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Characterization and Mutational Analysis of a Two-Polypeptide Bacteriocin Produced by Citrus Iyo-Derived *Lactobacillus brevis* 174A

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In the present study, we isolated a lactic acid bacterium (LAB) from a citrus iyo fruit and identified it as *Lactobacillus brevis*. This plant-derived LAB strain, designated 174A, produces bacteriocin consisting of two polypeptides designated brevicin 174A-*β* and 174A-*γ*. Although each polypeptide itself displays antibacterial activity, the ability is enhanced 100 fold by mixing both polypeptides at a 1 : 1 ratio. Significantly, brevicin 174A inhibits even the growth of several pathogenic bacteria that are more resistant to a lantibiotic bacteriocin, nisin A, which is commonly utilized as a preservative added to foodstuffs. Structural analysis of the 174A bacteriocin using a program that predicts secondary structure suggests that both component polypeptides have a positively charged N-terminal region, as well as two cysteine residues in both the N- and C-terminals. Judging from a mutational analysis of the antibacterial polypeptides, these unique amino acid sequences of 174A-*β* might be important for the expression of the synergistic activity that occurs in the presence of the two polypeptides combined.

Key words bacteriocin; *Lactobacillus brevis*; lactic acid bacteria

Bacteriocins, which are polypeptides displaying antibacterial activity and synthesized ribosomally, are produced in many bacterial species including some lactic acid bacteria (LABs).[1] Because of the emergence of multiple drug-resistant pathogenic bacteria and potential undesirable side effects of chemical food additives, there has been great interest in alternative uses of bacteriocin for medicines and the food industry. LAB, which is a typical probiotic, is traditionally utilized in dairy products and other fermented foods. Since LABs, which are non-pathogenic bacteria, have been used for long time to produce many kinds of fermented foods, LABs such as lactobacilli and lactococci are generally recognized as safe (GRAS) organisms, and they have attracted considerable attention for their industrial importance lately.[2]

We have previously isolated and identified more than 600 LAB strains from many kinds of plant sources such as vegetables, fruits, flowers, and medicinal plants. In the plant-derived LAB library, it has been demonstrated that some strains produce bacteriocins, enhance intestinal immunity, or improve constipation and liver function.[3–6] With these studies, we have shown that plant-derived LABs are superior to animal-derived LABs used to produce yoghurt and cheese, especially, from the viewpoint of tolerance to gastric and bile acids, immune-stimulating activity, and intestinal regulation action, not only in animals but also in humans.

In the present study, we isolated a LAB strain from citrus iyo and designated it the 174A strain. The strain, which produces a bacteriocin designated brevicin 174A, was identified as *Lactobacillus* (*Lb.*) *brevis* by determining the entire sequence of the 16S ribosomal RNA (rRNA)-endoding gene and the carbohydrate utilization. Brevicin 174A inhibits the growth of not only the closely related LAB but also pathogenic bacteria, like *Staphylococcus* (*S.*) *aureus*, *Listeria* (*L.*) *monocytogenes*, and *Streptococcus* (*S.*) *mutans*.

Bacteriocins are classified into two main classes, which are designated class I and class II.[2,7,8] The latter class bacteriocins are further grouped into four subclasses, IIa, IIb, IIc, and IId. The class IIb-type bacteriocins, which are composed of two different peptides, exhibits the synergistic antibacterial activity when equal amounts of both complementary peptides are present.[7,8] Some of the class IIb bacteriocins hardly display the antibacterial activity when the component peptides are individually used. However, the two polypeptide components of brevicin 174A, named brevicin 174A-*β* and 174A-*γ*, display antimicrobial activity at sub-micro molar concentrations. Furthermore, the antibacterial activity of mixture of both polypeptides increases about 100 times higher than that of individual polypeptide. Interestingly, the mixture inhibits the growth of some pathogenic bacteria even at lower concentrations than nisin A, suggesting that it may be widely used to prevent the corruption of foods. Although the 174A strain harbors several plasmids, the *Lb. brevis* 174A derivative, which lost a few plasmid, lost the productivity of the brevicin 174A and self-resistance to the own antibiotic products, like *Lb. brevis* 925A isolated from kimchi, a traditional Korean fermented dish made from Chinese cabbage.[3] The 925A strain has been found to produce a bacteriocin designated brevicin 925A. We have been demonstrated that the brevicin 925A-biosynthetic gene is located on a 64 kb size plasmid of four plasmids harbored in the 925A strain.

In general, the production of secondary metabolites, such as antibiotics and bacteriocins, is strain specific but not species specific. In the present study, to investigate whether a citrus-derived *Lb. brevis* 174A harbors the same bacteriocin biosynthetic gene cluster as kimchi-derived *Lb. brevis* 925A, we confirmed the presence of the brevicin 174A biosynthetic gene using *breC*, which is a bacteriocin biosynthetic gene from the

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925A strain, as a probe. We show that a 10 kb DNA fragment containing brevicin 174A-biosynthetic gene cluster from *Lb. brevis* 174A is completely the same as that from *Lb. brevis* 925A. We also refer to the synergistic antibacterial effect of brevicin 174A, which consists of two component polypeptides.

MATERIALS AND METHODS

Media and Growth Conditions A medium, de Man, Rogosa and Sharpe (MRS) broth (Merck), was used to grow LABs. Luria–Bertani (LB) medium[9] was used for *Escherichia* (*E.*) *coli*, *Staphylococcus*, and *Bacillus* species cultures. *L. monocytogenes* were cultured in a tryptic soy broth medium supplemented with 0.3% (w/v) yeast extract (TSBYE). *Streptococcus* strains were cultured in brain-heart infusion (BHI) medium (Fluka). For minimum inhibitory concentration (MIC) assay, LABs were cultured in MRS broth. TSBYE and Mueller–Hinton Broth (Becton Dickinson and Company) were used to culture genus *Listeria* and other bacteria, respectively. According to the requirements, ampicillin (100 μg/mL) and 1.5% (w/v) agar were added to each medium.

Isolation and Identification of Bacteriocin-Producing LAB Isolation of LABs from citrus iyo was performed as described previously.[3] Briefly, each piece of a plant sample (about 1 cm^2) was put into MRS broth, and an aliquot of the culture was plated for single-colony isolation. Gram staining, the production of organic acid, and catalase testing were carried out on the purified colonies prior to taxonomical identification of the isolated bacteria. The antibacterial activity test of supernatant fluid obtained by centrifugation of the LAB cultures was done by the agar well diffusion method.[10,11] Prior to the antibacterial assay, the supernatant fluid from the cultured medium was adjusted to pH 7.0 and filtered with a 0.22-μm pore size membrane filter (Advantec).

To identify taxonomically the isolated bacteriocin-producing LABs, we investigated the sugar fermentation profile of each strain using an API 50 CHL kit (bioMérieux). Furthermore, the entire 16S ribosomal DNA (rDNA) sequence of the collected LAB was determined as described previously[12–14] and compared with that of typical LAB obtained from the DNA Data Bank of Japan (DDBJ) website (http://www.ddbj.nig.ac.jp/). LAB species names were determined by analysis of the sequence alignment using the ClustalW program (http://clustalw.ddbj.nig.ac.jp/).

Production of Bacteriocin Non-producing Derivatives A bacteriocin-non-producing mutant from *Lb. brevis* 174A was isolated as described previously.[3] Briefly, the mutant from strain 174A, designated *Lb. brevis* 174A-Δp, was grown in MRS medium supplemented with novobiocin. The plasmid profile of mutant was confirmed by agarose gel electrophoresis.

DNA Preparation, Manipulation, and Sequencing An *E. coli* plasmid DNA was isolated using Wizard *Plus* Minipreps DNA Purification System (Promega) in accordance with the manufacturer's protocol. The chromosomal DNA from *Lb. brevis* were isolated from cultured cells as described previously.[3] The plasmids harbored in the LAB were purified using Genopure Plasmid Maxi Kit (Roche Diagnostics GmbH) according to manufacturer's instruction. The nucleotide sequence was determined with the ABI PRIZM 310 genetic analyzer, using the BigDye Terminator v1.1 Cycle Sequencing Kit in accordance with the manufacturer's protocol (Applied Biosystems). Genetic analysis was done using ATGC and GENETYX software (Genetyx Corporation). Open reading frames (ORFs) were predicted using the ORF Finder tool at NCBI (http://www.ncbi. nlm.nih.gov/gorf/gorf.html). Homology analyses were performed with the BLAST algorithm,[15] utilizing the non-redundant database provided by NCBI.

Cloning and Nucleotide Sequence Analysis of the Bacteriocin Biosynthetic Gene Cluster The restriction enzyme-digested plasmid DNA was fractionated using 0.5% (w/v) agarose gel electrophoresis and transferred to a Hybond-N^+ membrane (GE Healthcare), using the standard protocol.[9] A probe to clone the brevicin 174A biosynthetic gene cluster was amplified by the polymerase chain reaction (PCR) using a sense primer, 5′-ATG TAT AAA GAA TTA ACA GTT GAT GAA TTA GC-3′, and an antisense primer, 5′-TTA GTG CAT GCC GTG TAA GTT GTT AGA-3′. In this case, the plasmid DNA from *Lb. brevis* 925A was used as a template. The primers were designed on the basis of the nucleotide sequence of the *breC* gene from *Lb. brevis* 925A (accession no. AB370337). Labeling of the probe and detection were performed using an AlkPhos Direct Labeling and Detection Kit (GE Healthcare) in accordance with the manufacturer's instructions. After confirming the presence of the brevicin 174A biosynthetic gene in *Lb. brevis* 174A by southern hybridization and plasmid curing analysis, the entire biosynthetic gene cluster was PCR-amplified with a sense primer, 5′-GGT ACC CGG GGA TCC AGT TAT GGA TTT GCA CCA GAA CCT A-3′, and an antisense primer, 5′-CTT GCA TGC CTG CAG TTA AAC TAC CAG ACG CCA ATT GAG G-3′, which were designed on the basis of the brevicin 925A biosynthetic gene cluster (AB370337), and the nucleotide sequence of the resultant PCR product was determined.

Overexpression and Purification of Brevicin 174A-β and 174A-γ *breB* and *breC* genes from *Lb. brevis* 174A were amplified by PCR using a primer pair, 5′-CAC CCATATGAAG AAA AAA AAG AAA TAT AC-3′ (the underline indicates the *Nde*I cleavage site) and 5′-TAT TCTCGAGTTA TTT GTT ATT TAG GCA GC-3′ (the underline indicates the *Xho*I cleavage site) for *breB*, and 5′-CAC CCATATGAAA AAG AAG AAG AAA AAA GT-3′ (the underline indicates the *Nde*I cleavage site) and 5′-GAT TCTCGAGTTA GTG CAT GCC GTG TAA G T-3′ (the underline indicates the *Xho*I cleavage site) for *breC*, according to the determined brevicin 174A biosynthetic gene cluster. At this time, the primers were designed to remove the N-terminus signal sequences, which were included in the mature polypeptides encoded by the entire *breB* and *breC* genes. The amplified DNA fragments were inserted into the same sites of the pCold TF expression vector (TaKaRa), the protein expressed by which has a trigger factor (TF) tag at the N-terminus, to generate pCold-*breB* and pCold-*breC*. *E. coli* BL21 (DE3) harboring each plasmid was grown in LB medium at 37°C until the exponential phase of growth ($O.D._{600nm}$=0.6). Each culture was supplied with an isopropyl-β-D-thiogalactopyranoside at a concentration of 1 mM and kept at 15°C for 30 min to induce expression of the inserted gene. After an additional cultivation at 15°C overnight, the cells were harvested by centrifugation. Unless otherwise noted, purification was carried out at 4°C as follows. The *E. coli* cells were washed with an equilibration buffer containing 50 mM phosphate-Na, 300 mM NaCl, 20 mM imidazole, pH

7.4, and resuspended in the same buffer (20 mL per wet gram of cell). After cell disruption by sonication at 4°C, the cell debris was removed by centrifugation at 12000×*g* for 30 min. The resulting cell-free extract was applied on a His60 Ni Superflow Resin column (1.8×3.0 cm, Clontech) equilibrated previously with the same buffer. The column was washed with 10 bed volumes of equilibration buffer, and followed by the same bed volumes of wash buffer (50 mM phosphate-Na, 300 mM NaCl, 20 mM imidazole, pH 7.4). Elution was done with 10 bed volumes of elution buffer (50 mM phosphate-Na, 300 mM NaCl, 300 mM imidazole, pH 7.4). Concentration and buffer exchange of pooled fractions containing TF-tagged protein were performed with an ultrafiltration device (Amicon Ultra, Millipore) using Factor Xa buffer (50 mM Tris–HCl, 100 mM NaCl, 5 mM CaCl, pH 8.0). After Factor Xa digestion, ammonium sulfate was added to the sample until the concentration reached 1.8 M, and the resulting solution was applied to a Butyl-Sepharose 4 Fast Flow resin (GE Healthcare) equilibrated with factor Xa buffer containing 1.8 M ammonium sulfate. After eliminating the flow-through fraction, the resin was washed with 3 bed volumes of distilled water and eluted with 1/5 volumes of 0.1% (v/v) trifluoroacetic acid (TFA) until the eluate was clear and nearly free of bacteriocins. After confirming the purity of each polypeptide by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with the silver stain method, the identification of molecular weight by using the mass spectrometry analysis was done. The antibacterial activity of the purified fraction was confirmed by bioassay, and each bacteriocin was stored at 4°C until use. The polypeptide concentrations were determined using a Pierce BCA Protein Assay Kit (Thermo Scientific).

Construction of Bacteriocin Mutants The mutant polypeptides were also expressed as a fusion protein with a trigger factor using the pCold TF expression vector. The mutations were introduced into each polypeptide by site-directed mutagenesis using a PrimeSTAR Mutagenesis Basal Kit (TaKaRa), in accordance with the supplier's instructions, with two individually designed overlapping primers for each desired mutation (Table 1). Each generated vector was introduced into *E. coli* BL21 (DE3) for protein expression, and each mutant polypeptide was purified as described above.

Matrix Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF)/MS Analysis After desalting and freeze-drying each peptide, they were dissolved in TA buffer (50% acetonitrile/0.1% trifluoroacetic acid). A 0.5-μL portion of the sample was mixed with a same volume of matrix solution (α-cyano-4-hydroxycinnamic acid; CHCA 10 mg/mL in TA buffer) and followed by application on a MALDI target plate. The MS data was obtained using an autoflex II MALDI-TOF/TOF mass spectrometer (Bruker Daltonics) operated in the linear mode.

Determination of Antibacterial Activity Using a 96-well microtiter plate, the antibacterial activity of brevicin 174A was determined to be a MIC. The bacteriocins were filtrated by a 0.22-μm pore size filter (Advantec), followed by microtiter plate assay. A 150-μL portion of the medium was poured into each well of a plate with bacteriocin and a given test microorganism, the colony-forming units (CFU) of which were adjusted to 2–8×10^5/mL. After incubation at 37°C for 18 h or until obvious growth had developed (up to 30 or 42 h) in the control wells, the bacterial growth was monitored, and the MIC was defined as the lowest concentration at which no visible bacterial growth was observed. In the MIC assay, a commercial nisin A produced by *Lactococcus lactis* (Sigma-Aldrich), which contains 2.5% (w/w) nisin A, was used as a reference bacteriocin.

Table 1. Oligonucleotide Primers Used to Construct the Brevicin Mutants

Name	Sequence (5′→3′)	Mutant
β-Δ34-39-F	AAATGCAACTGGTGCACTGGCTGGA	ΔGXXXG mutants of 174A-β
β-Δ34-39-R	GCACCAGTTGCATTTCCCCAAGTACA	
β-Δ43-46-F	TATTCTTAACAACTTACACGGCATG	
β-Δ43-46-R	AAGTTGTTAAGAATACCCTTAACTGC	
γ-Δ15-30-F	TGGCGTTGGCATGGCTGGATTAGTT	ΔGXXXG mutants of 174A-γ
γ-Δ15-30-R	GCCATGCCAACGCCAAATGGAGAACC	
γ-Δ28-46-F	CATGGCTGCAATTAGCTGCCTAAAT	
γ-Δ28-46-R	CTAATTGCAGCCATGCCACCACCAAC	
β-Δ1-25-F	AGGTAGGATTGGAGGTTCTCCATTT	ΔN-terminal region mutant of 174A-β
β-Δ1-25-R	CCTCCAATCCTACCTTCGATACCACC	
γ-Δ1-11-F	AGGTAGGGGAAATGCAGCAACAGCC	ΔN-terminal region mutant of 174A-γ
γ-Δ1-11-R	GCATTTCCCCTACCTTCGATACCACC	
β-Δ1-5-F	AGGTAGGTATACCGGACCAAACTAC	ΔN-polyK mutant of 174A-β
β-Δ1-5-R	CCGGTATACCTACCTTCGATACCACC	
γ-Δ1-6-F	AGGTAGGGTAGCTTGTACTTGGGGA	ΔN-polyK mutant of 174A-γ
γ-Δ1-6-R	CAAGCTACCCTACCTTCGATACCACC	
β-C13S-F	CTACCGTAGCATGGTTAAATCAGGTG	C13S mutant of 174A-β
β-C13S-R	ACCATGCTACGGTAGTTTGGTCCGGT	
β-C51S-F	AATTAGCAGCCTAAATAACAAATAAC	C51S mutant of 174A-β
β-C51S-R	TTTAGGCTGCTAATTGCACCACCAAC	
γ-C9S-F	GTAGCTAGCACTTGGGGAAATGCAGCA	C9S mutant of 174A-γ
γ-C9S-R	CCAAGTGCTAGCTACTTTTTTCTTCTT	
γ-C44S-F	TTCACAAAGCGCGTCTAACAACTTAC	C44S mutant of 174A-γ
γ-C44S-R	GACGCGCTTTGTGAAACGCCCCAGAT	

RESULTS

Identification of a Bacteriocin-Producing LAB The supernatant fluid from the 174A strain grown in MRS broth, which is a LAB isolated from citrus iyo, exhibited antibacterial activity against some test microorganisms as listed in Table 1. The activity was resistant to heat treatment at 100°C for 10 min. However, the antibacterial activity was lost when treated with proteinase K and trypsin (data not shown), suggesting that the antibacterial substance, designated brevicin 174A, may be the polypeptide like bacteriocin. By the sugar utilization test and the 16S rDNA sequence analysis, it was shown that the 174A strain is identical with *Lb. brevis* (accession No. LC062086). The plasmid profile of *Lb. brevis* 174A observed by agarose gel electrophoresis suggests that the strain has several plasmids (Fig. 1).

When grown in the MRS medium supplemented with novobiocin, *Lb. brevis* 174A lost a few plasmids. The resulting mutant, designated 174A-Δp, lost both of the brevicin 174A productivity and resistance to the substance. To clone the brevicin 174A-biosynthetic gene in *Lb. brevis* 174A, we gathered plasmids harbored in *Lb. brevis* 174A. The brevicin 174A biosynthetic gene cluster (accession No. LC062087) was PCR-amplified using the plasmids mixture as template, and two primers designed by the information of the biosynthetic gene cluster found in *Lb. brevis* 925A.[3] The nucleotide sequence of the amplified 10 kb-DNA fragment was identical to the brevicin 925A biosynthetic gene cluster (Fig. 2). The 10-kb fragment was not amplified from the total DNA extracted from the mutant strain 174A-Δp, indicating that the brevicin 174A gene cluster is derived from one of several plasmid harbored in *Lb. brevis* 174A.

Although the 16S rDNA sequence of the 174A strain is the same as that of the 925A strain, the agarose gel electrophoretic profiles of plasmids and the sugar utilization profiles of both strains were different: the 925A strain can utilize 5-keto gluconate, but the 174A strain can not.

Gene Organization of the Brevicin 174A Biosynthetic Gene Cluster As shown in Fig. 2, the brevicin 174A biosynthetic gene cluster consists of 8 ORFs that were designated *breA* to *breH*. Judging from the BLAST homology search results, the following 5 ORFs in 8 ORFs must be essential components in class IIb bacteriocin biosynthetic gene clusters[2,7,8]: two bacteriocin structural genes that are considered to be co-transcribed (*breB* and *breC*), a specific ATP-binding cassette (ABC)-transporter with a polypeptide cleavage domain coding gene for brevicin polypeptides (*breH*) and its accessory protein (*breA*), and a bacteriocin immunity protein gene (*breE*). Furthermore, *breD* and *breG* encode predicted tran-

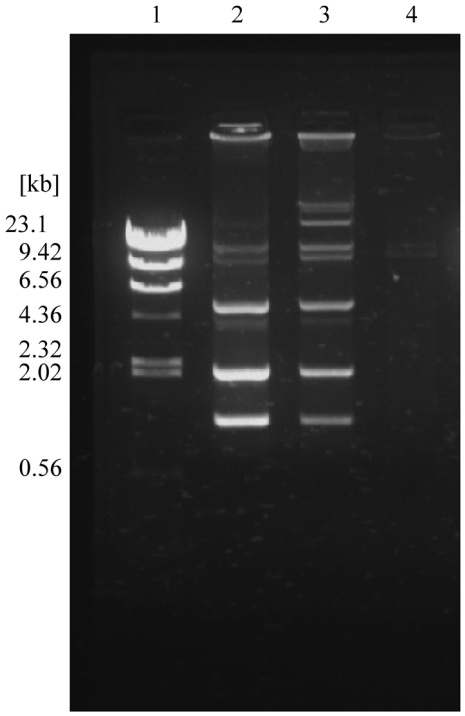
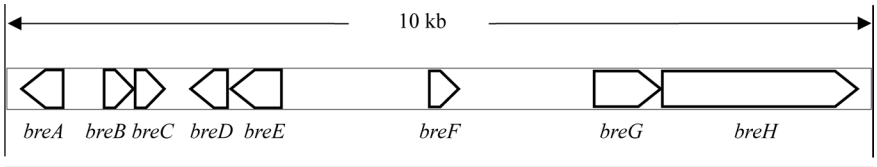


Fig. 1. Agarose Gel Electrophoretic Profile of Plasmids Harbored in the *Lb. brevis* Strains

Lanes: 1, λ/*Hin*dIII molecular mass marker; 2, plasmid isolated from *Lb. brevis* 925A; 3, plasmid isolated from *Lb. brevis* 174A; 4, plasmid isolated from *Lb. brevis* 174A-Δp.



Gene	Location[a]	Size (aa)	(proposal) Function
breA	952–1362 C	136	Bacteriocin transport accessory protein
breB	1939–2160	73	Brevicin 174A-β (leader 18 aa + 55 aa)
breC	2192–2401	69	Brevicin 174A-γ (leader 15 aa + 54 aa)
breD	2461–2658 C	65	Transcriptional regulator
breE	2669–3166 C	178	Immunity protein
breF	5454–5597	47	Unknown (bacteriocin-like peptide)
breG	7151–7783	210	Transcriptional regulator
breH	7850–10006	718	Bacteriocin ABC-transporter

a) C, Complementary sequence

Fig. 2. Gene Organization of the Brevicin 174A Biosynthetic Gene Cluster and Functions of the Genes

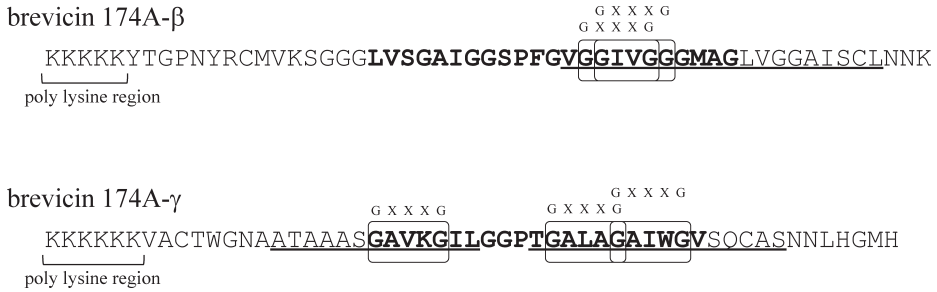


Fig. 3. Prediction of Secondary Structure and Transmembrane Helices of Both Brevicin 174A Polypeptides

The predicted transmembrane regions are represented by boldface letters. The underline and square box indicates the predicted α-helix and GXXXG motifs, respectively.

Table 2. Antibacterial Activity of Brevicin 174A Po1ypeptides and Nisin A

Species	Indicator strain	MIC (nM)			
		174A-β	174A-γ	$\beta+\gamma$	Nisin A
Gram-positive bacteria					
Bacillus cereus	ATCC 11778	ND[a)]	ND	ND	3000
Bacillus coagulans	NBRC 12583	400	400	100	400
Bacillus subtilis	ATCC 6633	ND	4200	2000	3000
Enterococcus avium	NBRC 100477	ND	ND	ND	1500
Enterococcus faecalis	NBRC 12964	ND	ND	ND	1500
Enterococcus hirae	NBRC 3128	ND	ND	ND	1500
Listeria monocytogenes	ATCC 7644	ND	ND	ND	3000
Listeria monocytogenes	ATCC 15313	ND	ND	1000	3000
Lactobacillus brevis	174A-Δp	400	500	5.0	60
Lactobacillus hilgardii	NBRC 15886	600	800	6.25	7.5
Lactobacillus plantarum	NBRC 3070	100	800	1.0	60
Lactobacillus sakei	NBRC 15893	ND	400	50	2.0
Lactococcus lactis ssp. *cremoris*	NBRC 100676	400	300	25	0.4
Leuconostoc mesenteroides ssp. *mesenteroides*	NBRC 100496	ND	ND	750	60
Pediococcus pavulus	NBRC 100673	100	1500	10	4.0
Staphylococcus aureus	IFO 12732	2000	ND	400	1500
Staphylococcus aureus	FDA 209-P	1500	ND	400	1500
Staphylococcus aureus	IID 1677 (MRSA)	ND	ND	ND	1500
Staphylococcus epidermidis	NBRC 12993	ND	ND	2000	3000
Streptococcus equinus	NBRC 12553	ND	ND	ND	750
Streptococcus mutans	MT 8148R	ND	ND	5000	ND
Streptococcus sobrinus	ATCC 27607	ND	ND	ND	ND
Gram-negative bacteria					
Escherichia coli	DH5α	ND	ND	ND	ND
Pseudomonas aeruginosa	PAO1	ND	ND	ND	ND

a) ND shows that the growth inhibition of bacterium tested were not detected at the concentration of 6000 nM.

scriptional regulators and deduced amino acid sequences that contain a helix-turn-helix DNA-binding motif (COG1476). The *breF* gene product, which seems to be bacteriocin-like short polypeptides, displays no significant similarity in the BLAST search database.

Purification of Recombinant Brevicin 174A-β and 174A-γ Polypeptides Each polypeptide was overproduced as a fusion protein with a trigger factor tag (also includes the His_6 tag) in *E. coli* and purified homogeneity using a His60 Ni Superflow Resin column. After Factor Xa digestion, brevicin polypeptides were purified again with additional Butyl-Sepharose column chromatography. Except for the mutant polypeptides with the N-terminal deletion, the purified polypeptides have additional first two N-terminal amino acid residues (His-Met-) coded by an *Nde*I site.

Antibacterial Activity and Synergistic Effects of Brevicin 174A Polypeptides As shown in Table 2, minimum inhibitory concentrations (MICs) of each component polypeptides of brevicin 174A were determined using *Lb. brevis* 174A-Δp as a test microorganism, indicating that the antibacterial activity of brevicin 174A-β (400 nM) was almost the same as that of 174A-γ (500 nM). Antibacterial activity analyses of both polypeptides were also performed on the other 23 test microorganisms, which indicated that the activity of each pair of polypeptide components of brevicin 174A to some sensitive microorganisms could be distinguished (Table 2). When all polypeptides were equally mixed, the antibacterial ability to *Lb. brevis* 174A-Δp was enhanced 100 fold as compared to each polypeptide alone. Furthermore, when synergistic activity of at least 6 μM for each polypeptide was attained, the

polypeptide mixture was newly observed to inhibit growth of *S. mutans*, *S. epidermidis*, and *L. monocytogenes* ATCC 15313. Although nisin A also inhibited effectively the growth of some LAB strains, it needed a higher amount to inhibit the growth of these pathogenic bacteria than that of brevicin 174A.

Prediction of Secondary Structure and Transmembrane Helices As shown in Fig. 3, secondary structures and transmembrane helices of brevicin 174A-β and 174A-γ were predicted by the phyre[2] program (http://www.sbg.bio.ic.ac.uk/~phyre2/html/page.cgi?id=index) and the TMHMM program (http://www.cbs.dtu.dk/services/TMHMM/), respectively. The amino acid region from lysine 21 to glycine 43 in brevicin 174A-β and that from glycine 21 to valine 41 in brevicin 174A-γ were predicted to be transmembrane regions by analysis with these programs. Two GXXXG motifs were found in the latter half of the transmembrane region that overlapped the α-helix in brevicin 174A-β. However, the transmembrane region lies across two different α-helices and contains GXXXG motifs at each overlap region in brevicin 174A-γ. This GXXXG sequence is a typical motif found in class IIb bacteriocins[8] and may mediate helix–helix interactions in target membrane proteins through interhelical van der Waals interactions and hydrogen bonds.[16–18]

Variations of the Activities of Mutant Polypeptides The antibacterial activities of mutant polypeptides are listed in Table 3. The GXXXG-deleted mutant polypeptides (ΔGXXXG: 174A-β Δ34–39 and Δ43–46; 174A-γ Δ15–30 and Δ28–46) and the N-terminal deletion mutants (ΔN-terminal region: 174A-β Δ1–25; 174A-γ Δ1–11), which consist of the C-terminus α-helix part only, had undetectable activities in both brevicin 174A polypeptides (data not shown). In the N-terminal poly lysine deletion mutants (ΔN-polyK: 174A-β Δ1–5; 174A-γ Δ1–6) and the cysteine to serine point mutants (174A-β C13S and C51S; 174A-γ C9S and C44S), although the synergistic effects of the 174A-β parental polypeptide and 174A-γ mutants against *Lb. brevis* 174A-Δp were only slightly decreased, those of the 174A-γ parental polypeptide and 174A-β mutants were dramatically decreased. A similar effect was observed on the antibacterial activity to *Lb. sakei* NBRC 15893.

DISCUSSION

The biosynthetic gene for brevicin 174A is located on the plasmid harbored in *Lb. brevis* 174A. The gene cluster was identical to that of brevicin 925A produced by *Lb. brevis* 925A isolated from kimchi by us previously.[3] However, the sugar utilization profiles of both strains were obviously different: the 925A strain can utilize 5-keto gluconate, but the 174A strain cannot. The electrophoresis profiles of plasmids harbored in each strain also slightly differed. Interestingly, part of the brevicin 174A-biosynthesizing gene cluster was the same as the adjacent region of the plantaricin 1.25β-encoding gene found in the chromosome of *Lb. plantarum* TMW1.25.[19] Judging from these results, the bacteriocin biosynthetic gene cluster may be diffused toward the related LAB species in nature. In fact, we have cloned and sequenced the gene cluster (accession no. AB454504) for the biosynthesis of a bacteriocin, designated mundticin 15-1A, from *Enterococcus* (*E.*) *mundtii* 15-1A.[4] The nucleotide sequence of the bacteriocin was identical to that of mundticin KS produced by *E. mundtii* NFRI 7393 (accession no. AB066267).[20]

In the present study, we suggest that the brevicin 174A gene cluster contains two transcriptional regulatory genes (*breD* and *breG*), in addition to five genes necessary for class IIb bacteriocin biosynthesis (*breA*, *breB*, *breC*, *breE*, and *breH*). It has been demonstrated in review reports that class IIb bacteriocins[7,8] are produced under the regulation of three regulatory systems,[21] which consist of an inducing peptide (IP) pheromone, a membrane-associated histidine protein kinase (HPK), and response regulators (RR). However, the predicted regulators encoded by *breD* and *breG*, which are necessary for brevicin 174A biosynthesis, do not have motifs or homologies to the above three-component response regulators. Analysis of the molecular mechanism that allows *breD* and *breG* to control the production of brevicin 174A and its immunity protein as a self-resistance factor is in progress.

In general, class IIb bacteriocins are synthesized together with a 15–30 residue N-terminal leader polypeptide.[8] Cleavage by the dedicated ABC transporter at the C-terminal double-glycine residues as a leader sequence, which contains a peptidase domain and is engaged in export of the bacteriocin, is necessary for generating the mature bacteriocin.[7,8] In fact, each sequence of the mature brevicin 925A-β and 925A-γ polypeptides, which is identical to that of the mature brevicin 174A-β and 174A-γ ones, respectively, starts with N-terminal poly-lysine residues that follow the double-glycine ended leader sequence.[3] Since the mature form of brevicin 174A-β is intermingled with that of brevicin 174A-γ in the supernatant fluid of the *Lb. brevis* 174A culture broth, each purified brevicin may be unsuitable for evaluating its individual antibacterial activity. Therefore, in the present study, we over-produced each polypeptide, using an *E. coli* host-vector system. We found that the deletion mutants of the GXXXG motif found in the predicted transmembrane helix lost their antibacterial activity, suggesting that the motif is essential for exhibiting the antibacterial activity of brevicin 174A (data not shown). As shown in Table 3, brevicin 174A-β is different from 174A-γ, with regard to the synergistic effects of deletion mutants of N-terminal poly-lysine residues and cysteine to serine point mutants. It is of interest that the mutations in the brevicin 174A-β molecule were more critical for their activities than were those in 174A-γ. The N-terminal poly-lysine residues may position themselves in the negatively charged surface of the membrane.[22] Judging from the decreased activity,

Table 3. Minimum Inhibitory Concentration of Brevicin 174A and Its Mutants

Mixture			MIC (nM)[a]	
174A-β		174A-γ	174A-Δp	*Lb. sakei*
Parental	×	—	400	2000
—	×	Parental	500	400
Parental	×	Parental	5	50
Parental	×	ΔN-polyK	8	1500
		C9S	60	1000
		C44S	20	1000
ΔN-polyK	×	Parental	ND[b]	ND
C13S			ND	ND
C51S			300	ND

a) *Lb. brevis* 174A-Δp and *Lb. sakei* NBRC 15893 were used as a test microorganism. *b*) ND shows that non-antibacterial activity was detected at MIC of parental 174A-γ.

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Sln1        --KR---GPN--CVGNFLGGLFAGAAAGVPLGPAGIVGGANLGMVGGALTCL--- 45
Abp118α     --KR---GPN--CVGNFLGGLFAGAAAGVPLGPAGIVGGANLGMVGGALTCL--- 45
174A-β      KKKKKYTGPNYRCMVKSGGLVSGAIGGSPFGVGGIVGGGMAGLVGGAISCLNNK 55
              *:   ***  *: :  ***.:** .* *:* .*****.  *:****::**


Sln2        KNGYGGSGNRWVHCGAGIVGGALIGAIGGPWSAVAGGISGGFASCH-------- 46
Abp118β     KNGYGGSGNRWVHCGAGIVGGALIGAIGGPWSAVAGGISGGFTSCR-------- 46
174A-γ      KKKKKKVACTWGNAATAAASGAVKGILGGPTGALAGAIWG-VSQCASNNLHGMH 53
            *:     .  * :..:. ..**: * :*** .*:**.* * .:.*
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Fig. 4. Alignment of the Amino Acid Sequence of Bacteriocins Similar to Brevicin 174A Polypeptides

Sequence alignment was performed by ClustalW program (http://clustalw.ddbj.nig.ac.jp/). The conserved residues are indicated by boldface letters and asterisk. The conservative and semi-conservative substitutions are donated with a colon and period, respectively. Sln1 and Sln2, and Abp118*α* and Abp118*β* are component polypeptides of salivaricin P[23] and ABP-118,[24] respectively.

this stabilizing effect might be necessary to brevicin 174A-*β* and is a contributor to the antibacterial activity of 174A-*γ*. The same results were obtained by the point substitutions on each N- and C-terminal cysteine residue, suggesting that maintenance of the functional structure through the disulfide bridge is important for the activities of both brevicin 174A component polypeptides, and particularly in 174A-*β*. It is notable that a similar result was observed in the activity against not only *Lb. brevis* 174A-Δp but also *Lb. sakei* NBRC 15893, which has less sensitivity to the 174A-*β* parental polypeptide than to 174A-Δp. These results indicate that synergistic effects depend on the 174A-*β* polypeptide, regardless of whether the polypeptide is active against the indicator strain or not.

Each of the two-component polypeptides, designated Sln1 and Sln2, of the *Lb. salivarius* DPC6005-produced salivaricin P,[23] which is a class IIb bacteriocin, displays antibacterial activity independently, like brevicin 174A. Although Sln1 has similarities with brevicin 174A-*β* (Fig. 4), Sln2 is different except for around the transmembrane helices of 174A-*γ*. Figure 3 shows the alignment of the ABP-118 component polypeptide produced by *Lb. salivarius* ssp. *salivarius* UCC118,[24] which indicates that ABP-118*α* is identical to Sln1, but ABP-118*β* differs from Sln2 in only two residues in the N-terminal region. Some of the following common characteristics have been found—positively charged N-terminal region, predicted transmembrane helix containing GXXXG motifs, and two cysteine residues at both the N- and C-terminal side—in the component polypeptides of at least these three bacteriocins. The N-terminal region of the *β*-polypeptide in lactococcin G is not strictly embedded in the hydrophobic or hydrophilic parts of the membrane.[8] The antibacterial activity of brevicin 174A was decreased by deletion of the positively charged residues at the N-terminal and of the cysteine residues. Judging from these results, the activity of brevicin 174A is likely to be proportionate to the structural stability of each component polypeptide. Brevicin 174A is effective against some pathogenic bacteria, such as *S. aureus*, *L. monocytogenes*, and *S. mutans*, suggesting that brevicin 174A is meaningful as a biopreservative and is also useful for understanding the action mechanism of class IIb bacteriocins.

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Conflict of Interest The authors declare no conflict of interest.

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