Sensitivity and Resistance of *Listeria monocytogenes* ATCC 19115, Scott A, and UAL500 to Nisin

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(Received for publication April 25, 1991)

ABSTRACT

Listeria monocytogenes ATCC 19115, Scott A, and UAL500 were evaluated for sensitivity to nisin (0 to 50 μg/ml) using a direct plating method. Nisin (10 μg/ml) decreased an initial population of *L. monocytogenes* (10^9 CFU per ml) by 6- to 7-log cycles. Sensitivity to nisin was enhanced by addition of 2% NaCl or by reduction of the medium pH from 6.5 to 5.5 with either hydrochloric or lactic acid. Mutants resistant to 50 μg/ml nisin were detected at frequencies of 10^{-6} to 10^{-8}. Nisin-resistant *L. monocytogenes* mutants should be expected to arise when nisin is used as an antimicrobial in food systems.

Ubiquitous distribution in the environment (*6*), ability to grow at refrigerated temperatures (*41*), ability to initiate growth at relatively low pH (*9,17*), and tolerance to high levels of NaCl (*36*) make *Listeria monocytogenes* a difficult pathogen to control in food. Fermented foods traditionally have been considered to be pathogen free due to various combinations of these inhibitory factors. However, *L. monocytogenes* has been shown to grow and survive the manufacture and storage of fermented foods made from raw materials contaminated with the organism. These products have included fermented meats (*4,18,25*) and a wide variety of cheeses and fermented milks (for a review see 33). Several authors have suggested the use of bacteriocin-producing starter cultures (specifically lactic acid bacteria) or their bacteriocins as a safeguard against *L. monocytogenes* in fermented and nonfermented foods (*4,19,32,34*).

Sensitivity of *L. monocytogenes* to nisin has been demonstrated (*3,8,19,31,38*). However, published reports quantifying the inhibitory effect of nisin toward *L. monocytogenes* have been relatively scant. In an initial study, it was noted that nisin-producing *Lactococcus lactis* subsp. lactis could inhibit *L. monocytogenes* by a deferred antagonism method (*19*). However, supernatant extracts were not inhibitory in well diffusion assays, and it was suggested that inadequate nisin concentrations were attained in broth. Using an inoculum of 10^5 CFU/ml in broth, Mohamed et al. (*31*) obtained complete inhibition of *L. monocytogenes* at 32 or 256 IU nisin per ml (ca. 1 or 7 μg purified nisin), depending upon the strain evaluated. At lower nisin concentrations, the population decreased initially to below detectable levels; however, upon further incubation, maximum bacterial numbers were achieved. Using similar methodology, Somers and Taylor (*11*) reported resistance to 500 IU nisin per ml (12.5 μg/ml) for several *L. monocytogenes* strains. *L. monocytogenes* Scott A was particularly resistant and, after a delay of a few days, was capable of growth at levels of 2,000 IU nisin per ml (ca. 50 μg/ml).

With classification of nisin as a GRAS substance and Food and Drug Administration approval for its use in pasteurized cheese spreads (*13*), we felt it essential that the effect of nisin on *L. monocytogenes* be clarified. The present study was conducted to determine the inhibitory effect of nisin on *L. monocytogenes* using direct plating methods, to determine whether nisin-resistant variants could be selected from a sensitive population, and to investigate the influence of traditional microbial inhibitors such as salt and pH on the effectiveness of nisin.

MATERIALS AND METHODS

Bacteria and culture conditions

Three *L. monocytogenes* strains, ATCC 19115 and clinical isolates Scott A and UAL500, were used to determine sensitivity to nisin. Two additional strains were used for controls in plasmid analysis. *L. monocytogenes* NCK157 (*29*) is an electro-transformant of strain F-5069 which contains the 4.4 kilobase pair plasmid GK12 (*27*). *L. monocytogenes* NCF-U2K3 is an isolate from a poultry processing plant which naturally carries a large molecular weight plasmid (ca. 60 kilobase pairs). Nisin-producing *L. lactis* subsp. *lactis* ATCC 11454 served as the nisin-resistant control. *L. lactis* subsp. *cremoris* ATCC 14365 or *L. lactis* subsp. *lactis* LM0230 were used as nisin-sensitive controls. *L. monocytogenes* strains were propagated in brain-heart infusion broth (BHI) at 37°C, while lactococci were cultured in M17 broth with 0.5% glucose at 30°C. When agar plates were required, 1.5% granulated agar was added to the broth media. Frozen stock cultures were streaked onto agar and an isolated colony was transferred to broth prior to use in each experiment. Media were obtained from Difco Laboratories (Detroit, MI) and were prepared following manufacturer's directions.

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Nisin sensitivity

The effect of nisin concentration on *L. monocytogenes* was determined essentially as described by Klaenhammer and Sanozky (*26*). Briefly, overnight cultures were inoculated (1%) into fresh broth, incubated 8 h (*L. monocytogenes*) or 6 h (lactococci), diluted in 10% BHI broth, and plated onto 20 ml BHI agar plates adjusted to pH 6.5 with 1 N HCl and supplemented with 0.1% Tween 20 (Magnus, Mabee and Reynard, Inc., New York) and purified nisin (0 to 50 μg/ml). For some experiments, the pH of BHI agar was reduced to pH 5.5 with 1 N HCl or 85% lactic acid (Fisher Scientific Co., Pittsburgh, PA) prior to autoclaving. Purified nisin (37 x 10^6 IU/g) was obtained from Aplin and Barrett, Ltd. (Trowbridge, England). A nisin stock solution (1.25 mg/ml), prepared in 0.02 N HCl and sterilized using a 0.22–μm low-protein-binding filter (Millex GV, Millipore Products Division, Bedford, MA), was used to adjust nisin levels in the sterile agar. Sodium chloride (Aldrich Chemical Co., Milwaukee, WI) was added to the agar at a level of 1, 2, or 3% for some experiments. BHI agar is formulated to contain 0.5% NaCl; therefore, final NaCl concentrations in the agar were 0.5 to 3.5%. CFU per milliliter were determined by spotting 25 μl of the appropriate dilutions made in 0.85% saline onto the surface of prepoured agar plates. Plates were incubated at 37°C for up to 4 d in sealed plastic bags.

Nisin stability

The stability of nisin in BHI agar at pH 6.5 and 5.5 was monitored. Uninoculated agar plates, containing various concentrations of nisin, were incubated under the test conditions of the experiment (37°C, 4 d). MICs were determined for *L. lactis* subsp. *lactis* LM0230 and *L. monocytogenes* Scott A on day 0 and day 4. An overnight culture was diluted 10-fold in 0.85% saline, and 2 μl spots were applied to the surface of the plates. Plates were incubated at the appropriate temperature and were scored for growth after 24 h. The MIC was defined as the lowest level of nisin which inhibited growth.

Plasmid analysis

Plasmids were extracted using a modified procedure of Anderson and McKay (*2*). Lysozyme concentration was increased to 15 mg/ml, and incubation was extended to 30 min at 37°C. Two phenol extractions were performed with the addition of 300 μl chloroform. DNA was allowed to precipitate in isopropanol at -20°C for 4 h. Plasmids were separated by gel electrophoresis using 0.8% agarose and a current of 3 to 4 V/cm.

RESULTS

Three strains of *L. monocytogenes* were evaluated for sensitivity to nisin by plating an initial population of 10^9 CFU per ml on BHI agar (pH 6.5) with nisin (0 to 50 μg/ml). A biphasic survival curve was observed for *L. monocytogenes* strains (Fig. 1). The number of survivors of *L. monocytogenes* decreased 6- to 7-log cycles as nisin levels increased to 10 μg/ml. At nisin concentrations between 10 and 50 μg/ml, a surviving population of approximately 100 to 1,000 CFU per ml was detected. Nisin-producing *L. lactis* subsp. *lactis* ATCC 11454 was not inhibited in this concentration range. Survivors of the nisin-sensitive indicator, *L. lactis* subsp. *cremoris* ATCC 14365 were not detected at nisin levels of greater than 0.5 μg/ml (data not shown).

To determine whether survivors had escaped the effect of nisin or were nisin-resistant mutants, single colonies of *L. monocytogenes* Scott A and ATCC 19115 were selected

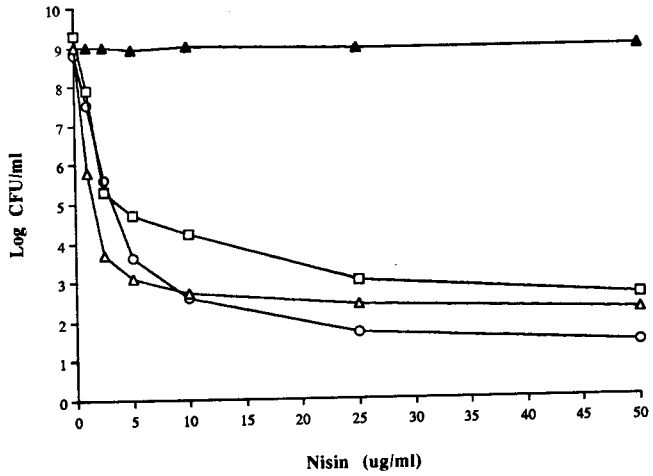

Figure 1. *Survival curves of* L. monocytogenes *Scott A (❑), ATCC 19115 (Δ), UAL500 (○), and nisin-resistant* L. lactis *subsp.* lactis *ATCC 11454 (▲), plated on BHI agar with 0.1% Tween 20 and various concentrations of purified nisin. Nisin-sensitive* L. lactis *subsp.* cremoris *ATCC 14365 was not detected at nisin levels of 0.5 μg/ml or greater.*

from plates containing 50 μg/ml nisin. These clones, designated LJH36 and LJH38, respectively, were subcultured in BHI broth and reevaluated for nisin susceptibility (Fig. 2). Both clones were more resistant to nisin than the parent strains, and each was only moderately inhibited at nisin levels of 50 μg/ml.

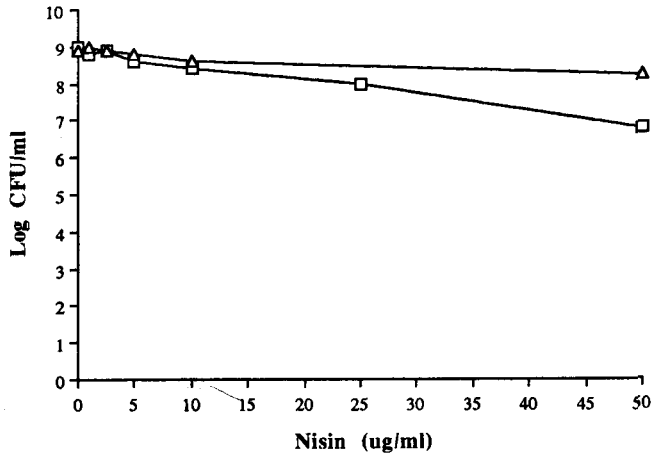

Figure 2. *Survival curves of nisin-resistant derivatives of* L. monocytogenes *Scott A (LJH36, ❑) and ATCC 19115 (LJH38, Δ) plated on BHI agar with 0.1% Tween 20 and various concentrations of purified nisin.*

Sodium chloride was added to BHI agar to give a final NaCl concentration of 2.5%. Increased NaCl levels in the medium did not inhibit any strain, with the exception of *L. lactis* subsp. *cremoris* ATCC 14365. Salt-tolerant *L. lactis* subsp. *lactis* LM0230 was substituted as the nisin-sensitive indicator strain in these experiments. The effectiveness of nisin appeared to be slightly enhanced by 2.5% NaCl at lower nisin concentrations (1 to 10 μg/ml). Little effect was noted for the subpopulation which grew at higher nisin concentrations (10 to 50 μg/ml) as shown for *L. monocytogenes* Scott A (Fig. 3). Results for other *L. monocytogenes* strains were essentially the same (data not shown).

To further investigate the effect of NaCl at lower nisin concentrations, levels of 0.5 to 3.5% NaCl were combined

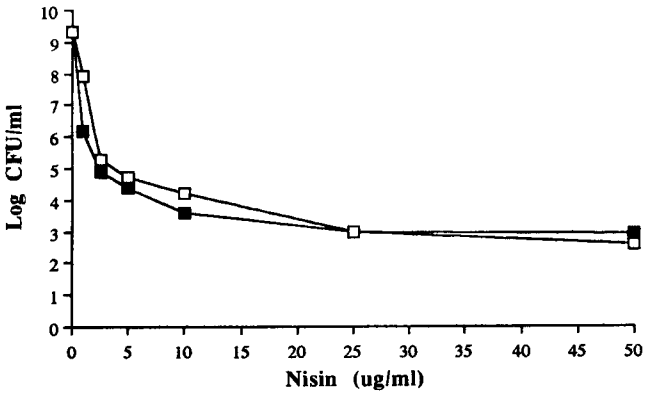

Figure 3. *Survival of* L. monocytogenes *Scott A in the presence of purified nisin and 0.5 (❑) or 2.5% (■) NaCl.*

with nisin concentrations of 0 to 2.5 μg/ml. Survival in the presence of nisin decreased as salt levels were increased, as illustrated for Scott A (Fig. 4). A final concentration of at least 2.5% NaCl was necessary before an effect was observed.

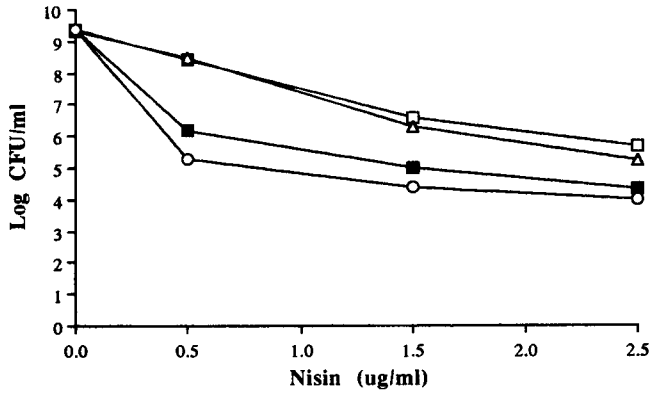

Figure 4. *Survival of* L. monocytogenes *Scott A in the presence of purified nisin and 0.5 (❑), 1.5 (Δ), 2.5 (■), and 3.5% (○) NaCl.*

The effect of pH on nisin activity was determined by reducing the medium pH from 6.5 to 5.5 with HCl or lactic acid (Table 1). Strain Scott A was chosen as representative for these experiments since the effect of nisin was similar for each of the *L. monocytogenes* strains in previous experiments. A lower pH enhanced the bactericidal effects of nisin. At 10 μg/ml nisin and pH 5.5, the population of *L. monocytogenes* Scott A was at the detection limits of the experiment (20 CFU per ml). This effect appeared to be pH dependent rather than acid dependent. Little difference was seen between HCl or lactic acid in the number of survivors at the lower pH. Increased effectiveness of nisin at pH 5.5 was not due to an increase in stability of the nisin molecule. Control experiments demonstrated that nisin was equally stable at both pH 6.5 and 5.5 under the conditions of this experiment. Growth of *L. monocytogenes* Scott A was slower at pH 5.5 in the absence of nisin, as demonstrated by slower colony development and smaller colony size. Bacterial counts in the absence of nisin were not lower at pH 5.5 than those at pH 6.5.

Plasmid DNA was not detected in any of the three nisin-sensitive or nine nisin-resistant strains tested (three shown, Fig. 5). Large and small molecular weight plasmids were detected in positive-control *L. monocytogenes* strains NCF-U2K3 and NCK157, respectively, demonstrating that the plasmid isolation technique was reliable.

TABLE 1. *Effect of medium pH on the sensitivity of* L. monocytogenes *Scott A to nisin.*

Purified nisin (μg/ml)	Survivors (log CFU/ml) pH 6.5	HCl pH 5.5	Lactate pH 5.5
0	9.1	9.2	9.2
1	7.9	6.2	6.8
2.5	5.8	3.4	3.2
5	4.6	2.4	2.1
10	4.0	1.3	<1.3
25	2.9	<1.3	<1.3
50	2.6	<1.3	<1.3

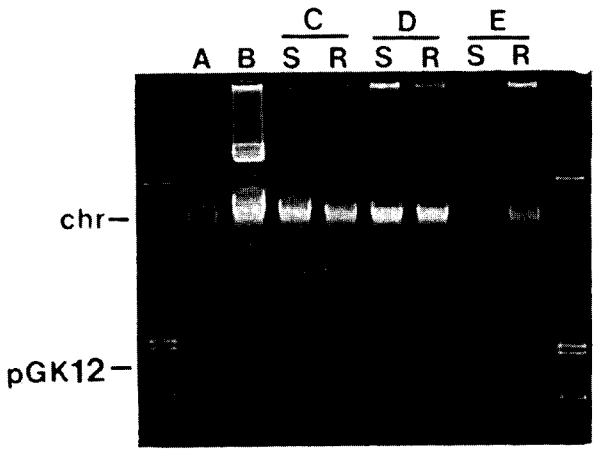

Figure 5. *Plasmid profiles of nisin-sensitive (S) and nisin-resistant (R)* L. monocytogenes *Scott A (C), ATCC 19115 (D), and UAL500 (E). Plasmid-containing control strains* L. monocytogenes *NCK157 (A) and NCF-U2K3 (B) are also shown. Outer lanes are* Escherichia coli *V517 molecular size plasmids (bands identified from top to bottom); 35, 5.1, 3.5, 3.0, 2.2, 1.7, 1.5, and 1.2 MDa. chr = chromosomal DNA.*

DISCUSSION

A direct plating method was employed to determine the sensitivity of *L. monocytogenes* to nisin. Nisin was effective in reducing the population of *L. monocytogenes* ATCC 19115, Scott A, and UAL500 by 6- to 7-log cycles at a level of 10 μg/ml. Addition of NaCl to the medium increased the bactericidal effect of nisin, particularly at concentrations of less than 10 μg/ml. A concentration of 2.5% NaCl in the medium was necessary before a significant effect was observed. *L. monocytogenes* fails to grow in heat-sterilized cabbage juice at NaCl levels greater than 5% (*5*) or in clarified cabbage juice at NaCl levels greater than or equal to 2.5% (*9*). Extended lag times and reduced acid production in clarified cabbage juice are observed in the presence of 1.5 and 2.0% NaCl but not at levels of 0.5 or 1.0% NaCl (*9*). These effects suggest that NaCl may simply make *L. monocytogenes* populations more susceptible to nisin by placing additional environmental stresses on the organism. However, more direct effects are also possible.

A decrease in the medium pH from 6.5 to 5.5 also increased the effectiveness of nisin. Mohamed et al. (*31*) observed an enhanced effect of nisin of pH 5.5 over that at pH 7.3 and attributed the effect to increased stability of the nisin molecule at the lower pH. At neutral or alkaline pH, nisin is both relatively insoluble and unstable (*22*), and it is possible that the effect observed by Mohamed et al. (*31*) was a combination of these factors. No difference was detected in nisin stability at pH 6.5 compared with pH 5.5 under the conditions of the present study.

Lactic and hydrochloric acids were similarly effective in reducing the number of survivors at a particular nisin concentration. Lactic acid has previously been shown to be more effective than HCl in inhibiting initiation of growth of *L. monocytogenes* (*10,12,39*).

Mutant strains of *L. monocytogenes* resistant to 50 μg/ml nisin were detected in our study at a frequency of 10^{-6} to 10^{-8}. This observation may explain previous reports of the growth of *L. monocytogenes* in broth at high levels of nisin after a lag of a few hours to a few days (*11,31*). In those studies, the nisin-sensitive subpopulation would have initially been eliminated, allowing a nisin-resistant population to become established upon extended incubation. However, in these cases, the sensitivity of initial and final cultures was not determined.

The development of acquired or spontaneous nisin resistance has been reported for *Streptococcus agalactiae* (*20*), *Staphylococcus aureus* (*7*), and *Clostridium butyricum* (*35*). Hirsch and Gringted (*21*) used spontaneous resistant mutants of *L. lactis* subsp. *cremoris* (formerly *Streptococcus cremoris*) to differentiate bacteriocins of different lactococci. The mechanism of spontaneous nisin resistance has never been determined.

Nisin resistance determinants have been linked to plasmid DNA in *L. lactis* subsp. *lactis* biovar *diacetylactis* (*30*) and in *L. lactis* subsp. *lactis* (*26,40*). Nisin resistance in the *L. monocytogenes* strains could not be explained by the presence of plasmid DNA. The gene from *L. lactis* subsp. *lactis* biovar *diacetylactis* has been subcloned (*14,37*) and sequenced (*15*), and encodes for a protein of 319 amino acids. The function of the protein is, as yet, unknown.

Currently, the only suggested protein involved in nisin resistance is an enzyme which destroys nisin activity. Nisinase has been reported in *Lactobacillus plantarum* (*28*), *L. lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris*, *Enterococcus faecalis*, betacoccus (*Leuconostoc* spp.; *16*), *S. aureus* (*7*), *Streptococcus salivarius* subsp. *thermophilus* (*1*), and several *Bacillus* spp. (*23*). Nisinase from *S. salivarius* subsp. *thermophilus* (*1*) and *Bacillus cereus* (*24*) was partially purified. The mechanism of resistance to nisin was not determined for *L. monocytogenes* in the current study. The possibility of a nisinase was not eliminated.

Nisin concentrations currently allowed in pasteurized cheese spreads (250 μg/ml; *13*) should be effective for control of *L. monocytogenes* at the population levels expected in food prepared under proper sanitary conditions. The use of nisin in combination with other food preservation techniques (addition of NaCl, decrease in pH) will be more effective in the control of *L. monocytogenes*. However, our observations demonstrated that resistant strains should be expected to arise when nisin is used as an antimicrobial in food, particularly when high levels of contamination are encountered. Further research into the mechanism of nisin resistance may shed insight into mode of action of nisin and allow development of strategies to minimize the occurrence of nisin-resistant populations. It is clear, however, that, despite its bactericidal effect, nisin cannot be solely relied upon to control *L. monocytogenes* in our food supply.

ACKNOWLEDGMENTS

We thank Drs. Cathy Donnelly, Peggy Foegeding, and Mike Stiles for providing the *L. monocytogenes* strains.

This investigation was supported in part by a research grant from The National Kraut Packers Association, Inc., St. Charles, IL.

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