Characterization of a Gene Cluster of Staphylococcus warneri ISK-1 Encoding the Biosynthesis of and Immunity to the Lantibiotic, Nukacin ISK-1

Yuji Asō, Toshihiro Sashihara, Jun-ichi Nagao, Youhei Kanemasa, Hanako Koga, Taku Hashimoto, Toshimasa Higuchi, Asahiko Adachi, Harumi Nomiyama, Ayako Ishizaki, Jiro Nakayama, and Kenji Sonomoto

Laboratory of Microbial Technology, Division of Microbial Science and Technology, Department of Bioscience and Biotechnology, Faculty of Agriculture, Graduate School, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan

Received March 1, 2004; Accepted May 7, 2004

We characterized a gene cluster in a plasmid designated pPI-1 of Staphylococcus warneri ISK-1 encoding the biosynthesis of and immunity to the lacticin-481 type lantibiotic, nukacin ISK-1. The DNA sequence suggested that the nukacin ISK-1 gene cluster consists of at least six genes, nukA (a structural gene), -M, -T, -F, -E, -G, and two open reading frames, ORF1 and ORF7. NukM and NukT were predicted to be involved in post-translational modification and secretion of nukacin ISK-1 respectively. NukF, -E, and -G were predicted to form a membrane complex which contributes to self-protection from nukacin ISK-1. Transcriptional analyses revealed that nukM through ORF7 comprises an operon, and that ORF1 is transcribed independently from downstream of nukA. The transcriptional levels of the nukA and nukM genes were enhanced by osmotic stress. The expression level of the nukA transcript was scarcely enhanced by nukacin ISK-1, suggesting that expression is not under the control of the autoregulatory circuit.

Key words: lantibiotic; bacteriocin; nukacin ISK-1; Staphylococcus; gene cluster

Lantibiotics are a class of bacteriocins that are antimicrobial polypeptides produced by a wide range of gram-positive bacteria. They are characterized by the presence of unusual amino acids such as dehydroalanine, dehydrobutyrine, lanthionine, and 3-methylanthionine, which are synthesized post-translationally. According to the structure imposed by the thioether rings of the lanthionine residues, they are classified into linear (type A) and globular (type B) groups. Type-A lantibiotics are further divided into three subgroups, viz., the nisin, lacticin-481, and lactococcin-S types, based on their primary structure similarities. Nukacin ISK-1 is a lacticin-481 type lantibiotic produced by Staphylococcus warneri ISK-1 (JCM 11004). The N-terminal amino acid sequence analysis, amino acid composition analysis, mass spectrum analysis, and structural gene sequence analysis suggested that nukacin ISK-1 consists of 27 amino acids including 2 molecules of lanthionine, 1 molecule of 3-methylanthionine, and 1 residue of dehydrobutyrine (Fig. 1).

The gene clusters of lacticin-481 type lantibiotics so far characterized commonly consist of a structural gene for a bacteriocin prepeptide (lanA), a gene for a modification enzyme involved in post-translational modification to generate unusual amino acids such as lanthionine (lanM), a gene for an ABC transporter protein involved in the secretion of the bacteriocin (lanT), and genes for a membrane protein complex involved in self-protection against its lantibiotic (lanFEG). Although the primary structures of lacticin-481 type lantibiotics are highly similar, there are some diversities in the organization of their gene clusters. For

Fig. 1. Proposed Structure of Nukacin ISK-1.

The dark shaded residues indicate modified amino acids. Ala-S-Ala, lanthionine; Abu-S-Ala, 3-methylanthionine; Dhb, dehydrobutyrine.
instance, the expression of the streptococcin A-FF22 gene cluster is controlled by a two-component regulatory system composed of ScnK and ScnR, while MutR, a transcription regulator, controls the expression of mutacin II in an unknown manner. In the case of nisin, the expression of the gene cluster is autoregulated by nisin itself as a signal transduction substance via a two-component regulatory system composed of NisR and NisK. No signal transduction substance has been found, however, in the lacticin-481 type gene cluster. Furthermore, some unique genes were found in the lacticin-481 type lantibiotic producers. In the lacticin 3147 producer Lactococcus lactis subsp. lactis DPC3147, ltnD encodes a biosynthetic protein of lacticin 3147, which shows similarity to dehydrogenase.

**Materials and Methods**

**Bacterial strains, media, and plasmids.** S. warneri ISK-1 (JCM 11004) isolated from long-aged Nukadoko and Pedicoccus pentosaceus JCM 5885 (formerly reported as Pedicoccus acidilactici JCM 5885) were grown in MRS medium (Oxoid, Hampshire, U.K.), as described previously. Escherichia coli JM 109 (Toyobo, Osaka, Japan) was grown in LB medium at 37 °C. Plasmid pUC18 (Toyobo) was used for cloning of the fragments of the nukacin ISK-1 biosynthetic gene cluster. For selection of transformants carrying pUC18 and its derivatives, ampicillin was used at concentrations of 25 mg/l.

**DNA cloning and sequence analysis.** Plasmid DNA of S. warneri ISK-1 was isolated according to the method developed by Anderson and McKay, except that 0.1 g/liter of N-acetylmuramidase (Seikagaku, Tokyo, Japan) was used for cell lysis. For cloning of the 4.5-kb XbaI fragment containing a part of the nukM and nukT genes, the XbaI digests of plasmid DNA of S. warneri ISK-1 were cloned into pUC18 at the XbaI site, and subsequently the XbaI genomic library was constructed. The positive clone harboring the 4.5-kb XbaI fragment was screened by colony hybridization using the 1-kb fragment containing the 3′-terminal region of the 3.6-kb HindIII fragment as a probe. To clone the 6-kb BcI- EcoRI fragment containing a part of nukT and nukF, -E, -G, and ORF7, the BclI and EcoRI digests of the plasmid DNA of S. warneri ISK-1 were cloned into pUC18 at the BamHI and EcoRI sites, and subsequently the BclI-EcoRI genomic library was constructed. The positive clone harboring the 6-kb BcI-EcoRI was screened by colony hybridization using the 0.4-kb fragment containing the 3′-terminal region of the 4.5-kb XbaI fragment as a probe. The nucleotide sequencing was done with a Thermo Sequenase Cy5.5 dye terminator cycle sequencing kit (Amersham Pharmacia Biotech, Uppsala, Sweden) and a Gene Rapid sequencer (Amersham Pharmacia Biotech). The sequence analysis and similarity searches were done as described previously.

**Northern blot hybridization, RT-PCR, and primer extension analysis.** Extraction of the total RNA from S. warneri ISK-1 and Northern blot hybridization were done as described previously. The probes for Northern blot hybridization were amplified by PCR with the total DNA of S. warneri ISK-1 and Northern blot hybridization were done as described previously. The probes for Northern blot hybridization were amplified by PCR with the total DNA of S. warneri ISK-1 and Northern blot hybridization were done as described previously. The probes for Northern blot hybridization were amplified by PCR with the total DNA of S. warneri ISK-1 and Northern blot hybridization were done as described previously. The probes for Northern blot hybridization were amplified by PCR with the total DNA of S. warneri ISK-1 and Northern blot hybridization were done as described previously. The probes for Northern blot hybridization were amplified by PCR with the total DNA of S. warneri ISK-1 and Northern blot hybridization were done as described previously. The probes for Northern blot hybridization were amplified by PCR with the total DNA of S. warneri ISK-1 and Northern blot hybridization were done as described previously.
region between nukA and ORF1. For primer extension analysis, nucleotide sequencing reactions were done with the following antisense oligonucleotides: 5′-ACTT-CAATGTCCTTCATAACTTTAGA-3′ and 5′-ATTGT-TCAACCTTAATGTGTTCA-3′, located in the 5′ regions of the nukA gene and the nukM gene respectively, using a T7 sequence quick denature plasmid sequencing kit (Amersham Pharmacia Biotech). For reverse transcription reactions, a primer extension system (Promega, Madison, Wisconsin, U.S.A.) was used. Briefly, the same oligonucleotides were labeled with [γ-32P]dATP using T4 polynucleotide kinase, annealed to 5 μg of RNA extracted from S. warneri ISK-1 in the hybridization buffer at 42 °C for 30 min, followed by denaturing at 95 °C for 5 min. Avian myeloblastosis virus reverse transcriptase (25 U, Amersham Pharmacia Biotech) and dNTPs were added to the samples. After a 30-min extension with the reverse transcriptase at 48 °C for 60 min, the samples were heated at 95 °C for 3 min and resolved on a 6% polyacrylamide gel for 2 h at 2,500 V.

The effect of nukacin ISK-1 on the transcriptional level of nukA. Partially purified nukacin ISK-1 was prepared according to the purification method. 5) Ten milliliters of S. warneri ISK-1 overnight culture was inoculated into 200 ml of fresh MRS with or without the partially purified nukacin ISK-1 at the minimum inhibitory concentration (MIC) for the indicator strain, P. pentosaceus JCM 5885, and incubated at 37 °C for 27 h. The MIC was determined according to the method reported previously. 20) The cells were harvested from the culture at intervals of 3 h. Subsequently, total RNA was extracted from the cells, and the transcriptional levels of nukA were compared by dot blot hybridization according to the method described previously. 6)

Nucleotide sequence accession number. The nucleotide sequence data given here was deposited in the EMBL, GenBank, and DDBJ databases under accession no. AB121757.

Results

Cloning and nucleotide sequence of the nukacin ISK-1 gene cluster

As shown in Fig. 2, we analyzed the 11.7-kb HindIII-EcoRI region of a plasmid designated pPI-1 of S. warneri ISK-1, including the 3.6-kb HindIII region reported previously. 6) Features of putative gene products encoded in the region are summarized in Table 1. In the region upstream of nukA, six open reading frames (ORFs) preceded by putative ribosome binding sites were found in the same orientation. The truncated nukM gene was reported in our previous study. 6) Because the four ORFs downstream of nukM showed similarities with the corresponding genes found in lacticin-481 type lantibiotics in terms of both nucleotide sequences and the predicted motifs of the deduced amino acid sequence, 9) they were termed nukT, -F, -E, and -G respectively.

The nukM gene encodes a 917-residue protein without a potential membrane-spanning region. The putative
The nukT gene is presumably translated from TTG as a start codon, and its putative product consists of 694 amino acids. The deduced nukT product possesses the N-terminal proteolytic domain for cleavage of the leader sequence of the precursor bacteriocin with the two conserved sequence motifs (QX\_4D/ECX\_2AX\_3MX\_3-MX\_4Y/FGX\_4I/L and HY/FY/VVX\_1O/LXDP\_21) (positions 6–30 and 90–107 respectively). NukT also contains a membrane-spanning domain consisting of four transmembrane helices in the middle region (approximately positions 157–311) and a C-terminal ATP-binding domain with Walker motifs A and B, GXXGXGKS/T and hhhh\_DEP/A (h is a hydrophobic residue)\_22,23) (positions 497–504 and 611–617 respectively). These findings suggested that the putative nukT translational product is involved in the secretion of nukacin ISK-1 as a membrane ABC transporter with processing activity.

The nukF, -E, and -G products consist of 291, 250, and 245 amino acids respectively. The nukG gene is presumably translated from TTG as a start codon as well as the nukT gene. The nukF, -E, and -G products showed high sequence similarities with a series of proteins encoded by the immunity gene cluster of lacticin-481 type lantibiotics. The nukF product possesses the consensus amino acid sequence of the ATP-binding domain with Walker motifs A and B (positions 38–45 and 153–159 respectively), and the nukE and -G products were predicted to be transmembrane proteins. These results suggested that NukF, -E, and -G form an ABC transporter-like complex which contributes to self-protection of S. warneri ISK-1 from nukacin ISK-1. The putative ORF7 product consists of 92 amino acids. The deduced amino acid sequence of ORF7 located immediately downstream of nukG showed significant similarities only to function-unknown proteins, e.g., ORF4\_\_24) found in the gene cluster of a lacticin-481 type lantibiotic, butyrivibriocin OR79A, from Butyrivibrio fibrisolvens OR79, and a putative immunity-related protein gene, rumH\_\_25) encoded on the locus of a lacticin-481 type lantibiotic, ruminococcin A, from Ruminococcus gnavus E1 (Fig. 3A). Interestingly, ORF4 and rumH, were also located immediately downstream of a gene cluster for immunity proteins. By a hydrophobic profile developed by Kyte and Doolittle,\_26) the putative ORF7 product was highly cationic (net charge of +7 at neutral pH) and was predicted to have three membrane-spanning domains (Fig. 3B). The ORF4 product and RumH show a similar hydrophobic
profile to the ORF7 product. Taken together, this suggested that the ORF7 product has the same function as the ORF4 product and RumH.

In the spacer regions between nukM, -T, -F, -E, -G, and ORF7, no terminator-like sequence was found. An inverted repeat, which is likely to act as a rho-independent transcription terminator, was found 42 bp downstream of ORF7. Downstream of ORF7, two ORFs (ORF8 and ORF9) were found in an opposite orientation to nukMTFEG-ORF7. The deduced amino acid sequences of ORF8 and ORF9 showed significant similarities to those of ORF2 and ORF1 of Staphylococcus simulans respectively, which are located in the flanking region of genes encoding the preprolysostaphin and lysostaphin immunity factor, and appear to be unrelated to the biosynthesis of nukacin ISK-1.

**Transcriptional analysis of the nukacin ISK-1 biosynthetic genes**

In the previous report, we indicated that nuka is transcribed independently. Northern blot analysis was done to confirm that nukM, -T, -F, -E, -G, and ORF7 were co-transcribed as anticipated from the nucleotide sequence. As shown in Fig. 4, a transcript of about 9-kb was detected by both nukM- and ORF7-specific probes, indicating that these genes are co-transcribed. Transcription of ORF1 was not detected by Northern blot analysis, but a 220-bp fragment was confirmed by RT-PCR with the primers, each corresponding to the internal region of ORF1 (data not shown), suggesting transcription of ORF1. On the other hand, although the transcription of the spacer region between ORF1 and nuka was investigated by RT-PCR with the primers, each corresponding to ORF1 and nuka, no PCR product was confirmed. This indicated that ORF1 was transcribed by the promoter upstream of ORF1 regardless of nuka. The transcription units of the nukacin ISK-1 gene cluster are summarized in Fig. 2.

The transcription start sites of nuka and nukMTFEG-ORF7 were precisely determined by primer extension analysis. The transcription start sites of nuka and nukM genes corresponded to a T located 31 bp and a T located 21 bp upstream from the ATG codon respectively (Figs. 5A and 6A, lane 1). As a result of characterization of the promoter, sequences with similarity to the constitutive σ70-like promoter were found 8 and 7 bp
upstream of the transcriptional start sites of nukA (−35/−10, TTTACA/GATATT) and nukM (−35/−10, TTTATA/TATGAT) respectively (Figs. 5B and 6B). The transcription start site of ORF1 could not be detected. This may be due to its low expression level (data not shown).

Our previous study indicated that the osmotic stress caused by NaCl, KCl, and sorbitol stimulates the production of nukacin ISK-1 and transcription of nukA.16) In this study, Northern blot analysis indicated that osmotic stress increased the transcriptional level of nukM approximately 4-fold at 8 h after cultivation as well as that of nukA (Fig. 7). The respective transcription start sites of nukA and nukMTFEG-ORF7 under the osmotic stress condition were identical to those under a non-stress condition, whereas the intensity of the bands was increased by salt stress in the cases of both nukA and nukMTFEG-ORF7 (Figs. 5A and 6A, lane 2). These results suggested that osmotic stress enhanced the transcription of nukA and nukMTFEG-ORF7, which resulted in an increase in nukacin ISK-1 production.
It has been reported that the production of some bacteriocins is controlled by positive feedback regulation.\(^{12}\) Hence we investigated whether the transcription of \(nukA\) was autoregulated by nukacin ISK-1. \(S.\ warneri\) ISK-1 was cultivated with or without the partially purified nukacin ISK-1, and subsequently the transcriptional levels of \(nukA\) were compared by Northern blotting using the total RNAs isolated from harvested cells. As a result, the addition of the partially purified nukacin ISK-1 little affected the transcriptional level of \(nukA\) as well as the bacteriocin production itself (Fig. 8). Hence it was suggested that the production of nukacin ISK-1 is not under the control of the autoregulatory circuit.

**Discussion**

Confirming the previous report,\(^{6}\) this study indicated that the nukacin ISK-1 gene cluster consists of at least the structural gene (\(nukA\)), a modification enzyme gene (\(nukM\)), an ABC transporter gene (\(nukT\)), genes encoding for self-protection (\(nukFEG\), ORF1, and ORF7. Those ORFs, except for ORF1 and ORF7, are commonly found in the biosynthetic gene clusters of lacticin-481 type lantibiotics.\(^{7-9}\) The ORF7 product showed sequence similarity (identity, 26\% and 32\%; similarity, 51\% and 57\%) to the ORF4 product\(^ {24}\) of \(B.\ fibrisolvens\) OR79 and RumH\(^ {25}\) of \(R.\ gnavus\) E1, which are putative accessory proteins encoded immediately downstream of the gene cluster for immunity complex to butyrivibrio cin OR79A and ruminococcin A respectively. Furthermore, the ORF7, ORF4, and RumH products showed significant similar hydrophobic profiles. Therefore, it was suggested that the ORF7 product, the ORF4 product, and RumH have similar functions. On the other hand, the gene cluster of lacticin 481 does not contain any kind of ORF like the ORF7 of nukacin ISK-1.

The transcriptional analyses in this study indicated that three transcripts were expressed in this putative nukacin ISK-1 gene cluster and that the transcriptional sites of \(nukA\) and \(nukMTFEG\)-ORF7 were located in the intergenic region of \(nukA\) and \(nukM\) (Fig. 2). ORF1 and \(nukA\) were transcribed independently, whereas the long transcript from \(nukM\) to ORF7 was expressed as an operon. In this study, we found that osmotic stress increased the transcriptional level of \(nukM\) as well as
that of nukA. In the previous report,\textsuperscript{16} it was suggested that increases in the transcriptional levels of nukA and nukMTFEG-ORF7 by osmotic stress resulted in increase in the productivity of nukacin ISK-1. The respective transcription start sites of nukA and nukMTFEG-ORF7 were not changed by osmotic stress when compared to a non-stress condition. This finding suggested that the promoters of nukA and nukMTFEG-ORF7 were not affected by osmotic stress. To understand the mechanism of enhancement of transcriptional levels of nukA and nukM by osmotic stress, we examined the effects of nukacin ISK-1 on the transcriptional level of nukA. The addition of the partially purified nukacin ISK-1 little affected the transcriptional level of nukA. Therefore the production of nukacin ISK-1 is not under the control of the autoregulatory circuit. This finding coincides with the fact that there is no inverted or direct repeat sequence around the promoter region, as has been commonly found in a promoter under the control of the autoregulatory circuit.\textsuperscript{28,29} In the case of nisin, the expression of the biosynthetic genes is controlled by the autoregulatory circuit via the histidine kinase NisK and the response regulator NisR.\textsuperscript{12} Such an autoregulatory circuit is involved in the expression of the biosynthetic genes of other lantibiotics such as the type-A lantibiotics, subtilin\textsuperscript{30} and streptococcin A-FF2\textsuperscript{20} and the type-B lantibiotic, mersacidin.\textsuperscript{31} On the other hand, some lantibiotic biosynthetic gene clusters possess genes encoding orphan transcription regulators. In the case of epidermin, a nisin-type lantibiotic, the expression of the biosynthetic genes is under an orphan transcription regulator, EpiQ.\textsuperscript{32,33} Mutacin II, a lacticin 481-type lantibiotic, is also produced under an orphan transcription regulator, MutR.\textsuperscript{11} But the mechanism of gene expression via these orphan transcription regulators is still unknown. Interestingly, the ORF1 product showed high similarity to response regulators of the AlgR/AgRA/LytR family.\textsuperscript{15} Those response regulators commonly function in response to phosphorelay signal transduction from histidine kinase and are encoded adjacent to the kinase gene. A histidine kinase-like gene was not found, however, in the flanking region of ORF1. The ORF1 product might be associated with a histidine kinase, which might function as an osmosensor, encoded by the chromosome of \textit{S. warneri} ISK-1. In the future, we hope to discover the mechanism of enhancement of the transcription of the nukA and nukM by osmotic stress and the relationship between the function of the ORF1 product and osmotic stress. These further investigations of the transcriptional mechanism of the nukacin ISK-1 gene cluster might reveal the function of orphan transcription regulators involved in the biosynthesis of lantibiotics.

Acknowledgment

This work was partially supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS) and the Novartis Foundation (Japan) for the Promotion of Science, a Sasakawa Scientific Research Grant from the Japan Science Society, and JSPS research fellowships.

References

13) McCauliffe, O., Hill, C., and Ross, R. P., Identification


