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Mode of Action of Nisin Z against *Listeria monocytogenes* Scott A Grown at High and Low Temperatures

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Nisin Z, a natural nisin variant, was recently isolated from *Lactococcus lactis* subspecies *lactis* NIZO 22186. The gene for this lantibiotic, designated *nisZ*, has been cloned, and its nucleotide sequence was found to be identical to that of the precursor nisin gene with the exception of a single mutation resulting in the substitution of Asn-27 for His-27 in the mature polypeptide (J. W. M. Mulders, I. J. Boerrigter, H. S. Rollema, R. J. Siezen, and W. M. de Vos, Eur. J. Biochem. 201:581–584, 1991). A K^+ electrode was used to investigate the effect of various environmental parameters on the action of nisin Z against *Listeria monocytogenes*. Addition of nisin Z resulted in immediate loss of cell K^+, depolarization of the cytoplasmic membrane, inhibition of respiratory activity, and hydrolysis and partial efflux of cellular ATP. The action of nisin Z was optimal at pH 6.0 and was significantly reduced by di- and trivalent cations. The lanthanide gadolinium (Gd^{3+}) was an efficient inhibitor and prevented nisin Z activity completely at a concentration of 0.2 mM. Nisin Z-induced loss of cell K^+ was reduced at low temperatures, presumably as a result of the increased ordering of the lipid hydrocarbon chains in the cytoplasmic membrane. In cells grown at 30°C, the action of nisin Z was prevented below 7°C, whereas in cells grown at 4°C nisin Z was able to induce K^+ leakage at this low temperature.

Lactic acid bacteria play an essential role in the majority of food fermentations, and many different strains are used as starter cultures for dairy, meat, vegetable, and bakery products. Lactic acid bacteria have long been known to produce antimicrobial proteins called bacteriocins (21, 23, 27, 36). Biochemical and genetical studies have defined four major classes of bacteriocins (24). The best-known and most studied bacteriocin produced by lactic acid bacteria is nisin A, which has been approved by the World Health Organization as a preservative in the food industry (22). The mature nisin A molecule is a protein with 34 amino acid residues which is produced by *Lactococcus lactis* subsp. *lactis*. Nisin is a lantibiotic, an antimicrobial protein which contains thioether amino acids (lanthionine [Ala-S-Ala]). These unusual amino acids are produced by modification of serine, threonine, and cysteine residues in the precursor molecule. The structural gene for nisin has been cloned and sequenced (24).

Nisin A has a broad activity spectrum and is active against a variety of gram-positive bacteria. It has been shown that nisin A permeabilizes the cytoplasmic membrane, thereby dissipating the membrane potential, inhibiting transport of amino acids, and causing release of accumulated amino acids from cells and membrane vesicles from bacteria such as *Staphylococcus aureus* and *Bacillus subtilis* (4, 25, 32, 33). The in vitro action of nisin A on liposomes and proteoliposomes was recently shown to be dependent on their phospholipid composition (13, 14). In addition, gram-negative bacteria are not sensitive to nisin A. It is likely that the outer membrane acts as a barrier to bacteriocins since membrane vesicles and osmotically shocked cells from the gram-negative bacterium *Escherichia coli* were affected by nisin A. This hypothesis was confirmed by Stevens et al. (34, 35), who showed an increased sensitivity of Tris-EDTA-treated gram-negative bacteria and lipopolysaccharide mutants of *Salmonella typhimurium* towards nisin A.

Recently, nisin Z, a natural nisin variant, was isolated from *L. lactis* subsp. *lactis* NIZO 22186. The gene for this lantibiotic, designated *nisZ*, has been cloned, and its nucleotide sequence was found to be identical to that of the precursor nisin gene, apart from a single mutation resulting in the substitution of Asn-27 for His-27 in the mature polypeptide. Nuclear magnetic resonance studies have indicated that it has a structure similar to that of nisin A (28).

Listeria monocytogenes is a gram-positive, facultatively anaerobic rod that has emerged in recent years as an important food-borne pathogen (11). Listeriosis can be life threatening to pregnant women, newborns, infants, and immunocompromised adults. *L. monocytogenes* has become a major concern to the food industry because of reports of listeriosis outbreaks associated with food (12). *L. monocytogenes* is considered to be ubiquitous in the environment and has been isolated from soils, plants, sewage, and water (7). It grows at temperatures ranging from 1 to 45°C and shows a high tolerance towards salt (11, 15). These characteristics make it difficult to control the growth of *L. monocytogenes* in foods. Nisin A (3, 8, 18, 22) and nisin Z (21) have been shown to be effective inhibitors of *L. monocytogenes*.

To investigate in more detail the potential of nisin Z as a natural preservative in various (fermented) food products, we focused on the action of nisin Z against *L. monocytogenes* Scott A grown at high and low temperatures. A potassium-selective electrode (6) was used to study the kinetics of cytoplasmic K^+ efflux induced by nisin Z. This allowed us to investigate in detail the effects of environmental parameters, i.e., pH and temperature, on the action of nisin Z. In addition, its effect on

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bioenergetic parameters such as proton motive force (PMF) generation, ATP generation, and oxygen consumption in *L. monocytogenes* has been investigated.

MATERIALS AND METHODS

Growth of bacteria. *L. monocytogenes* Scott A was grown at 30°C in brain heart infusion medium (BHI) containing 1% (wt/vol) glucose and harvested at an optical density at 650 nm of 0.6. Cells were washed and resuspended in 115 mM sodium phosphate (pH 6.0) unless indicated otherwise. This cell suspension (optical density at 650 nm of 20) was stored on ice and used within 3 h.

Determination of potassium content of cells. The variations in the K^+ content of the cells (K^+_{in}) were determined by measuring the changes in the K^+ concentration in the external medium (K^+_{out}) with a K^+-valinomycin-selective electrode. The voltmeter was connected to a computing device with which K^+_{in} can be directly determined from the value of K^+_{out} (6). The time resolution of the electrode is 4 s. The assays were performed at 30°C in 115 mM sodium phosphate (pH 6.0) containing 0.6 mM KCl and glucose (0.2% [wt/vol]) unless indicated otherwise. To estimate the total K^+ content of the bacteria, the cation was released from the cells by an excess amount of nisin Z. The amount of cation released was then estimated with the K^+ electrode.

Measurement of oxygen consumption. Oxygen consumption was measured polarographically with a Clark electrode connected to a Gilson oxygraph. Measurements were carried out at 30°C on cells incubated in 115 mM sodium phosphate (pH 6.0) containing glucose (0.2% [wt/vol]) and 0.6 mM KCl.

Measurement of cytoplasmic volume and determination of membrane potential. The cytoplasmic volume was determined with ^3H_2O (0.12 MBq/ml) and [^{14}C]sucrose (0.3 μM, 20 GBq/mmol) by the centrifugation technique (31). A value of 1 μl/mg of cells (dry weight) was found. The membrane potential was determined from the accumulation of [^{3}H]tetraphenylphosphonium ion (TPP^+) (10 μM, final concentration; 2.17 GBq/mmol). Cells were incubated at 30°C in 115 mM sodium phosphate (pH 6.0) in the presence of 0.2% glucose, 0.6 mM KCl, and 10 μM TPP^+. After 10 min of incubation, aliquots of 100 μl were taken (in triplicate), filtered on Whatman glass microfiber filters GF/F, and washed with 3 ml of the assay medium. TPP^+ uptake was corrected for unspecific binding by subtracting a blank obtained under identical conditions, except that the cells were pretreated with the protonophore 3,3′,4′,5-tetrachlorosalicylanilide (TCS; 10 μM, final concentration) before the addition of TPP^+.

ATP measurements and determination of extracellular P_i. Cells (1 mg [dry weight]/ml) were preincubated for 10 min at 30°C in 115 mM sodium phosphate (pH 6.0) or in 30 mM sodium piperazine-*N*,*N*′-bis(2-ethanesulfonate) (PIPES [pH 6.0]) containing glucose (0.2% [wt/vol]), 150 mM NaCl, and 0.6 mM KCl. The total amount of ATP, the extracellular ATP, and phosphate concentrations were determined as described by Chen et al. (9) and Guihard et al. (16).

Preparation of nisin Z. Nisin Z was purified by reverse-phase high-performance liquid chromatography as described previously (27) and stored in 0.05% (vol/vol) acetic acid at −20°C.

Materials. ^3H_2O (0.12 MBq/ml), [^{14}C]sucrose (20 GBq/mmol), and [^{3}H]TTP^+ (2.17 GBq/mmol) were purchased from the Commissariat à l'Energie Atomique (Saclay, France) and Amersham (Les Ulis, France). All materials were reagent grade and were obtained from commercial sources.

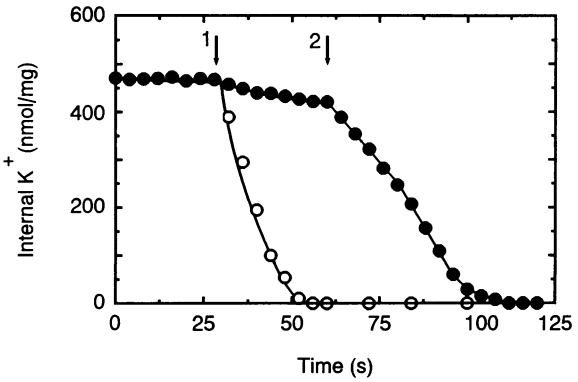

FIG. 1. Nisin Z-induced K^+ efflux from *L. monocytogenes*. Cells (1 mg [dry weight]/ml) were incubated at 30°C in 115 mM sodium phosphate (pH 6.0) containing glucose (0.2% [wt/vol]) and 0.6 mM KCl. Nisin Z (1 μg/ml) (open symbols) or TCS (10 μM, final concentration) (closed symbols) was added after 30 s (arrow 1). After 60 s, nisin Z was added (arrow 2) in the experiment with TCS. Internal K^+ is expressed in nmoles per milligram of cells (dry weight).

RESULTS

Nisin Z-induced K^+ efflux from *L. monocytogenes*. Freshly prepared *L. monocytogenes* Scott A cells contained 450 to 500 nmol of K^+ per mg of cells (dry weight) (450 to 500 mM). When these cells were kept concentrated on ice, they lost K^+. However, upon incubation at 30°C in the presence of glucose (0.2% [wt/vol]) and KCl (0.6 mM), they reaccumulated K^+ (data not shown). When the steady state of internal K^+ was attained, nisin Z was added (1 μg/ml; final concentration, 0.3 μM), and an immediate rapid efflux of K^+ (initial rate, >3.1 μmol/min/mg of cells [dry weight]) was observed such that the cells lost all K^+ in approximately 20 s (Fig. 1). Figure 2 shows that the lag time and the initial rate of K^+ efflux are dependent on the concentration of nisin Z. Efflux was observed for nisin Z concentrations as low as 0.02 μg/ml. However, at low concentrations of nisin Z, the internal K^+ level did not reach zero. This means either that, at low nisin Z concentrations, cells lose only part of their K^+ or that only a limited number of cells were hit and subsequently lost all their K^+. The latter hypothesis has been confirmed by comparing the residual level of internal K^+ (percentage of internal K^+) and the number of CFU (percent survival) after treatment with different concentrations of nisin Z (Fig. 3). Therefore, the efflux curves presented were normalized to 100% of cells hit. After normalization, we observed that the rate of efflux increased with increasing nisin Z concentrations (Fig. 2A). Because of the limit in accuracy in the K^+ electrode response, it was not possible to determine these rates for nisin Z concentrations higher than 0.6 μg/ml. Figure 2B shows that the lag time preceding K^+ efflux varied from 40 s at low nisin Z concentrations to <4 s for nisin Z concentrations of >0.2 μg/ml.

Dissipation of the PMF by adding the protonophore TCS (10 μM, final concentration) before nisin Z resulted in a small K^+ efflux (initial rate [V_i] of 0.04 μmol/min/mg of cells [dry weight]) from *L. monocytogenes*. The addition of nisin Z 30 s later resulted in a large acceleration of K^+ efflux (V_i = 1.39 μmol/min/mg of cells [dry weight]) (Fig. 1). The increased rate of K^+ efflux upon the addition of nisin Z to cells preincubated at pH 6.0 with TCS suggests that nisin Z is able to insert in the cytoplasmic membrane in the absence of a PMF. Comparison of the K^+ efflux rate in the absence and presence of a PMF

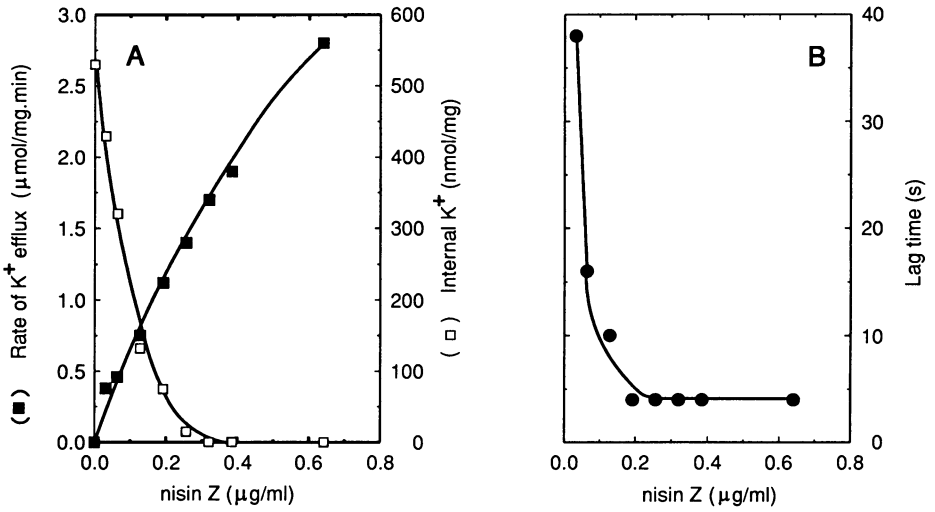

FIG. 2. Initial rate of K^+ efflux from *L. monocytogenes* (A) and corresponding lag time before K^+ efflux (B) as a function of nisin Z concentration. Cells (1 mg [dry weight]/ml) were incubated at 30°C in 115 mM sodium phosphate (pH 6.0) containing glucose (0.2% (wt/vol]) and 0.6 mM KCl. The lag time is the time elapsing between the addition of nisin Z and K^+ efflux. The K^+ efflux rate is expressed in micromoles per minute per milligram of cells (dry weight). This rate was corrected by taking into account the number of cells hit by nisin Z and normalized to 100% of the cells hit (see Fig. 3).

indicates that nisin Z is approximately threefold more active in the latter condition.

Effect of external pH on nisin Z-induced K^+ efflux from *L. monocytogenes*. K^+ efflux induced by nisin Z was influenced by the external pH. This pH dependence was analyzed for a concentration of nisin Z (1 μg/ml) at which all cells were hit. Nisin Z was able to induce K^+ efflux over a broad pH range (pH 5 to 8), with an optimum at pH 6. At higher pH values, the rate decreased gradually with increasing pH to reach, at pH 8, 50% of the value obtained at pH 6. A loss of activity was observed below pH 6, and at pH 5 to 5.5 the rate of K^+ efflux was only 37% of that at pH 6 (data not shown). Subsequent experiments were therefore performed at pH 6.

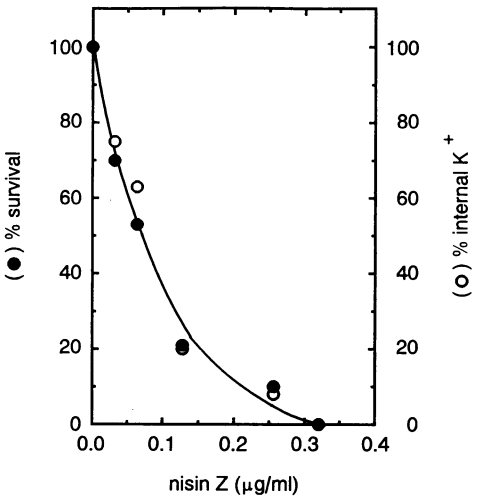

FIG. 3. Potassium content of *L. monocytogenes* cells and their viability as a function of nisin Z concentration. Cells (1 mg [dry weight]/ml) were incubated at 30°C in 115 mM sodium phosphate (pH 6.0) containing glucose (0.2% [wt/vol]) and 0.6 mM KCl. The internal K^+ content was determined with a K^+-selective electrode as described above, and viability (CFU) was determined by plating 0.1 ml of appropriate dilutions of the incubation mixture on BHI agar plates and subsequent incubation at 30°C.

Action of nisin Z against *L. monocytogenes* Scott A grown at 30 and 4°C. Since nisin Z acts on the cytoplasmic membrane, temperature effects on its effectiveness and kinetics of action at low temperatures can be expected. First, the action of nisin Z (1 μg/ml) as a function of the temperature was investigated in cells grown at 30°C (Fig. 4A). The rate of K^+ efflux decreased significantly with decreasing temperatures, and below 7°C, nisin Z was not able to induce K^+ loss from the cells. Furthermore, for temperatures lower than 18°C, a lag time was observed before the cells started to lose K^+ (Fig. 4B).

When cells grown at 4°C were assayed for nisin Z sensitivity, a similar pattern was observed, except that for a given temperature of assay, the rate of K^+ efflux was three times lower for cells grown at 4°C than for cells grown at 30°C (Fig. 4A). The rates of K^+ efflux also decreased with decreasing temperatures; however, the cells were sensitive towards nisin Z even at 4°C. Lag times before K^+ loss were also observed for temperatures below 15°C (Fig. 4B).

The Arrhenius plot of the initial rates of K^+ efflux from cells grown at 4°C and 30°C showed a break at approximately 8.5 and 14.5°C, respectively (data not shown). This difference in temperature of the break is likely to represent the difference in the temperature of transition (T_t) of the lipid hydrocarbon chains. Despite these differences in the break temperature, the energy of activation (E_A) for nisin Z-induced K^+ efflux was not very different: 0.65 and 0.97 kJ/mol above T_t and 1.7 and 3.0 kJ/mol below T_t for cells grown at 30 and 4°C, respectively.

Effect of di- and trivalent cations on nisin Z activity. The addition of increasing concentrations of $MgSO_4$ or $CaCl_2$ to cells incubated in 30 mM sodium PIPES (pH 6.0) before nisin Z was added decreased the initial rate of K^+ efflux. In the presence of 5 mM Mg^{2+} or 5 mM Ca^{2+}, the initial rate of K^+ efflux was decreased 31 and 60%, respectively (data not shown). The trivalent cation gadolinium (Gd^{3+}) was even more efficient and completely inhibited nisin Z activity at a concentration of 0.2 mM (Fig. 5A). Inhibition by Gd^{3+} was

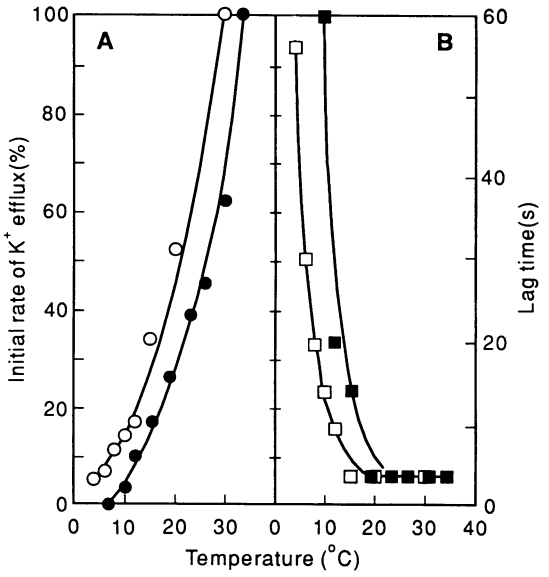

FIG. 4. Effect of temperature on initial rate of nisin Z-induced K^+ efflux (A) and lag time (B) in *L. monocytogenes* cells grown on BHI-glucose at 30°C (closed symbols) and 4°C (open symbols). Cells (1 mg [dry weight]/ml) were incubated in 115 mM sodium phosphate (pH 6.0) containing glucose (0.2% [wt/vol]) and 0.6 mM KCl. The rates of K^+ efflux in the presence of 1 μg of nisin Z per ml were normalized for 100% of the cells hit. The 100% values for nisin Z-induced K^+ efflux are 2.8 and 0.9 μmol/min/mg of cells (dry weight) for cells grown at 30 and 4°C, respectively.

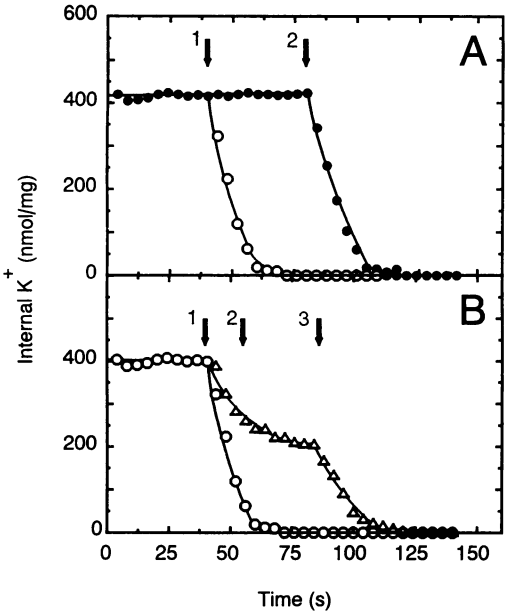

FIG. 5. Effect of gadolinium (Gd^{3+}) on the action of nisin Z against *L. monocytogenes*. Cells were washed and resuspended (1 mg [dry weight]/ml) in 30 mM sodium PIPES (pH 6.0) containing glucose (0.2% [wt/vol]), 100 mM NaCl, and 0.6 mM KCl. (A) In the control experiment (○), 1 μg of nisin Z per ml was added after 40 s (arrow 1). In a second experiment (●), Gd^{3+} (0.2 mM) was present, and nisin Z (arrow 1) and EDTA (1.5 mM) (arrow 2) were added as indicated. (B) In another experiment (△), nisin Z (0.3 μg/ml) (arrow 1) was added first, and then Gd^{3+} (0.2 mM) (arrow 2) and EDTA (1.5 mM) (arrow 3) were added. The control experiment (○) was performed as described for panel A. Internal K^+ is expressed as nanomoles per milligram of cells (dry weight).

reversed by chelation of Gd^{3+} with EDTA (1.5 mM). To investigate whether Gd^{3+} prevents the binding of nisin Z and/or the efflux of K^+ once nisin Z has reached the cytoplasmic membrane, Gd^{3+} was added after the efflux of K^+ had been initiated. This resulted in an arrest of the K^+ efflux, suggesting that the inhibitory effect of Gd^{3+} takes place even after nisin Z has been inserted in the cytoplasmic membrane (Fig. 5B). This inhibition was again reversed upon the addition of EDTA.

Effect of nisin Z on the energetic state of *L. monocytogenes* cells. The effect of nisin Z on several other vital cell functions was determined.

(i) Membrane potential dissipation by nisin Z. Energized cells of *L. monocytogenes* retain a membrane potential of −116 mV (inside negative) at pH 6. The addition of nisin Z resulted in a complete depolarization of the cytoplasmic membrane (data not shown). Direct measurement of the pH gradient was not possible because of the release of pH indicators ([^{14}C]acetate and carboxyfluorescein) from the cells via the nisin Z pores (data not shown). However, the large diameter of these pores (see below) will allow passage of protons, resulting in a dissipation of the pH gradient.

(ii) Inhibition of oxygen consumption by nisin Z. At 30°C and in the presence of glucose, the oxygen consumption of *L. monocytogenes* cells is 49 nmol/min/mg of cells (dry weight). The addition of nisin Z (1 μg/ml) resulted in a total inhibition of the respiratory activity. However, this inhibition became apparent only 1 min after the addition of nisin Z, while at that time K^+ efflux was already complete (data not shown).

(iii) Nisin Z induces a decrease in and release of internal ATP. Energized cells of *L. monocytogenes* contain 9 to 10 mM cytoplasmic ATP. The addition of nisin Z to energized cells resulted in an immediate decrease in ATP, and the cells were totally depleted of ATP within the first minute of nisin Z addition (Fig. 6A). However, this ATP decrease is not simply due to its release since only 40% of the total ATP was found in the external medium. Apparently, the nisin Z-induced decrease of the PMF is not responsible for the depletion of the remaining 60% of internal ATP since this depletion takes place far more rapidly than the one observed after depolarization of cells by TCS (10 μM) (Fig. 6A). The addition of nisin Z to cells depolarized by TCS (10 μM, final concentration) resulted in an efflux of 35% of ATP while the remaining intracellular ATP stayed constant (60% of total ATP), indicating that no hydrolysis of internal ATP took place (Fig. 6B) Nisin Z-induced hydrolysis and efflux of ATP were not associated with cell lysis since the optical density of the suspension was not affected (data not shown).

(iv) Nisin Z-induced efflux of P_i is responsible for ATP decrease. Figure 7 shows that nisin Z caused an efflux of P_i, which took place within the same time scale as the release of ATP. From the known cytoplasmic volume of *L. monocytogenes* (1 μl/mg of cells [dry weight]), we calculated that the cells had lost approximately 40 mM of P_i. No significant phosphate efflux and ATP hydrolysis were observed in TCS-treated cells.

DISCUSSION

The results of the studies presented above show that the addition of nisin Z to *L. monocytogenes* results in immediate loss of cell K^+, depolarization of the cytoplasmic membrane, inhibition of the respiratory activity, and hydrolysis and partial

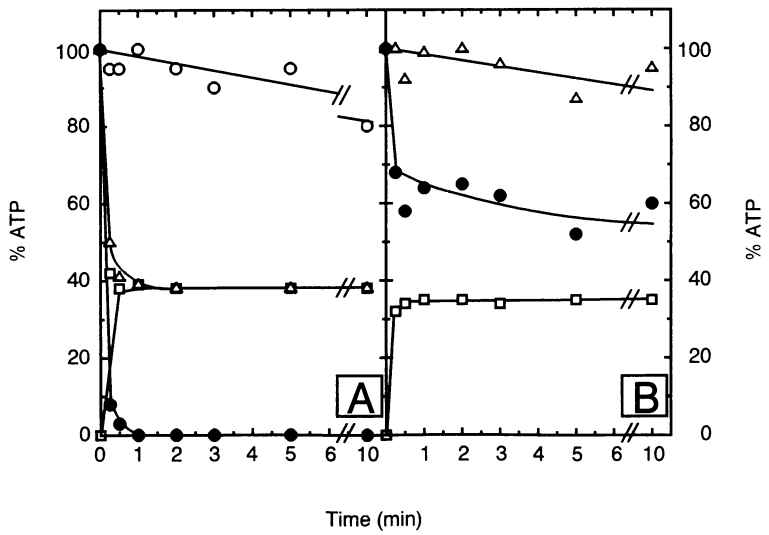

FIG. 6. Effect of the protonophore TCS and nisin Z on the intracellular ATP level in *L. monocytogenes*. Cells (1 mg [dry weight]/ml) were incubated in 115 mM sodium phosphate (pH 6.0) containing glucose (0.2% [wt/vol]) and 0.6 mM KCl. (A) TCS (10 μM, final concentration) or nisin Z (1 μg/ml) was added after preincubation for 15 min. (B) In a second experiment, TCS was added first followed by the addition of nisin Z. Symbols: cytoplasmic (total) ATP in the presence of TCS (○) and cytoplasmic ATP (●), extracellular ATP (□) and total ATP (△) in the presence of nisin Z. Values are represented as percentages of the initial intracellular ATP concentrations (100% is 9.8 mM).

efflux of internal ATP. These data indicate that the cytoplasmic membrane is the primary target for nisin Z.

It has been suggested that the cytoplasmic membrane is also the target for nisin A action (4, 32). Nisin A has been shown to form discrete-size pores in liposomes that allow the efflux of molecules with low molecular masses (14). Furthermore, Sahl et al. (33) observed that nisin A produced transient multistate pores with diameters ranging from 0.2 to 1.0 nm in black lipid membranes. Such pores would allow the passage of hydrophilic solutes with molecular masses up to 0.5 kDa. Results from nuclear magnetic resonance studies indicated that nisin Z has a structure similar to that of nisin A, except for a His residue which is replaced by an Asn residue in position 27 (28). It is therefore likely that the perturbations induced by nisin Z in the cytoplasmic membrane are due to the formation of pores. Interestingly, a nisin Z/cell ratio of approximately 10^5 is required for a complete K^+ efflux from the cells and for all cells to be hit, as judged from the analysis of the number of CFU. At higher nisin Z/cell ratios, the initial rate of K^+ efflux increased with added nisin Z and approached saturation levels with higher concentrations of nisin Z (>0.6 μg/ml), but clear saturation could not be observed, probably because of the limit in accuracy in the K^+ electrode response (Fig. 2A). The reason why such a high number of nisin Z molecules is needed is not clear. However, it is known that aggregation of nisin molecules is increased at high concentrations and high pH values (26). Therefore, we cannot exclude the possibility that even at pH 6, only part of the added nisin Z molecules are able to reach the cytoplasmic membrane (i.e., the nisin Z monomers). Furthermore, it was recently shown that nisin A interacts with negatively charged phospholipids in liposomes (14). This interaction, however, did not lead to a permeabilization of the liposomes, as judged by the absence of carboxyfluorescein leakage. Permeabilization was observed only in liposomes which contained zwitterionic phospholipids (14). If this observation is also valid for nisin Z and for its in vivo mode of action, then only part of the added nisin Z molecules, i.e., those not interacting with acidic phospholipids (approximately 85 to 90%, mainly phosphatidylglycerol and cardiolipin [30]) of the cytoplasmic membrane of *L. monocytogenes*, would be able to form pores. At lower nisin Z concentrations, a clear increase in the lag time before K^+ efflux was observed. This observation is in agreement with a model in which nisin Z molecules first bind to the cytoplasmic membrane, are subsequently inserted into this membrane, and finally form multicomponent pores. This lag time would reflect the time needed for the monomers to

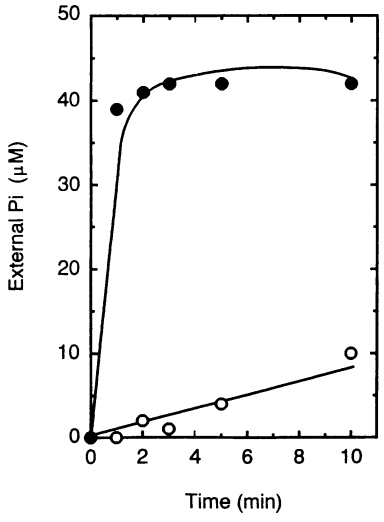

FIG. 7. Nisin Z-induced phosphate efflux from *L. monocytogenes*. Cells (1 mg [dry weight]/ml) were incubated in 30 mM sodium PIPES (pH 6.0) containing glucose (0.2% [wt/vol]), 100 mM NaCl, and 0.6 mM KCl. P_i efflux was measured upon the addition of TCS (10 μM, final concentration) (○) or nisin Z (1 μg/ml) (●).

diffuse laterally in the plane of the cytoplasmic membrane and to polymerize (29).

The action of nisin Z against *L. monocytogenes* is pH dependent, with an optimum at pH 6. The decrease in activity of nisin Z against *L. monocytogenes* at pH values below 6.0 might, however, be species specific. In experiments with another indicator organism, i.e., *Enterococcus faecalis*, nisin Z activity increased with acidic pH values as low as pH 5.0 (2). Recently, acidic external pH values as low as pH 5.5 appeared to stimulate the action of nisin A on *Escherichia coli* liposomes (14). This stimulation was even enhanced when an artificial pH gradient (alkaline inside) was imposed. Cells of *L. monocytogenes* have been shown to maintain a significant pH gradient (alkaline inside) over a broad pH range. This pH gradient increased from 0.5 to 1.8 pH units when the external pH decreased from pH 7 to 5.5 (8). The increased pH gradient at low external pH might contribute to nisin Z efficiency of pore formation as was observed for nisin A in an in vitro system (14).

Some information has been obtained on the minimum size of the nisin Z pores and on their ionic selectivity. Indeed, nisin Z caused an efflux of both cations (K^+) and anions (phosphate, ATP), suggesting that these pores do not show ionic selectivity. The fact that ATP is found in the external medium also indicates that these pores allow the efflux of hydrophilic compounds with molecular masses of approximately 0.5 kDa. Depletion of ATP is one of the primary consequences of nisin Z addition. However, ATP efflux was only partially responsible for this decrease since only 40% of the ATP was released in the external medium, the remaining 60% being hydrolyzed in the cytoplasm. The observed ATP hydrolysis induced by nisin Z is not due to PMF dissipation since it takes place far more rapidly than the ATP depletion in cells treated with the protonophore TCS (Fig. 6A). ATP hydrolysis is most likely caused by phosphate efflux (Fig. 7) and a subsequent shift in the ATP hydrolysis equilibrium. Equilibration of internal and external phosphate concentrations in the presence of TCS in cells incubated in 115 mM sodium phosphate (pH 6.0) most likely prevents a significant efflux of phosphate upon subsequent addition of nisin Z to these cells, thereby preventing ATP hydrolysis (Fig. 6B). Recently, a similar phenomenon has been described for the action of channel-forming colicins and phage proteins against *Escherichia coli* cells (16) and for the action of lactacin F, a bacteriocin produced by *Lactobacillus johnsonii*, against *Enterococcus faecalis* (1).

The presence of a PMF is not absolutely required for opening of the nisin Z pore, since K^+ and ATP efflux take place at pH 6.0 even in depolarized cells. However, the efficiency of pore formation is improved in the presence of a PMF, resulting in a threefold stimulation of the K^+ efflux rate and in an increased amount of ATP lost from the cells. This situation is very similar to that observed in vitro for nisin A. Bacteriocin-induced membrane permeabilization (i.e., carboxyfluorescein leakage) was observed in liposomes composed of *Escherichia coli* lipids (no PMF present) but stimulated in the presence of a PMF (negative and alkaline inside) (14). In TCS-treated cells of *L. monocytogenes*, the ATP efflux stopped approximately 1 min after nisin Z addition. To account for this arrest of efflux, we propose that the nisin Z pores are only transiently open, as was observed with nisin A in black lipid membranes (4) and with insect defensin A in *Micrococcus luteus* (10).

As mentioned above, there are numerous factors which contribute to or affect the action of bacteriocins. The divalent cations Mg^{2+} and Ca^{2+} have an inhibitory effect on the action of nisin Z against *L. monocytogenes*. These ions can bind to the negatively charged headgroups of phosphatidylglycerol and cardiolipin (19), which are the predominant phospholipids in the cytoplasmic membrane of *L. monocytogenes* (30) (see above). Neutralization of the negative charges induces a condensation of these lipids, resulting in a more rigid membrane (19), which most likely results in a decreased efficiency of nisin Z insertion and pore formation. The largest effect was observed for gadolinium (Gd^{3+}), and the addition of Gd^{3+}, once K^+ efflux had been initiated, even resulted in a closing of the nisin Z pores. The pores could however be opened again upon chelation of Gd^{3+} by EDTA. This closing of the pores by Gd^{3+} is more difficult to explain. Gd^{3+} has been shown to inhibit stretch-activated channels in eukaryotic and prokaryotic cells (5, 20). Whether Gd^{3+} closes the pores formed by nisin Z molecules directly, or indirectly by disturbing the interaction of the pore-forming molecules with the phospholipids in the bilayer such that the structure of the pore is modified, remains to be elucidated. It should be stressed that the pores formed by insect defensin A, a cationic peptide, show a similar dependence on Gd^{3+} (10).

The psychrotrophic bacterium *L. monocytogenes* can grow at low temperatures in coleslaw, milk, cheese, meat, and ready-to-use products (11, 12). The action of nisin Z (1 μg/ml) against *L. monocytogenes* cells was severely reduced at decreased temperatures. The ordering of the lipid hydrocarbon chains, which takes place when the temperature is decreased, and consequently the decrease of membrane fluidity (17, 19) are probably responsible for the decreased efficiency of nisin Z action. *L. monocytogenes* adapts to low-temperature growth by increasing the proportion of unsaturated fatty acyl chains of the lipids (17, 37), thereby maintaining an optimum fluidity. This adaptation is probably responsible for the remaining activity of nisin Z against these cells at 4°C. The activation energy (E_A) values for nisin Z-induced K^+ efflux above and below the presumed order-disorder transition of the lipids were 0.65 and 0.97 kJ/mol above T_t and 1.7 and 3.0 kJ/mol below T_t for cells grown at 30 and 4°C, respectively. This suggests first that the characteristics of the pores formed in cells grown at 30°C are similar to those formed in cells grown at 4°C. Second, since the E_A values are not very different above and below T_t, this suggests that properties of the channels once formed are little dependent on the state of the lipids. The values of E_A for nisin Z-induced K^+ efflux are compatible with those found for various channels (20) and for lactacin F-induced K^+ efflux from *Enterococcus faecalis* (1).

These findings may have important implications for the application of nisin Z and other bacteriocins, which have a comparable mode of action, as food preservatives. Depending on the growth history, i.e., growth temperature from food spoilage or food pathogens, which can come, for example, from water, soil, and air, these microorganisms might not be killed directly upon exposure to bacteriocins in foods stored at refrigeration temperatures. When the applied bacteriocin is not inactivated during storage, it will kill the target organism at the onset of growth, since at that point the organism has adapted the properties of the cytoplasmic membrane (i.e., membrane fluidity), resulting in optimal functioning of membrane proteins and thereby also allowing efficient insertion of bacteriocins into this membrane.

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