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Effect of *Bacillus cereus* Exocellular Factors on Human Intestinal Epithelial Cells

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ABSTRACT

To gain insight on the biological effects of the exocellular factors produced by *Bacillus cereus,* culture filtrate supernatants of different strains were coincubated with differentiated Caco-2 cells. Exocellular factors were able to detach enterocyte-like cells from the substratum after 1 h of incubation. In addition, microvilli effacing and dramatic changes on the cellular surface of enterocytes were found after incubation periods as short as 20 min. Since cell detachment was not inhibited by fetal calf serum, thiol activated cholesterol-binding cytolysin, cereolysin O, does not seem to be involved. Also, translocation of phosphatidylserine from the inner to the outer leaflets of the plasma membrane was demonstrated by using fluorescein isothiocyanate (FITC)-Annexin V. In contrast to the high capability of detaching Caco-2 cells shown by all the strains under study, the mitochondrial dehydrogenase activity was lowered by culture filtrate supernatants in a strain-dependent manner. For strain M2, the decrease in dehydrogenase activity was already evident after 30 min of incubation. Production of biologically active factors depends on the growth phase, and maximal activity was found in late exponential-early stationary phases. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of concentrated exocellular factors showed a very complex scenery supporting the multifactorial character of the biological activity of *B. cereus.*

Like other members of the genus *Bacillus,* it is generally accepted that the primary habitat of *Bacillus cereus* is the soil. However, due to the resistance of its spores to physicochemical stresses, this microorganism can be isolated from a wide variety of sources.

Contamination of foods by *B. cereus* constitutes not only an important cause of spoilage, but also, it is associated with both diarrheal and emetic syndromes. Diarrheal syndrome is characterized by abdominal pain, a profuse watery diarrhea, and rectal tenesmus that occur between 8 and 16 h after ingestion of food containing *B. cereus.* Nausea and vomits, starting 1 to 5 h after ingestion *(6, 28),* characterize the emetic syndrome. In addition, *B. cereus* can produce extraintestinal syndromes such as posttraumatic endophthalmitis *(11, 12, 18).*

Several biologically active factors have been described. Indeed, *B. cereus* is able to produce (i) cereolysin O, a thiol activated cholesterol-binding cytolysin *(3, 26)*; (ii) phospholipases *(11, 13, 25)*; (iii) emetic toxin or cereulide: a heat-stable cyclic dodecadepsipeptide with a molecular mass around 1.2 kDa *(1, 2, 32)*; (iv) hemolysin BL, a tripartite enterotoxin that requires all the three components (B, L1, and L2) for maximal activity *(7, 10–13, 18, 30)*; (v) the nonhemolytic enterotoxin complex, a complex of three subunits *(30)*; and (vi) hemolysin IV, which shows a strong effect on plasma membranes with a wide range of compositions *(11).* However, involvement of other factors cannot be ruled out, and some authors have suggested that adhesion of *B. cereus* spores to enterocytes might play a role in virulence *(4).*

To assess toxigenic activity of *B. cereus* isolates, different methods have been employed—e.g., hemagglutination assays *(23)*; immunological methods *(9, 16, 17, 20)*; in vivo assays *(2, 9, 10)*; polymerase chain reaction-based assays *(31)*; and assays employing eucaryotic cells in culture *(2, 25, 27, 30, 32, 36),* as well as assays employing spermatozoa *(5).*

In spite of the well-documented scientific evidence related to the pathogenic capability of *B. cereus,* mechanisms involved in the interaction between its exocellular virulence factors and host cells are not fully understood.

To elucidate interactions between *B. cereus* and the host in the digestive tract, the use of human enterocytes in culture seems to be an interesting approach. Among several available cell lines, Caco-2 cells (ATCC HTB-37) constitute a good reductionist model *(22).* Indeed, Caco-2 cells spontaneously differentiate in culture exhibiting characteristics typical of the intestinal epithelial cells—e.g., microvilli, tight junctions, and high levels of brush border-associated enzymes such as alkaline phosphatase *(35).* Due to the above-mentioned characteristics, Caco-2 cells have been used in several studies to mimic bacteria-host interactions in the intestinal tract *(14, 15, 19, 33, 34).*

The aim of the present work was to gain insight to the biological activity of the exocellular factors produced by enterotoxigenic strains of *B. cereus* isolated from foods by using human enterocytes in culture.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. *B. cereus* strains belong to the Cátedra de Microbiología General de la Facultad de Ciencias Exactas de la Universidad Nacional de La Plata and were isolated from skim milk powder (strains M2, 2, and 3), milk powder (strains 253 and 273), and infant formula (strain 11.3). Strain T2 was kindly provided by Dr. R. L. Buchanan and F. J. Schultz from the U.S. Department of Agriculture, Philadelphia, Pa. *(16, 17)*. Bacteria were grown in brain heart infusion (BHI) broth (Biokar Diagnostics, Beauvais, France) at 32°C for 16 h. Bacterial concentration was evaluated by means of optical density readings at 600 nm (Metrolab RC 325, Buenos Aires, Argentina). Cultures were centrifuged at 900 × *g* for 10 min, and supernatants were collected. To avoid damaging of Caco-2 cells by low pH, supernatants were neutralized to pH 7 with 0.1 N NaOH. Afterwards, bacteria were removed by filtration (0.45 μm). All strains under study were positive in the *Bacillus* diarrheal enterotoxin visual immunoassay (Tecra) performed according to the manufacturer's instructions.

Culture of enterocyte-like cells. Caco-2 cells were grown in Dulbecco modified Eagle medium supplemented with nonessential amino acids, penicillin (12 IU/ml), streptomycin (12 μg/ml), gentamicin (50 μg/ml), fungizone (1.25 μg/ml), and inactivated (30 min, 58°C) fetal calf serum (20% vol/vol). Monolayers were prepared in 24-well tissue culture plates (Iwaki Glass, Funabashi, Japan) by seeding 2×10^5 cells per well. Incubations were performed at 37°C in a 5% CO_2-95% air atmosphere. Culture medium was changed every 2 days. Assays were performed with cells at passages between 30 and 50 and with differentiated monolayers (21 days in culture).

Detachment of Caco-2 cells. Detachment of enterocyte-like cells was measured as reported previously *(16)*. Briefly, differentiated Caco-2 monolayers were coincubated with filter-sterilized supernatants (0.5 ml per well) at 37°C for different time intervals. Cells were washed twice with glucose-potassium-sodium solution (GKN; in g/liter: NaCl, 8; KCl, 0.4; glucose, 2; $NaH_2PO_4 \cdot H_2O$, 0.69; Na_2HPO_4, 1.57; pH 7.2 to 7.4), fixed for 1 min at room temperature with 2% formaldehyde in phosphate-buffered saline and washed again with GKN. Afterwards, cells were stained by incubating for 20 min at room temperature with 500 μl of crystal violet solution (0.13% crystal violet, 5% ethanol, and 2% formaldehyde in GKN). After being washed with GKN to eliminate stain excess, samples were treated with freshly prepared 50% ethanol for 1 h at room temperature. Absorbance was measured at 650 nm (Metrolab RC 325). Percentage of cell detachment was calculated as follows:

$$\text{Cell detachment } \% = 100 \times (\text{Ac} - \text{As})/\text{Ac}$$

where Ac = A_{650} of control cells, and As = A_{650} of sample cells.

Mitochondrial dehydrogenase activity. Conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium (MTT) to an insoluble purple formazan by mitochondrial dehydrogenases was assessed by means of a modification of a previously reported protocol *(21)*. After coincubation with culture filtrate supernatants, cells were detached by mechanical scraping and incubated with MTT (Sigma Chemical Co., St. Louis, Mo.) for 4 h at 37°C (final concentration 0.5 mg/ml in phosphate-buffered saline). Samples were centrifuged at 14,000 × *g* for 1 min, and stain was extracted with 0.1 N HCl in isopropanol. After centrifugation, absorbance was measured at 550 nm (reference wavelength 650 nm) (ELISA Plate Reader SLT Rainbow Reader, Wien, Austria). Percentage of remaining activity was calculated as 100 × *A*/Ac, where *A* is the absorbance of treated cells, and Ac is the absorbance of control cells.

Scanning electron microscopy. Cells were cultured on round glass coverslips (Marcel Blanc & Cie S.A. Le Mont, Lausanne, Switzerland). After coincubation at 37°C with culture filtrate supernatants, specimens were washed with GKN solution and fixed in 2.5% (vol/vol) glutaraldehyde (Riedel de Haen, Seelze, Germany) for 16 h at 4°C. Smears were dehydrated in a graded series of ethanol solutions. Finally, samples were critical point dried using CO_2 (Model CP30, Baltec), gold coated (Jeol FineCoat Sputter JFC-1100, Jeol Ltd., Akishima Tokyo, Japan), and examined using a Jeol model JSM-T100 scanning electron microscope (Jeol Ltd.).

Translocation of phosphatidylserine in plasma membrane. One of the earlier events of both apoptotic and necrotic pathways is the translocation of phosphatidylserine from the inner to the outer leaflet of the plasma membrane. The resulting loss of the plasma membrane asymmetry can be detected by using Annexin V, a Ca^{2+}-dependent phospholipid-binding protein that shows a high affinity for phosphatidylserine.

Detection of cells in which translocation of phosphatidylserine has occurred was performed by a modification of a previously reported method *(37)*. Briefly, cells cultured on glass coverslips were treated with culture filtrate supernatants for 1 h at 37°C, washed two times with phosphate-buffered saline, and incubated for 1 h at 37°C with 100 μl of binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4) and 5 μl of recombinant Annexin V (Pharmingen, San Diego, Calif.). After being washed with binding buffer, samples were mounted in glycerol 50% (vol/vol) and examined by fluorescent microscopy (Leica DMLB equipped with a Leica DC100 camera, Leica Microscopy Systems Ltd., CH Heerbrugg, Switzerland).

Statistical analysis. Statistical analysis of the variations was performed by analysis of variance (Tukey's test for least significant differences) and paired Student's *t* test (Systat, Inc., Evanston, Ill.).

RESULTS

Coincubation of Caco-2 monolayers with culture filtrate supernatants of *B. cereus* leads to cell detachment values over 70% for all strains under study (Fig. 1).

Figure 2 shows the effect of different concentrations of culture filtrate supernatants of strains M2 and T2 on Caco-2 monolayers. Even though, when applied at high concentrations, supernatants of both strains produce the same cell detachment, strain T2 showed higher biological activity at lower doses compared to strain M2 (the differences between strains M2 and T2 were significant with $P < 0.05$, for 20 and 40% relative concentration, respectively). It must be pointed out that cultures where supernatants were obtained had the same bacterial concentration in terms of optical density values at 600 nm.

Scanning electron microscopy of Caco-2 cells after 20 min of incubation with culture filtrate supernatants of strain M2 is depicted in Figure 3. Whereas controls coincubated with BHI broth showed typical microvilli of differentiated Caco-2 cells, the treatment with exocellular factors of strain M2 leads to microvilli effacing in some cells.

As shown in Figure 4, culture filtrate supernatants of strains M2, T2, and 273 induce translocation of phospha-

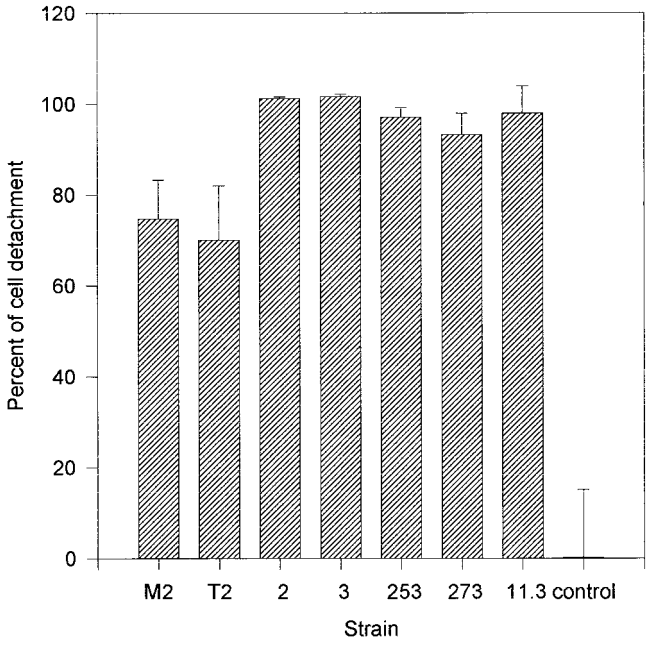

FIGURE 1. *Effect of* B. cereus *culture filtrate supernatants on Caco-2 cells. Bars represent percentage of cell detachment ± standard deviation (*n = *3). Controls were incubated with sterile BHI medium.*

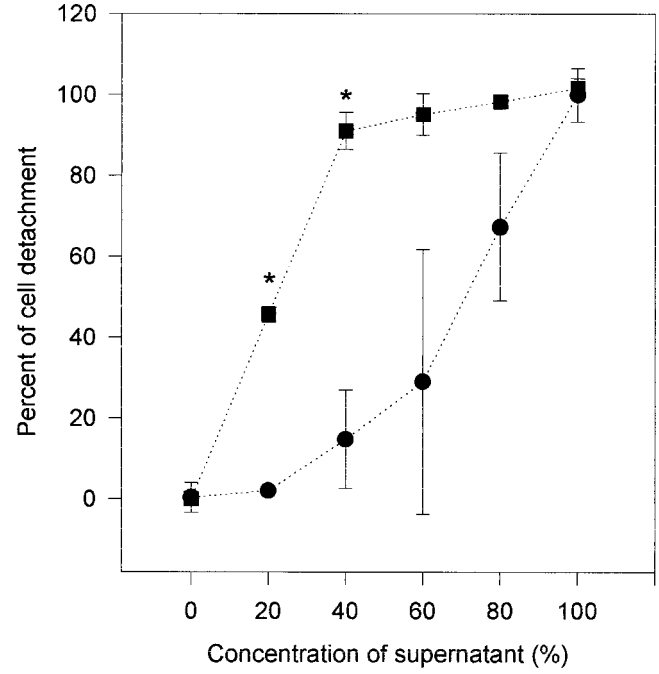

FIGURE 2. *Detachment of enterocyte-like cells (Caco-2) produced after coincubation with different concentrations of culture filtrate supernatants of* B. cereus *strains M2 and T2. Values represent averages from three determinations ± standard deviation. References: (■) strain T2, (●) strain M2, and (*) indicates significant differences between the two strains (*$P < 0.05$*).*

tidylserine from the inner to the outer leaflet of the plasma membrane of enterocyte-like cells. This leads to the binding of FITC-Annexin V.

Even though all strains under study were able to detach enterocyte-like cells, effect of culture filtrate supernatants on dehydrogenase activity was strongly strain dependent. After 1 h of incubation, percentages of dehydrogenase activity related to controls ranged from 16.3 ± 4.6% to 76.8 ± 10.9% for strains 2 and 273, respectively (Table 1). Note that strain 273 did not show significant differences with control ($P > 0.1$) in spite of its capacity to detach cells.

Coincubation of monolayers with culture filtrate supernatants of strain M2 for different time intervals (Fig. 5) showed that mitochondrial dehydrogenase activity was affected after incubation periods as short as 30 min. Controls of BHI did not produce significant changes.

Production of biologically active factors during growth of strain M2 showed that maximal biological activity of the supernatant, as assessed by MTT assay, was achieved for cultures at stationary phase (data not shown).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) performed with concentrated and dialyzed supernatants of strain M2 grown in BHI broth showed a very different pattern from that of concentrated uninoculated BHI. In addition to one diffuse band around 56 kDa shown in uninoculated BHI broth, seven different bands could be found in concentrate culture filtrate supernatants of strain M2. Large amounts of material with low molecular mass were also observed (data not shown).

DISCUSSION

B. cereus is able to produce intestinal disorders, but virulence factors have not been fully characterized. Commercial kits react with different molecules: whereas the Oxoid kit detects the L_2 component from the hemolysin BL that shows high biological activity in the vascular permeability assay, the Tecra kit reacts with two proteins codified by the bceT gene fragment that do not produce dermal reactions in rabbits *(8).* Although the above findings support the hypothesis that the products of the bceT gene are nontoxic *(31),* some authors have suggested that they are part of the nonhemolytic enterotoxic complex *(29).*

Concerning the heat-stable emetic toxin, its low molecular weight and low antigenicity make the development of immunological methods of detection difficult. For this factor, a colorimetric MTT-based assay that uses Hep-2 cells has been developed *(21).* However, some authors have suggested that the heat-stable toxin of *B. cereus* could be a product of the growth medium resulting from an enzymatic modification, a degradative modification, or both *(24).* When assayed on different cell lines, heated supernatants of *B. cereus* grown in rice-based medium showed a wide range of biological responses *(36);* thus, it is very difficult to extrapolate results from different authors.

The present work shows that supernatants from *B. cereus* cultures have a strong effect on cultured human enterocytes. Interaction between Caco-2 cells and plastic surfaces was affected, thus resulting in cell detachment. It should be noted that culture filtrate supernatants from nonpathogenic intestinal microorganisms (*Lactobacillus* spp. and *Bifidobacterium* spp.) did not affect enterocytes (data not shown). Although these findings do not rule out the likelihood that nonspecific factors are acting on Caco-2 cells, they support the idea that we are not dealing with a

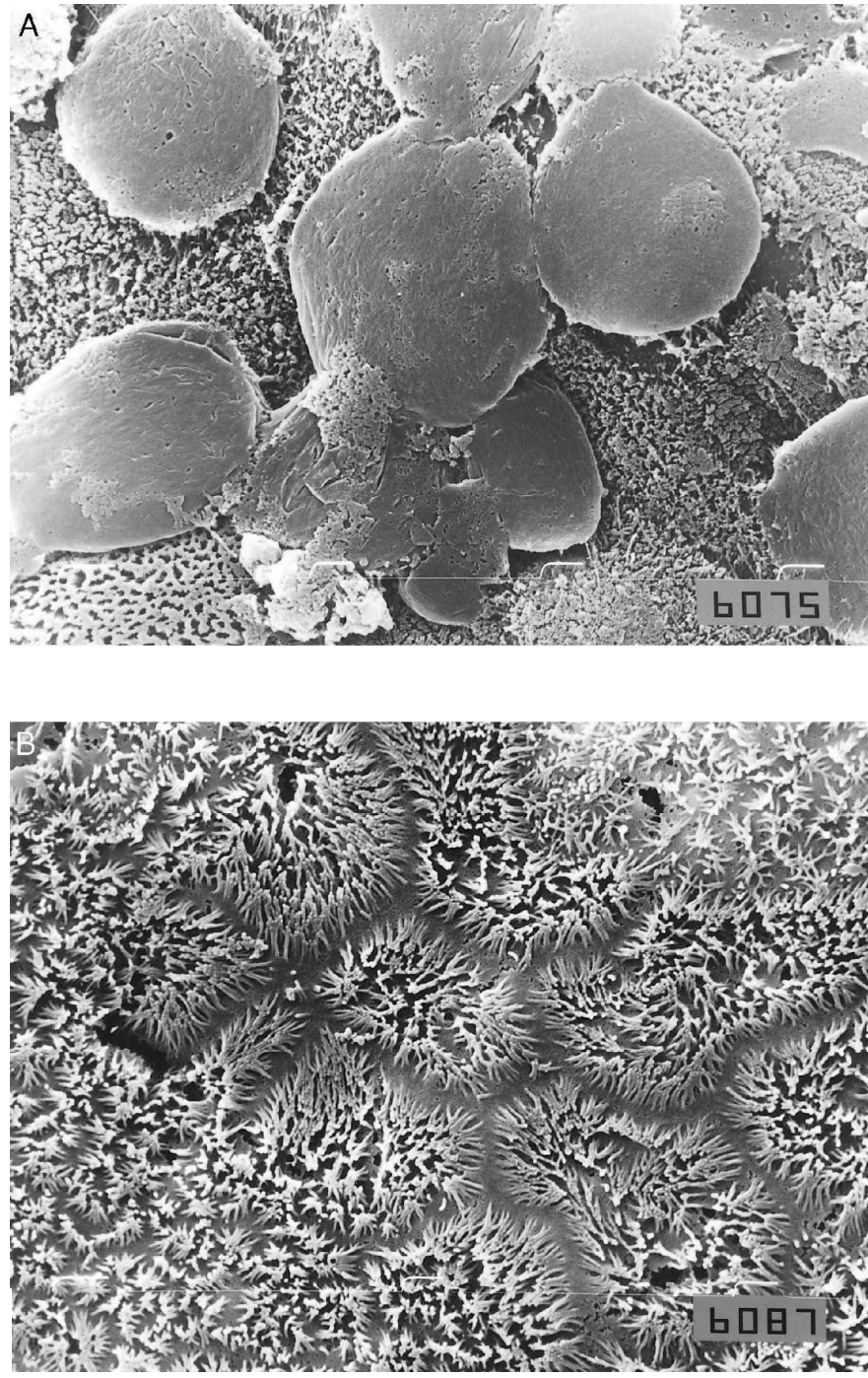

FIGURE 3. *Scanning electron micrography of differentiated Caco-2 cells coincubated for 20 min with (A) culture filtrate supernatant of strain M2 and (B) BHI medium.*

general mechanism of damage produced by any bacterial supernatant.

The effect of extracellular factors produced by *B. cereus* may not be ascribed to proteases, since Caco-2 monolayers coincubated with supernatants of *B. cereus* in the presence of 20% fetal calf serum also showed cell detachment (data not shown). In addition, since fetal calf serum contains cholesterol, involvement of cholesterol-binding toxins such as cereolysin O in this cytopathic effect can be ruled out. We also showed that the enterocyte surface was damaged, since microvilli effacing was observed. This effect could be observed after incubations as short as 20 min. It should be noted that these findings correlate with the decrease in dehydrogenase activity at short incubation periods (Fig. 5).

Translocation of phosphatidylserine to the external surface of enterocyte-like cells (Fig. 4) suggests induction of early events related either to apoptotic or necrotic pathways *(37).* These findings could be related to cytoskeleton disorganization, with another hypothesis being the effect of phospholipase C activity that disrupts eucaryotic cell membranes by hydrolysis of their constitutive phospholipids *(26).*

For all strains under study, exocellular factors were heat labile, and biological activity was completely abolished after 30 min at 100°C (data not shown). Therefore,

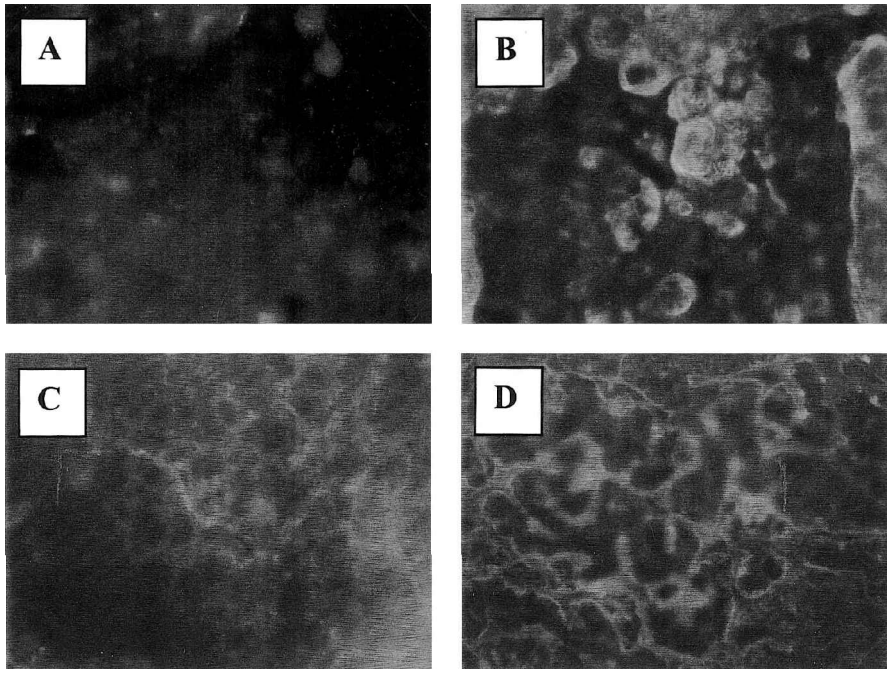

FIGURE 4. *Staining of Caco-2 cells with Annexin V-FITC. Cells were incubated for 1 h with BHI broth (A), culture filtrate supernatants of strains T2 (B), M2 (C), and 273 (D).*

emetic toxin does not seem to be involved in the biological activity of the strains under study.

In dose-response experiments, cells were not detached at low doses, but different strains led to different degrees of staining after decoloration steps (data not shown). These findings could be due to factors capable of modifying cell permeability without affecting cell interactions with the substrate. Furthermore, coincubation with supernatants leads to a diminution of the capability of MTT reduction in enterocyte-like cells, thus suggesting that biologically active factors affect mitochondrial activity.

Interestingly, strain T2 produces detachment of differentiated Caco-2 monolayers. These results are different from those previously reported for this strain with CHO and Hep-2 cells *(16, 17),* where no biological activity was found when culture filtrate supernatants of this strain were coincubated with these cell lines. It should be noted that the human intestinal epithelial cells used in our study are a widely accepted model of human intestinal epithelial cells. Although cytopathic effects can be detected with several cell lines, the use of Caco-2 cells would allow the detection of host-pathogen interactions typical of the intestinal tract.

Analysis of the proteins produced by strain M2 showed that culture filtrate supernatants of *B. cereus* constitute a complex system (data not shown). Therefore, two different approaches can be considered: (i) study of the effect of exocellular factors as a whole, and (ii) purification of different fractions and study of their biological activity. In the

TABLE 1. *Percentage of mitochondrial dehydrogenase activity remaining in Caco-2 monolayers coincubated for 1 h with culture filtrate supernatants of* B. cereus

Source of supernatant	% of dehydrogenase activity[a,b] ± standard deviation
Control (BHI broth)[c]	10.0 ± 14.2
M2	39.0 ± 11.9*
273	76.8 ± 10.9
123	64.1 ± 8.6**
253	46.2 ± 3.8*
3	28.8 ± 3.8*
2	16.3 ± 4.6*

[a] Percentage of dehydrogenase activity = (A_{550} sample/A_{550} control) × 100.

[b] Differences with the control at $*P < 0.002$ and $**P < 0.02$ levels.

[c] BHI, brain heart infusion.

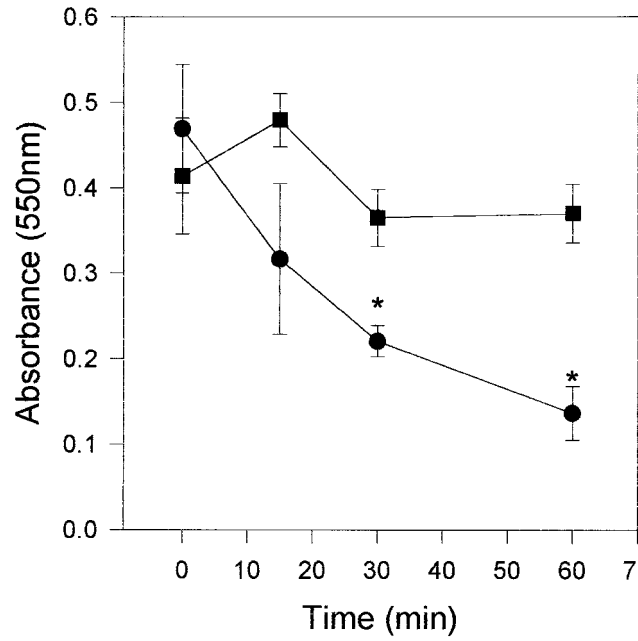

FIGURE 5. *Time course of MTT assay performed on Caco-2 cells coincubated with culture filtrate supernatants of strain M2. Results are averages of three replicates ± standard deviation. References: (■) control, (●) strain M2, and (*) indicates significant differences with the corresponding control (*P < 0.05).*

present study, we chose the former approach in an attempt to mimic the actual situation in the gut. However, to gain insight on the mechanisms associated with the action of virulence factors, studies with purified fractions must be conducted.

In short, we show that *B. cereus* exocellular factors produce dramatic changes in enterocyte-like cells. These changes encompass microvilli effacing, loss of plasma membrane asymmetry of mitochondrial activity, and cell detachment. Should all of these exocellular factors be responsible for the in vivo activity of *B. cereus* strains, it will be a matter for discussion and further studies will be required.

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