Nisin Resistance in *Listeria monocytogenes* ATCC 700302 Is a Complex Phenotype†

ALLISON D. CRANDALL‡ AND THOMAS J. MONTVILLE*

Department of Food Science, Cook College, New Jersey Agricultural Experiment Station, Rutgers, The State University of New Jersey, New Brunswick, New Jersey 08901-8520

Received 24 July 1997/Accepted 24 October 1997

Nisin resistance in *Listeria monocytogenes* ATCC 700302 is a complex phenotype involving alterations in both the cytoplasmic membrane and the cell wall and a requirement for divalent cations. In addition to a lower ratio of C16 to C18 fatty acids than in the wild-type strain (A. S. Mazzotta and T. J. Montville, J. Appl. Microbiol. 82: 32–38, 1997), this nisin-resistant (Nisr) strain contained significantly more zwitterionic phosphatidylethanolamine and less anionic phosphatidylglycerol and cardiolipin. The extraction of cardiolipin was enhanced by a penicillin-lysozyme step to disrupt the cell wall. This study is the first to quantify the phosphatidylethanolamine component of the *L. monocytogenes* cytoplasmic membrane. While these cytoplasmic membrane changes were induced by nisin, the Nisr strain also showed altered sensitivities to cell wall-acting compounds, even when grown in the absence of nisin, suggesting a constitutive alteration in the strain’s cell wall. A model which integrates the roles of the cell membrane, cell wall, and divalent cations is presented. Finally, nisin resistance in *L. monocytogenes* ATCC 700302 conferred cross-resistance to the class IIa bacteriocin pediocin PA-1 and the class IV leuconisin S.

*L. monocytogenes*, the causative agent of listeriosis, has resulted in numerous major food-borne outbreaks worldwide (16). The ability of *L. monocytogenes* to grow at temperatures ranging from 1 to 45°C (19, 41, 42), its high tolerance for salt (11), and its ability to initiate growth at a relatively low pH (19) make this pathogen particularly difficult to control in food. A novel approach to controlling *L. monocytogenes* in food is the use of antimicrobial bacteriocins from lactic acid bacteria (36). Nisin, a lantibion-containing peptide produced by certain strains of *Lactococcus lactis* (26) with antimicrobial activity against *L. monocytogenes* (2–4, 25), is a bacteriocin with many “generally recognized as safe” applications in the United States (12–14).

Nisin’s action stems from the disruption of the cell’s cytoplasmic membrane, as evidenced by the rapid efflux of small molecules from both whole cells and liposomes (1, 3, 18, 39, 44). As a result, nisin depletes the proton motive force (PMF) of sensitive cells and artificial liposomes (1, 3, 18, 39, 44). Anionic phospholipids play an important role in nisin’s interaction with membranes (9, 10, 30). On binding to anionic phospholipids, nisin causes a local perturbation of the lipid bilayer (10), followed by electrical potential (Δψ) or pH gradient (ΔpH)-enhanced insertion into the membrane to form a wedge-like pore (35).

Nisin’s efficacy as an antilisterial agent would be compromised by the emergence of nisin-resistant *L. monocytogenes* in a food-processing situation. The generation of nisin-resistant (Nisr) *L. monocytogenes* mutants in the laboratory is easily achieved by exposure to high concentrations of nisin (21, 32, 33). Given the role of the cytoplasmic membrane in nisin’s mechanism of action, several researchers have examined membrane compositional changes to explain nisin resistance in *L. monocytogenes*. Resistance has been correlated with both an altered fatty acid composition (32, 33) and an altered phospholipid composition (34). When grown in the presence of nisin, the Nisr strain (32) investigated in this study contains a lower ratio of C16 to C18 fatty acids than the wild-type or Nisr strain grown in the absence of nisin. In addition to membrane compositional changes, the cell wall may also be involved in nisin resistance in *L. monocytogenes* (8).

The objectives of this research were to investigate the roles of both the cytoplasmic membrane and the cell wall in a single nisin-resistant strain of *L. monocytogenes*, to investigate whether nisin resistance results in intrinsic resistance to other antimicrobials, and to develop a model of nisin’s interaction with nisin-resistant *L. monocytogenes*.

**MATERIALS AND METHODS**

**Bacterial cultures, growth media, and nisin stock preparations.** All bacterial stock cultures were maintained in their appropriate broth containing 20% glycerol at −80°C. *L. monocytogenes* Scott A was cultured in Trypticase soy broth without dextrose (BBL Microbiology Systems, Cockeysville, Md.), supplemented with 0.6% yeast extract (Difco Laboratories, Detroit, Mich.) and 0.5% glucose (Fisher Scientific Co., Fair Lawn, N.J.) (TSBYEG). The nisin-resistant strain of *L. monocytogenes* Scott A was isolated at 30°C after the wild-type strain was plated on TSBYE agar plates containing 1,000 IU of nisin preparation per ml (32). This Nisr strain was recently deposited in the American Type Culture Collection with accession no. ATCC 700302. The Nisr strain was maintained and cultured in TSBYE agar containing 1,000 IU of nisin per ml of *L. lactis* ATCC 11454, *L. lactis* ATCC 21053, *Leuconostoc parae mesenteroides* OX (28), and *Pediococcus acidilactici* PACI.0 (provided by J. L. Swezey, USDA Agricultural Research Service Midwest Area, Peoria, Ill.) were cultured in *Lactobacillus* MRS (Difco) broth. Working cultures were maintained at 4°C on agar slants solidified with 1.5% Bacto-Agar (Difco). All cultures were grown at 30°C. TSBYE diffusion agar contained 0.1% Tween 20 and was solidified with 1.5% Noble agar (Difco).

Nisin stock solutions were prepared from either pure nisin (a gift of Aplin and Barrett, Trowbridge, England) or nisin preparation (Sigma, St. Louis, Mo., or Aplin and Barrett Ltd.), as noted, in 0.2 N HCl-0.75% NaCl (nisin diluent) and autoclaved at 15 lb/in² for 15 min.
Identification and quantification of membrane phospholipids. Overnight cultures of wild-type cells and of Nisr cells grown in the presence of 1,000 IU of nisin preparation per ml were centrifuged; the cell pellets were resuspended in TSBYE containing 20% (vol/vol) sucrose, 0.2% (vol/vol) MgSO4 8 mg of l-lysine (Sigma) per ml, 1,000 U of penicillin G (Sigma) per ml and incubated for another 2 h at 30°C. The lysosome-penicillin treatment disrupted the cell wall, a step necessary for the complete extraction of cardiolipin from stationary-phase gram-positive bacteria (15). The cells were then pelleted again and the lipids were extracted as previously described (4).

The dried lipid extract was resuspended in chloroform and separated by high-performance thin-layer chromatography (HPTLC) (23) on Silica Gel 60 HPTLC plates with concentrating zones (E. Merck, Darmstadt, Germany). For two-dimensional analysis, the plates were developed first in chloroform-methanol-water (50:25:6, vol/vol) and then in chloroform-methanol-acetic acid-water (50:25:6, vol/vol). For one-dimensional analysis, the plates were developed with chloroform-methanol-acetic acid-water (50:25:6, vol/vol). Lipid spots were visualized by staining with iodine vapors. Phosphatidylglycerol and cardiolipin were identified on the basis of their relative mobilities compared with those of authentic standards, as well as by reaction with the specific detection reagents ninhydrin, Dragendorff, and molybdenum blue. The spot identified as phosphatidylyethanolamine stained ninhydrin positive, Dragendorff negative, and molybdenum blue positive. In one-chamber of the thin-layer chromatogram of a spiral replay (TLC), it migrated similarly to the amine-containing phospholipids phosphatidylethanolamine, phosphatidylethanolamine, and phosphatidylmonomethylethanolamine. This spot was identified as phosphatidylethanolamine following extraction from a one-dimensional silica gel plate, acid hydrolysis to yield the free phospholipid head group base, and comparison to similarly prepared standards separated on one-dimensional silica gel plate, acid hydrolysis to yield the free phospholipid head group base, and comparison to similarly prepared standards separated on one-dimensional silica gel plate, acid hydrolysis to yield the free phospholipid head group base, and comparison to similarly prepared standards separated on one-dimensional silica gel plate, acid hydrolysis to yield the free phospholipid head group base, and comparison to similarly prepared standards separated on one-dimensional silica gel plate, acid hydrolysis to yield the free phospholipid head group base, and comparison to similarly prepared standards separated on one-dimensional silica gel plate, acid hydrolysis to yield the free phospholipid head group base, and comparison to similarly 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Nisr cells grown in the absence of nisin for any of the phospholipids or between any of the strains for an unidentified amine-containing phospholipid and two other minor phospholipids were found.

Lysozyme sensitivity. In the absence of lysozyme, the wild-type and Nisr strains exhibited similar growth characteristics (Fig. 1), attaining a final optical density at 660 nm of approximately 0.5. In the presence of 4 mg of lysozyme per ml, the Nisr strain reached a higher final optical density of approximately 0.4, compared to 0.25 for the wild-type strain.

MIC determinations. The MICs of all the antibiotics tested for Nisr cells grown in the absence of nisin (data not shown) were not significantly different \((P < 0.05)\) from those for the Nisr strain grown in the presence of 1,000 IU of nisin preparation per ml (Table 2). The MIC of nisin for the Nisr strain was 1,423 ± 310 IU/ml, compared to 240 ± 61 IU/ml for the wild-type strain (means ± standard deviations). The MICs of benzylpenicillin and ampicillin for the Nisr strain were approximately 10-fold lower than those for the wild-type strain (significant at \(P < 0.001\)). The Nisr strain was also slightly more sensitive to cycloserine (significant at \(P < 0.001\)). In addition, small but significant \((P < 0.001)\) increases in the MICs of gramicidin S and gentamicin for the Nisr strain were observed. No significant differences in the MICs of any of the other antibiotics tested were found.

Transmission EM. No significant morphological differences between wild-type and Nisr cells were observed by transmission EM at \(×70,000\) magnification. Wild-type and Nisr cells contained cell walls of comparable thicknesses (approximately 360 nm).

PMF measurements. The PMFs of wild-type and Nisr L. monocytogenes were measured both in the absence and in the presence of nisin. Basal PMFs of \(-122 ± 11\) and \(-117 ± 13\) mV (means ± standard deviations) were measured for the wild-type and Nisr strains, respectively (Fig. 2).

TABLE 2. MICs of antimicrobials for wild-type L. monocytogenes grown in the absence of nisin and Nisr L. monocytogenes grown in the presence of nisin

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>Site of action</th>
<th>Wild type</th>
<th>Nisin resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzylopenicillin</td>
<td>Cell wall synthesis</td>
<td>0.26 ± 0.04A</td>
<td>0.03 ± 0.00B</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>Cell wall synthesis</td>
<td>0.33 ± 0.08A</td>
<td>0.05 ± 0.00B</td>
</tr>
<tr>
<td>Cycloserine</td>
<td>Cell wall synthesis</td>
<td>26.18 ± 4.01A</td>
<td>14.81 ± 2.47B</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>Cell wall synthesis</td>
<td>0.63 ± 0.11A</td>
<td>0.59 ± 0.11A</td>
</tr>
<tr>
<td>Gramicidin S</td>
<td>Cytoplasmic membrane</td>
<td>2.07 ± 0.25A</td>
<td>3.27 ± 0.64B</td>
</tr>
<tr>
<td>Nigericin</td>
<td>Cytoplasmic membrane</td>
<td>0.09 ± 0.02A</td>
<td>0.09 ± 0.01A</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>Protein synthesis (30S)</td>
<td>2.79 ± 0.30A</td>
<td>6.38 ± 1.26B</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>Protein synthesis (30S)</td>
<td>1.31 ± 0.57A</td>
<td>0.98 ± 0.30A</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>Protein synthesis (30S)</td>
<td>10.89 ± 1.33A</td>
<td>15.38 ± 2.68A</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>Protein synthesis (50S)</td>
<td>0.39 ± 0.07A</td>
<td>0.39 ± 0.10A</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>Protein synthesis (50S)</td>
<td>3.57 ± 0.77A</td>
<td>3.27 ± 0.98A</td>
</tr>
</tbody>
</table>

\(^{a}\) Values are means ± standard deviations. Different letters for the wild-type and Nisr strains for a given antimicrobial indicate statistically significant differences \((P < 0.001)\).
of different divalent cations was assessed. The viability of Nis\(^r\) cells suspended in MES buffer and treated with 60 IU of nisin per ml was reduced by 2.5 log cycles (Fig. 3). Inclusion of either 10 mM MgSO\(_4\), MgCl\(_2\), CaCl\(_2\), MnSO\(_4\), or BaCl\(_2\) reduced the lethality caused by nisin to an approximately 1-log reduction or less. In the absence of nisin, the divalent cations had no effect on cell viability. This effect was attributed to the divalent cation, as opposed to the anion, on the basis of the similar results obtained with MgSO\(_4\) and MgCl\(_2\) and the lack of effect of KCl and NaCl. The effect was confirmed to be due to the divalent cation by experiments involving EDTA, a chelator of divalent cations. Inclusion of 20 mM EDTA in any of the systems containing divalent cations increased the lethality caused by nisin to a 3- to 4-log reduction. In a parallel experiment with wild-type cells, nisin was equally lethal regardless of the presence or absence of divalent cations (data not shown). The metal salts and EDTA, either alone or in combination, had no effect on the viability of either strain.

A concentration dependence of the divalent cation magnesium on the ability of the Nis\(^r\) strain to resist nisin was demonstrated. Nisin, in the absence of MgSO\(_4\), caused an approximately 4.5-log reduction in both wild-type and Nis\(^r\) cell numbers (Fig. 4). For the wild-type strain, the log reduction caused by nisin remained constant at approximately 4, even in the presence of concentrations of MgSO\(_4\) up to 100 mM. With the Nis\(^r\) strain, as the concentration of MgSO\(_4\) was increased, the lethality caused by nisin decreased to approximately 1.5 log at 80 mM MgSO\(_4\).

**Determination of bacteriocin cross-resistance.** The sensitivity of the Nis\(^r\) strain to other lactic acid bacteria bacteriocins was assessed. Neither the wild-type nor the Nis\(^r\) strain was inhibited by *L. lactis* ATCC 21053, the control strain which does not produce bacteriocin (Table 3). As expected, the Nis\(^r\) strain was not inhibited by *L. lactis* ATCC 11454, the nisin-producing strain. The Nis\(^r\) strain was less sensitive to pediocin PA-1 and leuconocin S than the wild-type strain.

**DISCUSSION**

Nisin resistance in *L. monocytogenes* has been correlated with altered cytoplasmic membrane fatty acids (32, 33) and phospholipid compositions (34) and with alterations in the cell wall (8). This study identifies all of these changes, in addition to a requirement for divalent cations, in a single nisin-resistant
strain, demonstrating that resistance to nisin in *L. monocytogenes* occurs via a complex mechanism.

Alterations in the cytoplasmic membrane composition of nisin-resistant *L. monocytogenes* were first reported by Ming and Daeschel (33, 34). Their nisin-resistant mutant contained more straight-chain and fewer branched-chain fatty acids (33) and less phosphatidylglycerol and cardiolipin (34) than the wild type. Similar changes in membrane fatty acid composition, including increased long-chain fatty acids, decreased short-chain acids, and a lower C_{16}/C_{18} ratio, were found for Nisr *L. monocytogenes* ATCC 700302 (32). Both laboratories concluded that the observed fatty acid composition changes were consistent with a less fluid cytoplasmic membrane and that this increase in rigidity might prevent nisin from inserting into the membrane. In addition, we report here that the Nisr strain ATCC 700302 contained more of the zwitterionic phospholipid phosphatidylethanolamine and less of the anionic phospholipids phosphatidylglycerol and cardiolipin than the wild-type cells. A major difference between the Nisr strain ATCC 700302 and the nisin-resistant strain studied by Ming and Daeschel (34) is that phosphatidylethanolamine was a major component of the Nisr ATCC 700302 strain. While this study confirmed previous reports that the major phospholipids in *L. monocytogenes* are phosphatidylglycerol and cardiolipin (27, 31), it is the first to identify and quantify the phosphatidylethanolamine component of the cytoplasmic membrane of *L. monocytogenes*. Given the role that anionic phospholipids play in nisin’s interaction with membranes (10, 18, 30), a decrease in the net negative charge of the lipid bilayer might hinder nisin’s ability to bind and interact with the membrane.

Both the fatty acid composition change (32) and the alteration in membrane phospholipids were observed only when Nisr *L. monocytogenes* ATCC 700302 was grown in the presence of nisin, indicating that nisin induced these changes. This suggested that the cytoplasmic membrane alterations might not be the cell’s primary defense against nisin. We therefore looked for changes in the cell wall of the Nisr strain by evaluating the strain’s sensitivity to cell wall-acting compounds. The Nisr strain was more resistant than the wild type to lysozyme, which catalyzes the hydrolysis of the β-1,4 glycosidic bond between N-acetylmuramylglucosamine and N-acetylmuramylglucosamine of cell wall peptidoglycan, and more sensitive to the cell wall-acting antibiotics benzylpenicillin and ampicillin, which block the cross-linking reaction of peptidoglycan synthesis. These altered sensitivities suggest compositional changes in the cell wall of the Nisr strain. The nature of these compositional changes remains to be determined. In addition, small but significant (*P* < 0.001) increases in the MICs of gramicidin S and gentamicin for the Nisr strain were observed. The sites of action of gramicidin S and gentamicin are the cytoplasmic membrane and protein synthesis, respectively. Given that no other changes in sensitivities to other membrane-acting or protein synthesis-disrupting compounds were observed, the increase in resistance to gramicidin S and gentamicin might result from an alteration in the cell wall which prevents these compounds from reaching their targets. The altered sensitivities to lysozyme and the penicillins were observed even when the Nisr strain was cultured in the absence of nisin. Therefore, unlike the fatty acid composition change, the cell wall alterations are constitutive. No gross morphological changes in the cell wall of the Nisr strain were apparent by EM. The cell wall has also been implicated in nisin-resistant *Listeria innocua*, in which altered sensitivities to cell wall-acting antibiotics and enzymes and a thickened cell wall were observed (29). In addition, removal of the cell wall from nisin-resistant *L. monocytogenes* F6861 resulted in the loss of nisin resistance, suggesting that differences in the cell wall were responsible for resistance in this strain (7).

Next, to determine whether nisin causes significant membrane disruption in the Nisr strain, the cell’s PMF was measured. In the absence of nisin, the wild-type and Nisr strains of *L. monocytogenes* maintained similar basal PMFs of around −120 mV, indicating that the mutation to nisin resistance did not affect the Nisr strain’s ability to generate and maintain a PMF. Addition of nisin at 60 IU/ml to wild-type cells caused a 3-log reduction in cell viability and depleted the PMF by 88%. In the Nisr strain, nisin at 60 IU/ml reduced the PMF by only 21%. While this PMF reduction was statistically significant, the remaining PMF was still within the range required to support bacterial growth (22). The reduction of PMF in the Nisr strain indicates that some membrane disruption occurred in this strain, but this decrease was not sufficient to cause significant lethality.

In addition to changes in the cytoplasmic membrane and cell wall, the Nisr strain of *L. monocytogenes* required divalent cations to resist the inhibitory effect of nisin. The effect was dependent on the concentration of divalent cations. Abe et al. (1) found that di- and trivalent cations (Mg^{2+}, Ca^{2+}, and Gd^{3+}) decreased the nisin Z-induced rate of K^- efflux from whole cells of *L. monocytogenes* Scott A. They suggested that di- and trivalent cations might inhibit the electrostatic interactions between the positive charges on the nisin molecule and negatively charged phospholipid head groups. Alternatively or additionally, the neutralization of the negative head group charges may induce a condensation of these phospholipids, resulting in a more rigid membrane (1). In our study, divalent cations did not prevent nisin from killing wild-type cells. As for the role of divalent cations in protecting Nisr *L. monocytogenes* from nisin, it seems unlikely that the cations are interfering only with the electrostatic binding of nisin to the anionic phos-
pholipids. If this were simply the case, divalent cations would also be expected to prevent binding to and subsequent killing of wild-type cells. However, Nis' cells also have reduced amounts of the anionic phospholipids phosphatidylglycerol and cardiolipin in their membrane, as well as an altered fatty acid composition consistent with a more rigid membrane. These two membrane composition changes alone were still insufficient to prevent nisin from interacting with the Nis' cells in the absence of divalent cations. Perhaps divalent cations are required to sufficiently stabilize the altered Nis' cell's cytoplasmic membrane against disruption by nisin. This stabilization might involve interfering with nisin's binding or a specific interaction of the cations with membrane components or some combination of the two. Alternatively, the divalent cations may also play a role in the altered cell wall of the Nis' strain. Divalent cations interact extensively with cell wall components, especially the negatively charged teichoic acids. Perhaps the change in the cell wall of the Nis' strain requires additional divalent cations to either stabilize extra negative charge or prevent nisin from binding to anionic sites.

A model illustrating nisin's interaction with wild-type *L. monocytogenes* and how this interaction might be disrupted in the Nis' strain is presented in Fig. 5. With wild-type cells, nisin passes through the cell wall; binds to the cytoplasmic membrane, probably via electrostatic interactions with the anionic phospholipids phosphatidylglycerol and cardiolipin; and disrupts the membrane through the formation of pores. In the Nis' strain, cell wall alterations may prevent nisin from interacting with the cytoplasmic membrane. Nisin which does reach the membrane interacts with it to induce the changes in fatty acid and phospholipid composition. The bound nisin probably forms a limited number of pores, since the PMF of the Nis' strain was partially depleted by nisin. These pores, however, are not sufficient to cause significant lethality. Nisin's ability to bind to the cytoplasmic membrane of the Nis' strain may be hindered by the decrease in net negative charge of the membrane surface, and its ability to insert may be hampered by a decrease in membrane fluidity. Divalent cations, which are required by the Nis' strain to resist the effects of nisin, may play a role in stabilizing the cell wall and/or the cytoplasmic membrane or may prevent nisin from binding to the cell wall and/or the membrane. This model is consistent with data from a variety of sources and provides a conceptual framework for more-detailed studies of various aspects of the Nis' phenotype.

Finally, the Nis' strain of *L. monocytogenes* was also cross-resistant to the class IIa bacteriocin pediocin PA-1 and the class IV leuconocin S. Pediocin PA-1 is a 44-amino-acid protein whose sequence has been determined (24) and modeled into a three-dimensional structure (5) which predicts that initial binding to membranes is through electrostatic interactions. Leuconocin S is a small (molecular weight, <10,000) glycoprotein (28). Both leuconocin S and pediocin PA-1, like nisin, act against *L. monocytogenes* by depleting the PMF (4). *L. monocytogenes* mutants resistant to mesentericin 52, curvatin 13, and plantaricin were each also cross-resistant to the other bacteriocins (38). In addition, piscicolin 126-resistant mutants of *L. monocytogenes* which emerged in cheese made from milk containing the bacteriocin were also resistant to pediocin P02 (43). These reports of cross-resistance indicate that the use of multiple bacteriocins to achieve greater antibacterial efficacy (20) might not be feasible. The development of resistance to one of the bacteriocins in the combination might render the organism resistant to the others.

**ACKNOWLEDGMENTS**

Research in our laboratory and preparation of the manuscript were supported by state appropriations, U.S. Hatch Act Funds, and U.S. Department of Agriculture CSRS NRI Food Safety Program (no. 94-52100-5099). We sincerely thank Peter Cooke for the EM work and George Carman, Richard Ludescher, Karen Schaich, and Judith Storch for helpful discussion and comments.

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Nisin Resistance in Listeria Monocytogenes

Vol. 64, 1998


