APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 1994, p. 1006–1013
0099-2240/94/$04.00+0

Vol. 60, No. 3

Kinetic Studies of the Action of Lactacin F, a Bacteriocin Produced by *Lactobacillus johnsonii* That Forms Poration Complexes in the Cytoplasmic Membrane

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Received 27 September 1993/Accepted 10 December 1993

The bacteriocin lactacin F is bactericidal against *Lactobacillus delbrueckii*, *Lactobacillus helveticus*, and *Enterococcus faecalis*. Activity against *L. delbrueckii* was recently shown to be dependent on two peptides, LafA and LafX, which are encoded within the lactacin F operon (T. R. Klaenhammer, FEMS Microbiol. Rev. 12:39–87, 1993). It has been proposed that two peptides form an active lactacin F complex. In this study, the action of lactacin F against *E. faecalis* ATCC 19443 and the effects of various environmental parameters were investigated in detail. Addition of lactacin F induced the loss of K^+ from cells of *L. delbrueckii*, *Lactobacillus johnsonii* 88-4, and *E. faecalis*, while the lactacin F producer *L. johnsonii* VPI 11088 was not affected by the bacteriocin. Lactacin F caused an immediate loss of cellular K^+, depolarization of the cytoplasmic membrane, and hydrolysis of internal ATP in *E. faecalis*. Lactacin F induced loss of K^+ in 3,3′,4′,5-tetrachlorosalicylanilide-treated cells, indicating that pores are formed in the absence of a proton motive force. ATP hydrolysis was not due to dissipation of the proton motive force but was most likely caused by efflux of inorganic phosphate, resulting in a shift of the ATP hydrolysis equilibrium. Action of lactacin F was optimal at acidic pH values and was reduced in the presence of di- and trivalent cations. The lanthanide gadolinium (Gd^{3+}) prevented action of lactacin F completely at a concentration of 0.2 mM. Lactacin F-induced loss of cell K^+ was severely reduced at low temperatures, presumably as a result of increased ordering of the lipid hydrocarbon chains in the cytoplasmic membrane. In cells grown at 30°C, lactacin F action was prevented at temperatures below 10°C, and increasing lag times were observed at temperatures below 25°C. An examination of parameters that affected lactacin F action provided insights into the possible mechanisms by which peptide bacteriocins interact with the cytoplasmic membrane and form poration complexes.

Proteinaceous antimicrobial agents are produced widely by lactic acid bacteria (19). Biochemical and genetic studies have defined four major classes of bacteriocins, including lantibiotics, small heat-stable peptides, large heat-labile proteins, and complex proteins that require carbohydrate or lipid moieties for bacteriocin activity (20). The mechanisms of action of most of these bacteriocin classes have not been defined and probably include several distinct processes that affect cellular physiology or metabolism. However, mechanistic studies of selected members of two of the bacteriocin classes mentioned above, the lantibiotics (class I) and the small peptides (class II), have revealed that bacteriocin action occurs at the cytoplasmic membrane.

Nisin A, which is produced by *Lactococcus lactis*, is generally active against gram-positive bacteria. This lantibiotic acts on energized membrane vesicles to disrupt the proton motive force (PMF), inhibit transport of amino acids, and cause release of accumulated amino acids (6, 21, 28, 29). Nisin A also acts on artificial membranes, and this action is dependent on the lipid composition of the membranes (15, 16). Lactococcins A and B are small class II peptides which have more restricted host ranges and kill only closely related species. These bacteriocins alter membrane permeability, but unlike nisin their action and specificity are mediated by membrane receptor proteins (33, 34). Recently, Nissen-Meyer et al. (27) described a novel bacteriocin whose activity depends on the complementary action of two small peptides, α1 and β, which have molecular weights of 4,376 and 4,109, respectively. It has been predicted that the N-terminal halves of these two peptides form α-helices, and it has been suggested that these molecules are pore-forming toxins (27). However, mechanistic studies of the lactococcin G system have not been performed to confirm this hypothesis.

The lactobacilli are the most bacteriocinogenic lactic acid bacteria because of the diversity of their species and habitats. Many lactobacilli associated with intestinal tracts produce bacteriocins (5, 19), and these compounds probably affect the competitive abilities of these bacteria in intestinal tracts. *Lactobacillus johnsonii* VPI 11088, which was previously classified in the *Lactobacillus acidophilus* group (14), produces a bacteriocin, lactacin F, that has been the subject of extensive biochemical and genetic characterization (24–26). Molecular analysis of the lactacin F operon has defined three genes (*lafA*, *lafX*, and *lafZ*), two of which are required to elicit bactericidal action against *Lactobacillus delbrueckii* and *Enterococcus faecalis*. A combination of the LafA and LafX peptides in vitro elicits bactericidal activity, whereas neither peptide alone kills *L. delbrueckii* and *E. faecalis* indicator organisms (2, 13, 20).

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The requirement for two apparently structural genes and two peptides for bacteriocin activity is similar to requirements in the lactococcin M (32) and G systems (27).

Since to date no detailed information about the mode of action of the two component bacteriocins is available, we investigated the action of lactacin F against lactobacilli and enterococci. Our initial results indicated that lactacin F acts on the cytoplasmic membrane. A potassium-selective electrode (8) was used to study the kinetics of K^+ efflux induced by lactacin F. This allowed us to investigate in detail the effects of environmental parameters, including ionic strength, pH, and temperature, on the action of lactacin F. In addition, the effects of lactacin F on bioenergetic parameters, such as PMF generation and ATP synthesis in *E. faecalis*, were investigated.

MATERIALS AND METHODS

Growth of bacteria. *E. faecalis* ATCC 19433 was grown at 30°C in brain heart infusion medium containing 1% (wt/vol) glucose. *L. delbrueckii* subsp. *lactis* 970 (lactacin F sensitive [Laf^s]) and *L. johnsonii* VPI 11088 (Laf$^+$ Laf^r) and 88-4 (Laf$^-$ Laf^s) were propagated at 30°C in MRS (24). Cells were harvested at an optical density at 660 nm (OD_{660}) of 0.6, washed and resuspended in 115 mM sodium phosphate buffer (pH 6.0) (unless indicated otherwise) to an OD_{660} of 20, stored on ice, and used within 3 h.

Determination of the potassium content of cells. Variations in the intracellular K^+ contents of cells (K^+_{in}) were determined by measuring changes in the K^+ concentration in the external medium (K^+_{out}) with a K^+ electrode (8). The K^+ and reference electrodes were connected to a voltmeter and a computing device with which K^+_{in} could be directly determined from the value of K^+_{out}. The time resolution of the electrode is 4 s. The total K^+ content of bacteria was determined with the K^+ electrode after K^+ was released from the cells by cold osmotic shock or by treatment with an excess amount of nisin Z (2 μg/mg of protein). Assays were performed at 30°C in 10 or 115 mM sodium phosphate (pH 6.0) containing 0.6 mM KCl and 0.2% (wt/vol) glucose. In experiments with di- and trivalent cations the cells were harvested at an OD_{660} of 0.6, washed, and resuspended to a final OD_{660} of 20 in 30 mM sodium piperazine-*N*,*N*′-bis(2-ethanesulfonate) (PIPES) (pH 6.0) containing 100 mM NaCl, and the assay was performed in 10 mM sodium PIPES (pH 6.0) containing 0.2% (wt/vol) glucose and 0.6 mM KCl. Cells were incubated in the assay medium at an OD_{660} of 0.8, which corresponded to an internal volume of 1 μl and a protein content of 0.5 mg (3, 4).

Measurement of the membrane potential. The transmembrane electrical potential was determined with an electrode specific for the lipophilic cation tetraphenylphosphonium (final concentration, 4 μM), as described previously (30). Cells of *E. faecalis* (1.8 mg of protein per ml) were incubated at 30°C in 50 mM potassium phosphate (pH 6.0) in the presence of 0.2% glucose. By adding the K^+-H^+ exchanger nigericin (1.0 μM), the pH gradient (alkaline inside) was dissipated such that the PMF was composed of the membrane potential only (12).

ATP measurements and determination of extracellular inorganic phosphate content. Cells were harvested at an OD_{660} of 0.6, washed, and resuspended to a final OD_{660} of 20 in 30 mM sodium PIPES (pH 6.0) containing 100 mM NaCl. Cells (0.8 mg [dry weight] per ml) were preincubated for 10 min at 30°C in 10 mM sodium PIPES (pH 6.0) containing 0.2% (wt/vol) glucose and 0.6 mM KCl. The K^+ efflux was recorded with a K^+ electrode (see above), and aliquots were removed at specified time intervals to determine ATP and extracellular phosphate concentrations. To determine the total (internal and external) ATP content, 0.020 ml of the cell suspension was rapidly permeabilized by adding 0.08 ml of dimethyl sulfoxide. The suspension was diluted with 5.0 ml of sterile water, 0.1 ml was mixed with the luciferin-luciferase reagent, and the ATP content was determined with a Lumac luminometer. To determine the extracellular ATP concentration, 0.1 ml of the cell suspension was first diluted with 4.9 ml of assay buffer, and then 0.1 ml of the preparation was analyzed as described above. The assays were calibrated by using ATP solutions having known concentrations. To determine extracellular inorganic phosphate concentrations, 1.0-ml samples were centrifuged, and the supernatants were assayed for phosphate as described by Chen et al. (10) and Guihard et al. (17).

Preparation of lactacin F. Lactacin F was precipitated from *L. johnsonii* VPI 11088 culture supernatant with a 40% ammonium sulfate cut as described previously (25). The floating pellet was harvested, resuspended in 50 mM sodium phosphate (pH 7.0), and lyophilized. The dried samples were resuspended in 0.1% tetrafolic acid and stored at −20°C. Activity units (AUs) were determined with the sensitive indicator strain *L. delbrueckii* subsp. *lactis* ATCC 4797 by spotting twofold dilutions of lactacin F preparations onto cell lawns overlaid in MRS agar; 1 AU was defined as the highest dilution factor of a lactacin F-containing sample that still caused clearly visible zones of inhibition.

RESULTS

Lactacin F-induced K^+ efflux from lactobacilli and *E. faecalis*. Freshly prepared *E. faecalis* ATCC 19433 cells retained 420 nmol of K^+ per mg of protein (approximately 210 mM). When these cells were kept concentrated on ice, they lost K^+. However, in the presence of glucose and K^+ (0.6 mM) the cells reaccumulated K^+ when they were incubated at 30°C (data not shown). *L. johnsonii* and *L. delbrueckii* cells did not reaccumulate K^+, and the latter organism even exhibited spontaneous loss of cellular K^+. Addition of lactacin F (816 AU/mg of protein) resulted in an immediate stimulation of K^+ efflux from *L. delbrueckii* subsp. *lactis* 970 cells. *E. faecalis* cells started to lose K^+ after a lag time of 1 min, while *L. johnsonii* 88-4 (Laf$^-$ Laf^s) was affected only slightly since K^+ efflux was very slow. Cells of the producer strain, *L. johnsonii* VPI 11088 (Laf$^+$ Laf^r), were not affected (Fig. 1). These results indicated that the cytoplasmic membrane is the primary target for lactacin F and that the producer strain is resistant to membrane action by this bacteriocin. Since the physiology and bioenergetics of *E. faecalis* have been studied extensively (3, 4), we elected to utilize this organism for detailed studies of the mode of action of lactacin F and the effects of various environmental parameters.

Lag time and K^+ efflux rate as a function of lactacin F concentration at high and low ionic strengths. The effects of increasing concentrations of lactacin F on the rate of K^+ efflux and the lag time preceding this efflux were analyzed (Fig. 2). At low concentrations lactacin F induced K^+ efflux from *E. faecalis* cells in 115 mM sodium phosphate buffer (pH 6.0). The rate of K^+ efflux increased with the lactacin F concentration and became saturated at approximately 30 μl/mg of protein (1,500 AU/mg of protein) (Fig. 2A). Decreasing the ionic strength by using 10 mM sodium phosphate resulted in an eightfold stimulation of the K^+ efflux rate and a decrease in the lag time before K^+ efflux from 45 to 8 s (Fig. 2B). Under these conditions the bacteria lost all K^+, as judged from the internal K^+ contents.

Effect of detergents on the activity of lactacin F. Electrophoresis of isolated lactacin F preparations indicated that both

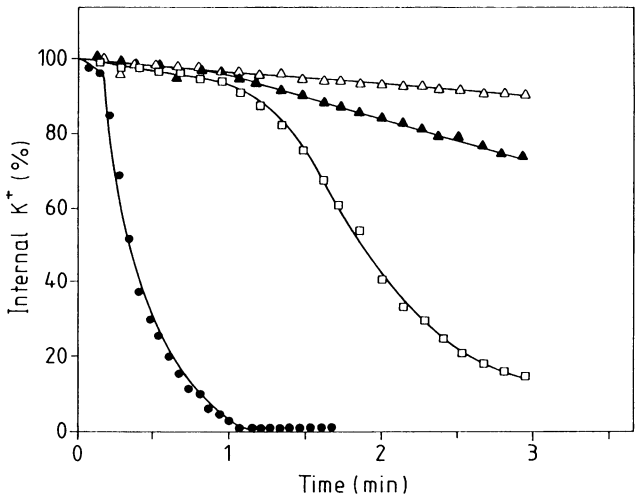

FIG. 1. Lactacin F-induced K^+ efflux from lactobacilli and *E. faecalis*. Cells were grown as described in the text, harvested at an OD_{660} of 0.6, and washed and resuspended in 115 mM sodium phosphate (pH 6.0). Lactacin F (816 AU/mg of protein) was added, and the K^+ efflux in buffer containing 0.2% (wt/vol) glucose and 0.6 mM KCl was determined with a K^+ electrode. The 100% values for cell K^+ concentration are approximately 160, 190, 200, and 225 mM for *L. delbrueckii* 970 (●), *L. johnsonii* VPI 11088 (△) and 88-4 (▲), and *E. faecalis* ATCC 19433 (□), respectively.

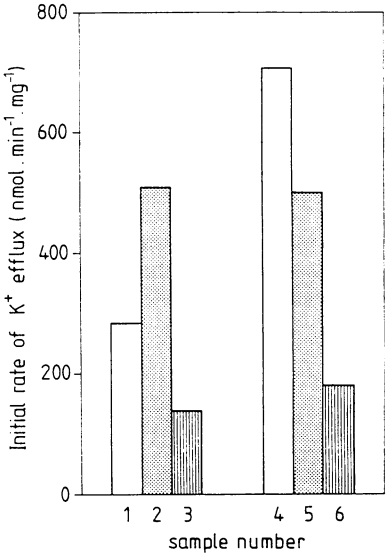

FIG. 3. Effect of detergents on lactacin F activity. The initial rates of K^+ efflux from *E. faecalis* cells in 10 mM sodium phosphate at pH 6.0 were determined after various treatments of a lactacin F suspension (51,200 AU/ml). Sample 1, control; sample 2, treatment with 1% Triton X-100; sample 3, treatment with 1% SDS; samples 4 through 6, same as samples 1 through 3, respectively, after preincubation for 8 min at 100°C. Portions (5 μl) of the various samples were added to the incubation mixtures (2.5 ml) so that the final concentrations of Triton X-100 (samples 2 and 5) and SDS (samples 3 and 6) were 0.002%. No effects on K^+ retention in cells were observed when detergents alone were added.

monomers and bacteriocin aggregates were present (25). Therefore, detergents were used to disrupt the aggregates. Low concentrations of Triton X-100 (final concentration, 0.002%) increased the rate of K^+ efflux induced by lactacin F from 280 to 520 nmol · min^{-1} · mg^{-1} (Fig. 3). Sodium dodecyl sulfate (SDS) (final concentration, 0.02%) did not stimulate lactacin F activity but rather inhibited it. Strikingly, heating the

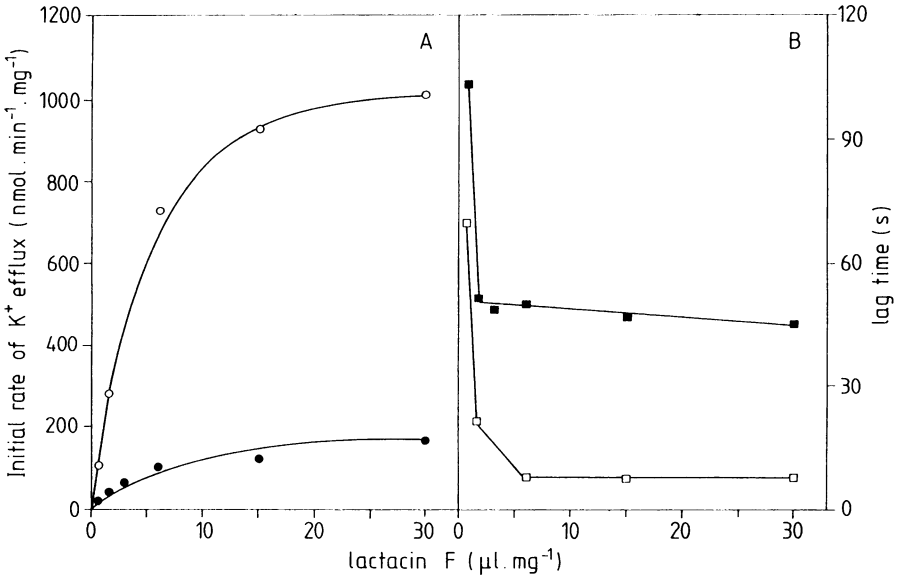

FIG. 2. Effect of ionic strength on lactacin F-induced K^+ efflux from *E. faecalis* (A) and the corresponding lag time (B). Lactacin F was added from a stock solution containing 51,200 AU/ml. Cells (1 mg of protein per ml) were incubated at 30°C in 115 mM sodium phosphate (solid symbols) or in 10 mM sodium phosphate (open symbols) at pH 6.0 in the presence of 0.2% (wt/vol) glucose and 0.6 mM KCl. Lag time was defined as the time that elapsed between lactacin F addition and K^+ efflux. The initial rate of K^+ efflux was determined as described in Materials and Methods.

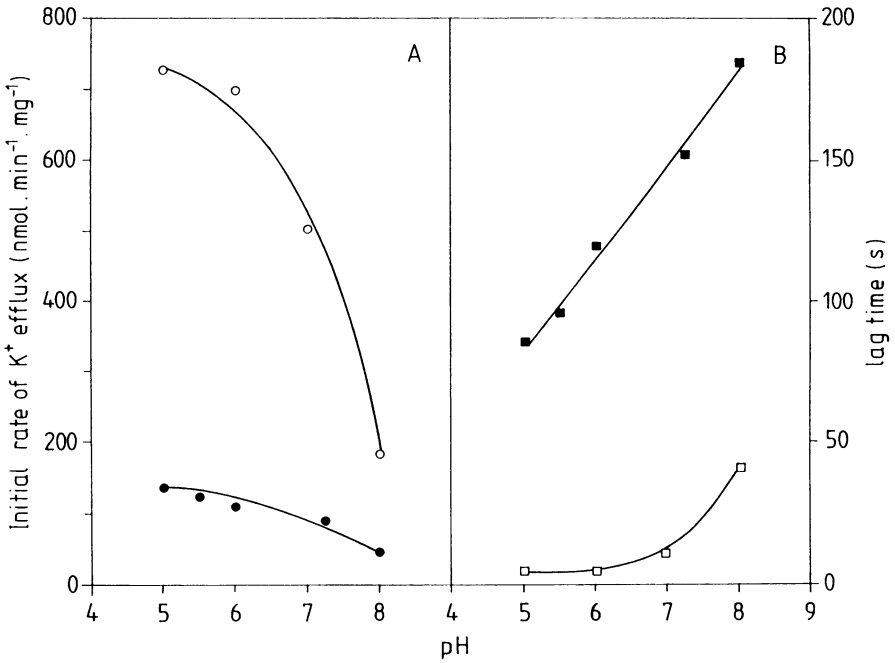

FIG. 4. Effect of pH on lactacin F-induced K^+ efflux from *E. faecalis*. Cells were grown, harvested, and resuspended in 115 mM sodium phosphate. Lactacin F (408 AU/mg protein)-induced K^+ efflux (A) and the corresponding lag time (B) were determined at different pH values in low-ionic-strength (10 mM) (open symbols) and high-ionic-strength (115 mM) (solid symbols) sodium phosphate buffers containing 0.2% (wt/vol) glucose and 0.6 mM KCl.

lactacin F preparation (8 min at 100°C) resulted in a significant increase in activity and enhancement of the K^+ efflux rate to 710 nmol · min^{-1} · mg^{-1}. Heating in the presence of Triton X-100 did not further stimulate lactacin F activity. Again, no marked effect was observed with SDS (Fig. 3).

Effect of pH on lactacin F-induced K^+ efflux from *E. faecalis* at high and low ionic strengths. The action of lactacin F is also strongly dependent on the pH of the medium. Lactacin F (408 AU/mg of protein)-induced K^+ efflux was maximal at pH 5 (Fig. 4A). The same dependence of lactacin F-induced K^+ efflux on pH was observed in high- and low-ionic-strength buffers. However, even at an alkaline pH (pH 8.0) lactacin F exhibited significant activity and the rate of K^+ efflux was approximately 25% of the value obtained at pH 5. The lag time before K^+ efflux was also severely affected by the pH (Fig. 4B). In high-ionic-strength buffer, the lag time decreased from 180 to 90 s when the pH was decreased from 8 to 6. In low-ionic-strength buffer the lag time, which was 38 s at pH 8, was not observed at acidic pH values (pH 6.0 or below). These results indicate that lactacin F is optimally active at low pH values.

Action of lactacin F in the absence of a PMF. The results of mode-of-action studies of narrow-spectrum bacteriocins lactococcins A and B produced by *Lactoccoccus lactis* subsp. *cremoris* indicated that these peptides were active in the absence of a PMF (33, 34). Therefore, we analyzed the action of lactacin F on cells treated with 3,3′,4′,5-tetrachlorosalicylanilide (TCS). In the presence of the protonophore TCS (10 μM) the membrane potential was completely dissipated (data not shown). The TCS-induced K^+ efflux rate (30 nmol · min^{-1} · mg^{-1}) from *E. faecalis* was extremely low compared with the lactacin F-induced efflux rate (Fig. 5). Addition of lactacin F after preincubation with TCS for 60 s resulted in a significant acceleration of K^+ efflux. Apparently, lactacin F-induced K^+ efflux takes place far more rapidly than K^+ exit

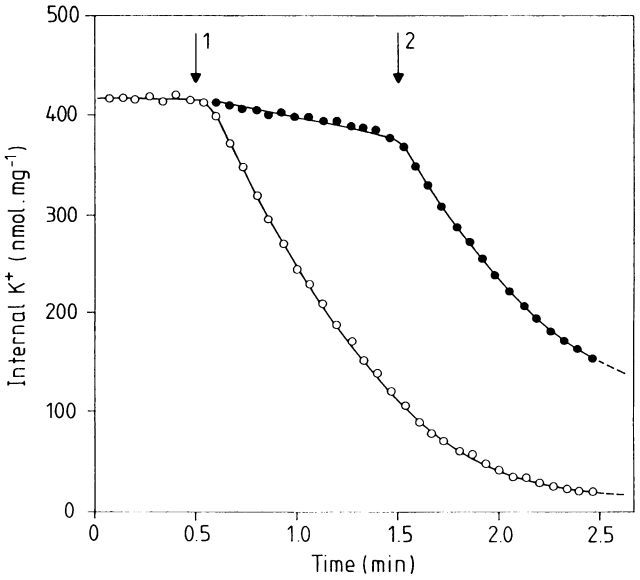

FIG. 5. K^+ efflux induced by lactacin F in the absence and presence of a PMF. Cells were grown and harvested as described in the text, and K^+ efflux was assayed in 10 mM sodium phosphate (pH 6.0) containing 0.2% glucose and 0.6 mM KCl. Lactacin F (408 AU/mg of protein) (○) or TCS (10 μM) (●) was added after 0.5 min (arrow 1). After 1 min lactacin F was added (arrow 2) in the experiment with TCS. K^+ efflux was determined as described in Materials and Methods.

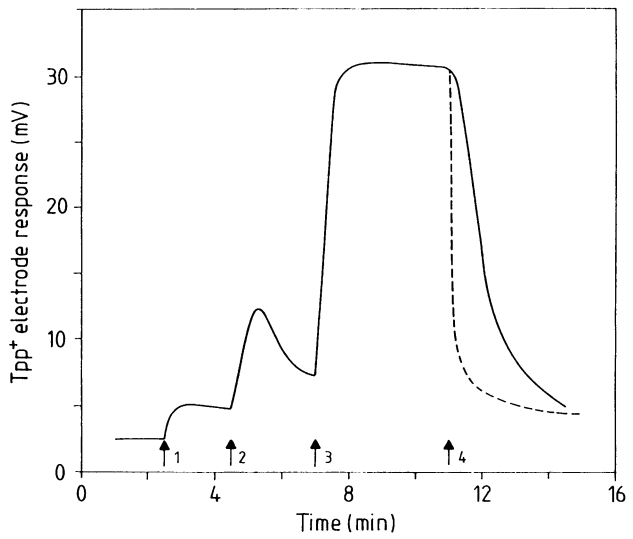

FIG. 6. Membrane potential changes induced by lactacin F. *E. faecalis* cells were grown in brain heart infusion medium at 30°C, harvested at an OD_{660} of 0.6, and washed and resuspended in 50 mM potassium phosphate (pH 6.0). Each incubation mixture contained the same buffer and cells (1.8 mg of protein per ml) (arrow 1); glucose (0.2%, wt/vol) (arrow 2), nigericin (1 μM) (arrow 3), and valinomycin (1 μM) (dashed line) or lactacin F (816 AU/mg of protein) were added (arrow 4) at the times indicated. An upward deflection reflects tetraphenylphosphonium ion (Tpp^+) uptake.

mediated by K^+ transport systems after dissipation of the PMF. The increased rate of K^+ efflux after lactacin F is added to cells preincubated with TCS indicates that lactacin F is active in the absence of a PMF. A comparison of the K^+ efflux rates indicated that lactacin F is equally active in the absence and presence of a PMF.

Effect of di- and trivalent cations on lactacin F activity. Addition of increasing concentrations of $MgSO_4$ or $CaCl_2$ decreased the initial rate of lactacin F-induced K^+ efflux. In the presence of 5 mM Mg^{2+} and 5 mM Ca^{2+}, the initial rate of K^+ efflux was decreased 25 and 55%, respectively (data not shown). The trivalent cation gadolinium (Gd^{3+}) was even more effective and completely inhibited lactacin F activity at a concentration of 0.2 mM. Inhibition by Gd^{3+} was reversed by addition of 1.5 mM EDTA (data not shown).

Effect of lactacin F on the energetic state of *E. faecalis* cells. The effects of lactacin F on other vital cell functions were also determined.

(i) Membrane potential dissipation by lactacin F. Energized cells of *E. faecalis* retained a membrane potential of −136 mV (inside negative) at pH 6 in the presence of 1.0 μM nigericin. Addition of lactacin F resulted in a complete depolarization of the cytoplasmic membrane (Fig. 6).

(ii) Lactacin F-induced phosphate efflux and hydrolysis of ATP. Energized cells of *E. faecalis* contain 8.6 mM cytoplasmic ATP. Depolarization of the cells by the addition of 10 μM TCS caused a slow decrease in the intracellular ATP concentration (Fig. 7A). Addition of lactacin F to energized cells resulted in a decrease in the intracellular ATP concentration, which decreased to 30% of its original level, while no ATP was found externally. The lactacin F-induced loss of a PMF was appar-

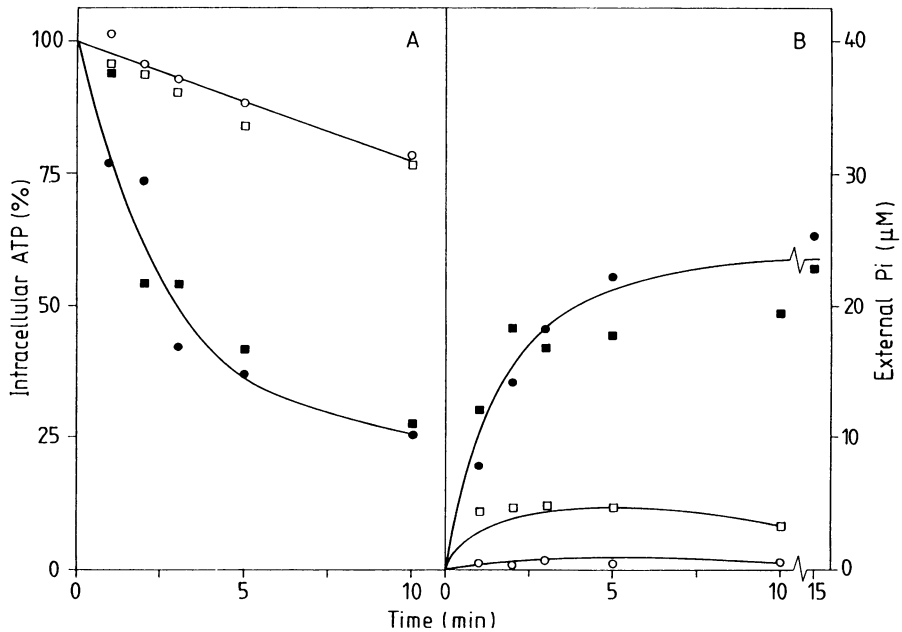

FIG. 7. Lactacin F-induced hydrolysis of internal ATP (A) and efflux of inorganic phosphate from *E. faecalis* (B). Cells were grown in brain heart infusion medium containing glucose at 30°C, harvested at an OD_{660} of 0.6, and washed and resuspended in 30 mM sodium PIPES (pH 6.0) containing 100 M NaCl. The assay was performed in 10 mM sodium PIPES (pH 6.0) containing 0.2% (wt/vol) glucose and 0.6 mM KCl. Symbols: ○, ATP concentration in control cells; □, ATP concentration in cells to which TCS (10 μM) was added after preincubation for 15 min; ●, ATP concentration in cells to which lactacin F (408 AU/mg of protein) was added after preincubation for 15 min. In a second experiment, TCS was added first and then lactacin F was added (■) (A). The values are percentages of the initial intracellular ATP concentration (the 100% value was 8.6 mM). The corresponding values for extracellular phosphate concentration are shown in panel B. Cytoplasmic ATP, extracellular ATP, and extracellular inorganic phosphate concentrations were determined as described in Materials and Methods.

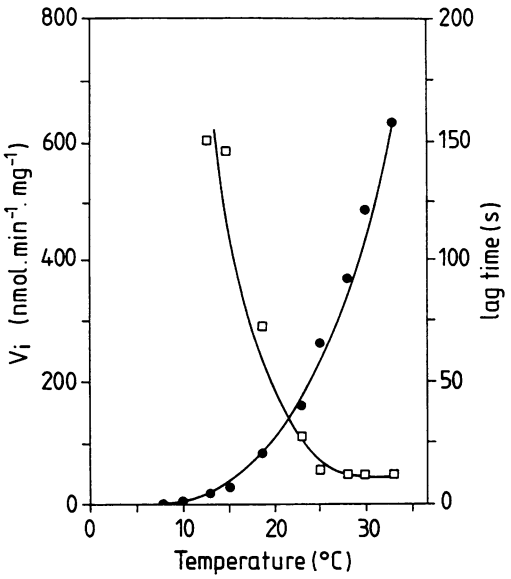

FIG. 8. Effect of temperature on the rate of lactacin F-induced K^+ efflux from *E. faecalis* (●) and the corresponding lag time (□). Cells were grown on brain heart infusion medium containing glucose at 30°C, harvested, and incubated (1 mg of protein per ml) in 10 mM sodium phosphate (pH 6.0) containing 0.2% glucose and 0.6 mM KCl. At different temperatures lactacin F (408 AU/mg of protein) was added, and the K^+ efflux (V_i, the initial rate of K^+ efflux) was determined as described in Materials and Methods.

ently not responsible since ATP depletion took place far more rapidly than the depletion expected from depolarization of the cells. This hypothesis was confirmed in a second experiment, in which TCS was added first and 10 min later lactacin F was added. Lactacin F induced ATP depletion to the same extent that it did in energized cells, and this depletion was much more pronounced than the depletion in cells treated with TCS alone (Fig. 7A). Figure 7B shows that, concomitant with ATP loss, lactacin F induced a rapid efflux of phosphate from the cells, while in the presence of TCS almost no efflux was observed. The amount of phosphate found in the medium indicated that the cells had lost approximately 25 mM phosphate.

Effect of temperature on the action of lactacin F against *E. faecalis*. Since lactacin F acts on the cytoplasmic membrane, the effectiveness of the bacteriocin and the kinetics of action should be affected by the conformation of the lipid hydrocarbon chains. Therefore, the action of lactacin F (408 AU/mg of protein) was investigated as a function of temperature in cells grown at 30°C (Fig. 8). The rate of K^+ efflux decreased significantly with decreasing temperatures. At temperatures below 10°C, lactacin F did not induce loss of K^+. At temperatures between 10 and 25°C a lag time was observed before the cells started to lose K^+, and this lag time increased with decreasing temperatures. Apparently, low-temperature-induced changes in membrane fluidity caused by increased ordering of the lipid hydrocarbon chains prevent insertion of lactacin F into the membrane. An Arrhenius representation of the initial rates of lactacin F-induced K^+ efflux from cells grown at 30°C showed a break at 22°C (data not shown). At temperatures above 22°C, the activation energy for lactacin F-induced K^+ efflux is 1.2 kJ/mol, while at temperatures below 22°C the activation energy is 1.7 kJ/mol. The activation energy values for lactacin F-induced K^+ efflux are comparable to those found for nisin Z (1) and for channels (18).

DISCUSSION

Our data show that addition of lactacin F to *E. faecalis* cells results in an immediate loss of cellular K^+, depolarization of the cytoplasmic membrane, and hydrolysis of internal ATP. These results demonstrate that the cytoplasmic membrane is the primary target for lactacin F. Several observations suggest that lactacin F does not disrupt the membrane structure by a detergent-like effect: (i) we did not observe any change in cell absorbance when lactacin F was added; (ii) lactacin F was only slightly active against *L. johnsonii* 88-4 and was not active at all against the producer strain, *L. johnsonii* VPI 11088; and (iii) although we observed an increased permeability toward ions, we could not detect any efflux of ATP. Therefore, the bactericidal effect of lactacin F is most likely due to the formation of pores in the cytoplasmic membrane. This is in agreement with the results of previous studies, which indicated that lactic acid bacterium bacteriocins belonging to classes I and II (20) act at the cytoplasmic membrane (6, 9, 15, 33, 34, 36). Since lactacin F is active against only a limited number of closely related *Lactobacillus* species and *E. faecalis* (20, 24, 25), it is likely that its activity is receptor mediated. In addition, the absence of activity observed in the producer strain suggests that pore formation may be prevented by the interaction of lactacin F with an immunity protein, as has been observed with other bacteriocin producers (20, 32–34).

The lactacin F-induced membrane permeability changes did not take place unless a critical amount of lactacin F was added to the cells (approximately 50 AU/10^9 cells). Strikingly, the efficiency of lactacin F-induced K^+ efflux in *E. faecalis* was approximately eightfold higher when the cells were incubated in a buffer having a low ionic strength (10 mM sodium phosphate) than when the cells were incubated in a buffer having a high ionic strength (115 mM sodium phosphate). Pretreating lactacin F with the detergent Triton X-100 also stimulated its permeabilizing effect. One possible explanation for this is that increasing the ionic strength favors the formation of lactacin F aggregates in solution, leading to a reduction in the number of peptides that can form pores. High-molecular-weight aggregates of lactacin F have indeed been observed previously, and the effective number of AU per milliliter increases after detergent treatment (25). These observations are also consistent with recent data obtained for insect defensins (11). These peptides form channels in the cytoplasmic membrane of *Micrococcus luteus*, and their bactericidal and permeabilizing effects are strongly enhanced by a decrease in ionic strength and pretreatment with a detergent. The magainin-2 bactericidal peptide also forms polymer aggregates in solution, and this process is stimulated by increasing the salt concentration (31).

The action of lactacin F against *E. faecalis* is clearly pH dependent and optimal at acidic pH values. Other bacteriocins also exhibit higher activity at acidic pH values. Nisins A and Z become more soluble and stable at low pH values, while at alkaline pH values aggregation resulting in a loss of activity can occur (22, 23). Recently, acidic external pH values as low as pH 5.5 appeared to stimulate the action of nisin against *Escherichia coli* liposomes (16). Experiments in which the action of nisin Z against *E. faecalis* was studied indicated that the activity of this molecule was highest at pH 5.0 (1). Absorption of various bacteriocins to cell surfaces of both producer and indicator bacteria appeared to be dependent on the pH (35). The pH values for optimum absorption of nisin A, pediocin AcH, sakacin A, and leuconocin Lcm1 were approximately 5.5 to 6.5. Since bacteriocin binding is not affected after cells are heated for 25 min at 70°C, it is assumed that binding first

occurs nonspecifically to the surface via the cell wall (35). Increased concentrations of bacteriocins in the cell wall due to lower pH values might enhance bacteriocin efficiency by providing more molecules which can interact directly, or indirectly via a receptor protein, with the cytoplasmic membrane.

The action of lactacin F is significantly influenced by the presence of divalent and trivalent cations since Mg^{2+} and Ca^{2+} partially prevented the efflux of potassium. Total inhibition was observed with Gd^{3+}, a lanthanide which is known to inhibit various stretch-activated channels in eucaryotic and procaryotic cells (7, 18). This inhibition could, however, be reversed by chelating the trivalent cation with EDTA. Lactostrepcin 5, a bacteriocin produced by *Streptococcus cremoris*, has been shown to induce efflux of K^+ and hydrolysis of internal ATP in target cells (36). The action of this bacteriocin was also severely reduced in the presence of high concentrations of Mg^{2+} and Ca^{2+} ions; this was attributed to the presumed stimulating effect of these ions on the membrane-bound H^+-pumping ATPase such that bacteriocin-induced H^+ leakage into the cells could be compensated for. We suggest that inhibition of pore formation by di- and trivalent cations can be explained if these ions bind to negatively charged phospholipids in the membrane, thereby negating electrostatic interactions between positive charges on the bacteriocins and the negatively charged headgroups of the phospholipid molecules.

A molecular analysis of the lactacin F operon in *L. johnsonii* recently indicated that three open reading frames are present (13). In addition to *lafA*, the lactacin F structural gene, expression of *lafX* is essential to lactacin F activity against *L. delbrueckii* subsp. *lactis* 970 and ATCC 4797 and *E. faecalis* ATCC 19433. In complementation reactions on agar, in which the LafA and LafX peptides were excreted by different clones, bactericidal action against *L. delbrueckii* 4997 occurred at the point where the two products diffused together (2, 20). This indicates that lactacin F activity occurs through the action of two peptides, LafA and LafX. Therefore, it is likely that the bactericidal action of lactacin F results from the complementation of the two peptides, which form poration complexes in the cytoplasmic membrane.

Permeabilization of the cytoplasmic membrane of *E. faecalis* did not take place immediately after lactacin F was added. A lag was observed, and this lag was strongly dependent on the concentration of lactacin F and on the temperature of incubation; the lower the concentration and the temperature were, the longer the lag time was. Since decreasing the temperature increases the ordering of lipid hydrocarbon chains, it is likely that this ordering slows down and even prevents insertion of the lactacin F molecules into the membrane. If, as proposed above, the pores are formed by complexes of LafA and LafX molecules and if these complexes are formed in the cytoplasmic membrane, then decreasing the temperature or the concentration of lactacin F may reduce the probability of forming these multicomponent pores.

Addition of lactacin F to *E. faecalis* also causes an efflux of inorganic phosphate and a loss (but no leakage) of ATP. This indicates that the pores are permeable to small ions but do not allow efflux of large molecules. ATP was shown to be hydrolyzed, and this hydrolysis was accompanied by depolarization of the cytoplasmic membrane. However, the observed ATP hydrolysis induced by lactacin F is not due to PMF dissipation since it takes place far more rapidly than ATP depletion in cells treated with the protonophore TCS (Fig. 7A). ATP hydrolysis is most likely caused by phosphate efflux and a subsequent shift in the ATP hydrolysis equilibrium. Recently, a similar phenomenon was described for the action of channel-forming colicins and phage proteins against *Escherichia coli* cells (17) and for the action of nisin Z, a natural nisin variant (23), against *Listeria monocytogenes* (1).

The induction of K^+ and phosphate efflux and the hydrolysis of internal ATP when lactacin F is added to cells treated with high concentrations of the protonophore TCS indicate that lactacin F action does not require the presence of a PMF (Fig. 5 and 7). A comparison of the rates of these processes induced by lactacin F in the absence and presence of a PMF shows that the PMF does not contribute to the efficiency of the pore formation process. The recently described and characterized bacteriocins lactococcin A and B, which are produced by *Lactococcus lactis* subsp. *cremoris*, also form pores in the cytoplasmic membranes of sensitive bacteria in the absence of a PMF (33, 34). The action of these bacteriocins is most likely also receptor mediated. This might indicate that receptor-mediated bacteriocin insertion into the membrane is very efficient, such that the PMF does not contribute to the process.

The results described above are in agreement with genetic data and the biochemical properties of peptides LafA and LafX. LafA and LafX possess most of the molecular features that characterize class II lactic acid bacterium bacteriocins, including a hydrophobic profile, an N-terminal extension with a consensus processing site, and a cationic C terminus harboring conserved sequences (12, 26). Therefore, lactacin F is similar to bacteriocins produced by *Lactococcus lactis* strains that appear to require two peptides for activity, lactococcin M (32) and lactococcin G (27). Taken as a whole, our results suggest that the bactericidal action of these two-component bacteriocins occurs through complementation of the two peptides, which form poration complexes in the cytoplasmic membrane, thereby dissipating ion gradients, causing hydrolysis of internal ATP, and resulting in inhibition of the growth of target microorganisms.

ACKNOWLEDGMENTS

We thank G. Guihard (CNRS, Orsay, France) for assistance and stimulating discussions, Sally McFall (North Carolina State University, Raleigh) for isolating lactacin F, and Rik van Veen (Agricultural University, Wageningen, The Netherlands) for the use of his tetraphenylphosphonium ion electrode equipment.

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