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The Bacteriocin Lactococcin A Specifically Increases Permeability of Lactococcal Cytoplasmic Membranes in a Voltage-Independent, Protein-Mediated Manner

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Lactococcin A is a bacteriocin produced by *Lactococcus lactis*. Its structural gene has recently been cloned and sequenced (M. J. van Belkum, B. J. Hayema, R. E. Jeeninga, J. Kok, and G. Venema, Appl. Environ. Microbiol. 57:492–498, 1991). Purified lactococcin A increased the permeability of the cytoplasmic membrane of *L. lactis* and dissipated the membrane potential. A significantly higher concentration of lactococcin A was needed to dissipate the membrane potential in an immune strain of *L. lactis*. Lactococcin A at low concentrations (0.029 μg/mg of protein) inhibited secondary and phosphate-bond driven transport of amino acids in sensitive cells and caused efflux of preaccumulated amino acids. Accumulation of amino acids by immune cells was not affected by this concentration of lactococcin A. Lactococcin A also inhibited proton motive force-driven leucine uptake and leucine counterflow in membrane vesicles of the sensitive strain but not in membrane vesicles of the immune strain. These observations indicate that lactococcin A makes the membrane permeable for leucine in the presence or absence of a proton motive force and that the immunity factor(s) is membrane linked. Membrane vesicles of *Clostridium acetobutylicum*, *Bacillus subtilis*, and *Escherichia coli* were not affected by lactococcin A, nor were liposomes derived from phospholipids of *L. lactis*. These results indicate that lactococcin A acts on the cytoplasmic membrane and is very specific towards lactococci. The combined results obtained with cells, vesicles, and liposomes suggest that the specificity of lactococcin A may be mediated by a receptor protein associated with the cytoplasmic membrane.

For many years it has been known that lactic acid bacteria produce inhibitory substances which are important in food fermentation and preservation. In several cases it has been shown that the antagonistic activity resulted from metabolic end products such as hydrogen peroxide, diacetyl, and organic acids (19). However, many strains of lactic acid bacteria also secrete antimicrobial proteins, termed bacteriocins. Although a large number of lactic acid bacterial bacteriocins have been described (13), little is known about their mode of action. The best-characterized bacteriocin produced by lactic acid bacteria is nisin, which belongs to the group of lantibiotics. It is produced by several strains of *Lactococcus lactis*. Nisin has a broad activity spectrum and is active against a variety of gram-positive bacteria. It has been shown that nisin disrupts the cytoplasmic membrane, thereby dissipating the membrane potential, inhibiting transport of amino acids, and causing release of accumulated amino acids from cells and cytoplasmic membrane vesicles derived from bacteria such as *Staphylococcus cohnii* and *Bacillus subtilis* (26). In addition, membrane vesicles and osmotically shocked cells of the gram-negative bacterium *Escherichia coli* were affected by nisin, suggesting that the outer membrane is a barrier for the lantibiotic (18). Nisin requires an energized membrane for its activity (8, 26), which appeared to be dependent on the phospholipid composition of membranes (8). At present, nisin is the only lactococcal bacteriocin commercially used in food preservation.

Another bacteriocin produced by lactic acid bacteria of which the mode of action has been analyzed to some extent, is lactostrepcin 5 (Las 5). Leakage of ATP and K^+ ions from *L. lactis* cells by Las 5 has been described by Zajdel et al. (36). Protoplasts and cells pretreated with trypsin were less affected by Las 5, and it has been suggested that a receptor in the cell wall was necessary for activity of this bacteriocin.

Recently, Holo et al. (10) have purified and characterized the bacteriocin lactococcin A, which is produced by an *L. lactis* subsp. *cremoris* strain. The amino acid sequence of this bacteriocin was determined, and its structural gene was subsequently cloned and sequenced. Lactococcin A, which acts only on lactococci, appeared to be identical to the bacteriocin encoded by one of the bacteriocin operons of p9B4-6 (10, 31). This 60-kb plasmid, isolated from *L. lactis* subsp. *cremoris* 9B4 (20), encodes bacteriocin production and immunity. The plasmid contains three operons coding for lactococcin A, B, and M, as well as for the corresponding immunity proteins (30–32). Lactococcin A is synthesized as a precursor of 75 amino acids and is processed by removal of 21 N-terminal amino acids to yield the mature molecule of 54 amino acids (10). *L. lactis* cells containing one of the genetic determinants for lactococcin A, B, or M were capable of inhibiting cells containing one of the other lactococcin determinants (30, 32).

Here we report that lactococcin A specifically increases the permeability of the cytoplasmic membrane of whole cells and membrane vesicles of *L. lactis* in a voltage-independent, protein-mediated manner. To our knowledge, lactococcin A is the first bacteriocin from a gram-positive bacterium of which not only the structural and immunity genes but also its mode of action are well characterized.

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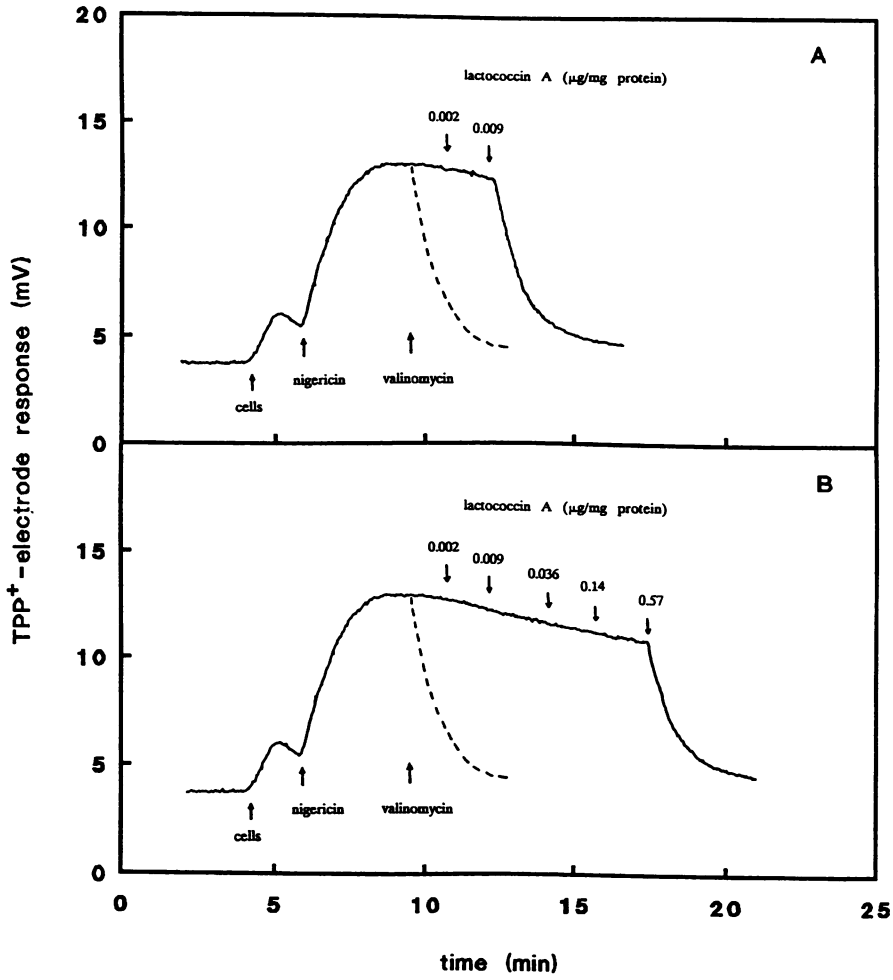

FIG. 1. Effects of lactococcin A on the membrane potential of energized cells of *L. lactis* IL1403 (A) and *L. lactis* IL1403(pMB563) (B). The membrane potential was measured in the presence of nigericin with a TPP^+ ion-selective electrode as described in Materials and Methods. The effect of valinomycin (1 μM) is indicated with the broken lines. Cells of IL1403 and IL1403(pMB563) were treated with increasing concentrations of lactococcin A.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and purification of lactococcin A. *L. lactis* subsp. *lactis* IL1403, *L. lactis* subsp. *lactis* ML3, and *L. lactis* subsp. *cremoris* Wg2 were grown at 30°C in MRS (2) broth containing 0.5% glucose. For maintaining the plasmid of *L. lactis* subsp. *lactis* IL1403 (pMB563), a selective concentration of erythromycin of 5 μg/ml was used. *E. coli* ML308-225 and *B. subtilis* W23 were grown as described by Kaback (12) and de Vrij et al. (3), respectively. *Clostridium acetobutylicum* NCIB 8025 was grown as described by Driessen et al. (5). Lactococcin A was purified as described previously (10) and stored at a concentration of 0.2 mg/ml in 60% ethanol–2.5 mM sodium phosphate (pH 7.3) at −20°C.

Chemicals. [1-^{14}C]2-α-amino-isobutyric acid (AIB) (59 mCi/mmol), L-[U-^{14}C]glutamate (285 mCi/mmol), and L-[U-^{14}C]leucine (312 mCi/mmol) were obtained from the Radiochemical Centre, Amersham, United Kingdom. L-Leucine, valinomycin, and nigericin were purchased from Sigma Chemical Co. (St. Louis, Mo.). All other chemicals were reagent grade and obtained from commercial sources.

Transport assays with whole cells and measurement of the membrane potential. *L. lactis* cells were grown to an optical density at 600 nm of 0.6 (MCP Vitraton, Vital Scientific, Dieren, The Netherlands); harvested; washed; concentrated 50-fold in buffer containing 50 mM potassium 2(*N*-morpholino)ethanesulfonic acid (MES) (pH 6), 50 mM KCl, and 2 mM $MgSO_4$; and stored on ice. Transport assays were performed at 30°C by adding 0.1 ml of cell suspension to 1.9 ml of the same buffer supplemented with 0.4% glucose as the energy source. The cells were incubated for 3 min before uptake was initiated by the addition of the radiolabeled substrates. At desired intervals, samples (0.1 ml) were taken from the incubation mixture, diluted in 2 ml of 0.1 M LiCl, filtered on a 0.45-μm-pore-size cellulose nitrate filter (Schleicher and Schuell, Dassel, Germany), and washed with 2 ml of 0.1 M LiCl. The filters were dried, and the radioactivity was measured by liquid scintillation spectrometry.

The membrane potential of the cells (inside negative) was monitored by the distribution of the lipophilic cation tetraphenylphosphonium (TPP^+) by using a TPP^+-selective electrode (29). TPP^+ was added to a concentration of 4 μM. By

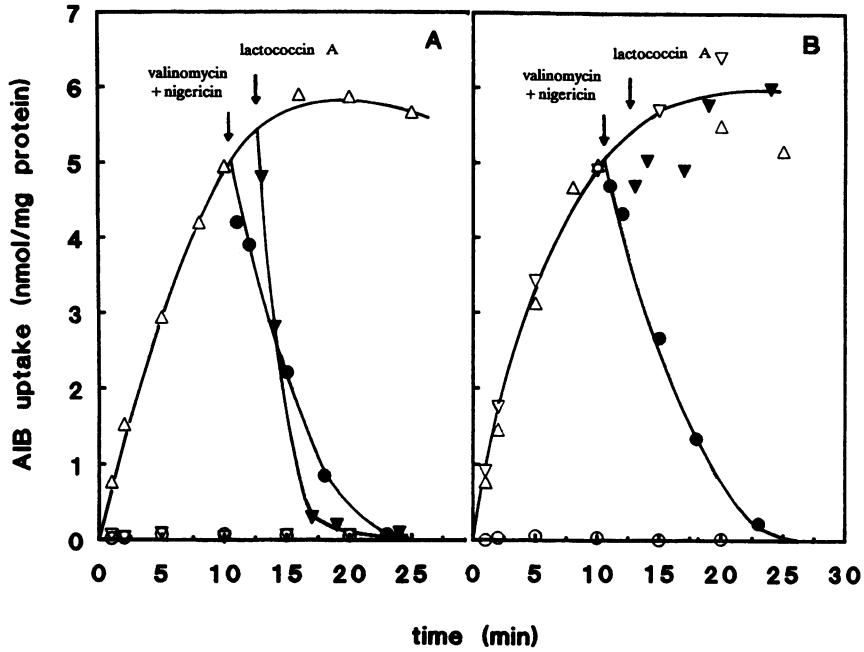

FIG. 2. Effects of lactococcin A on AIB uptake in energized cells of *L. lactis* IL1403 (A) and *L. lactis* IL1403(pMB563) (B). The assay was started by the addition of 8.5 μM ^{14}C-labeled AIB to the incubation mixture. At times indicated the suspension was subdivided into portions to which were added: valinomycin (1 μM) plus nigericin (0.5 μM) (●) or lactococcin A (0.029 μg/mg of protein) (▼). No additions were made to the control (△). Uptake by cells preincubated for 3 min with valinomycin plus nigericin (○) or lactococcin A (▽) is indicated.

the addition of the K^+-H^+ exchanger nigericin (0.5 μM), the pH gradient was dissipated such that the proton motive force was composed of the membrane potential only (4).

Preparation of vesicles and liposomes. Membrane vesicles of *L. lactis* and *C. acetobutylicum* were prepared according to the method of Otto et al. (21). The method for preparing *E. coli* membrane vesicles was described by Kaback (12) and that for *B. subtilis* membrane vesicles by Konings et al. (14). Lipids of *L. lactis* were isolated as described by Driessen et al. (6) and dissolved in $CHCL_3$-MeOH (9:1 [vol/vol]). The lipids were subsequently dried in vacuo and resuspended in a buffer containing 50 mM KP_i (pH 6) and 2 mM $MgSO_4$ at a concentration of 20 mg of lipid per ml by using a bath sonicator (Sonicor; Sonicon Instrument Corporation, Copiague, N.Y.). Liposomes were obtained by sonication of the lipids at 0°C (amplitude, 4 μm; eight cycles of 15 s of sonication with intervals of 45 s) with a probe sonicator (Soniprep 150; MSE Scientific Instruments, West-Sussex, United Kingdom) under a constant flow of N_2 and subsequently treated with valinomycin (0.1 nmol/mg of lipid). A K^+ diffusion potential was generated by diluting the liposome suspension 100-fold in a buffer containing 50 mM NaP_i (pH 6) and 2 mM $MgSO_4$. The membrane potential was monitored by the distribution of TPP^+ as described above.

Transport driven by an imposed proton motive force or by counterflow in membrane vesicles. To measure amino acid uptake driven by an imposed proton motive force, the membrane vesicles were incubated for 30 min in the presence of valinomycin (2 nmol/mg of protein), centrifuged, and resuspended in a buffer containing 20 mM KP_i (pH 6), 100 mM K acetate, and 2 mM $MgSO_4$. A membrane potential (inside negative) and a pH gradient (inside alkaline) were generated upon a 100-fold dilution of a concentrated membrane vesicle suspension (20 mg of protein per ml) in a buffer containing 20 mM NaP_i (pH 6) and 100 mM Na-piperazine-*N*,*N*′-bis(2-ethanesulfonate) (NaPIPES), supplemented with 2 mM $MgSO_4$. The assay was performed at 30°C in 200 μl and uptake of ^{14}C-labeled amino acids was monitored by filtering the membrane vesicle suspension and measuring the radioactivity as described above. For counterflow experiments, membrane vesicles were incubated for 3 h at 25°C in 50 mM KP_i (pH 6) containing 2 mM $MgSO_4$ and 5 mM leucine, unless indicated otherwise. The membrane vesicles were subsequently concentrated (20 mg of protein per ml), and 4 μl of the suspension was diluted 50-fold in 200 μl of 50 mM KP_i (pH 6), 2 mM $MgSO_4$, and 3 μM ^{14}C-labeled leucine. Uptake was monitored by filtration as described above.

RESULTS

Effect of lactococcin A on the membrane potential of whole cells. Lactococcin A specifically inhibits the growth of lactococcal strains (10). Since lactococcin A is a small hydrophobic polypeptide like several peptide antibiotics permeabilizing the cytoplasmic membrane (7, 8, 17, 26, 28), a likely target for its action could be the cytoplasmic membrane. To investigate its mode of action, the effect of increasing concentrations of lactococcin A on the membrane potential was measured in sensitive *L. lactis* IL1403 cells (Fig. 1), as well as in *L. lactis* IL1403(pMB563). The latter strain contains plasmid pMB563 that carries the immunity gene *lciA* specific for lactococcin A but does not produce lactococcin A (31). The *lciA* gene had been placed under control of the lactococcal promoter P59 (33) and gave rise to the same level of lactococcin A immunity, as did the plasmid containing the complete lactococcin A operon. The membrane potential of IL1403 was dissipated by a lactococcin A concentration of 0.009 μg/mg of protein at about the same rate as was observed upon addition of 1 μM valinomycin (Fig. 1A). In

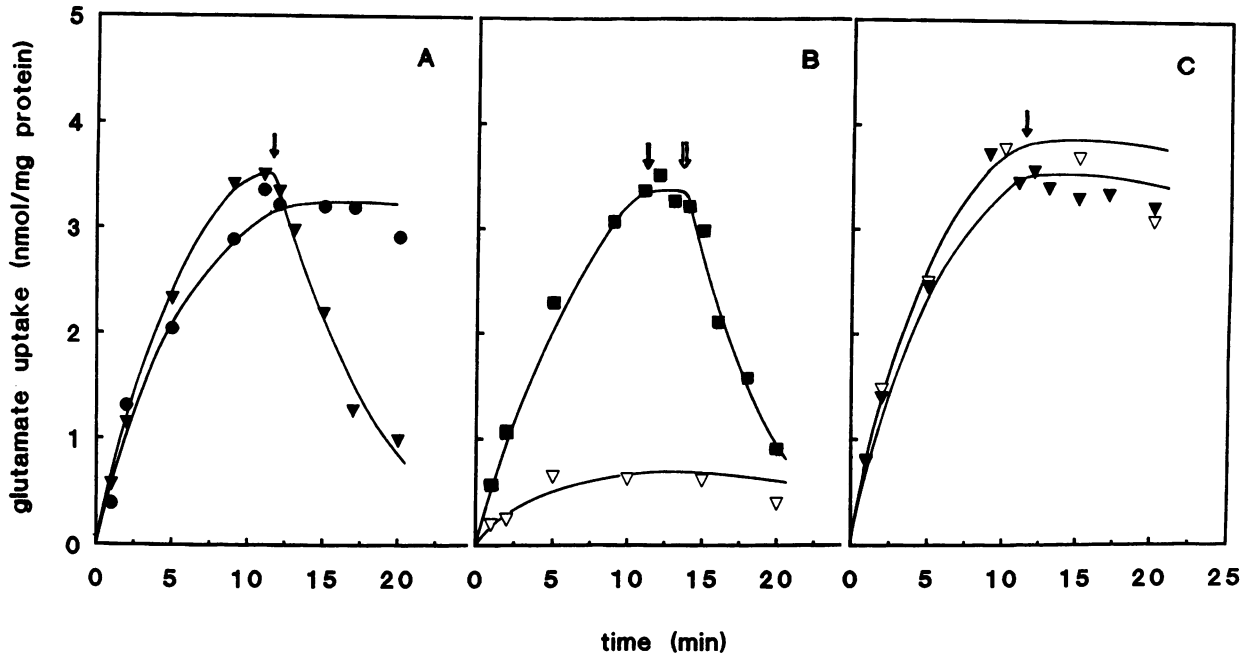

FIG. 3. Effects of lactococcin A on L-glutamate uptake in energized cells of *L. lactis* IL1403 (A and B) and *L. lactis* IL1403(pMB563) (C). In separate experiments valinomycin (1 μM) plus nigericin (0.5 μM) (●) or lactococcin A (0.029 μg/mg of protein) (▼) were added to the cell suspension of strain IL1403 or strain IL1403(pMB563) at the times indicated by the arrows. Uptake was also monitored in an experiment (B) in which first valinomycin (1 μM) plus nigericin (0.5 μM) and subsequently lactococcin A (0.029 μg/mg of protein) were added to the cell suspension (indicated with the closed and the open arrow, respectively) (■). Uptake in cells of IL1403 and IL1403(pMB563) preincubated for 3 min with lactococcin A (0.029 μg/mg of protein) for 3 min is indicated (▽). The assays were started by the addition of 1.75 μM ^{14}C-labeled glutamate.

contrast, the membrane potential of IL1403(pMB563) only dissipated by increasing the concentration of lactococcin A to 0.57 μg/mg of protein (Fig. 1B). These results reveal that lactococcin A has at the effective concentration an immediate effect on the membrane potential of intact cells of *L. lactis*, and that *lciA* renders *L. lactis* cells within certain limits insensitive to lactococcin A.

Influence of lactococcin A on amino acid uptake in whole cells. The dissipation of the membrane potential by lactococcin A suggests that it affects the permeability of the cytoplasmic membrane. To investigate this further, the effect of lactococcin A on amino acid transport was studied. A lactococcin A concentration of 0.029 μg/mg of cell protein dissipated the membrane potential of strain IL1403 but not of strain IL1403(pMB563) (Fig. 1). This concentration, which corresponds to approximately 600 lactococcin A molecules added per sensitive or immune cell, was chosen in the amino acid uptake experiments by whole cells of both strains. The effect of lactococcin A on the uptake of AIB, a nonmetabolizable analog of alanine, was studied in energized cells of IL1403 and IL1403(pMB563) (Fig. 2). AIB is taken up in symport with one proton and is driven by the proton motive force (15). Cells of both strains take up AIB at a high rate. Uptake of AIB was completely blocked by the uncoupling action of the ionophore combination valinomycin and nigericin (1 and 0.5 μM, respectively). Also, efflux of accumulated AIB upon addition of valinomycin and nigericin was observed (Fig. 2A and B). Lactococcin A had a very similar effect. Preincubating cells of IL1403 with lactococcin A completely inhibited AIB uptake and addition of lactococcin A to cells of IL1403 which had accumulated AIB resulted in efflux of AIB (Fig. 2A). Interestingly, this lactococcin A-induced efflux was even faster than the valinomycin-plus-nigericin-induced efflux of AIB. This result suggests that AIB efflux induced by lactococcin A was not only mediated by the carrier but also by leakage through the cytoplasmic membrane. In contrast, lactococcin A at a concentration of 0.029 μg/mg of protein had no effect on AIB uptake by cells of IL1403(pMB563), nor was lactococcin A-induced efflux of AIB observed (Fig. 2B).

To examine whether lactococcin A induced leakage of substrates through the cytoplasmic membrane, the effect of lactococcin A on the uptake of L-glutamate was studied (Fig. 3). Glutamate uptake in *L. lactis* is a phosphate bond-linked unidirectional uptake process which is not driven by the proton motive force (22). As expected, the addition of valinomycin plus nigericin to cells of strain IL1403 did not result in an efflux of accumulated glutamate (Fig. 3A). In contrast, efflux of accumulated glutamate was observed when strain IL1403 was treated with lactococcin A (0.029 μg/mg of protein), indicating that lactococcin A indeed affects the permeability of the cytoplasmic membrane in *L. lactis* (Fig. 3A).

Several bacteriocins have been described to have a voltage-dependent pore-forming ability (1, 8, 16, 17, 23, 27, 28, 35). To investigate whether this also applied to lactococcin A, cells of strain IL1403 were allowed to accumulate glutamate and were then treated with valinomycin and nigericin to collapse the proton motive force, and lactococcin A was subsequently added. Figure 3B shows that in the absence of a proton motive force lactococcin A-induced glutamate efflux still takes place and that the observed rate of efflux was very similar to the rate of efflux in the presence of a proton motive force (Fig. 3A). These observations imply that

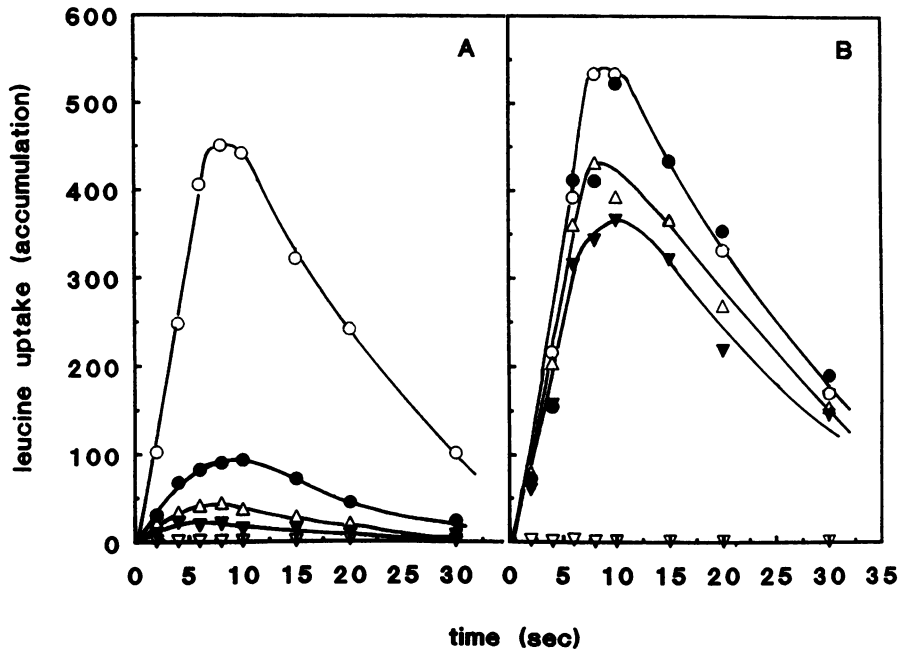

FIG. 4. Effects of lactococcin A on the uptake of leucine driven by an artificially imposed proton motive force in cytoplasmic membrane vesicles derived from *L. lactis* IL1403 (A) and *L. lactis* IL1403(pMB563) (B). Proton motive force-driven uptake was started by diluting the K acetate-loaded, valinomycin-treated membrane vesicles 100-fold in 20 mM NaP_i (pH 6), 100 mM NaPIPES, and 2 mM $MgSO_4$ containing 1.6 μM ^{14}C-labeled leucine. Symbols: ○, membrane vesicles not preincubated with lactococcin A; ●, △, and ▼, uptake after 10 min of preincubation of the membrane vesicles with lactococcin A at concentrations of 0.05, 0.12, and 0.25 μg/mg of protein, respectively. Uptake of leucine in the absence of an imposed proton motive force by diluting the membrane vesicles in 20 mM KP_i (pH 6), 100 mM K acetate, 2 mM $MgSO_4$, and ^{14}C-labeled leucine (▽) is shown.

the activity of lactococcin A in whole cells is not voltage dependent. Prolonged incubation of the cells with the ionophore combination did not change the rate of glutamate efflux mediated by lactococcin A (data not shown). Preincubating strain IL1403 with lactococcin A (0.029 μg/mg of protein) significantly decreased the uptake of glutamate (Fig. 3B). When the concentration of lactococcin A was increased to 0.57 μg/mg of protein during preincubation of the cells, the uptake of glutamate was completely inhibited (data not shown). The uptake of L-glutamate by cells of strain IL1403(pMB563) preincubated with lactococcin A (0.029 μg/mg of protein) or by cells to which lactococcin A was added after steady-state glutamate accumulation was not affected by lactococcin A (Fig. 3C).

Effect of lactococcin A on cytoplasmic membrane vesicles. The results described above suggest that the cytoplasmic membrane is the primary target for lactococcin A. This prompted us to examine the effect of lactococcin A on cytoplasmic membrane vesicles derived from strain IL1403 and strain IL1403(pMB563) carrying the immunity gene (Fig. 4). Uptake of leucine by membrane vesicles from lactococci can be driven by an artificially imposed proton motive force (6, 11). To our surprise, addition of lactococcin A to a concentration as high as 2.5 μg/mg of protein at time zero did not affect the uptake of leucine (data not shown). However, preincubation of the membrane vesicles with lactococcin A at 0.25 μg/mg of protein inhibited leucine uptake; the maximal inhibitory effect of lactococcin A on leucine uptake was reached after 5 min of preincubation (data not shown). Therefore, in all uptake experiments the membrane vesicles were preincubated with lactococcin A for at least 10 min before uptake was initiated. Lactococcin A strongly inhibited leucine uptake in membrane vesicles of strain IL1403, and this inhibition increased with increasing concentrations of lactococcin A (Fig. 4A). In contrast, membrane vesicles derived from IL1403(pMB563) were hardly affected by the addition of lactococcin A (Fig. 4B). These observations confirm that lactococcin A acts on the cytoplasmic membrane and indicate that the immunity of IL1403(pMB563) is linked to the cytoplasmic membrane.

The effect of lactococcin A on membrane vesicles of other lactococcal strains and bacterial species was also studied. Uptake of leucine in membrane vesicles derived from *L. lactis* subsp. *cremoris* Wg2 and of *L. lactis* subsp. *lactis* ML3 was also severely inhibited by lactococcin A (data not shown). In contrast, leucine uptake driven by an artificially imposed proton motive force in membrane vesicles of two other gram-positive bacteria, *Clostridium acetobutylicum* NCIB 8052 and *Bacillus subtilis* W23, and of the gram-negative bacterium *Escherichia coli* ML308-225 was not significantly inhibited by lactococcin A at a concentration which completely blocked leucine uptake by membrane vesicles of sensitive lactococci (0.25 μg/mg of protein) (data not shown). Apparently, the species-specific response to lactococcin A lies at the level of the cytoplasmic membrane.

The experiments described above were done with membrane vesicles in which a proton motive force was generated. The effect of lactococcin A on membrane vesicles of lactococci was also studied in the absence of a proton motive force. For that purpose, membrane vesicles were first equilibrated with 5 mM unlabeled leucine and subsequently diluted 50-fold in a medium containing 3.2 μM ^{14}C-labeled leucine. In this way, an outwardly directed leucine concentration gradient was imposed and leucine carrier-mediated

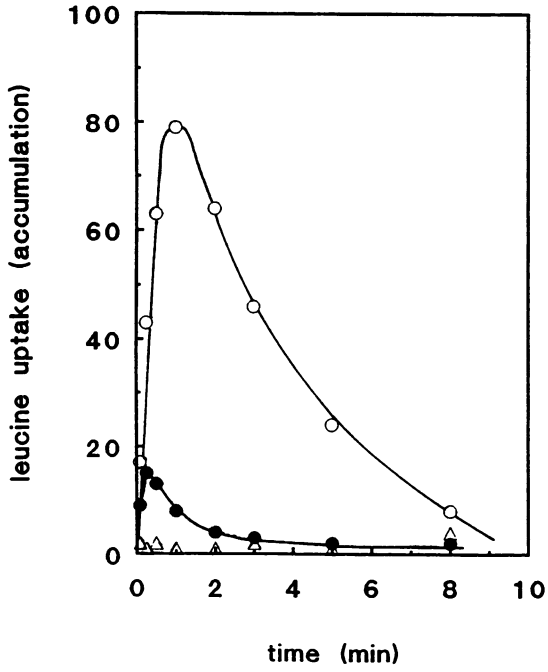

FIG. 5. Effects of lactococcin A on counterflow uptake of leucine by cytoplasmic membrane vesicles from *L. lactis* IL1403. Symbols: ○, membrane vesicles loaded with 5 mM leucine; △, membrane vesicles not loaded with leucine; ●, membrane vesicles loaded with 5 mM leucine and preincubated for 10 min with lactococcin A (0.25 μg/mg of protein). The assay was started by diluting the membrane vesicles 50-fold in 50 mM KP_i (pH 6), 2 mM $MgSO_4$, and 3.2 μM ^{14}C-labeled leucine.

exchange of nonradioactive leucine in the membrane vesicles for radioactive leucine in the medium could occur (Fig. 5) (6). Preincubation of the lactococcal membrane vesicles with lactococcin A (0.25 μg/mg of protein) severely inhibited accumulation of the labeled leucine (Fig. 5), indicating that lactococcin A caused leakage of unlabeled leucine out of the membrane vesicles. This result shows that lactococcin A also permeabilizes lactococcal membrane vesicles in the absence of a proton motive force and acts in a voltage-independent way.

Electron microscopy of whole cells and membrane vesicles of *L. lactis*. The morphology of cells and membrane vesicles treated with lactococcin A (0.029 protein and 0.25 μg/mg of protein, respectively) was examined by electron microscopy. No lysis or other morphological alterations could be observed in cells after incubation with lactococcin A, even when the incubation time with lactococcin A was prolonged to 45 min. The closed structure of the membrane vesicles remained unaffected by the treatment with lactococcin A, and no aggregation of membrane vesicles was observed. This observation, together with the fact that cells and membrane vesicles of *L. lactis* incubated with lactococcin A showed efflux of accumulated substrates, suggests that lactococcin A permeabilizes the cytoplasmic membrane by the formation of pores.

Effect of lactococcin A on liposomes. The results presented above indicate that lactococcin A acts on the cytoplasmic membrane of cells and membrane vesicles. To examine whether the action of lactococcin A depends on membrane proteins, the effect of lactococcin A on liposomes was studied. Liposomes were prepared from phospholipids derived from *L. lactis* ML3. Across the membrane of these liposomes, a valinomycin-induced K^+ diffusion potential was imposed and the membrane potential was monitored by measuring the distribution of TPP^+. Lactococcin A at concentrations as high as 40 μg/mg of lipid did not affect the artificially imposed membrane potential of the liposomes. Also preincubation of the liposomes with lactococcin A (0.5 μg/mg of lipid) did not decrease the membrane potential (Fig. 6). However, the addition of nigericin (0.5 μM) to the liposomes resulted in a rapid dissipation of the membrane potential (Fig. 6). These observations suggest that membrane proteins are required for the activity of lactococcin A.

DISCUSSION

The results presented in this paper suggest that lactococcin A, a small, basic, and relatively hydrophobic peptide, induces the formation of pores in the cytoplasmic membrane of cells of *L. lactis* which allow the free diffusion of ions and amino acids. It is important to note that for the uptake experiments, the concentration of lactococcin A was chosen such that only sensitive cells but not cells carrying the immunity gene were affected. Lactococcin A caused depolarization of the membrane potential, inhibited amino acid uptake, and induced the efflux of preaccumulated amino acids in sensitive cells and membrane vesicles of *L. lactis*. The observation that lactococcin A induced rapid efflux of glutamate, previously taken up by *L. lactis* via a phosphate bond-linked unidirectional uptake system, supplies evidence for pore formation in the cytoplasmic membrane. Glutamate efflux could have been caused by gross damage of the cytoplasmic membrane, but no such indication could be obtained by electron microscopic examination of cells and membrane vesicles treated with lactococcin A.

The uptake of AIB in *L. lactis* is driven by the proton motive force and can be completely blocked by treatment of the cells with lactococcin A. The activity of lactococcin A on whole cells was not voltage dependent, since lactococcin A caused efflux of glutamate from cells treated with valinomycin and nigericin. The counterflow experiments revealed that lactococcin A caused leakage of leucine from membrane vesicles indicating that also in membrane vesicles of *L. lactis* a proton motive force was not required for lactococcin A to be effective. Thus, the action of lactococcin A differs from the voltage-dependent activity that was first described for the pore-forming colicins (27) and, more recently, for several small, basic peptides such as nisin (8, 26), Pep-5 (17), and subtilin (28).

Lactococcin A exclusively inhibits the growth of lactococci (10), and this property was retained in the membrane vesicles from lactococci, as membrane vesicles derived from other bacterial species were refractory to the action of lactococcin A. Conceivably, the insensitivity of these vesicles might be due to a different phospholipid composition in their cytoplasmic membranes compared with that of lactococci (25). However, we consider this possibility unlikely, because liposomes prepared from phospholipids of *L. lactis* were not affected by lactococcin A, even not at high concentrations of the bacteriocin (Fig. 6). Apparently, a membrane-associated protein specific for lactococci acts as receptor for lactococcin A, or this putative protein participates in pore formation after interaction with the bacteriocin. This is in contrast to other small, broad-spectrum, pore-forming peptide antibiotics such as nisin (8), Pep-5 (17), subtilin (28), and AS-48 (7) which do not require a membrane receptor, as they also act on artificial membranes.

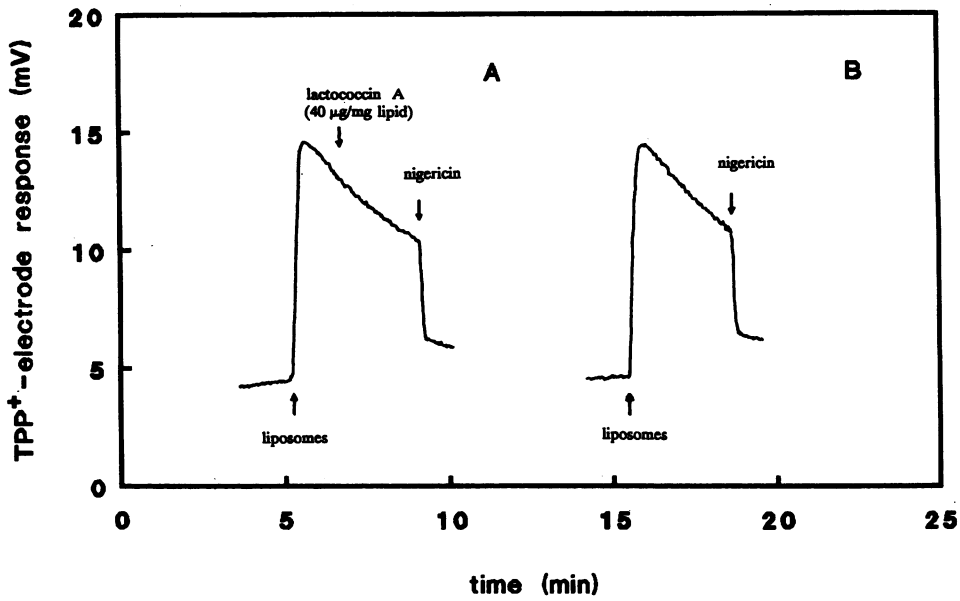

FIG. 6. Effects of lactococcin A on the artificially imposed membrane potential in liposomes of *L. lactis* ML3. Valinomycin-treated liposomes in 50 mM KP_i (pH 6) and 2 mM $MgSO_4$ were diluted 100-fold in 50 mM NaP_i (pH 6), 2 mM $MgSO_4$, and 4 μM TTP^+. The membrane potential was monitored with a TTP^+ electrode. (A) Effects of lactococcin A (40 μg/mg of lipid) on the membrane potential in liposomes; (B) effects of preincubation for 10 min of the liposomes with lactococcin A (0.5 μg/mg of lipid) on the generation of a membrane potential. The addition of nigericin (0.5 μM) is indicated.

Lactococcin A hardly had any effect on the membrane potential below a threshold concentration of the bacteriocin (Fig. 1). Above this concentration, the membrane potential of sensitive lactococcal cells dissipated immediately with rapid efflux of amino acids. In contrast to whole cells, membrane vesicles derived from *L. lactis* became leaky for ions and amino acids only after preincubation with a high concentration of lactococcin A. Although the reason for this difference in sensitivity between cells and membrane vesicles of *L. lactis* remains to be established, it is conceivable that the peptidoglycan layer targets lactococcin A to the membrane or to the putative receptor. Alternatively, the isolation of the lactococcal membrane vesicles may have resulted in partial loss of a receptor for lactococcin A.

The finding that the membrane potential of *L. lactis* IL1403(pMB563) could be dissipated only by high concentrations of lactococcin A indicates that the immunity system of IL1403(pMB563) can be overcome by high concentrations of lactococcin. This so-called immunity breakdown has also been reported for several colicins (26). The hydrophobicity plot of the immunity gene product of pMB563 does not resemble that of a typical hydrophobic membrane protein. Nevertheless, the fact that membrane vesicles derived from strain IL1403(pMB563) were not affected by lactococcin A at a concentration that did affect the sensitive strain IL1403 indicates that the immunity specified by pMB563 was still associated with the membrane. The immunity protein could either confer immunity by blocking the receptor or by preventing the pore formation in the membrane. The immunity proteins of some of the pore-forming colicins appear to be associated with the cytoplasmic membrane (9, 36).

Taken together, all these results point to a receptor-mediated action of lactococcin A on the cytoplasmic membrane of lactococci, which can be blocked specifically by the immunity protein. The efflux of essential compounds can explain the growth inhibition and ultimate death of lactococcal cells exposed to lactococcin A.

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