

Full Length Research Paper

Purification and characterization of a bacteriocin produced by *Lactococcus lactis* subsp. *lactis* PD6.9

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In this study, a bacteriocin produced by *Lactococcus lactis* subsp. *lactis* PD6.9 was purified, characterized and identified. The bacteriocin was purified to homogeneity from culture supernatant by cation exchange and reversed-phase liquid chromatography, and its molecular weight was determined by mass spectrometry. The presence of the nisin gene was confirmed by polymerase chain reaction (PCR) and DNA sequencing. The gene showed that it was a natural nisin variant, nisin Z, as indicated by substitution of an asparagine residue for histidine at position 27. The purified bacteriocin was biochemically pure, and the molecular weight was approximately 3329.571 Da. The peak of nisin Z production by *L. lactis* PD6.9 occurred after 5 h of culture during stationary phase. This bacteriocin demonstrated inhibitory activity towards significant foodborne pathogens and *Staphylococcus aureus* strains isolated from dairy cattle diagnosed with mastitis, it may be useful for future applications.

Key words: Antimicrobials, *Lactococcus lactis*, bacteriocins, identification, inhibitory activity.

INTRODUCTION

Lantibiotics are antimicrobial peptides that have attracted widespread scientific attention as promising safe and natural food additives and as potential therapeutic agents to combat medically significant bacteria and their multi-drug resistance (Field et al., 2008). These ribosomally synthesized peptides are distinguished by the presence of post-translationally modified amino acids such as dehydroalanine (Dha), dehydrobutyrine (Dhb) and eponymous lanthionine (Lan) and β -methyllanthionine

(MeLan) formed by thioether linkages between dehydrated amino acid residues and neighboring cysteines (Rink et al., 2007). Nisin A is a lantibiotic produced by *Lactococcus lactis*, has already been employed as a food preservative for long time and is licensed by 48 countries around the world (Delves-Broughton, 1990) and is one of the few bacteriocins to have been applied commercially (Bierbaum and Sahl, 2009). This peptide is suggested to be effective

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against many Gram-positive bacteria, including food-borne pathogens such as staphylococci, bacilli, clostridia and mycobacteria (Field et al., 2010). Some natural variants of nisin have been described as nisin Z (Mulders et al., 1991; De Vos et al., 1993), nisin F (De Kwaadsteniet et al., 2008), nisin Q (Zendo et al., 2003) produced by strains of *L. lactis*, nisin U (Wirawan et al., 2006) and nisin U2 (Piper et al., 2010) produced by *Streptococcus uberis* and *Streptococcus agalactiae*, respectively.

Potentially, the most significant application of lantibiotics may be in the treatment of antibiotic resistant pathogens. Nisin A has been shown to be active against a number of multidrug-resistant Gram-positive pathogens (Goldstein et al., 1998; Severina et al., 1998), including a wide range of mastitis-causing pathogens (Cotter et al., 2005a).

Mastitis is the inflammation of the mammary gland in response to bacterial invasion. Clinical mastitis results in alterations in milk composition and appearance and decreased milk production (Wu et al., 2007). Because of increased antibiotic resistance of mastitis pathogens (Wang et al., 2006), reduced responses to antibiotic therapy have become very common in veterinary practice (Cao et al., 2007). In addition, loss of milk due to discarding milk contaminated with antibiotics has been the reason why treatment of mastitis is not suggested during lactation (Wu et al., 2007).

We have previously described a natural isolate of *L. lactis* subsp. *lactis* strain PD6.9, which produces a bacteriocin possessing some important features (De Carvalho et al., 2006). Here, we reported the purification and identification of this bacteriocin designated as nisin Z. Purified nisin Z was tested against foodborne pathogens and others pathogenic bacteria, including *Staphylococcus aureus* strains responsible for bovine mastitis.

MATERIALS AND METHODS

Bacterial strains and culture conditions

L. lactis subsp. *lactis* PD6.9 was cultured in M17 broth (Oxoid) supplemented with 0.4% (w v⁻¹) glucose at 30°C. All target strains were grown in brain heart infusion (BHI) broth (Oxoid) at 37°C for 12 h before tests.

Bacteriocin production

L. lactis subsp. *lactis* PD6.9 (1% inoculum, v/v, standardized to OD_{600nm} = 0.6) was grown in M17 broth with glucose and incubated at 30°C, without agitation, for 24 h. Culture samples were collected each hour and bacterial growth (absorbance values, OD_{600nm}) and changes in culture pH were determined. Preparations of the cell-free culture supernatant collected (boiled and neutralized) were serially diluted and tested against indicator *L. lactis* IL1403 for determination of bacteriocin activity (expressed as BU mL⁻¹).

Bacteriocin activity testing

Quantitative determination of the antimicrobial activity of the bacteriocin was performed using a microtiter assay method (Holo et al., 1991). A twofold serial dilution (in medium) with 100 µL bacteriocin samples were prepared in a microtiter plate well containing 50 µL of culture medium to which 150 µL of a diluted culture of the target bacteria (approximately 10⁶ viable cells mL⁻¹) were added. The plate was incubated for 12 h, after which growth inhibition was measured turbidometrically at 620 nm with a microtiter plate reader (Labsystems iEMS reader MF; Labsystems, Helsinki, Finland). One bacteriocin unit (BU) was defined as the amount of bacteriocin that inhibited 50% growth of the target microorganism under these conditions.

To study the antimicrobial spectrum of the bacteriocin produced by *L. lactis* subsp. *lactis* PD6.9, a wide range of target organisms (Table 1) were used in the microtiter assay system.

DNA isolation, PCR and sequencing

Genomic DNA was isolated with Wizard Genomic DNA purification Kit (Promega, USA), applying the protocol for Gram-positive bacteria and using mutanolysin and lysozyme (Sigma-Aldrich, USA). Nucleotide sequencing was performed with the PCR products obtained from amplifications of genomic DNA of *L. lactis* subsp. *lactis* PD 6.9 using the following primers specific to nisin structural gene: nqf (5'-GTTCGAAGGAACACAAAATAAATT-3') and naqzr (5'-ACAGACCAGCATTATATTCTGC-3'); and to the *nisa* promoter region: pnisAf (5'-TTGAGTCTTAGACATACTTGAATGACC-3') and pnisAr (5'-CAATGACAAGTTGCTGTTTTCA-3'). Each PCR procedure was performed separately and differently. The PCR thermal cycle program included a pre-denaturation at 94°C for 2 min followed by 35 cycles, with a denaturation step at 94°C for 1 min, an annealing step for 30 s at 40°C (for primers sets nqf/naqzr) and 48°C (for primers sets pnisAf/pnisAr) followed by an extension step for 1 min at 72°C. A final extension was performed at 72°C for 7 min. PCR products were purified with the Gel Extraction Kit (Nucleospin® Gel and PCR clean up, Machery-Nagel, Germany) and sequenced using the BigDye Terminator v3.1 cycle Sequencing Kit and ABI Prism 377 DNA sequencing system (Applied Biosystems, United States). Sequences were aligned using BLAST software provided online by National Center for Biotechnology Information (USA).

Purification of bacteriocin

The supernatant from a 200 mL overnight culture (at 30°C in M17 supplemented with 0.4% glucose) of *L. lactis* subsp. *lactis* PD6.9 was collected. Ammonium sulfate (40 g per 100 mL) was added to the supernatant and agitated for 30 min at 4°C. The bacteriocin was then precipitated from the supernatant by centrifugation (10,000 ×g for 30 min at 4°C) and dissolved in 20 mL sterile distilled water, and the pH was adjusted to 3.5 with 1 M HCl. It was then passed through a 5 mL SP Sepharose Fast Flow column (GE Healthcare Biosciences, Uppsala) equilibrated with 10 mM acetic acid. The column was eluted with a stepwise gradient consisting of 10 mL each of 0.1, 0.3 and 1.0 M NaCl at 1 mL/min flow rate and stored on ice. The fractions displaying the highest bacteriocin activity were used for further purification. The purification was followed by reversed-phase chromatography using the Äkta Purifier fast protein liquid chromatography system. The most active fractions following cation exchange chromatography were applied to a reversed-phase column (Resource 15 RPC 3 mL; Pharmacia Biotechnology) equilibrated with 0.1% trifluoroacetic acid (TFA) in water. Elution was performed with a linear water-isopropanol gradient from 0 to 100% isopropanol containing 0.1% TFA (w v⁻¹). The most active

Table 1. Inhibition spectrum of nisin Z produced by *L. lactis* subsp. *lactis* PD6.9.

Indicator	Strain	Source or reference	BU (mL ⁻¹)
<i>Bacillus subtilis</i>	DSMZ347	LMGT	640
<i>Enterococcus faecalis</i>	v583	LMGT	160
<i>Lactococcus lactis</i>	IL1403	LMGT	10200
<i>Listeria innocua</i>	BL86/26B	LMGT	1280
<i>Listeria innocua</i>		CERELA	1280
<i>Listeria ivanovii</i>		CERELA	640
<i>Listeria monocytogenes</i>		CERELA	1280
<i>Listeria monocytogenes</i>	Scott A	CERELA	2560
<i>Micrococcus luteus</i>	4698	ATCC	640
<i>Micrococcus luteus</i>	10240	ATCC	5120
<i>Streptococcus pneumoniae</i>	TIGR4	LMGT	1280
<i>Staphylococcus aureus</i>	ME8245-3	LMGT	2560
<i>Staphylococcus aureus</i>	8452	LMGT	1280
<i>Staphylococcus aureus</i>	4759	Pinto, 2008	2560
<i>Staphylococcus aureus</i>	4052	Pinto, 2008	2560
<i>Staphylococcus aureus</i>	4784	Pinto, 2008	1280
<i>Staphylococcus aureus</i>	3870	Pinto, 2008	640
<i>Staphylococcus aureus</i>	4119	Pinto, 2008	5120
<i>Staphylococcus aureus</i>	3212	Pinto, 2008	640
<i>Staphylococcus aureus</i>	3702	Pinto, 2008	1280
<i>Staphylococcus aureus</i>	4716	Pinto, 2008	2560
<i>Staphylococcus aureus</i>	3975	Pinto, 2008	10240
<i>Staphylococcus aureus</i>	3129	Pinto, 2008	1280
<i>Escherichia coli</i>	14763	ATCC	NI
<i>Pseudomonas aeruginosa</i>		LMGT	NI

ATCC, American Type Culture Collection; CERELA, Reference Center for Lactobacilli; LMGT, Laboratory of Microbial Gene Technology (UMB); NI, no inhibition.

fractions were stored at 4°C for further analysis. The susceptible strain *L. lactis* IL1403 was used as the indicator strain in biological assay for bacteriocin quantification.

Mass spectrometry

The molecular weight of the purified bacteriocin was determined by mass spectrometry. Bacteriocin samples (active fractions) were mixed 1:1 with a solution of 15 mg α -cyano-4-hydroxycinnamic acid in 50% acetonitrile, 49.9% ethanol and 0.1% TFA and deposited on a ground steel matrix-assisted laser desorption ionization target. Mass spectra were recorded in the positive reflector mode with an Ultra Flex TOF/TOF (Bruker Daltonic GmbH, Bremen, Germany), using a pulsed ion extraction setting of 40 ns and an acceleration voltage of 25 kV.

Effect of heat and proteolytic enzymes on the stability of nisin Z

Samples of fractions purified were dispensed in micro tubes and treated separately in a water bath at 100°C for 15 min and 100°C for 30 min. After, samples were cooled and the residual activity was determined. The protein nature of the antimicrobial compounds was verified by treatment with the enzymes trypsin (Sigma-Aldrich) and

proteinase K (Finnzymes) at 10 mg mL⁻¹ concentration, in 0.01 M phosphate buffer at pH 7.0 and were added to 0.1 mL of purified fraction sample to give a 1 mg mL⁻¹ enzyme final concentration. The samples were filtered through 0.22 μ m pore-size filters (millipore) and incubated for 5 h at 37°C. The reactions were stopped by boiling the mixture for 3 min. The residual activity was tested.

RESULTS

Bacteriocin production

The kinetics of microbial growth and bacteriocin production of *L. lactis* subsp. *lactis* PD6.9 are presented in Figure 1. Exponential growth of *L. lactis* subsp. *lactis* took place during a period of approximately 4 h, and bacteriocin production began during the exponential phase (Figure 1). Its activity reached a maximum level (22.000 BU mL⁻¹) at 5 h of culture during stationary phase when the pH of the medium was below 4.5 (Figure 1). After 7 h of incubation, bacteriocin titres decrease by approximately 37%, and remained constant to 24 h (Figure 1).

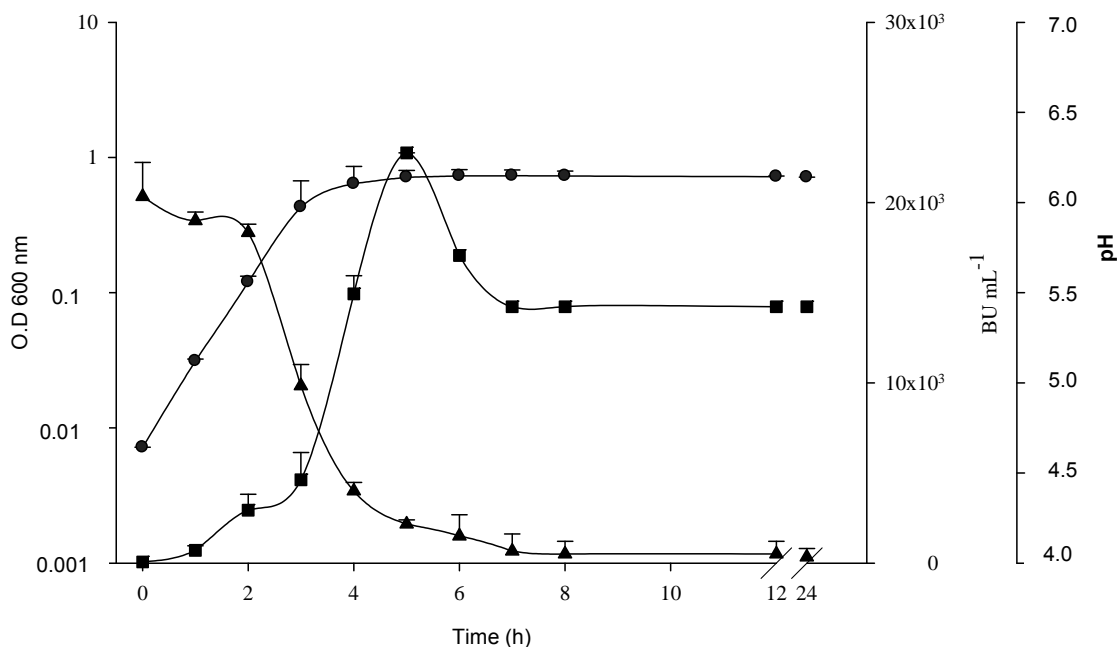


Figure 1. Production of bacteriocin during growth of *L. lactis* subsp. *lactis* PD6.9 in M17 broth at 30°C. Antimicrobial activity is presented as BU mL⁻¹ (■), change in optical density (●) and pH (▲) are indicated.

Table 2. purification of the bacteriocin produced by *L. lactis* subsp. *lactis* PD6.9.

Purification step	Volume (mL)	Recovery (%)	Protein concentration (mg mL ⁻¹) ^a	Antimicrobial activity (BU mL ⁻¹)	Specific activity (BU mg ⁻¹)	Increase in specific activity (fold)
Cell free culture supernatant	200	100	24.87	1.28 × 10 ³	51.46	1.0
Ammonium sulphate precipitate	20	20	4.44	2.56 × 10 ³	576.58	11
Ion-exchange chromatography	10	20	0.32	5.12 × 10 ³	1.6 × 10 ⁴	311
Reversed- phase chromatography	2	8	0.08	1.02 × 10 ⁴	1.28 × 10 ⁵	2490

^aThe protein concentration was determined by measuring the optical density at 280 nm.

Purification of bacteriocin and mass spectrometry

The cell free culture supernatant from a 0.2-L culture of *L. lactis* subsp. *lactis* PD6.9 grown overnight in M17 broth was used for bacteriocin purification. This supernatant was precipitate with ammonium sulfate and subsequently purified by cation exchange and reversed-phase chromatography (Table 2). The cell free culture supernatant contained 1280 bacteriocin units mL⁻¹ as determined with the indicator strain *L. lactis* IL1403 (Table 2). The specific activity of the bacteriocin was concentrated 10-fold from the cell free culture supernatant by ammonium sulphate precipitation. This concentration step resulted in a recovery of 20% of

activity. Upon a subsequent pre-purification step by ion exchange chromatography, the specific activity was about 300-fold higher than that of the cell free culture supernatant and the recovery was about 20%. The specific activity of the final purified bacteriocin eluted from the reversed-phase chromatography was about 2500-fold higher than that of the cell free culture supernatant, with a recovery of about 8%. The results of the purification procedure are summarized in Table 2.

The molecular mass of purified fractions 14 and 15 (Figure 2A) was determined by mass spectrometry to be 3329.571 Da (M+1, 3330. 6) (Figure 2B), which is close to the molecular mass of lantibiotic nisin Z, whose monoisotopic molecular mass is 3330.93 Da (Piper et al.,

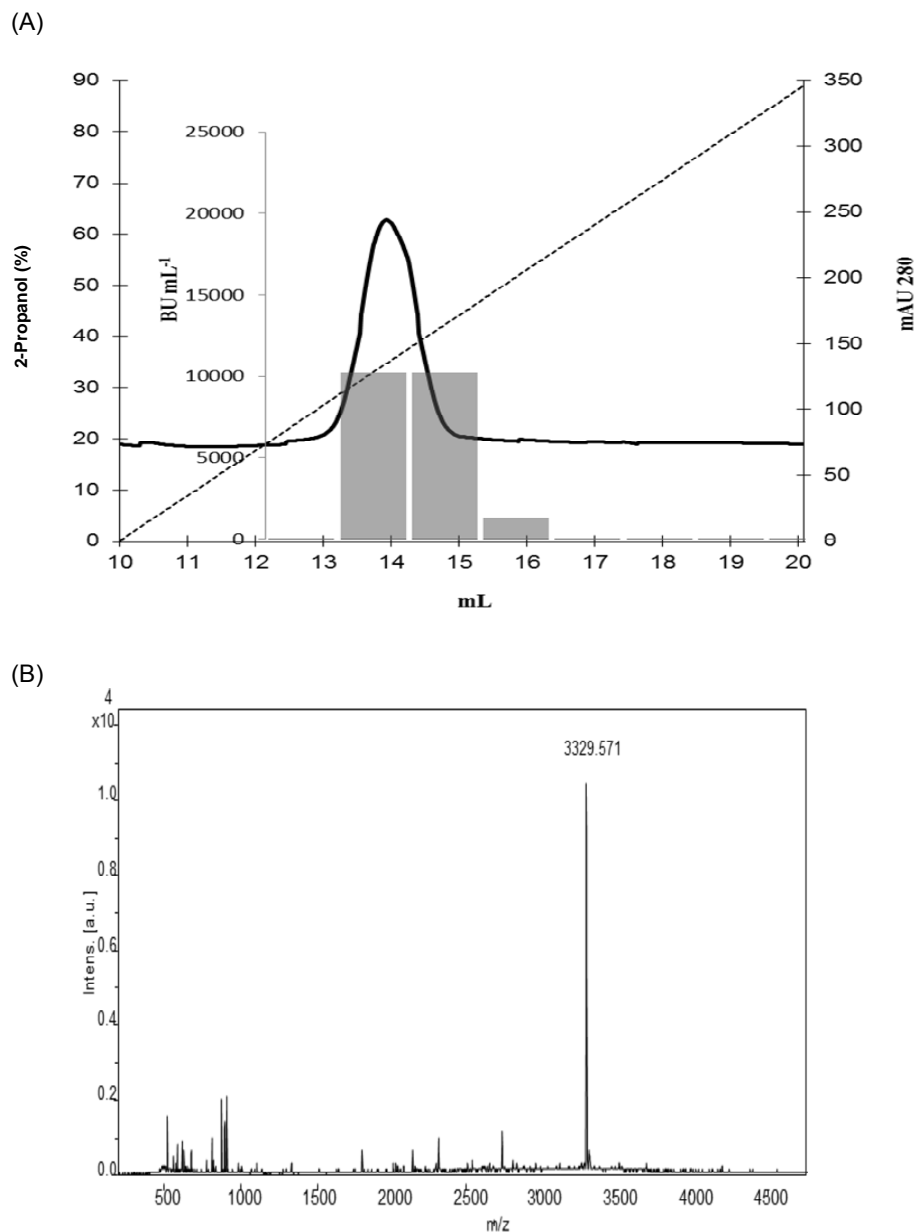


Figure 2. (A) Results of second reversed-phase chromatography of bacteriocin produced by *L. lactis* subsp. *lactis* PD6.9. Elution was performed by using a linear gradient of 0 to 100% 2-propanol containing 0.1% TFA. Solid line, absorbance at 280 nm; dashed line, isopropanol gradient; bars, bacteriocin units (BU) in active eluted fractions. (B) Mass spectrometry analysis of purified bacteriocin.

2010).

Effect of heat and proteolytic enzymes on the stability of nisin Z

The inhibitory action of purified fraction of nisin Z was inactivated when it was treated with trypsin and proteinase K. Furthermore, the activity of nisin was maintained after heat treatment at 100°C for 15 and 30

min (date not shown). These results demonstrated that nisin Z produced by *L. lactis* PD6.9 was heat stable.

PCR and sequencing nisin genes

PCR products obtained from amplifications of *L. lactis* subsp. *lactis* PD6.9 genomic DNA with primers specific to the nisin structural gene were subjected to nucleotide sequencing (Figure 3A). The results indicated that the

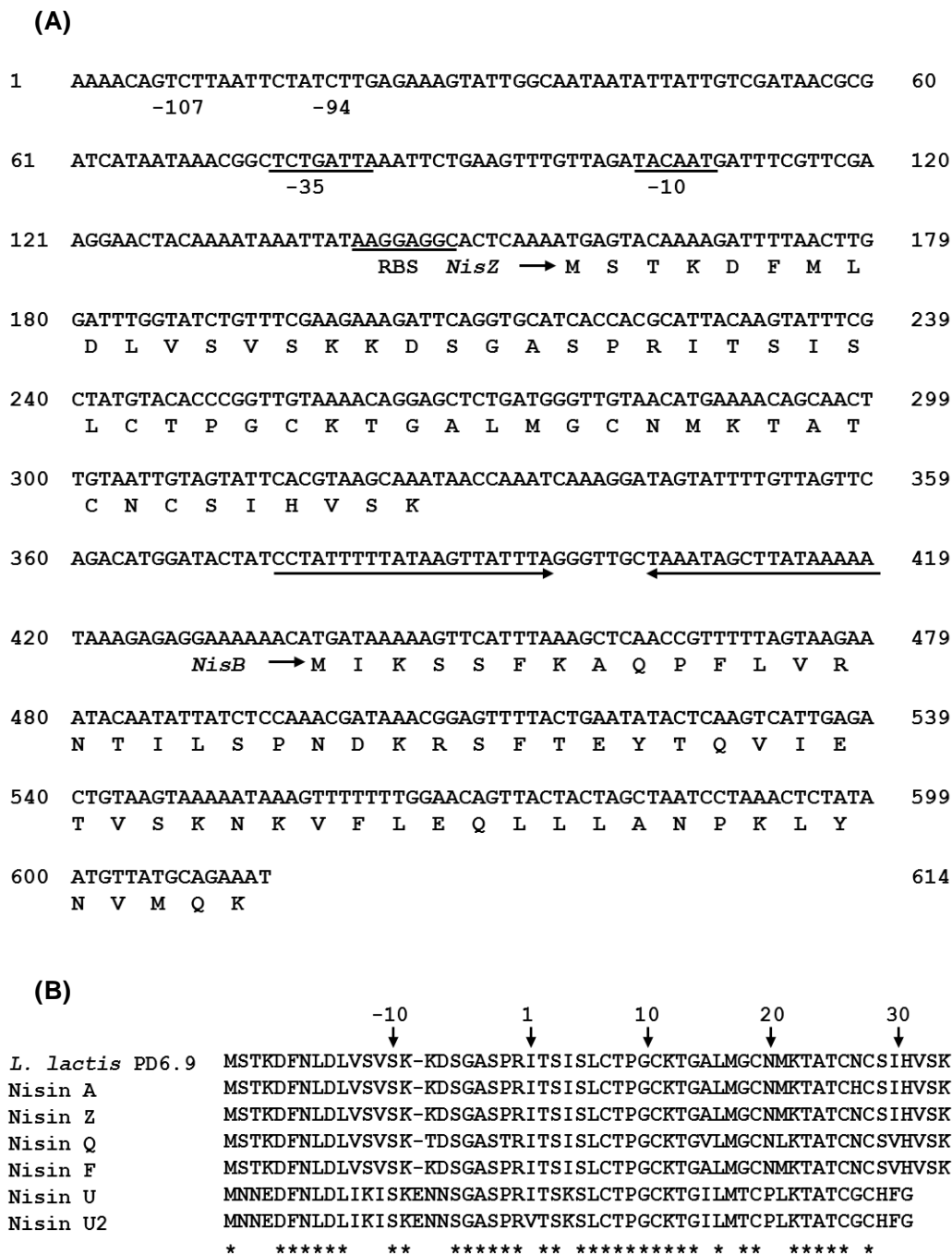


Figure 3. (A) Nucleotide sequence of the region encoding nisin Z in *L. lactis* subsp. *lactis* PD6.9 and deduced amino acid sequence. The putative -35 and -10 promoter regions and a putative ribosome binding site (RBS) are underlined. (B) Alignment of nisin Z in *L. lactis* subsp. *lactis* PD6.9 and homologous sequences of nisin A, Z, Q, F, U and U2 are obtained from GenBank data. Identical amino acid residues are indicated with an asterisk.

sequence of the PD6.9 nisin gene was identical to that of nisin Z (GenBank accession number AB375441.1). Homology with nisin A (GenBank accession number HM219853.1) was also recorded, except for a C-to-A transversion at position 148 (Figure 3A). This resulted in an asparagine (AAT) residue at position 27 in the nisin

peptide instead of histidine (CAT). The deduced amino acid sequence showed complete similarity (100% identity) to nisin Z (GenBank accession number P29559.1). This indicates that the bacteriocin produced by *L. lactis* subsp. *lactis* PD6.9 is a natural variant, nisin Z, as shown in Figure 3B. The nucleotide sequence of

the PCR fragment (amplified with primers *pnisAf* and *pnisAr*) containing the *nisA* promoter region had 99% identity to the sequence recorded for the promoter region encoding nisin Z (Gen Bank accession number Y13384.1). It has a consensus promoter characterized by sequences at -35 and -10 that are spaced by an average of 17 nucleotides. The promoter region upstream of the structural *nis* gene contains a TCT direct repeat with an 8-bp spacer region at positions -39 to -26 upstream of the transcription start site. It also contains a second TCT-N₈-TCT motif present upstream of the structural *nisZ* gene at positions -107 to -94 (Figure 3A).

Assay of bacteriocin activity

The inhibitory spectrum of nisin produced by *L. lactis* subsp. *lactis* PD6.9 is presented in Table 1. Members of several species of Gram-positive bacteria (*Lactococcus*, *Bacillus*, *Enterococcus*, *Listeria*, *Micrococcus*, *Streptococcus*, *Staphylococcus*) were susceptible to nisin Z, but species Gram-negative (*Escherichia coli* ATCC 14763 and *Pseudomonas aeruginosa*) were not affected (Table 1). The bacteriocin unit concentration varied considerably among the different target strains, with *L. lactis* IL1403, *Micrococcus luteus* ATCC 10240, *S. aureus* 4119 and *S.s aureus* 3975 seeming to be the organisms most sensitive to nisin Z, whereas *Enterococcus faecalis* v583 appeared to be less sensitive (Table 1). All the *S. aureus* strains (4749, 4052, 4784, 3870, 4119, 3212, 3702, 4716, 3975 and 3129) isolated from dairy cattle diagnosed with mastitis were sensitive to nisin Z (Table 1).

DISCUSSION

In the last few years, a variety of bacteria such as lactic acid bacteria have attracted attention for their production of compounds with potential uses in many fields. In this investigation, we have carried out the identification and purification of the antimicrobial compound produced by a naturally fermented salami isolate of lactic acid bacteria, *L. lactis* subsp. *lactis* PD6.9 (Maciel, 1998). In fact, the nisin production phenotype has been widely found among *L. lactis* strains from cheese, raw milk, grain, fish, fermented vegetable and river (Choi et al., 2000; Zendo et al., 2003; Mitra et al., 2005; De Kwaadsteniet et al., 2008). However, *L. lactis* PD6.9 was isolated from fermented salami, indicating that this product could be a good source of strains displaying enhanced outputs.

The bacteriocin produced by *L. lactis* PD6.9 displayed secondary metabolite kinetics, because the bacteriocin was produced during exponential growth phase and reached a maximum level at stationary phase. Extending stationary phase resulted in a decrease in bacteriocin production. This decrease could be due to the activity of

extracellular endogenous proteases induced within this growth phase.

Development of three steps purification procedure allowed the separation of bacteriocin and the reliability of each step were demonstrated by significant increase in the specific activity of bacteriocin. After mass spectrometry analysis of the purified bacteriocin, the apparent molecular mass was confirmed to be 3329.571 Da (M+1, 3330.6), corresponding the native form of nisin Z.

Heat stability and protease sensitivity is a key criterion for the characterization of an inhibitory substance such as bacteriocin. The heat stability and no activity of nisin Z was produced by *L. lactis* PD.69 when treated with trypsin and proteinase K. These were characteristics similar to those of other nisins (Matsusaki et al., 1998; Mitra et al., 2005; De Kwaadsteniet et al., 2008).

The deduced amino acid sequence of the PD6.9 nisin showed that it contained an asparagine at position 27 instead of a histidine as in nisin A. A BLAST search of GenBank sequences indicated that the PD6.9 nisin is a variant nisin Z. De Vos et al. (1993) reported that the His27Asn substitution resulted in a higher diffusion rate for nisin Z, that may be of practical significance, since many products to which nisin A is applied are diffusion limited.

Natural variants of a number of lantibiotics have been described (Cotter et al., 2005a). The existence of natural variants suggests that the identity of amino acids present at certain locations is flexible and it thus may be possible to generate mutants. These natural variants may highlight regions of lantibiotics that demonstrate a greater propensity and permissiveness to change, while comparisons of more distantly related peptides permit the identification of conserved regions that are likely to be essential for activity (Cotter et al., 2005a). In addition, nisin variants may have potential as novel antibiotics because, generally, it is not recommended to use the same compound both for food preservation and for antibiotic treatment (Lubelski et al., 2008).

The promoter sequences of *nisZ* of the PD6.9 strain was identical to be nisin A (GenBank accession number HM219853.1), contain a partially conserved region which could be involved in the transcriptional control function, the TCT-N₈-TCT motif present upstream of structural gene *nisZ* (Figure 2A). Chandrapati and O'Sullivan (2002) reported that the TCT-N₈-TCT motif present upstream of the structural *nisZ* gene is supposed to be involved in a co-operative binding of the NisR response regulator of the NisRK two-component regulatory system involved in the transcriptional control function (Figure 3A). They also reported a second TCT-N₈-TCT motif present upstream of *nisA* at positions -107 to -94, which was also shown in our results. This TCT repeat, together with the first one, is involved in the optimal binding of NisR (Trmčić et al., 2011).

The spectrum of target bacteria against which the nisin Z produced by PD6.9 strain is effective is also interesting.

Among the bacteria tested, the pathogens *L. monocytogenes*, *S. pneumoniae* and *S. aureus* were sensitive. Studies with nisin variants against a series of clinically significant pathogens have established differences in specific activities against selected targets (Piper et al., 2010). Piper et al. (2010) stated that nisin Z had an inhibitory effect against methicillin-resistant *S. aureus* (MRSA) and (heterogeneous) vancomycin intermediate *S. aureus* [(h)VISA]. In addition, most of the *S. aureus* strains that were more sensitive to nisin Z were isolated from bovine mastitis. This is significantly due to the severity of the clinical symptoms and mortality associated with this infection (Espeche et al., 2009). Mastitis is the main disease affecting dairy cattle herds in Brazil and worldwide (Pinto, 2008).

More recently, this area has received renewed attention, undoubtedly as a consequence of the ability of nisin to inhibit a wide range of mastitis-causing pathogens (Cao et al., 2007). The development of non-antibiotic formulations, such as bacteriocins, has the potential to reduce the dependence on antibiotics for prophylactic therapies in the future.

In short, *L. lactis* subsp. *lactis* PD6.9 displays a good potential for bacteriocin production. Because, *L. lactis* is generally recognized as safe for human health and *L. lactis* PD6.9 was obtained from a food source, its reintroduction in fermented meat should not impose technological problems for consumption of the final product. The bacteriocin produced by *L. lactis* PD6.9 is potentially active against foodborne pathogens, such as *S. aureus* and *L. monocytogenes*. The use of bacteriocin and bacteriocin-producing strains as starters or protective cultures in food preservation will contribute to the safe and wholesome food production. In addition, its bacteriocin showed an inhibitory effect against mastitis-causing pathogens, being attractive to replace antibiotics for prophylactic therapies. *L. lactis* PD6.9 is a new strain, originating from fermented products, presenting good production and desirable characteristics.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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