Food Sci. Biotechnol. Vol. 14, No. 1, pp. 49 ~ 57 (2005)

Food Science and Biotechnology

Characterization of Bacteriocin Produced by *Enterococcus faecium* MJ-14 Isolated from *Meju*

Sung-Mee Lim[1], Mi-Yeon Park* and Dong-Suck Chang

Department of Food Science and Technology, Pukyong National University, Busan 608-737, Korea
[1]Department of Hotel Culinary Arts, Tongmyong College, Busan, 608-740, Korea

Abstract *Enterococcus faecium* MJ-14, having strong antilisterial activity, was isolated from Korean fermented food, *Meju*. MJ-14 showed the same phenotypic characteristics, but different sugar utilization, as reference strain, *E. faecium* KCCM12118. It could utilize D-xylose, amygdaline, and gluconate, whereas *E. faecium* KCCM12118 could not. Optimal condition for bacteriocin production by *E. faecium* MJ-14 was at 37°C and pH 7.0. Bacteriocin activity appeared in mid exponential phase and increased rapidly up to stationary phase. Activity was significantly promoted in MRS broth containing 3.0% glucose, 1.5% lactose, 2.0% peptone, or 1.5% tryptone. Bacteriocins effectively inhibited *Enterococcus faecalis* and *Listeria* spp. of Gram-positive bacteria, and *Helicobacter pylori* of Gram-negative bacteria, but did not inhibit yeasts and molds. They were stable against heat (for 30 min at 100°C), pH (3.0-9.0), long-term storage (for 60 days at 4 or -20°C), and enzymatic digestion by catalase, proteinase K, papain, lysozyme, trypsin, chymotrypsin, and lipase, etc. Bacteriocin activity was completely inhibited by protease and pepsin, and 50% by α-amylase. Studies on PCR detection of enterocin structural genes revealed bacteriocins are identical to enterocins A and B.

Key words: *Meju*, Bacteriocin activity, *Listeria* spp., *Helicobacter pylori*

Introduction

In human intestinal lactic acid bacteria (LAB) are closely associated with the host's health, because they are an important biodefense factor in preventing colonization of pathogenic bacteria in the intestine (1). Therefore, some species of LAB have been claimed as probiotics, a live microbial feed supplement beneficially affecting the host animal by improving its intestinal microbial balance (2).

LAB are involved in the production of fermented foods, representing virtually all commodity groups including dairy, vegetable, fruit, meat, and cereal products, because they carry out this function by desirably altering the flavor, texture, and appearance of raw commodities. Preservation of fermented foods by LAB is primarily due to sugars being converted into organic acids, additionally causing a reduction in pH. LAB produce antimicrobial substances including hydrogen peroxide, diacetyl, metabolic products, and bacteriocin, which have potential to inhibit a variety of other microorganisms (3). Bacteriocins are ribosomally synthesized and extracellularly released peptides with antibacterial activities generally against bacteria closely related to the producer strains (4). The modes of action of bacteriocin elucidated to date include inhibition of cell wall formation, formation of pores in the cell membrane, resulting in the disruption of membrane potential with eventual lysis of the cell, and, finally, inhibition of nuclease activity involving RNase and/or DNase activity (5, 6). However, bacteriocin-producing strains are protected from the lethal action of their own products due to the mechanism of action of specific immunity proteins (7).

Many bacteriocins of LAB are active against many Gram-positive bacteria associated with food spoilage and food-borne pathogens such as *Listeria monocytogenes*, *Clostridium perfringens*, *Bacillus cereus*, and *Staphylococcus aureus*. Recently, bacteriocins, with a broad inhibitory spectrum of activity including unrelated species, have been shown to display both antiviral and antifungal activities *in vitro*, and also effective in experimental infections with multidrug-resistant strains (8).

Because food safety has become an increasingly important international concern, the application of antimicrobial peptides from LAB that target food pathogens without other adverse effects has received great attention. Because these peptides are degraded by the proteolytic enzymes of the gastrointestinal tract and appear to be nontoxic and nonantigenic to animals, they thus can be used to enhance the safety and shelf life of a variety of foods (9). For example, nisin, a bacteriocin produced by strains of *Lactococcus lactis* subsp. *lactis* is the only one to date that has been found with practical application in food technology; it is permitted as a food additive in approximately 50 countries. Much of the renewed interested in these substances appears to be a direct response to the perceived potential practical applications of these agents either to preservation of foods or to the prevention and treatment of bacterial infections (10, 11).

In this study a strain of *E. faecium* MJ-14 having specific antibacterial spectrum was isolated from *Meju*, and the bacteriocin produced by *E. faecium* MJ-14 was partially characterized to assess its use as a natural preservative in food production.

Materials and Methods

Media and reagent All media were purchased from Difco Co. (Sparks, MD, USA). The API CH 50 kit was obtained from bioMërieux Co. (Marcy I'Etoile, France)

*Corresponding author: Tel: 82-51-620-6414; Fax: 82-51-622-9248
E-mail: mypark@mail1.pknu.ac.kr
Received August 5, 2004; accepted October 26, 2004

and reagents and enzymes from Sigma-Aldrich (St. Louis, MO, USA). Horse serum was purchased from GIBCO (Gaithersburg, USA).

Indicator bacteria Gram-positive bacteria (*Bacillus* spp. *Lactobacillus* spp., *Leuconostoc* spp., *Enterococcus* spp., *Listeria* spp., *Streptococcus* spp.), Gram-negative bacteria (*Enterobacter* spp., *Eschericha coli*, *Helicobacter pylori*, *Salmonella* spp., *Vibrio* spp.), and some yeasts and molds were used as indicator microorganisms for evaluation of antimicrobial activity. All strains were obtained from American Type Culture Collection, Korean Collection for Type Cultures, and Korean Culture Center of Microorganisms, and propagated in brain heart infusion (BHI) broth at 37°C. *H. pylori* was cultured in BHI containing 10 % horse serum at 37°C for 48 hr under microaerobic condition (10% O_2) (anoxomat system, MART, Lichtenvoorde, Netherlands). Mold and yeast were cultured in potato dextrose agar (PDA) and YM agar at 25°C, respectively.

Isolation of bacteriocin-producing LAB *Meju* was purchased from local oriental grocery stores in Kyungnam and Busan. All samples were shredded using a sterilized knife and homogenized with sterile PBS for 5 min in a blender. Appropriate dilutions were prepared with PBS, and 1 mL each of serial 10-fold dilutions was spread onto MRS agar plate containing 1.0% $CaCO_3$. Colonies forming clear zone on the plate were selected, among which bacteriocin-producing strains were examined by the spot-on-lawn method (12). The culture supernatant (10 μL) of the selected strains was spotted onto a BHI agar plate (0.7% w/v) seeded with active growing cells (10^6 CFU/mL) of an indicator bacterium (*L. monocytogenes* KCTC 3569). After incubation at 37°C for 24 hr, the plates were observed for appearance of inhibitory zones around the colonies. Selected strain was characterized by physiological and biochemical tests according to the criteria of Bergey's Manual of Systematic Bacteriology (13) and The Prokaryotes (14). Cell morphology was observed by a optical microscope (DW-THN, DONGWON, Busan, Korea). Carbohydrate fermentation patterns were determined using the API 50 CH system (BioMerieux, Marcy lEtoile, France) according to the manufacturer's instructions.

Bacteriocin activity assay Critical dilution method for bacteriocin activity assay was conducted as modified by Pucci *et al.* (15). Culture supernatant was serially diluted, and 50 μL of each dilution was loaded onto a paper disk in a plate seeded with the bacterial suspension (10^6-10^7 CFU/mL). After incubation at 37°C for 18 hr, the bacteriocin activity was defined as the reciprocal of the highest dilution fold that produced a distinct inhibition zone against the indicator bacterium, and was multiplied by 20 (1 mL/50 μL) to obtain the bacteriocin units (BU) per mL.

Determination of optimal conditions for bacteriocin production

Temperature and pH: Cell growth and the bacteriocin activity in MRS broth, pH adjusted in the range of 5.0 to 8.0, were examined at 15, 25, 37, and 45°C for 40 hr. The cell growth was monitored turbidimetrically at 600 nm using a spectrophotometer (UV 1601, Shimazu, Kyoto, Japan), and bacteriocin activity was determined at 2 hr intervals by critical dilution method with *L. monocytogenes* KCTC3569 as an indicator bacterium.

Carbon and nitrogen sources: The influences of supplementing carbon and nitrogen sources on cell growth and bacteriocin activity were investigated in MRS broth for 12 hr at 37°C. The nutrients tested were as follows: carbon sources were glucose (2.0 to 4.0%), galactose (0 to 2.0%), lactose (0 to 2.0%), and maltose (0 to 2.0%), and nitrogen sources were peptone (1.0 to 3.0%), tryptone (0 to 2.0%), beef extract (0 to 2.0%), and yeast extract (0.5 to 2.5%).

Spectrum of antagonistic activity Spectrum of antagonistic activity was tested against a wide spectrum of LAB as well as food-borne pathogens, yeast, and mold by agar diffusion method (16). Briefly, indicator microorganisms were swabbed onto the surface of an agar plate, and wells (diameter, 8 mm) were made by punching into the agar. The wells were filled with culture supernatant, and the plate was incubated for 24 hr at temperatures suitable for the growth of indicator microorganisms as described earlier and examined for clear zones of inhibition.

Stability of bacteriocin against various treatments

Enzymes Culture supernatant (2 mL) was mixed with the following enzyme solution (2 mL) at a final concentration of 1 mg/mL: catalase (10 mM potassium phosphate, pH 7.0), pepsin (10 mM citrate, pH 2.0), α-amylase (50 mM sodium acetate, pH 6.0), proteinase K (50 mM Tris-HCl, pH 7.5), protease (50 mM Tris-HCl, pH 7.5), papain (10 mM potassium phosphate, pH 7.0), lysozyme (50 mM Tris-HCl, pH 7.0), lipase (50 mM Tris-HCl, pH 7.5), trypsin and chymotrypsin (50 mM Tris-HCl, pH 8.0). After incubation at 37°C for 1 hr, the enzyme was inactivated by heating for 10 min at 80°C.

Heating Culture supernatant (5 mL) was exposed to heat treatment (60 min at 50 and 70°C, 30 min at 100°C, and 15 min at 121°C).

pH Culture supernatant adjusted to various pH values (pH 2-10) was incubated for 1 hr at 37°C. After pH treatment, the reaction mixture was brought to neutral pH (6.5) and tested for residual the bacteriocin activity.

Storage To test the stability during storage, culture supernatant were stored for 60 days at -20, 4, and 25°C. After each treatment, residual the bacteriocin activity was determined by the critical dilution method using *L. monocytogenes* KCTC3569 as an indicator bacterium.

Polymerase chain reaction (PCR) detection of enterocin structural genes PCR amplification of known structural genes of enterocins A, B, P, L50A, and L50B (17) was performed with the specific primers listed in Table 1. The enriched culture solution was centrifuged at 9,000 × g for 5 min (HERMLE Z320, National Labnet Co., Woodbridge, NJ, USA), and the precipitate was resuspended with distilled water. The suspension was heated for 10 min in boiling water and recentrifuged. The obtained supernatant was used as a template DNA.

Table 1. Specific forward and reverse primers for the PCR detection of enterocin structural genes

Name	Forward primer	Reverse primer
Enterocin A	5'-GGTACCACTCATAGTGGAAA-3'	5'-CCCTGGAATTGCTCCACCTAA-3'
Enterocin B	5'-CAAAATGTAAAAGAATTAAGTACG-3'	5'-AGAGTATACATTTGCTAACCC-3'
Enterocin P	5'-GCTACGCGTTCATATGGTAAT-3'	5'-TCCTGCAATATTCTCTTTAGC-3'
Enterocin L50A	5'-ATGGGAGCAATCGCAAAATTA-3'	5'-TTTGTTAATTGCCCATCCTTC-3'
Enterocin L50B	5'-ATGGGAGCAATCGCAAAATTA-3'	5'-TAGCCATTTTTCAATTTGATC-3'

Reaction mixture for PCR was constituted with template DNA (4 μL), enterocin primer (20 pmol each), premix (10 μL, Takara Co., Otsu, Japan), and distilled water (2 μL). The reaction mixture was denatureated for 5 min at 95 and recycled 30 times for 30 sec at 95°C, 30 sec at 58, and 30 sec at 72°C. The amplified gene was electrophoresed with 1% agarose gel (40 mA, 100V). The gel was stained with ethidium bromide (EtBr), and the amplified gene was identified with UV transilluminator (VILBER LOURMAT TFX-20M, Sigma Co., St, Louis, MO, USA).

Results and Discussion

Identification of a bacteriocin-producing strain A total of 307 bacteriocin-producing LAB strains were isolated from Korean fermented food, *Meju*. Among the 11 strains showing inhibition activity against *L. monocytogenes* KCTC3569, MJ-14 was selected due to its strong antilisterial activity (results not shown). MJ-14, which showed the same phenotypic characteristics as the reference strain, *E. faecium* KCCM12118 (Table 2), was a catalase-negative, Gram-positive, facultatively anaerobic coccus with the ability to grow at 10-45°C, pH 5.0-9.0, and was tolerant at 5% salt. The strain fermented glucose into L-lactic acid and did not produce gas, but produced ammonia from the hydrolysis of arginine. In addition, it did not show urease and hemolysin activities on horse and sheep bloods. The strain also fermented D-xylose, amygdaline, and gluconate, whereas *E. faecium* KCCM1211d could not (Table 2). Although the results of API 50 CH bacterial identification kit showed differences in sugar utilization between the two strains, the authors tentatively identified the strain MJ-14

Table 2. Phenotypic characteristics and the utilization of various sugars of *E. faecium* MJ-14

Contents	MJ-14	KCCM 12118	Sugar	MJ-14	KCCM 12118	Sugar	MJ-14	KCCM 12118
Cell shape	Cocci	Cocci	Glycerol	-	-	Salicine	+	+
Gram staining	+[1)]	+	Erythritol	-	-	Cellobiose	+	+
Spores staining	-[2)]	-	D-Arabinose	-	-	Maltose	+	+
Acid-fast staining	-	-	L-Arabinose	+	+	Lactose	+	+
Motility	-	-	Ribose	+	+	Melibiose	-	-
Gas from glucose	-	-	D-Xylose	+	-	Saccharose	+	+
H_2S production	-	-	L-Xylose	-	-	Trehalose	+	+
Lactic acid	L[3)]	L	Adonitol	-	-	Inuline	-	-
Nitrate reduction	-	-	α-Methyl-xyloside	-	-	Melezitose	-	-
Methyl red	+	+	Galactose	+	+	D-Raffinose	-	-
Voges-Proskauer	-	-	D-Glucose	+	+	Amidon	-	-
Horse blood hemolysis	-	-	D-Fructose	+	+	Glycogene	-	-
Sheep blood hemolysis	-	-	D-Mannose	+	+	Xylitol	-	-
Catalase	-	-	L-Sorbose	-	-	β-Gentiobiose	+	+
Oxidase	-	-	Rhamnose	-	+	D-Turanose	-	-
Urease	-	-	Dulcitol	-	-	D-Lyxose	-	-
Arginine hydrolysis	+	+	Inositol	-	-	D-Tagatose	-	-
Growth in aerobic condition	+	+	Mannitol	+	+	D-Fucose	-	-
anaerobic	+	+	Sorbitol	-	-	L-Fucose	-	-
Growth at 10-45°C	+	+	α-Methyl-D-Mannoside	-	-	D-Arabitol	-	-
Growth at pH 5.0-9.0	+	+	α-Methyl-D-glucoside	-	-	L-Arabitol	-	-
10.0	-	-	N-Acetyl glucosamine	+	+	Gluconate	+	-
Growth in 1-5% NaCl	+	+	Amygdaline	+	-	2-ceto-gluconate	-	-
10% NaCl	-	-	Arbutine	+	+	5-ceto-gluconate	-	-
			Esculine	+	+			

[1)]Positive reaction.
[2)]Negative reaction.
[3)]Configuration of lactic acid produced from glucose.

as *E. faecium* MJ-14 according to phenotypic characteristics and T-value in API 50 CH results.

Siragusa (18) reported that a bovine intestinal bacterial isolate, identified as *E. hirae*, produced a bacteriocin against *Listeria* spp. Sabia *et al.* (19) reported that *E. casseliflavus* IM 416K1 showed the strongest antagonistic activity against *L. monocytogenes*. Parente and Hill (20) reported that *E. faecium* DPC1146 showed inhibitory activity against *L. innocua* and *L. monocytogenes*. Furthermore, *Lactococcus lactis* subsp. *lactics* (21), *Leuconostoc mesenteroides* subsp. *mesenteroides*, *Leuconostoc paramesenteroides*, and *Pediococcus pentosaceus* (22) were reported to produce bacteriocins against *L. monocytogenes*. These results are in accordance with ours and thus demonstrate that *Enterococcus* spp. could produce bacteriocins that have specific inhibitory activities against *Listeria* spp.

Optimal conditions for bacteriocin production To determine the optimal production conditions of bacteriocin produced by *E. faecium* MJ-14, temperature, pH, and carbon and nitrogen sources optimal for the bacterial growth and bacteriocin activity in the culture supernatant during its growth were investigated. The growth of *E. faecium* MJ-14 was better at 37°C than those at 15, 25, and 45°C. *E. faecium* MJ-14 showed 640 BU/mL at 37°C, while did not show bacteriocin activity for 40 hr at 15°C. At 25°C, the cell growth was normal, but showed bacteriocin activity 50% (320 BU/mL) of those at 37°C. And *E. faecium* MJ-14 showed weak bacteriocin activity at 45°C. The bacteriocin activity appeared in mid exponential phase and showed maximum at stationary phase (Fig. 1).

E. faecium MJ-14 showed 640 BU/mL at pH 6.0 and 7.0, while no activating at pH 5.0. At pH 8.0, the cell growth was normal, but bacteriocin activity was half (320 BU/mL) of those at pH 6.0 and 7.0. The bacteriocin activity appeared in mid exponential phase and increased rapidly up to stationary phase, and was most stable at pH 7.0 because maximum activity was maintained longer than at pH 6.0 (Fig. 2). These results agreed with those obtained by Biswas *et al.* (23) and Parente *et al.* (24). Biswas *et al.* (23) reported that pediocin production of *P. acidilactici* was highest in TGE broth at pH 6.5 at 30 or 37°C, and Parente *et al.* (24) reported that bacteriocin production by *Lactococcus lactis* subsp. *lactis* 140NWC was maximal between pH 6.0-6.5.

Effects of carbon (glucose, galactose, lactose, and maltose) and nitrogen (peptone, tryptone, and beef and yeast extracts) sources on the bacteriocin production were investigated. The bacteriocin activity was significantly promoted in MRS broth containing 3.0% glucose or 1.5% lactose compared with 640 BU/mL of the control (no addition), while galactose and maltose had almost no effect (Fig. 3). Among the nitrogen sources, 2.0-2.5% peptone and 1.5% tryptone greatly promoted the activity, whereas other sources were weakly affected (Fig. 4).

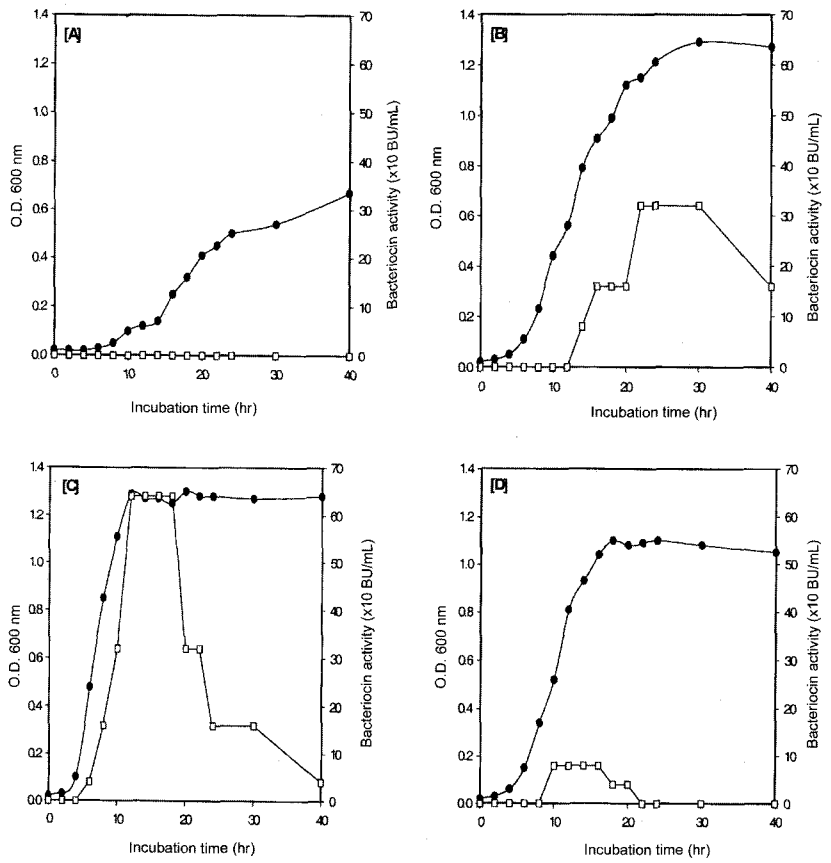

Fig. 1. Effects of incubation temperature on the production of the bacteriocin by *E. faecium* MJ-14. *E. faecium* MJ-14 was cultured at 15°C [A], 25°C [B], 37°C [C], and 45°C [D] in MRS broth, respectively. - ● - Bacterial growth; - □ -, Bacteriocin activity.

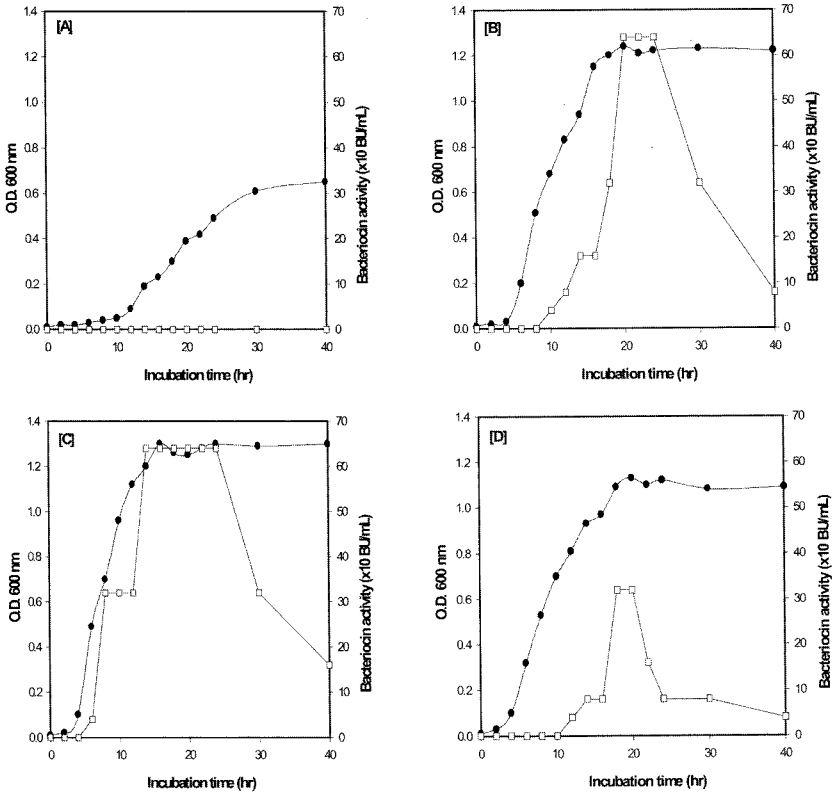

Fig. 2. Effects of initial pH on the production of the bacteriocin by *E. faecium* MJ-14. *E. faecium* MJ-14 was cultured at pH 5.0 [A], pH 6.0 [B], pH 7.0 [C], and pH 8.0 [D] in MRS broth at 37°C. - ● - Bacterial growth; - □ -, Bacteriocin activity.

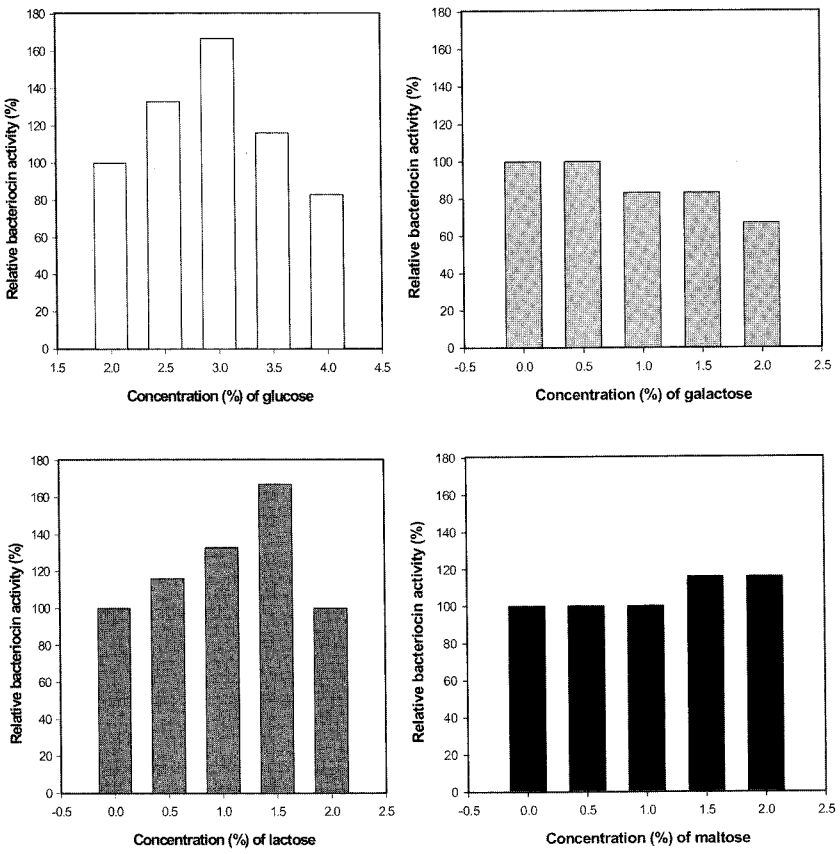

Fig. 3. Effects of carbon sources on the production of the bacteriocin by *E. faecium* MJ-14. The *E. faecium* MJ-14 was cultured at 37°C for 12 hr in MRS added carbon sources and bacteriocin activity was determined by critical dilution method.

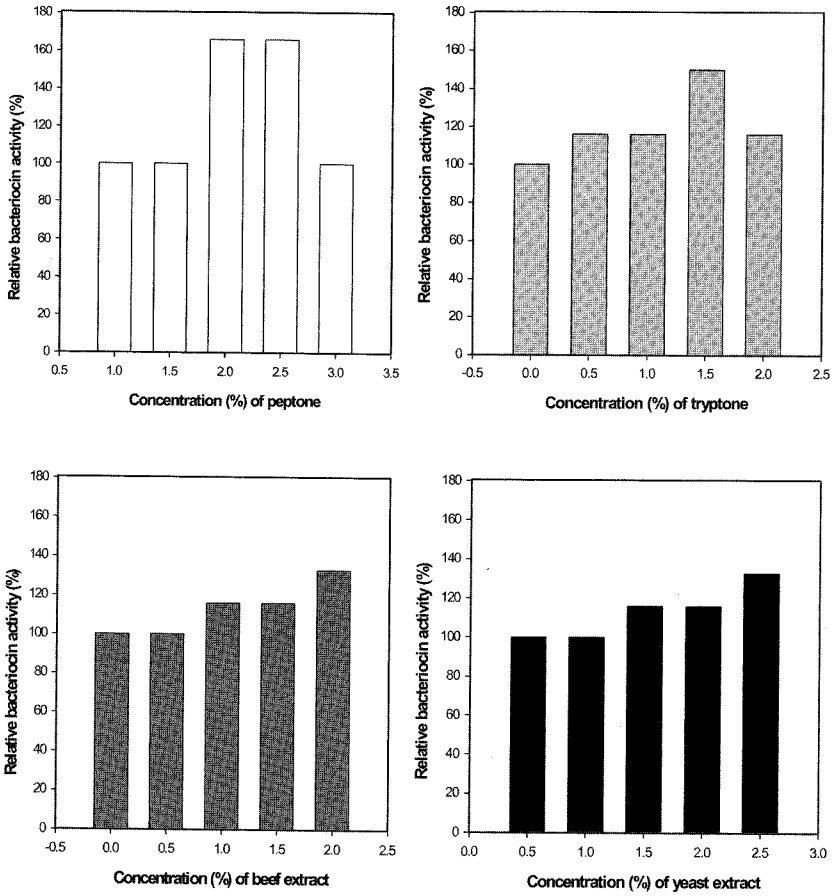

Fig. 4. Effects of nitrogen sources on the production of the bacteriocin by *E. faecium* MJ-14. The *E. faecium* MJ-14 was cultured at 37°C for 12 hr in MRS added nitrogen sources and bacteriocin activity was determined by critical dilution method.

Biswas *et al.* (23) reported pediocin production was stimulated with glucose and yeast extracts at 1.0% level in TGE broth, but not at 2.0% concentrations of these nutrients. Ogunbanwo *et al.* (25) reported that bacteriocin by *Lactobacillus brevis* OG1 was promoted by the addition of yeast extracts (3.0%) and glucose (1.0%) in the MRS broth. In the present study, the bacteriocin activity was promoted by 3.0% glucose and 1.5% lactose, but was rather suppressed at higher concentrations, in partial agreement with the results of Ogunbanwo *et al.* (25) and Biswas *et al.* (23).

Antagonistic spectrum of the bacteriocin The antagonistic spectrum of bacteriocin produced by *E. faecium* MJ-14 was investigated with various indicator microorganisms (Table 3). The bacteriocin had a narrow antibacterial activity against other *E. faecalis* and *Listeria* sp. of Gram-positive bacteria, and *H. pylori* of Gram-negative bacteria. No effect was observed against bacteria such as *S. aureus, Salmonella* sp., *Vibrio* sp., *Bacillus* sp., yeasts, and molds. The inhibitory spectra of bacteriocins of some *E. faecium* strains were similar to the results in the present study, generally inhibiting other enterococci, clostridia, and *Listeria* spp. (26, 27). Therefore, bacteriocin with a specific inhibitory spectrum against related LAB and food-borne pathogens such as *Listeria* spp. may be of interest, especially for use with resistant starter cultures. *L. monocytogenes* has been found in raw and ready-to-eat meat products, poultry, seafood, vegetables, and other foods (28, 29). If *E. faecium* MJ-14 is employed in meat and dairy fermentations, the bacteriocin would not adversely affect the lactic acid starter cultures, while selective inhibition against *L. monocytogenes* could occur.

Stability of bacteriocin against various treatments Effects of heat, pH, long term storage, and various enzymes on the bacteriocin activity of *E. faecium* MJ-14 against *L. monocytogenes* KCTC3569 as an indicator bacterium were investigated (Table 4). Although the bacteriocin activity was only 25% of the control after 15 min at 121°C, it remained constant after heat treatment for 30 min at 100°C. Heat stability of bacteriocin could be due to the formation of small globular structures and the occurrence of strongly hydrophobic regions, and stable cross-linkage (30).

The bacteriocin activity of *E. faecium* MJ-14 was also stable at a wide pH range, between pH 3.0-9.0. These results indicate that the bacteriocin can be useful in pasteurized food as well as acidic and non-acidic foods.

The bacteriocin activity was completely inhibited by protease or pepsin, and decreased 50% by α-amylase, whereas proteinase K, papain, lipase, lysozyme, trypsin, and chymotrypsin as well as catalase showed no effect, which confirmed that none of the inhibitory compounds were hydrogen peroxide. Therefore, the bacteriocin would be degraded by the proteolytic enzymes of the gastro-

Table 3. Antimicrobial spectrum of the bacteriocin produced by *E. faecium* MJ-14

Microorganisms	Indicator species	Inhibition zone[1)] (mm)
Gram-positive bacteria	*Bacillus cereus* ATCC 11778	-
	B. licheniformis KCTC 1918	-
	B. stearothermophilus ATCC 10149	-
	B. subtilis ATCC 35421	-
	Lactobacillus acidophilus KCTC 3168	-
	L. brevis KCTC 3102	-
	L. casei ATCC 25302	-
	L. paracaei ATCC 25302	-
	L. plantarum KCTC 1048	-
	Leuconostoc mesenteroides KCTC 3719	-
	Enterococcus faecalis KCTC 3206	+++
	Listeria monocytogenes KCTC 3569	+++
	L. innocua ATCC 33090	+++
	L. ivanovii ATCC 19119	+++
	Streptococcus lactis ATCC 1913	-
	Stapylococcus aureus ATCC 6538	-
Gram-negative bacteria	*Enterobacter aerogenes* ATCC 13480	-
	Escherichia coli ATCC 11229	-
	Escherichia coli O157 ATCC 43889	-
	Helicobacter pylori KCCM 41351	++
	Salmonella enteritidis ATCC 13076	-
	Sal. typhimurium KCTC 2514	-
	Vibrio parahaemolyticus KCTC 2471	-
	V. vulnificus KCTC 2982	-
molds	*Aspergillus oryzae* KCTC 6983	-
	Penicillium roqueforti KCCM 11269	-
yeasts	*Candida albicans* KCTC 7965	-
	Saccharomyces cerevisiae KCTC 7246	-
	Kluyveromyces marxianus KCCM 35458	-

[1)]+, 9-10 mm; ++, 11-15 mm; +++, more than 16 mm.

Table 4. Physico-chemical stability of the bacteriocin produced by *E. faecium* MJ-14

Treatment		Relative bacteriocin activity (%)
Control		100
Enzyme	Catalase	100
	Pepsin	0
	α-amylase	50
	Proteinase K	100
	Protease	0
	Papain	100
	Lysozyme	100
	Lipase	100
	Trypsin	100
	Chymotrypsin	100
Heating	50°C, 60min	100
	70°C, 60min	100
	100°C, 30min	100
	121°C, 15min	25
pH	2.0	25
	3.0-9.0	100
	10.0	50
Storage for 60 days	-20°C	100
	4°C	100
	25°C	50

intestinal tract and seem to be nontoxic to animals.

The bacteriocin activity was quite stable after storage for 60 days at -20 and 4°C, while at 25°C was significantly decreased. The bacteriocin of *E. faecium* MJ-14, therefore, can be used to inhibit the growths of psychrotrophic spoilage and food-borne pathogenic bacteria such as *L. monocytogens* in refrigerated foods.

Effects of physical and chemical treatments on bacteriocin activity were also reported by Laukova *et al.* (16). The bacteriocin activity produced by enterococci isolated from cow dung water was not affected by catalase, and decreased by hydrolytic enzymes. In addition, the activity was also retained after 30 min at 100°C. Arihara *et al.* (31) reported that the enterococcal bacteriocin was sensitive to several proteolytic enzymes, but stable against heat treatment (121°C for 20 min) and at a wide pH range (3.0-10.0). These results corresponded with those of the present study. Recently, consumers demand for more natural and minimally processed food has increased. From this point of view, bacteriocin has been shown to be safe and has potential as an effective natural food preservative, because it undergoes enzymatic digestion in gastro-intestinal environment without toxic or other adverse effects.

PCR detection of enterocin structural genes After

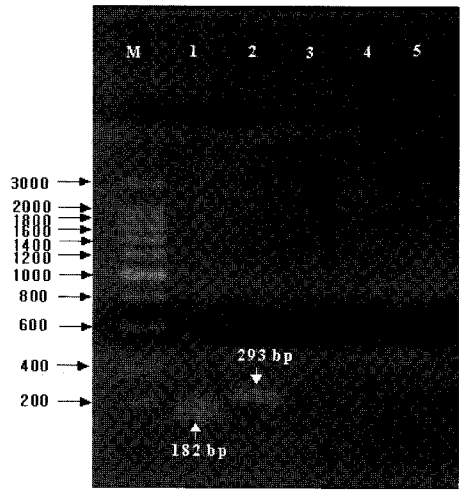

Fig. 5. PCR products with different enterocin primers. M, 200 bp ladder marker (Takara, Co.,). 1, PCR products with enterocin A primers. 2, PCR products with enterocin B primers. 3, PCR products with enterocin P primers. 4, PCR products with enterocin L50A primers. 5, PCR products with with enterocin L50B primers.

agarose gel electrophoresis, PCR products of about 182 and 293 bp, were amplified from the DNA of *E. faecium* MJ-14 with primers for enterocin A and B. However, no signals were obtained that corresponded with the enterocins P, L50A, and L50B (Fig. 5). PCR products of about 130, 160, and 120 bp were amplified from the DNA of *E. faecium* JCM 5804 with primers for enterocin A, B, and P (32). Herranz *et al.* (33) reported that agarose gel electrophoresis of PCR products allowed the visualization of two amplification bands of these sizes, suggesting that *E. faecium* P21 contains *ent* A (172 bp) and *ent* B (126 bp). In addition, the enterocins L50A and L50B were exclusively found among *E. faecium* (34).

References

1. Gilliand SE. Health and Nutrition benefits from lactic acid bacteria. FEMS Microbiol. Rev. 87: 175-178 (1990)
2. Fuller R. Probiotics in man and animals. J. Appl. Bacteriol. 66: 365-371 (1988)
3. Lindgren SW, Dobrogosz WJ. Antagonistic activities of lactic acid bacteria in food and feed fermentation. FEMS Microbiol. Rev. 87: 149-164 (1990)
4. Tagg JR, Dajani AS, Wannamaker LW. Bacteriocins of Gram-positive bacteria. Bacteriol. Rev. 40: 722-756 (1976)
5. Maria P. Ribosomally synthesized peptides with antimicrobial properties: biosynthesis, structure, function, and applications. Biotechnol. Adv. 21: 465-499 (2003)
6. Bruno ME, Montville TJ. Common mechanistic action of bacteriocins from lactic acid bacteria. Appl. Environ. Microbiol. 59: 3003-3010 (1993)
7. Tjakko A. Pore-forming bacteriocins of Gram-positive bacteria and self-protection mechanisms of producer organisms. FEMS Microbiol. Lett. 129: 1-10 (1995)
8. Marekova M, Laukova A, DeVuyst L, Skaugen M, Nes IF. Partial characterization of bacteriocins produced by environmental strain *Enterococcus faecium* EK13. J. Appl. Microbiol. 94: 523-530 (2003)
9. Cleveland B, Montville TJ, Nes IF, Chikindas ML. Bacteriocins: safe, natural antimicrobials for food preservation. Int. J. Food Microbiol. 71: 1-20 (2001)
10. Holzapfel WH, Geisen R, Schillinger U. Biological preservation of foods with reference to protective cultures, bacteriocins and food-grade enzymes. Int. J. Food Microbiol. 24: 343-362 (1995)
11. Schillinger U, Geisen R, Holzapfel WH. Potential of antagonistic microorganisms and bacteriocins for the biological preservation of foods. Trends Food Sci. Technol. 7: 158-164 (1996)
12. Mayr HA, Hedges AJ, Berkeley RC. Methods for studying bacteriocin. Vol. 7a, pp. 313-342. In: Methods in Microbiology. NY, Norris JR, Ribbons DW (eds). Academic Press, New York, NY, USA (1972)
13. Mundt JO. Enterococci. Vol. 2, pp. 1063-1065. In: Bergey's Manual of Systematic Bacteriology. Sneath PHA, Mair NS, Sharpe ME, Holt JG (eds). Williams & Wilkins, Baltimore, MD, USA (1986)
14. Devriese LA, Collins MD, Wirth R. The Genus *Enterococcus*. pp. 1465-1481. In: The Prokaryotes 2nd ed. Balows A, Trüper HG, Martin D, Wim H, Karl HS (eds). Springer-Verlag, New York, NY, USA (1992)
15. Pucci MJ, Vedamuthu ER, Kunka BS, Vandergergh PA. Inhibition of *Listeria monocytogenes* by using bacteriocin PA-1 produced by *Pediococcus acidilactici* PAC 1.0. Appl. Environ. Microbiol. 54: 2349-2353 (1988)
16. Laukova A, Czikkova S, Vasilkova Z, Juris P, Marekova M. Occurrence of bacteriocin production among environmental enterococci. Lett. Appl. Microbiol. 27: 178-182 (1998)
17. Vuyst LD, Moreno MRF, Revets H. Screening for enterocins and detection of hemolysis and vancomycin resistance in enterococci of different origins. Int. J. Food Microbiol. 84: 299-318 (2003)
18. Siragusa GR. Production of bacteriocin inhibitory to *Listeria* species by *Enterococcus hirae*. Appl. Environ. Microbiol. 58: 3508-3513 (1992)
19. Sabia C, Manicardi G, Messi P, Niederhausern S, Bondi M. Enterocin 416K1, and antilisterial bacteriocin produced by *Enterococcus casseliflavus* IM 416K1 isolated from Italian sausages. Int. J. Food Microbiol. 75: 163-170 (2002)
20. Parente E, Hill C. Characterization of enterocin 1146, a bacteriocin from *Enterococcus faecium* Inhibitory to *Listeria monocytogenes*. J. Food Prot. 55: 497-502 (1992)
21. Stecchini ML, Aquili V, Sarais I, Pitotti A. Inhibition of *Listeria monocytogenes* by *Lactococcus lactis* subsp. *lactis* isolated from Italian raw ham. J. Food Saf. 12: 295-302 (1992)
22. Kim JH. Inhibition of *Listeria monocytogenes* by bacteriocin(s) from lactic acid bacteria isolated from *Kimchi*. Agric. Chem. Biotechnol. 38: 302-307 (1995)
23. Biswas SR, Purbita R, Johnson MC, Ray B. Influence of growth conditions on the production of a bacteriocin, Pediocin AcH, by *Pediococcus acidilactici* H. Appl. Environ. Microbiol. 57: 1265-1267 (1991)
24. Parente E, Ricciardi A, Addario G. Influence of pH on growth and bacteriocin production by *Lactococcus lactis* subsp. *lactis* 140NWC during batch fermentation. Appl. Microbiol. Biotechnol. 41: 388-394 (1994)
25. Ogunbanwo ST, Sanni AI, Onilude AA. Influence of cultural conditions on the production of bacteriocin by *Lactobacillus brevis* OG1. Afr. J. Biotechnol. 2: 179-184 (2003)
26. Toit MD, Franz CM, Dicks LM, Holzapfel WH. Preliminary characterization of bacteriocin produced by *Enterococcus faecium* and *Enterococcus faecalis* isolated from pig feces. J. Appl. Microbiol. 88: 482-494 (2000)
27. Franz CM, Schillinger U, Holzapfel WH. Produced and characterization of enterocin 900, a bacteriocin produced by *Enterococcus faecium* BFE 900 from black olives. Int. J. Food Microbiol. 29: 255-270 (1996)
28. Kozak J, Balmer T, Byrne R, Fisher K. Prevalence of *Listeria monocytogenes* in foods: Incidence in dairy products. Food Control 7: 215-221 (1996)
29. James MJ. Prevalence of *Listeria* spp. in meat and poultry products. Food Control 7: 209-214 (1996)
30. Jung MY, Paik HD. Identification and partial characterization of lacticin JW3, a bacteriocin produced by *Lactococcus lactis* JW3 isolated from commercial Swiss cheese products. Food Sci. Biotechnol. 9: 116-123 (2000)
31. Arihara K, Robert GC, John BL. Characterization of bacteriocins from *Enterococcus faecium* with activity against *Listeria monocytogenes*. Int. J. Food Microbiol. 19: 123-134 (1993)
32. Park SH, Itoh K, Fujisawa T. Characteristics and identification of enterocins produced by *Enterococcus faecium* JCM 5804. J.

Appl. Microbiol. 95: 294-300 (2003)
33. Herranz C, Casaus P, Mukhopadhyay S, Martinez JM, Rodriguez JM, Nes IF, Hernandez PE, Cintas LM. *Enterococcus faecium* P21: a strain occurring naturally in dry-fermented sausages producing the class ll bacteriocins enterocin A and enterocin B. Food Microbiol. 18: 115-131 (2001)
34. Cintas LM, Casaus P, Holo H, Hernandez PE, Nes IF, Havarstein LS. Enterocins L50A and L50B, two novel bacteriocins from *Enterococcus faecium* L50, are related to staphylococcal hemolysins. J. Bacteriol. 180: 1988-1994 (1998)