Identification of the genes involved in the secretion and self-immunity of lacticin Q, an unmodified leaderless bacteriocin from Lactococcus lactis QU 5

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Lacticin Q (LnqQ) produced by Lactococcus lactis QU 5 is an unmodified linear bacteriocin, which is synthesized without an N-terminal leader peptide. In vitro synthesis and in vivo expression of LnqQ have revealed the intracellular toxicity of this leaderless peptide, as well as the necessity of a dedicated secretion and self-immunity system of producer cells. Further DNA sequencing and analysis have discovered 11 putative orf genes at the LnqQ locus. None of the orf genes showed similarities to any of the bacteriocin biosynthetic genes characterized to date; however, six orf genes (orf2q–7q), not including the structural gene (lnqQ), were highly conserved at the lacticin Z locus (orf2z–7z), which is a LnqQ homologue produced by L. lactis QU 14. ORF2q (ORF2z), the gene of which is located upstream of the structural gene, is a putative transcriptional regulator, whereas ORF6q and ORF7q (ORF6z and ORF7z) form a putative ATP-binding cassette transporter. The ORF3q–5q (ORF3z–5z) are all predicted to be membrane proteins with no clear functions. Co-expression of LnqQ and ORF3q–7q in a heterologous host allowed the extracellular production of LnqQ; additionally, the expression of ORF3q–7q rendered the host cells immune to LnqQ. This self-immunity was facilitated possibly by two means; firstly, by secreting the active LnqQ peptides, thus reducing the intracellular toxicity, and secondly, by protecting the host cells from extracellularly released LnqQ. This is the first report, to our knowledge, that describes intracellular toxicity of a leaderless bacteriocin and provides a rare example of biosynthetic genes that are required for bacteriocin secretion and immunity.

INTRODUCTION

Bacteriocins are ribosomally synthesized, extracellularly released antimicrobial peptides or proteins produced by a wide variety of bacteria (Klaenhammer, 1993). Small peptide bacteriocins of Gram-positive bacteria are classified mainly into two groups: the class I lantibiotics that contain post-translationally formed lanthionine or methyllanthionine bridges, and the non-lanthionine-containing class II bacteriocins (Cotter et al., 2005). Class II bacteriocins are generally small (<10 kDa), heat-stable peptides and are subdivided into several subclasses, including pediocin-like bacteriocins (class IIa), two-peptide bacteriocins (class IIb), cyclic bacteriocins (class Iic) and non-pediocin-like, one-peptide bacteriocins (class IIId) (Cotter et al., 2005).

Bacteriocin biosynthesis is carried out by several biosynthetic proteins that are encoded near the bacteriocin structural gene and which are usually organized in an operon (Nes et al., 1996). General functions of these proteins are (i) to mediate the maturation the bacteriocin peptide, (ii) to transport the bacteriocin outside the producer cells (secretion) and (iii) to protect the producer cells from their own bacteriocins (self-immunity). The majority of bacteriocins are synthesized as prepeptides that consist of an N-terminal leader sequence and a C-terminal propeptide, the latter of which undergoes post-translational modification events to become active. An N-terminal leader sequence plays key roles during this process, to prevent the prepeptide from being active inside the producer cell before secretion and to act as a recognition site for the post-translational modification and/or the dedicated transporter system.
(Chatterjee, et al., 2005; Nes, et al., 1996). Moreover, some
typical sequences have been recognized in N-terminal
leaders, so it is predictable which types of proteins are
involved in bacteriocin biosynthesis. There are, however,
some class IIId bacteriocins that are synthesized without an
N-terminal leader sequence and are thus called leaderless
bacteriocins. Enterocin L50, produced by Enterococcus
faecium L50, is an early identified two-peptide (EntL50A
and EntL50B), leaderless bacteriocin (Cintas, et al., 1998),
and some identical or homologous bacteriocins have been
found in other enterococcal species (Floriano, et al., 1998;
Martin-Platero, et al., 2006; Sánchez-Hidalgo, et al., 2003;
Yamamoto, et al., 2003). Enterocin L50 was successfully
expressed and secreted by two yeast hosts through their
general secretory pathways (Banta et al., 2009, 2010);
however, the genes required for its secretion and immunity
have not yet been determined.

We have identified two homologous leaderless bacteriocins,
lacticin Q (LnqQ) and lacticin Z (LnqZ), produced by
Lactococcus lactis QU 5 and L. lactis QU 14, respectively
(Fujita, et al., 2007; Iwatani et al., 2007). LnqQ/Z have no
significant similarity to any members of the enterocin L50
family; in contrast, they share some similarities (46–48 %)
to other leaderless bacteriocins, such as BHT-B from
Streptococcus rattus strain BHT (Hyink, et al., 2005), aureocin
A53 from Staphylococcus aureus strain A53 (Netz, et al., 2002)
and epidermicin NI01 from Staphylococcus epidermidis
strain 224 (Sandiford & Upton, 2012). Aureocin A53 and
epidermicin NI01 are both plasmid encoded and share
similarity genetic organizations of their gene loci, suggesting
the involvement of those genes (seven genes, not including
the structural gene) in the secretion and/or the immunity of
these two bacteriocins (Nascimento, et al., 2012; Sandiford
& Upton, 2012). On the other hand, the gene locus of BHT-B
shows a different organization from the two bacteriocin loci
mentioned above, thus indicating a variation of this type of
bacteriocin gene locus.

In this study, we firstly sequenced the flanking regions of
LqQ/LqZ structural genes (lnqQ/lnqZ); in addition, a
variety of heterologous expression of the gene(s) of interest
was performed to identify the genetic requirement for the
secretion and self-immunity of LnqQ/Z. We also focused
on the intracellular toxicity of this leaderless bacteriocin,
which is sometimes overlooked but is important for the
study of leaderless bacteriocin biosynthesis.

### METHODS

**Bacterial strains and culture conditions.** Bacterial strains and
plasmids used in this study are listed in Table 1. Lactococcus strains
were grown in M17 broth (Merck) containing 0.5 % (w/v) glucose
(GM17) at 30 °C. Bacillus coagulans JCM 2257 was grown in tryptic
soy broth (Difco Laboratories) supplemented with 0.6 % (w/v) yeast
extract (Nacalai Tesque) at 37 °C with shaking. Escherichia coli DH5α
was grown in Luria–Bertani broth (Difco) at 37 °C with shaking. The
agar media were prepared by the addition of 1.5 % (w/v) agar
(Nacalai Tesque) to the broth media, and overlay agars were prepared
by the addition of 1.0 % (w/v) agar to the broth media. As necessary,
culture media were supplemented with 10 μg chloramphenicol ml⁻¹
for *L. lactis* and 34 μg chloramphenicol ml⁻¹ and 100 μg ampicillin
ml⁻¹ for *E. coli*. Nisin induction was conducted by the addition of

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacterial strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. lactis</em> QU 5</td>
<td>LqQ producer</td>
<td>Fujita, et al. (2007)</td>
</tr>
<tr>
<td><em>L. lactis</em> QU 14</td>
<td>LqQ producer</td>
<td>Iwatani, et al. (2007)</td>
</tr>
<tr>
<td><em>L. lactis</em> subsp. cremoris NZ9000</td>
<td>Plasmid-free derivative of <em>L. lactis</em> subsp. cremoris MG1363, pepN::nisRK Bacteriocin indicator</td>
<td>Kuipers, et al. (1998)</td>
</tr>
<tr>
<td><em>B. coagulans</em> JCM 2257T</td>
<td>sucE44 ΔlacU169 (ψ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</td>
<td>Takara</td>
</tr>
<tr>
<td><em>E. coli</em> DH5α</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pGEM-T</td>
<td>Cloning vector</td>
<td>Promega</td>
</tr>
<tr>
<td>pTD1</td>
<td>Expression vector for cell-free translation; Amp’</td>
<td>Shimadzu</td>
</tr>
<tr>
<td>pTD1lnqQ</td>
<td>pTD1 derivative with lnqQ under the control of T7 promoter; Amp’</td>
<td>This study</td>
</tr>
<tr>
<td>pNZB8048</td>
<td>pWV01-based inducible expression vector carrying PnisA promoter; Cm’</td>
<td>Kuipers, et al. (1998)</td>
</tr>
<tr>
<td>pNZA</td>
<td>pNZB8048 derivative with nukA under the control of PnisA; Cm’</td>
<td>Aso, et al. (2004)</td>
</tr>
<tr>
<td>pNZlnqQ</td>
<td>pNZB8048 derivative with lnqQ under the control of PnisA; Cm’</td>
<td>This study</td>
</tr>
<tr>
<td>pMG36c</td>
<td>pWV01-based expression vector carrying the strong lactococcal promoter Pα2; Cm’</td>
<td>van de Guchte, et al. (1989)</td>
</tr>
<tr>
<td>pMGlnqQ</td>
<td>pMG36c derivative with lnqQ under the control of Pα2; Cm’</td>
<td>This study</td>
</tr>
<tr>
<td>plLNQ</td>
<td>pMGlnqQ derivative with orf38-7q downstream of lnqQ; Cm’</td>
<td>This study</td>
</tr>
<tr>
<td>plLNQQAQ</td>
<td>plLNQ derivative lacking lnqQ; Cm’</td>
<td>This study</td>
</tr>
</tbody>
</table>

*JCM, Japan Collection of Microorganisms.
10 ng nisin ml⁻¹ (Sigma-Aldrich) to the bacterial culture when OD₆₆₀ reached ~0.5. The bacterial growth (OD₆₆₀) was monitored by using a biophotorecorder (TVS602CA; Advantec) and the data were represented as the mean values ± SD of three independent experiments.

**DNA manipulations and transformation**. DNA manipulation and other molecular cloning techniques were performed basically as described by Sambrook & Russell (2001). KOD Plus DNA polymerase (Toyobo), Ex Taq polymerase (Takara Bio), Ligation-High (Toyobo) and restriction enzymes (Roche Applied Science) were used according to the suppliers’ instructions. QIAquick PCR Purification kit (Qiagen) was used to purify PCR products as well as the DNA fragments derived from other modification reactions. Synthetic oligonucleotides were obtained from Hokkaido System Science and DNA-sequencing reactions were conducted by FASMAC. All the transformations were carried out by electroporation using MicroPulser (Bio-Rad Laboratories) by following the procedures described by Holø & Nes (1995).

**DNA amplification, sequencing and analysis**. An inverse-PCR technique (Ochman et al., 1988) was applied to amplify the flanking regions of lnqQ and lnqZ. The total DNA fragments of *L. lactis* QU 5 and *L. lactis* QU 14 were isolated using the MagExtractor-Genome kit (Toyobo). Isolated DNA fragments were digested with BanHI, EcoRI, HindIII, SacI, SpeI or XbaI, and appropriate restriction enzymes were selected by Southern blotting and hybridization procedures (Sambrook & Russell, 2001), using the structural genes as probes. DNA fragments digested with the selected enzymes were then self-ligated and used as templates for inverse-PCR techniques. The amplified fragments were sequenced either directly or after cloning into pGEM-T vector (Promega). Based on the sequence information obtained, new sequence-specific primers were designed for further inverse-PCR and subsequent DNA sequencing. Computer analyses of DNA and protein sequences were performed by GENETYX-WIN ver.8.0 and ATGC ver.5.0 (Software Development). ORF prediction was performed using ORF Finder (http://www.ncbi.nlm.nih.gov/projects/orffinder/) and the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) of the National Center for Biotechnology Information. *SOSUI* (http://bp.nmp.nagoya-u.ac.jp/sosui/) and TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) programs were used for further characterization of individual ORFs.

**In vitro synthesis of LnQ**. The structural gene of LlnQ (lnqQ) was amplified by PCR with the primers lnqQ-F (5’-ATGGCAGGTTT-TA AAAAGTGATG-3’) and lnqQ-XbaI-R (5’-CCCTATATAGAATAC-TCTAGATGATC-3’) to add an XbaI site at the 3’ end. The amplified fragments were then digested with XbaI and ligated to the pTD1 (Shimadzu). The ligated product, pTDlnqQ, was introduced into *E. coli* DH5α for plasmid replication and DNA sequencing. A part of pTDlnqQ encompassing a T7 promoter, lnqQ, and a terminator region was amplified by PCR and was purified through the QIAquick PCR Purification kit. The purified DNA fragments were quantified by NanoDrop ND-1000 (Nano-Drop Technologies), 100 ng of which was used as the template DNA for *in vitro* DNA transcription using Thermo T7 RNA polymerase (Toyobo). *In vitro* translation of the reaction mixture was performed by Transdirect insect cell (Shimadzu), according to the supplier’s instructions, with a supplementary addition of 0.1 % (v/v, final concentration) Triton X-100 (Sigma-Aldrich) in the mixture. An *in vitro* transcription/translation using pTD1 as the DNA template was also carried out as a control reaction.

**Heterologous expression of lnqQ genes**. For the construction of pNZlnqQ, lnqQ was amplified with designed primers, Psrl-lnqQ-F (5’-TATAAGTTTGGTCGAGAGAAGAG-3’) and lnqQ-XbaI-R (sequence given previously), to add Psrl and XbaI sites at the 5’ and 3’ ends of the fragment, respectively. The amplified fragments were digested and ligated to the corresponding site of pNZ8048. The resulting plasmid, termed pNZlnqQ, was then introduced into *L. lactis* subsp. cremoris NZ9000 (*L. lactis* NZ9000), which was used as an expression host for nisin-controlled gene expression system (de Ruyter et al., 1996). Another recombinant plasmid pNZA, supplied from our laboratory collection, was also introduced into *L. lactis* NZ9000. This pNZ8048-based recombinant plasmid encodes a 57 aa prepeptide (NukA) of a type AII lantibiotic nukacin ISK-1, produced by *Staphylococcus warneri* ISK-1 (Aso et al., 2004). Because nukacin ISK-1 requires a modification enzyme (NukM) for its maturation (Nagao et al., 2005; Shioya et al., 2010), heterologously expressed NukA is solely inactive and thus, pNZA was used as a control plasmid for pNZlnqQ in this study.

For pMG36c-based plasmid construction, lnqQ was amplified with designed primers, SacI-lnqQ-F (5’-TGGATCTGCAAGAGAACAGA-CGTG-3’) and lnqQ-XbaI-R (sequence given previously), and then cloned into the corresponding site of pMG36c to generate a recombinant plasmid pMGlnqQ, which places lnqQ under the control of the lactococcil constitutive promoter *P*₃₂ (van de Guchte et al., 1989). The region orf₃q-7q was amplified with designed primers, XbaI-orf₃-F (5’-CTTTATATAGTTAATGATAATTGGTT-3’) and orf₇-SphI-R (5’-ATCTCGATGCTTTAATTACAAACAC-3’), to add XbaI and SphI sites at the 5’ and 3’ ends of the fragment, respectively. The amplified fragment was then digested and ligated to the corresponding site of pMGlnqQ, resulting in a rearrangement of lnqQ and orf₃q-7q under the control of *P*₃₂ on a newly constructed plasmid, pLNLQ. The digested fragment encompassing orf₃q-7q was also ligated to the corresponding site of pMG36c, to arrange these *orf* genes under the control of *P*₃₂, but this fragment lacked lnqQ (pLNLQ1). All the pMG36c-based recombinant plasmids were also introduced into *L. lactis* NZ9000 to enable the constitutive expression of the gene(s) of interest.

**Bacteriocin activity assay and LC/MS analysis**. Purification of LlnQ from the culture supernatants of *L. lactis* QU 5 and *L. lactis* NZ9000 recombinants was performed as described previously (Fujita et al., 2007). Culture supernatants were obtained by double-centrifugations of overnight cultures at 13 000 g for 5 min at 4 °C. The harvested cells were resuspended in 50 % (v/v) acetonitrile, containing 0.1 % (v/v) trifluoroacetic acid, and were homogenized by using Multi-beads shocker (Yasui Kikai) with a triplicate round of 2700 r.p.m. for 30 s at 4 °C. Cell-free extracts were obtained after the centrifugation of the homogenized mixture at 12 000 g for 5 min at 4 °C. For the bacteriocin assay and LC/MS analysis, the acetone-tritrifluoroacetic acid present in the cell-free extracts was removed by SpeedVac (Thermo Fisher Scientific K.K.) drying, and the precipitates were resuspended in adequate amounts of 50 mM sodium phosphate buffer (pH 6.0).

The colony-overlay assay with *B. coagulans* JCM 2257T as an indicator strain was simply performed to detect the extracellular antimicrobial activities of *L. lactis* NZ9000 recombinants. Bacteriocin activities of *in vitro* synthesis mixtures, culture supernatants or cell-free extracts were detected by the spot-on-lawn method, as described previously (Ehnahar et al., 2001). The self-immunity of *L. lactis* NZ9000 recombinants was also monitored by the spot-on-lawn method. Molecular mass analyses of *in vitro* synthesized- or heterologously expressed-LnQ were performed using the Agilent 1100 HPLC system (Agilent Technologies) equipped with JMS-TI010LC ESI-TOF MS (JEOL), according to a previous report (Zendo et al., 2008), but a higher gradient of 10 % (v/v) acetonitrile was applied.

**RESULTS**

**DNA sequence analyses of lnqQ/lnqZ gene loci**

As a consequence of sequencing the PCR products, a 16.7 kb nucleotide sequence of the regions flanking lnqQ/lnqZ.
and an 8.4 kb nucleotide sequence of the regions flanking \textit{lnqZ} were obtained. ORF analysis of the obtained sequences revealed 11 putative \textit{orf} genes in the vicinity of \textit{lnqQ} and nine putative \textit{orf} genes in the vicinity of \textit{lnqZ} that were temporarily named in number order from the 5' to the 3' end of the fragment (from \textit{orf1Q} to \textit{orf11Q} for the \textit{lnqQ} locus, and from \textit{orf1Z} to \textit{orf9Z} for the \textit{lnqZ} locus). A schematic overview of the two gene loci is illustrated in Fig. 1 and the annotated characteristics of deduced protein products are shown in Table 2.

At the \textit{lnqQ} locus, \textit{orf1q}, located 2 kb upstream of \textit{lnqQ}, encodes a collagen-binding protein that is found in the \textit{L. lactis} IL1403 genome. The GC content of this region is similar to that of the \textit{L. lactis} genome (mean 35 %), whereas the regions encompassing \textit{orf2q}–7q and \textit{orf8q}–11q show lower GC contents (means 26 and 31 %, respectively). The \textit{orf2q}, divergently located upstream of \textit{lnqQ}, encodes a putative transcriptional regulator, which shares some homology with TetR family proteins. The \textit{orf3q}–7q, located downstream of \textit{lnqQ}, are all oriented in the same direction, in which the \textit{orf3q}–6q are tightly organized in overlapping clusters and the \textit{orf7q} is located 66 bp downstream of \textit{orf6q}. Deduced products of \textit{orf3q}–5q are all predicted to be membrane proteins but with unknown functions, whereas \textit{orf6q} and \textit{orf7q} showed considerable similarities to ATP-binding cassette (ABC) transporter components. The \textit{orf8q} encodes a putative transcriptional regulator with a DNA-binding motif, and the products of \textit{orf9q}–11q are predicted to function in DNA replication and recombination.

The \textit{orf2q}–7q cluster was highly conserved at the \textit{lnqQ} locus (\textit{orf2z}–7z), with a constant nucleotide identity (ca 75 %). This similarity is, however, disrupted right outside the region (upstream of \textit{orf2q}/2z and downstream of \textit{orf7q}/7z). Similar to the \textit{lnqQ} locus, \textit{orf3z}–6z are organized in overlapping clusters, whereas \textit{orf7z} is located 68 bp downstream of \textit{orf6z}. The homologies between the deduced products of \textit{orf2q}–7q and \textit{orf2z}–7z ranged from 60 to 80 % (Table 2). \textit{ORF2q/2z, ORF3q/3z, ORF6q/6z and ORF7q/7z} showed approximately 80 % homology to each other, while relatively low homologies were observed in \textit{ORF4q/4z} (60 %) and \textit{ORF5q/5z} (69 %). Transmembrane topology predictions by \textit{SOSUI} and \textit{TMHMM} programs indicated the presence of multiple transmembrane sequences in each of the \textit{ORF3q}–6q and \textit{ORF3z}–6z, which strongly supported the annotated characteristics of these ORFs functioning as membrane proteins. \textit{ORF7q/7z} were both predicted to be ATP-binding proteins of an ABC transporter, which was confirmed by the presence of the two main motifs, Walker A and Walker B (Walker et al., 1982), in their primary structures (data not shown).

It is also noteworthy that some consensus sequences were recognized in the non-coding regions of \textit{lnqQ/Z} loci. The 200 bp upstream region of \textit{lnqQ/Z} is the most conserved region between the two loci (86 %), in which putative promoter sequences, −10 (TATAAT) and −35 (TAATAT), were found. This region also contains the consensus sequence of a 26 bp inverted repeat (5'–GTAGTGCTAAA–ATGGTTTAGACTAC–3') upstream of the −35 sequence. Another tight inverted repeat was recognized in the non-coding region between \textit{lnqQ/Z} and \textit{orf3q}/3z, which is, in contrast, considered as typical of a rho-independent transcription terminator because of the T-stretch following the stem–loop structure (data not shown).

As compared with other bacteriocin loci, there was no similarity observed between \textit{lnqQ/Z} loci and aureocin A53 or epidermicin N101 loci, whereas \textit{ORF6q/6z} and \textit{ORF7q/7z} share some homology to \textit{ORF3} and \textit{Abc2} at the \textit{bht-b} locus of \textit{Streptococcus rattus} BHT (Hyink et al., 2005). The organization of the \textit{bht-b} locus is similar to those of the \textit{lnqQ/Z} loci, in that three more genes (\textit{abc1}, \textit{orf1} and \textit{orf2}) are wedged between the structural gene (\textit{bht-b}) and ABC transporter genes (\textit{orf3} and \textit{abc2}) in the same orientation. However, no significant similarity was found between the three protein products (\textit{Abc1}, \textit{ORF1} and \textit{ORF2}) and \textit{ORF3q}–5q or \textit{ORF3z}–5z.

**Antimicrobial activity of in vitro synthesized LnqQ (sLnqQ)**

To demonstrate the post-translational antimicrobial activity of \textit{in vitro} \textit{sLnqQ}, the structural gene \textit{lnqQ} was solely cloned and applied to an \textit{in vitro} transcription/translation reaction. The reaction mixture containing \textit{sLnqQ} was spotted onto an indicator lawn of \textit{B. coagulans} JCM 2257T. As a result, a clear inhibition zone of growth was observed in a spot of \textit{sLnqQ}, which was, in contrast, not observed in a spot of the control.
reaction mixture (Fig. 2). Since sLnqQ was synthesized within insect cell extracts, the initial methionine of sLnqQ was not theoretically formylated; however, possibly due to the complexity or the small amount of the reaction mixture, the theoretical mass of sLnqQ (5898 Da) could not be detected by LC/MS analysis.

**Table 2.** Characteristics of the predicted ORFs of lnqQ/lnqZ gene loci

<table>
<thead>
<tr>
<th>ORF</th>
<th>Size (aa)</th>
<th>PI</th>
<th>TM</th>
<th>Characteristics of encoded protein (% identity), organism</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ORF1q</td>
<td>626</td>
<td>5.09</td>
<td>1</td>
<td>Collagen-binding protein (96 %), <em>L. lactis</em></td>
<td></td>
</tr>
<tr>
<td>ORF1z</td>
<td>185</td>
<td>9.10</td>
<td>2</td>
<td>Hypothetical protein (43 %), <em>E. faecium</em></td>
<td></td>
</tr>
<tr>
<td>ORF2q (LnqR)</td>
<td>191</td>
<td>5.66</td>
<td>0</td>
<td>TetR family transcriptional regulator (38 %), <em>Streptococcus galolyticus</em></td>
<td></td>
</tr>
<tr>
<td>ORF2z (LnzR)</td>
<td>192</td>
<td>5.93</td>
<td>0</td>
<td>ORF2q (78 %), <em>L. lactis</em> QU 5</td>
<td></td>
</tr>
<tr>
<td>LnqQ</td>
<td>53</td>
<td>10.57</td>
<td>0</td>
<td>Bacteriocin structural gene, aureocin A53 (48 %), <em>Staphylococcus aureus</em>; BHT-B (47 %), <em>Streptococcus rattus</em>; epidermicin NI01 (46 %), <em>Staphylococcus epidermidis</em></td>
<td>Netz et al. (2002); Hyink et al. (2005); Sandiford &amp; Upton (2012)</td>
</tr>
<tr>
<td>LnqZ</td>
<td>53</td>
<td>10.37</td>
<td>0</td>
<td>LnqQ (94 %), <em>L. lactis</em> QU 5</td>
<td>Iwatani et al. (2007)</td>
</tr>
<tr>
<td>ORF3q (LnqB)</td>
<td>79</td>
<td>10.59</td>
<td>3</td>
<td>Membrane permease protein (35 %), <em>Listeria welshimeri</em></td>
<td></td>
</tr>
<tr>
<td>ORF3z (LnzB)</td>
<td>82</td>
<td>10.24</td>
<td>3</td>
<td>ORF3q (77 %), <em>L. lactis</em> QU 5</td>
<td></td>
</tr>
<tr>
<td>ORF4q (LnqC)</td>
<td>159</td>
<td>9.82</td>
<td>0</td>
<td>Membrane-franked domain protein (27 %), <em>Xylanimonas cellulosilytica</em></td>
<td></td>
</tr>
<tr>
<td>ORF4z (LnzC)</td>
<td>172</td>
<td>10.37</td>
<td>2</td>
<td>ORF4q (60 %), <em>L. lactis</em> QU 5</td>
<td></td>
</tr>
<tr>
<td>ORF5q (LnqD)</td>
<td>432</td>
<td>9.56</td>
<td>6*/5†</td>
<td>Membrane-franked domain protein (24 %), <em>Lactobacillus buchneri</em></td>
<td></td>
</tr>
<tr>
<td>ORF5z (LnzD)</td>
<td>442</td>
<td>10.09</td>
<td>4*/5†</td>
<td>ORF5q (69 %), <em>L. lactis</em> QU 5</td>
<td>Hyink et al. (2005)</td>
</tr>
<tr>
<td>ORF6q (LnqE)</td>
<td>225</td>
<td>9.97</td>
<td>6</td>
<td>ABC-2 type transporter, ORF3 in <em>bht-b</em> locus (29 %), <em>Streptococcus rattus</em></td>
<td></td>
</tr>
<tr>
<td>ORF6z (LnzE)</td>
<td>220</td>
<td>10.04</td>
<td>5</td>
<td>ORF6q (81 %), <em>L. lactis</em> QU 5</td>
<td>Hyink et al. (2005)</td>
</tr>
<tr>
<td>ORF7q (LnqF)</td>
<td>264</td>
<td>6.44</td>
<td>0</td>
<td>ABC transporter, ATP-binding protein, Abc2 in <em>bht-b</em> locus (57 %), <em>Streptococcus rattus</em></td>
<td></td>
</tr>
<tr>
<td>ORF7z (LnzF)</td>
<td>263</td>
<td>5.80</td>
<td>0</td>
<td>ORF7q (78 %), <em>L. lactis</em> QU 5</td>
<td></td>
</tr>
<tr>
<td>ORF8q</td>
<td>227</td>
<td>7.70</td>
<td>0</td>
<td>Putative transcriptional regulator (55 %), <em>Streptococcus mutans</em></td>
<td></td>
</tr>
<tr>
<td>ORF8z</td>
<td>194</td>
<td>3.99</td>
<td>0</td>
<td>Prophage ps1 protein 16 (40 %), <em>L. lactis</em></td>
<td></td>
</tr>
<tr>
<td>ORF9q</td>
<td>443</td>
<td>5.93</td>
<td>0</td>
<td>Replication protein (32 %), <em>L. lactis</em></td>
<td></td>
</tr>
<tr>
<td>ORF9z</td>
<td>114</td>
<td>5.40</td>
<td>0</td>
<td>Prophage ps1 protein 15 (47 %), <em>L. lactis</em></td>
<td></td>
</tr>
<tr>
<td>ORF10q</td>
<td>375</td>
<td>9.89</td>
<td>0</td>
<td>DNA integrase/recombinase (43 %), <em>Streptococcus sp.</em></td>
<td></td>
</tr>
<tr>
<td>ORF11q</td>
<td>280</td>
<td>5.31</td>
<td>0</td>
<td>ATP/GTP binding protein (27 %), <em>Pseudomonas sp.</em></td>
<td></td>
</tr>
</tbody>
</table>

*Putative transmembrane sequences predicted by SOSUI.
†Putative transmembrane sequences predicted by TMHMM.

Intracellular toxicity of heterologously expressed LnqQ

Induced expression of *lnqQ* alone in *L. lactis* NZ9000 (pNZ*lnqQ*) resulted in growth suppression of the host cells (Fig. 3a). Within 2 h after nisin induction, *L. lactis* NZ9000 (pNZ*lnqQ*) drastically stopped growing and the optical density remained around 0.90 (mean), whereas the control strain harbouring pNZ8048 kept growing, to final OD 1.66 (mean). In addition, *L. lactis* NZ9000 (pNZA) expressing nukacin ISK-1 prepeptide (NukA) also kept growing without any considerable suppression.

Antimicrobial activity or the molecular mass of LnqQ was not detected in the 12 h cultured supernatant of *L. lactis* NZ9000 (pNZ*lnqQ*); in contrast, a clear antimicrobial
activity against *B. coagulans* JCM 2257^T^ was detected in the cell-free extract of nisin-induced *L. lactis* NZ9000 (pNZlnqQ) (Fig. 3b). LC/MS analysis also revealed the molecular mass of intact LnqQ in the cell-free extract of *L. lactis* NZ9000 (pNZlnqQ) (Fig. 3c), indicating that LnqQ was expressed, but was trapped intracellularly in the absence of a transporter that is dedicated to secret it outside the cell.

**LnqQ production by a heterologous host**

Considering the potential transcriptional terminator sequence between *lnqQ* and *orf3q–7q*, the gene fragment encompassing *orf3q–7q* was cloned into pMGlnqQ, resulting in the rearrangement of *lnqQ* and *orf3q–7q*, lacking the putative terminator sequence, in the same orientation under the control of P32 (pLNQ). In a colony-overlay assay, a clear inhibition zone was observed on the periphery of the colony of *L. lactis* NZ9000 (pLNQ), as well as for *L. lactis* QU 5 (LnqQ producer), which was not observed around the negative controls such as *L. lactis* NZ9000 (pMG36c), *L. lactis* NZ9000 (pMGlnqQ) or *L. lactis* NZ9000 (pLNQΔQ) (Fig. 4a). Thus, the heterologous production of LnqQ was only achieved by the co-expression of *lnqQ* and *orf3q–7q* (pLNQ). The 12 h cultured supernatants or cell-free extracts of tested strains were also spotted onto the indicator lawn of *B. coagulans* JCM 2257^T^ to confirm the expression and secretion of LnqQ. (Fig. 4b). Results showed that LnqQ activity in the culture supernatant was detected only from *L. lactis* NZ9000 (pLNQ), whereas the activity was detected in the cell-free extract of *L. lactis* NZ9000 (pMGlnqQ), which confirmed the expression of LnqQ in this strain. LC/MS analysis revealed the molecular mass of intact LnqQ (5928 Da) in the culture supernatant of *L. lactis* NZ9000 (pLNQ) (data not shown).
Host cell immunity against exogenous and endogenous LnqQ

The spot-on-lawn method was applied to evaluate the host cell immunity against exogenous LnqQ. The purified LnqQ solution, adjusted at 10 μM concentration, was serially twofold diluted and spotted onto the indicator lawn of L. lactis recombinants. Results showed that serial inhibition zones, even at the lowest concentration of LnqQ, were observed on the lawn of L. lactis NZ9000 (pMG36c). In contrast, L. lactis NZ9000 (pLNQ) showed an improved immunity, which was confirmed by both the smaller number of and the smaller size of the inhibition zones. Moreover, the immunity level was further improved in L. lactis NZ9000 (pLNQΔQ), which only expresses orf3q–7q (Fig. 5a).

The growth of the recombinant strains in liquid culture was also monitored to evaluate the intracellular toxicity of LnqQ in the host cells. L. lactis NZ9000 (pMGlnqQ), constitutively expressing lnqQ alone, showed a prolonged lag phase compared with the control strain (pMG36c). This prolonged lag phase was, however, no longer observed in L. lactis NZ9000 (pLNQ), the growth of which was almost similar to that of the control strain (Fig. 5b).

DISCUSSION

The biosynthesis of lactic acid bacteria bacteriocins is maintained by several biosynthetic proteins that are generally required for the post-translational modification (class I), processing and transport of bacteriocin, and self-immunity of the producer strain (Nes et al., 1996). An N-terminal leader peptide plays key roles in the sequential process, such as preventing intracellular activity of bacteriocin prepeptide or acting as a recognition signal either for modification or for the transport system (Chatterjee et al., 2005; Nes et al., 1996).

In this respect, we initially focused our interest on the intracellular toxicity of LqQ, as it is synthesized without an N-terminal leader peptide or intramolecular bridges (Fujita et al., 2007). The in vitro synthesis of the LnqQ peptide showed a clear inhibition zone on the indicator lawn (Fig. 2), which indicated that LnqQ is translated in an active form. Consequently, the overexpression of LnqQ caused intracellular toxicity in L. lactis NZ9000, as it triggered growth suppression of the host cells (Fig. 3a). In this experiment, we also performed the overexpression of the nukacin ISK-1 prepeptide (NukA), because nukacin ISK-1, a type AII lantibiotic, needs a modification enzyme (NukM) for its maturation (Nagao et al., 2005; Shioya et al., 2010) and thus, we assumed that no biological activity would result from NukA alone. This negative control reaction indicated that the overexpression of this small peptide (57 aa) does not trigger growth suppression of the host cells and thus highlighted the intracellular toxicity of LnqQ peptide. Previous work revealed that LnqQ damages cell membranes, even in the absence of specific receptors (Yoneyama et al., 2009a, b). This may explain the intracellular toxicity of this peptide, even though the mechanism of how it acts intracellularly remains to be elucidated. As well as induced expression, constitutive expression of lnqQ also caused growth suppression of the host cells. In comparison with the control strain, L. lactis NZ9000 (pMGlnqQ) underwent a prolonged lag phase that was followed by a certain level of growth recovery (Fig. 5b). It is noteworthy that the inoculation of the recovered cells into fresh culture medium also reproduced the same prolongation, following the recovery of host cell growth (data not shown). Meanwhile, the in vivo expression of lnqQ in L. lactis NZ9000 also revealed that LnqQ would not be secreted by a general secretory pathway of the host strain, indicating the presence of a dedicated transporter for LnqQ secretion.

The genetic similarity observed between lnqQ/lnqZ loci strongly suggested the involvement of ORF2q–7q (ORF2z–7z) in the production of these homologous bacteriocins. The ORF2q/2z, sharing similarities to putative transcriptional regulators, are potential regulators of LnqQ/Z production,
whereas ORF3q–7q (ORF3z–7z) are seemingly transcribed in the same operon and have functional roles in the secretion and/or the self-immunity of these bacteriocins. The latter hypothesis was confirmed by expression of orf3q–7q in a heterologous host L. lactis NZ9000. Constitutive expression of lnqQ and orf3q–7q in L. lactis NZ9000 (pLNQ) enabled the host cells to secrete LnqQ (Fig. 4); simultaneously, the expression of orf3q–7q rendered the host cells immune to LnqQ. This self-immunity is, in particular, facilitated by two means; firstly, secretion of LnqQ outside the cells, thus reducing the intracellular toxicity, and secondly, protection of the producer cells from extracellularly released LnqQ. The growth suppression caused by the expression of LnqQ alone (pMGLnqQ) was recovered by the additional expression of ORF3q–7q (Fig. 5b); furthermore, the recombinant cells exhibited enhanced immunity against exogenous LnqQ (Fig. 5a). The differential immunity levels observed in L. lactis NZ9000 pLNQ and L. lactis NZ9000 pLNQAQ can be explained by the differential amount of LnqQ peptide produced extracellularly. As L. lactis NZ9000 (pLNQ) both produced and secreted LnqQ, the cells were considered to be exposed to a higher concentration of LnqQ in the assay. Taken together, the orf3q–7q (orf3z–7z) are apparently involved in the biosynthesis of LnqQ (LnqZ), thus, these orf genes are hereafter referred to as lnqB–F (lnzB–F), respectively.

In the case of LsbB, a leaderless bacteriocin produced by a lactococcal strain, an ABC-type multi-drug efflux pump, termed LmrB, has been reported to facilitate the secretion of and immunity to the bacteriocin (Gajic et al., 2003). Remarkably, LmrB shares a high degree of similarities with EntQB and AurT that are dedicated transporters for enterocin Q and aureocin A70, respectively, even though there is no sequence similarity in the structures of LsbB, enterocin Q and aureocin A70 (Criado et al., 2006; Netz et al., 2001). Although none of the ORFs at the lnqQ/lnqZ loci showed similarity to LmrB, EntQB or AurT, it is likely that LnqE and LnqF (LnzE and LnzF) compose an ABC-transporter that has some homology to the product of orf3 and abc2 at the BHT-B locus. Despite the similarities of the bacteriocin structure, lnqQ/lnqZ loci have no similarity to aureocin A53 or epidermicin N101 gene loci. However, the fact that all those gene loci encode an ABC-type transporter proposes a potential common mechanism of leaderless bacteriocins, in which one dedicated ABC-transporter mediates the functions of secretion and self-immunity of cognate bacteriocin.

In many of the class II bacteriocins, a membrane-associated protein has been found encoded near a transporter gene. The function(s) of those accessory proteins has not yet been clarified but is considered as essential in maintaining the localization and/or the function of the transporter (Nes et al., 1996). This may explain the roles of LnqBCD (LnzBCD), as well as other membrane proteins found at BHT-B, aureocin A53 and epidermicin N101 loci, in the production of this type of leaderless bacteriocin, although this hypothesis remains to be elucidated.

The present study is, to the best of our knowledge, the first report describing intracellular toxicity of a leaderless bacteriocin by both in vitro and in vivo experiments. This study also provides a rare but highly conserved genetic organization that is required for bacteriocin secretion and self-immunity. In addition, ORF2q and ORF2z (hereafter named as LnqR and LnzR) are putative TetR family proteins that are rarely found in bacteriocin gene regulation. Further studies are now encouraged to clarify the individual function of LnqB–F (LnzB–F), as well as the activity of LnqR/LnzR in the production of LnqQ/LnzZ.

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not belong to the pediocin family of bacteriocins. Appl Environ Microbiol 64, 4883–4890.


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