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Cloning of the Gene Encoding a Novel Lantibiotic, Nukacin ISK-1, of *Staphylococcus warneri*

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Staphylococcus warneri ISK–1, we had reported as *Pediococcus* sp. ISK–1 previously, produces a novel bacteriocin, nukacin ISK–1. Edman degradation of the chemically reduced nukacin ISK–1 revealed a sequence of 27 amino acids, 7 of which were unidentified. Using single–specific–primer–PCR (SSP–PCR) product as a probe, a 3.6–kb *Hin*dIII fragment containing nukacin ISK–1 structural gene (*nukA*) was cloned and sequenced. The deduced amino acid sequence of nukacin ISK–1 revealed that it was comprised of 57–amino acids, including a 30–amino acid leader region. The propeptide sequence showed significant similarity to those of lacticin–481 type lantibiotics. It was expected that an active 27–residue nukacin ISK–1 contained two lanthionines, one 3–methyllanthionine, and one dehydrobutyrine. In the region upstream of *nukA*, a part of long open reading frame (ORF), designated as *nukM*, encoding a putative modification enzyme involved in the lantibiotic biosynthesis, was oriented in the opposite direction. In the region of downstream of *nukA*, ORF1 was found, in which the sequence of the putative translational product was similar to those of response regulatory proteins such as LytT of *Bacillus subtilis* and VirR of *Clostridium perfringens*.

INTRODUCTION

Some microorganisms produce proteinaceous antimicrobial substances called bacteriocins. Bacteriocins are generally a heterogeneous group of bactericidal proteins which are effective against species that are closely related to the producer strains. Some bacteriocins inhibit the growth of food–borne pathogenic bacteria such as *Listeria* and *Clostridium*, which makes them potential as natural food preservatives in the near future.

Nisin, the antimicrobial peptide produced by *Lactococcus lactis*, was discovered in 1928 (Rogers and Whittier, 1928) and many lantibiotics have been reported (Jung, 1991). Lantibiotics are class I bacteriocins which contain unusual amino acids such as dehydroalanine (Dha), dehydrobutyrine (Dhb), lanthionine, and 3–methyllanthionine (Sahl *et al.*, 1995). Lantibiotics are ribosomally synthesized as precursor peptides, which are post–translationally converted to the biologically active peptides through enzymatic modifications. First, serine and threonine residues in the propeptide portion of the

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precursor peptide are dehydrated to Dha and Dhb, respectively. The double bonds of some of the Dha and Dhb residues react with the thiol group of a neighboring cysteine residue to form the respective thioether rings of lanthionine and 3–methyllanthionine in an enzyme–mediated reaction. Finally, the active lantibiotics are released and secreted by proteolytic cleavage of the leader peptide (Kupke and Götz, 1996).

In addition to the thioether amino acids, some lantibiotics contain a variety of other modified amino acids, including lysino–alanine, 2–aminovinyl–D–cysteine, hydroxy–aspartic acid and D–alanine (Jung, 1991, Skaugen *et al.*, 1994). It has been considered that they play an important role to maintain the structure and the antimicrobial activity of lantibiotics. They also confer the antiprotease and pH tolerances due to the conformation of the thioether rings in the peptides. Thus, the modification enzymes for lantibiotics are attractive for biotechnological applications like protein engineering.

On the basis of the ring structures of lantibiotics, Jung (1991) proposed that they were divided into two types. Type A comprises those that are more elongated screw–shaped, amphiphilic polypeptides. Their primary bactericidal action is exerted by the formation of pores and depolarization of the bacterial cytoplasmic membrane. Type B comprises more compact, globular, and almost neutral amphiphilic polypeptides. Type–B lantibiotics show not only bactericidal activities but also inhibition of enzymatic activities (angiotensin–I converting enzyme or phospholipase A_2) (Fredenhagen *et al.*, 1990), inhibition of herpes simplex virus–1, and immune adjuvant (Naruse *et al.*, 1989, Brötz *et al.*, 1997). Moreover, type–A lantibiotics are classified with regard to their ring structure and their leader peptide with a characteristic cleavage site into three subtypes; nisin, lacticin–481, and lactocin–S types. The nisin type lantibiotics such as nisin A (Gross and Morell, 1971), nisin Z (Mulders *et al.*, 1991, Matsusaki *et al.*, 1996), epidermin (Allgaier *et al.*, 1986), Pep5 (Kellner *et al.*, 1991), and subtilin (Gross *et al.*, 1973) are the most widely investigated in terms of their mechanism of pore formation (Moll *et al.*, 1996), their biosynthesis(Siezen *et al.*, 1996), and the relationship between their structures and antibacterial activity (Moll *et al.*, 1996). The commercial application of these lantibiotics has been expected, nisin A being used as a natural food preservative in many countries (Delves–Broughton *et al.*, 1996). In the past decade other lantibiotics belonging to lacticin–481 type were also isolated and characterized. This subtype includes lacticin 481 (Piard *et al.*, 1993), bacteriocin J46 (Huot *et al.*, 1996), variacin (Pridmore *et al.*, 1996), streptococcin A–FF22 (Hynes *et al.*, 1993), streptococcin A–M49 (Hynes *et al.*, 1994), mutacin II (Woodruff *et al.*, 1998), butyrivibriocin OR79A (Kalmokoff *et al.*, 1999), and salivaricin A (Ross *et al.*, 1993). However, these lantibiotics have not been extensively characterized yet. They have been considered something different in the mechanism of biosynthesis judging from these gene clusters from those of nisin type lantibiotics (Siezen *et al.*, 1996).

Staphylococcus warneri ISK–1 was isolated in our laboratory from well–aged *Nukadoko*, a bed of fermented rice bran. This strain, which had been reported as *Pediococcus* sp. ISK–1 previously, produces a bacteriocin, nukacin ISK–1 (Kimura *et al.*, 1997, 1998). We reported the purification, the amino acid composition and sequence analysis of nukacin ISK–1, indicating that it is a peptide with a molecular mass of 2.9 kDa containing 3 molecules of lanthionine and/or 3–methyllanthionine. The N–terminal 7 amino acid residues were also determined as NH_2–K–K–K–S–G–V–I by Edman degra-

dation, the sequence of which showed high similarities to those of lacticin–481 type lantibiotics (Kimura *et al.*, 1998). However, we could not determine the primary structure of nukacin ISK–1 completely, because Edman degradation after 8 cycles was blocked due to the possible thioether bridges. Here, we report the amino acid sequence of the nukacin ISK–1 reduced by the treatment with alkaline mercaptoethanol and cloning of its structural gene. The results strongly suggest that nukacin ISK–1 is a novel lantibiotic belonging to lacticin–481 type. Other ORFs relating to nukacin ISK–1 biosynthesis are also described.

MATERIALS AND METHODOS

Bacterial strains, media and plasmid

Staphylococcus warneri ISK–1, which had been referred to as *Pediococcus* sp. ISK–1 (Kimura *et al.*, 1997, 1998), producing nukacin ISK–1 was used in this study. This strain was grown in MRS medium (Oxoid, Hampshire, United Kingdom) at 37 °C. *Escherichia coli* JM109 was grown in LB medium (Sambrook *et al.*, 1989) at 37 °C. When needed, 40 mg/liter ampicillin was added to medium.

A plasmid pUC18 (Toyobo, Osaka, Japan) was used for cloning of nukacin ISK–1 structural gene.

Amino acid sequence analysis of the reduced nukacin ISK–1

The chemical reduction of the purified nukacin ISK–1 was performed according to the method as described by Meyer *et al.* (1994). The reduced nukacin ISK–1 was further purified by high performance liquid chromatography (HPLC) under the condition described previously (Kimura *et al.*, 1998), and the amino acid sequence was analyzed by Edman degradation with an automated gas–phase protein sequencer (PSQ1; Shimadzu, Kyoto, Japan) with an on–line LC6A HPLC system (Shimadzu).

DNA manipulations

Total genomic DNA of ISK–1 strain was isolated by a combination of the methods of Marmur (1961) and Berns and Thomas (1965), except that 0.1 mg/ml of *N*–acetylmuramidase (Seikagaku, Tokyo, Japan) was used for cell lysis instead of lysozyme. Isolation of plasmids, digestion of DNA with restriction endonucleases, and agarose gel electrophoresis were carried out by standard procedures (Sambrook *et al.*, 1989) or as recommended by the manufacturers. The total DNA library of ISK–1 strain was constructed with pUC18 and completely digested genomic DNA using *Hin*dIII. The primers, or the cassette oligonucleotide (Takara, Tokyo, Japan) used for single–specific–primer–PCR (SSP–PCR) (Shyamala and Ames, 1993), are shown in Table 1. Prior to SSP–PCR, the total DNA of ISK–1 strain was completely digested with *Xba*I and the cassette at *Xba*I site was ligated to the total DNA fragments. They were then used as templates for PCR, which was performed with primers C–1 and PI–7 using *LA Taq* DNA polymerase (Takara), in the procedures outlined by the manufacturer, using the following conditions: denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and elongation at 72 °C for 2 min, through 30 cycles. Elution of PCR products from 1.2% agarose gel was performed after gel electrophoresis by using the Geneclean II Kit (Bio 101, Vista, CA,

Table 1. Sequence of oligonucleotides used in this study

Names	Sequences	Notes
PI-7	5'-CA(A/G)CA(A/C/G/T)GT(A/G)AA(A/G/T)AC(A/G)AA(C/T)TG(A/G)AA-3'	The synthetic oligonucleotide corresponding to the putative 3' region of nukacin ISK-1 structural gene
C-1	5'-GTACATATTGTCGTTAGAACGCGTAATACGA-3'	The primer corresponding to the cassette oligomer
Cassette	5'-OH GTACATATTGTCGTTAGAACGCGTAATACGACTCACTATAGGGAGAT-3' 3'-CATGTATAACAGCAATCTTGCGCATTATGCTGAGTGATATCCCTCTAGATC OH-5'	Cassette oligomer

The underlined letters indicate the sequence of *Xba*I site.

USA). The amplified PCR fragments were ligated into a pUC18 derivative T-vector, which was prepared by digestion with *Sma*I and the addition of a single T by *Taq* DNA polymerase. Transformation of *E. coli* was carried out by electroporation, as described by Dower *et al.* (1988). Cells were electroporated in a 0.2-cm cuvette with a Gene Pulser apparatus (Bio-Rad, Hercules, CA, USA) set at 25 μF, 200 Ω and 2.5 kV. After electroporation, cells were grown in SOC medium (Sambrook *et al.*, 1989) at 37 °C for 1 h, and then incubated in LB agar medium containing 40 mg/liter ampicillin.

Hybridization experiments

Hybridization was carried out as described by Southern (1975) under a stringent condition (68 °C). Colonies on LB plates were transferred to Hybond-N^+ nylon membrane (Amersham, Buckinghamshire, United Kingdom). The DNA probe used was the 720-bp product obtained by PCR with primers C-1 and PI-7. Preparation of digoxigenin-labeled probe and detection of hybridization signals on membranes were carried out with a DIG DNA Labeling and Detection Kit (Boehringer, Mannheim, Germany).

Nucleotide sequence analysis

DNA fragments to be sequenced were further subcloned into pUC18, and nested sets of deletion clones were generated by using *double-stranded* Nested Deletion Kit (Pharmacia Biotech, Uppsala, Sweden), and they were sequenced with an ALF express sequencer (Pharmacia Biotech). The sequencing reaction based on the method of Sanger *et al.* (1977) was performed with the Auto Sequencer Core Kit (Toyobo) according with the instructions of the manufacturer. The resulting nucleotide sequence was analyzed by SDC-GENETYX genetic information processing software (Software Development, Tokyo, Japan). Sequence similarity searches were performed with the BLAST (Basic Local Alignment Search Tool) program and the NCBI (National Center for Biotechnology Information) databases.

Nucleotide sequence accession number

The nucleotide sequence data determined here appear in the EMBL, GenBank, and DDBJ databases under accession no. AB034941.

RESULTS

Amino acid sequence analysis of the reduced nukacin ISK-1

As described previously (Kimura *et al.*, 1998), Edman degradation after 8 cycles was blocked due to possible thioether bridges. Therefore, nukacin ISK-1 was treated with alkaline mercaptoethanol and the sequence of 27 amino acids was determined as, NH_2-K-K-K-S-G-V-I-P-X-V-X-H-D-X-H-M-N-X-F-Q-F-V-F-X-X-X-S-COOH. Here, X indicates unidentified amino acids, probably modified post-translationally. The amino acid sequence of the reduced nukacin ISK-1 revealed a sequence similarity to those of lacticin-481 type lantibiotics.

Cloning of *nukA* from *Staphylococcus warneri* ISK-1

At first, for cloning of the nukacin ISK-1 structural gene (*nukA*), several oligonucleotides were synthesized based on the N-terminal 7-amino acids sequence of nukacin ISK-1, and they were used as probes and primers for Southern hybridization and SSP-PCR, respectively. However, the attempts were not successful using the degenerated oligonucleotides. Hence, primer PI-7 was designed based on the expected C-terminal sequence as -F-Q-F-V-F-T-C-C-S-COOH, because the C-terminal region of lacticin-481 type lantibiotics was highly conserved, -F-V-F-T-C-C-S-COOH. Then, SSP-PCR was performed with primers PI-7 and C-1, and cassette-ligated total DNA fragment of ISK-1 strain as template, resulting in successful amplification of a 720-bp product. Because of the lack of the phosphate of 5'-terminal of the cassette, the PCR product can not be amplified by C-1 primer alone. This product was purified from agarose gel after electrophoresis and cloned into a pUC18 derivative T-vector, and then used as a template for DNA sequencing. The nucleotide sequence of the PCR product corresponded to an amino acid sequence of the reduced nukacin ISK-1. Furthermore, the 720-bp PCR product was used as a probe to isolate and characterize a full-length *nukA* clone from genomic DNA library of ISK-1 strain. Finally, pPUH4 containing a 3.6-kb *Hin*dIII fragment hybridized with the probe was isolated by colony hybridization.

Nucleotide sequence of 3.6-kb *Hin*dIII fragment, *nukA*, *nukM*, and ORF1

The complete nucleotide sequence of the cloned 3.6-kb *Hin*dIII fragment was determined in both strands. The restriction map of the cloned fragment and the nucleotide sequence were shown in Figs. 1 and 2, respectively. In the 3.6-kb *Hin*dIII fragment, 174-bp open reading frame (ORF) containing nukacin ISK-1 structural gene (*nukA*) was found at position 2123 to 2296. In this ORF, two possible ATG start codons were found at positions 2123 and 2141. From the comparison of lacticin-481 type lantibiotics, position 2141 seemed to be the start codon. However, at 10 nucleotides upstream of position 2123, a possible ribosomal binding site (GGAG) was found. Hence, the *nukA* gene was predicted to be translated from position 2123. The deduced amino acid sequence corresponding to the N-terminal sequence of nukacin ISK-1 was found to start at residue 31 of the predicted product of the *nukA* gene. Thus, it seems that nukacin ISK-1 is synthesized as a 57-amino acid precursor peptide that is cleaved between Ala^{-1} and Lys^{+1} to form a 27-residue propeptide. The predicted propeptide sequence was in good agreement with the amino acid sequence obtained from the

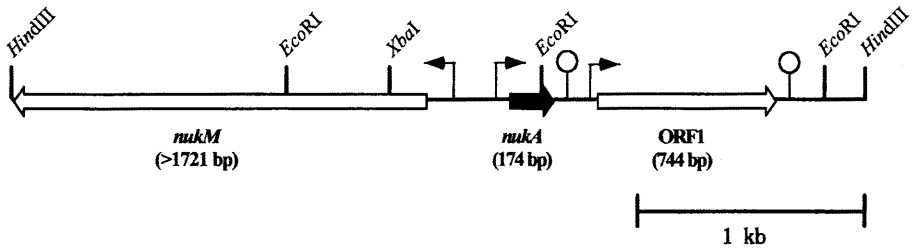

Fig. 1. Restriction map of the 3.6-kb *Hin*dIII fragment and organization of *nukA*, *nukM* and ORF1. The arrows represent promoters. The possible ρ-independent transcription terminators for *nukA* and ORF1 are illustrated by lollipop-like symbols.

reduced nukacin ISK-1, except for unidentified amino acids.

In the region upstream of *nukA*, a part of long ORF was found, designated as *nukM*, in the opposite orientation (starting from position 1721). The translational product of this truncated gene was similar to that of the 5'-terminal region of *lanM* (Rince *et al.*, 1994, Woodruff *et al.*, 1998) encoding a modification enzyme involved in the biosynthesis of lantibiotics, (25–44% identity for the N-terminal 572-amino acids). LanM is usually composed of more than 900 amino acids, suggesting that *nukM* contains a further 1-kb of DNA.

An ORF1 (744-bp), which could encode a protein of 247 residues with a molecular mass of 28.8 kDa, was also found at 428-bp downstream of *nukA*, and the sequence of the putative translational product was similar to various response regulator proteins such as LytT of *Bacillus subtilis* (28% identity, 48% similarity) (Gothel *et al.*, 1997) and VirR of *Clostridium perfringens* (23% identity, 44% similarity) (Shimizu *et al.*, 1994). Therefore, the motif program *Pfam*, developed by Sonnhammer *et al.* (1997), was used to search ORF1 translational product for its defined amino acid sequence motif. The search revealed that 112 residues at the N-terminal region of ORF1 (residues 4 to 115) corresponded to the response regulator receiver domain.

Several -35 to -10 consensus sequences of σ^{70}-dependent promoters were found in the region between *nukA* gene and truncated *nukM* gene on both strands, and upstream of ORF1 (Fig. 2). Stem-loop structures were found downstream of *nukA* and ORF1. They might act as a ρ-independent transcription terminator sequences (calculated free energy of formation, ΔG_0, -105.6, and 123.2 kJ·mol^{-1}, respectively) (Platt, 1986).

Comparison of amino acid sequence of nukacin ISK-1 precursor peptide with those of lacticin-481 type lantibiotics

The nukacin ISK-1 precursor peptide showed significant similarities to lacticin 481 (Piard *et al.*, 1993), bacteriocin J46 (Huot *et al.*, 1996), variacin (Pridmore *et al.*, 1996), streptococcin A-FF22 (Hynes *et al.*, 1993), streptococcin A-M49 (Hynes *et al.*, 1994), mutacin II (Woodruff *et al.*, 1998), butyrivibriocin OR79A (Kalmokoff *et al.*, 1999) and salivaricin A (Ross *et al.*, 1993) (Fig. 3). The position of the thioether bridge of these lacticin-481 type lantibiotics has not been determined yet except for lacticin 481 (Van

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      S  E  F  I  I  E  E  G  K  S  I  G  L  N  M  W  N  V  S  G  D  K  E  D  I  V  A  N  N  I  I  I
   1  TTCGAAAGTTTATAATAAAGAAGAGGAAAACTATACGGATTTAAGTAGGTTAAATGTCTTGGTAGAAAAAGTAGATACTGACGTAATAATTAATATTACG
      HindIII
      S  V  L  N  D  I  A  D  D  I  N  D  S  N  V  I  E  Y  G  Y  N  I  F  G  N  G  D  I  F  L  K  N  Q  S
 101  AATGATTTAACAGCTAACGTAGCAGTTATAACAGACTTAAATGATAAAGTATAGGTATTAAATATTTAGGTAATGGTAGTTATTTATTAAATAAAACTGA

      E  L  S  F  K  I  L  K  C  Q  F  L  L  D  K  L  S  Y  R  Q  T  K  E  L  F  V  N  L  P  N  K  L  L
 201  AAGATTACTTTTAAAATAATCAAATGTAACTTTGTTGTTTAGAAAGTTTCTTATAGAAACGCAAAAAAGATTTTTATGTAACTCACCCAAAAAGTTATTC

      H  H  V  T  N  N  K  I  D  K  V  S  V  S  Q  V  K  T  Y  F  Y  P  V  S  L  T  E  I  Q  K  I  E  S
 301  ACTACATGGCATAATAAAAATTATAGAAAATGGCTTTGTGAAACCTGGAATCATATTTTTATCCCATGACTTTCTCAAAGATAAACAAAATAAAGACTTA

      N  I  L  K  D  S  L  L  N  E  G  Y  E  E  L  K  N  F  L  S  K  R  K  N  A  Y  L  P  S  K  A  A  Q  I
 401  AATAATTGAATAGTGAATTATTTAAAAGTGGCATGAGAAGATCAAACAATTTGTTCGAGAAAGCGAATAAACGTATTTCTCCACTAAATCGTCGAACATA

      L  V  S  Y  E  I  T  S  R  P  L  I  R  V  E  V  E  S  S  T  K  K  I  Y  H  L  I  E  K  R  N  N  L
 501  ATTATGACTCATGAGTTAACATGAAGAACCATTTTAAGAGTGAAGATGAAGACTACTGCAAAAGAAATATATTACATCATAAAGAAAAGATAACAATTCT

      F  L  E  Y  G  Y  K  F  G  E  Q  I  D  E  I  F  V  E  P  S  F  R  R  K  K  D  N  E  M  Y  F  P  I
 601  TTATTAAGCATTGGTATAAATTTCGGAAGAACTTATAGAAGATATTTATGAAGCCCTCTTTTAGAGGAAAAAAATAGTAAAAGGTATATTTTACCCTATA

      H  D  N  R  K  V  R  V  L  R  K  E  F  K  I  D  D  R  N  P  N  S  I  T  R  E  H  K  N  F  V  G  G  L
 701  CTAGTAAAGAAAATTGAGAATGATTAGAGAAAAGCTTAAAATATAGTAGAGATAATCCTAATGAATATCAAGAAAGCACAAATAATTTATGAGGTGGATT

      I  G  S  I  D  G  G  Y  K  K  D  K  K  S  I  P  L  M  G  T  A  F  V  S  N  A  A  K  D  E  I  N  K
 801  ATAAGGTGAATATAGAGGTGGCATAAAAAACAGAAAAAACCTATAGCCATTGTAAGGCCATCGCTTATGTCTTAATCGTCGAAATAGAAGATATAAGAAA

      T  A  L  N  R  F  K  S  E  Y  I  S  T  H  F  I  T  E  L  D  I  L  I  P  F  N  S  S  C  L  I  N  E
 901  CATCGATTTAATGCTTTAAAACTAAGTATATACCTGCATACTTTATATCAAAGATTTAGTTAATTATAACCTTTTAATGACCTTGTGTCATATAAAAGCT

      F  H  L  D  S  I  N  L  L  Y  G  I  S  L  L  Y  G  L  R  K  Y  Y  M  N  I  K  N  K  S  T  P  Q  K  E
1001  TTACATTTAGTGAATATAAATTATTTATAGGATATCTGTTATTTATAGGGTTAGAAAACATTATGTATAAATAAAATAAAAATCTCCATCCAACGAAAAG

      I  Y  E  M  W  G  H  D  K  Y  D  I  F  K  Y  K  Y  Y  T  S  I  E  N  K  Q  F  S  Y  M  F  E  L  F
1101  ATATATAAGGTAGGTTGGCACCAGAAATATTAGATACTTAAATATGAATATCATACAACTATAAAGTAAAAAAACTTTTGACATGTATTTAAGATTCTTA

      E  L  F  F  S  D  N  E  L  S  R  P  K  Y  L  L  Q  S  K  D  T  T  V  K  T  V  A  K  G  G  H  L  D
1201  AGATTTTTCTTTGATAGTAAAAGATTACTGGATCCAAATATATTATTAACTCTAAATAGTCACCAATGAAATCAGTGGCGAAATGGAGGTACATTTAGAG

      G  L  I  S  I  H  C  I  T  E  K  E  T  K  I  L  N  A  E  L  L  K  K  K  E  Q  N  F  K  N  E  I  D  K
1301  GATTTTATGACTATACTGTTTAGCAAAGAAAAAGTCAAAAATAATTTAACCGAAGATTTTCAAAGAAAAAAAGAACTAACTTAAATAAAAGCTACAGAAA

      L  F  S  F  Y  S  N  L  T  Q  E  L  D  N  I  I  S  P  Y  S  K  N  L  E  E  Y  I  I  G  K  E  C  L
1401  GTTTTTGCTTTTTATACTCAAATTTCAAACGAGATTTAGTAAATAATAACTACCTATTGAAAATAAATTGAGAAGTATATATTAGGGAAAAAGTGTATTA

      E  E  D  F  Y  K  Y  R  E  E  P  T  N  G  N  L  K  K  N  L  R  K  E  N  I  L  V  I  L  S  K  G  C
1501  AGAAGTAGTTTTATAAATATCGCAAGAAGTCCTCATAAAGGTAACTCAAAAAACAAATTAGAAAAAAGTAATTAATTTTGTTAGTTACTAAAAGGTGTTT

      I  D  L  I  I  S  R  L  Y  N  W  D  I  L  T  N  L  E  Q  K  S  Y  K  K  L  I  F  N  S  F  G  R  F  Q
1601  ATAGATCTTATTATCTAGAATTCATCAAGGTTAGTTAATTACACAATTCAAGAACAAATCTCATAAAAAAATTTTATTTTAAACTTTTAGGAGATTTAAC
            ◄──── nukM
      E  V  K  I  N  N  M                RBS              -10                    -35
1701  AAGTTGAAATTACAACAAGTATCAATTATAGTAGAGGATAATAATTGATAGTATTAAGTATATTGCGTAATATATTTAAACGAAATTGTATAGAAAAGCT
1701  TTCAACTTTAATGTTGTTCATAGTTAATATCATCTCCTATTATTAACTATCATAATTCATATAACGCATTATATAAATTTGCTTTAACATATCTTTTCGA
                                                                                                  -10
1801  GCAAACTTTAATCAAAACTACACATACTTGTAAGGAGAAAATGTCACAATATTTTTATTGATCGAACGATGTTAAAGCGAAATGAAGATTATAACCATTA
1801  CGTTTGAAATTAGTTTTGATGTGTATGAACATTCCTCTTTTACAGTGTTATAAAAATAACTAGCTTGCTACAATTTCGCTTTACTTCTAATATTGGTAAT
        -35        -35        -10        -10                    -35                    -10
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                 -35
1901  GATTGGAGTATGATGTTCTATTTAAAACGTCGTCTTTGCCTTTTTGGATTTTGATTGTTTTAACATTCAAAGTTTAAAAATAAAATAAGTATATCTTTTG
1901  CTAACCTCATACTACAAGATAAATTTTGCAGCAGAAACGGAAAAACCTAAAACTAACAAAATTGTAAGTTTCAAATTTTTATTTTATTCATATAGAAAAC

2001  GTGAAATAGTGAGATTTATATTTAAAATTAATATATTTATTCACCAAATATGTTAAAATGTGAAACATTTATAGTACACTATAATTTGAACATTGTTTAT
2001  CACTTTATCACTCTAAATATAAATTTTTAATTATATAAATAAGTGGTTTATACAATTTTACACTTTGTAAATATCATGTGATATTAAACTTGTAACAAATA
                                 -35                    -10
2101  TTAAATTTAGGAGGTAACAAACATGGAAAATTCTAAAGTTATGAAGGACATTGAAGTAGCAAATTTATTAGAAGAGGTTCAAGAAGATGAATTGAATGAA
                RBS            M  E  N  S  K  V  M  K  D  I  E  V  A  N  L  L  E  E  V  Q  E  D  E  L  N  E
                               nukA ---------->
2201  GTCTTAGGAGCTAAGAAAAAGTCAGGAGTAATCCCAACTGTGTCACACGATTGCCATATGAATTCTTTCCAATTTGTATTTACTTGTTGTTCATAAAAAG
      V  L  G  A  K  K  K  S  G  V  I  P  T  V  S  H  D  C  H  M  N  S  F  Q  F  V  F  T  C  C  S  *
                 ↑
2301  GGGAATGTTGTAAAATCAGTAAAAATAACTTTAAGTGATACTTACTTTAGTTTTTTTATTGATTAACATGATACTAATATTATGGCATGGCGAAAATCCG
                                                                                                  - - - - -
2401  TAGTTTTGAAAAGAACTACGGATTTTTTATGAACCGTAAAACATATATAGTGTCTTTAGTTATTGTATTTTGGAGAGTATTGAAACCTTAATATTTTGTT
      ---->          <- - - - - -
2501  CTTTCCAGCTTTTATTAAGATAACTTCATCGAAGGCAATTTATTGGCAACAATTACTTGAAGTCTAGCAGATATACTTTACAAAATAATCTTACAATCTA

2601  TCAATTTAAAAAAATTTTAAAGTTCATTTAATAATAATGATTTAAAAAAATAATTGTGATATATTAATGAAGTAAGGTTGGTTTTAATTTTTTATTGATA
                                          -35                    -10
2701  AAGGAATGGGAAAATTGATTTTATGAAAAAAATAAATGCTATTATTATAGACGATAATGCAAGTACTAGAGAGGATTTAAAAGTAAATCTATCTTACTTC
              RBS              M  K  K  I  N  A  I  I  I  D  D  N  A  S  T  R  E  D  L  K  V  N  L  S  Y  F
                               ORF1 ---------->
2801  TCATTTGTTTCATGTAAAGGTACATTTAGTGAATATATTGAAGCCAAAAATACCCTAGATAAGAAAGATATAGACTTAATATTTTTAAAAATAAATACAA
      S  F  V  S  C  K  G  T  F  S  E  Y  I  E  A  K  N  T  L  D  K  K  D  I  D  L  I  F  L  K  I  N  T  N

2901  ATAACAACAAGTTTATAGATATAGCCAATAATATCAATTCTATACATAAACATATTCAAATAGTTTTTCTATCTTCTGATTCCAAACACGCACATAAAAG
       N  N  K  F  I  D  I  A  N  N  I  N  S  I  H  K  H  I  Q  I  V  F  L  S  S  D  S  K  H  A  H  K  S

3001  TTTTGAAGCTCATCCTTTTGACTATTTAATACAACCAGTAGAGCCTATTAAATTGGAGAAGTGCTTACTTCATTTCAAAAATAGATGCAGAAAAAAATTC
      F  E  A  H  P  F  D  Y  L  I  Q  P  V  E  P  I  K  L  E  K  C  L  L  H  F  K  N  R  C  R  K  K  F

3101  GATCTTAACAATCTTAAAAACAGAAAAATCGCCATTAAAAAGGGGAAATCTTTATATTTTTTAGAAATTGATAAAATTACAACAGCAGAATGTTGCGGTA
      D  L  N  N  L  K  N  R  K  I  A  I  K  K  G  K  S  L  Y  F  L  E  I  D  K  I  T  T  A  E  C  C  G  R

3201  GAAAAATAATTATCACTTTGGATGACAAAGAAACAGTTTCTTATAATGGCTCCTTAAAATATTTAGAAAAAAAACTCTATTCTTATGGATTTATTTTAGT
       K  I  I  I  T  L  D  D  K  E  T  V  S  Y  N  G  S  L  K  Y  L  E  K  K  L  Y  S  Y  G  F  I  L  V

3301  AAGTAGGTCATTACTTATTCCTTTAAAAAGTATTAAAAATATAATATTCGATAAGCATAGTAAATGCTATATTTTGAAATCGATTAAGAATAATACGGTT
      S  R  S  L  L  I  P  L  K  S  I  K  N  I  I  F  D  K  H  S  K  C  Y  I  L  K  S  I  K  N  N  T  V

3401  AAAATCACTCCAGAAAAGTATAAAAAAGTAAAGCAAACATTAAGTGAATTCAACTGGATTATTTAATCTGGTTCTATTACAAAAAATTAAAGTTTTAGAA
      K  I  T  P  E  K  Y  K  K  V  K  Q  T  L  S  E  F  N  W  I  I  *

3501  AATTCAGATATAACGTTATATTGTATTTTGAAAACAACTTATAATCTAGTTCCCCCATCTCCTTTACAAGAGATGGGGGGGGAACATTTGATTTTTTGTT
                                                                  - - - - ->          <- - - - -

3601  CAAAGTTTAAAGATAAAGCTT  3621
                   HindIII
```

Fig. 2. Nucleotide sequence of the 3.6-kb *Hin*dIII fragment containing the structural gene *nukA*, truncated *nukM* gene and ORF1, and the deduced amino acid sequences. The putative ribosome binding sequences are boxed and indicated by RBS. The putative transcriptional promoter sequences (-35 and -10) are underlined. Termination codons are indicated by asterisks. The vertical arrow indicates cleavage site of the propeptide to form active nukacin ISK-1. The horizontal dashed arrows below the nucleotide sequence indicate putative transcriptional terminators.

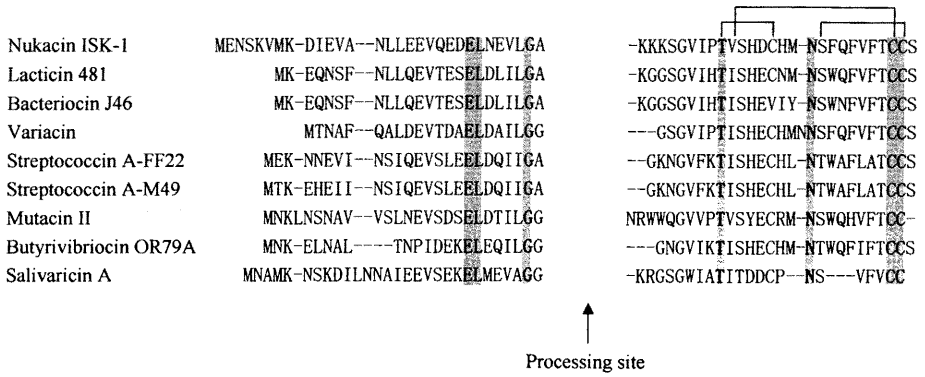

Fig. 3. Sequence comparison of the precursor of nukacin ISK-1 with those of lacticin-481 type lantibiotics. Identical residues are indicated in shaded boxes. The vertical arrow indicates processing site.

den Hooven *et al.*, 1996). The thioether bridging pattern of lacticin-481 type lantibiotics has been considered to be the same structure, because the positions of serine, threonine and cysteine, which should be converted to unusual amino acids such as dehydro amino acids and lanthionine, are highly conserved (Fig. 3). The molecular mass of nukacin ISK-1 determined by fast atom bombardment mass spectrometry (FAB-MS) was 2,960 Da (Kimura *et al.*, 1998). On the other hand, the calculated molecular mass of the propeptide portion of nukacin ISK-1 was 3,031 Da. Since nukacin ISK-1 contains 3 molecules of lanthionine and/or 3-methyllanthionine as described previously (Kimura *et al.*, 1998), the location of the thioether bridges was predicted to be between residues 9 and 14, 11 and 25, and 18 and 26 judging from the conserved residues. Threonine at residue 24 in the propeptide of nukacin ISK-1 could be converted to Dhb in the active form, because the residue could not be identified by amino acid sequence analysis of the reduced nukacin ISK-1 described above. In the case using this hypothesis, the calculated molecular mass of the active nukacin ISK-1 should be 2,959 Da, which was in good agreement with the molecular mass of that obtained by FAB-MS.

The proteolytic cleavage site of the leader peptide in the nukacin ISK-1 precursor peptide was not similar to the consensus sequence found in other type-A lantibiotics such as nisin and epidermin (X^{-4}-X^{-3}-Pro^{-2}-X^{-1}-X^{+1}, where residues X^{-4}, X^{-2}, and X^{+1} are hydrophobic, residue X^{-3} is negatively charged or polar, and residue X^{-1} is large and positively charged or polar) (Jung, 1991). The leader peptides of lacticin-481 type lantibiotics have a conserved negatively charged central part with several glutamic acid residues, and the cleavage site is characterized by Gly^{-2}-Ala/Gly^{-1} similar to that of non-lantibiotic bacteriocins of lactic acid bacteria (Klaenhammer, 1993) (Fig. 3).

DISCUSSION

Staphylococcus epidermidis produces some nisin type lantibiotics such as Pep5 (Kellner *et al.*, 1991), epidermin (Allgaier *et al.*, 1986), gallidermin (Schnell *et al.*, 1989),

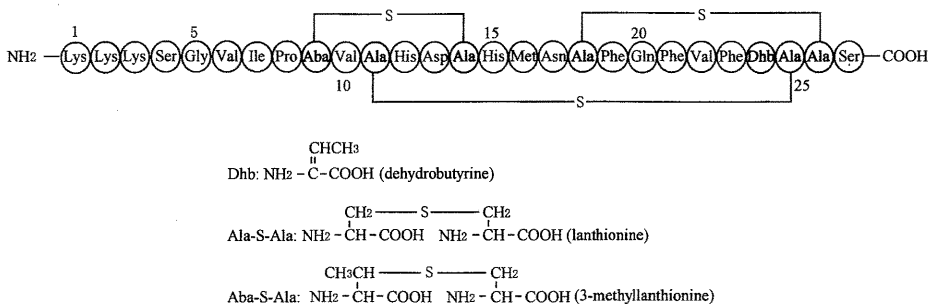

Fig. 4. Proposed structure of nukacin ISK–1. Ala–S–Ala, Aba–S–Ala, and Dhb indicate lanthionine, 3–methyllanthionine, and dehydrobutyrine, respectively.

and epilancin K7 (Van de Kamp *et al.*, 1995). In this study, we demonstrated strong evidence that nukacin ISK–1 was a novel lacticin–481 type lantibiotic. This is the first report that staphylococci produce lacticin–481 type lantibiotics. Sequence analysis of 3.6–kb *Hin*dIII fragment showed that nukacin ISK–1 was synthesized as a 57–amino acid precursor peptide. The prepeptide sequence was very similar to those of lacticin–481 type lantibiotics. From the sequence similarity of lacticin–481 type lantibiotics, it was predicted that the secondary structure of nukacin ISK–1 was almost the same as that of lacticin 481. The calculated molecular mass of the active nukacin ISK–1 based on the aforementioned assumption was in good agreement with that measured by FAB–MS. In Fig. 4, we are proposing the structure of nukacin ISK–1.

In the region upstream of *nukA*, a part of long ORF designated as *nukM*, which encodes a putative modification enzyme involved in the lantibiotic biosynthesis, was oriented in the opposite direction. In the past a few years, many gene clusters of lacticin–481 type lantibiotics have been characterized (Rince *et al.*, 1997, Chen *et al.*, 1999, McLaughlin *et al.*, 1999). The principal difference between the gene clusters found in lacticin–481 type lantibiotics and those of nisin type lantibiotics, is that the former gene clusters have no genes for proteins with a significant sequence similarity to LanB. LanB was elucidated as a dehydration enzyme in nisin type lantibiotics by *in vivo* experiment (Sen *et al.*, 1999). However, the former gene clusters contain a similar gene (*lanM*) in size to the LanB protein genes. Furthermore, the C–terminal half of the translational product (LanM) has sequence similarity to the LanC proteins including the 7 conserved segments (Siezen *et al.*, 1996). LanC seems to catalyze the formation of thioether rings between the dehydrated amino acids and cysteine residue. Although there is no obvious sequence similarity to the LanB proteins, it seems possible that LanM represents hybrid proteins, capable of catalyzing both of the reactions assumed for LanB and LanC. In biosynthesis of nisin and subtilin, it is revealed that post–translational modifications occur at a level of membrane–associated multimeric lanthionine synthetase complex (Siegers *et al.*, 1996, Kiesau *et al.*, 1997). Now, it is interesting to know how the multimeric lanthionine synthetase complex of lacticin–481 type lantibiotics is associated.

In the region downstream of *nukA*, ORF1, having a high similarity to the response regulator gene, was located. As found in other lantibiotic gene clusters such as nisin,

subtilin and epidermin, the transcription of some gene clusters involved in biosynthesis of lantibiotics are positively controlled by two-component regulatory system composed of response regulatory protein and histidine kinase, i.e. NisR and NisK in nisin (Kuipers *et al.*, 1995), SpaR and SpaK in subutilin (Klein *et al.*, 1993), and EpiQ in epidermin (Peschel *et al.*, 1993). However, the deduced amino acid sequence of ORF1 had low similarities to these regulatory proteins belonging to nisin type lantibiotics (20.5% identity for 220 amino acids of NisR, 19.8% identity for 81 amino acids of SpaR, and 19.5% identity for 82 amino acids of EpiQ). NisR and SpaR have both response regulator receiver domain and transcriptional regulator domain in N- and C-terminal regions, respectively. On the other hands, putative translational product of ORF1 has only a response regulator receiver domain in N-terminal region. However, VirR of *Clostridium perfringens*, which shows high structural similarity to ORF1 translational product, still acts as a transcriptional regulator (Shimizu *et al.*, 1994). Therefore, further investigation of nukacin ISK-1 gene clusters and further molecular analysis of biosynthetic genes are presently in progress.

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