Mutational Analysis of Putative Helix−Helix Interacting GxxxG-Motifs and Tryptophan Residues in the Two-Peptide Bacteriocin Lactococcin G[†]

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ABSTRACT: The membrane-permeabilizing two-peptide bacteriocin lactococcin G consists of two different peptides, LcnG-α and LcnG-*β*. The bacteriocin contains several tryptophan and tyrosine residues and three putative helix-helix interacting GxxxG-motifs, G_7xxxG_{11} and G_{18}xxxG_{22} in LcnG-α and G_{18}xxxG_{22} in LcnG-*β*. The tryptophan and tyrosine residues and residues in the GxxxG-motifs were altered by site-directed mutagenesis to analyze the structure and membrane-orientation of lactococcin G. Substituting the glycine residues at position 7 or 11 in the G_7xxxG_{11}-motif in LcnG-α with large hydrophobic or hydrophilic residues was highly detrimental, whereas small residues were tolerated. Qualitatively similar results were obtained for the G_{18}xxxG_{22}-motif in LcnG-*β*. In contrast, replacement of the glycine residues in the middle of these two motifs with large hydrophilic residues was tolerated. All mutations in the G_{18}xxxG_{22}-motif in LcnG-α were relatively well-tolerated, indicating that this motif is not involved in helix−helix interactions. The four aromatic residues in the N-terminal part of LcnG-*β* could individually be replaced by other aromatic residues, a hydrophilic positive residue, and a hydrophobic residue without a marked reduced activity, indicating that this region is structurally flexible and not embedded in a strictly hydrophobic or hydrophilic environment. The results are in accordance with a structural model where the G_7xxxG_{11}-motif in LcnG-α and the G_{18}xxxG_{22}-motif in LcnG-*β* interact and allow the two peptides to form a parallel transmembrane helix−helix structure, with the tryptophan-rich N-terminal part of LcnG-*β* positioned in the outer membrane interface and the cationic C-terminal end of LcnG-α inside the cell.

Gene-encoded antimicrobial peptides (AMPs)[1] produced by lactic acid bacteria (LAB) are often referred to as bacteriocins and are divided in two main classes (*1, 2*). The peptide bacteriocins found in class-I contain the modified amino acid residues lanthionine or *β*-methyllanthionine and are often referred to as lantibiotics (*1*). Class-II consists of the nonlanthionine-containing peptide bacteriocins and may, according to Cotter et al. (*1*), be further divided into four subclasses, class-IIa, -IIb, -IIc, and -IId. Class-IIa contains the antilisterial one-peptide *pediocin-like* bacteriocins that have similar amino acid sequences, class-IIc the cyclic bacteriocins, and class-IId the one-peptide noncyclic bacteriocins that show no sequence similarity to the *pediocin-like* bacteriocins (*1*). Class-IIb contains the unmodified two-peptide bacteriocins (reviewed in (*3*)). Common for all two-peptide bacteriocins is that they consist of two different peptides that must be present in about equal molar amounts to obtain optimal antimicrobial activity.

Since the first isolation of a class-IIb two-peptide bacteriocin, lactococcin G in 1992 (*4*), more than 10 other two-peptide bacteriocins have been characterized (*5*−*22*). The genes encoding the two peptides are always located next to each other in the same operon, along with the gene encoding the immunity protein, which protects the bacteriocin-producer from being killed by its own bacteriocin (*3*). The peptides are normally synthesized with an N-terminal double-glycine type leader sequence that is cleaved off by a dedicated ABC transporter at the C-terminal side of the conserved double-glycine motif (*3*). All two-peptide bacteriocins whose mode of action has been studied render membranes of sensitive cells permeable to small molecules (*15, 23*−*28*).

Lactococcin G is perhaps the most studied two-peptide bacteriocin (*4, 26, 27, 29*−*31*). It consists of a 39-residue α-peptide (termed LcnG-α) and a 35-residue *β*-peptide (termed LcnG-*β*). Neither of the two peptides show any activity when assayed individually (*27*). Circular dichroism (CD) studies show that LcnG-α and LcnG-*β* are relatively unstructured in water but become structured in the presence of trifluoroethanol (TFE) and membrane mimicking entities such as dodecylphosphocholine (DPC) micelles and liposomes (*29*). Moreover, the two peptides structure each other when in contact with membranes (such as liposomes), indicating that they interact physically with each other (*29*). Similar results have been obtained for the two two-peptide bacteriocins plantaricin EF and plantaricin JK (*32*). Recently,

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[1] Abbreviations: AMPs, antimicrobial peptides; LAB, lactic acid bacteria; LcnG, lactococcin G; CD, circular dichroism; TFE, trifluoroethanol; DPC, dodecylphosphocholine; NMR, nuclear magnetic resonance; MRS, de Man Rogosa Sharp; LB, Luria−Bertani; PCR, polymerase chain reaction; TFA, trifluoroacetic acid; MALDI-TOF, matrix assisted laser desorption ionization time-of-flight; MIC, minimum inhibitory concentration.

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FIGURE 1: Amino acid sequences of the unmodified (class-IIb) two-peptide bacteriocins that have been characterized. The GxxxG-motifs are marked with black background. Many of the two-peptide bacteriocins containing GxxxG-motifs also contain similar AxxxA- or SxxxS-motifs. Plantaricin Sβ and plantaricin NC8β have instead of the GxxxG-motif a similar AxxxA- and SxxxS-motif, respectively. The two-peptide bacteriocins leucocin H (*34*), lactococcin MN (*19*), and lactococcin MMT24 (*35*) are not included in the figure. Leucocin H has not been completely sequenced, but the partial sequence reveals possible GxxxG-motif. Also, lactococcin MN has possible GxxxG-motifs but is not included in the list because only the sequences of the preforms are known and the cleavage site is unknown. Lactococcin MMT24 has not been sequenced.

the three-dimensional structures of LcnG-α and LcnG-β were determined in the presence of DPC micelles and in TFE by the use of nuclear magnetic resonance (NMR) spectroscopy (*33*). LcnG-α had a well-defined N-terminal amphiphilic α-helix from residues 3 to 21 and a less well-defined hydrophilic α-helix from residues 24 to 34, while LcnG-β had an α-helix from about residues 9 to 19 and an α-helix (in the presence of TFE, but not in DPC) from residues 23 to 32 (*33*).

All two-peptide bacteriocins identified so far contain GxxxG-motifs (Figure 1). This motif is found to be significantly over-represented in transmembrane helices (*36*) and it is, along with the "GxxxG-like" motifs AxxxA and SxxxS, known to mediate helix−helix interactions in membrane proteins (*37*). It is believed that, in an α-helix, these two glycine residues will be on the same side of the helix and thereby create a flat interaction site that allows close contact between two α-helices, with extensive interhelical van der Waals interactions and the formation of stabilizing interhelical backbone Cα−H···O hydrogen bonds (*37, 38*). Lactococcin G contains three GxxxG-motifs (Figure 1), two in LcnG-α (G_7xxxG_{11} and $G_{18}xxxG_{22}$) and one in LcnG-β ($G_{18}xxxG_{22}$). The G_7xxxG_{11}-motif in LcnG-α and $G_{18}xxxG_{22}$-motif in LcnG-β are conserved in the three homologous two-peptide bacteriocins lactococcin G, lactococcin Q, and enterocin 1071 (Figure 1). Based on the predominant helical structure of LcnG-α and LcnG-β, it has been proposed that these two GxxxG-motifs interact and allow LcnG-α and LcnG-β to form a parallel transmembrane helix−helix structure (*33*). It was also proposed that the three tryptophan residues in the N-terminal part of LcnG-β position themselves in the membrane−water interface, because aromatic tryptophan and tyrosine residues in membrane proteins are often found in the interface region (*39*−*42*). In this study, residues in the GxxxG-motifs in lactococcin G have been altered by site-directed mutagenesis to see if the effect of these alterations is consistent with the proposed structural model. Moreover, tryptophan and tyrosine residues were also replaced with other residues to analyze how lactococcin G might position itself in target membranes.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. For production of the lactococcin G peptides (LcnG-α and LcnG-β) and their mutant variants, *Lactobacillus sake* Lb790 containing the two-plasmid expression system pSAK20/pLT100 (*31, 43, 44*) was grown without agitation at 30 °C in MRS (Oxoid) medium. Chloramphenicol and erythromycin were added for selection (each at a final concentration of 10 μg/mL), because the pSAK20 and pLT100 plasmids contain resistance markers for chloramphenicol and erythromycin, respectively. For growth on agar plates, the media was solidified by adding 1.5% (w/v) agar and the concentrations of the two antibiotics were reduced to 2 μg/mL chloramphenicol and 5 μg/mL erythromycin.

For amplifying the plasmid (pLT100) containing the gene encoding either LcnG-α, LcnG-β, or a modified version of these peptides, competent *Escherichia coli* DH5α cells (*45*) were transformed and grown with vigorous agitation at 37 °C in LB medium. Erythromycin was added to a final concentration of 150 μg/mL for selection, both in liquid media and on agar plates (solidified by adding 1.5% (w/v) agar).

When assaying the activity, *Lactococcus lactis* LL108, *L. lactis* MG1363, *L. lactis* IL1403, *Lactococcus* LMGT-2063 and *Lactococcus* LMGT-2077 were used as indicator organisms. They were all grown without agitation at 30 °C in M17

medium (Oxoid) supplemented with 0.4% (w/v) glucose and 0.1% (v/v) Tween 80.

Site-Directed Mutagenesis and Plasmid Isolation. All mutations were constructed using the Quick Change site-directed mutagenesis kit (Stratagene) and the *PfuTurbo* DNA polymerase (Stratagene). The plasmids pLT100α and pLT-100*β*, containing the gene encoding LcnG-α and LcnG-*β*, respectively, were used as templates in the PCR, which was performed on a Gene-Amp 2400 PCR system (Perkin-Elmer). About 40 ng plasmid template, 125 ng of each oligo nucleotide primer (Eurogenetec) and dNTPs (each to a final concentration of 0.05 mM, Stratagene) were mixed together in a 50 μL reaction mixture. After a 2 min hotstart at 95 °C, 2.5 *U PfuTurbo* DNA polymerase were added to the mixture. PCR was carried out with 16 cycles of denaturation for 45 s at 95 °C, annealing for 1 min at 48 °C and 7 min polymerization at 68 °C. To increase the mutation efficiency, the original template was then eliminated by a 1 h digestion at 37 °C with the restriction enzyme *Dpn*I. The nicked plasmid (containing the desired mutation) from the PCR was transformed into competent *E. coli* DH5α cells where the nick was sealed by the *E. coli* DNA repair system. The plasmids were isolated from the *E. coli* DH5α cells using the Plasmid DNA Purification kit (Macherey-Nagel) according to the manual.

The correct DNA sequences of the mutated plasmids were confirmed by automated DNA sequence determination, using an ABI PRISM 377 DNA sequencer and an ABI Prism Ready Reaction dye terminator cycle sequencing kit (Perkin-Elmer).

Preparation of Competent Cells and Cell Transformation. *E. coli* DH5α cells were made competent by treatment with $CaCl_2$ (*46*). Transformation of competent *E. coli* DH5α cells was performed according to the manual provided with the Quick Change site-directed mutagenesis kit (Stratagene).

Competent *L. sake* Lb790/pSAK20 cells were prepared as described by Aukrust et al. (*47*). Cells were cultured in MRS broth (Oxoid; supplemented with 2% (w/v) glycine and 10 μg/mL chloramphenicol) until an OD_{600} of 0.5−0.6 was reached. The cells were first washed with 1 mM $MgCl_2$ and then with 30% (w/v) polyethylene glycol 1500 (molecular weight range 1300−1600). Competent *L. sake* Lb790/pSAK20 cells were transformed by electroporation using a Gene Pulser and Pulse Controller unit (Bio-Rad Laboratories) as described previously (*47*).

Expression and Purification of Bacteriocins. A previously described two-plasmid expression system (based on the expression, processing and secretion of the class-IIa bacteriocin sakacin A) was used to produce the lactococcin G peptides and their mutant variants (*31, 43, 44*). The two plasmids, pSAK20 (a pVS2-derived plasmid) and pLT100 (a pLPV111-derived *E. coli*−*Lactobacillus* shuttle vector), were introduced into the bacteriocin-deficient strain *L. sake* Lb790. The pLT100 plasmids contain the gene encoding either LcnG-α or LcnG-*β* or a mutated version of these (termed pLT100α when containing the gene encoding LcnG-α and pLT100*β* when containing the gene encoding LcnG-*β*) and the gene encoding the lactococcin G-immunity protein. These genes have been placed under the control of the sakacin A-specific promoter and the structural gene has the N-terminal leader from sakacin A (*31, 43, 44*). pSAK20 contains the *orf4sapKRTE* operon needed for activation, processing, and secretion of the bacteriocin peptides (*43, 44*).

The peptides were purified essentially as described by Uteng et al. (*48*). The supernatant of a 0.5 L overnight culture was applied to a 5−6 mL SP Sepharose Fast Flow (GE Healthcare) cation exchange column that had been equilibrated with 50 mL of 20 mM sodium phosphate buffer, pH 6. After a wash with 100 mL of the sodium phosphate buffer, the peptides were eluted with 40 mL of the sodium phosphate buffer containing 1 M NaCl. Trifluoroacetic acid (TFA) and 2-propanol were added to the eluent to final concentrations of 0.1 and 5% (v/v), respectively, and the eluent was then applied to a reverse phase column (Resource RPC, GE Healthcare). The peptides were eluted using a linear 2-propanol gradient in 0.1% TFA. The purity of the peptides were analyzed by analytical reverse phase chromatography (using a μRPC C2/C18 ST 4.6/100 column (GE Healthcare) in the Äkta chromatography system (GE Healthcare) or a μRPC SC 2.1/10 C2/C18 column (GE Healthcare) in the SMART chromatography system (GE Healthcare)).

The molecular masses of the purified peptides were confirmed with a Voyager-DE RP matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer (Perseptive Biosystems), using α-cyano-4-hydroxycinnamic acid as matrix. The concentrations of purified peptides were determined by measuring the UV absorption at 280 nm and using the molar extinction coefficients calculated from the contributions of individual amino acid residues.

Activity Assays. All of the peptide variants in combination with the complementary wild-type peptide were assayed for antimicrobial activity, and the activity was quantified using a microtiter plate assay system as described previously (*4, 31*). M17 broth supplemented with 0.4% (w/v) glucose and 0.1% (v/v) Tween 80, the peptide combination (at 2-fold dilutions) and the indicator organism were added to a final volume of 200 μL in each well. Stationary phase cultures of the indicator organisms were diluted 1:50 before added to the microtiter plates. The microtiter plates were incubated for 4−7 h at 30 °C before the growth inhibition was measured spectrophotometrically at 600 nm (Microplate Reader, TECAN). The MIC-value (minimum inhibitory concentration) was defined as the total amount of peptides (the sum of both peptide (in 1:1 ratio) concentrations) that inhibited the growth by 50%.

RESULTS

Mutational Effects on the GxxxG-Motifs. To investigate whether the GxxxG-motifs in lactococcin G might be involved in helix−helix interactions between LcnG-α and LcnG-*β*, the glycine residues in these motifs were replaced with other small amino acid residues (Ala, Ser), large hydrophobic residues (Ile, Leu), or large hydrophilic residues (Lys, Gln), and the potencies of the peptide variants in combination with the complementary wild-type peptide were determined. There are also small residues in the middle of these GxxxG-motifs (i.e., Gly9 and Ala20 in LcnG-α and Gly20 in LcnG-*β*), and these residues were exchanged to determine whether or not small residues are important at these positions. A total of 36 versions of LcnG-α and 18 versions of LcnG-*β* were thereby constructed and assayed

Table 1: Relative MIC Values[a] of the Different Mutant Peptides Combined with the Complementary Wild-Type Peptide Assayed against *L. lactis* LL108

		mutations with the corresponding relative MIC values						
motif	position	Gly	Ala	Ser	Ile	Leu	Lys	Gln
α-G_7xxxG_{11}	G7	1	20	6	>1400	>1500	270	150
	G9	1	4	2	250	630	5	3
	G11	1	8	10	>1500	>1500	>1500	>1100
α-G_{18}xxxG_{22}	G18	1	2	1	2	6	10	9
	A20	1	1	1	2	2	9	1
	G22	1	2	1	2	2	17	13
β-G_{18}xxxG_{22}	G18	1	1	2	39	47	49	37
	G20	1	2	1	25	24	4	2
	G22	1	2	2	160	48	270	58

[a] The MIC values are given as relative values compared to the MIC value of the wild-type peptide combination (i.e., LcnG-α + LcnG-*β*, relative MIC value = 1, absolute MIC value = 0.07 nM). The MIC value is defined as the total amount of peptides (the sum of both peptide (in 1:1 ratio) concentrations) that inhibited the growth by 50%.

for activity. Three different indicator organisms were used when determining the activity (*Lactococcus lactis* LL108, *L. lactis* MG1363, and *Lactococcus* LMGT-2077) because mutational effects on activity may in some cases depend on the indicator strain (*49*−*52*). The activity of the peptide variants in combination with the complementary wild-type peptide was compared to the wild-type combination (i.e., LcnG-α + LcnG-*β*) and the results obtained when using *L. lactis* LL108 as indicator organism are summarized in Table 1. Concurring results were obtained using the other two strains (*L. lactis* MG1363 and *Lactococcus* LMGT-2077), and the results are available as Supporting Information. The results obtained with these two strains revealed the same general changes in activity as the results obtained with the strain *L. lactis* LL108.

The effect the mutations had on the antimicrobial activity varied considerably between the different GxxxG-motifs, dependent on the position of the residues substituted and the type of substitutions. Mutations made in the G_7xxxG_{11}-motif in LcnG-α were the most detrimental. Substituting the glycine residues at positions 7 or 11 with a large hydrophobic residue (Ile or Leu) resulted in inactivation (more than a 1400-fold reduction in activity) of the two-peptide bacteriocin (Table 1). Replacement with a large hydrophilic residue (Lys or Gln) was also very detrimental, reducing the activity by a factor of 150 to more than 1100 (Table 1). In contrast, replacement with a smaller amino acid residue (Ala or Ser) was relatively well-tolerated, causing a 6−20-fold reduction in activity (Table 1). Thus, there seems to be an environmental space restriction at positions 7 and 11. This is in contrast to position 9 in the middle of the GxxxG-motif, because this glycine residue could be replaced with a large hydrophilic residue (Lys, Gln) or with a small noncharged residue (Ala, Ser), with no significant loss of activity (only 2−5-fold reduction; Table 1). Although there was no marked space restriction at position 9, it seems that the position is in a hydrophilic environment because replacement with the hydrophobic residues (Ile, Leu) reduced the activity by more than 250-fold (Table 1).

The results obtained upon introducing mutations in the G_{18}xxxG_{22}-motif in LcnG-*β* were qualitatively similar to the results obtained with the G_7xxxG_{11}-motif in LcnG-α (Table 1). Large residues, irrespectively of whether they were hydrophobic (Ile, Leu) or hydrophilic (Lys, Gln), were not tolerated to the same extent as small residues (Ala, Ser) as replacements for the glycines at positions 18 and 22. Large hydrophilic residues (Lys, Gln), as well as small residues (Ala, Ser), were, however, well-tolerated (1−4-fold reduction in activity) as replacements for the glycine at position 20 in the middle of the motif, while large hydrophobic residues (Ile, Leu) were less well-tolerated (25-fold reduction in activity). Thus, as for the G_7xxxG_{11}-motif in LcnG-α, the first and last residue in the G_{18}xxxG_{22}-motif in LcnG-*β* has to be small, whereas the middle residue may be large but it has to be hydrophilic.

In marked contrast to mutations in the G_7xxxG_{11}- and G_{18}xxxG_{22}-motifs in LcnG-α and LcnG-*β*, respectively, mutations in the G_{18}xxxG_{22}-motif in LcnG-α were relatively well-tolerated, causing only 1−17-fold reduction in activity (Table 1). Notably, replacing the glycine residues with large isoleucine residues reduced the activity only 2-fold (Table 1), clearly indicating that this GxxxG-motif is not important in helix−helix interactions.

Effects of Trp- and Tyr- Mutations. Studies on membrane proteins and model peptides have revealed that the aromatic residues tyrosine and tryptophan are normally found in the membrane interface (*39*−*42*). These residues may, however, also be found in the hydrophobic part of the membrane. To gain insight into how LcnG-α and LcnG-*β* position themselves in target membranes, tryptophan and tyrosine residues were replaced with either a hydrophobic amino acid residue (Leu), a positively-charged hydrophilic residue (Arg), or another aromatic residue (Trp, Tyr or Phe). A total of 12 versions of LcnG-α and 20 versions of LcnG-*β* were therefore constructed, and the activity of the peptide variants in combination with the complementary wild-type peptide was determined. Five different indicator organisms were used when determining the activity. The activity of the peptide variants combined with the complementary wild-type peptide were compared to the wild-type combination (LcnG-α + LcnG-*β*) and the results obtained when using *L. lactis* LL108 as indicator organism are shown in Table 2. Concurring results were obtained using the four other indicator organisms (*L. lactis* MG1363, *L. lactis* IL1403, *Lactococcus* LMGT-2077, and *Lactococcus* LMGT-2063), and the results are available as Supporting Information. The results obtained with these four strains revealed the same general changes in activity as the results obtained with the strain *L. lactis* LL108.

Replacing any of the three tryptophan residues in the N-terminal part in LcnG-*β* (Trp3, Trp5, and Trp8) with another aromatic residue (Tyr, Phe), a hydrophilic positively-charged residue (Arg) or a hydrophobic residue (Leu) had

Table 2: Relative MIC Values[a] of the Different Mutant peptides Combined with the Complementary Wild-Type Peptide Assayed against *L. lactis* LL108

		mutations with the corresponding relative MIC values				
peptide	position	Trp	Tyr	Phe	Leu	Arg
α	W3	1	2	1	3	53
	Y15	3	1	1	20	870
	W16	1	1	2	1	2
β	W3	1	1	1	2	2
	W5	1	1	1	2	2
	W8	1	1	2	1	2
	Y13	1	1	1	7	3
	W32	1	2	1	50	40

[a] The MIC values are given as relative values compared to the MIC value of the wild-type peptide combination (i.e., LcnG-α + LcnG-*β*, relative MIC value = 1, absolute MIC value = 0.07 nM). The MIC value is defined as the total amount of peptides (the sum of both peptide (in 1:1 ratio) concentrations) that inhibited the growth by 50%.

only a marginal effect on the antimicrobial activity (1−2-fold reduction; Table 2). Also the tyrosine at position 13 in LcnG-*β* tolerated all the mutations fairly well (only 1−7-fold reduction), with perhaps a somewhat higher tolerance for the aromatic residues (Table 2). The ability to tolerate aromatic, hydrophobic, and hydrophilic residues in the N-terminal region of LcnG-*β* suggests structural flexibility and that this region is probably positioned in the membrane interface. The tryptophan residue at position 32 in LcnG-*β* could also be replaced with another aromatic residue (Tyr, Phe) without a marked detrimental effect on the activity, whereas replacement with hydrophilic arginine or hydrophobic leucine residues was relatively detrimental, reducing the activity 40−50-fold (Table 2). The preference of aromatic tyrosine to hydrophobic leucine suggests localization in or near the membrane interface.

Substituting the tryptophan residue at position 3 in LcnG-α revealed that this position tolerated both another aromatic residue (Tyr, Phe) and a hydrophobic leucine residue but not a hydrophilic arginine residue, suggesting that the N-terminal part of LcnG-α may be embedded partly or completely in the hydrophobic part of the membrane. Replacing the tyrosine residue at position 15 in LcnG-α with arginine was very detrimental, causing more than an 800-fold reduction in activity (Table 2), indicating that this residue probably is not in a highly hydrophilic environment. Replacement with a hydrophobic residue (Leu) was less-detrimental, causing a 20-fold reduction in activity, whereas replacement with another aromatic residue (Trp, Phe) was tolerated (1−3-fold reduction in activity; Table 2). Taken together, the results suggest that the tyrosine residue may be in the interface, near the hydrophobic part of the membrane. The neighboring residue (Trp16) tolerated all replacements and was in this manner similar to the tryptophan residues in the N-terminal part of LcnG-*β*, thus, likewise suggesting structural flexibility and positioning in or near the membrane interface.

DISCUSSION

Glycine to alanine or serine substitutions in GxxxG-motifs involved in helix−helix interactions are expected to be tolerated because AxxxA- and SxxxS-motifs are in fact also involved in helix−helix interactions in some transmembrane proteins, although these two motifs do not allow as close contact between two helices as the more common GxxxG-motif (*37*). In contrast, substituting the glycine residues with large hydrophobic or hydrophilic residues will clearly interfere with close helix−helix contact and thereby interfere with helix−helix interactions. These substitutions are consequently expected to be quite detrimental. Activity measurements of the different peptide versions in combination with the complementary wild-type peptide indicated that the G_7xxxG_{11}-motif in LcnG-α and the $G_{18}xxxG_{22}$-motif in LcnG-*β* are involved in helix−helix interactions between the two peptides. Replacing the glycine residues at position 7 or 11 in LcnG-α with leucine, isoleucine, lysine, or glutamine residues reduced the activity dramatically, whereas replacements with alanine or serine residues were relatively well-tolerated (Table 1). Similarly, substituting glycine residues at positions 18 and 22 in LcnG-*β* with leucine, isoleucine, lysine, or glutamine residues were much more detrimental than replacements with alanine and serine residues (Table 1). The fact that both large hydrophobic and large hydrophilic residues were quite detrimental indicates that it is the size of the residue that is the major cause of the detrimental effect of the mutations. Also, the glycine to alanine and serine mutations in these two motifs led to a somewhat reduced activity (1−20-fold reduction compared to the wild-type combination), and this may reflect that these, although small residues, to a somewhat lesser extent than glycine, allow close contact between GxxxG-motifs. Notably, replacing the glycine residues in the middle of the G_7xxxG_{11}-motif in LcnG-α (i.e., Gly9) and the $G_{18}xxxG_{22}$-motif in LcnG-*β* (i.e., Gly20) with a large hydrophilic residue was tolerated, whereas replacement with a large hydrophobic residue was relatively detrimental, indicating that hydrophilicity rather than size was important for the residues that face away from the putative helix−helix interaction site.

All mutations in the $G_{18}xxxG_{22}$-motif in LcnG-α were relatively well-tolerated, indicating that this GxxxG-motif is not involved in helix−helix interactions. This was also expected because this GxxxG-motif is not conserved in the homologous two-peptide bacteriocin enterocin 1071 (Figure 1). The results are in accordance with a proposed structural model for lactococcin G (*33*) where the G_7xxxG_{11}-motif in LcnG-α and the $G_{18}xxxG_{22}$-motif in LcnG-*β* interact and allow the two peptides to form a parallel transmembrane helix−helix structure (Figure 2). This model is also consistent with earlier mutagenesis studies on lactococcin G and enterocin 1071 (*31*), showing that a negatively-charged residue in the N-terminus of the α-peptide (as in enterocin 1071; Figure 1) is functionally equivalent to a negatively-charged residue at position 10 in the *β*-peptide (as in lactococcin G and Q; Figure 1). The detrimental effect of removing the N-terminal negative charge in the α-peptide of enterocin 1071 was neutralized by introducing a negative charge at position 10 in the *β*-peptide (*31*). In the proposed structural model (Figure 2), these two residues (i.e., residue 1 in the α-peptide and residue 10 in the *β*-peptide) are located near each other, and this may explain why these two residues seem to be functionally equivalent.

Tryptophan and tyrosine residues in membrane-associated proteins position themselves preferentially in the membrane interface (*39*−*42*). The four aromatic residues (Trp3, Trp5, Trp8, and Tyr13) in the N-terminal part of LcnG-*β* could individually be replaced with other aromatic residues (Trp,

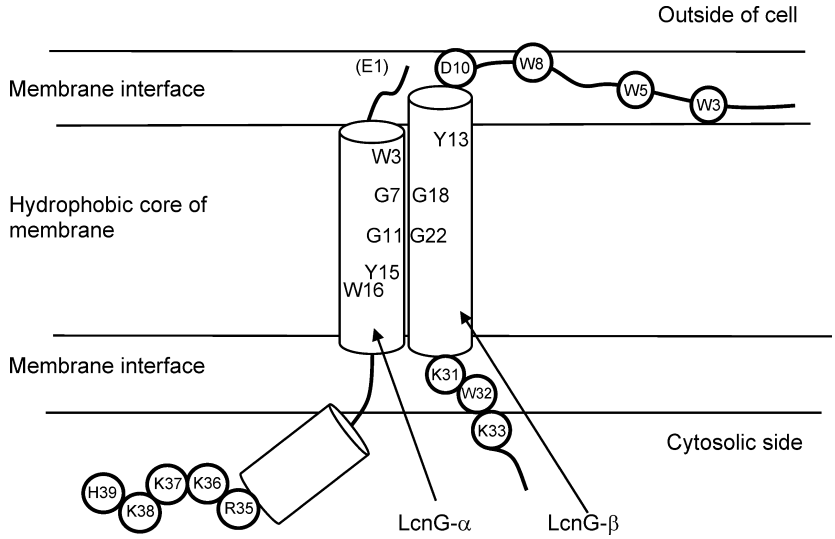

FIGURE 2: Structural model of lactococcin G and its orientation in target cell membranes. The two peptides (LcnG-α and LcnG-*β*) interact through the G_7xxxG_{11}-motif in LcnG-α and the $G_{18}xxxG_{22}$-motif in LcnG-*β* and form a transmembrane helix−helix structure. The highly positively-charged C-terminal end of LcnG-α is forced through the membrane by the transmembrane potential (negative inside). The model also places the functionally equivalent negatively-charged glutamic acid residue (shown as (E1)) in the α-peptide of enterocin 1071 and aspartic acid residue, D10, in the *β*-peptide of lactococcin G in close proximity to each other (see text for details). The tryptophan residues are according to the model placed in or near the membrane interfaces and are in agreement with the mutagenesis results.

Tyr, Phe), a hydrophilic positively-charged residue (Arg), or a hydrophobic residue (Leu) without a marked detrimental effect in the activity (Table 2). This remarkable tolerance for different types of residues in the N-terminal region of LcnG-*β* suggests that this region is structurally flexible and is not embedded in a strictly hydrophobic or hydrophilic environment. The results indicate that the region becomes positioned in the membrane interface, thus enabling the side chains of the hydrophilic and hydrophobic residues to "snorkel" into the hydrophilic and hydrophobic regions of the membrane, respectively (see reference (*40*) for "snorkel" effects).

These results, combined with the proposed structural model of lactococcin G and the striking clustering of three tryptophan residues among the first eight residues in LcnG-*β* and five positively-charged residues in the C-terminal end of LcnG-α, suggest that the cationic C-terminal end of LcnG-α is forced through the target cell membrane by the transmembrane potential (negative inside). The cationic C-terminus of LcnG-α becomes thereby positioned inside the cell, while the tryptophan-rich N-terminal region of LcnG-*β* becomes positioned in the outer membrane interface, and the helix−helix interacting region of LcnG-α and LcnG-*β* inserts as a transmembrane coil−coil segment (Figure 2). This orientation will position the tryptophan at position 3, the tyrosine at position 15, and the tryptophan at position 16 in LcnG-α in the hydrophobic part of the membrane. The tryptophan at position 3 will be near the outer membrane interface, and the tyrosine and tryptophan at positions 15 and 16, respectively, will be closer to the interface on the cytosolic side of the membrane. This is consistent with results showing that the tryptophan at position 3 could more readily be replaced with a hydrophobic leucine residue (3-fold reduction in activity) than with a hydrophilic arginine residue (53-fold reduction in activity), and that replacement of the tyrosine at position 15 with an arginine residue was highly detrimental (870-fold reduction in activity), while replacement with a leucine residue was tolerated to a greater extent (20-fold reduction in activity; Table 2). The tryptophan at position 16 in LcnG-α could, however, be replaced with an arginine residue, as well as with phenylalanine, tyrosine, and leucine residues, without a marked reduction in activity. In the proposed model, an arginine residue at positions 16 might be able to "snorkel" into the interface region, considering that its long side chain might enable its positively-charged group to reach 3−4 Å away from the bilayer center and into the hydrophilic membrane interface.

The fact that one of the three tryptophan residues in the N-terminal region of LcnG-*β* and one of the positively-charged lysine residues in the C-terminal end of LcnG-α may be removed (see ref (*31*) for the removal of the lysine), indicates that the residues do not interact in a structurally restricted manner (consistent with their proposed positioning in the membrane interface). Interestingly, also lactococcin Q and enterocin 1071 have tryptophan residues in the N-terminal region of their *β*-peptide (3 and 2 tryptophan residues, respectively; Figure 1) and cationic C-terminal residues in their α-peptide (5 and 4, respectively; Figure 1). It may thus be some degree of functional redundancy in having three N-terminal tryptophan residues and five C-terminal cationic residues, however, these residues may be involved in the initial interaction between the bacteriocin and the target membrane.

Recently, the individual three-dimensional structures of the two peptides that constitute the two-peptide bacteriocin plantaricin EF were determined by the use of NMR spectroscopy (Fimland et al., unpublished results). Both peptides were shown to adopt amphiphilic α-helices and it was proposed that they form a helix−helix structure involving the GxxxG-motifs that is also present in plantaricin EF. Indeed, GxxxG- or "GxxxG-like" motifs are present in all presently characterized peptides that are part of a two-peptide bacteriocin (Figure 1), suggesting that membrane-penetrating helix−helix structures formed by two peptides might be a common structure in most, if

not all, two-peptide bacteriocins. Such a structure might interact with an integrated membrane protein in an analogous fashion as seems to be the case for lactococcin A and the *pediocin-like* (class-IIa) bacteriocins (*53*). Lactococcin A and the *pediocin-like* bacteriocins bind (the latter probably via their helical region) to a part of the mannose phosphotransferase system that is embedded in the cell membrane. Helical interactions between peptide bacteriocins and integrated membrane (transport) proteins might thus be a common mechanism, whereby peptide bacteriocins cause membrane-leakage in target cells.

SUPPORTING INFORMATION AVAILABLE

Relative MIC values of different peptide variants (both GxxxG- and Trp/Tyr-mutations) combined with the complementary wild-type peptide assayed against various indicator organisms. This material is available free of charge via the Internet at http://pubs.acs.org.

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