Bacteriocins, which are polypeptides displaying antibacterial activity and synthesized ribosomally, are produced in many bacterial species including some lactic acid bacteria (LABs). 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.

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. 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, immunostimulating 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 brevis by determining the entire sequence of the 16S ribosomal RNA (rRNA)-encoding 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. 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, exhibit the synergistic antibacterial activity when equal amounts of both complementary peptides are present. 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. 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...
925A strain, as a probe. We show that a 10 kb DNA fragment containing brevicin 174A biosynthetic gene cluster from \textit{Lb. brevis} 174A is completely the same as that from \textit{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 \textit{Escherichia coli}, \textit{Staphylococcus}, and \textit{Bacillus} species cultures. \textit{L. monocytogenes} were cultured in a tryptic soy broth medium supplemented with 0.3% (w/v) yeast extract (TSBY). \textit{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 \textit{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.\(^5\) 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 nucleotide sequence using the ClustalW program (http://clustalw.ddbj.nig.ac.jp/).

**Production of Bacteriocin Non-producing Derivatives** A bacteriocin-non-producing mutant from \textit{Lb. brevis} 174A was isolated as described previously.\(^5\) Briefly, the mutant from strain 174A, designated \textit{Lb. brevis} 174A-Ap, 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 \textit{E. coli} plasmid DNA was isolated using Wizard Plus Miniprep DNA Purification System (Promega) in accordance with the manufacturer’s protocol. The chromosomal DNA from \textit{Lb. brevis} were isolated from cultured cells as described previously.\(^5\) 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'-ATGTTATAAAGATTTAACAATGATGAA TTACG-3', and an antisense primer, 5'-TTAGTGCAAATGTTAG-3'. In this case, the plasmid DNA from \textit{Lb. brevis} 925A was used as a template. The primers were designed on the basis of the nucleotide sequence of the \textit{breC} gene from \textit{Lb. brevis} 925A (accession no. AB370337). Labeling of the probe and detection were performed using an AlkBios 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 \textit{Lb. brevis} 174A by southern hybridization and plasmid curing analysis, the entire biosynthetic gene cluster was PCR-amplified with a sense primer, 5'-GGTACCCGGGATCC AGTTATGGATTGTCAACCCGAAACT3', and an antisense primer, 5'-CTTGCATGCTGTAGTTAACATCACG ACAAGATTAGAG-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** \textit{breB} and \textit{breC} genes from \textit{Lb. brevis} 174A were amplified by PCR using a primer pair, 5'-CACGATATGAA AAAAAAAAGAAATAC-3' (the underline indicates the NdeI cleavage site) and 5'-TATTCTCGAGTTATTTGATTAGT GCAGC-3' (the underline indicates the Xhol cleavage site) for \textit{breB}, and 5'-CACGATATGAAAAGAACAGG AAAAGATG-3' (the underline indicates the NdeI cleavage site) and 5'-GATATTCTCGAGTTATGGTATGACGC-3' (the underline indicates the Xhol cleavage site) for \textit{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 \textit{breB} and \textit{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-brecB and pCold-brecC. \textit{E. coli} BL21 (DE3) harboring each plasmid was grown in LB medium at 37°C until the exponential phase of growth (OD\(_{600nm}\)=0.6). Each culture was supplied with an isopropyl-\(\beta\)-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 \textit{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, 300 mM NaCl, 300 mM imidazole, pH 7.4). 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 eluting the flow-through fraction, the resin was washed with 3 bed volumes of distilled water and eluted with 10 bed volumes of elution buffer (50 mM phosphate-Na, 300 mM NaCl, 20 mM imidazole, pH 7.4). Elution was done by using the mass spectrometry analysis was done. The anti-bacterial activity of the purified fraction was confirmed by using the silver stain method, the identification of molecular weight was confirmed the linear mode.

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 | GCACAGGTCATGATCTTCCCAAGTAGA                         |              |
| β-Δ43-46-F | TATCTCTAACACCTACAGCGCATG                           |              |
| β-Δ43-46-R | AAGTTGTAAGAATACCTTAACTG                             |              |
| γ-Δ15-30-F | TGGCCTGTGCTGCTGATGGATATTGT                             | ΔGXXXG mutants of 174A-γ |
| γ-Δ15-30-R | GCCATGCCAACCGCAATGGGAAACC                           |              |
| γ-Δ28-46-F | CATGCTGCTAATTAGCTGCTTAAAT                         |              |
| γ-Δ28-46-R | CTAATTCGACCCATGGCACCACACAC                          |              |
| β-Δ1-25-F | AAGTTAGGATGGAGGTTCCTCCATTT                          | AN-terminal region mutant of 174A-β |
| β-Δ1-25-R | CTCCTAATCCCATCTTCTGATACCAC                          |              |
| γ-Δ11-11-F | AAGTTAGGGGAATGTCAGAACAGGCC                         | AN-terminal region mutant of 174A-γ |
| γ-Δ11-11-R | GCATTTCCCTACCTTCTGATACCAC                          |              |
| β-Δ1-5-F | AAGTTAGGATGATCCGGACACCACACT                         | AN-polyK mutant of 174A-β |
| β-Δ1-5-R | CGGTATACCTACCTTCTGATACCC                           |              |
| γ-Δ1-6-F | AAGTTAGGATGACTGCTACTTGGGA                            | AN-polyK mutant of 174A-γ |
| γ-Δ1-6-R | CAAGCTACTTCCATCTTCTGATACCAC                         |              |
| β-ΔC135-F | CTACCCGAGCATGTTTTAAATCAGGGT                        | C13S mutant of 174A-β |
| β-ΔC135-R | ACCATGCTAGGATTGCTGTTTGGTCGCGT                     | C13S mutant of 174A-β |
| β-ΔC515-F | AATTAGCAGCTAATAAACAAATAC                           | C51S mutant of 174A-β |
| β-ΔC515-R | TTGAGGAGTCAGTTGAGACACCAC                           |              |
| γ-ΔC9-F  | GTAGCTAGACTGCTGAGGAAATGAGCAGCA                     | C9S mutant of 174A-γ |
| γ-ΔC9-R  | CCAAGTCTAGCTCTTTTCTTCTT                             |              |
| γ-ΔC44S-F | TTTCAAAAGCGGCTCAACACTTAC                           | C44S mutant of 174A-γ |
| γ-ΔC44S-R | GACCGCGCTTGTAAAAACGCCACAT                          |              |
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. 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 bio-

| Gene | Location | 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. 1. Agarose Gel Electrophoretic Profile of Plasmids Harbored in the Lb. brevis Strains

1, λ/HindIII 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.

Fig. 2. Gene Organization of the Brevicin 174A Biosynthetic Gene Cluster and Functions of the Genes
criptional 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 His6 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 NdeI 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

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**Table 2. Antibacterial Activity of Brevicin 174A Polypeptides and Nisin A**

| Species                              | Indicator strain | MIC (nM) |
|--------------------------------------|-----------------|----------|
|                                      |                 | 174A-β   | 174A-γ   | β+γ | Nisin A |
| Gram-positive bacteria               |                 |          |          |     |        |
| *Bacillus cereus*                    | ATCC 11778      | ND       | 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     |
| *Pedococcus 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 12850      | ND       | ND       | ND  | 3000   |
| *Streptococcus mutans*               | MT 8148R        | ND       | ND       | 5000| ND     |
| *Streptococcus sobrinus*             | ATCC 27607      | ND       | ND       | ND  | ND     |

| Gram-negative bacteria               |                 |          |          |     |        |
| *Escherichia coli*                   | DH5e            | ND       | ND       | ND  | ND     |
| *Pseudomonas aeruginosa*             | PAO1            | ND       | ND       | ND  | ND     |

ND shows that the growth inhibition of bacterium tested were not detected at the concentration of 6000 nM.
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 phyre² 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 bacteriocins⁵⁰ 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 (AGXXG: 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 Δ1–11; 174A-γ Δ1–6), 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* NFRJ 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 *breF*). It has been demonstrated in review reports that class IIb bacteriocins⁴⁷ 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.⁵⁰ 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.⁷⁸ 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.⁵ 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.²⁰ Judging from the decreased activity,

| Mixture | MIC (nm) | Lb. sakei |
|---------|----------|----------|
| 174A-β  | 174A-γ   | 174A-Δp  |          |
| Parental| —        | 400      | 2000     |
|         | Parental | 500      | 400      |
| Parental| ×        | 10       | 5        |
| Parental| ΔN-polyK | 8        | 1500     |
| C9S     | 60       | 1000     |
| C44S    | 20       | 1000     |
| ΔN-polyK| Parental | ND      | 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.
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* NBCR 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, which is identical to Sln1, but ABP-118 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 helices 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.

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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