Mutations near the cleavage site of enterocin NKR-5-3B prepeptide reveal new insights into its biosynthesis

Rodney H. Perez,1† Haruki Sugino,1 Naoki Ishibashi,1 Takeshi Zendo,1,* Pongtep Wilaipun,2 Vichien Leelawatcharamas,3 Jiro Nakayama1 and Kenji Sonomoto1,4

Abstract

Enterocin NKR-5-3B (Ent53B) is a 64-residue novel circular bacteriocin synthesized from an 87-residue prepeptide. Albeit through a still unknown mechanism, the EnkB1234 biosynthetic enzyme complex processes the prepeptide to yield its mature active, circular form. To gain insights into the key region/residue that plays a role in Ent53 maturation, several mutations near the cleavage site on the precursor peptide were generated. The interaction of the precursor peptide and EnkB1234 appeared to be hydrophobic in nature. At the Leu1 position, only mutations with helix structure-promoting hydrophobic residues (Ala, Ile, Val or Phe) were able to yield the mature Ent53B derivative. In this study, we also highlight the possible conformation-stabilizing role of the Ent53B leader peptide on the precursor peptide for its interaction with its biosynthetic enzyme complex. Any truncations of the leader peptide moiety interfered in the processing of the prepeptide. However, when propeptides of other circular bacteriocins (circularin A, leucocyclicin Q or lactocyclicin Q) were cloned at the C-terminus of the leader peptide, EnkB1234 could not process them to yield a mature bacteriocin. Taken together, these findings offer new perspectives in understanding the possible molecular mechanism of the biosynthesis of this circular bacteriocin. These new perspectives will help advance our current understanding to eventually elucidate circular bacteriocin biosynthesis. Understanding the biosynthetic mechanism of circular bacteriocins will materialize their application potential.

INTRODUCTION

Bacteriocins are a huge family of ribosomally synthesized antimicrobial peptides produced by some bacteria, including the ‘generally regarded as safe’ (GRAS) lactic acid bacteria (LAB) [1, 2]. They are very potent antimicrobials that can target a narrow range of bacteria or can inhibit a broad range of sensitive strains depending on their type [1]. Many bacteriocins especially from LAB have been used for many years as food-grade food preservatives in many countries [3, 4], and their application as therapeutic antibiotics is now seriously being considered [5, 6].

Within the family of antimicrobial peptides, a group characterized by the head-to-tail circularization of their backbone, such as circular bacteriocins, has attracted more attention than their linear counterparts due to their superior structural stability [7, 8]. They have huge application potential as safe food preservatives because of their exceptional stability and broad antimicrobial spectra [9]. Moreover, it has been suggested that information on their biosynthetic mechanism (how they are cyclized from their linear precursor peptides) will provide novel strategies in bioengineering important bioactive molecules such as therapeutic compounds to adopt a circular structure thereby enhancing their stability [8, 10].

Bacteriocins are originally synthesized as inactive precursor peptides that contain an N-terminal leader sequence (also known as leader peptide) attached to a C-terminal propeptide. The bacteriocin prepeptide undergoes post-translational processing by its cognate biosynthetic enzymes to yield its active form [11]. Leader peptides play crucial roles in these enzymatic reactions. They function as
recognition sites for the biosynthetic enzymes responsible for directing the prepeptide towards maturation and translocation of the bacteriocin to the extracellular space [11, 12]. Leader peptides also function to protect the producing bacteria by keeping the bacteriocin in an inactive state while still in the intracellular space of the producing bacteria [11]. Leader peptides are also believed to interact with the prepeptide domain to ensure a suitable conformation, which is essential for enzyme–substrate interaction of the modification machinery [11, 13]. The roles of the leader peptide in lantibiotics and pediocin-like bacteriocins have been extensively studied. Most lantibiotic leader peptides share a conserved FNLDI sequence, which functions as recognition site of their cognate modification enzymes [14–16]. In the case of pediocin-like bacteriocins, double-glycine-type leader peptides were shown to serve as target signal for the proteolytic cleavage and translocation by their cognate ABC-transporter proteins [12, 17, 18]. However, the leader peptides of circular bacteriocins vary significantly in length and do not share a conserved motif among them, making it difficult to predict their role in the maturation process [19]. It is probably because of this that a new classification scheme among circular bacteriocins based on length of the leader peptide has been suggested [20].

Enterocin NKR-5-3B (Ent53B) is a 64-amino acid novel circular bacteriocin produced by Enterococcus faecium NKR-5-3 [21–24]. The Ent53B structural gene, enkB, encodes an 87-amino acid prepeptide, which consists of a 23-amino acid leader peptide and a 64-amino acid propeptide domain. The head-to-tail circularization of Ent53B occurs between the Leu1 and Trp64 [22]. We recently reported that the maturation of Ent53B from its precursor peptide requires cooperative functioning of four gene products, EnkB1234, that form an enzyme complex [21]. However, very little is known about the molecular mechanism of its biosynthesis. In a continued effort to unravel the mystery surrounding the biosynthesis of this circular bacteriocin, various mutations near the cleavage site of the Ent53B prepeptide, which included point mutations, truncations, and hybrid-circular peptide engineering, were performed to glean insight on its maturation.

METHODS

Bacterial strains and reagents
The strains and plasmids used in this study are summarized in Table 1. The expression host strain Enterococcus faecalis JH2-2 was cultivated in M17 medium supplemented with 0.5% glucose (GM17) and grown at 30°C. The cloning strain Escherichia coli DH5α was cultivated in Luria–Bertani (LB) medium (Becton Dickinson), with agitation at 37°C. The indicator strains, Lactococcus lactis ATCC 19435T, Pediococcus pentosaceus JCM 5885 and Lactobacillus sakei subsp. sakei JCM 1157T were cultured in MRS medium (Oxoid). Listeria innocua ATCC 33090T and Bacillus coagulans JCM 2257T were cultivated in LB medium and incubated at their respective optimum incubation conditions. Chloramphenicol (Cm) was used as an antibiotic marker in selective media at a final concentration of 10 µg ml⁻¹. All bacteria were stored at −80°C in their respective media with 30% (w/v) glycerol and cultivated twice before use.

Truncation and hybrid circular peptide construction
To gain insights into the essential Ent53B leader peptide regions, Ent53B precursor peptides with truncated leader peptides were constructed by inverse PCR using the plasmid pNK-B1234 as template DNA [21]. Molecular cloning techniques were performed using standard methods [25]. Briefly, inverse PCR using outward-facing primers to ensure the deletion of a specified region was performed. A list of these primers is provided in Table S1 (available in the online Supplementary Material). Amplified fragments were then phosphorylated using T4 kinase (Toyobo) and subsequently self-ligated using Ligation High version 2 (Toyobo). Resulting plasmid constructs were then transformed into Escherichia coli DH5α for plasmid storage and into Enterococcus faecalis JH2-2 for expression analysis. The resulting plasmids were confirmed by DNA sequencing.

Using specific primers (Table S1), chimeric precursors of circular bacteriocins were genetically constructed by cloning the propeptide of other circular bacteriocins: leucocyclicin Q (LcyQ) [26], lactocyclicin Q (LycQ) [27] and circularin A (CirA) [28], and fused to the Ent53B leader peptide. These primers amplified each respective propeptide region, and the respective phosphorylated fragments were individually ligated into the pNK-B1234 inverse PCR product lacking the Ent53B propeptide region. Resulting plasmids were confirmed by DNA sequencing and subsequently cloned into plasmid storage and expression hosts. Expression of these plasmids yielded chimeric precursor peptides with Ent53B leader peptide fused with each respective propeptide region of the above-mentioned circular bacteriocins.

Site-directed mutagenesis of the Ent53B precursor peptide
Mutations at selected residues in close proximity to the cleavage site in the Ent53B leader peptide and propeptide were introduced in the enkB structural gene through inverse PCR using specific primers (Table S2). Resulting amplified fragments were treated in the same way as described above to generate the desired plasmids, which were subsequently cloned into plasmid storage and expression hosts. Desired mutations in all plasmid constructs were confirmed by DNA sequencing.

RNA isolation and semi-quantitative reverse transcription PCR (RT-PCR)
Total RNA of each mutant was extracted using the RNasy kit (Qiagen) according to the manufacturer’s instructions. Extracted RNA was then treated with deoxyribonuclease I (Life Technologies) to ensure the absence of DNA. The treated RNA was then used for reverse transcription using SuperScript VILO cDNA Synthesis kit (Life Technologies). Synthesized cDNAs were then used as template for the RT-PCR to confirm the presence of messenger transcripts. Since
Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description*</th>
<th>Reference or source†</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Enterococcus faecalis</em> JH2-2</td>
<td>Plasmid-free derivative of <em>Enterococcus faecalis</em> JH-2; used as heterologous host for Ent53B expression</td>
<td>[21, 47]</td>
</tr>
<tr>
<td><em>Lactococcus lactis</em> ATCC 19435†</td>
<td>Bacteriocin indicator strain</td>
<td>ATCC</td>
</tr>
<tr>
<td><em>Listeria innocua</em> ATCC 33090†</td>
<td>Bacteriocin indicator strain</td>
<td>ATCC</td>
</tr>
<tr>
<td><em>Lactobacillus sakei</em> subsp. sakei JCM 1157†</td>
<td>Bacteriocin indicator strain</td>
<td>JCM</td>
</tr>
<tr>
<td><em>Pediococcus pentosaceus</em> JCM 5885</td>
<td>Bacteriocin indicator strain</td>
<td>JCM</td>
</tr>
<tr>
<td><em>Bacillus coagulans</em> JCM 2257†</td>
<td>Bacteriocin indicator strain</td>
<td>JCM</td>
</tr>
<tr>
<td><em>Escherichia coli</em> DH5α</td>
<td>Plasmid storage strain</td>
<td>Novagen</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pNK-B1234</td>
<td>Cm′, pMG36c derivative containing <em>enkB, B1234</em></td>
<td>[21]</td>
</tr>
<tr>
<td>p(Δ—22–1)</td>
<td>Cm′, pNK-B1234 derivative with leader moiety truncation at —22 to —1</td>
<td>This study</td>
</tr>
<tr>
<td>p(Δ—22–6)</td>
<td>Cm′, pNK-B1234 derivative with leader moiety truncation at —22 to —6</td>
<td>This study</td>
</tr>
<tr>
<td>p(Δ—22–11)</td>
<td>Cm′, pNK-B1234 derivative with leader moiety truncation at —22 to —11</td>
<td>This study</td>
</tr>
<tr>
<td>p(Δ—22–16)</td>
<td>Cm′, pNK-B1234 derivative with leader moiety truncation at —22 to —16</td>
<td>This study</td>
</tr>
<tr>
<td>pEnt53B-CirA</td>
<td>Cm′, pNK-B1234 derivative with Ent53B leader moiety and circularin A propeptide</td>
<td>This study</td>
</tr>
<tr>
<td>pEnt53B-LeuQ</td>
<td>Cm′, pNK-B1234 derivative with Ent53B leader moiety and leucocyclicin Q propeptide</td>
<td>This study</td>
</tr>
<tr>
<td>pEnt53B-LycQ</td>
<td>Cm′, pNK-B1234 derivative with Ent53B leader moiety and lactocyclicin Q propeptide</td>
<td>This study</td>
</tr>
</tbody>
</table>

*Cm′, Chloramphenicol resistance.
†ATCC, American Type Culture Collection, Rockville, MD USA; JCM, Japan Collection of Microorganisms, Wako, Japan.

The Ent53B precursor and its biosynthetic machinery are transcribed in a single polycistronic mRNA [21], introduction of mutations in the *enkB* gene should have no effect on the upstream genes within the *enkB* biosynthetic operon as long as these mutations will not affect the proper framing of these genes. This was easily confirmed through DNA sequencing. Hence, the primers used for the RT-PCR target the propeptide region in *enkB*, while for the chimeric peptide expressing mutants, primers targeting the leader peptide region were used.

**Detection, quantification assay and MIC determination of mature Ent53B and derivatives**

The production of mature Ent53B was evaluated using a direct-colony-overlay assay as described previously [21] with slight modification. Briefly, recombinant *Enterococcus faecalis* JH2-2 harbouring the respective plasmids were pre-cultivated in 5 ml GM17 medium containing 10 µg ml⁻¹ cm incubated at 30 °C overnight and sub-cultured into fresh medium containing the same antibiotic and kept under the same conditions for 24 h. The amount of cells of each recombinant strain was normalized to the same optical density (OD₆₀₀ of 3.0) using sterile distilled water. One-micro-litre aliquots of the normalized cell solutions were then spotted onto a GM17 agar lawn inoculated with 1% indicator strain. After overnight incubation at 30 °C, inhibition zones around the colonies were checked to confirm the production of mature Ent53B. Relative bioactivity (%) was determined by measuring the diameter of the zone of inhibition of each mutant and then dividing it by the size of the zone of inhibition of the wild-type (WT) and multiplying by 100. In the case of the Ent53B Leu1 derivatives, estimation of production was done using the electrospray ionization LC/MS (ESI-LC/MS) quantification system we described previously [24]. Briefly, the bacteriocin from a 10 ml culture supernatant was concentrated and partially purified using a Sep-Pak C18 cartridge (Waters). To further concentrate and standardize the fold concentration, the eluted fraction was evaporated using a Savant SPD1010 SpeedVac Concentrator (Thermo Scientific) until the eluate volume reached 100 µl. The solution was then applied to the ESI-LC/MS system to quantify the Ent53B derivatives using the ESI-LC/MS quantification protocol. All assays were done in triplicate.

Confirmation of the molecular weight (MW) of Ent53B and its derivatives was performed according to previously described methods [29, 30]. Briefly, a 10 ml culture supernatant was loaded onto an activated Sep-Pak C18 cartridge (Waters) to concentrate and partially purify the bacteriocin prior to ESI-LC/MS analysis.

To determine the minimum inhibitory concentrations (MICs) of the Ent53B Leu1 derivatives, the bacteriocins were purified from their respective phenotypes following our previously reported purification method of NKR-5-3 enterocins [22, 23] and were subsequently quantified using the BCA Protein Assay kit (Thermo Scientific). The MICs were determined using the spot-on-lawn assay, as described previously [21, 22], using serial dilutions of the purified bacteriocins of known concentrations against a set of indicator bacteria. MICs were defined as the minimum
concentrations that yielded clear zones of growth inhibition on the indicator lawns.

**Detection of possible intracellular accumulation of Ent53B precursor**

Assessment of possible intracellular accumulation of the Ent53B precursor peptide was done using the previously reported method with slight modifications [31, 32]. Briefly, recombinant strains showing negative Ent53B bioactivity were precultivated in GM17 containing 10 µg ml⁻¹ cm at 30 °C overnight and sub-cultured into fresh medium containing the same antibiotic with the same culture conditions for 7 h. Cells were then harvested by centrifugation, washed with 50 mM Tris-HCl (pH 7.4), and resuspended in 500 µl of the same buffer. Cells were then transferred to a 2 ml screw-cap tube containing 500 mg glass beads (diameter: 0.1 mm; Yasui Kikai) and shaken at 2700 r.p.m. for 3 min at 4 °C in a bead-beater machine (Multi-beads Shocker; Yasui Kikai) to lyse the cells. Cell-free extracts were then obtained from the lysed cells by centrifugation at 13 000 g at 4 °C for 5 min. The peptides in the cell-free extract were concentrated by passing the extract through a C18 ZipTip (Millipore) pre-wetted with 30 % acetonitrile (0.1 % trifluoroacetic acid [TFA]) and subsequently equilibrated with MilliQ water (0.1 % TFA). Elution was done using 60 % acetonitrile (0.1 % TFA) after washing the column with 30 % acetonitrile (0.1 % TFA). Five-microlitre aliquots of each concentrated sample were analysed using MALDI-TOF/MS (AXIMA-CFR plus Mass Spectrometer; Shimadzu) with α-cyano-4-hydroxy-cinnamic acid as the matrix.

**Computer analyses and secondary structure prediction**

DNA sequences were analysed for correct open reading frames using GENETYX-WIN software, version 8.0.1 (Genetyx). Secondary structure prediction of circular bacteriocins was done using both Jpred3 [33] and PSIPRED [34] protein structure prediction servers. To predict the secondary structure across the N- and C-termini, each sequence was submitted twice. First, the original sequence was submitted to identify coil regions, after which the sequence along the coil region was then swapped so that the coil region would become the new N-terminus. Next, the former N-terminal sequence was added to the C-terminus of the sequence. This new sequence was then resubmitted for analysis.

**RESULTS**

**Processing enzymes recognize both leader and propeptide regions**

All phenotypes expressing truncated leader peptides showed no activity against the indicator strain, suggesting hampered processing of the precursor peptide to yield the mature Ent53B. This is despite RT-PCR analysis confirming the presence of specific mRNA transcripts (Fig. 1). This led us to hypothesize that the biosynthetic enzymes of Ent53B

---

**Fig. 1.** Effect of leader peptide truncations on Ent53B production. (a) Amino acid sequence of the precursor peptide of Ent53B. (b) Different regions of Ent53B leader peptide were deleted through genetic engineering of the plasmid pNK-B1234. (c) RT-PCR analysis of the enkB gene confirmed its successful transcription. Ent53B production was determined via the direct-colony-overlay assay using *Lactobacillus sakei* subsp. *sakei* JCM 1157 as an indicator strain.
recognize the entire leader peptide region for prepeptide processing similar to that of lantibiotic biosynthesis [11, 35]. To confirm this hypothesis, we genetically constructed hybrid prepeptides using the plasmid pNK-B1234, wherein the Ent53B leader peptide region was attached to the N-terminus of the propeptide of other circular bacteriocins. However, the results obtained did not support our hypothesis. The mutant phenotypes expressing the chimeric prepeptides did not exhibit any activity against the indicator strain and further examination of their culture supernatants revealed that respective mature circular bacteriocins were not produced. This is despite successful transcription of specific genes as confirmed by RT-PCR (Fig. S1). These results suggested that the biosynthetic enzymes specifically recognize the Ent53B propeptide region along with its interaction with the leader peptide.

**Functionality of leader peptide residues involved in Ent53B maturation**

To glean insights on the critical residues near the cleavage site (-1 to -5 position) for their role in Ent53B maturation, mutations in these residues were genetically introduced to the Ent53B precursor peptide using site-directed mutagenesis. The mutations were specifically engineered to generate amino acids of different chemical properties (hydrophobic, hydrophilic uncharged, and hydrophilic charged residues) into the leader peptide moiety of the Ent53B precursor. The mutants showed variable effects on Ent53B production as indicated by the change in the inhibition zones relative to those of the WT (Table 2 and Fig. 2a), although RT-PCR analysis on all of the mutants showed comparable enkB operon transcription levels to that of WT (Fig. 2b).

For instance, when the Met at position -1 was mutated to hydrophobic residues, there were variable effects on the bioactivity exhibited by the phenotypes. A Met-1Val mutation completely disabled Ent53B production, but replacement with Ala in this position yielded a phenotype exhibiting a slightly reduced bioactivity. Substitution with Asp, a charged hydrophilic residue, at the Met-1 position led to a 115% increase in bioactivity, but Glu substitution (also a charged hydrophilic residue) yielded a phenotype with a slightly reduced bioactivity. Ser and Thr (both uncharged hydrophilic residues) replacements at the Met-1 position also had differential effects on bioactivity. The Thr mutant showed comparable activity to that of WT, but the Ser mutant had no activity. Interestingly, the Ala at position -3 was more tolerant to mutations. All substitutions at Ala-3 showed either comparable or increased bioactivity relative to WT. The only exception was the Ala-3Thr mutant, which had a 50% decrease in bioactivity (Table 2).

**Analysis of possible interactions of propeptide and processing enzymes**

Leader peptides among circular bacteriocins lack a conserved motif and they vary significantly even in their lengths (Fig. S2). However, secondary structure analysis of their mature forms revealed a common structural motif and position of helicity [36]. The head-to-tail ligation of circular bacteriocins occurs between two hydrophobic residues and is located within a helical structure (Fig. S3). Moreover, Leu or Val, both aliphatic residues with hydrophobic side-chains, is common at the first residue, and an aromatic residue with a hydrophobic side-chain (usually Trp) is usually at the last residue (Fig. S3).

Hoping to gain insights into the possible significance of the consensus on their structural motif and the hydrophobic residues at the ligation site, we genetically introduced mutations at the Leu1 position of the propeptide. Mutations with hydrophobic residues with the propensity of promoting helical structure formation (Ala, Ile, Val and Phe) at this position did not block Ent53B derivative production (Table 3). ESI-LC/MS analysis of the active compound purified from mutant supernatants revealed mass spectra MWs identical to the theoretical MWs of the respective derivatives (Fig. 3). When Leu1 was mutated to Glu, a strong helix former, this did not result in the production of a mature Ent53B derivative probably due to the negatively charged side-chain of Glu, which may have interfered with the propeptide’s interaction with biosynthetic enzyme(s). Furthermore, mutations at this position with a residue known to break helical structure (Pro or Gly) did not yield an Ent53B derivative (Table 3).

### Table 2. Effect of mutations in the leader peptide on Ent53B maturation

<table>
<thead>
<tr>
<th>Position</th>
<th>Hydrophobic</th>
<th>Hydrophilic, uncharged</th>
<th>Hydrophilic, charged</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>V</td>
<td>S</td>
</tr>
<tr>
<td>M-1</td>
<td>77</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>P-2</td>
<td>100</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>A-3</td>
<td>WT</td>
<td>100</td>
<td>115</td>
</tr>
<tr>
<td>G-4</td>
<td>0</td>
<td>0</td>
<td>62</td>
</tr>
<tr>
<td>V-5</td>
<td>0</td>
<td>WT</td>
<td>100</td>
</tr>
</tbody>
</table>

*Antimicrobial activity of each mutant relative to that of the wild-type (WT) calculated by taking the mean diameter of the inhibition zone from three independent assays.*
Production and bioactivity of Ent53B derivatives

Phenotypes of the Ent53B Leu1-mutated derivatives exhibited weaker bioactivities than WT against the indicator strain *Lactobacillus sakei* subsp. *sakei JCM 1157\(^T\) (Fig. 4a). Furthermore, these phenotypes also exhibited lower bacteriocin production compared to the WT. The Leu1Ile derivative was produced in 30\% lower amounts than the WT, whereas the Leu1Val derivative was only 2.3\% of the production amount of WT. Additionally, the Leu1Ala and Leu1Phe mutants had 13.17 and 48.04\% production rates, respectively (Fig. 4b).

In general, bioactivities of the Ent53B derivatives showed slightly higher minimum inhibitory concentration (MIC) values as compared to WT. Leu1Ile and Leu1Ala derivatives showed almost comparable specific activities to WT Ent53B against all indicator strains tested. Interestingly, Leu1Val and Leu1Phe derivatives showed noticeably weaker specific activities against *P. pentosaceus* JCM 5885 and *Listeria innocua* ATCC 33090\(^T\) but showed almost similar specific activities relative to WT Ent53B against other indicator strains (Table 4).

DISCUSSION

While the roles of the leader peptide in the maturation and transport of most bacteriocins (especially lantibiotics and pediocin-like bacteriocins) have been extensively studied, the role of the leader peptide in circular bacteriocins has remained a mystery. The lack of sequence homology and huge variation in their lengths make it difficult to predict their role in bacteriocin biosynthesis. Here, we shed mechanistic light on the possible interaction of the Ent53B precursor and its biosynthetic enzymes. Initially, the entire leader peptide region appeared to be essential in the interaction of the precursor peptide and its biosynthetic enzymes since any truncations of the leader moiety disabled the production of mature Ent53B (Fig. 1a), despite having successful transcription of the *enkB* gene cluster as confirmed by RT-PCR analysis (Fig. 1b).

Unlike lantibiotic biosynthesis, where interaction of biosynthetic enzymes and precursor peptides are mediated by specific residues in the leader peptides, a more complex mechanism seems to govern the interaction of the Ent53B prepeptide and its biosynthetic enzymes. Nisin A biosynthetic enzymes, NisC, NisB and NisT, have been shown to

---

Fig. 2. Variable effects of leader peptide mutations at the M-1 position on Ent53B maturation and production. (a) The leader moiety of Ent53B precursor peptide was genetically engineered using the plasmid pNK-B1234 to introduce mutations. Bacteriocin production was determined using the direct-colony-overlay assay with *Lactobacillus sakei* subsp. *sakei JCM 1157\(^T\) as an indicator strain. Variable effects of the mutations observed were based on mutant antimicrobial activity and compared to the WT (unchanged, enhanced, reduced and disabled). (b) Semi-quantitative RT-PCR analysis of the transcription of the *enkB* gene in all recombinant strains. RNA was isolated from the *Enterococcus faecalis* JH2-2 strain carrying the recombinant pNK-B1234 constructs. *Enterococcus faecalis* JH2-2 carrying WT pNK-B1234 was used as the control strain. The 16S rRNA gene was used as an endogenous control gene. Samples were electrophoresed on a 1\% agarose gel after 20 and 30 PCR cycles, respectively.
directly interact with the conserved domain FNLDV box in the leader peptide [11, 14, 35]. In fact, posttranslational modifications (dehydration and disulfide bridge formations) were successfully introduced to non-lantibiotic peptides when fused with the nisin leader peptide [15, 16, 37]. However, in the case of Ent53B, the biosynthetic enzyme complex appears to require both the leader moiety and its propeptide region since it failed to process hybrid precursor peptides containing the Ent53B leader moiety and propeptide region of other circular bacteriocins (Fig. S1). Alternatively, these findings tempted us to hypothesize that the function of the Ent53B leader peptide could be more to ensure a specific fold of the Ent53B precursor that is essential for its interaction to the biosynthetic enzyme complex. This notion is discussed further below.

It has been acknowledged that the specificity of the interaction of proteolytic enzymes and their substrates lies in the residues having close proximity to the cleavage site [11, 38,
For Ent53B, mutations of the leader peptide at the residues near the cleavage site, with amino acids of different chemical properties, had variable effects on Ent53B production as indicated by the change in the inhibition zones relative to those of the WT (Table 2 and Fig. 2a). However, to this end, it was still unclear whether the changes in the sizes of the inhibition zones were caused by the production of different molecules or fragments of Ent53B having different specific activities – more so because it has been reported that mutations of the lantibiotic Pep5 precursor led to the production of peptide fragments having antimicrobial activity, albeit lower than the WT Pep5 [40]. Thus, to rule out this possibility, we isolated and examined the mass spectra of the active compounds from the culture supernatants of the phenotypes and found identical mass spectra to the native Ent53B (data not shown). Thus, the changes of the sizes of the inhibition zones exhibited by the mutants were a result of the differences in Ent53B production. It can be inferred that these mutations probably resulted in the alteration of substrate conformation thereby reducing or enhancing the ability of the substrate to fit and interact with the substrate-binding cleft of the still unidentified enzyme(s). Additionally, these results support our theory that the role of the Ent53B leader peptide is more in the maintenance of a proper fold of the precursor peptide that is essential for its interaction with the biosynthetic enzymes. It should be noted that, unlike other groups of bacteriocins, secondary prediction analysis of the Ent53B leader peptide suggested it did not have the propensity to form helical structures. The importance of leader peptide α-helical structure in interacting with biosynthetic enzymes has been extensively evaluated. Nukacin ISK-1 biosynthetic enzymes critically recognize the α-helical orientation of the leader peptide of the NukA precursor peptide [41]. The carnobacteriocin B2 leader peptide has an amphipathic α-helical structure that plays an indispensable role in its maturation [42]. The α-helical conformation of the lacticin 481 leader peptide has also been suggested to be critical in its interaction with its synthetase [43]. All these findings support the theory presented above.

The phenotypes that exhibited no Ent53B production did not intracellularly accumulate the Ent53B prepeptide, suggesting that the linear Ent53B prepeptide is susceptible to unspecific protease digestion. We surmised that Ent53B maturation (cleavage of the leader peptide and the head-to-tail circularization) is a quick process. However, it still remains unclear whether the cleavage, head-to-tail circularization, and secretion are coupled processes. However, recently it was suggested that the head-to-tail circularization and secretion of leucocyclicin Q and garvicin ML are separate processes [32, 44]. However, it should be noted that leucocyclicin Q and garvicin ML belong to the short leader sequence group, and Ent53B belongs to the long leader sequence group [21]. It is still unclear whether there is a difference in their biosynthetic mechanisms.

On the other hand, mature forms of circular bacteriocins share a common structural motif. Their ligation sites occur between two hydrophobic residues and are located within a helical structure. It was previously suggested that the hydrophobic patches surrounding the head-to-tail circularization of circular bacteriocins are crucial for the interaction with their cognate biosynthetic enzyme(s) [36]. Mutations at the Leu1 position of Ent53B with hydrophobic residues with the propensity of promoting helical structure formation (Ala, Ile, Val and Phe) did not block the processing of precursor peptide yielding the mature Ent53B derivatives; however, substitution with non-helix-promoting residues and non-hydrophobic residues led to unsuccessful prepeptide processing (Table 3). Extensive mutagenesis within the core peptide of circular bacteriocins has not yet been done. Only the circular bacteriocin AS-48 has been subjected to some mutations at P1′ (Met1) and at the last C-terminal residue P70′ (Trp70) but this was limited to a single amino acid substitution. Met1Ala substitution of AS-48 yielded a phenotype that successfully produced a derivative although its production was strikingly low, while the mutant phenotype expressing the Trp70Ala substitution cannot efficiently process the AS-48 precursor peptide, which produced both linear and circular species of the bacteriocin [45]. It should be interesting to subject other circular bacteriocins, such as AS-48, to similar extensive mutagenesis to that used in this study to find the importance of helix-forming hydrophobic residues at this position. To the best of our knowledge, the data we present here are the first experimental evidence.

### Table 3. Effect of mutation at Leu1 position on the maturation of Ent53B

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Structure preference</th>
<th>Ent53B derivative production†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aliphatic with hydrophobic side-chain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leu</td>
<td>H</td>
<td>WT</td>
</tr>
<tr>
<td>Ile</td>
<td>i</td>
<td>+</td>
</tr>
<tr>
<td>Val</td>
<td>h</td>
<td>+</td>
</tr>
<tr>
<td>Ala</td>
<td>H</td>
<td>+</td>
</tr>
<tr>
<td>Aromatic with hydrophobic side-chain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phe</td>
<td>h</td>
<td>+</td>
</tr>
<tr>
<td>Tyr</td>
<td>b</td>
<td>–</td>
</tr>
<tr>
<td>Neutral side-chain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ser</td>
<td>i</td>
<td>–</td>
</tr>
<tr>
<td>Thr</td>
<td>i</td>
<td>–</td>
</tr>
<tr>
<td>Negative side-chain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glu</td>
<td>H</td>
<td>–</td>
</tr>
<tr>
<td>Helix-breaker</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gly</td>
<td>B</td>
<td>–</td>
</tr>
<tr>
<td>Pro</td>
<td>B</td>
<td>–</td>
</tr>
</tbody>
</table>

*Structure designations: H, strong former; h, former; i, weak former; i, indifferent; B, strong breaker; b, breaker.
†+, Appropriate derivative detected; –, no production of the derivative.
supporting the notion on the importance of the hydrophobic patches around the ligation site of circular bacteriocins.

Furthermore, our findings suggest that the interaction of the Ent53B prepeptide and its biosynthetic enzymes could be a hydrophobic-interaction-driven process instead of the electrostatic-interaction-driven process for other bacteriocins. It should be noted that the ability of most bacteriocin leader peptides (non-circular bacteriocins) to facilitate protein export is based on the electrostatic interactions of positively charged terminal amine groups of the leader peptides with the negatively charged phospholipid head groups of the cell membrane [38]. Thus, in the case of circular bacteriocins, their translocation to the extracellular space could also be driven through a hydrophobic interaction process. Until now, molecular details of the maturation processes of circular bacteriocins including their translocation to the extracellular space have remained unclear. The nature of interaction of the Ent53B precursor and its biosynthetic enzymes that we present here could encourage a more detailed study leading to the eventual elucidation of circular bacteriocin biosynthesis.

The strikingly low production of the Leu1 derivatives could be the result of inefficient processing of the precursor peptide. It is worth noting that Leu1Ile had the highest production among the derivatives. This is probably because Ile is the same size as Leu, and thus there would not be much effect on its interaction with the processing enzymes. On the other hand, the slight change of the specific activities of

Table 4. Antimicrobial activity of Ent53B Leu1 derivatives on selected indicator strains

<table>
<thead>
<tr>
<th>Indicator strain</th>
<th>Minimum inhibitory concentration, MIC (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
</tr>
<tr>
<td>Lactococcus lactis subsp. lactis ATCC 19435^2</td>
<td>0.01</td>
</tr>
<tr>
<td>Lactobacillus sakei subsp. sakei JCM 1157^3</td>
<td>0.01</td>
</tr>
<tr>
<td>P. pentosaceus JCM 5885</td>
<td>0.07</td>
</tr>
<tr>
<td>Listeria innocua ATCC 33090^4</td>
<td>0.03</td>
</tr>
<tr>
<td>B. coagulans JCM 2257^7</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Fig. 4. Quantified production of Ent53B Leu1 derivatives. (a) Bioactivity of Ent53B derivative mutant strains against Lactobacillus sakei subsp. sakei JCM 1157^1, the indicator strain. (b) Production of Ent53B derivatives was quantified via ESI-LC/MS. Relative production (%) was determined by dividing the production of each derivative by WT value. Error bars represent standard deviation from 3 independent assays.
Ent53B derivatives could be attributed to the impact of these substitutions on the structural conformation of these molecules. In the case of enterocin AS-48, substitution with Ala at the Met1 position led to helical fraying toward a less rigid turn-like structure of the AS-48 derivative that resulted in decreased bioactivity [45]. However, in this study, the effect of the Val and Phe substitutions on structural conformation was unlikely due to the enormous change in their specific activities against \( P. \) \textit{pentosaceus} JCM 5885 and \textit{Listeria innocua} ATCC 33090\(^1\) because their bioactivities remained intact against the other indicator strains (Table 4). It is highly probable that the mode of action of Ent53B against different sensitive bacteria strains varies, where a substitution may lead to decreased affinity of the strain-specific receptor. In fact, it has been suggested that circular bacteriocins have dual modes of action. Garvicin ML has been reported to utilize the maltose ABC transporter as a docking molecule for its bioactivity [46].

In conclusion, data presented here provide insights into the possible mechanism of Ent53B maturation. This study has offered perspectives on the conformation-stabilizing role of the Ent53B leader peptide and the possible hydrophobic-interaction-driven process of Ent53B maturation that could help advance our current understanding to eventually elucidate circular bacteriocin biosynthesis.

**Funding information**

This work was partially supported by the Japan Society for the Promotion of Science (JSPS) KAKENHI Grant 24380051, the JSPS-National Research Council of Thailand (NRCT) Core University Program on Development of Thermotolerant Microbial Resources and Their Applications, the Kato Memorial Bioscience Foundation, and the Novozymes Japan Research Fund.

**Acknowledgements**

We thank the Center for Advanced Instrumental and Educational Supports, Faculty of Agriculture, Kyushu University for access to the MALDI-TOF/MS instrument. We are also grateful to the Ministry of Education, Culture, Sports, Science and Technology of Japan for providing a scholarship grant to R. H. P.

**Conflicts of interest**

The authors declare that there are no conflicts of interest.

**References**


