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Characterization of a bacteriocin-like substance produced by *Bacillus amyloliquefaciens* isolated from the Brazilian Atlantic forest

Summary. A *Bacillus* strain producing a bacteriocin-like substance was characterized by biochemical profiling and 16S rDNA sequencing. Phylogenetic analysis indicated that the strain has high sequence similarity with *Bacillus amyloliquefaciens*. The antimicrobial substance was inhibitory to pathogenic and food-spoilage bacteria, such as *Listeria monocytogenes*, *Bacillus cereus*, *Serratia marcescens*, and *Pasteurella haemolytica*. It was stable over a wide temperature range, but lost activity when the temperature reached 121°C/15 min. Maximum activity was observed at acidic and neutral pH values, but not at alkaline pH. The antimicrobial substance was sensitive to the proteolytic action of trypsin, papain, proteinase K, and pronase E. Except for iturins, other antimicrobial peptides have not been described for *B. amyloliquefaciens*. The identification of a bacteriocin-like inhibitory substance active against *L. monocytogenes* addresses an important aspect of food protection. [**Int Microbiol** 2006; 9(2):111-118]

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Introduction

Bacteriocins are proteins or peptides with bactericidal activity towards species that are often closely related to the producer bacteria [29]. These substances, which are frequently metabolites produced by various gram-positive and gram-negative microorganisms [27], are heterogeneous compounds that display variable molecular weights, biochemical properties, inhibitory spectra, and mechanisms of action [14,21,25]. Due to their potential use as natural preservatives, bacteriocins produced by lactic acid bacteria have been the subject of intensive investigation in recent years [7,23], and many attempts are being made to incorporate bacteriocins into processes and products [25].

Several bacteriocins or bacteriocin-like substances (BLS) produced by the genus *Bacillus* have been reported [12,29]. The best-characterized are subtilin of *B. subtilis* [1,15], megacin of *B. megaterium* [32], bacteriocins of *B. cereus* [2,24], and bacteriocins of *B. thuringiensis* [9,26]. Although several bacteriocin strains produce bacteriocins that are active against a narrow spectrum of bacteria [26], others, including important pathogens such as *Listeria monocytogenes* and *Staphylococcus aureus*, produce bacteriocins with a broad spectrum of activity [24]. The ability to screen for a bacteriocine-like substance (BLS)-producing *Bacillus* strains

is of major interest in bacteriocin research since this genus produces a diverse array of antimicrobial peptides with several different basic chemical structures [10,28].

The Brazilian Atlantic forest is a unique ecosystem with enormous biological diversity. The forest has been reduced to less than 10% of its original size, but many efforts are now being made to conserve the remaining biodiversity of this region [19]. There are only a few studies on the microbial diversity of this region [8,22] but no specific reports on screening for biotechnologically relevant microorganisms. The objective of the present study was to evaluate the potential antimicrobial activity of bacteria isolated from native woodlands of southern Brazil. A BLS produced by a *Bacillus* sp. was identified and its antimicrobial spectrum and properties are described.

Materials and methods

Reagents and media. Trypticase soy broth (TSB) and nutrient broth (NB) were from Difco (Detroit, MI, USA), brain-heart infusion (BHI), and de Man-Rogosa-Shape (MRS) were from Oxoid (Basingstoke, UK). Papain, trypsin, proteinase K, and pronase E were from Sigma (St. Louis, MO, USA). All other media and reagents were from Merck (Darmstadt, Germany).

Bacterial cultures. Indicator strains listed in Table 1 are laboratory stocks obtained from different sources. The strains were kept frozen in 20% (v/v) glycerol at –20ºC until needed and then under the culture conditions indicated in Table 1.

Isolation of microorganisms. Soil samples were collected in the native woodlands of Santa Catarina State, near Santo Amaro da Imperatiz (27º24′S, 48º27′W), Brazil. This region corresponds to the southern part of the Brazilian Atlantic Forest. The samples were obtained by removing the

Table 1. Antimicrobial activity spectrum of the antimicrobial substance

Indicator organism	Media	Temp. (ºC)	Inhibition (mm)[a]
Gram-positive			
Bacillus cereus ATCC 9634	BHI	37	9.3
Bacillus subtilis ATCC 9372	BHI	37	9.5[b]
Corynebacterium fimi NCTC 7547	BHI	37	13.3
Lactobacillus acidophilus ATCC 4356	MRS	30	8.0[b]
Lactococcus lactis R704	MRS	30	–
Listeria innocua (food isolate)	BHI	37	25.5
Listeria monocytogenes ATCC 7644	BHI	37	9.3
Micrococcus luteus (clinical isolate)	BA	37	–
Enterococcus faecalis (clinical isolate)	BA	37	–
Streptococcus sp. (α-haemolytic)	BA	37	–
Staphylococcus 271	BHI	37	12.6[b]
Staphylococcus aureus (food isolate)	BHI	37	–
Staphylococcus aureus ATCC 25923	BHI	37	–
Staphylococcus intermedius (clinical isolate)	BHI	37	–
Rhodococcus sp.	BHI	37	17.6
Gram-negative			
Pasteurella haemolytica (clinical isolate)	BHI	37	12.5
Proteus vulgaris (clinical isolate)	BHI	37	9.0
Pseudomonas aeruginosa (clinical isolate)	BHI	37	–
Salmonella enteritidis (ATCC 13076)	BHI	37	–
Salmonella enteritidis (food isolate)	BHI	37	–
Salmonella gallinarium (clinical isolate)	BHI	37	12.8
Serratia marcescens (clinical isolate)	BHI	37	10.6
Erwinia carotovora (food isolate)	BHI	25	10.6[b]
Escherichia coli ATCC 25922	BHI	37	–
Escherichia coli (clinical isolate)	BHI	37	–
Yeast			
Candida kefir (food isolate)	MYG	37	–
Candida sp. (food isolate)	MYG	37	–
Kluyveromyces marxianus CBS 6556	MYG	37	–
Malassezia paquidermatis (clinical isolate)	MYG	37	–

[a]Aliquots of 20 ml were applied to discs, corresponding to 8 activity units (AU) per disc. Values are the diameter of the inhibition zone in mm around the disc and correspond to the means of three independent experiments.
[b]The inhibitory zone was hazy.
Media: BHI, brain hearth infusion; MRS, de Man-Rogosa-Sharpe agar; BA, sheep blood agar; MYG, malt yeast glucose.

leaf litter and collecting the top 10 cm of soil. Soil samples (100 g wet weight) were mixed with sterile water (1:1 w/v), homogenized for 15 min at 60 cycles in a laboratory blender (Seward, London, UK), and allowed to stand for 1 h at room temperature. One ml of this suspension was inoculated into 100 ml of nutrient broth, after which microbial growth was monitored by changes in the turbidity of the cultures. Aliquots of the cultures were inoculated onto nutrient agar plates, incubated at 30ºC, and single colonies were isolated and screened for antimicrobial activity.

Antimicrobial activity assay. Antimicrobial activity was detected by the agar-disk diffusion assay [13] and tested against all indicator strains. An 20-ml aliquot of culture supernatant filtrate was applied to disks (6 mm) placed on agar plates previously inoculated with a suspension of each indicator strain. The plates were incubated at the optimal temperature of the test organism. The bacteriocin titer was determined by the serial two-fold dilution method described in [20]. Activity was defined as the reciprocal of the dilution after the last serial dilution giving a zone of inhibition and expressed as activity units (AU) per milliliter.

Bacterial identification. Bacterial identification was based on 16S rRNA gene sequencing analysis and biochemical analysis using the API 50 CHB test kit with automated interpretation (APILAB Plus, bioMérieux, Marcy-l'Etoile, France). Additional morphological and physiological tests were carried out as described elsewhere [18].'

Cloned 16S rDNA was sequenced using DYEnamic ET Dye Terminator Cycle Kit for MegaBACE (a product from Amersham-Pharmacia Biotech, Buckinghamshire, England) with the primers 16S-27 (5'-GAGTTTGATCCTGGCTCAG-3') and 16S-1525 (5'-AGAAAGGAGGTGATCCAGCC-3') in an automatic sequencer (MegaBACE 1000, Amersham-Pharmacia Biotech). Phylogenetic data were obtained by alignment of the 41 different 16S rDNA sequences retrieved from the BLAST algorithm (National Center for Biotechnology Information [http://www.ncbi.nlm.nih.gov]), using the software CLUSTAL W version 1.8 [30] with the standard parameters. The alignments were visually corrected when necessary. Phylogenetic and molecular evolutionary analyses were conducted using MEGA, version 2.1 [16]. An unrooted phylogram was obtained by the neighbor-joining (NJ) method. An interior branch test was done (heuristic option, 1000 replications) to check the tree topology for robustness. Additionally, the Poisson correction was applied to NJ for distance estimation, and the complete deletion option was used in handling gaps or missing data obtained from the alignments.

BLS production under different conditions. The production of antimicrobial activity was tested in six different media: TSB, nutrient broth, BHI, Mueller-Hinton broth (MHB), lactose broth (3 g beef extract/l, 5 g peptone/l, 5 g lactose/l), and peptone water (8 g NaCl/l, 1 g peptone/l) at different incubation temperatures (30, 37, and 55ºC) for up to 48 h. At the end of each incubation period, antimicrobial activity was detected by the agar-disk diffusion assay and tested against *Bacillus cereus* and *Listeria monocytogenes* as indicator strains.

Preparation of BLS. To produce BLS for characterization, the selected strain was cultivated in 200 ml of BHI medium at 37ºC in a rotary shaker at 125 cycles per min for 24 h. The cells were harvested by centrifugation at 10,000 ×*g* for 15 min and culture supernatants were filtered through 0.22-µm membranes. The culture filtrate was precipitated with ammonium sulfate at 20% saturation and centrifuged at 10,000 ×*g* for 20 min at 4ºC. The pellet was resuspended in 10 mM sodium phosphate buffer (pH 6.0), and applied to a Sephadex G-100 column eluted with this same buffer. Active fractions were stored in sterile flasks at 4ºC until used for antimicrobial assays.

Sensitivity of antimicrobial activity to proteolytic enzymes and heat. The antimicrobial substance was treated at 37ºC for 1 h with 2 and 10 mg of the following enzymes/ml (final concentration): papain, trypsin, proteinase K, and pronase E. The samples were then boiled for 2 min to inactivate the enzyme. To analyze thermal stability, samples of bacteriocin were exposed to temperatures of 10, 40, 60, and 80ºC for 30 min, 100ºC for 5, 10, 15, 20, 30, 40, 50, and 60 min, and 121ºC/105 kPa for 15 min. After treatment, the samples were tested for antimicrobial activity against *L. monocytogenes* ATCC 7644.

Antimicrobial activity at different pH values. Samples of the antimicrobial substance were diluted in the following buffers (0.25 M): (pH 3.0), citrate (pH 4.0–5.0), phosphate (pH 6.0–8.0), and Tris (pH 9.0–10.0). After incubation for 2 h at 25ºC, the antimicrobial activity against *L. monocytogenes* was determined. Controls consisting of buffer alone were run in parallel.

Activity on polyacrylamide gels. Antimicrobial activity was detected on polyacrylamide gels as described previously [3]. Briefly, the samples were applied to 14% polyacrylamide gels and electrophoresed at 20 mA per gel. The gels were then washed with sterile distilled water to remove SDS and antimicrobial activity was tested against *L. monocytogenes*.

Results

Isolation and identification of bacteriocin-producing strain. Soil isolates were screened for antimicrobial activity against *Listeria monocytogenes*, *Bacillus cereus*, *Escherichia coli*, and *Salmonella enteritidis*. Four isolates that showed antimicrobial activity against at least two indicator strains were selected. Isolates I1 and I5 had antimicrobial activity against *L. monocytogenes* and *B. cereus*, while isolates I2 and I3 also inhibited *E. coli*. Since the inhibition zones of isolate I3 were the largest, it was selected for strain classification and its antimicrobial activity was characterized. The I3 strain was determined to be a gram-positive spore-forming bacterium with a positive catalase reaction. Based on morphological and biochemical examinations and the API 50 CHB results, strain I3 was identified as a *Bacillus* species.

Isolate I3 was classified to the species level based on sequence analysis of its 16S rDNA. CLUSTAL W and BLAST were used to compare the 800-bp sequence with 41 other 16S rRNA sequences from different *Bacillus* species present in GenBank. Clustering analysis obtained by the NJ method showed that the new isolate was taxonomically very close to *Bacillus amyloliquefaciens* (Fig. 1), having 99% sequence identity with *B. amyloliquefaciens*. This strain was assigned the number LBM 5006 and deposited in our culture collection.

Bacteriocin production. Strain LBM 5006 was oxically incubated in several media at different temperatures in a rotary shaker. Maximum antibacterial activity was evaluated during stationary growth phase by cultivation in BHI at 37ºC (Fig. 2A). While similar levels of activity were observed at 30ºC, growth was negligible and there was no antimicrobial activity at 55ºC. Antimicrobial activity was also produced during cultivation in lactose broth, MHB, TSB, and nutrient broth

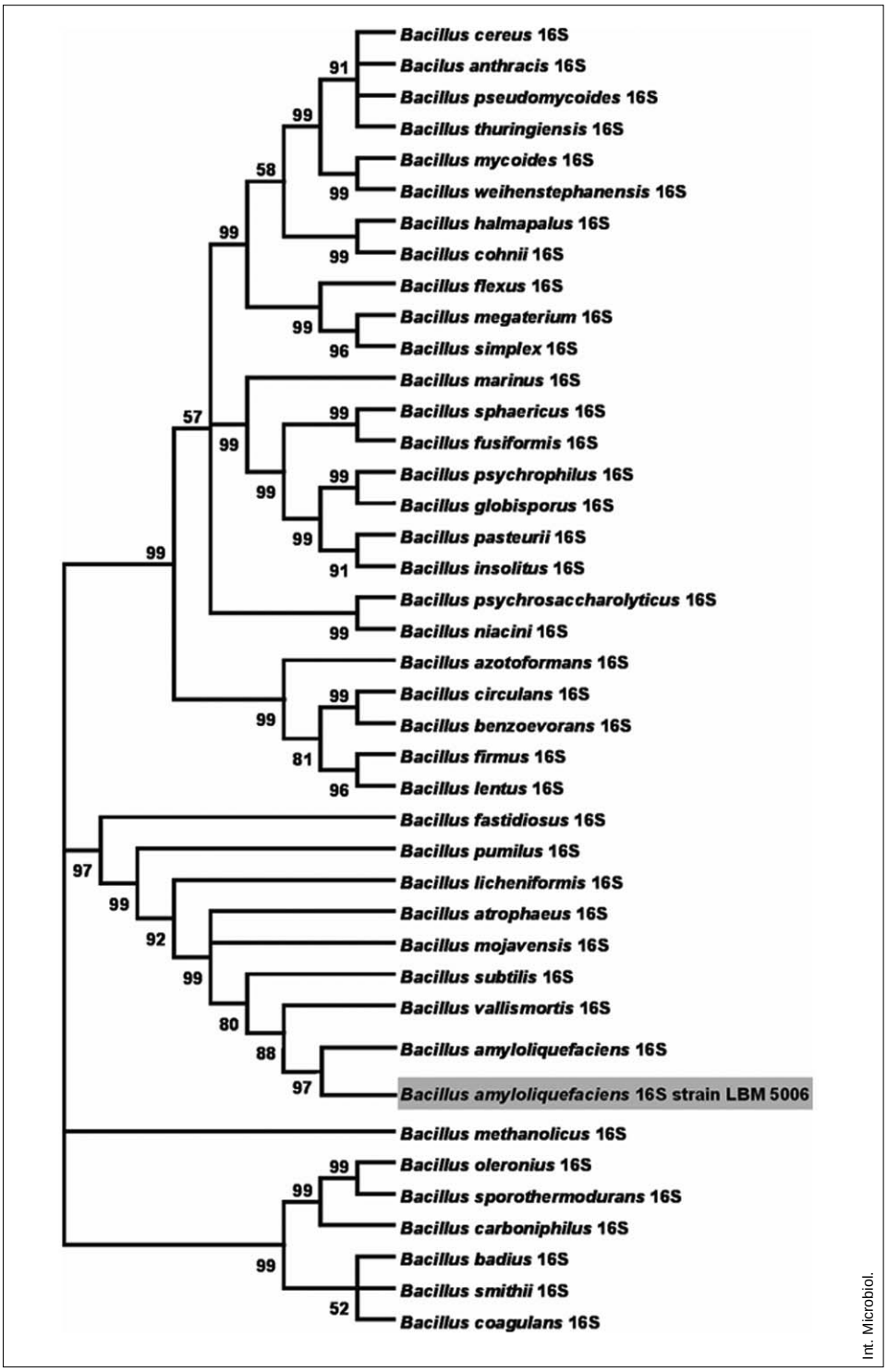

Fig. 1. Topological unrooted tree obtained by neighbor-joining analysis of the different *Bacillus* 16S rDNA sequences. *Bacillus amyloliquefaciens* strain LBM 5006 is indicated by a gray box in the phylogram. Interior branch analysis numbers (%) are shown in the nodes of the phylogram. Branches with interior branch analysis numbers < 50% were collapsed.

(Fig. 2B). No activity against either *B. cereus* or *L. monocytogenes* was detected when the strain was cultivated in peptone.

Inhibitory spectrum. Supernatant filtrate (400 AU/ml) was tested for the presence of antimicrobial activity against several microorganisms, including gram-positive and gram-negative bacteria and yeast. The results are shown in Table 1. Inhibitory activity was observed towards several gram-positive bacteria, including important pathogenic microorganisms such as *B. cereus* and *L. monocytogenes*. Among the gram-negative bacteria tested, some clinically relevant bacteria, such as *Pasteurella haemolytica*, *Proteus vulgaris*, and

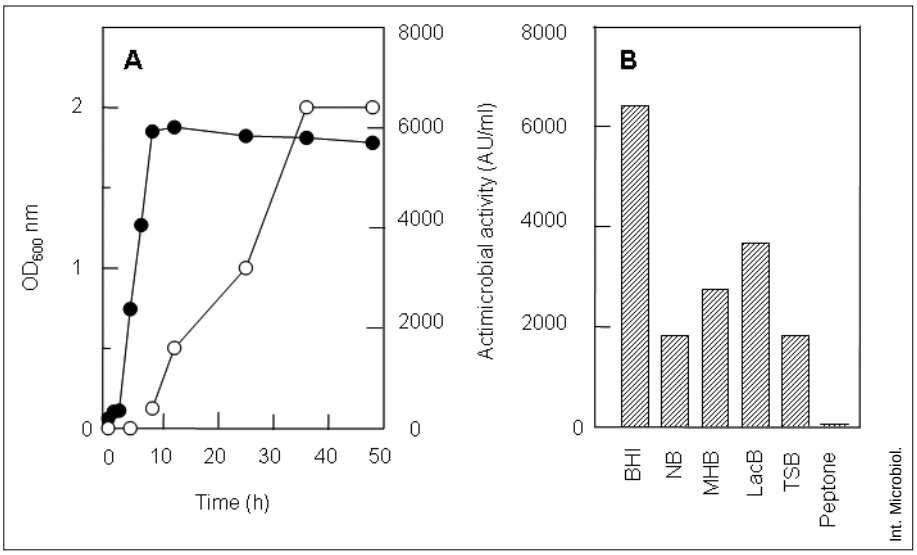

Fig. 2. (**A**) Growth (closed symbols) and antimicrobial activity (open circles) during cultivation of *Bacillus amyloliquefaciens* strain LBM 5006 at 37ºC in brain-heart infusion (BHI). (**B**) Antimicrobial activity at 48 h cultivation at 37ºC in BHI, nutrient broth (NB), Mueller-Hinton broth (MHB), lactose broth (LacB), tryptticase soy broth (TSB), and peptone.

Serratia marcescens, were inhibited. When the supernatant was concentrated (12,800 AU/ml), *Staphylococcus aureus*, *E. coli*, and *Enterococcus faecalis* were also inhibited (Table 2). None of the yeasts tested were inhibited, nor was the producer strain inhibited by its own antimicrobial substance.

Effect of enzymes, pH, and heat on antimicrobial activity. The effect on the antimicrobial substance (800 AU/ml) of the proteolytic enzymes papain, trypsin, proteinase K, and pronase E was evaluated by measuring residual activity against *L. monocytogenes* ATCC 7644 in the agar-disk diffusion assay. Antimicrobial activity was sensitive to all proteases tested, but only at an enzyme concentration of 10 mg/ml.

The heat sensitivity of the antimicrobial substance was determined by measuring its residual activity after incubation for 30 min at different temperatures. It was stable at temperatures up to 80°C, but residual activity decreased at 100°C (Table 3). All activity was lost after incubation at 121ºC

To evaluate the antimicrobial activity at different pHs, the bacteriocin was incubated at 25ºC at pH values between 3.0 and 10.0 for 2 h and activity was measured by the agar-disk diffusion assay against *L. monocytogenes* ATCC 7644. Activity was retained over a pH range of 3.0–8.0, but was lost at alkaline pH values (pH 9.0 and 10.0) (Fig. 3).

Activity on gels. The fraction containing antimicrobial activity was analyzed by SDS-PAGE, and a protein band

Table 2. Antimicrobial activity spectrum of the freeze-dryed concentrated antimicrobial substance

Indicator organism	Medium	Inhibition (mm)[a]
Staphylococcus aureus ATCC 25923	BHI	–
Staphylococcus aureus (food isolate)	BHI	10.5
Staphylococcus intermedius	BHI	–
Bacillus subtilis ATCC 9372	BHI	12.0
Enterococcus faecalis (clinical isolate)	BA	14.0
Salmonella enteritidis (food isolate)	BHI	–
Escherichia coli ATCC 25922	BHI	9.5
Escherichia coli (clinical isolate)	BHI	9.0
Candida sp. (food isolate)	MYG	–

[a]Aliquots of 20 ml were applied to discs, corresponding to 256 AU per disc. Values are the diameter of the inhibition zone in mm around the disc and correspond to the mean of three independent experiments. Media: BHI, brain hearth infusion; BA, sheep blood agar; MYG, malt yeast glucose. Growth at 37ºC.

Table 3. Influence of temperature on the activity of antimicrobial substance

Treatment	Residual activity (%)[a]
10ºC/30 min	100
40ºC/30 min	100
60ºC/30 min	100
80ºC/30 min	100
100ºC/30 min	67
100ºC/40 min	67
100ºC/50 min	42
100ºC/60 min	33
121ºC/15 min	0

[a]Residual activity compared with antimicrobial activity before the treatment. Data are means of three independent experiments.

with an apparent molecular mass of about 5 kDa was identified (Fig. 4). A single band coinciding with the location of antimicrobial activity was observed in the gel (Fig. 4).

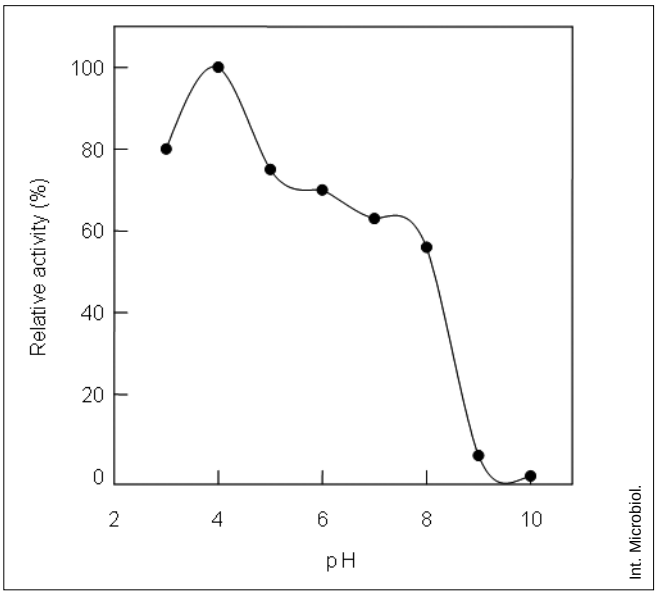

Fig. 3. Effect of pH on bacteriocin-like substance (BLS) activity. The antibacterial activity of BLS (800 AU ml^{-1}) was assayed at various pH values. Activity is expressed as the percentage of maximum activity determined against *L. monocytogenes*. Data are means of three independent experiments.

Discussion

In the present investigation, bacterial strains isolated from the soils of native woodlands of southern Brazil were subjected to antimicrobial assay in order to obtain antimicrobial substances. A newly isolated strain, identified as *Bacillus* sp., was found to be taxonomically very close to strains of *B. amyloliquefaciens*. This strain produces an antimicrobial substance that inhibits *B. cereus* and *L. monocytogenes*, among other relevant pathogenic bacteria. In addition, when concentrated by lyophilization, the antimicrobial substance was able to inhibit bacterial strains of *S. aureus*, *E. coli*, and *Enterococcus faecalis*, a very important property regarding food safety.

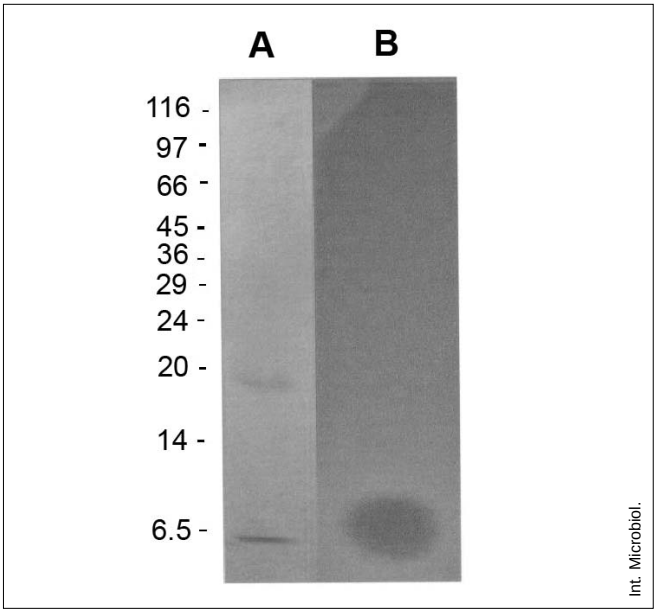

Fig. 4. Gel electrophoresis of the bacteriocin-like substance (BLS) of *Bacillus amyloliquefaciens* strain LBM 5006. Samples of the antimicrobial substance were submitted to SDS-PAGE and stained for proteins with Coomassie blue (lane A) or tested for antimicrobial activity against *L. monocytogenes* (lane B).

The antimicrobial substance was sensitive to all proteases tested, suggesting that a peptide moiety is associated with its activity. Although high protease concentrations were necessary to inactivate the antimicrobial activity, some antimicrobials produced by *Bacillus* spp. are cyclic peptides containing unusual amino acids, which are more resistant to proteases [31]. The substance showed thermal resistance, since a total loss of activity was observed only after incubation at 121ºC. This heat resistance, relative sensitivity to proteases, and low molecular weight are common to group I bacteriocins [4,14]. The resistance to heat and proteases resembled that of a BLS produced by *Bacillus licheniformis* P40 [5]. In addition, the antimicrobial compound was inactive at alkaline pH, showing maximum activity at acid pH values. Thus, the bacteriocin may have features in common with nisin, which is 228 times more soluble at pH 2 than at pH 8 [17].

The antimicrobial substance was produced when the isolate was cultured in BHI but not in peptone, suggesting that biosynthesis of the peptide requires a rich medium. By con-

trast, peptone appears to be a key nutrient for the production of antifungal compounds by *B. amyloliquefaciens* RC-2 [33]. Also, maximum activity was observed at stationary growth phase, which suggests that the antimicrobial peptide is produced as a secondary metabolite. However, it cannot be ruled out that the compound is the specific product of gene transcription and translation. *B. amyloliquefaciens* is closely related to *Bacillus subtilis*. The production of antimicrobial peptides by strains of *B. subtilis* is thought to be under complex genetic regulation. It has been observed that modifications of the culture conditions, including nitrogen source and pH, may induce the production of different peptide antibiotics [6]. Although many of the properties of *B. amyloliquefaciens* are similar to those of *B. subtilis*, its ability to produce an antagonist of microbial growth has not been frequently reported.

Bacillus spp. appear to be a relatively abundant source of antimicrobials, since many species of this genus synthesize antimicrobial peptides [10,28]. *B. amyloliquefaciens* is known to produce iturins, a family of cyclic lipopeptide antibiotics, [11]. These molecules have seven α-amino acids and one β-amino fatty acid, and iturin A is produced as a mixture of up to eight isomers [34]. *B. amyloliquefaciens* strains producing iturin A_2 have been used as biocontrol agents to suppress fungal plant pathogens [33,34]. The BLS isolated in this work may be a macrolactone rather than an iturinic peptide, since the latter are well-established antifungal agents with distinct properties [33,34]. Instead, identification and characterization of the bacteriocin-like substance produced by *B. amyloliquefaciens* strain LBM 5006 may represent a novel antimicrobial substance with potential applications in food safety, with important implications for the biological control of pathogenic and spoilage microorganisms, and thus for food protection.

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Caracterización de una substancia del tipo bacteriocina producida por *Bacillus amyloliquefaciens* aislado del bosque atlántico brasileño

Resumen. Se ha caracterizado una cepa de *Bacillus* productora de una substancia del tipo bacteriocina mediante perfiles bioquímicos y secuenciación del rDNA 16S. El análisis filogenético indicó que la cepa presentaba una gran similitud de secuencia con *Bacillus amyloliquefaciens*. La sustancia antimicrobiana era inhibitoria para algunas bacterias patógenas y causantes del deterioro de los alimentos como *Listeria monocytogenes*, *Bacillus cereus*, *Serratia marcescens* y *Pasteurella haemolytica*. La sustancia era estable en una amplia gama de temperaturas, pero perdía la actividad cuando la temperatura alcanzaba 121°C/15 min. La actividad máxima fue observada a valores de pH ácidos y neutros, pero no a pH alcalinos. La sustancia antimicrobiana era sensible a la acción proteolítica de la tripsina, la papaína, la proteinasa K y la pronasa E. Excepto las iturinaos, no se han descrito otros péptidos antimicrobianos de *B. amyloliquefaciens*. La identificación de una sustancia tipo bacteriocina activa contra *L. monocytogenes* cubre un aspecto importante en la protección de los alimentos. [**Int. Microbiol** 2006; 9(2):111-118]

Palabras clave: *Bacillus amyloliquefaciens* · actividad antimicrobiana · bacteriocina · péptido bioactivo

Caracterização de uma substância do tipo bacteriocina produzida por *Bacillus amyloliquefaciens* isolado da Mata Atlântica brasileira

Resumo. Uma linhagem de *Bacillus* produtora de uma substância tipo-bacteriocina foi caracterizada por perfil bioquímico e sequenciamento do rDNA 16S. A análise filogenética indicou que a linhagem tem una grande similitude de sequência com *Bacillus amyloliquefaciens*. A substância antimicrobiana inibiu bactérias patogênicas e deteriorantes como *Listeria monocytogenes*, *Bacillus cereus*, *Serratia marcescens* e *Pasteurella haemolytica*. A substância antimicrobiana foi estável num amplo intervalo de temperatura, mas perdeu atividade quando a temperatura foi de 121°C/15 min. A atividade máxima foi observada em valores de pH ácidos e neutros, mas não em pH alcalino. A substância antimicrobiana foi sensível frente à ação proteolítica da tripsina, da papaína, da proteinase K e da pronase E. Com excepção das iturinas, outros peptídeos antimicrobianos não estão descritos em *B. amyloliquefaciens*. A identificação de uma substância inibitória tipo bacteriocina com atividade contra *L. monocytogenes* atende a um aspecto importante da protecção dos alimentos. [**Int Microbiol** 2006; 9(2):111-118]

Palavras chave: *Bacillus amyloliquefaciens* · atividade antimicrobiana · bacteriocina · peptídeo bioativo