ORIGINAL ARTICLE

Purification and amino acid sequence of a bacteriocins produced by *Lactobacillus salivarius* K7 isolated from chicken intestine

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Abstract

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A bacteriocin-producing strain, *Lactobacillus* K7, was isolated from a chicken intestine. The inhibitory activity was determined by spot-on-lawn technique. Identification of the strain was performed by morphological, biochemical (API 50 CH kit) and molecular genetic (16S rDNA) basis. Bacteriocin purification processes were carried out by amberlite adsorption, cation exchange and reverse-phase high perform-

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ance liquid chromatography. N-terminal amino acid sequences were performed by Edman degradation. Molecular mass was determined by electrospray-ionization (ESI) mass spectrometry (MS). *Lactobacillus* K7 showed inhibitory activity against *Lactobacillus sakei* subsp. sakei JCM 1157^T, *Leuconostoc mesenteroides* subsp. *mesenteroides* JCM 6124^T and *Bacillus coagulans* JCM 2257^T. This strain was identified as *Lb. salivarius*. The antimicrobial substance was destroyed by proteolytic enzymes, indicating its proteinaceous structure designated as a bacteriocin type. The purification of bacteriocin by amberlite adsorption, cation exchange, and reverse-phase chromatography resulted in only one single active peak, which was designated FK22. Molecular weight of this fraction was 4331.70 Da. By amino acid sequence, this peptide was homology to Abp 118 beta produced by *Lb. salivarius* UCC118. In addition, *Lb. salivarius* UCC118 produced 2-peptide bacteriocin, which was Abp 118 alpha and beta. Based on the partial amino acid sequences of Abp 118 beta, specific primers were designed from nucleotide sequences according to data from GenBank. The result showed that the deduced peptide was high homology to 2-peptide bacteriocin, Abp 118 alpha and beta.

Key words : lactic acid bacteria, bacteriocin, chicken

Consumers have been concerned about chemicals used as preservatives in food because of their side effects. Therefore, natural antibiotics have been considered as alternatives (Soomro *et al.*, 2002). Lactic acid bacteria (LAB) are commonly found in many foods such as meat, vegetable and milk, sometimes as dominating microflora (Atrih *et al.*, 2001). Exploitation of LAB as a preservative agent is advantageous not only in improving the microbial safety of food but also as a probiotic in animals and humans to improve the balance of microflora and to inhibit pathogenic bacteria in intestinal tract (Soomro *et al.*, 2002). In addition, antimicrobial substances produced by LAB were reported to have the ability to stimulate a host immune response and to influence the metabolic activities (Salminen *et al.*, 1996). Its inhibitory activity is due to pH decrease, competition for substrates, and to a variety of antimicrobial compounds produced, including bacteriocins (Parente and Ricciardi, 1999).

Bacteriocins are ribosomally synthesized bacteriostatic or bactericidal proteins and peptides (Balla *et al.*, 2000). They are produced by many different bacterial species including many members of the lactic acid bacteria (O' Sullivan *et al.*, 2002). There has been interest to use bacteriocins produced by LAB in food industry because they are safe agents to prevent undesirable microorganisms (Uteng *et al.*, 2002). In this study, *Lactobacillus salivarius* K7 was isolated from chicken intestine from farms in Thailand. It produced interesting bacteriocins, which inhibit *B. coagulans, Leu. mesenteroides* and *Lb. sakei*. Therefore, the purification and identification of a bacteriocin produced by *Lb. salivarius* K7 isolated from chicken intestine was studied.

Materials and Methods

Strains and culture conditions

The strains used in this study are listed in Table 1. *Lb. salivarius* K7 was propagated in MRS broth (Difco, USA) at 30ºC. The indicator strains used in this study were grown in MRS and TSB-YE broth at 26-37ºC (Table 1). All strains were maintained at -80ºC in appropriate media containing 15% (v/v) glycerol.

E. coli JM109 was used as a host for cloning. It was grown in Luria-Bertani broth (Sambrook *et al.*, 2001), supplemented with 100 µg ampicillin at 37ºC, with vigorous agitation overnight.

Determination of inhibitory activity

The inhibitory activity was examined by spot-on-lawn test (Ennahar *et al.*, 1999). Cell-free supernatant (CFS) was adjusted to pH 5.5 with NaOH solution and sterilized by boiling for 5 min. The inhibitory activity was expressed in an arbitrary unit (AU/mL) by testing serial two-fold dilutions prepared in a 96-well micro litter plate (Greiner bio-one, Germany). The arbitrary unit (AU) was defined as the reciprocal of the highest dilution producing a clear zone of growth inhibit-

Table 1. Inhibitory activity of *Lb. salivarius* K7 in AU/mL against indicator strains.

Indicator strains	Media	Temperature	Inhibitory Activity (AU/mL)
Lactic acid bacteria			
Lactococcus lactis subsp. lactis ATCC 19435^T	MRS	30ºC	-
Lactococcus lactis subsp. cremoris TUA 1344L	MRS	30ºC	-
Lactobacillus plantarum ATCC 14917^T	MRS	30ºC	-
Pediococcus pentosaceous JCM 5885	MRS	30ºC	-
Lactobacillus sakei subsp. *sakei* JCM 1157^T	MRS	30ºC	1600
Leuconostoc mesenteroides subsp. *mesenteroides* JCM 6124^T	MRS	30ºC	200
Enterococcus feacalis JCM 5803^T	MRS	37ºC	-
Other gram positive bacteria			
Bacillus subtilis JCM 1465^T	TSBYE	30ºC*	-
Bacillus circulans JCM 2504^T	TSBYE	30ºC*	-
Bacillus coagulans JCM 2257^T	TSBYE	37ºC*	400
Bacillus cereus JCM 2152^T	TSBYE	30ºC*	-
Listeria innocua ATCC 33090^T	TSBYE	37ºC	-
Micrococcus leuteus IFO 12708	TSBYE	30ºC*	-
Gram negative bacteria			
Pseudomonas fluorescens JCM 5963^T	TSBYE	26ºC	-
Escherichia coli JM109	TSBYE	37ºC	-

ATCC = American Type Culture Collection, Rockville, Md
JCM = Japanese culture of Microorganisms, Wako, Japan
JM = Commercial strain from Toyobo, Osaka, Japan
TUA = Tokyu University of Agriculture, Tokyo Japan
IFO = Institute for Fermentation, Osaka, Japan

ion of the indicator strain. AU was calculated as (1000/10)D where D was the dilution factor (Parente *et al*., 1995)

Effect of enzyme, pH and heat stability

CFS was treated with proteolytic enzymes at a final concentration of 1 mg/mL as the following: trypsin (Sigma, St. Louis, Mo, U.S.A), alpha-chymotrypsin (Sigma), ficin (Sigma), protease type XIII (Sigma), pepsin (Sigma), papain (Merck, Darmstadt, Germany), proteinase K (Merck) and actinase E (Kaken Pharmaceutical, Tokyo, Japan). All samples were adjusted to pH 7 except that treated with pepsin, which was adjusted to pH 3. Samples were filter-sterilized using filter membrane (0.2 µm, polysulfone, Kanto chemical, Japan) and then incubated at 37ºC for 2 h. Residual enzyme activity was finally stopped by boiling for 5 min. (Ennahar *et al*., 1999). For pH stability, CFS samples were adjusted to a pH range of 3-10. All samples were incubated at 37ºC for 2 h and then adjusted to pH 5.5. For the study of thermal stability, CFS samples were adjusted to pH 5.5 and then boiled at 100ºC for 5 and 30 min and at 121ºC for 15 min. The remaining of bacteriocin activity was examined by spot-on-lawn method.

Morphological identification

Cellular morphology was determined by gram-stain reaction and examined by a phase contrast microscope (BH-2, Olympus, Japan). The catalase test was investigated by the method of Forbes *et al*., (1998).

Determination of the growth on different temperature, pH and NaCl concentrations

The ability of *Lb. salivarius* K7 to grow in salt was determined in MRS broth containing 6.5

and 18% salt. The growth of *Lb. salivarius* K7 at temperature 5, 10, 15, 30, 37, 42, 45 and 50ºC and at pH 4.5 and 9.6 were determined by inoculating the overnight culture in to MRS broth and observing the turbidity after incubating for 24 and 48 hr.

Biochemical test

API 50 CH kit (BioMerieux, France) was used to determine the carbohydrate fermentation pattern of the bacteriocin-producing strain.

Bacteriocin purification

Lb. salivarius K7 was grown in MRS broth at 42ºC until OD at 600 nm reached to 5. CFS was obtained by centrifugation at 6000 rpm for 10 min at 4ºC and their purification processes were carried out as described by Cintas *et al*., (2000). Bacteriocin adsorption was carried out by adding 20 g of Amberlite XAD-16 (Sigma, St. Luis, Mo) into 1 L of CFS and shaking at room temperature for 2 h. The Amberlite adsorbing hydrophobic substances were washed with 100 mL of distilled water, followed by 100 mL of 40% (v/v) ethanol in distilled water and, finally, the bacteriocin was eluted with 100 mL of 70% isopropanol in distilled water (containing 0.1% trifluoroacetic acid, TFA). The elutant was then evaporated to get rid of isopropanol and then diluted with 20 mM phosphate buffer pH 5.7. Subsequently, pH of the solution was adjusted to 5.7. The sample was then applied to SP-Sepharose fast flow cation-exchange column (Amersham Phamacia Biotech, Tokyo). The column was first washed with 20 mM phosphate buffer pH 5.7 and the elution was performed with a linear gradient from 0.25-1 M NaCl in 20 mM phosphate buffer pH 5.7. The bioactive fractions were subjected to a reverse phase HPLC (Shimadzu, Japan) using a reverse-phase column of polystyrene/divinyl-benzene (Amersham Bioscience) and elution was completed with acetronitrile containing 0.1% TFA with a linear gradient ranging from 0-80% for 30 min.

N-terminal amino acid sequence and molecular weight analysis

N-terminal amino acid sequence of bacteriocin produced by *Lb. salivarius* K7 was performed by Edman degradation on gas-phase automatic sequence analyzer (PSQ-1, Shimazu, Japan). Molecular weight of the bacteriocin was determined by electrospray-ionization (ESI) mass spectrometry (MS) (JMS-T100LC, JEOL, Japan).

Plasmid DNA and design for specific primers

pGem-T vector was use as a plasmid DNA vector for PCR cloning and sequencing. Oligonucleotide primers used in this study were listed in Table 2.

Isolation of DNA

Genomic DNA of *Lb. salivarius* K7 was isolated by using Genomic DNA purification kit (Toyobo, Japan). Plasmid DNA from *E. coli* JM109 transformant was extracted by using Mag extractor plasmid kit (Toyobo, Japan).

Table 2. Primers used for PCR amplification and DNA sequencing.

Primer set	Database
118a forward	5' GGA TGA TTA TCA TGA AGG A 3'
118a reverse	5' CTG ATG CTA AGT TAT CTT CAG T 3'
118b forward	5' TAG GTG GAG CAA ACT TAG GA 3'
118b reverse	5' CTT ACA CTT GAC ACT ACT TGA 3'
M13 universal	5' CAG GAA ACA GCT ATG ACC 3'
M13 reversal	5' AAC AGC TAT CAG CAT G 3'
8f	5' AGA GTT TGA TCA TGG CTC AG 3'
1510r	5' GTG AAG CTT ACG GCT ACC TTG TTA CGA CTT 3'

DNA amplification by PCR

PCR condition for amplification of 16S rDNA included: denaturation at 94ºC for 3 min., followed by 30 cycles of denaturation at 94ºC for 30 sec., annealing at 52ºC for 30 sec., and extension at 72ºC for 45 sec. and the final extension at 72ºC for 2 min. PCR reaction composed of 50 µL of 1x reaction buffer with 25mM $MgCl_2$, 2.5 mM deoxynucleoside triphosphate, 1 U of Taq polymerase (promega,USA), 20 pM of each primer (8f, 1510r) (Martinez-Murcia *et al*., 1995; Christine *et al*., 2002) and a genomic DNA of 50-200 ng which was used as a template for amplification.

To study structural gene of bacteriocin, DNA was amplified in 25 µl of 1x reaction buffer with 1.25 units of Premix Tag DNA polymerase (Ex Taq version, Takara Bio Inc, Japan) and 20 pM of each primer. Genomic DNA of 50-200 ng was used as template. The PCR program consisted of denaturation at 94ºC for 3 min, followed by 30 cycles including denaturation at 94ºC (30 s), annealing at 52ºC (30 s), and extension at 72ºC (45 s) and the final extension at 72ºC for 2 min.

Screening of *E. coli* JM109 positive clone was determined by colony PCR. Each clone was suspended in 20 µL of 1X reaction buffer with 25 mM $MgCl_2$, 2.5 mM deoxynucleotide triphosphate, 1 U of Taq polymerase (Promega, USA) 20 pM of each primer (M13 universal and M13 reversal). PCR condition was followed by denaturation at 94ºC for 5 min, 30 cycles including denaturation at 94ºC for 30s, annealing at 52ºC for 20 s, and extension at 72ºC for 1 min and the final extension at 72ºC for 7 min.

PCR cloning and DNA sequence analysis

The PCR product was purified by QIAquick PCR purification kit (Qiagen, USA) and cloned into pGem-T vector system (Promega, USA). The ligation product was transformed into *E.coli* JM109. Positive clone was screened by colony PCR as the method mentioned above. The DNA sequences of positive clones were analyzed by ABI PRISM 3730 XL sequencer with bigdye terminator version 3.1 (Marcogen Ltd. Korea). A data base search was done by BLAST program (GenBank).

Results and Discussion

Determination of inhibitory spectrum

CFS of *Lb. salivarius* K7 was neutralized with NaOH solution in order to eliminate the acids and then sterilized by boiling. Activity of the neutralized CFS was tested against gram-positive bacteria including *Leu. mesenteroides* subsp. *mesenteroides* JCM6124^T, *Lb. sakei* subsp. *sakei* JCM 1157^T and *B. coagulans* JCM 2257^T (Table 1). No inhibition toward gram-negative bacteria was shown. However, another strain, *Lb. salivarius* UCC118, exhibited wide spectrum activities. Its CFS displayed inhibition activity against both gram-positive and gram-negative bacteria such as *B. subtilis, B. cereus, B. thuringiensis, E. faecalis, E. feacium, Listeria monocytogenes, Staphylococcus aureus* and *Pseudomonas fluorescens* (Dunne *et al*., 1999). Moreover, *Lb. salivarius* M7 inhibited *Lb. fermentum, Lb. plantarum, Lb. acidophilus, L. monocytogenes L2, Streptococcus feacalis, S. epidermis* and *S. aureus* (Cataloluk and Gurakan, 2003).

Identification of *Lb. salivarius* K7

Lactobacillus K7 was found to be short rod and gram positive. It was catalase-negative and did not produce gas. The strain was able to grow in the pH range of 4.5-9.6, temperature of 30-45ºC and in 5% NaCl (Table 3). Based on the comparison of its characteristics with Bergey's manual (Kandler and Weiss, 1986), the isolate was classified as genus *Lactobacillus*. For species determination, biochemical identification was used. Carbohydrate fermentation patterns showed that *Lactobacillus* K7 was able to ferment fructose, glucose, galactose, lactose, mannitol, melibiose and raffinose (Table 4), indicating similarities to *Lb. salivarius* (Kandler and Weiss, 1986). Furthermore, identifications made by API database correlation indicated that this strain was 99.9% identified as *Lb. salivarius*. In order to support the conventional identification, the 16S rDNA gene sequence was investigated because this method is more discriminating (Chagnaud *et al*., 2001). Thus, 1500 bp of 16S rDNA was amplified. Comparison

Table 3. Morphology and physiology identification of *Lactobacillus* K7.

Test		Results
Gram's strain		Positive
Morphology		Short rod
Catalase		Negative
CO_2 production		-
Growt at	5ºC	-
	10ºC	-
	15ºC	-
	30ºC	+
	45ºC	+
	50ºC	-
NaCl	1% - 5%	+
	6.5%	-
	18%	-
pH 4.5		+
pH 9.6		+

Table 4. Biochemical identification of *Lactobacillus* K7

Method	Characteristics of the Strain
Fermentable sugar	Galactose, D-Glucose, D-Fructose, D-Mannose, Mannitol, Sorbitol, N Acetyl glucosamine, Esculine, Lactose, Melibiose, Saccharose and D-Raffinose
Non-fermentable sugar	Glyceral, Erithritol, D-Arabinose, L-Arabinose, Ribose, D-Xylose, Adonitol, Beta Methyl-xyloside, L-Sorbose, Rhamnose, Dulcitol, Inositol, Alpha Methyl-D-manoside, Alpha Methyl-D-glucoside, Amygdaline, Arbutine, Salicine, Cellobiose, Maltose, Trehalose, Inuline, Melezitose, Amidon, Glycogene, Xylitol, Beta Gentiobiose, D-Turanose, D-Lyxose, D-Tagatose, D-Fructose, L-Fructose, L-Arabitol, D-Arabitol, Gluconate, 2 ceto-gluconate, 5 ceto-gluconate

of the sequence with the database in GenBank by BLAST program, the microorganism was identified with 99% certainty to be *Lb. salivarius* (accession no. AY389803.1). Therefore, the strain was designated as *Lb. salivarius* K7.

Effect of enzymes, pH and heat on bacteriocin stability

CFS was treated with proteolytic enzymes, as showed in Table 5. The completely inactivated inhibitory activity indicated that it is a proteinaceous structure classified as a bacteriocin (Vaughan *et al.*, 2001). The inhibitory substance was heat stable after heat treatment at 100ºC for 30 min. However, when it was heated up to 121ºC (autoclave) for 15 min, the inhibitory activity was decreased to a half of its initial activity. It was found to be stable at the pH range of 4-7, but a higher activity was obtained at the high pH range of 8-10 and at the low pH of 3. It seems that its activities were rather different from other bacteriocins, exhibiting activity at low pH (Messens and De Vuyst, 2002)

Table 5. Effect of enzyme, pH and heat on the bacteriocin activity produced by *Lb. salivarius* K7 against *Lb. sakei* subsp. *sakei* JCM 1157^T.

Treatment	Activity (AU/mL)
Enzyme stability	
Untreated pH 3	800
Untreated pH 5.5	800
Untreated pH7	800
Trypsin	0
Alpha-chymotrypsin	0
Papain	0
Ficin	0
Actinase E	0
Proteinase k	0
Pepsin	0
Protease XIII	0
Heat stability	
100ºC at 5 min	800
100ºC at 30 min	800
121ºC at 15min	400
pH stability	
pH 3	1600
pH 4	800
pH 5	800
pH 5.5	800
pH 6	800
pH 7	800
pH 8	1600
pH 9	1600
pH 10	1600

Purification and structural analysis of bacteriocins

The bioactive fraction from cation-exchange technique was obtained by eluting with 0.25 M NaCl in 20 mM phosphate buffer of pH 5.7. Subsequently, the bioactive fraction was applied to reverse-phase HPLC. The chromatogram (220 nm absorbance) of bacteriocin produced by *Lb. salivarius* K7 revealed the highest active fraction at the retention time of 22 min (Figure 1). This fraction was subsequently designated FK22. This bioactive fraction was directly sequenced by Edman degradation. It showed 20 amino acid residues: Lys-Asn-Gly-Try-Gly-Gly-Ser-Gly-Asn-Arg-Gln-Val-Thr-Glu-Gly-Ala-Gly-Ile-Val-Gly. This amino acid sequence was analyzed by BLAST program in GenBank. The results showed that the amino acid sequence of FK22 was homology to bacteriocin Abp 118 beta (Figure 2) (Flynn *et al*., 2002).

Molecular weight of purified bacteriocin, fraction FK22, was performed using ESI mass spectrometry (Figure 3). The result showed that fraction FK22 corresponded to 4331.70 Da. This value was very similar to bacteriocin Abp 118 beta (4333.80 Da), which was produced by *Lb. salivarius* UCC 118 (Flynn *et al*., 2002).

Structural gene analysis by PCR and DNA sequencing

To obtain the complete amino acid sequence of bacteriocin FK22, its DNA sequence was determined. Based on the similarities of amino acid sequence between bacteriocin fraction FK22 and Abp 118 beta (Flynn *et al*., 20002), the specific primers were designed for PCR amplification of bacteriocin structural gene. Since Abp 118 beta consists of a 2-peptide bacteriocin, with two sets of primers, 118a and 118b were synthesized based on the sequence of bacteriocin Abp 118 alpha and beta (Flynn *et al*., 2002), respectively (Figure 4). After amplification, amplicons 277 bp and 340 bp were obtained by using primer sets 118a and 118b, respectively. The results revealed that nucleotide and amino acid sequences deduced from the nucleotide sequence of 340 bp region showed complete homology to bacteriocin Abp 118 beta (Figure 5,A), whereas 277 bp region showed high homology of 97.5% to Abp 118 alpha. Six nucleotides A, A, A, C, G and G of 277 bp region were different from Abp 118 alpha. These were G, T, T, T, A and A, which are located at position 47, 77, 100, 154, 163 and 190, respectively (Figure 5, B). The result showed that only 10 amino acid sequences at N-terminal deduced from 277 bp region was homology to abp 118 alpha. Flynn *et al*., (2002) reported that the structure of bacteriocin Abp 118 alpha is composed of 45 amino acid residues. It is remarkable that one nucleotide, T, at position 100 of 277 bp region (bold letter) (Figure 5, B) was deleted. Then nucleotide shift became TAG, which was stop codon. However, if one

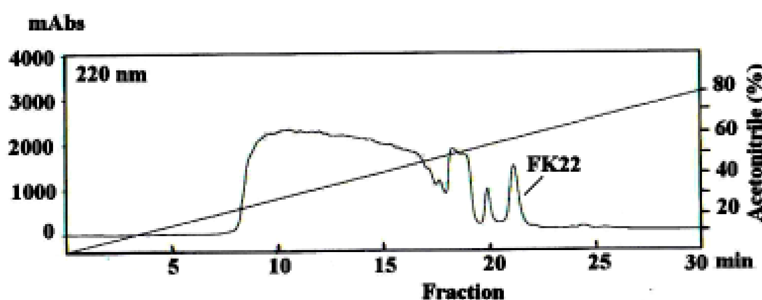

Figure 1. Chromatogram of bacteriocin purification at 220 nm from reverse-phase HPLC.

a) K22
FK22: (Edman degradation) KNGYGGSGNR QVTEGAGIVG
FK22: (deduced peptides): MKNLDKRFTIMTEDNLASVNGG KNGYGGSGNR WVHCGAGIVG GALIGAIGGP WSAVAGGISG GF TS CR
Abp118 beta: MKsNLDKRFTIMTEDNLASVNGG KNGYGGSGNR WVHCGAGIVG GALIGAIGGP WSAVAGGISG GF TS CR

b) 277 bp bacteriocin (*Lb. salivarius* K7)
277 bp bacteriocin: (deduced peptides): MMKETVLTECELAKVDGG KRGPNCVGN F*
Abp 118 alpha: MMKETVLTECELAKVDGG KRGPNCVGN F LGGLFAGAAA GVPLGPAGIV GGANLGMVGG ALTCL

Figure 2. Alignment of amino acid sequences deduced from DNA sequences compare to amino acid sequences in GenBank database

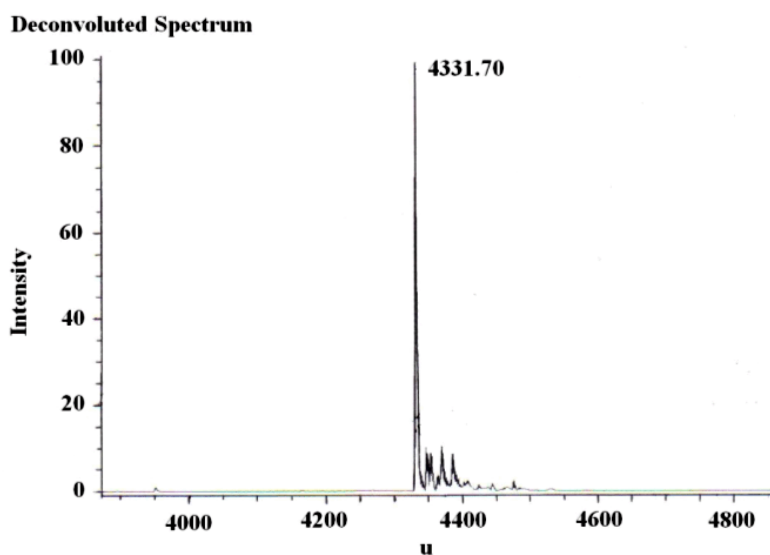

Figure 3. Molecular mass of FK22 determined by ESI mass spectrometry.

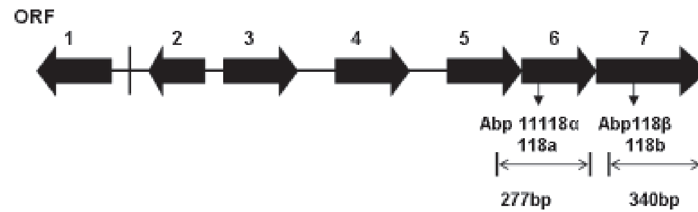

Figure 4. Oligonucleotide primers designed base on gene cluster of bacteriocin Abp 118 alpha and beta.

A) FK22

```
    Primer 118b forward
1   TAGGTGGAGCAAACTTAGGAATGGTAGGCGGAGCGCTTACTTGTTTATAATATAGGGGTG 61

61  ATTTAAGATGAAAAATCTAGATAAAAGATTCACAATTATGACTGAAGATAACTTAGCATC 121
     F  K  M  K  N  L  D  K  R  F  T  I  M  T  E  D  N  L  A  S

121 AGTAAATGGTGGTAAAAATGGTTATGGTGGTAGTGGAAATCGCTGGGTTCACTGTGGAGC 181
     V  N  G  G  K  N  G  Y  G  G  S  G  N  R  W  V  H  C  G  A

181 TGGCATCGTAGGTGGAGCTTTAATTGGAGCTATCGGTGGACCCTGGTCAGCCGTAGCGGG 241
     G  I  V  G  G  A  L  I  G  A  I  G  G  P  W  S  A  V  A  G

241 TGGAATTTCTGGTGGTTTTACAAGTTGCCGTTAAGATGAGTCTAATGAATTAGAAAATAA 301
     G  I  S  G  G  F  T  S  C  R  *

301 GAAATAAATCTATTTTTCTTCAAGTAGTGTCAAGTGTAAG  340
                        Primer 118b reverse
```

B) DNA sequences deduced to amino acid sequences (277 bp bacteriocin produced by *Lb. salivarius* K7)

```
    Primer 118a forward
1   GGATGATTATCATGATGAAGGAATTTACAGTATTGACAGAATGTGAATTAGCAAAGGTTG 61
    D  D  Y  H  D  E  G  I  Y  S  I  D  R  M  *  I  S  K  G  *
     M  I  I  M  M  K  E  F  T  V  L  T  E  C  E  L  A  K  V  D

61  ATGGTGGGAAACGTGGACCTAACTGTGTAGGTAACTTCTAGGTGGTCTATTTGCTGGAGC 121
     W  W  E  T  W  T  *  L  C  R  *  L  L  G  G  L  F  A  G  A
      G  G  K  R  G  P  N  C  V  G  N  F  *  V  V  Y  L  L  E  Q

121 AGCTGCAGGAGTTCCACTTGGACCAGCTGGTATCGTAGGTGGGGCAAACTTAGGAATGGT 181
    A  A  G  V  P  L  G  P  A  G  I  V  G  G  A  N  L  G  M  V
     L  Q  E  F  H  L  D  Q  L  V  S  *  V  G  Q  T  *  E  W  *

181 AGGCGGAGCGCTTACTTGTTTATAATATAGGGGTGATTTAAGATGAAAAATCTAGATAAA 241
    G  G  A  L  T  C  L  *  Y  R  G  D  L  R  *  K  I  *  I  K
     A  E  R  L  L  V  Y  N  I  G  V  I  *  D  E  K  S  R  *  K

241 AGATTCACAATTATGACTGAAGATAACTTAGCATCAG  277
                   Primer 118a reverse
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Figure 5. Amino acid sequences of bactericin produced by *Lb. salivarius* K7 deduced from DNA sequence: A) Amino acid sequences of bacteriocin FK22, B) 277 bp bacteriocin produced by *Lb. salivarius* K7 (The underline shows leader peptides and N-termainal amino acid sequences which produced 10 residues. The italic and bold letters shows frameshift of N-terminal amino acid residues.)

nucleotide, T, was added, the amino acid sequence of 277 bp would be homology to Abp 118 alpha; even six different nucleotides mentioned above did not affect its translation.

This data indicated that *Lb. salivarius* K7 could not produce the complete amino acid sequence of Abp 118 alpha. Dunne *et al*., (1999) reported that CFS of *Lb. salivarius* UCC118 was capable of inhibiting both gram-positive and gram-negative bacteria such as *B. subtilis, B. cereus, B. thuringiensis, E. faecalis, E. feacium, L. monocytogenes, S. aureus* and *Pseudomonas fluorescens*. Although *Lb. salivarius* K7 inhibited some of these gram-positive bacteria, it did not show any activity against gram-negative bacteria (Table 1). This difference of inhibition spectra exhibited by *Lb. salivarius* K7 and *Lb. salivarius* UCC118 seems to result from incomplete amino acid sequence of *Lb. salivarius* K7. In this regard, Ennahar *et al*., (2000) described that the N-terminal beta sheet structure of class IIa bacteriocins is of great significance for bacteriocin-membrane primary interaction. The C-terminal part is the main determinant of the target cell specificity and would also act as the key membrane-recognition region. It also indicated that class IIa bacteriocins with similar C-terminal sequences would display similar spectra of antimicrobial activity. Therefore, we suggested that 277 bp bacteriocin (*Lb. salivarius* K7) and Abp 118 alpha showed different inhibition spectra because of their difference in the C-terminal sequences.

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References

Atrih, A., Rekhif, N., Moir, A.J.G., Lebrihi, A., and Lefebvre, G. 2001. Mode of action, purification and amino acid sequence of plantaricin C19, an anti-Listeria bacteriocin produced by *Lactobacillus plantarum* C19. Inter. J. Food Microbiol., 68: 93-104.

Balla, E., Dicks, L.M.T., Du Toit, M., Van Der Merwe, M.J. and Holzapfel, W.H. 2000. Characterization and cloning of the genes encoding enterocin 1071A and enterocin 1071B, two antimicrobial peptides produced by Enterococcus feacalis BFE 1071. Appl. Environ. Microbiol., 66(4): 1298-1304.

Cataloluk., O. and Gurakan, C.G. 2003. Characterization of salivaricin B, A protein expressed by *Lactobacillus salivarius* M7. Turk. J. Biol., 27: 131-136.

Chagnaud, P., Machinis, K., Coutte, L.A., Marecat, A. and Mercenier, A. 2001. Rapid PCR-based procedure to identify lactic acid bacteria: application to six common *Lactobacillus* species. J. Microbiol. Methods, 44: 139-148.

Christine, F., Vaughan, E.E., De Vos, W.M. and Akkermans, A.D.L. 2002. Molecular monitoring of succession of bacterial communities in human neonates. Appl. Environ. Microbial. 68: 219-226.

Cintas, L. M., Casaus, P., Herranz, C., Havarstein, L. S., Holo, H., Hernadez, P.E. and Nes, I.F. 2000. Biochemical and genetic evidence that *Enterococcus feacium* L50 produces enterocin L50 A and L 50 B, the sec- dependent enterocin P, and a novel bacteriocin secreted without an N-terminal extension termed enterocin Q. J. Bacteriol., 182: 6806-6814.

Dunne, C., Murphy, L., Flynn, S., O'Mahony, L., O' Halloran, S., Feeney, M., Morrissey, D., Thornton, G., Fitzgerald, G., Daly, C., Kiely, B., Quigley, E.M.M., O' Sullivan, G.C., Shanahan, F. and Collins, J.K. 1999. Probiotics: from myth to reality. Demonstration of functionality in animal models of disease and in human clinical trial. Antonie van Leeuwenhoek, 76: 279-292.

Ennahar, S., Zendo, T., Sonomoto, K. and Ishizaki, A.1999. Investigation of bacetriocin production

and purification from *Nukadoko* Isolates displaying anitimicrobial activity. Jap. J. Lactic Acid Bacteria, 10: 29-36.

Ennahar, S., Sashihara, T., Sonomoto, K. and Ishizaki, A. 2000. Class II bacteriocins: biosynthesis, structure and activity. FEMS Microbiol. Rev., 24: 85-106.

Flynn, S., Sinderen, D.V., Thornton, G. M., Holo, H., Nes, I.F. and Collins, J.K. 2002. Characterization of the genetic locus responsible for the production of ABP-118, a novel bacteriocin produced by the probiotic bacterium *Lactobacillus salivarius* subsp. salivarius UCC118. Microbiol., 148: 973-984.

Forbes, B.A., Sahm, D.F. and Weissfeld, A.S. 1998. Bailay and Scott's Digostic Microbiology. 10th ed. Mosby, Inc., Missouri. 1074 pp.

Kandler, O. and Weiss, N. 1986. Regular, nonsporing gram-positive rods, pp 1208-1233. **In**: Sneath, P.H.A., Mair, N.S., Sharp M.E and Holt, J.G. (eds.). Bergey's Manual of systematic Bacteriology, vol. 2. The Williams and Wilkins Co., Baltimore.

Martinez-Murcia, A. J., Acinas, S. G. and Rodriguez-Valera, F. 1995. Evaluation of prokaryotic diversity by restrictase digestion of 16S rDNA directly amplified from hypersaline environments. FEMS Microbiol. Ecol. 17: 247-256.

Messens, W. and De Vuyst, L. 2002. Inhibitory substances produced by *Lactobacilli* isolated from sourdoughs - a review. Int. J. Food Microbiol., 72: 31-43.

O' Sullivan, L., Ross, R.P. and Hill, C. 2002. Potential of bacteriocin-producing lactic acid bacteria for improvements in food safety and quality. Biochemie, 84: 593-604.

Parente, E., Brienza, C., Moles, M. and Ricciardi, A. 1995. A comparison of methods for the measurement of bacteriocin activity. J. Microbiol. Methods, 22: 95-108.

Parente, E., and Ricciardi, A.1999. Production, recovery and purification of bacteriocins from lactic acid bacteria. App. Microbiol. Biotechnol., 52: 628-638.

Salminen, S., Isolauri, E. and Salminen, E. 1996. Clinical uses of probiotics for stabilizing the gut mucosal barrier: successful strains and future challenges. Antonie Van Leeuwenhoek, 70: 251-262.

Sambrook, J.L., Fritsch, E.F. and Maniatis, T. 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor. N.Y.

Soomro, A.H., Masud, T. and Anwaar, K. 2002. Role of lactic acid bacteria (LAB) in food preservation and human health - a review. Pakistan J. Nutrition, 1(1): 20-24.

Uteng, M., Hauge, H.H., Brondz, I., Meyer, J.N. and Fimland, G. 2002. Rapid two-step procedure for large-scale purification of pediocin-like bacteriocins and other cationic antimicrobial peptides from complex culture medium. Appl. Environ. Microbiol., 68: 952-956.

Vaughan, A., Eijsink, V.G.H., O' Sullivan, E.F., O' Hanlon, K. and Van Sinderen, D. 2001. An analysis of bacteriocins produced by lactic acid bacteria isolated from malted barley. J. Appl. Microbiol., 91: 131-138.