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Deletion of the *sigB* Gene in *Bacillus cereus* ATCC 14579 Leads to Hydrogen Peroxide Hyperresistance

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The *sigB* gene of *Bacillus cereus* ATCC 14579 encodes the alternative sigma factor σ^B. Deletion of *sigB* in *B. cereus* leads to hyperresistance to hydrogen peroxide. The expression of *katA*, which encodes one of the catalases of *B. cereus*, is upregulated in the *sigB* deletion mutant, and this may contribute to the hydrogen peroxide-resistant phenotype.

Bacillus cereus is a gram-positive bacterium that is a frequent cause of foodborne illnesses, with diarrheal and emetic symptoms (18, 22). It is also an important spoilage organism, especially in the dairy industry, as *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 (9).

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 (17). Recent complete-genome sequence studies confirmed the close genetic relationships between the organisms in the *B. cereus* group (21), and this 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* (16). 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 (24).

The alternative sigma factor σ^B, which is encoded by the *sigB* gene, has a central role in the stress responses of several gram-positive bacteria, including *Bacillus subtilis*, *Listeria monocytogenes*, *Staphylococcus aureus*, and *B. cereus* (26). In *B. cereus*, σ^B is activated upon stress exposure (including exposure to ethanol and osmotic upshock but most profoundly heat shock) and entry into stationary phase (6, 24). The phenotypic characterization of the *B. cereus sigB* deletion mutant revealed that σ^B plays a role in the adaptive heat stress response (24).

In other gram-positive bacteria, the deletion of *sigB* generally leads to a decreased resistance to reactive oxygen species (7, 8, 10, 13). The sole exception to this rule is provided in a report in which the increased resistance of a *sigB* deletion mutant of a *L. monocytogenes* strain towards the oxidative agent cumene hydroperoxide is described (19). However, neither quantitative nor mechanistic details were provided in this study.

In this study, we set out to assess the role of σ^B in *B. cereus* ATCC 14579 in resistance to the oxidizing agent hydrogen peroxide (H_2O_2).

***The B. cereus sigB* deletion mutant is hyperresistant to H_2O_2.** *B. cereus* ATCC 14579 and its isogenic *sigB* deletion mutant were grown aerobically as described earlier (24). For experiments under anaerobic conditions, *B. cereus* was precultured overnight (final optical density at 600 nm [OD_{600}] of 1.0 to 1.2) and grown until mid-exponential phase (OD_{600} of 0.2) in brain heart infusion (BHI) medium in tightly stoppered flasks, which were purged with N_2 gas for 45 min before inoculation. The indicator resazurin was added to the medium at a final concentration of 0.0002% (wt/vol). Upon inoculation of freshly purged BHI medium with an anaerobic overnight preculture, there were still minimal amounts of oxygen present in the medium, as indicated by the incomplete disappearance of the pink color of resazurin. After 2 h of culturing, however, the indicator dye had become completely colorless, indicating anaerobic conditions. The cultures were then grown for an additional 4-h period until the culture reached the mid-exponential growth phase, after which cells were either exposed to H_2O_2 or harvested for the catalase activity assay (see below). The H_2O_2 resistance of aerobically grown 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 in *B. cereus* ATCC 14579, prior to the addition of H_2O_2. When anaerobically grown, wild-type cells in the mid-exponential growth phase were exposed to 10 mM H_2O_2, no survivors could be detected, but when 2 mM H_2O_2 was added to the culture, a killing efficiency that was comparable to that of the exposure of aerobically grown wild-type cells to 10 mM H_2O_2 was observed. 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.

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 rate of heat-shocked *B. cereus* FM1400 cells after exposure to

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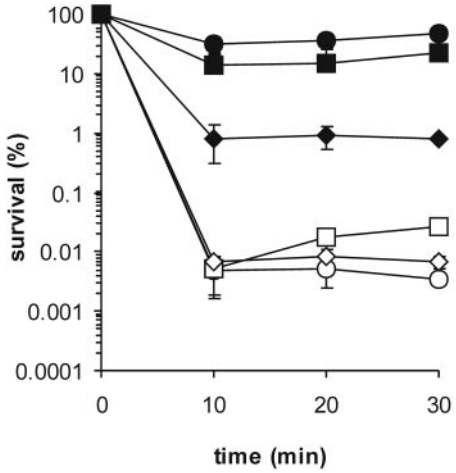

FIG. 1. Survival rates of *B. cereus* ATCC 14579 and *B. cereus* FM1400 during exposure to H_2O_2. The survival rates of *B. cereus* ATCC 14579 cells (open symbols) and the cells of *sigB* deletion mutant *B. cereus* FM1400 (closed symbols) were determined upon exposure to H_2O_2. Cells were grown until 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.

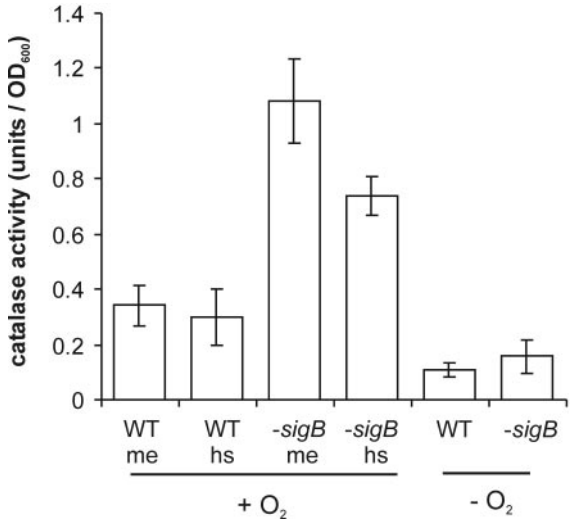

FIG. 2. Catalase activity in *B. cereus* ATCC 14579 and *B. cereus* FM1400. Levels of catalase activity of whole cells of *B. cereus* ATCC 14579 (WT) and *B. cereus* FM1400 (−*sigB*) were determined. Levels of catalase activity of aerobically ($+O_2$) grown cells in the mid-exponential growth phase in BHI (me) and after a 30-min heat shock from 30°C to 42°C (hs) were determined. Catalase activity of cells grown in anaerobic ($-O_2$) culture was also determined. 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.

H_2O_2 is twofold lower than that of cells of the mid-exponential growth phase. Under anaerobic conditions, the *sigB* deletion mutant is also more resistant to H_2O_2 than the parent strain, but the difference in survival rate is now approximately 100-fold, which is markedly less than that under aerobic conditions.

Increased catalase activity in the *sigB* deletion mutant. 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 (4, 12, 15). Under conditions for which H_2O_2 concentrations are high, catalase appears to be the most important cellular H_2O_2-scavenging enzyme (23).

The determination of the catalase activity of whole cells of *B. cereus* was performed based on previously described methodology (2, 5) with slight modifications. Two-milliliter (for aerobically grown cultures) or 10-milliliter (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 (13,000 × *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. One hundred microliters of the cell suspension was then added to a quartz cuvette containing 900 μl PBS with 44.4 mM H_2O_2 (the final concentration of H_2O_2 in the assay was 40 mM). After a mixing, the cuvette 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 as indicated by the value of the OD_{600} of the cell suspension. This assay was performed in triplicate for all tested conditions.

The assay of the catalase activity of whole cells revealed that during the mid-exponential growth phase, catalase activity in the *sigB* deletion mutant is threefold higher than that in the parent strain (Fig. 2). A heat shock from 30°C to 42°C has no effect on the cellular catalase activity of wild-type cells. This suggests that the catalase KatE, of which the structural gene is expressed only upon heat shock in a σ^B-dependent fashion (25), has a minor role in H_2O_2 resistance of *B. cereus*. 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 rate of heat-shocked *B. cereus* FM1400 cells. The catalase activity of anaerobically grown cells is clearly 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.

Expression of *katA*, which encodes the main vegetative-cell catalase, is upregulated in the *sigB* deletion mutant. In the *B. cereus* ATCC 14579 genome sequence (16), three open reading frames encoding catalases can be identified. We termed these catalase genes *katA*, *katB*, and *katE*. The original *B. cereus* genome sequence codes for these genes are *rzc06308*, *rzc07268*,

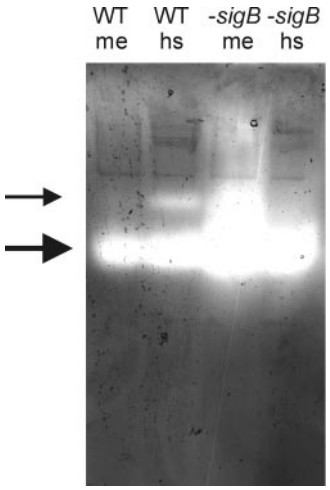

FIG. 3. Visualization of catalase activity on a native polyacrylamide gel. Cell-free protein extracts were isolated from *B. cereus* ATCC 14579 (WT) and *B. cereus* FM1400 (−*sigB*) cells in the mid-exponential growth phase in BHI (me) and after a 30-min exposure to 42°C (hs). Proteins (100 μg) were separated on a nondenaturing 10% polyacrylamide gel and stained for catalases as described by Woodbury et al. (27), 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.

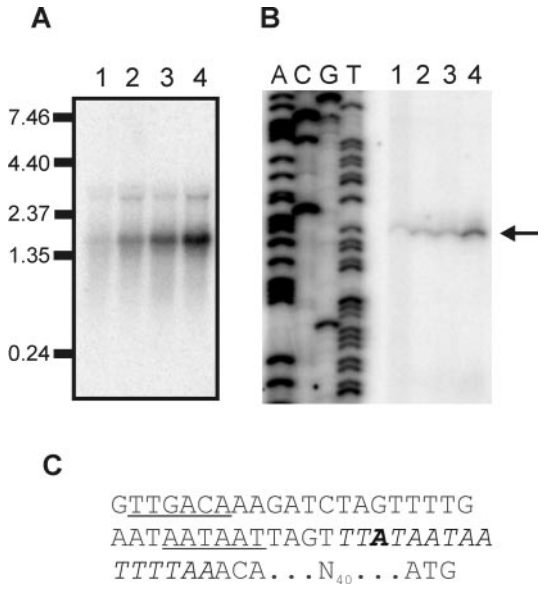

FIG. 4. Transcriptional analysis of *katA* of *B. cereus*. (A) Northern blot analysis of the transcription of *katA*. Total RNA was extracted from *B. cereus* ATCC 14579 and *B. cereus* FM1400 cells during mid-exponential growth in BHI (lane 1 and 3, respectively) and after a 10-min exposure to 42°C (lane 2 and 4, respectively). A ^{32}P-labeled internal PCR product of *katA* was used as a 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.

and *rzc01424*, respectively. *katE* was previously found to be expressed upon a heat shock in a σ^B-dependent fashion (25). 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. First, 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). Total proteins were isolated from *B. cereus* cultures as described previously (20), and crude cell-free protein extracts of *B. cereus* were obtained by pelleting insoluble material by centrifugation at 13,000 × *g* for 30 min. One hundred micrograms of cell-free protein extracts were separated on a native 10% polyacrylamide gel, and catalases were stained by the method described by Woodbury et al. (27), which results in yellow catalase bands against a dark-green background.

The highest level of activity staining is observed in the cell extract of the *B. cereus sigB* deletion mutant, while a heat shock leads to a decrease in 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, as no such band is present in the protein samples isolated during mid-exponential growth and from the *sigB* deletion mutant. This again indicates that KatE does not contribute significantly to the total catalase activity of the cell under these conditions.

As the expression of *katE* was determined previously (25), here the expression of *katA* and *katB* in *B. cereus* ATCC 14579 and its *sigB* deletion mutant during exponential growth and upon a heat shock from 30°C to 42°C was studied. The techniques used (Northern and primer extension analysis) are described in reference 24. *katB* is not expressed under the tested conditions, which makes *katA* the only catalase gene 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. 4A). *katA* is mainly transcribed as a monocistronic 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 (1). 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 (25). 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. 4B and C). 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 (11). 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 (12). A practically identical consensus PerR-binding sequence in *S. aureus* has been described (14). 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*, the deletion of *perR* leads to the upregulation of catalase activity and increased H_2O_2 resistance by the abolishment of the repression of *katA* transcription (3, 14). 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.

Concluding remarks. This study illustrates 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 these can provide crucial knowledge on the precise role of σ^B in the parental strains.

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