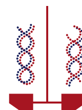

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Evaluation of leader peptides that affect the secretory ability of a multiple bacteriocin transporter, EnkT

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EnkT is a novel ATP-binding cassette (ABC) transporter responsible for secretion of four bacteriocins, enterocins NKR-5-3A, C, D, and Z (Ent53A, C, D, and Z), produced by *Enterococcus faecium* NKR-5-3. It is generally recognized that the secretion of a bacteriocin requires a dedicated ABC transporter, although molecular mechanisms of this secretion are yet to be revealed. In order to characterize the unique ability of EnkT to secrete multiple bacteriocins, the role of N-terminal leader peptides of bacteriocin precursors was evaluated using Ent53C precursor as a model. The 18-amino acid leader peptide of Ent53C (Lc) was modified by site-directed mutagenesis to generate various point mutations, truncations, or extensions, and substitutions with other leader peptides. The impact of these Lc mutations on Ent53C secretion was evaluated using a quantitative antimicrobial activity assay. We observed that Ent53C production increased with Ala substitution of the highly conserved C-terminal double glycine residues that are recognized as the cleavage site. In contrast, Ent53C antimicrobial activity decreased, with decrease in the length of the putative α-helix-forming region of Lc. Furthermore, EnkT recognized and transported Ent53C of the transformants possessing heterologous leader peptides of enterocin A, pediocin PA-1, brochocins A and B, and lactococcins Qα and Qβ. These results indicated that EnkT shows significant tolerance towards the sequence and length of leader peptides, to secrete multiple bacteriocins. This further demonstrates the functional diversity of bacteriocin ABC transporters and the importance of leader peptides as their recognition motif.

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[**Key words:** Lactic acid bacteria; Bacteriocin; Enterocin; ABC transporter; Leader peptide]

Bacteriocins are ribosomally synthesized, antimicrobial peptides produced by a wide variety of bacteria, including lactic acid bacteria (LAB) (1,2). In general, LAB bacteriocins exert bacteriostatic or bactericidal effects against gram-positive bacterial strains that may be closely related to the producers. Since they are bland and innocuous materials, with thermo- and low pH-stability, LAB bacteriocins are potential bio-preservatives that can act as safe and natural antimicrobial agents (3).

LAB bacteriocins are divided mainly into 2 classes (2,4). Class I bacteriocins, the lantibiotics, contain unusual amino acid residues such as lanthionine and dehydrated amino acid residues (5). Nisin A, the best-studied class I bacteriocin, is used as a food preservative in over 50 countries. In contrast, class II bacteriocins contain no unusual amino acid residues, and are further divided into 4 subclasses, based on their structure and properties. Class IIa bacteriocins are the anti-listerial peptides, possessing a conserved N-terminal YGNGVXC motif (6,7). Class IIb bacteriocins consist of two peptides, both of which are essential for their antimicrobial activity (8). Class IIc bacteriocins have a circular structure, formed by an N- to C-terminal covalent bond (9,10). The other class II bacteriocins are classified into class IId (11).

Typically, bacteriocin biosynthesis minimally requires genes encoding the bacteriocin precursor, an immunity protein, and a transporter. These genes are often located closely in the genome, as a gene cluster (12). Most bacteriocins are synthesized as precursors with an N-terminal leader peptide. The leader peptide cleavage and concomitant bacteriocin secretion across the cytoplasmic membrane is mediated by dedicated transporters (12). Several transporter proteins involved in bacteriocin secretion have been identified and characterized as ATP-binding cassette (ABC) transporters (4). These transporters consist of three domains: an N-terminal peptidase domain (PD) that cleaves the leader peptide, a transmembrane domain (TM) for translocation across the cytoplasmic membrane, and a C-terminal ATP-binding domain (ABD) that catalyzes ATP-dependent conformational change in the TM (13). Some transporters often require accessory proteins for bacteriocin secretion (14–16). Additionally, certain immunity proteins confer resistance to the bacteriocin-producing bacteria from their own bacteriocins, using adsorption and translocation-dependent mechanisms (17,18). Furthermore, additional regulatory proteins co-located in the bacteriocin-gene clusters often control bacteriocin production at the transcriptional level (19,20).

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The leader peptide of the bacteriocin precursor is known to be a recognition motif for PD cleavage since it contains a double glycine cleavage site (GG-site) that is conserved amongst all class II bacteriocins, except class IIc (13,21). Presumably, the leader peptide is also involved in the inactivation of bacteriocin activity, towards its producer cell. The leader peptides of lantibiotics are known to function as the recognition sites for post-translational modification enzymes (22–24). For example, studies on the nisin A production pathway show that the conserved FNLD motif of the leader peptide is important for interactions between the precursor, the dehydrogenase, and the cyclase, that is, NisA, NisB, and NisC, respectively (25–27). In contrast, class II bacteriocin production machinery does not require modification enzymes and thus, their leader peptides are involved in self-immunity and recognition by ABC transporters (28).

Enterococcus faecium NKR-5-3, a LAB strain isolated from a fermented Thai fish *Pla-ra*, produces five bacteriocins: enterocins NKR-5-3A, B, C, D, and Z (Ent53A, Ent53B, Ent53C, Ent53D, and Ent53Z) (29–31). Ent53A and Ent53Z belong to the two-peptide bacteriocin class IIb and show synergistic activity. Ent53B is a novel circular bacteriocin. Ent53C is an anti-listerial peptide belonging to class IIa bacteriocins and possesses the conserved, N-terminal YGNGL motif. Ent53D functions as an inducer of the bacteriocin production. In a previous study, Ent53A, Ent53C, Ent53D, and Ent53Z biosynthesis was attributed to the enterocin NKR-5-3ACDZ (Ent53ACDZ) gene cluster (32). The Ent53ACDZ gene cluster includes genes for a histidine kinase (*enkK*), a response regulator (*enkR*), and a transporter (*enkT*). In addition to these, the gene cluster also contains immunity genes, *enkIaz* and *enkIc*, that confer self-immunity against Ent53A/Z and Ent53C, respectively. In contrast, to Ent53ACDZ, the biosynthetic genes for immunity, cyclization, and secretion of Ent53B are located in a different locus, as a separate gene cluster (33).

Previously, *in silico* analysis predicted that EnkT is an ABC transporter, with an N-terminal PD, like the other ABC transporters involved in bacteriocin secretion (13). Furthermore, heterologous expression and gene disruption of EnkT showed that it mediates secretion of the four enterocins NKR-5-3 (Ent53A, Ent53C, Ent53D, and Ent53Z) without any accessory proteins, despite their varied structures and molecular sizes (32). These findings suggest that EnkT is a wide-range ABC transporter capable of secreting bacteriocins that belong to three different subclasses. However, the details of molecular function and recognition mechanisms used by ABC transporters, like EnkT, that are associated with secretion of class II bacteriocins, remain unexplained.

This study focusses on the importance of the leader peptide in the EnkT secretory mechanism. The leader peptide of the Ent53C precursor (EnkC) (Fig. 1) was mutated using heterologous expression-plasmid constructs with *enkT*, *enkC*, and *enkIc*. The influence of leader peptide mutation was evaluated by quantifying the amount of Ent53C secreted by transformants, containing various mutations.

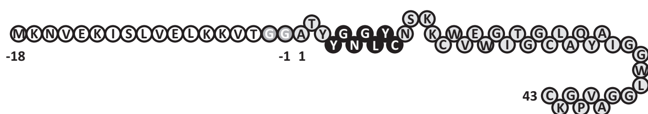

FIG. 1. Structure of the Ent53C precursor. Mature Ent53C is a class IIa bacteriocin consisting of 43 amino acid residues, including a conserved YGNGV motif, and forms two disulfide bonds (Cys positions 10/15 and 25/43). The precursor peptide of Ent53C possesses an N-terminal 18-amino-acid leader peptide. A C-terminal double glycine site (Gly at positions-1 and -2), is also widely conserved as a cleavage site, amongst class II bacteriocins.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions The strains used in this study are listed in Table 1. *Lactococcus lactis* NZ9000 (34) was stored at −80°C in M17 medium (Merck, Darmstadt, Germany) with 0.5% glucose (GM17) (Nacalai Tesque, Kyoto, Japan) and 15% glycerol (Nacalai Tesque), and propagated in GM17 medium at 30°C for 20 h, before using as a pre-culture. *E. faecalis* JCM 5803^T propagated in MRS medium (Oxoid, Basingstoke, United Kingdom) at 37°C for 18 h was used as an indicator strain for antimicrobial activity assay. The antibiotic chloramphenicol (Wako, Osaka, Japan) used for selection was added in the LB (BD, Sparks, MD, USA) and GM17 media at a concentration of 30 μg ml^{-1} (*Escherichia coli* DH5α) and 10 μg ml^{-1} (*L. lactis* NZ9000), respectively. The agar medium was prepared using 1.5 % (wt/vol) agar (Nacalai Tesque). *E. faecium* JCM 5804^T (enterocin A producer) (35), and *Pediococcus pentosaceus* TISTR 536 (pediocin PA-1 producer) (36) were cultivated in MRS medium at 37°C. *Brochothrix campestris* NBRC 15547^T (brochocin C producer) (37,38) and *L. lactis* QU 4 (lactococcin Q producer) (39) were cultivated at 30°C in APT media (BD) and MRS, respectively.

Plasmid construction and heterologous expression Plasmids and primers used in this study are listed in Tables 1 and 2. Molecular cloning was performed as described by Sambrook and Russell (40). KOD-plus-Ver.2 polymerase (Toyobo, Osaka, Japan) and Quick Taq HS polymerase (Toyobo) were used for PCR and colony PCR, respectively. The DNA fragments were purified using Expin PCR SV (GeneAll, Seoul, South Korea). *L. lactis* NZ9000 was transformed with pNK-TCI (Table 1), a pMG36c construct encoding a structural gene (*enkC*), an immunity gene (*enkIc*), and a transporter gene (*enkT*), as described previously (32).

The leader peptide of Ent53C was mutated by inverse PCR, using pNK-TCI as a template and oligonucleotide primers engineered with the required point mutation (Table 2). These PCR amplified fragments were phosphorylated by T4 kinase (Toyobo, Osaka, Japan) and self-ligated by Ligation high Ver. 2 (Toyobo). The resulting plasmids were termed as pC-E14A, pC-K13A, pC-I12N, pC-E8A, pC-L7Q, pC-K6A, pC-V4Q, pC-G2A, and pC-G1AG2A, based on their amino acid substitution position and corresponding amino acid residues. Deletion and extension mutant constructs of the Ent53C leader peptide were prepared by inverse PCR, using pNK-TCI as a template and primers engineered for respective deletion and extension. Subsequently, these amplified fragments were phosphorylated and self-ligated, as described above. The resulting plasmids were termed pC-N-3, pC-N-6, pC-N-9, pC-N-10, pC-N-12, pC-N-15, pC-C-5, and pC-3A, based on the length and position of addition/deletion mutation.

The respective fragments of the genes encoding the leader peptides of Ent53A (*enkA*), Ent53D (*enkD*), Ent53Z (*enkZ*), enterocin A (*entA*), pediocin PA-1 (*pedA*), brochocin A (*brcA*), brochocin B (*brcB*), lactococcin Qα (*laqA*), and lactococcin Qβ (*laqB*) were amplified using gene-specific primers and the respective total genomic DNA template from their producer strain, using a method described previously (32). These fragments were phosphorylated and ligated into pNK-TCI lacking the leader peptide region of *enkC*, which was obtained by inverse PCR using primers Inv-enkC-F and Inv-enkC-R. The resulting plasmids were termed pNK-T-LaCI, pNK-T-LdCI, pNK-T-LzCI, pNK-T-LeCI, pNK-T-LpCI, pNK-T-LbaCI, pNK-T-LbbCI, pNK-T-LqaCI, and pNK-T-LqbCI.

All the engineered plasmid constructs were first cloned in *E. coli* DH5α and transferred into *L. lactis* NZ9000 for heterologous expression.

Partial purification of Ent53C secreted by transformants Ent53C was purified from the culture supernatants of various transformants by Amberlite XAD-16 (Sigma-Aldrich, St. Louis, MO, USA), as described previously (32). Ent53C elution was performed using 70% (vol/vol) isopropanol (Nacalai Tesque) with 0.1% (vol/vol) trifluoroacetic acid (Nacalai Tesque). Solvents were removed from the eluate using a rotary evaporator. The remaining moisture was removed by lyophilization, and the resulting pellets were dissolved by 10% (vol/vol) dimethyl sulfoxide (Nacalai Tesque) solution. These partially purified solutions obtained from the leader peptide mutants were used as working samples.

Evaluation of the secretory ability of EnkT using antimicrobial activity assay The secretory ability of EnkT was evaluated by measuring the antimicrobial activity of Ent53C, which is secreted into the culture supernatant. Ent53C was purified from the culture supernatants of various transformants, as described above, and the antimicrobial activity was assayed by the spot-on-lawn method (41). Briefly, 10 μl of a 2-fold dilution of purified Ent53C, was spotted on a double layer culture. The upper layer consisted of 10 ml of Lactobacilli Agar AOAC (LAA; BD) inoculated with 50 μl of an indicator strain culture, and the bottom layer was formed by 10 ml of MRS with 1.5% (wt/vol) agar. After overnight incubation, the bacterial lawns were checked for inhibition zones. The activity titer, expressed in arbitrary activity units (AUs) per milliliter of purified Ent53C, was defined as the reciprocal of the highest Ent53C dilution causing a clear zone of growth inhibition in the indicator lawn. *E. faecalis* JCM 5803^T was used as an indicator strain for the spot-on-lawn assay. The activity units were defined as the amount of Ent53C secreted by EnkT, and these tests were performed in triplicates, independently. Each sample was also analyzed by liquid chromatography/electrospray ionization time-of-flight mass spectrometry (LC/ESI-TOF-MS) to confirm proper Ent53C production according to the method previously described (42).

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