CHAPTER IV

Mutational analysis and chemical modification of lactococcin B, a bacteriocin produced by *Lactococcus lactis*.

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Mutational analysis and chemical modification of lactococcin B, a bacteriocin produced by Lactococcus lactis.

SUMMARY

Using site directed mutagenesis the single cysteine residue at position 24 of lactococcin B was replaced by all other possible amino acids. Most of these mutant molecules retained activity, with the exception of those in which cysteine was replaced by a positively charged amino acid. This would seem in agreement with the fact that treatment of the wild-type molecule with HgCl₂ resulted in inactivation of the molecule. The introduction of a positive charge at position 24 by HgCl₂ is the only factor that causes inactivation of lactococcin B, as treatment of the bacteriocin with other oxidative chemicals did not interfere with the ability of lactococcin B to dissipate the membrane potential of sensitive cells. The results imply that inactive lactococcin B could still bind to its receptor. It could be replaced by an active bacteriocin molecule, resulting in dissipation of the membrane potential. In this case there is a lag-time between addition of the active bacteriocin and dissipation of the potential.

INTRODUCTION

The past several years have seen an explosion of research activity on bacteriocins produced by lactic acid bacteria (LAB) (4,7,8-10,15). As this field continues to expand, our knowledge about LAB and their bacteriocins is increasing rapidly, owing to detailed studies of the peptides, their mechanism of action, and the immunity-, processing- and secretion systems involved. Lactococccins are bacteriocins produced by Lactococcus lactis. The genes specifying lactococcin A, B and M production, immunity and secretion are located on plasmid p9B4-6 and have been sequenced (23,24,26). Lactococcin A and lactococcin B specifically inhibit the growth of lactococci and their modes of action have been studied. Both are small cationic, hydrophobic peptides that structurally resemble several peptide antibiotics permeabilizing membranes (5,6,12,13,21). At lactococcin concentrations that did not affect immune cells, both bacteriocins rapidly dissipated the membrane potential of glucose-energized sensitive cells of L.lactis and caused efflux of preaccumulated amino acids (25,27). Efflux was a direct consequence of permeability changes in the cytoplasmic membrane, and was not caused by the dissipation of the proton motive force (pmf). Both lactococccins are able to permeabilize the cytoplasmic membrane in a voltage-independent manner, most likely by the formation of pores. It is believed that both lactococccins require a proteinaceous receptor for their insertion into the cytoplasmic membrane (25,27,28).

A prerequisite for lactococcin B activity is that its only cysteine residue (Cys24) is in the reduced state (27). Partially purified (and, thus, oxidized) lactococcin B was almost inactive on whole cells. Only after addition of dithiothreitol (DTT; 5 mM) was lactococcin B capable of dissipating the membrane potential of sensitive whole cells. Cys24 in DTT-treated lactococcin B is oxidized by HgCl₂, resulting in the inactivation of the bacteriocin, while HgCl₂-oxidized lactococcin B could be reactivated by DTT. It was postulated that the reduced state of the Cys24 residue was required for receptor recognition, or, alternatively, that the oxidized state of Cys24 changed the structure of the molecule in such a way that its insertion and/or pore-forming abilities had been affected. In this study we have used site directed mutagenesis together with chemical modification of Cys24 to investigate these possibilities.
Table 1: Bacterial strains and plasmids.

<table>
<thead>
<tr>
<th>Bacterial strain or plasmid</th>
<th>Relevant properties</th>
<th>source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacteria</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Lactococcus lactis</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL1403</td>
<td>plasmid free strain, indicator strain for lactococcin B</td>
<td>2</td>
</tr>
<tr>
<td><em>E.coli</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JM103</td>
<td></td>
<td>29</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
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<td></td>
</tr>
<tr>
<td>pMB580</td>
<td>Em', containing the lactococcin B operon</td>
<td>26</td>
</tr>
<tr>
<td>pUC19</td>
<td>Amp', cloning vector</td>
<td>29</td>
</tr>
<tr>
<td>pMG36CT</td>
<td>Cm', cloning vector</td>
<td>C.M. Franke</td>
</tr>
<tr>
<td>pUCIcnB</td>
<td>Amp', containing the lactococcin B operon</td>
<td>this work</td>
</tr>
</tbody>
</table>

Em: erythromycin; Cm: chloramphenicol; Amp: ampicillin; r: resistance.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids and growth conditions.**

The bacterial strains and plasmids used are listed in Table 1. *L.lactis* was cultured in M17 broth (22) supplemented with 0.5 % glucose (GM17) or in MRS broth (3) containing 0.5% glucose. For solid plates, 1.5% agar was used. *E.coli* was cultured on TY medium and plates (17). Erythromycin (Em) and chloramphenicol (Cm) were used at final concentrations of 5 µg/ml for *L.lactis*, and 100 µg/ml and 10 µg/ml for *E.coli*, respectively.

**Molecular cloning, DNA sequencing, and primer synthesis.**

Transformation of *L.lactis* and *E.coli* was done as described earlier (28). General DNA cloning and manipulation techniques were carried out essentially as described by Sambrook *et al.* (18). DNA sequencing was done on double-stranded DNA by the dideoxy chain-termination method (19), using the T7 sequencing kit and protocol (Pharmacia, Uppsala, Sweden). Synthetic oligo deoxynucleotides were synthesized with an Applied Biosystems 381A DNA synthesizer (Foster City, Calif.). The sequences of the primers were as follows (the position of degenerate nucleotides is indicated between brackets; the position corresponding to the position of the cysteine residue is shown in italic; the *Sca*I site in the primer is underlined):

KOVI0: 5'-GGTATAAAAGATATCATAGTACTGGAAA-AACAATAT(TCA)AAACAGACAATTG-3';
KOVI1: 5'-GGTATAAAAGATATCATAGTACTGGAAA-AACAATAC(TCAG)(TA)AAACAGACAATTG-3';
KOVI2: 5'-GGTATAAAAGATATCATAGTACTGGAAA-AACAATAA(TCAG)(TC)AAACAGACAATTG-3';
KOVI3: 5'-GGTATAAAAGATATCATAGTACTGGAAA-AACAATAG(TCAG)(TG)AAACAGACAATTG-3';
KOVI4: 5'-GGTATAAAAGATATCATAGTACTGGAAA-AACAATATGGAAAAACAGACAATTG-3';

**Bacteriocin assay.**

Overlayers on colonies were done as follows: colonies were treated with chloroform vapor for 15 min. After subsequent exposure of the plates to air for 30 min, M17 softagar seeded with 0.1% of an overnight culture of the indicator *L.lactis* IL1403 was poured onto the plates. After 12-14 hrs. of incubation at 30 °C, the plates were examined for zones of growth inhibition (halos). The sizes of the halos were measured from the edge of a bacteriocin-producing colony to the edge of the inhibition zone.
Construction of mutants of lactococcin B.

The codon for Cys24 in the lactococcin B gene was changed, using a two-step PCR strategy, into codons for all other possible amino acids. For this, four degenerate primers were used. One primer (KOV14) was used to replace Cys24 by tryptophan. The lactococcin B operon from pMB580 was cloned in pUC19 as an EcoRI-XbaI fragment to give pUClnB. This plasmid was used as a template in the first PCR step using the mutation primers and the universal primer (Boehringer Mannheim GmbH, Germany) (Fig. 1). The products obtained in the five different reactions were purified by Qia prep PCR columns (Qiagen GmbH, Hilden, Germany) and used as a primer, together with the reverse primer (Boehringer), on pUClnB in a second PCR step (Fig. 1). The purified

Figure 1: Two step PCR strategy for obtaining \textit{lcnB} with a changed codon at position 24 of the molecule. For details of the procedure see Materials and Methods. \textit{Amp}': ampicillin-resistance marker; \textit{Ori}: origin of replication; \textit{uni}: universal primer; \textit{rev}: reverse primer; \textit{lollipop}: position of the mutation.

PCR products were restricted with the enzymes \textit{EcoRI} and \textit{XbaI} and cloned in pUC19. The ligation mixture was used to transform \textit{E.coli} and the proper constructs were selected by screening for the presence of a \textit{ScaI} site, introduced by the mutation primers. Inserts containing a \textit{ScaI} site were sequenced to determine which mutation they contained and transferred to pMG36CT using the \textit{EcoRI} and \textit{XbaI} sites. The set of 19 mutants and the wild type gene, all in pMG36CT, were introduced in \textit{L.lactis} which was subsequently screened for bacteriocin activity in a colony-overlay assay.

Measurement of cytoplasmic membrane potential.

The change in cytoplasmic membrane potential of cells of \textit{L.lactis} was monitored by the distribution of the lipophilic cation tetraphenylphosphonium (TPP') as described before (27).

Chemical modification of lactococcin B.

Modification of Cys24 was performed with the following chemicals:
cysteine, N-ethylmaleimide (NEM), p-mercuribenzoate (pMB), performic acid, CuSO4, and HgCl2. Conditions were optimal for reaction of only the sulphydryl-group with the oxidative chemicals and are described in reference 14, with the exception of performic acid, which, under these conditions, also reacts with methionine. To examine whether the chemicals had reacted with the cysteine, the molecule was subsequently treated with HgCl2 which would inactivate the bacteriocin when it had not reacted with the other oxidative chemicals. These data indicated that all chemicals had reacted with Cys24 (data not shown).

RESULTS

Cys24 is not essential for lactococcin B activity.

Using a two step PCR procedure with degenerate primers, the gene encoding lactococcin B was mutated such that Cys24 was replaced by one of all other 19 amino acids. The mutated variants of \textit{lcnB} together with the lactococcin B immunity gene, \textit{lciB}, were introduced in \textit{L.lactis} IL1403 and the transformants were tested for lactococcin B activity in a colony-overlay assay. As shown in Table 2, all mutants produce active bacteriocin as judged by the presence of a halo around the colonies, with the exception of those mutants in which Cys24 has been replaced by a positively charged residue: Cys24Arg, Cys24His, and Cys24Lys. Apparently, a cysteine residue at position 24 is not required for lactococcin B to be active. All strains were also immune to the mutant lactococcin B they produce, except for the strain producing lactococcin B(Cys24Thr). Colonies producing lactococcin B(Cys24Thr) were initially very small. It appeared that the plasmid contained one extra mutations: by one base substitution a stop was introduced in the fourth codon of the immunity gene (data not shown). The number of transformants

Table 2: Activity of lactococcin B variants mutated at
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Mutational analysis and chemical modification of lactococcin B

Mutational analysis and chemical modification of lactococcin B residue 24.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Activity a</th>
<th>Mutant</th>
<th>Activity a</th>
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<tbody>
<tr>
<td>Cys (wt)</td>
<td>+</td>
<td>Lys</td>
<td>-</td>
</tr>
<tr>
<td>Ala</td>
<td>+++</td>
<td>Met</td>
<td>++</td>
</tr>
<tr>
<td>Arg</td>
<td>-</td>
<td>Phe</td>
<td>++</td>
</tr>
<tr>
<td>Asn</td>
<td>++</td>
<td>Pro</td>
<td>++</td>
</tr>
<tr>
<td>Asp</td>
<td>++</td>
<td>Ser</td>
<td>++</td>
</tr>
<tr>
<td>Gln</td>
<td>++</td>
<td>Thr</td>
<td>++</td>
</tr>
<tr>
<td>Glu</td>
<td>++</td>
<td>Trp</td>
<td>++</td>
</tr>
<tr>
<td>His</td>
<td>-</td>
<td>Tyr</td>
<td>++</td>
</tr>
<tr>
<td>Ile</td>
<td>++</td>
<td>Val</td>
<td>++</td>
</tr>
<tr>
<td>Leu</td>
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*a Activity was measured as a zone of inhibition (halo) around a colony producing bacteriocin using IL1403 as the indicator strain. -: no halo; +: halo 1-6 mm; ++: halo 6-12 mm; +++: halo 12-18 mm.

obtained with the plasmid carrying the mutated immunity gene was the same as that obtained with a plasmid carrying a functional immunity gene (data not shown).

A chemically introduced positive charge at position 24 abolishes lactococcin B activity.

Lactococcin B can be inactivated by oxidation of its only cysteine residue by HgCl₂ (27). Other oxidative chemicals specific for cysteine were tested to examine whether the lactococcin B molecule could be oxidized while retaining activity. The effect of the oxidized bacteriocin on the cytoplasmic membrane potential, as measured by the distribution over this membrane of the lipophilic cation tetraphenylphosphonium (TPP⁺), was tested. As documented previously, lactococcin B, when fully reduced by DTT, dissipates the membrane potential of sensitive cells of L. lactis (Fig. 2A). When oxidized by HgCl₂, activity is lost completely (Fig. 2B). This can be explained by the acquisition of a positive charge at the position of Cys24 (see Fig. 2B, inset) and is in agreement with the observation that lactococcin B mutants, in which Cys24 is replaced by a positively charged amino acid, are inactive (see above). To examine whether lactococcin B could be oxidized with other chemicals in the absence of introducing a positive charge, the bacteriocin was reacted with cysteine, NEM, pMB, or CuSO₄. As can be seen in Figures 2C through 2F, none of these oxidative compounds affected lactococcin B activity. In the inset of these figures the chemical bond that is formed upon oxidation is shown. These results indicate that it is the presence of a positive charge at this position that abolishes bacteriocin activity. Treatment of lactococcin B with performic acid results in loss of activity (Fig. 2G), although in this case no positive charge is introduced at the Cys24 position (see inset in Fig. 2G). Also, the lactococcin B molecule containing the Cys24Ala substitution was inactivated by performic acid (data not shown), indicating that the inactivation was not caused by oxidation of the cysteine residue.

Lactococcin B(Cys24Ala) dissipates the membrane potential of sensitive cells.

The effect of one of the lactococcin B mutants on the membrane potential was investigated. The Cys24Ala mutant was chosen, since it produced the largest halo in the overlay assay (see Table 2). Figure 3A shows that lactococcin B(Cys24Ala) dissipated the cytoplasmic membrane potential of sensitive cells. As expected, addition of HgCl₂ to lactococcin B(Cys24Ala) had no effect on its activity (Fig. 3B), since this molecule no longer contains the oxidizable cysteine residue.
Figure 2: Effect of lactococcin B treated with various chemicals on the membrane potential of energized cells of \textit{L. lactis} IL1403. All curves have been normalized to that of the positive control, addition of valinomycin (Fig. 2H). In the insets the chemical bond formed upon oxidation is presented. Lactococcin B was treated with dithiothreitol (A), HgCl$_2$ (B), cysteine (C), N-ethyl-maleimide (D), p-mercury-benzoate (E), CuSO$_4$ (F), and performic acid (G). The treated lactococcin B was added when the membrane potential had reached its optimum (at the time indicated by the arrow). Differences between the final membrane potential of two different curves after dissipation with lactococcin B were insignificant.
HgCl₂-inactivated lactococcin B blocks the immediate action of subsequently added active lactococcin B.

Approximately 30 sec. after HgCl₂-inactivated lactococcin B was added to the cells (Fig. 3C, at the time indicated by the first arrow), active (DTT-treated) lactococcin B was added (Fig. 3C, at the time indicated by the second arrow). Instead of an immediate dissipation of the membrane potential, there was a lag-time of approximately 25 sec. before dissipation started. Moreover, dissipation was much slower than when only active (DTT-treated) lactococcin B had been added (compare Fig. 2A and 3C). To exclude that DTT caused this effect it was added to cells pretreated with HgCl₂-inactivated lactococcin B. As can be seen in Fig. 3D, DTT had no effect on the membrane potential of these cells. Apparently, dissipation of the membrane potential occurred as the result of the addition of the active bacteriocin and not because of activation of the HgCl₂-inactivated molecules. This conclusion is in agreement with the fact that the active Cys24Ala mutant is still able to dissipate the membrane potential after the lag time of approximately 25 sec. (Fig. 3E).

Figure 3: Effect of various lactococcin B derivatives on the membrane potential of energized cells of *Lactis* IL1403. All curves have been normalized to that of the positive control, addition of valinomycin (Fig. 3F). Additions (at the time indicated by the arrow) were: lactococcin B(Cys24Ala) (A), HgCl₂-inactivated lactococcin B(Cys24Ala) (B), HgCl₂-inactivated lactococcin B at the time of the first arrow, and DTT-treated lactococcin B at the time of the second arrow (C), HgCl₂-inactivated lactococcin B at the time of the first arrow, and DTT at the time of the second arrow (D), HgCl₂-inactivated lactococcin B at the time of the first arrow, and lactococcin B(Cys24Ala) at the time of the second arrow (E), valinomycin, as the positive control (F). The bars in panels C and E indicate the lag time between addition of lactococcin B and the start of the membrane potential dissipation. Differences between the final membrane potential of two different curves after dissipation with lactococcin B were insignificant.

**DISCUSSION**

The two step PCR procedure used to construct the mutants of lactococcin B produced the correct mutants.
with a frequency of 80%. The other 20% contained extra mutations, such as frame shift mutations and/or nucleotide substitutions. One example is the plasmid encoding lactococcin B(Cys24Thr). In this plasmid, a stop was introduced in the fourth codon of the immunity gene by one base substitution. The transformants carrying this plasmid initially grew poorly, but gradually restored their normal growth potential. This resumption of growth (tolerance) is frequently observed when sensitive cells are exposed to lactococcin (24,26) and may be due to a change in lipid composition.

We reported previously that lactococcin B is only active when its single cysteine residue is in the reduced state (27). Here we show that this cysteine can be replaced by all other amino acids, except the positively charged ones, without negating bacteriocin activity. Also, the bacteriocin molecule is still active when oxidized with oxidative chemicals other than HgCl₂ (except performic acid). Since oxidation of cysteine residue 24 by HgCl₂ results in a molecule with a positive charge at this position (see the inset of Fig. 2B), apparently no positive charge at position 24 is tolerable for activity of lactococcin B. Clearly, Cys24 is not essential for bacteriocin activity. In this regard lactococcin B resembles a group of thiol-activated toxins in which the reduced state of the cysteine residue appears to be essential for the generation of functional lesions in toxin-treated membranes, but in which the cysteine residue can be replaced with other amino acids without loss of activity (1). The fact that Cys24 can even be replaced by proline or glycine, two residues able to induce large structural changes, probably implies that this part of the molecule is not directly involved in the action mechanism of the bacteriocin.

Addition of active lactococcin B after addition of HgCl₂-inactivated lactococcin B results in a lag-time before the onset of membrane potential dissipation. We conclude from this result that the inactive bacteriocin is still able to bind to the bacteriocin receptor, but that active lactococcin B can replace the inactive molecule at the receptor. Since a lag-time is observed between addition of active lactococcin B and start of membrane potential dissipation, apparently some time is needed before the exchange occurs. Alternatively, the exchange starts immediately, but it takes some time before enough molecules have exchanged to dissipate the membrane potential. Another possibility is that HgCl₂-inactivated molecules insert into the membrane, but form a closed pore. Addition of active lactococcin B might then result in the formation of mixed-pores consisting of both inactive and active lactococcin B molecules, which would open again at a certain ratio of inactive:active molecules. If this interpretation would be correct, the lag-time would represent the time needed to reach the critical ratio of inactive:active molecules and would agree with the observation that the membrane potential dissipated more slowly, which might be due to the lower efficiency of solute transport through the mixed pores.

A 21-amino-acid-sequence between residues Ala30 and Phe50 in lactococcin A could possibly form a membrane-spanning α-helix (11; Fig. 4A).

![Figure 4: Single letter amino acid sequence of mature lactococcin A (A) and lactococcin B (B). The amino acid sequences that could possibly form a transmembrane helix (lactococcin A) or an amphiphilic α-helix (lactococcin B) are underlined. The cysteine residue in lactococcin B is double underlined. The methionine residues are circled.](image-url)
membrane spanning helix is not obvious. However, a possible weak amphiphilic helix is present between residue 28 and 46 in the C-terminus of lactococcin B (hydrophobic moment <µH>=0.35), at a position similar to that of the transmembrane helix in lactococcin A (Fig. 4B).

Treatment of lactococcin B with performic acid resulted in the complete loss of bacteriocin activity, most probably due to the oxidation of the two methionine residues in the lactococcin B molecule, which are oxidized under the applied conditions (14). One of the methionine residues is present in the amphiphilic helix (Met38; Fig. 4B). Oxidation of this residue would destroy the amphipathic nature of the helix. Alternatively, oxidation of the methionine residue at position six of the molecule could interfere with activity, for instance by blocking receptor binding.

ACKNOWLEDGEMENTS

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LITERATURE CITED