Functional Genetic Analysis of the GarML Gene Cluster in *Lactococcus garvieae* DCC43 Gives New Insights into Circular Bacteriocin Biosynthesis

Christina Gabrielsen, Dag A. Brede, Zhian Salehian, Ingolf F. Nes, Dzung B. Diep

Department of Chemistry, Biotechnology and Food Science, Laboratory of Microbial Gene Technology and Food Microbiology, Norwegian University of Life Sciences (UMB), Ås, Norway

Garvicin ML (GarML) is a circular bacteriocin produced by *Lactococcus garvieae* DCC43. The recently published draft genome of this strain allowed determination of the genetic background for bacteriocin production. Bioinformatic analysis identified a gene cluster consisting of nine open reading frames likely involved in the production of and immunity to GarML. The *garA* gene encodes the bacteriocin precursor, *garX* a large transmembrane protein, *garBCDE* a putative immunity protein (*garB*) followed by an ATPase and two transmembrane proteins, and *garFGH* a putative ABC transporter complex. Functional genetic analysis revealed that deletion of *garFGH* had no effect on sensitivity to or production of GarML. In contrast, deletion of *garBCDE* or inactivation of *garX* resulted in high-level sensitivity to GarML and completely abolished production of active bacteriocin. Mass spectrometry of culture supernatants revealed that wild-type cultures contained the mature circular form as well as the linear forms of the bacteriocin, both with and without the three-amino-acid leader sequence, while bacteriocin-negative mutants contained only the linear forms. These results indicate that cleavage of the leader peptide precedes circularization and is likely performed by a functional entity separate from the GarML gene cluster. To our knowledge, this is the first conclusive evidence for these processes being separated in time. Loss of immunity and antimicrobial activity in addition to our inability to detect the circular bacteriocin in the Δ *garBCDE* and *garX*::pCG47 mutants demonstrate that both these units are indispensable for GarML biosynthesis as well as immunity. Furthermore, the results indicate that these genes are implicated in the circularization of the bacteriocin and that their functions are probably interlinked.

Bacteriocins are ribosomally encoded antimicrobial peptides that are produced by bacteria and display activity against other bacterial strains (1). The production of bacteriocins is a widespread trait among lactic acid bacteria, which produce numerous bacteriocins varying in size, structure, physicochemical properties, and inhibitory spectrum. These bacteriocins are classified into two main classes: the class I bacteriocins, which contain posttranslationally modified amino acids, and the class II bacteriocins, which are heat-stable unmodified bacteriocins (2).

Garvicin ML (GarML) is a potent bacteriocin produced by *Lactococcus garvieae* DCC43 (3, 4) that is a member of the class IIc circular bacteriocins, characterized by their N- to C-terminal covalent link forming a circular backbone (5). These antimicrobial peptides are attracting much interest because of their physicochemical characteristics, namely, high pH tolerance and thermal stability as well as resistance to proteases, which make this class of bacteriocins highly promising as drugs or natural preservatives for diverse applications in medicine and food technology.

Circular bacteriocins are synthesized as linear precursors with a leader peptide of variable size (2 to 35 amino acid residues) that is cleaved off at some point during the maturation process, which also includes circularization of the peptide and export out of the producer cell (5). Although several of the circular bacteriocins have now been genetically characterized, the enzymes responsible for the different steps of maturation and the mechanisms and potential coupling of the processes are still unknown (6).

To date, there have been characterized 9 circular bacteriocins, which are subdivided into two classes (2). Subclass i contains cationic peptides with a high isoelectric point (pI close to 10) and comprises the majority of the known circular bacteriocins: garvi-

cin ML (3), enterocin AS-48 (7), circularin A (8), carnocyclin A (9), uberolysin (10), lactocyclin Q (11), and leucocyclin (12). These peptides display limited sequence identity, but homology modeling based on the known structures of enterocin AS-48 and carnocyclin A indicates that the members of this subclass share a conserved three-dimensional (3D) structure (13). This structure consists of 4 or 5 α -helices forming a compact globular bundle around a hydrophobic core, where several positively charged residues located on the surface of the structure are thought to attract the peptides to the membranes of the target cells (13). Subclass ii circular bacteriocins differ in that they share high sequence identity, contain more acidic residues, and hence have a lower isoelectric point (pI close to 5). These include gasserin A (14) and butyrylvibriocin AR10 (15). Previously, the circular peptide subtilisin A (16) was considered to belong to this subclass; however, this and a few other circular peptides, including the sporulation killing factor (SKF) of *Bacillus thuringiensis* (17), contain thioether linkages and are structurally and genetically distinct from the circular bacteriocins. These peptides have therefore been

Received 23 September 2013 Accepted 2 December 2013

Published ahead of print 13 December 2013

Address correspondence to Dzung B. Diep, dzung.diep@nmbu.no.

Supplemental material for this article may be found at <http://dx.doi.org/10.1128/JB.01115-13>.

Copyright © 2014, American Society for Microbiology. All Rights Reserved.

doi:10.1128/JB.01115-13

reclassified as members of a novel subclass of peptides known as the sacitobiotics (18).

For most of the circular bacteriocins, the respective gene clusters have been described (19–23). A minimal set of 5 to 7 genes is generally required for production of and immunity to the bacteriocin, including the bacteriocin structural gene and a gene encoding a small hydrophobic high-pI peptide which constitutes the cognate immunity protein that provides a basal immunity level (19). Furthermore, 2 to 4 genes encoding putative membrane proteins, one of which contains the domain of unknown function 95 (DUF95) as well as an ATPase, make up the minimal set of genes for biosynthesis and immunity. These membrane proteins may form a transporter complex responsible for exporting the peptide out of the producer cell. Additionally, most clusters contain 3 or 4 genes thought to encode another ABC transporter, which in several cases has been shown to be nonessential for bacteriocin production and immunity (19, 20, 22) but may, however, provide an increased level of immunity and enhance the production of the bacteriocin (21). Although the sequence similarities between the encoded proteins of different circular bacteriocin gene clusters are generally low, there appears to be strong functional conservation in the biosynthetic machinery and mechanisms of this class of bacteriocins. However, the precise roles of the encoded proteins in the biosynthesis and export of and immunity to circular bacteriocins remain to be elucidated.

In this study, we identified the gene cluster responsible for production of the circular bacteriocin garvicin ML (GarML) in the producer strain *L. garvieae* DCC43 and functionally analyzed the production of this bacteriocin at the gene and protein levels, which has provided new insights into the biosynthesis of subclass i circular bacteriocins.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table S1 in the supplemental material. Lactococci were routinely grown in M17 medium (Oxoid, Hampshire, United Kingdom) supplemented with 0.4% (wt/vol) glucose (GM17) at 30°C. *Escherichia coli* strains used for cloning purposes were grown in lysogeny broth (LB) at 37°C with shaking at 225 rpm. Tetracycline was added to a final concentration of 5 µg ml⁻¹ when required for selection of *Lactococcus* transformants. Ampicillin was added to a final concentration of 100 µg ml⁻¹, and kanamycin to 50 µg ml⁻¹, when required for selection of *E. coli* transformants.

Bacteriocin purification and assays. The bacteriocin garvicin ML was concentrated 100-fold from the supernatant of the producing strain *L. garvieae* DCC43, grown in MRS medium (Oxoid, Hampshire, United Kingdom), by precipitation with 45% (wt/vol) ammonium sulfate and subsequently resuspended in sterile deionized water. Bacteriocin activity was determined using microtiter plate assays (MPA) as described previously (24), where one bacteriocin unit (BU) was defined as the amount of bacteriocin required to produce 50% growth inhibition in 200 µl of culture against the indicator strain *Lactococcus lactis* IL1403 (25). Production of and immunity to bacteriocin were assayed by agar diffusion tests (ADTs), where 1 µl ml⁻¹ of the overnight culture of the indicator strain was added to GM17 soft agar and poured into plates. After solidification and drying, circular wells were punched out and bacteriocin solution added. The plates were incubated overnight at the appropriate temperature, and the area of growth inhibition was subsequently measured.

Bioinformatic analysis. The entire genome of the bacteriocin producer *L. garvieae* DCC43 was previously sequenced and assembled *de novo* by Gabrielsen et al. (26). From the known nucleotide sequence of the GarML structural gene (GenBank accession no. GU205098) (3), the

GarML precursor gene (*garA*) was localized to contig 1 of the *L. garvieae* DCC43 draft genome (GenBank accession no. AMQ500000000). Open reading frames (ORFs) in the draft genome located close to the GarML precursor gene were predicted using CLC DNA workbench 6.0.2, allowing for all possible start codons, using the bacterial genetic code (11) and including small (>50 codons) ORFs.

Searches for similarity of the putative proteins encoded in the GarML cluster to proteins of other circular bacteriocin clusters (known sequences) or to other known proteins (nonredundant protein database) were performed by protein BLAST alignment (27), with an E value cutoff of 0.01. Conserved domains were identified similarly through the NCBI Conserved Domain Database (28), with an E value threshold of 0.01.

The physicochemical properties of the putative proteins, including theoretical isoelectric point (pI), average molecular weight, aliphatic index (AI), and grand average of hydropathicity (GRAVY), were predicted using ProtParam (29). The putative cellular localizations of the proteins were predicted using PSORTb v3.0.2 (30).

For the proteins with significant structural homologues in the Protein Data Bank (PDB), i.e., GarML, the 3D structure of the protein was modeled based on Tcoffee multiple alignment (31) and SWISS-model homology modeling (32) using the closest homolog as the template, which for GarML is the solution structure of carnosycin A (PDB accession no. 2KJF). For the proteins which had no close sequence or structural homologues, the secondary structure was predicted using either MEMSAT3-SVM (33), for the putative transmembrane proteins, or the Jpred server (34), for the soluble proteins.

Cloning and functional analysis of the GarML gene cluster. Cloning techniques, in general, were performed according to the methods of Sambrook and Russell (35). Cloning was performed mainly in *E. coli* before introduction of plasmids into lactococci. Transformations of lactococci were performed as described by Holo and Nes (36). Plasmid isolation was carried out using an EZNA plasmid miniprep kit (Omega Bio-Tek, Inc.) or a Qiagen Plasmid Midi kit (Qiagen). Restriction enzymes, calf intestinal alkaline phosphatase, T4 polynucleotide kinase, and T4 DNA ligase (New England BioLabs Inc.) were used according to the supplier's instructions. Oligonucleotides were supplied by Invitrogen Life Technologies. PCR was performed using Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific), and PCR products were purified using NucleoSpin extract II (Macherey-Nagel).

Vectors used for deletion of the two operons (*garBCDE* and *garFGH*) were created by PCR amplification of approximately 1-kb fragments flanking the respective operons, using primers listed in Table S1 in the supplemental material. The flanking sequences of each operon were subjected to splicing by overhang extension PCR using the outer primer pairs, and the resulting fragments were subsequently ligated into pCR-Blunt IITOP. A *Sna*BI/*Sma*I fragment of pAS222 (37) containing the lactococcal thermosensitive replicon *repA* and a tetracycline resistance marker was then blunt-end cloned into the *Sma*I site, creating deletion vectors pCG44 and pCG45. The deletion vectors were introduced into *L. garvieae* DCC43, and selection for deletion of *garBCDE* and *garFGH* by double crossover was carried out as described previously (38). PCR and sequencing were used to confirm the deletion of each operon.

To inactivate *garX*, a 600-bp internal fragment of this gene (primer pair *garX-F/garX-R*) was cloned into pAS222, creating vector pCG47. As a control, a similarly sized fragment of an intergenic region (primer pair *IGR-F/IGR-R*) was cloned into pAS222 to create pCG48. The vectors were transformed into *L. garvieae* DCC43, and selection for integration was performed as described previously. The correct integration of the vectors at either locus was confirmed by PCR and sequencing, while incubation at 37°C combined with antibiotic selection was used for all subsequent assays.

The *L. garvieae* DCC43 Δ *garFGH*, Δ *garBCDE*, and *garX*::pCG47 mutants were functionally characterized with respect to growth, immunity, and production of bacteriocin. Growth was evaluated by 24-h kinetic measurement of growth (optical density at 600 nm [OD₆₀₀]) of 100-fold-

diluted cultures in GM17 medium by use of a SPECTROstar Nano microplate reader (BMG Labtech). Immunity to GarML was quantified by MPA and ADT against concentrated GarML from the wild-type producer strain supernatant, while production of bacteriocin was measured by ADT using *L. lactis* IL1403 as the indicator strain.

Preparation of supernatant for mass spectrometry (MS). Cell cultures were grown to stationary phase before separation of supernatant and cells by centrifugation at $6,500 \times g$ for 10 min. Supernatants were concentrated by ammonium sulfate precipitation, as described previously, and resuspended in 1 ml of 10 mM Tris-HCl buffer (pH 7.5). Supernatant samples were subsequently filtered, desalted, and concentrated using columns with a 10-kDa size limit (Amicon Ultra-2 centrifugal filter unit with Ultracel-10 membrane; Millipore) followed by centrifugation at $6,000 \times g$ for 10 min and washing twice with 10 mM Tris-HCl (pH 7.5) before elution.

In-tip digestion. Before use, Stage columns were prepared, conditioned, and equilibrated as described previously (39). A volume of 2.5 μ l immobilized trypsin slurry (Poroszyme; Applied Biosystems) was added, and the trypsin part of the column was packed/equilibrated by pressing through two 10- μ l volumes of 50 mM $(\text{NH}_4)_2\text{HCO}_3$. The protein solution was mixed with a $0.1 \times$ volume of 500 mM $(\text{NH}_4)_2\text{HCO}_3$ and pressed through the column. The column (Stage column plus immobilized trypsin) was rinsed twice with 10 μ l 0.1% trifluoroacetic acid (TFA), which simultaneously inactivated the enzyme. Tryptic peptides were eluted from the column by using 70% acetonitrile (ACN)-0.03% TFA. Prior to MS analysis, the eluate was evaporated and the peptides dissolved in 10 μ l loading buffer.

LC-MS analysis. The peptides were separated in a 50-cm by 75- μ m-internal-diameter Acclaim PepMap column by use of a linear 3 to 40% ACN gradient over 90 min, with a flow rate of 300 nl min⁻¹. A nano-liquid chromatography (nano-LC) (Dionex Ultimate 3000 UPLC) system was interfaced with a Q Exactive quadrupole orbitrap mass spectrometer (Thermo Scientific) acquiring positive-ion mass spectra in data-dependent (dd) mode. For every full scan at resolution 70,000, 10 MS/MS scans at resolution 17,500 were recorded. The dd settings were as follows: automatic gain control (AGC) target, 1×10^5 ions; maximum injection time, 50 ms; normalized collision energy, 30; and isolation width, 2.0 *m/z*.

Nucleotide sequence accession number. The new ORFs and updated annotation of the GarML gene cluster were appended to the draft genome of *L. garvieae* DCC43 (GenBank accession no. [AMQS000000000](#)).

RESULTS

Sequence and bioinformatic analyses of the GarML gene cluster.

The genome of the GarML producer strain *L. garvieae* DCC43 was previously sequenced and *de novo* assembled to allow determination of the genetic determinants behind GarML production and immunity (26). A 264-bp fragment encompassing the structural gene of the GarML precursor (189 bp) and some surrounding sequence was previously determined by reverse genetics (3), and from this known nucleotide sequence (GenBank accession no. [GU205098](#)), the GarML precursor gene was localized to contig 1 of the *L. garvieae* DCC43 draft genome (GenBank accession no. [AMQS000000000](#)). This contig is 175.6 kb long and contains chromosomal genes (e.g., genes for a ribosomal protein cluster, tRNA synthetase, and the RNA polymerase alpha subunit), thus establishing that the GarML gene cluster is chromosomally encoded.

In the vicinity of the GarML precursor gene, henceforth referred to as *garA*, in accordance with suggested nomenclature (40), we identified eight ORFs that appeared to be involved in production of and immunity to GarML, based on similarity to gene clusters of other circular bacteriocins (Fig. 1). A single ORF named *garX* is located 61 bp downstream of and on the same strand as *garA*, while the remaining ORFs, *garBCDE* and *garFGH*,

are located upstream, on the opposite strand. The predicted physicochemical characteristics, secondary structure, subcellular localization, and conserved domains of the putative proteins encoded by the GarML gene cluster are summarized in Table 1.

The genetic organization suggests that *garA* and *garX* form independent transcriptional units. However, *garBCDE* and *garFGH* appear to constitute operons that are regulated by translational coupling, as the end of *garC* overlaps (by 11 bp) the start of *garD*, and the *garF*, *garG*, and *garH* ORFs also overlap by 1 and 4 bp, respectively (Fig. 1).

The putative protein encoded by *garB* was predicted to constitute a small (6,514.12 Da), hydrophobic, high-pI protein with a predicted secondary structure of two alpha-helices, likely to be membrane localized. These characteristics concur with those of immunity proteins of other circular bacteriocins (19, 20, 22), and we therefore predicted *garB* to encode the cognate immunity protein to GarML.

Using BLAST to search for homologues of the putative proteins encoded by the GarML gene cluster (Table 1), specifically among other circular bacteriocins, we found that GarC is similar to CclT, CirD, and AS-48D, of the carnocyclin A, circularin A, and enterocin AS-48 gene clusters, respectively, which are all predicted soluble ATP-binding proteins. The protein displays a high degree of homology to members of the P-loop NTPase domain superfamily, containing a nucleotide binding site (formed by Walker A, Walker B, Q loop, D loop, and H loop motifs) as well as an ABC transporter signature motif (LSGGQ). The evidence thus suggests that *garC* encodes the ATPase subunit of an ABC transporter complex.

GarD displays homology to CclC of the carnocyclin A gene cluster. The protein was predicted to consist of four transmembrane helices, indicating that it is a membrane protein. Interestingly, *garD* was found to contain a conserved domain of unknown function, DUF95, which has been found in the gene clusters of almost all genetically characterized circular bacteriocins to date. DUF95 proteins share a similar overall structure of several predicted transmembrane helices and are believed to constitute integral membrane proteins, but the function of this family of proteins is unknown (Pfam database accession no. PF01944).

The proteins encoded by the *garFGH* operon display a high degree of similarity to AS-48FGH and CclFGH of the enterocin AS-48 and carnocyclin A gene clusters, which are thought to encode ABC transporter complexes involved in bacteriocin immunity (21). GarH displays a high degree of homology to the SalY multidomain (permease component of an ABC-type antimicrobial peptide transport system) and is predicted to have a total of four transmembrane helices, three of which are constituted by the C-terminal FtsX domain found in many permease proteins. The protein also contains a MacB-like PCD, i.e., a periplasmic core domain found in a variety of ABC transporters. GarG (like GarC) displays a high degree of homology to members of the P-loop NTPase domain superfamily, contains the same motifs, and is therefore thought to encode the ATPase component of this ABC transporter complex. GarF, on the other hand, is predicted to contain a leader sequence and displays similarity to the RND MFP multidomain, which is a membrane fusion protein component of the RND (resistance, nodulation, and cell division) family of transporters. These proteins, including GarF, contain biotinyl/lipoyl-like domains. Based on these results, we propose that *garFGH* encodes an ABC transporter, with *garG* encoding a soluble

FIG 1 Comparative analysis of the GarML gene cluster relative to other circular bacteriocin gene clusters. Sequence identity between the encoded proteins is indicated by gray blocks.

ATP-binding protein, *garF* encoding the transmembrane permease component, and *garH* encoding an accessory periplasmic protein.

The perhaps most enigmatic gene in the GarML gene cluster is *garX*, which is the only ORF carried on the same strand as the GarML precursor gene. It is a relatively large gene (1,434 bp) that encodes a 56,564-Da protein. Secondary structure prediction suggests a structure of 8 to 12 transmembrane helices separated by intracellular and extracellular segments of various sizes. This putative protein has no significant sequence similarity to any known proteins or conserved domains, including those of other circular bacteriocin clusters, and as such, bioinformatic analysis cannot

give any more clues to the possible function of this protein. However, similar large putative membrane proteins, which could have related functions, are found in several other circular bacteriocin gene clusters (Fig. 1).

Comparative analysis of the GarML gene cluster. Comparative analysis of the GarML gene cluster to the gene clusters of other circular bacteriocins reveals that although there is limited sequence identity of the encoded proteins (average of 20 to 30% pairwise identity), there are many shared traits and commonalities between the encoded proteins (Fig. 1). The GarML gene cluster appears to be most similar to the enterocin AS-48 (GenBank accession no. Y12234 and AJ438950) and carnocyclin A (GenBank

accession no. [EU624394](#)) gene clusters, which also encode the peptides with which GarML has the highest sequence identity. However, the inverted organization of the *garBCDE* and *garFGH* versus *garA* and *garX* units in *L. garvieae* DCC43 appears to be unique.

Functional analysis of the GarML gene cluster. To investigate the biological roles of the different genes of the GarML cluster, we proceeded to make deletion mutants of the putative operons of the GarML gene cluster. For the Δ *garBCDE* and Δ *garFGH* mutants, this was readily achieved by a classical double-crossover approach. For *garX*, we failed to delete the gene after several attempts. Instead, a gene inactivation approach was used to generate the *garX*::pCG47 strain. We then determined the impact of these genotypes on the production, immunity, and growth of *L. garvieae* DCC43, the results of which are displayed in [Fig. 2](#) and summarized in [Table 2](#).

The deletion of the first operon in the GarML gene cluster, *garFGH*, encoding a putative ABC transporter complex, appeared to have no major impact on either growth of the strain or immunity to or extracellular production of GarML. The immunity was determined to be at the wild-type level (MIC_{50} of 320 BU ml⁻¹), as was the extracellular production of GarML. These results suggest that this putative ABC transporter is not required for immunity or production of bacteriocin.

The second operon in the GarML gene cluster, *garBCDE*, encodes the putative immunity protein as well as two putative membrane proteins and an ATPase. Deletion of this operon had dramatic effects on the phenotype of the producer strain. First, production of GarML was not detected in the Δ *garBCDE* mutant ([Fig. 2](#)). Furthermore, it was shown that the immunity of this deletion mutant was lowered 250-fold (MIC_{50} = 1.25 BU ml⁻¹; that of the wild-type producer strain was 320 BU ml⁻¹), which is 4-fold lower than the sensitivity level of the indicator strain, *L. lactis* IL1403 (5 BU ml⁻¹). Together, these results provide strong evidence that the *garBCDE* operon is vital not only for biosynthesis but also for immunity to GarML.

Lastly, the *garX*::pCG47 mutant also displayed loss of GarML production and lowered immunity, with an approximately 8- to 16-fold reduction in immunity toward GarML compared to the wild-type producer ([Fig. 2](#)). This mutant was less sensitive to GarML than the Δ *garBCDE* mutant, but even so, it is evident that GarX also contributes in some manner to immunity toward extracellularly added GarML.

Proteomic analysis of GarML biosynthesis. To explore the roles of the *garBCDE* and *garX* operons in biosynthesis of GarML at the protein level, we performed mass spectrometry analysis of culture supernatants of wild-type producer *L. garvieae* DCC43 and the deletion/knockout mutants.

For wild-type producer cells and the bacteriocin-positive Δ *garFGH* mutant, we were able to detect specific trypsin fragments diagnostic for the circular mature bacteriocin as well as the full-length linear precursor peptide and the processed linear precursor peptide (i.e., without the leader sequence) ([Table 2](#); see [Fig. S1](#) in the supplemental material). In contrast, only the linear forms were detected for the Δ *garBCDE* and *garX*::pCG47 mutants, which is consistent with the fact that these mutants produce no active bacteriocin ([Fig. 2](#)). Together, these results indicate that the circular structure of the bacteriocin is essential for its antimicrobial activity and that both GarBCDE and GarX are involved in the

TABLE 1 Bioinformatic analysis of the putative proteins encoded by the GarML gene cluster

Gene	Locus tag	Protein ID	No. of aa	Homologs according to BLASTp	Structure prediction by MEMSAT//PRED ^a	Localization (confidence) by PSORTb	ProtParam results			Domains/superfamilies according to NCBI CDD	Putative function	
							pI	M _w	GRAVY			AI
garH	C426_0084	EKF52509.1	398	AS-48H, CcH, CtrI	4 TM helices, periplasmic domain	Membrane (10.0)	8.7	43,901	0.1	109.9	MacB PCD, FtsX, SalY	ABC transporter component
garG	C426_0085	EKF52510.1	227	AS-48G, CcG, CtrH		Membrane (10.0)	5.2	25,788	−0.3	100.4	P-loop NTPase domain, SalX	ABC transporter ATPase
garF	C426_0086	EKF52511.1	399	AS-48F, CcF		Unknown	5.9	44,142	−0.4	97.6	RND MFP, biotinyl/lipoyl domain	ABC transporter component
garE	C426_00001	ERL47808.1	160	Low/no similarity with other proteins	5 TM helices	Membrane (10.0)	9.7	18,119	1.2	166.3		Membrane protein
garD	C426_00002	ERL47809.1	167	CdcC	4 TM helices	Membrane (10.0)	9.5	18,948	1.0	141.8	DUF95	Membrane protein DUF95
garC	C426_0087	EKF52512.1	213	CcT, CtrD, AS-48D		Cytoplasmic (7.5)	5.7	24,476	−0.2	105.2	P-loop NTPase domain, SalX	ABC transporter ATPase
garB	C426_00003	ERL47810.1	55	Low/no similarity with other proteins	2 helices	Membrane (9.55)	10.2	6,514	1.3	166.6		Immunity protein
garA	C426_0088	EKF52513.1	63	GarML		Membrane (9.55)	10.1	6,025	0.9	115.8		Bacteriocin precursor
garX	C426_0089	EKF52514.1	477	Low/no similarity with other proteins	8–12 TM helices	Membrane (10.0)	9.6	56,564	0.7	138.8		Membrane protein

^a TM, transmembrane.

FIG 2 Immunity and antimicrobial activity of *L. garvieae* DCC43 wild type and deletion/knockout mutants. Displayed in the top panels are results of agar diffusion tests (ADTs) of 100-fold-concentrated GarML from the wild-type producer strain against the indicated strains. Displayed in the lower panels are results of ADTs of 20-fold-concentrated supernatants of the indicated strains against the GarML-sensitive strain *L. lactis* IL1403.

circularization step that eventually gives rise to mature and active GarML.

DISCUSSION

In this work, we identified and characterized the gene cluster responsible for biosynthesis of and immunity to the circular bacteriocin GarML, which is chromosomally encoded in the producer strain *L. garvieae* DCC43. Bioinformatic analysis suggests that the gene cluster contains four transcriptional units: the monocistronic *garA* (bacteriocin precursor) and *garX* (transmembrane protein) genes and the polycistronic *garBCDE* (immunity protein and putative transport complex) and *garFGH* (putative ABC transporter) operons (Fig. 1 and Table 1). The gene cluster shares several traits, both in genetic organization and in the putative functions of the encoded proteins, with other subclass i circular bacteriocin gene clusters (Fig. 1). Functional analysis combined with mass spectrometry of deletion mutants of these operons revealed new insights into the biosynthesis of GarML, which may thus apply to subclass i circular bacteriocins in general.

Deletion of the *garFGH* operon did not have any significant effect on either the production of mature GarML or immunity (Fig. 2), and thus this operon is evidently nonessential to GarML biosynthesis. The corresponding genes (*AS-48FGH*) in the producer strain of enterocin AS-48 were not at first identified as part

of the gene cluster encoding this bacteriocin; however, they were later shown to provide a small increase in both production of and immunity to enterocin AS-48 (21). It is possible that GarFGH functions as a secondary transporter of GarML to prevent extracellular GarML from penetrating the cell envelope, in a manner similar to the immunity-providing ABC transporters of many lantibiotics (41). However, our experimental results did not support this notion, as the presence of the intact *garFGH* operon did not compensate for the loss of immunity resulting from deletion of the *garBCDE* operon or the *garX* gene. The corresponding ABC transporters encoded by the carnocyclin A and circularin A gene clusters have also been shown to be nonessential for bacteriocin production and immunity (20, 22). Thus, we are unable to ascribe any function to the GarFGH complex at this time. However, based on the apparent conservation of these ABC transporters between circular bacteriocin subclass i gene clusters (Fig. 1), in addition to the known function of the corresponding complex in enterocin AS-48 (21), it is possible that an independent function or accessory role of GarFGH will be elucidated at a later stage.

By mass spectrometry, we were able to detect all three forms of the GarML peptide in the culture supernatant of the wild-type producer strain (Table 2; see Fig. S1 in the supplemental material) and the bacteriocin-positive Δ *garFGH* mutant (results not shown): the mature circular peptide as well as the full-length linear peptide with the three-amino-acid leader sequence (MFD) and the linear peptide without the leader sequence. As expected, we were not able to detect the circular form of GarML in the bacteriocin-negative Δ *garBCDE* and *garX*::pCG47 mutants, although both these mutants contained the linear forms of GarML. Based on these observations, it appears likely that both the Δ *garBCDE* and *garX*::pCG47 mutants have lost the ability to perform the circularization step, which appears to be essential for the antimicrobial activity of GarML.

For most class II linear bacteriocins, including lactococcin G (class IIb) and lactococcin A (class IIc), a dedicated membrane-located ABC transporter is responsible for the removal of the leader sequence from the precursor peptide and the concurrent export of the mature peptide out of the cell (42–44). For class II circular bacteriocins like GarML, however, little is known about the process behind their maturation. Nevertheless, it is generally believed that the maturation of circular bacteriocins consists of three events, i.e., removal of the leader peptide, circularization, and export, and that these three events take place either in a de-

TABLE 2 Effects of deletions/insertions in the GarML gene cluster on cell growth and immunity to or production of GarML

Genotype	Growth ^a	Sensitivity ^b	Antimicrobial activity ^c	Peptide species ^d
DCC43 wild type	+	—	+	pre, lin, cir
DCC43 Δ <i>garFGH</i>	+	—	+	pre, lin, cir
DCC43 Δ <i>garBCDE</i>	+	+	—	pre, lin
DCC43 <i>garX</i> ::pCG47	+	+	—	pre, lin
DCC43 IGR::pCG48	+	—	+	pre, lin, cir

^a Growth was determined by measuring the OD₆₀₀ over time. +, no significant difference in growth rate and maximum cell density compared to those of the control strain was observed.

^b Sensitivity was measured by ADT, using the strains as indicators against 100-fold-concentrated GarML from the producer strain supernatant.

^c Antimicrobial activity was determined by ADT of 20-fold-concentrated supernatant against the indicator strain *L. lactis* IL1403 or IL1403(pAS222).

^d Peptide species detected in the supernatant of the strain by LC-MS/MS. pre, precursor peptide with leader sequence; lin, linear peptide without leader sequence; cir, circular mature peptide.

finer order in a stepwise fashion or in a coordinated one-step reaction. However, the specific sequence of events and potential coupling of these processes are not known (6, 45). In the present study, we have shown that, at least for garvicin ML, removal of the leader sequence and circularization are two separate processes, as we could detect the linear processed form of GarML (without a leader) in the two bacteriocin-negative mutants and also in the bacteriocin-positive cells. To our knowledge, this is the first time it has been demonstrated that these processes are independent and, importantly, that leader sequence cleavage takes place before circularization.

Furthermore, as the linear peptides were detected in all three mutants (Δ garFGH, Δ garBCDE, and garX::pCG47) and in wild-type cells, this observation suggests that leader sequence cleavage is most likely executed by a separate functional entity, i.e., one not encoded by garX, garFGH, or garBCDE. However, no candidate protease- or peptidase-encoding genes are located in the vicinity of the GarML gene cluster. The cleavage of the leader sequence must therefore be performed by an as yet unidentified, specific or nonspecific peptidase. Similar ideas have been suggested previously for enterocin AS-48 (46, 47) and may explain the apparent problems in expressing circular bacteriocins outside the genera or species of the original producer (23, 46) and in obtaining stable heterologous production (20). Resolving this issue could therefore render efficient heterologous expression of circular bacteriocins in industrially relevant species possible.

The detection of linear forms of circular bacteriocins in the supernatant of the producing strain has previously been taken into account for the circularization reaction taking place on the extracellular side of the cytoplasmic membrane (48). However, in the case of GarML, the results indicate that cell autolysis can explain detection of these species in the culture supernatant, as linear forms of GarML in a few cases were not detectable in supernatants from cells grown in sucrose-stabilized media (results not shown). Consequently, we cannot make any assumptions as to the intra- or extracellular localization of the steps in GarML biosynthesis.

Functional analysis of the Δ garBCDE and garX::pCG47 mutants revealed that production of functional bacteriocin and immunity were impaired in both strains. This is analogous to the situation for enterocin AS-48, where AS-48B and AS-48C (similar to garX and garD, respectively) have been implicated in biogenesis and immunity (49). Moreover, mass spectrometry confirmed that neither the Δ garBCDE nor garX::pCG47 mutant produced the mature circular bacteriocin. The results thus imply that both GarBCDE and GarX are involved specifically in the circularization step of GarML biosynthesis, which has not been demonstrated previously for any other circular bacteriocin. The functions of GarBCDE and GarX appear to be nonoverlapping, as neither compensates to any significant extent for inactivation of the other, which would be the expected outcome if these proteins either could perform circularization or provide immunity separately. If one operon were responsible for circularization and the other for immunity, one would expect to observe immunity without production in one strain and to detect circular bacteriocin and/or observe detrimental growth effects in the strain without the immunity determinant(s), which was not the case. Our results strongly suggest that circularization does not occur, and that immunity is severely affected, without the presence of both GarBCDE and GarX. Consequently, it is likely that these proteins are linked, either functionally or by regulation, to provide both immu-

FIG 3 Proposed model for biosynthesis of GarML in *L. garvieae* DCC43. The N-terminal three amino acid residues (represented by mauve spheres) of the precursor peptide are cleaved off by an unknown peptidase. The linear peptide is subsequently exported out of the cell, with concomitant circularization by a GarCDE and GarX multicomplex. The mature and active bacteriocin is then released into the extracellular space. GarB is likely the primary immunity determinant providing host self-protection toward GarML; however, full immunity requires the entire multicomplex.

nity to and biosynthesis of GarML. In other circular bacteriocin clusters, the genes corresponding to garX and garD are carried by the same polycistronic transcript (19, 20) (Fig. 1), thus supporting the possible link between the encoded proteins. Coproduction and/or coregulation of the immunity and biosynthetic machinery is indeed common in bacteriocin production (41, 50), forming a strategy which is clearly advantageous for the producer organism. In the GarML gene cluster, however, there are no encoded proteins which display similarity to known regulatory domains. We therefore believe that GarX and GarBCDE are functionally linked, possibly by forming a multicomplex in the cell membrane, to perform circularization of the linear peptide into the mature circular form, most likely concomitant with export out of the cell.

In conclusion, based on these results, a possible model for GarML biosynthesis is postulated (Fig. 3). In this model, the N-terminal three amino acid residues (MFD) of the precursor peptide are cleaved off by an unknown peptidase. The linear peptide is subsequently exported out of the cell, with concomitant circularization by a GarCDE and GarX multicomplex, which could act in a defined coupled sequence or as a single functional unit. The mature and active bacteriocin is then released into the extracellular space. By analogy to other circular bacteriocin immunity proteins (19, 20, 22), GarB likely constitutes the primary immunity determinant, but full immunity requires the entire multicomplex.

ACKNOWLEDGMENTS

This work was supported by a grant from the Norwegian University of Life Sciences.

We thank Morten Skaugen for performing MS analysis.

REFERENCES

- Klaenhammer TR. 1993. Genetics of bacteriocins produced by lactic acid bacteria. *FEMS Microbiol. Rev.* 12:39–85.
- Cotter PD, Hill C, Ross RP. 2005. Bacteriocins: developing innate immunity for food. *Nat. Rev. Microbiol.* 3:777–788. <http://dx.doi.org/10.1038/nrmicro1273>.
- Borrero J, Brede DA, Skaugen M, Diep DB, Herranz C, Nes IF, Cintas LM, Hernandez PE. 2011. Characterization of garvicin ML, a novel circular bacteriocin produced by *Lactococcus garvieae* DCC43, isolated from mallard ducks (*Anas platyrhynchos*). *Appl. Environ. Microbiol.* 77:369–373. <http://dx.doi.org/10.1128/AEM.01173-10>.
- Sanchez J, Basanta A, Gomez-Sala B, Herranz C, Cintas LM, Hernandez PE. 2007. Antimicrobial and safety aspects, and biotechnological potential of bacteriocinogenic enterococci isolated from mallard ducks (*Anas platyrhynchos*). *Int. J. Food Microbiol.* 117:295–305. <http://dx.doi.org/10.1016/j.ijfoodmicro.2007.04.012>.
- van Belkum MJ, Martin-Visscher LA, Vederas JC. 2011. Structure and genetics of circular bacteriocins. *Trends Microbiol.* 19:411–418. <http://dx.doi.org/10.1016/j.tim.2011.04.004>.
- Conlan BF, Gillon AD, Craik DJ, Anderson MA. 2010. Circular proteins and mechanisms of cyclization. *Biopolymers* 94:573–583. <http://dx.doi.org/10.1002/bip.21422>.
- Maqueda M, Galvez A, Bueno MM, Sanchez-Barrena MJ, Gonzalez C, Albert A, Rico M, Valdivia E. 2004. Peptide AS-48: prototype of a new class of cyclic bacteriocins. *Curr. Protein Pept. Sci.* 5:399–416. <http://dx.doi.org/10.2174/1389203043379567>.
- Kawai Y, Kemperman R, Kok J, Saito T. 2004. The circular bacteriocins gasserin A and circularin A. *Curr. Protein Pept. Sci.* 5:393–398. <http://dx.doi.org/10.2174/1389203043379549>.
- Martin-Visscher LA, van Belkum MJ, Garneau-Tsodikova S, Whittall RM, Zheng J, McMullen LM, Vederas JC. 2008. Isolation and characterization of carnocyclin A, a novel circular bacteriocin produced by *Carnobacterium maltaromaticum* UAL307. *Appl. Environ. Microbiol.* 74:4756–4763. <http://dx.doi.org/10.1128/AEM.00817-08>.
- Wirawan RE, Swanson KM, Kleffmann T, Jack RW, Tagg JR. 2007. Uberolysin: a novel cyclic bacteriocin produced by *Streptococcus uberis*. *Microbiology* 153:1619–1630. <http://dx.doi.org/10.1099/mic.0.2006/005967-0>.
- Sawa N, Zendo T, Kiyofuji J, Fujita K, Himeno K, Nakayama J, Sonomoto K. 2009. Identification and characterization of lactocyclin Q, a novel cyclic bacteriocin produced by *Lactococcus* sp. strain QU 12. *Appl. Environ. Microbiol.* 75:1552–1558. <http://dx.doi.org/10.1128/AEM.02299-08>.
- Masuda Y, Ono H, Kitagawa H, Ito H, Mu F, Sawa N, Zendo T, Sonomoto K. 2011. Identification and characterization of leucocyclin Q, a novel cyclic bacteriocin produced by *Leuconostoc mesenteroides* TK41401. *Appl. Environ. Microbiol.* 77:8164–8170. <http://dx.doi.org/10.1128/AEM.06348-11>.
- Martin-Visscher LA, Gong X, Duszyk M, Vederas JC. 2009. The three-dimensional structure of carnocyclin A reveals that many circular bacteriocins share a common structural motif. *J. Biol. Chem.* 284:28674–28681. <http://dx.doi.org/10.1074/jbc.M109.036459>.
- Kawai Y, Saito T, Kitazawa H, Itoh T. 1998. Gasserin A; an uncommon cyclic bacteriocin produced by *Lactobacillus gasserii* LA39 linked at N- and C-terminal ends. *Biosci. Biotechnol. Biochem.* 62:2438–2440. <http://dx.doi.org/10.1271/bbb.62.2438>.
- Kalmokoff ML, Cyr TD, Hefford MA, Whitford MF, Teather RM. 2003. Butyrylviobriocin AR10, a new cyclic bacteriocin produced by the ruminal anaerobe *Butyrivibrio fibrisolvens* AR10: characterization of the gene and peptide. *Can. J. Microbiol.* 49:763–773. <http://dx.doi.org/10.1139/w03-101>.
- Babasaki K, Takao T, Shimonishi Y, Kurahashi K. 1985. Subtilisin A, a new antibiotic peptide produced by *Bacillus subtilis* 168: isolation, structural analysis, and biogenesis. *J. Biochem.* 98:585–603.
- Liu WT, Yang YL, Xu Y, Lamsa A, Haste NM, Yang JY, Ng J, Gonzalez D, Ellermeier CD, Straight PD, Pevzner PA, Pogliano J, Nizet V, Pogliano K, Dorrestein PC. 2010. Imaging mass spectrometry of intraspecies metabolic exchange revealed the cannibalistic factors of *Bacillus subtilis*. *Proc. Natl. Acad. Sci. U. S. A.* 107:16286–16290. <http://dx.doi.org/10.1073/pnas.1008368107>.
- Murphy K, O'Sullivan O, Rea MC, Cotter PD, Ross RP, Hill C. 2011. Genome mining for radical SAM protein determinants reveals multiple
- sacitibiotic-like gene clusters. *PLoS One* 6:e20852. <http://dx.doi.org/10.1371/journal.pone.0020852>.
- Martinez-Bueno M, Valdivia E, Galvez A, Coyette J, Maqueda M. 1998. Analysis of the gene cluster involved in production and immunity of the peptide antibiotic AS-48 in *Enterococcus faecalis*. *Mol. Microbiol.* 27:347–358. <http://dx.doi.org/10.1046/j.1365-2958.1998.00682.x>.
- Kemperman R, Jonker M, Nauta A, Kuipers OP, Kok J. 2003. Functional analysis of the gene cluster involved in production of the bacteriocin circularin A by *Clostridium beijerinckii* ATCC 25752. *Appl. Environ. Microbiol.* 69:5839–5848. <http://dx.doi.org/10.1128/AEM.69.10.5839-5848.2003>.
- Diaz M, Valdivia E, Martinez-Bueno M, Fernandez M, Soler-Gonzalez AS, Ramirez-Rodrigo H, Maqueda M. 2003. Characterization of a new operon, as-48EFGH, from the as-48 gene cluster involved in immunity to enterocin AS-48. *Appl. Environ. Microbiol.* 69:1229–1236. <http://dx.doi.org/10.1128/AEM.69.2.1229-1236.2003>.
- Belkum M, Martin-Visscher L, Vederas J. 2010. Cloning and characterization of the gene cluster involved in the production of the circular bacteriocin carnocyclin A. *Probiotics Antimicrob. Proteins* 2:218–225. <http://dx.doi.org/10.1007/s12602-010-9056-1>.
- Kawai Y, Kusnadi J, Kemperman R, Kok J, Ito Y, Endo M, Arakawa K, Uchida H, Nishimura J, Kitazawa H, Saito T. 2009. DNA sequencing and homologous expression of a small peptide conferring immunity to gasserin A, a circular bacteriocin produced by *Lactobacillus gasserii* LA39. *Appl. Environ. Microbiol.* 75:1324–1330. <http://dx.doi.org/10.1128/AEM.02485-08>.
- Gabrielsen C, Brede DA, Hernandez PE, Nes IF, Diep DB. 2012. The maltose ABC transporter in *Lactococcus lactis* facilitates high level sensitivity to the circular bacteriocin garvicin ML. *Antimicrob. Agents Chemother.* 56:2908–2915. <http://dx.doi.org/10.1128/AAC.00314-12>.
- Chopin A, Chopin MC, Moillo-Batt A, Langella P. 1984. Two plasmid-determined restriction and modification systems in *Streptococcus lactis*. *Plasmid* 11:260–263. [http://dx.doi.org/10.1016/0147-619X\(84\)90033-7](http://dx.doi.org/10.1016/0147-619X(84)90033-7).
- Gabrielsen C, Brede DA, Hernandez PE, Nes IF, Diep DB. 2012. Genome sequence of the bacteriocin-producing strain *Lactococcus garvieae* DCC43. *J. Bacteriol.* 194:6976–6977. <http://dx.doi.org/10.1128/JB.01864-12>.
- Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. *J. Mol. Biol.* 215:403–410.
- Marchler-Bauer A, Zheng C, Chitsaz F, Derbyshire MK, Geer LY, Geer RC, Gonzales NR, Gwadz M, Hurwitz DJ, Lanczycki CJ, Lu F, Lu S, Marchler GH, Song JS, Thanki N, Yamashita RA, Zhang D, Bryant SH. 2013. CDD: conserved domains and protein three-dimensional structure. *Nucleic Acids Res.* 41:D348–D352. <http://dx.doi.org/10.1093/nar/gks1243>.
- Wilkins MR, Gasteiger E, Bairoch A, Sanchez JC, Williams KL, Appel RD, Hochstrasser DF. 1999. Protein identification and analysis tools in the ExPASy server. *Methods Mol. Biol.* 112:531–552.
- Yu NY, Wagner JR, Laird MR, Melli G, Rey S, Lo R, Dao P, Sahinalp SC, Ester M, Foster LJ, Brinkman FS. 2010. PSORTb 3.0: improved protein subcellular localization prediction with refined localization subcategories and predictive capabilities for all prokaryotes. *Bioinformatics* 26:1608–1615. <http://dx.doi.org/10.1093/bioinformatics/btq249>.
- Notredame C, Higgins DG, Heringa J. 2000. T-Coffee: a novel method for fast and accurate multiple sequence alignment. *J. Mol. Biol.* 302:205–217. <http://dx.doi.org/10.1006/jmbi.2000.4042>.
- Arnold K, Bordoli L, Kopp J, Schwede T. 2006. The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. *Bioinformatics* 22:195–201. <http://dx.doi.org/10.1093/bioinformatics/bti770>.
- Nugent T, Jones DT. 2009. Transmembrane protein topology prediction using support vector machines. *BMC Bioinformatics* 10:159. <http://dx.doi.org/10.1186/1471-2105-10-159>.
- Cole C, Barber JD, Barton GJ. 2008. The Jpred 3 secondary structure prediction server. *Nucleic Acids Res.* 36:W197–W201. <http://dx.doi.org/10.1093/nar/gkn238>.
- Sambrook J, Russell DW. 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Holo H, Nes IF. 1989. High-frequency transformation, by electroporation, of *Lactococcus lactis* subsp. *cremoris* grown with glycine in osmotically stabilized media. *Appl. Environ. Microbiol.* 55:3119–3123.
- Jonsson M, Saleihan Z, Nes IF, Holo H. 2009. Construction and characterization of three lactate dehydrogenase-negative *Enterococcus faecalis*

- V583 mutants. *Appl. Environ. Microbiol.* 75:4901–4903. <http://dx.doi.org/10.1128/AEM.00344-09>.
38. Biswas I, Gruss A, Ehrlich SD, Maguin E. 1993. High-efficiency gene inactivation and replacement system for Gram-positive bacteria. *J. Bacteriol.* 175:3628–3635.
 39. Rappsilber J, Ishihama Y, Mann M. 2003. Stop and go extraction tips for matrix-assisted laser desorption/ionization, nanoelectrospray, and LC/MS sample pretreatment in proteomics. *Anal. Chem.* 75:663–670. <http://dx.doi.org/10.1021/ac026117i>.
 40. Arnison PG, Bibb MJ, Bierbaum G, Bowers AA, Bugni TS, Bulaj G, Camarero JA, Campopiano DJ, Challis GL, Clardy J, Cotter PD, Craik DJ, Dawson M, Dittmann E, Donadio S, Dorrestein PC, Entian KD, Fischbach MA, Garavelli JS, Goransson U, Gruber CW, Haft DH, Hemscheidt TK, Hertweck C, Hill C, Horswill AR, Jaspars M, Kelly WL, Klinman JP, Kuipers OP, Link AJ, Liu W, Marahiel MA, Mitchell DA, Moll GN, Moore BS, Muller R, Nair SK, Nes IF, Norris GE, Olivera BM, Onaka H, Patchett ML, Piel J, Reaney MJ, Rebuffat S, Ross RP, Sahl HG, Schmidt EW, Selsted ME, Severinov K, Shen B, Sivonen K, Smith L, Stein T, Sussmuth RD, Tagg JR, Tang GL, Truman AW, Vederas JC, Walsh CT, Walton JD, Wenzel SC, Willey JM, van der Donk WA. 2013. Ribosomally synthesized and post-translationally modified peptide natural products: overview and recommendations for a universal nomenclature. *Nat. Prod. Rep.* 30:108–160. <http://dx.doi.org/10.1039/c2np20085f>.
 41. Draper LA, Ross RP, Hill C, Cotter PD. 2008. Lantibiotic immunity. *Curr. Protein Pept. Sci.* 9:39–49. <http://dx.doi.org/10.2174/138920308783565750>.
 42. Havarstein LS, Diep DB, Nes IF. 1995. A family of bacteriocin ABC transporters carry out proteolytic processing of their substrates concomitant with export. *Mol. Microbiol.* 16:229–240. <http://dx.doi.org/10.1111/j.1365-2958.1995.tb02295.x>.
 43. Stoddard GW, Petzel JP, Vanbelkum MJ, Kok J, McKay LL. 1992. Molecular analyses of the lactococcin A gene cluster from *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis*. *Appl. Environ. Microbiol.* 58:1952–1961.
 44. Franke CM, Tiemersma J, Venema G, Kok J. 1999. Membrane topology of the lactococcal bacteriocin ATP-binding cassette transporter protein LcnC—involvement of LcnC in lactococcin A maturation. *J. Biol. Chem.* 274:8484–8490. <http://dx.doi.org/10.1074/jbc.274.13.8484>.
 45. Maqueda M, Sanchez-Hidalgo M, Fernandez M, Montalban-Lopez M, Valdivia E, Martinez-Bueno M. 2008. Genetic features of circular bacteriocins produced by Gram-positive bacteria. *FEMS Microbiol. Rev.* 32:2–22. <http://dx.doi.org/10.1111/j.1574-6976.2007.00087.x>.
 46. Fernandez M, Martinez-Bueno M, Martin MC, Valdivia E, Maqueda M. 2007. Heterologous expression of enterocin AS-48 in several strains of lactic acid bacteria. *J. Appl. Microbiol.* 102:1350–1361. <http://dx.doi.org/10.1111/j.1365-2672.2006.03194.x>.
 47. Montalban-Lopez M, Sanchez-Hidalgo M, Cebrian R, Maqueda M. 2012. Discovering the bacterial circular proteins: bacteriocins, cyanobactins, and pilins. *J. Biol. Chem.* 287:27007–27013. <http://dx.doi.org/10.1074/jbc.R112.354688>.
 48. Cebrian R, Maqueda M, Neira JL, Valdivia E, Martinez-Bueno M, Montalban-Lopez M. 2010. Insights into the functionality of the putative residues involved in enterocin AS-48 maturation. *Appl. Environ. Microbiol.* 76:7268–7276. <http://dx.doi.org/10.1128/AEM.01154-10>.
 49. Fernandez M, Sanchez-Hidalgo M, Garcia-Quintans N, Martinez-Bueno M, Valdivia E, Lopez P, Maqueda M. 2008. Processing of as-48ABC RNA in AS-48 enterocin production by *Enterococcus faecalis*. *J. Bacteriol.* 190:240–250. <http://dx.doi.org/10.1128/JB.01528-07>.
 50. Nes IF, Diep DB, Havarstein LS, Brurberg MB, Eijsink V, Holo H. 1996. Biosynthesis of bacteriocins in lactic acid bacteria. *Antonie Van Leeuwenhoek* 70:113–128. <http://dx.doi.org/10.1007/BF00395929>.