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Bacteriocin inhibition of two glucose transport systems in *Listeria monocytogenes*

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B.L. WAITE, G.R. SIRAGUSA AND R.W. HUTKINS. 1998. *Listeria monocytogenes* transports glucose by proton motive force-mediated and phosphoenolpyruvate-dependent phosphotransferase systems (PEP-dependent PTS). Inhibition of both systems by nisin, pediocin JD and leuconosin S is reported here for four strains of *L. monocytogenes*. Intracellular and extracellular adenosine triphosphate (ATP) and extracellular inorganic phosphate were measured in energized *L. monocytogenes* Scott A cells to determine whether inhibition of the PEP-dependent PTS might occur as a result of bacteriocin-induced leakage of intracellular components. Addition of nisin resulted in a decrease in intracellular ATP with an increase in extracellular ATP. Leuconosin S and pediocin JD induced a depletion of intracellular ATP. ATP efflux was low for the leuconosin S-treated cells and barely detectable for pediocin JD-treated cells. Addition of nisin, leuconosin S and pediocin JD induced efflux of inorganic phosphate. It appears that bacteriocin-mediated inhibition of the glucose PEP-dependent PTS occurs as a result of hydrolysis or efflux of ATP, PEP and other essential molecules from *L. monocytogenes* cells.

INTRODUCTION

Since the emergence of *Listeria monocytogenes* as a food-borne pathogen, significant efforts have been directed toward preventing its presence and growth in food products. Bacteriocins, in particular, have been promoted as potential antimicrobial agents against the growth of *L. monocytogenes* in foods. Naturally produced by many Gram-positive bacteria, bacteriocins are small antimicrobial peptides that have an inhibitory effect on other, usually closely related, species of bacteria. Leuconosin S, nisins A and Z and pediocin JD are among the bacteriocins reported to inhibit the growth of *L. monocytogenes* (Bruno *et al.* 1992; Christensen and Hutkins 1992; Lewus *et al.* 1992; Bruno and Montville 1993; Abee *et al.* 1994a; Winkowski *et al.* 1994). Although exact mechanisms of inhibition are not entirely known, it is generally accepted that bacteriocins inhibit growth of sensitive bacteria by disrupting the integrity of the cell membrane. One mechanism of inhibition common among bacteriocins is the dissipation of proton motive force (PMF) (Bruno *et al.* 1992; Christensen and Hutkins 1992; Bruno and Montville 1993; Venema *et al.* 1993; Abee *et al.* 1994b; Montville and Bruno 1994; Winkowski *et al.* 1994; Montville *et al.* 1995). In addition to dissipation of PMF, bacteriocins are reported to deplete intracellular adenosine triphosphate (ATP), either through leakage or hydrolysis, and to cause efflux of intracellular inorganic phosphate (P_i) (Abee *et al.* 1994a; Abee *et al.* 1994b; Winkowski *et al.* 1994; Chen and Montville 1995). Some bacteriocins also inhibit uptake and induce efflux of nutrients such as amino acids and sugars (van Belkum *et al.* 1991; Venema *et al.* 1993; Christensen and Hutkins 1994).

Two models were recently proposed to describe the interaction of bacteriocin monomers and the cytoplasmic membrane (Montville *et al.* 1995). The detergent disruption model predicts random insertion of bacteriocin monomers into the membrane with a resulting disruption of the phospholipid bilayer. In the poration complex model, bacteriocin monomers bind and insert into the membrane, followed by congregation of other monomers in a barrel stave formation. Such interaction of monomers results in pore formation within the membrane (Montville *et al.* 1995). While either model is consistent with the membrane disruption and loss of PMF observed in bacteriocin-sensitive cells, evidence of saturation

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kinetics and bacteriocin inhibition mechanisms, such as leakage of intracellular ATP and other large molecules, favour the poration complex model (Winkowski *et al.* 1994; Montville *et al.* 1995). Although it has been shown that bacteriocins dissipate PMF in sensitive cells, other inhibitory effects probably occur which are either a result of PMF dissipation or independent of any effect on PMF.

Recently it was reported that *L. monocytogenes* uses both a low affinity ($K_m = 2{\cdot}9$ mmol l^{-1}) PMF-mediated transport system and a high affinity ($K_m = 0{\cdot}11$ mmol l^{-1}) phosphoenolpyruvate-dependent phosphotransferase system (PEP-dependent PTS) to transport glucose into the cell (Parker and Hutkins 1997). Premaratne *et al.* (1991) reported that glucose is the preferred carbon source for *L. monocytogenes* Scott A and that 0·5% was required for cells to reach an A_{660} greater than 1·0. In addition, it has been suggested that the ability of *L. monocytogenes* to grow at low temperatures is due to a cold-resistant glucose transport system (Wilkins *et al.* 1972). As the ability of *L. monocytogenes* to grow in foods may be related to its glucose transport systems, the effects of leuconosin S, nisin and pediocin JD on glucose transport were investigated in four *L. monocytogenes* strains. To determine whether inhibition of the PEP-dependent PTS might occur as a result of bacteriocin-induced leakage or hydrolysis of intracellular components, intracellular and extracellular ATP and extracellular P_i were measured in *L. monocytogenes* Scott A.

MATERIALS AND METHODS

Bacterial strains

Listeria monocytogenes strains Scott A, Murray B, Brie 1 and V7 were grown in Tryptic Soy Broth with 0·5% Yeast Extract (Difco Laboratories, Detroit, MI, USA) at 37 °C. *Pediococcus acidilactici* JD1–23 was grown in MRS Broth (Difco) at 37 °C and *Leuconostoc paramesenteroides* OX was grown in APT Broth (Difco) with 1·5% glucose at 30 °C. All cells were grown aerobically without shaking. Bacterial strains were maintained at −20 °C in 25% glycerol.

Listeria monocytogenes Scott A and *Ped. acidilactici* JD1-23 were from the UN-L culture collection (Lincoln, NE, USA). *Listeria monocytogenes* Brie 1, Murray B and V7 were from the USDA Meat Animal Research Center culture collection (Clay Center, NE, USA). *Leuconostoc paramesenteroides* OX was generously provided by T.J. Montville (Rutgers-The State University of New Jersey, NJ, USA).

Bacteriocins

Pediocin JD and leuconosin S were isolated as described previously (Christensen and Hutkins 1992; Lewus *et al.* 1992). When necessary, both bacteriocins were inactivated by trypsin (0·2 mg ml^{-1}) (Sigma, St Louis, MO, USA, Type III). Nisin solutions were prepared by thoroughly dissolving 1 g of Nisaplin™ (10^6 IU g^{-1}) (Aplin & Barrett, Trowbridge, UK) in 11 ml 0·02 mol l^{-1} HCl. This mixture was centrifuged for 10 min at 10 000 *g* (Montville 1996). The supernatant fluid was removed and the pH adjusted to 5·3 with 1 mol l^{-1} NaOH. When necessary, nisin was inactivated by increasing the pH to 10·0 and autoclaving (121 °C, 15 min). The pH of inactivated nisin was readjusted to 5·3 (1 mol l^{-1} HCl) before use in assays. Bacteriocin activity was measured against each *L. monocytogenes* strain using the modified critical dilution method described previously (Christensen and Hutkins 1992). For pediocin JD and leuconosin S, arbitrary units were assigned to each strain using the reciprocal of the highest dilution to inhibit growth.

Glucose uptake assays

Log phase *L. monocytogenes* cells were harvested by centrifugation (10 min at 10 000 *g*), washed twice with 50 mmol l^{-1} MOPS (3-[*N*-morpholino]propanesulphonic acid) buffer, pH 6·5, and resuspended to an A_{625} of approximately 1·0. Each assay consisted of three different treatments: untreated control cells, active bacteriocin-treated cells and inactive bacteriocin-treated cells. Bacteriocins and inactive bacteriocins were added to cell suspensions 5 min prior to the start of the assays. Bacteriocin levels were based on concentrations necessary to abolish the PMF of strain Scott A (Bruno *et al.* 1992; Christensen and Hutkins 1992; Bruno and Montville 1993). Glucose uptake assays were started by addition of [^{14}C]glucose (3·7 mCi $mmol^{-1}$; Sigma Radiochemicals, St. Louis, MO, USA) to cell suspensions to give a final concentration of either 15 mmol l^{-1} (PMF-mediated transport) or 0·5 mmol l^{-1} (PEP-dependent PTS). Mixtures were incubated at room temperature and, at regular intervals, 1 ml samples of cell suspension were removed and centrifuged through silicon oil (12 000 *g* for 2 min) (Christensen and Hutkins 1994). Radioactivity was determined by liquid scintillation counting as described previously (Parker and Hutkins 1997). Representative results from at least two replicate experiments are shown.

To determine whether glucose was effluxing from bacteriocin-treated cells, uptake assays using [^{3}H]2-deoxyglucose (5·1 mCi mmol l^{-1}; Sigma Radiochemicals) were conducted on *L. monocytogenes* Scott A. The protocol for these assays was as for the glucose uptake assays except that at time zero, 2-deoxyglucose was added (0·5 mmol l^{-1} final concentration) to start the reaction. A 1 ml sample was taken at 5 min and active bacteriocins were added at 6 min. Following the addition of bacteriocin, 1 ml samples were taken at regular intervals.

ATP assays

ATP was measured by the method of Siragusa *et al.* (1995) with the following modifications. Log phase *L. monocytogenes* Scott A cells were harvested by centrifugation (15 min, 1480 *g*), washed once and resuspended in 50 mmol l^{-1} MOPS buffer, pH 6·5, to an A_{625} of 0·02. Cells were divided into 1 ml portions for different treatments (untreated control, nisin, leuconosin S, pediocin JD). At time zero, glucose was added to each cell mixture (0·5 mmol l^{-1} final concentration). After 5 min of incubation, a 50 μl sample was removed, placed in a Filtravette™ (New Horizon Diagnostics, Columbia, MD, USA) and 50 μl of buffer was added. The mixture was immediately pushed through the filter. Filtrate was collected for extracellular measurements; cells retained on the filter were used for intracellular ATP measurements. At 6 min, active bacteriocins were added to the cell suspension. At regular intervals, 50 μl samples were removed and filtered as described above. For determination of intracellular ATP levels, 50 μl of NRB™ (nucleotide-releasing agent for bacteria; Lumac B.V., The Netherlands) and 50 μl of luciferin/luciferase reagent (New Horizon Diagnostics) were added to each Filtravette™. Cells and reagents were rapidly aspirated three times and the Filtravette™ placed in a Model 3550 microluminometer (New Horizon Diagnostics). Light emission was integrated over 10 s and results were recorded as relative light units (RLU). For extracellular measurements, 50 μl of luciferin/luciferase reagent were added to 50 μl of filtrate in a disposable assay well (RemovaWell©, Dynatech Laboratories, Chantilly, VA, USA). This mixture was aspirated quickly and the assay well placed in the microluminometer. Extracellular RLU readings were adjusted to account for dilution by buffer. Standard curves were prepared by serial dilution of an ATP standard (2 pg ml^{-1}) (Lumac) in NRB™ for intracellular ATP and in 50 mmol l^{-1} MOPS buffer, pH 6·5, for extracellular ATP. Standards were read in the same manner as for extracellular ATP.

Inorganic phosphate measurement

Listeria monocytogenes Scott A cells were grown to mid-log phase, harvested (10 000 *g* for 10 min), washed twice and resuspended in 50 mmol l^{-1} MOPS buffer, pH 6·5, to A_{625} = 1·0. Aliquots of cells for each treatment (control, nisin, pediocin JD and leuconosin S) were prepared and, at time zero, glucose was added to give a final concentration of 1·0 mmol l^{-1}. At 5 min, 1 ml was removed and centrifuged through silicon oil (12 000 *g* for 2 min). Nisin, pediocin JD and leuconosin S were added after 6 min. At regular intervals following the addition of bacteriocins, 1 ml samples of each treatment were removed and centrifuged through silicon oil. For each sample, 500 μl of supernatant fluid were used to determine extracellular inorganic phosphate by the method of Chen *et al.* (1956).

Other procedures

Protein concentrations of *L. monocytogenes* cells, nisin, pediocin JD and leuconosin S were done by the Lowry method (Lowry *et al.* 1951). Nisaplin™ was provided by Aplin and Barrett Ltd. All chemicals were obtained from Sigma Chemical Co. except where noted.

RESULTS

Bacteriocin activity assays

All three bacteriocins inhibited growth of *L. monocytogenes* strains Brie 1, Murray B, Scott A and V7. The four strains did not differ in their sensitivity to nisin. However, differences in sensitivity were observed for pediocin JD and leuconosin S, with Brie 1 being the most sensitive strain and V7 the least sensitive (Table 1). For pediocin JD and leuconosin S, arbitrary units were assigned separately to each *L. monocytogenes* strain. For glucose uptake, ATP and P_i assays with nisin, 2600 IU ml^{-1} were used while leuconosin S and pediocin JD were used at 150 and 180 AU ml^{-1}, respectively.

Glucose uptake

It had previously been shown (Parker and Hutkins 1997) that the activities of the low and high affinity glucose transport systems present in *L. monocytogenes* could be estimated independently by employing either 15 or 0·5 mmol l^{-1} glucose in the assay mixture. Although the higher glucose concentration would also activate the high affinity PTS system (K_m = 0·11 mmol l^{-1}), the latter system transports glucose at rates nearly 10 times less than the low affinity PMF system and would therefore not be expected to contribute much to the total glucose uptake rate by the low affinity, high velocity system. In the presence of high glucose concentrations, glucose uptake was inhibited by nisin, pediocin JD and leuconosin S in all four strains of *L. monocytogenes*, suggesting an effect on the PMF-dependent glucose transport system. In *L. monocytogenes* Scott A, nisin decreased initial glucose uptake to a lesser degree than did pediocin JD and leuconosin S (Fig. 1). However, final accumulations of glucose were similar for nisin-, pediocin JD- and leuconosin S-treated *L. monocytogenes* cells at approximately 35% of the untreated control. Lower initial glucose uptake with similar final intracellular glucose concentrations in nisin-treated cells may be due to the requirement of nisin for an energized membrane (Ruhr and Sahl 1985). The inhibitory effects of nisin may have increased as the cells metabolized glucose.

The glucose PEP-dependent PTS, which functions at low

Table 1 Bacteriocin inhibition of *Listeria monocytogenes* strains

	Highest inhibitory dilution			AU ml^{-1}*		
Strain	Nisin	Pediocin JD	Leuconosin S	Nisin	Pediocin JD	Leuconosin S
Brie 1	1:8	1:256	1:128	267	8533	4267
Murray B	1:8	1:32	1:64	267	1067	2133
Scott A	1:8	1:64	1:64	267	2133	2133
V7	1:8	1:8	1:4	267	267	134

*For *L. monocytogenes* Scott A, one arbitrary unit (AU) was equivalent to 341 IU (nisin) or 0·50 μg of protein (leuconosin S and pediocin JD).

glucose concentrations, was severely inhibited by all three bacteriocins for all strains of *L. monocytogenes*. As with the PMF-mediated glucose transport system, nisin decreased initial glucose uptake in *L. monocytogenes* Scott A less than did pediocin JD and leuconosin S (Fig. 2). Again, this is probably due to the energy requirement of nisin. Unlike results from the uptake assays of the PMF-mediated transport system, final accumulations of glucose were lower in pediocin JD- and leuconosin S-treated cells (5% of control) than in nisin-treated cells (19% of control) for all four *L. monocytogenes* strains.

Interestingly, although both uptake systems were inhibited by nisin, pediocin JD and leuconosin S, active transport may have occurred in bacteriocin-treated cells. Intracellular concentrations of glucose were higher than extracellular concentrations for all bacteriocin treatments in both the PMF-mediated and PEP-dependent PTS glucose uptakes (data not shown). For both glucose transport systems, no inhibition was observed in cells treated with inactivated nisin, pediocin JD or leuconosin S (Figs 1 and 2).

Addition of nisin did not cause efflux of accumulated [^{3}H]2-deoxyglucose in *L. monocytogenes* Scott A while both pediocin JD and leuconosin S caused a 30% decrease in intracellular levels of this non-metabolizable glucose analogue (data not shown).

ATP measurements

Following addition of 0·5 mmol l^{-1} glucose, untreated *L. monocytogenes* Scott A cells showed a steady increase in intracellular ATP with no detectable ATP in the extracellular media (Fig. 3). Addition of nisin to cells caused a decrease in intracellular ATP and a 400-fold increase in extracellular ATP. Upon addition of pediocin JD, *L. monocytogenes* Scott A cells showed a marked decrease in intracellular ATP concentrations with a slight increase in extracellular ATP levels. Addition of leuconosin S caused a decrease in intracellular ATP, also with a slight increase in extracellular ATP. Ten minutes after the addition of bacteriocin, pediocin JD- and leuconosin S-treated cells had lower final intracellular ATP concentrations than did nisin-treated cells.

Extracellular inorganic phosphate measurement

Little P_i (39 nmol mg^{-1} cell protein) was present in the extracellular media of energized *L. monocytogenes* Scott A control cells. However, after the addition of nisin, an increase in extracellular P_i to a final concentration of 373 nmol mg^{-1} cell protein was observed. Leuconosin S and pediocin JD also caused an increase in extracellular P_i to 189 nmol mg^{-1} cell protein and 239 nmol mg^{-1} cell protein, respectively. Increases observed after the addition of leuconosin S and pediocin JD proceeded at a slower rate with lower final concentrations than those observed with the addition of nisin (Fig. 4).

DISCUSSION

Nisin, pediocin JD and leuconosin S dissipate either or both components of the PMF ($\Delta\psi$, ΔpH) in *L. monocytogenes* Scott A (Bruno *et al.* 1992; Christensen and Hutkins 1992; Bruno and Montville 1993). Levels of nisin, pediocin JD and leuconosin S used in these assays were equivalent to or higher than levels reported to totally dissipate PMF in the Scott A strain (2·5 μg ml^{-1}, A_{660} = 1; 180 AU ml^{-1}, A_{625} = 1·0 and 47·5 μg ml^{-1}, A_{660} = 1·0–1·1, respectively) (Bruno *et al.* 1992; Christensen and Hutkins 1992; Bruno and Montville 1993). It was therefore anticipated that the addition of nisin, pediocin JD or leuconosin S to *L. monocytogenes* cells would inhibit glucose transport by the PMF-mediated system. Results of PMF-mediated glucose uptake assays demonstrate that such inhibition did occur.

We report that nisin, pediocin JD and leuconosin S were also able to inhibit glucose transport by the PEP-dependent PTS in the absence of a PMF. This glucose transport system does not rely on PMF and has been reported to increase in

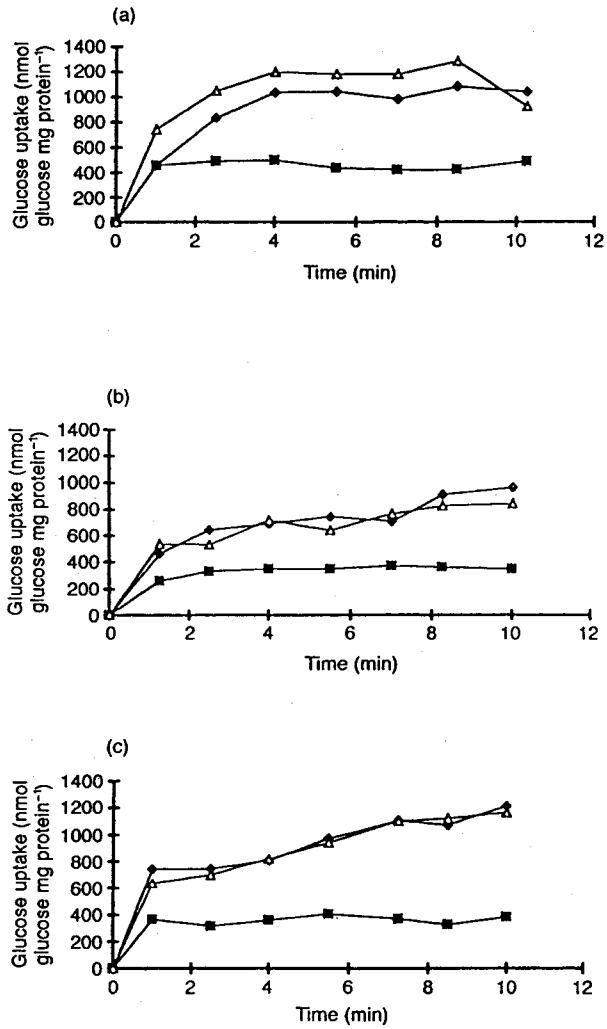

Fig. 1 Proton motive force-mediated glucose transport by *Listeria monocytogenes* Scott A in the presence of active and inactive bacteriocin. Cells were incubated with (a) nisin, (b) pediocin JD or (c) leuconosin S for 5 min prior to starting the assay. [^{14}C]-glucose (15 mmol l^{-1} final concentration) was added at time zero. Intracellular glucose levels were adjusted for non-specific binding. The figure shows nmol of glucose transported mg^{-1} of cell protein by control cells (◆), bacteriocin-treated cells (■) and inactive bacteriocin-treated cells (△). Similar results were seen for *L. monocytogenes* strains V7, Brie 1 and Murray B

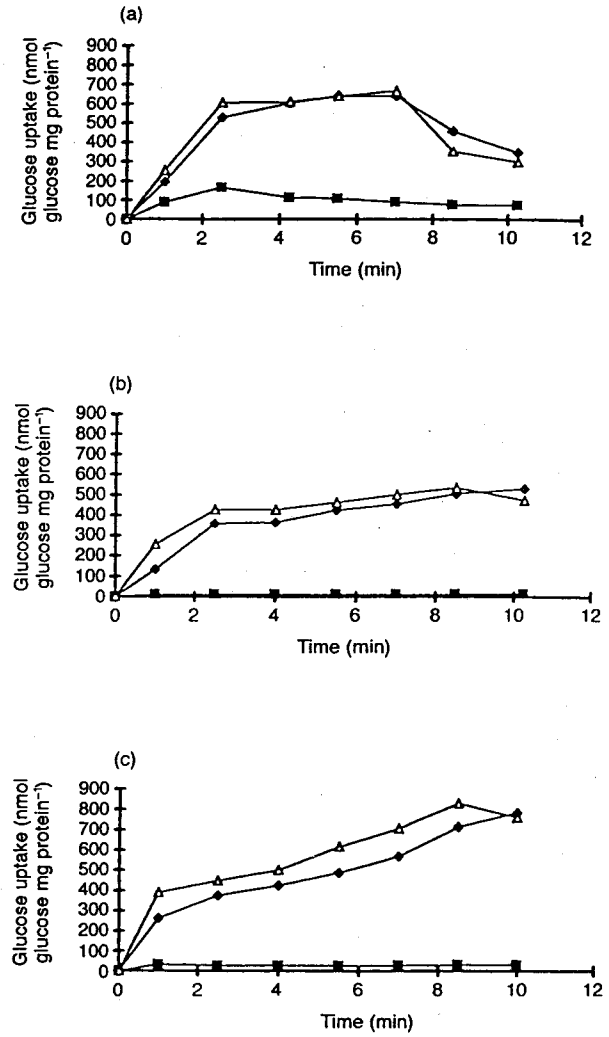

Fig. 2 Glucose transport by the phosphoenolpyruvate-dependent phosphotransferase system in *Listeria monocytogenes* Scott A in the presence of active and inactive bacteriocin. Cells were incubated with (a) nisin, (b) pediocin JD or (c) leuconosin S for 5 min prior to starting the assay. [^{14}C]-glucose (0·5 mmol l^{-1} final concentration) was added at time zero. Intracellular glucose levels were adjusted for non-specific binding. The figure shows nmol of glucose transported mg^{-1} of cell protein by control cells (◆), bacteriocin-treated cells (■) and inactive bacteriocin-treated cells (△). Similar results were seen for *L. monocytogenes* strains V7, Brie 1 and Murray B

activity in the absence of a PMF in both Gram-positive and Gram-negative cells (Reider *et al.* 1979; Kordel and Sahl 1986). Efflux of glucose may account for a small portion of the apparent glucose transport inhibition, but assays with a non-metabolizable glucose analogue demonstrated that efflux is not equivalent to the decrease in intracellular glucose observed in bacteriocin-treated cells.

Transport of glucose by the PEP-dependent PTS relies on high energy phosphate groups, donated by phosphoenolpyruvate (PEP), and the activity of PTS enzymes. Inhibition of the PEP-dependent PTS by nisin, pediocin JD and leuconosin S may be due to insufficient available PEP or to direct inhibition of the PTS enzymes. Studies on the effects of PEP 5, a bacteriocin produced by *Staphylococcus epidermidis* 5, found that PEP-dependent PTS activity of

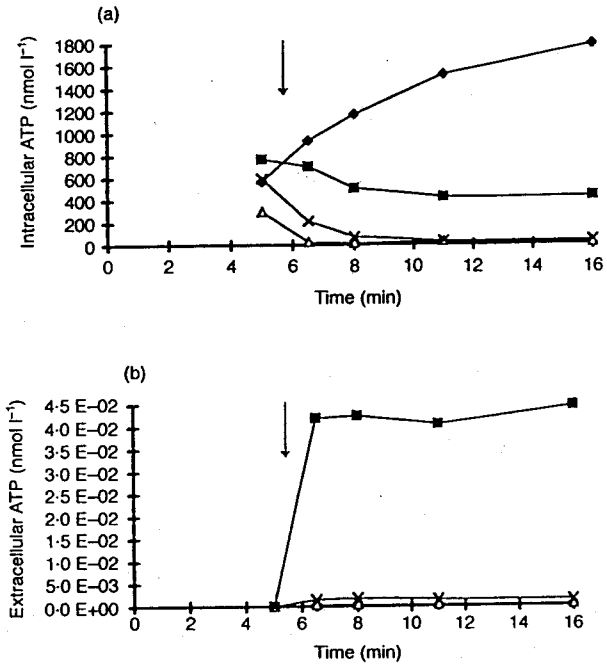

Fig. 3 (a) Intracellular and (b) extracellular adenosine triphosphate (ATP) levels in *Listeria monocytogenes* Scott A. Glucose (0·5 mmol l^{-1} final concentration) was added to cell suspensions and the suspensions incubated at room temperature for 5 min before removing the first sample for ATP determination. Samples were taken at regular intervals following the addition of active nisin (■), pediocin JD (△) and leuconosin S (×) at 6 min (↓). ◆, Control cells

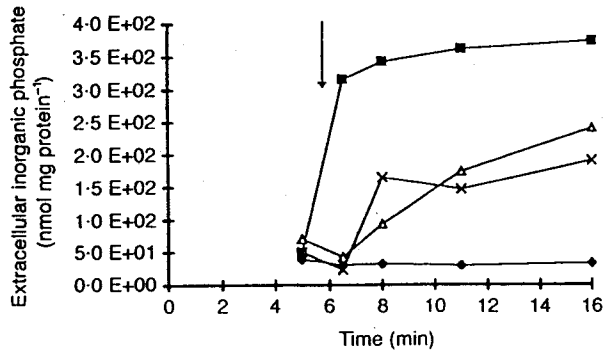

Fig. 4 Extracellular inorganic phosphate (P_i) in *Listeria monocytogenes* Scott A. Glucose (1·0 mmol l^{-1} final concentration) was added to cell suspensions and the suspensions incubated at room temperature for 5 min. A 1-ml sample was removed after 5 min and spun through silicon oil. Samples were taken at regular intervals following the addition of active nisin (■), pediocin JD (△) and leuconosin S (×) at 6 min (↓). ◆, Control cells. Supernatant fluids from centrifuged samples were assayed for determination of P_i

sensitive cells increased in the presence of this bacteriocin and excess PEP (Kordel and Sahl 1986). This indicates that inhibition observed in these PEP-dependent PTS uptake assays is due to the loss of intracellular PEP in the bacteriocin-treated cells rather than to direct inhibition of any particular enzyme. Very low levels of active transport by the PEP-dependent PTS were observed, indicating that PTS enzymes are still active in bacteriocin-treated *L. monocytogenes* cells.

The decrease in intracellular ATP and concomitant increase in extracellular ATP observed when *L. monocytogenes* cells were treated with nisin indicate that intact ATP is leaking from the cells. These results are similar to the findings of Abee *et al.* (1994a) and Winkowski *et al.* (1994) although the efflux in these studies (> 100%) was much greater than the 40% reported for nisin Z and 20% reported for nisin A. Cells treated with pediocin JD and leuconosin S had less efflux of intact ATP (1·5% and 41·1% of initial levels, respectively) although an almost total depletion of intracellular ATP did occur. The rapid decrease in intracellular ATP due to addition of pediocin JD is similar to results seen with pediocin PA-1, a bacteriocin that is probably identical to pediocin JD (Chen and Montville 1995).

Measurement of intracellular and extracellular ATP and extracellular P_i demonstrates that these three bacteriocins have differing modes of action. Nisin, a lantibiotic or Class I bacteriocin, appears to form pores that are large enough to allow intact ATP and P_i to leak from the cell. In contrast, the lower efflux of ATP and P_i from leuconosin S- (Class IV bacteriocin) treated cells and efflux of P_i from pediocin JD- (Class IIa) treated cells indicate a different or more transient type of pore formation than that found with nisin. It appears that ATP is hydrolysed in *L. monocytogenes* Scott A cells treated with pediocin JD or leuconosin S. This may be in an attempt to maintain a PMF, as suggested by the Montville groups (Winkowski *et al.* 1994; Chen and Montville 1995), or due to a shift in the equilibrium between ATP and ADP + P_i from loss of P_i, as suggested by Abee *et al.* (1994a).

Loss of intracellular ATP due to leakage or hydrolysis may be responsible for the inhibition of the PEP-dependent PTS. As ATP is lost, the high energy phosphate on PEP could be transferred to ADP (via pyruvate kinase) to maintain cellular ATP levels rather than to Enzyme I of the PTS. As P_i is lost through efflux, PEP may likewise be hydrolysed to maintain an intracellular pool of P_i. Similarly, if ATP is leaking from the cell, as is the case with nisin and to a lesser degree with pediocin JD and leuconosin S, certainly PEP, which is much smaller than ATP, could also leak from the cell. In fact, preliminary experiments demonstrate that efflux of PEP does occur in *L. monocytogenes* Scott A cells treated with nisin, pediocin JD and leuconosin S.

In this study, we demonstrate that PMF-mediated glucose transport and PEP-dependent PTS glucose transport are inhibited in *L. monocytogenes* by nisin, pediocin JD and leu-

conosin S. Loss of PMF is probably the cause of inhibition of the low affinity, PMF-mediated glucose transport system. Efflux of intracellular ATP and P_i suggests that inhibition of the high affinity glucose transport system is due to the loss of intracellular metabolites, such as PEP and ATP, rather than to the effects of PMF dissipation.

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