Identification of surface proteins involved in the adhesion of a probiotic *Bacillus cereus* strain to mucin and fibronectin

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Several *Bacillus* strains isolated from commercial probiotic preparations were identified at the species level, and their adhesion capabilities to three different model intestinal surfaces (mucin, Matrigel and Caco-2 cells) were assessed. In general, adhesion of spores was higher than that of vegetative cells to the three matrices, and overall strain *Bacillus cereus*CH displayed the best adhesion. Different biochemical treatments revealed that surface proteins of *B. cereus*CH were involved in the adhesion properties of the strain. Surface-associated proteins from vegetative cells and spores of *B. cereus*CH were extracted and identified, and some proteins such as S-layer components, flagellin and cell-bound proteases were found to bind to mucin or fibronectin. These facts suggest that those proteins might play important roles in the interaction of this probiotic *Bacillus* strain within the human gastrointestinal tract.

INTRODUCTION

Members of the genus *Bacillus* are aerobic or facultatively aerobic, endospore-forming and rod-shaped Gram-positive bacteria which are saprophytic and widely distributed in nature, particularly in the soil. One of the industrial advantages of the genus *Bacillus* is its capacity to produce spores, which can be stored for a long time over a wide range of temperatures. In addition, spores have an intrinsic resistance to industrial processes and to the conditions of the gastrointestinal tract (GIT) (Mazza, 1994).

These spore-forming bacteria do not belong to the commensal microbiota present in the human GIT. However, several health food supplements and therapeutic products that contain one or more *Bacillus* strains/species have been consumed by humans since the second half of the twentieth century (Ciffo, 1984). In fact, only a few of the 100 species contained in the genus *Bacillus* are used as probiotics in humans, such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus coagulans*, *Bacillus toyoi* (cereus), *Bacillus natto* (subtilis), *Bacillus claussii*, *Bacillus pumilus* and *Bacillus cereus* (Urdaci et al., 2004).

Many characteristics of probiotic *Bacillus* strains differ from those of other probiotic bacteria, including *Bifidobacterium* spp. and *Lactobacillus* spp. In addition, the mechanisms responsible for their beneficial effects on the host have remained relatively unexplored until fairly recently (Hong et al., 2005; Urdaci et al., 2004). For example, it remains unclear whether vegetative cells, spores or both are responsible for their probiotic effects. There is no clear evidence of GIT colonization by probiotic *Bacillus* strains, although it has been shown that a small percentage of *Bacillus* spores are able to germinate, replicate and produce new spores within the small intestine (Cartman et al., 2008; Duc et al., 2004). Moreover, spores interact with the gut-associated lymphoid tissue (GALT), and are detected in Peyer’s patches and in the cytoplasm of M cells (Duc et al., 2003). In this context, it has been shown that only the administration of *B. subtilis* in combination with *Bacteroides fragilis* led to proper development of the GALT in the appendices of germ-free rabbits. This effect was not observed with other bacterial combinations (Rhee et al., 2004). Therefore, some *Bacillus* strains may not be transient passengers through the mammal GIT, but they may have an intimate interaction with GIT cells and the resident microbiota (Hong et al., 2005).

Although being a controversial issue, adhesion to intestinal surfaces is believed to be an important probiotic char-
acteristic, as proposed by the FAO/WHO (Araya et al., 2002). This trait is often dependent on the existence of certain surface proteins, including mannoside-specific lectins or S-layers, that bind to specific receptors present in the GIT mucosa (Tallon et al., 2007). Some surface-associated proteins present in probiotic bacteria have been described and characterized in recent years, mainly in the genus Lactobacillus (Velez et al., 2007), but almost nothing is known about the genus Bacillus.

The use of Bacillus in probiotic products has raised a number of questions, including their safety (Hong et al., 2008; Sorokulova et al., 2008). Use of B. cereus as a probiotic is particularly controversial, since some non-probiotic strains produce either diarrheal or emetic syndrome, two kinds of food poisoning, by the production of up to six enterotoxins (Kramer & Gilbert, 1989). B. cereus IP5832, included in products such as Bactisubtil or Paciflor, has been used as a probiotic for many years, but it has been recently banned in Europe. However, this strain is still sold as a probiotic for humans in several countries all over the world. Nowadays, B. cereus var. toyoi is licensed by the European Food Safety Authority (EFSA) for use in animal feed (Règlement (CE) No. 1143/2007).

In the present work, the adhesion properties of 14 probiotic or potentially probiotic Bacillus strains, isolated from different products, were studied using either vegetative cell or spore preparations. The surface proteins involved in adhesion in the most adherent strain, B. cereusCH, were characterized.

**METHODS**

Bacterial strains, cell lines and growth conditions. The probiotic and non-probiotic Bacillus strains used in the present study are listed in Table 1, as well as their source of isolation and manufacturer when available. Strains were recovered by resuspension of part of the material in PBS, followed by dilution and serial plating in Mueller–Hinton broth (MH; Becton Dickinson) supplemented with 1.5 % (w/v) agar. Strains of Bacillus spp. were routinely grown aerobically at 37 °C in MH. Frozen stock cultures were stored at −80 °C in MH supplemented with 18 % (v/v) glycerol.

The Caco-2 line was routinely grown in Dulbecco’s modified Eagle’s minimal essential medium (DMEM; Sigma-Aldrich) supplemented with 10 % (w/v) heat-inactivated fetal bovine serum (GibcoBRL) (DMEM complete medium, DMEMC) plus penicillin and streptomycin (5000 IU ml⁻¹ and 5000 μg ml⁻¹; Sigma-Aldrich), at 37 °C in an atmosphere of 5 % (v/v) CO₂. Cells were cultured until confluence in 25 cm² tissue culture flasks (Nunc). For adhesion assays, 10⁵ cells per well were seeded in 12-well culture plates (Nunc) and cultivated, with a daily change of the culture medium, until confluence, which was normally achieved at 10–12 days.

Strain identification. Strains of Bacillus spp. were identified by partial 16S rDNA sequencing. Briefly, the 16S rDNA gene was amplified with the universal primers 20F (5’-AGAGTTTGATCCTGCGGAG-3’) and 1500R (5’-GGTACCTTGTTACGACTT-3’) and the sequences of the first 600 bp of the PCR products were obtained. These sequences were used to query the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/) and to retrieve the identification at the species level for each strain.

Preparation of vegetative cells. For the adhesion assays, bacteria were grown in MH at 37 °C until early stationary phase. Sporulation rate, monitored by Gram staining, was zero at this point. Growth curves and OD₆₀₀-c.f.u. ml⁻¹ equivalences were done for each strain (data not shown). Aliquots of 1 ml were sampled from the cultures and harvested by centrifugation at 6000 g for 5 min. Pellets were washed twice with 1 ml sterile PBS, resuspended in the same buffer and adjusted to approximately 2 × 10⁶ c.f.u. ml⁻¹.

Preparation of spores. For the production of spores, Difco Sporulation broth (DS) (Becton Dickinson) was employed. Cells were first grown overnight in MH and the culture was used to inoculate 150 ml DS adjusted to pH 7 at 1 % (v/v). Cultures were then incubated at 37 °C for 3–6 days and spores were harvested by centrifugation at 10 000 g for 10 min. Spores were washed ten times in ice-cold sterile distilled water and resuspended in 8 ml sterile distilled water. The adequateness of the preparations was established by stain and microscopic examination. Each spore suspension was titrated to determine the number of c.f.u. ml⁻¹ and stored at −20 °C.

Adhesion assays. Adhesion to porcine gastric mucin (Type III, Sigma-Aldrich), Matrigel (from mouse sarcoma; BD Biosciences) and Caco-2 cells, and yeast-agglutination assays were performed as described before (Tallon et al., 2007), using the following starting bacterial amounts: 10⁷ c.f.u. per well (96-well plates, Nunc) were used for mucin; 10⁶ c.f.u. per glass chamber slide (Lab-Tek Chamber Slides, Nunc) for Matrigel, and 50 bacteria per cell (12-well plates, Nunc) for Caco-2 cells.

Matrigel is a commercial preparation containing laminin and type IV collagen as major components followed by heparan sulphate proteoglycans, entactin and nidogen. Matrigel is used to form gel-like structures resembling the lamina densa zone of basement membranes (Kleinman et al., 1986). In the case of vegetative cells, the bacterial cultures used for adhesion assays were in the early stationary phase of growth, which was normally achieved after 18 h of culture. Assays were performed at least in triplicate, being done each time in duplicate, and results are expressed as the mean ± SD.

Enzymic and biochemical pretreatments of bacteria. Cells were grown to early stationary phase to avoid the presence of endospores, and 1 ml of culture was centrifuged for 5 min at 6000 g, washed with sterile PBS and resuspended in the same volume of the following treatment solutions: (i) trypsin, 2 mg ml⁻¹ in PBS; (ii) lithium chloride (LiCl), 5 M in PBS; (iii) trypsin solution followed by the lithium chloride solution and (iv) NaOH, 0.01 M. Trypsin was chosen since it digests proteins located at the bacterial surface, while LiCl and NaOH are able to extract bacterial cell-wall-associated and S-layer proteins (Messner et al., 1997). Trypsin suspensions were incubated for 1 h at 37 °C, while LiCl and NaOH treatments were performed at 4 °C for 30 min with gentle agitation. Cells were then harvested by centrifugation and washed twice in sterile PBS before being resuspended to their original volume in PBS. In all the experiments, an aliquot of each bacterial suspension without chemical or enzymic treatment was used as a control sample.

Extraction of surface-associated proteins

Vegetative cells. B. cereusCH was grown in volumes of 150 ml and incubated at 37 °C with a constant agitation at 150 r.p.m. The growth was stopped at different times (5, 7, 9, 18 and 24 h) by putting the cultures in ice, in order to collect cells at several exponential- and stationary-phase points of their growth curve. Cells were harvested by centrifugation (5000 g, 10 min) and washed once with 45 ml PBS. Surface proteins were extracted from the final pellets with 10 ml 0.01 M NaOH and suspensions were kept at 4 °C for 1 h with gentle agitation. The protease inhibitors EDTA and PMSF were added to all
the solutions at final concentrations of 5 mM and 1 mM, respectively. After treatments, cells were removed by centrifugation and supernatants were recovered and dialysed against 3 l distilled water for 48 h at 4 °C, with two water changes per day, finally being lyophilized. Total protein extracts, used as controls, were obtained by sonication in ice using a VibraCell 75021 ultrasonic processor equipped with a 3 mm microtip (Fisher Scientific Bioblock) for three to seven cycles of 3 min (amplitude 12, duty 33 %) and samples were finally centrifuged (10 min, 10,000 g, 4 °C).

**RESULTS**

**Protein binding assays.** Fibronectin (BD Biosciences), 50 µg per well, or mucin (partially purified type II porcine gastric mucin; Sigma-Aldrich), 3 mg per well, in PBS was first bound to 96-well sterile polystyrene plates. Plates were incubated at 37 °C for 1 h, followed by an overnight incubation at 4 °C. Non-bound protein was then removed, and wells were washed twice with PBS, blocked with 1 % (w/v) BSA in PBS at 37 °C for 2 h, and washed again twice with PBS. Then 100 µg of surface protein extracts, resuspended in PBS at pH 7, from *B. cereus* at different growth phases was added to the wells and incubated at 37 °C for 2 h. After the incubation period, wells were washed five times in 20 mM NH₄HCO₃ in order to eliminate unbound proteins. Then 60 µl of 1 % (w/v) SDS was added to each well and incubated at 37 °C for 2 h with gentle agitation. Wells were then dried and bound proteins were solubilized in 1 x Laemmli buffer without SDS (Laemmli, 1970). Proteins were analysed by SDS-PAGE and visualized by standard silver staining.

**Characterization of the adhesion properties**

The adhesion abilities of the *Bacillus* vegetative cells and spores were characterized *in vitro* using mucin, Caco-2 cell monolayers and Matrigel as matrices. Globally, vegetative cells adhered less well than did the corresponding spores (Table 1), strain *B. cereus* being the strain that displayed the best adhesion. The rest of the strains showed lower adhesion, except for cases, such as *B. subtilis* B, which displayed good adhesion to Matrigel and Caco-2 cells, and *B. cereus* KF1, which adhered moderately to mucin.

With regard to spores, *B. cereus* showed the best adhesion to mucin, followed by *B. endophyticus* UCM B-5715 and *B. subtilis* S (Table 1), while *B. endophyticus* displayed the best adhesion to Matrigel and Caco-2 cells. *B. cereus* spores also showed good adhesion to Caco-2 cells, but their adhesion to Matrigel was average in comparison to the other strains. None of the strains agglutinated the yeast *Saccharomyces cerevisiae*, which indicated the absence of mannose-like lectins on the bacterial surface.

**Nature of the factors involved in the adhesion of *B. cereus* to the mucosal surfaces**

The strong adhesion properties of both vegetative cells and spores of *B. cereus* prompted us to further research the possible factors involved. Adhesion to mucin increased following the growth phase, being maximal for vegetative cells at 18 h. After this point, adhesion continued to increase, due mainly to bacterial sporulation (data not shown). For this reason, experiments with vegetative cells were performed after 18 h of culture.

In order to establish the class of molecules involved in the adhesion of strain *B. cereus*, different treatments, which did not affect the viability of either vegetative cells or spores (data not shown), were applied to vegetative cells and spores. In general, treatments with trypsin, LiCl, trypsin plus LiCl or NaOH produced a decrease in the adhesion to the three surfaces (Fig. 1). In addition, a drastic reduction of the adhesion of spores to mucin was observed after sonication, glass bead or NaOH treatments (Fig. 2). These results suggested that surface proteins were involved in the adhesion of the strain *B. cereus* to the three matrices.

**Identification of proteins involved in the adhesion of *B. cereus* cells and spores**

Surface proteins extracted with NaOH at different growth phases, or by sonication in the case of spores, were resolved by SDS-PAGE (Figs 3a and 4a). Among the proteins detected, S-layer, flagellin, response regulator, spore coat proteins and some surface-associated proteases were identified (Table 2). In order to assess the affinity of these proteins to mucin and fibronectin, binding assays were performed (Figs 3b and 4b). For vegetative cell extracts, with mucin as coated matrix, only flagellin (labelled BC1,
Table 1. Bacterial strains used in this study and their adhesion properties

Adhesion results in columns 4–9 are expressed as the mean of at least three independent experiments ± SD.

<table>
<thead>
<tr>
<th>Bacillus strain*</th>
<th>Source of isolation</th>
<th>Manufacturer/country</th>
<th>Mucin (c.f.u. per well)</th>
<th>Matrigel (bacteria or spores per field)</th>
<th>Caco-2 cells (bacteria or spores per cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Vegetative cells</td>
<td>Spores</td>
<td>Vegetative cells</td>
</tr>
<tr>
<td>B. cereus DM-423</td>
<td>Cerebiogen</td>
<td>Keda Drugs Trade/China</td>
<td>8 ± 1</td>
<td>1736 ± 833</td>
<td>582 ± 300</td>
</tr>
<tr>
<td>B. cereus CH</td>
<td>Yuanshou Capsule</td>
<td>Anyang Yuanshou Biopharmaceutical/China</td>
<td>10 726 ± 2 600</td>
<td>21 450 ± 5000</td>
<td>2 000 ± 186</td>
</tr>
<tr>
<td>B. cereus KD</td>
<td>Neutraline</td>
<td>Russia</td>
<td>1 401 ± 69</td>
<td>2 196 ± 682</td>
<td>435 ± 250</td>
</tr>
<tr>
<td>B. cereus KF1</td>
<td>Kefir</td>
<td>Vietnam</td>
<td>5 065 ± 3 200</td>
<td>4 043 ± 1500</td>
<td>338 ± 139</td>
</tr>
<tr>
<td>B. coagulans AV1</td>
<td>Avi-Bac WS</td>
<td>Probyin/Vietnam</td>
<td>183 ± 24</td>
<td>1 556 ± 950</td>
<td>78 ± 50</td>
</tr>
<tr>
<td>B. subtilis 3</td>
<td>Biosporin</td>
<td>Biofarm/Ukraine</td>
<td>10 ± 0</td>
<td>1 166 ± 250</td>
<td>15 ± 1</td>
</tr>
<tr>
<td>B. subtilis 26D</td>
<td>Phyto sporin</td>
<td>Biocontrol/Ukraine</td>
<td>15 ± 1</td>
<td>1 626 ± 480</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>B. subtilis 63Z</td>
<td>Plant probiotic</td>
<td>Ukraine</td>
<td>747 ± 300</td>
<td>1 611 ± 867</td>
<td>403 ± 150</td>
</tr>
<tr>
<td>B. subtilis B</td>
<td>Bioplus 2B</td>
<td>Christian Hansen/Denmark</td>
<td>5 ± 2</td>
<td>1 893 ± 956</td>
<td>1 157 ± 279</td>
</tr>
<tr>
<td>B. subtilis S</td>
<td>Sporobacterine</td>
<td>Immunopreparat/Russia</td>
<td>300 ± 86</td>
<td>2 285 ± 1300</td>
<td>217 ± 76</td>
</tr>
<tr>
<td>B. subtilis UCM B-5113</td>
<td>Soil, Carpathian Mountains</td>
<td>Russia</td>
<td>284 ± 17</td>
<td>9 700 ± 5000</td>
<td>142 ± 50</td>
</tr>
<tr>
<td>B. endophyticus UCM B-5715†</td>
<td>Healthy cotton plants</td>
<td>Tajikistan</td>
<td>2 ± 0</td>
<td>15 242 ± 5000</td>
<td>734 ± 200</td>
</tr>
<tr>
<td>B. licheniformis 31</td>
<td>Biosporin</td>
<td>Biofarm/Ukraine</td>
<td>1 982 ± 650</td>
<td>400 ± 24</td>
<td>350 ± 71</td>
</tr>
<tr>
<td>B. pumilus Nha</td>
<td>Biosubtyl</td>
<td>Biophar Company/Vietnam</td>
<td>12 ± 7</td>
<td>4 350 ± 1226</td>
<td>57 ± 6</td>
</tr>
</tbody>
</table>

*UCM, Ukranian Collection of Micro-organisms.
that other proteins also bound mucin and fibronectin: a 91.6 kDa S-layer protein (BC3), aminopeptidase (BC4), aureolysin (BC5) and metalloprotease (BC6) (Fig. 3b). In contrast, the 54 kDa S-layer protein (BC2), and the spore-associated proteins (BC7, BC8 and BC9) did not adhere (Fig. 3a).

For spore extracts, no protein was observed after Coomassie staining (data not shown). Further silver staining evidenced the presence of several bands, making their identification from the SDS-PAGE gels difficult (Fig. 4b). Thus, a clear association between SDS-PAGE analysis and binding experiments cannot be concluded for any protein. Molecular mass comparison suggests the possibility that the response regulator (BS4) might bind to mucin and fibronectin, but further research is needed to confirm this. The rest of the proteins that were majority components of the protein extract, i.e. zinc proteinase aureolysin (BS2), S-layer proteins (BS1, BS3), small acid-soluble spore protein (BS5) and proteinase VCA0223 (BS6), were not detected in the binding assays.

**DISCUSSION**

The absence of studies regarding the adhesion abilities of probiotic Bacillus strains, a property possibly responsible for some of their probiotic effects, prompted us to carry out the present investigation. The adhesion of several Bacillus strains that are main components of the microbiological formulation of various probiotic products has been tested using three different in vitro models: mucin, Matrigel and Caco-2 cells. B. cereus CH proved to be the most adherent strain; hence it was chosen for further identification of the proteins involved in adhesion to GIT surfaces.

**Adhesion properties vary with the matrix**

Adhesion was found to be dependent on the matrix as, for example, in the case of B. subtilis B strain. This strain displayed good adhesion to Matrigel, while its adhesion to mucin was poor. Adhesion was also dependent on the species and on the strain; hence, among the different B. cereus strains, B. cereus DM-423 vegetative cells displayed poor adhesion to mucin, while B. cereus CH vegetative cells showed the highest. This strain-dependent variation was also observed for B. subtilis. In general, adhesion was higher for spores than for vegetative cells, with a few exceptions. This observation agrees with previous studies, which showed that the adhesion of spores to both hydrophilic and hydrophobic surfaces is greater when compared to that of vegetative cells (Ronner et al., 1990). It is also known that Bacillus spores can adhere to several kinds of surfaces, including stainless steel (Peng et al., 2001), and that this characteristic is proportional to their hydrophobicity (Faillie et al., 2002; Ronner et al., 1990).
Proteins are involved in the adhesion of \textit{B. cereus}_{CH} to the different matrices

The adhesion results obtained after the incubation of \textit{B. cereus}_{CH} vegetative cells with trypsin, LiCl and NaOH strongly pointed to the involvement of proteins except when the target matrix was mucin, which agrees with previous studies (Ouwehand et al., 2001; Sanchez et al., 2008). One should also take into account that pretreatments could have also affected some properties of the bacterial surface. For example, NaOH might hydrolyse the D-Ala residues in teichoic acids, thus affecting the charges present on the surface of the cell.

Few data are available concerning adhesion of \textit{Bacillus} spores to different surfaces. Most of these studies principally focused on the adhesion to materials used in the food industry (glass, stainless steel, Teflon, etc.), and to intestinal surfaces, including Caco2 cells (Andersson et al., 1998; Faille et al., 2002; Peng et al., 2001; Wijnands et al., 2007). Concerning \textit{B. cereus}_{CH} spores, all the treatments caused a marked reduction of adhesion to mucin. It is known that the spore coat and the exosporium, the main protein components of the spore, have a role in spore adhesion (Du et al., 2005; Faille et al., 2007). Thus, proteins present in the surface of \textit{B. cereus}_{CH} may be responsible for the high adhesion properties of these spores.

Identification of proteins associated with the \textit{B. cereus}_{CH} surface

Among the proteins identified on the surface of vegetative cells/spores of the \textit{B. cereus}_{CH}, bands labelled BC2, BC3, BS1 and BS3 were identified as S-layer proteins. Bacterial S-layers are assemblies of identical glycoprotein subunits (with molecular masses ranging from 30 to 200 kDa) that form crystalline structures on the surface of a wide variety of prokaryotic micro-organisms, establishing a kind of
proteins could not be identified by MS, and tandem mass spectrometry (MS/MS) was performed. In the latter case, fragmented peptides allowing the identification of the protein and the score of the analysis are given.

### Table 2. Identification of B. cereus<sub>CH</sub> surface proteins in vegetative cells and spores

For proteins identified by mass spectrometry (MS), matched peptides, sequence coverage and score of the analysis are given. Some proteins could not be identified by MS, and tandem mass spectrometry (MS/MS) was performed. In the latter case, fragmented peptides allowing the identification of the protein and the score of the analysis are given.

| Label* | Putative function | pI  | Mol. mass (kDa) | Accession no.† | Match‡ | Cov§ | Score|| MS/MS¶ | Score# | Binding** |
|--------|-------------------|-----|----------------|----------------|--------|-------|-------|---------|---------|-----------|
| BC1    | Flagellin         | 8.4 | 43.0           | AAZ22698       | 21     | 58    | 222   | –       | –       | +         |
| BC2    | Surface layer protein | 5.6 | 54.0           | Q74P34         | –      | –     | –     | 5       | 138     | –         |
| BC3    | S-layer protein, EA1 protein | 5.9 | 91.6           | Q63FB8         | –      | –     | –     | 2       | 152     | +         |
| BC4    | Aminopeptidase    | 8.8 | 50.6           | Q74P34         | 11     | 31    | 107   | –       | –       | +         |
| BC5    | Zinc metalloprotease aureolysin | 6.3 | 62.5           | Q4MI07         | –      | –     | –     | 8       | 473     | +         |
| BC6    | Cell-envelope-bound metalloprotease | 4.5 | 20.8           | Q4MSA2         | –      | –     | –     | 2       | 78      | –         |
| BC7    | Spore coat-associated protein N (fragment) | 5.5 | 22.1           | AAR20933       | –      | –     | –     | 5       | 205     | –         |
| BC8    | Spore coat-associated protein N (fragment) | 5.5 | 22.1           | AAR20933       | –      | –     | –     | 7       | 463     | –         |
| BC9    | Spore coat-associated protein | 4.6 | 21.7           | 166982078      | –      | –     | –     | 6       | 334     | –         |
| BS1    | S-layer protein, EA1 protein | 6.1 | 91.6           | Q4MWF5         | –      | –     | –     | 3       | 460     | –         |
| BS2    | Zinc metalloprotease aureolysin | 6.3 | 62.5           | Q4MI07         | –      | –     | –     | 3       | 108     | –         |
| BS3    | S-layer protein, EA1 protein | 5.7 | 91.3           | Q68XP9         | –      | –     | –     | 2       | 49      | –         |
| BS4    | Response regulator | 5.8 | 42.8           | Q74NU6         | –      | –     | –     | 3       | 129     | ?         |
| BS5    | Small, acid-soluble spore protein, gamma-type | 9.3 | 9.7            | SASG_BACCE     | –      | –     | –     | 1       | 33      | –         |
| BS6    | Proteinase VCA0223 | 5.3 | 86.6           | Q4MSA5         | 13     | 20    | 103   | –       | –       | –         |

*Labels refer to proteins marked in Figs 3 and 4. 
†Protein accession numbers. 
‡Number of observed peptides contributing to the percentage of amino acid coverage in the theoretical protein. 
§Percentage of amino acid coverage in the theoretical protein. 
||MOWSE MS score. All protein scores are significant ($P<0.05$). 
¶Number of fragmented peptides that allowed identification of the protein. 
#MOWSE MS/MS score. All protein scores are significant ($P<0.05$). 
**Proteins that bound mucin and fibronectin are indicated by +. Binding of protein BS4 to mucin and fibronectin is speculative.
growth. From the resulting literature it is not clear whether the exosporium or certain spore appendices have a role in adhesion (Egelseer et al., 1995; Hachisuka et al., 1984; Husmark & Ronner, 1990; Stabnikova et al., 1995). Since vegetative cells lack such structures, it would be very interesting to establish whether the spore coat and the exosporium are or are not related to the in vivo adhesion of the probiotic Bacillus strains.

Surface-associated proteins bind to mucin and fibronectin

Finally, among all the proteins present in the extract obtained from B. cereus CH vegetative cells, a 90 kDa S-layer, an aminopeptidase, a flagellin and a cell-envelope-bound metalloprotease were found to specifically bind to mucin and fibronectin by relative mobility in electrophoresis. Mucin is a group of glycoproteins that form most of the epithelial secretions, whereas fibronectin, a membrane basal component, is a fibrous protein present on the GIT surface following injuries of diverse origin, and pathogenic bacteria can exploit this situation to cause infection. Probiotic bacteria might interfere with pathogen adhesion by adhering to such proteins, but as yet this point remains very speculative. Since lyophilization of the surface extracts might irreversibly denature proteins such as S-layer proteins and flagellins, the data concerning the binding of surface proteins to both matrices must be taken as preliminary. In addition, relative mobility can lead to misinterpretations if an underproduced protein, for example a second flagellin, is responsible for the binding. However, these surface proteins might have an important role in the adhesion of probiotic B. cereus CH strain to the GIT. Further genetic research will allow us to define those roles.

Conclusions

To sum up, the probiotic Bacillus strains tested in this study showed different adhesion properties, and globally spores adhered better than did the corresponding vegetative cells, B. cereus CH being the most adherent strain. Biochemical pretreatments suggested that surface-associated proteins were involved in the adhesion of this strain to the different matrices. These proteins were identified as S-layer, flagellin, cell-wall associated proteases and spore coat-associated proteins, and some of them adhered to mucin and fibronectin. These proteins are thus important targets for elucidating the molecular cross-talk between probiotic Bacillus species and the human host.

ACKNOWLEDGEMENTS

Borja Sánchez was the recipient of a Clarín postdoctoral contract from the Gobierno del Principado de Asturias funded by the Plan de Ciencia, Tecnología e Innovación (PCTI) de Asturias 2006–2009. S. A. was the recipient of a postdoctoral contract from the Fundación Ramón Areces.

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Edited by: D. A. Mills