October 1992

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APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1992, p. 3312–3315
0099-2240/92/103312-04$02.00/0

Vol. 58, No. 10

Collapse of the Proton Motive Force in *Listeria monocytogenes* Caused by a Bacteriocin Produced by *Pediococcus acidilactici*†

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Received 5 June 1992/Accepted 22 July 1992

The effect of pediocin JD, a bacteriocin produced by *Pediococcus acidilactici* JD1-23, on the proton motive force and proton permeability of resting whole cells of *Listeria monocytogenes* Scott A was determined. Control cells, treated with trypsin-inactivated bacteriocin at a pH of 5.3 to 6.1, maintained a pH gradient and a membrane potential of approximately 0.65 pH unit and 75 mV, respectively. However, these gradients were rapidly dissipated in cells after exposure to pediocin JD, even though no cell lysis had occurred. The pH gradient and membrane potential of the producer cells were also unaffected by the bacteriocin. Whole cells treated with bacteriocin were twice as permeable to protons as control cells were. The results suggest that the inhibitory action of pediocin JD against *L. monocytogenes* is directed at the cytoplasmic membrane and that inhibition of *L. monocytogenes* may be caused by the collapse of one or both of the individual components of the proton motive force.

Bacteriocins produced by lactic acid bacteria have attracted much recent interest because of their antimicrobial activity against many food spoilage and pathogenic bacteria (7). Because lactic acid bacteria naturally occur in a wide range of food products, including vegetables, meats, and cheeses, the use of bacteriocins produced by lactic acid bacteria could serve as a natural means of food preservation. For example, the bacteriocin nisin, which is produced by strains of *Lactococcus lactis* subsp. *lactis*, has gained approval for use in processed cheese products (9).

Only recently have mechanisms by which nisin and other lactococcal bacteriocins inhibit bacteria been proposed. By using liposomes and proteoliposomes, Gao et al. (10) reported that nisin depolarized membranes and dissipated the membrane potential ($\Delta\psi$) and the pH gradient (ΔpH) in a voltage-dependent manner. Similarly, van Belkum et al. (18) recently provided evidence that the *L. lactis* subsp. *cremoris*-produced bacteriocin lactococcin A also increased proton permeability and decreased the $\Delta\psi$ in sensitive *L. lactis* whole cells and membrane vesicles but in a voltage-independent process.

In contrast to these and other reports on the lactococcus-produced bacteriocins, relatively little is known regarding the mode of action of bacteriocins produced by *Pediococcus* species. *Pediococcus*-produced bacteriocins have advantages as food preservatives, since many have antimicrobial activity against *Listeria monocytogenes* and *Clostridium botulinum* and may inhibit these pathogens in actual food systems (3, 4, 8, 14, 15).

In this report, we show evidence that a pediococcal bacteriocin, pediocin JD, like nisin and lactococcal bacteriocins, also acts at the cytoplasmic membrane level. This bacteriocin caused the collapse of the proton motive force (Δp) and its individual components, the membrane potential and the pH gradient, and also increased the proton permeability in cells of *L. monocytogenes* Scott A.

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† Published as paper no. 9970, Journal Series, Nebraska Agricultural Experiment Station, Lincoln, NE 68583-0919.

MATERIALS AND METHODS

Organisms and growth conditions. *Pediococcus acidilactici* JD1-23, an organism isolated from a commercial culture and previously reported (16) to produce a heat-stable, proteinaceous antilisterial bacteriocin (called pediocin JD), was routinely grown in MRS broth (Difco Laboratories, Detroit, Mich.) at 37°C. *L. monocytogenes* Scott A was grown in tryptic soy broth (Difco) containing 0.5% yeast extract (TSBYE) at 37°C.

Bacteriocin preparation and assays. Bacteriocin was obtained from 24-h *P. acidilactici* cultures. Cell suspensions were centrifuged (6,300 × *g*, 15 min), and the supernatant was removed. The supernatant was adjusted to different pH levels with HCl or NaOH and filter sterilized by using 0.45-μm-pore-size filter discs (Acrodisc; Gelman Sciences, Ann Arbor, Mich.). In some experiments, portions were first adjusted to pH 6.5 and treated with trypsin for 25 min (type III; Sigma Chemical Co., St. Louis, Mo.) at room temperature and at a final concentration of 0.2 mg of trypsin per ml of bacteriocin preparation. Concentrated bacteriocin was prepared by the addition of ammonium sulfate to *P. acidilactici* culture supernatants to 60% saturation. After 24 h at 4°C, the precipitate was collected by centrifugation, resuspended in distilled water, and dialyzed for 18 h against distilled water.

To measure bacteriocin activity, the critical dilution method of Barefoot and Klaenhammer (1) was used with modifications. A 1-ml portion of bacteriocin preparation was serially diluted, and 30 μl of each dilution was placed in wells made in tryptic soy agar (containing 0.5% yeast extract) plates. The plates were overlaid with 0.1 ml of a 16-h *L. monocytogenes* Scott A culture (containing approximately 10^8 cells per ml), which was suspended in 4.0 ml of TSBYE containing 0.6% agar. Plates were allowed to dry in a laminar-flow hood for 30 min and then inverted and incubated for 24 h at 37°C. The highest dilution that caused a discernible zone of inhibition (>9 mm) on the *Listeria* lawn represented 1 arbitrary unit.

pH gradient and membrane potential measurements. The ΔpH and $\Delta\psi$ were measured as described by Kashket et al. (13) and Hutkins and Ponne (11). Cells of *L. monocytogenes*

TABLE 1. Effect of pediocin JD on ΔpH, Δψ, and Δp in *L. monocytogenes* Scott A

pH_{out}[a]	Active bacteriocin			Inactive bacteriocin[b]		
	ΔpH	Δψ (mV)	Δp[c] (mV)	ΔpH	Δψ (mV)	Δp[c] (mV)
5.3	0.05	9	12	0.65	75	113
5.7	0.09	33	38	0.64	72	110
6.1	0.08	0	0	0.69	85	125

[a] pH_{out}, medium pH.
[b] Trypsin treated.
[c] Calculated as 59ΔpH + Δψ.

TABLE 2. Effect of pediocin JD on ΔpH, Δψ, and Δp in *P. acidilactici* JD1-23

pH_{out}[a]	Active bacteriocin			Inactive bacteriocin[b]		
	ΔpH	Δψ (mV)	Δp[c] (mV)	ΔpH	Δψ (mV)	Δp[c] (mV)
5.3	0.25	48	62	0.24	44	59
5.7	0.19	64	75	0.20	59	71
6.1	0.09	69	74	0.10	68	73

[a] pH_{out}, medium pH.
[b] Trypsin treated.
[c] Calculated as 59ΔpH + Δψ.

or, in some experiments, *P. acidilactici* were grown for 16 h, harvested by centrifugation, and resuspended in fresh medium (TSBYE or MRS) adjusted with 1 N HCl to pH 5.3, 5.7, or 6.1. Resuspended cells (usually 10 ml), at a cell density of 0.25 mg (dry weight) of cells per ml, were then treated with either active bacteriocin (180 arbitrary units per ml), trypsin-treated (inactivated) bacteriocin, or sterile untreated MRS medium. The pHs of the mixtures were readjusted, if necessary, to give identical pH values for all samples within each treatment. Samples were incubated for 30 min at room temperature. To separate 9.0-ml portions from each treatment was added either 0.01 μM (final concentration) [^{3}H]tetraphenylphosphonium bromide (TPP, 10 mCi/μmol) or 0.03 mM (final concentration) [^{14}C]benzoic acid (7.3 mCi/mmol), to estimate the ΔpH and Δψ, respectively. In some experiments (at pH 5.3), 0.004 mM [^{14}C]acetylsalicylic acid (57.4 mCi/mmol) was used instead of benzoic acid. Butanol (5%) was added to parallel TPP-treated samples to account for nonspecific TPP binding. TPP- and benzoate-treated (or acetylsalicylate-treated) cells were incubated for 30 and 10 min, respectively, and 1.0-ml portions were added to 1.5-ml microcentrifuge tubes containing 0.5 ml of silicon oil. Tubes were centrifuged (12,000 × *g*, 1.5 min), and pellet and supernatant samples were removed and counted by using a liquid scintillation counter (model LS 3801; Beckman Instruments, Fullerton, Calif.) as previously described (11). The intracellular volume (3.80 μl/mg [dry weight] of cells) of *P. acidilactici* was determined by using ^3H_2O and [^{3}H]polyethylene glycol as previously described (12). The intracellular volume of *L. monocytogenes* was reported previously (12) as 2.97 μl/mg [dry weight] of cells. The ΔpH, Δψ, and Δp were calculated as described by Kashket et al. (13) and represent the average of at least four duplications. For simplicity, negative signs were omitted for the calculated Δp values. All radioisotopes were purchased from New England Nuclear Corp., Boston, Mass.

Proton permeability. Overnight 200-ml cultures of *L. monocytogenes* were harvested by centrifugation, washed twice in 150 mM KCl buffer (pH 5.9), and resuspended in the same buffer to give 10 ml of cells containing approximately 40 mg (dry weight) of cells. Cells (4 ml) were dispensed into a vial, a pH electrode was inserted into the vial, and the pH was allowed to equilibrate under constant stirring. A pH meter (model 145; Corning, Medfield, Mass.) was adjusted to deliver a deflection of 0.18 pH unit on an attached chart recorder. Twenty microliters of active (480 arbitrary units per μl) or trypsin-inactivated bacteriocin was added, and after a steady baseline was reached, 10 to 20 μl of 10 mM HCl was added to start the assay and the pH was monitored for up to 10 min. The rate of influx of protons was calculated as the $t_{1/2}$, or the amount of time after the acid pulse required for the pH to return halfway to the original point, on the basis of extrapolation of the initial alkalinization rates.

RESULTS AND DISCUSSION

The addition of the bacteriocin pediocin JD to resting cells of *L. monocytogenes* at pH 6.1 led to the virtual collapse of the ΔpH, Δψ, and Δp, as shown in Table 1. Similar results also occurred at pH 5.3 and 5.7, although at the latter pH the Δψ was decreased somewhat less than at pH 5.3 or 6.1. As expected, no effect on these gradients was observed in the absence of bacteriocin or when the bacteriocin had been inactivated by trypsin treatment, nor was the producer organism affected by the bacteriocin (Table 2). Although pediococcal bacteriocins reportedly bind to producer as well as other insensitive organisms, the inability of pediocin JD to dissipate the Δp in the producer cells may be due to the lack of specific cytoplasmic receptors, as suggested by Bhunia et al. (5, 6). Recently, van Belkum et al. (18) reported that high concentrations of bacteriocin dissipated the Δψ even in immune cells. The effect of the bacteriocin on the ΔpH, and on the Δψ to a lesser extent, also appeared to be dose dependent (Fig. 1).

During the time course of these experiments (usually less than 1 h), there was no decrease in the optical density of the bacteriocin-treated, nongrowing cell suspensions, indicating that cell lysis had not occurred. This finding is consistent with other reports on the action of pediococcal bacteriocins,

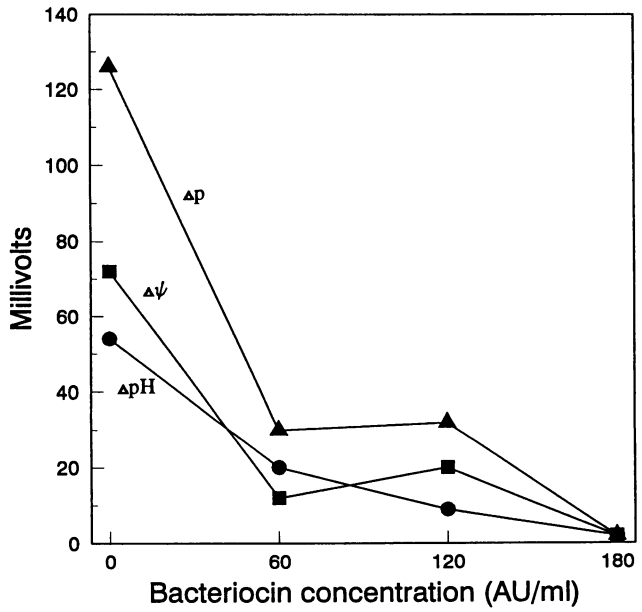

FIG. 1. Effect of bacteriocin concentration on the ΔpH, Δψ, and Δp in *L. monocytogenes* Scott A at pH 5.7. AU, arbitrary units.

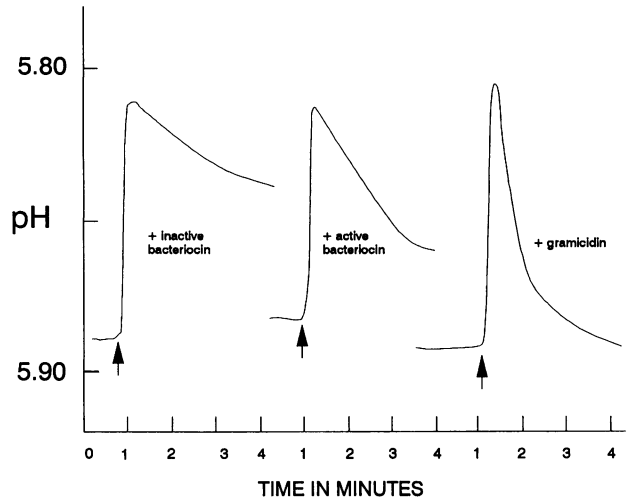

FIG. 2. Proton permeability by cells of *L. monocytogenes* exposed to active or inactivated bacteriocin. Cells were prepared as described in the text, and an acid pulse was added at the times indicated by the arrows. The gramicidin concentration was 5 μg/ml.

which show that these bacteriocins generally do not cause significant cell lysis in sensitive organisms (6, 15). In addition, bacteriocin added to cells during the early log phase of growth led to growth inhibition but not cell lysis (data not shown). Thus, it is argued that the collapse of the Δp, as measured by isotope distribution methods, was caused by specific bacteriocin-mediated effects on the cell membrane rather than having occurred as a result of general cell lysis. The increased proton permeability in the bacteriocin-treated cells (Fig. 2) further supports this hypothesis, since cultures containing lysed cells would not be expected to accumulate protons at the observed rates. (*L. monocytogenes* Scott A showed proton permeabilities [$t_{1/2}$] of 2.0 ± 0.4 min [n = 6] and 4.6 ± 0.7 min [n = 5] when treated with active and inactive [trypsin-treated] bacteriocin, respectively, at pH 5.9.)

L. monocytogenes was previously reported to maintain a relatively constant ΔpH of 0.5 to 0.7 pH unit over a pH range of 5.0 to 6.0 (12). Even at a very low pH (<5.0), *Listeria* cells remained viable as long as a ΔpH could be maintained. Therefore, the known tolerance of *L. monocytogenes* to low-pH, high-acid environments may be associated with the ability of this organism to sustain a relatively large and constant ΔpH. Although pediocin JD, in fact, may inhibit *L. monocytogenes* Scott A by causing the intracellular pH (pH_{in}) to decrease, that the organism also tolerates a low pH_{in} suggests that the overall collapse of the Δp and its components may be more critical. Since pediocin JD is active against *Listeria* cells even at near-neutral pH (3, 4, 16), when the pH_{in} would also be near neutral and the ΔpH is low (12), it would appear more likely that the bacteriocin acts as a general proton uncoupler in *L. monocytogenes*, resulting in the dissipation of essential proton, and perhaps other ion, gradients. Additionally, the increased permeability of whole cells to protons (Fig. 2) is typical of results obtained with protonophore-treated cells (2).

Although the mode of action of other pediococcal bacteriocins has not been fully established, it has been suggested that these bacteriocins cause generalized membrane destabilization or damage (6) similar to those effects described for lactococcus-produced bacteriocins (10, 17, 19). In one study (6), pediocin AcH, produced by *P. acidilactici*, was shown to cause leakage of potassium ions and UV-absorbing materials and made sensitive cells permeable to *o*-nitrophenyl-β-D-galactopyranoside, but no other membrane-directed effects were reported and cells were not lysed. These findings, however, are consistent with the action of a membrane-integrating, ionophorelike inhibitor, which dissipates ion gradients, and result in proton influx, as reported in this study. Although the role of the Δp in driving transport of sugars or other nutrients has not been studied in *L. monocytogenes*, preliminary experiments (data not shown) indicate that pediocin JD inhibited the active transport of glucose. Whether this bacteriocin-mediated inhibition was caused as a result of the collapse of the Δp (i.e., by inhibition of a Δp-driven transport system) or by inhibition of some other transport process has not yet been determined. Experiments with pediocin JD-treated membrane vesicles are currently in progress and will further clarify the nature of these observations.

ADDENDUM

Recently, Bruno et al. (6a) reported depletion of the proton motive force in nisin-treated *L. monocytogenes* in a concentration-dependent manner. In addition, the magnitudes of the decreases of the ΔpH, Δψ, and Δp were similar to those reported in the present report.

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