APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 1995, p. 992–997 Vol. 61, No. 3
0099-2240/95/$04.00+0

Suppression of *Listeria monocytogenes* Colonization following Adsorption of Nisin onto Silica Surfaces

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Received 1 November 1994/Accepted 3 January 1995

Nisin is an antimicrobial peptide proven to be an effective inhibitor of gram-positive bacteria. It is known that nisin can adsorb to various surfaces and still retain much of its original activity (M. A. Daeschel, J. McGuire, and H. Al-Makhlafi, J. Food Prot. 55:731–735, 1992). In this study, nisin films were allowed to form on silanized silica surfaces and then exposed to medium containing *Listeria monocytogenes*. Representative areas were selected from each surface, and images of resident listeriae were obtained at 4-h intervals for 12 h. During this time, cells on surfaces that had been in contact with a high concentration of nisin (1.0 mg/ml) exhibited no signs of growth and many displayed evidence of cellular deterioration. Surfaces treated with a lower concentration of nisin (0.1 mg/ml) had a smaller degree of inhibition. In contrast, both protein-free surfaces and those with films of heat-inactivated nisin allowed attached *L. monocytogenes* cells to grow and reproduce. These studies, when repeated with a nisin-resistant strain of *L. monocytogenes*, resulted in no inhibition of growth on surfaces with adsorbed nisin. The bactericidal effect of adsorbed nisin was also studied with iodonitrotetrazolium violet, a tetrazolium salt, which is reduced to a red formazan crystal by viable bacteria. Crystals were visible in 95% of the cells adhered to control surfaces but were present in less than 20% of the cells on surfaces with adsorbed nisin. These data indicate that adsorbed nisin may have potential for use as a food grade antimicrobial agent on food contact surfaces.

Nisin is a polypeptide (molecular weight, 3,510) that is synthesized by *Lactococcus lactis* subsp. *lactis*. Since its discovery in the early 1900s, nisin has proven to be an effective inhibitor of gram-positive bacteria (22, 42). It is now approved for use in 57 countries around the world (25) and has been affirmed as generally recognized as safe in the United States (16). Nisin is nontoxic and nonantigenic to humans (18) and can be ingested in quantities of up to 3.3×10^7 U/kg of body weight with no adverse effects (2). It is inactivated by proteolytic enzymes in the digestive tract (10, 23, 27) and therefore is assumed to be safe for use as a food preservative.

Numerous studies have documented the efficacy of nisin for controlling spoilage bacteria in foods. Its most significant application, however, may be in the inhibition of pathogenic organisms in raw and processed foods. *Listeria monocytogenes* has been implicated in several outbreaks of foodborne illness (3, 17, 26). This pathogen is widely distributed in the environment and is of special concern since it can exist under diverse conditions. It is capable of growing in salt concentrations as high as 10% (45) and can tolerate a pH range of 5.0 to 9.0 (9, 45). Additionally, it is known to survive and grow at refrigeration temperatures (48).

L. monocytogenes has been isolated from all types of food-processing environments (11, 20) and is capable of adhering to a variety of food contact surfaces (24, 37). This can create problems for food processors, since attached microorganisms exhibit increased resistance to sanitizers and other antimicrobial agents (33, 34). Under favorable conditions, these adhered cells are able to proliferate (37). When this colonization is accompanied by extracellular polysaccharide production, a biofilm is created. Accumulation of biofilms results in a variety of problems for food processors, including increased fluid frictional resistance and decreased heat transfer efficiency (5). The most serious concern, however, is potential contamination of food products.

Nisin can inhibit the growth of *L. monocytogenes* (4, 21) and has proven to be an effective antimicrobial agent when incorporated into a food system (15, 28). As little as 100 U of nisin per ml in a pH 6.6 phosphate buffer (67 mM) can decrease a population of *L. monocytogenes* from 10^6 to 10^3 CFU/ml in 24 h (40). Since microbial contamination of food contact surfaces is an ongoing concern, the food industry has dedicated considerable resources toward finding a solution. A novel approach to this problem involves adsorption of an antimicrobial agent, such as nisin, onto food-processing surfaces.

There is an abundance of literature describing the interactions of proteins at interfaces; however, little is known about the adsorption of biologically active proteins such as nisin. Protein surfaces typically contain both hydrophilic and hydrophobic regions, as well as positively and negatively charged groups. This structural diversity contributes to the high surface activity of proteins and their increased capacity for adsorption. Once a protein has adsorbed to a surface, it is often thermodynamically favorable for the molecule to undergo some degree of structural rearrangement. The degree to which any protein changes its conformation when adsorbed depends not only on the protein but on the type of surface and surrounding medium as well. Even small variations in pH and ionic strength can alter the surface charge of a protein, thus influencing its conformation and subsequent behavior at an interface. One way of quantifying the unfolding that occurs after adsorption is by measuring the surfactant-mediated elutability of adsorbed protein (32). With this procedure, proteins that have undergone relatively large conformational changes show relatively high resistance to removal upon exposure to a surfactant.

Allowing nisin to adsorb to food contact surfaces may have the potential to prevent unwanted colonization of pathogenic

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organisms like *L. monocytogenes*, thereby contributing to a safer food supply. Earlier research has demonstrated that nisin can retain antimicrobial activity when in an adsorbed state (13). The purpose of this study was to quantify the mass and activity of adsorbed nisin on surfaces with different hydrophobicities and to correlate these results with the observed antimicrobial effect.

MATERIALS AND METHODS

Bacterial culture. *L. monocytogenes* Scott A was obtained from C. Donnelly, University of Vermont. Stable, nisin-resistant mutant strain R-2000 was derived from *L. monocytogenes* Scott A as described previously (39). This strain is resistant to a nisin activity of 2,000 U/ml in brain heart infusion broth (Difco Laboratories, Detroit, Mich.). Cultures were prepared for inoculation by subculturing into protein-free medium consisting of 21 g of RPMI 1640 (Sigma, St. Louis, Mo.) per liter and 20 g of Casamino Acids (Difco) per liter and supplemented with 10 g of glucose per liter. These cultures were grown at 25°C with transfers every 24 h for 2 days prior to the start of each experiment. Inoculation density was approximately 10^9 cells per ml as enumerated on brain heart infusion agar.

Preparation of hydrophobic surfaces. Polished silicon wafers (1-0-0 orientation; resistivity, 0.4 to 0.7 Ω/cm; phosphorus doped) were obtained from Wacker Siltronics (Portland, Oreg.). Each surface was circular and had an area of 1 cm^2. These wafers have proven to be ideal for electro-optical investigation of protein adsorption (30, 47) because of the optical flatness, smoothness, and specularity of their surfaces. Protein films that form on silica surfaces are the outcome of true adsorption events rather than simple entrapment caused by surface irregularities. Silica surfaces were chemically modified to mimic hydrophilic and hydrophobic properties of food contact material. The surfaces were treated with dichlorodimethylsilane (0.01 or 0.1%) in xylene to create two surface types with different hydrophobicities in accordance with the procedure of Krisdhasima et al. (30). This process was employed since bacterial adhesion is significantly influenced by the hydrophobicity of the surface (1, 44).

Adsorption protocol. Nisin (lots NP 26/2 and NP 72) was obtained from Aplin and Barrett (Dorset, United Kingdom) with an activity of 5×10^7 U/g. Nisin solutions were prepared in pH 7 phosphate buffer (0.01 M) by first dissolving nisin in a monobasic phosphate buffer (0.01 M) and then adding sufficient dibasic phosphate buffer (0.01 M) to produce a solution with a final pH of 7. For the control samples, nisin (1.0 mg/ml, pH 7) was inactivated by autoclaving at 121°C for 3 h. Inactivity was confirmed by an agar well diffusion bioassay with *Pediococcus pentosaceus* FBB-61-2 as the sensitive indicator strain (12).

To prepare a material with adsorbed nisin, silanized silica surfaces were immersed in solubilized nisin for 8 h at 25°C. Surfaces were rinsed in 20 ml of phosphate buffer to remove nonadsorbed nisin. Care was taken during all transfers to prevent the surfaces from drying. Once prepared, the surfaces could be tested for activity by bioassay (12) or used as a substrate for adhesion of *L. monocytogenes*.

Ellipsometric measurement of adsorbed mass of nisin. In this study, an L116 C automated ellipsometer (Gaertner Scientific Corp., Chicago, Ill.) was used to continuously measure the adsorbed mass of nisin films formed on silanized silica surfaces in situ. The adsorbed mass of nisin on each surface was calculated from film optical properties by a Lorentz-Lorenz relationship as described by Krisdhasima et al. (31). For this purpose, it is necessary to know the protein's specific volume and molecular weight/molar refractivity ratio. For nisin, the specific volume (0.818 cm^3/g) and molar weight/molar refractivity ratio (3.777 g-cm^3/mol) were estimated by the method of Pethig (41), with unusual amino acid residues treated as neutral rather than hydrophobic or hydrophilic.

Silanized silica surfaces were placed in phosphate buffer to obtain an initial bare-surface reading. The buffer was then carefully removed, a nisin solution was added, and the adsorption process was allowed to continue for 4 h. Loosely bound nisin molecules were removed by in situ rinsing with buffer before the final adsorbed-mass value was recorded.

Bioassay for nisin activity. Determination of activity levels for adsorbed nisin was based on a bioassay procedure (12) that was slightly modified to accommodate solid samples. Bioassay plates were prepared by autoclaving MRS medium (Difco) at 121°C for 15 min and then inoculating the tempered medium with 0.1% *P. pentosaceus* FBB 61-2 as the sensitive indicator strain. The medium was poured into petri plates to a depth of 5 mm and stored at 4°C until needed. The activity of adsorbed nisin was determined by placing each disk face down on the medium. Each bioassay plate included standards that were prepared by placing 10 μl of a known nisin concentration onto a silanized disk. All samples were tested in triplicate. Bioassay plates containing experimental samples were kept at 4°C for 24 h and then incubated at 37°C for outgrowth of the indicator. Nisin activity was quantified on each disk by measuring the width of the zone of inhibition. The $\log_{10}$ concentration of each nisin control (in units per milliliter) was plotted against the square of the corresponding zone width to obtain a line ($R > 0.98$) for calculating the nisin activities of the samples.

Imaging of attached *L. monocytogenes*. Images were obtained with a Cohu camera (Cohu Inc., San Diego, Calif.) mounted on an Epistar incident-light microscope (Cambridge Instruments). The incoming video signal was digitized to 8 bits of accuracy per pixel by a Visionplus-AT board (Imaging Technology Inc.) before storage in frame memory. The frame grabber's display logic then converted the pixels back into an analog format for video monitor display. Each stored image had a resolution of 640 by 480 pixels with a range of 256 gray values. Ten to twenty images (of 2,500 μm^2 each) were digitized from each surface. Cells were enumerated, and their lengths and areas were measured and averaged by Image-Pro Plus (Silver Spring, Md.) processing software including a descriptive statistics program.

To study the growth of adhered bacteria, surfaces with adsorbed nisin were transferred to a petri dish containing a 24-h culture of *L. monocytogenes*. Cells were allowed to adsorb to each surface for 3 h before being gently rinsed in phosphate buffer and prepared for microscopic image analysis. Wet surfaces were placed on a slide, covered with a coverslip, and scanned at a magnification of ×100 with an oil immersion lens until a representative field of adhered bacteria was located. An image of the chosen field was then routinely recorded for 12 h. Periodically, brain heart infusion broth was added dropwise to the edge of the coverslip to provide nutrients for the growing bacteria. Each surface type was tested in duplicate, and the areas of at least 15 representative cells per image were measured. The average change in cell area was converted into a percentage based on the initial size of each cell.

Cell viability. Iodonitrotetrazolium violet (INT; Sigma) was prepared as a 0.4% (wt/vol) solution with distilled water. This solution was added to an *L. monocytogenes* culture at a ratio of 1 ml of INT to 10 ml of culture and kept for 1 h at 25°C. During this time, the 2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyltetrazolium chloride was reduced to iodonitrotetrazolium formazan by the electron transport system of actively respiring cells (49). The optically dense formazan crystals were observed by bright-field microscopy. This provided a convenient means of differentiating viable cells (darkly stained) from nonviable bacteria that did not contain formazan crystals.

RESULTS

Ellipsometry studies enabled construction of an isotherm showing a general increase in adsorbed mass with increasing solution concentrations of nisin (Fig. 1). Values of adsorbed mass were reproducible at low nisin concentrations but became increasingly more difficult to replicate at higher concentrations. Most attempts to determine the adsorbed mass of nisin at 1.0 mg/ml failed to produce results, suggesting that nisin molecules at that concentration did not adsorb onto the surface in a simple monolayer. After each 4 h of nisin-surface contact, the surfaces were rinsed for 30 min in buffer and then a final adsorbed mass was recorded. Postrinse values were significantly lower, indicating that many nisin molecules were only loosely held and thus easily removed during rinsing. These observations were true for both surface types; however, adsorbed-mass values were generally higher on higher-hydrophobicity surfaces.

The activity of nisin at these interfaces was largely dependent on surface hydrophobicity. Low-hydrophobicity surfaces generally displayed more nisin activity than higher-hydrophobicity surfaces across a range of nisin concentrations (Fig. 2), despite the finding that nisin adsorbed in greater amounts on the more hydrophobic surfaces.

Surfaces with active nisin clearly inhibited cellular growth and most likely killed the listeriae which had adhered. This was confirmed when the cells were exposed to INT, a tetrazolium salt that can be used to detect actively respiring bacteria. The images in Fig. 3 show formazan crystals produced when INT was reduced by the electron transport chain of viable *L. monocytogenes* cells. Figure 3A shows cells adhered to a surface covered with inactivated nisin. The rapid INT uptake and uniform staining gave clear evidence that these bacteria were alive and actively respiring. However, in Fig. 3B, the cells that had adhered to a nisin-coated surface showed no evidence of INT uptake, indicating that they were no longer viable.

Surfaces were allowed to contact solutions with low and high nisin concentrations (0.1 and 1.0 mg/ml) to test the efficacy of adsorbed nisin against *L. monocytogenes*. The photomicrographs in Fig. 4 depict the cellular integrity of adhered *L. monocytogenes* cells on a hydrophobic surface exposed to 1.0

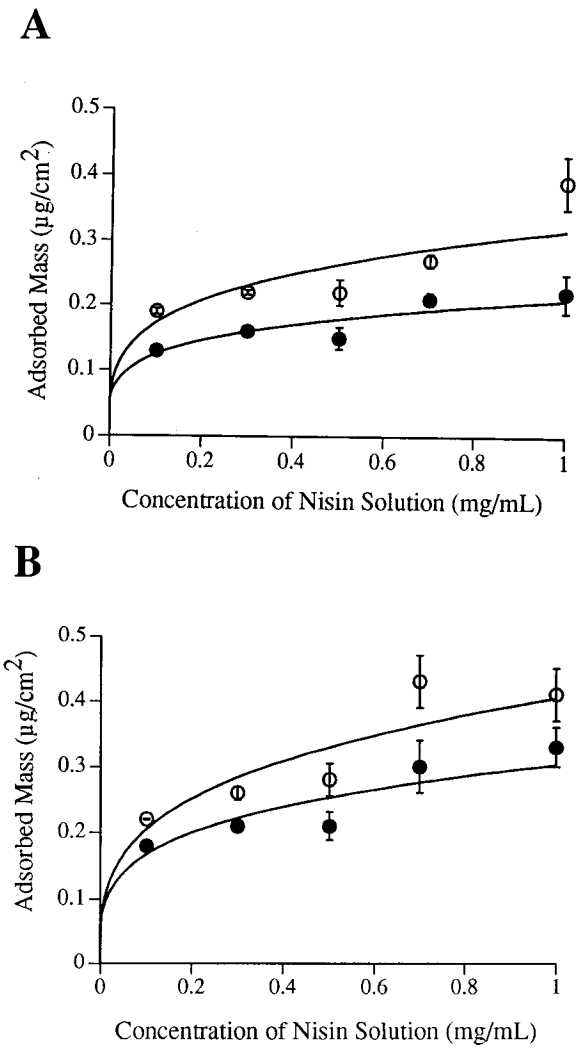

FIG. 1. Adsorbed mass of nisin on low (A)- and high (B)-hydrophobicity surfaces as a function of nisin concentration before (○) and after (●) a buffer rinse.

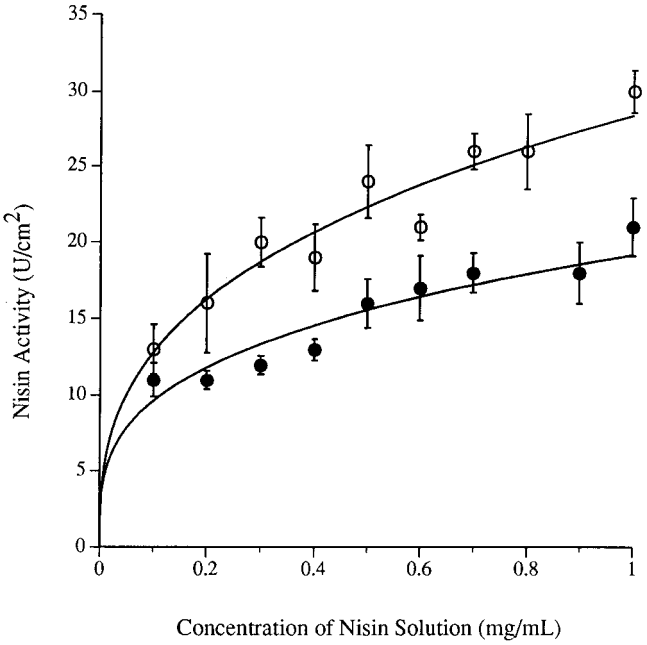

FIG. 2. Activity of adsorbed nisin (as quantified by bioassay) on low (○)- and high (●)-hydrophobicity surfaces following 8 h of contact with nisin solutions of various concentrations.

mg of nisin per ml for 8 h. Figure 4A is the image after the initial adhesion of *L. monocytogenes*, while Fig. 4B was obtained 4 h later. The number of adhered cells and their average cell length remained essentially unchanged. No cells appeared to be dividing; however, several seemed to be losing their cellular integrity and were visibly fading from the surface. The image in Fig. 4C was obtained 8 h after contact with *L. monocytogenes*. The adhered cells had not increased in size, and there was no evidence of cellular division. More of the cells were beginning to appear as empty shells remaining in their former positions. After 12 h (Fig. 4D), there was still no increase in cell length among the attached cells, nor had any cells undergone reproduction. The average decreases in total area occupied by attached cells were 4% (±6%) on highly hydrophobic surfaces and 9% (±6%) on lower-hydrophobicity surfaces. About 20% of the cells were still viable on each surface. Surfaces exposed to a lower concentration of nisin (0.1 mg/ml) before exposure to *L. monocytogenes* produced similar results. The cells neither grew nor reproduced in 12 h.

The images displayed in Fig. 5 were obtained after adhesion of cells onto a surface that had been exposed to inactivated nisin for 8 h. In Fig. 5A, several cells appeared to be almost ready to divide. *L. monocytogenes* cells retained some degree of motility even when they were adhered; thus, active cells sometimes appeared blurred when they were not entirely in the plane of focus as the image was captured. The same field was observed 4 h later (Fig. 5B). The average cell length of the adhered cells had visibly increased, and more cells appeared to be ready to divide. After 8 h (Fig. 5C), the cells had continued to grow and many were beginning to divide. No cells showed any signs of lysis or disintegration as was seen when the cells were exposed to surfaces with adsorbed nisin. After 12 h (Fig. 5D), many of the attached cells had begun to divide. All cells appeared healthy and continued to show signs of growth. Cells that attached to surfaces with heat-inactivated nisin had an average increase in area of 34% (±6%) on highly hydrophobic surfaces but only 27% (±6%) on lower-hydrophobicity surfaces. More than 95% of the attached cells were viable on all of the surfaces tested with INT. The adsorbed mass of heat-inactivated nisin was only slightly lower than that for nisin adsorbed in its native state, regardless of the hydrophobicity of the surface.

The number of adhered cells and their capacity to grow and reproduce were heavily influenced by conditions at the inter-

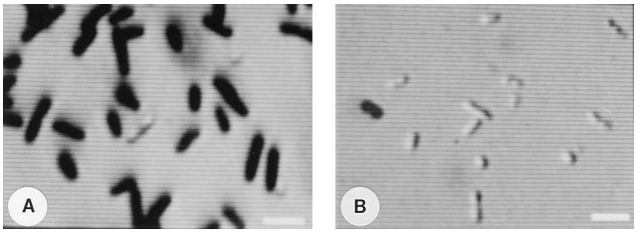

FIG. 3. *L. monocytogenes* adhered to hydrophobic silanized silica surfaces with adsorbed nisin after exposure to INT for 1 h at 25°C. Surfaces were treated with inactivated nisin (1.0 mg/ml) (A) or active nisin (1.0 mg/ml) (B). Bars, 3 µm.

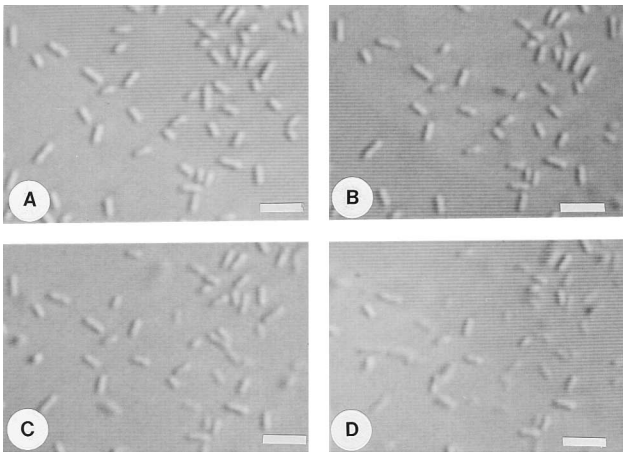

FIG. 4. *L. monocytogenes* adhered to a silanized silica surface (0.1% dichlorodimethylsilane) with adsorbed nisin following 0 (A), 4 (B), 8 (C), or 12 (D) h of cell contact. Bars, 3 μm.

face (Table 1). Surfaces treated to be highly hydrophobic consistently had more adhered cells than did less hydrophobic surfaces. Protein-free surfaces evoked greater adhesion than nisin-covered surfaces, and surfaces exposed to heat-inactivated nisin had higher levels of cell attachment than did surfaces exposed to active nisin at the same concentration (1.0 mg/ml). Lower concentrations of nisin produced films that allowed more cells to attach.

These studies were repeated with a nisin-resistant strain of *L. monocytogenes*. The growth and reproduction of the resistant strain were not inhibited by the presence of adsorbed nisin on the surface (Table 1). The area of the attached cells increased at a rate comparable to that of nisin-sensitive *L. monocytogenes* cells on both protein-free surfaces and surfaces with heat-inactivated nisin. The number of cells adhered to each surface, however, was generally two to three times higher for the nisin-resistant strain.

DISCUSSION

Little is known about the antimicrobial behavior of nisin when it is adsorbed to a surface. Previous research has shown that adsorbed nisin can retain its antimicrobial activity (13), but the actual mechanism has not been determined. Current thinking about the antimicrobial mechanism of nisin in solution suggests that nisin moves through the cell wall, integrates into the cell membrane, and forms a pore (7, 19, 29, 43).

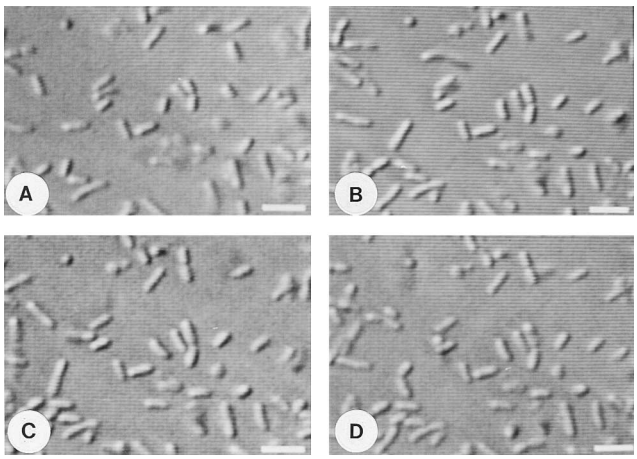

FIG. 5. *L. monocytogenes* adhered to a silanized silica surface (0.1% dichlorodimethylsilane) with inactivated nisin following 0 (A), 4 (B), 8 (C), or 12 (D) h of cell contact. Bars, 3 μm.

These results are consistent with previous reports of nisin's mode of action. *L. monocytogenes* cells exposed to surfaces with adsorbed nisin not only failed to grow, but several appeared to undergo lysis, leaving only empty shells adhered to the surface. Most of the remaining cells failed to reduce the INT salt, suggesting that complete failure of their electron transport systems had occurred. Since the quantity of formazan crystals within each bacterial cell is generally considered to be a function of respiratory intensity (49), it might be argued that the cells lacking formazan crystals were merely in a state of activity below the level of detectability. However, previous work has shown that the maximum number of actively respiring bacteria is obtained within a few minutes and that this number does not change significantly, even with prolonged INT exposure (49). This strongly supports the hypothesis that nonstained cells did not simply experience slower rates of metabolism but, instead, had sustained lethal amounts of membrane damage directly attributable to the lytic effects of nisin.

It has been consistently shown that nisin is effective against populations of *L. monocytogenes* (12, 15, 22, 40). The results of this study demonstrated that adsorbed nisin can decrease cellular adhesion and can be lethal to *L. monocytogenes* cells which do attach. To exert its antimicrobial effect, however, the nisin molecule must first cross the cell membrane. Because of nisin's small size, this can occur only after desorption has taken place. As with most proteins, nisin undoubtedly experiences some degree of distortion at the molecular level when it adsorbs. Nisin is considered to be a hydrophobic molecule and possibly would experience a larger change in conformation when adsorbing to a hydrophobic surface than when adsorbing to a hydrophilic one (30, 46). Nisin that adsorbs to a surface in an antimicrobially ineffective or nondesorbable conformation would theoretically be unavailable. Therefore, surface-induced changes in conformation may account for the reduced amount of nisin activity exhibited on hydrophobic surfaces, as shown in Fig. 2.

Cellular adhesion is greatest on hydrophobic surfaces (1). This relates to the low level of interfacial energy between water and hydrophilic materials, which serves to prevent adhesion of hydrophilic bacteria such as *L. monocytogenes* (1, 8, 14). In this study, cellular adhesion to low-hydrophobicity surfaces was significantly lower than adhesion to higher-hydrophobicity surfaces.

The structural changes that occur when a protein adsorbs to a surface may result in exposure of previously hidden residues to solution, thereby allowing interfacial free energy (35, 38) to be further reduced by adsorption of a loosely bound outer layer. If this type of multilayer formation occurs during nisin adsorption, then the adsorbed mass of nisin films after rinsing would be significantly lower than before rinsing, as shown in Fig. 1.

It is also possible that nisin molecules on the surface associate intermolecularly to form multimers. Liu and Hansen used sodium dodecyl sulfate-polyacrylamide gel electrophoresis to document the spontaneous formation of nisin dimers in concentrated solutions (2.0 mg/ml) (36). Formation of nisin multimers would also be consistent with the isotherms in Fig. 1.

In bacterial enumeration studies, however, adsorbed nisin films were not subjected to extensive rinsing either before or after incubation with cells. This resulted in similar numbers of attached cells on both surface types, regardless of hydropho-

TABLE 1. Attachment of sensitive and resistant strains of *L. monocytogenes* to nisin films and protein-free surfaces

Substrate for cell adhesion	Surface hydrophobicity	Sensitive		Resistant	
		Adhered cells (10^6 cells/cm^2)[a]	Increase in cell area (%)	Adhered cells (10^6 cells/cm^2)[b]	Increase in cell area (%)
Protein free	Low	7.1	28 ± 6	24	20 ± 10
	High	8.9	30 ± 9	24	44 ± 10
Inactive nisin	Low	4.8	27 ± 6	13	ND[c]
	High	6.4	34 ± 6	15	ND
Nisin at 0.1 mg/ml	Low	5.2	0 ± 6	20	32 ± 10
	High	6.0	0 ± 6	22	16 ± 8
Nisin at 1.0 mg/ml	Low	2.4	−9 ± 5	15	15 ± 8
	High	3.0	−4 ± 6	18	ND

[a] Standard error of the mean, 5.4×10^5 cells per cm^2.
[b] Standard error of the mean, 3.1×10^6 cells per cm^2.
[c] ND, not done.

bicity (Table 1). This effect was shown in previous studies of attachment by *L. monocytogenes* (6). A comparison of gentle and vigorous (300 ml/min, 4 min) rinsing protocols after adhesion of cells revealed that a more vigorous rinse produced lower cell counts on all of the surfaces tested, with significantly more cells detached from hydrophilic surfaces.

The range of food contact surfaces to which microorganisms may adhere varies from relatively hydrophilic (glass and steel) to more hydrophobic (plastics and polymers). On the basis of our observations, the potential applications of nisin may be more suited for hydrophobic surfaces. However, this does not exclude hydrophilic surfaces, as our data show.

This research visually explored the lytic effect of adsorbed nisin on *L. monocytogenes* cells and thus further supports the hypothesis that the use of adsorbed nisin as an antimicrobial agent on food contact surfaces may indeed be feasible.

ACKNOWLEDGMENTS

This work was supported in part by The Western Center for Dairy Proteins Research and Technology and the USDA National Research Initiative Competitive Grants Program (Food Safety).

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