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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  $\alpha$  and  $\beta$ . Biologically active, synthetic lactococcin G was used to study the mode of action on sensitive cells of *Lactococcus lactis*. The  $\alpha$  and  $\beta$  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  $\alpha$  and  $\beta$  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  $\alpha$  and  $\beta$ , 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-to-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 <sup>86</sup>Rb<sup>+</sup>. 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.** <sup>86</sup>Rubidium (<sup>86</sup>Rb<sup>+</sup>) (1 mCi/1.1 mg), <sup>22</sup>Na (1 mCi/3.7 mg), <sup>32</sup>P<sub>i</sub> (1 mCi/0.11 nmol), and [<sup>14</sup>C]choline (7.2 mCi/mmol) were obtained from Amersham UK. Gramicidin A' was obtained from Sigma (St. Louis, Mo.). The bacteriocin peptides were dissolved in 55% (vol/vol) isopropanol and 0.1% (vol/vol) trifluoroacetic acid and mixed in a 1-to-1 ratio.

**Peptide synthesis, purification, and analysis of the polypeptides.** The lactococcin G  $\alpha$  and  $\beta$  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 growth phase.

**Bacteriocin assay.** Bacteriocin activity was measured as previously described (14). Briefly, 200  $\mu$ l of culture medium (supplemented with 0.1% [vol/vol] Tween 80), bacteriocin fractions at twofold dilutions, and the indicator strain ( $A_{600} =$

0.1) were added to each well of a microtiter plate. After 3 h of incubation at 30°C, growth inhibition of the indicator strain was measured spectrophotometrically at 600 nm with a microplate reader (Titertek Multiskan MCC/340 MKII, type 347).

**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'*-bis(2-ethanesulfonic acid)] (pH 7.0) and 0.5% glucose for 2 min at 30°C, and subsequently <sup>86</sup>Rb<sup>+</sup> (0.45  $\mu$ Ci/ml) was added. Lactococcin G was added after 20 min and, at intervals, samples were filtered over 0.45- $\mu$ m-pore-size cellulose nitrate filters (Millipore Corp.) and washed twice with 2 ml of ice-cold 0.1 M LiCl as described previously (4). <sup>22</sup>Na and [<sup>14</sup>C]choline were loaded into the cells by overnight incubation of a 30- $\mu$ l suspension of cells (140  $\mu$ g of protein) in medium without glucose containing either 4  $\mu$ Ci of <sup>22</sup>Na or 2  $\mu$ Ci of [<sup>14</sup>C]choline. Ion-loaded cells were diluted in 800  $\mu$ l of 50 mM KP<sub>i</sub> (pH 7.0), and 100- $\mu$ l samples were filtered and washed as above. The lactococcin G-mediated cation efflux rate depended on the initial intracellular concentration of radiolabelled cation.

**Phosphate flux assays.** Efflux of unlabelled phosphate was measured as follows. Cells in 50 mM Na-PIPES, pH 7.0, were incubated at 30°C (5.3 mg of protein/ml). At time points up to 10 min, samples of 340  $\mu$ l were subjected to silicon oil centrifugation (6), separating the cells from the buffer. The amount of released phosphate in the supernatant fraction was determined as described by Rouser et al. (18). For phosphate (<sup>32</sup>P<sub>i</sub>) transport, cells were suspended in 50 mM MES (morpholineethanesulfonic acid) buffer, pH 7.0 (300  $\mu$ g of protein/ml), and supplemented with <sup>32</sup>P<sub>i</sub> (4.7  $\mu$ Ci/ml). In order to avoid glucose-(bis)phosphate formation, cells were energized with 0.5% (wt/vol) malate (17). After a 5-min uptake, lactococcin G (40 nM) was added either directly or after the addition of valinomycin and nigericin (0.2  $\mu$ M each), which blocked further uptake of <sup>32</sup>P<sub>i</sub>. Samples were filtered and washed with LiCl as described above. Radioactivity was measured by liquid scintillation counting in a Packard Tri-Carb 460 CD counter (Packard Instruments Corp.).

**Measurements of proton motive force.** Generation of a transmembrane electrical potential ( $\Delta\Psi$ ) was measured with the fluorescent probe 3,3'-dipropylthiadicarbocyanine iodide [DiSC<sub>3</sub>(5)]. Cells were used only directly after isolation. The transmembrane pH gradient ( $\Delta$ pH) was measured by loading the cells with the fluorescent pH indicator 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) as described previously (9).

**Miscellaneous methods.** Protein measurements were performed according to the method of Lowry et al. (7). ATP depletion was induced by incubation for 30 min at 30°C with freshly prepared 20 mM deoxyglucose.

### 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 (<sup>22</sup>Na<sup>+</sup>). When diluted into buffer, the cells only slowly released the <sup>22</sup>Na<sup>+</sup> (Fig. 1A). <sup>22</sup>Na<sup>+</sup> efflux was not in-

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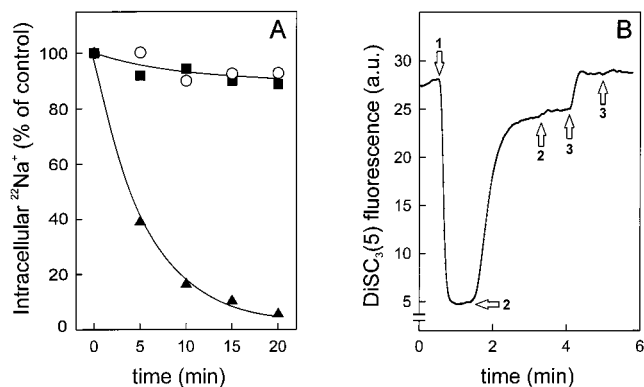


FIG. 1. Lactococcin G causes passage of sodium ions. (A) *L. lactis* cells were loaded with  $^{22}\text{Na}^+$  as described in Materials and Methods. At time zero, cells were diluted in 50 mM  $\text{KP}_i$  to 174  $\mu\text{g}$  of protein/ml, and either 50 nM lactococcin G ( $\blacktriangle$ ), 0.25  $\mu\text{M}$  valinomycin ( $\blacksquare$ ), or solvent ( $\circ$ ) was added. (B) In *L. lactis* cells (28  $\mu\text{g}$  of protein/ml) suspended in 50 mM Na-PIPES, pH 7.0, a  $\Delta\Psi$  was generated by 0.25  $\mu\text{M}$  valinomycin (arrow 1), after which 20 nM lactococcin G (arrows 2) and 6.7  $\mu\text{M}$  nisin (arrows 3) were added.

duced by the addition of solvent or valinomycin. In contrast, lactococcin G addition resulted in rapid and complete release of sodium ions (Fig. 1A). Parallel control experiments with cells loaded overnight by diffusion with  $^{86}\text{Rb}^+$  showed that lactococcin G induced  $^{86}\text{Rb}^+$  efflux rates comparable to those of  $^{22}\text{Na}^+$  (data not shown). This implies that lactococcin G is not only specific for potassium ions (10) but conducts sodium ion movements as well.

Lactococcin G also dissipates the valinomycin-induced  $\Delta\Psi$  of cells suspended in 50 mM Na-PIPES buffer as monitored by the use of the cyanine dye DiSC<sub>3</sub>(5) (Fig. 1B). This implies that the valinomycin-mediated potassium ion efflux is counteracted by a lactococcin G-mediated sodium ion influx. Dissipation, however, is not complete. The intracellular potassium concentration can be as high as 800 mM (15), leaving the possibility open that the ion concentration on the outside is too low to counterbalance the valinomycin-induced potassium flux. Indeed, lactococcin G virtually completely dissipated the  $\Delta\Psi$  generated in 300 mM sodium or potassium (data not shown).

When cells were diluted in 50 mM  $\text{KP}_i$  (Fig. 2B) or in 50 mM Na-PIPES (Fig. 2C), addition of lactococcin G resulted in generation of  $\Delta\Psi$  akin to the valinomycin-induced  $\Delta\Psi$  when cells were diluted into 50 mM  $\text{KP}_i$  (Fig. 2A). When the sodium ion concentration on the outside was raised from 50 to 300 mM, addition of lactococcin G did not result in the generation of a  $\Delta\Psi$  (Fig. 2D). Similar results were obtained when sodium was replaced by potassium ion (data not shown). The  $\Delta\Psi$  was not induced when only  $\alpha$  peptide or  $\beta$  peptide was added to cells, nor when prior to lactococcin G addition an excess of the nonspecific pore-forming bacteriocin nisin (6.7  $\mu\text{M}$ ) was added. Addition of valinomycin after  $\Delta\Psi$  induction by lactococcin G did not affect the  $\Delta\Psi$  (data not shown).

**Lactococcin G induces choline flux.** In order to define more precisely the specificity of lactococcin G pores, experiments similar to those above were performed with the choline ion. Lactococcin G causes efflux of [ $^{14}\text{C}$ ]choline from cells which have been loaded overnight by diffusion (Fig. 3). Likewise it dissipates—although not completely—the  $\Delta\Psi$  induced by valinomycin in cells suspended in 50 mM choline chloride (data not shown). Similar results were obtained with lithium, cesium, tetramethyl-ammonium, or Tris as the counteracting cation (data not shown), which demonstrates that lactococcin G permeabilizes the membrane for a broad range of monovalent cations. The rate of lactococcin G-mediated dissipation of the

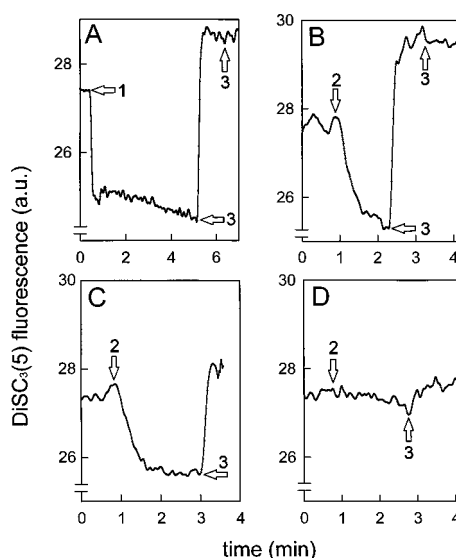


FIG. 2. Lactococcin G can induce a  $\Delta\Psi$ . *L. lactis* cells were suspended at pH 7.0 either in 50 mM  $\text{KP}_i$  (A and B), in 50 mM Na-PIPES (C), or in 300 mM Na-PIPES (D). The final cell protein concentration was 28  $\mu\text{g}/\text{ml}$ . Valinomycin at 0.2  $\mu\text{M}$  (arrow 1) and 20 nM lactococcin G (arrows 2) generated a  $\Delta\Psi$ , whereas excess (6.7  $\mu\text{M}$ ) nisin (arrows 3) dissipated the  $\Delta\Psi$ .

$\Delta\Psi$  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  $\text{MgCl}_2$ , 50 mM  $\text{MgSO}_4$ , or 50 mM bis(Tris)-propane (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 effect the release of accumulated  $^{32}\text{P}_i$  from malate-energized cells (data not shown). This demonstrates that lactococcin G does not

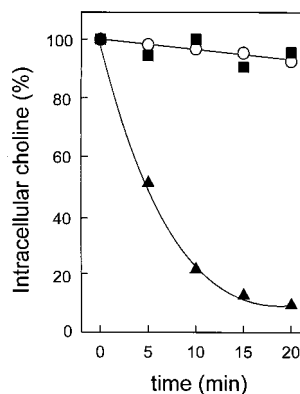


FIG. 3. Lactococcin G causes choline flux. *L. lactis* cells, loaded with [ $^{14}\text{C}$ ]choline, were diluted in 50 mM  $\text{KP}_i$  (174  $\mu\text{g}$  protein/ml) at time zero, and either 50 nM lactococcin G ( $\blacktriangle$ ), 30  $\mu\text{M}$  carbonyl cyanide *m*-chlorophenylhydrazone ( $\blacksquare$ ), or solvent ( $\circ$ ) was added.

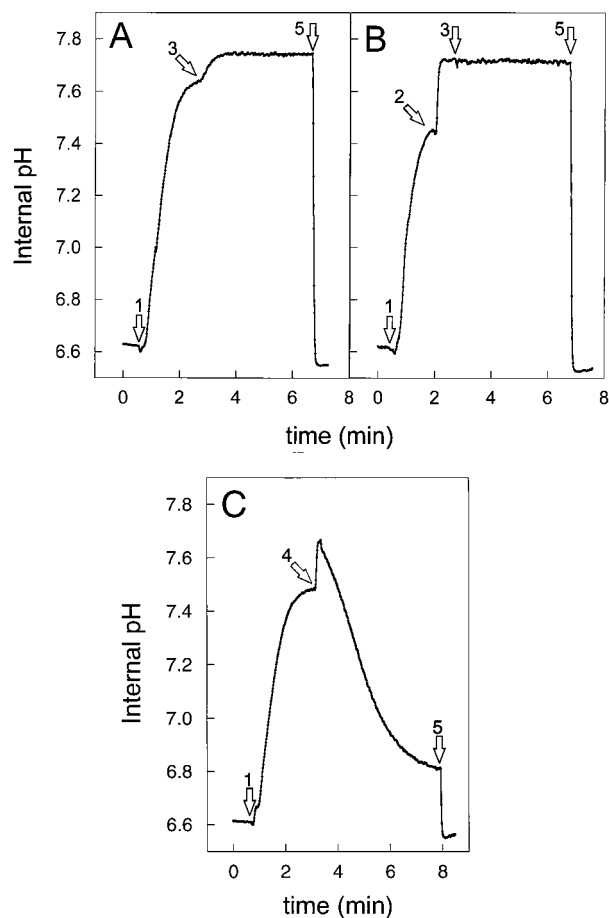


FIG. 4. Lactococcin G causes an increase in the  $\Delta$ pH. Cells (28  $\mu$ g of protein/ml) loaded with BCECF were energized in 50 mM  $\text{KPi}$ , pH 6.5, with 0.5% glucose (arrows 1). (A) Addition of 20 nM lactococcin G (arrow 3) and 0.2  $\mu$ M nigericin (arrow 5). (B) Addition of 0.2  $\mu$ M valinomycin (arrow 2) and 20 nM lactococcin G (arrow 3), followed by 0.2  $\mu$ M nigericin (arrow 5). (C) Addition of 12 nM gramicidin A' (arrow 4) followed by addition of 0.2  $\mu$ M nigericin (arrow 5).

conduct the transmembrane movement of one of the major cytosolic constituents.

**Lactococcin G causes an increase in the  $\Delta$ pH.** Previous experiments with cells loaded with the fluorescent pH indicator BCECF showed that after addition of valinomycin, lactococcin G is unable to dissipate the  $\Delta$ pH (10). These experiments were performed in the presence of valinomycin to assure that the  $\Delta$ pH 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  $\Delta\Psi$  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  $\Delta$ pH. 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  $\Delta$ pH in *L. lactis* cells, but this process is immediately followed by a steep decrease in  $\Delta$ pH (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  $\text{K}^+/\text{H}_3\text{O}^+$  ratio was  $1.6 \times 10^5$ . In order to investigate possible competition of  $\text{K}^+$  and  $\text{H}_3\text{O}^+$ , cells were suspended in 20 mM bis(Tris)-propane, pH 6.5, instead of 50 mM  $\text{KPi}$ . 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  $\Delta$ pH was observed after addition of lactococcin G, which confirms lactococcin G's inability to conduct protons.

**The lactococcin G  $\alpha$  and  $\beta$  peptides can interact independently with intact cells.** Lactococcin G is active against cells only when both the  $\alpha$  and  $\beta$  peptides are present, preferentially in stoichiometric amounts (10; see also Table 1). However, growth inhibition is also observed when the cells are first pretreated with one peptide, followed by extensive washing, and subsequently supplemented with the complementary peptide (Table 1). This suggests that the  $\alpha$  peptide alone, as well as the  $\beta$  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  $\alpha$  and  $\beta$  peptides at pH 5.3 caused efflux of  $^{86}\text{Rb}^+$  from cells. On the other hand, pretreatment of the cells with one peptide at pH 5.3 followed by the addition of the complementary peptide caused an attenuation of  $^{86}\text{Rb}^+$  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 pretreated 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  $^{86}\text{Rb}^+$  efflux. The rate of  $^{86}\text{Rb}^+$  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  $6.69 \pm 0.07$  (mean  $\pm$  standard deviation). When cells loaded with  $^{86}\text{Rb}^+$  were depleted of ATP by incubation in the presence of deoxyglucose prior to lactococcin G addition, the same pH dependence of lactococcin G-induced efflux of  $^{86}\text{Rb}^+$  was observed

TABLE 1.  $\alpha$  and  $\beta$  peptides can interact independently with cells<sup>a</sup>

Treatment	IC <sub>50</sub> (nM)	<sup>86</sup> Rb <sup>+</sup> efflux
$\alpha$	>29.0	—
$\beta$	>29.0	—
$\alpha + \beta$	$0.05 \pm 0.01$	+
$\beta$ Cells + $\alpha$	$0.06 \pm 0.01$	+
$\alpha$ Cells + $\beta$	$0.05 \pm 0$	+
$\alpha$ Cells + $\beta$ cells	NA <sup>b</sup>	—

<sup>a</sup>  $\beta$  cells and  $\alpha$  cells, cells pretreated with  $\beta$  and  $\alpha$  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  $\mu$ g of cellular protein/ml followed by four washes. IC<sub>50</sub>, 50% inhibitory concentration. IC<sub>50</sub> and  $^{86}\text{Rb}^+$  efflux were determined as described in Materials and Methods.

<sup>b</sup> NA, no activity.

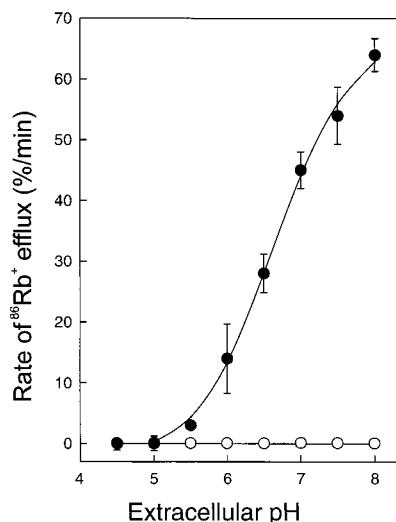


FIG. 5. pH dependence of lactococcin G. *L. lactis* cells (0.25  $\mu\text{g}/\text{ml}$ ) that had taken up  $^{86}\text{Rb}^+$ , as described in Materials and Methods, were subsequently subjected at various pHs to lactococcin G (58 nM) (●) or to solvent (○), and initial  $^{86}\text{Rb}^+$  efflux was measured. The apparent pK was  $6.69 \pm 0.07$ .

(data not shown). These results suggest that 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  $\alpha$  and  $\beta$  peptides are predicted to form an  $\alpha$ -helical amphipathic structure (14). The premixing-dependent activity at low pH observed in our complementation studies indicates that the peptides interact. It seems therefore that a complex of  $\alpha$  and  $\beta$  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, other 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  $\text{Na}^+$  gradient and  $\Delta\Psi$  (10), and—indirectly—ATP depletion (10). Consequently,  $\text{Na}^+$ -coupled transport and  $\Delta\Psi$ - 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  $\Delta\text{pH}$ , causing  $\text{P}_i$  efflux, nor being active on liposomes. Acidocin J1132 (19) and thermophilin 13 (8) dissipate both  $\Delta\text{pH}$  and  $\Delta\Psi$ . 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<sub>a</sub> in that range. The very C-terminal residue of the  $\alpha$  peptide is the only histi-

dine 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  $\alpha$  and  $\beta$  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  $\alpha$  peptide of lactococcin G, whether the pH range can be extended and the target specificity can be modified.

## ACKNOWLEDGMENTS

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