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Enterotoxigenic gene profiles of *Bacillus cereus* and *Bacillus megaterium* isolates recovered from honey

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ABSTRACT

One hundred and thirty two *Bacillus cereus* and 52 *Bacillus megaterium* isolates from honeys were evaluated for the presence of genes encoding enterotoxin HBL, enterotoxin-T, cytotoxin K and the NHE complex, respectively. The relationship between hemolytic and coagulase activity and its correlation with the presence of the four mentioned enterotoxins was determined by principal component analysis (PCA). PCA in *B. cereus* revealed a positive correlation among free coagulase, hemolysis and the presence of genes *hblA*, *hblB*, *hblC*, *hblD* (HBL complex) and *bce*T (enterotoxin-T), but no correlation with the clumping factor (bound coagulase) and the presence of sequences of the NHE complex. On the other hand, PCA in *B. megaterium* showed a high positive correlation between coagulase (bound and free) and the haemolytic activity but no correlation in relation to the presence of genes of the HBL complex, cytotoxin K, enterotoxin T and the NHE complex. To our knowledge, this is the first report of the detection of cytotoxin K and of the NHE complex genes in *B. megaterium.* The relationship between the coagulase activity and the presence of virulence factors has not been described before in the genus *Bacillus,* being this work the first report of this correlation. Interestingly, the presence of the *cytK* gene was almost independent of the presence of the rest of virulence factors herein analyzed both in *B. cereus* and *B. megaterium* populations. Our results suggest that honey could be a possible vehicle for foodborne illness due to the presence of toxigenic *B. cereus* and *B. megaterium* strains containing different virulence factors.

Key words: *Bacillus cereus,* honey, hemolysis, coagulase activity, enterotoxins, *Bacillus megaterium*, virulence factors

RESUMEN

Búsqueda de factores de virulencia en cepas de *Bacillus cereus* y *de Bacillus megaterium* aisladas de miel. Se evaluaron 132 aislamientos de *Bacillus cereus* y 52 de *Bacillus megaterium* provenientes de mieles de distintos orígenes geográficos para investigar la presencia de secuencias de ADN relacionadas con genes de virulencia y su posible correlación con la actividad hemolítica y coagulasa. Con respecto a los genes de virulencia, se analizaron por PCR secuencias de ADN de los genes *nhe* (A, B y C), HBL (A, B, C, D), *cytK* y *bceT*. La relación entre las variables fue evaluada mediante un análisis de componentes principales, donde se encontró que los aislamientos de *B. cereus* mostraron una correlación positiva entre actividad de coagulasa (coagulasa libre) y presencia de los genes del complejo HBL y *bceT*, mientras que en *B. megaterium* se halló una alta correlación positiva entre actividad de coagulasa (libre y fija) y actividad hemolítica, pero no se observó correlación significativa entre la presencia de genes de virulencia y dichas actividades. Este estudio constituye el primer registro de la presencia de los genes *cyt K* y NHE en cepas de *B. megaterium* y el primer trabajo que analiza la relación entre la actividad de coagulasa y la presencia de genes de virulencia en *B. cereus* y *B. megaterium*. La presencia del gen *cytK* en ambas especies resultó totalmente independiente del resto de los factores de virulencia analizados. Nuestros hallazgos sugieren que la miel podría vehiculizar enfermedades transmisibles por alimentos debido a la presencia de cepas de *B. cereus* y *B. megaterium* potencialmente tóxicas.

Palabras clave: *Bacillus cereus*, miel, hemólisis, actividad de coagulasa, enterotoxinas, *Bacillus megaterium*, factores de virulencia

INTRODUCTION

Honey quality is influenced by microorganisms, particularly spore-forming bacteria and yeast (35). Aerobic mesophylic spore-forming bacteria of the genera *Paenibacillus, Bacillus* and *Brevibacillus* are commonly found in honey (5, 35). Several *Bacillus* species, including *Bacillus cereus (sensu lato* group*)*, *Bacillus megaterium*, *Bacillus coagulans, Bacillus subtilis,* and *Bacillus licheniformis,* have been frequently isolated from honeys (4, 5, 14, 15). The presence of *Bacillus* species was reported, in Argentinean honeys, being *B. cereus* (28%) and *B. megaterium* (14%) the most prevalent ones (4, 5, 24, 25).

The *Bacillus cereus* group (also called *B. cereus sensu lato*) consists of six closely related species: *B. cereus, Bacillus anthracis*, *Bacillus thuringiensis*, *Bacillus mycoides*, *Bacillus pseudomycoides,* and *Bacillus weihenstephanensis* (22, 39), being *B. cereus (sensu stricto)* a common food-poisoning organism and the most prevalent one

in industrial processes. It is classified into hazard category 2 and has been associated with two forms of food poisoning, named emetic and diarrheal diseases, as well as with non-intestinal pathologies (12, 19). *B. cereus* (*sensu stricto*) produces four main enterotoxins, named hemolysin BL (HBL), enterotoxin-T, non-hemolytic enterotoxin complex (NHE) and cytotoxin K, and has been involved in some human infectious processes (16, 21, 31). Hemolysin BL (HBL) is a protein complex of three distinct protein subunits: B, L_1 and L_2, which is considered one of the most important virulence factor of *B. cereus* (6). All three parts are required for biological effects and together possess hemolytic, cytotoxic, dermonecrotic, and vascular permeability activities, including fluid accumulation in ligated rabbit ileal loops (7). None of the individual subunit components have toxic activity. Enterotoxin-T is a simple component toxin with biological activities related to diarrheal enterotoxins, such as fluid accumulation and permeability in rabbit ileal loops and cytotoxic activities (3). NHE is a three-component enterotoxin responsible for the diarrheal food-poisoning syndrome. NHE is composed of three genes (*nheA*, *nheB* and *nheC*), that constitute one operon, being all the three components necessary for cytotoxic activity (23). Cytotoxin K (*cytK*) causes a diarrheal syndrome with necrotic, hemolytic and cytotoxic effects on the intestinal ephithelium (13) and it was first characterized in *B. cereus* strains that caused a severe poisoning outbreak (26). In addition, virulence of the so-called emetic strains is related to cereulide (2, 11), a thermostable cyclic peptide responsible for fatal cases of food-borne infections (9). *B. megaterium* is another gram positive spore-forming bacterium that produces several enzymes and antibiotic-like compounds and has been found in diverse habitats including honey (1, 25, 28, 29, 38). Taylor *et al.* (36) reported the production of a novel heat-stable toxin in *B. megaterium* strain F98/3079, while the presence of diarrheagenic genes *bceT* (enterotoxin-T), *hblC*, *hblA*, and *hblD* (HBL complex) was detected in one out of two *B. megaterium* strains tested by Rowan *et al.* (34). On the other hand, hemolytic and coagulase activities were observed in 77% and 74% of *B. megaterium* isolates from honeys (25). Coagulase activity is most often associated with pathogenic staphylococci or yersiniae and is not ordinarily reported as a property of the *Bacillus* species. Because coagulase may be correlated with virulence of certain organisms (10), it seemed appropriate to investigate the matter further. The objectives of the present study are: (i) to document the existence of coagulase activity in *B. cereus* isolates from honeys and (ii) to assess the distribution of homologous sequences of gene components encoding for the HBL complex, enterotoxin-T, cytotoxin K and the NHE complex in *B. cereus* and *B. megaterium* populations and its correlation with coagulase and hemolytic activities.

MATERIALS AND METHODS

Strain collection

A collection of 132 *B. cereus* and 52 *B. megaterium* strains isolated from honey samples from different geographical areas in Argentina was used for this study. In addition, isolates of both species from honeys from Italy, USA, Mexico, Brazil and France and reference strains were also studied. Further details of these organisms are available in López and Alippi (24, 25).

All the isolates were grown in tryptic soy agar (TSA) at 30 °C, maintained on TSA at 4 °C and stored in tryptic soy broth (TSB) plus 20% glycerol v/v at -80 °C.

Hemolytic and coagulase activities

Data for the hemolytic activity and production of a discontinuous hemolytic pattern on blood agar plates were detailed in previous studies (24, 25). The 132 *B. cereus* strains were evaluated for coagulase activity in rabbit plasma as reported by López and Alippi (25). Briefly, *B. cereus* cultures were grown in brain heart infusion broth (BHI) (Merck) for 24 h at 36 ± 1 °C. The tube procedure for determination for both free and bound coagulase was done by mixing 100 µl of broth culture with 300 µl rabbit plasma (Britania) in a sterile screw-capped tube. Tubes were incubated at 36 ± 1 °C and observed at various time intervals for clot formation. The degree of coagulation was noted as complete lack of clot formation, partial or incomplete clot formation, or complete or firm clot formation. The slide procedure was used to determine bound coagulase enzyme (clumping factor) by mixing with a wooden stick a single bacterial colony from a 24 h culture in BHI agar in saline in a clean microscope slide with one drop of rabbit plasma. In positive cases, a visible clumping of cells appeared within 20 seconds. Results from *B. megaterium* strains (n = 52) were detailed previously (23, 24).

Detection of enterotoxin genes in *B. cereus* and *B. megaterium* by PCR

Total genomic DNA was isolated from 24h-cultures grown on TSA using the rapid procedure described previously by López and Alippi (23). All strains were screened for the presence of genes encoding enterotoxin-T (*bceT*) (18), cytotoxin K (*cytk*) (11) and hemolysin BL (*hblA*, *hblB*, *hblC*, *hblD*) (18, 21, 33) and non hemolytic enterotoxin (*nheA*, *nheB*, *nheC*) (16) complexes by PCR.

PCR conditions for primers B f/r; B' f/r, L-1 f/r; L-2 f/r and ET f/r were carried out in a final volume of 25 µl as previously reported (21), while PCR conditions for CKF2/CKR5 for the detection of cytotoxin K, and NAF/R, NBF/R and NCF/R primers for the detection of the NHE complex were conducted in 50 µl volumes according to Ehling-Schulz and co-workers (11) and Guinine-bretiere *et al.* (16), respectively. *B. cereus* F4430/73 strain with well-characterized toxin profiles (16) was used as positive control in all PCR runs.

DNA amplifications were performed in a thermal cycler (Mastercycler personal; Eppendorf Hamburg, Germany). Amplicons were analyzed by 1.6% (W/V) agarose gel, in TBE buffer, stained with SYBR® Safe (Invitrogen, Argentina) for 2 h at 80 V using 100 bp ladder (Promega) as a molecular weight marker. Gels were photographed on the visi-blue transilluminator (Safe imager™) using a digital image capture gel documentation system (Digi Doc-it, UVP, v. 1.1.25).

Principal component analysis (PCA)

The correlation among the presence of DNA sequences corresponding to the genes *hblA*, *hblB*, *hblC*, *hblD*, *nheA*, *nheB*, *nheC*, *cytK*, and *bce*T; and hemolytic and coagulase activity was evaluated by principal component analysis (PCA). Results of hemolysis and production of a discontinuous hemolytic activity

pattern were coded as 1 (hemolytic activity) or 0 (lack of hemolytic activity). Results for coagulase activity were coded as 1 (coagulase activity) or 0 (lack of coagulase activity). In addition, results of PCRs for the presence of the four enterotoxins listed in Tables 1 and 2 were ranked as 1 (PCR positive) or 0 (PCR negative). Data were not normalized and software MSVP 3.1 (Kovach Computing Services) was used to analyze the data set by performing PCA. Plots of the first two principal components scores represent the best 2D representation of natural variance in the data. In biplots vector variables represent the positive direction of the variable axes, therefore variables forming an acute angle are positively correlated, whereas those forming an obtuse angle are negatively correlated and right angles indicate uncorrelated variables. All the variable vectors meet at the centroid, which represents the means for all the variables (27).

RESULTS

Hemolytic and coagulase activities

Data for the hemolytic activity in *B. cereus* showed that 95% of isolates from honey presented hemolytic activity, and within these strains, 11% produced a discontinuous hemolytic pattern (Tables 1 and 3). In the case of *B. megaterium*, hemolytic activity was shown by 77% of isolates, and within this group, 10% produced a discontinuos hemolytic pattern (Table 2). These results are summarized in Tables 1, 2, and 3 and previous studies (24, 25). All the *B. cereus* strains that presented a discontinuous hemolytic pattern presented at least 3 target genes for the HBL complex (Table 1), and in the case of *B. megaterium,* at least 2 target genes (Table 2) with the exception of the Bm2 strain.

Coagulase activity was observed as clot formation in 106 strains (80%) of *B. cereus* and among the positive strains, 57 (54%) presented the clumping factor (Table 1) while in the case of *B. megaterium,* coagulase activity was observed as clot formation on 74% of strains out of a total of 53 strains and among the positive strains, only 49% presented the clumping factor (Table 2).

Detection of enterotoxin genes in *B. cereus* and *B. megaterium* by PCR

In *B. cereus* isolates (n = 132) the presence of the *hblA* gene encoding for the B part of the HBL complex was detected in 110 strains (83%), the gen *hbl*B encoding for the B' part in 75 strains (57%), the gen encoding for the L_1 part (*hblD*) in 109 strains (83%) and the gene encoding for the L_2 part (*hbl*C) in 84 strains (64%), respectively. Two percent of the isolates presented the L1 and L2 components (Lytic components of the HBL complex), while 4% of the isolates showed B and B' components (binding components of HBL complex). In addition, all four HBL genes (*hblA*, *hblB, hblC and hblD)* were detected in 55 strains (42%) (Tables 1 and 3 and Figure1A). The results of the PCR analysis of the NHE complex revealed that 47 strains (56%) yielded the expected *nhe*A-PCR product of 755 bp; 35 (12%) strains yielded the expected *nheC*-PCR product of 683bp, but only 13 strains (10%) presented the expected *nheB*-PCR product of 743 bp (Tables 1 and 3 and Figure 1C). The presence of genes encoding for the enterotoxin-T was observed in 96 strains (73%) of our collection and the presence of the CytK-PCR product of 421 bp was detected in 53% of the 133 strains tested (Table 1, Figures 1B and 1 A, respectively).

In our *B. megaterium* collection, results for the HBL complex showed that the presence of part B was observed in 13 strains (27%), of part B' in 3 strains (6%), of part L_1 in 12 strains (23%) and of part L_2 in 3 strains (6%). Only one strain (Bm8) presented all four components of the HBL complex and was correlated with the presence of a discontinuous hemolytic pattern (Table 2 and Figure 1A). The presence of the *bceT* gene encoding for enterotoxin-T was detected in 9 strains (17%) (Table 2 and figure 1B). Variable results were obtained for *B. megaterium* in relation to the NHE complex, the presence of the *nheA* gene was observed in 3 strains (6%), the *nheC* gene in 7 strains (13%) (Table 2 and Figure 1C), and none of the isolates presented the *nheB* gene (Table 2).

Principal Component Analysis

The relationship among hemolytic and coagulase activities and the presence of the four enterotoxins studied was determined by PCA.

The PCA of 132 *B. cereus* strains isolated from honeys revealed that the total variance of the system was defined by the first 3 principal components, which accounted for 76%, 5% and 3% of the variance, respectively. Biplot analysis (PC1 *vs.* PC2) showed a positive correlation ($p = 0.0001$) among free coagulase, hemolysis and the presence of genes *hblA*, *hblB*, *hblC*, *hblD* (HBL complex) and *bceT* (enterotoxin-T) but no correlation with the clumping factor (bound coagulase) (Figure 2A). Seventeen per cent of the strains presented the 4 genes of the HBL complex, and within this group, 71% showed coagulase activity (free coagulase) (Figure 2A) but there was no correlation between genes belonging to the NHE complex and those of the HBL complex. Biplot of PC1 *vs.* PC3 showed that the presence of the *cyt*K gene was almost independent of the presence of the rest of virulence factors herein analyzed (Figure 2B).

The PCA for 53 strains of *B megaterium* showed that the percentages of the variation were explained by the first 2 principal components that accounted for 62% and 9%, respectively. Biplots showed high positive correlation ($p = 0.0001$) between coagulase and hemolytic activities (Figure 3). Seventeen strains presented both activities (the strains have been placed inside a ring for visual clarity) (Figure 3). However, no correlation was observed between the presence of enterotoxin genes *hbl* (*A, B, C, D*), *cyt*K, *bce*T and *nhe* and these activities (Figure 3).

Table 1. Screening of virulence genes and coagulase and haemolytic activities of *Bacillus cereus* isolates from honey.

Number of strains	HBL complex				NHE complex			*bceT*	*cytK*	Free coagulase	Clumping Factor	Haemolysis*
	hblA	*hblB*	*hblC*	*hblD*	*nheA*	*nheB*	*nheC*					
ATCC 1778	-	-	-	-	-	-	-	-	-	-	-	+
F4433/73	+	+	+	+	+	+	+	+	+	ND	ND	ND
3	+	+	+	+	+	+	+	+	+	+a	-b	+
1	+	+	+	+	+	+	+	+	-	+a	+a	+
1	+	+	+	+	+	+	+	-	+	+a	-b	+
1	+	+	+	+	+	+	+	-	+	+a	+a	+
1	+	+	+	+	+	+	+	-	-	+a	-b	+
6	+	+	+	+	+	-	+	+	+	+a	-b	+
8	+	+	+	+	+	-	+	+	+	+a	+a	+
1	+	+	+	+	+	-	+	+	+	+a	+a	D
3	+	+	+	+	+	-	+	+	+	-b	-b	+
2	+	+	+	+	+	-	+	+	+	+a	+a	-
1	+	+	+	+	+	-	+	+	-	-b	+a	+
4	+	+	+	+	+	-	+	+	-	+a	+a	+
2	+	+	+	+	+	-	+	+	-	+a	-b	+
1	+	+	+	+	+	-	+	+	-	+a	-b	D
1	+	+	+	+	+	-	+	+	-	-b	-b	+
3	+	+	+	+	+	-	+	-	+	+a	-b	+
1	+	+	+	+	+	-	+	-	+	+a	+a	+
1	+	+	+	+	+	-	+	-	-	+a	+a	D
3	+	+	+	+	-	-	+	+	+	+a	-b	+
3	+	+	+	+	-	-	+	+	+	+a	+a	+
3	+	+	+	+	-	-	+	+	+	-b	-b	+
1	+	+	+	+	-	-	+	+	+	+a	-b	D
1	+	+	+	+	-	-	+	+	+	+a	+a	D
1	+	+	+	+	-	-	+	+	-	+a	-b	D
1	+	+	+	+	-	-	+	+	-	+a	+a	D
1	+	+	+	+	-	-	+	+	-	+a	-b	+
1	+	+	+	+	+	-	-	+	+	+a	+a	D
1	+	+	+	+	-	-	+	-	-	-b	+a	+
1	+	+	+	+	-	-	+	-	-	+a	+a	+
1	+	+	-	+	+	+	+	+	-	+a	+a	+
1	+	+	-	+	+	+	+	-	-	-b	-b	+
1	+	+	-	+	+	-	+	+	+	+a	-b	D
1	+	+	-	+	+	-	+	+	-	+a	-b	-
2	+	+	-	+	+	-	+	+	-	+a	-b	+
1	+	+	-	+	+	-	+	-	+	+a	-b	+
1	+	+	-	+	-	-	+	+	+	-b	-b	+
2	+	+	-	+	-	-	+	+	-	-b	-b	+
1	+	+	-	+	-	-	+	-	+	+a	-b	D
1	+	+	-	+	-	-	+	-	-	+a	+a	+
3	+	-	+	+	+	-	+	+	+	+a	+a	+
1	+	-	+	+	+	-	+	+	+	-b	-b	+
2	+	-	+	+	+	-	+	+	-	-b	-b	+
1	+	-	+	+	+	-	+	+	-	+a	+a	+
1	+	-	+	+	+	-	+	+	-	+a	+a	D
1	+	-	+	+	+	-	+	-	+	-b	-b	-

Table 1. Continuation

Number of strains	HBL complex				NHE complex			*bceT*	*cytK*	Free coagulase	Clumping Factor	Haemolysis*
	hblA	*hblB*	*hblC*	*hblD*	*nheA*	*nheB*	*nheC*					
1	+	-	+	+	-	-	+	+	+	+a	-b	+
1	+	-	+	+	-	-	+	+	-	+a	-b	+
1	+	-	+	+	-	-	+	+	-	+a	+a	+
1	+	-	+	+	-	-	-	+	+	+a	+a	D
1	+	-	+	+	-	-	-	+	-	+a	-b	D
1	+	-	+	+	-	-	-	-	-	+a	+a	+
1	-	+	+	+	+	-	+	+	+	+a	-b	+
1	-	+	+	+	+	-	+	+	+	+a	+a	+
1	-	+	+	+	+	-	+	+	-	+a	+a	+
1	+	+	-	-	+	+	+	-	+	+a	+a	+
1	+	+	-	-	+	-	-	+	+	+a	+a	+
1	+	+	-	-	+	-	-	-	-	+a	+a	+
1	+	+	-	-	-	-	-	+	+	+a	+a	+
1	+	-	-	+	+	+	+	+	+	-b	-b	+
1	+	-	-	+	+	-	+	+	+	-b	-b	+
1	+	-	-	+	+	-	+	+	+	+a	+a	-
3	+	-	-	+	+	-	+	-	+	-b	-b	+
1	+	-	-	+	-	-	+	+	+	+a	+a	+
1	+	-	-	+	-	-	+	+	+	+a	-b	+
3	+	-	-	+	-	-	+	+	-	+a	+a	+
1	+	-	-	+	-	-	+	+	-	+a	-b	+
1	-	-	+	+	+	-	+	+	+	+a	-b	+
1	-	-	+	+	+	-	+	+	+	+a	+a	+
1	-	-	+	+	-	-	+	-	-	+a	+a	+
1	-	-	+	+	-	-	-	-	-	+a	-b	+
1	+	-	+	-	-	-	+	+	-	+a	-b	+
1	+	-	+	-	-	-	+	-	-	+a	-b	-
1	+	-	-	-	+	+	+	+	+	+a	+a	+
1	+	-	-	-	+	-	+	+	+	+a	-b	+
1	+	-	-	-	-	-	-	+	-	+a	+a	+
3	+	-	-	-	-	-	-	-	-	+a	+a	+
1	+	-	-	-	-	-	-	-	-	+a	-b	+
1	+	-	-	-	-	-	-	-	-	-b	-b	+
1	-	-	+	-	-	-	+	+	+	+a	-b	+
1	-	-	+	-	-	-	+	+	-	+a	-b	+
1	-	-	+	-	-	-	+	-	-	-b	-b	+
1	-	-	-	+	+	-	+	-	+	-b	-b	-
1	-	-	-	+	-	-	-	+	-	+a	+a	+
1	-	-	-	+	-	-	-	+	-	+a	-b	+
2	-	-	-	+	-	-	-	-	-	-b	-b	+
1	-	-	-	-	+	-	-	-	+	+a	-b	+
1	-	-	-	-	-	-	-	+	-	+a	+a	+
1	-	-	-	-	-	-	-	+	-	-b	-b	+
1	-	-	-	-	-	-	-	-	-	+a	+a	-
1	-	-	-	-	-	-	-	-	-	+a	-b	+

ND: not determined; +a: coagulase activity; -b: lack of coagulase activity; + PCR: product of the expected size was observed; -: no PCR product was observed; D: production of discontinuous haemolytic pattern on blood agar plates. ∅: number of strains that share the same genotypic and phenotypic characteristics listed here; *: data obtained from previous results (23).

DISCUSSION

According to previous studies on our *B. cereus* collection isolated from honeys (24), data for the hemolytic activity showed that 95% of isolates presented hemolysis, and within these strains, 11% of them produced a discontinuous hemolytic pattern (Table 3). In the case of *B. megaterium*, hemolytic activity was shown by 77% of isolates, and within this group, 10% produced a discontinuous hemolytic pattern (25). According to Beecher and Wong (6), the presence of a discontinuous hemolytic pattern is usually correlated with the presence of hemolysin BL in *B. cereus.* Another study by Pruβ *et al.* (32), found that all but one of the strains within the *B. cereus* group that were positive for *hblA* exhibited hemolytic activity (Table 3). Thaenthanee *et al*. (37) found that among all tested *B. cereus* strains carrying the three *hbl* genes, more than 60% displayed a discontinuous hemolytic pattern (Table 3). These results were correlated with those herein observed where 57% of the strains showing a discontinuous hemolytic pattern presented the 4 components of the HBL complex and the rest (43%), at least 3 components (Tables 1 and 3). In the case of *B. megaterium,* the strains showing discontinuous hemolytic pattern presented at least 2 target genes (Table 2) with the exception of the Bm2 strain. Coagulase activity was

Table 2. Screening of virulence genes and coagulase and haemolytic activities of *Bacillus megaterium* isolates from honey.

Number of strains	HBL complex				NHE complex			*bceT*	*cytK*	Clumping* Factor	Free* Coagulase	Haemolysis*
	hblA	*hblB*	*hblC*	*hblD*	*nheA*	*nheB*	*nheC*					
NRRL B- 939	-	-	-	+	-	-	-	-	-	-b	-b	-
1	+	+	+	+	-	-	+	+	+	+a	+a	D
1	+	+	-	-	-	-	-	-	-	+a	+a	D
1	+	+	-	-	-	-	-	-	-	-b	-b	-
1	+	-	+	-	-	-	-	+	-	+a	+a	+
1	+	-	-	+	+	-	+	+	-	-b	+a	+
1	+	-	-	+	-	-	-	-	-	-b	-b	+
1	+	-	-	+	-	-	-	-	-	-b	+a	+
1	-	-	+	+	-	-	+	+	+	+a	+a	D
1	+	-	-	-	-	-	-	+	-	+a	+a	-
1	+	-	-	-	-	-	-	-	-	+a	+a	+
3	+	-	-	-	-	-	-	-	-	-b	+a	+
1	+	-	-	-	-	-	-	-	-	-b	-b	+
1	-	-	-	+	-	-	-	+	-	+a	+a	+
1	-	-	-	+	-	-	-	-	-	+a	+a	+
1	-	-	+	-	-	-	-	+	-	+a	+a	-
3	-	-	+	-	-	-	-	-	-	-b	+a	+
2	-	-	+	-	-	-	-	-	-	+a	+a	+
1	-	-	+	-	-	-	-	-	-	-b	-b	+
1	-	-	-	-	+	-	+	-	-	+a	+a	-
1	-	-	-	-	+	-	+	-	+	+a	+a	+
2	-	-	-	-	-	-	+	-	-	+a	+a	-
1	-	-	-	-	-	-	-	+	-	+a	+a	+
1	-	-	-	-	-	-	-	+	-	-b	-b	-
1	-	-	-	-	-	-	-	-	+	+a	+a	+
1	-	-	-	-	-	-	-	-	-	+a	+a	D
5	-	-	-	-	-	-	-	-	-	-b	-b	+
8	-	-	-	-	-	-	-	-	-	-b	+a	+
3	-	-	-	-	-	-	-	-	-	+a	+a	+
2	-	-	-	-	-	-	-	-	-	-b	+a	-
3	-	-	-	-	-	-	-	-	-	-b	-b	-

ND: not determined; +a: coagulase activity; -b: Lack of coagulase activity; + PCR: product of the expected size was observed; -: no PCR product was observed; D: production of discontinuous haemolytic pattern on blood agar plates. $^{\varnothing}$: number of strains that share the same genotypic and phenotypic characteristics listed here. * data obtained from previous results (24).

observed as clot formation in 106 *B. cereus* strains (80%) and among the positive strains, 57 (54%) presented the clumping factor. Our previous studies on the *B. megaterium* collection from honeys showed that coagulase activity was observed as clot formation in 74% of strains out of a total of 53 strains and, among the positive strains, only 49% presented the clumping factor (25). The coagulase activity has been broadly studied in *Staphylococcus aureus* and is considered the most important virulence factor in this species (10, 20). *In vitro* assays for testing the ability to clump plasma via the activity of the clumping factor (bound coagulase) (CF) and coagulase (free coagulase) has not been described in *B. cereus.*

The presence of the *hblA* gene encoding for the B part of the HBL complex, the gen *hblB* encoding for the B' part, the gen encoding for the L_1 part (*hblD*), and the gene encoding for the L_2 part (*hblC*) was detected in 83%, 57%, 83%, and 64% of the isolates tested, respectively (Table 3). Two percent of the isolates presented the L1 and L2 components of the HBL complex, while 4% of the isolates showed B and B' components (Tables 1 and 3). In addition, all three HBL genes were detected in 42 % of strains (Tables 1 and 3). In relation to the NHE complex, 56% of strains yielded the expected *nheA*-PCR product; 12% the expected *nheC*-PCR product, but only 10% presented the expected *nheB*-PCR (Tables 1 and 3). The high distribution of these genes within *B. cereus* populations isolated from honey samples are in agreement with results obtained with *B. cereus* strains isolated from different sources (8, 16, 21, 34, 40) and are summarized in Table 3. The presence of genes encoding for the enterotoxin-T was observed in 73% of our strains (Tables 1 and 3) while other workers (16) (21) have reported that most of the *B. cereus* strains from food-poisoning outbreaks carried the *bce*T genes in a proportion of 91% and 57% of positive strains, respectively (Table 3). The presence of the *cyt*K-PCR product of 421 bp was detected in 53% of our collection (Tables 1 and 3), while Guinebretiere *et al.* (16) found that 92% of strains carried the *cytK* gene (Table 3).

In our *B. megaterium* collection, the presence of part B, part B', part L_1 and part L_2 was detected on 27%, 6%, 23% and 6% of the strains and only one strain (Bm8) presented all four components of the HBL complex that was correlated with the presence of a discontinuous hemolytic pattern. The presence of the *bceT* gene encoding for the enterotoxin-T was detected in 9 strains (17%). Rowan *et al.* (34) found that one *B. megaterium* strain out of a total of 2 analyzed presented genes associated with the HBL complex and *Bcet*. In relation to the NHE complex, the presence of the *nhe*A and *nheC* genes was observed in 36% and 13% of strains, respectively, while none of the isolates presented the *nheB* gene.

Phelps and McKillip (30) detected by PCR individual enterotoxin genes within the *Bacillaceae* family in some but not all the strains tested that were shown to produce enterotoxins. In addition, Pruβ *et al.* (32) determined the prevalence of the HBL complex in all species of the *B. cereus* group, where most of the strains studied carried *hblA,* but its presence was not related to a certain species or a specific environment. These studies emphasize the need to design assays for *Bacillus* species that do not rely on a single virulence determinant.

The PCA in our *B. cereus* collection revealed a positive correlation among free coagulase, hemolysis and the presence of genes *hblA*, *hblB*, *hblC*, *hblD* (HBL complex) and *bceT* (enterotoxin-T), but no correlation with the clumping factor (bound coagulase) and the presence of

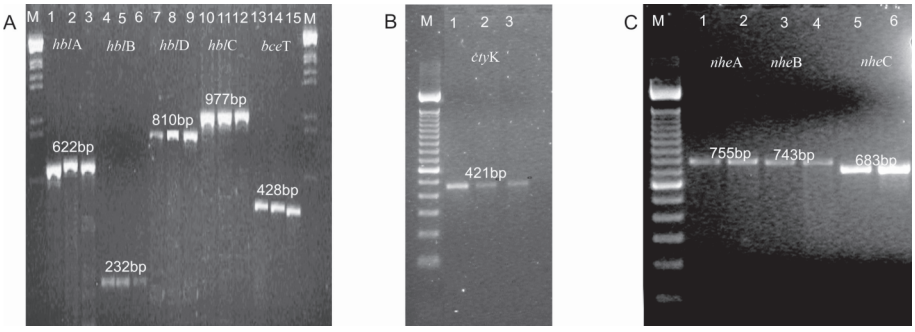

Figure 1. A) PCR patterns obtained with the various PCR primers sets for the detection of the enterotoxin HBL complex and enterotoxin-T and various *B. cereus* and *B. megaterium* strains. Lanes (from left to right): M: molecular size marker λ Eco *Hind* III Promega; 1 to 3: primer pair B, strains Bc20, Bc21and Bc37; 4 to 6: primer pair B', strains Bc 20, Bc 21 and Bc37; 7 to 9: primer pair L-1, strains Bc20, Bc21 and Bc37; lanes 10 to 12: primer pair L-2, strains Bc20, Bc21 and Bc37; 13 to15: primer pair ET, strains Bc20, Bc21 and Bc37; M: molecular size marker I eco*Hind*III, Promega. B) PCR products obtained with the primers sets targeting the cytotoxin K gene (CK F2 and CK R5) in *B. megaterium* strains. Lanes (from left to right): M: molecular size marker 100 bp ladder Promega, Argentina; lanes 1 to 3: Bm1, Bm8 and Bm9. C) PCR patterns obtained with the various PCR primers sets for the detection of the enterotoxin NHE complex in *B. cereus* strains. Lanes (from left to right): M: molecular size marker λ Eco *Hind* III Promega; 1 to 2: primer pair *nheA*, strains Bc30 and Bc80; 3 to 4: primer pair *NheB*, strains Bc84 and Bc100; 5 to 6: primer pair *NheC*, strains Bc 101 and Bc32.

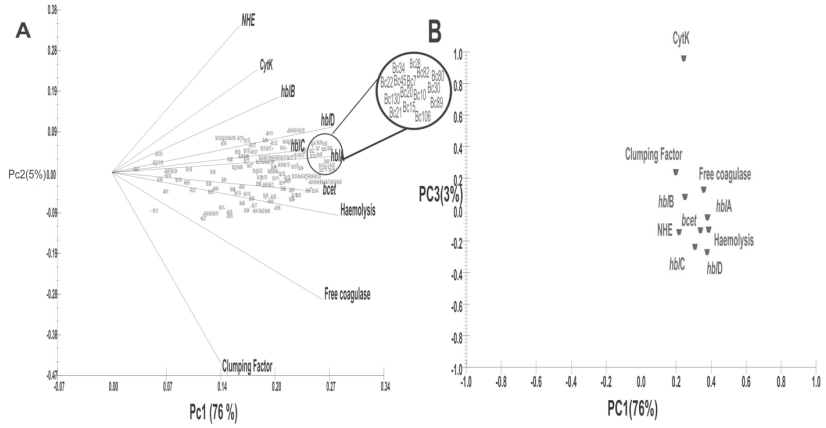

Figure 2. Biplots for the entire data set of 133 *B. cereus* isolates from honey. (A) PC1 *vs.* PC2. For visual clarity, strains positive for all enterotoxins and free coagulase activity are included in a circle. (B) PC1 *vs.* PC3. The percentages of the variation explained by principal components are indicated in parentheses.

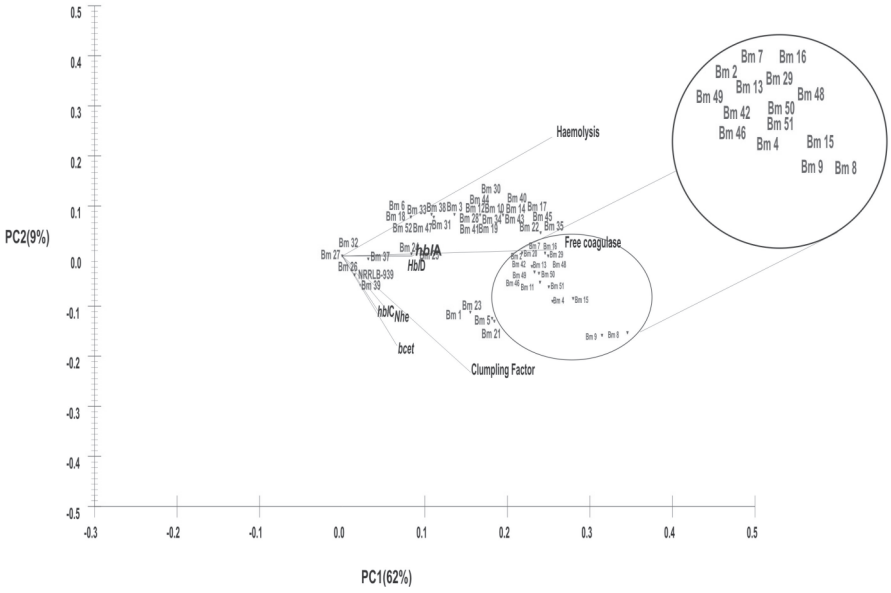

Figure 3. Biplots for the entire data set of 52 *B. megaterium* isolates from honey. PC1 *vs.* PC2. A circle has been placed around strains positive for coagulase (bound and free coagulase) and hemolytic activities. The percentages of the variation explained by principal components are indicated in parentheses.

sequences of the NHE complex. This lack of correlation was also observed by Minnaard *et al.* (27) in *B. cereus* strains isolated from different sources. On the other hand, PCA in *B. megaterium* showed a high positive correlation between coagulase (bound and free) and hemolytic activity but no correlation in relation to the presence of genes of the HBL complex, cytotoxin K, enterotoxin T and NHE complex.

Its interesting to point out that the presence of the *cytK* gene was almost independent of the presence of the rest of virulence factors analyzed here, both in *B. cereus* (Figure 2B) and *B. megaterium* populations.

In conclusion, our results show the associations among free coagulase activity and the presence of other virulence factors as specific DNA sequences of enterotoxins and/or hemolytic activity in *B. cereus* populations isolated from honey samples. It is important to point out that this study is the first report of the detection of genes of cytotoxin K (*cytK*) and the genes of the non-hemolytic enterotoxin complex (NHE) in *B. megaterium* and the first screen-

Table 3. Comparison of results obtained with our *B. cereus* collection with those reported by others authors regarding haemolytic activity, production of a discontinuous haemolytic pattern, and detection of enterotoxin genes by PCR.

References	This work and López & Alippi (24)	Beecher & Wong (6)	Borge *et al.* (8)	Guinebretiere *et al.* (16)	in'Veld *et al.* (20)	Pruβ *et al.* (32)	Rowan *et al.* (34)	Thaenthanee *et al.* (37)		Zahner *et al.* (40)
Total of Isolates tested	132	17	11	37	86	23	11	339	222	65
HBL complex										
hblA	110 (83%)	ND	6 (55%)	27 (92%)	57(66%)	9 (39%)	10 (91%)	224 (65%)	222 (100%)	58 (89%)
hblB	75 (57%)	ND	6 (55%)	22 (59%)	9 (10%)	ND	ND	ND	ND	ND
hblC	84 (64%)	ND	6 (55%)	27 (92%)	53 (62%)	ND	10 (91%)	228 (67%)	222 (100%)	ND
hblD	109 (83%)	ND	6 (55%)	27 (92%)	56 (65%)	ND	ND	226 (66%)	222 (100%)	ND
Features tested*										
NHE complex										
nheA	47 (56%)	ND	11 (100%)	36 (97%)	ND	ND	ND	ND	ND	ND
nheB	13 (10%)	ND	11 (100%)	36 (97%)	ND	ND	ND	ND	ND	ND
nheC	35 (12%)	ND	11 (100%)	36 (97%)	ND	ND	ND	ND	ND	ND
Cytotoxin K (*cyt*K)	70 (53%)	ND	ND	27 (92%)	ND	ND	ND	ND	ND	ND
Enterotoxin-T (*bce*T)	96 (73%)	ND	10 (91%)	21 (57%)	53 (62%)	ND	5 (45%)	ND	ND	53 (82%)
Haemolytic activity	125 (95%)	ND	ND	ND	23 (27%)	23 (100%)	ND	ND	12 (5%)	ND
Discontinuous haemolytic pattern	14 (11%)	11 (65%)	ND	ND	17 (18%)	ND	ND	ND	210 (95%)	ND

*Only positive results are listed here. ND: not determined

ing of virulence factors in *B. cereus* and *B. megaterium* isolates from honey.

In addition, an association between coagulase and hemolytic activities was observed in *B. megaterium* that did not correlate with the presence of specific DNA sequences of enterotoxins. The potential risk of enterotoxins produced by *B. megaterium* has been noted in honey and their significance in other foods remains to be determined.

Until now, honey is not known to have been involved in outbreaks of disease cause by *B. cereus*, but our results suggest that honey could be a possible vehicle for foodborne illness. Further studies involving cell infection assays with the collection of *B. cereus* and *B. megaterium* from honey on human enterocytes are needed in order to corroborate the correlation between the presence of virulence genes and biological activity.

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