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Characterization of Emetic *Bacillus weihenstephanensis*, a New Cereulide-Producing Bacterium†

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Cereulide production has until now been restricted to the species *Bacillus cereus*. Here we report on two psychrotolerant *Bacillus weihenstephanensis* strains, MC67 and MC118, that produce cereulide. The strains are atypical with regard to pheno- and genotypic characteristics normally used for identification of emetic *B. cereus* strains. MC67 and MC118 produced cereulide at temperatures of as low as 8°C.

Bacillus cereus can cause food-related diarrhea through the production of the nonhemolytic and hemolytic enterotoxin complexes, Nhe and Hbl, respectively, and emesis through the production of the toxin cereulide (D-*O*-Leu-D-Ala-L-*O*-Val-L-Val)$_3$ (14). Ehling-Schulz et al. (8) demonstrated that cereulide formation by *B. cereus* is restricted to a single evolutionary lineage of mesophilic strains, and the genetic determinants are located on a plasmid, pBCE4810 (10). Recently, one emetic psychrotolerant *B. cereus* strain has been reported (2). However, whether this psychrotolerant strain is a *Bacillus weihenstephanensis* strain (24) was not specified (2). The increasing demand for convenience foods such as cooked, chilled, ready-to-eat foods raises the question of whether psychrotolerant *B. cereus* and *B. weihenstephanensis* present a health risk in these food products because of their ability to survive heat treatment and grow at refrigeration temperatures (7, 30). The objectives of the current work were to investigate the occurrence of cereulide producers among 921 environmental isolates of the *B. cereus* group, to characterize the cereulide producers with regard to psychrotolerance, and to compare them to well-known cereulide producers at the pheno- and genotypic levels.

Screening for cereulide producers. A total of 921 *B. cereus* group isolates (Table 1) were screened by a PCR assay for the emetic character (9), using DNA prepared as described previously (19). Only two strains, MC67 and MC118, showed the emetic character. The two strains originated from different soil samples (within 1 m^2) at the same location, a sandy loam on the island of Møn, Denmark (20).

Identification of emetic *Bacillus weihenstephanensis*. The identified emetic strains, MC67 and MC118, both grew at 6°C and not at 43°C on brain heart infusion (BHI) agar (Oxoid). PCR analysis (12, 34) revealed that the strains possessed the 16S rRNA gene signature for psychrotolerance and the cold shock protein gene *cspA*. Thus, MC67 and MC118 should be affiliated with *B. weihenstephanensis* strains (24), and to our knowledge they are the first strains of this species that have been shown to be emetic.

Typing and sequencing of emetic *Bacillus weihenstephanensis* strains. Ehling-Schulz et al. (8) suggested that random amplified polymorphic DNA (RAPD) PCR typing may be useful for rapid identification of potential emetic strains. RAPD_1 PCR (26) and profile analysis with Bionumerics version 1.01 (Applied Maths, Kortrijk, Belgium), using the parameters described elsewhere (8), showed that MC67 and MC118 were identical but were different from the mesophilic emetic strains (Table 2) and from 20 randomly chosen nonemetic *B. weihenstephanensis* strains (results for 10 strains are shown in Fig. 1). The RAPD_1 profiles were not suitable for rapid identification of psychrotolerant emetic strains. More RAPD profiles of emetic *B. weihenstephanensis* strains from other origins are required to show whether RAPD typing is a useful screening tool for identification of potential emetic psychrotolerant strains. Sequence analysis of multiple genes of mesophilic emetic *B. cereus* originating from different countries has shown high similarity between strains (8). Analysis of PCR-amplified DNA sequences (8, 29), using Clustal W (31), of the 16S rRNA gene (1,580 nucleotides [nt]), the 16 to 23S rRNA gene spacer (791 nt), and the *spoIIIAC-spoIIIAB* sporulation gene fragments (547 nt) as well as the partial cereulide peptide synthetase gene *cesB* (1091 nt) showed that MC67 and MC118 are 100% identical. The partial *cesB* gene was amplified as proposed by Ehling-Schulz et al. (11), with the modifications of changing the annealing temperatures to 50°C during the first five cycles and increasing the last 25 cycles to 30 cycles. Purification of DNA and sequencing were as described previously (35). The GenBank accession numbers used for comparison with the 16S rRNA, the 16 to 23S rRNA, and the *spoIIIAC-spoIIIAB* gene sequences of MC67/MC118 were Z84575 to -94 and Y18473 (24); AJ577274 to -92, AJ578036, AY920248 to -50, and AY920252 to 3 (5); AY758318 to -37 and AY758342 to -49 (8); AY277557 (15); AB021199 (13); AF290547 (32); AE016877 (21); and AM062685 to -6, AE017225, AE017334, and AE017355. The sequence analysis using Clustal W (31) showed

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TABLE 1. *B. cereus* group isolates used in the emetic screening[a]

No. of strains	Origin	Site
390[b]	Sandy loam on Møn, Denmark	Soil
245	Curly kale fields[c]	Soil
196	Curly kale fields	Lower leaves
90	Curly kale fields	Upper leaves

[a] The strains were from 18 different Danish localities and were isolated from lower and upper leaves of curly kale phylloplane (*Brascia olearcea acephala*) and from soil of curly kale phylloplane fields.
[b] Ninety of these strains (including strain MC8, MC10, MC17, MC21, MC26, MC35, MC37, MC42, MC48, MC51, MC58, MC59, MC70, MC73, MC78, MC80, MC84, MC87, MC89, and MC90 used for RAPD_1 typing) were identified as *B. weihenstephanensis* (20).
[c] Curly kale fields from 17 localities throughout Denmark.

TABLE 2. Emetic *B. cereus* reference strains used

Strain[a]	Origin	Reference
F4810/72[b]	Emetic food poisoning, United Kingdom	33
NC7401[b]	Emetic food poisoning, Japan	1
NS117[c]	Spruce tree, Norway	18
F3080B/84[c]	Emetic food poisoning, United Kingdom	28
F5881[c]	Emetic food poisoning, United Kingdom	4
RIVM-BC68[c]	Feces, The Netherlands	3
B203[c]	Rice mush, Finland	22

[a] The strains are listed with original strain identification numbers.
[b] From the HAMBI Culture Collection, University of Helsinki, Helsinki, Finland.
[c] Kindly provided by Maria A. Andersson, Department of Applied Chemistry and Microbiology, Division of Microbiology, University of Helsinki, Helsinki, Finland.

that MC67 and MC118 were more related (but not identical) to the psychrotolerant *B. weihenstephanensis* and *B. mycoides* strains than to the mesophilic *B. cereus* group strains (*B. thuringiensis*, *B. anthracis*, and *B. cereus*), including the clonal group of mesophilic emetic strains (8). The 16S rRNA and 16 to 23S rRNA gene sequences of MC67 and MC118 differed by 1 and 1 to 3 nt from the respective sequences of psychrotolerant *B. mycoides* (the *spoIIIAC-spoIIIAB* sequences of *B. mycoides* are not available). The 16S rRNA, the 16 to 23S rRNA, and the *spoIIIAC-spoIIIAB* gene sequences of MC67/MC118 differed by 1 to 2, 2, and 7 nt from the respective sequences of *B. weihenstephanensis*; by 5 to 7, 19 to 23, and 49 to 61 nt from those of *B. cereus* (emetic and nonemetic); by 5 to 6, 21, and 59 nt from those of *B. thuringiensis*; and by 7 to 8, 21, and 59 nt from those of *B. anthracis*. Thus, the sequence data suggest that MC67 and MC118 are closely related to *B. weihenstephanensis* and *B. mycoides.* However, MC67 and MC118 are most likely *B. weihenstephanensis* strains, taking into consideration the colony morphology, the fact that other species of the *B. cereus* group such as *B. cereus* are heterogeneous (8) and display more or less sequence variability in similar genes between strains, and the limited number of *B. weihenstephanensis* and *B. mycoides* sequences available in the databases. The *cesB* gene, which is a peptide synthetase gene involved in cereulide production, is highly conserved (single nucleotide difference) in mesophilic emetic *B. cereus* strains, indicating a relatively recent acquisition of the emetic genes (8). Interestingly, the *cesB* gene fragment of MC67 and MC118 showed only 92% identity to the *cesB* gene from F4810/72 (GenBank accession number AY691650) (11). The translated CesB amino acid sequence was highly conserved at the N-terminal half, while the C-terminal half was variable (see Table S1 in the supplemental material). The variation in the *cesB* gene between the psychrotolerant and the mesophilic strains suggest that their separation is not a recent event. The *cesB* gene is located on a plasmid in mesophilic emetic *B. cereus* (10), and thus transfer of the emetic plasmid to other bacteria is possible and needs to be further investigated.

Examination for amylase activity, salicin fermentation, hemolysis, and enterotoxins. MC67 and MC118 differed from the mesophilic emetic isolates (Table 3) with regard to some of the traditional phenotypic characteristics of emetic strains, as analyzed by methods described elsewhere (27). Further, they differed genotypically by harboring the Hbl enterotoxin complex genes *hblA* and *hblD* (8). Our results highlight the precautions which need to be taken when screening for emetic isolates based upon phenotypic traits such as starch hydrolysis and salicin fermentation. Andersson et al. (3) proposed lack of hemolysis as an indicator for emetic strains, and this is also in accordance with our results using the proposed method (3). PCR examination for the enterotoxin genes *hblA*, *hblC*, and *hblD* was performed as described elsewhere (16). The L_2 component (HblC) could not be detected using the BCET-RPLA kit as recommended by the manufacturer (Oxoid), using a growth temperature of 32°C.

Cereulide production at different temperatures. Production of cereulide at refrigeration temperatures is critical in relation to food safety, since cereulide will not be destroyed during food processing. To evaluate the risk of cereulide production, MC67, MC118, and the mesophilic strains (Table 2) were grown aerobically on BHI agar (Oxoid) for 10 days at 8, 12, 15, and 25°C. Cereulide was extracted from bacterial mass with 96% ethanol and sonication for 30 min. Cell debris was removed at 17,000 × *g* for 5 min. Liquid chromatography–high-

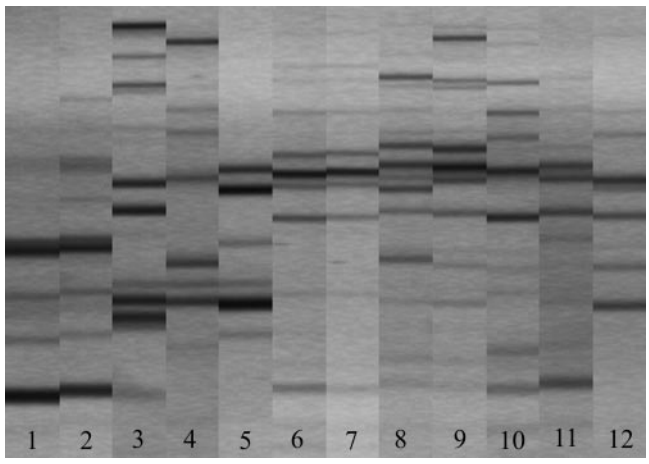

FIG. 1. RAPD_1 profiles of the emetic *B. weihenstephanensis* strains MC67 and MC118 compared to those of emetic *B. cereus* strains and nonemetic *B. weihenstephanensis* strains from a sandy loam on the island Møn in Denmark. Lanes 1 and 2, emetic *B. cereus* strains F5881 and NS117, respectively; lanes 3 to 5, nonemetic *B. weihenstephanensis* strains MC73, MC59, and MC8, respectively; lanes 6 and 7, emetic *B. weihenstephanensis* strains MC67 and MC118, respectively; lanes 8 to 12, nonemetic *B. weihenstephanensis* strains MC84, MC37, MC10, MC58, and MC17, respectively.

TABLE 3. Characteristics of the emetic *B. weihenstephanensis* strains MC67 and MC118 compared to those of emetic *B. cereus* reference strains

Strain(s)	Reaction[a]						
	Phenotypic tests			Hbl complex			
	Salicin fermentation	Starch hydrolysis	Hemolysis	BCET-RPLA[b] L_2 component	*hblA*	*hblC*	*hblD*
MC67	+	+	−	−	+	−	(+)[c]
MC118	+	+	−	−	+	−	(+)[c]
F4810/72, NC7401, NS117, F3080B/84, F5881, B203, RIVM-BC68	−	−	−/(+)	ND	−	−	−

[a] +, positive reaction; (+), weak positive reaction; −, negative reaction; ND, not determined.
[b] BCET-RPLA, *Bacillus cereus* enterotoxin-reversed passive latex agglutination assay.
[c] Results were variable.

resolution mass spectrometry (LC-HR-MS) for verification and quantification of cereulide was performed essentially as described elsewhere (17), using the equipment described previously (25) (for details, see Appendix). The MS in-source fragmentation spectrum which was obtained from the ethanol extracts of MC67 and MC118 could be superimposed on the spectrum of the emetic reference strain F4810/72. All the ca. 40 major ions originating from cleavage of the peptide and ester bonds were in the same ratios (results not shown), which indicates that the compound produced by MC67 and MC118 is similar to cereulide. The biological activity of cereulide produced by MC67 and MC118 was confirmed by measurement of the metabolic activity of Chinese hamster ovary (CHO-K1) cells upon exposure to heated ethanol extracts (heated for 10 min at 100°C) as described elsewhere (6), using the WST-1 cell proliferation assay as described by the manufacturer (Roche, Hvidovre, Denmark). MC67 and MC118 were the only emetic strains that were able to grow and produce cereulide at 8°C (Table 4). Compared to the mesophilic strains, MC67 and MC118 produced large amounts of cereulide at 25°C, which indicates no coherence between temperature growth profile and cereulide production (Table 4). Cereulide was not produced at critical concentrations for food poisoning (23) at temperatures of 8 to 15°C. However, unknown factors, such as temperature abuse, the food matrix, and interactions with other bacteria, which were not tested in this work might provoke cereulide production. Therefore, the risk of food poisoning from psychrotolerant emetic strains in refrigerated foods needs to be further investigated.

TABLE 4. Cereulide production by *B. weihenstephanensis* MC67 and MC118 compared to that of reference strains of emetic *B. cereus*[a]

Strain	Cereulide production (μg/g biomass [wet wt]) at:			
	8°C	12°C	15°C	25°C
MC67	0.1	0.3	2.1	530
MC118	0.1	0.1	1.4	606
F4810/72	NG[b]	1.2	0.4	95[d]
NC7401	NG	1.0	0.3	117
NS117	NG	0.3	0.2	353
F3080B/84	NG	NG	0.4[c]	94[e]
F5881	NG	1.7	0.8	211
RIVM-BC68	NG	0.2	0.1	24
B203	NG	1.7	1.1	62

[a] Strains were grown on BHI agar and incubated aerobically for 10 days at four different temperatures, and cereulide production was determined by LC-HR-MS.
[b] NG, no growth.
[c] Standard deviation, 0.1 μg/g.
[d] Standard deviation, 18 μg/g.
[e] Standard deviation, 76 μg/g.

Nucleotide sequence accession numbers. The identical sequences of MC67 and MC118 were deposited in GenBank under accession numbers DQ345789, DQ345790, DQ345791, and DQ345792 for *spoIIIAC-spoIIIAB*, *cesB*, the 16S rRNA gene, and the 16 to 23S rRNA gene, respectively.

APPENDIX

LC-HR-MS was performed using an Agilent Zorbax SB-CN column (150 by 2 mm [inner diameter] by 5 μm) and the equipment described previously (25). A linear water-CH_3CN gradient system (H_2O buffered with 10 mM $HCOONH_4$ and 20 mM HCOOH and CH_3CN buffered with 20 mM HCOOH) at a flow rate of 0.3 ml/min was used, starting at 50% CH_3CN, increasing to 100% for 12 min, and staying at 100% for 3 min before reverting to the starting conditions. Samples were analyzed in electrospray ionization positive mode at a resolution of >7,000 (half peak height) (25) and with data being centroid spectra from *m/z* 200 to 1,500. Three scan functions (1 s each) were used: (i) with a potential difference between the skimmers of 50 to 60 V (no fragmentation), (ii) with a difference of 100 to 125 V (high fragmentation), and (iii) the spray from the lock spray probe (second electrospray ionization spray) for on-line mass correction. The responses of valinomycin and cereulide in LC-HR-MS have been shown to be very similar (17). Valinomycin was used as an internal standard at 0.82 μg/ml. Cereulide and valinomycin were detected from the first scan function of their reconstructed ion chromatograms ($\pm m/z$ 0.05) of the ammoniated adducts $(M + NH_4)^+$ at *m/z* 1,170.7125 and 1,128.6655, respectively. The detection limit (on column, first scan function) for valinomycin was ca. 80 pg/2 μl at a signal-to-noise ration of 10. The identity of cereulide in the samples was confirmed from the second scan function which gave significant in-source fragmentation (>40 ions) to validate the primary structure of the depsipeptide.

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