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Mechanistic Properties of the Two-Component Bacteriocin Lactococcin G

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Lactococcin G is a bacteriocin whose activity depends on the complementary action of two peptides, termed α and β. Biologically active, synthetic lactococcin G was used to study the mode of action on sensitive cells of Lactococcus lactis. The α and β peptides can bind independently to the target cell surface, but activity requires the complementary peptide. Once bound to the cell surface, the peptides cannot be displaced to the surfaces of other cells. A complex of α and β peptides forms a transmembrane pore that conducts monovalent cations but not protons. Efflux of potassium ions is observed only above pH 5.0, and the rate of efflux increases steeply with the pH. The consequences of cation fluxes for the viability of the target cells are discussed.

Bacteriocins produced by lactic acid bacteria are peptides displaying bactericidal activity against closely related gram-positive bacteria. In terms of applications, bacteriocins are interesting as preservatives of food products. Lactococcin G activity depends on two peptides, termed α and β, that consist of 39 and 35 amino acids, respectively (14). Bactericidal activity is observed only in the presence of both peptides, and optimal activity is observed when the peptides are present in a 1:1 ratio (10). Biologically active lactococcin G can also be obtained by solid-phase peptide synthesis (10). Addition of lactococcin G to sensitive cells results in a collapse of the transmembrane electrical potential but not in dissipation of the transmembrane pH gradient (10). Such cells show a rapid depletion of cellular ATP and release intracellular potassium, as monitored through the use of 86Rb. These studies have led to the suggestion that the loss of cell viability results from futile cycling of potassium ions via a lactococcin G-induced potassium-specific pore and the ATP-dependent uptake of potassium ions. We now show that lactococcin G not only causes the release of potassium ions but that it has a broader specificity for monovalent cations.

MATERIALS AND METHODS

Materials. 86Rb (specific activity 10 mCi/mM) (30.0 Ci/mM), 72Na (1 Ci/3.7 mg), 32P (1 Ci/0.11 nmol), and [14C]choline (7.2 mCi/mM) were obtained from Amersham UK. Gramicidin A and trifluoroacetic acid and mixed in a 1-to-1 ratio.

Peptide synthesis, purification, and analysis of the polypeptides. The lactococcin G α and β peptides were prepared by solid-phase synthesis, purified, and analyzed as described previously (10).

Strains and culture conditions. Lactococcus lactis LMG 2081 (10) and L. lactis IL 1403 (3) were used as nonsensitive and sensitive strains, respectively. Both strains were grown at 30°C in M17 broth (Oxoid) without lactose but supplemented with 0.5% (wt/vol) glucose or, alternatively, with both 0.25% (wt/vol) glucose and 0.25% (wt/vol) L-malate. Cells were harvested in the logarithmic

Cation flux assays. Rubidium transport and lactococcin G-mediated rubidium efflux were measured as described previously (10). Cells (0.25 mg of protein/ml) suspended in an appropriate buffer were preenergized in a solution containing 50 mM Na-PiPES [piperazine-N,N,N-tris(2-carboxyethyl)-5(and-6)-carboxylfluorescein (BCEF) as described previously (9).

RESULTS

Lactococcin G elicits cellular release of monovalent cations. Since lactococcin G induces the release of potassium ions from sensitive cells, it has been suggested that it acts as a potassium-conducting pore (10). To determine the ion specificity of lactococcin G, cells were loaded overnight with radioactive sodium ions (22Na). When diluted into buffer, the cells only slowly released the 22Na+ (Fig. 1A). 22Na+ efflux was not in-

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Lactococcin G causes efflux of \( [14C] \) choline from cells which were diluted in 50 mM KPi, to 174 \( \mu \)g of protein/ml, and either 50 nM lactococcin G (\( \bullet \)), 0.25 \( \mu \)M valinomycin (\( \blacksquare \)), or solvent (C) was added. (B) In \( L. \) lactis cells (28 \( \mu \)g of protein/ml) suspended in 50 mM Na-PIPES, pH 7.0, a \( \Delta \Psi \) was generated by 0.25 \( \mu \)M valinomycin (arrow 1), after which 20 nM lactococcin G (arrows 2) and 6.7 \( \mu \)M nisin (arrows 3) were added.

\( \Delta \Psi \) indicates that the conductivity of potassium, sodium, cesium, or lithium is slightly higher than that of choline or tetramethylammonium and much higher than of Tris (data not shown). In contrast, lactococcin G does not at all dissipate the \( \Delta \Psi \) induced by valinomycin in cells suspended either in 50 mM MgCl\(_2\), 50 mM MgSO\(_4\), or 50 mM bis(Tris)-propionate (data not shown). This clearly indicates that lactococcin G has conductance for neither the anions tested here nor the divalent cations.

Lactococcin G does not mediate phosphate efflux. To investigate whether lactococcin G’s inability to conduct anions also extends to phosphate, release of cellular phosphate was analyzed. Phosphate transport in \( L. \) lactis is unidirectional and is likely ATP dependent (16). Intracellular phosphate concentrations can be as high as 140 mM (16). Lactococcin G was unable to induce the release of cellular phosphate, nor did it affect the release of accumulated \( ^3P \) from malate-energized cells (data not shown). This demonstrates that lactococcin G does not
conduct the transmembrane movement of one of the major cytosolic constituents.

**Lactococcin G causes an increase in the ΔpH.** Previous experiments with cells loaded with the fluorescent pH indicator BCECF showed that after addition of valinomycin, lactococcin G is unable to dissipate the ΔpH (10). These experiments were performed in the presence of valinomycin to assure that the ΔpH is the sole component of the proton motive force. As shown in Fig. 4A, addition of lactococcin G to glucose-energized cells results in an elevation of the intracellular pH, as monitored by the fluorescence of cells with entrapped BCECF. This effect is likely due to the dissipation of the ΔΨ and enhanced proton extrusion by the F_0F_1 ATPase and was not observed when cells were pretreated with valinomycin (Fig. 4B). The lactococcin G action may be comparable to the effect of gramicidin A’ on the ΔpH. Gramicidin A dimers form water-filled channels specific for small cations and—in contrast to lactococcin G—the conductivity for protons can be up to 150 times higher than for sodium ions (13). Initially, gramicidin A’ causes a rapid increase of the ΔpH in *L. lactis* cells, but this process is immediately followed by a steep decrease in ΔpH (Fig. 4C). This clearly shows that gramicidin channels functionally differ from lactococcin G pores and further suggests that lactococcin G does not conduct proton movements at a significant rate. In the above experiments, the K^+ /H_2O^+ ratio was 1.6 × 10^2. In order to investigate possible competition of K^+ and H_2O^+, cells were suspended in 20 mM bis(Tris)-propane, pH 6.5, instead of 50 mM KP_i. A control experiment showed that the presence of bis(Tris)-propane does not affect lactococcin G activity in terms of permeation of small monovalent cations. No change in ΔpH was observed after addition of lactococcin G, which confirms lactococcin G’s inability to conduct protons.

**The lactococcin G α and β peptides can interact independently with intact cells.** Lactococcin G is active against cells only when both the α and β peptides are present, preferentially in stoichiometric amounts (10; see also Table 1). However, growth inhibition is also observed when the cells are first pre-treated with one peptide, followed by extensive washing, and subsequently supplemented with the complementary peptide (Table 1). This suggests that the α peptide alone, as well as the β peptide, can interact stably with the target cell surface, without losing its potential bactericidal activity in this idle state. In agreement with the data presented above, cells treated with one peptide can accumulate rubidium ions, and only when the complementary peptide is added does rubidium ion efflux occur (Table 1). In contrast, no lactococcin G activity is observed when cells treated with one peptide are mixed with cells treated with the complementary peptide. This demonstrates that the lactococcin G peptide is unable to diffuse to another cell once it is bound to the cell surface.

**Lactococcin G action strongly depends on the extracellular pH.** We noted that a premix of both α and β peptides at pH 5.5 caused efflux of ^86^Rb^+^ from cells. On the other hand, pretreatment of the cells with one peptide at pH 5.5 followed by the addition of the complementary peptide caused an attenuation of ^86^Rb^+^ uptake, rather than efflux (data not shown). This difference between premixing and no premixing of the peptides was not observed at pH 6.8 or when cells were both pre-treated with one peptide and washed at pH 5.3 and then subsequently supplemented with the complementary peptide at pH 6.8. These data are indicative of the effect of pH on the interaction of the two peptides. Figure 5 shows the pH dependence of lactococcin G-mediated ^86^Rb^+^ efflux. The rate of ^86^Rb^+^ efflux increased with pH, whereas in the absence of lactococcin G no efflux occurred. Curve fitting of the pH dependence data suggests an apparent pK of 6.69 ± 0.07 (mean ± standard deviation). When cells loaded with ^86^Rb^+^ were depleted of ATP by incubation in the presence of deoxy-glucose prior to lactococcin G addition, the same pH dependence of lactococcin G-induced efflux of ^86^Rb^+^ was observed.

**TABLE 1. α and β peptides can interact independently with cells.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IC_{50} (nM) ^86^Rb^+^ efflux</th>
</tr>
</thead>
<tbody>
<tr>
<td>α</td>
<td>&gt;29.0</td>
</tr>
<tr>
<td>β</td>
<td>&gt;29.0</td>
</tr>
<tr>
<td>α + β</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>β Cells + α</td>
<td>0.06 ± 0.01</td>
</tr>
<tr>
<td>α Cells + β</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>α Cells + β cells</td>
<td>NA^b</td>
</tr>
</tbody>
</table>

^a^ β cells and α cells, cells pretreated with β and α peptides, respectively. Peptide-treated cells were obtained by treating cells in medium of pH 6.8 for 3 min with 29 nM peptide at 220 µg of cellular protein/ml followed by four washes. IC_{50}, 50% inhibitory concentration. IC_{50} and ^86^Rb^+^ efflux were determined as described in Materials and Methods.

^b^ NA, no activity.
Activity increases with the pH, with an apparent pK of about 6.7. Lactococcin G is pH dependent and that deprotonation of an amino acid chain with a pK of 6.7 is critical for the mode of action.

**DISCUSSION**

Most bacteriocins are one-peptide systems. The present work provides studies on the mechanism of action of a two-component bacteriocin, lactococcin G. Both the α and β peptides are predicted to form an α-helical amphipathic structure (14). The premix-dependent activity at low pH observed in our complementation studies indicates that the peptides interact. It seems therefore that a complex of α and β peptides forms a transmembrane pore. In *L. lactis* cells, potassium, sodium, and phosphate are the most abundant inorganic ions. Here we demonstrate that lactococcin G not only induces potassium ion efflux (10) but also sodium and other monovalent cation (in)flux. On the other hand, neither phosphate, another anion, nor divalent cation conductance by lactococcin G was observed. The highest conductivity was measured for potassium and sodium ions. Studies in the present work support previous data (10) indicating that lactococcin G does not conduct protons.

Lactococcin G causes an influx of sodium ions into the cells (Fig. 1B). Intracellular sodium is known to be cytotoxic, although the mechanism of this toxicity is incompletely understood (2). The extensive cation fluxes into and out of the cell cause an osmotic imbalance of the cell's turgor pressure, a collapse of both the transmembrane Na⁺ gradient and ΔΨ (10), and indirectly—ATP depletion (10). Consequently, Na⁺-coupled transport and ΔΨ- and ATP-requiring (transport) processes are arrested. The above effects together explain the bactericidal activity.

Lactococcin G differs from some other two-component systems by neither dissipating ΔpH, causing F⁻ efflux, nor being active on liposomes. Acidocin J1132 (19) and thermophilin 13 (8) dissipate both ΔpH and ΔΨ. Lactacin F (1) seems to cause efflux of both potassium and inorganic phosphate.

Lactococcin G exhibits a prominent pH dependence. The activity increases with the pH, with an apparent pK of about 6.7. Histidine is the only amino acid with a pK of that range. The very C-terminal residue of the α peptide is the only histidine in lactococcin G. Therefore, involvement of this histidine in pH dependence seems very likely. The pH dependence strongly suggests that this histidine needs to be deprotonated before functional membrane interaction is possible or before a functional complex can be formed between the α and β peptides. Strikingly, in the case of lactococcin A (5) and lactocin S (11), the last C-terminal residues are two histidines. Lactocin S is active only below pH 6.0 (12), suggesting that in this case the two C-terminal histidines of lactocin S both need to be protonated for interaction with the target cell surface. It will be of interest to establish, by mutagenesis studies on the α peptide of lactococcin G, whether the pH range can be extended and the target specificity can be modified.

**ACKNOWLEDGMENTS**

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