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The Alternative Sigma Factor σ^B of *Bacillus cereus*: Response to Stress and Role in Heat Adaptation

Willem van Schaik,[1,2] Marcel H. Tempelaars,[1,2] Jeroen A. Wouters,[1,2]† Willem M. de Vos,[1] and Tjakko Abee[1,2]*

Wageningen Centre for Food Sciences,[1] and Laboratory of Food Microbiology, Wageningen University,[2] Wageningen, The Netherlands

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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.

Bacillus cereus is a spore-forming gram-positive rod that is increasingly being recognized as a food-borne 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 food-borne 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 food-borne pathogen. Vegetative cells of *B. cereus* also play an important role in the pathogenesis of food-borne 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 food-borne 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 food-borne 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

* Corresponding author. Mailing address: Laboratory of Food Microbiology, Wageningen University, Bomenweg 2, 6703 HD Wageningen, The Netherlands. Phone: 31-317-484981. Fax: 31-317-484978. E-mail: tjakko.abee@wur.nl.

† Present address: Department Flavour, Nutrition and Ingredients, NIZO Food Research, 6710 BA Ede, The Netherlands.

TABLE 1. Plasmids used in this study

Plasmid	Relevant properties	Source or 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

(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 σ^B 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.

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.

TABLE 2. Oligonucleotides used in this study

Oligonucleotide	Sequence[a]
OEcSigBF	GCAGCCATGGTGGAAATCCAATCTCAACCT
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.

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 *Nco*I site and an *Xho*I 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-logarithmic 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 × *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.

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 × *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 *Xba*I and *Bam*HI, 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 *Xma*I and *Eco*RI, 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 *Xba*I, 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-morpholinepropanesulfonic 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 [α-^{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.

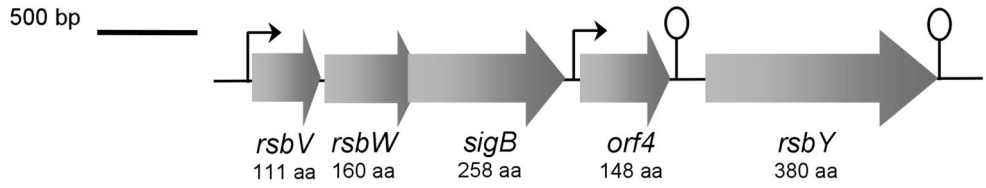
Primer extension analysis was performed as described by Kuipers et al. (24). The oligonucleotides used were PERsbV and PEOrf4, which are complementary to *rsbV* and *orf4*, respectively. Four picomoles of primer was added to 50 μg of RNA in reaction buffer containing 10 nmol of dCTP, 10 nmol of dGTP, 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-logarithmic 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 σ^B 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



%identity with					
B. anthracis Ames	95%	98%	96%	96%	96%
B. cereus ATCC 10987	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 homologues 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 homologue (*rsbP*) is not located directly downstream of the *sigB* operon like *rsbY* in the *B. cereus* group.

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 homologues 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 homologues 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* homologue (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 homologue 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 homologue 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.

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 homologues 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 homologues in *B. cereus* ATCC 14579.

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

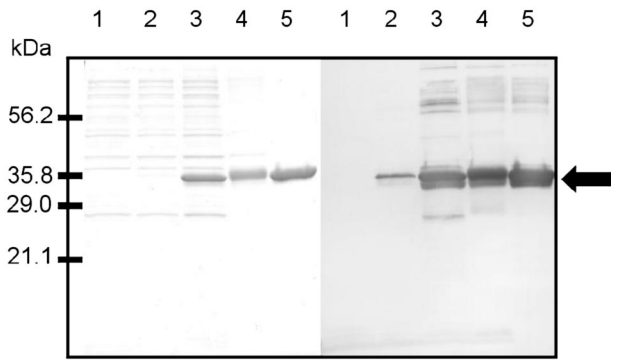


FIG. 2. Overproduction and purification of σ^B. (Left panel) SDS-PAGE of protein extracts from *E. coli* BL21-Codonplus-(DE3)-RIL carrying either pET28-b or pMT01. (Right panel) Immunodetection of σ^B with anti-σ^B antiserum. Ten micrograms of protein was loaded for each sample. Lane 1, crude protein 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.

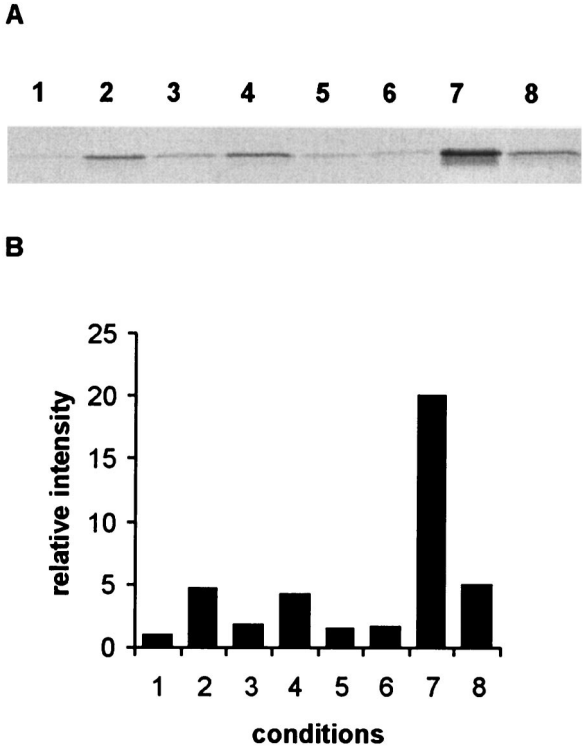


FIG. 3. Stress-induced activation of σ^B in *B. cereus*. (A) Cellular σ^B levels upon exposure to stress. Protein extracts from mid-logarithmic-phase *B. cereus* cells (lane 1) and stressed *B. cereus* cells were prepared as described in Materials and Methods. *B. cereus* cells in the mid-logarithmic 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-logarithmic-phase culture was defined as 1.

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. 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-logarithmic 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-logarithmic 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. 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 homologue 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

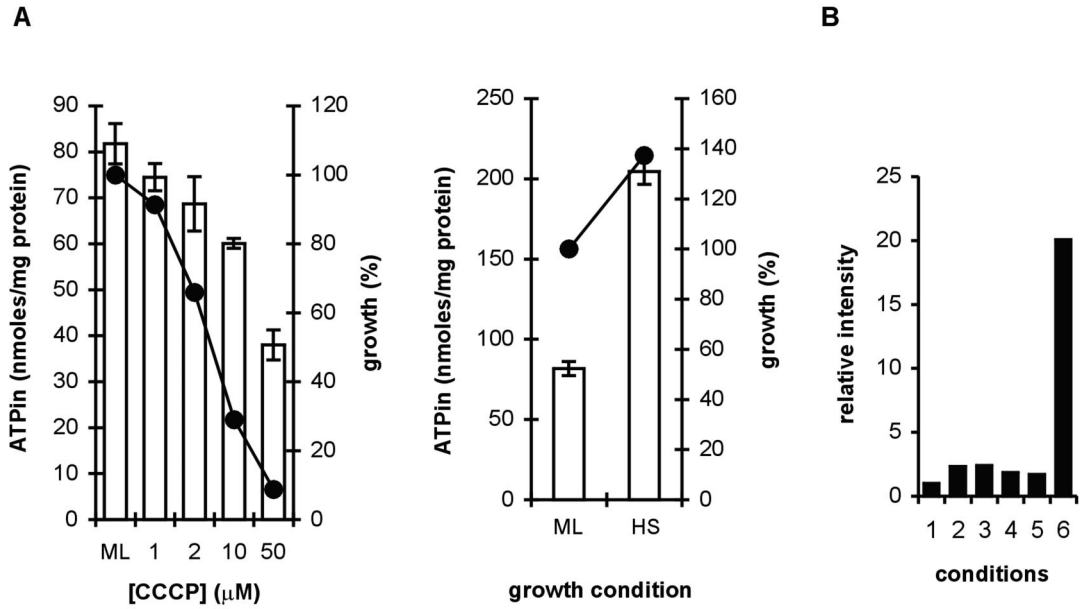


FIG. 4. Effects of CCCP on growth, the intracellular concentration of ATP, and σ^B expression of *B. cereus*. (A) Growth (circles) and intracellular ATP concentration (bars) in *B. cereus* cells from a mid-logarithmic-phase culture (ML) after exposure to 1, 2, 10, and 50 μM CCCP (left panel) and after 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.

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-logarithmic 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-logarithmic growth phase no transcription of the *sigB* operon was observed, results which corresponded to the barely detectable σ^B levels in the Western blot experiments. In the RNA isolated from wild-type cells after 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-logarithmic growth but are activated upon a heat shock. 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

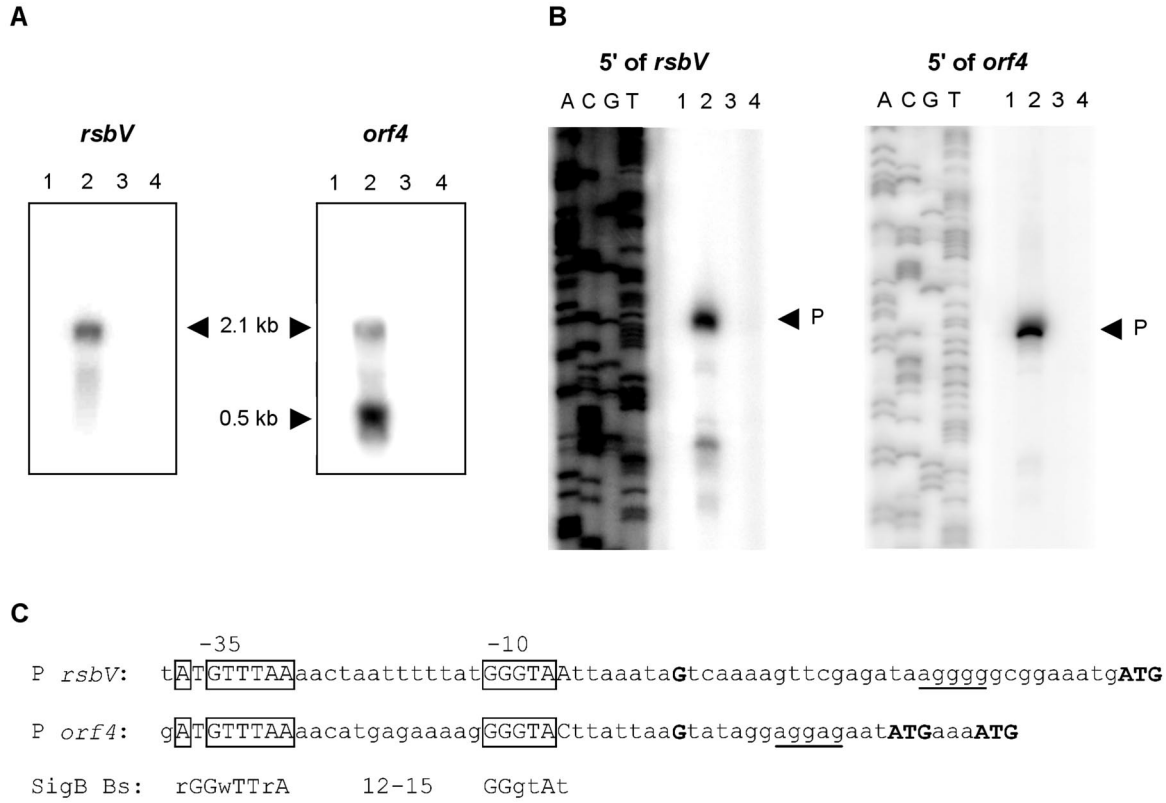


FIG. 5. Analysis of transcription of the *sigB* operon in *B. cereus*. (A) Northern blot analysis of transcription of the *sigB* operon. Total RNA was extracted from *B. cereus* ATCC 14579 and *B. cereus* FM1400 cells during mid-logarithmic growth in BHI medium (lanes 1 and 3, respectively) and after 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 logarithmic 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.

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*.

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-logarithmic 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-logarithmic 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

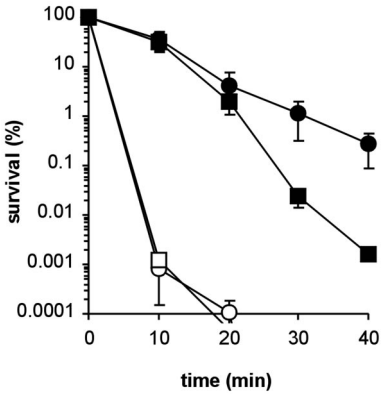


FIG. 6. Survival of of *B. cereus* ATCC 14579 cells (circles) and *B. cereus* FM1400 cells (squares) in the mid-logarithmic growth phase at 30°C (open symbols) and after pretreatment at 42°C for 30 min before exposure to 50°C (solid symbols). The averages of three independent experiments are shown. The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

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 ($\leq$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 σ^B 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 homologues, 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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REFERENCES

1. **Becker, L. A., M. S. Cetin, R. W. Hutkins, and A. K. Benson.** 1998. Identification of the gene encoding the alternative sigma factor σ^B from *Listeria monocytogenes* and its role in osmotolerance. J. Bacteriol. **180:**4547–4554.
2. **Beecher, D. J., T. W. Olsen, E. B. Somers, and A. C. Wong.** 2000. Evidence for contribution of tripartite hemolysin BL, phosphatidylcholine-preferring phospholipase C, and collagenase to virulence of *Bacillus cereus* endophthalmitis. Infect. Immun. **68:**5269–5276.
3. **Benson, A. K., and W. G. Haldenwang.** 1993. Regulation of σ^B levels and activity in *Bacillus subtilis.* J. Bacteriol. **175:**2347–2356.
4. **Bron, S.** 1990. Plasmids, p. 75–174. *In* C. R. Harwood and S. M. Cutting (ed.), Molecular biological methods for *Bacillus.* John Wiley & Sons Ltd., Chichester, Great Britain.
5. **Chan, P. F., S. J. Foster, E. Ingham, and M. O. Clements.** 1998. The *Staphylococcus aureus* alternative sigma factor σ^B controls the environmental stress response but not starvation survival or pathogenicity in a mouse abscess model. J. Bacteriol. **180:**6082–6089.
6. **Deora, R., T. Tseng, and T. K. Misra.** 1997. Alternative transcription factor σ^{SB} of *Staphylococcus aureus*: characterization and role in transcription of the global regulatory locus *sar.* J. Bacteriol. **179:**6355–6359.
7. **Ferreira, A., C. P. O'Byrne, and K. J. Boor.** 2001. Role of σ^B in heat, ethanol, acid, and oxidative stress resistance and during carbon starvation in *Listeria monocytogenes.* Appl. Environ. Microbiol. **67:**4454–4457.
8. **Ferreira, A., D. Sue, C. P. O'Byrne, and K. J. Boor.** 2003. Role of *Listeria monocytogenes* σ^B in survival of lethal acidic conditions and in the acquired acid tolerance response. Appl. Environ. Microbiol. **69:**2692–2698.
9. **Fouet, A., O. Namy, and G. Lambert.** 2000. Characterization of the operon encoding the alternative σ^B factor from *Bacillus anthracis* and its role in virulence. J. Bacteriol. **182:**5036–5045.
10. **Gahan, C. G., and C. Hill.** 1999. The relationship between acid stress response and virulence in *Salmonella typhimurium* and *Listeria monocytogenes.* Int. J. Food Microbiol. **50:**93–100.
11. **Gaur, A. H., C. C. Patrick, J. A. McCullers, P. M. Flynn, T. A. Pearson, B. I. Razzouk, S. J. Thompson, and J. L. Shenep.** 2001. *Bacillus cereus* bacteremia and meningitis in immunocompromised children. Clin. Infect. Dis. **32:**1456–1462.
12. **Gaur, A. H., and J. L. Shenep.** 2001. The expanding spectrum of disease caused by *Bacillus cereus.* Pediatr. Infect. Dis. J. **20:**533–534.
13. **Gitt, M. A., L. F. Wang, and R. H. Doi.** 1985. A strong sequence homology exists between the major RNA polymerase sigma factors of *Bacillus subtilis* and *Escherichia coli.* J. Biol. Chem. **260:**7178–7185.
14. **Granum, P. E.** 2001. *Bacillus cereus*, p. 327–336. *In* M. P. Doyle, L. R. Beuchat, and T. J. Montville (ed.), Food microbiology: fundamentals and frontiers, 2nd ed. American Society for Microbiology, Washington, D.C.
15. **Haldenwang, W. G., and R. Losick.** 1980. Novel RNA polymerase sigma factor from *Bacillus subtilis.* Proc. Natl. Acad. Sci. **77:**7000–7004.
16. **Hecker, M., and S. Engelmann.** 2000. Proteomics, DNA arrays and the analysis of still unknown regulons and unknown proteins of *Bacillus subtilis* and pathogenic gram-positive bacteria. Int. J. Med. Microbiol. **290:**123–134.
17. **Helgason, E., O. A. Okstad, D. A. Caugant, H. A. Johansen, A. Fouet, M. Mock, I. Hegna, and A. B. Kolsto.** 2000. *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis*—one species on the basis of genetic evidence. Appl. Environ. Microbiol. **66:**2627–2630.
18. **Helmann, J. D., and C. P. J. Moran.** 2002. RNA polymerase and sigma factors, p. 289–312. *In* A. L. Sonenshein, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and its closest relatives: from genes to cells. ASM Press, Washington, D.C.
19. **Helmann, J. D., M. F. Wu, P. A. Kobel, F. J. Gamo, M. Wilson, M. M. Morshedi, M. Navre, and C. Paddon.** 2001. Global transcriptional response of *Bacillus subtilis* to heat shock. J. Bacteriol. **183:**7318–7328.
20. **Ivanova, N., A. Sorokin, I. Anderson, N. Galleron, B. Candelon, V. Kapatral, A. Bhattacharyya, G. Reznik, N. Mikhailova, A. Lapidus, L. Chu, M. Mazur, E. Goltsman, N. Larsen, M. D'Souza, T. Walunas, Y. Grechkin, G. Pusch, R. Haselkorn, M. Fonstein, E. S. Dusko, R. Overbeek, and N. Kyrpides.** 2003. Genome sequence of *Bacillus cereus* and comparative analysis with *Bacillus anthracis.* Nature **423:**87–91.
21. **Kalman, S., M. L. Duncan, S. M. Thomas, and C. W. Price.** 1990. Similar organization of the *sigB* and *spoIIA* operons encoding alternate sigma factors of *Bacillus subtilis* RNA polymerase. J. Bacteriol. **172:**5575–5585.
22. **Keim, P., and K. L. Smith.** 2002. *Bacillus anthracis* evolution and epidemiology. Curr. Top. Microbiol. Immunol. **271:**21–32.
23. **Kotiranta, A., K. Lounatmaa, and M. Haapasalo.** 2000. Epidemiology and pathogenesis of *Bacillus cereus* infections. Microbes Infect. **2:**189–198.
24. **Kuipers, O. P., M. M. Beerthuyzen, R. J. Siezen, and W. M. de Vos.** 1993. Characterization of the nisin gene cluster nisABTCIPR of *Lactococcus lactis.* Requirement of expression of the *nisA* and *nisI* genes for development of immunity. Eur. J. Biochem. **216:**281–291.
25. **Kullik, I., and P. Giachino.** 1997. The alternative sigma factor σ^B in *Staphylococcus aureus*: regulation of the *sigB* operon in response to growth phase and heat shock. Arch. Microbiol. **167:**151–159.
26. **Kunst, F., N. Ogasawara, I. Moszer, A. M. Albertini, G. Alloni, V. Azevedo, M. G. Bertero, P. Bessieres, A. Bolotin, S. Borchert, R. Borriss, L. Boursier, A. Brans, M. Braun, S. C. Brignell, S. Bron, S. Brouillet, C. V. Bruschi, B. Caldwell, V. Capuano, N. M. Carter, S. K. Choi, J. J. Codani, I. F. Connerton, and A. Danchin.** 1997. The complete genome sequence of the gram-positive bacterium *Bacillus subtilis.* Nature **390:**249–256.
27. **Lereclus, D., H. Agaisse, M. Gominet, S. Salamitou, and V. Sanchis.** 1996. Identification of a *Bacillus thuringiensis* gene that positively regulates transcription of the phosphatidylinositol-specific phospholipase C gene at the onset of the stationary phase. J. Bacteriol. **178:**2749–2756.
28. **Lereclus, D., H. Agaisse, C. Grandvalet, S. Salamitou, and M. Gominet.** 2000. Regulation of toxin and virulence gene transcription in *Bacillus thuringiensis.* Int. J. Med. Microbiol. **290:**295–299.
29. **Lu, J., Y. Nogi, and H. Takami.** 2001. *Oceanobacillus iheyensis* gen. nov., sp. nov., a deep-sea extremely halotolerant and alkaliphilic species isolated from a depth of 1050 m on the Iheya Ridge. FEMS Microbiol Lett. **205:**291–297.
30. **Lund, T., M. L. De Buyser, and P. E. Granum.** 2000. A new cytotoxin from *Bacillus cereus* that may cause necrotic enteritis. Mol. Microbiol. **38:**254–261.
31. **McKillip, J. L.** 2000. Prevalence and expression of enterotoxins in *Bacillus cereus* and other *Bacillus* spp., a literature review. Antonie Leeuwenhoek **77:**393–399.
32. **Namy, O., M. Mock, and A. Fouet.** 1999. Co-existence of *clpB* and *clpC* in the *Bacillaceae.* FEMS Microbiol. Lett. **173:**297–302.
33. **Periago, P. M., W. van Schaik, T. Abee, and J. A. Wouters.** 2002. Identification of proteins involved in the heat stress response of *Bacillus cereus* ATCC 14579. Appl. Environ. Microbiol. **68:**3486–3495.
34. **Petersohn, A., M. Brigulla, S. Haas, J. D. Hoheisel, U. Volker, and M. Hecker.** 2001. Global analysis of the general stress response of *Bacillus subtilis.* J. Bacteriol. **183:**5617–5631.
35. **Price, C. W.** 2000. Protective function and regulation of the general stress response in *Bacillus subtilis* and related gram-positive bacteria, p. 179–197. *In* G. Storz and R. Hengge-Aronis (ed.), Bacterial stress response. ASM Press, Washigton, D.C.
36. **Price, C. W.** 2002. General stress response, p. 369–384. *In* A. L. Sonenshein, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and its closest relatives: from genes to cells. ASM Press, Washington, D.C.
37. **Price, C. W., P. Fawcett, H. Ceremonie, N. Su, C. K. Murphy, and P. Youngman.** 2001. Genome-wide analysis of the general stress response in *Bacillus subtilis.* Mol. Microbiol. **41:**757–774.
38. **Radnedge, L., P. G. Agron, K. K. Hill, P. J. Jackson, L. O. Ticknor, P. Keim, and G. L. Andersen.** 2003. Genome differences that distinguish *Bacillus*

anthracis from *Bacillus cereus* and *Bacillus thuringiensis.* Appl. Environ. Microbiol. **69:**2755–2764.

39. **Read, T. D., S. N. Peterson, N. Tourasse, L. W. Baillie, I. T. Paulsen, K. E. Nelson, H. Tettelin, D. E. Fouts, J. A. Eisen, S. R. Gill, E. K. Holtzapple, O. A. Okstad, E. Helgason, J. Rilstone, M. Wu, J. F. Kolonay, M. J. Beanan, R. J. Dodson, L. M. Brinkac, M. Gwinn, R. T. DeBoy, R. Madpu, S. C. Daugherty, A. S. Durkin, D. H. Haft, W. C. Nelson, J. D. Peterson, M. Pop, H. M. Khouri, D. Radune, J. L. Benton, Y. Mahamoud, L. Jiang, I. R. Hance, J. F. Weidman, K. J. Berry, R. D. Plaut, A. M. Wolf, K. L. Watkins, W. C. Nierman, A. Hazen, R. Cline, C. Redmond, J. E. Thwaite, O. White, S. L. Salzberg, B. Thomason, A. M. Friedlander, T. M. Koehler, P. C. Hanna, A. B. Kolsto, and C. M. Fraser.** 2003. The genome sequence of *Bacillus anthracis* Ames and comparison to closely related bacteria. Nature **423:**81–86.
40. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
41. **Schnepf, E., N. Crickmore, J. Van Rie, D. Lereclus, J. Baum, J. Feitelson, D. R. Zeigler, and D. H. Dean.** 1998. *Bacillus thuringiensis* and its pesticidal crystal proteins. Microbiol. Mol. Biol. Rev. **62:**775–806.
42. **Strickland, M. S., N. E. Thompson, and R. R. Burgess.** 1988. Structure and function of the σ^{70} subunit of *Escherichia coli* RNA polymerase. Monoclonal antibodies: localization of epitopes by peptide mapping and effects on transcription. Biochemistry **27:**5755–5762.
43. **Takami, H., Y. Takaki, and I. Uchiyama.** 2002. Genome sequence of *Oceanobacillus iheyensis* isolated from the Iheya Ridge and its unexpected adaptive capabilities to extreme environments. Nucleic Acids Res. **30:**3927–3935.
44. **Trieu-Cuot, P., C. Carlier, C. Poyart-Salmeron, and P. Courvalin.** 1991. An integrative vector exploiting the transposition properties of Tn*1545* for insertional mutagenesis and cloning of genes from gram-positive bacteria. Gene **106:**21–27.
45. **Van Kranenburg, R., J. D. Marugg, I. I. van Swam, N. J. Willem, and W. M. de Vos.** 1997. Molecular characterization of the plasmid-encoded *eps* gene cluster essential for exopolysaccharide biosynthesis in *Lactococcus lactis.* Mol. Microbiol. **24:**387–397.
46. **Vijay, K., M. S. Brody, E. Fredlund, and C. W. Price.** 2000. A PP2C phosphatase containing a PAS domain is required to convey signals of energy stress to the σ^B transcription factor of *Bacillus subtilis.* Mol. Microbiol. **35:**180–188.
47. **Voelker, U., B. Maul, and M. Hecker.** 1999. Expression of the σ^B-dependent general stress regulon confers multiple stress resistance in *Bacillus subtilis.* J. Bacteriol. **181:**3942–3948.
48. **Voelker, U., A. Voelker, B. Maul, M. Hecker, A. Dufour, and W. G. Haldenwang.** 1995. Separate mechanisms activate σ^B of *Bacillus subtilis* in response to environmental and metabolic stresses. J. Bacteriol. **177:**3771–3780.
49. **Volz, K.** 1993. Structural conservation in the CheY superfamily. Biochemistry **32:**11741–11753.
50. **Wu, S., H. de Lencastre, and A. Tomasz.** 1996. Sigma-B, a putative operon encoding alternate sigma factor of *Staphylococcus aureus* RNA polymerase: molecular cloning and DNA sequencing. J. Bacteriol. **178:**6036–6042.
51. **Zhang, S., J. M. Scott, and W. G. Haldenwang.** 2001. Loss of ribosomal protein L11 blocks stress activation of the *Bacillus subtilis* transcription factor σ^B. J. Bacteriol. **183:**2316–2321.