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ScienceDirect
International Journal of Food Microbiology 116 (2007) 197–206
INTERNATIONAL JOURNAL OF Food Microbiology
www.elsevier.com/locate/ijfoodmicro

Virulence of *Bacillus cereus*: A multivariate analysis

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Received 1 June 2006; received in revised form 28 December 2006; accepted 29 December 2006

Abstract

Biological activity and presence of DNA sequences related to virulence genes were studied in 21 strains of the *Bacillus cereus* group. The activity of spent culture supernatants and the effect of infection by vegetative bacterial cells were assessed on cultured human enterocytes (Caco-2 cells). The effect of extracellular factors on the detachment, necrosis and mitochondrial dehydrogenase activity of cultured human enterocytes was studied. Hemolytic activity on rabbit red blood cells was also evaluated and the effect of direct procaryotic–eucaryotic interactions was assessed in infection assays with vegetative bacterial cells.

Concerning virulence genes, presence of the DNA sequences corresponding to the genes *entS*, *entFM*, *nhe* (*A*, *B* and *C*), *sph*, *hbl* (*A*, *B*, *C* and *D*), *piplC* and *bceT* was assessed by PCR. Ribopatterns were determined by an automated riboprinting analysis after digestion of the DNA with *Eco*RI. Principal component analysis and biplots were used to address the relationship between variables.

Results showed a wide range of biological activities: decrease in mitochondrial dehydrogenase activity, necrosis, cell detachment and hemolytic activity. These effects were strain-dependent. Concerning the occurrence of the DNA sequences tested, different patterns were found. In addition, ribotyping showed that strains under study grouped into two main clusters. One of these clusters includes all the strains that were positive for all the DNA sequences tested.

Positive and negative correlations between variables under study were evidenced. Interestingly, high detaching strains were positively correlated with the presence of the sequences *entS*, *nheC* and *sph*. Within gene complexes, high correlation was found between sequences of the *hbl* complex. In contrast, sequences of the *nhe* complex were not correlated. Some strains clustered together in the biplots. These strains were positive for all the DNA sequences tested and they were able to detach enterocytes upon infection.

Our results highlight the multifactorial character of the virulence of the *B. cereus* group and show the correlation between ribopatterns, occurrence of toxin genes and biological activity of the strains under study.

Keywords: *Bacillus cereus*; Virulence; Caco-2 cells; Biplot; Virulence genes; Multivariate analysis

1. Introduction

The *Bacillus cereus* group constitutes a very homogeneous cluster within the *Bacillus* genus. This group (often called *B. cereus sensu lato*) comprises six species: *B. cereus*, *Bacillus anthracis*, *Bacillus thuringiensis*, *Bacillus mycoides*, *Bacillus pseudomycoides* and *Bacillus weihenstephanensis* (Jensen et al., 2003).

B. cereus (*sensu stricto*) is an opportunistic sporeforming microorganism able to produce intestinal and non-intestinal pathologies (Shoeni and Lee Wong, 2005). Virulence of this microorganism has been ascribed to different extracellular factors. Two of these virulence factors are protein complexes, i.e. the hemolysin BL (HBL) (Beecher et al., 1995; Beecher and Lee Wong, 2000) and the non-hemolytic enterotoxin (NHE,

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doi:10.1016/j.ijfoodmicro.2006.12.013

Lund and Granum, 1997). Other factors are single gene products encoded by *entFM* (enterotoxin FM), *cytK* (cytolysin K) and *bceT* (*B. cereus* enterotoxin) (Fagerlund et al., 2004; Lund et al., 2000). Products from other genes such as phosphatidylinositol specific phospholipase (*piplC*), enterotoxin S (*entS*), sphingomyelinase (*sph*), cereolysin O (clo) are also involved in pathogenesis (Alouf, 2000; Beecher et al., 2000; Granum and Nissen, 1993). Virulence of the so called emetic strains is related to cereulide, a thermostable cyclic dodecadepsipeptide synthesized by a non-ribosomal peptide synthetase encoded in *ces* genes (Agata et al., 1994; Ehling-Schulz et al., 2004; Horwod et al., 2004). In addition, a cell-bound metalloprotease (camelysin) also seems to be involved in virulence (Fricke et al., 2001).

In recent years, research in the field of *B. cereus* virulence is starting to unravel a multifactorial system where direct bacteria–cell interactions could also play a main role (Minnaard et al., 2004). *In vitro* assays have demonstrated that coincubation of cultured human enterocytes with either spent culture supernatants or vegetative cells of *B. cereus* leads to cytopathic effects. These effects include cell detachment, decrease in mitochondrial dehydrogenase activity, dramatical morphological changes and actin disassembly (Minnaard et al., 2001, 2004). Furthermore, some strains have demonstrated the ability to invade monolayers of cultured enterocytes thus adding another dimension to the mechanisms responsible for *B. cereus* virulence (Minnaard et al., 2004).

Study of the population structure of microorganisms belonging to the *B. cereus* group has been based on sequence analysis of housekeeping genes (Ko et al., 2004; Apetroaie et al., 2005), ribotyping (Grif et al., 2003), RAPD-PCR (Ghelardi et al., 2002), analysis of virulence genes (Ehling-Schulz et al., 2005) and phenotypic characteristics (Guttmann and Ellar, 2000). Experimental evidence suggests correlation between the presence of some genes and the biological activity of the microorganisms. Indeed, simultaneous presence of *hbl* genes is associated with discontinuous hemolytic pattern, a key characteristic of hemolysin BL (Thaenthanee et al., 2005). PCR results regarding genes *hbl*, *nhe* and *bceT* have been correlated with results obtained by using immunological commercial kits (Guinebretière et al., 2002; Rowan et al., 2003) and toxicity on Hep-2 cells (Rowan et al., 2003; Ehling-Schulz et al., 2005).

Correlation between genotypic and phenotypic markers allows for gaining further insight on the molecular epidemiology and virulence mechanisms of this microorganism. In this context, the aim of the present study was to determine the relationships between riboprint patterns, presence of virulence genes and biological activity on cultured human enterocytes of *B. cereus* strains by means of a multivariate analysis.

2. Materials and methods

2.1. Strains and culture media

Strains used in the present study are listed in Table 1. Bacteria were grown in 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, Argentina). Cultures were centrifuged at 900 ×*g* for 10 min and supernatants were collected. In order 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 (BDE) Visual Immunoassay (TECRA International Pty Ltd. Australia) performed according to the manufacturer's instructions.

Table 1
Nomenclature and origin of the strains studied in the present paper

Strain	Source	Reference
2	Skim milk powder	Minnaard et al. (2001)
114	Infant formula	Minnaard et al. (2001)
273	Infant formula	Minnaard et al. (2001)
113	Infant formula	Minnaard et al. (2001)
253	Infant formula	Minnaard et al. (2001)
M2	Skim milk powder	Minnaard et al. (2001)
3	Skim milk powder	Minnaard et al. (2001)
313	Infant formula	This paper
124	Infant formula	This paper
123	Infant formula	This paper
93	Infant formula	This paper
T1	Unknown	Buchanan and Schultz (1992)
T2	Unknown	Buchanan and Schultz (1992)
E2[a]	Unknown	Buchanan and Schultz (1992)
Watertown	Unknown	Buchanan and Schultz (1992)
A7	Unknown	Buchanan and Schultz (1992)
M845	Infant formula	This paper
M74	Infant formula	This paper
B10502	Food-poisoning outbreak	Minnaard et al. (2004)
ATCC 10876	ATCC	–
ATCC 13061	ATCC	–

[a] All the strains except strain E2 (*Bacillus thuringiensis*) belong to the *Bacillus cereus* species.

2.2. Ribotyping

Ribotyping was performed using the standard method of the automated ribotyping device RiboPrinter System (Dupont Qualicon, USA), as described by Bruce (1996) and according to the manufacturer's instructions. Bacteria were grown overnight at 37 °C on Tryptic Soy Agar (TSA, Merck). The automated process includes bacterial cell lysis and cleavage of the DNA using the restriction endonuclease *Eco*RI. DNA fragments were hybridized with a DNA probe harbouring the genes for the small and large rRNA subunits of *Escherichia coli*.

2.3. PCR

Chromosomal DNA was extracted using GFX Genomic Blood DNA Purification Kit (Amersham Biosciences Corp, New Jersey, USA) following the manufacturer's instructions. Quality of DNA extracted was controlled by electrophoresis on 1% (w/v) agarose (Invitrogen Corp., California, USA.) gel using 1× TAE buffer (40 mM Tris-acetate, 1 mM EDTA) and ethidium bromide (0.5 mg/ml). All strains were screened for the

Table 2
PCR primers and annealing temperatures used in this study

Target	Primer	Primer sequence (5′–3′)[a]	Annealing temperature (°C)[a]	Size of the PCR product (bp)
sph	Ph1 F	CGTGCCGATTTAATTGGGGC	58	558
	Ph2 F	CAATGTTTTAAACATGGATGCG		
bceT	ETF	TTACATTACCAGGACGTGCTT	56	428
	ETR	TGTTTGTGATTGTAATTCAGG		
entFM	ENTA F	ATGAAAAAAGTAATTTGCAGG	52	1269
	ENTB R	TTAGTATGCTTTTGTGTAACC		
entS	TY123 F	GGTTTAGCAGCAGCTTCTGTAGCTGGCG	60	581
	TY125 R	GTTTCGTTAGATACAGCAGAACCACC		
piplC	PC105 F	CGCTATCAATGGACCATGG	57	569
	PC106 R	GGACTATTCCATGCTGTACC		
nheA	*nheA* 344S F	TACGCTAAGGAGGGGCA	55	499
	nheA 843A R	GTTTTTATTGCTTCATCGGCT		
nheB	*nheB* 1500 S F	CTATCAGCACTTATGGCAG	55	769
	nheB 2269 A R	ACTCCTAGCGGTGTTCC		
nheC	*nheC* 2820 S F	CGGTAGTGATTGCTGGG	55	581
	nheC 3401 S R	CAGCATTCGTACTTGCCAA		
hblA	*HblA* F	AAGCAATGGAATACAATGGG	56	1154
	HblA R	AGAATCTAAATCATGCCACTGC		
hblB	*HblB* F	AAGCAATGGAATACAATGGG	58	2684
	HblB R	AATATGTCCCAGTACACCCG		
hblC	*HblC* F	GATACTAATGTGGCAACTGC	58	740
	HblC R	TTGAGACTGCTGTCTAGTTG		
hblD	*HblD* F	ACCGGTAACACTATTCTAGC	58	829
	HblD R	GAGTCCATATGCTTAGATGC		

F: forward; R: reverse.
[a] Ghelardi et al. (2002) and Guinebretière et al. (2002).

presence of the genes *sph* (sphingomyelinase), *bceT* (*B. cereus* enterotoxin), *entFM* (enterotoxin FM), *ent*S (enterotoxin S), *piplC* (phosphatidylinositol phospholipase C) and components of the NHE (non-hemolytic enterotoxin) and HBL (hemolysin BL) complexes. Primers and annealing temperatures used in PCR are listed in Table 2.

DNA amplifications were performed in an Eppendorf Mastercycler gradient equipment (Eppendorf-Netheler-Hinz

Table 3
Biological activity of the strains under study

Strain	% detach[a]	Model[b]	Detach inf[c]	% MTT[d]	Necrosis[e]	Hemolysis[f]
2	101.2 (0.37)	H	−	16.3 (4.6)	44 (19)	40 (34)
114	97.2 (0.14)	L	−	nt	33 (5)	64 (0)
273	93.3 (4.7)	H	−	76.8 (10.9)	14 (4)	48 (23)
B10502	6.1 (0.1)	N	+	nt	nt	128 (0)
T2	99 (11.9)	L	−	116.8 (0.8)	6 (4)	40 (34)
113	98 (5.9)	H	−	nt	58 (4)	160 (135)
253	97.1 (2.0)	H	−	46.2 (3.8)	12 (4)	64 (0)
E2	84.0 (5.4)	H	−	32.9 (6.1)	nt	72 (79)
M2	74.7 (8.5)	L	−	39.5 (11.9)	14 (5)	80 (68)
3	101.6 (0.6)	H	−	28.8 (3.8)	78 (7)	40 (34)
Watertown	101.3 (0.4)	H	+	9.7 (5.7)	nt	72 (79)
ATCC 10876	100.0 (0.3)	H	+	nt	77 (10)	192 (90)
A7	92.7 (4.2)	H	+	9.2 (0.9)	nt	128 (0)
T1	97.8 (1.7)	H	+	38.6 (20.3)	31 (7)	80 (68)
ATCC 13061	98.3 (0.1)	H	+	nt	nt	96 (45)

Experiments were run in duplicate. Standard deviation is indicated in parentheses. For further details see Materials and Methods.
nt: not tested.
[a] Percentage of detachment of Caco-2 cells after 1 h incubation with undiluted cell free spent culture supernatants.
[b] Dose–response model for the effect of cell free spent culture supernatants of *Bacillus cereus* on enterocytes. H: high detaching, L: low detaching, N: non-detaching. For further details see Fig. 1.
[c] Detachment of cultured human enterocytes after 3 h infection with 10^8 CFU of vegetative cells.
[d] Percentage of remaining mitochondrial dehydrogenase activity (MTT assay) after incubation with undiluted cell free spent culture supernatants for 1 h at 37 °C.
[e] Percentage of necrotic cells after 1 h (37 °C) incubation with 1/32 dilutions of spent culture supernatants.
[f] Hemolysis titter of spent culture supernatants from 16 h-old cultures of *Bacillus cereus*. Assays were performed with rabbit red blood cells.

GmbH, Hamburg, Germany). Mix reaction contained: PCR buffer (20 mM TRIS–HCl, 50 mM KCl, pH: 8.4), $MgCl_2$ 1.5 mM, dNTPs 0.2 mM each one, *Taq* DNA polymerase 1 U (Invitrogen Corp.), primers 1 μM each one and 50 ng DNA in a final volume of 10 μl. PCR amplification was run under the following conditions: denaturation at 94 °C for 3 min, 30 cycles of: denaturation at 94 °C for 30 s, primer annealing at the corresponding temperature for 30 s, and DNA extension at 72 °C for 30 s. A final extension was added at 72 °C for 5 min. Amplicons were analyzed by 1% (w/v) agarose gel electrophoresis for 1 h at 90 V, using Ladder 100 of 400 bp (productos Bio-Lógicos, Bernal, Argentina) as a molecular weight marker.

2.4. Culture of enterocyte-like cells

Caco-2 cells (Fogh et al., 1977) were grown in DMEM medium (GIBCO BRL Life Technologies Rockville, MD. USA) supplemented with non-essential aminoacids, penicillin (12 IU/ml), streptomycin (12 μg/ml), gentamicine (50 μg/ml), fungizone (1.25 μg/ml) and inactivated (30 min, 58 °C) fetal calf serum (15% v/v). Monolayers were prepared in 24-well tissue culture plates (Greiner Bio-One, Germany) by seeding 7×10^4 cells per well. Incubations were performed at 37 °C in a 5% CO_2–95% air atmosphere. Assays were performed with cells at passages between 49 and 55. Differentiated cells (15 days in culture) were used throughout.

2.5. Cell infection assays

Infection of cultured human enterocytes was performed as described previously (Minnaard et al., 2004). Briefly, cell monolayers were washed twice with PBS before the infection assays were performed. Bacterial cultures (37 °C for 3 h) were centrifuged and the pellet was resuspended in DMEM (GIBCO BRL Life Technologies Rockville, MD. USA) containing 100 μg/ml cloramphenicol. After being counted in an hemocytometer, bacterial concentration was adjusted to 10^8 CFU/ml (multiplicity of infection = 100) and suspensions were added to the monolayers and incubated for 3 h at 37 °C in a 5% CO_2–95% air atmosphere. In these conditions, no bacterial growth was observed during the period of the experiment and bacteria remained viable. Cell detachment was determined as described below.

2.6. Cell detachment

Detachment of enterocyte-like cells was measured as reported previously (Minnaard et al., 2001). In this protocol, differentiated Caco-2 monolayers were coincubated with filter sterilized supernatants (0.5 ml per well) at 37 °C for 2 h. Cells were washed twice with PBS, fixed for 1 min at room temperature with 2% formaldehyde in PBS and washed again with PBS. 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 PBS). After being washed with PBS in order 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, Argentina). Percentage of cell detachment was calculated as follows: cell

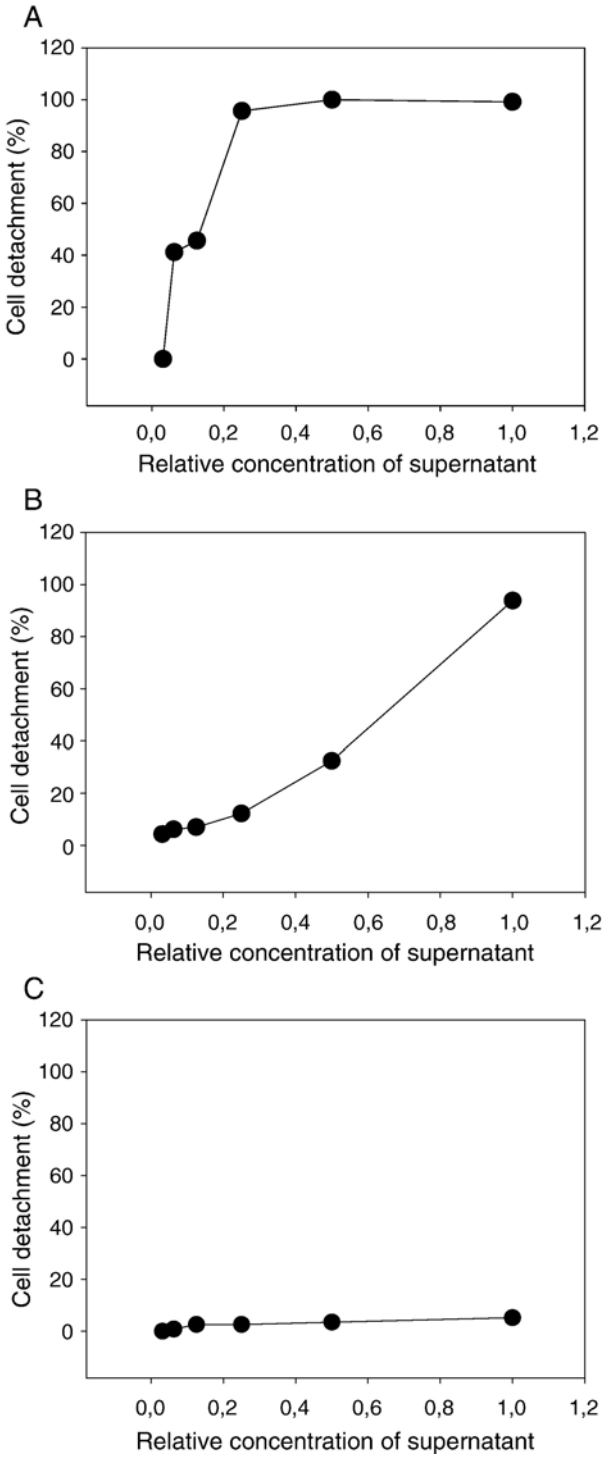

Fig. 1. Effect of the concentration of *Bacillus cereus* culture filtrate supernatants on cultured human enterocytes. Strains: 2 (A), T2 (B) and B10502 (C). Relative concentration of supernatant equal to 1 indicates undiluted supernatants. Dilutions of spent culture supernatants were done in BHI broth.

Table 4
PCR analysis of virulence genes and biological activity of strains under study

Strain	Gene												Model [a]	Detach inf [b]
			nhe complex				*hbl* complex							
	entS	*entFM*	*nheA*	*nheB*	*nheC*	*sph*	*hblA*	*hblB*	*hblC*	*hblD*	*piplC*	*bceT*		
2	+	−	−	+	+	+	−	−	−	+	−	+	H	−
114	+	+	+	+	+	+	−	−	−	+	+	−	L	−
273	+	+	+	+	+	+	+	+	+	+	+	+	H	−
253	+	+	+	+	+	+	+	+	+	+	+	+	H	−
124	+	+	+	+	+	+	+	+	+	+	+	+	H	−
T1	+	+	+	+	+	+	+	+	+	+	+	+	H	+
123	+	+	+	+	+	+	+	+	+	+	+	+	nt	nt [c]
B10502	−	+	+	−	−	+	−	−	−	−	−	−	N	+
T2	−	+	−	+	+	+	+	+	+	−	−	+	L	−
113	+	+	+	+	+	+	+	−	+	+	+	+	H	−
E2	+	−	+	+	+	+	−	−	+	−	−	−	H	−
M2	−	+	−	+	−	+	−	−	−	−	+	−	L	−
3	−	+	+	+	+	+	−	−	−	−	−	−	H	−
Watertown	−	+	−	−	−	+	−	−	−	−	+	−	H	−
313	−	+	−	+	−	+	−	−	−	−	−	−	L	+
M845	−	+	−	+	+	+	−	−	−	−	−	−	L	+
A7	−	+	−	+	+	+	−	−	−	−	−	−	H	+
M74	+	+	−	+	+	+	−	−	−	−	−	−	H	−
93	−	−	−	+	−	−	−	−	+	+	−	−	N	−
ATCC 13061	−	+	−	+	+	+	−	−	−	−	+	−	H	+
ATCC 10876	+	−	+	+	+	+	+	+	+	+	+	+	H	+

[a] Dose–response model for the effect of cell free spent culture supernatants on enterocytes. H: high detaching, L: low detaching, N: non-detaching. For further details refer to Fig. 1.
[b] Ability to detach enterocytes upon infection.
[c] Not tested.

detachment % = 100 × (Ac − As) / Ac, where Ac: A_{650} of control cells and As: A_{650} of treated cells.

2.7. 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 as previously reported (Minnaard et al., 2004). Briefly, after being incubated with culture filtrate supernatants, cells were detached by mechanical scraping and incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium (MTT, Sigma Chemical Co., St. Louis, MO, USA) for 4 h at 37 °C (final concentration 0.5 mg/ml in PBS). 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 (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.

2.8. Necrosis

Labeling with propidium iodide was performed according to a previously published method (Minnaard et al., 2004). After treatment for 1 h at 37 °C with spent culture supernatants diluted 1/32 (to prevent cell detachment) cells were washed twice with PBS. Afterwards, 1 μg/ml of propidium iodide and 100 μg/ml RNase were added in 100 μl of PBS. Cells were incubated at room temperature for 15 min and then samples were mounted in 50% glycerol in PBS and analyzed by conventional fluorescence microscopy using a Leica DMLB microscope coupled to a Leica DC 100 camera (Leica Microscopy Systems Ltd., CH Heerbrugg, Switzerland).

2.9. Hemolysis

Filtered spent culture supernatants obtained as indicated for the cell detachment assays were used throughout. Samples were serially diluted in BHI broth in a 96-well plate. Red blood cells were collected from the marginal ear vein of a New Zealand White rabbit using trisodium citrate as anticoagulant. Suspensions of rabbit red blood cells in PBS (2%) were added and plates were incubated at room temperature for 1 h. Hemolysis titers were determined by both visual inspection and by absorbance readings at 600 nm.

2.10. Detection of non-hemolytic enterotoxin (Nhe)

Spent culture supernatants were tested for the presence of NheA by using the Tecra BDE kit (Tecra Diagnostics) according to the instructions of the manufacturers.

2.11. Statistical analysis

Clustering analysis was performed by using UPGMA and Dice coefficients. Biplot analysis (Gabriel and Odoroff, 1990) was conducted with PCR results and biological activity

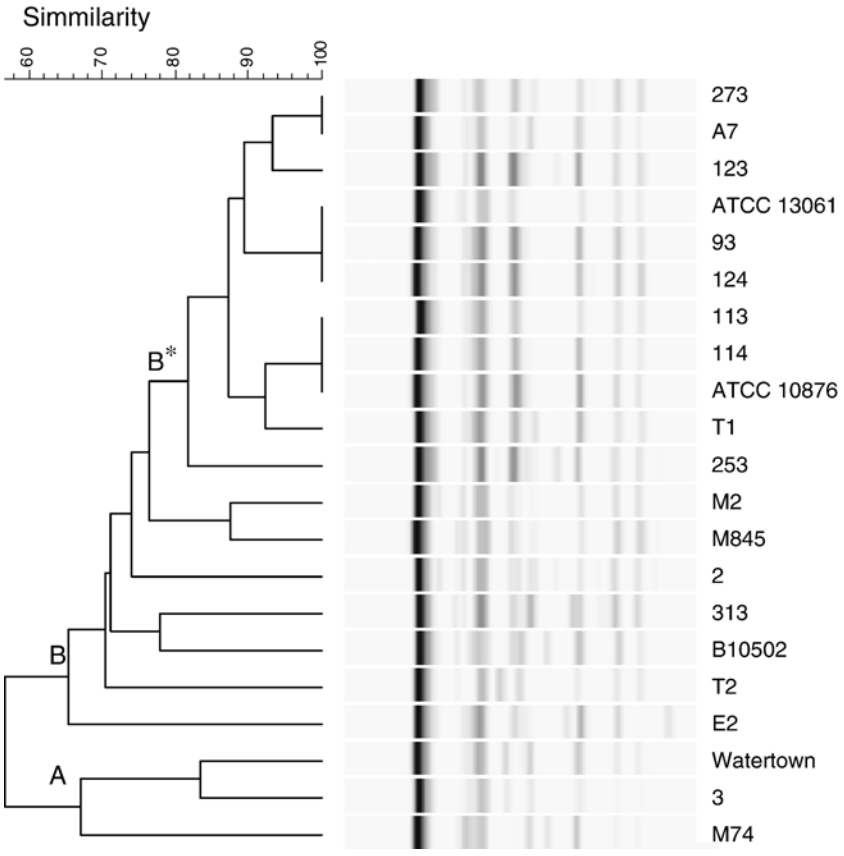

Fig. 2. Ribopatterns and cluster analysis of the strains under study. Dendrograms were generated by UPGMA based on Dice coefficients.

(detachment upon infection and model of cell detachment). Results of cell detachment upon infection were coded as 1 (detaching strain) and 0 (non-detaching strain). Results of the model of cell detachment by extracellular factors were coded as 1 (high detaching strain), 0.5 (low detaching strain) and 0 (non-detaching strain). In biplots, vector variables represent the positive direction of the variable axes. The lengths of these vectors approximate the standard deviation of the variables. In our study, genes found in all the strains will lead to a vector of length equal to zero at the centre of the graph (centroid). The angle between two variable vectors approximates the arc cosine of the correlation between those variables. Therefore, variables forming an acute angle are positively correlated whereas those forming an obtuse angle are negatively correlated. Right angles indicate uncorrelated variables. Each strain is denoted by a circle whose coordinates correspond to the scores for principal components. All the variable vectors meet at the centroid, which represents the means for all the variables. Thus, a strain located at this point can be considered as an "average" strain. InfoStat software (Grupo InfoStat, Córdoba, FCA, Universidad Nacional de Córdoba, Argentina) was used throughout.

3. Results

The effect of extracellular factors of *B. cereus* on human intestinal epithelial cells (Caco-2 cells) was examined. When spent culture supernatants were incubated with cultured human enterocytes, dramatic cytopathic effects were observed. Indeed, most of the strains under study lead to high percentages (>70%) of cell detachment (Table 3). Only extracellular factors from strain B10502 were unable to detach enterocytes in the conditions of the assay (1 h, 37 °C). Dose–response studies led to a further insight on the effect of extracellular factors of *B. cereus*. These analyses allowed for the definition of three dose–response curves. Strains whose extracellular factors were unable to detach enterocytes were classified as non-detaching (e.g. strain B10502). Strains showing full detachment in at least two serial dilutions were classified as high detaching (e.g. strain 2). Finally, strains whose detaching activity was highly affected by dilutions were termed low detaching strains (e.g. strain T2). Representative examples of these three dose–response curves are shown in Fig. 1 and results for all strains studied are shown in Table 4. Besides the ability to detach enterocytes, reduction of mitochondrial dehydrogenase activity, different percentages of cell necrosis and titters of hemolysins were observed. As shown in Table 3, after incubation of enterocytes with spent culture supernatants of some strains, remaining mitochondrial dehydrogenase activity was very low (e.g. 9.2±0.9 and 9.7±5.7% for strains A7 and Watertown respectively). In contrast, this activity was not significantly modified by strain T2. Percentages of necrotic cells were also strain-dependent. Indeed, values that ranged from 78±7 to 6±4% were observed for strains 3 and T2 respectively. Titters

of hemolysins present in spent culture supernatants ranged from 40 to 192 although we observed high inter-experiment variability for some strains (Table 3).

Interestingly, some strains were able to produce cell detachment when vegetative cells infected human enterocytes for 3 h, even in the presence of chloramphenicol (Table 3).

Ribotyping and cluster analysis of 21 strains (Dice coefficient) are shown in Fig. 2. Sixteen different profiles were found. These profiles constituted two clusters: A (3 strains) and B (18 strains) the last one having several sub-clusters. Clusters A and B joined at around 60% similarity. Some strains showed the same ribopattern. Further analysis showed that strains belonging to cluster B* were positive for all the DNA sequences tested (see below).

Next we determined the presence of sequences related to genes associated to the virulence of *B. cereus*. Results of the analysis of 12 genes are shown in Table 4 and Fig. 3. All the genes gave amplification fragments of expected sizes. Five strains were positive for all the sequences tested. All these strains belong to cluster B* (Fig. 2 and Table 4). Most strains were positive for the sequence of the sphingomyelinase (*sph*). Ten strains presented at least one of the sequences of the hemolysin BL (*hbl*) and 20 strains presented at least one of the sequences of the non-hemolytic enterotoxin (*nhe*). Noteworthy, we never found the pattern − − + for the genes *nheA*, *nheB* and *nheC* respectively (Table 4).

To gain further insight on the relationship between the presence of the sequences tested and biological activity of the strains we conducted principal component analysis with both virulence genes and biological activity. We considered that the ability to detach cultured enterocytes upon infection and the model of cell detachment contained the most comprehensive information on the biological activity. These two characteristics include the effect of direct bacteria–cell interaction (detachment upon infection) and the effect or extracellular factors (model of cell detachment).

Correlation probability matrix of the principal component (PC) analysis is shown in Table 5. Significant positive and negative correlations were found between some variables. Ability to detach enterocytes upon infection shows a trend of negative correlation with the presence of sequences *entS* and *nheB* ($p=0.07$). Concerning the model of detachment by extracellular factors, we found significant positive correlations ($p<0.05$) with sequences *entS*, *nheC* and *sph*. There were also trends of positive correlations with sequences *piplC* and *bceT* ($p=0.076$ and 0.070 respectively). It is worth to note the lack of significant correlation between sequences of the NHE complex. In contrast, sequences of the *hbl* complex were highly correlated.

Biplot analysis allowed to confirm the relationship between different variables as well as to define groups of strains (Fig. 3). Strains 113, 253, 273, 124, ATCC 10876 and T1 consistently clustered together (group I) in different graphs, i.e. PC1 vs PC2, PC1 vs PC3 and PC1 vs PC4. These 6 strains are all high detaching strains and they were positive for all the gene sequences tested. This fact is reflected by the position of the strains with respect to the vector of the variables. In contrast, they constitute a heterogeneous group concerning the ability to detach enterocytes upon infection (Table 4). Interestingly, strains B10502 and Watertown clustered together and constitute a separate pair in PC1 vs PC3 and PC1 vs PC4 plots. These strains were positive for a

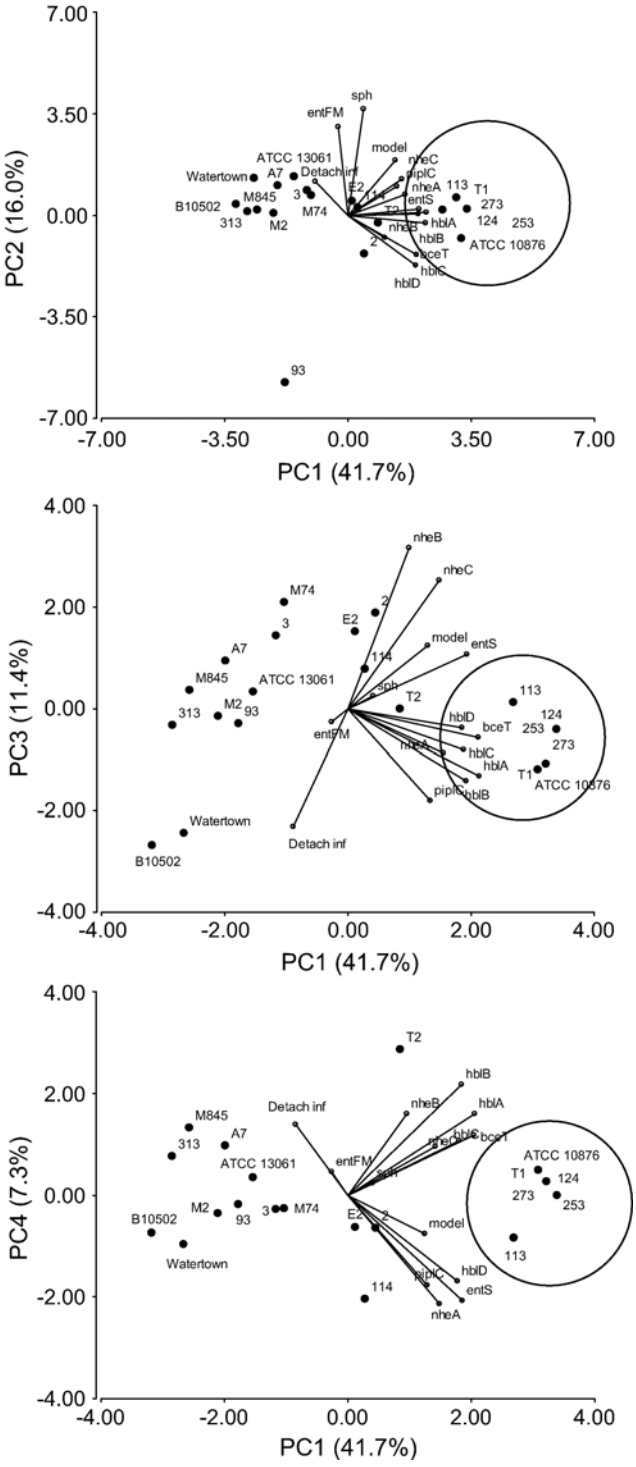

Fig. 3. Biplot: genes and biological activity on cultured human enterocytes. Strains positive for all the DNA sequences tested (group I in the text) are included in a circle. The percentages of the variation explained by principal components (PC1 to PC4) are indicated in parentheses.

Table 5
Multivariate analysis of virulence genes and biological activity of strains under study

A	Detach inf	Model	*ent*S	*ent*FM	*nhe*A	*nhe*B	*nhe*C	*sph*	*hbl*A	*hbl*B	*hbl*C	*hbl*D	*pipl*C	*bce*T
Detach inf	1.00													
Model	–0.14	1.00												
*ent*S	–0.41	0.54	1.00											
*ent*FM	0.06	0.09	–0.14	1.00										
*nhe*A	–0.20	0.27	0.60	0.14	1.00									
*nhe*B	–0.41	0.22	0.33	–0.14	0.00	1.00								
*nhe*C	–0.24	0.54	0.58	0.08	0.35	0.58	1.00							
sph	0.19	0.46	0.23	0.55	0.23	–0.08	0.40	1.00						
*hbl*A	–0.17	0.35	0.52	0.01	0.52	0.24	0.42	0.17	1.00					
*hbl*B	–0.09	0.29	0.44	–0.03	0.44	0.22	0.38	0.15	0.89	1.00				
*hbl*C	–0.33	0.20	0.50	–0.18	0.50	0.30	0.29	–0.25	0.81	0.72	1.00			
*hbl*D	–0.33	0.20	0.70	–0.46	0.50	0.30	0.29	–0.25	0.60	0.50	0.60	1.00		
*pipl*C	0.00	0.40	0.40	0.14	0.40	0.00	0.12	0.23	0.52	0.44	0.30	0.50	1.00	
*bce*T	–0.25	0.41	0.61	–0.23	0.41	0.27	0.47	0.19	0.90	0.80	0.70	0.70	0.41	1.00

B	Detach inf	Model	*ent*S	*ent*FM	*nhe*A	*nhe*B	*nhe*C	*sph*	*hbl*A	*hbl*B	*hbl*C	*hbl*D	*pipl*C	*bce*T
Detach inf														
Model	0.5629													
*ent*S	0.0739	0.0141												
*ent*FM	0.8108	0.6922	0.5560											
*nhe*A	0.3880	0.2502	0.0052	0.5560										
*nhe*B	0.0739	0.3408	0.1510	0.5560	>0.9999									
*nhe*C	0.3171	0.0130	0.0077	0.7347	0.1346	0.0077								
sph	0.4291	0.0393	0.3306	0.0127	0.3306	0.7486	0.0828							
*hbl*A	0.4705	0.1264	0.0177	0.9510	0.0177	0.2986	0.0627	0.4780						
*hbl*B	0.7088	0.2079	0.0544	0.8982	0.0544	0.3553	0.1004	0.5274	<0.0001					
*hbl*C	0.1577	0.3900	0.0239	0.4401	0.0239	0.1964	0.2147	0.2806	<0.0001	0.0003				
*hbl*D	0.1577	0.3900	0.0005	0.0391	0.0239	0.1964	0.2147	0.2806	0.0051	0.0233	0.0056			
*pipl*C	>0.9999	0.0769	0.0806	0.5560	0.0806	>0.9999	0.6278	0.3306	0.0177	0.0544	0.1964	0.0239		
*bce*T	0.2878	0.0704	0.0041	0.3322	0.0739	0.2457	0.0359	0.4291	<0.0001	<0.0001	0.0006	0.0006	0.0739	

Significant correlations ($p<0.05$) and trends ($0.07<p<0.09$) are indicated in grey.

few gene sequences and they were able to detach enterocytes upon infection.

4. Discussion

Virulence of *B. cereus* is related to several genes. These genes encode for different toxins responsible for the biological effects of this microorganism. Transcription of virulence genes is under control of the pleiotropic regulon PlcR and this control implies a link between bacterial growth and expression of virulence genes (Gohar et al., 2002; Slamti et al., 2004).

In vitro assays have demonstrated the ability of *B. cereus* and its extracellular factors to produce strongly biological effects on different systems (Minnaard et al., 2001, 2004; Agata et al., 1995; Jackson, 1993; Lund and Granum, 1997; Andersson et al., 1998; Finlay et al., 1999).

Here we report for the first time a multivariate analysis taking into account ribotyping, virulence genes and biological activity of the strains. We found diversity with respect to ribopatterns, virulence genes as well as strain-dependent biological effects.

Ribotyping showed the heterogeneity within the strains under study. Indeed, 16 patterns were found thus indicating polymorphism in the rRNA sequences (Fig. 2). All the strains belonging to group I of the biplot also belong to the cluster B* of the ribotyping (Figs. 2 and 3). This correlation indicates the usefulness of the ribotyping for grouping of strains. However, as previously reported, ribopatterns generated with a single restriction enzyme could lead to heterogeneous groups (Grif et al., 2003). Therefore, we performed further analysis by including other genotypic and phenotypic markers.

We assumed that the ability of extracellular factors to detach human enterocytes upon infection is an important virulence trait. Interestingly there was a trend of positive correlation between the model of cell detachment and the presence of the *piplC* sequence (Table 5 and Fig. 3). Even though the presence of a sequence neither guarantees that the gene is active nor that it is expressed, this correlation makes sense from a mechanistic point of view. In fact, phosphatidylinositol phospholipase C activity could contribute to the destabilization of the plasma membrane thus inducing hemolysis and cytolysis but also, it is involved in signaling pathways related to cytoskeleton assembly (Henderson et al., 1999). Both activities could account for the capacity to detach enterocytes after bacteria–cell contact (Table 3) as well as the reported effect on the cytoskeleton (Minnaard et al., 2004). However, some strains able to produce dramatic effects on the cytoskeleton are situated in the opposite side of the detachment vector (e.g. strain 2). These findings account for the multifactorial character of the *B. cereus* virulence.

As reported previously, operon *nhe* is present in almost all the strains of *B. cereus* (Lindbäck et al., 2004; Guinebretière et al., 2002). Accordingly, we found amplicons with at least one *nhe* sequence in 20 out of 21 strains studied (95%).

The sequence of the gene *nheA* (whose product is detected by the TECRA kit) was present in 11 out of 21 strains. However, all the strains under study were positive when tested by the ELISA assay. This paradoxical result could indicate either that the primer sequence selected for the *nheA* gene is not appropriate or that the kit is detecting products other than those from this gene. Variations in the gene sequences could affect binding sites in the target DNA leading to false negative results. Indeed, sequence variations that prevent their detection by PCR have been reported (Thaenthanee et al., 2005).

Interestingly, we detected 9 strains that do not carry the *hbl* gene (e.g. strain B10502). A recently published paper (Ehling-Schulz et al., 2005) showed that emetic strains do not carry *hbl* genes and provided evidence for a clonal population structure of cereulide-producing emetic *B. cereus*. Even though neither cereulide production nor *ces* genes were tested in the present work, strain B10502 was associated to an emetic outbreak (data not shown).

Regarding biological activity, some strains sharing relevant biological activities grouped in a definite region of the biplot. However, strains showing other important virulence factors were scattered in different regions of the graph. For example, strains M2, T1, 2 and ATCC 10876 are able to invade Caco-2 monolayers and strain B10502 is a non-invading strain (Minnaard et al., 2004).

Our results underline the multifactorial character of *B. cereus* virulence and show the association between ribopatterns, the presence of specific DNA sequences and biological activities involved in the virulence of this microorganism. This analysis allows the finding of highly related genes as well as to direct studies on the virulence factors of *B. cereus*.

Acknowledgements

Authors are grateful to R. Buchanan and F. Schultz for providing strains T1, T2, E2, Watertown and A7. Strain B10502 was kindly provided by the Laboratorio Central de Salud Pública de la Provincia de Buenos Aires, Argentina.

Hollmann and L. Delfederico are recipients of fellowships from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, Argentina). Dr. L Semorile is member of the Research Career of Comisión de Investigaciones Científicas de la Provincia de Buenos Aires (CIC-PBA). P.F. Pérez is a member of the Carrera de Investigador Científico y Tecnológico del Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET, Argentina). This work was partially financed by the International Foundation for Science, the Agencia de Promoción Científica y Tecnológica (ANPCyT, Argentina) and CONICET. Authors are indebted to Yves Tirilly, from the Ecole Supérieure de Microbiologie et Sécurité Alimentaire de Brest (ESMISAB), for help and encouragement.

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