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 clausii*, *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-

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Abbreviation: GIT, gastrointestinal tract.

acteristic, as proposed by the FAO/WHO (Araya *et al.*, 2002). This trait is often dependent on the existence of certain surface proteins, including mannose-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 diarrhoeal 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. cereus*_{CH}, 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 resuspending 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 rRNA gene was amplified with the universal primers 20F (5'-AGAGTTTGAT-CATGGCTCAG-3') and 1500R (5'-GGTTACCTTGTTACGACTT-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 × 10⁸ 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; 10⁸ 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. cereus*_{CH} 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).

Spores. Surface proteins were extracted by three different methods. First, the spore solutions were sonicated in ice (Vibracell 75021 ultrasonic processor, Fisher Scientific Bioblock) for three to seven cycles of 3 min (amplitude 12, duty 33 %). After sonication, samples were centrifuged for 5 min at 9000 g and the supernatants containing the surface proteins were kept. Second, 1.5 ml aliquots of spore suspensions were harvested by centrifugation and mixed with 200 µl PBS containing glass beads. Tubes were agitated in a Hybaid Ribolyser for 30 s (speed 3) and the supernatants were recovered as mentioned above. Third, 5 mg of dry spores was dissolved in 500 µl 0.01 M NaOH and the suspension was kept at 4 °C for 1 h with gentle agitation. All the extraction buffers were supplemented with EDTA and PMSF at final concentrations of 5 mM and 1 mM, respectively.

Protein analysis. The amount of protein present in the different extracts was measured with the BCA Protein Assay kit (Pierce). Approximately 40 µg protein was boiled for 2 min in 1 × Laemmli buffer. Samples were analysed by SDS-PAGE as described by Laemmli (1970) using a polyacrylamide concentration of 12.5 % (w/v), and proteins were identified by peptide mass fingerprinting or tandem mass spectrometry in a MALDI Q-ToF Premier instrument (Waters).

Protein binding assays. Fibronectin (BD Biosciences), 50 µg per well, or mucin (partially purified type III 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*_{CH} 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 × Laemmli buffer without SDS (Laemmli, 1970). Proteins were analysed by SDS-PAGE and visualized by standard silver staining.

RESULTS

Fourteen *Bacillus* strains were isolated principally from commercial probiotic products destined for human nutrition, mainly in Asia. All these strains belonged to the species claimed to be contained in each product, as determined by specific 16S rRNA gene amplification, sequencing, and comparison against the NCBI genomic database. Six strains were identified as *B. subtilis*, four as *B. cereus*, and the rest as one of the following species: *B. coagulans*, *Bacillus endophyticus*, *B. licheniformis* and *Bacillus pumilus* (Table 1). A good spore yield, near to 100 %, was observed for all the strains except *B. coagulans* B8.

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*_{CH} 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*_{CH} showed the best adhesion to mucin, followed by *B. endophyticus* UCM B-5715^T and *B. subtilis* S (Table 1), while *B. endophyticus* displayed the best adhesion to Matrigel and Caco-2 cells. *B. cereus*_{CH} 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*_{CH} to the mucosal surfaces

The strong adhesion properties of both vegetative cells and spores of *B. cereus*_{CH} 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*_{CH}, 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*_{CH} to the three matrices.

Identification of proteins involved in the adhesion of *B. cereus*_{CH} cells and spores

Surface proteins extracted with NaOH at different growth phases, or by sonicating 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 $\pm$ SD.

<i>Bacillus</i> strain*	Source of isolation	Manufacturer/country	Mucin (c.f.u. per well)		Matrigel (bacteria or spores per field)		Caco-2 cells (bacteria or spores per cell)	
			Vegetative cells	Spores	Vegetative cells	Spores	Vegetative cells	Spores
<i>B. cereus</i> DM-423	Cerebiogen	Keda Drugs Trade/China	8 $\pm$ 1	1 736 $\pm$ 833	582 $\pm$ 300	455 $\pm$ 152	0.04 $\pm$ 0.01	1.25 $\pm$ 0.03
<i>B. cereus</i> _{CH}	Yuanshou Capsule	Anyang Yuanshou Bio-pharmaceutical/China	10 726 $\pm$ 2 600	21 450 $\pm$ 5000	2 000 $\pm$ 186	788 $\pm$ 163	2.00 $\pm$ 1.10	4.66 $\pm$ 0.72
<i>B. cereus</i> _N	Neutraline	Russia	1 401 $\pm$ 69	2 196 $\pm$ 682	435 $\pm$ 250	127 $\pm$ 64	0.04 $\pm$ 0.03	2.06 $\pm$ 0.44
<i>B. cereus</i> KF1	Kefir	Vietnam	5 065 $\pm$ 3 200	4 043 $\pm$ 1500	338 $\pm$ 139	131 $\pm$ 45	0.08 $\pm$ 0.04	0.21 $\pm$ 0.05
<i>B. coagulans</i> AV1	Avi-Bac WS	Probyn/Vietnam	183 $\pm$ 24	1 556 $\pm$ 950	78 $\pm$ 50	289 $\pm$ 49	0.35 $\pm$ 0.09	1.03 $\pm$ 0.80
<i>B. subtilis</i> 3	Biosporin	Biofarm/Ukraine	10 $\pm$ 0	1 166 $\pm$ 250	15 $\pm$ 1	415 $\pm$ 72	0.01 $\pm$ 0.00	0.72 $\pm$ 0.25
<i>B. subtilis</i> 26D	Phytosporin	Biocontrol/Ukraine	15 $\pm$ 1	1 626 $\pm$ 480	0 $\pm$ 0	1 177 $\pm$ 228	0.06 $\pm$ 0.01	1.38 $\pm$ 0.41
<i>B. subtilis</i> 63Z	Plant probiotic	Ukraine	747 $\pm$ 300	1 611 $\pm$ 867	403 $\pm$ 150	512 $\pm$ 87	0.03 $\pm$ 0.01	0.54 $\pm$ 0.10
<i>B. subtilis</i> B	Bioplus 2B	Christian Hansen/Denmark	5 $\pm$ 2	1 893 $\pm$ 956	1 157 $\pm$ 279	277 $\pm$ 80	1.10 $\pm$ 0.20	2.02 $\pm$ 0.62
<i>B. subtilis</i> S	Sporobacterine	Immunopreparat/Russia	300 $\pm$ 86	2 285 $\pm$ 1300	217 $\pm$ 76	1 121 $\pm$ 316	0.06 $\pm$ 0.01	3.60 $\pm$ 1.00
<i>B. subtilis</i> UCM B-5113	Soil, Carpathian Mountains	Russia	284 $\pm$ 17	9 700 $\pm$ 5000	142 $\pm$ 50	385 $\pm$ 186	0.01 $\pm$ 0.00	1.19 $\pm$ 0.72
<i>B. endophyticus</i> UCM B-5715 ^T	Healthy cotton plants	Tajikistan	2 $\pm$ 0	15 242 $\pm$ 5000	734 $\pm$ 200	2 732 $\pm$ 414	0.10 $\pm$ 0.00	8.45 $\pm$ 1.40
<i>B. licheniformis</i> 31	Biosporin	Biofarm/Ukraine	1 982 $\pm$ 650	400 $\pm$ 24	350 $\pm$ 71	372 $\pm$ 87	0.10 $\pm$ 0.03	1.75 $\pm$ 0.00
<i>B. pumilus</i> Nha	Biosubtyl	Biophar Company/Vietnam	12 $\pm$ 7	4 350 $\pm$ 1226	57 $\pm$ 6	1 118 $\pm$ 213	0.31 $\pm$ 0.17	1.29 $\pm$ 0.20

*UCM, Ukrainian Collection of Micro-organisms.

Fig. 1. Effect of (1) trypsin, (2) lithium chloride, (3) trypsin plus lithium chloride and (4) NaOH on adhesion of *B. cereus*_{CH} vegetative cells to (a) mucin, (b) Matrigel and (c) Caco-2 cells. See Methods for details. N, no treatment.

Fig. 3b) was detected after Coomassie staining, while no protein was visualized when the coated matrix was fibronectin (data not shown). This indicates the higher affinity of this protein for mucin as compared to fibronectin. Subsequent silver staining, which classically leads to detecting lower protein quantities, demonstrated

Fig. 2. Adhesion of *B. cereus*_{CH} spores to mucin after treatment with (1) three sonication cycles, (2) seven sonication cycles, (3) glass beads, (4) NaOH and (5) LiCl. See Methods for details. N, no treatment.

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 (Faille *et al.*, 2002; Ronner *et al.*, 1990).

Fig. 3. (a) Representative SDS-PAGE gel showing the surface protein profiles of *B. cereus*_{CH} vegetative cells after 5, 7, 9, 18 and 24 hours of culture. Lane 1, total protein extract. The sporulation rate of the culture (%) is indicated above the gel. (b) SDS-PAGE gel showing the surface-associated proteins obtained from vegetative cells at stationary phase that bound to mucin and fibronectin. Band names correspond to those indicated in Table 2. The presence/absence of bovine serum albumin (BSA) in the binding assays is denoted by the symbols + and –, respectively.

Proteins are involved in the adhesion of *B. cereus*_{CH} to the different matrices

The adhesion results obtained after the incubation of *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 *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 *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 *B. cereus*_{CH} may be responsible for the high adhesion properties of these spores.

Fig. 4. (a) Representative SDS-PAGE gel with the most abundant proteins on the surface of *B. cereus*_{CH} spores. Proteins were extracted by three different methods as indicated in Methods. Lane 1, total protein extract; lanes 2 and 3, sonication treatments (40 µg and 20 µg); lanes 4 and 5, glass bead treatment (40 µg and 20 µg); (b) SDS-PAGE gel showing the surface-associated proteins obtained from spores that bound to mucin (Muc) and fibronectin (Fib). Band names correspond to those indicated in Table 2.

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

Among the proteins identified on the surface of vegetative cells/spores of the *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

Table 2. Identification of *B. cereus*_{CH} 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 metalloproteinase 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	–	–	–	13	460	–
BS2	Zinc metalloproteinase aureolysin	6.3	62.5	Q4MI07	–	–	–	3	108	–
BS3	S-layer protein, EA1 protein	5.7	91.3	Q6SXP9	–	–	–	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.

protective lattice (Egelseer *et al.*, 1995; Sleytr *et al.*, 1993). Around 18 species of *Bacillus* have been shown to possess S-layers and, in *B. cereus*, S-layer proteins have been linked to the adhesion to the host in vegetative cells and to the resistance to phagocytosis (Kotiranta *et al.*, 2000; Sidhu & Olsen, 1997). Taking these facts into account, it is postulated that S-layer proteins are involved in the adhesion of *B. cereus*_{CH} to mucin.

Bands BC4, BC5, BC6, BS2 and BS6 were identified as cell-wall-associated proteases. Since the biological function of proteases is to hydrolyse specific peptide bonds, it is not unexpected that such proteins are involved in the (at least temporary) adhesion to protein compounds present on the surfaces assayed in this work (mucin, Matrigel and Caco-2 cells). Band BC1 was identified as flagellin, a protein that arranges itself in subunits to form the flagellum filament (Kuwajima *et al.*, 1986; Nuijten *et al.*, 1990). Bacterial flagella comprise a basal body, a hook and a filament, the latter being responsible for up to 98% of the mass. In addition flagella are responsible for bacterial chemotaxis

(LaVallie & Stahl, 1989). In a recent study it was found that a *B. cereus* strain, knocked-out for the *flhA* gene, which encodes a component of the flagellum-apparatus formation, showed lower adhesion to both Caco-2 and HeLa cell lines (Ramarao & Lereclus, 2006), suggesting an involvement of flagella in adhesion. In addition, monomeric flagellin obtained from *Escherichia coli* Nissle 1917 is recognized by the innate immune system via Toll-like receptor 5 (Jacchieri *et al.*, 2003). Toll-like receptors (TLRs) play a pivotal role as primary sensors of commensal and pathogen micro-organisms, determining whether host antimicrobial defences are triggered or not (Pandey & Agrawal, 2006; Vinderola *et al.*, 2005). Flagellins on the surface of probiotic strains have been shown to be responsible for some beneficial effects (Schlee *et al.*, 2007); this may be true in the case of *B. cereus*_{CH}.

Three other bands, BC7, BC8 and BS5, showed homology with spore-coat-associated proteins. It is not contradictory to find such proteins in vegetative cells, since spores begin their formation process in the early stationary phase of

growth. From the scientific 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.

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REFERENCES

- Andersson, A., Granum, P. E. & Ronner, U. (1998). The adhesion of *Bacillus cereus* spores to epithelial cells might be an additional virulence mechanism. *Int J Food Microbiol* **39**, 93–99.
- Araya, M., Morelli, L., Reid, G., Sanders, M. E. & Stanton, C. (2002). Guidelines for the evaluation of probiotics in foods. FAO/WHO Report (http://www.who.int/foodsafety/fs_management/en/probiotic_guidelines.pdf).
- Cartman, S. T., La Ragione, R. M. & Woodward, M. J. (2008). *Bacillus subtilis* spores germinate in the chicken gastrointestinal tract. *Appl Environ Microbiol* **74**, 5254–5258.
- Ciffo, F. (1984). Determination of the spectrum of antibiotic resistance of the “*Bacillus subtilis*” strains of Enterogermina. *Chemioterapia* **3**, 45–52.
- Du, C., Chan, W. C., McKeithan, T. W. & Nickerson, K. W. (2005). Surface display of recombinant proteins on *Bacillus thuringiensis* spores. *Appl Environ Microbiol* **71**, 3337–3341.
- Duc, L. H., Hong, H. A. & Cutting, S. M. (2003). Germination of the spore in the gastrointestinal tract provides a novel route for heterologous antigen presentation. *Vaccine* **21**, 4215–4224.
- Duc, L. H., Hong, H. A., Barbosa, T. M., Henriques, A. O. & Cutting, S. M. (2004). Characterization of *Bacillus* probiotics available for human use. *Appl Environ Microbiol* **70**, 2161–2171.
- Egelseer, E., Schocher, I., Sara, M. & Sleytr, U. B. (1995). The S-layer from *Bacillus stearothermophilus* DSM 2358 functions as an adhesion site for a high-molecular-weight amylase. *J Bacteriol* **177**, 1444–1451.
- Faille, C., Jullien, C., Fontaine, F., Bellon-Fontaine, M. N., Slomianny, C. & Benezech, T. (2002). Adhesion of *Bacillus* spores and *Escherichia coli* cells to inert surfaces: role of surface hydrophobicity. *Can J Microbiol* **48**, 728–738.
- Faille, C., Tauveron, G., Le Gentil-Lelievre, C. & Slomianny, C. (2007). Occurrence of *Bacillus cereus* spores with a damaged exosporium: consequences on the spore adhesion on surfaces of food processing lines. *J Food Prot* **70**, 2346–2353.
- Hachisuka, Y., Kozuka, S. & Tsujikawa, M. (1984). Exosporia and appendages of spores of *Bacillus* species. *Microbiol Immunol* **28**, 619–624.
- Hong, H. A., Duc, L. H. & Cutting, S. M. (2005). The use of bacterial spore formers as probiotics. *FEMS Microbiol Rev* **29**, 813–835.
- Hong, H. A., Huang, J. M., Khaneja, R., Hiep, L. V., Urdaci, M. C. & Cutting, S. M. (2008). The safety of *Bacillus subtilis* and *Bacillus indicus* as food probiotics. *J Appl Microbiol* **105**, 510–520.
- Husmark, U. & Ronner, U. (1990). Forces involved in adhesion of *Bacillus cereus* spores to solid surfaces under different environmental conditions. *J Appl Bacteriol* **69**, 557–562.
- Jacchieri, S. G., Torquato, R. & Brentani, R. R. (2003). Structural study of binding of flagellin by Toll-like receptor 5. *J Bacteriol* **185**, 4243–4247.
- Kleinman, H. K., McGarvey, M. L., Hassell, J. R., Star, V. L., Cannon, F. B., Laurie, G. W. & Martin, G. R. (1986). Basement membrane complexes with biological activity. *Biochemistry* **25**, 312–318.
- Kotiranta, A., Lounatmaa, K. & Haapasalo, M. (2000). Epidemiology and pathogenesis of *Bacillus cereus* infections. *Microbes Infect* **2**, 189–198.
- Kramer, J. M. & Gilbert, R. J. (1989). *Bacillus cereus* and other *Bacillus* species. In *Foodborne Bacterial Pathogens*, pp. 21–70. Edited by M. P. Doyle. New York & Basel: Marcel Dekker.
- Kuwajima, G., Asaka, J., Fujiwara, T., Fujiwara, T., Node, K. & Kondo, E. (1986). Nucleotide sequence of the *hag* gene encoding flagellin of *Escherichia coli*. *J Bacteriol* **168**, 1479–1483.

- Laemmli, U. K. (1970).** Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- LaVallie, E. R. & Stahl, M. L. (1989).** Cloning of the flagellin gene from *Bacillus subtilis* and complementation studies of an in vitro-derived deletion mutation. *J Bacteriol* **171**, 3085–3094.
- Mazza, P. (1994).** The use of *Bacillus subtilis* as an antidiarrhoeal microorganism. *Boll Chim Farm* **133**, 3–18.
- Messner, P., Allmaier, G., Schaffer, C., Wugeditsch, T., Lortal, S., Konig, H., Niemetz, R. & Dorner, M. (1997).** Biochemistry of S-layers. *FEMS Microbiol Rev* **20**, 25–46.
- Nuijten, P. J., van Asten, F. J., Gaastra, W. & van der Zeijst, B. A. (1990).** Structural and functional analysis of two *Campylobacter jejuni* flagellin genes. *J Biol Chem* **265**, 17798–17804.
- Ouwehand, A. C., Tuomola, E. M., Lee, Y. K. & Salminen, S. (2001).** Microbial interactions to intestinal mucosal models. *Methods Enzymol* **337**, 200–212.
- Pandey, S. & Agrawal, D. K. (2006).** Immunobiology of Toll-like receptors: emerging trends. *Immunol Cell Biol* **84**, 333–341.
- Peng, J. S., Tsai, W. C. & Chou, C. C. (2001).** Surface characteristics of *Bacillus cereus* and its adhesion to stainless steel. *Int J Food Microbiol* **65**, 105–111.
- Ramarao, N. & Lereclus, D. (2006).** Adhesion and cytotoxicity of *Bacillus cereus* and *Bacillus thuringiensis* to epithelial cells are FlhA and PlcR dependent, respectively. *Microbes Infect* **8**, 1483–1491.
- Rhee, K. J., Sethupathi, P., Driks, A., Lanning, D. K. & Knight, K. L. (2004).** Role of commensal bacteria in development of gut-associated lymphoid tissues and preimmune antibody repertoire. *J Immunol* **172**, 1118–1124.
- Ronner, U., Husmark, U. & Henriksson, A. (1990).** Adhesion of *Bacillus* spores in relation to hydrophobicity. *J Appl Bacteriol* **69**, 550–556.
- Sanchez, B., Bressollier, P. & Urdaci, M. C. (2008).** Exported proteins in probiotic bacteria: adhesion to intestinal surfaces, host immunomodulation and molecular cross-talking with the host. *FEMS Immunol Med Microbiol* **54**, 1–17.
- Schlee, M., Wehkamp, J., Altenhoefer, A., Oelschlaeger, T. A., Stange, E. F. & Fellermann, K. (2007).** Induction of human beta-defensin 2 by the probiotic *Escherichia coli* Nissle 1917 is mediated through flagellin. *Infect Immun* **75**, 2399–2407.
- Sidhu, M. S. & Olsen, I. (1997).** S-layers of *Bacillus* species. *Microbiology* **143**, 1039–1052.
- Sleytr, U. B., Messner, P., Pum, D. & Sara, M. (1993).** Crystalline bacterial cell surface layers. *Mol Microbiol* **10**, 911–916.
- Sorokulova, I. B., Pinchuk, I. V., Denayrolles, M., Osipova, I. G., Huang, J. M., Cutting, S. M. & Urdaci, M. C. (2008).** The safety of two *Bacillus* probiotic strains for human use. *Dig Dis Sci* **53**, 954–963.
- Stabnikova, E. V., Selezneva, M. V., Ivanov, V. N. & Reva, O. (1995).** Theoretical and experimental screening of microbial component of biopreparation using for bioremediation of soil contaminated with oil. *Appl Biochem Microbiol* **31**, 534–540.
- Tallon, R., Arias, S., Bressollier, P. & Urdaci, M. C. (2007).** Strain- and matrix-dependent adhesion of *Lactobacillus plantarum* is mediated by proteinaceous bacterial compounds. *J Appl Microbiol* **102**, 442–451.
- Urdaci, M. C., Bressollier, P. & Pinchuk, I. (2004).** *Bacillus clausii* probiotic strains: antimicrobial and immunomodulatory activities. *J Clin Gastroenterol* **38**, S86–S90.
- Velez, M. P., De Keersmaecker, S. C. & Vanderleyden, J. (2007).** Adherence factors of *Lactobacillus* in the human gastrointestinal tract. *FEMS Microbiol Lett* **276**, 140–148.
- Vinderola, G., Matar, C. & Perdigon, G. (2005).** Role of intestinal epithelial cells in immune effects mediated by gram-positive probiotic bacteria: involvement of Toll-like receptors. *Clin Diagn Lab Immunol* **12**, 1075–1084.
- Wijnands, L. M., Dufrenne, J. B., van Leusden, F. M. & Abbe, T. (2007).** Germination of *Bacillus cereus* spores is induced by germinants from differentiated Caco-2 cells, a human cell line mimicking the epithelial cells of the small intestine. *Appl Environ Microbiol* **73**, 5052–5054.

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