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 σ^A -promoted transcription of *spo0A* was found to be crucial for cereulide production, while the σ^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.

Received 23 September 2008

Revised 27 November 2008

Accepted 8 December 2008

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 pre-formed 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

Abbreviation: RT-qPCR, quantitative real-time polymerase chain reaction.

A supplementary table of the oligonucleotides used for PCR and RT-qPCR is available with the online version of this paper.

hence toxin gene expression is independent of the PlcR regulon and involves other regulatory pathways (Agaïsse *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 phosphorelay 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 & Lazazzera, 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 10^3 c.f.u. 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 conjugative suicide vector pAT113, giving rise to pAT113Δ*plcR*/spc, pAT113Δ*spo0A*/spc and pAT113Δ*abrB*/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-qPCR 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* F48Δ*plcR*, F48Δ*spo0A* and F48Δ*abrB*, respectively. For construction of an *abrB*/*spo0A* double mutant, the spectinomycin-resistance cassette of pAT113Δ*spo0A*/spc was replaced by a chloramphenicol-resistance cassette, which was obtained from pAD123 using the oligonucleotides Cm-F and Cm-R (Table S1). The new construct pAT113Δ*spo0A*/cm was then transformed into F48Δ*abrB* by conjugation, yielding the double mutant F48Δ*abrB*Δ*spo0A*. For complementation of the *spo0A* null mutant, two different plasmids were constructed, one with *spo0A* and its σ^H -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*/ σ^A σ^H -Pro and pAD/*spo0A*/ σ^H -Pro. These 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* F48Δ*spo0A*AcomAH and F48Δ*spo0A*AcomH.

Construction of *B. cereus* strains overexpressing AbrB and AbrB*.

The shuttle vector pAD/PcspA, 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 using 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/PcspA, giving pAD/*abrB*/PcspA and pAD/*abrB**/PcspA, 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

Table 1. Plasmids and strains

Plasmid or strain	Description	Source or reference
Plasmids		
TOPO pCR 2.1	PCR cloning vector; Amp ^r Kan ^r	Invitrogen
pUC1318Spc	Cloning vector with Spc-resistance cassette; Amp ^r Spc ^r	Mesnage <i>et al.</i> (1997)
pAD123	Shuttle vector for Gram-positive hosts, containing a promoter-less <i>gfpmut3a</i> ; Amp ^r Cm ^r	Dunn & Handelsman (1999)
pAD/spo0A/ $\sigma^A\sigma^H$ -Pro	pAD123 derivative containing <i>spo0A</i> and its two promoter regions (lacking <i>gfp</i>); Amp ^r Cm ^r	This study
pAD/spo0A/ σ^H -Pro	pAD123 derivative containing <i>spo0A</i> and its σ^H promoter region (lacking <i>gfp</i>); Amp ^r Cm ^r	This study
pAD/PcspA	pAD123 derivative containing the promoter region of the cold-shock protein CspA in front of <i>gfp</i> ; Amp ^r Cm ^r	M. Dommel
pAD/abrB/PcspA	pAD123 derivative containing the <i>cspA</i> promoter region in front of the <i>abrB</i> gene (lacking <i>gfp</i>); Amp ^r Cm ^r	This study
pAD/abrB*/PcspA	pAD123 derivative containing the <i>cspA</i> promoter region in front of the <i>abrB</i> -like gene from pBCE (lacking <i>gfp</i>); Amp ^r Cm ^r	This study
pXen1	Shuttle vector containing a promoterless luciferase (<i>luxABCDE</i>) for Gram-positive hosts; Amp ^r Cm ^r	Francis <i>et al.</i> (2000)
pXen/Pces	pXen1 derivative containing the <i>ces</i> promoter region fused to <i>lux</i> ; Amp ^r Cm ^r	M. Dommel
pXen/Pces/erm	pXen1 derivative containing the <i>ces</i> promoter region fused to <i>lux</i> and an additional Erm-resistance cassette; Amp ^r Erm ^r	This study
pAT113	Conjugative suicide vector for <i>Bacillus</i> ; Kan ^r Erm ^r	Trieu-Cuot <i>et al.</i> (1991)
pAT113 Δ spo0A/spc	pAT113 derivative containing the up- and downstream flanking regions of <i>spo0A</i> and the Spc-resistance cassette; Spc ^r Erm ^r Kan ^r	This study
pAT113 Δ spo0A/cm	pAT113 derivative containing the up- and downstream flanking regions of <i>spo0A</i> and the Cm-resistance cassette; Cm ^r Erm ^r Kan ^r	This study
pAT113 Δ plcR/spc	pAT113 derivative containing the up- and downstream flanking regions of <i>plcR</i> and the Spc resistance cassette; Spc ^r Erm ^r Kan ^r	This study
pAT113 Δ abrB/spc	pAT113 derivative containing the up- and downstream flanking regions of <i>abrB</i> and the Spc-resistance cassette; Spc ^r Erm ^r Kan ^r	This study
pET28b	<i>E. coli</i> expression vector; Kan ^r	Novagen
pET28b + <i>abrB</i>	pET28b derivative carrying the <i>abrB</i> gene of <i>B. cereus</i> F4810/72 fused to the His-tag; Kan ^r	This study
pET28b + <i>abrB*</i>	pET28b derivative carrying the <i>abrB*</i> gene of <i>B. cereus</i> F4810/72 fused to the His-tag; Kan ^r	This study
Strains		
<i>E. coli</i>		
TOP10 and INV110	General cloning strains	Invitrogen
JM83/pRK24	Donor strain for conjugation; Tra ⁺ Mob ⁺ Amp ^R	Trieu-Cuot <i>et al.</i> (1987)
BL21	Protein expression strain	Novagen
BL21/ <i>abrB</i>	BL21 harbouring pET28b + <i>abrB</i> for <i>AbrB</i> overexpression	This study
BL21/ <i>abrB*</i>	BL21 harbouring pET28b + <i>abrB*</i> for <i>AbrB*</i> overexpression	This study
<i>B. cereus</i>		
F4810/72 (AH187)	Emetic reference strain	UK (PHLS)
F48 Δ plcR	<i>plcR</i> deletion mutant of F4810/72; Spc ^r	This study
F48 Δ spo0A	<i>spo0A</i> deletion mutant of F4810/72; Spc ^r	This study
F48 Δ abrB	<i>abrB</i> deletion mutant of F4810/72; Spc ^r	This study
F48 Δ abrB Δ spo0A	<i>abrB</i> and <i>spo0A</i> deletion mutant of F4810/72; Spc ^r Cm ^r	This study
F48 Δ spo0AcomAH	F48 Δ spo0A complemented with pAD/spo0A/ $\sigma^A\sigma^H$ -Pro; Amp ^r Cm ^r Spc ^r	This study
F48 Δ spo0AcomH	F48 Δ spo0A complemented with pAD/spo0A/ σ^H -Pro; Amp ^r Cm ^r Spc ^r	This study
F48pAD/ <i>abrB</i>	F4810/72 harbouring pAD/ <i>abrB</i> /PcspA; Amp ^r Cm ^r	This study
F48pAD/ <i>abrB*</i>	F4810/72 harbouring pAD/ <i>abrB*</i> /PcspA; Amp ^r Cm ^r	This study
F4810/72pLux	F4810/72 harbouring pXen/Pces; Amp ^r Cm ^r	This study
F48 Δ spo0ApLux	F48 Δ spo0A harbouring pXen/Pces; Amp ^r Cm ^r Spc ^r	This study
F48 Δ abrBpLux	F48 Δ abrB harbouring pXen/Pces; Amp ^r Cm ^r Spc ^r	This study
F48 Δ abrB Δ spo0ApLux	F48 Δ abrB Δ spo0A harbouring pXen/Pces/erm; Amp ^r Cm ^r Spc ^r Erm ^r	This study

B. cereus F4810/72 was grown in parallel under the same conditions, as control.

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 (twofold) 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 × 10⁵ 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 A₄₅₀ 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) treatment at 37 °C for 45 min. After chloroform extraction and precipitation with ethanol, RNA was quantified by measuring the absorbance at 260 nm and 280 nm. The absence of DNA was tested by using the purified RNA as template in a standard PCR with 16S rDNA universal primers (Martineau *et al.*, 1996).

Reverse transcription (RT) was performed using 100 ng total RNA, 2 pmol gene-specific reverse primer (Table S1), 0.5 mM of each dNTP, 5 mM DTT, 100 U SuperScript II and 20 U RNaseOUT (all from Invitrogen). The mixture including RNA, dNTPs and primer was incubated continuously from 75 °C to 45 °C for 1 min each, before adding the enzymes and incubating the reaction mix at 42 °C for 1 h.

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 SmartCycler (Cepheid) 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 (*T_m*) 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 *C_t* values of the sample and a calibrator for the target gene and the reference gene *rrn* (16S rDNA). *C_t* values of the wild-type strain F4810/72 at an OD₆₀₀ 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 luciferase genes *luxABCDE*, 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 promoter-less 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 B containing 7.5 mM imidazole. Finally, AbrB-His₆ or AbrB*-His₆ was eluted with buffer B 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 Vivaspinn 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

Fig. 1. (a) Growth, measured as OD₆₀₀, of the wild-type F4810/72 (□), the *plcR* null mutant (×) and the *spo0A* null mutant (●) incubated in LB medium at 30 °C. (b) Colony morphology of *B. cereus* F4810/72, F48 $\Delta plcR$ and F48 $\Delta spo0A$, 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.

not shown). In contrast, the *spo0A* null mutant grew to a lower maximum OD₆₀₀ (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 σ^H 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 σ^A is recognized by the housekeeping sigma factor σ^A during vegetative growth, P σ^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. 2. Cereulide production of *B. cereus* F4810/72, the deletion mutants F48 $\Delta plcR$, F48 $\Delta spo0A$, F48 $\Delta abrB$ and F48 $\Delta abrB\Delta spo0A$ and the complemented strains F48 $\Delta spo0AcomAH$ and F48 $\Delta spo0AcomH$, 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 %.

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 OD₆₀₀ 4 to OD₆₀₀ 15), determined by RT-qPCR. (b) Transcript levels of *abrB* in early growth stages: transition from lag to exponential phase (OD₆₀₀ 0.05), early exponential phase (OD₆₀₀ 0.2) and mid-exponential phase (OD₆₀₀ 10). The transcript levels shown represent four different datasets normalized to *rnn* transcript levels.

gene with both its promoter regions ($P\sigma^A$ and $P\sigma^H$) and the other with only $P\sigma^H$ 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, F48 $\Delta spo0A$ comAH and F48 $\Delta spo0A$ comH, were able to sporulate (data not shown), but only F48 $\Delta spo0A$ comAH was toxic to HEp-2 cells, while F48 $\Delta spo0A$ comH (bearing $P\sigma^H$ but not $P\sigma^A$) 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 $P\sigma^H$ -directed transcription of *spo0A*. The cereulide titre of *B. cereus* F48 $\Delta spo0A$ comAH was comparable to the wild-type, whereas F48 $\Delta spo0A$ comH produced significantly less toxin (data not shown). These data demonstrate that cereulide production is indeed affected by σ^A -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 'OA-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 (F48 $\Delta abrB$) and the double mutant F48 $\Delta abrB\Delta spo0A$ 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, F48 $\Delta spo0A$, F48 $\Delta abrB$ and the double mutant F48 $\Delta abrB\Delta spo0A$ 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

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%.

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. 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).

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 displayed 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 Bongiorno *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 F48 Δ *abrB* Δ *spo0A* 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 phosphorylation, but not by the *B. cereus* specific virulence regulator PlcR. 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*.

ACKNOWLEDGEMENTS

We would like to thank Sophie Davison for helpful discussions and assistance with mutagenesis, and Romy Renner for excellent technical support. We also thank Dr Daniel Zeigler (Bacillus Genetic Stock Center) for the gift of pAD123, Dr Kevin Francis (Xenogen Corporation) for the gift of pXen1 and Dr Patrice Courvalin (Institut Pasteur) for kindly supplying us with *E. coli* JM83/pRK24 and pAT113. This work was supported in part by the FEI (Forschungskreis der Ernährungsindustrie eV, Bonn), the AiF (Arbeitskreis für industrielle Forschung), and the Ministry of Economics and Technology (project no. 15186N).

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Edited by: Hanne Ingmer