier geholpen, waarvoor dank. Jullie hebben het toch maar een hele tijd met me moeten uithouden, en dat betekent vals gezang, ongevraagde Hans Teeuwen imitaties, gags die bij mij minstens twee jaar "running" kunnen blijven, stapels papier op onhandige plaatsen en PCR-apparaten die bij hun eerste contact met mij direct uit elkaar spatten. Ook de andere collega's van de leerstoelgroep Levensmiddelenmicrobiologie (met name mijn kamergenoten Dorette, Birgit en Kaouther) wil ik bedanken voor de uitstekende werksfeer die ze hebben gecreëerd. Ook mijn WCFS-collega's, in het bijzonder de *B. cereus* AIO's Luc en Ynte, wil ik bedanken voor de prettige samenwerking.

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CURRICULUM VITAE

Willem van Schaik werd op 18 februari 1975 geboren te Zwolle. In 1993 behaalde hij zijn VWO-diploma op het Gymnasium Celeanum in diezelfde plaats. In september van dat jaar startte hij met de studie Levensmiddelentechnologie aan de Landbouwuniversiteit Wageningen (tegenwoordig Wageningen Universiteit). Van september tot april 1997 voerde hij een afstudeervak uit bij de leerstoelgroep Levensmiddelenmicrobiologie met als onderwerp de vorming van biogene amines tijdens de fermentatie van vis onder begeleding van Dr. Rob Nout en Maria Dapkevicius. Vervolgens heeft hij van mei tot november 1997 een stage uitgevoerd in het laboratorium van Prof. Colin Hill, University College Cork (Ierland) over de stress-respons en de bacteriocine-resistentie van *Listeria monocytogenes*. Van november 1997 tot augustus 1998 rondde hij zijn studie af met een afstudeervak bij de leerstoelgroep Industriële Microbiologie over de enzymatische oxidatie en analyse van galactomannanen, onder begeleiding van Dr. Sybe Hartmans. In augustus 1998 werd de studie Levensmiddelentechnologie *cum laude* afgesloten.

Van augustus 1998 tot november 2000 was Willem van Schaik aangesteld als junior onderzoeker bij de afdeling Biotechnologie van Numico Research te Wageningen. Hier deed hij onderzoek voor de ontwikkeling van een nieuwe generatie probiotische producten.

In november 2000 startte Willem van Schaik met zijn promotie-onderzoek dat werd uitgevoerd aan de leerstoelgroep Levensmiddelenmicrobiologie van Wageningen Universiteit met als onderwerp de rol van de alternatieve sigma factor σ^B in de stress-respons van *Bacillus cereus*. Dit onderzoek was onderdeel van het onderzoeksprogramma van het Wageningen Centre for Food Sciences. Dit proefschrift is het tastbare resultaat van zijn promotie-onderzoek.

Vanaf maart tot eind 2005 is Willem van Schaik aangesteld als post-doctoraal onderzoeker bij de leerstoelgroep Levensmiddelenmicrobiologie van Wageningen Universiteit.

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