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Biofilm Formation and Cell Surface Properties among Pathogenic and Nonpathogenic Strains of the *Bacillus cereus* Group▽†

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Biofilm formation by 102 *Bacillus cereus* and *B. thuringiensis* strains was determined. Strains isolated from soil or involved in digestive tract infections were efficient biofilm formers, whereas strains isolated from other diseases were poor biofilm formers. Cell surface hydrophobicity, the presence of an S layer, and adhesion to epithelial cells were also examined.

The *Bacillus cereus* group includes *B. cereus* sensu stricto, *B. anthracis*, and *B. thuringiensis*, three genetically close pathogenic species. Based on genetic evidence, it has been suggested that they could represent one species (7). *B. cereus* sensu stricto is itself an opportunistic human pathogen occasionally found to cause various diseases such as endophthalmitis or periodontitis but is more frequently involved in gastrointestinal diseases with diarrheal or emetic syndromes (4, 12). Emetic syndromes result from the presence of cereulide, a heat-stable toxin produced in food before ingestion, whereas diarrheal syndromes require survival of the bacterium in the host digestive tract. *B. thuringiensis* is an insect pathogen, and *B. anthracis* causes anthrax, a lethal human disease.

The persistent contamination of industrial food processing systems by *B. cereus* (12) may facilitate its involvement in gastroenteritis. This persistence is due to spores, which may survive pasteurization, heating, and gamma-ray irradiation (9, 13), and to biofilms, which have been shown to be highly resistant to cleaning procedures (18). Biofilms are also suspected to be involved in bacterial pathogenicity, as they may form on host epithelia (15).

In this study, we wanted to test whether biofilm formation by species of the *B. cereus* group could be connected to the pathogenicity of the bacterium. For this purpose, we screened a collection of 102 pathogenic (diarrheal, emetic, and oral diseases) and nonpathogenic strains of *B. cereus* and *B. thuringiensis* for their capability to form biofilms. As adhesion to inert or living surfaces is a prerequisite for biofilm formation, we have investigated relationships within our collection of strains between biofilm formation and cell surface hydrophobicity, the presence of an S-layer, or adhesion to epithelial cells.

Biofilm-forming capacity in microtiter plates. *B. cereus* and *B. thuringiensis* strains were assayed for biofilm formation by the use of 96-well polyvinylchloride (PVC) microtiter plates (Falcon 35911) and LB medium containing bactopeptone at 30°C as described earlier (2). Under these conditions, the sporulation level was less than 10% after 72 h of culture. Biofilms were stained with crystal violet, and the dye was subsequently solubilized (2). The optical density at 595 nm (OD_{595}) of the solubilized dye ranged from 0.0 to 2.8, and the OD_{595} threshold value over which strains were considered to be significant biofilm formers was fixed at 0.5. As shown earlier, strong differences between strains with respect to biofilm formation were found (27). However, grouping strains according to their origins revealed significant differences in biofilm production between groups (Fig. 1A). After 72 h of incubation, 37.5% to 47% of *B. thuringiensis* and nonclinical and diarrheal *B. cereus* strains had formed biofilms whereas none of the emetic and oral diseases strains had done so, although some emetic strains formed small transient biofilms after 24 h (see Table S2 in the supplementary material). The homogeneity of the emetic group can be explained by its clonal population structure (5). Strains causing oral diseases were all collected from patients with periodontitis disease, with the exception of one strain (AH817) collected from a lichen planus (see Table S1 in the supplementary material). These strains were genetically close but belonged to at least four different serotypes (7). Nonclinical strains of *B. cereus* were isolated as spores from soil samples and might have included pathogens or symbionts of the insect gut (8). Biofilms may be beneficial to entomopathogenic or diarrheal *B. cereus* or *B. thuringiensis* strains by conferring protection against antimicrobial agents and enhancing persistence in the host digestive tract. In contrast, emetic strains are not gut colonizers, as the cereulide is produced outside from the host. Similarly, periodontal strains do not need to form biofilms de novo on host tissues, as they are not involved in dental plaque initiation (10).

Presence of an S layer. The S layer, a regularly ordered protein layer, is the outermost cell envelope component of

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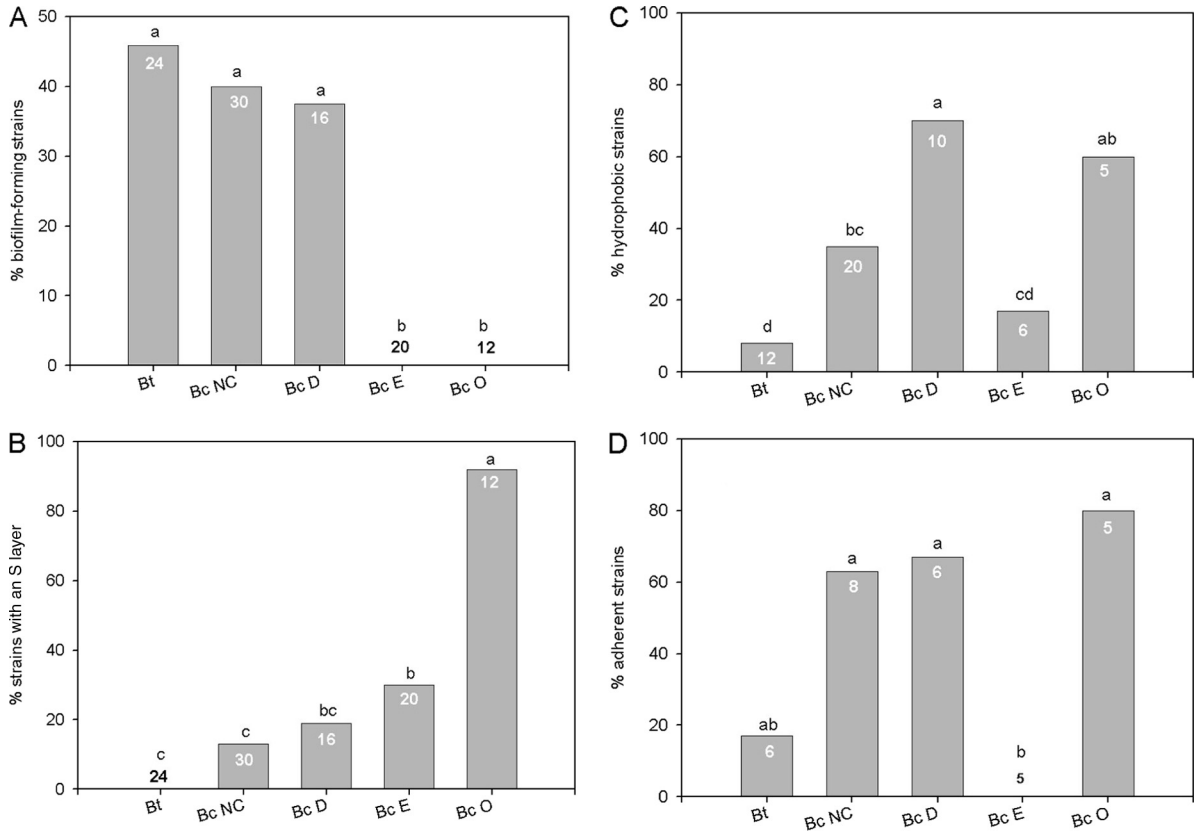

FIG. 1. Bar plots of the frequencies of phenotypes in the different *B. thuringiensis* and *B. cereus* groups. (A) Frequencies of strains found to have formed biofilms (after 72 h of incubation) with an OD_{595} of >0.5 after crystal violet staining. (B) Frequencies of strains displaying an S layer. (C) Frequencies of strains with a hydrophobicity index (LnK) greater than 3. (D) Frequencies of strains adherent to HeLa cells. Figure symbols: bars sharing the same letters (a, b, and c) represent results that were not significantly ($P < 0.05$) different, as determined by a c^2 test; the numbers within bars indicate sample sizes. Bc, *B. cereus*; Bt, *B. thuringiensis*; NC, nonclinical; D, diarrheal; E, emetic; O, oral.

numerous archaea and bacteria (22). The presence of an S layer has been described for *B. anthracis* (6, 16) and for some *B. cereus* and *B. thuringiensis* strains (11, 14, 17, 24). The presence of the S layer was assayed using the 102 strains tested for biofilm formation by Western blot analysis as described previously (16). None of the 24 *B. thuringiensis* strains tested here possessed an S layer; however, of the 12 *B. cereus* oral disease strains, all 11 of the periodontal strains, but not the lichen planus strain, had one. Between these extremes, from 13% to 30% of the nonclinical, diarrheal, and emetic *B. cereus* strains exhibited an S layer. The differences were significant (Fig. 1B), showing that the presence of the S layer was dependent upon strain origin. The S-layer presence was negatively correlated with biofilm formation in PVC microtiter plates (Pearson coefficient $r = -0.28$ [$P < 0.01$]).

Cell surface hydrophobicity. The hydrophobic properties of planktonic cells grown in LB medium at 30°C and harvested in the early stationary phase at an OD of 3.0 were determined for a subset of 53 strains selected from the 102 strains screened for biofilm formation and S-layer presence. We used an assay examining bacterial adhesion to hydrocarbon (3, 21), which measures the distribution of cells between an aqueous and a hydrophobic phase. The equilibrium constant K was calculated as previously described (21) and is expressed as the hydrophobicity index LnK, which spanned a range from −0.38 to 4.58. A strain was considered hydrophobic when the LnK value was greater than 3. Whereas 70% of the diarrheal isolates displayed a hydrophobic surface, only 17% of the emetic strains were hydrophobic, and this difference was significant (Fig. 1C). For the *B. thuringiensis*, nonclinical *B. cereus*, and *B. cereus* oral disease groups, the frequencies of hydrophobic strains were 8%, 35%, and 60%, respectively. Therefore, the diarrheal and oral diseases groups, both of which are involved in mammal tissue infections, displayed the highest hydrophobic scores. The S layer has been suggested to play an important role in attachment to surfaces (23) and has been reported to be involved in cell surface hydrophobicity in studies of *B. cereus* (11) and lactobacilli (25, 26). However, in our study, hydrophobicity was not positively correlated with biofilm formation in PVC microtiter plates ($r = -0.23$ [not significant]) or with S-layer presence ($r = 0.124$ [not significant]).

Adhesion of *B. cereus* and *B. thuringiensis* strains to epithelial cells. In mammals, epithelial cells are the first and major cell type encountered by microorganisms in the mucosa (20) and are the main site of host-pathogen interactions. Adhesion to HeLa epithelial cells was assessed as previously described (19) for a subset of 30 strains selected within the 102 strains screened for biofilm formation and S-layer presence. These 30 strains were all also included in the cell surface hydrophobicity assay. The level of adhesion ranged from 0.003 bacterium per

HeLa cell to 1 bacterium per HeLa cell. A strain was considered positive for adhesion to epithelial cells when more than 0.07 bacteria were found to bind to each HeLa cell. Within the oral diseases group, all the periodontal strains were positive for adhesion to epithelial cells, supporting the hypothesis that these strains may be actively involved in dental plaque development. In contrast, the emetic strains, which are not found in the host, were not adherent to epithelial cells. The difference between the *B. cereus* oral diseases and emetic groups was significant (Fig. 1D). The results determined for the *B. cereus* diarrheal and nonclinical groups, in which 67% and 63%, respectively, of the strains were positive for adhesion, were also significantly different from those determined for the emetic group. Finally, 16% of the *B. thuringiensis* strains were positive for adhesion. Whereas no correlation was found between adhesion to epithelial HeLa cells and biofilm formation in experiments using PVC microtiter plates or cell surface hydrophobicity, adhesion was positively correlated with the presence of an S layer ($r = 0.335$ [$P < 0.05$]). Previous studies, carried out using four strains, suggested that the *B. cereus* S layer may promote interactions with human polymorphonuclear leukocytes and other host tissues (11). Here we confirm this link, using 30 strains. In addition, S layers can play an important role in adhesion to extracellular matrix components such as collagens and laminin in *Lactobacillus crispatus* (1).

In conclusion, our study shows that the ability to form biofilms in PVC microtiter plates at 30°C in LB medium is strongly dependent on the strain origin in the *B. cereus* group. Strains involved in gut colonization were better biofilms formers. Cell surface hydrophobicity, the presence of an S layer, and adhesion on HeLa epithelial cells were not positively correlated to biofilm formation. However, periodontal strains exhibited specific properties compared to strains of other groups: the presence of an S layer, inability to form a biofilm, and strong adhesion on HeLa cells.

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