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ORIGINAL ARTICLE

Partial characterization of bacteriocin-like compounds from two strains of *Bacillus cereus* with biological activity against *Paenibacillus larvae,* the causal agent of American Foulbrood disease

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Significant and Impact of the Study: An Integrated Pest Management (IPM) approach is needed to ensure the sustainability of the beekeeping industry due to the increasing demand for organic honey and the reduction of dependence on antibiotics. Biocontrol agents produced by bacteria isolated from apiarian sources seem promising and able to combine with an IPM strategy. The most significant findings of this study are the characterization of bacteriocin-like compounds (BLIS) obtained from two strains of *Bacillus cereus* isolated from honey. Both BLIS have a narrow activity range and highly inhibit the growth of *Paenibacillus larvae,* the causal agent of American Foulbrood disease of honey bees.

Keywords
American Foulbrood, *Bacillus cereus*, biocontrol, bacteriocin-like inhibitory substances, *Paenibacillus larvae*.

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Abstract

American Foulbrood (AFB), caused by the spore-forming Gram-positive bacterium *Paenibacillus larvae,* is the most severe bacterial disease affecting honeybees worldwide. Two bacterial isolates showing specific inhibitory activity against *P. larvae* were identified as *Bacillus cereus* by 16S rDNA sequencing. Antagonistic compounds were obtained from cell-free supernatants of strains m6c and m387 growing on Trypticase Soy Broth and concentrated by NH_4SO_4 precipitation, ultrafiltration and butanol extraction. Both compounds were characterized as bacteriocin-like inhibitory substances (BLIS). BLISm6c and BLISm387 were stable at 70°C for 30 min and active in the pH range from 3 to 7. The antibacterial activity was completely lost at pH values higher than 8 or temperatures >80°C. Both BLIS have a narrow activity range and highly inhibit the growth of *P. larvae.* BLISm6c and BLISm387 differ from each other and other BLIS reportedly produced by *B. cereus* with regard to their molecular weights, antibacterial activity, minimal inhibitory concentration values and sensitivity to degradative enzymes. The findings of this study suggest that BLISm6c and BLISm387 can potentially be used to control AFB.

Introduction

Honey bees are the most important pollinating insect worldwide and are threatened by several pathogens. American Foulbrood (AFB) is the most devastating bacterial disease affecting honeybee brood worldwide caused by the spore-forming Gram-positive bacterium *Paenibacillus larvae* (Genersch 2010). AFB occurs in temperate or subtropical regions throughout the world and can lead to huge losses in the apicultural economy and significantly impact pollination.

In many countries, AFB-infected colonies are destroyed by burning the bees, brood combs, and other wooden or plastic beehive components. However, in most American honey-producing countries, the antibiotic oxytetracycline (OTC) has been used by beekeepers to prevent and control AFB. Antibiotic treatment is an alternative to the burning of infected beehives in areas where disease

incidence is high. The widespread use of OTC favoured natural selection of resistant bacteria carrying tetracycline-resistant plasmids, disequilibrium in the microbiota of the beehive and honey contamination (Alippi *et al.* 2014). Also, in December 2016, the United States FDA will fully enforce Guidance #213 which entails new laws limiting the use of agricultural antibiotics (including tetracycline) that is on the list for Veterinary-Feed-Directive. This situation reinforces the need for alternative treatments for AFB prevention and control, including several biological control agents with various modes of action including the production of antibiotics, antibiotic-like compounds, bacteriocins, enzymes and lipopeptides.

Bacteriocins are ribosomally synthesized antibacterial proteinaceous compounds released extracellularly by bacteria that interfere with the growth of other bacteria, including those closely related to the producing bacterium, by inhibition of cell wall biosynthesis and/or pore formation (Heng *et al.* 2007). In contrast, bacteriocin-like inhibitory substances (BLIS) are defined as antimicrobials where ribosomal synthesis is presumed but are not well characterized (Abriouel *et al.* 2011). In addition, there are other antimicrobial components, such as bacterial lipopeptides, produced by most *Bacillus* species that are secondary metabolites produced by nonribosomal peptide synthetases that exhibit a broad-spectrum antimicrobial activity that can be used as biocontrol agents (Abriouel *et al.* 2011; Cochrane and Vederas 2014). Some bacteriocins from bacilli (e.g. subtilins, subtilosins, mersacidin, sublancin, ericins, lichenin, lichenicidin, haloduracin, coagulin and megacins) have been characterized (Abriouel *et al.* 2011). Furthermore, members of the *Bacillus cereus* group are capable of producing bacteriocins and BLIS. Examples include thuricins, entomocins, tolworthcins and bacthurincins produced by the entomopathogen *Bacillus thuringiensis*, and cereins, cerexins and BLIS produced by the ubiquitous *B. cereus* (Abriouel *et al.* 2011). Also, *B. cereus* is responsible for intestinal pathologies in humans by producing cereulide and extracellular factors, while *B. thuringiensis* is an insect pathogen by producing δ-endotoxins (Stenfors Arnesen *et al.* 2008).

In a previous study, we isolated *Bacillus* and *Brevibacillus* species from honey that exhibited inhibitory effects against *P. larvae in vitro* (Alippi and Reynaldi 2006). Subsequently, in the case of some strains, we related this activity to the production of BLIS. The aims of the present study were to characterize BLIS produced by *B. cereus* strains, m6c and m387, that showed a high level of inhibitory activity against *P. larvae* and to evaluate their antibacterial spectrum against a collection of Gram-positive and Gram-negative bacteria.

Results and discussion

Bacterial identities were confirmed by sequencing the 16S rDNA of both antagonistic strains m6c and m387. The partial bacterial 16S rDNA gene sequences were subjected to a BLAST-N search to identify sequences with the highest similarity. The analysis confirmed that both bacterial strains belong to *B. cereus.* Sequences were deposited in the DDBJ/EMBL/GenBank database under Accession Numbers: KP005456.1 for *B. cereus* m6c and KP005455.1 for *B. cereus* m387, respectively.

BLISm6c and BLISm387 showed inhibitory activity against *P. larvae* ATCC 9545 with inhibition areas of $375{\cdot}01 \pm 26{\cdot}68$ mm^2 and $660{\cdot}31 \pm 25{\cdot}75$ mm^2 respectively (Fig. 1). BLISm6c was bacteriostatic at a dilution of 1 : 16 and bactericidal at a dilution of 1 : 4, while BLISm387 was bactericidal at a dilution of 1 : 4 without bacteriostatic activity at higher dilutions. Protein concentration as determined by Bradford (means of three independent replications) was $0{\cdot}22 \pm 0{\cdot}12$ mg ml^{-1} for BLISm6c and $0{\cdot}27 \pm 0{\cdot}10$ mg ml^{-1} for BLISm387. These discrepancies between replications could be due to the incorporation of $(NH_4)_2SO_4$ manually that can vary the yield of protein precipitation.

An electrophoretic analysis of the proteins present in BLISm6c showed three bands having apparent molecular weights of about 6·2, 14·4 and 17·1 kDa respectively (Fig. 2, lane 4). The proteins present in BLISm387 showed several bands, detected in the part of the gel corresponding to the molecular weights of about 6·2 kDa, between 6·2 and 17·1, 21·4 and >26·6 kDa respectively (Fig. 2, lane 3). Both BLISm6c and BLISm387 were able

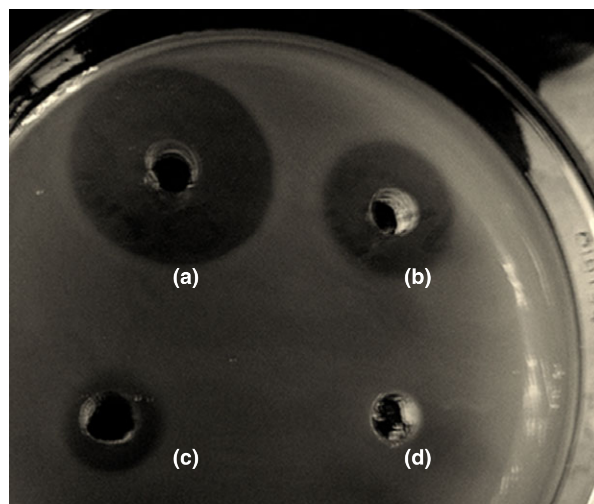

Figure 1 Inhibitory activity of bacteriocin-like inhibitory substances (BLIS) BLISm387 and BLISm6c respectively against *Paenibacillus larvae* ATCC 9545 by the well diffusion technique. (a) BLISm387; (b) BLISm6c; (c) BLISm387 dilution 1 : 4; (d) Control.

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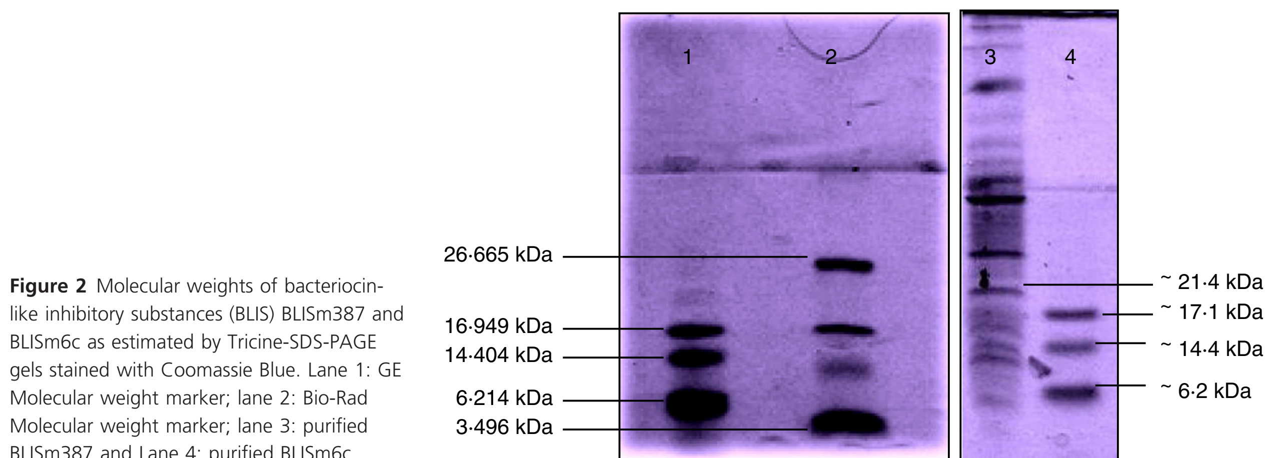

Figure 2 Molecular weights of bacteriocin-like inhibitory substances (BLIS) BLISm387 and BLISm6c as estimated by Tricine-SDS-PAGE gels stained with Coomassie Blue. Lane 1: GE Molecular weight marker; lane 2: Bio-Rad Molecular weight marker; lane 3: purified BLISm387 and Lane 4: purified BLISm6c.

to pass through dialysis membranes of 14 kDa-molecular weight cut-off (MWCO) and 17 kDa MWCO, showing halos of inhibition similar to controls; indicating that the primary antagonistic compound should have a molecular weight under 14 kDa. Also, as determined by ultrafiltration with Amicon® Ultra-15 centrifugal filters units with a membrane of 3 KDa, both BLIS should have molecular weights higher than 3 kDa. These results are consistent with one band of approx. 6·2 KDa observed in the Tricine-SDS-PAGE gel for BLISm6c (Fig. 2, lane 4) and four bands between 6·2 kDa and under 14·4 kDa in the case of BLISm387 (Fig. 2, lane 3). BLISm387 showed higher inhibition than BLISm6c (Fig. 1). It might be explained by the presence of several active proteins between 6·2 and 14·4 kDa (Fig. 2, lane 3) while the activity of BLISm6c was restricted to an active protein of about 6·2 kDa (Fig. 2, lane 4). These results are not conclusive to differentiate between single peptides or multisubunit peptides.

BLISm6c and BLISm387 differ from each other and other BLIS reported by other authors produced by *B. cereus* by their molecular weights. Cerein 7 has a molecular weight of 3·9 kDa (Oscáriz *et al.* 1999) while cerein MRX1 (Sebei *et al.* 2007) has a molecular weight of 3·4 kDa (Sebei *et al.* 2007). On the other hand, cerein GN105 (Naclerio *et al.* 1993), and cerein 8A (Bizani *et al.* 2005) have molecular weights of 9 and 26 kDa, respectively. BLISm6c and BLISm387 are therefore considered to be two newly identified BLIS produced by *B. cereus*.

The antibacterial activity of BLISm6c was completely lost after treatment with chymotrypsin while a partial loss of activity was observed after treatment with lipase, papain, pepsin, proteinase K, ribonuclease, RNase and trypsin (Table 1). In contrast, the antibacterial activity of BLISm387 was completely lost after treatment with chymotrypsin, ribonuclease and RNase. BLISm387 could contain some phosphoester or phosphodiester bond sensitive to ribonuclease and RNase. Nevertheless, further studies are needed to decipher the actual effect of ribonucleases on the antagonistic activity of BLISm387. Moreover, no reduction in activity was observed after treatment with lipase, and partial loss of activity was observed after treatment with papain, pepsin and proteinase K (Table 1). Also, the antibacterial activity of both BLIS was not affected when treated for 3 min at 100°C or when the buffer was added.

Table 1 Sensitivity to different degradative enzymes

Treatment	BLISm6c residual activity (%)*	BLISm387 residual activity (%)*
Chymotrypsin	0	0
Lipase	70·88 ± 2·42	85·28 ± 25·76
Papain	70·81 ± 3·82	53·70 ± 6·28
Pepsine	74·68 ± 2·95	60·69 ± 3·49
Proteinase K	65·75 ± 7·37	74·39 ± 5·14
Ribonuclease	84·96 ± 2·79	0
RNase	82·17 ± 10·29	0
Trypsine	63·47 ± 3·44	58·50 ± 0·06

BLIS, bacteriocin-like inhibitory substances.
*Residual activity compared to the antibacterial activity before the enzymatic treatment. Data are means of three independent experiments.

Other *B. cereus* bacteriocins and BLIS were also sensitive to proteolytic enzymes confirming their proteinaceous nature (Naclerio *et al.* 1993; Oscáriz and Pisabarro 2000; Bizani *et al.* 2005; Sebei *et al.* 2007; Senbagam *et al.* 2013). The digestion patterns of the BLIS described here were not identical nor did the patterns match those of previously reported BLIS, that is, the only proteolytic enzyme that completely inhibited the activity of BLISm6c, and BLISm387 was chymotrypsin.

Both BLIS maintained their inhibitory activity when treated at temperatures of 60°C for 60 min and 70°C for 30 min. The antibacterial activity was completely lost when treated at temperatures higher than 80°C for 30 min or

Table 2 Effect of pH on antibacterial activity

pH	BLISm6c residual activity (%)*	BLISm387 residual activity (%)*
3	85·85 ± 2·71	93·29 ± 12·90
4	78·63 ± 12·30	88·46 ± 10·34
7	100	100
8	0	0
10	0	0

BLIS, bacteriocin-like inhibitory substances.
*Residual activity compared to the antibacterial activity before the treatment during 1 h at room temperature and then neutralized to pH 7. Data are means of three independent experiments.

121°C for 15 min. Similar results were reported for cerein (Naclerio *et al.* 1993) and cerein 8A (Bizani *et al.* 2005).

Our results indicate that both BLIS remain stable and maintain their inhibitory activity at 37°C, which is the temperature of the bee brood (Genersch 2010). BLISm6c and BLISm387 were stable in the pH range from 3 to 7, but the antibacterial activity was completely lost at pH values higher than 8 (Table 2). These results suggest that the stability of both BLIS at acidic conditions benefits the delivery of the active compounds to young larvae through royal jelly (pH ranging 3·4–4·5). As the target of AFB infection is young larvae that feed on royal jelly, the stability at low pH is a critical point to consider in the study of the pharmacokinetics of BLIS or any antibiotic in the hive. Also, BLISm6c and BLISm387 were tested for toxicity to honey bee larvae by previously described methods (Reynaldi *et al.* 2008). Both BLIS were nontoxic for larvae when compared with the standard brood mortality ranging from 10 to 14% (Minnaard *et al.* 2013).

Table 3 shows that within Gram-positive bacteria, the inhibitory activity range of both BLIS was rather narrow and mainly limited to *P. larvae* strains (10 from a total of 17 positives for BLISm6c and 12 from a total of 17 positives for BLISm387). The activity spectrum clearly shows that BLISm6c and BLISm387 are active against strains of *P. larvae* belonging to genotype ERIC I except strain PL15. Moreover, all *P. larvae* strains belonging to genotype ERIC IV were insensitive. In relation to *P. larvae* strains belonging to genotype ERIC II, BLISm387 was active against two strains tested (*P. larvae* SAG 290 and *P. larvae* SAG 10230) while BLISm6c was active only on *P. larvae* SAG 290.

Also, both BLIS showed antibacterial activity against *Bacillus mycoides* ATCC 10206, *Bacillus subtilis* ATCC 10783, *B. thuringiensis* ATCC 10792, and *Paenibacillus macerans* ATCC 8244. Moreover, BLISm387 also inhibited *B. cereus* ATCC 11778 and *Bacillus megaterium* NRRL B-939. In contrast, all the Gram-negative bacteria tested ($n = 12$) were insensitive to both BLIS. In general, BLISm387 showed higher inhibition areas than BLISm6c (Table 3). Our results are in agreement with previously reported studies that demonstrated that antimicrobial compounds obtained from *B. cereus* strains differ in their antibacterial activity (Shoji *et al.* 1976; Oscáriz and Pisabarro 2000; Risøen *et al.* 2004; Bizani *et al.* 2005; Sebei *et al.* 2007). For instance, cerein 8A inhibits several pathogenic and food spoilage micro-organisms including *Listeria monocytogenes* and *B. cereus* strains (Bizani *et al.* 2005). Cerexins show activity against Gram-positive bacteria including species of *Streptococcus* and *B. subtilis* (Shoji *et al.* 1976) while cerein GN105 is active exclusively against other *B. cereus* strains. In contrast, cerein 7 (Oscáriz and Pisabarro 2000), cerein MRX1 (Sebei *et al.* 2007) and a BLIS from *B. cereus* type strain ATCC 14579 (Risøen *et al.* 2004) showed a broader spectrum of antagonistic activity. It is important to point out that BLISm6c and BLISm387 showed antagonistic activity only against *P. larvae* genotypes ERIC I and ERIC II, while genotype ERIC IV was insensitive to both compounds. Our results demonstrated that BLISm6c and BLISm387 are distinct from each other because of differences in molecular weight, sensitivity to enzymes, activity ranges and MIC values, suggesting that they not be the same peptide responsible for the inhibitory activity against *P. larvae*. In some *Bacillus* species, a multiproduction of nonribosomal cyclic lipopeptide responsible for antifungal effects was described (Mora *et al.* 2015). Furthermore, the presence of genes *fen*D (fengycin), *srf*AA (surfactin) and *itu*D (iturin) were detected by PCR in *B. cereus* strain m6c while genes *srf*AA (surfactin), *bmy*B (bacillomycin) and *itu*D (iturin) were found in *B. cereus* strain m387 (Data not shown).

Although some studies reported the antagonism between *B. cereus* and *P. larvae*, none of them have characterized the antimicrobial compounds produced (Alippi and Reynaldi 2006; Evans and Armstrong 2006; Yoshiyama and Kimura 2009). To our knowledge, this is the first description of two BLIS produced by *B. cereus* strains isolated from honey that exhibit strong inhibition effects and high specificity on *P. larvae* growth. The findings of this study suggest that BLISm6c and BLISm387 can potentially be used to control AFB. Both seem promising and able to combine with an IPM strategy. Nevertheless, more research is needed to examine their mechanisms of action and guarantee their safe employment.

Materials and methods

Bacterial strains, culture conditions and genotypic characterization

Paenibacillus larvae strains were cultured in MYPGP agar at 37°C for 24–48 h while *B. cereus* strains were grown on trypticase soy agar (TSA; Britania, Buenos

Table 3 Culture conditions of bacteria and antibacterial spectrum of BLISm6c and BLISm387 as determined by the well diffusion technique

Indicator strain	Rep-PCR	Source*	Growth conditions	BLISm6c	BLISm387
Gram-positive bacteria					
Bacillus cereus m6c, Argentina		UB-CIDEFI[(a)]	TSA- 32°C	−†	−
B. cereus m387, Argentina		UB-CIDEFI[(a)]	TSA- 32°C	−	−
B. cereus		ATCC 11778	TSA- 32°C	−	++
Bacillus circulans		ATCC 4515	MYPGP- 37°C	−	−
Bacillus coagulans		ATCC 35670	TSA- 30°C	−	−
Bacillus licheniformis		NRRL B-1001	TSA- 30°C	−	−
Bacillus megaterium		NRRL B-939	TSA- 30°C	−	+
Bacillus mycoides		ATCC 10206	TSA- 30°C	+	++
Bacillus pumilus		ATCC 7061	TSA- 30°C	−	−
Bacillus subtilis		ATCC 10783	TSA- 30°C	+	+
Bacillus thuringiensis		ATCC 10792	TSA- 32°C	++	++
Brevibacillus laterosporus		CCT 31	MYPGP- 37°C	−	−
Enterococcus faecalis		ATCC 29212	TSA- 37°C	−	−
Paenibacillus alvei		NRRL B-383	TSA- 37°C	−	−
Paenibacillus amylolyticus		NRRL B-142	TSA- 37°C	−	−
Paenibacillus apiarius		ATCC 29575	MYPGP- 37°C	−	−
Paenibacillus macerans		ATCC 8244	TSA- 30°C	+	+
Paenibacillus larvae	ERIC I	ATCC 9545	MYPGP- 37°C	+++	+++
P. larvae PL 4 Argentina	ERIC I	UB-CIDEFI[(b)]	MYPGP- 37°C	++	++
P. larvae PL15 Argentina	ERIC I	UB-CIDEFI[(b)]	MYPGP- 37°C	−	−
P. larvae PL 27 Argentina	ERIC I	UB-CIDEFI[(b)]	MYPGP- 37°C	++	+
P. larvae PL 38 Argentina	ERIC I	UB-CIDEFI[(b)]	MYPGP- 37°C	++	+++
P. larvae PL 45 France	ERIC I	UB-CIDEFI[(b)]	MYPGP- 37°C	+	++
P. larvae PL 58 Sweden	ERIC I	UB-CIDEFI[(a)]	MYPGP- 37°C	++	+
P. larvae PL 99 Argentina	ERIC I	UB-CIDEFI[(b)]	MYPGP- 37°C	++	+++
P. larvae PL 444 Argentina	ERIC I	UB-CIDEFI[(a)]	MYPGP- 37°C	++	++
P. larvae PL 450 Argentina	ERIC I	UB-CIDEFI[(a)]	MYPGP- 37°C	++	++
P. larvae PL 452 Italy	ERIC I	UB-CIDEFI[(a)]	MYPGP- 37°C	++	+
P. larvae PL 454 Cuba	ERIC I	UB-CIDEFI[(a)]	MYPGP- 37°C	++	+
P. larvae SAG 290 Chile	ERIC II	UB-CIDEFI[(a)]	MYPGP- 37°C	++	+
P. larvae SAG 10230 Chile	ERIC II	UB-CIDEFI[(a)]	MYPGP- 37°C	−	+
P. larvae	ERIC IV	ATCC 14154	MYPGP- 37°C	−	−
P. larvae	ERIC IV	CCM 38	MYPGP- 37°C	−	−
P. larvae	ERIC IV	ATCC 13537	MYPGP- 37°C	−	−
Paenibacillus polymyxa		NRRL B-510		−	−
Gram-negative bacteria					
Agrobacterium rubi F266, Argentina		UB-CIDEFI[(c)]	TSA- 28°C	−	−
Agrobacterium tumefaciens bv. 1 F268, Argentina		UB-CIDEFI[(c)]	TSA- 28°C	−	−
Agrobacterium vitis K306		NBRC 15142	TSA- 28°C	−	−
Clavibacter michiganensis subsp. *michiganensis* F 528, Argentina		UB-CIDEFI[(c)]	TSA- 28°C	−	−
Escherichia coli		ATCC 25922	TSA- 37°C	−	−
Pantoea ananatis F 521, Argentina		UB-CIDEFI[(c)]	TSA- 28°C	−	−
Pseudomonas aeruginosa		ATCC 27853	King B- 37°C	−	−
Pseudomonas corrugata		NCPPB 2445	King B- 28°C	−	−
Pseudomonas syringae pv. *tomato* F491, Argentina		UB-CIDEFI[(c)]	King B- 28°C	−	−
Pseudomonas putida		NRRL B-252	King B- 28°C	−	−
Xanthomonas campestris pv. *campestris* F467, Argentina		UB-CIDEFI[(c)]	TSA- 28°C	−	−

BLIS, bacteriocin-like inhibitory substances.

*UB-CIDEFI: Unidad de Bacteriología-Centro de Investigaciones de Fitopatología, Facultad de Ciencias Agrarias y Forestales, Universidad Nacional de La Plata, La Plata, Argentina: [(a)]honey, [(b)]larval remains or scales, [(c)]plant material; ATCC, American Type Culture Collection, Rockville, MD; CCM, Czech Collection of Microorganisms, Brno, Czech Republic; CCT, Coleçao de Culturas Tropical, Fundaçao André Tosello, Campinas, SP, Brazil; NBRC, Biological Resource Center, National Institute of Technology and Evaluation, Kisarazu, Chiba, Japan; NCPPB, National Collection of Plant Pathogenic Bacteria, Harpenden, UK; NRRL, Northern Utilization Research and Development Division, Peoria, IL; SAG, Servicio Agrícola Ganadero, Chile.

†Scores were assigned based on the areas of inhibition: (−): No zone; (+): Clear zone 28–64 mm^2; (++): Clear zone 65–299 mm^2, (+++): Clear zone ≥300 mm^2. Data are from triplicate trials.

Aires, Argentina) for 24 h at 32°C. All the bacterial strains used and their growth conditions are listed in Table 3. Bacteria were kept as frozen cultures in 20% (v/v) glycerol in broth media according to requirements of each species (Table 3).

Bacillus cereus strains m6c and m387 were isolated from honey samples and preliminarily tested for antibacterial activity against *P. larvae* (Alippi and Reynaldi 2006). Both strains were further identified by sequencing the 16S rDNA. Universal primers used for 16S rDNA sequence analysis were 27f (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492r (5′-TACGGYTACCTTGTTACGACTT-3′). PCR was performed under the following conditions: 94°C for 1 min, 35 cycles of (94°C for 45 s, 55°C for 60 s and 72°C for 60 s), 72°C for 5 min. The purified PCR products of approx. 1400 bp were sequenced by the dideoxy termination method by the commercial services of Macrogen Inc. (Seoul, Korea). Homology searches were performed with BLAST-N (http://www.ncbi.nlm.nih.gov).

Antibacterial agent purification

Overnight cultures of each *B. cereus* strain growing in trypticase soy broth (TSB) were inoculated (2% v/v) in 5 ml of TSB and incubated at 32°C in a rotary shaker at 125 cycles per min for 16 h. The cells were harvested by centrifugation at 12 500 ***g*** for 15 min at 4°C (Beckman Coulter Allegra 2IR, Brea, CA) and culture supernatants were filtered through 0·22-μm membranes. Filtrates were precipitated with ammonium sulphate at 65% saturation (g ml^{-1}) at 4°C. The precipitate was dissolved with 20 ml phosphate buffer saline pH = 6·8 (PBS). Then, the volume was reduced to one hundredth by ultrafiltration with Amicon® Ultra-15 centrifugal filters units with a membrane of 3 kDa (Millipore, Billerica, MA) at 5000 ***g*** (Heraeus Biofuge Stratus; Thermo Scientific, Waltham, MA) at room temperature for 45 min. Each concentrated sample was extracted with butyl alcohol (2 : 3 ratio of butyl alcohol: sample) at room temperature for 2 h with gentle agitation. The butyl alcohol was evaporated at 40°C in an atmosphere of 25 mmHg in a rotoevaporator (Heidolph vvmicro, Schwabach, Germany) and the resulting pellet was suspended in one hundredth of the original volume of PBS. The extracts obtained by this procedure were named BLISm6c and BLISm387 for the compounds obtained from *B. cereus* strain m6c and *B. cereus* strain m387, respectively.

Antibacterial activity assay

Inhibitory activity was evaluated by a modified well diffusion technique (Alippi and Reynaldi 2006). Briefly, 12 ml of MYPGP were poured onto 90 mm Petri plates as a basal medium. Vegetative cells of *P. larvae* ATCC 9545 grown on MYPGP agar at 37°C for 48 h were suspended in sterile distilled water and adjusted to approx. $1{\cdot}2 \times 10^9$ CFU ml^{-1} (equivalent to a Mc Farland standard of 4). Then, 1 ml was added to 9 ml of 0·6% MYPGP agar (maintained at 45°C) and poured over basal medium. Twofold dilutions of the purified samples were spotted onto a Petri dish by placing 10 μl into each well. The plates were then preincubated at 4°C for 1 h to allow diffusion (Oscáriz *et al.* 1999; Oscáriz and Pisabarro 2000) and finally incubated at 37°C for 48 h to allow the growth of the indicator strain. The diameter of the inhibition halos was measured after 24 and 48 h with a calliper and areas were calculated. All assays were performed in triplicate. One activity unit (AU) was defined as the reciprocal of the dilution after the last serial dilution that produced a detectable inhibitory zone and is expressed as activity units per millilitre (AU ml^{-1}) (Bizani and Brandelli 2002).

BLIS characterization

Protein concentration

Protein concentration was determined according to Bradford (1976) using bovine serum albumin as standard. The results obtained are means of three replications.

Molecular weight estimation

The molecular weight of each BLIS was estimated by Tricine-sodium dodecyl sulphate-polyacrylamide gel electrophoresis (Tricine-SDS-PAGE) as described by Schägger and von Jagow (1987) using a 16·5% (w/v) separating gel, 10% (v/v) space gel and 4% (v/v) stacking gel. Electrophoresis was conducted in a Mini-Protean Tetra system (Bio-Rad Laboratories, Hercules, CA) at a constant voltage of 100 V. As weight standards, Polypeptide SDS-PAGE standard (Bio-Rad, Hercules, CA) and LMW-SDS Marker Kit (GE Healthcare Life Sciences, Buenos Aires, Argentina) were used. Gels were stained with Coomassie Brilliant Blue R-250 (Sigma-Aldrich, St. Louis, MO).

Also, molecular weight estimation of each BLIS was evaluated by diffusion through dialysis membranes of 14·0 and 17·0 kDa MWCO (Sigma) respectively. For this purpose, we used the well diffusion technique as previously described placing sterile pieces of the MWCO in the agar, whereas controls were left uncovered. Then, 10 μl of each BLIS was spotted over the MWCO. Inhibition areas in the wells covered with the MWCO were considered as an indication that molecular weights were under 14·0 or 17·0 kDa, respectively.

Effects of enzymes, heat and pH on antibacterial activity

Each BLIS sample was tested for sensitivity to different degradative enzymes (except for RNase (Biodynamics,

Buenos Aires, Argentina), all enzymes obtained from Sigma-Aldrich) as described by Cherif *et al.* (2008). Briefly, 50 AU ml^{-1} was incubated at 37°C for 1 h with chymotrypsin, lipase, pepsin, proteinase K, ribonuclease, RNase and trypsin, at a final concentration of 1 mg ml^{-1}, in the correspondent buffer as recommended by the supplier. For papain, the mix was incubated at 40°C for 1 h. Following incubation, the enzymes were inactivated at 100°C for 3 min. Untreated BLIS plus buffer, buffer, buffer treated at 100°C for 3 min and BLIS plus buffer treated at 100°C for 3 min served as controls.

Thermal stability was determined by incubation of BLIS aliquots at 60°C, 70°C, 80°C, 100°C for 30 and 60 min and by autoclaving at 121°C for 15 min.

The effect of pH was tested by adjusting BLIS aliquots to pH values of 3, 4, 8 and 10 with sterile 1 N NaOH or ClH, incubated at room temperature for 1 h, and then neutralized to pH 7.

After each treatment, residual BLIS activity was tested by the spot test assay with *P. larvae* ATCC 9545 as indicator strain. Residual activity (RA) was calculated by using the following formula: RA = 100% × A_2/A_1, where A is the area of the inhibition halo before treatment (A_1) and after treatment (A_2). All the experiments were performed in triplicate including their respective controls.

Determination of bactericidal and bacteriostatic activity

The bactericidal and bacteriostatic activity of BLIS determined by a broth macrodilution method using *P. larvae* ATCC 9545 as a reference strain. The inoculum was prepared as suspensions in MYPGP broth from a 48-h agar plate and adjusted to $1{\cdot}5 \times 10^8$ CFU ml^{-1}. Ninety microlitres of inoculum were mixed with 10 μl of serial dilutions (1 : 2, 1 : 4, 1 : 8 and 1 : 16) of each BLIS and then incubated at 37°C for 48 h under constant agitation. After the incubation period, the optical density at 600 nm was measured and compared to controls. The highest dilution of BLIS at which no growth of the indicator strain was recorded. Ten microlitres of each sample was spread onto MYPGP agar and incubated at 37°C for 48 h to check the viability of bacterial cells.

Antibacterial spectrum of BLIS

The antibacterial spectrum of BLISm6c and BLISm387 was determined against a collection of Gram-positive and Gram-negative bacteria (Table 3) by using the well diffusion technique. All tests were performed in triplicate. Sensitivity was recorded in a four-level scale: (−) no inhibition of the growth of the bacterial strain tested; (+) low inhibition of bacterial growth (Clear zone areas between 28–64 mm^2); (++) high inhibition of bacterial growth (Clear zone areas between 65–299 mm^2); and (+++) very high inhibition of bacterial growth (Clear zone area ≥300 mm^2).

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Conflict of Interest

No conflict of interest is declared.

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