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Common Mechanistic Action of Bacteriocins from Lactic Acid Bacteria†

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The influence of four bacteriocins from lactic acid bacteria on the proton motive force (PMF) of sensitive organisms was investigated. Pediocin PA-1 (20 μg/ml) and leuconocin S (48.5 μg/ml) mediated total or major PMF dissipation of energized *Listeria monocytogenes* Scott A cells in a concentration-dependent manner, as has been shown for nisin. Lactacin F (13.5 μg/ml) caused 87% PMF depletion of energized *Lactobacillus delbrueckii* ATCC 4797 cells, also in a concentration-dependent fashion. The energy requirements for the activity of these four bacteriocins were determined by using the ionophores nigericin and valinomycin to carry out partial and specific deenergization of the target organisms. Pediocin PA-1, leuconocin S, and lactacin F acted in an energy-independent manner, whereas the activity of nisin was confirmed to be energy dependent. These results together with published reports on other bacteriocins suggest that the bacteriocins of lactic acid bacteria share a common mechanism, the depletion of PMF.

Bacteriocins from Generally Recognized As Safe (GRAS) lactic acid bacteria (LAB) have aroused a great deal of interest as a novel approach to control food-borne pathogens in foods (17, 29, 34, 37, 51, 60, 61). Certainly, such interest is partially attributed to antimicrobial properties of some LAB bacteriocins against *Listeria monocytogenes* (40), which, in turn, caused a dramatic increase of reports on bacteriocin-mediated antilisterial activity (10, 17, 19, 43, 53, 58, 68, 69). Bacteriocins are biologically active proteins exhibiting antimicrobial properties against other bacterial species which are usually closely related to the producer organism (29, 62). LAB bacteriocins are, in general, small cationic proteins (30 to 60 amino acid residues) with high isoelectric points and amphiphilic characteristics. Research has focused primarily on the purification, amino acid sequencing, and description of genetic determinants of LAB bacteriocins (5, 9, 20, 21, 23, 24, 26, 37, 38, 41, 45–47, 49, 65). Much less is known about their fundamental mechanism of action. The recent and comprehensive review on LAB bacteriocins by Nettles and Barefoot (48) reports no mechanistic studies for at least two-thirds of the bacteriocins covered. The mechanistic studies which have been done are summarized below.

Nisin, produced by some strains of *Lactococcus lactis*, is the best-characterized LAB bacteriocin. Nisin's structure was first elucidated by Gross and Morell (16), and the structural gene of nisin has been cloned and sequenced (9, 26). Nisin dissipates the membrane potential ($\Delta\psi$) in cells of sensitive organisms (55) and causes proton motive force (PMF) depletion of artificially energized liposomes (15) and of whole cells of *L. monocytogenes* (8) and *Clostridium sporogenes* (50). Moreover, the activity of nisin depends on energized membranes of susceptible organisms (15, 33, 55). Lactococcin A, another LAB bacteriocin produced by *L. lactis* subsp. *cremoris*, has been cloned and sequenced (25), and its mechanism of action has been studied (66). It dissipates the $\Delta\psi$, inhibits the uptake of amino acids, and causes the efflux of preaccumulated amino acids in sensitive cells. The permeabilization of cytoplasmic membranes and membrane vesicles of *L. lactis* by lactococcin A occurs in a voltage-independent fashion. Lactococcin B, produced by *L. lactis* subsp. *cremoris* 9B4, dissipates the PMF of sensitive organisms (67). Moreover, lactococcin B causes the efflux of glutamate from the cytoplasm of target cells in an energy-independent manner. Pediocin JD, a pediococcal bacteriocin from *Pediococcus acidilactici* JD1-23, collapses the PMF of *L. monocytogenes* and increases its membrane permeability to protons (11). Lactacin F, a bacteriocin produced by *Lactobacillus johnsonii*, has been cloned and sequenced (46). It is a small hydrophobic peptide with two cysteines within its 56 amino acid residues. The mechanism of lactacin F has also been studied by Abee et al. (1). Lactacin F induces membrane permeability, leading to potassium efflux and PMF dissipation, in *Enterococcus faecalis* cells. Membrane pores do not allow an efflux of ATP, but intracellular ATP is slowly reduced in apparently futile efforts to regenerate PMF. Lactacin F also induced potassium efflux from *Lactobacillus delbrueckii* (1). Lactostrepcin 5, a bacteriocin from *Streptococcus cremoris* 202, induces the leakage of potassium ions and ATP from the cytoplasm of sensitive cells and inhibits the synthesis of DNA, RNA, and protein (70).

The bacteriocin literature led us to the hypothesis that bacteriocins from LAB share a common mechanism of action, which is the dissipation of the PMF in sensitive cells. To test the PMF depletion hypothesis, we determined the influence of pediocin PA-1, leuconocin S, and lactacin F on the PMF of *L. monocytogenes*. In contrast to a previous report (34), lactacin F was not effective against *L. monocytogenes*. Therefore, the effects of this bacteriocin on PMF were extended to determine the mechanism of action on its most sensitive indicator strain, *L. delbrueckii*. The energy requirements for the activity of pediocin PA-1, leuconocin S, lactacin F, and nisin were also investigated.

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MATERIALS AND METHODS

Bacterial strains and culture conditions. *L. monocytogenes* Scott A and FRI-LM 103M, obtained from the Food Research Institute, Madison, Wis., were maintained on slants of Trypticase soy agar. Working cultures of *Leuconostoc paramesenteroides* Ox, a bacteriocin producer previously isolated from retail meat (35), and *L. delbrueckii* ATCC 4797 were made as stabs in APT agar and Lactobacilli MRS agar (Difco Laboratories, Detroit, Mich.) prepared with 0.5% glucose, respectively. All working slants were maintained at 4°C and transferred monthly for a maximum of four transfers before preparation of fresh working cultures. *L. johnsonii* 11088 and its Bac^- derivative *L. johnsonii* 88-4 were maintained and propagated as described previously (45). *Lactobacillus acidophilus* 11088 has recently been reclassified as *L. johnsonii* 11088 (13).

For the determination of the pH gradient (ΔpH) and $\Delta\psi$, *L. monocytogenes* was grown aerobically to the mid-log phase at 30°C with shaking (150 rpm) in Trypticase soy broth formulated to contain 0.5% glucose and 0.6% yeast extract. For the same purposes, *L. delbrueckii* ATCC 4797 was grown aerobically to the mid-log phase at 30°C without agitation in Lactobacilli MRS medium made from the standard formulation but without ammonium citrate, sodium acetate, or dipotassium phosphate. The medium components were dissolved in 0.1 M morpholineethanesulfonic acid (MES) buffer which had a pH of 7.1 after autoclaving. The mid-log-phase cells of *L. delbrueckii* ATCC 4797 were harvested by centrifugation (28,373 × *g* for 10 min) and resuspended in fresh modified MRS medium for 30 min at 30°C. From this point on, the preparation of the cells from both organisms for assaying ΔpH and $\Delta\psi$ was the same. Briefly, the mid-log-phase cells were harvested by centrifugation (20,845 × *g* for 20 min), washed once in 0.9% NaCl, and resuspended in 0.1 M MES buffer containing 10 mM $MgSO_4 \cdot 7H_2O$ and 10 mM KCl at pH 6.5 for *L. monocytogenes* strains and pH 5.5 for *L. delbrueckii* ATCC 4797 to an A_{660} of 1.0 to 1.1 (10^9 CFU/ml).

Bacteriocin preparation. (i) Crude leuconocin S. *L. paramesenteroides* Ox was grown in 1.0 liter of APT broth (5% initial inoculum) by batch fermentation (Queue Systems, Parkersburg, W.Va.) at pH 7.0 and 30°C for 27 h, which are optimal conditions for leuconocin S production (4). The cells were separated by centrifugation (13,182 × *g* for 20 min), and the culture supernatant (500 ml) was subjected to ammonium sulfate precipitation (60% saturation) at 4°C. After 24 h, the material was centrifuged (13,182 × *g* for 20 min), and the pellet was resuspended in 7.0 ml of 0.1 M MES buffer at pH 6.5. The concentrated bacteriocin suspension was centrifuged (13,000 × *g* for 3 min), and the supernatant, which contained most of the bacteriocin activity, was used in the PMF assays.

(ii) Crude lactacin F. *L. johnsonii* 11088 was grown to the stationary phase in MRS broth at 37°C. The cells were separated by centrifugation, and the supernatant was subjected to ammonium sulfate precipitation (40% saturation). After centrifugation, the pellet was resuspended in 8 M urea and dialyzed against deionized water. The material containing lactacin F activity was lyophilized and resuspended in 3.0 ml of 0.1% trifluoroacetic acid. The concentrated bacteriocin suspension was centrifuged (13,000 × *g* for 3 min), and the supernatant, which contained most of the bacteriocin activity, was used in the PMF assays with *L. monocytogenes* FRI-LM 103M as the target organism.

(iii) Purified lactacin F and control sample. Lactacin F purified to homogeneity was the generous gift of T. Klaenhammer and J. Greene (North Carolina State University, Raleigh). They also provided control material obtained by processing in the same manner as the culture supernatant from *L. johnsonii* 88-4, a bacteriocin-negative derivative from *L. johnsonii* 11088 in which the lactacin F operon has been deleted (3, 46). The control material had no activity against *L. delbrueckii* ATCC 4797, as determined by the critical dilution assay.

(iv) Pediocin PA-1. Pediocin PA-1, purified to homogeneity (23), was generously provided by P. Vandenbergh and J. Henderson, Quest International (Sarasota, Fla.). The pure protein was dissolved to desired concentrations in 0.02 N HCl–0.75% NaCl (pH 4.9).

(v) Nisin. Nisin was purified to homogeneity and kindly provided by Aplin and Barrett Ltd. (Trowbridge, England). Purified nisin was dissolved to desired concentrations in 0.02 N HCl–0.75% NaCl (pH 4.9).

Critical dilution assay for bacteriocin activity. Activity was determined by a plate diffusion titering technique. Briefly, *L. monocytogenes* was grown overnight at 30°C and diluted to 10^4 to 10^5 CFU/ml in Trypticase soy broth containing 0.5% glucose, 0.6% yeast extract, and only 0.8% Noble agar as suggested by Bhunia et al. (5). Ten milliliters of this inoculum was overlaid onto plates previously prepared with a bottom layer (10 ml) of the same medium containing 1.5% agar. *L. delbrueckii* ATCC 4797 was prepared similarly in modified Lactobacilli MRS medium. Each bacteriocin sample was twofold serially diluted, and 10 μl from each dilution was spotted onto a lawn of the appropriate sensitive organism. The plates were incubated for 24 to 48 h at 30°C. One arbitrary unit (AU) was defined as the reciprocal of the highest dilution which gave rise to a discernible zone of inhibition.

Bacteriocin inactivation. Nisin (250 μg/ml) and pediocin PA-1 (2,000 μg/ml) solutions, with pH values adjusted to 10, were autoclaved for 30 min. After the pH was lowered to 4.9, residual bacteriocin activity was determined. Similarly, crude leuconocin S had its pH increased to 10.5 before being boiled for 30 min. Residual bacteriocin activity was also determined after the pH was lowered to 6.5. Crude lactacin F was boiled for 30 min and assayed for bacteriocin activity.

Cidal action of the bacteriocins. *L. monocytogenes* Scott A and *L. delbrueckii* ATCC 4797 cells were grown as described for the determination of ΔpH and $\Delta\psi$. Cell suspensions were treated with the appropriate bacteriocin for 45 min. Equal volumes of the bacteriocin diluents were added to untreated controls. The cell suspensions were diluted and plated in triplicate. The plates were incubated at 30°C for 48 h, and the colonies were enumerated.

Protein determination. The protein concentrations of leuconocin S and lactacin F samples were determined by the Bio-Rad Bradford protein assay as directed by the manufacturer, with bovine plasma gamma globulin as the standard. Nisin and pediocin PA-1 were pure solid proteins whose concentrations were determined gravimetrically.

Determination of V_i. The intracellular volume (V_i) of *L. delbrueckii* ATCC 4797 was determined at pH 5.5 from the distribution of ^3H-labeled water and ^{14}C-labeled inulin as described by Rottenberg (54). The calculated V_i was 1.66 ± 0.29 μl/mg (dry weight) of cells. For *L. monocytogenes* Scott A, a V_i of 1.80 μl/mg (dry weight) was previously established by using the same methodology (8). This value was also used for the strain FRI-LM 103M in the calculation of the $\Delta\psi$ and ΔpH.

Determination of ΔpH and $\Delta\psi$. The $\Delta\psi$ (interior negative)

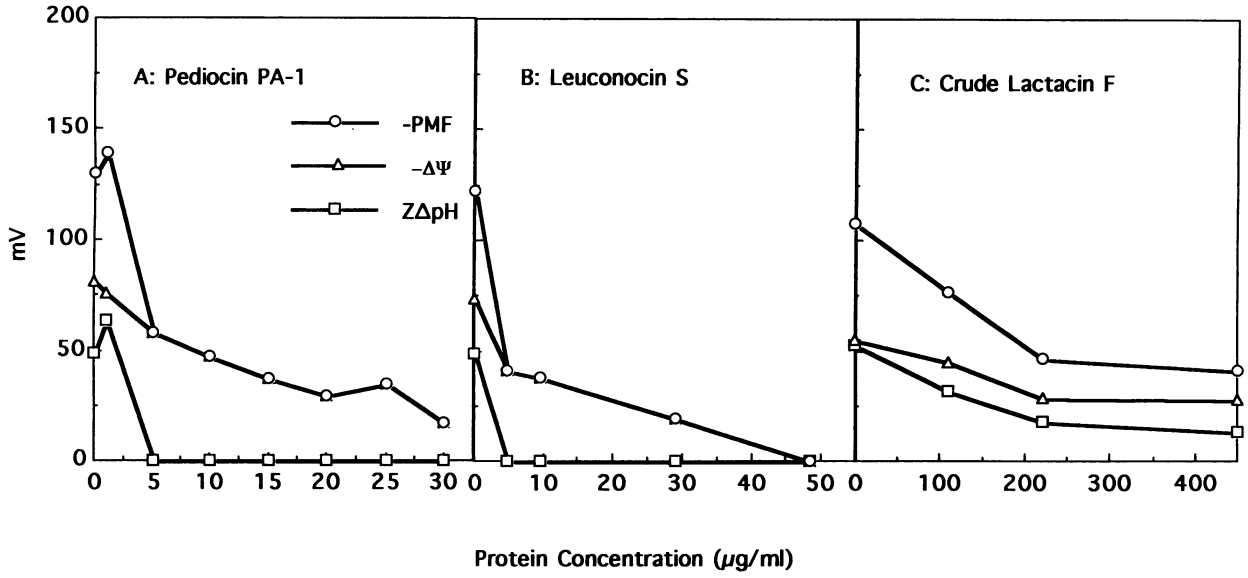

FIG. 1. Concentration-dependent action of LAB bacteriocins on $Z\Delta pH$, $\Delta\psi$, and total PMF in energized cells of *L. monocytogenes* Scott A (A and B) and FRI-LM 103 M (C) at pH 6.5. Datum points represent the average of at least two experiments done in duplicate.

and ΔpH (interior alkaline) were determined by the transmembrane distribution of ^{3}H-labeled tetraphenylphosphonium bromide (TPP) and ^{14}C-labeled salicylic acid, respectively, as described by Rottenberg (54). Total PMF was calculated from the equation $PMF = \Delta\psi - Z\Delta pH$, where $Z = 2.3(RT/F)$ and has a value of 59 mV at 25°C (R is the gas constant, T is the absolute temperature, and F is the Faraday constant). Corrections for nonspecific label binding and radioactivity attributable to extracellular fluid trapped in the pellets were determined from the extent of label accumulation in control cells treated with 7% butanol for 60 min. The counts from control samples were subtracted from the experimental values (28).

Cells resuspended in 0.1 M MES buffer containing 10 mM $MgSO_4 \cdot 7H_2O$ and 10 mM KCl were first energized for 15 min by adding glucose to a final concentration of 10 mM. Energized cells (1 ml) were immediately dispensed into microcentrifuge tubes containing 5.0 μM ^{14}C-labeled salicylic acid (0.25 μCi) and ^{3}H-labeled water (2.5 μCi) or 4.5 μM ^{3}H-labeled TPP (1.0 μCi) and incubated for 45 min at room temperature (20 to 22°C). In experiments with all bacteriocins, energized cells were dispensed into tubes containing the test compound and incubated for 30 min at room temperature before the addition and equilibration (15 min) of the probes. Separate experiments (data not shown) demonstrated that the probes equilibrated within 5 min. An equal volume of either bacteriocin diluent or heat-inactivated bacteriocin was added to the untreated controls. After incubation of the cells with the probes, 0.4 ml of silicone oil (96% mixture with octane; final density, 1.02 g/ml) was added to the suspension. The separation of the cells and subsequent radioactivity measurement of ΔpH and Δψ samples were done as described previously (8).

Deenergization experimental design. The deenergization experiments were performed by using the ionophores nigericin and valinomycin, which specifically deplete the chemical ($Z\Delta pH$) and the electrical ($\Delta\psi$) components of PMF, respectively. Energized cells were treated with the appropriate ionophore for 30 min. The bacteriocin was then added to the ionophore-treated cells and further incubated for 30 min prior to the addition and 15-min equilibration of the probes at room temperature. Appropriate volumes of ionophore diluent (95% ethanol) were added to untreated controls incubated for 45 min. Bacteriocin diluents were added to ionophore controls, which were, in turn, incubated for 60 min (including the probe equilibration time). There were no detectable differences between the PMF values for the ionophore controls incubated for either 60 or 75 min (data not shown).

Chemicals. Radioactively labeled [^{3}H]TPP (45.0 Ci/mmol), [^{14}C]salicylic acid (56.0 mCi/mmol), [^{3}H]water (1.0 mCi/ml), and [^{14}C]inulin (2.0 mCi/g) were purchased from Du Pont Co. (Wilmington, Del.). Silicone oil (density, 1.05 g/ml) and anhydrous octane were obtained from Aldrich Chemical Co., Inc. (Milwaukee, Wis.). Trypticase soy broth without dextrose was purchased from Becton Dickinson (Cockeysville, Md.). Noble agar, APT broth, and the components for Lactobacilli MRS medium were obtained from Difco. Glucose, perchloric acid, potassium chloride, magnesium sulfate, ammonium sulfate (enzyme grade), and butanol were purchased from Fisher Scientific Co. (Pittsburgh, Pa.). The Bio-Rad protein assay was obtained from Bio-Rad Laboratories (Richmond, Calif.). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.).

RESULTS

Figure 1A shows the influence of increasing pediocin PA-1 concentrations on the PMF of energized *L. monocytogenes* Scott A cells. The addition of 5.0 μg of pediocin PA-1 per ml to energized cells of *L. monocytogenes* Scott A led to the complete dissipation of $Z\Delta pH$, whereas $\Delta\psi$ decreased from −81 to −58 mV. Increasing concentrations of pediocin PA-1 caused further reductions in $\Delta\psi$, which was not totally dissipated even at 100 μg/ml (data not shown). Upon pediocin PA-1 addition (20 μg/ml, 256 AU/ml), the culture viability of *L. monocytogenes* Scott A decreased by 4 log cycles during the PMF assay time. Heat-inactivated pediocin PA-1

(20 μg/ml, 0 AU/ml) caused a negligible (10 mV) reduction in PMF.

The action of leuconocin S on the PMF of *L. monocytogenes* Scott A is illustrated in Fig. 1B. The bacteriocin activity of the supernatant from controlled batch fermentation of *L. paramesenteroides* Ox was 800 AU/ml against *L. monocytogenes* Scott A. Ammonium sulfate precipitation increased the activity of leuconocin S 64-fold (51,200 AU/ml). The protein content of this crude leuconocin S preparation was 0.97 mg/ml. The addition of 4.9 μg of total protein per ml to energized cells of *L. monocytogenes* Scott A caused the complete collapse of ZΔpH and significant reduction of Δψ. The highest concentration of leuconocin S tested (48.5 μg/ml; 2,560 AU/ml) caused total PMF collapse and reduced the culture viability of *L. monocytogenes* Scott A by 2 log cycles during the PMF assay period. The activity of leuconocin S against *L. monocytogenes* Scott A was reduced from 51,200 to 400 AU/ml upon heat treatment. Total PMF determined in the presence of heat-treated leuconocin S (48.5 μg/ml, 20 AU/ml) was only slightly lower (18%) than total PMF obtained in the presence of leuconocin S diluent.

Lactacin F lacks activity against *L. monocytogenes* Scott A, but the strain FRI-LM 103M was reported to be sensitive (34). However, the activity of crude lactacin F against strain FRI-LM 103M was low (400 AU/ml) for a relatively high protein content (15 mg/ml). The influence of crude lactacin F on the PMF of strain FRI-LM 103M is shown in Fig. 1C. At 450 μg/ml (12 AU/ml), crude lactacin F caused PMF dissipation from −107 to −41 mV. Nevertheless, both ZΔpH and Δψ were only partially depleted. Heat-treated lactacin F (450 μg/ml, 0 AU/ml) caused a 42% reduction in total PMF. Therefore, the actual PMF reduction by bacteriocin activity on cells of strain FRI-LM 103M was apparently very small if not negligible.

These findings prompted us to evaluate a more purified lactacin F preparation to reduce any nonspecific action on PMF caused by samples with high protein concentrations. The protein content of purified lactacin F was 2.7 mg/ml. Purified lactacin F had no activity against *L. monocytogenes* FRI-LM 103M as determined by the critical dilution assay.

Our laboratory previously reported that *Aeromonas hydrophila* K144, a gram-negative food-borne pathogen, is sensitive to nisin, pediocin PA-1, leuconocin S, and lactacin F (35). However, these assays were performed by using the spot deferred-antagonism method. Considering the misleading sensitivity of *L. monocytogenes* FRI-LM 103M to crude lactacin F, we assayed the bacteriocins used in this study for activity against *A. hydrophila*. *A. hydrophila* K144 was sensitive only to pure nisin. No inhibition by pure pediocin PA-1, purified lactacin F, or crude leuconocin S was detected by the critical dilution assay (data not shown).

Lactacin F-mediated PMF dissipation in *L. delbrueckii* ATCC 4797 cells. Although lactacin F lacked activity against *L. monocytogenes* Scott A and FRI-LM 103M, it is very active against *L. delbrueckii* ATCC 4797 (45, 47) and depletes PMF in *E. faecalis* (1). Therefore, the influence of lactacin F on the PMF of *L. delbrueckii* ATCC 4797 was investigated to thoroughly test the PMF depletion hypothesis and is illustrated in Fig. 2. The critical dilution assay of purified lactacin F on *L. delbrueckii* ATCC 4797 yielded 102,400 AU/ml. The influence of purified lactacin F on the PMF of *L. delbrueckii* ATCC 4797 was determined at pH 5.5 because at an external pH of 6.5, the cells maintained a negative ZΔpH (data not shown). At pH 5.5, the cells maintained PMF levels around −130 mV, which is within the range previously reported in the literature for LAB (27, 28).

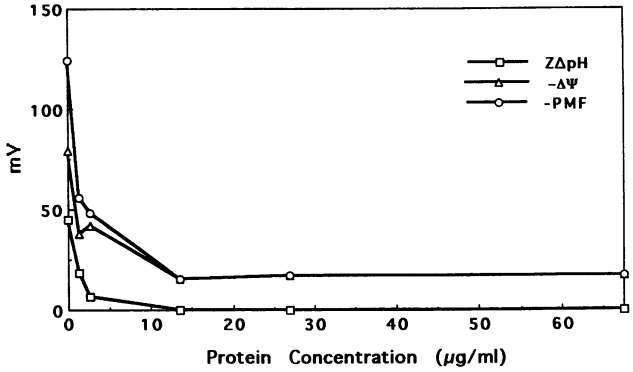

FIG. 2. Concentration-dependent action of purified lactacin F on ZΔpH, Δψ, and total PMF of energized cells of *L. delbrueckii* ATCC 4797 at pH 5.5. Datum points represent the average of at least two experiments done in duplicate.

The addition of purified lactacin F at 2.7 μg/ml (102 AU/ml) to energized cells of *L. delbrueckii* ATCC 4797 caused nearly complete dissipation of ZΔpH and significant reduction of Δψ. After the addition of increasing concentrations of purified lactacin F, the ZΔpH collapsed and the Δψ underwent further reduction. A residual Δψ around −20 mV persisted even when the purified lactacin F concentration was elevated to 270 μg/ml (data not shown). Exposure of the cells to purified lactacin F (67.5 μg/ml, 2,560 AU/ml) caused a 1.5-log-cycle reduction in the culture viability of *L. delbrueckii* ATCC 4797 during the PMF assay period. To exclude nonspecific action on the membrane by purified lactacin F preparation, we examined the control material isolated by the same procedure from the *lacF* Bac$^-$ mutant *L. johnsonii* 88-4 (46).

Energy requirements for bacteriocin-mediated PMF dissipation. The PMF-depleting antimicrobial proteins classified as colicins and defensins are characterized by their energy-dependent or energy-independent mode of action (7, 14, 30, 52, 57, 64). The energy requirements for nisin have been determined in other organisms but not *L. monocytogenes*. To investigate the energy requirements for the action of nisin, pediocin PA-1, leuconocin S, and lactacin F, energized cells of sensitive organisms were partially deenergized by using the ionophores nigericin and valinomycin in separate experiments.

The results from specific deenergization of *L. monocytogenes* Scott A with nigericin and valinomycin are summarized in Tables 1 and 2, respectively. Nigericin carries out

TABLE 1. Deenergization experiments with 2.0 μM nigericin on the PMF of energized *L. monocytogenes* Scott A cells at pH 6.5[a]

Treatment	ZΔpH (mV)	Δψ (mV)	PMF (mV)
Control	60 ± 18	−78 ± 10	−137 ± 24
Nigericin	0 ± 0	−109 ± 7	−109 ± 7
Nigericin + 2.5 μg of nisin per ml	0 ± 0	−14 ± 18	−14 ± 18
Nigericin + 20 μg of pediocin PA-1 per ml	0 ± 0	−35 ± 1	−35 ± 1
Nigericin + 48.5 μg of leuconocin S per ml	0 ± 0	0 ± 0	0 ± 0

[a] The results are expressed as the means ± standard deviations of at least three experiments conducted in buffer containing 10 mM KCl.

TABLE 2. Deenergization experiments with 5.0 μM valinomycin on the PMF of energized *L. monocytogenes* Scott A cells at pH 6.5[a]

Treatment	ZΔpH (mV)	Δψ (mV)	PMF (mV)
Control	53 ± 22	−75 ± 15	−128 ± 31
Valinomycin	42 ± 17	0 ± 0	−42 ± 17
Valinomycin + 2.5 μg of nisin per ml	40 ± 12	0 ± 0	−40 ± 12
Valinomycin + 20 μg of pediocin PA-1 per ml	0 ± 0	0 ± 0	0 ± 0
Valinomycin + 48.5 μg of leuconocin S per ml	0 ± 0	0 ± 0	0 ± 0

[a] The results are expressed as the means ± standard deviations of at least three experiments conducted in buffer containing 100 mM KCl.

the antiport of K^+ for H^+ and dissipates pH gradients in an electroneutral fashion (18). The addition of 2.0 μM nigericin to energized cells of *L. monocytogenes* Scott A caused total dissipation of ZΔpH and a 40% increase in residual Δψ as the cells tried to compensate for the ZΔpH collapse. The addition of 2.5 μg of nisin per ml, a concentration previously shown to collapse PMF in fully energized cells (8), to nigericin-treated cells caused drastic but not complete dissipation of the Δψ (Table 1). In the deenergization experiments with valinomycin (Table 2), which mediates the electrogenic uniport of K^+ with concurrent dissipation of Δψ (18), the addition of 5.0 μM valinomycin to energized *L. monocytogenes* Scott A cells caused complete depletion of Δψ and a 20% reduction in ZΔpH. In the same fashion, the addition of 2.5 μg of nisin per ml to valinomycin-treated cells caused negligible dissipation of ZΔpH. Therefore, in the presence of a ZΔpH as low as 42 ± 17 mV, no major reductions in total PMF were observed upon nisin addition. These results are also in accordance with an energy-dependent mode of action.

For pediocin PA-1, the addition of 20 μg/ml to nigericin-treated *L. monocytogenes* Scott A cells caused significant reduction of Δψ (Table 1) to a final value comparable to that obtained when the same concentration of pediocin PA-1 was added to fully energized cells (Fig. 1A). Remarkably, the addition of 20 μg of pediocin PA-1 per ml to valinomycin-treated cells caused the complete depletion of the remaining ZΔpH (Table 2). These findings suggest an energy-independent mode of action by pediocin PA-1 since a total PMF as low as −42 ± 17 mV was completely dissipated.

The addition of leuconocin S, at a concentration that causes total collapse of PMF (48.5 μg/ml), to nigericin-treated cells led to complete dissipation of Δψ (Table 1). In the deenergization experiments with valinomycin (Table 2), the addition of leuconocin S at the same concentration to valinomycin-treated cells caused the complete depletion of the low ZΔpH. These results are also consistent with leuconocin S activity being energy independent. The final effect in PMF reduction by leuconocin S is the same whether the cell membrane is fully or partially energized.

The results from specific deenergization of *L. delbrueckii* ATCC 4797 with nigericin and valinomycin at pH 5.5 are presented in Tables 3 and 4, respectively. The addition of purified lactacin F at 67.5 μg/ml to nigericin-treated cells (Table 3) caused reduction of Δψ to a residual value similar to that obtained when this concentration of purified lactacin F was added to fully energized cells (Fig. 2). *L. delbrueckii* is more resistant to valinomycin than *L. monocytogenes*.

TABLE 3. Deenergization experiments with 2.0 μM nigericin on the PMF of energized *L. delbrueckii* ATCC 4797 cells at pH 5.5[a]

Additive	ZΔpH (mV)	Δψ (mV)	PMF (mV)
Control	56 ± 4	−73 ± 8	−129 ± 9
Nigericin	0 ± 0	−95 ± 8	−95 ± 8
Nigericin + 67.5 μg of lactacin F per ml	0 ± 0	−15 ± 13	−15 ± 13

[a] The results are expressed as the means ± standard deviations of four experiments conducted in buffer containing 10 mM KCl.

Therefore, a higher valinomycin concentration of 75 μM was used and caused drastic but not total dissipation of Δψ in *L. delbrueckii* ATCC 4797. Nevertheless, the total PMF of valinomycin-treated cells was only −56 ± 17 mV and was entirely dissipated by the subsequent addition of purified lactacin F at 67.5 μg/ml. The combined results from Tables 3 and 4 indicate that lactacin F activity is energy independent. The PMF control levels (Table 4) for *L. delbrueckii* ATCC 4797 were lower than those obtained for nigericin studies (Table 3) because the buffer system used for valinomycin experiments contained 200 mM KCl.

DISCUSSION

PMF is the driving force for many vital energy-demanding processes in the cytoplasmic membrane, notably the accumulation of ions and metabolites and the synthesis of ATP (18, 39). During the PMF assay period, the culture viability of *L. monocytogenes* Scott A decreased by 4 log cycles upon the addition of pure pediocin PA-1 (20 μg/ml; 4.3 μM), which demonstrates a strong cidal effect by this bacteriocin at low concentrations and also indicates that the magnitude of the residual Δψ (around −30 mV) is insufficient to maintain culture viability. Christensen and Hutkins (11) have recently demonstrated that pediocin JD, a bacteriocin produced by *P. acidilactici* JD1-23, causes total PMF dissipation in *L. monocytogenes* Scott A. Moreover, Bhunia et al. (6) reported that pediocin AcH induces the efflux of potassium ions and UV light-absorbing materials from the cytoplasm of susceptible cells which is consistent with the PMF depletion hypothesis. It has recently become clear that pediocin PA-1 and pediocin AcH are the same protein (23, 38, 42). Lozano et al. (37) pointed out the similarities between the amino acid sequences of the LAB bacteriocins pediocin PA-1 (23), leucocin A-UAL 187 (20), and curvacin A and sakacin P (63), especially in their N-terminal regions. On the basis of the results with pediocin PA-1 and the structure-function relationships, it is likely that sakacin P, leucocin A-UAL 187, and curvacin A all mediate PMF depletion in cells of sensitive organisms.

To investigate putative nonspecific alteration of PMF by

TABLE 4. Deenergization experiments with 75 μM valinomycin on the PMF of energized *L. delbrueckii* ATCC 4797 cells at pH 5.5[a]

Additive	ZΔpH (mV)	Δψ (mV)	PMF (mV)
Control	41 ± 11	−57 ± 9	−98 ± 8
Valinomycin	41 ± 7	−15 ± 11	−56 ± 17
Valinomycin + 67.5 μg of lactacin F per ml	0 ± 0	0 ± 0	0 ± 0

[a] The results are expressed as the means ± standard deviations of four experiments conducted in buffer containing 200 mM KCl.

inactive bacteriocin preparations, the PMF of the target organisms tested was examined in the presence of heat-inactivated bacteriocin samples. Nonspecific effects are those obtained by the detergent-like properties of amphiphilic proteins when they are present in relatively high concentrations in crude or partially purified preparations. As expected for purified bacteriocin preparations, which exert high activity at micromolar concentrations, nonspecific action on PMF by pure pediocin PA-1 was negligible. On the other hand, nonspecific action on the membrane by other proteins present in the crude leuconocin S preparation probably accounts for the 18% decrease in PMF observed upon the addition of heat-inactivated leuconocin S. Nevertheless, this is a small reduction considering an estimated standard deviation of 20 mV for the PMF methodology in our laboratory. A preliminary observation of the ability of leuconocin S to partially dissipate PMF in *Lactobacillus sake* did not exclude the possibility of nonspecific action (35).

Heat-inactivated crude lactacin F caused almost as much PMF reduction in *L. monocytogenes* FRI-LM 103M as the active preparation. Because the purified lactacin F had no activity against this organism, these findings strongly suggest that PMF reduction by crude lactacin F was due entirely to nonspecific action and that the activity (400 AU/ml) against strain FRI-LM 103M determined by the critical dilution assay was an artifact and not attributable to specific lactacin F activity.

Nonspecific action of the purified lactacin F preparation on *L. delbrueckii* was ruled out by examining the effect of *lacF* Bac^- mutant control material on the PMF. Therefore, the drastic dissipation of PMF by purified lactacin F at 13.5 μg/ml was with certainty entirely attributed to bacteriocin activity on the cell membrane of *L. delbrueckii* ATCC 4797. Lactacin F is known to be a membrane-active peptide that causes leakage of potassium ions and depletes the PMF in *E. faecalis* (1).

The above results point to the importance of running critical controls to evaluate putative nonspecific action on the PMF caused by inactive bacteriocin preparations, especially when dealing with samples having high protein concentrations and low specific activities. Nonspecific membrane action by bacteriocins has been previously reported by Kordel and Sahl (32), who observed that at high concentrations, nisin and Pep 5 induced ATP leakage in insensitive eucaryotic cell membranes. However, this is not the characteristic mechanism of action of these bacteriocins, which induce prompt and complete release of intracellular compounds in sensitive bacterial cells at micromolar concentrations. The authors argued that the ATP efflux from eucaryotic cells resulted from the destabilizing nonspecific effects of high peptide concentrations on the membrane (i.e., peptide pressure).

We have demonstrated that pediocin PA-1, leuconocin S, lactacin F, and nisin (8), representative of the four major groups of LAB, have a common mechanism of action. They mediate total or major dissipation of PMF in energized cells of sensitive organisms in a concentration-dependent manner. Their action was first observed on the $Z\Delta pH$ component of PMF, which was totally dissipated by bacteriocin concentrations lower than those required to bring $\Delta\psi$ to zero.

Because of the fundamental role PMF plays in bacterial energy metabolism, PMF dissipation would ultimately cause the death of the cells. Low intracellular levels of ATP, the inability to carry out the active transport of nutrients, and the inability to maintain sufficient concentrations of cofactors, such as K^+ and Mg^{2+}, as a direct result of PMF collapse would all contribute to growth inhibition and death of the cells (30). Moreover, the complex process of growth has been shown to occur with PMF levels ranging from −100 to −200 mV (22). In our studies, exposure of the sensitive cells to bacteriocins at concentrations that dissipate PMF caused at least a 1.5-log-cycle reduction in culture viability.

Nigericin and valinomycin were used to carry out partial and specific dissipation of PMF in the deenergization experiments. The use of common ionophores to control either one or both components of PMF has been extensively reported (2, 12, 15, 44, 66). For nisin, the higher standard deviation obtained in the nigericin experiments (Table 1) can be explained by variations observed in the magnitude of the $\Delta\psi$ generated upon nigericin treatment and its direct effect on nisin activity. When the $\Delta\psi$ was −95 mV, the addition of nisin caused partial reduction of the remaining $\Delta\psi$. However, for a $\Delta\psi$ of around −110 mV, nisin addition led to total depolarization of the cell membrane. These results indicate not only energy dependence but also the possibility of a threshold $\Delta\psi$ required for nisin action. Subtilin and Pep 5, other PMF-depleting antimicrobial peptides structurally similar to nisin, have a threshold potential of −90 to −100 mV for activity (31, 36, 56, 59). Previous investigations have shown that energized membranes are needed to drive an effective interaction of nisin with artificial liposomes and membrane vesicles of sensitive organisms (15, 55). Van Belkum et al. (66) demonstrated that lactococcin A increases the permeability of the cell membrane in whole cells and membrane vesicles of sensitive lactococci in a voltage-independent manner. More specifically, the PMF-independent efflux of accumulated glutamate induced by lactococcin A occurred in cells whose PMF was totally depleted by pretreatment with valinomycin and nigericin. Similar experiments showed that lactococcin B causes the efflux of glutamate in sensitive cells in an energy-independent fashion (67).

We have demonstrated for the first time that the PMF-depleting bacteriocins pediocin PA-1, leuconocin S, and lactacin F act in an energy-independent fashion and confirmed the energy requirements for nisin activity. These studies ultimately establish the similarity of LAB bacteriocins to other PMF-depleting antimicrobial proteins such as colicins and defensins. LAB bacteriocins act by the common mechanism of PMF dissipation and can be further divided into two major subclasses on the basis of their energy requirements for activity.

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