Characterization of a Bacteriocin Produced by Enterococcus faecalis N1-33 and Its Application as a Food Preservative

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ABSTRACT

A bacteriocin-producing strain, N1-33, isolated from fermented bamboo shoot was identified as Enterococcus faecalis. The pH-adjusted culture supernatant of this strain consisted of several peptides with bacteriocin activity, and the supernatant inhibited the growth of pathogenic bacteria such as Listeria monocytogenes. The major peptide with bacteriocin activity was purified, and the first 39 amino acid residues of the bacteriocin were found to be identical to enterocin MR10A produced by E. faecalis MRR10-3. Addition of the pH-adjusted and concentrated culture supernatant of strain N1-33 caused a marked reduction in the growth of Bacillus cereus in custard cream and L. monocytogenes in pickled cucumber. These results suggest the potential use of the bacteriocin produced by strain N1-33 in food biopreservation.

Bacteriocins are ribosomally synthesized peptides displaying antimicrobial activity against bacterial strains closely related to the producer strain (16). Some bacteriocins produced by lactic acid bacteria show a broad inhibitory spectrum against Bacillus cereus (1, 12) and Listeria monocytogenes (15, 23). B. cereus is an aerobic, spore-forming bacterium that is widely distributed in the environment, contaminating many types of food. This bacterium is one of the leading causes of food poisoning in the industrialized world and causes gastrointestinal disorders (14, 21). L. monocytogenes is a gram-positive, nonsporulating pathogenic bacterium with a widespread presence in nature, affecting a wide range of domestic and wild animals, as well as humans (9, 19). L. monocytogenes is of particular concern for manufacturers of refrigerated, ready-to-eat food products, largely because of its wide distribution in the environment and its ability to grow on a variety of foods at refrigeration temperatures (5). Bacteriocins that control the growth of these bacteria could prove effective for quality stabilization and would be potential candidates for biopreservation of several foods sold in markets.

Nisin A, a typical bacteriocin produced by Lactococcus lactis, is one of the most popular merchandised bacteriocins and is used as an antagonistic additive in food industries of more than 70 countries worldwide. However, the use of nisin A is limited because it exhibits antimicrobial activity only under acidic conditions, and the antimicrobial spectrum is not versatile (6, 18). Although many bacteriocins other than nisin A have been reported, adequate information is lacking for the application of these bacteriocins to food industries. Bacteriocins that are more useful are expected, and many research groups are screening lactic acid bacterial strains, with the goal of producing new bacteriocins (13, 27).

Our group has screened traditional fermented foods in Thailand to obtain strains that produce bacteriocins against B. cereus and L. monocytogenes. We isolated a bacteriocin-positive strain, numbered N1-33, from a fermented bamboo shoot. The culture filtrate of strain N1-33 has shown a wide antibacterial spectrum, heat stability, stability in neutral pH conditions, and sensitivity to proteases (2). In this report, we describe the identification of the N1-33 strain, and purification and characterization of the bacteriocin produced by this strain. To investigate the effect of the bacteriocin activity of N1-33 as a food preservative in actual food, we examined the effects of the addition of the N1-33 culture supernatant on the growth of B. cereus in custard cream and L. monocytogenes in pickled cucumber.

MATERIALS AND METHODS

Bacterial strains and media. Bacterial strains used as indicators for antibacterial activity in this study are listed in Table 1. All lactic acid bacterial strains, including N1-33, were grown in deMan Rogosa Sharpe broth (Merck, Darmstadt, Germany) at 30°C, without agitation. The remaining bacterial strains were grown in Trypticase–yeast extract–glucose (TYG) broth containing 1.0% tryptone (Difco, Becton Dickinson, Sparks, MD), 0.5% yeast extract (Difco, Becton Dickinson), 1.0% glucose, and 0.5% NaCl at 37°C, with vigorous agitation, unless stated otherwise. At least two separate experiments were conducted for each test organism. TYG medium (pH 6.9) was also used for antibacterial tests in this study, including the microbe count in actual food.
TABLE 1. Antibacterial activity of Enterococcus faecalis N1-33 culture filtrate against various strains of bacteria by the agar-well diffusion method

<table>
<thead>
<tr>
<th>Indicator species</th>
<th>Strain⁵</th>
<th>C</th>
<th>10× C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus subtilis</td>
<td>IAM1069</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>OCC199</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>B. cereus</td>
<td>IFO13494</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>B. cereus</td>
<td>ATCC 10876</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>B. cereus</td>
<td>ATCC 10987</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>B. cereus</td>
<td>OCC200</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>B. cereus</td>
<td>OCC201</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>ATCC 6538P</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>S. aureus</td>
<td>IFO12732(P)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Lactobacillus plantarum</td>
<td>JCM1057</td>
<td>−</td>
<td>+++</td>
</tr>
<tr>
<td>Lactococcus lactis subsp. lactis</td>
<td>JCM7638</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L. lactis subsp. lactis</td>
<td>NRIC1074</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Pediococcus acidilactici</td>
<td>OCC-M</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Leuconostoc mesenteroides subsp. mesenteroides</td>
<td>JCM6124</td>
<td>+</td>
<td>+++</td>
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<tr>
<td>Enterococcus faecalis</td>
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<td>JCM1649</td>
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<td>−</td>
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<td>Salmonella typhimurium</td>
<td>IFO12529</td>
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<tr>
<td>Pseudomonas aeruginosa</td>
<td>IFO12689</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Listeria monocytogenes</td>
<td>OCC197</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>L. monocytogenes</td>
<td>OCC198</td>
<td>+</td>
<td>+++</td>
</tr>
</tbody>
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① IAM, Institute of Applied Microbiology Culture Collection, University of Tokyo (Tokyo, Japan); OCC, Ohmomo Culture Collection (Ibaraki, Japan); IFO, Institute for Fermentation (Osaka, Japan); ATCC, American Type Culture Collection (Rockville, MD); JCM, Japan Collection of Microorganisms (Saitama, Japan); NRIC, NODAI Culture Collection Center, Tokyo University of Agriculture (Tokyo, Japan).

② C, N1-33 culture filtrate was used as a sample; 10× C, 10× N1-33 culture filtrate was used as a sample; −, has no antibacterial activity (size of inhibition zone less than 8 mm); +, has antibacterial activity (size of inhibition zone between 8 and 12 mm); ++, has antibacterial activity (size of inhibition zone between 12 and 20 mm); ++++, has antibacterial activity (size of inhibition zone more than 20 mm).

Identification of strain N1-33. Strain N1-33 was identified based on cell morphology examined by microscopy, Gram staining, catalase reaction, and other identification tests described in Bergey’s Manual of Systematic Bacteriology (28). Gas production from glucose under anaerobic conditions was tested in the bacteria grown at 30°C for 72 h in deMan Rogosa Sharpe medium supplemented with ammonium sulfate instead of ammonium citrate. The carbohydrate fermentation was determined by the API 50 CHL system as specified by the manufacturer (bioMérieux, Marcy l’Etoile, France). Total DNA of the bacteriocin-producer strain was obtained by the alkaline lysis method (3). The 16S rRNA gene was amplified and sequenced as described previously (26, 31).

Preparation of supernatant from N1-33 culture. To prepare culture supernatant of strain N1-33, the bacterial culture was anaerobically grown in TYG broth containing 0.5% calcium carbonate at 30°C for 16 h, and then centrifuged at 12,000 × g for 20 min. The culture supernatant was adjusted to pH 6.5 and passed through a 0.45-μm sterile filter (Advantec/Toyo Roshi Kaisha, Tokyo, Japan). This preparation was termed “N1-33 filtrate” and was used to detect the antibacterial activity.

To prepare the 10-fold-concentrated N1-33 filtrate, a culture of strain N1-33 was grown in corn broth at 30°C for 24 h and then centrifuged at 12,000 × g for 20 min. The culture supernatant was adjusted to pH 6.5, and lactic acid was further removed by using an ultrafiltration membrane (molecular weight cutoff of 3,000; Pellicon 2 module PLBC 3K, Millipore, Billerica, MA) in order to prevent the influence of the antibacterial activity by concentrated lactic acid under pH 6.5 conditions. The concentrated supernatant was further concentrated by a rotary evaporator to 1/10 volume of the initial culture supernatant and termed “10× N1-33 filtrate.”

Bacteriocin activity assays in agar medium. The agar-well diffusion method (29) was used to detect the antibacterial activity of the N1-33 filtrate. Each indicator strain was cultivated in an appropriate liquid media at various temperatures and then inoculated into TYG agar (ca. 10⁵ CFU/ml). Wells (diameters of 8 mm) were hollowed out, and each agar well was filled with 100 μl of N1-33 filtrate. The plates were incubated overnight at 30°C. The inhibition zones around the wells were then measured. To test the precise activity against gram-positive strains that show resistance to N1-33 filtrate by the agar diffusion method described above, 10× N1-33 filtrate was used instead of N1-33 filtrate. One hundred microliters of 10× N1-33 filtrate was placed into the wells in the agar plate containing each indicator strain (ca. 10⁶ CFU/ml).

Bacteriocin activity of the purified bacteriocin was quantitatively measured by using an improved agar-well diffusion method. Two hundred microliters of each fraction was passed through a 0.45-μm-size-pore sterile filter (Advantec/Toyo Roshi Kaisha) and placed into agar wells (diameters of 8 mm) in a plate containing Pediococcus acidilactici OCC-M (ca. 10⁵ CFU/ml) as an indicator strain. The plate was incubated at 30°C for 24 h, and then the diameter of the growth inhibition zone of the indicator strain around the agar well was measured. The area of the growth inhibition zone was calculated from the diameter, and the value obtained was used to determine the bacteriocin activity. The activity unit (AU) was determined by using nisin A (Sigma, St. Louis, MO) as an activity standard. Nisin A standard solution (1 mg/ml) was prepared in 0.02 N HCl, and the activity was defined as 1,000 AU/ml.

Bacteriocin purification. The bacteriocin was purified from 1 liter of corn broth cultures of strain N1-33. All chromatographic purification steps were carried out at room temperature. The strain was grown to the early stationary phase for 16 h at 30°C, with the lower limit of the culture pH controlled to pH 6.0 by 0.5 N NaOH, and then the cells were removed by centrifugation at 12,000 × g for 20 min. The bacteriocin was precipitated from the culture supernatant by addition of 80% ammonium sulfate at 4°C. After centrifugation at 10,000 × g for 30 min, the pellet was
dissolved in 100 ml of 20 mM sodium phosphate buffer (pH 5.9) (buffer A). The solution was applied to a 12-ml SP-Sepharose fast-flow cation-exchange column (GE Healthcare UK, Buckinghamshire, England) equilibrated with buffer A. The column was washed with 20 ml of buffer A before the bacteriocin was eluted with 80 ml of the same buffer containing 1 M NaCl. The active fractions were pooled and loaded onto a 10-ml octyl-Sepharose CL-4B column (GE Healthcare UK) equilibrated with 10% (vol/vol) ammonium sulfate in buffer A. The column was washed with 20 ml of the same buffer, after which the activity was eluted with 100 ml of buffer A containing 70% (vol/vol) ethanol. The active fraction was concentrated by using a rotary evaporator making the final volume 10 ml, which was further diluted to 50 ml with 0.1% (vol/vol) trifluoroacetic acid in water. This solution was further purified by high-performance liquid chromatography (HPLC; model SPD-6A, Shimadzu, Kyoto, Japan) by using a Capcel Pak C_{18} MG II reverse-phase column (10-mm inner diameter and 150-mm length; Shiseido, Tokyo, Japan) equilibrated with 0.1% trifluoroacetic acid in water (solvent A). The mobile phase used was a linear gradient from 100% solvent A to 45% solvent B (100% acetonitrile containing 0.1% trifluoroacetic acid) in 10 min, which was followed by a linear gradient from 45 to 60% solvent B in 40 min at 30°C, with a flow rate of 2.5 ml/min. Peptides were detected at 230 nm. Fractions with high bacteriocin activity were mixed and repurified by the reverse-phase column. Purified bacteriocins were freeze dried under vacuum, dissolved with 5 ml of water, and stored at −20°C.

The bacteriocin activity in each purification step was assayed by the agar-well diffusion method described above. Fractions eluted from HPLC were evaporated, dissolved with 400 μl of water, and applied for agar well diffusion method.

The protein concentrations of purified bacteriocin were determined according to Lowry’s method (20).

N-terminal amino acid sequencing analysis and molecular mass spectrometry. The N-terminal amino acid sequences of HPLC-purified bacteriocins were analyzed via automated Edman degradation by using a PPSQ-21 protein sequence (Shimadzu) in the Hipep laboratory (Tokyo, Japan). The sequences were used to retrieve similar sequences from a database by using a FASTA search. The molecular masses of HPLC-purified bacteriocins were characterized by Shimadzu Biotech (Tsukuba, Ibaraki, Japan) by using matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI TOF-MS; AXIMA-CFR, Shimadzu).

Application of the bacteriocin added to actual food systems. All equipment for preparing food samples were sanitized or sterilized. For the application of the bacteriocin to actual food systems, the 10× N1-33 filtrate was used as the bacteriocin sample, and the experiments were performed in triplicate. Microbiological data were converted to log CFU per gram. The mean log CFU per gram in food was compared by using the Student’s t-test. Differences were considered significant when P < 0.05.

To test the bacteriocin against B. cereus in custard cream, the ingredients for custard cream were obtained from markets. The custard cream was made from 69.5% (wt/wt) milk, 15.5% (wt/wt) sugar, 2.0% (wt/wt) cornstarch, 2.0% (wt/wt) soft flour, and 11.0% (wt/wt) egg yolk, and 140-g aliquots of raw custard cream were dispensed into conical beakers. The raw custard cream was mixed with 10× N1-33 filtrate at the rate of 0, 0.4, and 0.6% (vol/vol). The samples were stirred on a hot stirrer (150°C) for 15 min, so that the final temperature of the custard cream reached 90°C, and then cooled at room temperature. Ten-gram aliquots of the custard cream were dispersed into six sterilized petri dishes (90 mm in diameter). Cells of 16-h cultures of B. cereus IFO13494 were collected and suspended in 0.85% sterile NaCl solution. The suspension was serially diluted and adjusted to approximately 4 log CFU/ml, and 10 μl of the diluted solution was inoculated into 10 g of each custard cream sample. Samples were then incubated at 25°C. The growth of B. cereus was analyzed on day(s) 0, 1, and 3 of aerobic storage by the standard microbiology method (30).

For assay of the bacteriocin against L. monocytogenes in pickled cucumber, cucumbers were purchased from a market (Nihonbashi, Tokyo, Japan), washed, soaked in 70°C water for 1 min to sterilize, and transversely cut into 2-cm-long pieces, which were then brined with the solution containing 5% NaCl and 0.3% sodium glutamate at the rate of 1:1 (cucumber:brine solution [wt/wt]) for about 16 h. The brined cucumbers were divided into three groups for treatment and were brined again in a solution containing 2% NaCl and 0.3% sodium glutamate at the rate of 1:1 (cucumber:brine solution [wt/wt]). The 10× N1-33 filtrate was added to the second brine solution at the rate of 0% and 0.3% (vol/vol). Cells of 16-h cultures of L. monocytogenes OCC197 were collected and suspended in 0.85% sterile NaCl solution. The suspension was serially diluted and adjusted to approximately 4 log CFU/ml, and 20 μl of the diluted solution was inoculated into 100 g of each pickled cucumber sample. Samples were then stored at 10°C. The growth of L. monocytogenes and the pH were analyzed on day(s) 0, 4, and 7 of storage, as described above.

Nucleotide sequence accession number. The sequencing of the 16S rRNA gene of strain N1-33 has been assigned accession number AB292313.

RESULTS

Identification of strain N1-33. The bacteriocin-producing strain N1-33 was a gram-positive and catalase-negative coccus that did not produce gas from glucose. The strain had β-galactosidase activity and was able to grow from 14 to 48°C, at pH 9.6, and in 6.5% NaCl. Based on these results, the strain was identified as belonging to the genus Enterococcus. The carbohydrate fermentation pattern showed that the strain produced acid from sorbitol and melizitose but not from L-arabinose; these findings are characteristic of E. faecalis but not E. faecium. In addition, the 16S rDNA sequence from N1-33 had a similarity value of more than 99.9% with the type strain, E. faecalis V583 (DNA Data Bank of Japan accession no. AE016830). Based on these biochemical and morphological characteristics, the strain was identified as E. faecalis, and further designated as E. faecalis N1-33.

Antibacterial activity spectrum of the bacteriocin produced by strain N1-33. The culture supernatant of E. faecalis N1-33 showed a wide spectrum of inhibitory ac-
tivity against gram-positive bacteria by the agar-well diffusion method (Table 1). The N1-33 filtrate inhibited growth of strains of *Bacillus subtilis*, *Staphylococcus aureus*, and *L. monocytogenes*, but did not inhibit strains of *E. faecalis*, which is the same species as strain N1-33, or two of the five strains of *B. cereus* (ATCC 10876 and OCC201). Similarly, *L. lactis* ICM7638 was sensitive to the N1-33 filtrate although *L. lactis* NRIC1074 was resistant. On the other hand, the 10× N1-33 filtrate inhibited the growth of all gram-positive indicator strains. None of the gram-negative bacteria assayed was inhibited.

As *Pediococcus acidilactici* OCC-M showed the greatest sensitivity to the bacteriocin, it was used as the target strain in bacteriocin purification.

### Purification and characterization of the bacteriocin.

The bacteriocin was purified from culture supernatant by ammonium sulfate precipitation, cation-exchange chromatography, and hydrophobic-interaction chromatography (Table 2). A part of the active fractions obtained by hydrophobic interaction chromatography was applied to reverse-phase HPLC and yielded several peaks with various specific activities (Fig. 1). Two major peaks, termed “P5” and “P10,” and eluting about 50.5 and 54.3% acetonitrile concentration, respectively, were further analyzed. P5 (1,055 μg) and P10 (650 μg) were obtained from 1 liter of N1-33 culture supernatant, respectively. The specific activity of bacteriocins in P5 and P10 was 1.97 and 1.61 AU/μg, respectively, when nisin A was used as a standard. MALDI-TOF MS of P5 and P10 gave molecular masses of 5,215.92 Da (m/z = 5,216.92) and 5,199.68 Da (m/z = 5,200.68), respectively (Fig. 2).

N-terminal amino acid sequence analyses of P5 and P10 were carried out by automated Edman degradation, and the first 39 amino acids for P5 and first 30 amino acids for P10 were determined. The N-terminal amino acid sequences and molecular masses showed that the peptides in P5 and P10 may differ in the presence or absence of an oxidized amino acid residue and should be identical substances.

The FASTA database homology search showed that the N-terminal amino acid sequence was identical to that of enterocin MR10A (EntMR10A) produced by *E. faecalis* MRR10–3 (4), and showed 97.4% identity with enterocin L50A (EntL50A) produced by *E. faecium* L50 (8). Based on the molecular mass of 5,201.58 Da for EntMR10A, we concluded that the peptides contained in P5 and P10 were identical to EntMR10A.

### Effect of the N1-33 filtrate on *B. cereus* IFO13494 inoculated in custard cream.

To test whether the N1-33 culture filtrate could be used to regulate pathogenic bacteria in foods, the effect of the culture filtrate on the growth of *B. cereus* inoculated in custard cream was examined. After inoculating *B. cereus* IFO13494 into 10-g samples of each custard cream, 22 to 81 CFU were obtained. The results are presented in Figure 3. When stored at 25°C, samples containing 0.4% (vol/wt) and 0.6% (vol/wt) of the 10× N1-33 filtrate showed a remarkable reduction in viable counts after 3 days of storage. Few viable *Bacillus* colonies (<10 CFU/g) were detected in custard cream in the presence of the 10× N1-33 filtrate, although the colonies counted in controls increased to reach log 9 CFU/g. The pH ranges of these custard cream samples were near neutral, between pH 6.64 and 6.99. Viable cell counts of 10× N1-33 filtrate-added samples were significantly (P < 0.05) lower compared with controls in all cases. These results indicated that the bacteriocin not only possessed antibacterial activity at neutral pH, but also heat resistance when applied under actual food-processing conditions.

### Effect of the bacteriocin on *L. monocytogenes* OCC197 inoculated in pickled cucumber.

The effect of the N1-33 culture filtrate on the growth of *L. monocytogenes* inoculated in pickled cucumber was examined as a model of uncooked food. Actual CFU of *L. monocytogenes* OCC197 inoculated into 100 g of pickled cucumber, excluding the negative control, was about log 2 CFU. After 4 and 7 days of storage, the number of *L. monocytogenes*...
FIGURE 2. Mass analysis of two major bacteriocin peaks, termed "P5" and "P10." (A) P5 chart: a mass peak at m/z 5,216.92. (B) P10 chart: a mass peak at m/z 5,200.68.

 FIGURE 3. Changes in populations of B. cereus IFO13494 inoculated into custard cream treated with 10× N1-33 filtrate during incubation at 25°C for 3 days. Data are shown as means ± standard deviations of three independent experiments. □, blank; △, 0.4% 10× N1-33 filtrate; ◆, 0.6% 10× N1-33 filtrate.

FIGURE 4. Changes in populations of L. monocytogenes OCC197 inoculated into pickled cucumber treated with 10× N1-33 filtrate during incubation at 10°C for 7 days. Data are shown as means ± standard deviations of three independent experiments. □, negative control (noninoculated L. monocytogenes); ◆, blank; ◆, 0.3% 10× N1-33 filtrate.

in the pickled cucumbers prepared with 0.3% 10× N1-33 filtrate was significantly (P < 0.05) lower than that in the blank samples (Fig. 4). These results indicate that the N1-33 culture filtrate can inhibit the growth of L. monocytogenes even in an actual food model.

DISCUSSION

In this study, the bacteriocin-producing strain E. faecalis N1-33 was identified, and the bacteriocins produced by the strain were purified and characterized. The purification of the bacteriocin activity by reverse-phase HPLC showed several peaks with antibacterial activity. The N-terminal amino acid sequences of two peptides contained in two major peaks were identical, although the molecular masses of these substances showed a difference of 16 Da.

The homology search revealed that the N-terminal amino acid sequences of the peptides were identical with those of EntMR10A (4), and showed 97.4% homology with EntL50A (8). Earlier studies (4, 8) have identified several unique features of EntMR10A and EntL50A. One is the existence of another bacteriocin that has a similar structure to these bacteriocins produced by identical strains. Enterocin MR10B (EntMR10B) showed 74.4% identity to EntMR10A (4) and enterocin L50B (EntL50B) showed 72.0% identity to EntL50A (8). Although it is possible that EntMR10B is present in a culture filtrate of the N1-33 strain, we isolated only a bacteriocin identical to EntMR10A. In the case of E. faecium L50, when EntL50A and EntL50B are mixed in a ratio of approximately 1:1, their antimicrobial effect is much greater than the additive effect of the two bacteriocins acting independently. In addition, this strain produces multiple bacteriocins other than EntL50A and EntL50B. In the case of E. faecalis N1-33, the reverse-phase HPLC profile of partially purified fractions also showed multiple peptides with antibacterial activity. Such active peptides may contribute to the wide antibacterial spectrum of the N1-33 culture filtrate.

Another feature is that these bacteriocins are categorized into class IIb bacteriocins, the enterocin subclasses of class II bacteriocin (10, 17, 24). EntMR10A, EntMR10B, EntL50A, and EntL50B (http://aem.asm.org/cgi/content/full/69/3/) possess many features of class IIb bacteriocin. For example, they are small, heat-stable, devoid of leader peptides, and thus have been assigned to class IIb (4, 8, 10,
Despite a single–amino acid substitution (Aps38Glu), the antibacterial spectra of EntMR10A did not agree with EntL50A. EntMR10A strongly inhibited the growth of B. cereus strains, whereas EntL50A had no antibacterial effect on B. cereus (4, 8). Several enterocins that showed antibacterial activity against B. cereus strains have been reported to date, including enterocin P produced by E. faecium P13 (7), enterocin EJ97 produced by E. faecalis EJ97 (12, 25), and enterocin AS-48 produced by E. faecalis S-48 (11, 22). However, these enterocins were not similar to either EntMR10A or EntL50A with regard to structure or features. The N1-33 culture filtrate showed strong antibacterial activity against B. cereus and, for that reason, the N1-33 culture filtrate would likely involve EntMR10A. The purified fraction containing EntMR10A and EntMR10B showed weak activity against Escherichia coli (4); however, no activity against E. coli was observed in the 10× N1-33 filtrate. EntMR10A and EntMR10B were prepared from the culture in brain heart infusion broth, and the activity against E. coli was also assayed on a plate of the same medium. In this study, TYG broth was used for the assay. The productivity of the bacteriocin or sensitivity of E. coli against the bacteriocin may be influenced by the medium.

Although the primary amino acid sequences of the two major bacteriocins isolated from the N1-33 culture filtrate were identical, their molecular masses were found to be different, i.e., 5,215.92 and 5,199.68 Da. This difference is because of oxidation of an amino acid residue in the heavier bacteriocin (5,215.92 Da). There is no description of the difference in activity of oxidized and non-oxidized EntMR10A in the preceding literature (4). Whether the difference stemmed from characteristics of the strain itself, related to the culture condition, or occurred during purification process remains to be determined.

E. faecalis N1-33 was isolated in Thailand, and the bacteriocin produced by the strain is the same or a variant of enterocins MR10A and L50A produced by strains isolated in Spain. The fact that this type of Enterococcus bacterium is widely dispersed around the world is suggested by the wide antibacterial spectrum of bacteriocins. One of the properties of the N1-33 filtrate that makes it of particular interest as a food preservative is its ability to inhibit or obstruct the growth of B. cereus strains and L. monocytogenes strains at a near-neutral pH. Focusing on this point, we applied the N1-33 filtrate in model foods such as custard cream (for B. cereus) and pickled cucumber (for L. monocytogenes), because these foods have a neutral pH and the main bacterial contaminants of these foods are thought to be the previously mentioned strains. Our results showed that the N1-33 filtrate entirely inhibited the growth of indicator B. cereus IFO13494 for at least 3 days in custard cream, and of L. monocytogenes OCC197 for 7 days in pickled cucumber. The greatest standard deviation value obtained for L. monocytogenes after 7 days in the 10× N1-33 filtrate–containing sample was most likely caused by differences in the appearance frequency of the resistant strains in each sample. The addition of the N1-33 culture filtrate extended the shelf lives of custard cream and pickled cucumber. These findings indicate that the bacteriocin of N1-33 has antimicrobial activity at neutral pH conditions not only in media, but also in actual food systems. Furthermore, the bacteriocin is heat stable, as demonstrated by its ability to withstand the cooking process for custard cream. In conclusion, the bacteriocin, which is identical to EntMR10A (8), was purified, and the N-terminal was sequenced from the culture supernatant of the strain N1-33. The N1-33 culture filtrate containing bacteriocins shows antibacterial activity in actual food systems under neutral pH conditions, inhibiting the growth of B. cereus in custard cream and L. monocytogenes in pickled cucumber, respectively. Isolation of the genes necessary for bacteriocin production and sequencing of the structural gene will be useful in determining the complete primary structure of the bacteriocin. This work is in progress.

REFERENCES


