

Identification and Characterization of Leucocyclicin Q, a Novel Cyclic Bacteriocin Produced by *Leuconostoc mesenteroides* TK41401[∇]

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The culture supernatant of *Leuconostoc mesenteroides* TK41401, isolated from Japanese pickles, possessed antimicrobial activity against broad range of a bacterial genera and particularly strong activity against *Bacillus coagulans*, the major contaminant of pickles. An antimicrobial peptide was purified in three chromatographic steps, and its molecular mass was determined to be 6,115.59 Da by electrospray ionization time-of-flight mass spectrometry (ESI-TOF MS). The primary structure of this peptide was determined by amino acid and DNA sequencing, and these analyses revealed that it was translated as a 63-residue precursor. This precursor showed high similarity to the precursor of lactocyclicin Q, a cyclic bacteriocin produced by *Lactococcus* sp. strain QU 12. The molecular weight calculated after cyclization, which was presumed to involve the same process as in lactocyclicin Q (between L3 and W63), agreed with that estimated by ESI-TOF MS. This peptide was proved to be a novel cyclic bacteriocin, and it was termed leucocyclicin Q. The antimicrobial spectrum of this bacteriocin clearly differed from that of lactocyclicin Q, even though their primary structures were quite similar. This is the first report of a cyclic bacteriocin produced by a strain of the genus *Leuconostoc*.

Fermented foods represent one of the most common examples of biopreservation. Many microorganisms contribute to the taste, flavor, and preservation of these foods. Lactic acid bacteria (LAB) are known to play important roles in preservation by producing various antimicrobial substances that eliminate other competing microorganisms during the fermentation process (6). Some LAB produce different types of bacteriocins, which are antimicrobial peptides exhibiting bactericidal or bacteriostatic effects on Gram-positive bacteria, including food-borne pathogens, but not against eukaryotic cells (13, 37). In general, LAB bacteriocins are highly resistant to high temperatures and low pH but are easily digested by human proteolytic enzymes. These beneficial characteristics have led to the utilization of bacteriocins as biopreservatives and bacteriocin-producing LAB as starter cultures for fermented foods, such as pickles (10, 22).

LAB bacteriocins are mainly grouped into two classes according to the classification approach of Cotter et al. (13). Class I bacteriocins, so-called lantibiotics, are heat-stable post-translationally modified peptides containing multiple rings that are bridged by lanthionine or 3-methyl lanthionine residues (30). Class II bacteriocins are small, heat-stable nonlantibiotic peptides and are further divided into four subgroups (13, 31, 32). Class IIa bacteriocins are *Listeria*-active peptides contain-

ing the consensus sequence YGNQVXC at the N terminus (18, 20). Class IIb bacteriocins comprise two peptides, both of which are required for complete antimicrobial activity (33). Class IIc bacteriocins are cyclic bacteriocins, such as enterocin AS-48 (27) and lactocyclicin Q (36). Class IId bacteriocins are the other class II bacteriocins, including enterocin P, which is processed and secreted by the *sec* pathway (9), and lactacin Q, which is secreted without a leader sequence (21, 40).

Nisin is the most typical bacteriocin and is used as a food preservative around the world (26). Nisin possesses strong, wide-spectrum antimicrobial activity, but some bacteriocins are known to show higher activity against particular species than nisin, such as class IIa bacteriocins against *Listeria*. Many potent LAB bacteriocins have therefore been studied, and their use as biopreservatives is anticipated. When nisin is added to certain fermented foods or when a nisin-producing strain is used as a starter culture, it sometimes eliminates not only harmful or pathogenic bacteria but also the bacteria responsible for the food's original taste, thereby triggering a decline in food quality. On the other hand, if bacteriocin-producing inhabitant LAB or the bacteriocins can be utilized, they may be able to control bacteria in the fermented foods more effectively without affecting any essential bacteria and their flavors.

Recently, some reports on LAB cyclic bacteriocins (28), such as enterocin AS-48 (27), gassericin A (24, 25), reuterin 6 (23), uberolysin (39), and lactocyclicin Q (36), have been published. These bacteriocins are known to be translated as linear precursors with N-terminal leader sequences of various lengths and then mature by removal of the leader sequences and formation of peptide bonds between the N-terminal and C-ter-

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minimal residues. In general, cyclic bacteriocins show much higher stability when exposed to pH stress, high temperature, and proteolytic digestion (5, 29, 36). Because of these characteristics, LAB cyclic bacteriocins are expected to emerge as novel bio-preservatives succeeding nisin. Enterocin AS-48 is the most extensively studied LAB cyclic bacteriocin, in terms of both its biosynthesis and its application as a biopreservative (8, 16, 19, 35).

In this report, we describe the identification and characterization of leucocyclin Q, a novel cyclic bacteriocin produced by *Leuconostoc mesenteroides* TK41401, isolated from Japanese pickles. Leucocyclin Q has a cyclic structure in which N and C termini are bound to each other and comprises 61 amino acid residues that show high homology to lactocyclin Q, a cyclic bacteriocin produced by *Lactococcus* sp. strain QU 12. Leucocyclin Q shows a unique antimicrobial spectrum that differs from that of lactocyclin Q and shows high stability against several bacterial proteases.

MATERIALS AND METHODS

Bacterial strains and media. *L. mesenteroides* TK41401, a bacteriocin-producing strain, was isolated from pickled red turnips and identified by 16S rRNA gene sequence analysis. The strain was stored at -80°C in de Man, Rogosa, and Sharpe (MRS) medium (Oxoid, Basingstoke, United Kingdom) with 15% glycerol and propagated in MRS medium at 30°C for 18 h before use. Indicator strains for the determination of antimicrobial activities were propagated at the respective appropriate temperatures (30°C or 37°C) for 18 h before use. LAB strains were grown in MRS medium, and the other Gram-positive indicator strains were grown in tryptic soy broth (BD, Sparks, MD) supplemented with 0.6% yeast extract (Nacalai Tesque, Kyoto, Japan). *Escherichia coli* strains DH5α and JM109 were grown in Luria-Bertani (LB) medium (BD), and transformants of *E. coli* DH5α were selected on LB agar plates containing 50 mg/liter isopropyl-β-D-thiogalactopyranoside and 30 mg/liter ampicillin.

Determination of bacteriocin activity. Bacteriocin activity was determined by the spot-on-lawn method as previously described (21). *Bacillus coagulans* JCM 2257^T was used as an indicator strain unless otherwise stated. Briefly, 10 μl of a bacteriocin preparation was spotted onto a double-layered agar plate containing 5 ml of *Lactobacilli* agar AOAC (BD) inoculated with an overnight culture of an indicator strain as the upper layer and 10 ml of MRS medium with 1.2% agar as the bottom layer. After overnight incubation at the temperatures appropriate for the indicator strains, bacterial lawns were analyzed for inhibition zones. In each purification step, the activity titer was defined as the reciprocal of the highest dilution that yielded a clear zone of growth inhibition in the indicator lawn; this value was expressed in arbitrary activity units (AU) per milliliter of bacteriocin preparation. Purified bacteriocin was diluted with 0.1% (vol/vol) Tween 80 solution to the appropriate concentrations and tested for antimicrobial activity against various indicator strains as described above. The MIC was defined as the minimum bacteriocin concentration that yielded clear zones of growth inhibition in the indicator lawn. All activity tests were performed in duplicate.

Purification procedure for bacteriocin. Bacteriocin purification was carried out in a three-step procedure using the supernatant of a 1-liter culture of *L. mesenteroides* TK41401 grown for 20 h in MRS broth at 30°C. The cell-free supernatant was mixed with 25 g of Amberlite XAD-16 (Sigma-Aldrich, St. Louis, MO), a synthetic hydrophobic resin previously activated with 50% (vol/vol) isopropanol, and equilibrated with distilled water. The mixture was gently shaken at 4°C overnight and transferred to a column (10-mm internal diameter, 200-mm length). The mixture was washed with distilled water and 40% ethanol, followed by elution of the bacteriocin with 200 ml of 80% isopropanol containing 0.1% trifluoroacetic acid (TFA). The active eluted solution was placed in a rotary evaporator (Tokyo Rikakikai, Tokyo, Japan) to remove the isopropanol. The resulting solution was then diluted with 50 mM sodium phosphate buffer (PB; pH 5.7) to 100 ml and loaded onto an SP-Sepharose Fast Flow cation-exchange column (15-mm internal diameter, 100-mm length; GE Healthcare, Uppsala, Sweden) pre-equilibrated with PB. The column was then subjected to serial washes with 100 ml PB. The bacteriocin was eluted with 40 ml of PB containing 0.25 M and 0.5 M NaCl. For further purification, the active eluted solution was applied to a reverse-phase column (Resource RPC 3-ml; GE Healthcare) incorporated in the LC-2000Plus high-performance liquid chromatography (HPLC) system (Jasco, Tokyo, Japan), and eluted with a gradient of MilliQ water-

TABLE 1. Oligonucleotide primers used to obtain the structural gene of leucocyclin Q

Primer name	Sequence (5'-3') ^a
dAKA-F1	TGGGCNACNAARGCNGC
dAKA-F2	AARCAYGARGGNAARGC
dAKA-R2	GCYTTNCYTCRTGYTT
dAKA-R3	CCANGCDATNGCNGC
1stMup13-f	TTAACTATGCGGCATCAGA
1stMup13-r	TAATGTGAGTTAGCTCACT
Mup13-f	AAGGCGATTAAGTTGGGTA
Mup13-r	GTATGTTGTGTGGAATTGTG
s-M13-f	GTA AACGACGGCCAGT
s-M13-r	TTCACACAGGAAACAGG
aka-F1	GATATCGTTCAGTTAATTTGGC
aka-F2	GAGATTA AACCAATTATTGAGCCTG
aka-R1	CAAATCTTTTACCACGCAATAGC
aka-R3	CTAATAATCTTTGCTTACGTTCTTC
Lcy-F	TTTGTTGTCGTA AACATCTTATCTT
Lcy-R	GACGCAACAATACCAGTAGAAG

^a In degenerate primers, N, R, Y, and D indicate A/T/G/C, A/G, T/C, and A/T/G, respectively.

acetonitrile containing 0.1% TFA at a flow rate of 1 ml/min as follows: 0 to 10 min, 30% (vol/vol); 10 to 40 min, 30 to 60% (vol/vol); and 40 to 45 min, 60 to 100% (vol/vol) acetonitrile. The active fraction was subjected to RP-HPLC again, followed by thorough removal of acetonitrile in a Speed-Vac concentrator (Savants, Farmingdale, NY). The purified bacteriocin solution was used for MIC determination, and further characterization, such as amino acid sequence analysis and stability assessment, is described below. The antimicrobial activity of the fractions was determined as described above for each purification step. The protein concentration (mg/ml) of each fraction was estimated from the A₂₈₀ by using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) according to the manufacturer's instructions. The concentration of purified bacteriocin was also determined using a Pierce BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA). These concentrations were significantly equivalent, and the former was used to determine the MICs.

Sensitivity analyses against peptidases. The peptidase sensitivity of leucocyclin Q was determined for pepsin, trypsin, α-chymotrypsin, proteinase K, carboxypeptidase Y, and Asp-N peptidase (all from Sigma-Aldrich). All enzymes were dissolved in the respective appropriate buffers with optimal pH, and the solutions were filter sterilized and added to the purified leucocyclin Q at an enzyme-to-peptide molar ratio of 1:100. Following incubation at 37°C for 4 h, the reaction samples were heated at 100°C for 5 min to denature the enzymes. The residual antimicrobial activities were determined by agar diffusion assay as described in a previous study (3) by using *Lactobacillus sakei* subsp. *sakei* JCM 1157^T as an indicator strain. A sample without enzyme treatments was used as the negative control. All sensitivity tests were performed in duplicate.

Mass spectrometry and amino acid sequencing. Molecular mass analyses of the purified bacteriocin and fragments were conducted by electrospray ionization time-of-flight mass spectrometry (ESI-TOF MS) using a JMS-T100LC mass spectrometer (JEOL, Tokyo, Japan). The N-terminal amino acid sequences of the purified bacteriocin and fragments were determined by Edman degradation using a PPSQ-21 gas-phase automatic protein sequencer (Shimadzu, Kyoto, Japan). Fragmentation with 2-(2'-nitrophenylsulfonyl)-3-methyl-3-bromoindole-9-one (BNPS-skatole) was performed as previously described (36) to obtain a part of the amino acid sequence. All fragments were fractionated by HPLC as described above and used for subsequent analyses.

Analysis of the genes encoding the bacteriocins. DNA manipulations were performed as described in a previous report (21). DNA polymerases, restriction enzymes, and other DNA-modifying enzymes were used according to the manufacturers' instructions. The total DNA of the strain TK41041 was extracted from cells treated with lysozyme (Seikagaku, Tokyo, Japan) and cetyltrimethylammonium bromide (Wako, Osaka, Japan) according to the procedures described previously (21) and used as a template for PCR and subsequent procedures. The total DNA of strain TK4101 was digested by the restriction enzymes BamHI, EcoRI, SacI, KpnI, and XbaI, and digested DNA fragments were subsequently ligated into the pUC18 cloning vector (Toyobo, Osaka, Japan) or self-ligated for use as templates in nested anchor rapid PCR (NAR-PCR) and inverse PCR. The oligonucleotide primers used in this study are listed in Table 1. The degenerate primers dAKA-F1, F2, R2, and R3, designed from the amino

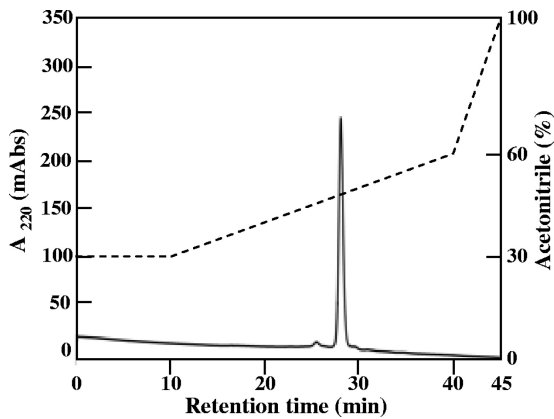


FIG. 1. Second reverse-phase HPLC of a bacteriocin from *L. mesenteroides* TK41401. The broken line indicates the acetonitrile gradient.

acid sequence obtained for the bacteriocin, were used for NAR-PCR as anchor primers. NAR-PCRs were performed using *Taq* DNA polymerase (Promega, Madison, WI) under the following conditions: denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at the optimal temperature for each primer for 30 s, and polymerization at 72°C for 2 min. The PCR fragments were cloned into the pGEM-T vector (Promega) and introduced into *E. coli* DH5 α by electroporation in a MicroPulser apparatus (Bio-Rad Laboratories, Hercules, CA) according to the procedure recommended by the manufacturer. DNA sequencing was carried out by FASMAC Co., Ltd. (Kanagawa, Japan). To obtain the upstream and downstream sequences of the bacteriocin structural gene, inverse PCR was performed with *Taq* DNA polymerase by using the primers aka-F1, F2, R1, and R3, designed from the DNA sequence obtained by NAR-PCR, under the following conditions: denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at the optimal temperature for each primer for 30 s, and polymerization at 72°C for 2 min. The products obtained by inverse PCR were cloned and sequenced as described above. On the basis of sequences obtained from the inverse PCR products, the new specific primers *Lcy-F* and *Lcy-R* were synthesized and used to confirm the DNA sequence around the structural gene by using the procedures described above.

Computer analysis of DNA and amino acid sequences. The DNA and amino acid sequences obtained were analyzed with GENETYX-WIN software, version 8.0.1 (Genetyx, Tokyo, Japan). Database searches were performed using the BLAST program of the National Center for Biotechnology Information (NCBI; <http://www.ncbi.nlm.nih.gov/BLAST/>).

Nucleotide sequence accession number. The nucleotide sequence determined in this study has been deposited in the DDBJ database under the accession no. AB649282.

RESULTS

Purification and molecular mass analysis of leucocyclin Q.

The bacteriocin produced by *L. mesenteroides* TK41401 was purified by a three-step procedure, which included hydrophobic interaction, cation-exchange chromatography, and reverse-phase HPLC. Most of the activity in the culture supernatant was recovered by hydrophobic interaction chromatography,

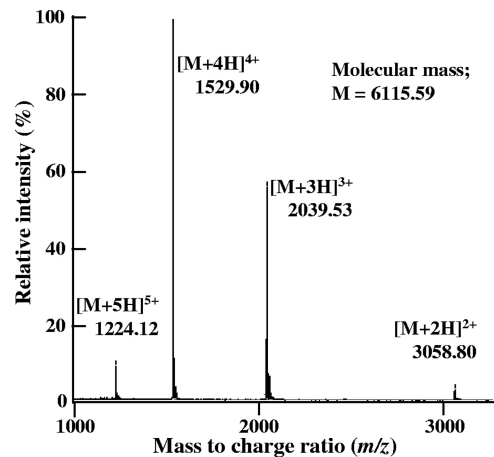


FIG. 2. ESI-TOF mass spectrum of purified leucocyclin Q. Multiple charged molecular ions are indicated. The molecular mass was calculated from the values of the major peak of tetravalent ions.

and the bacteriocin activity was primarily recovered in the 0.5 M NaCl fraction of the cation-exchange chromatography. This fraction was subsequently subjected to reverse-phase HPLC. One peak with antimicrobial activity was obtained, and the active fraction was subjected to RP-HPLC again (Fig. 1). The final yield of the peptide obtained in these purification steps was 25.6% from culture supernatant activity; the details are summarized in Table 2. ESI-TOF MS analysis was performed using the purified peptide, and the molecular mass of this peptide was determined to be 6,115.59 Da (Fig. 2). This molecular mass was not identical to that of any known bacteriocin. Therefore, we concluded that this peptide was a novel bacteriocin and designated it as leucocyclin Q.

Amino acid sequencing analysis of leucocyclin Q. Amino acid sequencing of purified leucocyclin Q was performed, but the Edman degradation did not proceed. This suggested that either the N-terminal amino acid is blocked by some modification or that leucocyclin Q possesses cyclic structure binding between the N and C termini. To obtain partial amino acid sequences, purified leucocyclin Q was fragmented by BNPS-skatole treatment, and the fragments were purified by HPLC. One fragment (2,437.24 Da) was subjected to amino acid sequencing, and a 23-residue amino acid sequence of leucocyclin Q was obtained. C-terminal tryptophan residues were cut by BNPS-skatole treatment. Considering tryptophan residues at both the N and C termini of the fragment, a 25-residue amino acid sequence was identified as (W)ATKAALATAETIVKHEGKAAAI(W). Based on this partial sequence, degenerate primers (Table 1) were designed and used for NAR-PCR.

TABLE 2. Purification of leucocyclin Q produced by *L. mesenteroides* TK41401

Step	Vol (ml)	Total activity (AU) ^a	Yield (%)	Sp act (AU/mg)	Purification (fold)
Supernatant	1,000	1.60×10^6	100	8.85×10	1.00
Amberlite XAD-16	200	1.28×10^6	80	6.88×10^3	77.8
SP-Sepharose	100	1.28×10^6	80	1.66×10^4	188
RP-HPLC	2	4.10×10^5	25.6	7.40×10^4	836

^a Antimicrobial activity (in arbitrary units [AU]) was assayed by the spot-on-lawn method using *B. coagulans* JCM 2257^T as an indicator strain.

1 ATATCCATTTTTTAAATCTTTAGAAAAATTTTTTGGAGGATTTAATATGTTCTTGGTAAAT 60
M F L V N
61 CAGTTAGGGATTTCAAAAATCGTTAGCTAATACTATTCTTGGTGCAATTGCTGTTGGTAAAT 120
Q L G I S K S L A N T I L G A I A V G N
121 TTGGCCAGTTGGTTATTAGCTTTGGTTCTGCTCCGGGTTGGGCAACAAAAGCAGCACTT 180
L A S W L L A L V P G P G W A T K A A L
181 GCGACAGCTGAAACAATTGTGAAGCATGAAGGAAAAGCAGCTGCTATTGCGTGGTAAAAA 240
A T A E T I V K H E G K A A A I A W *
241 GATTTGAACATAAATCTTTTTTGAAGTGAAGGAAAAGTCTGATAACTGTTGATGATATTAC 300

FIG. 3. Nucleotide sequence of the region encoding leucocyclin Q and the deduced amino acid sequence. The putative ribosome binding site is underlined. The structural gene is in italics, and the asterisk indicates the stop codon.

DNA sequencing analysis to elucidate the structure gene. NAR-PCRs were performed using degenerate primers as anchor primers, and the downstream DNA sequence of the structure gene of leucocyclin Q was successfully obtained. Based on this sequence, specific primers were constructed for inverse PCR, and a DNA sequence of around 700 bp was obtained using these primers. Sequencing analysis revealed some open reading frames, including *lcyQ*, encoding the precursor of leucocyclin Q (Fig. 3), which consisted of 63 amino acid residues. The calculated mass of this precursor was 6,411.50 Da, which is higher than the mass of purified leucocyclin Q determined by ESI-TOF MS, suggesting that it contained a leader sequence at the N terminus.

The database search we performed showed that leucocyclin Q was a novel bacteriocin, and its precursor showed high and characteristic homology (72%) with that of lactocyclin Q, a known cyclic bacteriocin produced by *Lactococcus* sp. strain QU 12 (Fig. 4). The precursor of lactocyclin Q contains an N-terminal leader sequence of two amino acid residues, and cyclization occurs between L3 and 63W with cleavage of the leader sequence (36). When this process was applied to leucocyclin Q, the calculated mass after considering the cleavage of the leader sequence (MF) and cyclization (-18 Da) was 6,115.13 Da, which agreed with that obtained by ESI-TOF MS. These results suggest that leucocyclin Q is a novel cyclic bacteriocin.

Antimicrobial spectrum of leucocyclin Q. Purified leucocyclin Q was tested for antimicrobial activity against various indicator strains by using the spot-on-lawn method (Table 3). As described above, although the leucocyclin Q structure showed very high similarity to lactocyclin Q, their antimicrobial spectra were very dissimilar. Both bacteriocins showed quite strong activities against *Lactobacillus sakei* and *Bacillus coagulans* and weak activities against Gram-negative bacteria. In addition, leucocyclin Q possessed higher activity against a broad range of Gram-positive bacteria, such as *Lactococcus*, *Weissella paramesenteroides*, *Pediococcus dextrinicus*, *Enterococcus*, *Streptococcus*, and *Leuconostoc*, than lactocyclin Q.

On the other hand, lactocyclin Q possessed higher activity against other Gram-positive bacteria, such as *Weissella cibaria*, *Pediococcus pentosaceus*, and *Bacillus subtilis*. Interestingly, both producer strains showed quite higher tolerance to each bacteriocin than their related species.

Sensitivity against peptidases. As shown in Table 4, leucocyclin Q was sensitive to digestion by human digestive enzymes such as pepsin and α -chymotrypsin but was tolerant to trypsin. On the other hand, leucocyclin Q showed high stability against bacterial peptidases, with the exception of proteinase K. It is noteworthy that leucocyclin Q showed no susceptibility to carboxypeptidase Y, which digests peptides from C-terminal residues.

TABLE 3. Antimicrobial spectra of leucocyclin Q and lactocyclin Q

Indicator strain ^a	MIC (μ M) ^b	
	Leucocyclin Q	Lactocyclin Q
<i>Lactococcus lactis</i> subsp. <i>lactis</i> JCM 7638	0.038	0.141
<i>L. lactis</i> subsp. <i>lactis</i> ATCC 19435 ^T	0.038	0.141
<i>Lactococcus</i> sp. QU 12	1.203	22.90
<i>Lactobacillus sakei</i> subsp. <i>sakei</i> JCM 1157 ^T	0.038	0.015
<i>Weissella cibaria</i> JCM 12495 ^T	2.405	0.710
<i>W. paramesenteroides</i> JCM 9890 ^T	0.075	ND
<i>Pediococcus pentosaceus</i> JCM 5885	2.405	0.550
<i>P. dextrinicus</i> JCM 5887 ^T	0.038	0.141
<i>Enterococcus faecium</i> JCM 5804 ^T	0.038	0.710
<i>E. faecalis</i> JCM 5803 ^T	0.150	0.260
<i>Streptococcus salivarius</i> JCM 5707 ^T	2.405	22.90
<i>S. bovis</i> JCM 5802 ^T	0.601	22.90
<i>S. mutans</i> JCM 5705 ^T	19.25	NA
<i>Bacillus coagulans</i> JCM 2257 ^T	0.075	0.015
<i>B. subtilis</i> subsp. <i>subtilis</i> JCM 1465 ^T	0.601	0.064
<i>B. cereus</i> JCM 2152 ^T	2.405	11.40
<i>Kocuria rhizophila</i> NBRC 12708	9.623	5.70
<i>Listeria innocua</i> ATCC 33090 ^T	0.601	1.03
<i>Leuconostoc mesenteroides</i> subsp. <i>mesenteroides</i> JCM 6124 ^T	0.300	1.03
<i>L. mesenteroides</i> TK41401	NA	22.90
<i>Escherichia coli</i> JM109	38.49	34.30
<i>E. coli</i> NBRC 3301	38.49	17.30
<i>Staphylococcus aureus</i> subsp. <i>aureus</i> ATCC 12600 ^T	NA	91.60

^a ATCC, American Type Culture Collection, Rockville, MD.; JCM, Japan Collection of Microorganisms, Saitama, Japan; NBRC, NITE Biological Resource Center, Chiba, Japan.

^b The antimicrobial spectrum of lactocyclin Q was drawn from the previous report (36). Only the MIC against *L. mesenteroides* TK41401 was determined in this study. ND, not determined; NA, no activity even with maximum concentrations of leucocyclin Q and lactocyclin Q (38.49 and 91.60 μ M, respectively).

1 3 63
Leucocyclin Q MFLVNQLG I SKSLANT I LGA I AVGNLASWLLALVPGPWATKAALATAET I VKHEGKAAA I AW
M L LG TILGAI AVGNLASW LALVPGPWGA KA LATA IVKH GKAAA AW
Lactocyclin Q MKL I DHLGAPRWAVDT I LGA I AVGNLASWLLALVPGPWAVKAGLATAAAA I VKHGKAAAAAAW

FIG. 4. Alignment of leucocyclin Q and lactocyclin Q. The middle line indicates identical amino acid residues.

TABLE 4. Peptidase sensitivity of leucocyclin Q

Peptidase	Diam (mm) ^a
Control (no peptidase)	23
Trypsin	23
Pepsin	0
α -Chymotrypsin	0
Proteinase K	0
Carboxypeptidase Y	23
Asp-N peptidase	23

^a Enzyme treatments were performed at 37°C for 4 h, and *L. sakei* subsp. *sakei* JCM 1157^T was used as the indicator strain. Diameters were based on the clear zones of inhibition.

DISCUSSION

In recent years, LAB cyclic bacteriocins have been carefully evaluated worldwide as the next generation of antimicrobial compounds because of their unusual characteristics. The search for novel LAB cyclic bacteriocins has resulted in discoveries of several bacteriocins produced by different genera, such as enterocin AS-48 from *Enterococcus*, uberolysin from *Streptococcus*, gassericin A, acidocin B, and reuterin 6 from *Lactobacillus*, carnocyclin A from *Carnobacterium* (29), and lactocyclin Q and garvicin ML (5) from *Lactococcus*. To the best of our knowledge, there are no reports to date on cyclic bacteriocins produced by members of the genus *Leuconostoc*. This study therefore presents the first report on cyclic bacteriocins produced by a member of this genus.

Leucocyclin Q was purified from the culture supernatant of *L. mesenteroides* TK41401 isolated from Japanese pickles by a three-step purification method and subsequently subjected to structure analyses. Edman degradation analysis, NAR-PCR, and inverse PCR were performed to obtain the structural gene of leucocyclin Q (Fig. 3). The amino acid sequence of its precursor was deduced from this DNA information, and it showed very high similarity to that of lactocyclin Q (Fig. 4). Lactocyclin Q contains a leader sequence of two amino acids, and cyclization occurs between L3 and W63 with concomitant cleavage of the leader sequence. Leucocyclin Q also contains a two-amino-acid leader sequence. In addition, the N and C termini of the cleavage peptide are flanked by leucine and tryptophan, respectively, the same amino acids flanking the termini of lactocyclin Q. The calculated mass of mature leucocyclin Q after cyclization and cleavage of the leader sequence agreed with the molecular mass obtained by ESI-TOF MS. These results indicate that leucocyclin Q is a novel cyclic bacteriocin. In addition to lactocyclin Q and leucocyclin Q, circularin A (24) and uberolysin (39) are known to possess short leader sequences, consisting of three and six residues, respectively. Other cyclic bacteriocins such as enterocin AS-48 possess long leader sequences, more than 20 amino acid residues (28). Regardless of the length of the leader sequences, there are certain differences in their secretion and cyclization processes, although little information is available regarding the cleavage reaction for maturation and function of these sequences. As predicted in a previous report on lactocyclin Q (36), leucocyclin Q possesses a KXXXXXXW sequence conserved at the C termini of many LAB cyclic bacteriocins (Fig. 4), which may be linked to the cyclization process. In addition, a leucine residue constitutes the N terminus of cleaved leuco-

cyclin Q, which is the same in most cyclic bacteriocins. The structure of leucocyclin Q also supported the prediction that these N and C termini might be important factors for secretion and cyclization and may serve as recognition sites for the cyclization enzyme.

The cyclization mechanisms of cyclic peptides, including those of LAB bacteriocins, are not well characterized. The enzymes McjB and McjC were recently shown to be essential for the cyclization of microcin J25 (17), a cyclic bacteriocin produced by *E. coli* AY 25 (4, 38). In contrast to most LAB cyclic bacteriocins, microcin J25 does not possess aromatic amino acids in the N or C termini, indicating that LAB cyclic bacteriocins possess an alternative cyclization mechanism. Eukaryotic animals and plants are also known to produce cyclic antibiotic peptides, such as defensin in animals and cyclotide in plants (7, 12, 14). These eukaryotic cyclic peptides include both N-terminal and C-terminal extensions in their linear precursor peptides, and both extensions are thought to function as processing sites and/or recognition sites in ligation reactions (15). There have been no reports on LAB cyclic bacteriocins with C-terminal extensions, and it is certain that the cyclization mechanism differs from that of eukaryotic cyclic peptides, while the N-terminal leader sequences are linked to their individual cyclization processes. In general, cyclotides possess longer N-terminal extensions, such as those in some LAB cyclic bacteriocins, and this region is possibly processed twice, once before and once during cyclization (1, 2, 15). Therefore, long leader sequences of cyclic bacteriocins can be considered tags that are recognized by a certain carrier that shuttles precursors to a cyclization factory. After cleavage of this tag, the remaining short leader sequence plays an important role in cyclization. On the other hand, bacteriocins with short leader sequences may use different systems to transfer the precursor to the appropriate cyclization position, because they do not possess long leader sequences that can act as tags.

Lactocyclin Q shows high antimicrobial activity against *Bacillus* and *Lactococcus*, and other cyclic bacteriocins show different spectra of activity. Enterocin AS-48 exerts strong antimicrobial activity against some Gram-positive bacteria, such as *Bacillus*, *Staphylococcus*, and *Listeria*, and also against some Gram-negative bacteria, such as *Salmonella* and *E. coli* (1, 2). The spectrum of leucocyclin Q differed from the spectra of these previously reported cyclic bacteriocins, exerting high activity against a very broad range of Gram-positive bacteria, with especially strong activity against *L. lactis*, *L. sakei*, *W. paramesenteroides*, *P. dextrinicus*, *Enterococcus faecium*, and *B. coagulans* but no activity against *Staphylococcus aureus*. Leucocyclin Q also exerted significant activity against Gram-negative bacteria when employed in high concentrations (Table 3). Although the primary structure of leucocyclin Q was similar to that of lactocyclin Q, the antimicrobial spectrum was quite different. In addition, the predicted secondary structure of leucocyclin Q included four α -helices, a structure similar to that of lactocyclin Q. The antimicrobial mechanism of cyclic bacteriocins has not yet been completely characterized. Further elucidation of the mode of action of these bacteriocins and structure analyses will help further our knowledge of LAB cyclic bacteriocin.

As shown in Table 3, *L. mesenteroides* TK41401 showed significantly high cross-immunity against lactocyclin Q, to the same extent as the original producer strain, *Lactococcus* sp. strain QU 12. This cross-immunity must depend on the leucocyclin Q immunity system, because another strain, *L. mesenteroides* subsp. *mesenteroides* JCM 6124^T, a non-bacteriocin-producing strain, showed quite high sensitivity. On the other hand, *Lactococcus* sp. strain QU 12 did not exhibit any immunity against leucocyclin Q significantly higher than that of strains of other genera; instead, it was approximately 40-fold more tolerant than other *Lactococcus* strains, including a nisin-producing strain (JCM 7638). These results suggest that the self-immunity systems of these strains employ a common mechanism that influences their comparatively lower susceptibilities to both cyclic bacteriocins.

In general, cyclic bacteriocins are known to possess extremely high stability against several stresses (11, 24, 27). For instance, lactocyclin Q maintains full activity after pH changes ranging from pH 3.0 to 9.0 and after exposure to 121°C for 15 min under acidic conditions. In addition, lactocyclin Q possesses high resistance against digestive enzymes, such as α -chymotrypsin, trypsin, pepsin, and proteinase K. When leucocyclin Q was exposed to stresses such as acidic, basic, or heat stress, it also exhibited remarkable stability (data not shown). Moreover, as shown in Table 4, leucocyclin Q exhibited noteworthy resistance against digestive enzymes and against bacterial peptidases. In contrast to lactocyclin Q (36), leucocyclin Q was not resistant to α -chymotrypsin or pepsin but exhibited significant resistance to trypsin.

Leuconostoc is one of the major LAB and can be isolated from a variety of fermented vegetables, such as Japanese pickles and sauerkraut (34). *L. mesenteroides* TK41401 produces a promising novel cyclic bacteriocin, leucocyclin Q, which exhibits remarkable antimicrobial activity and great stability against several stresses but is at the same time safely degradable by human digestive enzymes. Because of this useful bacteriocin production, it is expected that *L. mesenteroides* TK41401 will be used as a novel starter culture for fermented vegetables, and leucocyclin Q itself also holds enormous potential as a biopreservative. In addition, leucocyclin Q is a significantly potent research subject not only in applied food microbiology but also for the study of LAB cyclic bacteriocin biosynthesis, which is still not well characterized. Future studies will open a new platform for the application of LAB cyclic bacteriocins.

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