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Protamine-Induced Permeabilization of Cell Envelopes of Gram-Positive and Gram-Negative Bacteria

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The inhibitory effect of the cationic peptide protamine on *Listeria monocytogenes*, *Escherichia coli*, and *Shewanella putrefaciens* has been studied in detail. The addition of protamine (10 to 1,000 μg/ml) resulted in inhibition of oxygen consumption after less than 1 min and loss of intracellular carboxyfluorescein and ATP after 2 to 5 min. Maximum antibacterial activity was reached at alkaline pH and in the absence of divalent cations. The efficient permeabilization of cell envelopes of both gram-positive and gram-negative bacteria suggests that protamine causes a general disruption of the cell envelope, leading to a rapid and nonspecific efflux of low- and high-molecular-weight compounds.

Protamine found in salmon spermatozoan nuclei (salmine) is a basic peptide (pI > 10) with 32 amino acids, of which 21 are arginine (3). Protamine has antimicrobial activity against a broad spectrum of bacteria and fungi (9, 11, 13, 17), and this is considered to be a result of its polycationic nature. The broad antimicrobial spectrum of protamine and the fact that protamine is naturally occurring and nontoxic to humans (10) make it a promising biological alternative to chemical preservatives and disinfectants.

In a previous study, protamine was shown to alter the cell morphology of *Listeria monocytogenes* and *Shewanella putrefaciens* and form large holes in the cell envelope (14). The exact antibacterial mechanism of protamine, however, is not known but may be a general disruption of the cell envelope, e.g., a perturbation of the phospholipid dynamics at the membrane surface (12, 26) or, as described for other antibacterial cationic peptides, the formation of pores in the cytoplasmic membrane (1, 6, 16).

The purpose of the present study was to investigate, at a cellular level, how protamine exerts its antibacterial action against *L. monocytogenes*, *Escherichia coli*, and *S. putrefaciens*.

Determination of oxygen consumption. The effects of pH and the presence of Ca^{2+} ($CaCl_2 \cdot 2H_2O$) (catalog no. 2382; Merck) and Mg^{2+} ($MgCl_2 \cdot 6H_2O$) (catalog no. 5832; Merck) on the bactericidal activity of protamine were tested by measurements of oxygen consumption.

L. monocytogenes Scott A (27), *S. putrefaciens* (15), and *E. coli* O157:H7 (ATCC 43895) were grown in tryptone soy broth (TSB) (catalog no. CM129; Oxoid) at 25°C. Bacteria were harvested (10 min at 2,000 × *g*) at an absorbance at 450 nm (A_{450}) of 0.9 and washed once in 50 mM potassium phosphate buffer (pH 7.0) and once in 50 mM HEPES (pH 7.0). The pellet was resuspended in the 50 mM HEPES buffer to an A_{600} of 20, stored on ice, and used within 5 h.

Oxygen uptake by bacteria was measured at 25°C with a Clark-type oxygen probe (biological oxygen monitor, model 5300; YSI Co., Inc., Yellow Springs, Ohio) immersed in a magnetically stirred sample chamber containing 50 mM MES (2-[*N*-morpholino]ethanesulfonic acid) (pH 6.0) or 50 mM HEPES buffer (pH 8.0 or 7.0). The pH was adjusted with potassium hydroxide. The bacterial suspension was injected into the chamber at a final bacterial concentration of approximately 10^{10} CFU/ml. After stabilization of oxygen consumption, substrate was injected, followed by addition of protamine (catalog no. P 4005; Sigma). Glucose (6 mM) was added as substrate to *L. monocytogenes* and *E. coli*, and lactate (20 mM) was used as substrate for *S. putrefaciens*.

The effect of cations on the activity of protamine was evaluated by addition of various concentrations of $MgCl_2$ and/or $CaCl_2$ (0 to 10 mM) to the cell suspension before addition of substrate and protamine. The effects of chelation of divalent cations were tested by adding EDTA (10 mM), either before or after protamine addition, to a cell suspension with a total concentration of cations of 5 mM.

Measurement of cF efflux. Bacteria were grown in TSB at 5 and 25°C, harvested as described above, washed twice in 50 mM potassium phosphate buffer (pH 7), and resuspended in the same buffer (A_{450} = 0.8). Carboxyfluorescein (cF) diacetate (Molecular Probes, Inc.) dissolved in acetone (5 mg/ml) was added to a final concentration of 0.54 μM. Bacteria grown at 5°C were loaded with cF for 1 h at 15°C, and cells grown at 25°C were loaded for 10 min at 30°C (5). Efflux assays were performed at 5 and 25°C. Protamine (0, 10, 100, and 1,000 μg/ml) was added to cells loaded with cF, and samples (200 μl) were withdrawn at appropriate time intervals. Bacteria were removed by centrifugation (2 min), and the supernatants (150 μl) were transferred to wells of a microtiter plate. Fluorescence was measured in a luminescence spectrometer (model LS50B; Perkin-Elmer) at room temperature with excitation and emission wavelengths of 490 and 515 nm, respectively, and slit widths of 2.5 nm. Leakage of intracellular cF due to protamine treatment was evaluated relative to the maximum leakage, which was found by lysing cells with dimethyl sulfoxide (DMSO) (catalog no. D 5879; Sigma).

ATP measurements. Cells were harvested and washed as described above and preincubated before protamine addition

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for 10 min at 25°C in 50 mM MES (pH 6.0) or 50 mM HEPES (pH 7.0 or 8.0) buffer containing 0.2% (wt/vol) glucose.

Total ATP and extracellular ATP were determined with a luminometer (Lumac/3M Biocounter M2010A) as described by Abee et al. (2). Intracellular ATP concentrations were calculated by using the intracellular volumes of 3 and 2 μl/mg of protein for *L. monocytogenes* and *E. coli*, respectively (7, 23). The amount of protein was determined by the method of Lowry et al. (19), with bovine serum albumin as a standard.

Leakage of β-galactosidase. *E. coli* was grown at 25°C in veal infusion broth (catalog no. 0344-17-6; Difco) with lactose (2 g/liter) to an A_{450} of 0.9. Cells were harvested and washed as described above and resuspended in 50 mM potassium phosphate buffer (A_{450} = 0.8). Protamine was added to the cell suspension, and samples (100 μl) were withdrawn after appropriate time intervals and mixed with 100 μl of 3 mM *o*-nitrophenyl-β-D-galactoside (ONPG) (catalog no. N 1127; Sigma). The hydrolysis of ONPG was measured at A_{405} after a 1-min incubation (18). Total β-galactosidase activity was measured with bacteria lysed by ultrasonication with eight 30-s cycles at 5 A (MSE Soniprep 150 Ultrasonic Disintegrator; Scientific Instruments, Crawley, United Kingdom) in the presence or absence of protamine to exclude any interference of the cationic peptide with the enzyme activity.

Impedance assay for bacteriocidal activity. Bacteria were grown in TSB at 5, 10, 15, 25, or 30°C to an A_{450} of 0.9 and then were harvested by centrifugation (2,000 × *g*, 10 min), washed twice with 50 mM sterile phosphate buffer (pH 7.0), and resuspended in the same buffer (A_{450} = 1.0). Protamine was added to the cell suspension. To assess bactericidal activity, samples were withdrawn after a 30-min incubation and inoculated in Bactometer wells (Bactometer B123-2; bioMérieux UK Ltd., Hampshire, United Kingdom) (13). The detection time was determined and converted to a colony count by using a calibration curve (13). By this procedure, colony counts were not made directly on the protamine-treated suspensions as protamine caused significant clumping of the bacterial cells.

Fatty acid composition. *L. monocytogenes* and *S. putrefaciens* were grown in TSB at 5, 15, and 25°C until mid-exponential phase (A_{600} = 0.6) and washed twice (2,000 × *g*, 10 min) in 50 mM potassium phosphate (pH 6.8) containing 5 mM $MgSO_4$. Total lipid was extracted from the bacterial pellets by the method described by Bligh and Dyer (4). Methyl esters were analyzed in a gas chromatograph (model Sigma 3B; Perkin-Elmer) with a flame ionization detector on a polar column with a temperature profile from 175 to 225°C. Sample components were identified by comparison with retention times of standard methyl esters.

Effect of pH and divalent cations on the activity of protamine. The effect of protamine on oxygen consumption of *L. monocytogenes*, *E. coli*, and *S. putrefaciens* is shown in Fig. 1. The oxygen consumption by *L. monocytogenes* was not, at the concentrations tested, inhibited at pH 6, whereas significant inhibition was observed at pH 7 and 8 (Fig. 1A). Maximum effect was observed at pH 8, at which even the lowest concentration of protamine (10 μg/ml) resulted in 70% inhibition of oxygen consumption. A similar pattern of inhibition was observed with the gram-negative bacteria, *E. coli* (Fig. 1B) and *S. putrefaciens* (Fig. 1C). Oxygen consumption was, for all three species, completely inhibited at pH 8 in the presence of 1,000 μg of protamine per ml.

The increase in activity of protamine at high pHs may be caused by an increase in the amount of negatively charged groups on the cell envelope, which facilitates the electrostatic interactions between the cell envelope and protamine (pI > 10).

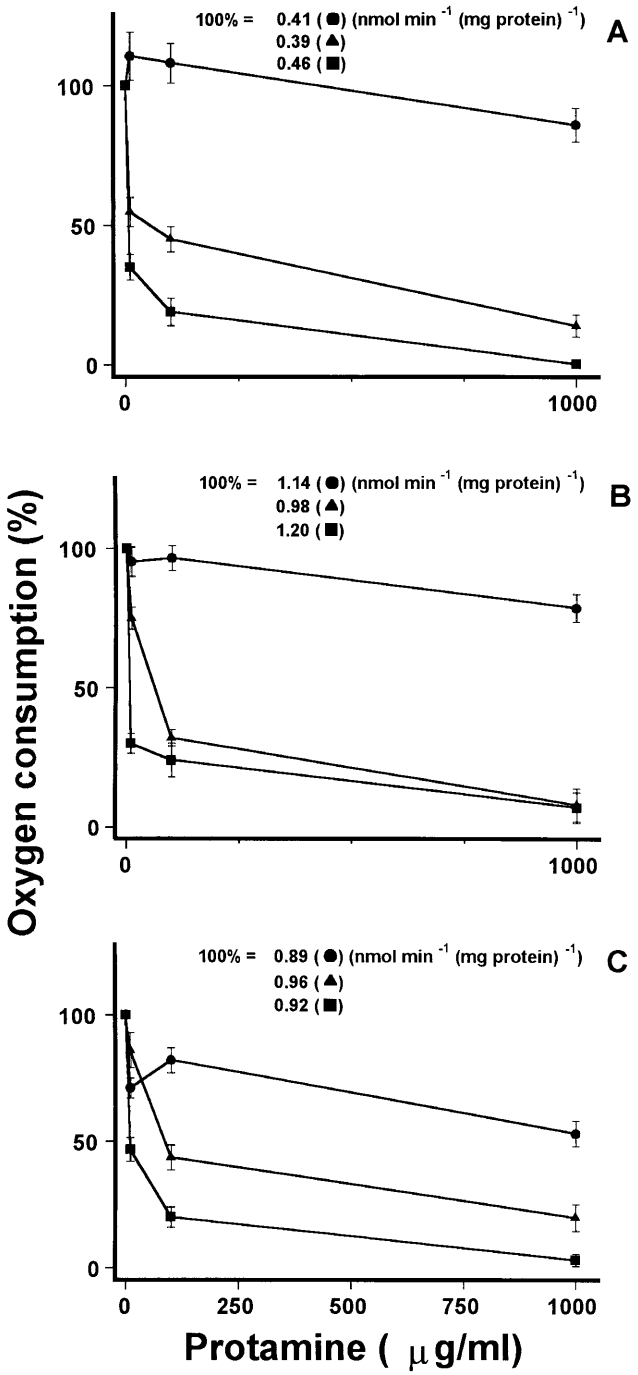

FIG. 1. Inhibitory effect of protamine on oxygen consumption, shown relative to the oxygen consumption of untreated cells. (A) *L. monocytogenes*; (B) *E. coli*; (C) *S. putrefaciens*. ●, pH 6; ▲, pH 7; ■, pH 8. Vertical bars, standard deviations (n = 3).

The inhibition of oxygen consumption by protamine for all three strains was prevented in the presence of either Ca^{2+} or Mg^{2+} (Table 1). The reduced action of protamine in the presence of 5 mM $CaCl_2$ or $MgCl_2$ could, for all the strains analyzed, be restored upon addition of 10 mM EDTA (data not shown). To prevent the activity of 1,000 μg of protamine/ml against *L. monocytogenes*, 20 mM divalent cations were required at pH 8.0. For the two gram-negative strains, signifi-

TABLE 1. Minimum concentrations of $CaCl_2$ or $MgCl_2$ preventing the inhibitory effect of protamine on oxygen consumption

Strain	Protamine concn (μg/ml)	Cation concn (mM)	
		pH 7.0	pH 8.0
L. monocytogenes	100	5	15
	1,000	10	20
E. coli	100	1	2.5
	1,000	2.5	5
S. putrefaciens	100	2.5	5
	1,000	5	15

cantly lower concentrations of Ca^{2+} or Mg^{2+} prevented the inhibition by protamine under all conditions analyzed (Table 1). This points to action of protamine on the outer membrane, as Ca^{2+} and Mg^{2+} are commonly found as counter-ions for the phosphoryl groups of lipopolysaccharide and phospholipids and are necessary for salt-bridging the macromolecules within the bilayer matrix (24). Divalent cations may also shield negatively charged phospholipids in the cytoplasmic membrane and peptidoglycan (8), thereby additionally diminishing the inhibitory effect of protamine. In addition, the overall structure of these surface layers may be stabilized in the presence of divalent cations (20, 22).

Leakage of intracellular compounds. The effect of protamine on retention of cF (376.3 g/mol), ATP, and β-galactosidase in bacteria was determined to assess the influence of protamine on the permeability properties of the cytoplasmic membrane. The protamine concentration needed to introduce cF leakage in *L. monocytogenes* was lower than that for *E. coli* and *S. putrefaciens* (Table 2). *L. monocytogenes* lost 88% of cF after a 30-min treatment with 10 μg of protamine per ml, whereas cF leakage (approximately 90%) from *S. putrefaciens* and *E. coli* was observed after incubation for 30 min with 100 and 1,000 μg of protamine per ml, respectively (Table 2). Leakage of cF due to addition of protamine occurred within 5 min for *L. monocytogenes* and within 5 to 10 min for the gram-negative bacteria.

Energized cells of *L. monocytogenes* (pH 7.0, 25°C) contained 8.0 mM cytoplasmic ATP, and the addition of protamine (1,000 μg/ml) resulted in a decrease in intracellular ATP (Fig. 2A) to 15% of the original level, whereas the level of external ATP increased, apparently due to the leakage of intracellular ATP. Hydrolysis of the extracellular ATP was observed 10 to 15 min after protamine addition. The cytoplasmic concentration of ATP in energized *E. coli* cells (pH 7, 25°C) was 8.5 mM. As a result of protamine treatment (1,000 μg/ml), the intracellular ATP was reduced to 3% of the original level and the extracellular ATP concentration increased (Fig. 2B).

TABLE 2. Leakage of intracellular cF from bacteria grown at 25°C and treated with protamine for 30 min at pH 7.0

Protamine concn (μg/ml)	% Efflux[a]		
	L. monocytogenes	*E. coli*	*S. putrefaciens*
0	34 ± 3	46 ± 4	30 ± 7
10	88 ± 8	58 ± 8	45 ± 2
100	94 ± 8	62 ± 14	90 ± 2
1,000	102 ± 7	98 ± 2	89 ± 12

[a] Efflux values (means ± standard deviations) ($n = 3$) are given as percentages relative to the total leakage determined after cell lysis with DMSO.

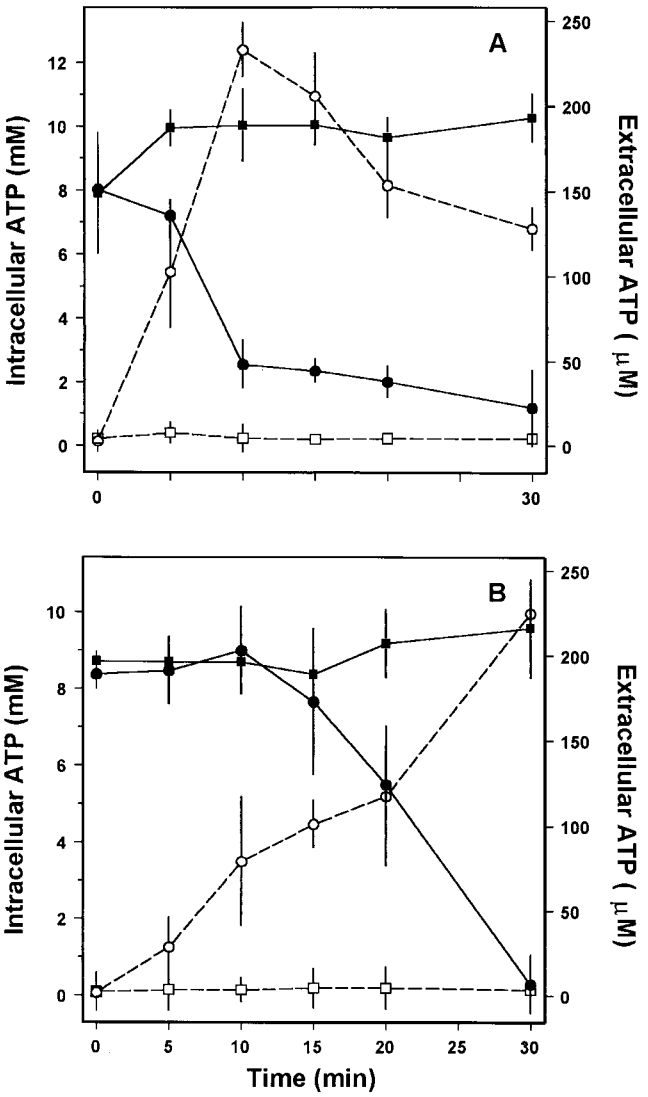

FIG. 2. Protamine-induced efflux of intracellular ATP from *L. monocytogenes* (A) and *E. coli* (B). Cells were preincubated 10 min with glucose at pH 7.0 and 25°C before addition of protamine. ■, intracellular ATP plus no protamine; ●, intracellular ATP plus 1,000 μg of protamine per ml; □, extracellular ATP plus no protamine; ○, extracellular ATP plus 1,000 μg of protamine per ml. Vertical bars, 90% confidence intervals ($n = 3$).

Leakage of intracellular ATP from *E. coli* was slower than the observed leakage from *L. monocytogenes*, and no hydrolysis of extracellular ATP occurred during the 30-min test period. In these experiments, the total amount of ATP remained approximately the same during the exposure to protamine, which indicates that the decrease in intracellular ATP was caused by efflux of ATP. The protamine-induced leakage of ATP was dependent on pH and the protamine concentration, with the most pronounced effect at high pH (data not shown).

E. coli was used to investigate whether protamine-induced permeabilization would allow the leakage of intracellular enzymes such as β-galactosidase. Indeed, extracellular activity of β-galactosidase was observed after approximately 5 min when *E. coli* cells were incubated with concentrations of protamine higher than 100 μg/ml (pH 7.0). Increasing the protamine concentration caused an increase in extracellular β-galactosidase activity. Complete release of β-galactosidase was ob-

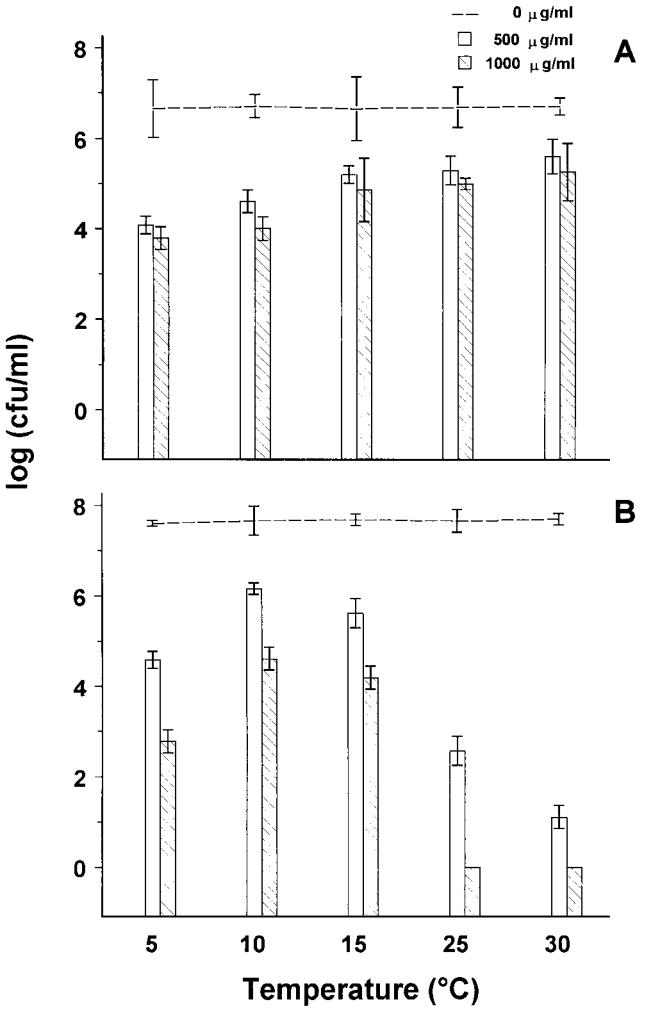

FIG. 3. Effect of temperature on the loss in viability of cells caused by different concentrations of protamine, as indicated by impedance measurements. (A) *L. monocytogenes*; (B) *S. putrefaciens*. Vertical bars, standard deviations (n = 2).

served 8 min after the addition of 1,000 μg of protamine per ml.

Permeabilization of the cytoplasmic membrane did not take place immediately after addition of protamine, and a lag time was observed. This lag time was increased at low concentrations of protamine, at low pH values, and in the presence of divalent cations. The lag time observed for the two gram-negative bacteria was longer than the lag time for *L. monocytogenes*, which may be explained by the permeability barrier function of the outer membrane (21).

Effect of temperature on inhibitory activity of protamine. The effect of temperature on protamine action was analyzed by exposing bacteria to protamine for 30 min at the temperature at which they were grown. Exposure of *L. monocytogenes* to 500 or 1,000 μg of protamine per ml resulted in a reduction in bacterial numbers, which was most pronounced at 5 and 10°C, at which the cell number decreased from 6×10^6 to 1×10^4 CFU/ml (Fig. 3A). The results were completely different for *S. putrefaciens* (Fig. 3B), which was most sensitive to protamine when grown and assayed at high temperatures; thus, cells grown and assayed at 25 or 30°C did not survive exposure to 1,000 μg of protamine per ml. Cells grown and assayed at 10°C showed significantly increased resistance, as approximately 10^4 CFU/ml survived exposure to 1,000 μg of protamine per ml. Thus, the antibacterial efficiency of protamine varies with the growth temperature of the test organisms (Fig. 3; Table 3). Further, the antibacterial effect of protamine therefore does not seem to vary systematically with the growth rate, and other species-specific parameters may influence the antibacterial activity of protamine as well.

As the bactericidal activity of protamine varied depending on the growth temperature of the bacteria, the influence of growth temperature on protamine-induced leakage was determined. Bacteria grown at 5 or 25°C were loaded with cF, and the effect of protamine on cF leakage from these cells incubated at 5 and 25°C was analyzed (Table 3). *L. monocytogenes* grown at 25°C was more resistant to 10 and 100 μg protamine per ml at both assay temperatures than were cells grown at 5°C, since leakage of cF was significantly higher from the low-temperature-grown cells.

Microorganisms grown at low temperatures adapt the composition of the cytoplasmic membrane to maintain its fluidity (25), and we therefore sought to determine if differences in membrane fatty acid composition could explain the different temperature dependencies. Membrane fatty acid analysis revealed that the majority of fatty acids in *L. monocytogenes* were anteiso 15:0 (60%) and anteiso 17:0 (27%) in 30°C-grown cells. In cells cultured at 5°C, the amount of short-chain fatty acids increased; i.e., 17:0 decreased to 4% and 15:0 increased to 82%. *S. putrefaciens* contained four major fatty acids; iso 15:0, 16:0, 16:1, and 18:1. Cells cultured at 5°C contained 13% 16:0 and 38% 16:1. In cells cultured at 25°C, 16:0 accounted for 66% and 16:1 for 33%, suggesting that in *S. putrefaciens* fluidity of the membrane at low temperatures is maintained by decreasing the amount of saturated fatty acids. Thus, both *L. monocytogenes* and *S. putrefaciens* modify the fatty acid composition of the membrane, dependent on growth temperature, but their different responses to protamine, dependent on growth temperature, can apparently not be related to a general influence of membrane fluidity.

Conclusions. The results presented in this work show that the addition of protamine to gram-positive as well as gram-negative bacteria causes nonspecific efflux of intracellular components such as cF, ATP, and the enzyme β-galactosidase and inhibition of the respiratory activity. In summary, the mechanism of protamine differs from that of pore-forming peptides, including bacteriocins produced by lactic acid bacteria (1), as the polycationic peptides most likely interact with anionic cell

TABLE 3. Effect of temperature on protamine-induced cF efflux

Test temp (°C)	Protamine concn (μg/ml)	% Efflux[a]			
		L. monocytogenes growth temp		*S. putrefaciens* growth temp	
		5°C	25°C	5°C	25 C
5	10	85 ± 5	57 ± 7	2 ± 10	8 ± 7
	100	99 ± 3	76 ± 10	47 ± 4	69 ± 8
	1,000	103 ± 8	101 ± 6	98 ± 8	111 ± 10
25	10	83 ± 9	58 ± 6	2 ± 10	5 ± 5
	100	101 ± 9	75 ± 2	51 ± 8	60 ± 5
	1,000	100 ± 10	103 ± 9	108 ± 7	105 ± 3

[a] Efflux values (means ± standard deviations) (n = 3) are given relative to total efflux determined after cell lysis with DMSO. Values have been corrected for the amount of cF detected extracellularly after incubation of cells for 30 min in the absence of protamine.

wall components and anionic phospholipids in the cytoplasmic membrane, thereby inducing condensation, resulting in disruption of these cell wall layers and killing of both gram-positive and gram-negative bacteria.

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