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Modifications of Membrane Phospholipid Composition in Nisin-Resistant *Listeria monocytogenes* Scott A

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A nisin-resistant (NIS^r) variant of *Listeria monocytogenes* Scott A was isolated by stepwise exposure to increasing concentrations of nisin in brain heart infusion (BHI) broth. The NIS^r strain was about 12 times more resistant to nisin than was the wild-type (WT) strain. Accordingly, higher nisin concentrations were required to dissipate both components of the proton motive force in the NIS^r strain than in the WT strain. Comparison of the membrane fatty acyl composition of the sensitive strain with that of its NIS^r derivative revealed no significant differences. From phospholipid head group composition analysis and phospholipid biosynthesis measurements during growth in the absence and presence of nisin, it could be inferred that the NIS^r strain produces relatively more phosphatidylglycerol (PG) and less diphosphatidylglycerol (DPG) than the parent strain does. Monolayer studies with pure lipid extracts from both strains showed that nisin interacted more efficiently with lipids derived from the WT strain than with those derived from the NIS^r strain, reflecting qualitative differences in nisin sensitivity. Involvement of the cell wall in acquisition of nisin resistance was excluded, since the WT and NIS^r strains showed a comparable sensitivity to lysozyme. Recently, it has been demonstrated that nisin penetrates more deeply into lipid monolayers of DPG than those of other lipids including PG, phosphatidylcholine, phosphatidylethanolamine, monogalactosyldiacylglycerol, and digalactosyldiacylglycerol (R. A. Demel, T. Peelen, R. J. Siezen, B. de Kruijff, and O. P. Kuipers, Eur. J. Biochem. 235:267–274, 1996). Collectively, the mechanism of nisin resistance in this *L. monocytogenes* NIS^r strain is attributed to a reduction in the DPG content of the cytoplasmic membrane.

Nisin is a 34-amino-acid polypeptide bacteriocin (lantibiotic) produced by *Lactococcus lactis* subsp. *lactis*. Since its discovery in the 1920s, nisin has proven to be an effective inhibitor of a broad spectrum of gram-positive bacteria, including the foodborne pathogen *Listeria monocytogenes*. It is the only bacteriocin produced commercially with a legal status for use as a food additive (10). Two natural variants of nisin are known, nisin A and nisin Z, which are equally distributed among nisin-producing *L. lactis* strains (14). Nisin A and nisin Z differ by a single substitution at position 27, with His in nisin A and Asn in nisin Z (38). The nisin molecule has an amphiphathic character, the N-terminal part of the nisin molecule contains a relatively large number of hydrophobic residues, whereas the C-terminal part is more hydrophilic. Nisin is cationic due to the presence of three lysine residues and one (in nisin Z) or two (in nisin A) histidine residues and the absence of glutamate and aspartate. The pK values of the side chain groups in histidine and lysine residues are 6.5 and 10.0, respectively, and the net charge of nisin is therefore pH dependent (24, 30).

The primary target of nisin in sensitive vegetative bacteria is the energy-transducing cytoplasmic membrane. Addition of nisin to gram-positive bacteria results in an efflux of essential small cytoplasmic components (amino acids, monovalent cations, ATP), depletion of both components of the proton motive force ($\Delta\psi$ and ΔpH), and cessation of biosynthesis (1, 6). Nisin has been shown to act on several species of gram-negative bacteria, provided that the integrity or barrier function of the outer membrane is first disrupted (25). Nisin does not require a specific protein receptor to exert its antimicrobial activity. Experiments with intact cells, cytoplasmic membrane vesicles, and liposomes indicate that a *trans*-negative $\Delta\psi$ of sufficient magnitude (15) is necessary for the activity of nisin. Studies by Gao et al. (17) and García Garcerá et al. (18), however, demonstrated that a ΔpH (inside alkaline) is dissipated by nisin in the absence of a $\Delta\psi$, suggesting that ΔpH is as efficient as $\Delta\psi$ in promoting nisin action. In addition, the threshold $\Delta\psi$ is influenced by the nisin concentration and the phospholipid composition of the cytoplasmic membrane (15, 17, 18, 28).

Microorganisms are often able to overcome the inhibitory effect of antibiotics and preservatives. The emergence of nisin-resistant (NIS^r) mutants, which are generated when nisin-sensitive (NIS^s) cells are exposed to relatively high nisin concentrations, has been described for several *Lactobacillus* spp., *Streptococcus* spp., *Leuconostoc* spp., *Bacillus* spp., and *Clostridium* spp. and for *Staphylococcus aureus*, and *L. monocytogenes* (5, 7, 8, 36, 37). Harris et al. (20) detected mutant strains of *L. monocytogenes*, at frequencies of 10^{-6} and 10^{-8}, which were able grow at nisin concentrations 5 to 10 times higher than was the original population, indicating the potential for NIS^r variants to arise from widespread application of the antibiotic. Insight into the mechanism of NIS^r is important because it will provide a rational basis for its application in the food industry. Several mechanisms of NIS^r in gram-positive bacteria can be envisaged. In view of the consensus that the bacterial membrane is the site of nisin action, the NIS^r phenotype may develop from alterations in the cell envelope (9, 32, 36, 37).

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In the present study, we investigated the mechanism of NIS^r in *L. monocytogenes* by comparing both fatty acyl and head group compositions of membrane phospholipids in NIS^s and NIS^r *L. monocytogenes* Scott A. Furthermore, the capability of both strains to synthesize phospholipids during exposure to nisin has been investigated. Monolayer studies with pure lipids extracts from both strains have been used to assess specific interactions of nisin with lipids in further detail. Combined, these approaches provide important new information on the role of membranes in NIS^r.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The wild-type (WT) strain of *L. monocytogenes* Scott A and its NIS^r derivative were grown in brain heart infusion (BHI) broth. Stock cultures were maintained in the same medium solidified with agar. The NIS^r strain was acquired by stepwise increases in the exposure to nisin A (0.6, 1.0, 1.6, 2.6, 3.4, 5.0, 7.0, 10, 15, and 30 mg/liter); the resistant strain is 10 to 15 times more resistant than the parent strain but fails to grow in BHI broth containing 40 mg of nisin per liter. Although the NIS^r was stable for at least 10 passages through nisin-free medium (24 h intervals), nisin was always incorporated into BHI agar at 10 mg/liter for routine subculture and maintenance of the mutant strain.

Cultures were grown in Erlenmeyer flasks containing 20% of the flask volume of medium at 7 or 30°C in a gyratory incubator (New Brunswick Scientific, Edison, N.J.) at 150 rpm. Growth was followed by measurement of turbidity at 600 or 620 nm or by plate counting on tryptic soy agar (TSA).

Determination of MICs. *L. monocytogenes* was grown overnight at 30°C in BHI broth and inoculated at approximately 10^5 cells per ml into different wells of a microtiter plate which contained fresh BHI with increasing nisin concentrations. The MIC was determined as the lowest nisin concentration which prevented growth after incubation for 24 h at 30°C.

Measurement of intracellular pH and membrane potential in intact cells. The intracellular pH was determined with the fluorescent probe 5- (and 6-)carboxyfluorescein diacetate succinimidyl ester, as described by Breeuwer et al. (4), in 50 mM potassium phosphate buffer (pH 7.2). Assays were performed in the presence of valinomycin (1 μM) to dissipate the membrane potential.

The $\Delta\psi$ (inside alkaline) was monitored by quenching the potential-sensitive fluorescent probe 3,3-dipropylthiadicarbocyanine ($DiSC_3[5]$) (excitation wavelength, 643 nm; emission wavelength, 666 nm). Membrane potential measurements were performed in 50 mM K-HEPES buffer (pH 7.2) in the presence of nigericin (1 μM) to prevent the generation of a transmembrane pH gradient. Fluorescence measurements were made with a Perkin-Elmer LS 50 spectrofluorimeter at 30°C with continuous stirring.

Isolation, fractionation, and identification of lipids. *L. monocytogenes* cultures were grown in BHI broth until the mid-exponential phase (optical density at 600 nm [OD_{600}] = 0.7), sedimented by centrifugation, and washed twice in 50 mM potassium phosphate buffer (pH 6.9) containing 5 mM $MgSO_4$, and the total lipids were extracted from cell pellets by the method of Bligh and Dyer (3) as detailed by Kates (26).

It has been suggested that gram-positive bacteria should be pretreated with lysozyme for a full extraction of lipids, particularly diphosphatidylglycerol (DPG), from stationary-phase cultures (13, 16). However, these studies show that in log-phase cultures, good extraction can be obtained without lysozyme, and so no lysozyme treatment was used.

Individual lipids were separated from total lipid extracts by thin-layer chromatography (TLC). Initially, the phospholipids were tentatively identified by comparing their mobilities with those of standards in one- and two-dimensional TLC on commercial precoated silica gel 60A plates (Whatman, Clifton, N.J.) and homemade plates containing silica gel 60H (Merck, Darmstadt, Germany) in the following solvent systems: chloroform-methanol-acetic acid-water (85:15:10:3.5 by volume), chloroform-methanol-water (65:25:4 by volume), chloroform–methanol–28% ammonia (65:25:4 by volume), chloroform-methanol-28% ammonia (65:35:5 by volume), chloroform-methanol-acetone-acetic acid-water (6:2:8:2:1 by volume), and chloroform-methanol-acetone-acetic acid-water (10:2:4:2:1 by volume). Individual lipids on TLC plates were visualized with the following stains: iodine vapor (general lipid detection), ninhydrin (amino groups), α-naphthol (sugar moieties), and phosphate stain (phosphate groups) (26). Phospholipids were quantified by phosphorus analysis, using the modified Bartlett (2) assay.

Fatty acid analysis. Aliquots of the phospholipid extract were used for the preparation of fatty acid methyl esters by transmethylation with concentrated 2.5% (vol/vol) H_2SO_4 in anhydrous methanol. The fatty acid methyl esters were extracted with petroleum ether (boiling point, 60 to 80°C) and subsequently separated isothermally at 165°C by gas-liquid chromatography in a Perkin-Elmer F33 gas chromatograph equipped with a glass column (1 m by 4 mm [inner diameter]) packed with 5% (wt/wt) SP2100 (Supelco, Bellefont, Pa.) as the stationary phase. Fatty acid methyl ester compositions were determined by measuring peak areas after identification by using authentic standards and comparison of retention times.

Phospholipid radiolabelling and extraction. Cells were grown overnight, inoculated into 80 ml of fresh medium, and allowed to grow to an OD_{600} of about 0.2. The culture was divided into two 40-ml portions, which were pulse-labelled at time zero with 52 μl of [1-^{14}C]acetate (final concentration, 9.62 kBq/ml; 4.8 μM), and 2.0 mg of nisin A per liter (i.e., 80 μl of a sterile stock solution in 0.02 M HCl containing 1,000 mg of nisin A per liter) was immediately added to one flask and 80 μl of 0.02 M HCl was added to the other flask, which served as the control. Rates of [^{14}C]acetate incorporation into lipids were determined as follows. Samples (3 ml) of the labelled cultures were withdrawn at different time intervals, and the lipids were extracted by rapidly mixing the culture sample with 11.25 ml of methanol-chloroform (2:1, vol/vol). After at least 1 h, a further 3.75 ml of chloroform and 3.75 ml of water were added. Subsequently, the mixture was centrifuged briefly, the upper phase was removed, and the chloroform layer was washed with 14 ml of methanol-water (10:9, vol/vol). The final washed chloroform extracts were evaporated to dryness under N_2, and the lipid residue was redissolved in 400 μl of chloroform.

Radioactivity measurements. The radioactivity in total lipid extracts was determined by transferring 40 μl of each of the lipid extracts into plastic scintillation vials. The solvent was evaporated, 10 ml of Ecolite (ICN, Irvine, Calif.) was added as the scintillant, and the radioactivity was measured with a Wallac 1211 Rackbeta liquid scintillation counter (LKB, Biochrom). To determine the radioactivity in individual lipids, the remainder of each lipid was separated by TLC on homemade plates containing silica gel 60H with chloroform-methanol-acetic acid-water (85:15:10:3.5 by volume) as the solvent system. To aid the visualization of phospholipid spots, approximately 0.1 mg of carrier lipid was added to the lipid samples before chromatography. The areas of individual spots were scraped into scintillation vials for determination of the radioactivity as above.

Lysozyme action. Early-stationary-phase cultures of the WT and NIS^r strains were inoculated into fresh BHI broth (10%, vol/vol), and growth was monitored at 30°C by measuring the turbidity at 620 nm with a Novaspec II spectrometer (Pharmacia Biotech). At an OD_{620} of 0.5 to 0.6, either 2 or 5 mg of egg white lysozyme per ml was added to the cultures, and the OD_{620} was recorded every 30 min for 4 h. In addition, after 1 and 3 h, appropriate serial dilutions of the samples were plated on TSA. The plates were incubated for 2 to 3 days at 30°C.

Monolayer experiments. The Wilhelmy plate method was used to measure nisin-induced changes in the surface pressure of purified bacterial total lipid extract. Purified lipid was obtained by conversion of the anionic lipids to their sodium salts and removal of proteins from the lipid extract by silica column chromatography (27, 43). Analysis by TLC showed that the lipid composition was not affected upon purification. Monolayers were formed by spreading a lipid-containing chloroform solution on the air/buffer (10 mM Tris [pH 7.4]) interface to an initial surface pressure between 18 and 40 mN/m. Nisin Z at a saturating concentration (1.2 μg/ml) was added through a small injection hole to the subphase, which was stirred continuously. The time-dependent surface pressure increase after the addition of nisin was measured until a stable surface pressure was reached. The experiments were performed at room temperature.

Protein determination. Protein concentrations were determined by the method of Lowry et al. (31) with bovine serum albumin as the standard.

Statistical evaluation. Statistical analysis of data was performed by a two-tailed Student *t*-test for unpaired observations. Differences were considered significant if $P < 0.05$. Values are expressed as the mean ± standard deviation.

Chemicals. Sodium [1-^{14}C]acetate (2.0 GBq mmol^{-1}) was purchased from Amersham (Little Chalfont, United Kingdom). BHI broth and TSA were from Difco Laboratories (Detroit, Mich.). Nisin A was obtained from Sigma (St. Louis, Mo.) as Nisaplin, which contains 2.5% (wt/wt) nisin. Nisin Z was produced and purified as previously described (38). Fluorescent dyes were obtained from Molecular Probes Europe B.V. (Leiden, The Netherlands). All other chemicals were reagent grade and were obtained from commercial sources.

RESULTS

Effect of nisin on bioenergetic parameters in WT and NIS^r *L. monocytogenes* Scott A. Nisin MICs for both *L. monocytogenes* strains were determined in BHI broth. The MIC for the WT strain was 3.2 ± 0.2 mg/liter, compared with 37.5 ± 2.5 mg/liter for the NIS^r strain. The influence of nisin on the ΔpH and $\Delta\psi$ was determined at an external pH of 7.2, which is the pH of BHI broth. Upon addition of 1.3 mg of nisin per liter, an immediate dissipation of the ΔpH was observed in WT *L. monocytogenes* Scott A (Fig. 1). This nisin concentration had no effect on ΔpH in the NIS^r strain. Titration with nisin resulted in a gradual decrease of the intracellular pH in the latter strain, and a complete collapse of the ΔpH was observed after the addition of 11.7 mg of nisin per liter (Fig. 1). The $\Delta\psi$ in the WT strain collapsed after the addition of 0.25 mg of nisin per liter, whereas the $\Delta\psi$ in the NIS^r strain was only slightly affected by this nisin concentration, requiring a final nisin con-

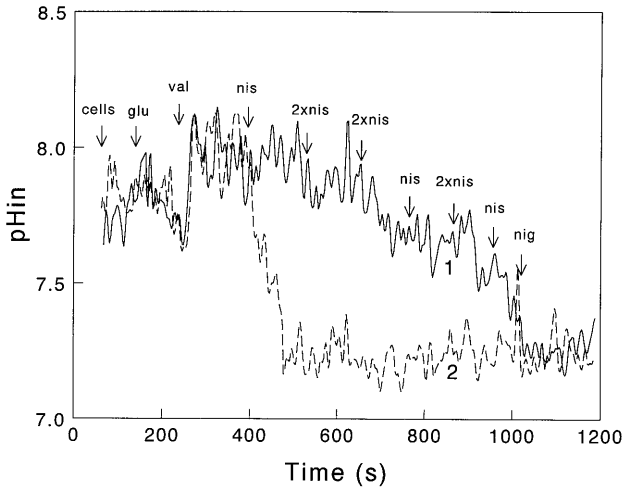

FIG. 1. Effect of nisin on the intracellular pH of glucose-energized cells of WT and NIS^r *L. monocytogenes* Scott A. Harvested cells were washed and resuspended in 50 mM K-HEPES buffer (pH 8.0) and incubated for 10 min at 30°C in the presence of 1 μM of 5- (and 6-)carboxyfluorescein diacetate succinimidyl ester. The cells were washed in 50 mM potassium phosphate buffer (pH 7.2), and the nonconjugated fluorescent probe was eliminated by incubation of the cells in the presence of 0.5% (wt/vol) glucose at 30°C followed by washing in 50 mM potassium phosphate buffer (pH 7.2). At the times indicated, cells (final OD_{620}, 0.067), 0.5% (wt/vol) glucose (glu), 1 μM valinomycin (val), 1.3 mg of nisin per liter (nis) or 2.6 mg of nisin per liter (2xnis), and 1 μM nigericin (nig) were added to the phosphate buffer. Assays were performed with the NIS^r (trace 1) and WT (trace 2) strains.

centration of 0.5 mg/liter for complete collapse of the $\Delta\psi$ (Fig. 2). Valinomycin completely abolished the $\Delta\psi$ in both strains. These results demonstrate that nisin susceptibility in BHI broth correlates with the effect of nisin on bioenergetic parameters in *L. monocytogenes* Scott A.

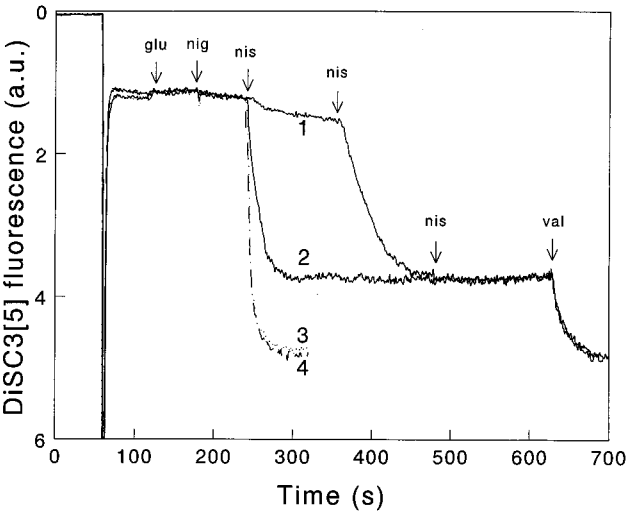

FIG. 2. Effect of nisin on the membrane potential of glucose-energized cells of WT and NIS^r *L. monocytogenes* Scott A. Cells were grown in BHI broth and then washed and resuspended in 50 mM K-HEPES buffer (pH 7.2). Cell suspensions of NIS^r (trace 1) and WT (trace 2) strains were diluted to an OD_{620} of 0.25, and $DiSC_3[5]$ was added to a final concentration of 5 μM. The following additions were made at the times indicated by the arrows: 0.5% (wt/vol) glucose (glu), 1 μM nigericin (nig), 0.25 mg of nisin per liter (nis), and 1 μM valinomycin (val). The dashed lines show the results of separate experiments with NIS^r (trace 3) and WT (trace 4) strains, in which 1 μM valinomycin was added.

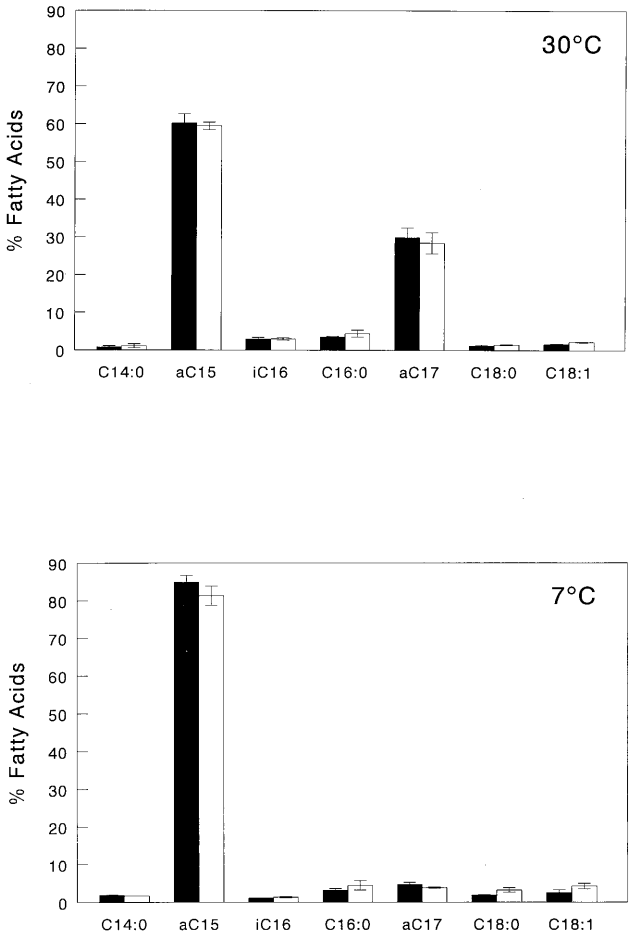

FIG. 3. Fatty acid compositions of parental (WT) (solid bars) and resistant (NIS^r) (open bars) strains of *L. monocytogenes* Scott A. Values are the means and standard deviation of analysis in duplicate of lipids extracted from three separate cultures grown in BHI broth at 30°C or at 7°C until the late exponential growth phase; a, anteiso-branched; i, iso-branched.

Membrane composition of WT and NIS^r *L. monocytogenes* grown at 30 and 7°C. When nisin is used as a biopreservative in minimally processed ready-to-eat refrigerated foods, the psychrotroph *L. monocytogenes* is exposed to nisin at low temperature. The membrane fatty acid and phospholipid head group composition of the mutant and parent strains were therefore determined in cells grown in BHI broth at both high (30°C) and low (7°C) temperatures. The primary response of the fatty acid composition in both WT and NIS^r *L. monocytogenes* Scott A to a decrease in growth temperature appeared to be a marked increase in anteiso-$C_{15:0}$ at the expense of anteiso-$C_{17:0}$ (Fig. 3), which is in agreement with reports in the literature (39, 40). For a given temperature, only very small differences between the fatty acid compositions of the WT and the NIS^r strains were detected: the NIS^r mutant contained more $C_{16:0}$, $C_{18:0}$, and $C_{18:1}$ and less of the branched-chain fatty acids anteiso-$C_{15:0}$ and anteiso-$C_{17:0}$ than did the WT strain at both temperatures (Fig. 3). However, statistical analysis indicated that these differences in fatty acid composition were not significant at the 95% confidence level.

The major phospholipids in *L. monocytogenes* Scott A were identified as being phosphatidylglycerol (PG) and DPG and a phospholipid that stained positively with α-naphthol, i.e., a phosphoglycolipid (PGL) (Table 1). The remaining phospho-

TABLE 1. Phospholipid composition of WT and NIS^r *L. monocytogenes* Scott A[a]

Phospholipid	Phospholipid composition (wt % phosphorus content) in[b]:			
	WT		NIS^r	
	30°C	7°C	30°C	7°C
PG	62.3 ± 0.54	62.1 ± 0.57	68.0 ± 0.64	69.1 ± 0.27
PGL	9.4 ± 0.06	9.4 ± 0.13	8.8 ± 0.05	8.9 ± 0.18
DPG	12.7 ± 0.05	12.8 ± 0.31	9.6 ± 0.12	9.8 ± 0.05
Others[c]	15.6 ± 0.25	15.7 ± 0.39	13.6 ± 0.60	12.2 ± 0.29

[a] Cultures were grown in BHI broth with shaking at 30 or 7°C until the mid-exponential phase (OD_{600} = 0.7).

[b] Values are the mean for triplicate samples of independent cultures ± standard deviation.

[c] Represent several minor phospholipids and may include phosphatidylserine, (amino)phosphoglycolipids, phosphatidylinositol, and bisphosphatidylglycerolphosphate.

lipids were minor components, and no further attempt was made to identify them. Data from previous studies indicate the presence of PG, DPG, and a PGL but give no firm description of the minor phospholipids in *L. monocytogenes*; possible identities include phosphatidylserine, (amino)phosphoglycolipids, phosphatidylinositol, and bisphosphatidylglycerolphosphate (13, 29, 34, 37, 39). Statistical analysis of the phospholipid content of either the WT or NIS^r strain grown at 30 and 7°C revealed no temperature-dependent differences for either strain. In comparison, there was a significant increase in PG and a decrease in the other phospholipids in the NIS^r strain compared to the WT strain at both growth temperatures (Table 1).

Phospholipid biosynthesis in WT and NIS^r *L. monocytogenes* Scott A during growth in the absence and presence of nisin. A radioactive precursor of lipid biosynthesis, [^{14}C]acetate, was used to quantitate the lipid biosynthesis in the two strains during exposure to nisin. Nisin was added to an exponentially growing culture together with the label at time zero. It was established that a nisin concentration of 2.0 mg/liter was the optimal concentration for the pulse-labelling experiments, since this concentration was high enough to inhibit growth of the WT strain but did not cause cell lysis. In the control experiment (no nisin present), the culture continued growing exponentially, whereas in the presence of nisin, growth stopped and resumed after a lag period of approximately 140 min (Fig. 4A). During this period, the viable cell counts remained constant (data not shown). The incorporation of [^{14}C]acetate into the lipids of the WT strain in BHI broth occurred at a rate roughly twice that of incorporation in WT growing in BHI broth plus nisin (Fig. 4A). The cells that continued growing 140 min after nisin addition were subcultured to determine the MIC of nisin; the results indicated that these cells had not developed resistance during this period (data not shown).

The rate of lipid biosynthesis in the NIS^r strain growing in BHI broth in the absence of nisin was comparable to that of WT growing under the same conditions. Growth of the NIS^r strain in the presence of nisin was indistinguishable from that in the absence of nisin. Accordingly, the addition of nisin to the culture medium did not affect the rate of [^{14}C]acetate incorporation into the lipids of the NIS^r strain (Fig. 4B).

Biosynthesis of the major individual phospholipids PG and DPG in WT and NIS^r *L. monocytogenes* Scott A during nisin exposure. The biosynthesis of PG and DPG, as measured by the incorporation rates of [^{14}C]acetate, during growth of WT in the absence of nisin were higher than those of cultures growing in the presence of nisin (Fig. 5A and B), which is consistent with the effect of nisin on total lipid biosynthesis (Fig. 4A). Furthermore, the amount of radioactivity incorporated in DPG relative to PG (i.e. the DPG/PG ratio) remained more or less unaffected upon nisin addition. This result implies that nisin does not directly affect phospholipid biosynthesis in WT *L. monocytogenes*. The incorporation rates of [^{14}C]acetate in both PG and DPG of the NIS^r strain were similar with or without nisin (Fig. 5C and D). However, the NIS^r strain incorporates more [^{14}C]acetate in PG and less in DPG than does the WT strain, which indicates that more PG and less DPG is produced in the NIS^r strain.

Sensitivity of WT and NIS^r *L. monocytogenes* Scott A to lysozyme. Recently, it has been demonstrated that NIS^r is based on cell wall changes in some *Listeria* strains (9, 32). Lysozyme addition (final concentration, 2 mg/ml) during exponential growth of the WT and NIS^r strains in BHI broth resulted in growth inhibition of both strains. At this lysozyme

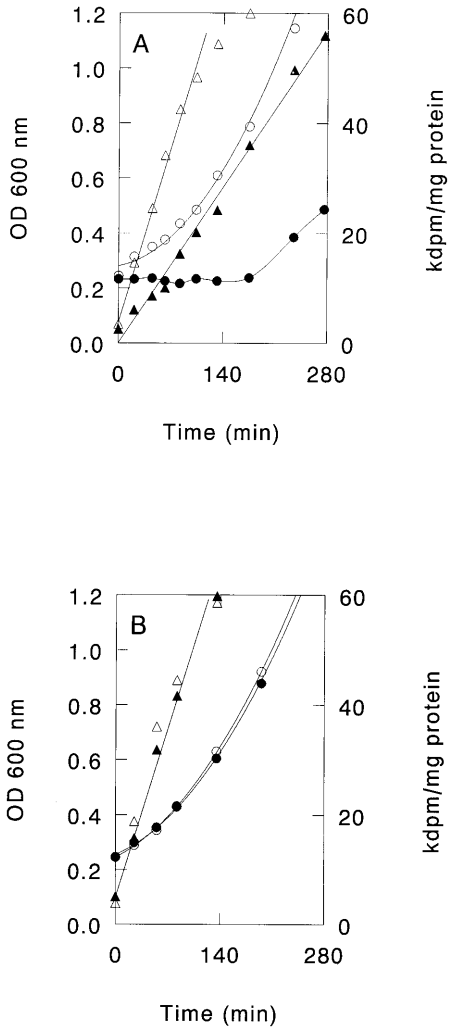

FIG. 4. Incorporation of [^{14}C]acetate in phospholipids of WT (A) and NIS^r (B) *L. monocytogenes* Scott A during growth at 30°C in the absence or presence of nisin. The radioactive precursor [^{14}C]acetate (9.6 kBq/ml; 4.8 μM) was added to cultures growing exponentially in BHI broth together with nisin (2 mg/liter) at time zero if required. Samples were removed for lipid extraction and subsequent determination of ^{14}C incorporation (triangles). Growth was monitored as OD_{600} (circles). Open and solid symbols represent the results in the absence and presence of nisin, respectively.

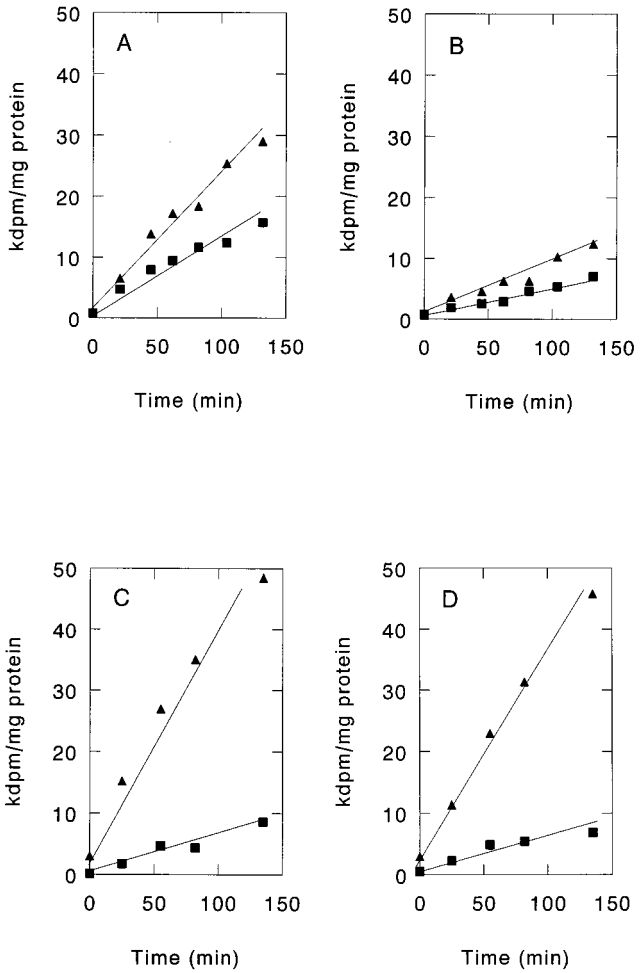

FIG. 5. Effect of nisin on PG and DPG synthesis in WT and NIS^r *L. monocytogenes*. *L. monocytogenes* Scott A cultures growing exponentially at 30°C were incubated with [^{14}C]acetate (9.6 kBq/ml; 4.8 μM), and at intervals samples were extracted, the individual phospholipids were fractionated by TLC, and the incorporation of [^{14}C]acetate into PG (▲) and DPG (■) was measured. (A and B) Rates of incorporation in the WT strain in the absence and presence, respectively, of nisin (2.0 mg/liter); (C and D) rates of incorporation into the NIS^r strain in the absence and presence, respectively, of nisin.

concentration, both WT and NIS^r numbers decreased by ca. 0.5 log unit over 3 h. The effect of 5 mg of lysozyme per ml on log-phase cultures was a greater viability loss for the WT and NIS^r strains; a reduction of ca. 1 log unit was recorded for both strains (data not shown). These results indicate that the NIS^r and WT strains have a comparable sensitivity toward lysozyme.

Interaction between nisin Z and lipid monolayers of WT and NIS^r *L. monocytogenes*. Monolayers of pure lipids from WT and NIS^r *L. monocytogenes* were formed to determine specific interactions of nisin with the lipids. For these experiments, purified nisin Z was used instead of nisin A. The MICs of both nisin A and nisin Z for the WT and NIS^r strains were the same (data not shown). The changes in surface pressure after the addition of nisin to the aqueous subphase are interpreted as being a result of the insertion of nisin into the lipid phase. In the absence of a lipid monolayer, nisin maximally increases the surface pressure up to 16 mN/m at the concentration used (data not shown). This indicates that at an initial surface pressure higher than 16 mN/m, the increases in surface pressure after the addition of nisin to the subphase of the lipid monolayer are a direct consequence of nisin-lipid interactions. The increase in surface pressure is smaller at higher initial surface pressures due to the tighter lipid packing of the monolayer; surface pressures between 30 and 35 mN/m are believed to be relevant for biological membranes (12).

Monolayers of WT lipid extracts showed large pressure increases after the addition of nisin (Fig. 6). The pressure changes induced by nisin in monolayers of lipid of the NIS^r strain were significantly lower. The results show that nisin interacts more efficiently with the lipids of WT strain membranes than those of the NIS^r strain, which mirrors the difference in nisin sensitivity of the bacterial strains. Considering the effect of external pH on nisin efficiency, monolayers were also formed with lipids from both strains on a subphase of 10 mM Tris buffer (pH 6.0) and the effect of nisin addition was evaluated and compared to results obtained with a subphase of 10 mM Tris buffer (pH 7.4). Pressure changes at pH 6.0 were lower than at pH 7.4; at an initial surface pressure of 22 mN/m, the pressure change was reduced by 15 to 20% for both strains, and at an initial surface pressure of 31 mN/m, this reduction was 30 to 40% (data not shown). These results suggest that a lower pH reduces the nisin-lipid interaction in monolayer studies. Divalent cations (i.e., Mg^{2+} and Ca^{2+}) have an inhibitory effect on the action of nisin Z against *L. monocytogenes*, which has been interpreted as a neutralization of the negatively charged phospholipid head groups (1). The effect of divalent cations on nisin-lipid interactions was tested by adding 10 mM $MgSO_4$ to the subphase of monolayers of lipids from the WT and NIS^r strains. The pressure increases recorded with monolayers after the addition of nisin to a subphase containing $MgSO_4$, at an initial pressure of 32 mN/m, were reduced to approximately 50% compared to the low-ionic-strength condition for both strains (data not shown). For comparison, the influence of Mg^{2+} ions on nisin MICs in BHI broth was determined. The MIC for the WT strain (3.2 ± 0.2 mg/liter) rose with increasing $MgSO_4$ concentrations and reached 4.0 ± 0.25 mg/liter at an $MgSO_4$ concentration of 10 mM. For the NIS^r strain, this increase was from 37.5 ± 2.5 (no $MgSO_4$) to 50 ± 4 mg/liter in the presence of 10 mM $MgSO_4$). In the presence of 250 mM $MgSO_4$, the nisin MIC for the WT strain was still

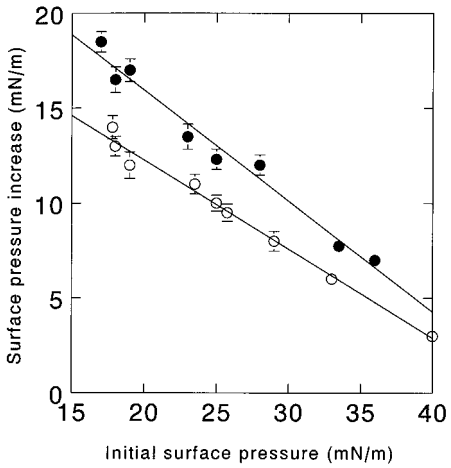

FIG. 6. Effect of nisin on surface pressure of isolated lipids of WT and NIS^r *L. monocytogenes*. Increases in surface pressure were determined after the injection of nisin Z underneath monolayers of purified total lipids extracted from WT (●) and NIS^r (○) strains of *L. monocytogenes* Scott A at different initial surface pressures. Nisin Z (41 μg) was injected in a subphase comprising 35 ml of 10 mM Tris (pH 7.4).

three to four times lower (i.e., about 10 mg/liter) than the value determined for the NIS^r strain in the absence of $MgSO_4$ (data not shown), which indicates that very high concentrations of divalent cations must be applied to the WT strain to reach the same resistance level as with the NIS^r strain.

DISCUSSION

In the present study, we showed that higher nisin concentrations were required to dissipate the ΔpH and $\Delta\psi$ in a NIS^r variant of *L. monocytogenes* Scott A compared to its parent WT strain, which correlates with the NIS^s status of the strains in BHI broth. This suggest a possible role for the energy-transducing cytoplasmic membrane in the acquisition of NIS^r in *L. monocytogenes* Scott A. The phenomenon of NIS^r in *L. monocytogenes* Scott A was studied in detail with a focus on membrane composition.

Comparison of the fatty acid composition at one particular temperature of the NIS^r isolate of *L. monocytogenes* Scott A with that of the WT strain at both growth temperatures tested (7 and 30°C) revealed no significant differences. Ming and Daeschel (37) found that their stable nisin-resistant mutant of *L. monocytogenes* contained a greater proportion of straight-chain fatty acids whereas the parent contained more branched-chain fatty acids; no changes in unsaturation of lipid acyl chains were reported. However, the specific growth rate of their resistant mutant was decreased, especially at low temperature, which could indicate a less flexible membrane, whereas our mutant exhibited the same growth characteristics as the parent strain at both 7 and 30°C (data not shown). Furthermore, the proton motive forces generated ($\Delta\psi$ and ΔpH) in the WT and the NIS^r strains are similar, indicating that bioenergetics is not affected in the latter strain (Fig. 1 and 2).

The phospholipid compositions of the WT and NIS^r strains did not alter significantly with growth temperature, which agrees with data described for other bacteria (42). However, significant changes in the proportional ratios of the phospholipids in WT and NIS^r *L. monocytogenes* were found, with the most prominent alteration in the NIS^r strain compared to the WT strain being the higher ratio between PG and DPG (approximately 7 compared to 5 [Table 1]). This observation was matched by the results of the phospholipid metabolism studies, which demonstrated that the incorporation rate of [^{14}C]acetate in DPG relative to PG is significantly reduced in the mutant strain (Fig. 5). A direct comparison of the DPG/PG ratios calculated from the mass values given in Table 1 with those obtained from the metabolism studies (Fig. 5) is not justified because the former are based on phosphorus content whereas the latter represent [^{14}C]acetate incorporation in the carbon skeleton; therefore, differences in the rate of turnover of different parts of the molecule, particularly the fatty acyl chains, would distort the values obtained. The finding that the NIS^r strain produces relatively more PG and less DPG than does the WT strain could be due to decreased activity of the enzyme DPG synthetase, which forms DPG by condensation of two PG molecules (41). It is conceivable that our NIS^r strain has reduced DPG synthetase activity, because the shifts in phospholipid composition were also observed when this strain is grown for various generations in the absence of nisin (data not shown).

It has been proposed that both electrostatic and hydrophobic interactions play an important role in the interaction between nisin and membranes (15, 33). Therefore, the interaction between nisin and pure lipids from the WT and NIS^r strains was studied by the lipid-monolayer technique. This technique has proven to be a valuable tool in the study of membrane-active compounds (11). The monolayer results (Fig. 6) show that the pressure increase induced by nisin Z was higher for WT than for NIS^r lipid monolayers, reflecting qualitatively the difference in nisin sensitivity. At an initial pressure of 32 mN/m, which is thought to be a relevant surface pressure of biological membranes (12), the pressure increase is 6.0 and 9.5 mN/m for the NIS^r and WT strains, respectively. The presence of the divalent cation Mg^{2+} resulted in a weaker interaction of nisin with monolayers of extracted lipids from the wild-type strain and its mutant. These ions also reduced nisin MICs (data not shown). This is most probably a consequence of the interaction of Mg^{2+} with the negatively charged head groups of the phospholipids. This interaction might result in neutralization of the negatively charged phospholipids (i.e., PG and DPG) in *L. monocytogenes*, which can reduce the efficiency of nisin action. Second, in the presence of divalent cations, a tighter packing of the acyl chains is introduced (21, 27), which can hinder nisin insertion. The effect of pH on the activity of nisin on lipid monolayers, i.e., that a lower pH reduces the nisin-lipid interactions, does not agree with the results obtained in vivo (1, 22, 23). An interpretation could be that a $\Delta\psi$ and/or ΔpH present in vegetative cells enhances nisin activity at acidic pH values.

In a previous study (13), it has been demonstrated that nisin penetrates more deeply into lipid monolayers of DPG than into those of other lipids including PG, phosphatidylcholine, phosphatidylethanolamine, monogalactosyldiacylglycerol, and digalactosyldiacylglycerol. Moreover, monolayers formed of lipid extracts of *Micrococcus flavus* were much more strongly penetrated than were those of *L. monocytogenes* (13). *M. flavus* is extremely sensitive to nisin compared to several other gram-positive bacteria (14), and, typically, the *M. flavus* membrane contains relatively large amounts of DPG and PG, i.e., 32 and 60% of the total phospholipid extracted, respectively (13, 39). For the *L. monocytogenes* strain used by Demel et al. (13), these values were 7% DPG and 51% PG. Although both PG and DPG are negatively charged and therefore can interact with the cationic nisin molecule, the interaction with DPG is apparently much stronger; this may be linked to its high charge density and to its specific charge distribution (13, 19, 33). Using a nisin variant containing a tryptophan at position 30, Martin et al. (33) showed that the N-terminal part of nisin penetrated very deeply into the hydrophobic part of DPG-containing lipid bilayers.

In conclusion, this study provides additional understanding of the mechanism of nisin resistance in *L. monocytogenes*. The results demonstrate that phospholipid head group alterations, particularly in the content of DPG, are the basis of a nisin-resistant variant of *L. monocytogenes* Scott A. However, the acquisition of nisin resistance in *Listeria* is probably a complex and multiple phenomenon and may differ among strains, as is suggested by reports on alterations in fatty acid composition (35, 36) and cell wall changes (9, 32) in nisin-resistant listeriae.

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REFERENCES

1. **Abee, T., F. M. Rombouts, J. Hugenholtz, G. Guihard, and L. Letellier.** 1994. Mode of action of nisin Z against *Listeria monocytogenes* Scott A at high and low temperatures. Appl. Environ. Microbiol. **60:**1962–1968.
2. **Bartlett, G. R.** 1959. Phosphorus assay in column chromatography. J. Biol. Chem. **234:**466–468.
3. **Bligh, E. G., and W. J. Dyer.** 1959. A rapid method of total lipid extraction

and purification. Can. J. Biochem. Physiol. **37:**911–917.
4. **Breeuwer, P., J.-L. Drocourt, F. M. Rombouts, and T. Abee.** 1996. A novel method for continuous determination of the intracellular pH in bacteria with the internally conjugated fluorescent probe 5- (and 6-)carboxyfluorescein succinimidyl ester. Appl. Environ. Microbiol. **62:**178–183.
5. **Breuer, B., and F. Radler.** 1996. Inducible resistance against nisin in *Lactobacillus casei*. Arch. Microbiol. **65:**114–118.
6. **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.
7. **Daeschel, M. A., D.-S. Jung, and B. T. Watson.** 1991. Controlling wine malolactic fermentation with nisin and nisin-resistant strains of *Leuconostoc oenos*. Appl. Environ. Microbiol. **57:**601–603.
8. **Davies, E. A., and M. R. Adams.** 1994. Resistance of *Listeria monocytogenes* to the bacteriocin nisin. Int. J. Food Microbiol. **21:**341–347.
9. **Davies, E. A., M. B. Falahee, and M. R. Adams.** 1996. Involvement of the cell envelope of *Listeria monocytogenes* in the acquisition of nisin resistance. J. Appl. Bacteriol. **81:**139–146.
10. **Delves-Broughton, J.** 1990. Nisin and its uses as a food preservative. Food Technol. **44:**100–112, 117.
11. **Demel, R. A.** 1994. Physicochemical methods in the study of biomembranes. Monomolecular layers in the study of biomembranes. Subcell. Biochem. **23:**83–120.
12. **Demel, R. A., W. S. M. Geurts van Kessel, R. F. A. de Zwaal, B. Roelofsen, and L. L. M. van Deenen.** 1975. Relation between various phospholipase actions on human red cell membranes and the interfacial phospholipid pressure in monolayers. Biochim. Biophys. Acta **406:**97–107.
13. **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.
14. **De Vos, W. M., J. W. M. Mulders, R. J. Siezen, J. Hugenholtz, and O. P. Kuipers.** 1993. Properties of nisin Z and the distribution of its gene, *nisZ*, in *Lactococcus lactis*. Appl. Environ. Microbiol. **59:**213–218.
15. **Driessen, A. J. M., H. W. van den Hooven, W. Kuiper, M. van der Kamp, H.-G. Sahl, R. N. Konings, and W. N. Konings.** 1995. Mechanistic studies of lantibiotic-induced permeabilization of phospholipid vesicles. Biochemistry **34:**1606–1614.
16. **Filgueiras, M. H., and J. A. F. op den Kamp.** 1980. Cardiolipin, a major phospholipid of Gram-positive bacteria that is not readily extractable. Biochim. Biophys. Acta **620:**332–337.
17. **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.
18. **García Garcerá, M. J., M. G. L. Elferink, A. J. M. Driessen, and W. N. Konings.** 1993. *In vitro* pore-forming activity of the lantibiotic nisin. Eur. J. Biochem. **212:**417–422.
19. **Giffard, C. J., S. Ladha, A. R. Mackie, D. C. Clark, and D. Saunders.** 1996. Interaction of nisin with planar lipid bilayers monitored by fluorescence recovery after photobleaching. J. Membr. Biol. **151:**293–300.
20. **Harris, L. J., H. P. Fleming, and T. R. Klaenhammer.** 1991. Sensitivity and resistance of *Listeria monocytogenes* ATCC 19115, Scott A and UAL500 to nisin. J. Food Prot. **54:**836–840.
21. **Harwood, J. L., and N. J. Russell.** 1984. Lipids in plants and microbes. George Allen and Unwin, London, United Kingdom.
22. **Huot, E., C. Barrena-Gonzales, and H. Petitdemange.** 1996. Comparative effectiveness of nisin and bacteriocin J46 at different pH values. Lett. Appl. Microbiol. **22:**76–79.
23. **Hurst, A.** 1981. Nisin. Adv. Appl. Microbiol. **27:**85–122.
24. **Jack, R. W., J. R. Tagg, and B. Ray.** 1995. Bacteriocins of gram-positive bacteria. Microbiol. Rev. **59:**171–200.
25. **Kalchayanand, N., M. B. Hanilin, and B. Ray.** 1992. Sublethal injury makes Gram-negative and resistant Gram-positive bacteria sensitive to the bacteriocins, pediocin AcH and nisin. Lett. Appl. Microbiol. **15:**239–243.
26. **Kates, M.** 1986. Techniques of lipidology: isolation, analysis and identification of lipids, 2nd ed. Elsevier Biomedical Press, Amsterdam, The Netherlands.
27. **Killian, J. A., M. C. Koorengevel, J. A. Bouwstra, G. Gooris, W. Dowhan, and B. de Kruiff.** 1994. Effect of divalent cations on lipid organization of cardiolipin isolated from *Escherichia coli* strain AH930. Biochim. Biophys. Acta **1189:**225–232.
28. **Kordel, M., F. Schüller, and H.-G. Sahl.** 1989. Interaction of the pore-forming peptide antibiotic Pep 5, nisin and subtilin with non-energized liposomes. FEBS Lett. **244:**99–102.
29. **Kosaric, N., and K. K. Carroll.** 1971. Phospholipids of *Listeria monocytogenes*. Biochim. Biophys. Acta **239:**428–442.
30. **Liu, W., and J. Hansen.** 1990. Some chemical and physical properties of nisin, a small-protein antibiotic produced by *Lactococcus lactis*. Appl. Environ. Microbiol. **56:**2551–2558.
31. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
32. **Maisnier-Patin, S., and J. Richard.** 1996. Cell wall changes in nisin-resistant variants of *Listeria innocua* grown in the presence of high nisin concentrations. FEMS Microbiol. Lett. **140:**29–35.
33. **Martin, I., J.-M. Ruysschaert, D. Sanders, and C. J. Giffard.** 1996. Interaction of nisin with membranes revealed by fluorescence quenching of an introduced tryptophan. Eur. J. Biochem. **239:**156–164.
34. **Mastronicolis, S. K., J. B. German, and G. M. Smith.** 1996. Diversity of the polar lipids of the food-borne pathogen *Listeria monocytogenes*. Lipids **31:** 635–640.
35. **Mazzotta, A. S., and T. J. Montville.** 1997. Nisin induces changes in membrane fatty acid composition of *Listeria monocytogenes* nisin-resistant strains at 10°C and 30°C. J. Appl. Microbiol. **82:**32–38.
36. **Ming, X., and M. A. Daeschel.** 1993. Nisin resistance of foodborne bacteria and the specific responses of *Listeria monocytogenes* Scott A. J. Food Prot. **56:**944–948.
37. **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.
38. **Mulders, J. W. M., I. J. Boerrigter, H. S. Rollema, R. J. Siezen, and W. M. de Vos.** 1991. Identification and characterization of the lantibiotic nisin Z, a natural nisin variant. Eur. J. Biochem. **201:**581–584.
39. **O'Leary, W. M., and S. G. Wilkinson.** 1988. Gram-positive bacteria, p. 117–201. *In* C. Ratledge and S. G. Wilkinson (ed.), Microbial lipids, vol. 1. Academic Press, Ltd., London, United Kingdom.
40. **Püttmann, M., N. Ade, and H. Hof.** 1993. Dependence of fatty acid composition of *Listeria* spp. on growth temperature. Res. Microbiol. **144:**279–283.
41. **Raetz, C. H. R.** 1986. Molecular genetics of membrane phospholipid synthesis in *Escherichia coli*. Annu. Rev. Genet. **42:**614–659.
42. **Russell, N. J.** 1984. Mechanisms of thermal adaptation in bacteria: blueprints for survival. Trends Biochem. Sci. **9:**108–112.
43. **Van't Hof, R., W. van Klompenburg, M. Pilon, A. Kozubek, G. de Korte-Kool, R. A. Demel, P. J. Weisbeek, and B. de Kruijff.** 1993. The transit sequence mediates the specific interaction of the precursor of ferredoxin with chloroplast envelope membrane lipids. J. Biol. Chem. **266:**4037–4042.