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The Lactococcin G Immunity Protein Recognizes Specific Regions in Both Peptides Constituting the Two-Peptide Bacteriocin Lactococcin G▽

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Lactococcin G and enterocin 1071 are two homologous two-peptide bacteriocins. Expression vectors containing the gene encoding the putative lactococcin G immunity protein (*lagC*) or the gene encoding the enterocin 1071 immunity protein (*entI*) were constructed and introduced into strains sensitive to one or both of the bacteriocins. Strains that were sensitive to lactococcin G became immune to lactococcin G when expressing the putative lactococcin G immunity protein, indicating that the *lagC* gene in fact encodes a protein involved in lactococcin G immunity. To determine which peptide or parts of the peptide(s) of each bacteriocin that are recognized by the cognate immunity protein, combinations of wild-type peptides and hybrid peptides from the two bacteriocins were assayed against strains expressing either of the two immunity proteins. The lactococcin G immunity protein rendered the enterococcus strain but not the lactococcus strains resistant to enterocin 1071, indicating that the functionality of the immunity protein depends on a cellular component. Moreover, regions important for recognition by the immunity protein were identified in both peptides (Lcn-α and Lcn-β) constituting lactococcin G. These regions include the N-terminal end of Lcn-α (residues 1 to 13) and the C-terminal part of Lcn-β (residues 14 to 24). According to a previously proposed structural model of lactococcin G, these regions will be positioned adjacent to each other in the transmembrane helix-helix structure, and the model thus accommodates the present results.

Lactic acid bacteria (LAB) produce ribosomally synthesized antimicrobial peptides, generally referred to as bacteriocins. There are two main classes of these bacteriocins (8, 22): the class I bacteriocins (often referred to as lantibiotics) that contain the modified amino acid residues lanthionine and/or β-methyllanthionine and the class II bacteriocins that lack modified residues (8). The class II bacteriocins are further divided into four subclasses, IIa, IIb, IIc, and IId (8). Class IIa contains the pediocin-like bacteriocins, which have very similar amino acid sequences, class IIc consists of the cyclic bacteriocins, and the one-peptide, noncyclic bacteriocins that show no sequence similarity to the pediocin-like bacteriocins are placed in class IId (8). The unmodified two-peptide bacteriocins are placed in class IIb. They are unique in that they consist of two different peptides, both of which must be present, in about equal amounts, to obtain optimal antimicrobial activity (25). More than 10 two-peptide bacteriocins have been isolated and characterized (see reference 25 for original references) since the first isolation of such a bacteriocin (lactococcin G) in 1992 (21). For the two-peptide bacteriocins that have been genetically characterized, the genes encoding the two bacteriocin peptides are always found next to each other in the same operon, along with the gene encoding the immunity protein that protects the bacteriocin producer from being killed by its own bacteriocin.

Lactococcin G is perhaps the best-characterized two-peptide bacteriocin (12, 18, 19, 21, 24, 26, 27). It consists of the 39-residue α peptide (termed Lcn-α) and the 35-residue β peptide (termed Lcn-β) (Fig. 1A). Like all two-peptide bacteriocins whose mode of action has been studied, lactococcin G causes cell death by rendering the membranes of target cells permeable to various ions (18, 19). Nuclear magnetic resonance (NMR) and circular dichroism (CD) spectroscopy have revealed that the two lactococcin G peptides adopt mainly α-helical structures when they are individually exposed to membrane-like entities (12, 27). Based on the NMR structures and findings from site-directed mutagenesis studies, a structural model of lactococcin G has recently been proposed (23, 26, 27). In this model, the two complementary peptides form parallel helices that span the target cell membrane. The helix-helix segment consists of the N-terminal region of Lcn-α (from about Trp-3 to Gly-22) and the C-terminal region of Lcn-β (from about Tyr-13 to Trp-32). The model also proposes that the cationic C-terminal end (residues 35 to 39, R-K-K-K-H) of Lcn-α is unstructured and forced through the target cell membrane by the membrane potential, thereby positioning the C termini of the two peptides inside the target-cell (Fig. 2). The tryptophan-rich N-terminal end of Lcn-β is also proposed to be relatively unstructured and to position itself in the outer membrane interface, thus forcing the N termini of the two peptides to remain on the outer side of the target cell membrane and the helix-helix segment to transverse the membrane (Fig. 2). This proposed structure is presumably also valid for the two-peptide bacteriocins enterocin 1071 (4, 5, 11), enterocin C (17), and lactococcin Q (32), since their sequence similarities to lactococcin G (lactococcin G has about 88 and 57% sequence identity to lactococcin Q and enterocin 1071, respectively; enterocin 1071 and enterocin C are identical except for one

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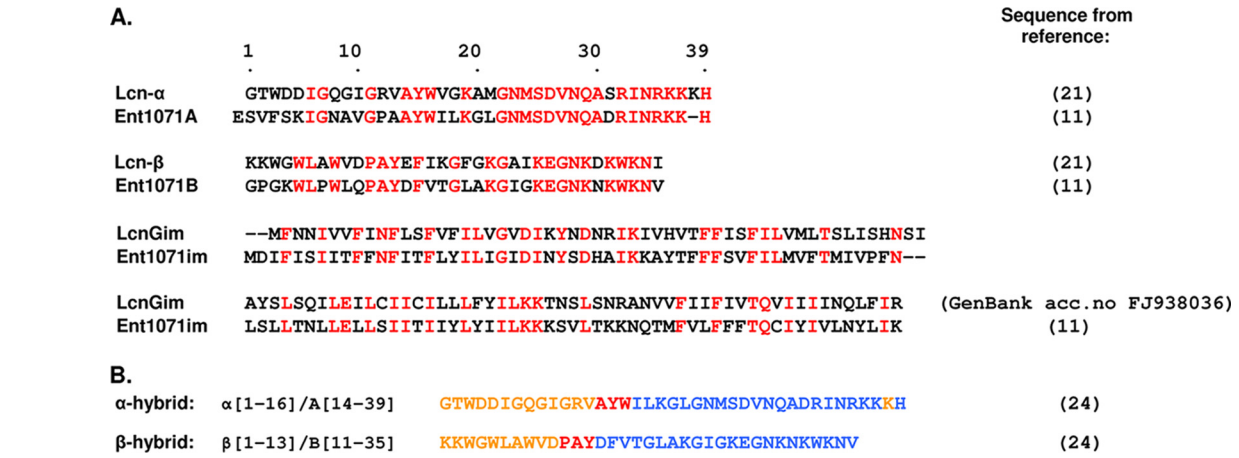

FIG. 1. (A) Amino acid sequence alignment of enterocin 1071 (peptides Ent1071A and Ent1071B) and lactococcin G (peptides Lcn-α and Lcn-β) and the cognate immunity proteins (Ent1071im and LcnGim, respectively). Ent1071A and Lcn-α show 59% sequence identity, whereas Ent1071B and Lcn-β show 54% sequence identity. The immunity proteins consist of 110 amino acid residues each and show 38% sequence identity. Identical amino acid residues are colored in red. (B) Amino acid sequences of the two hybrid peptides. The Lcn-α–Ent1071A hybrid peptide (α-hybrid) is termed α[1–16]/A[14–39] in this study (it is designated α2–4 in reference 24). Residues are numbered according to the corresponding amino acid positions in Lcn-α (Fig. 1A). Residues in orange are derived from Lcn-α, and residues in blue are derived from Ent1071A. The overlapping region (i.e., residues 14 to 16) is marked in red, and this region consists of residues that are identical in Lcn-α and Ent1071A. The α-hybrid peptide contains an additional lysine residue in the C-terminal end derived from Lcn-α (see reference 24 for the construction of the hybrid peptide). The Lcn-β–Ent1071B hybrid peptide (β-hybrid) is termed β[1–13]/B[11–35] in this study (it is designated β1–6 in reference 24). Residues in orange are derived from Lcn-β, and residues in blue are derived from Ent1071B. The overlapping region (i.e., residues 11 to 13) is marked in red, and residues in this region are identical in Lcn-β and Ent1071B.

residue) indicate that these four bacteriocins have similar three-dimensional structures.

The sequence of the lactococcin G operon (GenBank accession no. FJ938036) has been determined, and a gene (*lagC*) encoding the putative lactococcin G immunity protein has been identified downstream of the two genes encoding the two lactococcin G peptides. Downstream of the two genes encoding the two enterocin 1071 peptides, a gene (*entI*) encoding the enterocin 1071 immunity protein has been identified (4, 11). The putative lactococcin G immunity protein shows 38% amino acid sequence identity to the enterocin 1071 immunity protein (the sequence of which was obtained from Franz et al. [11]), and both proteins consist of 110 amino acid residues (Fig. 1A). The aim of this study was to identify which peptides or which parts of the peptides of the two-peptide bacteriocins lactococcin G and enterocin 1071 are recognized by these immunity proteins. To achieve this, combinations of wild-type lactococcin G peptides, wild-type enterocin 1071 peptides, and hybrid lactococcin-enterocin peptides were assayed against sensitive strains that were transformed with an expression plasmid carrying either the lactococcin G or the enterocin 1071 immunity gene.

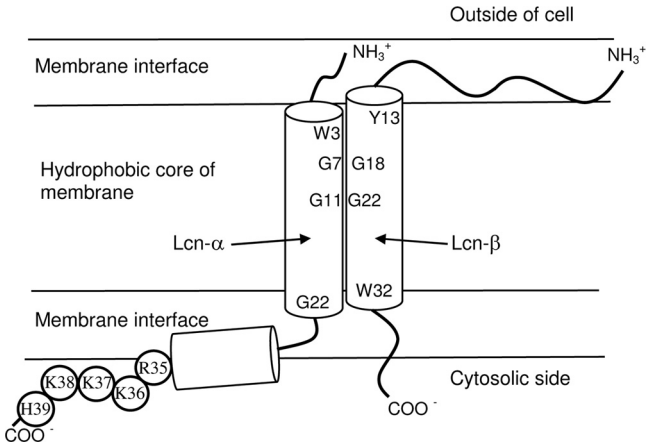

FIG. 2. Proposed structural model of lactococcin G. The two peptides (Lcn-α and Lcn-β) form a transmembrane helix-helix structure, with the flexible tryptophan-rich N-terminal end of Lcn-β positioned in the outer membrane interface and the unstructured, highly cationic C-terminal end of Lcn-α inside the target cell membrane. The transmembrane helix-helix segment consists of the N-terminal region of Lcn-α (from about Trp-3 to Gly-22) and the C-terminal region of Lcn-β (from about Tyr-13 to Trp-32). (Adapted from reference 26 with permission of the publisher. Copyright 2008 American Chemical Society.)

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Escherichia coli* DH5α cells were grown with vigorous agitation at 37°C in Luria-Bertani medium. For *E. coli* DH5α containing plasmid pLT100, pMG36e, pLcnGim, or pEnt1071im, erythromycin was added to a final concentration of 150 μg/ml for selection. For *E. coli* cells containing the plasmid pGEM-7Zf(+) (Promega) or pGEM-T Easy (Promega), ampicillin was added to a final concentration of 100 μg/ml. For growth on agar plates, the medium was solidified by the addition of 1.5% (wt/vol) agar.

Lactococcus lactis LMGT-2081 is a naturally lactococcin G-producing strain and was used to isolate template DNA for amplification of the lactococcin G immunity gene by PCR. The strain was grown without agitation at 30°C in GM17 medium (M17 medium [Oxoid] supplemented with 0.4% [wt/vol] glucose).

Lactobacillus sakei Lb790, containing the two-plasmid expression system used for production of different bacteriocin peptide variants, was grown without agitation at 30°C in MRS broth (Oxoid). The two plasmids pSAK20 and pLT100 contain markers of resistance to chloramphenicol and erythromycin, respectively. Consequently, these antibiotics were added to the growth medium, each at a final concentration of 10 μg/ml. For growth on agar plates, the medium was solidified with 1.5% (wt/vol) agar and the antibiotic concentrations were reduced to 2 μg/ml for erythromycin and 5 μg/ml for chloramphenicol.

TABLE 1. Strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Relevant characteristic(s)[a] or sequence	Source or reference
Strains		
L. lactis LMGT-2081	Wild-type lactococcin G producer	21
Lactococcus strain LMGT-2077	Lactococcin G sensitive; enterocin 1071 sensitive; Bac^+	LMGT[b]
L. lactis IL1403	Lactococcin G sensitive; enterocin 1071 sensitive; plasmid free	6, 7
E. faecalis LMGT-2333	Enterocin 1071 sensitive; Bac^+	LMGT
L. sakei Lb790	Wild-type strain used for heterologous expression of peptide bacteriocins; Bac^-	2, 3
E. coli DH5α	Host strain for plasmids	Gibco BRL
Plasmids		
pGEM-7Zf(+)	3.0-kb cloning vector; Amp^r	Promega
pGEM-T easy	3.0-kb linearized cloning vector; Amp^r	Promega
pUC57/*entI*	3.1-kb cloning vector containing the *entI* gene	GenScript Corp.
pLT100	5.2-kb plasmid used for heterologous expression of bacteriocin peptides; contains the gene encoding either one of the wild-type peptides or one of the hybrid peptides; *E. coli*/*L. sakei* shuttle vector; Ery^r	24
pSAK20	11.8-kb plasmid used for heterologous expression of bacteriocin peptides; contains the genes necessary for transcriptional activation and processing of the class IIa bacteriocin sakacin A; Cam^r	2, 3
pMG36e	*E. coli*/LAB shuttle vector; carries P32 promoter for expression in LAB; Ery^r	31
pLcnGim	pMG36e-derived plasmid containing the *lagC* gene for expression of the lactococcin G immunity protein; Ery^r	This study
pEnt1071im	pMG36e-derived plasmid containing the *entI* gene for expression of the enterocin 1071 immunity protein; Ery^r	This study
Primers		
LcnGimF[c]	5′-CGGGATCCCGGTTAGATTAAATAAGGCAAGTAG-3′	Eurogentec
LcnGimR[c]	5′-AAGAATTCCGCTATACAAGCTACTCCACAATC-3′	Eurogentec
Ent1071imF[d]	5′-TATGAGCTCTTTTTATTATTTTTAAGGAGATAAATATGG-3′	Eurogentec
Ent1071imR[d]	5′-TATTCTAGATAATTTACTTAATTAAATAGTTC-3′	Eurogentec

[a] Bac^-, non-bacteriocin producing; Bac^+, bacteriocin producing; Amp^r, ampicillin resistant; Ery^r, erythromycin resistant; Cam^r, chloramphenicol resistant; *lagC*, gene encoding the lactococcin G immunity protein; *entI*, gene encoding the enterocin 1071 immunity protein (sequence from Franz et al. [11]).

[b] LMGT, Laboratory of Microbial Gene Technology, Ås, Norway.

[c] Used to amplify the lactococcin G immunity gene from chromosomal DNA isolated from the naturally lactococcin G-producing strain *L. lactis* LMGT-2081. The BamHI and EcoRI restriction sites in the sequences of primers LcnGimF and LcnGimR, respectively, are underlined. The primers were designed based on the DNA sequence of the lactococcin G operon (GenBank accession no. FJ938036).

[d] Used to amplify the enterocin 1071 immunity gene from the pUC57/*entI* plasmid (GenScript Corporation). The SacI and XbaI restriction sites in the sequences of primers Ent1071imF and Ent1071imR, respectively, are underlined.

The indicator organisms used in the activity assay were *Lactococcus* strain LMGT-2077, *L. lactis* MG1363, and *Enterococcus faecalis* LMGT-2333. The lactococcus strains were grown without agitation at 30°C in GM17 medium containing 0.1% (vol/vol) Tween 80, whereas *E. faecalis* LMGT-2333 was grown without agitation in MRS broth (Oxoid) at 30°C. When the indicator strains contained the plasmid pMG36e or the plasmid carrying the lactococcin G immunity gene (pLcnGim) or the enterocin 1071 immunity gene (pEnt1071im), erythromycin was added to the growth medium (2 μg/ml erythromycin for the lactococcus strains and 5 μg/ml erythromycin for the enterococcus strain) to ensure that the plasmids were kept in the cells.

Construction of plasmids containing either the lactococcin G or the enterocin 1071 immunity gene. To construct the plasmid containing the lactococcin G immunity gene, the putative immunity gene (*lagC*) was amplified by PCR using a PTC-200 Peltier thermal cycler (MJ Research), *Taq* DNA polymerase (Fermentas), and chromosomal DNA from the naturally lactococcin G-producing strain *L. lactis* LMGT-2081 as the template. The 50-μl PCR mixture contained about 50 ng of the template, 200 ng of each primer (Eurogentec), each deoxynucleoside triphosphate (Stratagene) at a final concentration of 0.1 mM, and 2.5 U of *Taq* DNA polymerase. The primers used in the PCR are listed in Table 1 and were termed LcnGimR and LcnGimF. The immunity gene was amplified over 30 cycles of denaturation for 45 s at 95°C, annealing for 1 min at 48°C, and polymerization for 2 min at 72°C. The resulting PCR product was sequenced for verification, and the verified PCR product was cloned into pGEM-7Zf(+) (Promega) by using the BamHI and EcoRI restriction sites of both the PCR product and the plasmid. The lactococcin G immunity gene was then cloned into the *E. coli*/LAB shuttle vector pMG36e (31) by using the SacI and XbaI restriction sites of both the pGEM-7Zf(+) and pMG36e plasmids. The resulting plasmid was termed pLcnGim.

A pUC57 plasmid containing the enterocin 1071 immunity gene (*entI*, the sequence of which was obtained from Franz et al. [11]) was synthesized by GenScript Corporation (NJ) and was used as a template to amplify the enterocin 1071 immunity gene with *Taq* DNA polymerase (Fermentas). The PCR was performed under the same conditions employed for the amplification of the lactococcin G immunity gene. The primers (Eurogentec) used in the PCR are listed in Table 1 and were termed Ent1071imR and Ent1071imF. The resulting PCR product was sequenced for verification, and the verified PCR product was cloned into a pGEM-T Easy vector (Promega) before it was cloned into pMG36e by using the SacI and XbaI restriction sites of the PCR product and the pMG36e plasmid. The resulting plasmid was termed pEnt1071im.

Preparation of competent cells and cell transformation. Competent *E. coli* DH5α cells were prepared using the $CaCl_2$ method (28), and competent cells were transformed by heat shock at 42°C for 90 s. *L. sakei* Lb790/pSAK20 cells were made electrocompetent basically as described by Aukrust et al. (1). The cells were grown in MRS broth (Oxoid) supplemented with 2% (wt/vol) glycine and 10 μg/ml chloramphenicol to an optical density at 600 nm of 0.5 to 0.6. The cells were washed and transformed by electroporation using a Gene Pulser apparatus and a Pulse Controller unit (Bio-Rad Laboratories) as described in procedure 2 by Aukrust et al. (1). Electrocompetent *Lactococcus* strain LMGT-2077 and *L. lactis* MG1363 were prepared and transformed by electroporation as described by Holo and Nes (14). Electrocompetent *E. faecalis* LMGT-2333 was prepared and transformed by electroporation as described by Shepard and Gilmore (29), except that the cells were grown in SGM17 growth medium containing 6% (wt/vol) glycine.

Expression and purification of the bacteriocin peptides. Peptides were produced by a two-plasmid expression system using *L. sakei* Lb790/pSAK20 cells transformed with previously constructed plasmids (derived from pLT100) carrying the gene encoding either one of the wild-type peptides (Lcn-α, Lcn-β, Ent1071A, or Ent1071B) or one of the hybrid peptides (the Lcn-α–Ent1071A hybrid designated α[1–16]/A[14–39] or the Lcn-β–Ent1071B hybrid designated β[1–13]/B[11–35]) (24). The two-plasmid expression system is based on the production and secretion of the class IIa bacteriocin sakacin A (2, 3). The peptides were purified using a cation-exchange column, followed by reverse-

TABLE 2. MICs of wild-type bacteriocins for strains not expressing immunity proteins and degrees of protection conferred by immunity proteins

Bacteriocin	Strain	MIC[a] (nM) without immunity	Degree of protection[b] conferred by:	
			LcnGim	Ent1071im
Lactococcin G (Lcn-α and Lcn-β)	*L. lactis* MG1363	0.15	2,200	2,700
	Lactococcus strain LMGT-2077	0.12	14,000	>63,000
	E. faecalis LMGT-2333	>20,000	−	−
Enterocin 1071 (Ent1071A and Ent1071B)	*L. lactis* MG1363	0.5	1.6	400
	Lactococcus strain LMGT-2077	4.4	40	3,000
	E. faecalis LMGT-2333	0.24	46,000	54,000
Plantaricin EF	*L. lactis* MG1363	30	1	1
	Lactococcus strain LMGT-2077	76	1	2
	E. faecalis LMGT-2333	>400	−	−

[a] MICs of wild-type bacteriocins for three indicator strains not expressing the two immunity proteins are shown. The MICs were defined as the total amount of peptides (the sum of concentrations of both peptides in a 1:1 ratio) that inhibited growth by 50%. The given MICs are the averages of three independent measurements, and standard deviations were typically below 50%. The given MICs were obtained for strains carrying the unmodified pMG36e plasmid. MICs similar to those for strains carrying the pMG36e plasmid were obtained for the wild-type native strain (i.e., without the pMG36e plasmid).

[b] Degree of protection against the bacteriocin conferred by expression of either the lactococcin G or the enterocin 1071 immunity protein (LcnGim or Ent1071im). The degree of protection was defined as the MIC obtained for a strain expressing the immunity gene divided by the MIC obtained for the same strain containing the unmodified pMG36e plasmid. − indicates that the strain is not sensitive toward the bacteriocin at the tested concentrations and, consequently, no degree of protection was determined.

phase chromatography, as described previously (24, 30). Depending on the peptide purified, between 100 and 500 μg of peptide per liter of culture was obtained. The purity of the peptides was determined to be >80% by analytical reverse-phase chromatography using a μRPC SC 2.1/10 C_2/C_{18} column (GE Healthcare) in the SMART chromatography system (GE Healthcare).

The concentrations of the peptides were calculated by using UV absorption at 280 nm and the molar extinction coefficients calculated from the individual amino acid residues.

Activity assays. Combinations of the wild-type peptides and hybrid peptides were assayed for antimicrobial activity by using a microtiter plate assay system as described previously (21). The peptide combination (in twofold dilutions) and the indicator strain (*Lactococcus* strain LMGT-2077, *L. lactis* MG1363, or *E. faecalis* LMGT-2333) were added to a final volume of 200 μl in each well. Growth medium for the lactococcus strains was GM17 containing 0.1% (vol/vol) Tween 80, whereas *E. faecalis* LMGT-2333 was grown in MRS broth. Stationary-phase cultures of the lactococcus strains were diluted 1:50 before being added to the microtiter plates, and the plates were incubated for 5 h at 30°C. Likewise, stationary-phase cultures of *E. faecalis* LMGT-2333 were diluted 1:200 and incubated overnight at 30°C. Growth inhibition was measured spectrophotometrically at 600 nm with a microplate reader (Tecan), and the MIC was determined. The MIC was defined as the total amount of peptides (the sum of concentrations of both peptides in a 1:1 ratio) that inhibited growth by 50%. The degree of protection conferred by the two immunity proteins was evaluated by comparing the MICs obtained for an indicator strain expressing the immunity gene and the MICs obtained for the same strain containing the unmodified pMG36e plasmid.

RESULTS AND DISCUSSION

The putative lactococcin G immunity gene (*lagC*) encodes a protein conferring immunity toward lactococcin G. The gene encoding the enterocin 1071 immunity protein (*entI*) has been shown previously to encode a protein that confers immunity toward enterocin 1071 in an enterocin 1071-sensitive strain (11). In the lactococcin G operon, the *lagC* gene encodes the putative lactococcin G immunity protein (13, 20). Based on the DNA sequence of the operon (GenBank accession no. FJ938036), PCR primers were designed to amplify the putative lactococcin G immunity gene from a natural lactococcin G producer (*L. lactis* LMGT-2081). The PCR product was cloned into the pMG36e expression vector and sequenced. The DNA sequence was identical to that of the putative lactococcin G immunity gene (*lagC*) identified by Nes et al. (GenBank accession no. FJ938036) (20). Bacterial strains sensitive to lactococcin G and/or enterocin 1071 were transformed with an expression plasmid carrying the gene encoding the putative lactococcin G immunity protein (the plasmid termed pLcnGim) or the enterocin 1071 immunity protein (the plasmid termed pEnt1071im). These strains were then assayed against different combinations of wild-type lactococcin G peptides, wild-type enterocin 1071 peptides, and hybrid peptides from lactococcin G and enterocin 1071 in order to determine which peptides and which parts of the peptides are involved in the recognition of the bacteriocins by the cognate immunity proteins. The degree of protection was evaluated by comparing the MICs obtained for an indicator strain expressing the immunity protein with the MICs obtained for the same strain containing the unmodified pMG36e plasmid.

Both immunity proteins conferred immunity toward the cognate bacteriocin (Table 2). For strains expressing the putative lactococcin G immunity protein, 2,000- to 14,000-fold increases (depending on the strain) (Table 2) in the concentrations of the two lactococcin G peptides (i.e., Lcn-α and Lcn-β) were required to obtain antimicrobial activity similar to that obtained for the strains not expressing the putative immunity protein, indicating that the *lagC* gene in fact encodes the protein involved in lactococcin G immunity. For strains expressing the enterocin 1071 immunity protein, 400- to 54,000-fold increases (depending on the strain) (Table 2) in the concentrations of the two enterocin 1071 peptides (i.e., Ent1071A and Ent1071B) were necessary to obtain the same antimicrobial effect obtained for the strains not expressing the immunity protein. Neither of the two immunity proteins protected the lactococcus strains against the two-peptide bacteriocin plantaricin EF, a bacteriocin which is unrelated to lactococcin G and enterocin 1071 (Table 2). The immunity proteins, however, provided some degree of cross-immunity to the homologous

TABLE 3. MICs of different peptide combinations for the two lactococcus strains not expressing the two immunity proteins and MICs obtained when the two strains expressed either the lactococcin G or the enterocin 1071 immunity protein

Peptide combination	MIC[a] (nM) for *L. lactis* MG1363			MIC[a] (nM) for *Lactococcus* strain LMGT-2077		
	Without immunity[b]	With LcnGim	With Ent1071im	Without immunity[b]	With LcnGim	With Ent1071im
Lcn-α and β[1–13]/B[11–35]	16	1,100	1,000	44	2,000	11,000
α[1–16]/A[14–39] and β[1–13]/B[11–35]	19	930	1,200	39	4,000	16,000
Ent1071A and β[1–13]/B[11–35]	0.4	0.5	0.7	2.9	13	8,000
Ent1071A and Lcn-β	0.16	0.7	1.2	0.77	2,000	17,000
Ent1071A and Ent1071B	0.5	0.8	200	4.4	170	13,000
Lcn-α and Lcn-β	0.15	330	400	0.12	1,700	>7,600
α[1–16]/A[14–39] and Lcn-β	1.0	490	500	2.5	4,400	>18,000

[a] The MICs were defined as the total amount of peptides (the sum of concentrations of both peptides in a 1:1 ratio) that inhibited growth by 50%. The given MICs are the averages of three independent measurements, and standard deviations were typically below 50%. LcnGim and Ent1071im, lactococcin G and enterocin 1071 immunity proteins.

[b] MIC for strain carrying the unmodified pMG36e plasmid.

bacteriocins lactococcin G and enterocin 1071. The enterocin 1071 immunity protein protected the lactococcus strains against both enterocin 1071 and lactococcin G, while the lactococcin G immunity protein protected the enterococcus strain, but not the lactococcus strains, against enterocin 1071 (Table 2).

The functionality of the lactococcin G immunity protein depends on a cellular component. It has been speculated previously that the two-peptide bacteriocins recognize target cells through a receptor in the target cell membrane (26). Recently, it was shown that a component of the mannose phosphotransferase system (mannose-PTS) is the target cell receptor for several class II bacteriocins such as lactococcin A and the pediocin-like bacteriocins (9). It was also demonstrated that the bacteriocin-mannose-PTS complex is recognized directly by the cognate bacteriocin immunity protein but only when the bacteriocin is bound to the mannose-PTS (9). No such interactions between the mannose-PTS, bacteriocins, and the cognate immunity proteins has been detected for the two-peptide bacteriocins lactococcin G and plantaricin EF (9), suggesting that lactococcin G and other two-peptide bacteriocins may use another cellular component as a target receptor. The fact that the lactococcin G immunity protein confers immunity (46,000-fold protection) toward enterocin 1071 in the enterococcus strain but does not confer immunity to the same extent (only 2- to 40-fold protection, depending on the strain) in the lactococcus strains (Table 2) indicates that a cellular component is indeed required for the lactococcin G immunity protein to function. The lactococcin G immunity protein was clearly expressed in the lactococcus strains, since these strains were well protected against lactococcin G (with 2,000- to 14,000-fold protection, depending on the strain) (Table 2).

Regions in Lcn-α and Lcn-β that are recognized by the lactococcin G immunity protein. To identify which of the peptides or which parts of the peptides of lactococcin G and enterocin 1071 are recognized by the cognate immunity proteins, the antimicrobial activities of Lcn-α, Ent1071A, and an Lcn-α–Ent1071A hybrid peptide in combination with either Lcn-β, Ent1071B, or an Lcn-β–Ent1071B hybrid peptide were assayed for two strains (*L. lactis* MG1363 and *Lactococcus* strain LMGT-2077) expressing either the lactococcin G or the enterocin 1071 immunity protein (Table 3). The amino acid sequences of the two hybrid peptides are shown in Fig. 1B. The Lcn-α–Ent1071A hybrid peptide (termed α[1–16]/A[14–39]) consists of the N-terminal end of Lcn-α (residues 1 to 16) and the C-terminal end of Ent1071A (residues 14 to 39). Residues 14 to 16 in Lcn-α and Ent1071A are identical. The Lcn-β–Ent1071B hybrid peptide (termed β[1–13]/B[11–35]) consists of the N-terminal end of Lcn-β (residues 1 to 13) and the C-terminal end of Ent1071B (residues 11 to 35). Residues 11 to 13 in Lcn-β and Ent1071B are identical. Of the nine possible peptide combinations, two combinations, Lcn-α+Ent1071B and α[1–16]/A[14–39]+Ent1071B, could not be evaluated because their antimicrobial activities were too low (with MICs of 500 to 1,000 nM). Data for these two peptide combinations are consequently not included in Table 3.

The lactococcin G immunity protein clearly protected both indicator strains efficiently against both the Lcn-α+β[1–13]/B[11–35] and the α[1–16]/A[14–39]+β[1–13]/B[11–35] combinations but did not protect the two strains against the Ent1071A+β[1–13]/B[11–35] combination (Table 3). There are consequently residues in the N-terminal region (up to and including residue 13) of Lcn-α that are important for recognition of lactococcin G by the immunity protein, and these residues are absent in the corresponding N-terminal region of Ent1071A. The relevant residues in the N-terminal region of Lcn-α are G1, T2, W3, D4, D5, Q8, G9, I10, R12, and V13 (Fig. 1A).

The lactococcin G immunity protein did not protect the two strains efficiently against enterocin 1071 (i.e., Ent1071A and Ent1071B) or the *L. lactis* MG1363 strain against the Ent1071A+Lcn-β combination (Table 3). This was expected, since both the Ent1071A+Ent1071B and the Ent1071A+Lcn-β combinations lack all of the above-mentioned residues that are present in the N-terminal region of Lcn-α. Unexpectedly, the lactococcin G immunity protein did, however, protect *Lactococcus* strain LMGT-2077 against the Ent1071A+Lcn-β combination (Table 3). Residues on the C-terminal side of residue 13 in Lcn-β (because the immunity protein protected against Ent1071A+Lcn-β but not against Ent1071A+β[1–13]/B[11–35] [Table 3]) apparently compensate for the lack of the above-mentioned residues in the N-terminal region of Lcn-α. Residues on the C-terminal side of residue 13 in Lcn-β that are not present at the corresponding positions in Ent1071B are E14, I16, K17, F19, G20, A23, I24, D30, and I35. The two latter residues are probably not important for recognition, since sim-

ilar residues are present at corresponding positions in Ent1071B (N instead of D and V instead of I) and earlier mutagenesis results revealed that D30-to-N and I35-to-V mutations in Lcn-β do not affect the activity of lactococcin G (24).

According to the recently proposed structural model of lactococcin G (Fig. 2), the sequences covering residues 1 to 13 and 14 to 24 in Lcn-α and Lcn-β, respectively, will become adjacent to each other in the active lactococcin G complex. These sequences or parts of these sequences, as predicted by the structure, will form one protein-interacting entity in lactococcin G, and this may explain how residues in both sequences are (directly or indirectly) recognized by the lactococcin G immunity protein. The proposed structural model thus accommodates the present results showing that the lactococcin G immunity protein recognizes residues in sequences covering positions 1 to 13 and 14 to 24 in Lcn-α and Lcn-β, respectively.

The enterocin 1071 immunity protein protects lactococcus strains against both enterocin 1071 and lactococcin G, and its functionality depends on a cellular component. The enterocin 1071 immunity protein protected *Lactococcus* strain LMGT-2077 against all tested peptide combinations and the *L. lactis* MG1363 strain against only some of the combinations (Table 3). The immunity protein protected the latter strain against the Ent1071A+Ent1071B and Lcn-α+Lcn-β wild-type combinations but not against the Ent1071A+Lcn-β and the Ent1071A+β[1–13]/B[11–35] hybrid combinations, even though the four combinations were highly and equally toxic toward the strain (Table 3). This finding indicates (i) that the peptide-induced process that leads to cell toxicity is by itself not necessarily sufficient to trigger the functioning of the immunity proteins and (ii) that the two complementary peptides are recognized as one physical entity (since both the Lcn-α+Lcn-β and Ent1071A+Ent1071B wild-type combinations were recognized by the immunity protein but the Ent1071A+Lcn-β hybrid combination was not, even though the three combinations are equally toxic). It is noteworthy that the enterocin 1071 immunity protein protected the *L. lactis* MG1363 strain against the α[1–16]/A[14–39]+Lcn-β and α[1–16]/A[14–39]+β[1–13]/B[11–35] hybrid combinations but not the similar Ent1071A+Lcn-β and Ent1071A+β[1–13]/B[11–35] hybrid combinations (Table 3). It thus appears that, like the lactococcin G immunity protein, the enterocin 1071 immunity protein requires residues present in the N-terminal region (residues 1 to 13) of Lcn-α but absent in Ent1071A for the recognition of lactococcin G as well as lactococcin-enterocin hybrids when the immunity protein is present in the *L. lactis* MG1363 strain. The fact that the Ent1071A+Lcn-β and Ent1071A+β[1–13]/B[11–35] combinations were recognized differently by the two indicator strains indicates that the functionality of the enterocin 1071 immunity protein also depends on a cellular component.

It has also been shown previously that the functionality of the immunity proteins for the pediocin-like (class IIa) bacteriocins depends on a cellular component (10, 15, 16). This component is most likely the membrane-embedded MptC and/or MptD subunit of the mannose-PTS, with which the pediocin-like bacteriocins interact, thereby triggering the cognate immunity proteins to bind to the bacteriocin-mannose-PTS complex (9). Interactions between the pediocin-like bacteriocins and the MptC and/or MptD subunit apparently alter the conformation of the mannose-PTS in a manner that results in membrane leakage, and this leakage is blocked by the binding of cognate immunity proteins to the bacteriocin-mannose-PTS complex. The two-peptide bacteriocins may possibly function in an analogous manner. By interacting with an integrated membrane (transport) protein that functions as the bacteriocin receptor, two-peptide bacteriocins may induce membrane leakage, which in turn is blocked by the binding of the cognate immunity proteins to the bacteriocin-receptor complex. The functionality of the immunity proteins would then be dependent on a cellular component—the bacteriocin receptor—and the immunity proteins would then specifically recognize the cognate bacteriocins indirectly through the conformational alteration the bacteriocins induce in the receptor. One may speculate further that the conformational alterations induced by the bacteriocins may vary to some extent depending on modifications/mutations in the bacteriocins and that this variation may influence how optimally the immunity proteins are able to recognize the bacteriocins. Interestingly, the enterocin 1071 immunity protein protected *Lactococcus* strain LMGT-2077 against all tested peptide combinations, and one may speculate that the cellular component present in this strain has more optimal interaction with the different hybrid peptide combinations than the components present in the other strains analyzed.

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