

Identification and Partial Characterization of the Nonribosomal Peptide Synthetase Gene Responsible for Cereulide Production in Emetic *Bacillus cereus*

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Cereulide, a depsipeptide structurally related to valinomycin, is responsible for the emetic type of gastrointestinal disease caused by *Bacillus cereus*. Due to its chemical structure, (D-O-Leu-D-Ala-L-O-Val-L-Val)₃, cereulide might be synthesized nonribosomally. Therefore, degenerate PCR primers targeted to conserved sequence motifs of known nonribosomal peptide synthetase (NRPS) genes were used to amplify gene fragments from a cereulide-producing *B. cereus* strain. Sequence analysis of one of the amplicons revealed a DNA fragment whose putative gene product showed significant homology to valine activation NRPS modules. The sequences of the flanking regions of this DNA fragment revealed a complete module that is predicted to activate valine, as well as a putative carboxyl-terminal thioesterase domain of the NRPS gene. Disruption of the peptide synthetase gene by insertion of a kanamycin cassette through homologous recombination produced cereulide-deficient mutants. The valine-activating module was highly conserved when sequences from nine emetic *B. cereus* strains isolated from diverse geographical locations were compared. Primers were designed based on the NRPS sequence, and the resulting PCR assay, targeting the *ces* gene, was tested by using a panel of 143 *B. cereus* group strains and 40 strains of other bacterial species showing PCR bands specific for only the cereulide-producing *B. cereus* strains.

Bacillus cereus is a gram-positive spore-forming food pathogen that causes two types of food poisoning syndromes: emesis and diarrhea. It is an increasing problem, especially in heat-treated food, such as convenience food and food used in catering, because of its resistance to pasteurization and antimicrobial agents. The true incidence of *B. cereus* food poisoning is unknown for a number of reasons, including misdiagnosis of the illness, which may be symptomatically similar to other types of food poisoning.

The emetic syndrome is mainly characterized by vomiting a few hours after ingestion of the contaminated food. In the diarrheal syndrome, the symptoms appear 8 to 16 h after ingestion and include abdominal pain and diarrhea. Different enterotoxins are the causative agents of the diarrheal syndrome. These toxins are comparatively well characterized at the molecular level (for a review see reference 15), and molecular diagnostic assays are available (14, 19). Far less is known about the emesis-causing molecule cereulide. Cereulide is a small, heat-stable dodecadepsipeptide which, for instance, was shown to be involved in fulminate liver failure in a human case (23). It has been shown to cause cellular damage in animal models and is toxic for mitochondria by acting as a potassium ionophore (2, 27, 36). Recently, it has been reported that

cereulide inhibits human natural killer cells and might therefore have an immunomodulating effect (30). Based on its structure, (D-O-Leu-D-Ala-L-O-Val-L-Val)₃, one could expect cereulide to be synthesized nonribosomally. Alternating peptide and ester bonds, as well as D-amino acids and a cyclic structure, are often found in nonribosomal peptide synthetase (NRPS) products (24).

NRPSs are large multifunctional proteins with a modular organization. One module contains all catalytic activities which are necessary for incorporation of one amino acid residue into the peptide product. According to the “colinearity rule” (24, 48) the order of modules usually corresponds directly to the order of amino acids in the peptide product. The core of each module is the adenylation domain (A domain) that activates the cognate amino acid as an adenylate. The activated substrate is covalently bound to the thiolation domain (T domain) or peptidyl carrier protein. Chain elongation of the peptide is catalyzed by condensation domains (C domains) that are located at the N-terminal ends of modules accepting acyl groups from the preceding module (40). Following peptide bond formation, the peptidyl moiety is transferred to and condensed with the downstream carrier protein-bound monomer. A carboxyl-terminal thioesterase domain catalyzes the release (and in some cases even oligomerization and/or cyclization) of the mature NRPS-bound peptide product (44). The A and T domains contain highly conserved core motifs that can be used for a universal PCR approach to identify parts of NRPSs (24, 48).

The aim of this work was to identify genes potentially in-

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TABLE 1. Bacterial species used to test the specificity of the PCR assay

Bacterial species	No. of strains tested
Bacillus cereus group	
<i>Bacillus cereus</i> ^a	114
<i>Bacillus anthracis</i>	7
<i>Bacillus thuringiensis</i>	6
<i>Bacillus mycoides</i>	6
<i>Bacillus pseudomyoides</i>	3
<i>Bacillus weihenstephanensis</i>	7
Other <i>Bacillus</i> spp.	
<i>Bacillus brevis</i>	3
<i>Bacillus subtilis</i>	1
<i>Bacillus licheniformis</i>	3
<i>Bacillus amyloliquefaciens</i>	1
Non-<i>Bacillus</i> species	
<i>Staphylococcus aureus</i>	10
<i>Staphylococcus equorum</i>	1
<i>Clostridium perfringens</i>	3
<i>Listeria monocytogenes</i>	6
<i>Campylobacter</i> sp.	3
<i>Escherichia coli</i> (including serovar O157).....	4
<i>Salmonella</i> sp.	6
<i>Yersinia enterocolitica</i>	7

^a Including 30 cereulide-producing isolates identified by the HEP-2 cytotoxicity assay and LC-MS analysis.

involved in cereulide production by a PCR screening assay by using degenerate primers directed against conserved motifs of known NRPSs (24) in order to gain insight into the synthesis of cereulide and to provide the basis for the development of molecular detection systems for cereulide producers.

MATERIALS AND METHODS

Strains and plasmids. The cereulide-producing *B. cereus* strains F4810/72 (18), NC7401 (1), and MHI 87 (35) were used to search for the genetic locus responsible for the production of the emetic toxin cereulide. Cells were grown on plate count plates or in broth at 30°C. *Escherichia coli* strains were grown at 37°C in Luria-Bertani medium with the appropriate antibiotics. Bacterial strains used to test the specificity of the *ces* gene PCR assay are listed in Table 1, and additional information on the origins of cereulide-producing strains is provided in Table 2. Plasmids used in this study are listed in Table 3.

Measurement of toxicity and LC-MS analysis of cereulide-producing strains. Emetic toxin production by *B. cereus* strains included in this study was determined by a modification of the cytotoxicity assay described previously (12). In brief, *B. cereus* isolates were grown in 20 ml of skim milk medium for 18 h, and

after autoclaving, an aliquot of the preparation was serially diluted (twofold) in 96-well plates by using Earle's minimal essential cell culture medium supplemented with 1% fetal calf serum, 1% (vol/vol) sodium pyruvate (100 mmol/liter), 2% (vol/vol) L-glutamine (200 mmol/liter), 0.2% (vol/vol) penicillin-streptomycin (10,000 U/ml), and 2% ethanol as a diluent. Immediately after this, HEP-2 cells (0.15 ml; 10⁵ cells per well) were added, and the plates were incubated for 48 h at 37°C in a 5% CO₂ atmosphere. Toxicity titers were determined by using the cell proliferation reagent WST-1 (Roche Diagnostics) essentially as described by Dietrich et al. (9). In addition, all strains were tested by using the boar semen assay as a second bioassay (5). Cereulide production by all strains that were positive in the cytotoxicity assay was confirmed by liquid chromatography (LC)-ion trap mass spectrometry (MS) as described previously (18).

Isolation of DNA. DNA from gram-positive bacteria was prepared by using an AquaPure genomic DNA isolation kit (Bio-Rad, Munich, Germany). DNA from gram-negative bacteria was prepared by suspending cells from one colony in sterile water. The suspension was heated at 95°C for 3 min and then placed on ice. After centrifugation the supernatant was used as the template for PCR or stored at -20°C. For inverse PCR and Southern analysis, total chromosomal DNA was isolated by phenol-chloroform extraction as described previously (10). Preparation of plasmid DNA was performed by using standard procedures.

Primer design. Degenerate oligonucleotide primers targeting highly conserved motifs of known nonribosomal peptide synthetases (6, 24) were used to amplify and identify putative NRPS gene fragments in *B. cereus*. Inverse PCR and module jumping, with a combination of a specific primer located in the known sequence and a degenerate primer targeting conserved motifs in the flanking regions (for details concerning the latter technique see reference 29), were used to obtain sequence information from the flanking regions of the DNA fragments. Based on the sequence information derived from the amplified DNA fragments, specific primers and probes for detection of emetic *B. cereus* strains were designed. Sequence information for all primers used in this study (except primers used for inverse PCR and control PCR) is shown in Table 4.

Cloning of PCR amplicons and DNA sequencing. Amplification products obtained from PCR performed with degenerate primers and amplicons obtained from module jumping were subcloned in the TOPO TA vector (Invitrogen, Karlsruhe, Germany) and sequenced. Primers CesF1 and CesR1 were used to amplify a 2.2-kb DNA fragment from nine cereulide-producing *B. cereus* strains in order to study the molecular diversity of the *ces* (cereulide synthetase) gene. PCR amplicons were purified with a QIAquick PCR purification kit (QIAGEN) and were directly sequenced (Sequiseq, Ebersberg, Germany) by using a DNA DyeDeoxy terminator cycle sequencing kit (Applied Biosystems). PCR products obtained by inverse PCR were either subcloned in TOPO TA or directly sequenced.

Sequence analysis. The resulting sequences were searched against the sequenced genomes of *B. cereus* strains ATCC 14579^T and ATCC 10987, *Bacillus anthracis* strains A2012, Ames, Kruger B, and Western NA, *Bacillus halodurans*, and *Bacillus subtilis* and against the National Center for Biotechnology Information (NCBI) nonredundant protein database by using the Basic Local Alignment Search Tool (BLAST). All sequence similarity searches were performed by using BLASTX and BLASTP at the NCBI website (3, 4). The sequencing analysis software package Vector NTI (Informax Inc.) was used to generate the contig sequence from the sequenced PCR products obtained by inverse PCR and module jumping. The software packages Clustal X and TREECON were used for sequence alignment and cluster analysis (43, 47).

Southern analysis. Chromosomal DNA was digested with different restriction enzymes. The fragments were separated on a 1% agarose gel and blotted onto

TABLE 2. Origins of cereulide-producing *B. cereus* strains

Strain(s) ^a	Origin
WSBC 10879, WSBC 10880.....	Rice
MHI 87, MHI 135, MHI 294, M UHDAM IIFI (1), UHDAM IIFI(2), UHDAM IIFI(3).....	Baby food
F3080B/87, F3350/87, F3605/73, F3752A/86, F3942/87, F4108/89, F4426/94, F47/94, F5881/94, F6921/94, MHI 280, MHI 297, MHI 1305.....	Food remnants associated with emetic outbreaks
F3351/87, F3876/87.....	Human feces (patient)
F4552/75, F4810/72 (SMR-178), NC7401.....	Vomit (patient)
UHDAM IH41385.....	Dialysis liquid
UHDAM NS58, UHDAM NS88, UHDAM NS115.....	Spruce tree

^a MHI, *B. cereus* culture collection at the Institute of Hygiene and Technology of Food of Animal Origin, Ludwig-Maximilians-Universität München, Munich, Germany; NC, Nagoya City Public Health Research Institute, Nagoya, Japan; UHDAM, Department of Applied Chemistry and Microbiology, University of Helsinki, Helsinki, Finland; WSBC, Weihenstephan *B. cereus* culture collection at the Department of Biosciences, Technical University of Munich, Munich, Germany. F strains were obtained from the Public Health Laboratory Service, London, United Kingdom.

TABLE 3. Plasmids used in this study

Plasmid	Description	Use	Source
pMK4	<i>B. subtilis</i> - <i>E. coli</i> shuttle vector; Cm ^r	Testing of transformation efficiency	Sullivan et al. ^a
pCR2.1-TOPO	Topoisomerase I-activated cloning vector with 3' overhangs; Kan ^r Amp ^r	Subcloning and construction of nonreplicable plasmids for insertion mutagenesis	Invitrogen
pME3	pCR2.1-TOPO containing a 0.7-kb putative NRPS fragment		This study
pME11	pCR2.1-TOPO containing a 0.7-kb <i>ces</i> PCR fragment		This study
pMEC	pCR2.1-TOPO containing a 1.3-kb <i>ces</i> PCR fragment	Insertion mutagenesis	This study

^a See reference 42.

nitrocellulose. Southern analysis (37) was performed by using digoxigenin-labeled probes (Roche, Mannheim, Germany) directed against two different domains of *ces*. The probes were obtained from the emetic reference strain F4810/72 by PCR by using the oligonucleotide primers described in Table 4.

Preparation of electrocompetent cells and insertion mutagenesis. The electrocompetence of emetic *B. cereus* strains was tested by using replicative plasmid pMK4 (42). Sixteen cereulide-producing *B. cereus* strains were cultivated overnight in LB broth at 30°C and 180 rpm. One milliliter of an overnight culture was transferred into 100 ml of LB medium (with 2% [wt/wt] glycine) and incubated at 30°C and 180 rpm until the optical density at 600 nm was 0.4 to 0.7. Cells were harvested by centrifugation, and the pellets were washed with increasing concentrations of ice-cold glycerol (2.5, 5, and 10%), resuspended in precooled electroporation buffer (10% glycerol), and shock frozen in liquid nitrogen. Electroporation was carried out by using a Bio-Rad Gene Pulser with a Pulse Controller (Bio-Rad). Electrocompetent cell suspensions, including the plasmid DNA, were transferred into precooled electroporation cuvettes (Ecu-102; PeqLab Biotechnologie, Erlangen, Germany) and exposed to an electric pulse (voltage, 2.0 kV; initial field strength, 5,000 V/cm; capacitance, 25 µF; resistance, 200 Ω). Cells were overlaid with LB medium and incubated at 30°C and 160 rpm for 2 h. Transformants were selected on LB agar supplemented with chloramphenicol (5 µg/ml). For insertion mutagenesis, amplicons obtained from degenerative PCR and module jumping were subcloned into pCR2.1-TOPO, and the resulting nonreplicative plasmids pME11 and pMEC10 were sequenced. *B. cereus* strain F4810/72 was transformed with these disruption plasmids as described above, and integration of the plasmids into the chromosome was forced by selection for antibiotic-resistant mutants on LB agar supplemented with kanamycin (50 µg/ml). Successful recombination was checked by PCR by using specific primers for the *kan* gene and additional primers located in the flanking region of the target DNA.

***ces* gene-specific PCR.** Each PCR mixture (50 µl) contained each deoxynucleoside triphosphate at a concentration of 0.8 mM, 1.5 mM MgCl₂, 50 pM oligonucleotide primer CesF1 (Table 4), 50 pM oligonucleotide primer CesR2 (Table

4), 1.25 U of ThermoStart *Taq* DNA polymerase (ABgene, Epsom, United Kingdom), 5 µl of 10× polymerase buffer (ABgene), and 1 µl of template DNA. The PCR protocol started with a denaturation step consisting of 15 min at 95°C; this was followed by five cycles of 1 min at 95°C, 75 s at 53°C, and 50 s at 72°C, 25 cycles of 1 min at 95°C, 75 s at 58°C, and 50 s at 72°C, and finally an elongation step consisting of 72°C for 5 min. All strains used for evaluation of the assay were checked with the CesF1-CesR2 primer set. Selected strains were amplified in parallel with 16S rRNA primers (41) as a positive control.

Nucleotide sequence accession number. The nucleotide sequence (5.2 kb) of *B. cereus* F4810/72 described in this paper has been deposited in the GenBank database under accession no. AY691650.

RESULTS

Identification of putative peptide synthetase genes in *B. cereus*. To identify the genetic locus responsible for cereulide production in *B. cereus*, degenerate PCR primers targeted to conserved sequence motifs of known NRPS genes were used with chromosomal DNA derived from emetic and nonemetic strains. The use of degenerate primers targeting the A3 and A7 core motifs resulted in products of the expected size (700 bp) in all strains tested (data not shown). The 700-bp gene fragments amplified from the cereulide-producing *B. cereus* strain F4810/72 were subcloned in pCR 2.1 TOPO TA (Invitrogen) and sequenced. The resulting sequences were searched against NCBI's nonredundant database by using the BLASTX or BLASTP algorithms (3). One of the 20 clones analyzed (pME11) showed high homology to the valine activation do-

TABLE 4. Oligonucleotide primers used in this study^a

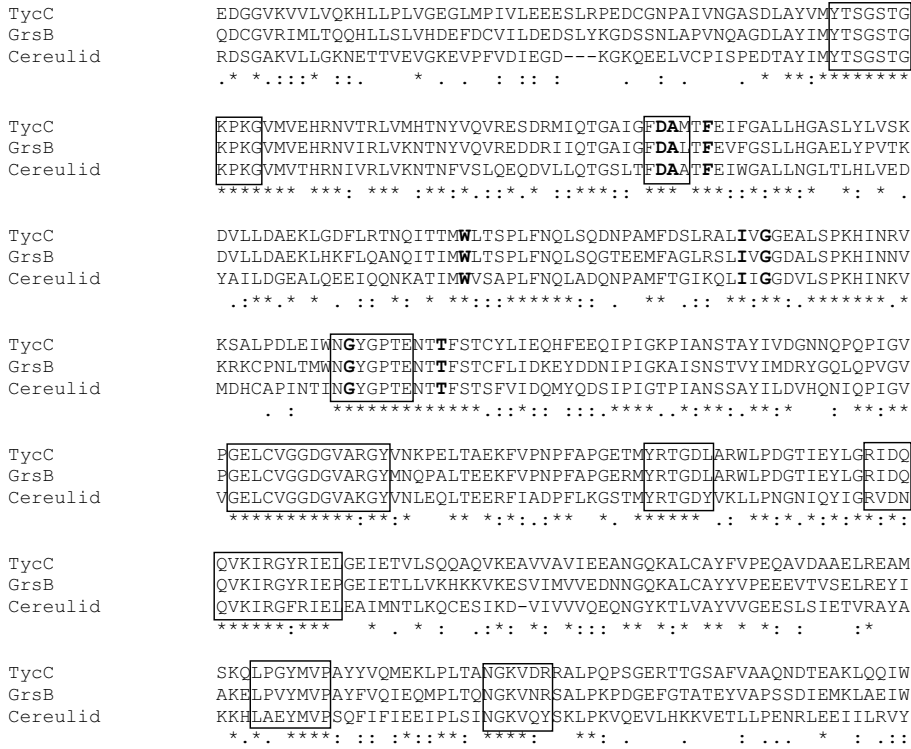
Primer	Sequence (5'-3')	Use	Source
PSF	GG(AT)C(AGT)AC(ACT)GG(ACT)(AC)A(AGCT)CC(ACT)AA(AG)GG	Degenerate PCR (A domain)	Carnio et al. ^b
PSR2	GGCA(GT)CCAT(CT)T(CT)GCCA(AG)GTC(AGCT)CC(GT)GT	Degenerate PCR (A domain)	Carnio et al. ^b
F_C3	GCA(CT)CA(CT)AT(ACT)AT(ACT)TC(AGCT)GA(CT)GG(AGCT)TGG	Module jumping (C-A domain)	This study
R_T1	C(AGT)A(GT)(AGT)A(AG)(AT)GA(AG)TG(ACT)CC(AC)CC	Module jumping (A-T domain)	This study
F_Vall	GAACCTTGAACAATTAACAGAAG	Module jumping (A-T domain)	This study
CesF1	GGTGACACATTATCATATAAGGTG	Molecular diversity; cereulide-specific PCR assay	This study
CesR1	GTTTTCTGGTAACAGCGTTTCTAC	Molecular diversity	This study
CesR2	GTAAGCGAACCTGTCTGTAACAACA	Module jumping (C-A domain); cereulide-specific PCR assay	This study
8-26/56	AGAGTTTGATCCTGGCTCA	16S rRNA gene amplification (positive control)	Stackebrandt and Liesack ^c
1511-1493	CGGCTACCTTGTTACGAC	16S rRNA gene amplification (positive control)	Stackebrandt and Liesack ^c
CF1	CCAATTTTCCAAGTGATGATGGG	Probe for hybridization (C domain)	This study
CR1	CAATAATTGTTTCAGGCGAACGCA	Probe for hybridization (C domain)	This study
AF1	TGTTGTTACAGACAGGTTTCGCTTAC	Probe for hybridization (A domain)	This study
AR1	GTTCCAATCGGAATGCTGTCTTG	Probe for hybridization (A domain)	This study

^a Primers used for inverse PCR and control sequencing reactions are not shown.

^b See reference 6.

^c See reference 41.

A



B

Code	Activated AA	Biosynthetic template
1 DAFWIGGT 8	?	Ces, <i>B. cereus</i> this study
1 DAFWIGGT 8	valine	GrsB-M2-Val Gramicidin synthetase B, LicB-M1-Val lichenysin synthetase B, SrfAB-M1-Val/Ile surfactin synthetase, TycC-M4-Val tyrocidine synthetase 3
1 D?FWIGGT 8	valine	FenE-M2-Val fengycin synthetase, Pps3-M2-Val fengycin synthetase
1 DILQLGLI 8	?	Gps putative NRPS module, <i>B. cereus</i> this study
1 DILQLGLI 8	glycine	Cda2-M2-Gly CDA peptide synthetase II, DhbF-M1-Gly siderophore 2,3-dihydroxybenzo, NosC-M2-Gly Nostopeptolide synthetase
1 DILQLGLV 8		SafA-M1-3h4mPhe saframycin Mx1 synthetase A

FIG. 1. Comparison of putative NRPS of cereulide-producing *B. cereus* F4810/72 with known NRPSs. (A) Alignment of a putative valine-activating A domain detected in cereulide-producing *B. cereus* F4810/72 by degenerative PCR with the A domains of valine-activating modules from GrsB and TycA of *B. brevis* (28, 46). Conserved core motifs are indicated by boxes, and amino acids that bind specific positions are indicated by boldface type. (B) The selectivity-conferring code for the A domain (38) was used to identify the eight specific signature amino acids of the potential NRPS in cereulide-forming *B. cereus*. The signature sequences of pME11 (designated Ces) and pME3 (designated Gps) were compared to database sequences of known NRPSs (7). AA, amino acid.

main of gramicidin S synthetase (Swissprot database accession no. P14688; 56% identity, 126 of 222 aligned amino acids) and tyrocidine synthetase (Swissprot database accession no. 030409; 57% identity, 127 of 222 aligned amino acids) from *Bacillus brevis* (28, 46) (Fig. 1). Two other clones (pME3 and pME13) with identical sequences showed significant homology to a glycine-activating module from DhbF (Fig. 2) encoded by a gene belonging to the NRPS complex responsible for cat-

eholic siderophore itoic acid production (EMBL accession no. AF184977; 69% identity, 154 of 223 aligned amino acids) in *B. subtilis* (25). The deduced amino acid sequences of pME11 and pME3 were used to predict their substrate specificities by the method of Stachelhaus et al. (38) and Challis et al. (7). The primary sequences of the Phe-activating A domain of gramicidin S and the translated sequences of pME3 and pME11 were aligned by using ClustalX (43), and the signature amino

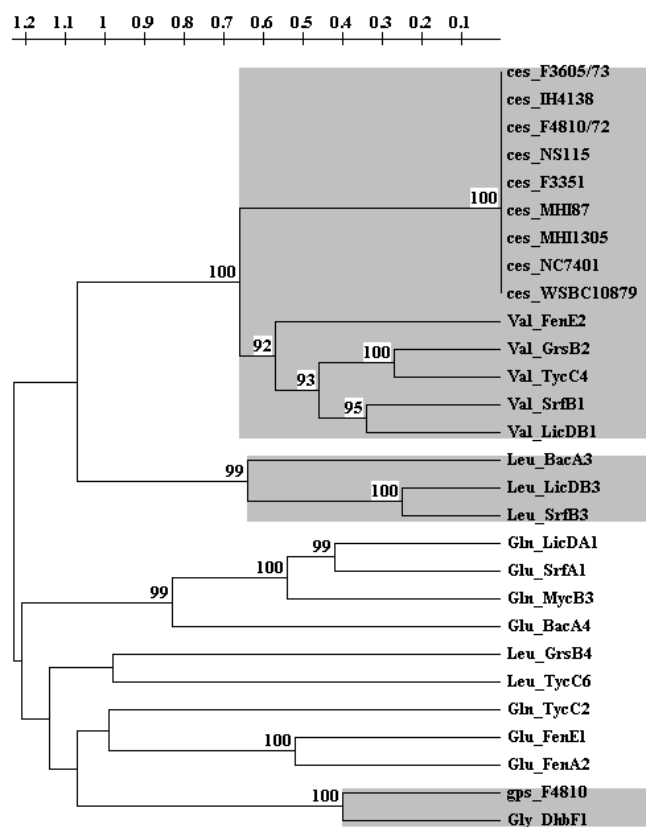


FIG. 2. Cluster analysis of nonribosomal peptide synthetase modules of *Bacillus*. Amino acid sequences derived from A domains of known *Bacillus* NRPSs and putative NRPS modules from F4810/72 obtained by degenerative PCR were aligned, and the regions between the A3 and A7 core motifs, which have been shown to confer substrate specificity (38), were used for cluster analysis. The tree was constructed with TREECON (47) by using the unweighted pair group method with arithmetic averages. All bootstrap values of >80% (100 bootstrap replicates) are shown at the nodes. Abbreviations: Bac, bacitracin synthetase; DhbF, siderophore synthetase; FenE, fengycin synthetase; Grs, gramicidin S synthetase; LicD, lichenysin synthetase; Myc, mycosubtilin synthetase; Srf, surfactin synthetase; Tyc, tyrocidine A synthetase; ces, putative cereulide synthetase; gps, putative NRPS derived from F4810/72 by degenerate PCR.

acids conferring substrate recognition were compared to a database of 198 known NRPS adenylation domains whose substrate specificity has been determined experimentally (<http://raynam.chm.jhu.edu/~nrps>). The eight amino acids lining the putative binding pocket of the A domain from pME11 were identical to the residues of the valine-activating module of peptide synthetases in *B. brevis* (GrsB and TycC) (28, 46), *Bacillus licheniformis* (LicB) (21), and *B. subtilis* (SrfAB) (8), while the eight residues predicted from pME3 were identical to the residues of the glycine-activating module of DhbF of *B. subtilis* (25) (Fig. 1). A comparison of the sequence of pME11 with the sequenced genome of *B. cereus* ATCC 14597^T yielded no homologous genes, but sequences of several other NRPS modules, including a putative glycine-activating module, were recognized (data not shown). Alignment and cluster analysis of A domains from known *Bacillus* NRPS genes and translated sequences from pME3 and pME11 derived from *B. cereus*

F4810/72 showed that the A domains cluster according to their predicted substrate specificities (Fig. 2).

Sequence analysis of the identified peptide synthetase gene.

The flanking regions of the DNA fragment from pME11 were sequenced by using a combination of a specific primer located in the known sequence and a degenerate primer located in a conserved domain in the putative flanking regions (module jumping) and by inverse PCR. The resulting 5-kb fragment was sequenced, and the deduced protein sequence was analyzed by using the protein analysis module of the HUSAR Bioinformatics lab at Deutsche Krebsforschungszentrum (<http://genome.dfkz-heidelberg.de>) and the BLAST conserved motif search tool at NCBI's website (<http://www.ncbi.nlm.nih.gov>). The corresponding open reading frame that was identified, designated *ces* (cereulide synthetase), contained a complete putative valine-activating module that had the typical domain organization of NRPS genes (Fig. 3). Strong homology to sequences encoding thioesterases, which are commonly found at the C terminal of bacterial NRPS genes, was observed in the C-terminal portion of *ces*. In the 5' region of the putative valine activation module the T domain and a predicted A10 core motif of the preceding monomer were found. In addition, sequence motifs typically found in ketoreductase and dehydrogenase genes were detected at the N-terminal end of the *ces* gene fragment. In addition to the Rossmann fold motif (Fig. 3), the typical short-chain dehydrogenase/reductase catalytic Tyr and Ser residues were found (data not shown).

Database searches of sequenced genomes showed that the *ces* gene fragment is not found in the non-cereulide-producing *B. cereus* strains ATCC 14579^T and ATCC 10987 and is not present in *B. anthracis* strains A2012, Ames, Kruger B, and Western NA. Southern analysis of DNA from the cereulide-producing strain F4810/72 that was restricted with different restriction enzymes revealed a single band when a probe that targets the A domain or the C domain of the valine module was used, while no bands were observed for *B. cereus* ATCC 14579^T (Fig. 4) or other non-cereulide-producing *B. cereus* group strains (data not shown).

Primers CesF1 and CesR1 were designed to amplify a 2.2-kb fragment of the *ces* peptide synthetase gene of cereulide-producing *B. cereus* strains (Fig. 3). Direct sequencing of the DNA amplicons of nine different cereulide producers from diverse geographical locations revealed nearly identical sequences of this peptide synthetase for all of the strains sequenced (data not shown). The deduced primary amino acid sequences of all strains were identical, since the only polymorphism detected in the nucleic acid sequence of two strains was located at a wobble position (Fig. 2).

Insertion inactivation of the putative cereulide synthetase gene. Because not all *B. cereus* strains are easily transformable, 16 cereulide-producing *B. cereus* strains were chosen to test their transformability. The food isolate MHI 87 showed the highest transformation efficiency, while the highly toxic food poisoning strains MHI1305, MHI 280, and MHI 297 were not transformable by electroporation, even when different electroporation conditions and different methods for competent cell preparation were used (data not shown). Since the emetic reference strain F4810/72 isolated from a food poisoning case showed a reasonable transformation efficiency, it was chosen for an insertion mutagenesis experiment. A PCR amplicon

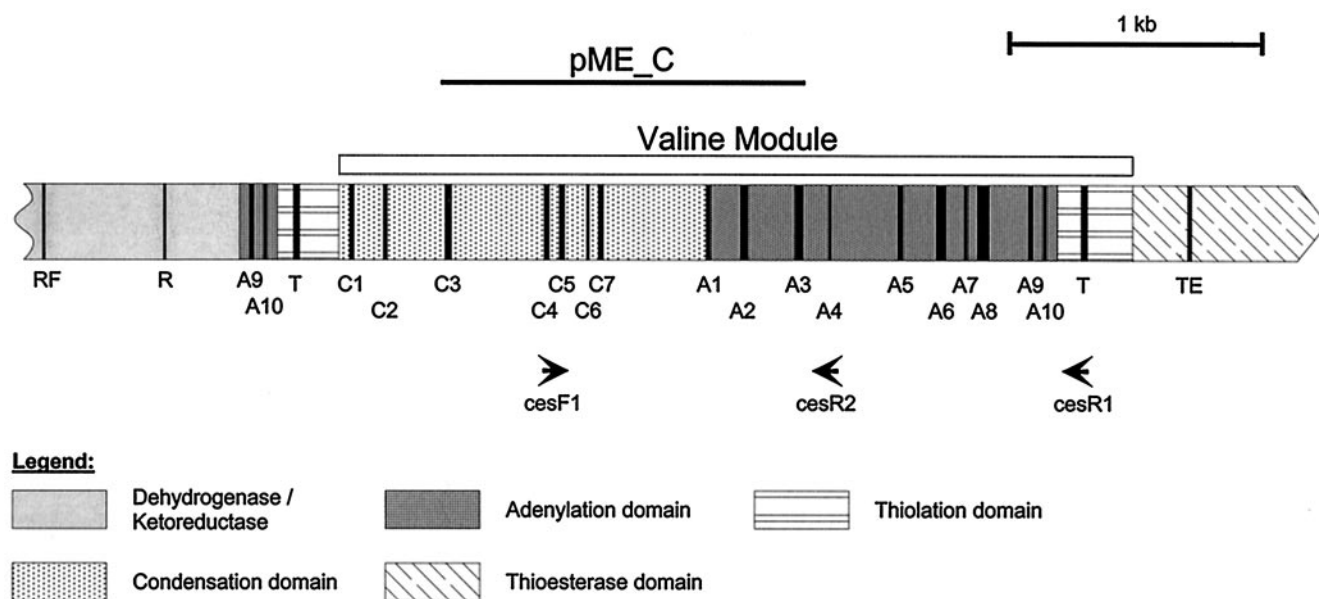


FIG. 3. Organization and structure of the C-terminal region of cereulide synthetase. The highly conserved core motifs of the peptide synthetase and signature sequences of a short-chain dehydrogenase/reductase (Rossmann fold [RF] motif for cofactor binding and catalytic residues [R] typically found in a short-chain dehydrogenase/reductase [33]) upstream of the valine-activating module are indicated by bars. A1 to A10 and C1 to C5 are highly conserved core sequences in the adenylation domain and the condensation domain, respectively; T indicates the site of 4'-phosphopantetheine binding and substrate acylation located in the thiolation domain; and TE indicates the conserved core motif of thioesterases (for a more detailed description of functional domains see reference 24). pME_C is the DNA fragment used for insertion mutagenesis, and its position is indicated by a bar. The arrows indicate the binding positions of primers used for studying the molecular diversity of the *ces* gene (CesF1 and CesR1) and in the cereulide-specific PCR assay (CesF1 and CesR2).

obtained by module jumping was cloned into TOPO TA, resulting in plasmid pMEC. Since this plasmid is unable to replicate in gram-positive hosts, it was used for insertion mutagenesis (Fig. 3). Mutants were selected on agar plates containing kanamycin, and correct integration of the plasmid in the genome of the antibiotic-resistant cells was demonstrated by PCR (data not shown). Analysis of integrants with the HEp-2 cytotoxicity assay and LC-MS showed that disruption of the C

domain yielded a cereulide-deficient phenotype. Wild-type cells were highly toxic (cytotoxicity titer, >160), while the mutant F4810cesCint was nontoxic when it was tested in the HEp-2 cell assay. Quantification of cereulide production by LC-MS revealed a cereulide production level of $0.4 \mu\text{g mg (wet weight)}^{-1}$ for wild-type cells, while $0.01 \mu\text{g}$ of cereulide per mg $(\text{wet weight})^{-1}$ was measured in the mutant.

Development of a primer system for identification of cereulide-producing *B. cereus* strains. The *ces* gene sequence from F4810/72 was aligned with valine activation modules from *B. brevis* and predicted NRPS modules from the genomic sequence of *B. cereus* ATCC 14579^T and pME3 in order to design primers specific for the *ces* gene. These primers were directed to regions of the peptide synthetase gene that did not belong to conserved functional core motifs. The forward primer was located in the C domain, while the reverse primer was located in the A domain. The approximate positions of these primers, which amplified a 1,271-bp DNA product, are shown in Fig. 3.

The PCR system developed was tested by using 114 *B. cereus* isolates from clinical cases, foods, and environments, and cross-reactivity was tested for closely related members of the *B. cereus* group, as well as for other bacilli and known food pathogens. A total of 143 *B. cereus* group strains and 40 isolates of other species were included in this survey (Table 1). All 30 cereulide-producing *B. cereus* isolates (Table 2) were detected by the assay, while no non-cereulide-producing isolates gave any signal in the PCR assay. To check for the absence of PCR-inhibiting substances or any inadequacies of the PCR

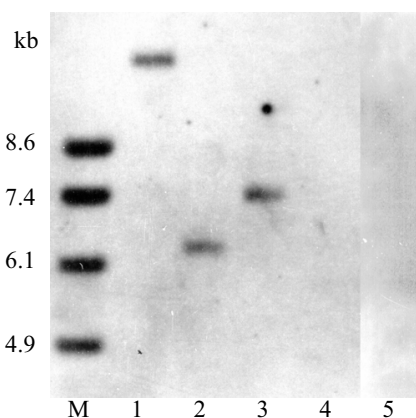


FIG. 4. Southern analysis of chromosomal DNA from the cereulide-producing *B. cereus* strain F4810/72 and the cereulide-negative strain ATCC 14579^T restricted with BamHI (lanes 1 and 4), DraI (lanes 2 and 5), and HpaI (lane 3) and hybridized to a probe from the A domain of the valine module. Lanes 1 to 3, strain F4810/72 (*B. cereus* emetic reference strain); lanes 4 and 5, strain ATCC 14579^T.

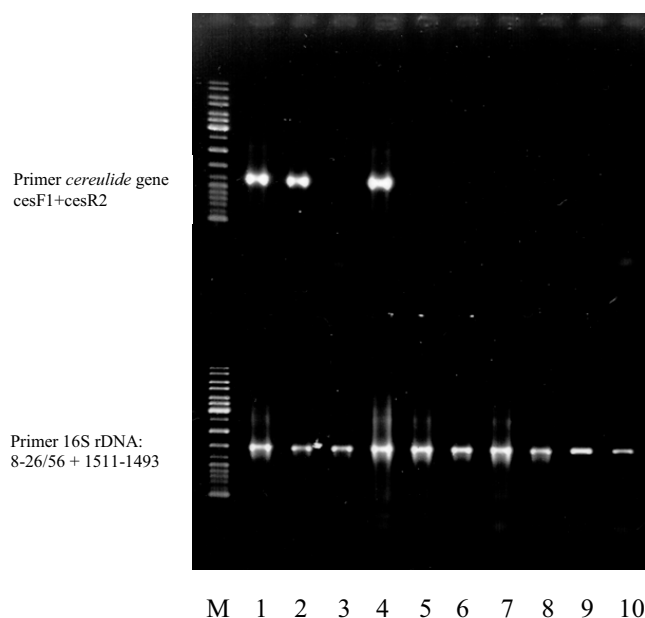


FIG. 5. PCR assay for identification of cereulide-producing *B. cereus*: gel electrophoresis of PCR products amplified with primers CesF1 and CesR2 (upper portion) and with primers 8–26/56 and 1511–1493 (lower portion) from purified DNA of different food pathogens. Primers CesF1 and CesR2 specifically detect cereulide-producing *B. cereus*, while primers 8–26/56 and 1511–1493 derived from *E. coli* (41) target 16S rRNA genes. Lane 1, *B. cereus* F4810/72 (emetic reference strain derived from vomit of a patient with food poisoning); lane 2, *B. cereus* MHI 280 (cereulide producer isolated from food remnants connected to food poisoning); lanes 3 and 5, non-cereulide-producing *B. cereus* food isolates; lane 4, cereulide-producing *B. cereus* food isolate; lane 6, *B. cereus* ATCC 14579^T; lane 7, *B. anthracis* Sterne (= CIP7702); lane 8, *B. brevis* ATCC 9999; lane 9, *Staphylococcus aureus* WS2604; lane 10, *Salmonella enteritidis* WS2863; lane M, marker ladder mixture (MBI Fermentas).

assay, selected isolates were amplified in parallel with universal primers targeting the 16S rRNA genes. Figure 5 shows the results of PCR amplification of target DNA with the cereulide-specific primers and the results of the control PCR.

DISCUSSION

Identification of the cereulide peptide synthetase (*ces*) gene.

We identified the genetic locus involved in cereulide synthesis by using a previously described PCR method (45) and degenerate primers derived from conserved core sequences of known NRPSs. One of the subcloned DNA fragments (pME11) obtained by degenerative PCR showed a high level of homology to the valine-activating A domain of gramicidin S. Substrate-binding pocket analysis suggested that the deduced adenylation domain of pME11 indeed activates valine [$P = 0.003$] (Fig. 1). Southern blot analysis with different restriction enzymes and probes that target the putative valine activation module indicated that only one copy of this module is present in the genome of the cereulide-producing *B. cereus* strain F4810/72, while this module is not found in the non-cereulide-producing strain ATCC 14579^T (Fig. 4) or in other non-cereulide-producing members of the *B. cereus* group (data not shown). Since the emetic toxin cereulide contains a valine

residue, it is tempting to speculate that the genetic locus identified belongs to the gene cluster responsible for cereulide production in *B. cereus*. In addition, in silico analysis of sequenced genomes of members of the *B. cereus* group showed that the NRPS gene identified is not found in non-cereulide-producing organisms. Further support for the hypothesis that the NRPS identified contributes to the synthesis of cereulide was provided by insertion mutagenesis experiments. Disruption of the corresponding genetic locus produced cereulide-deficient mutants. Based on these results, we suggest that cereulide is synthesized nonribosomally by the peptide synthetase Ces. Quite recently, Horwood et al. (20) described a small 500-bp gene fragment which was amplified from two emetic *B. cereus* strains with general NRPS primers and showed sequence homology to the NRPS gene. They suggested that this fragment is associated with cereulide synthesis. However, no substrate specificity was determined for this small DNA fragment, nor have any knockout experiments been performed to provide evidence for their suggestion. A comparison of the *ces* gene reported here and this 500-bp gene fragment did not support the hypothesis that this DNA fragment has a potential role in cereulide synthesis. In general, many peptide synthetases have been found in the *B. cereus* genomic sequence (11). In this study, for instance, we identified several putative peptide synthetase gene fragments in emetic *B. cereus* (e.g., the putative glycine-activating module Gps [Fig. 1B and 2]) which are, based on their predicted substrate specificity, not involved in cereulide synthesis.

Directly downstream of the valine activation module a stretch of 254 amino acids (29 kDa) with sequence similarity to thioesterases was identified, and it is assumed that cereulide synthesis is completed by activity of this domain. In bacteria, release of the completed peptide chain from the peptide synthetase is catalyzed by a thioesterase with a relative molecular mass of 28 to 35 kDa (24, 48), which is in a domain located at the carboxyl terminus of the NRPS. Due to the chemical structure of cereulide ($[D-O\text{-Leu-D-Ala-L-O-Val-L-Val}]_3$) and the colinearity rule (24, 48), an L-O-Val activation module is expected to be located directly upstream of the valine-activating module. In contrast to the valine-activating module that has the typical domain organization of NRPS, the preceding module seems to have a dehydrogenase/ketoreductase domain inserted between the A8 and A9 core motifs. Signature sequences typical of genes encoding short-chain dehydrogenases and ketoreductases (33) were found in the N-terminal region of the *ces* gene fragment sequenced. Insertions in the A domain between core motifs A8 and A9 have been reported for the EB domain, which is involved in the incorporation of D-2-hydroxyisovaleric acid in enniatin (17), as well as for specialized oxidoreductase domains (for a review see reference 39). Kuse et al. (22) reported that L-leucine and L-valine are the precursors of cereulide. They speculated that O-amino acids are converted into α -amino acids and then reduced to D-O-leucine and L-O-valine or are transaminated to D-alanine. The observed dehydrogenase/ketoreductase catalytic residues in the cereulide synthetase are consistent with this hypothesis. However, sequencing and analysis of the entire NRPS operon(s) are necessary to support this hypothesis and to elucidate the biochemical pathway of cereulide formation in vivo.

ces is highly conserved in cereulide-producing *B. cereus* strains. PCR primers derived from the *ces* gene fragment were designed to study the molecular diversity of the genetic locus responsible for cereulide production in a variety of *B. cereus* strains. In contrast to *B. cereus* enterotoxins that show great diversity at the molecular level (16), the partially sequenced cereulide synthetase (approximately 2.2 kb) was highly conserved among 10 emetic strains (Fig. 2). This sequence was not found in the nonemetic strain *B. cereus* ATCC 14579^T (Fig. 4) or in other nonemetic *B. cereus* group strains tested by Southern blot analysis (data not shown). These results are in accordance with phenotypic and genotypic studies that revealed a close relationship among emetic isolates (10a, 31) while enterotoxin production is commonly found in the different members of the *B. cereus* group and in other *Bacillus* species (13, 32, 34). In contrast to many *B. cereus* strains which possess enterotoxin genes but do not express them (16, 32), all strains tested so far that carry the *ces* gene, as determined by PCR and Southern analysis, also produce cereulide.

***ces*-specific PCR assay for identification of cereulide-producing *B. cereus* strains.** The molecular basis of cereulide synthesis was unknown previously, and the possibilities for fast and reliable detection of cereulide-producing *B. cereus* strains were therefore limited. Three principal methods for detection of the emetic toxin have been described during the last few years: a cytotoxicity assay, LC-MS analysis, and a sperm-based bioassay (5, 12, 18). These assays are rather difficult to perform on a routine basis and require 1 day to 1 week along with precultivation and laborious sample preparation. Until now, attempts to develop detection systems at an immunological level, such as the systems that are commercially available for *B. cereus* enterotoxins, failed because of the low antigenic potential of cereulide itself (26). Recently, two PCR systems for detection of emetic strains have been described (10, 20). One assay has been shown to be specific for emetic strains, but the coding potential or the function of the target sequence is unknown, because it did not show any homology to NRPS genes or significant homology to any database sequences (10). The second PCR assay which targets a putative NRPS gene is clearly not specific for cereulide producers, since it amplifies gene fragments from some nonemetic strains too (20). Due to these limitations of the recently described PCR systems, the sequence information for the *ces* gene was used to develop an improved molecular detection system that (i) directly targets the gene involved in cereulide synthesis and (ii) specifically detects emetic strains.

In conclusion, here we report on a valine module of the peptide synthetase *ces* gene which is involved in the production of cereulide via the nonribosomal pathway. This finding forms the starting point for a detailed study of the biosynthetic genes responsible for cereulide formation. Based on the unusual structure of the partially characterized L-*O*-Val activation module, it is expected that it will require quite some biochemical effort to unravel the biochemistry of cereulide synthesis, especially synthesis of the inserted heterocompounds D-*O*-Leu and L-*O*-Val.

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