Physical Characteristics of Spores of Food-Associated Isolates of the Bacillus cereus Group

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All 47 food-borne isolates of Bacillus cereus sensu stricto, as well as 10 of 12 food-borne, enterotoxigenic isolates of Bacillus thuringiensis, possessed appendages. Spores were moderately to highly hydrophobic, and each had a net negative charge. These characteristics indicate that spores of food-associated B. thuringiensis and not only B. cereus sensu stricto have high potential to adhere to inert surfaces.

Bacillus cereus is a worldwide food-borne pathogen causing diarrheal or emetic-type illnesses. The presumptive toxins have been identified in each case (2, 9, 11). We recently reported that the diarrheal type was the more common toxigenic type in U.S. retail rice and seafood (5, 24). Spores of B. thuringiensis and B. mycoides were also isolated from rice. Best known for the insecticidal activity of its parasporal crystals, Bacillus thuringiensis has been associated with gastroenteritis and isolated in rare cases from outbreaks of food-borne illness (17). Isolates of B. thuringiensis, including those isolated from commercial insecticides, have been shown to produce one or both of the enterotoxins HBL and NHE (6, 14, 18, 22, 23, 28).

Bacterial spores can adhere to inert surfaces of food processing equipment due to their surface properties and structures (8, 29). Spores of certain Clostridium and Bacillus species possess appendage-like structures (1, 12, 15, 19) which may contribute to biofilm formation (3, 30). Previous studies of the physical properties of spores of the B. cereus group have focused primarily on environmental isolates. Here the spore morphology, hydrophobic characteristics, and net negative charge of food-borne and food poisoning-associated isolates of the B. cereus group were investigated including potentially enterotoxigenic B. thuringiensis.

The diarrheal-type B. cereus strains 85 and 133, B. thuringiensis strains 105 and 129, and B. mycoides strain 157 isolated from rice were examined in detail in this study. These B. cereus and B. thuringiensis strains were selected on the basis of the presence of the nhe or hbl gene along with the ability to produce the corresponding gene product at elevated titers as previously described (5). Bacillus subtilis (ATCC 6633) was used as a comparative reference in the surface charge and hydrophobicity studies. In addition to the above isolates, the presence of appendages on spores of the following was also examined by negative staining: 32 isolates of diarrheal B. cereus isolated from seafood (24), an NHE-positive control strain (ATCC 1230/88), 12 emetic-type B. cereus isolates from food poisoning outbreaks (20), and 10 food-borne isolates of B. thuringiensis (in addition to the above two) (5). Spores were produced as previously described (4).

The spores of B. thuringiensis were separated from the inclusion bodies (IB) by centrifuging in step gradients of 0.6, 1.0, 1.4, and 1.8 g/ml sucrose. Cleaned spore suspensions in sodium-potassium phosphate buffer were diluted to an A500 of 0.4. Two to three ml of the diluted suspension was layered on top of the gradient. The gradients were centrifuged for 2.5 h in a swinging bucket rotor (Dupont-Sorvall) at 450 × g at 10°C. A visible white layer of spores was collected from the bottom with a Pasteur pipette. The layer was washed with 0.85% saline (at least twice) and stored in the same at 4°C. Following these procedures, spore suspensions had <20% inclusions (relative to spores) as observed by phase-contrast microscopy.

Spore hydrophobicity was measured using the bacterial adhesion to hydrocarbon (BATH) assay (26) modified from the observations reported elsewhere (16). The mean and the standard error were calculated from a minimum of seven measurements. ζ potentials of the spores were measured in a Malvern Zetasizer model nano 2S (Malvern Instruments, Westborough, MA) using the Smoluchowski equation (26). The spores were suspended in saline (0.15 M) at a pH of 6 to 7. The ζ potentials were determined from a mean value of five measurements.

Spores of two diarrheal B. cereus strains, two B. thuringiensis strains, and one B. mycoides strain were examined by transmission electron microscopy (TEM). Appendages were observed on B. cereus (Fig. 1a, b, and c) and B. thuringiensis (Fig. 1d and e) but not B. mycoides (Fig. 1f). In contrast, exosporia were seen in all the isolates examined (Fig. 1). Similar observations for environmental and clinical samples of these species have been reported by others (13, 16, 21, 29). The number of appendages observed here varied among strains. In the case of B. cereus these ranged from three to four (isolate 133) to four to nine (isolate 85). On the other hand, B. thuringiensis 129 had a higher number (12 to 18) of appendages per spore. The appendage length for B. cereus varied from 0.45 to 3.8 µm. Appendages appeared tube-like in appearance, with an average diameter of 13.6 nm (Fig. 1b) as determined by Image J software (http://rsb.info.nih.gov/ij). For each species, examined appendages were often lophotrichous (Fig. 1e) though peritrichous appendages were more common. All 35 food-borne B. cereus isolates

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examined in this study and one *B. cereus* NHE control strain possessed appendages, as did 12 of 12 food poisoning-associated, emetic-type *B. cereus* isolates. Appendages are a common but not universal feature of the *B. cereus* group. Whether the number and length of spore appendages of the *B. cereus* group are species associated or due to their fragility and loss during the preparation procedures (16, 30) remains a possibility. That all 47 *B. cereus sensu stricto* isolates examined here possessed spore appendages suggests that these structures are characteristic of this species. There is some controversy as to their role in adhesion (27).

Large differences in the relative hydrophobicity of five food-borne isolates representing three *Bacillus* species of the *B. cereus* group were not observed among the species examined (Table 1). The hydrophobicity values for the isolates tested were in a narrow range of 42.4 to 55.6%. Similar values for spores of *B. cereus sensu stricto* have been reported by others (16). *B. subtilis* ATCC 6633 was included for comparative purposes. Its relative hydrophobicity (Table 1) was the lowest among spores examined and similar to that reported by Husmark and Ronner (16) for this strain. Using the same procedures, Doyle et al. (7) reported adherence values of 38.3% and 61.4% for two isolates of *B. thuringiensis*, compared to 42.4% and 55.6% observed here for *B. thuringiensis* (Table 1). From

FIG. 1. Electron micrographs of *Bacillus cereus* group spores. (a) Shadowed image of *B. cereus* 85 showing appendages and exosporium; (b) appendage alone; (c) *B. cereus* 133 showing appendage and exosporium; (d) *B. thuringiensis* 129 showing appendage, exosporium, and inclusion; (e) negative stain of *B. thuringiensis* 129 showing lophotrichous appendages; (f) shadowed image of *B. mycoides* 157 showing exosporium and lack of appendages.
the values obtained here, our isolates can be classified to be moderately to highly hydrophobic. Exosporia have been proposed to be responsible for surface hydrophobicity of spores (16, 25). As mentioned above, exosporia were observed here in all isolates examined.

A lower charge indicates a higher adhering ability of spores (10, 16). The electrophoretic mobility of spores was determined by the ζ potential (Table 1). The greatest negative charges of −12.8 (strain 129) and −12.2 (strain 105) were observed among B. thuringiensis isolates, followed by −10.9 (strain 133) and −10.6 (strain 85) among the two strains of B. cereus. B. mycoides had a slightly less negative charge (−8.2). No significant difference (P > 0.05) in the net charge was observed here between B. thuringiensis isolates before and after separation of inclusions (not shown). B. subtilis ATCC 6633 was included for comparative purposes and was the most strongly negatively charged as determined by the ζ potential (Table 1).

The results obtained here indicate that the physical characteristics of Bacillus cereus spores are independent of the source, i.e., food versus environmental. Further, to our knowledge, this is the first report of appendages on food-associated, potentially enterotoxigenic B. thuringiensis. The spore surface properties of isolates of B. cereus sensu stricto described herein, as well as those of B. thuringiensis, i.e., an exosporium, low ζ potential, hydrophobicity, and the presence of appendages, may account for the persistence of B. cereus on food processing equipment and likely contribute to biofilm formation on food contact surfaces.

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REFERENCES


TABLE 1. Relative hydrophobicity and charge of spores of food-borne isolates of the B. cereus group

<table>
<thead>
<tr>
<th>Species and strain</th>
<th>Relative hydrophobicity (% ± SE)</th>
<th>Zeta potential (mV ± SD)</th>
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<tr>
<td>B. cereus 85</td>
<td>44.4 ± 4.9</td>
<td>−10.6 ± 1.04</td>
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<tr>
<td>B. cereus 133</td>
<td>51.9 ± 4.4</td>
<td>−10.9 ± 0.63</td>
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<tr>
<td>B. thuringiensis 105</td>
<td>55.6 ± 3.0</td>
<td>−12.2 ± 3.23</td>
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<tr>
<td>B. thuringiensis 129</td>
<td>42.4 ± 4.2</td>
<td>−12.8 ± 2.55</td>
</tr>
<tr>
<td>B. mycoides 157</td>
<td>51.5 ± 3.8</td>
<td>−8.18 ± 0.94</td>
</tr>
<tr>
<td>B. subtilis ATCC 6633</td>
<td>14.1 ± 2.18</td>
<td>−26.8 ± 0.71</td>
</tr>
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