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Full Length Research Paper

A probiotic bacterium, *Pediococcus pentosaceus* OZF, isolated from human breast milk produces pediocin AcH/PA-1

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***Pediococcus pentosaceus* OZF, originally isolated from healthy human breast milk, produces antimicrobial activities against many gram-positive bacterial species, including the food borne pathogen, *Listeria monocytogenes*. A bacteriocin was purified to homogeneity from the supernatant of exponentially growing cells using ion-exchange and reversed-phase chromatography. The purification resulted in a high recovery and an approximately 224.000 fold increase in specific activity. The molecular mass of the bacteriocin was 4,623 Da as determined by electrospray ionization mass spectrometry. Based on the DNA sequences of the pediocin AcH/PA-1 operon, specific primers were synthesized and used to determine whether the purified pediocin was identical to another previously reported bacteriocin. Results show that the bacteriocin produced by *P. pentosaceus* OZF was identical to pediocin AcH/PA-1. We thus refer to the identified bacteriocin as pediocin AcH/PA-1 in order to avoid confusion. The value of this report is that the producer strain is shown to be distinct from the pediocin PA-1 producer (*P. pentosaceus* PA-1) based on a sugar fermentation profile.**

Key words: *Pediococcus pentosaceus*, pediocin, purification, characterization.

INTRODUCTION

Health-promoting bacteria, commonly referred to as probiotics, have been shown to improve the intestinal microbial balance and the properties of the indigenous microflora (Mattila-Sandholm et al., 1999). Probiotics have been defined as living microorganisms which upon ingestion in adequate numbers exert positive health effects beyond inherent basic nutrition (FAO/WHO working group, 2001). One of the desirable properties of a probiotic strain is the ability to produce antimicrobial substances such as bacteriocins (Mattila-Sandholm et al., 1999).

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Many gram-positive bacteria secrete ribosomally synthesized antimicrobial polypeptides which are generally referred to as bacteriocins (Klaenhammer, 1993; Nes et al., 1996; Nissen-Meyer and Nes, 1997). Most of the bacteriocins produced by gram-positive bacteria are from lactic acid bacteria (LAB) (Ennahar et al., 2000; Garneau et al., 2002). Many bacteriocins produced by LAB have been discovered over many years and some are particularly interesting and offer promising prospects due to their potential applications in the food industry as natural food preservatives and antimicrobial agents (Galvez et al., 2007; Helander et al., 1997).

One important and well studied subclass of bacteriocins is the pediocin family of bacteriocins (Nes et al., 1996) a name derived from the first and most extensively studied member of this family, pediocin PA-1. The pediocin family members are small, heat-stable, membrane

active peptides which contain no lanthionine and possess a YGNGVxC consensus motif. Members of this class have a number of features in common, including a very strong antimicrobial activity against *Listeria* species (Nes et al., 1996). The food-borne pathogen *Listeria monocytogenes* is a major concern in the contamination of food because of its ability to grow at low temperature and a wide range of pH values in a variety of dairy semi-preserved and fresh meat products (Farber and Peterkin, 1991; Klaenhammer, 1993; Nissen-Meyer and Nes, 1997). Several pediocin PA-1-producing LAB strains have been isolated and identified (Bennick et al., 1997; Bhunia et al., 1988; Daba et al., 1994; Ennahar et al., 1996; Gonzalez and Kunka, 1987; Henderson et al., 1992; Hoover et al., 1988; Lozano et al., 1992; Luchansky et al., 1992; Rodriguez et al., 1997; Schved et al., 1993). However, in many cases, the isolated bacteriocin named as distinct names before establishing conclusively that the bacteriocin was distinct from any previously reported. Thus there are a confusing number of names of bacteriocins obtained under various growth conditions and reported from geographically scattered areas of the world, but they all refer to the same bacteriocin, and this suggests that bacteriocin genes are widely conserved while being widespread in a number of species (Martinez et al., 2000). As the biochemical and genetic characterization of bacteriocins is more firmly established, the number of reports on the production of what is likely the same bacteriocin from various belonging to different genera and species, obtained from different food substrates and different geographical origin will most certainly increase (Andersson et al., 1998; Herbin et al., 1997; Papathanosopoulos et al., 1997).

So far, only a limited number of bacteriocin-producing LAB really originate from humans, although, most of them have been isolated from ingested foods or related products (Flynn et al., 2002). These bacteriocins can be used as selective antimicrobials not only in food preservation but also in preventive or therapeutic medical practice, to inhibit pathogens without undesirable alterations in the normal flora (Gillor et al., 2008). Localized bacteriocin production is an important attribute of probiotic strains, enabling them to become established and to dominate their environment. In addition, it has been proven that bacteriocin-producing LAB can effectively suppress the growth of *Listeria* in mice and to eradicate its presence (Corr et al., 2007). There is increasing interest in isolating new bacteriocin-producing strains of human origin that could be developed for probiotic effects and inhibition of pathogenic bacteria in the gut. For that reason, identification and characteri-zation of bacteriocin-producing LAB of human origin are required in order to develop probiotic bacteria with diverse antimicrobial potential. In such cases, bacteriocin can be used as a food preservatives while the strains are used as probiotic.

In this work, the biochemical and genetic evidence demonstrating that a probiotic bacterium *Pediococcus pentosaceus* OZF which was originally isolated from healthy human breast milk produces pediocin AcH/PA-1 was presented.

MATERIALS AND METHODS

Bacterial strains and their growth condition

The bacteriocin producer strain isolated from human breast milk obtained from healthy volunteer mother was identified as *P. pentosaceus* by API 50 CHL (Bio Me´rieux, l'Etole, France). Bacterial identification was further confirmed in polymerase chain reaction (PCR). The primers used for the amplification of 16S rDNA region were fB (50 to 70) = 5'(TAA CAC ATG CAA GTC GAA CG) 3' and 1492r = 5'(TAC CTT GTT ACG ACT T) 3' (Escalante et al., 2001; Osborne et al., 2005). Amplified PCR fragments were purified by the use of PCR purification kit (Roche, agarose gel DNA extraction kit) and were sequenced by REFGEN Biotechnology (METU Technocity, Ankara, Turkey). Basic local alignment search tool (BLAST) was used to compare the sequences against the nucleotide database in the National Centre for Biotechnology Information (NCBI) (http:// www.ncbi.nlm.nih.gov.tr/BLAST). The bacterial strains used as indicator organism in the antimicrobial activity and their growth conditions are listed in Table 1. All LAB strains were grown in MRS broth and agar plates were made by adding 1.5% agar to the broth. Cultures of LAB and non-lactic cultures were maintained in appropriate broths with 25% (v/v) sterile glycerol and stored at -20°C.

Physico-chemical properties of crude bacteriocin

P. pentosaceus OZF was grown in MRS broth at 35°C for 18 h and the culture broth was centrifuged to remove cells. To exclude antagonistic effects caused by low pH value, hydrogen peroxide and bacteriophage, the heated culture supernatant fluid was adjusted to pH value of 7.0 by adding 1 N NaOH and treatment with catalase. The retention of activity in the supernatant following exposure to several pH values, heat and enzymes (1 mg/ml) was evaluated as described (Bhunia et al., 1991). To determine the sensitivity of bacteriocin preparation to proteolytic and other enzymes, the enzymes listed in Table 2 were dissolved in sterile 4 mM phosphate buffer, pH value of 7.0, at a concentration of 1 mg/ml. The control samples contained only buffer solution. Supernatant treated with enzyme were incubated at 35°C for 1 h and residual bacteriocin activities were determined by spot-on-lawn method. The thermal stability of bacteriocin was determined by heating the preparations at 100°C for 5, 10 and 15 min, or by autoclaving at 121°C for 15 min, cooling and assaying for activity. To determine the activity of bacteriocin at different pH values, the supernatant was adjusted with sterile 10 mM NaOH or 10 mM HCl to pH values between 3 to 12. Samples were then maintained for 2 h at 25°C; 24 h at 25°C; 20 min at 100°C and bacteriocin activity was checked using spot on lawn method.

Bacteriocin antimicrobial activity assay

Quantitative determination of the antimicrobial activity of the bacteriocin in cell-free culture supernatant (CFS) was performed in a microtitre plate assay system (Holo et al., 1991). Each well in the microtitre plate contained 200 µl MRS broth (pH 6.2), bacteriocin fractions at two fold dilutions and the indicator organism, *Lactobacillus sakei* NCDO 2714 (10^4 times dilution of an overnight culture). The microtitre plate cultures were incubated overnight (16 to 20 h) at 30°C, after which growth inhibition of the indicator organism was measured spectrophotometrically at 620 nm using a Dynatech microplate reader. One bacteriocin unit (BU) was defined as

Table 1. Bacterial strains and their growth conditions.

Bacterial strain	Growth medium	Growth condition
B. cereus ATCC 9139 B	LB broth	37ºC 18 h
C.divergens NCDO 2306^T	MRS broth	30ºC 18 h
C. piscicola NCDO 2762^T	MRS broth	30ºC 18 h
E. avium UA83	MRS broth	37ºC 18 h
E. faecalis V583	MRS broth	37ºC 18 h
E.faecium T136 LMGT 2384 (Enterocin A producer)	MRS broth	37ºC 18 h
L.plantarum LMGT 2003 (Daeschel NC State)	MRS broth	30ºC 18 h
L. sakei L 83T 2356 (Sakacin P producer)	MRS broth	30ºC 18 h
L. sakei NCDO 2708	MRS broth	30ºC 18 h
L. sakei NCDO 2714	MRS broth	30ºC 18 h
L. lactis NCDO 1403 (Nisin producer) broth	GM17	30ºC 18 h
L. lactis NCDO 533	MRS broth	30ºC 18 h
L. mesenteroides NCDO 529	MRS broth	30ºC 18 h
L. innocua BI86/26B broth	GM17	30ºC 18 h
L. monocytogenes EGDe broth	GM17	30ºC 18 h
P. acidilactici PAC-1.0 (Daeschel NC State)	MRS broth	37ºC 18 h
P. acidilactici NCDO 521	MRS broth	37ºC 18 h
P.pentosaceus NCDO 992	MRS broth	37ºC 18 h
P. pentosaceus OZF	MRS broth	37ºC 18 h
S. aureus ATCC 14458	LB broth	37ºC 18 h

Table 2. Effect of pH values, enzymes and heat treatments on antimicrobial activity of *P. pentosaceus* OZF.

Treatment	Activity [a]
pH value	2-11
Enzyme	
Catalase	+
Lysozyme	+
Lipase	+
Ribonuclease A	+
Amylase	+
Trypsin	-
Chymotrypsin	-
Ficin	-
Proteinase K	-
Heat	
100 ºC for 5 min	+
100 ºC for 10 min	+
100 ºC for 15 min	+
121 ºC for 15 min	+

[a]Culture supernatant was adjusted to pH value of 5.0 (except for pH treatment) and was used for different treatment. Retention of activity was tested by spot-on-lawn method against *L. sakei* NCDO 2714, incubating the plate at 35 ºC for 18 h and examining the presence (+) or absence (-) of zone of growth inhibition.

the amount of the bacteriocin causing 50% growth inhibition (50% of the turbidity of the control culture without bacteriocin) in this assay.

Purification of bacteriocin of cultures through cation exchanger followed by reverse-phase chromatography of peptides

Bacteriocin was purified from 500 ml cultures of *P. Pentosaceus* OZF grown to the stationary phase (18 h at 35ºC in MRS). The cells were pelleted by centrifugation at 10000 g for 20 min at 4ºC, after which supernatant was passed through a 5 ml SP sepharose fast flow column (GE Healthcare Biosciences, Uppsala, Sweeden) equilibrated with 10 mM glacial acetic acid. The column was washed with 50 ml of 20 mM sodium phosphate (pH 6.8) and was eluted with a stepwise gradient consisting of 50 ml of 0.1 NaCl, 50 ml of 1M NaCl and 10 ml of 6 M Guanidin HCl at a flow rate of 1 ml per min. All fractions except the second 10 ml 0.1 M NaCl fraction contained bacteriocin activity and the highest activity was found in the 50 ml 1 M NaCl elute. Subsequent purification was performed by using reversed phase chromatography with an Äkta purifier fast protein liquid chromatography system. The most active fraction (1 M NaCl eluate) from the ion-exchange chromatography step was applied to the reversed phase column (Resource I; Pharmacia Biotechnology) equilibriated with 0.1/trifluoroacetic acid (TFA) in water. Elution was performed by using a 30 column volume (CV) linear gradient from 0 to 100% 2-propanol containing 0.1% TFA and 2 ml fractions were collected. The fractions having the highest activity were combined and diluted in sterile water (final volume, 20 ml) and then, they were applied to a Source 5RPC ST 4.6/415 column (Pharmacia Biotechnology) and eluted with a 5 CV linear gradient as described earlier in 1 ml fractions. The fractions were assayed for antimicrobial activity and the most active fraction (fraction 21) which coincided with the single peak of absorbance at 280 nm were stored at -20ºC until further analysis.

Table 3. Inhibition spectrum of purified bacteriocin.

Strain	Inhibition[a]	MIC (nM) [b]
B. cereus ATCC 9139 B	+	101.88
C. divergens NCDO 2306^T	+	<0.40
C.piscicola NCDO 2762^T	+	<0.40
E.avium UM83	+	<0.40
E. faecalis V583	+	6.37
E. faecium T136 LMGT2384 (Enterocin A producer)	-	
L. plantarum LMGT 2003 (Daeschel NC State)	+	<0.40
L. sakei L 83 LMGT 2356 (Sakacin P producer)	-	
L. sakei NCDO 2708	-	
L.sakei NCDO 2714	+	0.01
L. lactic IL 1403 (Nisin producer)	-	
L.lactis NCDO 533	+	<0.40
L. mesenteroides NCDO 529	-	
L. innocua Bl86/26B	+	12.73
L. monocytogenes EGDe	+	6.37
P. acidilactici NCDO 521	+	12.73
P.pentosaceus NCDO 992	+	1.59
S.aureus ATCC 14458	-	

[a]+, inhibition; -, no inhibition. Antimicrobial tests with *P. pentosaceus* OZF were performed by using diferrent assays. The MICs were not determined for insensitive indicators; [b]MICs of purified bacteriocin.

Mass spectrometry

Determination of the molecular mass of the purified bacteriocin by mass spectrometry was performed as described previously (Diep et al., 2006). Briefly, a bacteriocin sample (Fraction 21) was mixed 1:1 with a saturated solution of α-cyano-4-hydroxycinnamic acid in 0.1% TFA-acetonitrile (2:1) and deposited on a ground steel matrix-assisted laser desorption ionization target. Mass spectra were recorded in reflection mode with an ultraflex TOF/TOF (Daltonics), using a pulsed ion extraction setting of 40 ns and an accelaration voltage of 25 kV. The spectra displayed the accumulated signals of 200 laser shots with the laser power adjusted to just above the threshold level.

Antimicrobial spectrum of purified bacteriocin

MIC (Minimum Inhibitory Concentration) values of the purified bacte-riocin for the sensitive strains listed in Table 3 were determined by using a microtiter plate assays system (Holo et al., 1991).

PCR and DNA sequencing

P. pentosaceus OZF was tested by PCR for the presence of gene encoding pediocin. Genomic DNA was extracted from overnight cultures by the promega genomic DNA isolation kit following the instructions given by the manufacturer, while plasmid DNA was extracted by the use of Qiagen plasmid miniprep kit (Qiagen, Germany). In addition, plasmid safe ATP-dependent Dnase kit (Epicentre Biotechnologies, USA) was used as a final clean up of plasmid from the contaminated DNA for to known whether or not bacteriocin was encoded by plasmid or genomic DNA. Pediocin PA-1 specific primers designed from the single strand DNA sequence of the region of *P. acidilactici* PAC1.0 plasmid containing pediocin PA-1 genes were used in the experiment (Rodriguez et al., 1997). The DNA sequences of the coding strand primer (primer G1) was 5'-AAA ATA TCT AAC TAA TAC TTC-3', while that of the complementary strand primer (primer A2) was 5'-TAA AAA GAT ATT TGA CCA AAA-3'. The 5' end of primer G1 was 91 nucleotides upstream from the start of pedA and the 5' end of primer A2 was 33 nucleotides downstream from the start of pedC. The primers used in the PCR amplified a 711 bp DNA fragment containing pedA and pedB genes from the whole DNA of the *P. acidilactici* PAC1.0. The primers G1 and A2 were synthesized and supplied by Invitrogen, at a concentration of 100 µM, respectively. PCR conditions were selected according to the criteria of Martinez et al. (1998) and Rodriguez et al. (1997). PCR was carried out in 50 µl reaction volumes in a sterile 200 µl PCR tubes. The PCR reaction mixture comprised of 500 ng genomic DNA, 2.5 mM of each dNTPs, primer G1 and A2 at 100 µm concentrations and 2.5 µl phusion. Same reaction condition was used for the plasmid DNA which was cleaned up from genomic DNA using Epicentre kit. PCR consisted of an initialization step of 1 cycle at 98°C for 1.5 min; PCR amplification (24 cycles) at 98°C for 10 s, 45°C for 30 s and 72°C for 15 s and final extension (1 cycle) at 72°C for 1 min. Amplification was carried out in Eppendorf gradient thermal cycler (programmed as described earlier) and was visualized in 1% agarose gels. The gels were electrophoresed at 100 V for 1 h using 5 µl 1 kbp DNA ladder plus (Fermentas, Finland) as a standard molecular mass marker. On completion of the run, the gel was stained with ethidium bromide, and visualized.

For sequence analysis, PCR product was purified by using the PCR clean up gel extraction kit (NucleoSpin, Macherey-Nagel, Germany). After purification, sequence PCR was done with only reverse primer (primer A2), which consisted of an initialization step (1 cycle) at 96°C for 1 min; PCR amplification (25 cycles) at 95°C for 10 s, 50°C for 5 s and 60°C for 4 min. PCR product was precipitated with ethanol and used for sequencing which was done by using a BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems, United States), and finally, the PCR poduct was

Table 4. Oligonucleotite primers used in PCR.

Primer	Sequence (5'-3')
1F	CCCTCTAAAAGCCTGCTGAGGGC
1R	AGCGTCTTTCTTTTCGGGTTTCCAGG
2F	AGCATTGGCTGTCAAACAAGCCG
2R	AAAGCCCGGAGATGGTGCGG
3F	ACTGCGTTGATAGGCCAGGTTTCA
3R	GGGCCAACATGTAATCGGAGCCA
4F	ATGCCGAAGCTGTGCGTGCT
4R	TCACCGTGCTGTTCTTGAGGCT
5F	AGCCGTCCTGGAGTAACTCAACA
5R	TGACGGGAAAAGGGATCACGAGC
6F	AGCAGCTTTCGAGTTTCCCCACT
6R	TGCCAGGTTTATGAAGATTCTCTGCAC
G1	AAAATATCTAACTAATACTTC
A2	TAAAAAGATATTTGACCAAAA

sequenced by using the ABI prism 377 DNA sequencing system (Applied Biosystems, United States). The sequence of PCR product was assembled by using the BioEdit software, version 7.0.0 and similarity search was done by using BLAST at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

For determining whether or not the plasmid responsible for pediocin production was identical to pediocin AcH/PA-1 plasmid, new 6 specific primers were synthesized based on the sequences of pediocin producing plasmid genome (Table 4). Primers were designed by use of geneious basic 4.8.5. web program based on *P. acidilactici* H pSMB74 completed genome (8971 bp) which produced pediocin AcH/PA-1. *P. acidilactici* PAC-1, Daeschel NC State was used as a control for pediocin AcH/PA-1 producer. PCR was carried out in 50 µl reaction volumes in a sterile 200 µl PCR tubes. The PCR reaction mixture comprised of 500 ng plasmid DNA, 2.5 mM of each dNTPs, primer F and R at 100 µm concentrations and 2.5 µl phusion. PCR consisted of an initialization step of 1 cycle at 98°C for 1.5 min; PCR amplification (24 cycles) at 98°C for 10 s, 55°C for 30 s and 72°C for 15 s and a final extension (1 cycle) at 72°C for 1 min. Amplification was carried out in Eppendorf gradient thermal cycler (programmed as described previously) and was visualized in 1% agarose gels. The gels were electrophoresed at 100 V for 1 h using 5 µl 1 kbp DNA ladder plus (Fermentas, Finland) as a standard molecular mass marker.

RESULTS

The strain was isolated from human breast milk and was identified as *P. pentosaceus* by both biochemical tests and API 50 CHL (BioMèrieux). The identification was further confirmed by 16S rRNA sequence analysis and the obtained sequence was registered in the GenBank database system (http://www.ncbi.nlm.nih.gov.tr/BLAST) under accession number 1337739 (706 bp).

Physico-chemical properties of purified bacteriocin

The activity of the bacteriocin produced by *P. pentosaceus* OZF was neutralized by protease (α-chymotrypsin, trypsin and proteinase K or with pepsin) treatment but was resistant to heat (Table 2). Treatment with lipolytic or amylolytic enzymes had no effect on the bacteriocin activity. The antagonistic activity of culture supernatant from strain *P. pentosaceus* OZF was stable at pH values between 2 to 11 for 24 h at 25°C. The preparations also showed detectable activity between pH values of 2 and 5 after 15 min of exposure to 121°C. Heating the bacteriocin for 5, 10 and 15 min at 100°C did not affect the activity when compared with an unheated control (Table 2).

Purification of bacteriocin

Results from the purification of the bacteriocin from the culture of *P. pentosaceus* OZF grown at 35°C in 500 ml MRS broth are summarized in Table 5. The fraction from the first run on the reverse phase column showing the highest activity was collected and re-chromotographed on the same column. A single absorbance peak, was obtained which was coincident with the peak of biological activity (Figure 1a). The final specific activity of the pure bacteriocin was approximately 224,000 fold greater than in the culture supernatant, and the recovery of activity was 200% (fraction 21). The protein concentration in this fraction was estimated to be 80 µg/ml. Study of this fraction by mass spectrometry, confirmed the purity of the sample and showed the mono-isotrophic molecular mass of the isolated bacteriocin to be 4,623 Da (Figure 1b) that is identical to pediocin PA-1 (Rodriguez et al., 2002).

Antimicrobial spectrum of purified bacteriocin

The MICs of the purified bacteriocin determined by micro-titer plate assay against selected indicator microorganisms

Table 5. Purification of bacteriocin (pediocin).

Purification step	Volume (ml)	Recovery (%)	Protein concentration (mg/ml) [a]	Antimicrobial activity (bacteriocin units/ml)	Specific activity (bacteriocin units/mg)	Increase in specific activity (fold)
Culture supernatant	500	100	17.8	1.3×10^4	730	1
Ion -exchange chromatography	50	40	0.15	5.1×10^4	3.4×10^5	466
First reversed-phase chromatography	2	100	0.33	3.3×10^6	1×10^7	13,700
Second reversed-phase chromatography	1	200	0.08	1.3×10^7	1.63×10^8	224,000

[a]The protein concentration was determined either by determining the optical density at 280 nm or by using the calculated absorbance value for purified bacteriocin (from the second reversed-phase chromotography fraction) (1 mg/ml = 3.075).

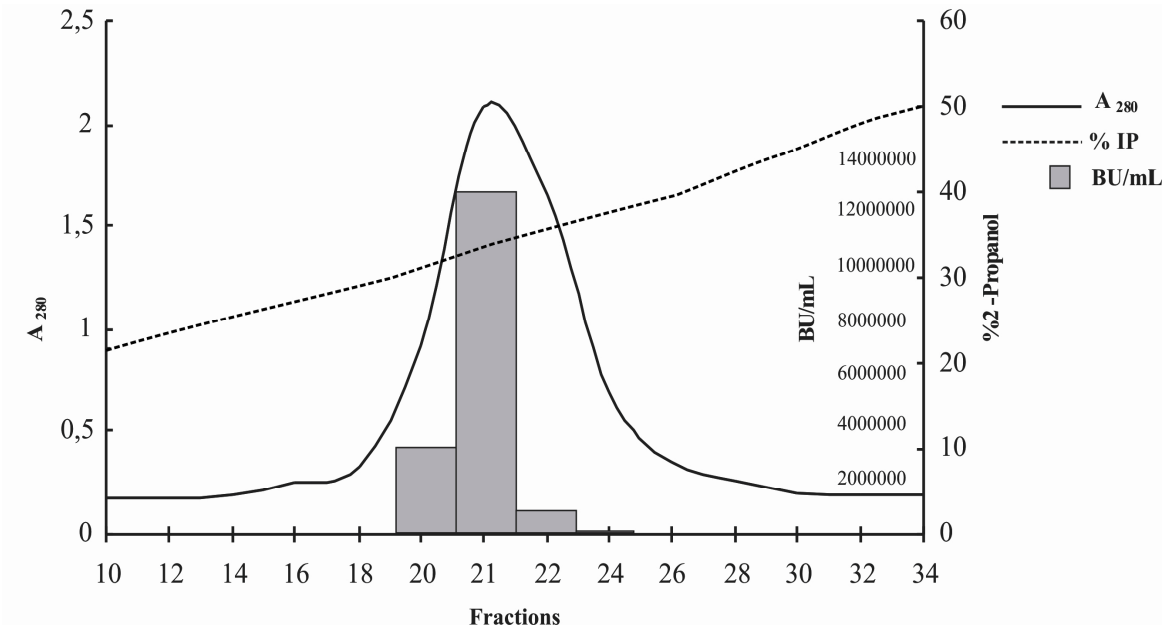

Figure 1. (A) Second reversed-phase chromatography step. Elution was performed using a 30-CV linear gradient of 0 to 100% 2-propanol containing 0.1% TFA. Solid line, absorbance at 280 nm; dashed line, isopropanol gradient; bars, bacteriocin units (BU) in eluted active fractions.

are given in Table 3. Most of the LAB, spoilage and food borne pathogenic bacteria tested were inhibited by the purified bacteriocin, with MICs ranging from 0.01- 102 nM. The antimicrobial spectrum of the bacteriocin producer was also tested with the same indicators using the over-lay assay.

Identification of the gene locus of the bacteriocin

The possibility that the purified bacteriocin was identical to pediocin PA-1/AcH was considered. So in order to test this hypothesis, pediocin PA-1 specific primers P1 and P2 for pediocin PA-1/AcH were used to amplify and sequence a 711 bp fragment [from 91 bp upstream of pedA to 33 bp downstream of the translational start of pedC (GenBank: M83924)]. The PCR product was observed only with plasmid DNA template of the *P. pentosaceus* OZF. The agarose gel analysis of the PCR products identified a PCR DNA fragment of approximately 706 as expected bp length (Figure 2). This plasmid-encoded PCR product was then sequenced and a DNA sequence search was done by BLAST at the NCBI. The search showed that the DNA sequence of PCR product was identical to pediocin AcH/PA-1 gene. The nucleotide sequence showed 99% similarity to the published sequences for pediocin operons on the genomes of *P. acidilactici* strain MTCC 5101 plasmid pCP289 ped operon (GenBank: GQ214404.1), *P. acidilactici* strain K10 pediocin operon (GenBank:

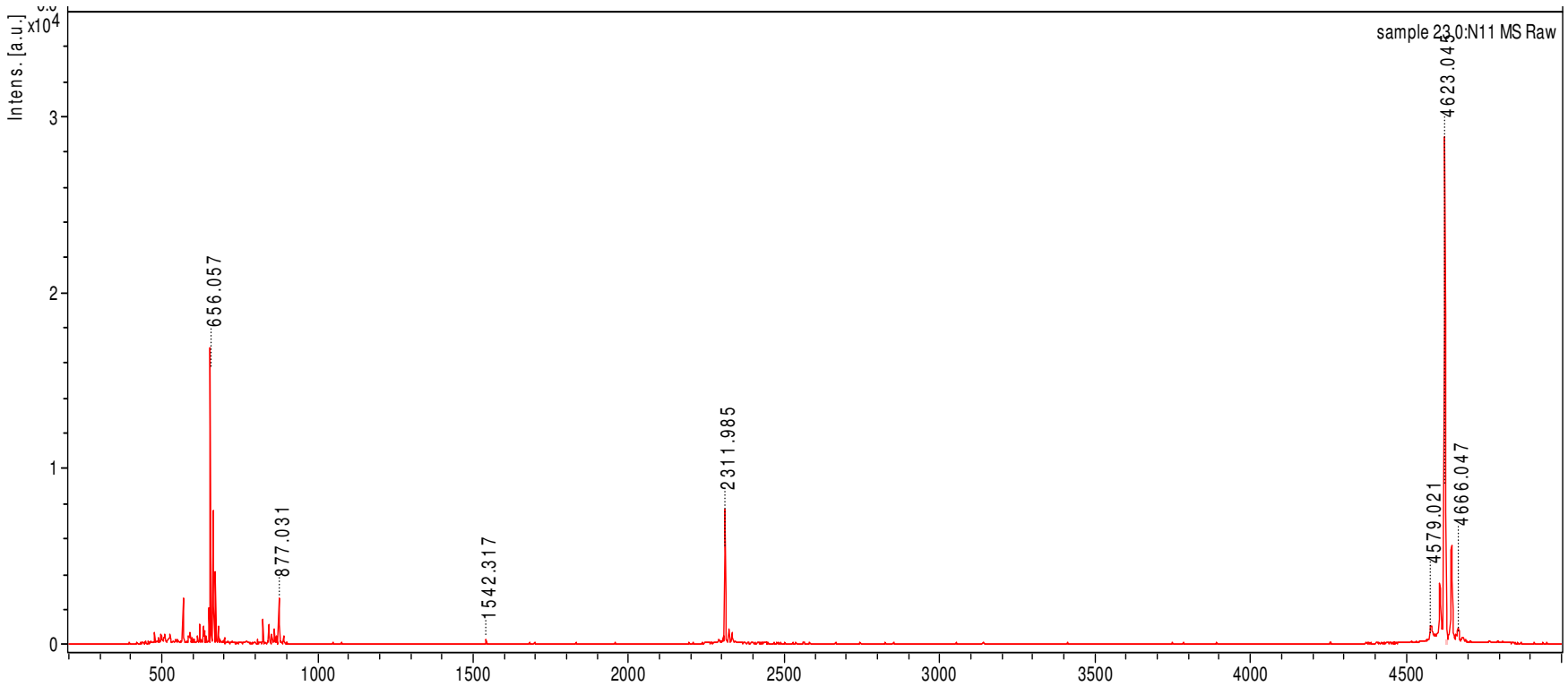

Figure 1. (B) Electrospray ionization mass spectrometry analysis of fraction 21 (Figure 1A); Intens, intensity.

AY705375.1), *P. acidilactici* H plasmid pSMB74 (GenBank: U02482), *P. acidilactici* PAC1.0 (GenBank: M839240) and *P. pentosaceus* (GenBank: AY316525).

P. pentosaceus OZF contains four plasmids with length of 14,8-8,9-6,8 and 3,4 kb. Based on the sequences of the pSMB74 encoded pediocin AcH/PA-1 plasmid of *P. acidilactici* H, new specific primers were synthesized and used to determine whether the plasmids might be identical. The various PCR products ranged between 1024 and 1476 bp (Figure 2), and showed that pediocin PA-1 producing *P. acidilactici* PAC 1.0 and the *P. pentosaceus* OZF strains, respectively had a similar genetic organization of the plasmid associated bacteriocin gene.

It should be emphasized that our strain isolated from a human source is different from the food-derived pediocin PA-1 producer (*P. acidilactici* PAC 1.0) and ie depends on the utilization of the four carbohdrates in their fermentation in the API 50 CHL test. *P. pentosaceus* OZF was shown to use maltose and lactose, while *P. acidilactici* PAC1 did not. Moreover, *P. acidilactici* PAC1 used methylαDmono-piranosid and saccharose, while *P. pentosaceus* OZF did not.

DISCUSSION

Bacteriocins produced by the LAB are attractive to the food industry because they may be used as natural biopreservatives and contribute to the improvement of the microbiological quality of foods. Also, the use of these ribosomally synthesized anti- microbial peptides may allow a significant reduction in the level of chemical preservatives and/or in the intensity of the physical treatments currently employed during food processing. Therefore, they might also help to provide healthier foods. Recently, it was shown

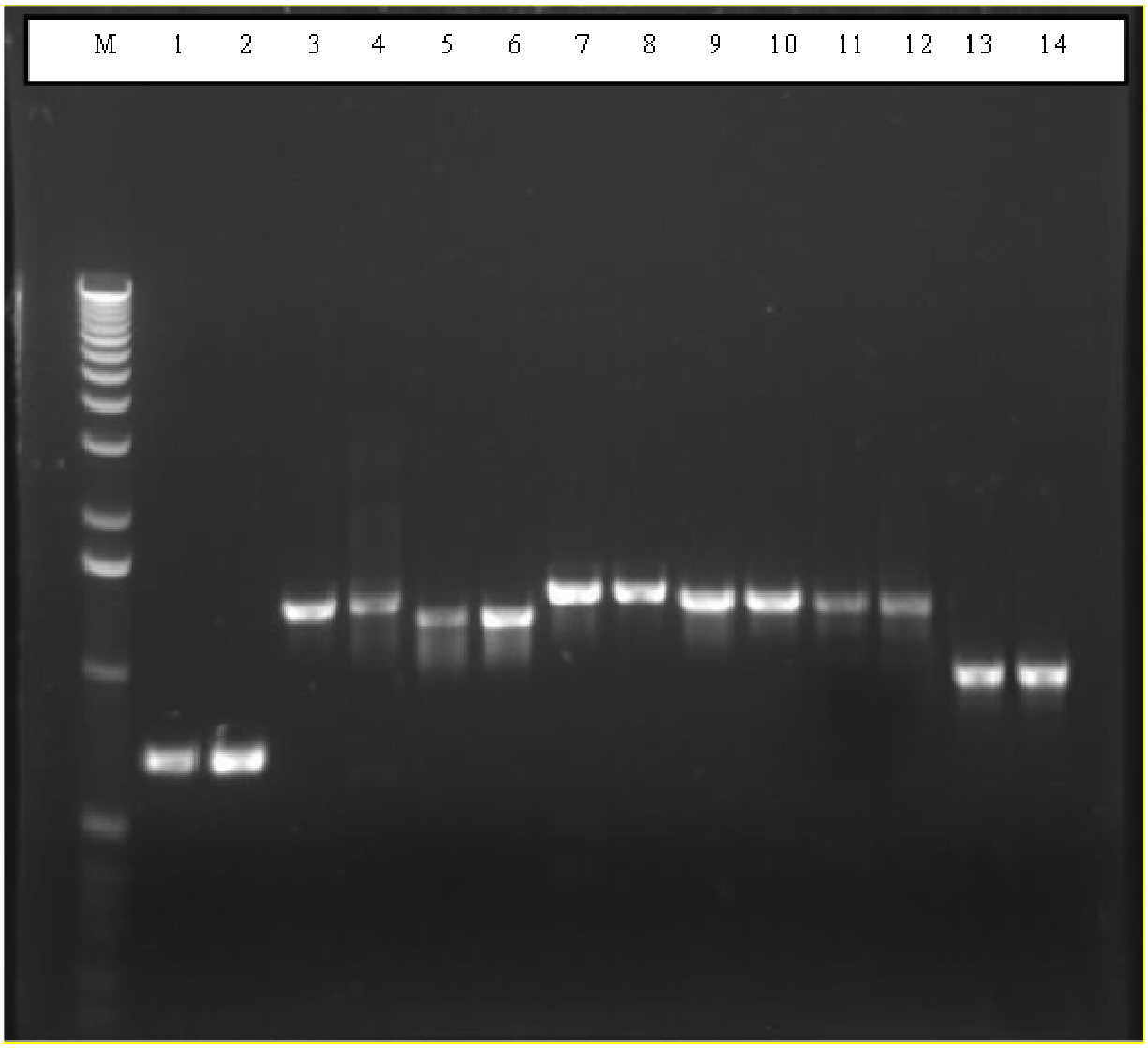

Figure 2. Agarose gel electrophoresis of specific PCR products obtained by use of the designed primers. Lane M, 1 kbp DNA ladder, Fermentas; lane 1, 711 bp PCR products of *P. pentosaceus* OZF plasmid DNA amplified with ped gene specific primers; lane 2, 711 bp PCR products of *P. acidilactici* PAC 1.0 plasmid DNA amplified with ped gene specific primers; lane 3, 1400 bp PCR products of *P. pentosaceus* OZF plasmid DNA amplified with F1/R1 specific primers; lane 4, 1400 bp PCR products of *P. acidilactici* PAC 1.0 plasmid DNA amplified with F1/R1 specific primers; lane 5, 1401 bp PCR products of *P. pentosaceus* OZF plasmid DNA amplified with F2/R2 specific primers; lane 6, 1401 bp PCR products of *P. acidilactici* PAC 1.0 plasmid DNA amplified with F2/R2 specific primers; lane 7, 1476 bp PCR products of *P. pentosaceus* OZF plasmid DNA amplified with F3/R3 specific primers; lane 8, 1476 bp PCR products of *P. acidilactici* PAC 1.0 plasmid DNA amplified with F3/R3 specific primers; lane 9, 1435 bp PCR products of *P. pentosaceus* OZF plasmid DNA amplified with F4/R4 specific primers; lane 10, 1435 bp PCR products of *P. acidilactici* PAC 1.0 plasmid DNA amplified with F4/R4 specific primers; lane 11, 1412 bp PCR products of *P. pentosaceus* OZF plasmid DNA amplified with F5/R5 specific primers; lane 12, 1412 bp PCR products of *P. acidilactici* PAC 1.0 plasmid DNA amplified with F5/R5 specific primers; lane 13, 1025 bp PCR products of *P. pentosaceus* OZF plasmid DNA amplified with F6/R6 specific primers; lane 14, 1025 bp PCR products of *P. acidilactici* PAC 1.0 plasmid DNA amplified with F6/R6 specific primers.

that a bacteriocin producing probiotic *Lactobacillus salivarius* strain was able to prevent *L. monocytogenes* infection in mice (Corr et al., 2007). Bacteriocin in the gut enabled, the bacterium to efficiently eradicate the presence of *L. monocytogenes*. This shows how important bacteriocin production can be for the successful establishment of probiotic bacteria.

The *P. pentosaceus* OZF bacteriocin was active over a wide range of pH values and was stable to various heat treatments. This heat and pH stability may be useful if the

bacteriocin is to be used as an antimicrobial agent in fermented or thermally processed foods. Like other class IIa bacteriocins, the pediocin produced by the strain inhibits *Pediococcus*, *Leuconostocs*, *Lactobacillus*, *Enterococcus*, *Listeria*, *Bacillus* and *Carnobacterium* species (Eijsink et al., 1998). The bacteriocin may have potential use as a food additive in the food industry, particularly processed food, in which some *Bacillus* species are potential spoilage microorganisms.

The purification of the bacteriocin from the liquid medium in our work was based on the method used by Lozano et al. (1992). The pediocin was purified by this protocol to high purity and yield. The molecular mass of the bacteriocin was determined by mass spectrometry to be 4,623 Da. A peptide mass similarity search using the TagIdent tool on the ExPASy server (http://au.expasy.org/tools/tagident) suggested a match with a previously reported bacteriocin, Pediocin PA-1.

The biological activity against *L. monocytogenes* and the results of the mass determination by MS were strong indications that the P. pentosaceus strain producing the bacteriocin could be a pediocin PA-1 producer. A PCR technique with specific primers was used to detect the pediocin PA-1 operon in this strain. Most class IIa bacteriocin genes are known to be located on plasmids (as opposed to being incorporated in the host genome). Graham and McCay (1985) were the first to report that a, pediocin production phenotype in *P. pentosaceus* FBB63 is associated with a plasmid of 15.7 kb. Association of bacteriocins with plasmid DNAs sizes of 19.4 and 8.3 kb in other *P. pentosaceus* strains have been reported by Daeschel and Klaenhammer (1985) and Hoover et al. (1988), respectively. In this study, the location of our bacteriocin gene was associated with a plasmid DNA determined by PCR using pediocin PA-1 gene specific primers. By sequencing the PCR product of bacteriocin gene sequence identity 99% sequence identity to pediocin PA-1/AcH. Confirmation of this level of identity to pediocin AcH/PA-1 was done by PCR using six different primer sets not previously used, and which were designed from the *P. acidilactici* H plasmid pSMB74 and compared with a pediocin PA-1/AcH producer strain of *P. acidilactici* PAC 1.0. The PCR products showed that bacteriocin produced by *P. pentosaceus* OZF seemed to be identical to theknown bacteriocin named pediocin PA-1/AcH and isolated from *P. acidilactici* PAC 1.0.

Almost all class IIa bacteriocin producing strains that have been characterized so far are isolated from food or related products. There are however few reports of class IIa bacteriocin producing strains having a human origin (Biri et al., 2010; Flynn et al., 2002; Millette et al., 2008; Oh et al., 2000). The ability of bacteriocin-producing LAB to overcome the harsh low pH values of gastric juice and the detergent effect of bile salts, and to arrive viably at a site in the gastrointestinal tract where its establishment and growth are relatively optimal, providing a function in the host that enables antimicrobiosis and immunmodulation, are the important criteria in defining probiotic strains (Ljungh and Wadström, 2006). In these respect, the strain in this study met all the criteria (Osmanagaoglu et al., 2010), and it has a localized bacteriocin production ability which is also an important probiotic feature.

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