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Interaction between *Bacillus cereus* and Cultured Human Enterocytes: Effect of Calcium, Cell Differentiation, and Bacterial Extracellular Factors

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ABSTRACT

Bacillus cereus interaction with cultured human enterocytes and the signaling pathways responsible for the biological effects of the infection were investigated. Results demonstrate that calcium depletion increases the ability of strains T1 and 2 to invade cells. Bacteria associated in greater extent to undifferentiated enterocytes and extracellular factors from strain 2 increased its own association and invasion. Inhibitors of signaling pathways related to phosphorylated lipids (U73122 and wortmannin) were able to significantly reduce cytoskeleton disruption induced by *B. cereus* infection. Adhesion of strain T1 decreased in the presence of U73122 and of wortmannin, as well as when those inhibitors were used together. In contrast, invasion values were diminished only by U73122. Results show that different factors are involved in the interaction between *B. cereus* and cultured human enterocytes. Following infection, disruption of the cytoskeleton could facilitate invasion of the eukaryotic cells.

Bacillus cereus is a spore-forming microorganism responsible for foodborne outbreaks and associated with different pathologies such as emetic and diarrheic syndromes, endophthalmitis, endocarditis, osteomyelitis, oral cavity infections, septicemia, peritonitis, pneumonia, and meningitis *(10)*. It usually leads to self-limited pathologies, although severe and fatal cases have been reported *(3, 18, 34, 52)*.

Within the context of intestinal illness there are two syndromes: emetic and diarrheic. Emetic syndrome is related to cereulide, a cyclic dodecadepsipeptide produced by a nonribosomal peptide synthetase *(2, 49)*. In contrast, diarrheic syndrome is a multifactorial process associated to several extracellular factors *(2, 5, 6, 29, 33, 46)*.

Virulence factors can be secreted during vegetative growth in the small intestine *(22)*, and their production is regulated by the PlcR regulon, which is the major virulence regulator in *B. cereus (1, 31)*. It is associated with a quorum sensing system related to the virulence gene expression such as *hbl*, *nhe*, and *cyt-K (21)*. Activation depends on PapR, a 48-amino-acid peptide, encoded downstream of *plcR (45)*. Although not all the virulence factors are under the control of *plcR*, this regulation is relevant for the virulence in insect and mouse infection *(44)*. Other regulatory systems include HlyIIR transcriptional regulator *(13)*, ferric uptake regulator (Fur) *(46)*, the ResDE two-component system, and the Fnr transcriptional regulator *(35, 46)*.

Even though *B. cereus* virulence was traditionally ascribed to toxin production, it has been demonstrated that adhesion of spores to host's cells could also contribute to virulence *(4)*. In addition, further studies demonstrated that adhesion to or invasion of enterocytes could also contribute to *B. cereus* virulence *(39)*. Although flagellar protein FlhA is involved in the adhesion process, adhesion is not related to motility, and its expression is not controlled by PlcR *(42)*. As for other bacterial pathogens, interaction of *B. cereus* with the host′s cells could trigger signals that are relevant for both the biological effect and the fate of the microorganisms in the host's cells *(7, 41, 48, 50)*.

The present study sought to gain further insight on the interaction between *B. cereus* and cultured human enterocytes as well as on the signaling pathways responsible for the biological effects following this interaction.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *B. cereus* strain 2 was isolated from infant formula *(38)*. Strain T1 was isolated from a food poisoning outbreak *(12)*. Microorganisms were stored at −80°C with 0.3 M sucrose as cryoprotectant. Prior to the experiments, bacteria were cultured for 16 h at 32°C with agitation in brain heart infusion broth (Biokar Diagnostics, Beauvais, France) supplemented with 0.1% (wt/vol) glucose (BHIG). Afterward, microorganisms were inoculated (4%, vol/vol) in 5 ml of BHIG and incubated with agitation at 32°C for 3 h. Bacteria were harvested by centrifugation (900 × *g* for 10 min).

Culture of Caco-2 cells. Caco-2 cells *(20)* were routinely grown in Dulbecco's modified Eagle's minimum essential medium

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(DMEM; 25 mM glucose; Life Technologies, Cergy, France), supplemented with 15% (vol/vol) heat-inactivated (30 min, 56°C) fetal calf serum (PAA Laboratories GmbH, Pasching, Austria) and 1% (vol/vol) nonessential 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 at 37°C in an atmosphere of 5% CO_2 and 95% air. Assays were performed with cells at passages between 56 and 62. Cells at late postconfluence (15 days in culture) were used except when otherwise indicated.

Cell association assays. Bacterial cultures were centrifuged and pellets were suspended in DMEM containing 100 μg/ml choramphenicol. As reported *(39),* in these conditions bacterial viability is preserved but no bacterial growth occurs. Bacterial concentration was adjusted to $A_{600nm} = 1$ (this represents 1×10^8 CFU ml^{-1}). Cell monolayers were washed twice with PBS before the infection assays, and bacterial suspensions were added to the monolayers (multiplicity of infection = 100 bacteria per cell) and incubated for 2 h at 37°C in an atmosphere of 5% CO_2 and 95% air. To evaluate association (adhering plus invading bacteria), monolayers were exhaustively washed with PBS and incubated with 1 ml of distilled water per well to lyse eukaryotic cells. Serial dilutions of the samples were plated onto nutrient agar (Biokar Diagnostics) and incubated at 37°C for 16 h. Invasion was assessed by the aminoglycoside protection assay as previously reported *(39).* Briefly, monolayers were washed three times with PBS and then 1 ml of gentamicin (100 μg/ml in PBS) was added per well. After being incubated for 1 h at 37°C, the monolayers were washed twice and lysed with 1 ml of distilled water for 1 h at 37°C. Serial dilutions of the suspensions were plated onto nutrient agar as indicated above. All the infection assays were performed in fetal calf serum–free medium.

Calcium effect. Infection was performed as described above in Ca^{2+}-free DMEM (Sigma Chemical Co., St. Louis, MO) or the same medium supplemented with 200 mg/liter $CaCl_2$. Before infection assay, cells were washed twice with Ca^{2+}-free DMEM and preincubated at 37°C in an atmosphere of 5% CO_2 and 95% air for 1 h in the same medium.

Cell differentiation effect. Association and invasion were evaluated as described above by using cells at different stages of differentiation (4, 9, 15, and 21 days in culture).

Effect of bacterial extracellular factors on infection. Filter-sterilized (0.45-μm pore size) spent culture supernatants (SCS) were obtained from 3-h-old culture of strain 2 in BHIG broth. Under these conditions, neutral SCS were obtained. After filtration, no bacteria were found in the SCS as assessed by plate counts.

Suspensions of strain 2 in DMEM (0.5 ml, 2×10^8 CFU ml^{-1}) were added to cell monolayers. Next, 0.5 ml per well of SCS dilutions in sterile BHIG was added in order to test SCS volumes ranging from 0 (0.5 ml of bacterial suspension in DMEM plus 0.5 ml of BHIG) to 0.5 (0.5 ml of bacterial suspension in DMEM plus 0.5 ml of SCS). Plates were incubated in an atmosphere of 5% CO_2 and 95% air. Under these conditions, no cell detachment was observed.

Signaling pathways. In order to identify the signaling pathways involved in the infection process, a pharmacological approach was conducted with the following inhibitors: genistein, a tyrosine kinase inhibitor *(32)*; U73122, an inhibitor of phospholipase C that blocks mobilization of intracellular Ca^{2+} *(47)*; filipin III, which disrupts caveolar structure *(40)*; 2-hydroxypropyl-β-cyclodextrin, which specifically removes cholesterol from the plasma membrane *(40)*; calphostin C, an inhibitor of conventional protein kinase C and novel protein kinase C *(43)*; dantrolene, which blocks release of Ca^{2+} from intracellular stores *(27)*; wortmannin, a phosphatidylinositol 3-kinase (PI3K) inhibitor *(24)*; nocodazole, a microtubule-depolymerizing drug *(8)*; and staurosporine, a protein kinase inhibitor *(9).*

Labeling of the F-actin cytoskeleton was performed with fluorescein-isothiocyanate phalloidin *(39).* Briefly, monolayers of Caco-2 cells cultured on glass coverslips (Assistent, Karl Hecht KG, Sondhein, Germany) were infected with *B. cereus* vegetative cells as indicated above in the presence of genistein (49.5 μg ml^{-1}), filipin III (14.4 μg ml^{-1}), 2-hydroxypropyl-β-cyclodextrin (7.3 μg ml^{-1}), calphostin (0.009 μg ml^{-1}), dantrolene (5.8 μg ml^{-1}), wortmannin (0.26 μg ml^{-1}), nocodazole (5.7 μg ml^{-1}), U73122 (9.3 μg ml^{-1}), or staurosporin (37 ng ml^{-1}). All the inhibitors were from Sigma Chemical. Concentrated solutions of the inhibitors were prepared in order to minimize the solvent volume added to the wells. After infection, cells were washed with PBS and fixed with 3% (vol/vol) paraformaldehyde (Riedel-de Haën, Seelze, Germany) in PBS for 15 min. Afterward, samples were treated with 50 mM NH_4Cl for 10 min, permeabilized for 4 min with Triton X-100, and incubated for 45 min with fluorescein-isothiocyanate phalloidin (all from Sigma Chemical). Samples were mounted in glycerol 50% (vol/vol) (Baker, Tultitán, Mexico) containing 0.1% (wt/vol) sodium azide (Sigma Chemical) in PBS and examined by conventional epifluorescence microscopy using a Leica DMLB microscope coupled to a Leica DC 100 camera (Leica Microscopy Systems Ltd., CH Heerbrugg, Switzerland). Analysis on a minimum of six images (300 cells per image) was performed by an operator unaware of the experimental conditions. Cells showing no fluorescein-isothiocyanate labeling, rounding, or uneven distribution of F-actin were considered to be damaged cells *(39).*

Measurement of TEER. Caco-2 cells were grown in 24-well plates with culture plate inserts (BD, Cockeysville, MD) until they were differentiated as described above. Afterward, cells were infected as described, and transepithelial electrical resistance (TEER) was assessed by means of a Millicell ERS-2 Volt-Ohm meter (Millipore, Billerica, MA). Uninfected cells were used as controls.

Statistical analysis. Statistical analysis was performed by means of Student's *t* test by using InfoStat version 2011(Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina).

RESULTS

Effect of calcium on association. To examine the role of Ca^{2+} in the interaction between *B. cereus* and cultured human enterocytes, we performed infection assays in Ca^{2+}-free DMEM medium in order to weaken Ca^{2+}-dependent intercellular junctions. Results showed that absence of Ca^{2+} did not modify association values of strains T1 and 2. As shown in Figure 1A and 1C, average values of associated bacteria were around 8×10^6 CFU ml^{-1} and 5×10^6 CFU ml^{-1} for strains 2 and T1, respectively. Interestingly, Ca^{2+} depletion significantly increased ability of both strains to invade Caco-2 monolayers (Fig. 1B and 1D). Indeed, invasion values obtained in the absence of Ca^{2+} were $8.0 \pm 1.0 \times 10^5$ CFU ml^{-1} for strain 2 and $6.3 \pm 1.0 \times 10^5$

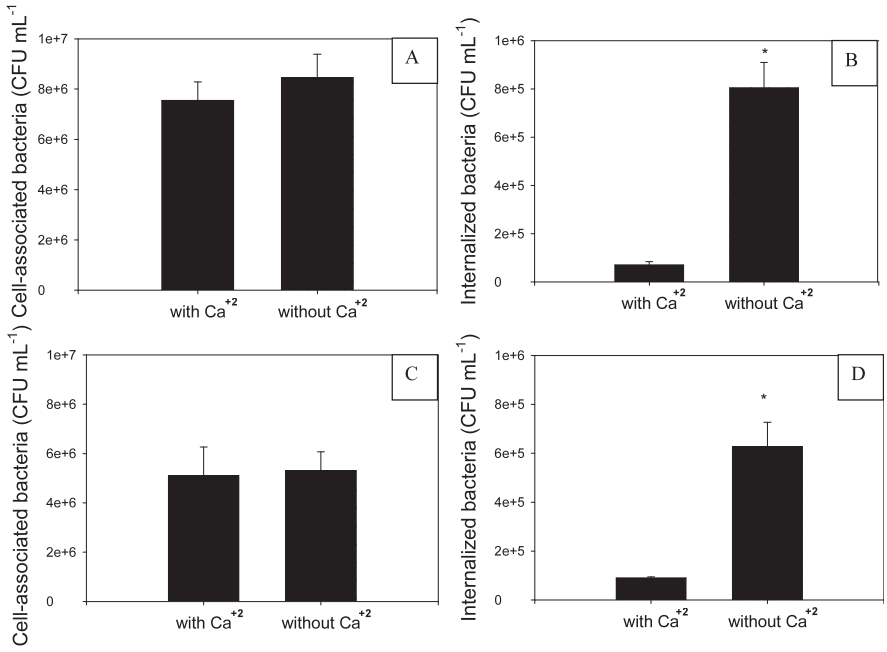

FIGURE 1. *Effect of Ca^{2+} depletion on the association of* B. cereus *to Caco-2 cells. Infection was performed in DMEM supplemented with chloramphenicol (100 μg/ml) at 37°C in an atmosphere of 5% CO_2 and 95% air for 2 h. (A and B) Strain 2; (C and D) strain T1. Error bars represent standard deviations. Results show a representative experiment from three independent assays. * Significant differences (*$P < 0.05$*).*

CFU ml^{-1} for strain T1. In the presence of Ca^{2+}, invasion values were $7.0 \pm 1.4 \times 10^4$ CFU ml^{-1} and $9.0 \pm 0.4 \times 10^4$ CFU ml^{-1} for strains 2 and T1, respectively.

Measurement of TEER showed that Ca^{2+} depletion significantly decreases ($P = 0.02$) electrical resistance from 750 ± 86 Ω cm^2 to 611 ± 86 Ω cm^2. Infection of monolayers with 1×10^8 CFU ml^{-1} per well leads to TEER values of 447 ± 24 Ω cm^2 and 356 ± 6 Ω cm^2 for strains 2 and T1, respectively.

Effect of cell differentiation on association. Given that expression of cell surface molecules depends on differentiation status, association assays were performed with confluent monolayers at different days in culture.

As shown in Figure 2, assays performed with undifferentiated cells (4 days in culture) led to the highest values of association (Fig. 2A and 2B) and invasion (Fig. 2C and 2D). No significant differences were found between assays performed with cells cultured for 9, 15, and 21 days ($P > 0.1$).

Effect of bacterial extracellular factors on association. Taking into account that *B. cereus* virulence is related to both extracellular factors and direct bacteria-enterocyte interactions, we performed assays in the presence of SCS of *B. cereus*.

When infection of Caco-2 cells by strain 2 was conducted in the presence of different ratios of its own SCS (pH = 6.7), a dose-response behavior was observed both in association and invasion values (Fig. 3A and 3B) ($P < 0.01$). Note that SCS from T1 strain completely detached cells; thus, the effect of extracellular factors on association could not be conducted with this strain (data not shown).

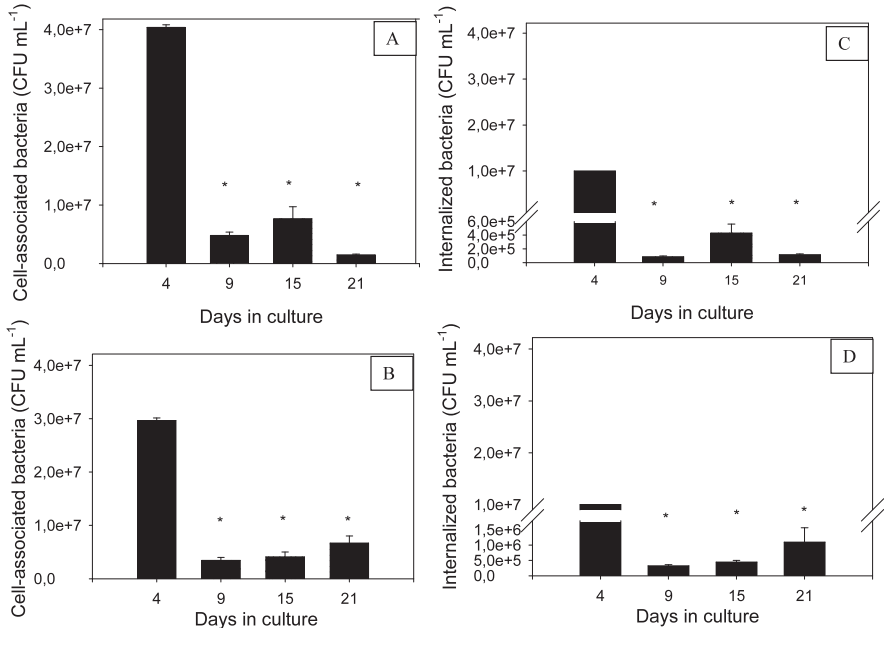

FIGURE 2. *Caco-2 cells after different numbers of days of culture were used to evaluate* B. cereus *association and invasion. Infection was performed in DMEM supplemented with chloramphenicol (100 μg/ml) at 37°C in an atmosphere of 5% CO_2 and 95% air for 2 h. (A and C) Strain 2; (B and D) strain T1. Error bars show standard deviations. Results show a representative experiment from three independent assays. * Significant differences (*$P < 0.05$*) with the cells cultured for 4 days.*

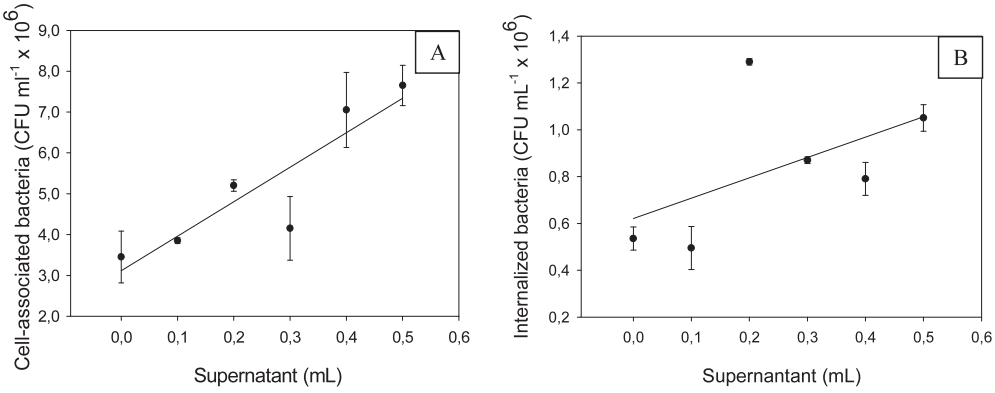

FIGURE 3. *Effect of spent culture supernatant of strain 2 on the association (A) and invasion (B) of* B. cereus *strain 2 to Caco-2 cells. Infection was performed in DMEM supplemented with chloramphenicol (100 μg/ml) at 37°C in an atmosphere of 5% CO_2 and 95% air for 2 h. Error bars represent standard deviations and are not shown when smaller than the symbols. Results show a representative experiment from three independent assays.*

Signaling pathways involved in the biological effects following infection of enterocytes by *B. cereus*. To assess the signaling pathways involved in the biological effects triggered by the infection of cultured human enterocytes by *B. cereus,* experiments with the invading strain T1 were conducted in the presence of inhibitors of different signaling cascades. Strain T1 was selected because it led to dramatic effects on the cytoskeleton of Caco-2 cells in adhesion-invasion assays *(39).*

Results showed that dantrolene, wortmannin, U73122, and staurosporin were able to significantly reduce cytoskeleton disruption that follows infection of enterocytes by *B. cereus* (Fig. 4A). Infection of Caco-2 cells with *B. cereus* strain T1 leads to different levels of alterations of the actin network (Fig. 4C). As an example, we show that, in the presence of U73122, damage was diminished as compared with controls without inhibitor (Fig. 4D). Control incubated with DMEM alone shows normal cell morphology and F-actin distribution (Fig. 4B). Incubation of cells with the inhibitors alone did not lead to cell damage (data not shown).

Because the above-mentioned compounds selectively inhibit signaling pathways related to phosphorylated lipids, we decided to perform further studies with wortmannin and U73122, which inhibit early steps of these activation cascades.

Association of strain T1 decreased in the presence of U73122 and of wortmannin and also when those inhibitors were used together, as compared with control (Table 1). In contrast, invasion values were diminished by U73122, and they were not affected by wortmannin (Table 1). The effects of the inhibitors on the invading ability of strain T1 were reflected by changes in the ratio of invading to associated bacteria. Indeed, this ratio was lower as compared with control for U73122 and when both inhibitors were used together ($P < 0.05$) (Table 1).

DISCUSSION

We have demonstrated that adhesion or invasion are involved in *B. cereus* virulence *(37, 39).* Therefore, we conducted the present study in order to gain further insight into the interaction between *B. cereus* and cultured human enterocytes.

Our work deals with bacteria in vegetative forms (not with spores); these are relevant for intestinal infection since it has been suggested that toxins could be produced in situ in the intestinal lumen *(4).* In addition, other studies have demonstrated that *B. cereus* in its vegetative form is able to survive passage through the acidic conditions of the stomach *(51).*

Infection of cultured human enterocytes by *B. cereus* leads to the disruption of the F-actin network *(39).* This effect could be ascribed to the induction of changes in the apical domain of the enterocyte, which could facilitate invasion of the monolayer *(39).*

Interestingly, infection of differentiated cells leads to a decrease of TEER, thus indicating that permeability of tight junctions (TJs) is affected by infection. These results suggest that basolateral regions of the monolayers could be involved in invasion. These findings correlate with published results obtained by laser confocal microscopy showing that some internalized bacteria are situated at the intercellular region *(39).* Certainly, evidence shown in the present work does not rule out entry of microorganisms through the apical domain but identifies the basolateral domain as one of the regions probably related to the invasion process.

Our results showed that *B. cereus* entry into cultured human enterocytes decreases in differentiated cells. These findings are in agreement with those obtained with other pathogens such as *Yersinia* spp., enteropathogenic *E. coli,* and *Listeria monocytogenes (19, 23).* This phenomenon is closely related to the transition process from undifferentiated to differentiated cells, in which a pivotal change in cell organization is the establishment of polarization, which delineates specific cell domains *(17).* It is known that β-integrins and cadherins are used as receptors for adhering or invading microorganisms *(11).* It has been demonstrated that these molecules, involved in cell-to-cell contact, are localized at the apical domain in proliferating cells. At late postconfluence these molecules relocalize themselves at the cell-to-cell junction and the basal domain *(23, 25).*

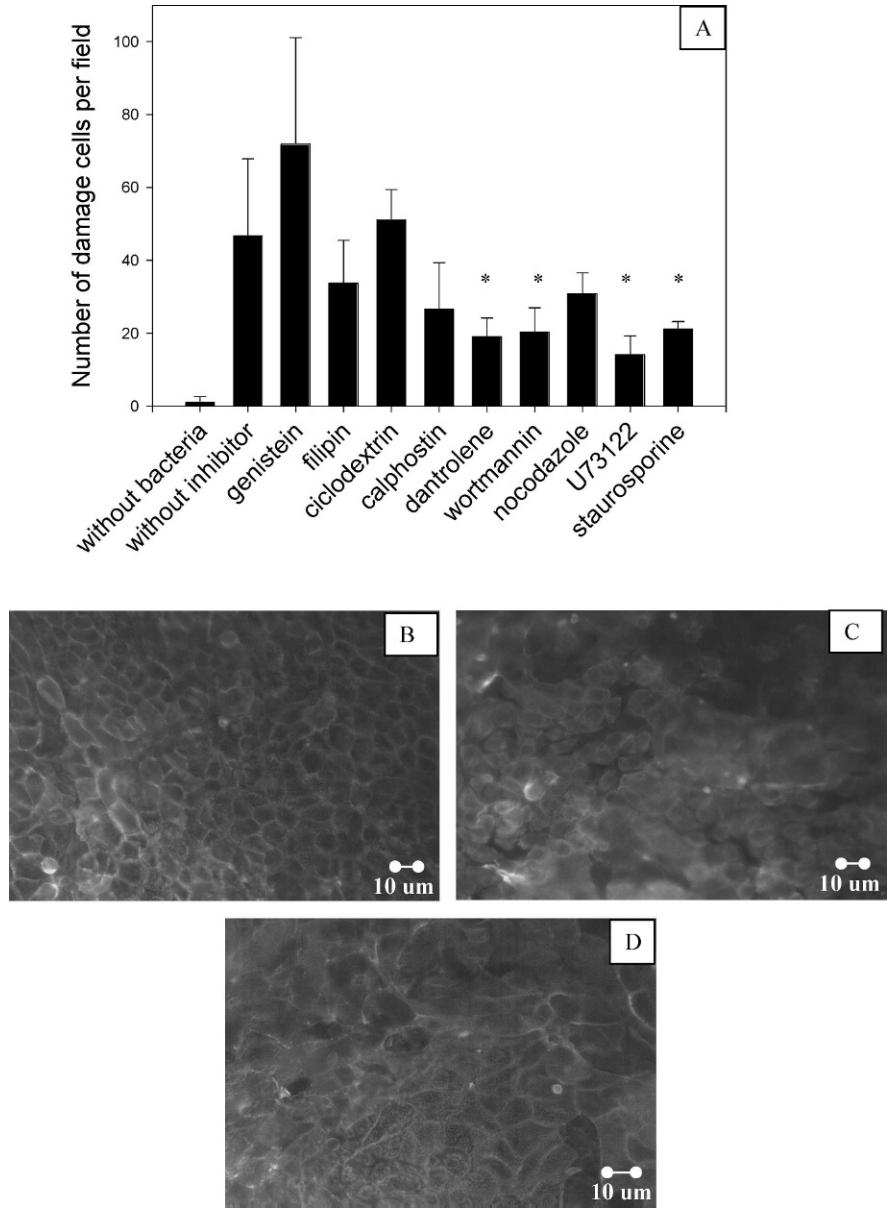

FIGURE 4. *Effect of inhibitors on F-actin disorganization after infection of Caco-2 cells with 1 × 10^8 CFU ml^{-1} of* B. cereus *strain T1. Results show a representative experiment from three independent assays. *, Significant difference (*$P < 0.05$*) with the corresponding control without inhibitor. References for micrographs: without bacteria (B), without inhibitor (C), infection in the presence of U73122 (9.3 μg ml^{-1}) (D). Bar = 10 μm.*

Therefore, in differentiated cells, those receptors are not accessible for adhesion of luminal microorganisms unless cell junctions are disrupted *(25)*.

Infection of enterocytes by *B. cereus* strain 2 in the presence of its own extracellular factors significantly increased association and invasion. These findings could be related to the modification of TJs by secreted factors present in the SCS *(38)*.

Note that concentrations of SCS and experimental conditions used in the present study were selected in order

TABLE 1. *Effect of wortmannin and U73122 on the association and invasion values upon Caco-2 cell infection*[a]

	No. of bacteria (CFU ml^{-1})[b]		
	Association	Invasion	% invading bacteria[c]
Control	5.9 ± 1.1 × 10^6	4.1 ± 0.2 × 10^5	7.0 ± 0.4
U73122	2.7 ± 0.9 × 10^{6}*[d]	1.0 ± 0.6 × 10^{5}*	3.7 ± 2.5*
Wortmannin	4.4 ± 0.3 × 10^{6}*	4.3 ± 0.3 × 10^5	9.9 ± 0.8*
U73122 and wortmannin	6.8 ± 2.9 × 10^{5}*	2.3 ± 1.7 × 10^{4}*	5.4 ± 1.0*

[a] Infection was performed in DMEM supplemented with chloramphenicol (100 μg/ml) at 37°C in an atmosphere of 5% CO_2 and 95% air for 2 h. Multiplicity of infection was 100 bacteria per cell.
[b] Values are means ± standard deviations of triplicate determinations in a representative experiment from three independent assays.
[c] Percentage was calculated as 100 × (invading bacteria/associated bacteria).
[d] * Significant difference ($P < 0.05$) with control.

to avoid cell detachment of cultured enterocytes. However, these low concentrations could lead to a moderate impairment of the cell-to-cell contacts, thus allowing microorganisms to gain access to the basolateral domain. In addition, expression of cell surface molecules could be modified by extracellular factors *(36)*.

Both extracellular and intracellular compartments are crucial for Ca^{2+} homeostasis: extracellular medium and intracellular stores *(28)*. Extracellular Ca^{2+} is required for homotypic interactions of E-cadherin, and it is relevant for the formation of the junctional complex. Intracellular Ca^{2+} is involved in the actin polymeration process, and it is critical for the biogenesis of cell-to-cell contacts *(30)*. These facts must be considered for the understanding of the signaling mechanisms involved in the formation of TJs where protein kinase C plays a central role *(17)*. Other protein kinases (e.g., protein kinase A) are present in the maintenance and assembly of TJs. Since protein kinase C is critical for mobilizing intracellular Ca^{2+} during TJs biogenesis, heterotrimeric G proteins could also be involved in this process *(17)*.

Signaling via phosphorylated lipids is a key step for the virulence of many pathogens such as uropathogenic *E. coli, Yersinia* spp., *Salmonella enterica, L. monocytogenes, Shigella flexneri,* and *Mycobacterium marinum (26, 41)*. Phagocytosis of several microorganisms is associated to phosphoinositides that bind to cytoeskeletal proteins like ezrin, radixin, and moesin *(15, 16, 54)*.

Our results constitute the first evidence of the role played by phospholipase C and PI3K in *B. cereus* adhesion to cultured enterocytes. Invasion is mainly related to the activation of the phospholipase C pathway since U73122 (phospholipase C inhibitor) significantly reduced the ratio of invading bacteria. It is known that bacterial pathogens take advantage of the scaffolding role of phosphatidylinositol to recruit entry site effectors required for promoting (or inhibiting) bacterial invasion, or to modify the maturation of their intracellular compartments *(14, 41)*. Note that phosphatidylinositol is relevant for the lung epithelial cell internalization of a *B. cereus* group member: *B. anthracis* spores *(53)*.

As expected, cell damage also decreases by inhibition of release of the intracellular Ca^{2+} stores (dantrolene) or inhibition of protein kinase C (staurosporine). These findings suggest that Ca^{2+} (extracellular and intracellular) is involved in the invasion of enterocytes by *B. cereus*.

In summary, the results obtained show that, after interaction between vegetative *B. cereus* and enterocytes, activation of signaling cascades related to phosphorylated lipids leads to significant changes of the cytoskeleton, which in turn could facilitate invasion of the monolayers.

Even though further studies are necessary to completely unravel the mechanisms involved in *B. cereus* pathogenesis, the present study will contribute to the understanding of the interaction between *B. cereus* and the host in the context of intestinal infections.

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