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In vitro interaction between *Bacillus megaterium* strains and Caco-2 cells

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Summary. To further our understanding of the virulence potential of *Bacillus megaterium* strains, cell association and invasion assays were conducted in vitro by infecting human enterocytes (Caco-2 cells) with 53 strains of this bacterium isolated from honey. Two series of experiments were performed: (i) necrosis and cell detachment assays with the supernatants of bacterial culture filtrates from 16-h cultures and (ii) adhesion/invasion assays in which cultured enterocytes incubated with bacteria from 3-h cultures were resuspended in Dulbecco's modified Eagle's medium and chloramphenicol. The detachment of Caco-2 cells was evaluated by staining the cells with crystal violet. Necrosis was assessed by fluorescence microscopy of cells labeled with propidium iodide. Association (adhesion plus invasion) was determined by plate counts and invasion in an aminoglycoside protection assay. The results showed that spent culture supernatants detached and necrotized Caco-2 cells in a strain-dependent manner. Seven out of 53 *B. megaterium* filtered culture supernatants caused complete cell detachment. Suspensions of these same bacterial strains adhered and invaded enterocytes in 2-h infection experiments. To our knowledge, this is the first report on the interaction between *B. megaterium* and intestinal epithelial Caco-2 cells. [**Int Microbiol** 2013; 16(1):27-33]

Keywords: *Bacillus megaterium* · Caco-2 cells · adhesion · cellular necrosis · spent culture supernatant · honey

Introduction

Due to its natural properties and to industrial control measures, commercially sold honey has minimal microbial contamination. Nevertheless, it may contain yeasts and spore-forming bacteria [41]. Although vegetative forms of disease-causing bacteria have not been found in honey, bacterial spores, when present, can remain viable for long periods without losing their infectivity.

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Several studies have shown that *Bacillus* and *Paenibacillus* species, including *Bacillus cereus*, *B. thuringiensis*, *B. licheniformis, B. pumilus, B. megaterium, B. coagulans, P. alvei*, and *P. larvae* [2,3,11,17,35,45], are commonly detected in honey. While *P. larvae* and *P. alvei* are associated with different honeybee diseases, *B. subtilis, B. pumilus, B. cereus, B. licheniformis, B. circulans*, and *B. megaterium* are predominant in the digestive tracts of larvae and honey bees [3,41]. *Bacillus cereus* and *B. megaterium* are also common in soils, dust, and flower surfaces. In Argentinian honeys, the most prevalent spore-forming species are *P. larvae, P. alvei, B. cereus, B. pumilus*, and *B. megaterium* [2,3,21,22].

Bacillus megaterium is an aerobic, gram-positive, spore-forming bacterium that in addition to honey has been isolated from various food sources, i.e., shellfish, raw meat,

rice, corn, and soymilk [1–3,18,31]. The antibiotic-like compounds produced by *B. megaterium* include megacins and other inhibitory substances [4,28,32]. To date, *B. megaterium* has not been associated with any human disease, but its pathogenic potential is evidenced by the presence in its genome of several toxin-encoding genes, i.e., *bceT* (enterotoxin-T), *hblC, hblA, hblD* (HBL complex), and *nheA, nheB, nheC* (NHE complex) [23,34,38]. In addition, the production of a novel heat-stable toxin by *B. megaterium* strain F98/3079 has been reported [43]. Of particular relevance to this study is the detection of both hemolytic and coagulase activities in 39 *B. megaterium* isolates obtained from honey samples of different origins [22].

The pathogenic potential of bacteria and the biological activity of bacterial toxins have been studied in vitro in a variety of cell culture systems [14]. As a cell line with differentiation markers characteristic of mature intestinal epithelial cells, enterocyte-like Caco-2 cells (ATCC HTB-37) are a well-recognized in vitro model system. These markers include functional tight junctions, microvilli, and a number of brush border-associated enzymes and transporters, i.e., peptidases, esterases, P-glycoproteins, and uptake transporters for amino acids, bile acids, and carboxylic acids [36,39]. Consequently, Caco-2 cells have been used to mimic bacteria-host interactions in the intestinal tract [6,7,12,16,25,26,33]. They were chosen in our study of the virulence potential of *B. megaterium* strains isolated from honey because the intestinal epithelium is rapidly exposed to potential foodborne pathogens. Although interactions between *Bacillus* spp. and cultured cells, e.g., Caco-2, human epithelial type 2 cells (Hep-2), and cell lines derived from cervical cancer (HeLa), have been investigated [26,37,38], to our knowledge, ours is the first study to focus on *B. megaterium*.

Materials and methods

Bacterial strains, media and culture conditions. Fifty-three strains of *B. megaterium* were evaluated. Except for *B. megaterium* NRRL B-939, all strains were isolated from honey (49 from Argentina [Bm1–Bm21 and Bm25–Bm52], 2 from Brazil [Bm23 and Bm24], and 1 from France [Bm22], as previously described [22]). The bacteria were stored at –80 °C in tryptone soy broth with 20 % (v/v) glycerol as cryoprotectant. Prior to the experiments, each strain was cultured in nutrient broth (NB) under constant agitation at 32 °C for 16 h. Afterwards, they were inoculated (2 % v/v) in 5 ml of NB and incubated under constant agitation at 32 °C for 3 h or 16 h, according to the assay conditions. Bacteria were harvested by centrifugation (900 ×*g* for 10 min).

Culture of Caco-2 cells. The human colon cancer cell line Caco-2 was routinely grown in Dulbecco's modified Eagle's medium (DMEM) (25 mM glucose) (Life Technologies, Carlsbad, CA, USA), supplemented with 15 % (v/v) heat-inactivated (56 ºC, 30 min) fetal calf serum (FCS, PAA Laboratories, Pasching, Austria), 12 IU penicillin/ml-12 µg streptomycin/ml (Life Technologies), and 1 % (v/v) non-essential amino acids (Life Technologies). Monolayers were prepared in 24-well tissue culture plates (Greiner Bio One, Frickenhausen, Germany) by seeding 7×10^4 cells per well. Experiments and cell maintenance were carried out in an atmosphere of 5 % CO_2 at 37 °C. Assays were performed with differentiated cells (late post-confluence, 15 days of culture).

Detachment of Caco-2 cells. Detachment of enterocyte-like cells was studied as reported previously [25]. Briefly, filter-sterilized (0.45-µm pore size) spent culture supernatants (SCSs) were obtained from 16-h bacterial cultures in NB. Differentiated Caco-2 monolayers were incubated with 0.5 ml of serially diluted SCSs in NB (ratios of SCSs/total volume ranged from 0/0.5 to 0.5/0.5 per well) at 37 °C for 1 h. The cells were washed twice with phosphate buffered saline (PBS) (pH 7.2), fixed at room temperature with 2 % (v/v) formaldehyde in PBS for 1 min, and washed again with PBS. Afterwards, the cells were stained by incubating them with 500 µl of a crystal violet solution (0.13 % [w/v] crystal violet, 5 % [v/v] ethanol, and 2 % [v/v] formaldehyde in PBS) at room temperature for 20 min. After a PBS wash to remove excess stain, the samples were treated with freshly prepared 50 % (v/v) ethanol at room temperature for 1 h. Absorbance was measured in a microplate reader at 620 nm (BioTek, Winooski, Vermont, USA). All experiments were performed in triplicate. The percentage of cell detachment was calculated as follows: cell detachment % = 100 × (Ac – As)/Ac, where Ac is the A_{620} of control cells and As the A_{620} of sample cells.

Necrosis. Necrosis was assessed according to a modification of a previously published method [47], using, short incubation periods to prevent cell detachment (37 °C, 20 min). After their incubation with SCSs from 16-h bacterial cultures in NB, the cells were washed twice with binding buffer containing 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 125 mM NaCl, 2.5 mM CaCl2, (pH 7.2), and 0.2 % (p/v) gelatin. One µg of propidium iodide in 100 µl of binding buffer was then added to each well, and the plates were placed on ice for 15 min. The samples were subsequently mounted in 50 % (v/v) glycerol in PBS and analyzed by conventional fluorescence microscopy using a Leica DMLB microscope coupled to a Leica DC 100 camera (Leica Microscopy Systems, Heerbrugg, Switzerland).

Cell association assays. Bacterial cultures (37 °C, 3 h) were centrifuged and the pellets were suspended in 1 ml of DMEM containing 100 µg chloramphenicol/ml. As reported by Minnaard et al. [26], under these conditions the bacteria remain viable but do not grow. The bacterial concentration was adjusted to 10^8 colony-forming units (CFU)/ml. The cell monolayers were washed twice with PBS, treated with the bacterial suspensions (multiplicity of infection = 100 bacteria per cell), and incubated in an atmosphere of 5 % CO_2 at 37 °C for 2 h. To evaluate cell association (adhering plus invading bacteria), the monolayers were exhaustively washed with PBS and then incubated with 1 ml of distilled water per well, which lysed only the eukaryotic cells. The bacteria present in the lysate were counted by plating appropriate dilutions on nutrient agar and incubating the samples at 32 °C for 16 h.

Cell invasion assays. Enterocyte invasion was assessed by the aminoglycoside protection assay as previously reported [13], using gentamicin to kill non-internalized bacteria. Briefly, after infection, monolayers washed three times with PBS were treated with 1 ml of gentamicin

(100 µg/ml in PBS) per well, and incubated at 37 °C for 1 h. The monolayers were then washed twice with PBS, lysed in 1 ml of distilled water, and maintained at 37 °C for 1 h. Serial dilutions of the suspensions were plated on nutrient agar as described above. To assess the presence of spores, aliquots of each sample were heat treated in a block heater (Nova Etica, Vargem Grande Paulista, SP, Brazil) at 60 °C for 30 min, and appropriate dilutions were plated onto nutrient agar. Strains were considered to be invasive only when the number of survivors after gentamicin treatment was significantly higher than the number after thermal treatment [26]. All infection assays were performed in FCS-free medium.

PCR analysis. Strains that detached enterocyte-like cells were screened by polymerase chain reaction (PCR) for the presence of enterotoxic genes (*entFM*, *entS*, and *piplC*) and *sph* (sphingomyelinase) gene according to the procedures described by Ghelardi and co-workers [10]. DNA was amplified in a thermal cycler (Eppendorff Mastercycler, Eppendorf AG, Hamburg, Germany) using the same PCR primers and conditions as previously reported [10]. PCR products were resolved in 1.6 % agarose gels in 0.5× TBE buffer, stained with GelRed (genBiotech SRL, Buenos Aires, Argentina), and visualized with a UV transilluminator (UVP, Upland, California, USA). Gel images were digitalized and photographed using a digital image capture gel documentation system (Digi Doc-it, UVP, v. 1.1.25, Upland, California, USA).

Statistical analysis. Microsoft Office Excel (version 2003) was used for the statistical analysis. The results of the experiments were compared by means of two-tailed Student's t-test, with $P < 0.05$ considered statistically significant.

Results

Detachment of Caco-2 cells. Exploratory experiments analyzing the effect of different *B. megaterium* SCSs on fully differentiated Caco-2 cells showed that the filtered culture supernatants of seven out of the 53 bacterial strains tested caused complete cell detachment, with no evidence of biological activity in the remaining 46 strains (data not shown). The SCSs of these strains, i.e., Bm1, Bm8, Bm9, Bm10, Bm29, Bm30 and Bm51, all isolated from Argentinian honeys, were used for further studies.

Differences in the biological activity of the seven SCSs at low doses were observed. At a SCS/total volume ratio of 0.2/0.5, the biological activities of strains Bm8, Bm29, Bm30 and Bm51 were higher than those of strains Bm1, Bm9 and Bm10 ($P < 0.05$). For all of the strains under study, undiluted supernatants (ratios 0.5/0.5) caused the total detachment of Caco-2 cell monolayers (Fig. 1).

Necrosis. Cell necrosis was evaluated with SCSs prepared from 16-h cultures of strains Bm1, Bm8, Bm10, Bm29,

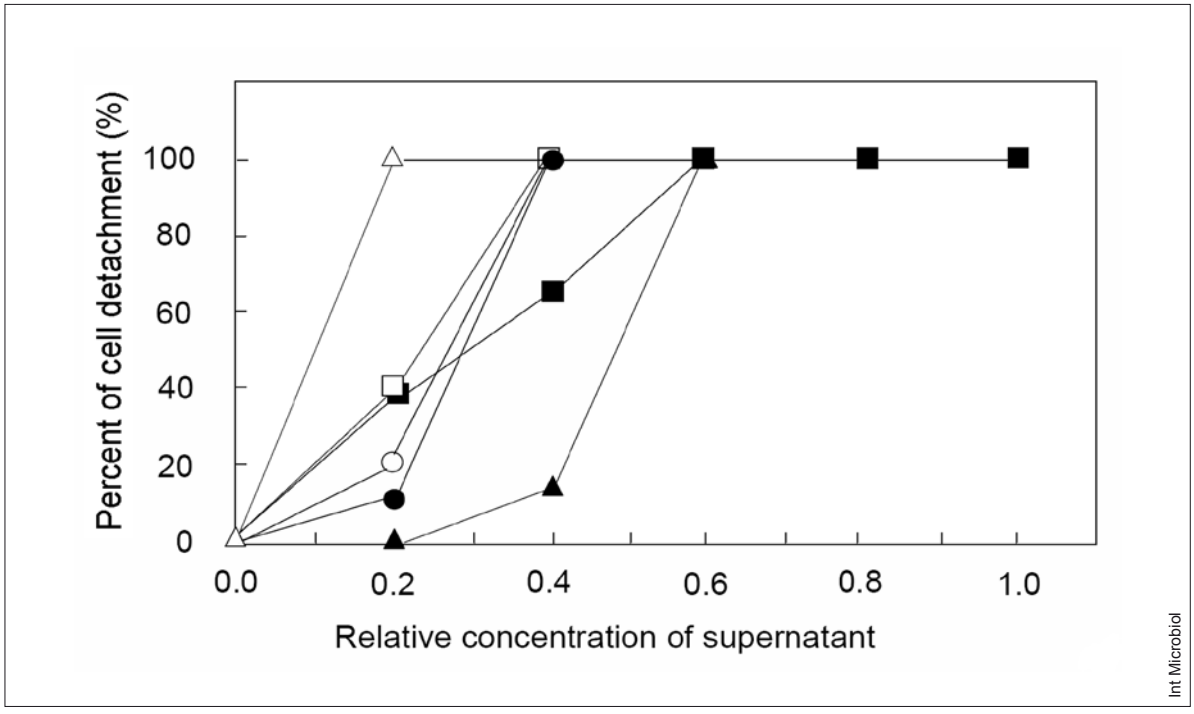

Fig. 1. Detachment of Caco-2 cells after their co-incubation (1 h at 37 °C) with different concentrations of filtered culture supernatants of *Bacillus megaterium* strains: Bm1 (closed triangles), Bm8 (open squares), Bm9 (open circles), Bm10 (closed circles), Bm30 (closed squares), Bm29 and Bm51 (open triangles; the same symbol is used for Bm29 and Bm51 strains because they induced the same responses). All symbols overlap at 0 and 100 % cell detachment. A relative concentration of supernatant equal to 1 indicates undiluted supernatants. Spent cultures were diluted in NB.

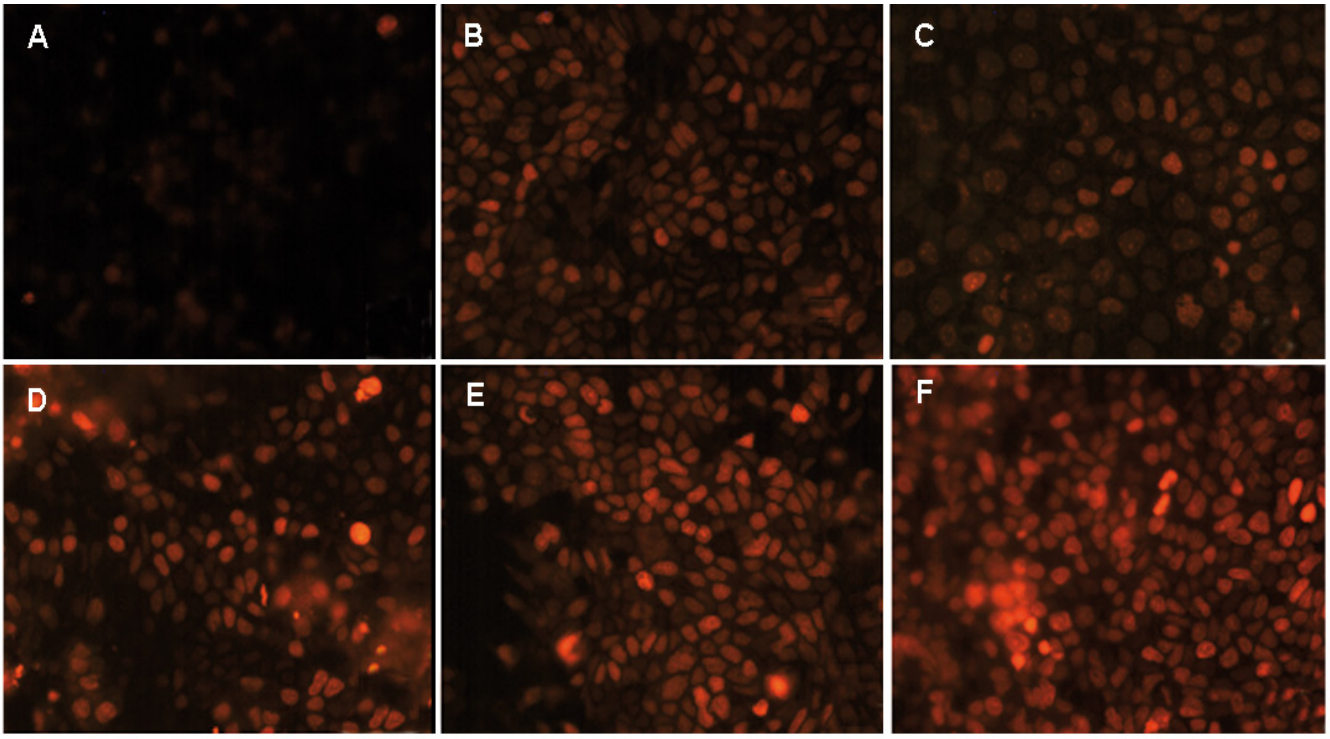

Fig. 2. Fluorescence microscopy of Caco-2 cells co-incubated at 37 °C for 20 min with filtered culture supernatants of strains Bm1 (**B**), Bm8 (**C**), Bm10 (**D**), Bm29 (**E**), and Bm30 (**F**). The control was co-incubated with NB medium only (**A**). Cells were labeled with propidium iodide (PI). Red cells indicate the nuclei of necrotic cells (40×). All micrographs are representative of three independent experiments conducted with successive cell passages.

and Bm30 (Fig. 2). When briefly (20 min) incubated with the cell monolayers, none of these strains induced detachment. Instead, high ratios of necrotic cells were obtained (Fig. 2B–D). By contrast, only a few necrotic cells were seen in untreated control monolayers (Fig. 2A).

Cell association and invasion. Infection assays were conducted only with strains whose supernatants detached Caco-2 cells. Strains Bm1, Bm9 and Bm10 associated with the monolayers at levels significantly higher than strains Bm29, Bm51 and Bm8 ($P < 0.05$) (Fig. 3A). Strain Bm30 was not included in these assays because it was gentamicin resistant and thus inappropriate for the assessment of invasion in the aminoglycoside protection assay.

As seen in Fig. 3B, strains Bm1 and Bm10 were the most invasive ($P < 0.05$). The number of bacteria penetrating the cells was 1×10^4 CFU/ml for strain Bm1 and 6×10^3 CFU/ml for strain Bm10. For strains Bm8, Bm9 and Bm29, about 4×10^3 CFU/ml invaded the Caco-2 cells while strain Bm51 was not internalized at all (Fig. 3B). Note that the experiments were performed with 3-h cultures to minimize the presence of sporulated microorganisms. For all six strains evaluated, the number of survivors was significantly ($P < 0.05$) higher after gentamicin treatment than after thermal treatment (60 °C, 30 min).

PCR analysis. Strains Bm8, Bm9, Bm10, and Bm51 were positive for all four enterotoxic genes tested, i.e., *sph, entFM, entS, and piplC*, as determined by PCR. Strain Bm29 was positive for *entS, sph* and *piplC*, and strain Bm1 for *entS* and *piplC* (data not shown).

Discussion

Bacteria belonging to the *B. cereus* group have been associated with outbreaks of foodborne illness and other diseases [42]. Their pathogenicity can be related to the production of several toxins [5,8–10,15,20,40,42]. In this work, we focused on *B. megaterium*, since genes encoding biologically active molecules have also been found in strains of the bacterium isolated in Argentinian honeys [23].

In our study, high doses of SCSs from seven strains of *B. megaterium* completely detached monolayers of cultured enterocytes and led to cell necrosis. Furthermore, six of

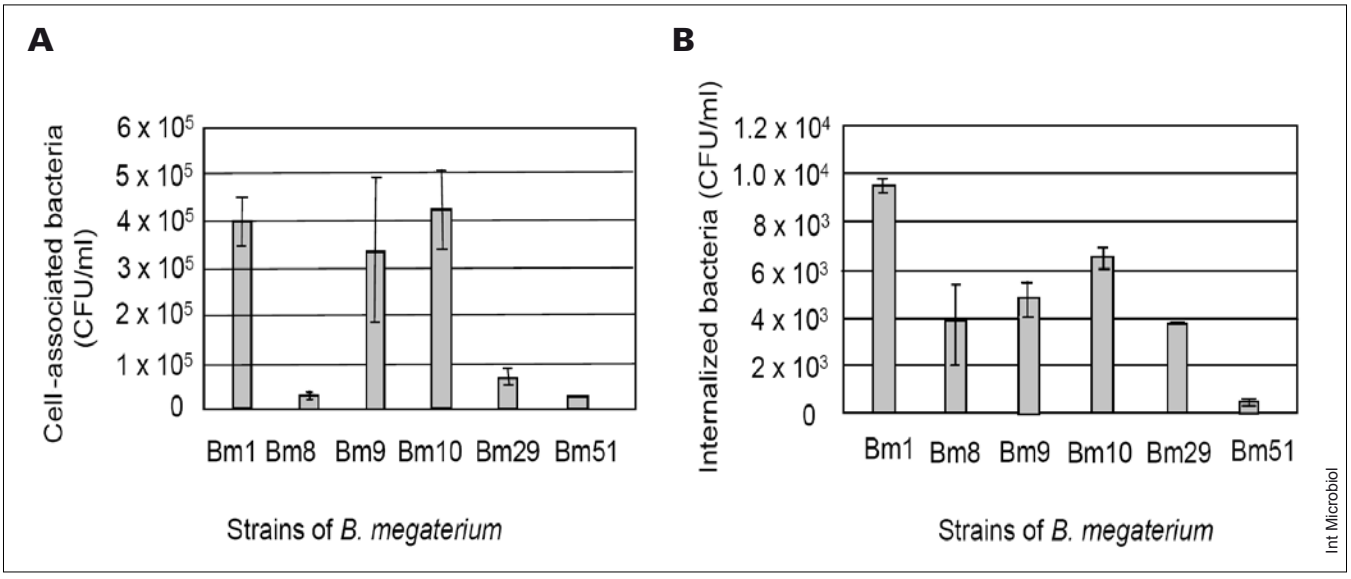

Fig. 3. Cell-association (**A**) and cell-invasion (**B**) assays of six different *Bacillus megaterium* strains with fully differentiated Caco-2 cells. Values ± standard deviations are from a representative experiment with three replicates.

those detachment-inducing strains were also capable of cell invasion (one strain could not be tested, see above). These findings demonstrated that the induction of cell detachment by extracellular factors secreted by *B. megaterium* is not a general property of this species, which is in agreement with findings published for *B. cereus* [25,27]. From the dose-response curves obtained in cell detachment assays with *B. cereus* SCSs, Minnaard and co-workers defined three types of strains: (i) high detaching (HD) strains detach cells even at low doses; (ii) low detaching (LD) strains detach cells at high doses; and (iii) non-detaching (ND) strains [27]. Accordingly, in the present study of *B. megaterium*, two HD (strains Bm 29 and Bm 51) and five LD (strains Bm 1, Bm 8, Bm 9, Bm 10 and Bm 30) strains were identified, whereas the remaining 46 strains were ND. The biological activity of the SCSs from the seven HD and LD strains may depend on secreted extracellular factors. The SCSs of strains Bm9 and Bm51 resulted in cell detachment within a few minutes, indicative of their stronger biological activity than the SCSs of the other five isolates, and could therefore not be assayed for their ability to cause cell necrosis.

All *B. megaterium* strains that led to enterocyte detachment had been previously reported to have hemolytic and coagulase activities [22,23]. Note that *B. cereus, B. thuringiensis* and *B. megaterium* share several sequences encoding virulence factors [10,23]. Besides the enterotoxic genes s*ph, entFM*, *entS*, and *piplC* detected in this work, sequences of the HBL complex, the *nheC* gene from the NHE complex, and *bceT* and *cytK* genes had been previously detected in the LD strains Bm8 and Bm9 [23]. HD strains Bm29 and Bm51 were positive for the enterotoxic genes *sph, entS, and piplC* and *sph*, *entFM, entS*, and *piplC*, respectively, while LD Bm10 was positive for all four enterotoxic genes, and LD Bm1 was positive for only two of the genes tested. By contrast, Bm30 was negative for all the virulence genes tested here and in a previous study [23].

Adhesion is a key event in the pathogenicity of many bacteria. Adhered bacteria either remain on the cell surface (e.g., diffusely adherent *E. coli* [DAEC]; enteropathogenic *E. coli* [EPEC]) or subsequently invade the cell (e.g., enteroinvasive *E. coli* [EIEC]; *Shigella* spp.; *Yersinia* spp.; *Listeria* spp.) [6,24,29,44,46].

The values obtained for *B. megaterium* in the association and invasion assays were in the range of those previously reported for *B. cereus* [26]. Note that, while the LD strains Bm8 and Bm29 had the lowest association values, their internalization efficiencies (radio of invading to associated bacteria) were relatively high (0.16 and 0.06, respectively).

Bacteria that adhere to eukaryotic cells can trigger a biological response by interacting with host cell surface receptors linked to signaling pathways [13]. In vitro, this ability depends on both the differentiation status of the host cells, which determines receptor expression, and the

growth phase at which the bacteria were harvested [19]. We found that cultured human enterocytes infected with *B. megaterium* suspensions prepared from 3-h cultures led to cell detachment (data not shown). These findings are in agreement with those reported for *B. cereus* infection of cultured human enterocytes [26]. In addition, we were able to show that cell detachment following infection with bacterial vegetative cells was inhibited when the assays were performed in the presence of chloramphenicol, suggesting that protein synthesis was necessary for a maximal biological effect.

The ability of *B. megaterium* to attach to and subsequently invade enterocytes may reflect its pathogenic potential. This conclusion supports a previous study showing that *B. megaterium* strains isolated from infant formula are not only able to adhere to and invade epithelial cells but are also positive for the presence of enterotoxins [38].

To our knowledge, this is the first report that demonstrates strain-dependent biological effects following the infection of cultured human enterocytes by *B. megaterium* isolates, and the biological activity of spent culture supernatants. Our results are consistent with the multifactorial character of *B. megaterium* virulence. Although further studies are necessary to completely unravel the pathogenic potential of this species, our work provides the first step in the study of the interaction between *B. megaterium* and its host in the context of intestinal infections.

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Competing interest. None declared.

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