Nisin Inhibits Several Gram-Positive, Mastitis-Causing Pathogens

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ABSTRACT
Organisms known to cause bovine mastitis, Enterococcus faecalis ssp. liquefaciens ATCC 27959, Staphylococcus aureus ATCC 29740, Streptococcus agalactiae ATCC 27956, Streptococcus equinus ATCC 27960, Streptococcus dysgalactiae ATCC 27957, Streptococcus uberis ATCC 27958, and the neotype Staphylococcus epidermidis ATCC 14990 were examined for their susceptibility to the small peptide antibiotic, nisin. Using a disc assay, minimum inhibitory concentrations of nisin ranged from 10 to 250 μg/ml among the strains. Examination of the antimicrobial effect of 50 μg/ml nisin in milk showed nisin inhibited all gram-positive pathogens tested.

INTRODUCTION
Mastitis is one of the most widespread and costly diseases affecting dairy herds (1). Morse (13) reported that nearly 50% of cows suffer at least one outbreak of clinical mastitis per lactation. Control of the disease involves hygienic practices such as teat dipping and infusion of antibiotic drugs into the udder. Gilmore (5) estimated 33 million antibiotic treatments are given each year in the United States. Once present, antibiotics cannot be removed from milk (9), so milk from treated cows cannot be sold for 3 to 5 d after treatment. The cost of discarding milk containing antibiotics is significant (14), but the procedure is necessary to protect the estimated 5 to 10% of adult Americans who show hypersensitivity to antibiotic drugs (4) as well as to protect starter cultures used in milk processing. Various on-farm screening tests have been developed to determine when milk is free of antibiotics, adding to overhead costs. More significantly, these tests occasionally yield false positive or false negative results (16).

Nisin is a short peptide antibiotic produced by some strains of Lactococcus lactis ssp. lactis. The protein exerts a bactericidal effect on many gram-positive organisms but is not effective against gram-negative bacteria. Nisin is nontoxic to humans and is readily broken down by digestive enzymes when consumed (7). Hypersensitivity to nisin has not been recorded. It has been used as a food preservative in other countries since 1954 (8) and has recently gained approval in the United States for use in certain dairy products (3). These features make nisin a good candidate for mastitis research, because many mastitis infections involve gram-positive pathogens (2) and nisin-containing milk does not present a threat to consumer health.

The objective of this study was to determine if several species of mastitis-causing pathogens were susceptible to nisin in vitro.

MATERIALS AND METHODS

Bacterial Strains
Enterococcus faecalis ssp. liquefaciens ATCC 27959, Staphylococcus aureus ATCC 29740, Staphylococcus epidermidis ATCC 14990, Streptococcus agalactiae ATCC 27956, Streptococcus equinus ATCC 27960, Streptococcus dysgalactiae ATCC 27957, and Streptococcus uberis ATCC 27958 were obtained from American Type Culture Collection (Rockville, MD). All ATCC cultures used, except the neotype strain of Staph. epidermidis, originated from bovine udder infections. Escherichia coli V517 (11) was received from Larry McKay (University of Minnesota). The staphylococci were grown in nutrient broth (5% peptone, 3% beef extract, pH 6.8) supplemented with .5%...
TABLE 1. Minimum inhibitory concentration\(^1\) of nisin on mastitis-causing organisms.

<table>
<thead>
<tr>
<th>Strain</th>
<th>(µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enterococcus faecalis ssp. liquefaciens ATCC 27959</td>
<td>250</td>
</tr>
<tr>
<td>Staphylococcus aureus ATCC 29740</td>
<td>50</td>
</tr>
<tr>
<td>Staphylococcus epidermidis ATCC 14990</td>
<td>100</td>
</tr>
<tr>
<td>Streptococcus agalactiae ATCC 27956</td>
<td>10</td>
</tr>
<tr>
<td>Streptococcus equinus ATCC 27960</td>
<td>50</td>
</tr>
<tr>
<td>Streptococcus dysgalactiae ATCC 27957</td>
<td>100</td>
</tr>
<tr>
<td>Streptococcus uberis ATCC 27958</td>
<td>100</td>
</tr>
<tr>
<td>Escherichia coli V517</td>
<td>NO(^2)</td>
</tr>
</tbody>
</table>

\(^1\)Minimum nisin concentration showing a 13.5 mm zone of inhibition to organism after 24 h growth at 37°C on BHI containing .5% yeast extract.

\(^2\)None observed.

yeast extract. All other strains were kept in brain-heart infusion (BHI) broth (BBL, Cockeysville, MD) supplemented with .5% yeast extract. Cultures were maintained by bi-weekly transfers, grown at 37°C, and stored at 4°C.

Disc Assays

Disc assays were performed with the following modifications to the method of Barry and Thornsberry (12); a stock nisin (Aplin and Barrett Ltd., Wiltshire, UK, 3.7 x 10^7 IU/g) solution of 10 mg/ml was prepared in double deionized water. Test solutions contained 0, 10, 50, 100, 250, 500, 750, 1000, 2500, 5000, 7500, and 10,000 µg/ml nisin. All solutions were sterilized using a .45-µm syringe-mounted filter. Fresh nisin solutions were prepared immediately before use. Midlog phase cells were obtained by making a 1% inoculation into fresh media from an overnight culture. Cells were then incubated at 37°C until absorbance at 600 nm reached .5 to .7. Optical densities were measured using a Bausch and Lomb Spectronic 20 (Milton Roy Co., Rochester, NY). Disc assays were performed on BHI media with .5% yeast extract. Cells were spread onto the media with sterile cotton applicators and the plates were allowed to dry for 2 to 3 min. Thirteen-millimeter filter paper discs (Difco, Detroit, MI) were then dipped into the appropriate nisin solution until saturated, touched lightly to the container wall to remove excess fluid, and immediately placed upon the inoculated plate. After 24 h of incubation at 37°C, zones of inhibition were measured to the nearest millimeter. The minimum inhibitory concentration (MIC) was the lowest nisin concentration showing a 13.5-mm zone of inhibition to the organism. All experiments were performed in duplicate.

Assays in Milk

Activity of nisin in milk was tested on Escherichia coli V517, Staphylococcus aureus ATCC 29740, Staphylococcus epidermidis ATCC 14990, Streptococcus agalactiae ATCC 27956, and Streptococcus dysgalactiae ATCC 27957. Cultures were standardized to an absorbance of .5 to .7 as described for the disc assay. For each strain four milk tubes were used, two control and two containing 50 µg/ml of sterile nisin solution. Milk robes contained 10 ml of 11% NDM that was sterilized by steaming for 45 min, then heated to 245°F at 12 psi for 12 min. At time zero, 1% inoculations were made into milk tubes, and cell counts of each strain were performed by plating onto BHI plus .5% yeast extract. Plates and milk tubes were incubated at 37°C for 24 h and cell counts made at 0, 6, and 24 h. Plate counts were performed in duplicate.

RESULTS AND DISCUSSION

Results of the disc assay are shown in Table 1. All species examined except E. coli, the negative control, were inhibited by nisin. Susceptibilities ranged from 10 to 250 µg/ml of the antibiotic. As has been observed previously (8), E. faecalis was the most resistant gram-positive organism tested. High susceptibility was observed with Strep. agalactiae; this also has been noted before (6). The other gram-positive pathogens showed markedly similar susceptibilities.
TABLE 2. Antimicrobial effect of 50 μg/ml nisin in milk on mastitis-causing organisms.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Nisin</th>
<th>0 h</th>
<th>6 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus aureus</em> ATCC 29740</td>
<td>0</td>
<td>4.4 x 10^6</td>
<td>1.8 x 10^8</td>
<td>6.6 x 10^8</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>4.9 x 10^6</td>
<td>0</td>
<td>EAPC^1 30</td>
</tr>
<tr>
<td><em>Staphylococcus epidermidis</em> ATCC 14990</td>
<td>0</td>
<td>1.2 x 10^6</td>
<td>1.2 x 10^8</td>
<td>2.8 x 10^8</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>1.4 x 10^6</td>
<td>EAPC^1 30</td>
<td>EAPC^1 2.5</td>
</tr>
<tr>
<td><em>Streptococcus agalactiae</em> ATCC 27956</td>
<td>0</td>
<td>2.5 x 10^5</td>
<td>3.1 x 10^8</td>
<td>7.2 x 10^7</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>6.5 x 10^3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>Streptococcus dysgalactiae</em> ATCC 27957</td>
<td>0</td>
<td>2.3 x 10^6</td>
<td>2.9 x 10^8</td>
<td>4.5 x 10^8</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>2.7 x 10^6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>Escherichia coli</em> V517</td>
<td>0</td>
<td>5.2 x 10^6</td>
<td>4.7 x 10^8</td>
<td>5.8 x 10^8</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>7.5 x 10^6</td>
<td>3.5 x 10^8</td>
<td>5.8 x 10^8</td>
</tr>
</tbody>
</table>

^1 Estimated aerobic plate count.

Results of the milk assay (Table 2) indicated that all gram-positive species tested were strongly inhibited at 50 μg/ml of nisin. This concentration was lower than some of the MIC determined on the disc assay. The difference between the milk and disc assay data may be a result of the homogeneous distribution of the antibiotic in liquid. Diffusion of nisin from the discs into the agar during the disc assay would diminish the actual nisin concentration near the disc thus resulting in higher MIC.

Table 2 also demonstrates that nisin retained activity in milk, although *Staph. aureus* did show a small increase in cell numbers at 24 h. These cells may represent resistant mutants or be indicative of slightly decreased antibiotic activity in milk (10, 15).

CONCLUSION

Although disc assays may not reflect actual MIC values, results indicate that nisin is effective for inhibition of gram-positive, mastitis-causing bacteria in vitro. In vivo studies need to be performed to determine whether nisin would be a useful therapeutic agent for treatment of mastitis caused by gram-positive organisms. Nisin would not, however, be useful for treating mastitis involving gram-negative pathogens.

ACKNOWLEDGMENTS

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REFERENCES


