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Correlation of Bioenergetic Parameters with Cell Death in *Listeria monocytogenes* Cells Exposed to Nisin†

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In *Listeria monocytogenes*, nisin induced ATP efflux, reduced the intracellular ATP concentration within 1 min, and dissipated the proton motive force within 2 min. Efflux accounted for only 20% of the ATP depletion, suggesting that ATP hydrolysis also occurred. ATP efflux depended on nisin concentration and followed saturation kinetics. These results suggest that nisin breaches the membrane permeability barrier in a manner more consistent with pore formation than with a nonspecific detergent-like membrane destabilization.

Nisin is a bacteriocin produced by some strains of *Lactococcus lactis* (10, 11). The primary target of nisin's action appears to be the cytoplasmic membrane, because the addition of nisin increases the permeability of membranes to ions and small molecular compounds in whole cells and liposomes (12, 13, 18). Furthermore, nisin induces a drop in the main components of the proton motive force (PMF), the membrane potential ($\Delta\Psi$), and the pH gradient (ΔpH) of sensitive cells or artificial liposomes (2, 6). The dissipation of the PMF results in cell inactivation and death. PMF dissipation seems to be a common step among different lactic acid bacterium bacteriocins in the process of exerting their antimicrobial action (3).

Different lines of evidence suggest that nisin breaches the membrane permeability of sensitive cells by pore formation through the barrel stave mechanism (7, 20). This process entails three steps (16): water-soluble nisin monomers bind to the membrane, they insert into the membrane, and then the monomers oligomerize to form a pore. An alternative mechanism proposed for nisin action is generalized membrane solubilization caused by the detergent-like properties of the amphiphilic molecule (18). This mechanism would result in an instantaneous depletion of the PMF and a complete loss of ATP to the external medium. If nisin acts by pore formation, however, a minimum time and a minimum concentration of the bacteriocin should be required. In this study, we investigated the time course of nisin's action in *Listeria monocytogenes* cells. We present evidence that nisin causes ATP efflux and depletes intracellular ATP contents and PMF within 1 and 2 min, respectively. Furthermore, a critical amount of nisin was required to induce these effects.

L. monocytogenes Scott A cells were grown aerobically to mid-log phase at 30°C, harvested by centrifugation, washed, and resuspended in 0.1 M MES buffer containing 10 mM KCl and 10 mM $MgSO_4 \cdot 7H_2O$ at pH 6.5 (except where otherwise specified) to an A_{660} of 1.0 to 1.1, as previously described (3). Resuspended cells were energized for 15 min by adding glucose to a final concentration of 10 mM. Pure nisin (provided by Aplin and Barret Ltd., Trowbridge, England) was dissolved in 0.02 N HCl–0.75% NaCl (pH 5.0) to the desired concentration and added at specific times.

Nisin action on cell viability. Nisin-treated bacterial cells were plated on Trypticase Soy Agar and enumerated after 24 h at 30°C. The addition of nisin (1.5 μg/ml) to *L. monocytogenes* cells reduced the initial population by 2.6 log cycles within 2 to 4 min (Fig. 1A). Treatment of the energized cells with different nisin concentrations for 12 min shows that nisin concentrations of 1.5 μg/ml or higher decreased viability by >99.0% (see Fig. 2A).

Nisin action on PMF. Our laboratory has previously shown that 2.5 μg of nisin per ml collapses the PMF in *L. monocytogenes* cells, whereas lower nisin concentrations affect mainly the ΔpH (2). In this study, we investigated the time course of nisin action on the PMF of *L. monocytogenes* cells at a concentration of 1.5 μg/ml by using ^{14}C-labeled salicylic acid and ^{3}H-labeled TPP^+, as previously described (3). PMF was calculated from the equation $PMF = \Delta\Psi - Z\Delta pH$, where Z equals 2.3 (RT/F) and R, T, and F (the Faraday constant) are as described previously (9). For simplicity, negative signs were omitted for the calculated PMF values. As shown in Fig. 1B, the addition of 1.5 μg of nisin per ml collapsed $Z\Delta$pH within 2 min, while the value of $\Delta\Psi$ decreased from 72.8 ± 5.1 to 49.3 ± 3.9 mV.

Nisin action on ATP levels. Since the dissipation of the PMF may result in decreasing ATP levels, we investigated the ATP content of nisin-treated *L. monocytogenes* cells. ATP concentrations were determined by the luciferin-luciferase bioluminescence assay (Sigma). Cells were prepared as described above, except that they were resuspended in 0.1 M MES buffer containing 10 mM KCl only. The internal ATP concentration was estimated indirectly by subtracting the external ATP concentration from the total ATP concentration as described by Guihard et al. (8). The amount of light emitted was recorded with a Lumac/3M biocounter M2010. ATP content is expressed in nanomoles per milligram of cells (dry weight) (CDW). Adenine nucleotide concentrations were determined as described by Guihard et al. (8).

Time-dependence assays showed (Fig. 1C) that the internal ATP level in *L. monocytogenes* (2.5 ± 0.8 nmol/mg of CDW) fell 80% within 1 min after the addition of 1.5 μg of nisin per ml. This drop in internal ATP was concomitant with ATP efflux. The efflux in ATP observed after the addition of 1.5 μg of nisin per ml (0.4 ± 0.1 nmol/mg of CDW) corresponded to only 20% of the intracellular ATP lost. Nisin-induced ATP efflux is consistent with Sahl et al.'s (20) observations on black lipid membranes. These researchers predicted that nisin could

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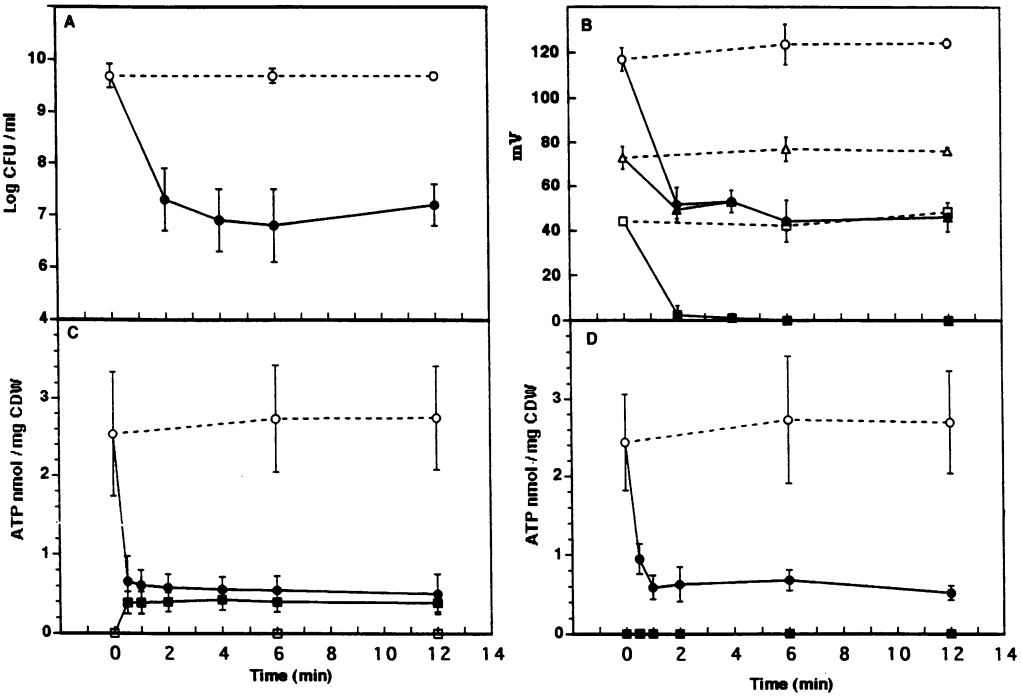

FIG. 1. Kinetics of nisin action on *L. monocytogenes* cells. (A) cell viability; (B) PMF dissipation; (C and D) ATP levels. All solid symbols represent the effect of 1.5 μg of nisin per ml (A, B, and C) and 2.0 μM nigericin (D). All open symbols represent the controls (diluent added). Symbols: for panel A, ●, *L. monocytogenes* viable cell count; for panel B, ●, total PMF; ▲, $\Delta\Psi$; ■, $Z\Delta$pH; for panels C and D, ●, internal ATP; ■, external ATP. Vertical bars represent the standard deviations of three independent experiments done in duplicate.

form a pore with a maximum diameter of 1 nm, which should allow the permeation of molecules with molecular masses up to 600 Da. Nisin also decreases the internal ATP levels in *Clostridium sporogenes* (17) and in *Lactobacillus* and *Pediococcus* spp. (15). Similar results were observed for the bacteriocins Pep 5 and lactostrepcin 5 (19, 23). Recently, Abee et al. (1) reported that lactacin F induces ATP hydrolysis in sensitive cells, although no ATP efflux was detected. In our studies, efflux of ATP accounted for only 20% of the intracellular ATP lost, suggesting that ATP hydrolysis also occurs. The nature of the internal ATP concentration assays, which are calculated by subtracting external ATP from total ATP, makes it possible to underestimate the amount of ATP efflux if ATP was hydrolyzed to ADP and AMP in the external medium. This would lead to overestimation of the percentage of ATP hydrolyzed inside the cells. However, the total concentration of external adenine nucleotides (ATP, ADP, and AMP) after nisin addition which may have resulted from efflux and/or external hydrolysis (data not shown) was only 37% of the intracellular ATP lost. This result demonstrates that most of the ATP was hydrolyzed intracellularly.

Hydrolysis of ATP can be explained, among other possibilities, from the conversion of ATP to regenerate the decreased PMF and/or as a shift in ATP hydrolysis equilibrium resulting from phosphate efflux (8). To distinguish between those mechanisms, we used the protonophore nigericin, which dissipates the ΔpH in an electroneutral manner (9). The addition of 2.0 μM nigericin to energized *L. monocytogenes* cells caused total dissipation of $Z\Delta$pH and increased $\Delta\Psi$ levels to 110 mV (2). In the present experiments, we observed that the addition of nigericin resulted in a reduction of the internal ATP content of *L. monocytogenes* cells (76%) (Fig. 1D). However, no ATP efflux was detected and no loss of *L. monocytogenes* cell viability occurred (data not shown), suggesting that the decrease in intracellular ATP levels observed must be primarily the result of ATP hydrolysis in order to regenerate a proton gradient. We suggest that pores formed by nisin allow efflux of protons and other small molecules. The concomitant ATP hydrolysis represents the cell's futile attempt to maintain PMF.

The second line of evidence favoring pore formation is that nisin action demonstrates saturation kinetics (Fig. 2B and C). The action of many other bacteriocins is concentration dependent and saturable (2, 4, 5, 7, 19, 21, 22). The results presented in Fig. 2 suggest that nisin concentrations of ≥1.5 μg/ml are required to significantly reduce the viability of *L. monocytogenes* populations. At this concentration, nisin causes maximum ATP depletion and maximum ATP efflux. We have previously reported that higher nisin concentrations (2.5 μg/ml) were required to dissipate both components of the PMF ($\Delta\Psi$ and ΔpH) (2). In the present study, we observed that increasing nisin concentrations results in a greater decrease in cell viability. These observations suggest that the concentration of nisin molecules per cell might determine the extent of its biological activity. This could occur by increasing the size of exclusion limit of the pores, by increasing the number of pores, or by some other mechanism.

The results discussed above suggest that, similar to observations in model liposomes (7), nisin may be acting through a poration mechanism in *L. monocytogenes* cells. These results are inconsistent with a generalized solubilization mechanism which would result in an all-or-none lysis of the cells, with a sudden collapse of the bioenergetic parameters and no saturation kinetics. Nisin may belong to a broader group of pore-forming peptides, including defensins, cecropins, mellittins, and magainins (14), some of which form nonspecific pores

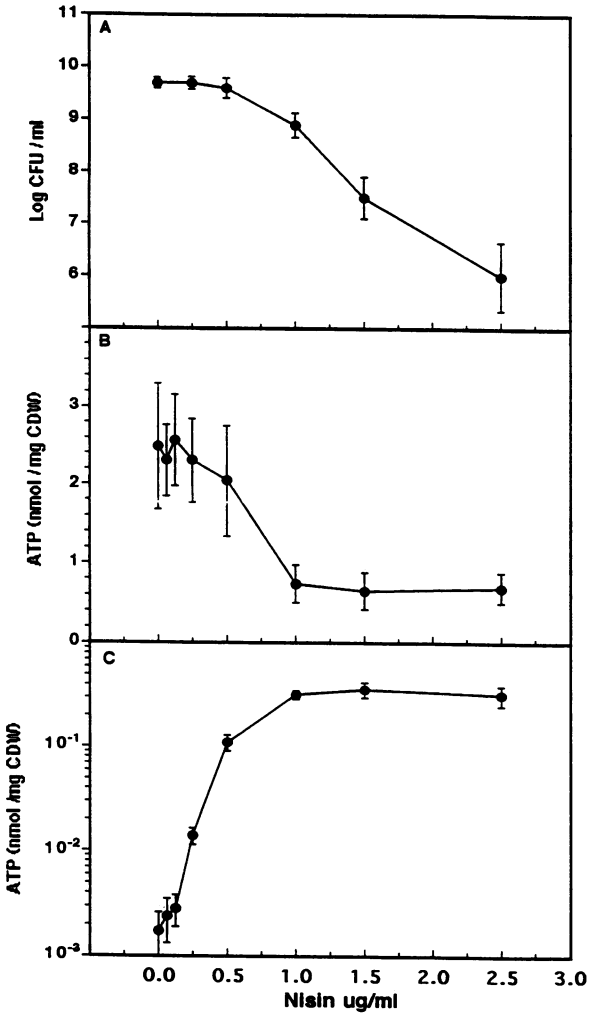

FIG. 2. Concentration-dependent action of nisin. (A) cell viability; (B), internal ATP depletion; (C) external ATP levels. Vertical bars represent the standard deviations of three independent experiments done in duplicate.

in the membranes of target organisms by the barrel stave mechanism (16).

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REFERENCES

1. **Abee, T., T. R. Klaenhammer, and L. Letellier.** 1994. Kinetic studies of the action of lactacin F, a bacteriocin produced by *Lactobacillus johnsonii* that forms poration complexes in the cytoplasmic membrane. Appl. Environ. Microbiol. **60:**1006–1013.
2. **Bruno, M. E. C., A. Kaiser, and T. J. Montville.** 1992. Depletion of proton motive force by nisin in *Listeria monocytogenes* cells. Appl. Environ. Microbiol. **58:**2255–2259.
3. **Bruno, M. E. C., and T. J. Montville.** 1993. Common mechanistic action of bacteriocins from lactic acid bacteria. Appl. Environ. Microbiol. **59:**3003–3010.
4. **Chikindas, M. L., M. J. García-Garcerá, A. J. M. Driessen, A. M. Ledeboer, J. Nissen-Meyer, I. F. Nes, T. Abee, W. N. Konings, and G. Venema.** 1993. Pediocin PA-1, a bacteriocin from *Pediococcus acidilactici* PAC1.0, forms hydrophilic pores in the cytoplasmic membrane of target cells. Appl. Environ. Microbiol. **59:**3577–3584.
5. **Christensen, D. P., and R. W. Hutkins.** 1992. Collapse of the proton motive force in *Listeria monocytogenes* caused by a bacteriocin produced by *Pediococcus acidilactici*. Appl. Environ. Microbiol. **58:**3312–3315.
6. **Gao, F. H., T. Abee, and W. N. Konings.** 1991. Mechanism of action of the peptide antibiotic nisin in liposomes and cytochrome *c* oxidase-containing proteoliposomes. Appl. Environ. Microbiol. **57:**2164–2170.
7. **Garcia-Garcera, M. J. G., M. G. L. Elferink, J. M. Driessen, and W. N. Konings.** 1993. *In vitro* pore-forming activity of the lantibiotic nisin. Role of PMF force and lipid composition. Eur. J. Biochem. **212:**417–422.
8. **Guihard, G., H. Benedetti, M. Besnard, and L. Letellier.** 1993. Phosphate efflux through the channels formed by colicins and phage T5 in *Escherichia coli* cells is responsible for the fall in cytoplasmic ATP. J. Biol. Chem. **268:**17775–17780.
9. **Harold, F. M.** 1986. The vital force: a study of bioenergetics. W. H. Freeman and Co., New York.
10. **Hurst, A.** 1981. Nisin. Adv. Appl. Microbiol. **27:**85–123.
11. **Klaenhammer, T. R.** 1993. Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiol. Rev. **12:**39–86.
12. **Kordel, M., and H. G. Sahl.** 1986. Susceptibility of bacterial, eukaryotic and artificial membranes to the disruptive action of the cationic peptides Pep 5 and nisin. FEMS Microbiol. Lett. **34:**139–144.
13. **Kordel, M., F. Schuller, and H. G. Sahl.** 1989. Interaction of the pore-forming peptide antibiotic Pep 5, nisin and subtilin with nonenergized liposomes. FEBS Lett. **244:**99–102.
14. **Montville, T. J., and A. Kaiser.** 1993. Antimicrobial proteins: classification, nomenclature, diversity, and relationship to bacteriocins, p. 1–22. *In* D. G. Hoover, and L. R. Steenson (ed.), Bacteriocins of lactic acid bacteria. Academic Press, New York.
15. **Ogden, K., and M. J. Waites.** 1986. The action of nisin on beer spoilage lactic acid bacteria. J. Inst. Brew. **92:**463–467.
16. **Ojcius, D. M., and J. D. E. Young.** 1991. Cytolytic pore forming proteins and peptides: is there a common structural motif? Trends Biochem. Sci. **16:**225–229.
17. **Okereke, A., and T. J. Montville.** 1992. Nisin dissipates the proton motive force of the obligate anaerobe *Clostridium sporogenes* PA 3679. Appl. Environ. Microbiol. **58:**2463–2467.
18. **Ruhr, E., and H. G. Sahl.** 1985. Mode of action of the peptide antibiotic nisin and influence on the membrane potential of whole cells and cytoplasmic and artificial membrane vesicles. Antimicrob. Agents Chemother. **27:**841–845.
19. **Sahl, H. G., and H. Brandis.** 1983. Efflux of low-M_r substances from the cytoplasm of sensitive cells caused by the staphylococcinlike agent pep 5. FEMS Microbiol. Lett. **16:**75–79.
20. **Sahl, H. G., M. Kordel, and R. Benz.** 1987. Voltage-dependent depolarization of bacterial membranes and artificial lipid bilayers by the peptide antibiotic nisin. Arch. Microbiol. **149:**120–124.
21. **van Belkum, M. J., J. Kok, G. Venema, H. Holo, I. F. Nes, W. N. Konings, and T. Abee.** 1991. The bacteriocin lactococcin A specifically increases permeability of lactococcal cytoplasmic membranes in a voltage-independent, protein-mediated manner. J. Bacteriol. **173:**7934–7941.
22. **Venema, K., T. Abee, A. J. Haandrikman, K. J. Keenholts, J. Kok, W. N. Konings, and G. Venema.** 1993. Mode of action of lactococcin B, a thiol-activated bacteriocin from *Lactococcus lactis*. Appl. Environ. Microbiol. **59:**1041–1048.
23. **Zajdel, J. K., P. Ceclowski, and W. T. Dobrzanski.** 1985. Mechanism of action of lactostrepcin 5, a bacteriocin produced by *Streptococcus cremoris* 202. Appl. Environ. Microbiol. **49:**969–974.