A New Phylogenetic Cluster of Cereulide-Producing *Bacillus cereus* Strains[▽]

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Phenotypic and molecular studies have established that cereulide-producing strains of *Bacillus cereus* are a distinct and probably recently emerged clone within the *Bacillus* population. We analyzed a set of *B. cereus* strains, both cereulide producers and nonproducers, by multilocus sequence typing. Consistent with earlier reports, nonproducers demonstrated high heterogeneity. Most cereulide-producing strains and all flagellar antigen type H1 strains were allocated to the known sequence type of exclusively emetic *B. cereus* strains. Several cereulide-producing strains, however, were recovered at a new phylogenetic location, all of which were serotype H3 or H12. We hypothesize that the group of cereulide producers is diversifying progressively, probably by lateral transfer of the corresponding gene complex.

Bacillus cereus is often associated with food contamination, food spoilage, and food poisoning. The clinical presentation takes one of two forms, emetic and diarrheal. It is well established that the emetic type of food poisoning is associated with a single heat-stable exotoxin, cereulide (2, 8, 22). Diarrheal food poisoning, on the other hand, can be caused by several thermolabile enterotoxins (6).

Agata et al. have shown that most cereulide-producing strains belong to serovar H1, with the exception of a few that belong to serotypes H3 and H12 (1). Thus, cereulide producers appear to be a homogeneous group. Phenetic and molecular typing studies have established that cereulide-producing *B. cereus* strains belong to a highly homogeneous cluster (9), which was also suggested by earlier reports of identical ribopatterns of 16S and 23S rRNA sequences from emetic strains (5, 15, 17). Multilocus sequence typing (MLST) has assigned all analyzed cereulide-producing strains, isolated from cases of both food poisoning and the environment, to single-sequence type (ST) 26 (16). Recently, however, a few cereulide-producing strains have been reported to possess unique ribopatterns and to demonstrate a variation in the housekeeping gene *adk* (4).

This unexpected finding leaves open the question of how widely the capacity for emetic toxin production is distributed among *B. cereus* strains. Should these findings be considered minor discrepancies, or is heterogeneity among cereulide producers real? To address these questions, we analyzed *B. cereus* strains isolated from a variety of sources, both cereulide-producing and cereulide-nonproducing strains, using MLST, and compared the phylogenetic allocation of the cereulide-producing strains with their serotypes.

MATERIALS AND METHODS

Bacterial strains and serotyping. A collection of 52 strains of *B. cereus*, 25 cereulide-producing strains and 27 cereulide-nonproducing strains, was used in this study. The strains were collected from cases of emetic and diarrheal food poisoning, during regular food hygiene inspections, and from the environment (river, water, and soil sources). These strains were isolated between 1974 and 2005, mainly in Japan (Tables 1 and 2). Flagellar antigen serotyping was performed by the H antigen agglutination method described by Taylor and Gilbert (19).

Toxin analyses. Cereulide production was tested by the vacuole response assay as described by Agata et al. (3). Enterotoxin production was tested by using a reversed passive latex agglutination *B. cereus* enterotoxin test kit (Oxoid, Basingstoke, United Kingdom) for HBL (hemolytic enterotoxin), using instructions provided by the manufacturer.

Molecular analyses. DNA was extracted according to the instructions for using a Promega Wizard genomic DNA purification kit (Promega K. K., Tokyo, Japan) after culture growth in 5 ml Luria-Bertani broth. MLST was performed as described on the *B. cereus* MLST Web site (<http://pubmlst.org/bcereus>). PCR amplification was performed on an Astec PC 802 thermal cycler (Astec, Fukuoka, Japan) as follows: 94°C for 5 min, 35 cycles of denaturation at 94°C for 1 min, annealing for 1 min (the recommended annealing temperature for each primer set was used), and extension at 72°C for 1 min, with final extension at 72°C for 7 min. The PCR amplicons were extracted following agarose gel electrophoresis using a QIAquick gel extraction kit (QIAGEN K.K., Tokyo, Japan). Sequencing was performed using a GenomeLab dye terminator cycle sequencing Quick Start kit (Beckman Coulter, Fullerton, CA) with the same primers used for the amplification, in both forward and reverse directions, and the reaction products were separated using a Beckman Coulter CEQ 2000 XL DNA analysis system (Beckman Coulter, Fullerton, CA). DNA sequences were assembled using Sequencher software (Gene Codes, Ann Arbor, MI).

Data analyses. The completed sequences were compared with those in the *B. cereus* MLST database. All new sequences were submitted to the database, and relevant data are available at <http://pubmlst.org/bcereus>. Concatenated sequences of all STs were downloaded from the database, and a neighbor-joining (NJ) tree was constructed using Clustal X (20) and visualized using Njplot (14). Split decomposition analysis was performed using the Web version of Splits Tree software on the MLST Web site (<http://pubmlst.org>) (12). eBURST analysis was performed using the Web tool at <http://www.mlst.net/> (18).

RESULTS

All cereulide-producing strains of *B. cereus* were serotyped into one of three groups, H1, H3, and H12. In contrast, many cereulide-nonproducing strains could not be assigned to any of the 18 established serotypes described by Taylor and Gilbert

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TABLE 1. Characteristics of cereulide-nonproducing strains of *B. cereus*

Strain ID	Source ^a	Year	Origin	Flagellar antigen ^b	Enterotoxin production
NC 1273	Food*	1993	Switzerland	ND	+
NC 1275	Food*	1991	India	ND	+
NC 1272	Food*	1993	Switzerland	H11	+
NC 1263	Food*	1989	India	H11	+
NC 1269	Food*	1991	India	ND	+
B-4ac	Stool*	Unknown	Belgium	ND	+
PHLS 2668	Food*	1990	Netherlands	H11	+
NC 1212	Stool	1996	Japan	ND	+
NC 1216	Food	1996	Japan	ND	+
NC 1225	Stool	1996	Japan	ND	+
NC 1235	Unknown	1996	Japan	ND	+
NC 1292	Food	1999	Japan	ND	+
NC 382	Stool	1982	Japan	ND	+
NC 383	Stool	1982	Japan	H11	+
NC 390	Stool	1982	Japan	ND	+
NC 392	Stool	1982	Japan	H11	+
NC 401	Food	1982	Japan	H14	+
NC 422	Stool	1982	Japan	ND	+
NC 466	Food	1982	Japan	ND	+
NC 478	Food	1983	Japan	H11	+
NC 492	Food	1983	Japan	ND	+
NC 499	Food	1983	Japan	ND	+
NC 536	River	1983	Japan	ND	+
NC 537	River	1983	Japan	ND	+
NC 538	River	1983	Japan	ND	+
NC 543	Food	1983	Japan	ND	+
NC 545	Food	1983	Japan	H11	+

^a * indicates strains are from cases of diarrheal food poisoning; entries without asterisks indicate that strains were obtained from regular analyses of food handlers and commercial food.

^b ND, not detected, i.e., did not fall into any of the 18 serotypes described by Taylor and Gilbert (17).

(19), except for a few strains belonging to serotype H11. When tested for enterotoxin production, all cereulide-nonproducing strains were HBL positive, whereas all cereulide-producing strains were HBL negative (Table 1 and 2).

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

Strain ID	Source ^a	Year	Origin	Flagellar antigen	Enterotoxin production
NC 7401	Stool/food*	1974	Japan	H1	—
G 0501	Vomit/food*	2005	Japan	H1	—
NC 1247	Food*	1996	Japan	H1	—
NC 1291	Food*	1999	Japan	H1	—
NC 1363	Stool*	2001	Japan	H1	—
NC-G15	Stool*	1995	Japan	H1	—
NC-I 55	Stool*	1980	Japan	H1	—
NC 92	Stool	1978	Japan	H1	—
NC 199	Environment	1979	Japan	H1	—
NC 444	Environment	1982	Japan	H1	—
NC 524	Environment	1983	Japan	H1	—
NC 529	Environment	1983	Japan	H1	—
NC 530	Environment	1983	Japan	H1	—
NC 541	Environment	1983	Japan	H1	—
NC 604	Environment	1990	Japan	H1	—
NC 622	Environment	1990	Japan	H1	—
NC 1237	Food	1996	Japan	H1	—
NC 1239	Food	1996	Japan	H1	—
NC 1274	Vomit*	1997	India	H3	—
NC-std	Food	Unknown	UK	H3	—
NC 526	Environment	1983	Japan	H3	—
NC 610	Environment	1990	Japan	H3	—
NC 1276	Food	1997	India	H3	—
NC 111	Food	1983	Japan	H12	—
NC 113	Environment	1983	Japan	H12	—

^a * indicates that strains were obtained from cases of emetic type food poisoning; entries without asterisks indicate that strains were obtained from regular analyses of food handlers and commercial food. Environment refers to strains found in stream water, soil, and lake water sources.

TABLE 3. Changes in the allelic profiles of cereulide-producing strains of *B. cereus*

No. of strains	Serotype	ST	Allelic profile						
			<i>glp</i>	<i>gmk</i>	<i>ilv</i>	<i>pta</i>	<i>pur</i>	<i>pyc</i>	<i>tpi</i>
16	H1	26	3	2	31	5	16	3	4
1	H1	165	3	44	31	5	16	3	4
5	H3	144	67	2	63	5	36	3	4
2	H12	164	3	2	63	5	36	3	4

MLST revealed that cereulide-nonproducing strains were highly diverse, and 13 new STs were identified (data not shown). Four strains were allocated to ST 142. Another six strains were recovered in ST 24, and four of them were serotype H11, but not all H11 strains were ST 24. MLST analysis of cereulide-producing strains separated them into four STs (Table 3). Sequence types 165, 164, and 144 were new to the database. Two of these STs (ST 165 and 144) were single-nucleotide variants of their parent types, ST 26 and ST 164, respectively (data not shown). It is noteworthy that all strains allocated to ST 26 and its variant ST 165 were serotype H1. All strains in ST 144 were serotype H3, and all strains in ST 164 were serotype H12 (Table 3).

Phylogenetic analysis placed all emetic toxin-producing strains in Clade 1, as designated by Priest, et al. (16), clustered at two distinct loci: the previously known ST 26 with the newly identified variant ST 165 and the new ST 144/ST 164 locus (Fig. 1). The new cluster ST 144/ST 164 is located in the neighboring lineage to *B. anthracis*, distinct from the previously identified cluster at ST 26. Cereulide-nonproducing strains, from both the environment and from cases of food poisoning, were distributed in Clade 2, as designated by Priest, et al. (16), with only two exceptions. These strains had no significant relationships with each other and did not form a recognizable cluster (Fig. 2). We further subjected the group of emetic strains to eBURST analysis (implementation of the BURST [based upon related sequence types] algorithm) to look for phylogenetic links in their recent evolutionary history (18). However, the two clusters did not link even when the number of identical loci for group definition was decreased from the standard 6 out of 7 to 3 out of 7 (Fig. 3).

DISCUSSION

Cereulide-nonproducing strains of *B. cereus* isolated from cases of food poisoning or the environment showed a high level of genetic diversity. Consistent with previously published results (16), they were distributed mainly in Clade 2, in close phylogenetic relationship to *Bacillus thuringiensis*. On the contrary, published reports have thus far assigned all analyzed cereulide-producing strains to a single phylogenetic cluster (9, 16). Based on MLST analysis, these strains have been assigned exclusively to ST 26 (16). Our study identified a strain with a new single mutation in ST 26, creating the new variant ST 165. We also recovered several cereulide-producing strains whose genetic sequences were at a location distinct from ST 26, in the lineage most closely related to the lineage populated by *B. anthracis*. Analysis by eBURST showed that this second cluster of cereulide-producing strains represents an independent

an effort to shed some light on genetic diversity in a geographically distinct region. Interestingly, all non-Japanese strains in our collection (total of three) fell into the newly identified cluster, together with a few Japanese strains. This distribution shows the rarity of ST 144/ST 164 strains and the predominance of ST 26 emetic strains worldwide.

Comparison of MLST analysis to flagellar typing provided a link between phylogenetic allocation and serotype. All strains of serotype H1 belonged to ST 26, while the newly identified cluster was formed exclusively of strains with serotypes H3 and H12. Our findings provide a link between phylogenetic location and flagellar serotype insofar as the clusters are distinguishable by their flagellar H serotype. It appears not only that cluster ST 26 (serotype H1) strains outnumber those of the new cluster ST 144/ST 164 (serotype H3/H12) but also that cluster ST 26 includes almost all food poisoning-associated strains. This correlates with the observations by Agata et al. that serotype H1 strains produce higher amounts of cereulide (1).

Recently published complete sequences of the cereulide synthetase gene cluster (7) and plasmid profiling work in emetic strains (11) as well as our unpublished data have shown that the operon responsible for cereulide production is situated on a large plasmid. Thorsen et al. recently reported that *B. weihenstephanensis* strains could produce cereulide (21). The fact that the capacity for cereulide production is shared by two phylogenetically distinct *B. cereus* clusters and by *B. weihenstephanensis* strains suggests that the plasmid can be subjected to lateral transfer.

It has been hypothesized that cereulide-producing strains, like *B. anthracis*, represent a recently emerged clone in the *B. cereus* population (9). So far, no substantial heterogeneity has been found among strains in the *B. anthracis* population (10, 13). The data presented here suggest that cereulide-producing strains are progressively diversifying.

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