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Identification of *Bacillus cereus* Group Species Associated with Food Poisoning Outbreaks in British Columbia, Canada▽

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Food poisoning laboratories identify *Bacillus cereus* using routine methods that may not differentiate all *Bacillus cereus* group species. We recharacterized *Bacillus* food-poisoning strains from 39 outbreaks and identified *B. cereus* in 23 outbreaks, *B. thuringiensis* in 4, *B. mycoides* in 1, and mixed strains of *Bacillus* in 11 outbreaks.

The genus *Bacillus* currently consists of at least 148 validly named species (List of Prokaryotic Names with Standing in Nomenclature—Genus *Bacillus* [http://www.bacterio.cict.fr/b/bacillus.html]). The *Bacillus cereus* group contains five species, *B. cereus* sensu stricto, *B. thuringiensis*, *B. anthracis*, *B. mycoides*, and *B. weihenstephanensis* (16, 19, 22). They are difficult to discern using standard biochemical schemes, chemotaxonomic methods, or phylogenetically relevant target genes (1, 2), and many distinguishing pathogenicity markers in this group can be attributed to mobile plasmids (18, 21, 22, 23). *B. cereus* sensu stricto carries the plasmid-borne emetic toxin cereulide (*ces*) (7, 13, 14), and *B. thuringiensis* carries insecticidal crystal protein (ICP) (*cry*) genes on one or more plasmids (3, 5, 6). The differentiation of *B. cereus* group members using molecular techniques is not routine in food-poisoning diagnostic methods and may cause underreporting of species such as *B. thuringiensis* (1, 8). In 2005, *Bacillus* was identified in a food-poisoning event implicating imported strawberries. The isolate was initially identified phenotypically as *B. cereus* and then later by PCR as *B. thuringiensis*. To assess the proportion of food poisonings caused by different *B. cereus* group species, 155 *B. cereus* group-like isolates collected from food or clinical specimens in food-borne outbreaks between 1991 and 2005 were characterized using molecular and phenotypic typing methods.

Frozen isolates were retrieved onto blood agar plates and incubated at 35°C for 24 h. Phenotypic characterization was conducted following established procedures (12). No *B. cereus* group-like bacterium reviewed here was consistent with *B. weihenstephanensis* or *B. anthracis* (data not shown).

DNA was extracted by lysing pure culture in a heating block at 102°C for 10 min. Microcentrifuged supernatant was frozen at −80°C until required. Pathogenicity genes for emetic cereulide toxin (nonribosomal peptide synthetase [NRPS]) and ICP (*cry1* or *cry2*) were detected in multiplex PCR assays (7, 10) shown in Fig. 1. Each master mix contained 0.8 μM of each primer, hot start master mix, diethyl pyrocarbonate water (20 μl), and 5 μl of DNA. The PCR products were loaded onto 2% agarose gels made with 0.5× Tris-borate-EDTA buffer and ethidium bromide (1 μg ml^{-1}). The gels were electrophoresed at 120 V for 30 min and then visualized on a Bio-Rad Gel Doc 2000.

Strains positive for NRPS were designated as *B. cereus*, those positive for ICP (by microscopy or PCR) as *B. thuringiensis*, those with rhizoidal growth on nutrient agar as *B. mycoides*, and all other strains with the typical *B. cereus* phenotype as *B. cereus* $NRPS^-$ ICP^-. PCR-negative isolates were further examined for ICP crystals using transmission electron microscopy (TEM) since *B. thuringiensis* strains may carry one to six *cry* genes and there is no universal method available to detect all *cry* genes (there are currently more than 150 *cry1* toxins) (17; *Bacillus thuringiensis* toxin nomenclature [http://www.lifesci.sussex.ac.uk/Home/Neil_Crickmore/Bt/]). Samples of *B. cereus* group bacteria were prepared for electron microscopy by fixation with 2% glutaraldehyde and 1% para-

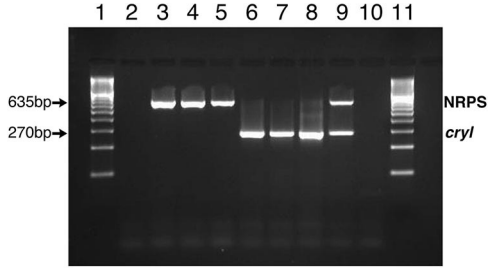

FIG. 1. Multiplex PCR of NRPS and *cry1* to distinguish between *B. cereus* and *B. thuringiensis*. Lanes 1 and 11, 100-bp ladder; lane 2, *B. cereus* $NRPS^-$ (emesis isolate 8-91-71); lane 3, *B. cereus* (fried rice isolate 28-240-5); lane 4, *B. cereus* (chow mein isolate 28-251-1); lane 5, *B. cereus* (stool isolate 36-254-1); lane 6, *B. thuringiensis* (stool isolate 36-254-2); lane 7, *B. thuringiensis* (strawberry isolate 43-05-36-1); lane 8, *B. thuringiensis* (mixed-salad isolate 41-04-259-1); lane 9, positive control; lane 10, negative control.

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TABLE 1. *Bacillus* food-borne illness outbreaks and isolate summary relating to *Bacillus* species retrieved from cryopreservation in this study

Species	No. of outbreaks	No. of people at risk	No. of ill people	No. of *Bacillus*-positive food specimens	No. of *Bacillus*-positive clinical specimens	No. of isolates tested
B. cereus NRPS$^+$	5	14	12	9	0	7
B. cereus NRPS$^-$ ICP$^-$	18	518	115	36	4	55
B. thuringiensis	4	22	14	5	0	20
B. mycoides	1	2	2	3	0	2
Mixed *Bacillus* spp.	11	78	57	38	3	71[b]
Total *Bacillus* species[a]	39	634 (18, 3)	200 (5.5, 2)	91 (2.3, 2)	7 (0.2, 0)	155 (4, 2)

[a] Values in parentheses are the average and median, respectively.
[b] *Bacillus* species identified: *B. cereus* NRPS$^+$, $n = 31$; *B. cereus* NRPS$^-$ ICP$^-$, $n = 35$; *B. thuringiensis*, $n = 3$; and *B. mycoides*, $n = 2$.

formaldehyde, adsorbed to a glow-discharged carbon-coated Formvar film on a 400-mesh copper grid for 1 min, and negatively contrasted with 2% methylamine vanadate (NanoVan; Nanoprobes, Yaphank, NY). Specimens were imaged in an FEI Tecnai 20 TEM operated at 200 kV at nominal instrument magnifications of 5,000 to 9,600 times with image acquisition done using an AMT Advantage XR 12 charge-coupled-device camera. The majority of the 155 food-poisoning isolates were identified as *B. cereus* NRPS$^-$ ICP$^-$ (58%, $n = 90$), as no plasmid markers for emetic toxin or ICPs were detected or visualized. Thirty-eight (24.5%) isolates were identified as *B. cereus* NRPS$^+$, and 23 (15%) isolates identified as *B. thuringiensis* were positive for either *cry1*, *cry2*, or both (in one case, ICPs were detected only by TEM). A small number (2.6%) were identified as *B. mycoides* based on rhizoid growth demonstrated on nutrient agar. In summary (Table 1), *B. cereus* isolates were identified in 23 of the 39 outbreaks (*B. cereus* NRPS$^+$ in 5 [12.8%] and *B. cereus* NRPS$^-$ ICP$^-$ in 18 [46.1%]), *B. thuringiensis* in 4 (10.3%), *B. mycoides* in 1 (2.6%), and mixed species of *Bacillus* in 11 (28.2%).

Records of food-poisoning investigations were reviewed, and epidemiological information included the number of people that ate the implicated meal (at risk), the number ill, and the onset and duration of the illness and symptoms. Data were available for 108 individuals, collected in 37 of the 39 investigations. The attack rate was 32%, the gender distribution was equal, and the mean age was 37 years (standard error, 1.9; median, 37 years; range, 2 to 75 years). The average time to symptom onset was 6 h, with the duration ranging from 1 to 144 h (median, 16 h). The predominant symptoms are shown in Table 2. When symptom data from ill individuals were separated by *Bacillus* species identified (excluding outbreaks involving mixed *Bacillus* species), the symptom profiles between species were significantly different (Pearson's coefficient, $P < 0.0001$). There was a significant correlation found between the symptom of vomiting and the identification of *B. cereus* NRPS$^+$ isolates (Fisher's exact test [right-tailed], $P = 0.038$). This was not true when NRPS gene presence was compared to any other symptom. Twenty-five (64%) investigations were traced back to the consumption of Asian foods, with 65% associated with restaurant foods, 17% with foods obtained at retail stores, and 7.5% with foods prepared at home. Raw foods accounted for 11.5% of the food poisonings and included fruit, green salads, and raw oysters.

Multiple *Bacillus* species were detected in more than 25% of the outbreaks. On average, four *Bacillus*-positive isolates were collected in each investigation (median, two isolates). If additional isolates for each sample had been collected and cryopreserved, more heterogeneous *Bacillus* populations in foods and clinical samples could potentially have been detected. In this study, 23 *B. thuringiensis* isolates were identified as the only *Bacillus* spp. associated with four food-poisoning outbreaks. The initial incorrect isolate identification may have occurred from a failure to test for ICP or from the failure of the culture to produce recognizable ICP under light microscopy. Standard methods that either do not differentiate *B. cereus* from *B. thuringiensis* (8) or specify that a 3- to 7-day culture followed by staining and microscopic examination for ICP be performed (12) are not as sensitive or rapid as detecting *cry* genes by PCR (unpublished data) to discriminate between these *Bacillus* species. Although *B. thuringiensis* is not considered a food-borne pathogen (11, 24), and is rarely found linked to food-borne (15) or other (4, 8, 20) human illness this pesticide is currently under review by the European Union (9). This study suggests that there is an association between this bacterium and previously recognized food-borne gastrointestinal illnesses.

The rapid identification of *Bacillus* species implicated in food poisonings can be facilitated by PCR. We recommend the use of PCR in tandem with phenotypic tests to assist in the

TABLE 2. Symptom profiles for ill individuals associated with *Bacillus* species identified in food-borne illness outbreaks

Species	% of individuals with indicated symptom						
	Nausea	Diarrhea	Abdominal cramps	Vomiting	Fever	Headache	Prostration
All *Bacillus* species ($n = 108$)	61	67	82	74	17	5	6
B. cereus NRPS$^+$ ($n = 12$)	50	50	92	67	0	0	25
B. cereus NRPS$^-$ ICP$^-$ ($n = 41$)	54	68	83	63	5	10	29
B. mycoides ($n = 2$)	0	100	100	0	100	0	0
B. thuringiensis ($n = 13$)	62	54	46	85	8	8	0

identification of all *B. cereus* group species implicated in foodborne illnesses.

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