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# A Novel Lactococcal Bacteriocin Whose Activity Depends on the Complementary Action of Two Peptides

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**A lactococcal bacteriocin, termed lactococcin G, was purified to homogeneity by a simple four-step purification procedure that includes ammonium sulfate precipitation, binding to a cation exchanger and octyl-Sepharose CL-4B, and reverse-phase chromatography. The final yield was about 20%, and nearly a 7,000-fold increase in the specific activity was obtained. The bacteriocin activity was associated with three peptides, termed $\alpha_1$, $\alpha_2$, and β, which were separated by reverse-phase chromatography. Judging from their amino acid sequences, $\alpha_1$ and $\alpha_2$ were the same gene product. Differences in their configurations presumably resulted in $\alpha_2$ having a slightly lower affinity for the reverse-phase column than $\alpha_1$ and a reduced bacteriocin activity when combined with β. Bacteriocin activity required the complementary action of both the α and the β peptides. When neither $\alpha_1$ nor β was in excess, about 0.3 nM $\alpha_1$ and 0.04 nM β induced 50% growth inhibition, suggesting that they might interact in a 7:1 or 8:1 ratio. As judged by the amino acid sequence, $\alpha_1$ has an isoelectric point of 10.9, an extinction coefficient of $1.3 \times 10^4$ $M^{-1}$ $cm^{-1}$, and a molecular weight of 4,346 (39 amino acid residues long). Similarly, β has an isoelectric point of 10.4, an extinction coefficient of $2.4 \times 10^4$ $M^{-1}$ $cm^{-1}$, and a molecular weight of 4110 (35 amino acid residues long). Molecular weights of 4,376 and 4,109 for $\alpha_1$ and β, respectively, were obtained by mass spectrometry. The N-terminal halves of both the α and the β peptides may form amphiphilic α-helices, suggesting that the peptides are pore-forming toxins that create cell membrane channels through a "barrel-stave" mechanism. The C-terminal halves of both peptides consist largely of polar amino acids.**

Bacteriocins are proteins that show bactericidal activity towards bacteria that are closely related to the bacteriocin-producing species (25). Because of their potential use as antibacterial agents, bacteriocins have been the subject of much research. In recent years, there has been considerable interest especially in bacteriocins from lactic acid bacteria (LAB), because of their potential as food and feed additives.

The LAB bacteriocins appear to be structurally quite different from the colicins of *Escherichia coli* (7, 14). LAB bacteriocins are usually small peptides, seldom containing more than 60 amino acids, while colicins are proteins of 300 to 800 amino acids. On the basis of their structure, LAB bacteriocins may be divided into two groups. The first group contains the so-called lantibiotics, which have been known for a long time (6, 11). Lantibiotics consist of a polypeptide chain that has been posttranslationally modified. The modified amino acids are lanthionine and methyllanthionine and their precursors dehydroalanine and dehydrobutyrine. Among the lantibiotics, nisin is by far the most studied (1, 4, 12), although three new LAB lantibiotics recently have been purified and characterized (15, 16, 21, 24). The second group of LAB bacteriocins contains those that consist of one short unmodified polypeptide chain, such as lactococcin A (10, 27), leucocin A-UAL 187 (8), lactacin F (17, 18), pediocin PA-1 (9, 19), sakacin P, and curvacin A (26). These bacteriocins contain between 35 and 60 amino acid residues and have a high isoelectric point, often above 10. Many of these bacteriocins share significant amino acid sequence homology over relatively large regions, indicating that these regions are of fundamental importance for activity (19). Lactococcin A, which does not show any apparent amino acid sequence homology to other isolated LAB bacteriocins, has been shown to induce cell death by permeabilizing the membrane of susceptible cells (28).

The antimicrobial activity of the bacteriocins that have been studied so far is due to the action of a single peptide. However, we describe a novel lactococcal bacteriocin, termed lactococcin G, whose activity depends on the complementary action of two distinct peptides. Both peptides may form amphiphilic α-helices, suggesting that lactococcin G is a pore-forming toxin. A relatively simple and rapid purification procedure by which bacteriocins from LAB in general may be purified was devised for the purification of both of these peptides to homogeneity, and their amino acid sequences were determined.

## MATERIALS AND METHODS

**Bacterial strains and media.** The bacteriocin producer *Lactococcus lactis* LMG 2081 was obtained from J. Narvhus, Agricultural University, Ås, Norway. The indicator organism used in the bacteriocin assay was *L. lactis* subsp. *lactis* IL 1403 (2). Both strains were grown at 30°C in M17 broth (Oxoid) without lactose but supplemented with 0.4% (wt/vol) glucose. M17 broth was also supplemented with Tween 80 to a final concentration of 0.1% (vol/vol) when strain LMG 2081 was cultured for the production of bacteriocin.

**Bacteriocin assay.** The bacteriocin was quantified in a microtiter plate assay system (5). Each well of the microtiter plate contained 200 μl of M17 broth (supplemented with 0.4% [wt/vol] glucose and 0.1% [vol/vol] Tween 80), bacteriocin fractions at twofold dilutions, and the indicator organ-

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TABLE 1. Purification of lactococcin G

| Fraction | Vol (ml) | Total $A_{280}$[a] | Total activity (BU) ($10^6$) | Sp act[b] | Increase in sp act[b] | Yield (%) |
|---|---|---|---|---|---|---|
| Culture supernatant | 2,000 | 67,000 | 30 | 450 | 1 | 100 |
| Ammonium sulfate precipitation (fraction I) | 250 | 1,050 | 17 | 16,000 | 35 | 57 |
| Binding to cation exchanger (fraction II) | 40 | 5.8 | 6 | $1 \times 10^6$ | 2,200 | 20 |
| Binding to octyl-Sepharose CL-4B (fraction III) | 10 | 2.5 | 6 | $2.5 \times 10^6$ | 5,600 | 20 |
| Reverse-phase chromatography (fraction IV) | 1 | 1.5 | 5 | $3 \times 10^6$ | 6,700 | 17 |

[a] Total $A_{280}$ is the $A_{280}$ multiplied by the volume in milliliters.
[b] Specific activity is bacteriocin units (BU) divided by the $A_{280}$.

ism, *L. lactis* subsp. *lactis* IL 1403 ($A_{600}$ = 0.1). The microtiter plate cultures were incubated for 3 h at 30°C, after which growth inhibition of the indicator organism was measured spectrophotometrically at 600 nm by use of a Dynatech microplate reader. One bacteriocin unit was defined as the amount of bacteriocin that inhibited the growth of the indicator organism by 50% (50% of the turbidity of the control culture without bacteriocin).

**Bacteriocin purification.** All the purification steps were performed at room temperature, and all the chromatographic equipment was obtained from Pharmacia-LKB Biotechnology (Uppsala, Sweden). The bacteriocin was purified from 2-liter cultures of *L. lactis* LMG 2081 grown to the late exponential-early stationary phase. The cells were removed by centrifugation at 4,000 × *g* for 15 min at 4°C, and 400 g of ammonium sulfate per liter of culture supernatant was added. The protein precipitate was pelleted by centrifugation at 7,000 × *g* for 20 min and solubilized in 20 mM sodium phosphate buffer (pH 5.7) (buffer A; 250 ml/2-liter culture) (fraction I). Fraction I was applied at a flow rate of about 10 ml/min to a 7-ml S-Sepharose Fast Flow cation-exchange column equilibrated with buffer A. After subsequent washing of the column with 20 ml of buffer A, the bacteriocin was eluted from the column with 40 ml of 1 M NaCl in buffer A (fraction II). Ammonium sulfate was added to fraction II to a final concentration of 10% (wt/vol), after which the fraction was applied at a flow rate of about 4 ml/min to a 2-ml octyl-Sepharose CL-4B column equilibrated with 10% (wt/vol) ammonium sulfate in buffer A. The column was washed with 8 ml of buffer A, after which the bacteriocin was eluted from the column with 10 ml of 70% (vol/vol) ethanol and 30% buffer A (fraction III). Fraction III was diluted to 50 ml with $H_2O$ containing 0.1% (vol/vol) trifluoroacetic acid (TFA) and subsequently applied to a $C_2/C_{18}$ reverse-phase column (PepRPC HR 5/5) equilibrated with 2-propanol–$H_2O$ (10:90) containing 0.1% TFA. The bacteriocin was eluted with a linear gradient ranging from 30 to 50% 2-propanol containing 0.1% TFA (fraction IV). The bacteriocin peptides eluting from the reverse-phase column (fraction IV) were in some cases diluted four to fivefold with $H_2O$ containing 0.1% TFA and rechromatographed on the reverse-phase column. Purified bacteriocin was stored in 50 to 60% 2-propanol and/or ethanol containing 0.1% TFA at −20°C.

**Mass spectroscopy analysis.** Mass spectroscopy analysis of peptides was performed with a Bioion mass analyzer (Applied Biosystems, Uppsala, Sweden) as described earlier (23). Peptide fractions were dissolved in 50 to 100 μl of TFA containing 20% acetonitrile. From each fraction, 5 μl was loaded onto a target, and data were accumulated for 10 min at 16 kV.

**Amino acid sequencing.** The amino acid sequences were determined by Edman degradation with an Applied Biosystems (Foster City, Calif.) 477A automatic sequencer with an on-line 120A phenylthiohydantoin amino acid analyzer (3).

## RESULTS

**Purification of bacteriocin.** Initial screening showed that *L. lactis* LMG 2081 produced antagonistic activity towards various LAB and a number of different clostridia. This strain produced a bacteriocin constitutively during growth, although maximum activity was found in the culture medium at the very end of the exponential or early stationary phase of growth. The activity started to decrease after a few hours in the stationary phase (data not shown). The stability of the bacteriocin in the culture depended on the bacteriocin-producing strain. With other strains that produced the same bacteriocin, maximum activity was found in the middle of the exponential phase, whereas little if any activity was found towards the end of this growth phase (data not shown). This result may have been due to the cell envelope-associated proteinase produced by these strains, as this proteinase rapidly degrades the bacteriocin when present in the culture medium (unpublished results).

The purification scheme developed for isolating the bacteriocin for sequencing is shown in Table 1. It was necessary to add Tween 80 (final concentration, 0.1% [vol/vol]) to the culture medium before ammonium sulfate precipitation to effect binding of the bacteriocin to the cation exchanger in the subsequent purification step. Tween 80 was also included in assays for bacteriocin activity (see Materials and Methods), as it increased the sensitivity of the assays 2- to 10-fold (data not shown). The bacteriocin was concentrated eightfold from the culture medium by ammonium sulfate precipitation. This concentration resulted in a 30- to 40-fold increase in the specific activity and a recovery of about 60% of the activity (Table 1, fraction I). By subsequently binding the bacteriocin in fraction I to a cation exchanger and eluting it with 1 M NaCl, we obtained an increase in the specific activity of more than 2,000-fold (Table 1, fraction II). From this stage on, the yield remained at about 20% (Table 1). The specific activity increased 5,000- to 6,000-fold after binding of the bacteriocin in fraction II to octyl-Sepharose CL-4B and elution with 70% ethanol (Table 1, fraction III). When fraction III was applied to the reverse-phase column and eluted with a steep propanol gradient (6%/ml), the bacteriocin activity coeluted with an absorbance peak at about 40% propanol (results not shown). This absorbance peak, however, did not appear to be entirely homogeneous, as two shoulders could be discerned on both sides of the main peak.

**Dependence of bacteriocin activity on the complementary action of two peptides.** Upon rechromatography of fraction IV, this time with elution of the bacteriocin activity by use of a shallow propanol gradient (0.5%/ml), four absorbance

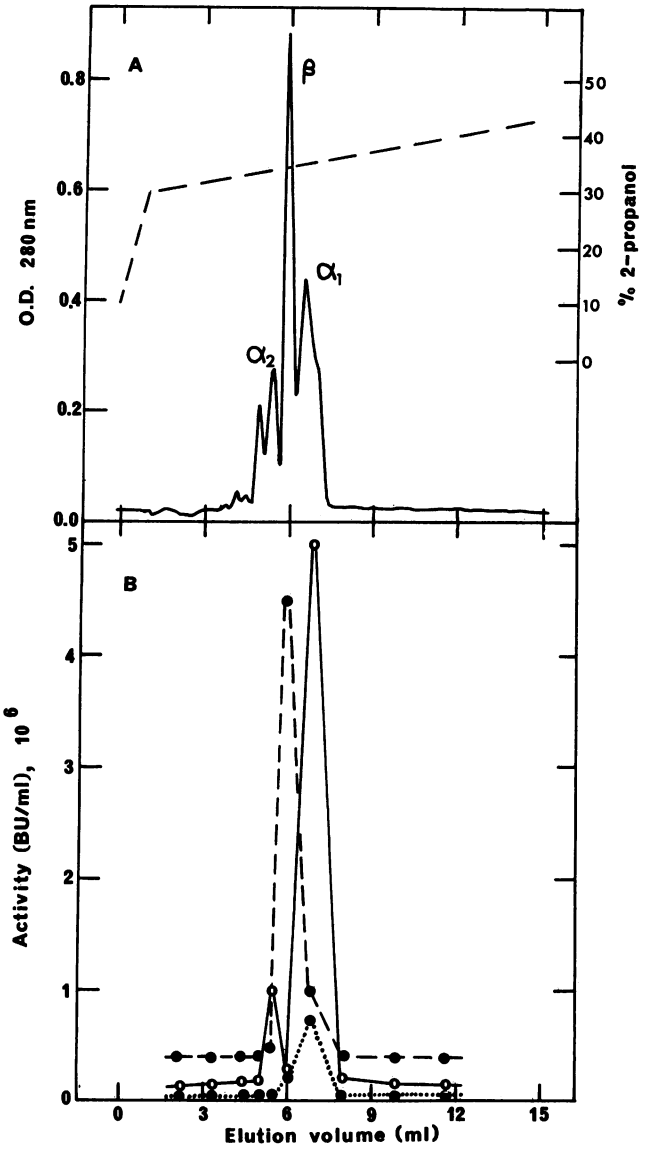


FIG. 1. Reverse-phase chromatography of lactococcin G (fraction IV). (A) Optical density (O.D.) profile (——) and propanol gradient (– – –). (B) Bacteriocin activity without complementation (···●···), with complementation with $\alpha_1$ (– –●– –), and with complementation with β (○). The amount applied to the column represents that obtained from approximately 2 liters of culture. BU, bacteriocin units.

peaks were obtained (Fig. 1). The last three of these peaks were termed $\alpha_2$, β, and $\alpha_1$, in the order in which they eluted from the column (Fig. 1). Most of the bacteriocin activity eluted together with the $\alpha_1$ absorbance peak, but the total activity was greatly reduced compared with that applied to the column (Fig. 1). However, when an aliquot of the fraction containing $\alpha_1$ was added to each of the column fractions, there was a complete recovery of the activity in the fraction containing β (Fig. 1). Similarly, there was a complete recovery of the activity in the fraction containing $\alpha_1$ and, to a lesser extent in the fraction containing $\alpha_2$, when an aliquot of the fraction containing β was added to each fraction (Fig. 1).

Each peptide, $\alpha_1$, $\alpha_2$, and β, was purified to homogeneity by rechromatography on the reverse-phase column (Fig. 2). Whereas relatively little activity was seen when each peptide was assayed alone for bacteriocin activity, the activity was recovered when the β peptide was complemented with the $\alpha_1$ peptide and to a lesser extent with the $\alpha_2$ peptide (Fig. 2). No additional increase in the bacteriocin activity was seen when the $\alpha_2$ peptide was added to fractions containing the β and $\alpha_1$ peptides (data not shown). Thus, the complementary action of two peptides, an α peptide and a β peptide, was necessary to obtain bacteriocin activity.

A small amount of β, which presumably had not been entirely separated from $\alpha_1$ on the previous reverse-phase column, was detected upon rechromatography of $\alpha_1$ (Fig. 2C). A small optical density peak apparently due to $\alpha_2$ and a minor peak eluting ahead of $\alpha_2$—similar to the optical density peak that eluted slightly ahead of $\alpha_2$ in Fig. 1—were also detected (Fig. 2C). It is unlikely that these two latter peaks were due to incomplete separation from $\alpha_1$ on the previous reverse-phase column, since they did not contaminate the β preparation to the same extent (Fig. 2B). Upon rechromatography of purified $\alpha_1$ after storage in 50% propanol for 3 months at −20°C, 30 to 40% of the peptides eluted as expected for $\alpha_1$, whereas 30 to 40% eluted as expected for $\alpha_2$ and 20 to 30% eluted as expected for the optical density peak that eluted slightly ahead of $\alpha_2$ in Fig. 1 (data not shown). As much as 10% of purified $\alpha_1$ eluted as expected for $\alpha_2$ upon rechromatography after storage for 24 h (data not shown). This result suggests that $\alpha_2$ and the component that eluted ahead of $\alpha_2$ were derived from $\alpha_1$.

**Mass spectroscopy analysis of the α and β peptides.** To determine the molecular weights of $\alpha_1$ and β and confirm their purities after the final reverse-phase chromatography step (Fig. 2), we analyzed these peptides by plasma desorption mass spectroscopy. Single peaks with molecular weights of 4,376 and 4,109 were observed for $\alpha_1$ and β, respectively (data not shown).

**Amino acid sequences of the α and β peptides.** The complete amino acid sequences of the $\alpha_1$ (39 amino acid residues) and β (35 amino acid residues) peptides and a major part of the sequence of the $\alpha_2$ peptide are shown in Fig. 3. Less of the $\alpha_2$ peptide was produced, so the sequence determination for this peptide was more difficult than that for $\alpha_1$ and β. For $\alpha_2$, neither residue 16 nor the last C-terminal amino acids—from residue 32 on—were determined. This quantity amounts to between 5 and 10 undetermined residues, since the total number of residues in $\alpha_2$, as judged by the amino acid composition (data not shown), is between 35 and 40. With the qualification that not all the residues in $\alpha_2$ were unambiguously determined, it appears from the sequences that $\alpha_2$ is identical to $\alpha_1$ (Fig. 3), which is consistent with the apparent formation of $\alpha_2$ peptides upon rechromatography of $\alpha_1$ on the reverse-phase column.

**Relative amounts of α and β necessary to obtain bacteriocin activity.** The concentrations of $\alpha_1$ and β that in combination inhibited the growth of the indicator organism by 50% were determined, and the results were plotted as an isobologram (Fig. 4). When $\alpha_1$ was present in excess (greater than 1.3 nM; 0.25 pmol per well), the presence of β at a concentration of 0.02 nM (0.004 pmol per well) resulted in 50% growth inhibition (Fig. 4). Similarly, when β was present in excess (greater than 0.15 nM; 0.03 pmol per well), the presence of $\alpha_1$ at a concentration of about 0.15 nM (0.03 pmol per well) resulted in 50% growth inhibition (Fig. 4). Thus, to obtain 50% growth inhibition in the presence of an excess of the complementary peptide, seven- to eightfold more $\alpha_1$ than β

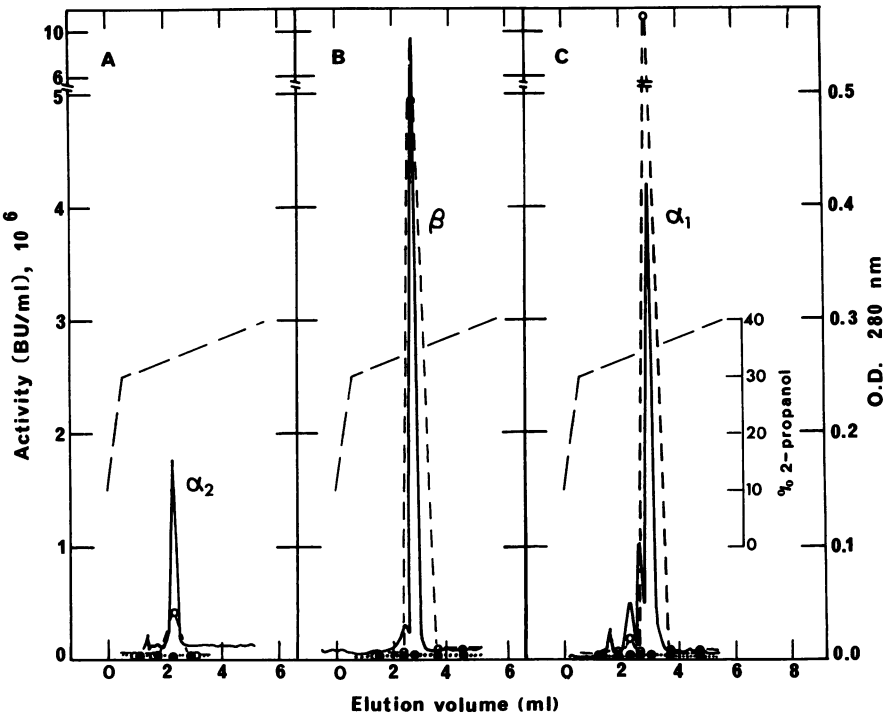


FIG. 2. Reverse-phase chromatography of $\alpha_2$ (A), β (B), and $\alpha_1$ (C). The optical density profile and propanol gradient are as in Fig. 1. Symbols: ●, bacteriocin activity without complementation; ○, bacteriocin activity with complementation with $\alpha_1$ (B) or with β (A and C). The amount applied to the column represents that obtained from approximately 2 liters of culture. BU, bacteriocin units.

was needed. When neither $\alpha_1$ nor β was present in excess, the concentrations that resulted in 50% growth inhibition were 0.3 nM (0.06 pmol per well) for $\alpha_1$ and 0.04 nM (0.008 pmol per well) for β (Fig. 4). Again, there was seven- to eightfold more $\alpha_1$ than β. The concentrations that inhibited growth by 50% appeared to be unaffected by the number of target cells present (within a 30-fold range in cell number), as the same concentrations of $\alpha_1$ and β resulted in 50% growth inhibition, irrespective of whether the cell density was such that the $A_{600}$ was 0.01 or 0.3 (data not shown).

## DISCUSSION

To our knowledge, this study is the first purification and characterization of a bacteriocin activity that requires the complementary action of two distinct peptides. As judged by reverse-phase chromatography, mass spectrometry, and amino acid sequencing, the bacteriocin peptides were purified to homogeneity by the purification procedure described in this study. We have now used this procedure for the purification of eight other bacteriocins (including nisin) produced by LAB, indicating that the procedure is in general applicable for the purification of low-molecular-weight bacteriocins. The addition of Tween 80 to the microplate wells in assays for bacteriocin activity increased the sensitivity of the assays 2- to 10-fold. The effect of Tween 80 could be due to a reduction of the binding of bacteriocin to the plastic microplate wells, a stabilization of a favorable configuration of the bacteriocin molecules, and/or a sensitization of the target cell, perhaps through destabilization of the cell membrane.

Three optical density peaks associated with bacteriocin activity were obtained by reverse-phase chromatography in the final purification step. The peptides associated with the three optical density peaks were termed $\alpha_1$, $\alpha_2$, and β. The bacteriocin activity was due to the complementary action of an α peptide and the β peptide. In combination with the β peptide, $\alpha_1$ yielded a much higher bacteriocin activity than $\alpha_2$. When purified $\alpha_1$ was rechromatographed on a reverse-phase column, some of it eluted as expected for $\alpha_2$. This result suggests that $\alpha_1$ and $\alpha_2$ may in fact be the same peptide but that they differ in their configuration in a manner that results in $\alpha_2$ having a slightly lower affinity for the reverse-phase column than $\alpha_1$ and a reduced bacteriocin activity when combined with β. This view was supported by the amino acid sequencing data. The $\alpha_2$ peptide, which contains between 35 and 40 amino acid residues, as judged from the amino acid composition (unpublished results), was sequenced to amino acid residue 31, and this sequence appeared to be identical to the corresponding sequence in the $\alpha_1$ peptide.

As judged by amino acid sequencing, $\alpha_1$ contained 39 amino acid residues, and its molecular weight should be 4,346. A molecular weight of 4,376 was obtained by mass spectrometry, indicating that the peptide is not grossly modified. Judging from its sequence, β contains 35 amino acid residues, and its molecular weight should be 4,110. This molecular weight is in good agreement with the molecular weight of 4,109 obtained by mass spectrometry, indicating that the peptide is not modified. From the amino acid sequence, the isoelectric point and extinction coefficient of $\alpha_1$ were calculated to be 10.9 and $1.3 \times 10^4$ $M^{-1}$ $cm^{-1}$, respectively; for the β peptide, the isoelectric point and extinction coefficient were calculated to be 10.4 and $2.4 \times 10^4$ $M^{-1}$ $cm^{-1}$, respectively.

The amino acid sequences of $\alpha_1$ and β are such that these peptides are likely to be pore-forming toxins that create cell membrane channels through a "barrel-stave" mechanism

| | 1 | | | | 5 | | | | | 10 | | | | | 15 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| $\alpha_1$: N | (Gly) | Thr | Trp | Asp | Asp | Ile | Gly | Gln | Gly | Ile | Gly | Arg | Val | Ala | Tyr |
| $\alpha_2$: N | (Gly) | (Thr) | Trp | (Asp) | Asp | Ile | Gly | Gln | Gly | Ile | Gly | Arg | Val | Ala | Tyr |
| β: N | Lys | Lys | Trp | Gly | Trp | Leu | Ala | Trp | Val | Asp | Pro | Ala | Tyr | Glu | Phe |

| | 16 | | | | 20 | | | | | 25 | | | | | 30 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| $\alpha_1$: | Trp | Val | Gly | Lys | Ala | Met | Gly | Asn | Met | Ser | Asp | Val | Asn | Gln | Ala |
| $\alpha_2$: | ? | Val | Gly | Lys | Ala | Met | Gly | Asn | (Met) | (Ser) | Asp | Val | Asn | Gln | Ala |
| β: | Ile | Lys | Gly | Phe | Gly | Lys | Gly | Ala | Ile | Lys | Glu | Gly | Asn | Lys | Asp |

| | 31 | | | | 35 | | | | |
|---|---|---|---|---|---|---|---|---|---|
| $\alpha_1$: | Ser | Arg | Ile | Asn | Arg | Lys | Lys | Lys (His) | C |
| $\alpha_2$: | Ser | | | | | | | | |
| β: | Lys | Trp | Lys | Asn | Ile | C | | | |

FIG. 3. Amino acid sequences of $\alpha_1$, $\alpha_2$, and β. Amino acids not identified with certainty, although most probable, are in parentheses. A question mark indicates an unidentified amino acid. N and C indicate the N- and C-terminal ends of the peptide, respectively.

and thus produce an ionic imbalance in the cell (20). A region in $\alpha_1$ starting with amino acid residue 3 and ending with residue 27 may form an amphiphilic α-helix, as is evident when this sequence is displayed on an Edmundson α-helical wheel (Fig. 5A). The polar amino acids are found almost completely on one side of the α-helix, whereas the nonpolar residues are found on the opposite side of the helix (Fig. 5A). The amphiphilic distribution of the amino acids in this region is nearly perfect, the only exception being glycine (residue 9), which appears on the hydrophobic side (Fig. 5A). However, glycine may be considered relatively neutral with respect to its hydrophilic-hydrophobic character. Moreover, the substitution of an amino acid by one of an opposite hydrophobicity may not represent an intolerable disruption of the amphiphilic character of a peptide (20). The 25-amino-acid-long amphiphilic region in $\alpha_1$ may allow peptide monomers to oligomerize into membrane-spanning pores in such a manner that the nonpolar side of the α-helix faces the membrane lipids, whereas the polar side faces towards the center of the pore (13, 20). The amphiphilic region in $\alpha_1$ should be long enough to span a membrane, as a minimum of about 20 residues is needed to form a membrane-spanning α-helix (13, 20). Ten of the 12 C-terminal amino acid residues, starting with position 28 and ending with position 39, in $\alpha_1$ are polar, and the last 5 of these are basic (Arg-Lys-Lys-Lys-His-COOH). In this respect, it is interesting to note that lactococcin A and lactocin S, bacteriocins produced by *Lactococcus lactis* subsp. *cremoris* and *Lactobacillus sake*, respectively, also contain basic C termini, the last 2 C-terminal amino acids of which are histidine for both (10, 16). It seems likely that the 11-amino-acid-long polar C-terminal region in $\alpha_1$ does not penetrate the membrane. One may speculate that its function is to recognize bacteriocin-binding sites on the target cells and thereby create a local high concentration of bacteriocin monomers on the outside of the cell membrane. This high concentration near the membrane could then induce oligomerization of the monomers into transmembrane pores. Another possible function of the polar C-terminal region might be to stabilize a correct peptide configuration in a hydrophilic environment. The first 2 N-terminal amino acid residues in $\alpha_1$ are polar and may possibly be the part of the pore that is located inside the cell.

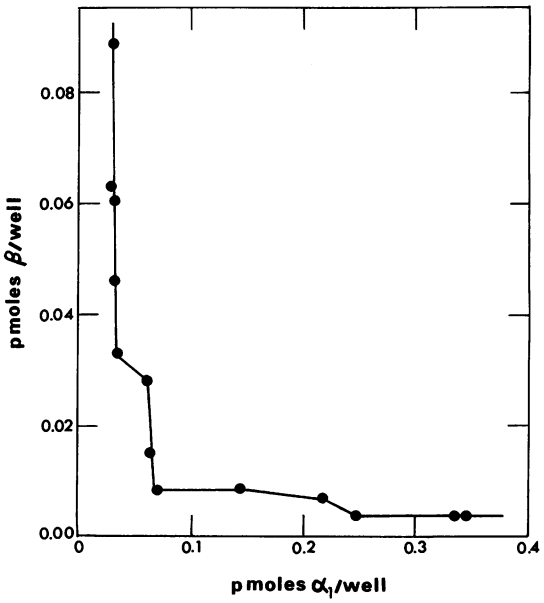


FIG. 4. Amounts of $\alpha_1$ and β that in combination inhibited the growth of the indicator strain by 50%.

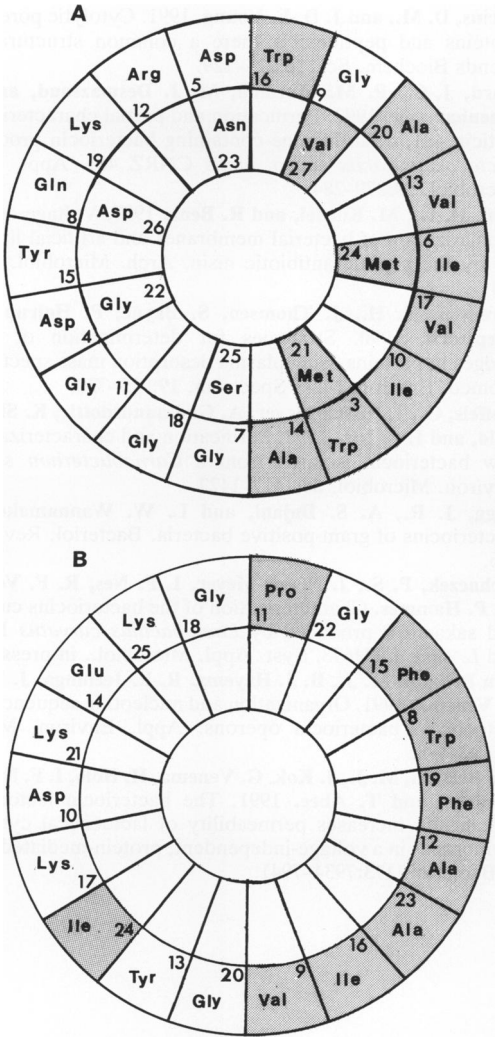


FIG. 5. Edmundson α-helical wheel representation of the amphiphilic regions in $\alpha_1$ (A) and β (B). For $\alpha_1$, the amphiphilic region shown starts with residue 3 and ends with residue 27; for β, it starts with residue 8 and ends with residue 25. The shaded areas indicate nonpolar residues, and the nonshaded areas indicate polar residues.

The situation is similar for the β peptide. An amphiphilic α-helix may be formed in the region starting with amino acid residue 8 and ending with residue 25 (Fig. 5B). In this region, there are two exceptions to a perfect amphiphilic amino acid distribution: glycine (residue 22), which appears on the hydrophobic side, and isoleucine (residue 24), which appears on the hydrophilic side. The former should not be a major problem, because of the neutral character of glycine. One would expect that proline at position 11 would cause a break or bend in the α-helical structure of the amphiphilic region. The region spans 18 amino acid residues, an amount that may be less than that required to span the cell membrane. However, in front of the amphiphilic region there are 5 nonpolar amino acids, starting with residue 3 and ending with residue 7, which presumably also are part of the transmembrane region. The hydrophilic amino acid, lysine, is found at positions 1 and 2 at the N terminus, and only 2 of 10 residues, starting with position 26, in the C-terminal part of the molecule are hydrophobic. As with $\alpha_1$, one might expect that these regions will be located outside the membrane, the long polar C-terminal region being on the outside of the cell. Although other LAB-produced bacteriocins that have been sequenced do not appear to have such a marked amphiphilic distribution of amino acids as $\alpha_1$ and β, such a distribution is seen in the C-terminal parts of lactococcin A (10) and lactocin S (16). There is evidence that lactococcin A permeabilizes target cell membranes (28), as also appears to be the case for nisin (22).

The concentrations of $\alpha_1$ and β that inhibited the growth of the indicator cells by 50% were, respectively, 0.15 and 0.02 nM when the complementing peptide was present in excess. When neither was present in excess, the concentrations were, respectively, 0.3 and 0.04 nM. Thus, seven to eightfold more $\alpha_1$ than β was needed. If the two peptides associate with the target cells with equal efficiency, then this ratio may reflect the fact that $\alpha_1$ and β interact in an approximately 8:1 ratio, for instance, in pore formation. The presence of approximately 40 β molecules per target cell together with an excess of $\alpha_1$ molecules is enough to induce 50% growth inhibition. The number of β molecules that interact with a target cell may possibly be even smaller, since the concentration of bacteriocin that inhibited growth by 50% appeared to be unaffected by the number of target cells present within a 30-fold range.

We have termed the present bacteriocin lactococcin G. The fact that its activity depends on the complementary action of two relatively small peptides, α and β, whose primary structures clearly suggest functional domains, makes this bacteriocin an attractive model for studying structural-functional relationships of pore-forming molecules.

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