Structure analysis of the two-peptide bacteriocin lactococcin G by introducing D-amino acid residues

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The importance of 3D structuring in the N- and C-terminal ends of the two peptides (39-mer LcnG- α and 35-mer LcnG- β) that constitute the two-peptide bacteriocin lactococcin G was analysed by replacing residues in the end regions with the corresponding D-isomeric residues. When assayed for antibacterial activity in combination with the complementary wild-type peptide, LcnG- α with four D-residues in its C-terminal region and LcnG- β with four D-residues in either its N- or its C-terminal region were relatively active (two- to 20-fold reduction in activity). 3D structuring of the C-terminal region in LcnG- α and the C- and N-terminal regions in LcnG- β is thus not particularly critical for retaining antibacterial activity, indicating that the 3D structure of these regions is not vital for interpeptide interactions or for interactions between the peptides and cellular components. The 3D structure of the N-terminal region in LcnG- α may be more important, as LcnG- α with four N-terminal D-residues was the least active of these four peptides (10- to 100-fold reduction in activity). The results are consistent with a proposed structural model of lactococcin G in which LcnG- α and - β form a transmembrane parallel helix–helix structure involving approximately 20 residues in each peptide, starting near the N terminus of LcnG- α and at about residue 13 in LcnG- β . Upon expressing the lactococcin G immunity protein, sensitive target cells became resistant to all of these D-residue-containing peptides. The end regions of the two lactococcin G peptides are consequently not involved in essential structure-dependent interactions with the immunity protein. The relatively high activity of most of the D-residue-containing peptides suggests that bacteriocins with increased resistance to exopeptidases may be generated by replacing their N- and C-terminal residues with D-residues.

Received 22 January 2010
Revised 24 February 2010
Accepted 28 February 2010

INTRODUCTION

Lactococcin G is a ribosomally synthesized unmodified two-peptide (class IIb) bacteriocin (antimicrobial peptide) produced by some lactococci strains (Nissen-Meyer *et al.*, 1992). It was the first two-peptide bacteriocin to be isolated and is perhaps the one that at present is best characterized (Hauge *et al.*, 1998; Moll *et al.*, 1996, 1998; Nissen-Meyer *et al.*, 1992; Oppegård *et al.*, 2007, 2008; Rogne *et al.*, 2008). The bacteriocin consists of two peptides, LcnG- α (39 amino acid residues) and LcnG- β (35 amino acid residues) (Fig. 1), and both peptides must be present in about equal molar amounts in order to obtain optimal antimicrobial activity (Moll *et al.*, 1996; Nissen-Meyer *et al.*, 1992). The genes encoding the two peptides are located next to each other on the same operon, along with the gene encoding the lactococcin G immunity protein (Håvarstein *et al.*, 1995; Nes *et al.*, 1995), which protects the producer cell

from being killed by its own bacteriocin. The bacteriocin causes cell death by inducing membrane leakage of small monovalent cations, such as Na⁺, K⁺, Li⁺, Cs⁺ and Rb⁺, but not H⁺ (Moll *et al.*, 1996, 1998). Circular dichroism (CD) and NMR spectroscopy show that the two peptides, LcnG- α and LcnG- β , are unstructured in aqueous environments, but adopt mainly α -helical structures when they are individually exposed to membranes or membrane-mimicking environments (Hauge *et al.*, 1998; Rogne *et al.*, 2008). Moreover, when the two peptides together are exposed to membrane-like entities such as liposomes, additional α -helical structuring is attained, indicating that the two peptides interact and form an active complex when exposed to membranes (Hauge *et al.*, 1998).

Based on the NMR structures of the individual lactococcin G peptides (Rogne *et al.*, 2008) and site-directed mutagenesis results (Oppegård *et al.*, 2007, 2008), a model of the active complex has been proposed (Nissen-Meyer *et al.*, 2009; Oppegård *et al.*, 2008; Rogne *et al.*, 2008) (Fig. 2). The model proposes that the two peptides form a parallel helix–helix structure that spans the target-cell membrane.

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Abbreviation: man-PTS, mannose phosphotransferase.

	1	10	20	30	39
LcnG- α	G	TWDDIGQ	GIGRVAYWVGK	AMGNMSDVNQ	ASRINRKKKH
LcnG- β	K	KKGWLA	WVDPAYEF	IKGFGK	GAIKEGNKDKWKNI

Fig. 1. Amino acid sequences of the two peptides, LcnG- α and LcnG- β , constituting the two-peptide bacteriocin lactococcin G. LcnG- α consists of 39 residues and LcnG- β consists of 35 residues.

The helix-helix segment consists of the N-terminal half of LcnG- α (from about Trp-3 to Gly-22) and the C-terminal half of LcnG- β (from about Tyr-13 to Trp-32). The model also entails that the cationic C-terminal end (residues 35–39: R-K-K-K-H) of LcnG- α is relatively unstructured and forced through the target-cell membrane by the membrane potential, thereby positioning the C-terminal ends of the two peptides inside the target cell (Fig. 2). The tryptophan-rich N-terminal end of LcnG- β is proposed to be relatively unstructured and to position itself in the outer membrane interface, thus forcing the N-terminal ends 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). It should be noted that the proposed model is based on the NMR structures of the individual peptides, as there is at present no NMR structural data that reveal how the two peptides interact and structure each other. It is consequently possible that the apparently unstructured cationic C- and tryptophan-rich N-terminal ends of, respectively, LcnG- α and LcnG- β become structured when the two peptides interact with each other upon contact with target membranes. To determine whether there is in fact a high degree of local structural flexibility in these end regions as the model proposes, the activity of LcnG- α and - β containing D-amino acid residues in these regions has been analysed. In addition, the peptides containing D-isomeric residues were assayed against strains expressing the lactococcin G immunity protein to determine whether a structuring in these regions is important for recognition of lactococcin G by its immunity protein.

METHODS

Bacterial strains and growth conditions. For production of the wild-type lactococcin G peptides, *Lactobacillus sake* Lb790 containing the heterologous two-plasmid expression system pSAK20/pLT100 (Axelsson & Holck, 1995; Axelsson *et al.*, 1998; Oppegård *et al.*, 2007) was grown without agitation at 30 °C in MRS (de Man, Rogosa and Sharpe) broth (Oxoid). Chloramphenicol and erythromycin were added, each to a final concentration of 10 µg ml⁻¹, since pSAK20 and pLT100 contain resistance markers for chloramphenicol and erythromycin, respectively. The indicator organisms used in the bacteriocin activity assays were *Lactococcus* sp. LMGT-2077, *Lactococcus lactis* IL1403 and *L. lactis* MG1363. All strains were grown without agitation at 30 °C in M17 medium (Oxoid) supplemented with glucose and Tween 80 to final concentrations of 0.4% (w/v) and 0.1% (v/v), respectively. The supplemented M17 medium is referred to as GM17. When the indicator strains *L. lactis* MG1363 and *Lactococcus* sp. LMGT-2077 contained the unmodified pMG36e plasmid (van de Guchte *et al.*, 1989) or the pLcnGim expression plasmid (Oppegård *et al.*, 2010), erythromycin was added to the growth media to a final concentration of 2 µg ml⁻¹ to ensure that the plasmids remained in the cells.

Production and purification of peptides. The wild-type lactococcin G peptides were produced by a heterologous expression system (Axelsson & Holck, 1995; Axelsson *et al.*, 1998; Oppegård *et al.*, 2007) and purified using a two-step purification method as previously described (Oppegård *et al.*, 2007; Uteng *et al.*, 2002). The six peptides containing D-amino acid residues were synthesized and purified to more than 90% purity by GenScript (NJ, USA). Two modified α -peptides, termed α -D4 and D4- α , were synthesized. α -D4 contained four D-amino acid residues in the C-terminal end: Lys-36, Lys-37, Lys-38 and His-39 (Fig. 1). In D4- α , four of the five N-terminal amino acid residues (Thr-2, Trp-3, Asp-4 and Asp-5) were

Fig. 2. Proposed model of lactococcin G. The two peptides (LcnG- α and LcnG- β) interact and form a transmembrane helix-helix structure. The tryptophan-rich N-terminal end of LcnG- β and the cationic C-terminal end of LcnG- α are proposed to be relatively flexible. The residues in the proposed flexible regions that were exchanged with the corresponding D-amino acid residues are marked with circles and their one-letter amino acid code. The glycine residues in positions 1 and 4 in LcnG- α and - β , respectively, are non-chiral and were consequently not replaced.

replaced with the corresponding D-residues (Gly-1 is not chiral). Four modified β -peptides were synthesized: D3- β , D4- β , D7- β and β -D4. In D3- β , the three N-terminal amino acid residues (Lys-1, Lys-2 and Trp-3) were replaced with the corresponding D-residues. In D4- β , four of the five N-terminal amino acid residues (Lys-1, Lys-2, Trp-3 and Trp-5) were replaced with the corresponding D-residues (Gly-4 is not chiral). D7- β contained seven D-residues among the eight N-terminal residues (Lys-1, Lys-2, Trp-3, Trp-5, Leu-6, Ala-7 and Trp-8) (Fig. 1). In β -D4, the four C-terminal amino acid residues (Trp-32, Lys-33, Asn-34 and Ile-35) were replaced with the corresponding D-residues.

The concentrations of 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.

Antimicrobial activity assay. The antimicrobial activity of the different peptides was tested using a 96-well microtitre plate-based activity assay, as previously described (Nissen-Meyer *et al.*, 1992; Oppegård *et al.*, 2007). Briefly, each well in a 96-well microtitre plate contained 200 μ l GM17 media with an indicator strain and twofold dilutions of the combination of peptides being tested. Overnight cultures of the indicator strains were diluted 1:50 before being added to the assay. The plates were then incubated for approximately 5 h at 30 °C before growth inhibition was determined by measuring the cell density spectrophotometrically at 600 nm (Microplate Reader, TECAN). The MIC value was defined as the total amount of peptides [the sum of both peptide (in a 1:1 ratio) concentrations] that inhibited growth by 50%. The reported MIC values are the mean of at least three independent measurements, unless stated otherwise. Inhibition of the antimicrobial activity caused by D7- β was determined by measuring the activity of wild-type LcnG- α and LcnG- β (in a 1:1 molar ratio) together with different amounts of D7- β . The degree of protection conferred by the lactococcin G immunity protein was evaluated by comparing the MIC values obtained with an indicator strain expressing the immunity protein with the MIC values obtained with the same strain containing the unmodified pMG36e plasmid.

RESULTS AND DISCUSSION

The recently proposed structural model of lactococcin G entails that the C- and N-terminal ends of LcnG- α and - β , respectively, are relatively unstructured (Nissen-Meyer *et al.*, 2009; Oppegård *et al.*, 2008; Rogne *et al.*, 2008). To analyse whether the end regions of LcnG- α and - β are in fact structurally flexible, LcnG- α and - β containing D-amino acid residues in the C- and N-terminal ends were synthesized and the antimicrobial activity of these D-amino acid-containing peptides was determined in combination with the complementary wild-type peptide. Replacement of L- with D-amino acid residues in a region of a peptide will clearly interfere with any 3D structure-dependent (chiral) interactions involving the region and should consequently lead to reduced antimicrobial activity.

Replacement of N- and C-terminal residues in LcnG- α with D-residues

For LcnG- α , replacement of the four C-terminal cationic residues (Lys-36, Lys-37, Lys-38 and His-39) with the corresponding D-residues was tolerated relatively well [three- to ninefold activity reduction (i.e. relative MICs of 3–9), depending on the indicator strain; Table 1]. Consequently, the four C-terminal residues are apparently not involved in vital 3D structure-dependent interactions. The lack of an apparent 3D structure in these C-terminal residues is consistent with the NMR structure of LcnG- α (determined in the absence of LcnG- β), where the C-terminal part, from Arg-35 onwards, is unstructured. Moreover, the results indicate that this C-terminal stretch also remains unstructured after LcnG- α interacts with LcnG- β , with the formation of the active complex. The

Table 1. MIC values of the wild-type LcnG- α +LcnG- β combination and of the different D-amino acid-containing peptides in combination with the complementary wild-type peptide

Peptide combination	MIC value (nM)*			Relative MIC†		
	<i>Lactococcus</i> sp. LMGT-2077	<i>L. lactis</i> MG1363	<i>L. lactis</i> IL1403	<i>Lactococcus</i> sp. LMGT-2077	<i>L. lactis</i> MG1363	<i>L. lactis</i> IL1403
$\alpha + \beta$	0.2 $\pm$ 0.1	0.14 $\pm$ 0.04	0.04 $\pm$ 0.01	1	1	1
α -D4 + β	1.7 $\pm$ 0.8	0.5 $\pm$ 0.1	0.12 $\pm$ 0.02	8–9	4	3
D4- α + β	22 $\pm$ 7	1.4 $\pm$ 0.4	0.93 $\pm$ 0.06	110	10	23
α + D3- β	0.4 $\pm$ 0.2	0.21 $\pm$ 0.05	0.09 $\pm$ 0.01	2	1–2	2
α + D4- β	1.0 $\pm$ 0.3	0.27 $\pm$ 0.05	0.17 $\pm$ 0.04	5	2	4
α + D7- β	2400 $\pm$ 100	1500 $\pm$ 400	600 $\pm$ 400	12 000	11 000	15 000
α + β -D4	4.4 $\pm$ 2.3	0.68 $\pm$ 0.08	0.6 $\pm$ 0.1	22	5	15

*The MIC value was defined as the total amount of peptides [the sum of both peptide (in a 1:1 ratio) concentrations] that inhibited growth by 50%. All MIC values are given as the mean $\pm$ SD of at least three independent measurements. Both the LMGT-2077 and MG1363 strains contained the unmodified pMG36e plasmid (van de Guchte *et al.*, 1989). Similar MIC values were obtained for strains containing the pMG36e plasmid and for strains without the plasmid. All peptide combinations were assayed against three indicator strains.

†Relative MIC was defined as the MIC value obtained for the peptide combination containing D-residues divided by the MIC value obtained for the wild-type combination.

results are in agreement with the model of the active complex (Fig. 2), which entails that the highly cationic C-terminal stretch of LcnG- α is unstructured and not involved in interactions with LcnG- β . According to the model, the main function of the C-terminal stretch of LcnG- α is to force, by use of the transmembrane potential, the C-terminal ends of the active complex through the target-cell membrane, and thereby insert the helix-helix segment in a transmembrane position (Fig. 2). Replacement of four of the five N-terminal residues in LcnG- α (Thr-2, Trp-3, Asp-4 and Asp-5) with the corresponding D-residues was more detrimental (10- to 110-fold reduction in antimicrobial activity, depending on the indicator strain; Table 1) than replacements in the C-terminal end. Introducing D-residues at the beginning of LcnG- α thus seems to interfere with some 3D structure-dependent interactions. This is consistent with the structural model, since it proposes that the helix-helix interaction between LcnG- α and - β starts near residue 3 in LcnG- α (Nissen-Meyer *et al.*, 2009; Oppegård *et al.*, 2008; Rogne *et al.*, 2008).

Replacement of N- and C-terminal residues in LcnG- β with D-residues

For LcnG- β , replacement of the first three (in the D3- β peptide) or four of the first five (in the D4- β peptide) N-terminal residues with the corresponding D-residues was tolerated relatively well, causing only a two- to fivefold reduction in antimicrobial activity (i.e. relative MICs of 2–5; Table 1). Replacement of the four C-terminal residues (Trp-32, Lys-33, Asn-34 and Ile-35) with the corresponding D-residues was somewhat more detrimental, causing a five- to 22-fold reduction, depending on the indicator strain (relative MICs of 5–22; Table 1). It thus appears that the first five residues in LcnG- β are not involved in 3D structure-dependent interactions of great importance for the antimicrobial activity of lactococcin G. Consistent with the proposed structural model, these residues are consequently involved neither in interactions between LcnG- α and LcnG- β nor in 3D structural interactions with cellular components. The slightly more detrimental effect of replacing the four C-terminal residues, residues 32–35, with the corresponding D-residues could be due to some interference with the helix-helix structure, which, according to the structural model, is thought to start, going from the C-terminal end, at about residue 32 in LcnG- β . The relatively high activity of most of the D-residue-containing peptides suggests that bacteriocins with increased resistance to exopeptidases may be generated by replacing their N- and C-terminal residues with the corresponding D-residues. Several studies have shown that peptides become increasingly resistant to proteolytic degradation upon introducing D-amino acid residues in the C- and/or N-terminal ends (Donermeyer & Allen, 1989; Powell *et al.*, 1993; Tugyi *et al.*, 2005).

The peptide (D7- β) in which seven of the first eight amino acid residues in LcnG- β were replaced with the

corresponding D-amino acid residues had drastically reduced antimicrobial activity when combined with LcnG- α (11 000- to 15 000-fold reduction in activity, depending on the indicator strain; Table 1). Adding D-residues in positions 6, 7 and 8, as well as in positions 1, 2, 3 and 5 in LcnG- β , thus clearly causes structural perturbations that are detrimental to the function of lactococcin G, whereas having D-residues in only four of the first five positions is well tolerated.

According to the proposed structure (Fig. 2), the tryptophan-rich N-terminal region (Lys-1 to Trp-8) of LcnG- β is not directly involved in the interaction with LcnG- α , and one might thus speculate that the D7- β peptide could function as a competitive inhibitor of LcnG- β . This was tested by measuring the antimicrobial activity of the wild-type LcnG- α + LcnG- β combination (in a 1 : 1 molar ratio) together with an excess of D7- β . A 20-fold excess of D7- β compared with LcnG- β reduced the antimicrobial activity seven- to 20-fold, and a 100-fold excess reduced the antimicrobial activity 40- to 70-fold (depending on the indicator strain; Table 2). One interpretation of the effective inhibition (i.e. reduction in activity) observed when adding an excess of D7- β to the wild-type combination is that D7- β efficiently competes with wild-type LcnG- β in binding to LcnG- α , which in turn suggests that the N-terminal part (Lys-1 to Trp-8) of LcnG- β is not directly involved in interactions with LcnG- α , consistent with the model presented in Fig. 2.

Activity of combinations of D-residue-containing peptides

The antimicrobial activity of all eight possible combinations of the different D-amino acid-containing peptides was also determined (Table 3). Two of the combinations (α -D4 + D7- β and D4- α + D7- β) are not included in Table 3, since their antimicrobial activities were so low that they

Table 2. The D7- β peptide inhibits the activity of the wild-type lactococcin G combination (i.e. LcnG- α + LcnG- β)

Ratio D7- β : LcnG- β	Relative MIC*		
	<i>Lactococcus</i> sp. LMGT-2077	<i>L. lactis</i> MG1363	<i>L. lactis</i> IL1403
4 : 1	2	1	2
20 : 1	20	7	7
100 : 1	40	40	70

*The relative MIC value was defined as the MIC value obtained for the wild-type combination together with an excess of D7- β divided by the MIC value for the wild-type combination (without D7-LcnG- β present). The two complementary wild-type peptides were added in a 1 : 1 molar ratio. All standard deviations for the measured MIC values were below 50 %.

could not be accurately determined (~100 000-fold or more reduction in activity compared with the LcnG- α +LcnG- β wild-type combination). The relative MIC values (i.e. the fold reduction in antimicrobial activity compared with the LcnG- α +LcnG- β wild-type combination) obtained for four of the combinations (α -D4+D3- β , α -D4+D4- β , α -D4+ β -D4 and D4- α +D3- β) were as one might expect. This was because their relative MIC values were similar to the product of the relative MIC values that were obtained when the D-residue-containing peptides were individually assayed together with the complementary wild-type peptide. For instance, the relative MICs for the α -D4+D3- β combination, when assayed against the LMGT-2077, MG1363 and IL1403 strains, were 20, 7 and 5, respectively, whereas the product of the relative MIC values for the α -D4+LcnG- β and LcnG- α +D3- β combinations were 18, 8 and 6, respectively (Table 3). For the two combinations D4- α +D4- β and D4- α + β -D4, the antimicrobial activity was, however, much lower (in some cases more than 10-fold lower) than expected from the activity obtained with individual D-residue-containing peptides in combination with the complementary wild-type peptide (Table 3). For instance, the relative MICs for the D4- α +D4- β combination when assayed against the LMGT-2077, MG1363 and IL1403 strains were >10 000, 200 and 200, respectively, whereas the products of the relative MIC values for the D4- α +LcnG- β and LcnG- α +D4- β combinations were 550, 20 and 92, respectively (Table 3). This suggests that the D-residue-containing regions interfere negatively with each other when D4- α is combined with either β -D4 or D4- β . Interestingly, D4- α and β -D4 are the only peptides that have D-residues in or close to regions which, according to the proposed structure, are involved in interpeptide helix-helix interactions (Fig. 2).

The end regions of LcnG- α and - β are not involved in structural interactions with the immunity protein

To determine whether the C- and N-terminal regions of LcnG- α and - β are involved in any direct or indirect essential structure-dependent interaction with the lactococcin G immunity protein, the antimicrobial activity of the peptides containing D-residues was assayed against two lactococci strains expressing the lactococcin G immunity protein. Of the six different peptide combinations tested, the α +D7- β combination could not be evaluated because of its low antimicrobial activity, and the combination is consequently not included in Table 4. For the five remaining combinations, the MIC values increased 100- to 1000-fold when the target cells expressed the lactococcin G immunity protein (Tables 1 and 4). Moreover, the MIC values obtained with combinations with D-residue-containing peptides were always equal to or higher than the MIC value obtained with the wild-type combination (Table 4). The results thus clearly show that the end regions of the two lactococcin G peptides are not involved in any essential direct or indirect structure-dependent interactions between lactococcin G and its immunity protein.

It has been shown that the pediocin-like bacteriocins and the one-peptide bacteriocin lactococcin A bind to a part of the mannose phosphotransferase (man-PTS) system that is embedded in the cell membrane, thereby apparently structurally perturbing the transferase in a manner that leads to membrane leakage (Diep *et al.*, 2007). It has also been shown that the cognate immunity protein recognizes and binds to the bacteriocin-man-PTS complex, and thereby prevents bacteriocin-induced cell death. It has been speculated that lactococcin G also induces cell death

Table 3. MIC values of all the different combinations of D-amino acid-containing peptides assayed against three indicator strains

Peptide combination	MIC value (nM)*			Relative MIC†		
	<i>Lactococcus</i> sp. LMGT-2077	<i>L. lactis</i> MG1363	<i>L. lactis</i> IL1403	<i>Lactococcus</i> sp. LMGT-2077	<i>L. lactis</i> MG1363	<i>L. lactis</i> IL1403
α -D4+D3- β	4.0±0.8	1.0±0.3	0.2±0.1	20 (18)	7 (8)	5 (6)
α -D4+D4- β	7±3	1.4±0.2	0.5±0.1	35 (45)	10 (8)	13 (12)
α -D4+ β -D4	44±11	12±1	3.1±0.3	220 (198)	86 (20)	78 (45)
D4- α +D3- β	30±15	1.9±0.9	1.5±0.1	150 (220)	14 (20)	38 (48)
D4- α +D4- β	≥2000‡	29±5	8±2	>10 000 (550)	200 (20)	200 (92)
D4- α + β -D4	≥1000‡	140±70	170±80	>5000 (2420)	1000 (50)	4300 (345)

*The MIC value was defined as the total amount of peptides [the sum of both peptide (in a 1:1 ratio) concentrations] that inhibited growth by 50%. MIC values are given as the mean±SD of at least three independent measurements. The two strains LMGT-2077 and MG1363 contained the unmodified pMG36e plasmid (see also Table 1, first footnote). All peptide combinations were assayed against three indicator strains.

†Relative MIC was defined as the MIC value obtained for the peptide combination containing D-residues divided by the MIC value obtained for the wild-type combination. Relative MIC values in parentheses are the product of the relative MIC values obtained when assaying the individual D-amino acid variant with the complementary wild-type peptide (Table 1).

‡For these combinations, two independent activity measurements were performed and the MIC value was set to be equal to or higher than the lowest obtained MIC value.

Table 4. MIC values of the wild-type LcnG- α +LcnG- β combination and of the different D-amino acid-containing peptides in combination with the complementary wild-type peptide

Peptide combination	MIC value (nM)*	
	<i>Lactococcus</i> sp. LMGT-2077/pLcnGim	<i>L. lactis</i> MG1363/pLcnGim
$\alpha + \beta$	800 $\pm$ 400	100 $\pm$ 50
α -D4 + β	1600 $\pm$ 400	180 $\pm$ 80
D4- α + β	1700 $\pm$ 800	>500
α + D3- β	1700 $\pm$ 800	300 $\pm$ 300
α + D4- β	1800 $\pm$ 500	1400 $\pm$ 800
α + β -D4	1500 $\pm$ 400	300 $\pm$ 200

*The MIC value was defined as the total amount of peptides [the sum of both peptide (in 1:1 ratio) concentrations] that inhibited growth by 50%. MIC values are given as the mean $\pm$ SD of at least three independent measurements. For evaluating the fold protection, compare MIC values with the corresponding MIC values (Table 1) obtained with these strains without the expression of the immunity protein (i.e. cells with only the unmodified pMG36e plasmid). All peptide combinations were assayed against two indicator strains expressing the lactococcin G immunity protein.

by binding to a membrane-associated receptor and that the lactococcin G immunity protein protects cells by binding to the bacteriocin–receptor complex (Oppegård *et al.*, 2008). Indeed, recent studies with the lactococcin G immunity protein have shown that its functionality depends on a cellular component/receptor (Oppegård *et al.*, 2010). Moreover, it has been shown that regions in the N-terminal end of LcnG- α (residues 1–13) and the mid part of LcnG- β (residues 14–24) are especially important for the recognition of lactococcin G by its immunity protein (Oppegård *et al.*, 2010). The fact that the immunity protein protected cells efficiently against the D-residue-containing peptides indicates that the first five and last four residues in LcnG- α and - β are not vital for the recognition of lactococcin G by its immunity protein. Taken together, the results suggest that regions between about residues 5 and 13 in LcnG- α and between residues 14 and 24 in LcnG- β , which according to the structural model would be in the N-terminal half of the helix–helix segment (Fig. 2), are essential for the recognition of lactococcin G by its immunity protein.

A previous site-directed mutagenesis study revealed that residues in the C-terminal parts of LcnG- α and - β (Gly-20 to His-39, and Lys-25 to Ile-35, respectively) do not influence the target-cell specificity of lactococcin G and are thus not important for receptor recognition (Oppegård *et al.*, 2007). This, combined with the fact that D4- β was relatively active (together with LcnG- α) and that site-directed mutagenesis studies show that the region covering residues 1–8 in LcnG- β is structurally flexible (Oppegård

et al., 2007), suggests that the receptor-interacting region in lactococcin G is also in the N-terminal part of the helix–helix segment. The residues in lactococcin G that are recognized by the receptor and the immunity protein might thus be one and the same, as seems to be the case for lactococcin A and the pediocin-like bacteriocins. The immunity proteins for lactococcin A and the pediocin-like bacteriocins apparently recognize structural perturbations induced in their receptor (i.e. the man-PTS) upon binding of the bacteriocin to the receptor, and this in turn leads to binding of the immunity protein to the bacteriocin–receptor complex (Diep *et al.*, 2007). The recognition of these bacteriocins by their immunity proteins appears therefore to be indirect (by way of the bacteriocin receptor), and the regions in these bacteriocins that are recognized by their receptor and their immunity proteins are thus apparently one and the same.

ACKNOWLEDGEMENTS

This work was supported by EMBIO, steering board for research in molecular biology, biotechnology, and bioinformatics at the University of Oslo, and by the Norwegian Research Council.

REFERENCES

- Axelsson, L. & Holck, A. (1995). The genes involved in production of and immunity to sakacin A, a bacteriocin from *Lactobacillus sake* Lb706. *J Bacteriol* **177**, 2125–2137.
- Axelsson, L., Katla, T., Bjørnslett, M., Eijsink, V. G. & Holck, A. (1998). A system for heterologous expression of bacteriocins in *Lactobacillus sake*. *FEMS Microbiol Lett* **168**, 137–143.
- Diep, D. B., Skaugen, M., Salehian, Z., Holo, H. & Nes, I. F. (2007). Common mechanisms of target cell recognition and immunity for class II bacteriocins. *Proc Natl Acad Sci U S A* **104**, 2384–2389.
- Donermeyer, D. L. & Allen, P. M. (1989). Binding to Ia protects an immunogenic peptide from proteolytic degradation. *J Immunol* **142**, 1063–1068.
- Hauge, H. H., Nissen-Meyer, J., Nes, I. F. & Eijsink, V. G. (1998). Amphiphilic α -helices are important structural motifs in the α and β peptides that constitute the bacteriocin lactococcin G – enhancement of helix formation upon α – β interaction. *Eur J Biochem* **251**, 565–572.
- Håvarstein, L. S., Diep, D. B. & Nes, I. F. (1995). A family of bacteriocin ABC transporters carry out proteolytic processing of their substrates concomitant with export. *Mol Microbiol* **16**, 229–240.
- Moll, G., Ubbink-Kok, T., Hildeng, H. H., Nissen-Meyer, J., Nes, I. F., Konings, W. N. & Driessen, A. J. (1996). Lactococcin G is a potassium ion-conducting, two-component bacteriocin. *J Bacteriol* **178**, 600–605.
- Moll, G., Hildeng, H. H., Nissen-Meyer, J., Nes, I. F., Konings, W. N. & Driessen, A. J. (1998). Mechanistic properties of the two-component bacteriocin lactococcin G. *J Bacteriol* **180**, 96–99.
- Nes, I. F., Håvarstein, L. S. & Holo, H. (1995). Genetics of non-lantibiotic bacteriocins. *Dev Biol Stand* **85**, 645–651.
- Nissen-Meyer, J., Holo, H., Håvarstein, L. S., Sletten, K. & Nes, I. F. (1992). A novel lactococcal bacteriocin whose activity depends on the complementary action of two peptides. *J Bacteriol* **174**, 5686–5692.

- Nissen-Meyer, J., Rogne, P., Oppegård, C., Haugen, H. S. & Kristiansen, P. E. (2009). Structure–function relationships of the non-lanthionine-containing peptide (class II) bacteriocins produced by Gram-positive bacteria. *Curr Pharm Biotechnol* **10**, 19–37.
- Oppegård, C., Fimland, G., Thorbek, L. & Nissen-Meyer, J. (2007). Analysis of the two-peptide bacteriocins lactococcin G and enterocin 1071 by site-directed mutagenesis. *Appl Environ Microbiol* **73**, 2931–2938.
- Oppegård, C., Schmidt, J., Kristiansen, P. E. & Nissen-Meyer, J. (2008). Mutational analysis of putative helix–helix interacting GxxxG-motifs and tryptophan residues in the two-peptide bacteriocin lactococcin G. *Biochemistry* **47**, 5242–5249.
- Oppegård, C., Emanuelsen, L., Thorbek, L., Fimland, G. & Nissen-Meyer, J. (2010). The lactococcin G immunity protein recognizes specific regions in both peptides constituting the two-peptide bacteriocin lactococcin G. *Appl Environ Microbiol* **76**, 1267–1273.
- Powell, M. F., Stewart, T., Otvos, L., Jr, Urge, L., Gaeta, F. C., Sette, A., Arrhenius, T., Thomson, D., Soda, K. & Colon, S. M. (1993). Peptide stability in drug development. II. Effect of single amino acid substitution and glycosylation on peptide reactivity in human serum. *Pharm Res* **10**, 1268–1273.
- Rogne, P., Fimland, G., Nissen-Meyer, J. & Kristiansen, P. E. (2008). Three-dimensional structure of the two peptides that constitute the two-peptide bacteriocin lactococcin G. *Biochim Biophys Acta* **1784**, 543–554.
- Tugyi, R., Uray, K., Ivan, D., Fellingner, E., Perkins, A. & Hudecz, F. (2005). Partial D-amino acid substitution: improved enzymatic stability and preserved Ab recognition of a MUC2 epitope peptide. *Proc Natl Acad Sci U S A* **102**, 413–418.
- Uteng, M., Hauge, H. H., Brondz, I., Nissen-Meyer, J. & Fimland, G. (2002). Rapid two-step procedure for large-scale purification of pediocin-like bacteriocins and other cationic antimicrobial peptides from complex culture medium. *Appl Environ Microbiol* **68**, 952–956.
- van de Guchte, M., van der Vossen, J. M., Kok, J. & Venema, G. (1989). Construction of a lactococcal expression vector: expression of hen egg white lysozyme in *Lactococcus lactis* subsp. *lactis*. *Appl Environ Microbiol* **55**, 224–228.

Edited by: V. Eijsink