Cereulide synthesis in emetic *Bacillus cereus* is controlled by the transition state regulator AbrB, but not by the virulence regulator PlcR

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Cereulide, a depsipeptide structurally related to the antibiotic valinomycin, is responsible for the emetic type of gastrointestinal disease caused by *Bacillus cereus*. Recently, it has been shown that cereulide is produced non-ribosomally by the plasmid-encoded peptide synthetase Ces. Using deletion mutants of the emetic reference strain *B. cereus* F4810/72, the influence of the well-known transcription factors PlcR, Spo0A and AbrB on cereulide production and on the transcription of the cereulide synthetase gene cluster was investigated. Our data demonstrate that cereulide synthesis is independent of the *B. cereus* specific virulence regulator PlcR but belongs to the Spo0A-AbrB regulon. Although cereulide production turned out to be independent of sporulation, it required the activity of the sporulation factor Spo0A. The $\sigma^S$-promoted transcription of spo0A was found to be crucial for cereulide production, while the $\sigma^H$-driven transcription of spo0A did not affect cereulide synthesis. Overexpression of the transition state factor AbrB in *B. cereus* F4810/72 resulted in a non-toxic phenotype. Moreover, AbrB was shown to bind efficiently to the main promoter region of the ces operon, indicating that AbrB acts as a repressor of cereulide production by negatively affecting ces transcription.

INTRODUCTION

The endospore-forming bacterium *Bacillus cereus* is increasingly recognized as a food-borne pathogen causing diarrhoea or emesis. These two types of gastrointestinal disease are associated with very different virulence factors. Several enterotoxins such as haemolysin (Hbl), non-haemolytic enterotoxin (Nhe) and cytotoxin CytK, which are known to be produced during vegetative growth in the small intestine of the host, are responsible for the diarrhoeal symptoms. In contrast, emesis is triggered by the single, heat-stable peptide toxin cereulide, which is preformed in contaminated food (for reviews see Ehling-Schulz et al., 2004; Stenfors Arnesen et al., 2008). Cereulide is a cyclic dodecadepsipeptide that causes cellular damage by acting as a potassium ionophore (Agata et al., 1995; Mikkola et al., 1999). It is synthesized enzymically by a non-ribosomal peptide synthetase encoded by the ces genes (Ehling-Schulz et al., 2005; Magarvey et al., 2006). The 24 kb ces gene cluster is located on a megaplasmid (pBCE) and is flanked by genetic regions homologous to the pXO1 toxin plasmid of *Bacillus anthracis* (Ehling-Schulz et al., 2006). Detailed sequence analysis of the entire pBCE revealed its close relation to a 272 kb plasmid from *B. cereus* isolates linked to periodontal disease. This new group of pXO1-like plasmids share a highly conserved core region containing genes involved in plasmid replication and maintenance, sporulation and germination, and a formaldehyde-detoxification locus (Rasko et al., 2007).

The ces structural genes show little molecular diversity; however, the toxicity of the emetic *B. cereus* varies strongly among different strains (Carlin et al., 2006; Ehling-Schulz et al., 2005). This suggests the existence of precise mechanisms governing cereulide synthesis in emetic *B. cereus*. In non-emetic *B. cereus* group strains many putative virulence factors, such as proteases, phospholipases, haemolysins and enterotoxins, have been shown to be transcribed under the control of the pleiotropic regulator PlcR (Gohar et al., 2008; Okstad et al., 1999). In *B. anthracis*, however, PlcR is inactive due to a nonsense mutation in the plcR gene and
hence toxin gene expression is independent of the PlcR regulon and involves other regulatory pathways (Agaisse et al., 1999; Fouet & Mock, 2006; Mignot et al., 2001). Interestingly, some strains of the B. cereus group, which also have a mutated copy of the plcR gene, display a reduced haemolytic and cytotoxic phenotype (Fricker et al., 2007; Slamti et al., 2004). The role of PlcR in emetic toxin production, as well as the involvement of other regulatory factors in the pathogenicity of emetic B. cereus, is so far undefined. Besides PlcR, the key regulator of sporulation, Spo0A was investigated with respect to cereulide regulation, as a possible correlation between sporulation and toxin production is under debate. Spo0A has a profound effect on global gene expression of Bacillus species. It influences the transcription of over 500 Bacillus subtilis genes, including 121 that are controlled directly by this master regulator (Fawcett et al., 2000; Molle et al., 2003). Once activated by a complex phosphorylation including several histidine kinases (Jiang et al., 2000), the response regulator Spo0A not only induces the onset of sporulation, but is also involved in the transcriptional regulation of various other stationary-phase processes, such as protease production, competence or biofilm production (Albano et al., 1987; Ferrari et al., 1986; Hamon & Lazazzeria, 2001). A crucial and early role of phosphorylated Spo0A is the repression of abrB transcription. AbrB acts as a ‘transition state regulator’ mainly by suppressing stationary-phase gene expression during the exponential growth phase. In B. subtilis, a wide range of AbrB-controlled genes have been identified that are involved in the production of antibiotics, degradative enzymes, motility, sporulation, development of competence and many other metabolic and physiological processes (for a review see Phillips & Strauch, 2002). Moreover, in B. anthracis AbrB was shown to regulate toxin gene expression of the pXO1-encoded virulence factors (Saile & Koehler, 2002).

The aim of this work was to decipher the potential role of the master regulators PlcR, Spo0A and AbrB in cereulide production of the emetic reference strain B. cereus F4810/72. Several mutant strains were constructed and characterized with respect to their phenotype, cytotoxicity and ces gene expression, to gain insights into the regulatory mechanism influencing cereulide synthesis. Our results show that toxin formation is not controlled by the B. cereus group specific virulence gene regulator PlcR, but by the AbrB repressor, which plays a pivotal role in growth-phase-dependent gene expression.

**METHODS**

**Bacterial strains, growth condition and plasmids.** The emetic reference strain B. cereus F4810/72 and mutant strains were routinely grown on Luria–Bertani (LB) agar plates or in LB broth at 30 °C. The selective plating medium MYP agar (mannitol-egg yolk-polymyxin agar, Oxoid) was used for phenotypic characterization of mutant strains. For toxin tests and RNA extraction 100 ml LB was inoculated with approximately 10^7 cfu ml^{-1} from an overnight pre-culture and cultures were incubated in 500 ml baffled flasks with rotary shaking (150 r.p.m.). Escherichia coli TOP10 and the non-methylating strain INV110, which were used as general cloning hosts, were cultured in LB broth or on LB plates at 37 °C. E. coli BL21 was used for protein expression and E. coli JM83/pRK24 served as the donor in conjugation experiments. Antibiotics were added to the media when appropriate: 100 µg ampicillin ml^{-1}, 100 µg spectinomycin ml^{-1}, 100 µg polymyxin B ml^{-1}, 50 µg kanamycin ml^{-1}, 10 µg erythromycin ml^{-1}, 5 µg chloramphenicol ml^{-1}. All strains and plasmids used in this study are listed in Table 1.

**Construction of deletion mutants and complemented strains.** To create plcR, spo0A and abrB null mutants, TOPO vector constructs were cloned which carried the spectinomycin-resistance cassette flanked by ~1.2 kb B. cereus DNA fragments, representing the upstream and downstream sequences of the gene to be deleted. The flanking regions of plcR, spo0A and abrB were amplified by conventional PCR using the primers shown in Supplementary Table S1 (available with the online version of this paper). PCR products were restricted according to the sites introduced by the primers and were, together with the spectinomycin-resistance cassette from pUC1318Spc, ligated into TOPO pCR 2.1. Inserts were checked by PCR, restriction analysis and sequencing. Constructs were then excised from TOPO and inserted into the multiple cloning site of the conjugal suicide vector pAT113, giving rise to pAT113SpcR/spc, pAT113Spa0A1/spc and pAT113AabrB/spc (see Table 1 for details). These plasmids were transformed into E. coli JM83/pRK24 and the resulting strains were used for transconjugal transfer into B. cereus F4810/72. Conjugation was carried out using a mating procedure previously described (Pezard et al., 1991). Transconjugants were screened for spectinomycin resistance and erythromycin sensitivity. PCR and RT-PCR confirmed gene deletion and integration of the resistance cassette resulting from a double-crossover recombination event. The plcR, spo0A and abrB null mutants were designated B. cereus F48AplcR, F48Aspo0A and F48AabrB, respectively. For construction of an abrB/spo0A double mutant, the spectinomycin-resistance cassette of pAT113Spa0A1/spc was replaced by a chloramphenicol-resistance cassette, which was obtained from pAD123 using the oligonucleotides Cm-F and Cm-R (Table S1). The newly constructed pAT113Spa0A1/cm was then transformed into F48AabrB by conjugation, yielding the double mutant F48AabrBSpao0A1. For complementation of the spo0A null mutant, two different plasmids were constructed, one with spo0A and its d^-dependent promoter and one with spo0A plus its two promoters. The spo0A gene including either 121 bp or 513 bp upstream region was PCR amplified with Pfu polymerase from genomic DNA of B. cereus F4810/72 using the primer pairs spo0A_F/pro1/spo0A_R and spo0A_F/pro2/spo0A_R, respectively (Table S1). After restriction, PCR products were cloned into the EcoRI-HindIII sites of the shuttle vector pAD123, releasing gfp and yielding pAD/spo0A/d^-Pro and pAD/spo0A/d^-Pro. The two plasmids were introduced into the B. cereus spo0A null mutant by electroporation as previously described (Ehling-Schulz et al., 2005). PCR analyses with spo0A-specific primers confirmed the presence of the plasmid in the recombinant strains, designated B. cereus F48Aspo0AcomAH and F48Aspo0AcomH.

**Construction of B. cereus strains overexpressing AbrB and AbrB*.** The shuttle vector pAD/Pcsa, harbouring the cold-shock-inducible promoter of cspA, was used for overexpression of the target proteins AbrB (locus-tag: BcAH187_A0046) and AbrB* (locus-tag: BcAH187_pCER270_0167). The promoterless abrB and abrB* genes were amplified by PCR with the primers abrB_F/abrB_R and abrB_F/abrB_*R, respectively (Table S1). The restricted products were ligated between the SacI and HindIII sites of pAD/Pcsa, giving pAD/abrB/Pcsa and pAD/abrB*/Pcsa, respectively. Each plasmid was electroporated into B. cereus F4810/72, resulting in B. cereus F48pAD/abrB and F48pAD/abrB*. The strains were incubated in LB medium at 15 °C for 4 days to overexpress AbrB and AbrB* under the control of the cold-inducible cspA promoter. The parental strain
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<th>Plasmid or strain</th>
<th>Description</th>
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**Cytotoxicity test.** Cereulide production of *B. cereus* strains was determined using a slightly modified protocol of the HEP-2 cytotoxicity assay described by Finlay et al. (1999). Samples of *B. cereus* cultures were taken during the stationary phase (either after 24 h of incubation at 30 °C or after 4 days at 15 °C) and autoclaved (15 min at 120 °C) to denature heat-labile toxins. Samples were serially diluted (two-fold) in 96-well microtitre plates using Eagle’s minimum essential medium with Earle salts (supplemented with 1% fetal calf serum, 1% sodium pyruvate and 0.4% penicillin-streptomycin) and 2% ethanol as a solvent for cereulide. Dilutions of valinomycin (1–500 ng ml⁻¹) served as an internal standard. After addition of HEP-2 cells (6 × 10⁴ cells ml⁻¹), the plates were incubated for 48 h at 37 °C in a 5% CO₂ atmosphere. Subsequently, the tetrazolium salt WST-1 (Roche) was added to detect the mitochondrial activity of viable cells. After 20 min incubation at 37 °C the A₅₇₀ of each well was determined in a microtitre plate reader. From the resulting dose–response curve the toxin titres were calculated as described previously (Dietrich et al., 1999). All values were normalized to the cytotoxicity of the parental strain *B. cereus* F4810/72, which was set at 100%.

**Total RNA isolation and cDNA synthesis.** Cells were disrupted with 0.1 mm zirconium-silica beads in a Ribolyser and RNA was isolated using TRIzol (Invitrogen) according to the manufacturer’s instructions. Residual DNA was removed with RQ1 DNase (Promega) or by fusing their C-termini to the His-tag coding sequence of the vector. For protein overexpression the *E. coli* strain BL21 carrying the appropriate construct pET28b + abrB or pET28b + abrB* was grown in LB containing kanamycin at 37 °C. At OD₆₀₀ ~0.5, IPTG was added to a final concentration of 1 mM and growth continued for 3 h. Bacteria were harvested by centrifugation (6000 r.p.m., 15 min), washed once in buffer A (50 mM Tris pH 7.6, 50 mM KCl, 1 mM DTT, 0.1 mM PMSF) and resuspended in buffer B (300 mM NaCl, 50 mM sodium phosphate). Cells were disrupted by passage through a French press (140 MPa) and cell debris removed by centrifugation (15 300 r.p.m., 2 × 15 min, 4 °C). The crude extract containing the soluble protein fraction was incubated with an equal volume of TALON metal-affinity resin (Clontech) for 2 h at 15 °C. The beads were then washed ten times with buffer B, followed by several washing steps with buffer C containing 7.5 mM imidazole. Finally, AbrB-His₅ or AbrB*-His₅ was eluted with buffer C containing 150 mM imidazole. The protein concentration was determined by the Bradford method (RotiNanoquant) and the purity of eluted fractions was checked by SDS-PAGE and Coomassie blue staining (data not shown). Protein fractions were concentrated and dialysed with buffer A using Vivaspin 500 filter units (Sartorius).

**Quantitative real-time PCR (RT-qPCR).** Reactions including cDNA (equivalent to 10 ng RNA), 80 nM of each primer and qPCR Mastermix with SYBR Green I (Abgene) were run on the StepOnePlus (Applied Biosystems) using the following parameters: polymerase activation at 95 °C for 15 min followed by 40 cycles of amplification (95 °C for 30 s; annealing temperature (Tₐ) for 30 s; 72 °C for 45 s). Specificity of the reactions was affirmed by melt-curve analysis of the amplified products.

Relative expression of the cereulide synthetase structural gene *cesA* and the regulator gene *abrB* was calculated by the REST method (Pfaffl, 2001; Pfaffl et al., 2002) using the difference in Cₜ values of the sample and a calibrator for the target gene and the reference gene *rrn* (16S rDNA). Cₜ values of the wild-type strain F4810/72 at an OD₆₅₀ of 1 (cesA) or 10 (abrB) was set as the calibrator for each respective gene.

**ces-promoter fusions and luciferase assay.** To determine promoter activity, the vector pXEN/Pces (M. Dommel & M. Ehling-Schulz, unpublished data), which contains a lux fusion between the ces promoter region and the lucferase genes *luxABCD*, was used. The plasmid was introduced into *B. cereus* F4810/72, F48Δspo0A and F48ΔabrB by electroporation, giving rise to F4810/72pLux, F48Δspo0ApLux and F48ΔabrBpLux, respectively. A derivative of pXEN/Pces carrying an additional erythromycin-resistance cassette (pXEN/Pces/erm) was used for transformation of the double mutant F48ΔabrBΔspo0A. For measurement of the ces promoter activity in different genetic backgrounds, overnight cultures of *B. cereus* strains containing pXEN/Pces were diluted (1:20), distributed into wells of a 96-well microtitre plate (µClear white; Greiner Bio-One) and incubated at 30 °C, with shaking at 400 r.p.m. Optical density (585 nm) and bioluminescence (490 nm), shown as relative light units (RLU), were measured in parallel using a Wallac Victor 1420 multilabel counter (Perkin Elmer Life Sciences). *B. cereus* F4810/72 harbouring a promoterless luciferase construct was used as negative control.

**Purification of AbrB and AbrB*.** The *abrB* gene and the *abrB* gene of *B. cereus* F4810/72 were amplified using the oligonucleotides abrB_F_Nco/abrB_R_Xho and abrB* F_Nco/abrB*/R_Xho, respectively (Table S1). The digested PCR products were cloned into pET28b by fusing their C-termini to the His-tag coding sequence of the vector. For protein overexpression the *E. coli* strain BL21 carrying the appropriate construct pET28b + abrB or pET28b + abrB* was grown in LB containing kanamycin at 37 °C. At OD₆₀₀ ~0.5, IPTG was added to a final concentration of 1 mM and growth continued for 3 h. Bacteria were harvested by centrifugation (6000 r.p.m., 15 min), washed once in buffer A (50 mM Tris pH 7.6, 50 mM KCl, 1 mM DTT, 0.1 mM PMSF) and resuspended in buffer B (300 mM NaCl, 50 mM sodium phosphate). Cells were disrupted by passage through a French press (140 MPa) and cell debris removed by centrifugation (15 300 r.p.m., 2 × 15 min, 4 °C). The crude extract containing the soluble protein fraction was incubated with an equal volume of TALON metal-affinity resin (Clontech) for 2 h at 15 °C. The beads were then washed ten times with buffer B, followed by several washing steps with buffer C containing 7.5 mM imidazole. Finally, AbrB-His₅ or AbrB*-His₅ was eluted with buffer C containing 150 mM imidazole. The protein concentration was determined by the Bradford method (RotiNanoquant) and the purity of eluted fractions was checked by SDS-PAGE and Coomassie blue staining (data not shown). Protein fractions were concentrated and dialysed with buffer A using Vivaspin 500 filter units (Sartorius).

**Gel mobility shift assay.** For the DNA-binding assay a cesP promoter region probe (~360 bp) was generated by PCR amplification of *B. cereus* F4810/72 DNA using the primer pair cesP_F_EMSA and cesP_R_EMSA (Table S1). As a negative probe, a randomly chosen PCR product (~300 bp) was used, which was amplified from genomic *B. cereus* DNA. The binding reactions contained different amounts of AbrB or AbrB* protein (2–14 pmol) and 1 pmol of each DNA probe (cesP promoter and a negative DNA probe) in 20 μl binding buffer (5 mM Tris/HCl, 75 mM KCl, 0.25 mM EDTA, 0.05 % Triton-X 100, 6.25 % glycerol, 0.1 mM DTT). Samples were incubated at 25 °C for 30 min before being loaded onto a 10 % native polyacrylamide gel, which was run in prechilled 1 × TBE buffer at 120 V for 3 h at 20 °C. Gels were stained in ethidium bromide solution for 5 min and DNA was visualized by UV irradiation.

**RESULTS**

**Growth characteristics of *plcR* and *spo0A* null mutants.** The transcription regulator genes *plcR* and *spo0A* of *B. cereus* F4810/72 were identified on the basis of sequence homology to the annotated strain *B. cereus* ATCC 10987 (GenBank accession no. NC_003909) and deletion mutants were constructed by replacing the respective genes with a spectinomycin-resistance cassette. Phenotypic characterization of these strains showed that the *plcR* null mutant and the parental strain had similar growth rates in LB (Fig. 1a) and initiated sporulation in late-exponential phase, which was observed by phase-contrast microscopy (data not shown).
not shown). In contrast, the spo0A null mutant grew to a lower maximum OD$_{600}$ (Fig. 1a) and was unable to sporulate; rather the cell density decreased after 18 h due to cell lysis (data not shown).

Both mutants revealed atypical morphologies on selective plating medium and could therefore be easily distinguished from the B. cereus F4810/72 wild-type (Fig. 1b). While the latter showed typical pink-coloured colonies with a precipitation zone on MYP agar, colonies of the plcR null mutant were not surrounded by a precipitation zone due to the downregulated phospholipase C activity in the absence of its regulator PlcR. The spo0A null mutant, however, showed a characteristic precipitation zone due to egg yolk hydrolysis, but colonies were shinier and darker in colour than the wild-type (Fig. 1b).

**Deletion of spo0A results in a cereulide-deficient phenotype**

To study the role of PlcR and Spo0A in cereulide production, toxin titres of wild-type and knockout mutant cultures at stationary phase were determined using the HEp-2 cell bioassay. The results indicated that the plcR null mutant displayed the same toxicity as the B. cereus F4810/72 wild-type strain. In contrast, the spo0A null mutant revealed a cereulide-deficient phenotype when tested in the HEp-2 assay (Fig. 2). Further samples of the spo0A null mutant taken at different time points during growth (from 10 h to 30 h) were also non-toxic in the HEp-2 assay (data not shown).

**Transcription of cesA in the spo0A null mutant**

For further characterization of the spo0A null mutant, transcriptional analysis of the cereulide synthetase gene cesA was carried out by RT-qPCR. Samples for RNA isolation were taken throughout growth and relative expression of cesA, normalized to the 16S rDNA gene rrn, was determined in comparison to transcript levels of the parental strain. The cesA transcript of the wild-type was only detectable in the exponential phase of growth, peaking in the mid-exponential phase (Fig. 3a). In contrast, cesA transcription in the spo0A null mutant was drastically reduced, representing a 17-fold downregulation compared to the parental strain (Fig. 3a). These data suggest that Spo0A has a positive effect on the transcription of the ces gene cluster.

**Spo0A affects cereulide synthesis independently of its σH promoter**

The transcription of spo0A occurs from two promoters, which induce spo0A transcription in different phases of the growth cycle. While the weak promoter $P^s_A$ is recognized by the housekeeping sigma factor $\sigma^A$ during vegetative growth, $P^s_H$ is strongly induced during the initial phase of sporulation (Chibazakura et al., 1991; Predich et al., 1992). In order to investigate whether early or late spo0A transcription is necessary for ces transcription, two different pAD vector constructs were used for complementation of the cereulide-deficient spo0A mutant: one containing the spo0A

![Fig. 1. (a) Growth, measured as OD$_{600}$, of the wild-type F4810/72 (□), the plcR null mutant (x) and the spo0A null mutant (●) incubated in LB medium at 30°C. (b) Colony morphology of B. cereus F4810/72, F48ΔplcR and F48Δspo0A, grown on MYP agar for 24 h at 30°C. Due to egg yolk hydrolysis by phospholipase C a typical precipitation zone surrounds the parental strain and the spo0A mutant, but not the plcR mutant lacking the regulator.](image)

![Fig. 2. Cereulide production of B. cereus F4810/72, the deletion mutants F48ΔplcR, F48Δspo0A, F48ΔabrB and F48ΔΔspo0A and the complemented strains F48Δspo0AcomAH and F48ΔΔspo0AcomH, as determined by the HEp-2 bioassay. Strains were grown in LB medium for 24 h at 30°C. The cytotoxicity of the wild-type strain was set to 100%.](image)
gene with both its promoter regions (PoA and PoH) and the other with only PoH upstream of spo0A. Plasmids were introduced into the B. cereus spo0A null mutant by electroporation and the complemented strains characterized with respect to growth and cytotoxicity. Both strains, F48Δspo0AcomAH and F48Δspo0AcomH, were able to sporulate (data not shown), but only F48Δspo0AcomAH was toxic to HEp-2 cells, while F48Δspo0AcomH (bearing PoH but not PoA) revealed a very low cytotoxicity (Fig. 2). Additionally, B. cereus F4810/72 and the two complemented strains of the spo0A null mutant were grown in the presence of 2% glucose, which is known to repress PoH-directed transcription of spo0A. The cereulide titre of B. cereus F48Δspo0AcomAH was comparable to the wild-type, whereas F48Δspo0AcomH produced significantly less toxin (data not shown). These data demonstrate that cereulide production is indeed affected by PoA-directed spo0A transcription during vegetative growth.

**AbrB is a negative regulator of cereulide synthesis**

Since *in silico* analysis of the ces promoter region did not reveal a putative ‘0A-box’, Spo0A may regulate ces indirectly, for example by repressing the transition state regulator AbrB. To investigate *abrB* expression and its relationship to Spo0A in *B. cereus* F4810/72, *abrB* transcript levels of the parental strain and the spo0A deletion mutant were measured in different growth phases using RT-qPCR. As shown in Fig. 3(b), *abrB* transcription of the wild-type peaked during the transition from lag to exponential phase and decreased strongly later in the growth cycle. In contrast, the *abrB* transcript level of the spo0A null mutant was not repressed but remained stable throughout growth (Fig. 3b). Hence, it is possible that the repression of the cereulide synthetase in the spo0A null mutant is due solely to the elevated AbrB level in this strain. Therefore, a *B. cereus* mutant overexpressing AbrB was constructed, by cloning the *abrB* gene downstream of an inducible promoter in the pAD123 vector, which was then introduced into *B. cereus* F4810/72 by electroporation. The HEp-2 cell assay revealed that AbrB overexpression resulted in a completely non-toxic phenotype (Fig. 4b), suggesting that AbrB represses cereulide synthesis. Interestingly, besides the chromosomal *abrB* gene, *B. cereus* F4810/72 possesses a plasmid-encoded copy (locus tag BcAH187_pCER270_0167; here designated *abrB*), revealing 61% identity and 81% similarity on the amino acid sequence level (Fig. 4a). However, analysis of an *AbrB*-overexpressing strain showed similar cytotoxicity as the wild-type (Fig. 4b), implying a minor impact of *AbrB* in cereulide production.

For further characterization of the regulation of cereulide formation by AbrB, an *abrB* null mutant (F48ΔabrB) and the double mutant F48ΔabrBΔspo0A were constructed. Both mutants were found to be toxic when tested on HEp-2 cells (Fig. 2). To determine *ces* promoter activity in these strains, the vector pXen/Pces, containing the *ces* promoter region fused to the luciferase operon, was introduced into *B. cereus* F4810/72 wild-type, F48Δspo0A, F48ΔabrB and the double mutant F48ΔabrBΔspo0A by electroporation. Strains were incubated in microtitre plates at 30°C, and bioluminescence and optical density were monitored throughout growth. According to its luminescence, the wild-type strain showed a strictly regulated *ces* promoter activity, peaking at 15 h of incubation, which corresponds to the exponential growth phase (Fig. 5). As expected, the *spo0A* null mutant displayed no *ces* promoter activity. In contrast, *ces* expression of the *abrB* null mutant seemed elevated at the beginning of growth, as luminescence was stronger and occurred earlier. Also, the *abrB/spo0A* double mutant revealed higher *ces* promoter activity compared to the wild-type in the early growth phase; however, luminescence remained rather low during the rest of the growth cycle, probably due to the poor growth rate of this strain (Fig. 5).

**AbrB binds to the ces promoter region**

To examine binding of AbrB and AbrB* to the *ces* promoter region, a non-radioactive gel mobility shift assay.

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![Fig. 3. RT-qPCR analysis of cesA and abrB in the parental strain B. cereus F4810/72 and its spo0A null derivative. (a) Transcript levels of cesA during exponential growth phase (from OD_{600} 4 to OD_{600} 15), determined by RT-qPCR. (b) Transcript levels of abrB in early growth stages: transition from lag to exponential phase (OD_{600} 0.05), early exponential phase (OD_{600} 0.2) and mid-exponential phase (OD_{600} 10). The transcript levels shown represent four different datasets normalized to *rrn* transcript levels.**
was used. The AbrB protein and its plasmid-encoded homologue AbrB* were purified and DNA-binding activity of these proteins to the cesP promoter region containing the central ces promoter (Dommel, 2008) was examined. The AbrB protein was found to bind to the cesP promoter region, while no binding could be detected for a randomly chosen DNA probe (Fig. 6a). For the cesP promoter region a complete shift could already be seen with 10 pmol of AbrB protein (Fig. 6a), whereas AbrB* did not bind to the ces promoter probe (Fig. 6b). These results demonstrate that AbrB, but not the plasmid-encoded AbrB*, is able to bind to the main promoter of the cereulide synthetase gene operon and thus may act as a direct repressor of cereulide expression.

Fig. 4. Repression of ces by AbrB but not by AbrB*. (a) Sequence alignment of the chromosomally encoded AbrB and the plasmid-encoded AbrB* of B. cereus F4810/72. (b) Cytotoxicity of wild-type B. cereus F4810/72 and the protein-overexpressing strains F48pAD/abrB and F48pAD/abrB*, as determined by the HEp-2 bioassay. Strains were grown at 15 °C for 4 days to induce protein overexpression. The cytotoxicity of the wild-type strain was set to 100 %.

Fig. 5. ces promoter activity measured as bioluminescence of the following B. cereus strains harbouring the ces promoter lux fusion (pXen/Pces): B. cereus F4810/72pLux (□), F48Δspo0ApLux (○), F48ΔabrBpLux (▲) and F48ΔabrBΔspo0ApLux (■). Bioluminescence intensity is indicated by relative light units (RLU) in relation to the optical density (OD₅₈₅).

Fig. 6. In vitro binding of (a) the AbrB protein and (b) the AbrB* protein to the cesP promoter region. Lanes of the gel mobility shift assays: 1, no protein; 2, 2 pmol; 3, 4 pmol; 4, 6 pmol; 5, 8 pmol; 6, 10 pmol; 7, 12 pmol; 8, 14 pmol of AbrB. The different AbrB or AbrB* amounts were mixed with both 1 pmol of the 360 bp cesP promoter probe and 1 pmol of a 300 bp randomly chosen DNA fragment (neg).
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**DISCUSSION**

Due to its stability the emetic toxin accumulates during growth of *B. cereus*, reaching high levels in late stationary phase. However, transcription of the peptide synthetase genes responsible for cereulide production is strictly growth phase dependent (Fig. 3b), suggesting the involvement of specific regulatory processes in cereulide expression. While enterotoxin expression of *B. cereus* has been studied in detail (for a review see Stenfors Arnesen et al., 2008), so far nothing is known about the molecular regulation of the emetic toxin cereulide. Our data demonstrate that the pleiotropic regulator PlcR does not affect cereulide synthesis, since the plcR null mutant was as cytotoxic as the wild-type (Fig. 2) and revealed high transcript levels of the cereulide synthetase gene *cesA* (data not shown). Furthermore, no highly conserved palindromic PlcR box-like sequence (Agaisse et al., 1999) could be found in the promoter region of the cereulide synthetase gene operon. Hence, PlcR, which has been shown to activate most of the extracellular virulence factors in *B. cereus* and *Bacillus thuringiensis* (Agaisse et al., 1999; Gohar et al., 2008), is not involved in cereulide formation in emetic *B. cereus*. In contrast, the master response regulator Spo0A seems to play an important role in the expression of the emetic toxin, as the spo0A null mutant resulted in a toxin-deficient phenotype (Fig. 2). Complementation of the *B. cereus* spo0A null mutant with spo0A plus its two promoters revealed that cereulide production depends mainly on σ^A^-induced spo0A expression (Fig. 2). As it has been shown that the σ^A^-dependent promoter of spo0A is expressed during vegetative growth, while the stronger σ^H^-dependent promoter is induced during the initial stages of sporulation (Chibazakura et al., 1991; Predich et al., 1992), the regulation of cereulide expression occurs early in the growth cycle. Since the promoter region of the *ces* gene cluster lacks a typical ‘0A box’ (Strauch et al., 1990), direct activation of *ces* expression by Spo0A is unlikely. However, one of the first functions of Spo0A is the repression of the transcription factor AbrB, achieved by low levels of phosphorylated Spo0A (Jiang et al., 2000; Trach & Hoch, 1993). Expression of *abrB* in *B. subtilis* was found to be highly growth phase dependent, reaching a maximum during the transition from lag phase to exponential growth and becoming undetectable in mid-exponential phase (O’Reilly & Devine, 1997). Our results indicate that AbrB of *B. cereus*, which is 85 % identical to the corresponding protein of *B. subtilis*, is regulated similarly, showing a strict growth-cycle-dependent expression and negative regulation by Spo0A (Fig. 3b). Analysing cereulide synthesis, an *abrB*-overexpressing strain turned out to be non-toxic (Fig. 2), while *ces* expression of an *abrB* null mutant was enhanced in early exponential phase (Fig. 5). Furthermore, the toxin formation inhibited in the spo0A null mutant was largely restored by additionally deleting *abrB* (Fig. 2). Thus, it appears that the major role of Spo0A in cereulide production is its negative regulation of *abrB*. *In vitro* DNA-binding studies showed that AbrB binds efficiently to the *ces* promoter region, confirming its possible role as a direct repressor of the cereulide peptide synthetase operon (Fig. 6a).

Besides the chromosomal *abrB* gene, *B. cereus* F4810/72 harbours an *abrB*-like gene, which is located on the plasmid pBCE. This paralogue was also found on the megaplasmid of *B. cereus* ATCC 10987 and on pXO1 of *B. anthracis* (Rasko et al., 2004; Saile & Koehler, 2002). While the pXO1 copy of *abrB* in *B. anthracis* is truncated and thus probably not functional, this is not the case for the pBCE-encoded *abrB* of *B. cereus* F4810/72. However, the *abrB*-overexpressing strain revealed a toxicity level similar to the wild-type and no binding of AbrB to the *ces* promoter region could be detected *in vitro* (Figs 4b and 6b), suggesting a minor role of this protein in the regulation of cereulide synthesis.

In addition to AbrB, one of its target genes, the sporulation sigma factor σ^H* (SigH), was investigated with respect to its potential effect on cereulide production. SigH, which plays an important role in post-exponential-phase gene expression (Britton et al., 2002; Weir et al., 1991), was also reported to be involved in toxin expression of *B. anthracis* by activating the virulence gene regulator AtxA (Hadjifrangiskou et al., 2007). However, recent work by Bongiorni et al. (2008) did not support a direct role of SigH in atxA expression. Likewise, our investigations showed no distinct correlation between SigH and cereulide expression (data not shown), and *in silico* analysis did not reveal a consensus sequence recognized by the σ^H* RNA polymerase in the promoter region of the *ces* gene cluster. Therefore, it is tempting to speculate that SigH is not directly linked to *ces* gene regulation.

The toxic but non-sporulating double mutant F481abrBAspo0A indicates that emetic toxin production, though regulated by Spo0A, is completely independent of later sporulation processes and does not require full sporulation. It is likely that besides the Spo0A-AbrB regulon there is a range of other external and internal factors controlling the toxin producing potential of emetic *B. cereus*. For instance, *ces* expression was shown to be strongly growth phase dependent and no transcript levels were detectable in stationary phase even in the *abrB* null mutant (Fig. 5). This suggests that there are AbrB-independent modes of control, possibly linked to environmental conditions and nutrient availability, that might play a role in *ces* gene expression and inhibit cereulide formation in later growth phases. In *Bacillus* species several peptide biosynthesis genes have been identified, which are controlled by the interaction of multiple regulators (Marahiel et al., 1993; Sanchez & Olmos, 2004). Moreover, in *B. anthracis* the virulence gene regulator AtxA has been shown to be involved in a complex regulatory network connecting plasmid and chromosome gene expression (for reviews see Fouet & Mock, 2006; Perego & Hoch, 2008). It is possible that similar regulatory pathways exist in *B. cereus* and that regulation of the *ces* genes, which are located on a pXO1-like megaplasmid, is
Another example of cross-regulatory interaction of chromosomally and plasmid-encoded genes.

In conclusion, this study provides an initial insight into the presumably complex regulatory networks controlling cereulide synthesis of emetic B. cereus. Our data show that cereulide synthesis is controlled by the Spo0A phosphorelay, but not by the B. cereus specific virulence regulator PkcR. The global transition state regulator AbrB was identified as an important factor repressing cereulide production in early exponential phase, presumably by direct binding to the main promoter region of the cereulide synthetase operon. However, it is expected that substantial additional work will be necessary to fully decipher the pathways governing cereulide synthesis in emetic B. cereus.

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REFERENCES


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