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Temperature- and Surfactant-Induced Membrane Modifications That Alter *Listeria monocytogenes* Nisin Sensitivity by Different Mechanisms†

Jie Li, Michael L. Chikindas, Richard D. Ludescher, and Thomas J. Montville*

Department of Food Science, Cook College, The New Jersey Agricultural Experiment Station, Rutgers, The State University of New Jersey, New Brunswick, New Jersey 08901

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Nisin interacts with target membranes in four sequential steps: binding, insertion, aggregation, and pore formation. Alterations in membrane composition might influence any of these steps. We hypothesized that cold temperatures (10°C) and surfactant (0.1% Tween 20) in the growth medium would influence *Listeria monocytogenes* membrane lipid composition, membrane fluidity, and, as a result, sensitivity to nisin. Compared to the membranes of cells grown at 30°C, those of *L. monocytogenes* grown at 10°C had increased amounts of shorter, branched-chain fatty acids, increased fluidity (as measured by fluorescence anisotropy), and increased nisin sensitivity. When 0.1% Tween 20 was included in the medium and the cells were cultured at 30°C, there were complex changes in lipid composition. They did not influence membrane fluidity but nonetheless increased nisin sensitivity. Further investigation found that these cells had an increased ability to bind radioactively labeled nisin. This suggests that the modification of the surfactant-adapted cell membrane increased nisin sensitivity at the binding step and demonstrates that each of the four steps can contribute to nisin sensitivity.

Listeria monocytogenes is a gram-positive, non-spore-forming food-borne pathogen that can grow at refrigeration temperatures (31, 41). *L. monocytogenes* has a sophisticated strategy to overcome environmental stresses, such as cold (23, 38), heat (35), an acidic environment (27), and osmotic shock (4). *L. monocytogenes* is of particular interest with respect to food safety due to its psychrotrophic nature (41), which may result in the enrichment of *L. monocytogenes* in foods at refrigeration temperature (15). This organism can pose a serious health threat to high-risk populations, such as immunocompromised individuals, newborns, and pregnant women. (42).

Nisin is used as an antimicrobial agent against *L. monocytogenes*. The bactericidal activity of nisin is due to pore formation in the bacterial membrane (12), which occurs through a four-step process of binding, insertion, aggregation, and pore formation. The cell's sensitivity to nisin is influenced by the membrane's lipid composition (24), which might act on any of the four steps. Nisin-resistant *L. monocytogenes* has a lowered cellular phospholipid content and an altered membrane fatty acid composition. (11, 24, 25).

The cytoplasmic membrane is the primary barrier between the cell and its environment. Environmental factors, especially temperature, influence membrane fluidity by modifying the membrane's lipid composition (19, 23). The process of maintaining fluidity, called homeoviscous adaptation (34, 29, 45), is critical for cell growth at nonoptimal temperatures (5, 20). The concept of membrane fluidity covers the thermal mobility of membrane proteins as well as the microviscosity of the membrane lipid bilayer (33). However, the primary way that bacteria maintain constant membrane fluidity at different growth temperatures is by adjusting their fatty acid compositions (1, 5, 29). For instance, there are higher proportions of short-chain and/or branched-chain fatty acids and unsaturated fatty acids in cold-tolerant bacteria. A variety of other environmental factors, such as low water activity, low pH, and the presence of chemicals and antimicrobials, influence membrane fluidity (7, 22, 39). Thus, the ability of bacteria to change their membrane fluidities determines to some extent how well a bacterium tolerates certain environmental stresses. The influence of temperature on the membrane is well documented. In contrast, little is known about how Tween 20, a surfactant and medium supplement widely used in laboratory studies of bacteriocins, influences homeoviscous adaptation. Tween 20 improves the well diffusion method for nisin quantification (28, 44). It also synergistically improves the antimicrobial effects of nisin and other bacteriocins (2, 44). The mechanism by which Tween 20 influences the cells' membranes and/or the mode of bacteriocin action is unknown.

This paper examines how alterations of membrane properties influence the nisin-membrane interaction and cellular sensitivity to nisin. We hypothesized that cold temperatures (10°C) and surfactant (0.1% Tween 20) in the growth medium would influence *L. monocytogenes* membrane lipid composition, membrane fluidity, and, as a result, sensitivity to nisin. This study reports the effects of temperature and surfactant on *L. monocytogenes* membrane lipid composition, membrane fluidity, nisin binding capacity, and consequently nisin sensitivity.

* Corresponding author. Mailing address: Department of Food Science, Cook College, The New Jersey Agricultural Experiment Station, Rutgers, The State University of New Jersey, 65 Dudley Rd., New Brunswick, NJ 08901. Phone: (732) 932-9611, ext. 222. Fax: (732) 932-6776. E-mail: montville@aesop.rutgers.edu.

† T.J.M. dedicates this, his centennial paper, to his teachers R. Brightbill, J. McGarry, M. Solberg, and A. J. Sinskey and to his graduate students, who have taught him the most.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. *L. monocytogenes* Scott A was cultured in buffered (0.1 M potassium phosphate buffer, pH 6.2) brain heart infusion (B-BHI) broth at 30°C. The stock culture was stored at −70°C in B-BHI

broth containing 20% glycerol. Working cultures were maintained on agar slants at 4°C and recultured biweekly. Pure nisin powder was a gift from Aplin and Barret (Trowbridge, England). Nisin stock solutions were prepared by using nisin diluent (0.2 M HCl containing 0.75% NaCl) and then autoclaved at 15 lb/in^2 for 15 min. *L. monocytogenes* was cultured at either 30 or 10°C. A supplement of 0.1% Tween 20 in the growth medium at 30°C was used for observation of the surfactant's effect on the cells. Since cells did not grow at 10°C in the presence of 0.1% Tween 20, the effect of this condition could not be examined.

Membrane lipid extraction. Cells grown under the different conditions were collected at mid-log phase (optical density at 630 nm [OD_{630}], ~0.7 to 0.8) by centrifugation (20,000 × *g*, 10 min, 4°C) and washed twice with 0.1% peptone water (Difco). Total lipids from *L. monocytogenes* were extracted in accordance with the procedure of Bligh and Dyer (6) as described by New (26) and modified by Winkowski et al. (43). Briefly, the cell pellet was resuspended in chloroform–methanol–0.1 N HCl (1:2:0.8 by volume) and vortexed for 1 min every 10 min in the presence of 6 g of glass beads (acid washed, <106 μm in diameter; Sigma) for a total of 1 h. Then, 0.1 N HCl and chloroform were added to achieve a final chloroform/methanol/0.1 N HCl ratio of 1:1:1. Phase separation was achieved by centrifugation at 2,000 × *g* for 5 min. After the chloroform phase was collected, the remaining water phase was washed for a second time with chloroform. The solvent phase of the second extraction was pooled with the previous one. The combined chloroform phases were neutralized with 1 M NH_4OH in methanol and dried under a stream of nitrogen. The dried lipid film was resuspended in chloroform-methanol (9:1, vol/vol) and stored at −20°C for up to 2 weeks.

GLC analysis of membrane fatty acids. The fatty acid analysis was carried out by Microbial ID, Inc. (Newark, Del.), by gas-liquid chromatography (GLC). GLC analysis of fatty acids was performed following their conversion to apolar methyl ester derivatives. Briefly, fatty acid methyl esters from lipid extracts were prepared by saponification and methylation of fatty acid molecules cleaved from the lipids before they were extracted into hexane–*tert*-butyl ether (1:1). They were further separated on a phenylmethyl silicone fused silica capillary column, with hydrogen as the carrier gas and nitrogen as the makeup gas. The fatty acids were identified with the aid of standards. The profile of the fatty acid composition included data on the retention time, response, area and height, equivalent chain length values, peak name, and percentage of each fatty acid. The data were collected and quantified by Microbial ID, Inc., with the MIDI Sherlock system. The MIS software was used with the HP/Agilent Technologies model 6890 GC for optimal analysis of fatty acid methyl esters by GC (technical note no. 101, Microbial ID, Inc.).

Total phosphorus analysis. Phospholipids were quantified by the method of Bartlett (3) as described in the Avanti Polar Lipids, Inc., catalog. Briefly, in 15-ml test tubes, 0.45 ml of 10 N H_2SO_4 was added to the phosphorus standards (Sigma, St. Louis, Mo.) or the samples, followed by heating at 200°C for 25 min for combustion. Then, 150 μl of 30% H_2O_2 (Fisher Scientific, Fairlawn, N.J.) was added. After peroxide was decomposed by a 30-min incubation at 200°C, 3.9 ml of deionized water, 0.5 ml of 2.5% ammonium molybdate(VI) tetrahydrate (Sigma) solution, and 0.5 ml of 10% ascorbic acid solution were added sequentially with intermittent vortexing. All the tubes were heated in boiling water for 7 min before the OD_{830} was determined. The OD_{830} values of the standards were used to generate a standard curve of phosphorus concentration, and the sample phosphorus concentration was calculated from the standard curve.

Steady-state fluorescence anisotropy. Membrane lipid vesicles derived from cells grown under different conditions were used to study membrane fluidity. Membrane fluidity was determined by monitoring the steady-state fluorescence anisotropy of a fluorescent membrane fluidity probe, 1,6-diphenyl-1,3,5-hexatriene (DPH), which is widely used in membrane fluidity studies due to its outstanding properties as a fluidity probe (32). Large unilamellar vesicles were constructed from lipid extracts obtained from *L. monocytogenes* grown under different conditions in accordance with the procedures of Hromy and Carman (21), with modification (43). Basically, 3- to 6-mg lipid extracts in chloroform-methanol (9:1) were dried under a stream of nitrogen gas in a siliconized microcentrifuge tube followed by vacuum drying for 2 h to remove any residual solvent. The final dry lipid film was resuspended in 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (pH 6.5) containing 50 mM *n*-octyl-β-D-glucopyranoside (Sigma) to achieve a lipid/detergent ratio of 14:1. The lipid-detergent mixture was vortexed continuously for 1 h before it was loaded onto a Sephadex G-50 superfine column (0.7 by 10 cm), which had been equilibrated with 50 mM MES buffer. The column was eluted with MES buffer. Large unilamellar vesicles were collected at the void volume. The vesicles were stored on ice for up to 3 h until use.

Steady-state fluorescence anisotropy of the DPH-labeled membrane vesicles was determined with a Perkin-Elmer LS-50B luminescence spectrometer equipped with a xenon light source, a thermostatted cuvette holder, and rotatable polarizers. The samples were excited with vertically polarized light at 360 nm, and the fluorescence intensity of the emission light at 430 nm was measured through a polarizer both vertically (I_{vv}) and horizontally (I_{vh}) relative to the excitation light. The cutoff filter was set at 390 nm for the emission light. The temperature of the cuvette was maintained at 25°C unless stated otherwise. The grating factor (G) of the instrument under these conditions was 1.27. The slit width was 10 nm for both excitation and emission light.

For the anisotropy determination, 0.1 to 0.2 μmol of lipid vesicles from the preparation described above was added to 2 ml of 50 mM MES buffer (pH 6.5) in a cuvette and mixed well. The fluorescence intensity of unlabeled cells (I'^0_{vv} and I'^0_{vh}) was monitored before addition of the fluorescence probe. Then, DPH in tetrahydrofuran (Sigma-Aldrich Corp., St. Louis, Mo.) was added to the liposome system (final concentration, 5×10^{-7} M) to achieve a lipid/probe ratio of about 250:1. The intensity of polarized emission light from labeled lipid vesicles (I'_{vv} and I'_{vh}) was monitored after about 8 to 10 min of incubation of the lipid-probe mixture at 25°C. Anisotropy (r) of labeled liposomes was calculated and corrected by the equation

$$r = \frac{(I'_{vv} - I'^0_{vv}) - G \cdot (I'_{vh} - I'^0_{vh})}{(I'_{vv} - I'^0_{vv}) + 2 \cdot G \cdot (I'_{vh} - I'^0_{vh})}$$

where G is a constant of 1.27.

Evaluation of membrane sensitivity to nisin by using CF efflux. The 5′(6′)-carboxyfluorescein (CF; Sigma) efflux assay is widely used to characterize the interaction of antimicrobials and lipid membrane vesicles, giving insight into the antimicrobials' modes of action (17, 43). Large unilamellar vesicles loaded with fluorescent dye were constructed from *L. monocytogenes* grown under different conditions. The dry lipid film was resuspended as described above, except that the buffer also contained 50 mM CF. The same separation steps were used to collect CF-loaded liposomes.

Nisin-induced CF leakage from the lipid vesicles was monitored with a Perkin-Elmer LB50 spectrofluorometer with an excitation wavelength of 490 nm, an emission wavelength of 516 nm, and a slit width of 5 nm. The temperature was 25°C if not stated otherwise. Liposomes were added to 2 ml of a 50 mM MES buffer (pH 6.5) system and mixed well by pipetting. A stable baseline fluorescence signal (F_0) contributed by free untrapped CF was determined before detergent or nisin was added. To evaluate the efficiency of dye entrapment, 0.1% Triton X-100 was added to completely disrupt the vesicles and the fluorescence intensity (F_∞) was determined. Nisin-induced CF leakage (F_t) was monitored after the addition of nisin. The change in fluorescence intensity in the system was kinetically monitored for 300 s. The final percentage of CF efflux was calculated by the following formula:

$$\%\ \text{CF efflux} = \frac{F_t - F_0}{F_\infty - F_0} \times 100$$

The initial efflux rate was calculated as the slope of the tangent line of the CF release curve in the first 30 s.

Evaluation of nisin sensitivity. Cell sensitivity to nisin was determined by monitoring the inhibitory effect of nisin on cell growth. *L. monocytogenes* cells grown at 30 and 10°C and in the presence of 0.1% Tween 20 at 30°C were collected at mid-log phase and used as inocula. Cells were washed with 0.1% peptone water (Difco) before they were inoculated into fresh medium with nisin at a concentration of 0, 50, 100, or 500 IU/ml. This experiment was carried out in a 96-well microplate incubated at 30°C. The OD_{630} of the culture was kinetically monitored for 48 h with a Dynex MLX microplate reader with a temperature control unit. For confirmation of the sensitivity as determined by kinetic growth data, the well diffusion assay was used to examine how cold influenced cell sensitivity to nisin. Cells adapted to 10 and 30°C were used to seed BHI agar. Wells containing a series of different concentrations of nisin were punched into the agar. The plates were incubated at 10 and 30°C, respectively, for inhibition zone development.

Radioactive labeling of nisin. Nisin was labeled with [^{14}C]formaldehyde on the ε-group of the lysine residue, in accordance with the method originally described by Dottavio-Martin and Ravel (13) with modification. Briefly, 500 μg of pure nisin was dissolved in 500 μl of 50 mM MES-KOH (Sigma) buffer (pH 6.5) containing 100 mM K_2SO_4 (Fisher Scientific), 20 mM $NaCNBH_3$ (J.T. Baker, Philipsburg, N.J.), and [^{14}C]formaldehyde (56.0 mCi/mmol) (Perkin-Elmer Life Science, Boston, Mass.). The ratio of [^{14}C]formaldehyde to nisin was kept at 2:1 in the system. The labeling was performed at room temperature for 1 h with continuous stirring. Labeled nisin was then separated from the free isotope by passing the whole system through a column of Sephadex G-50 (Sigma) packed in an Econo-pack 10 empty column (Bio-Rad, Richmond, Calif.), which was equilibrated with 0.05% (vol/vol) acetic acid. Labeled nisin was collected at 1.05 dpm/

TABLE 1. Fatty acid composition of membranes from *L. monocytogenes* grown under different conditions[a]

Fatty acid	% Total fatty acid under indicated growth condition(s)		
	30°C + Tween 20	30°C	10°C
n-12:0	4.2 ± 5.8		1.6 ± 0.2
i-14:0	1.1 ± 0.1	1.2 ± 0.1	1.8 ± 0.4
n-14:0	13.0 ± 1.6	3.8 ± 0.2	3.0 ± 2.1
i-15:0	8.0 ± 1.0	13.5 ± 0.9	11.5 ± 1.0
a-15:0	24.6 ± 3.0	36.4 ± 2.3	44.0 ± 3.3
i-16:0	2.4 ± 0.3	3.0 ± 0.0	3.2 ± 0.5
n-16:0	11.6 ± 4.0	7.7 ± 0.6	3.5 ± 0.4
i-17:0	2.3 ± 0.5	3.6 ± 0.4	1.4 ± 0.2
a-17:0	10.4 ± 1.7	15.2 ± 1.6	13.0 ± 1.2
n-18:0	8.0 ± 3.4	4.9 ± 1.8	3.8 ± 0.1

[a] Results are the means of two replicates with SD indicated.

nmol. This corresponded to a labeling efficiency of about 85%. The ^{14}C-labeled nisin was stored in 0.05% (vol/vol) acetic acid at −20°C.

Nisin binding. Membrane vesicles were prepared from *Listeria* cells grown at 30°C with or without Tween 20. To determine the binding of nisin to these two different membranes, 400 to 500 μM lipid vesicles, which were made according to the methods described in the previous section, were mixed with different amounts of labeled nisin. They were incubated at room temperature for 5 min. Then, within 10 s, 150 μl of the reaction mixture was put into a ProbeQuant G-50 prepacked minicolumn (Amersham Bioscience, Piscataway, N.J.) and centrifuged (1 min, 300 × *g*) through the column to separate the nisin-membrane complex from the free unbound nisin. The amount of nisin bound to the lipid vesicles was determined by scintillation counting. Three control tubes with lipid vesicles only were used to determine the efficiency of lipid vesicle recovery by total phosphorus analysis. The results were corrected for the 5% of the labeled nisin that bound nonspecifically to the membrane. At least 80% of the nisin-liposome complexes were recovered in the filtrates. A Langmuir binding isotherm was constructed by plotting bound nisin (in nanomoles per nanomole of P_i) against free nisin (in nanomoles per nanomole of P_i) at equilibrium. Bound nisin* (in nanomoles per nanomole of P_i) is usually regarded as *r**, which means that the molar ratio of bound nisin to 60% of the total phospholipids (the amount of lipid molecules present in the outer leaflet in the membrane), is defined as *r**.

Statistical analysis. Experiments were repeated at least twice and were reproducible. Standard deviations (50) were calculated for statistical comparisons. Probabilities (*P*) of less than 0.05 were considered significant by Student's *t* test.

RESULTS

Temperature and surfactant alter the fatty acid composition of *L. monocytogenes* membranes. Fatty acid profiles generated from cells grown at 30 and 10°C and at 30°C with Tween 20 are listed in Tables 1 and 2. The most abundant fatty acids found in the membrane lipids derived under all growth conditions were i-$C_{15:0}$, a-$C_{15:0}$, and a-$C_{17:0}$. This result is consistent with previous work (24). Other saturated fatty acids, such as $C_{18:0}$, $C_{16:0}$, and $C_{14:0}$, were also abundant. When the cells were grown at 10°C, the amount of a-$C_{15:0}$ in the membrane increased to 44.0% of total fatty acids (Table 1). Concurrently, there was a decrease in n-$C_{16:0}$ and i-$C_{17:0}$. No obvious change was observed for the other fatty acid species. Growth at the lower temperature resulted in both a higher C_{15}/C_{17} ratio and a higher branched-chain/straight-chain fatty acid ratio (Table 2). This means that there were more shorter-, branched-chain fatty acids in the membranes of cells grown at 10°C than in those of cells grown at 30°C. Also, a transformation of iso configuration into anteiso configuration was observed as an increase in the anteiso/iso fatty acid ratio. All the changes induced by cold were consistent with a decreased melting point of the membrane phospholipids. The fatty acid profile of *L. monocytogenes* grown at 30°C with Tween 20 was quite different from that of cells grown in the absence of Tween 20. Both $C_{15:0}$ and $C_{17:0}$ decreased, as did the iso and anteiso configurations (Table 2). The amount of the straight-chain, even-numbered, fatty acids of different chain lengths increased. The increased amount of straight-chain fatty acids would be expected to elevate the membrane's phase transition temperature, leading to higher membrane rigidity. In contrast, the C_{15}/C_{17} ratio and the anteiso/iso ratio of the membrane fatty acids increased, which might contribute to a lower membrane phase transition temperature.

Membranes with modified membrane fatty acid composition have altered membrane fluidity. When assayed at 25°C, the anisotropy of the membrane lipid vesicles from the cells grown at 10°C was lower than that of the cells grown at 30°C (Fig. 1A). This means that membranes from cells grown at 10°C were more fluid at 25°C than those from cells grown at 30°C. On the other hand, the presence of Tween 20 in the growth medium caused no significant changes in the membrane rigidity (Fig. 1B). Unlike whole cells, pure lipid vesicles cannot change their membrane composition in response to environmental factors. Therefore, changing the assay temperature or adding the surfactant to the vesicles during the anisotropy assay would mimic the instantaneous effects of external conditions on the cell membrane. The lower assay temperature (10°C) significantly increased the membrane rigidity (anisotropy increased from 0.134 to 0.236) of the lipid vesicles (Fig. 1A). In contrast, membrane rigidity decreased (anisotropy decreased from 0.134 to 0.057) when Tween 20 was added to the lipid vesicles (Fig. 1B).

Modification of lipid composition and membrane fluidity influence nisin-induced membrane permeability. Nisin-membrane interactions in CF-loaded lipid vesicles derived from *L. monocytogenes* are well studied (43). CF efflux was used to evaluate the sensitivity of the lipid vesicle to nisin. The leakage of the fluorophore from the membranes of different cell sources was studied by comparing the kinetic curves of induced

TABLE 2. Fatty acid profile analysis based on chain length and fatty acid chain configuration[a]

Growth condition	% Total fatty acid		C_{15}/C_{17} ratio	% Total fatty acid		Anteiso/iso ratio	% Total fatty acid		Branched-/straight-chain ratio
	C_{15}	C_{17}		Anteiso	Iso		Branched chain	Straight chain	
30°C + Tween 20	32.3 ± 4.9	12.7 ± 2.3	2.5 ± 0.0 A	37.5 ± 2.1	15.0 ± 1.5	2.5 ± 0.1 B	52.6 ± 3.6	37.5 ± 0.1	1.4 ± 0.1 A
30°C	44.9 ± 3.2	18.8 ± 2.0	2.7 ± 0.1 B	52.5 ± 2.6	23.0 ± 1.2	2.3 ± 0.0 A	75.6 ± 3.8	16.7 ± 2.6	4.6 ± 0.9 B
10°C	55.4 ± 2.3	14.3 ± 0.9	3.9 ± 0.1 C	59.0 ± 6.1	19.8 ± 0.6	3.0 ± 0.4 C	78.5 ± 5.5	12.5 ± 1.7	7.2 ± 1.8 C

[a] Results are the means of two replicates with SD indicated. Only fatty acids that exceed 1% of total membrane fatty acid were listed. Ratios with different capital letters are statistically significantly different ($P < 0.05$).

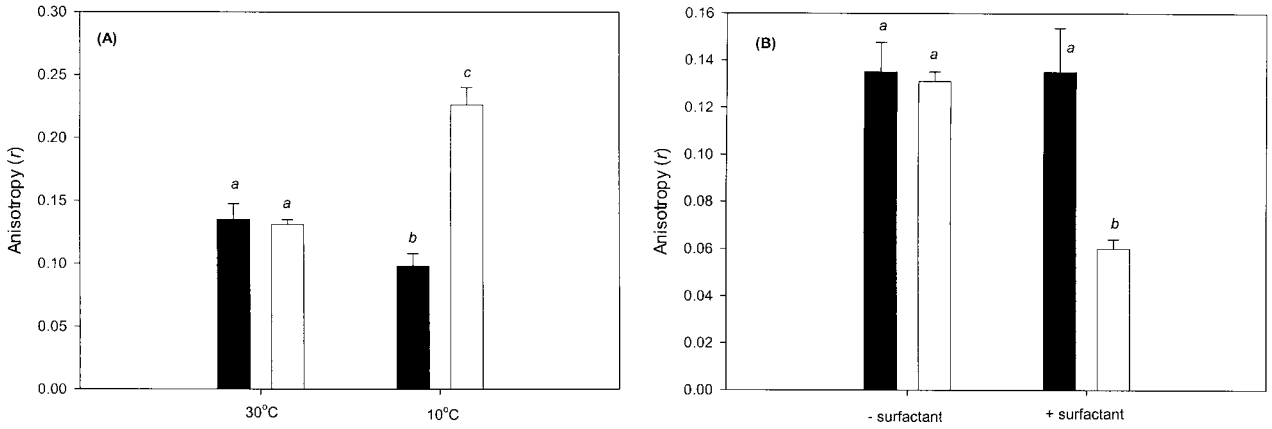


FIG. 1. Influence of temperature and surfactant on the membrane fluidity of lipid vesicles derived from *L. monocytogenes* grown under different conditions. The long-term (■) and instantaneous (□) effects of cold (A) and the surfactant (B) were evaluated. The long-term effect was obtained by growing the cells at different temperatures (A) and under different surfactant conditions (B) before the lipid vesicles were constructed from the lipid extracts from those cells. The vesicles were assayed for anisotropy at 25°C. The instantaneous effect was observed by changing the anisotropy assay temperature from 25 to 10°C or by adding 0.1% Tween 20 to the lipid vesicles during the anisotropy assay. Lipid vesicles for the experiment on the instantaneous effect were derived from cells grown at 30°C. Differences in the lowercase letters indicate that anisotropy data are significantly different ($P < 0.5$). Error bars represent the maximum variance observed in the studies.

CF leakage from the liposomes. Nisin caused nonlinear CF leakage with different final percentages of CF efflux, depending on the cell source of the lipids (data not shown). As shown from the slopes of the curves, the final percentage of CF efflux increased concurrently with the nisin applied. Nisin-induced CF efflux was influenced by the lipid composition of the vesicles, which, in turn, was determined by the growth conditions of the source cells. This lipid compositional dependence in membrane permeability was manifested in two ways (Fig. 2). First, in terms of nisin-induced CF efflux, given equal amounts of nisin per unit of lipid (nisin/lipid ratio, in moles per mole), surfactant-adapted membranes had the highest CF leakage. CF efflux induced from the membranes of cells grown at 30°C was the lowest compared to that induced from membranes under the other two growth conditions. From another point of view, to reach the same level of CF efflux, lipid vesicles from the surfactant-adapted cells required the lowest nisin/lipid ratio, while vesicles of the normal cells needed the highest. Both findings suggest that surfactant and cold made the cell membrane more vulnerable to nisin. The instantaneous effect of cold and surfactant, respectively, on CF efflux was also studied by lowering the assay temperature to 10°C and by adding 0.1% Tween 20 to the lipid vesicles from cells grown at 30°C. Cold reduced the percentage of CF efflux, while surfactant increased the efflux (data not shown).

Cell sensitivity to nisin was determined by the modifications of the membrane properties. Cell growth in the presence of different nisin concentrations is a function of nisin sensitivity. By comparing the inhibitory effects of nisin on cell growth curves, we obtained information on the sensitivity of cells adapted to different conditions. As shown in Fig. 3, when cells were grown without nisin, there was essentially no difference in growth curves, regardless of the sources of the cells. When cold-adapted cells were compared with normal cells, the growth curves generated with different concentrations of nisin had longer lag times (Fig. 3A and B). This established that cold-adapted cells had increased nisin sensitivity relative to normal cells. Surfactant-adapted cells were also sensitized to nisin (Fig. 3A and C). In addition to the above results, well diffusion assays also showed that the cold-adapted cells were more sensitive to nisin than normal cells (data not shown).

Influence of surfactant-induced changes on nisin-membrane binding. The surfactant-adapted cell membrane, compared with the normal (nonadapted) cell membrane, had increased sensitivity to nisin but no change in membrane fluidity. To determine if these results might be explained by changes in nisin binding, the binding affinity of nisin with the membranes of the surfactant-adapted and nonadapted cells was studied by using [^{14}C]formaldehyde-labeled nisin. Nisin-binding effi-

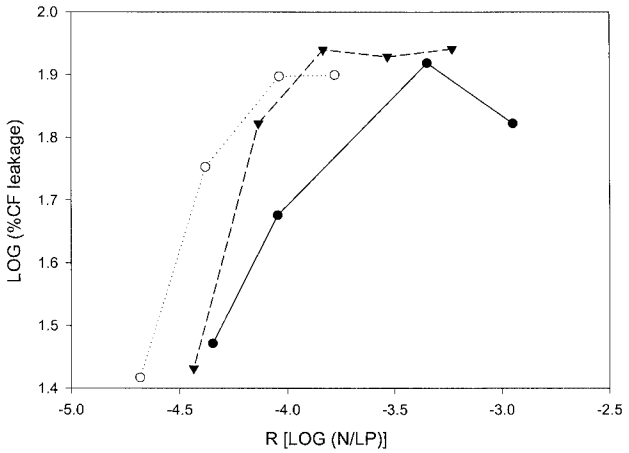
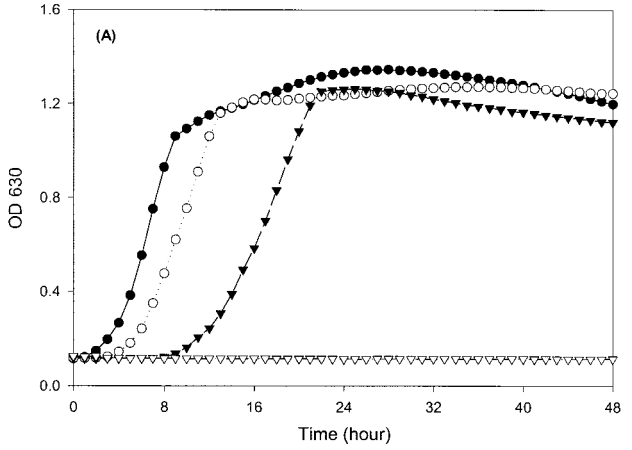
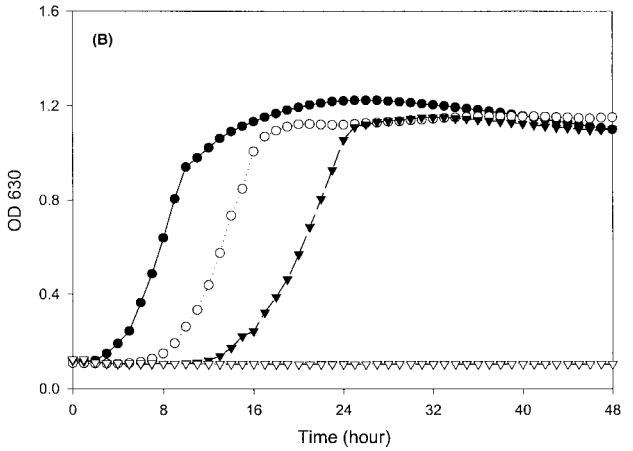


FIG. 2. Nisin concentration dependence in nisin-induced CF efflux from CF-loaded liposomes. The liposomes used were derived from *L. monocytogenes* grown under different conditions, namely, at 30 (●) and 10°C (▼) and at 30°C with the addition of 0.1% Tween 20 (○). R (N/LP), ratio of the amount of nisin per unit of lipid (moles per mole) in the assay system; % CF leakage, final percentage of CF efflux after nisin was added to the system for 300 s. The assay temperature was 25°C.





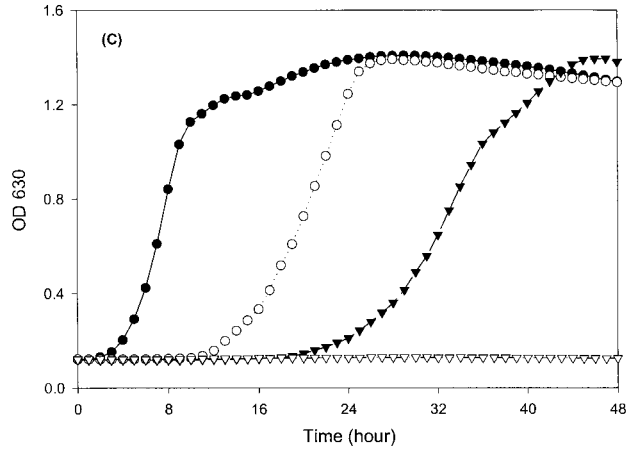
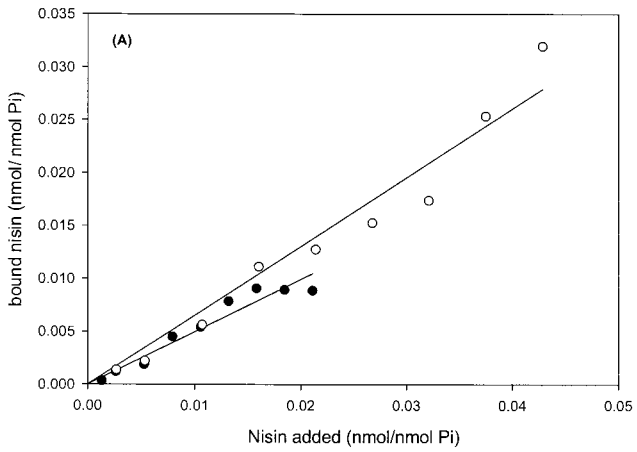


FIG. 3. Growth curves of *L. monocytogenes* with different nisin concentrations to evaluate cell sensitivity. Cells grown under different conditions were used as inocula and were inoculated into the same fresh B-BHI broth containing nisin at concentrations of 0 (●), 50 (○), 100 (▼), and 500 (▽) IU/ml. The cells were incubated at 30°C for 48 h. Growth curves for mid-log-phase cells originally grown at 30°C without surfactant (A), cells originally grown at 10°C (B), and cells that were originally adapted to the presence of 0.1% Tween 20 at 30°C (C) are shown.

ciency was concentration dependent for membranes from both non-surfactant-adapted and surfactant-adapted cells (Fig. 4 A). For lipid vesicles derived from the surfactant-adapted cells, the binding efficiency was about 65%, which was the slope of the linear regression curve. It was about 50% for the lipid vesicles of non-surfactant-adapted cells. When plotted as binding isotherms to describe the equilibrium state (Fig. 4 B), the lipid vesicles from the surfactant-adapted cells had higher nisin binding affinity than the nonadapted cells in terms of partition efficiency (bound nisin/free nisin ratio) at equilibrium conditions. The nonadapted cells reached saturation at about 0.015 nmol of nisin/nmol of P_i, while the surfactant-treated cells did not. The binding capacity of the surfactant-adapted membrane was also higher. For any free nisin concentration higher than 6 μM at equilibrium, the ratio of nisin bound to the membrane of non-surfactant-adapted cells remained at 1:60 (0.016) with respect to the number of phospholipid molecules in the membrane (Fig. 4B). However, the ratio of nisin bound to the surfactant-adapted membrane was at least twice as high. The



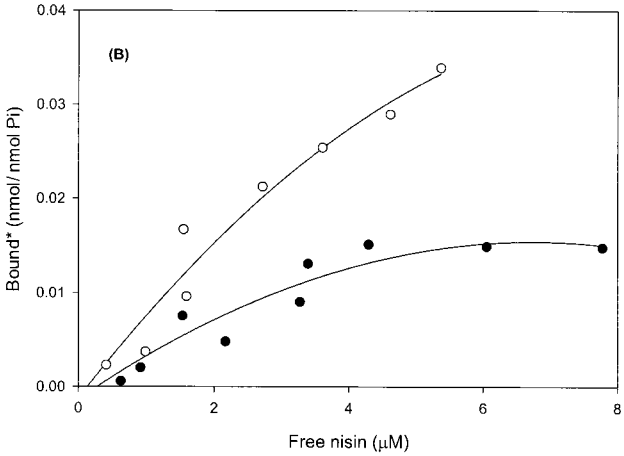


FIG. 4. Nisin binding isotherms for the lipid vesicles derived from *L. monocytogenes* grown with or without 0.1% Tween 20. Binding of nisin (A) and binding isotherms of nisin (B) to the lipid vesicles from the surfactant-adapted cells (○) and nonadapted cells (●) are shown.

data clearly demonstrate increased binding affinity between nisin and the surfactant-adapted cell membranes.

DISCUSSION

This study provides another example of the homeostatic ability of *L. monocytogenes*, extends it to a surfactant, and links it to nisin sensitivity. The comparison of the instantaneous and the long-term effects of the environmental factors on both membrane fluidity and membrane permeability demonstrated that the changes induced by long-term treatment and instant treatment were opposite to each other (Fig. 1). The cells act against the influence of the environmental factors they encounter. Generally, fatty acid composition changes in three ways in response to temperature fluctuation: acyl chain length, the degree of saturation, and the branch position of the fatty acids (36, 37). The major cell membrane response to temperature changes for *L. monocytogenes*, for instance, is the alteration in the fatty acid component of the membrane's lipids; changes in the head group composition are generally minor (29, 30). The fatty acid compositions determined for cells grown at 30 and 10°C reported here are consistent with published data (24).

Low temperatures induced shorter, branched-chained fatty acids and higher ratios of fatty acids with anteiso configurations to those with iso configurations (Table 1). This finding confirms those of previous reports (15, 23). The critical role of a-C_{15} in maintaining the membrane fluidity of the cells grown at low temperature (1) could also be confirmed by an increased a-C_{15}/a-C_{17} ratio in the fatty acid profile of the cells grown at 10°C (data not shown). Those changes in the fatty acid composition suggest a membrane with a decreased lipid membrane melting point. Therefore, cold-adapted cell membranes do adopt a lower gel-fluid phase transition temperature to maintain sufficient membrane fluidity at low temperature. When assayed at 25°C, membranes from cold-adapted cells had higher fluidity than those from cells grown at 30°C. When assayed at 10°C, the anisotropy of cells grown at 10°C was the same as that of the cells grown at 30°C and assayed at 30°C (data not shown). Thus, they had the same membrane fluidities at their growth temperatures, demonstrating the cells' homeostatic ability. The CF leakage of the membranes from cold-adapted cells also suggested that increased membrane fluidity made the membrane more sensitive to nisin. Furthermore, the increased nisin sensitivity of lipid vesicles derived from cold-adapted cells was seen in the intact cells (Fig. 3). Cell sensitivity to nisin at 10°C was also checked by another method of a different principle, the well diffusion assay, because during the 48-h incubation in the 96-well plate reader at 30°C, the cold-adapted cells might lose their differences in membrane composition; changes in membrane composition are reversible and may have occurred in a few hours (1, 18). In the diffusion assay, cold-adapted cells were still more sensitive to nisin than normal cells were (data not shown), confirming that cold sensitizes the cells to nisin.

This paper is the first to report the fatty acid composition of *L. monocytogenes* grown in the presence of 0.1% Tween 20. The results regarding the surfactant effect on the cell membrane lipid composition were initially difficult to interpret. The presence of 0.1% Tween 20 changed the membrane lipid composition. It was hard to predict the overall effect of the surfactant on the membrane phase transition temperature, but anisotropy demonstrated that the membrane fluidity of the surfactant-adapted cells remained unchanged. Nisin-induced CF efflux from the liposomes of surfactant-adapted cells increased (Fig. 2). The surfactant-adapted cells were also more sensitive to nisin in vivo. Considering that the instantaneous effect of surfactant on the membrane was to increase membrane fluidity (Fig. 1), the surfactant-adapted cells might not entirely offset the immediate effect posed by Tween 20. Thus, the surfactant-adapted cells were sensitized to nisin at both the membrane (in vitro) and whole-cell (in vivo) levels even though there was no change in membrane fluidity.

Although the changes in the membrane composition of the surfactant-adapted cells had no influence on membrane fluidity, they nevertheless enhanced nisin-membrane interaction. According to the generally accepted wedge model (8, 14, 40), nisin acts on the membrane through four steps: binding, insertion, aggregation, and pore formation. Aggregation, insertion, and pore formation are influenced by membrane fluidity, which is determined in microorganisms mainly by membrane lipid acyl chains (37). Binding, however is the very first step in the nisin-membrane interaction and may be a major determinant of antimicrobial activity (10). The binding of nisin to the target membrane is driven by the surface concentration and determined by the electrostatic interaction between the cationic peptide and the anionic membrane (9, 10).

We predicted that the binding step would be influenced by the membrane lipid composition of surfactant-adapted cells. The head groups of the phospholipids, for instance, influence nisin-membrane interaction. Higher anionic phospholipid contents in the membrane improved nisin-membrane electrostatic interaction, resulting in higher membrane sensitivity to nisin (10, 16). We therefore analyzed the phospholipid composition of the surfactant-adapted cells. No obvious increase in the anionic phospholipid was observed (data not shown). Nonetheless, we examined the binding of nisin to lipid vesicles derived from both surfactant-adapted cells and nonadapted cells by using radioactively labeled nisin. The equilibrium binding isotherms of the two different membranes were analyzed. There was an increased binding affinity between nisin and surfactant-adapted membrane. Nisin binding sites on the non-surfactant-adapted membranes became saturated at a free nisin concentration of 6 μM. There was a maximum of one binding site for every 60 phospholipid head groups. However, the nisin/phospholipid ratio was higher for the surfactant-adapted membranes, reaching 1:30 at saturation. The saturation ratios for nisin-membrane binding observed in our study were lower than those previously reported (43). This is probably because nisin molecules that loosely bound to the membrane were removed during the minispin column separation step (10). Since this would affect both the surfactant-treated and control membranes, it would not alter their relative binding capacities.

Our studies suggest that long-term treatment with low temperature could sensitize the cell to nisin through modification of membrane fluidity, while the surfactant sensitizes the cell by changing the membrane lipid composition to improve nisin-membrane binding efficiency.

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REFERENCES

1. **Annous, B. A., L. A. Becker, D. O. Bayles, D. P. Labeda, and B. J. Wilkinson.** 1997. Critical role of anteiso-$C_{15:0}$ fatty acid in the growth of *Listeria monocytogenes* at low temperatures. Appl. Environ. Microbiol. **63:**3887–3894.
2. **Baranova, I. P., I. Kozlova, V. A. Grushina, and N. S. Egorov.** 1980. Method of determining the concentration of the antibiotic nisin. Antibiotiki **25:**266–267.
3. **Bartlett, G. R.** 1959. Phosphorus assay in column chromatography. J. Biol. Chem. **234:**466–468.
4. **Begot, C., I. Lebert, and A. Lebert.** 1997. Variability of the response of 66 *Listeria monocytogenes* and *Listeria innocua* strains to different growth conditions. Food Microbiol. **14:**403–412.
5. **Berry, E. D., and P. M. Foegeding.** 1997. Cold temperature adaptation and growth of microorganisms. J. Food Prot. **60:**1583–1594.
6. **Bligh, E. G., and W. J. Dyer.** 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. **37:**911–917.
7. **Brailoiu, E., A. Margineanu, and M. D. Miyamoto.** 1998. Angiotensin II and related peptides alter liposomal membrane fluidity. Biochem. Mol. Biol. Int. **44:**203–209.
8. **Breukink, E., and B. de Kruijff.** 1999. The lantibiotic nisin, a special case or not? Biochim. Biophys. Acta **1462:**223–234.
9. **Breukink, E., P. Ganz, B. de Kruijff, and J. Seelig.** 2000. Binding of nisin Z to bilayer vesicles as determined with isothermal titration calorimetry. Biochemistry **39:**10247–10254.
10. **Breukink, E., C. van Kraaij, R. A. Demel, R. J. Siezen, O. P. Kuipers, and B. de Kruijff.** 1997. The C-terminal region of nisin is responsible for the initial interaction of nisin with the target membrane. Biochemistry **36:**6968–6976.
11. **Crandall, A. D., and T. J. Montville.** 1998. Nisin resistance in *Listeria monocytogenes* ATCC 700302 is a complex phenotype. Appl. Environ. Microbiol. **64:**231–237.
12. **Demel, R. A., T. Peelen, R. J. Siezen, B. de Kruijff, and O. P. Kuipers.** 1996. Nisin Z, mutant nisin Z and lacticin 481 interactions with anionic lipids correlate with antimicrobial activity: a monolayer study. Eur. J. Biochem. **235:**267–274.
13. **Dottavio-Martin, D., and J. M. Ravel.** 1978. Radiolabeling of proteins by reductive alkylation with ^{14}C-formaldehyde and sodium cyanoborohydride. Anal. Biochem. **87:**562–565.
14. **Driessen, A. J., H. W. van den Hooven, W. Kuiper, K. M. Van De, H. G. Sahl, R. N. Konings, and W. N. Konings.** 1995. Mechanistic studies of lantibiotic-induced permeabilization of phospholipid vesicles. Biochemistry **34:**1606–1614.
15. **Edgcomb, M. R., S. Sirimanne, B. J. Wilkinson, P. Drouin, and R. D. Morse.** 2000. Electron paramagnetic resonance studies of the membrane fluidity of the foodborne pathogenic psychrotroph *Listeria monocytogenes*. Biochim. Biophys. Acta **1463:**31–42.
16. **El Jastimi, R., K. Edwards, and M. Lafleur.** 1999. Characterization of permeability and morphological perturbations induced by nisin on phosphatidylcholine membranes. Biophys. J. **77:**842–852.
17. **Grant, E., Jr., T. J. Beeler, K. M. Taylor, K. Gable, and M. A. Roseman.** 1992. Mechanism of magainin 2a induced permeabilization of phospholipid vesicles. Biochemistry **31:**9912–9918.
18. **Guerzoni, M. E., R. Lanciotti, and P. S. Cocconcelli.** 2001. Alteration in cellular fatty acid composition as a response to salt, acid, oxidative and thermal stresses in *Lactobacillus helveticus*. Microbiology **147:**2255–2264.
19. **Guillot, A., D. Obis, and M. Y. Mistou.** 2000. Fatty acid membrane composition and activation of glycine-betaine transport in *Lactococcus lactis* subjected to osmotic stress. Int. J. Food Microbiol. **55:**47–51.
20. **Hazel, J. R.** 1995. Thermal adaptation in biological membranes: is homeoviscous adaptation the explanation? Annu. Rev. Physiol. **57:**19–42.
21. **Hromy, J. M., and G. M. Carman.** 1986. Reconstitution of *Saccharomyces cerevisiae* phosphatidylserine synthase into phospholipid vesicles: modulation of activity by phospholipids. J. Biol. Chem. **261:**15572–15576.
22. **Martin, C. E., and G. A. Thompson, Jr.** 1978. Use of fluorescence polarization to monitor intracellular membrane changes during temperature acclimation. Correlation with lipid compositional and ultrastructural changes. Biochemistry **17:**3581–3586.
23. **Mastronicolis, S. K., J. B. German, N. Megoulas, E. Petrou, P. Foka, and G. M. Smith.** 1998. Influence of cold shock on the fatty-acid composition of different lipid classes of the food-borne pathogen *Listeria monocytogenes*. Food Microbiol. **15:**299–306.
24. **Mazzotta, A. S., and T. J. Montville.** 1997. Nisin induces changes in membrane fatty acid composition of *Listeria monocytogenes* nisin-resistant strains at 10 degrees C and 30 degrees C. J. Appl. Microbiol. **82:**32–38.
25. **Ming, X., and M. A. Daeschel.** 1995. Correlation of cellular phospholipid content with nisin resistance of *Listeria monocytogenes* Scott A. J. Food Prot. **58:**416–420.
26. **New, R. R. C.** 1992. Characterization of liposomes., p. 105–162. *In* R. R. C. New (ed.), Liposomes: a practical approach. IRL Press, Oxford, England.
27. **Pagan, R., S. Condon, and F. J. Sala.** 1997. Effects of several factors on the heat-shock-induced thermotolerance of *Listeria monocytogenes*. Appl. Environ. Microbiol. **63:**3225–3232.
28. **Rogers, A. M., and T. J. Montville.** 1991. Improved agar diffusion assay for nisin quantification. Food Biotechnol. **5:**161–168.
29. **Russell, N. J.** 1993. Physiology and molecular biology of psychrophilic microorganisms, p. 203–224. *In* R. A. Herbert and R. J. Sharp (ed.), Molecular biology and bio/technology of extremophiles. Blackie, Glasgow, United Kingdom.
30. **Russell, N. J., R. I. Evans, P. F. ter Steeg, J. Hellemons, A. Verheul, and T. Abee.** 1995. Membranes as a target for stress adaptation. Int. J. Food Microbiol. **28:**255–261.
31. **Ryser, E. T., and E. H. Marth.** 1991. Listeria, listeriosis, and food safety, p. 1–632. Marcel Dekker, Inc., New York, N.Y.
32. **Shinitzky, M., and Y. Barenholz.** 1978. Fluidity parameters of lipid regions determined by fluorescence polarization. Biochim. Biophys. Acta **515:**367–394.
33. **Shinitzky, M., and M. Inbar.** 1976. Microviscosity parameters and protein mobility in biological membranes. Biochim. Biophys. Acta **433:**133–149.
34. **Sinensky, M.** 1974. Homeoviscous adaptation—a homeostatic process that regulates the viscosity of membrane lipids in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **71:**522–525.
35. **Smith, J. L., B. S. Marmer, and R. C. Benedict.** 1991. Influence of growth temperature on injury and death of *Listeria monocytogenes* Scott A during a mild heat treatment. J. Food Prot. **54:**166–169.
36. **Suutari, M., and S. Laakso.** 1992. Unsaturated and branched chain-fatty acids in temperature adaptation of *Bacillus subtilis* and *Bacillus megaterium*. Biochim. Biophys. Acta **1126:**119–124.
37. **Suutari, M., and S. Laakso.** 1994. Microbial fatty acids and thermal adaptation. Crit. Rev. Microbiol. **20:**285–328.
38. **Thieringer, H. A., P. G. Jones, and M. Inouye.** 1998. Cold shock and adaptation. Bioessays **20:**49–57.
39. **Tourdot-Marechal, R., D. Gaboriau, L. Beney, and C. Divies.** 2000. Membrane fluidity of stressed cells of *Oenococcus oeni*. Int. J. Food Microbiol. **55:**269–273.
40. **van Kraaij, C., E. Breukink, M. A. Noordermeer, R. A. Demel, R. J. Siezen, O. P. Kuipers, and B. de Kruijff.** 1998. Pore formation by nisin involves translocation of its C-terminal part across the membrane. Biochemistry **37:**16033–16040.
41. **Walker, S. J., P. Archer, and J. G. Banks.** 1990. Growth of *Listeria monocytogenes* at refrigeration temperatures. J. Appl. Bacteriol. **68:**157–162.
42. **Winkowski, K., A. D. Crandall, and T. J. Montville.** 1993. Inhibition of *Listeria monocytogenes* by *Lactobacillus bavaricus* MN in beef systems at refrigeration temperatures. Appl. Environ. Microbiol. **59:**2552–2557.
43. **Winkowski, K., R. D. Ludescher, and T. J. Montville.** 1996. Physiochemical characterization of the nisin-membrane interaction with liposomes derived from *Listeria monocytogenes*. Appl. Environ. Microbiol. **62:**323–327.
44. **Wolf, C. E., and W. R. Gibbons.** 1996. Improved method for quantification of the bacteriocin nisin. J. Appl. Bacteriol. **80:**453–457.
45. **Zaritsky, A., A. H. Parola, M. Abdah, and H. Masalha.** 1985. Homeoviscous adaptation, growth rate, and morphogenesis in bacteria. Biophys. J. **48:**337–339.