APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1996, p. 4536–4541 Vol. 62, No. 12
0099-2240/96/$04.00+0

Mode of Action of a Lysostaphin-Like Bacteriolytic Agent Produced by *Streptococcus zooepidemicus* 4881

R. S. SIMMONDS,[1]* L. PEARSON,[1] R. C. KENNEDY,[2] AND J. R. TAGG[1]

Department of Microbiology, University of Otago, Dunedin, New Zealand[1] and Department of Microbiology and Immunology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73190[2]

Received 17 June 1996/Accepted 1 August 1996

Electron microscopy of zoocin A-treated sensitive streptococcus cells revealed cytoplasmic disruption and ultimately complete rupture of the cell wall. Culture viability and optical density were shown to decrease rapidly and simultaneously in *Streptococcus pyogenes* FF22 but less quickly in the relatively more resistant *Streptococcus mutans* 10449. Zoocin A was shown to cleave hexaglycine in a colorimetric cell-free microtiter assay system, and it is concluded that the killing action of zoocin A, like that of lysostaphin, is most probably the result of direct cleavage of the peptidoglycan cross-links in the cell wall. The relationship between sensitivity to zoocin A and the peptidoglycan cross-linkage structure of *Streptococcus zooepidemicus*, *Lactococcus* spp., *S. pyogenes*, *Streptococcus gordonii*, *Streptococcus oralis*, *S. mutans*, and *Streptococcus rattus* has been evaluated.

The Lancefield group C streptococcus *Streptococcus zooepidemicus* 4881 has been shown to produce inhibitory activity against strains of a variety of streptococcal species, including all *Streptococcus pyogenes* strains, all mutans streptococcal strains except *Streptococcus rattus*, and all *S. zooepidemicus* strains other than strain 4881 itself (11). The molecule responsible for this activity, zoocin A, was subsequently purified and characterized (25). Sequencing and analysis of the structural gene (*zooA*) for zoocin A revealed an open reading frame corresponding to 285 amino acids (26). N-terminal amino acid sequencing of mature zoocin A suggested that the 285-amino-acid prepeptide is cleaved to yield a 262-amino-acid active product with an estimated molecular mass of 27,877 Da. Comparison of the predicted amino acid sequence of zoocin A with other published sequences revealed a number of homologous regions and led us to propose that zoocin A is a domain-structured molecule, possessing a catalytic domain in the N-terminal portion of the molecule and a substrate recognition domain in the C-terminal region. The proposed catalytic domain has considerable homology to sequences of proteins from both gram-negative and gram-positive bacteria, including the relatively well characterized endopeptidase lysostaphin (26).

The most intensively studied bacteriocins are the colicins produced by *Escherichia coli*. These kill bacteria in a two-step process, the first being adsorption to a cell surface receptor in the outer membrane and the second representing the production of irreversible widespread biochemical changes within the cell (15, 17). In contrast, the mechanisms of action of bacteriocins produced by gram-positive bacteria are less well known. Lantibiotics, the lanthionine-containing peptide bacteriocins produced by certain gram-positive bacteria, have been shown to insert into the bacterial membrane, resulting in the formation of pores (2), which in turn leads to a loss of proton motive force and subsequent lysis of the cell. Other small, nonlantibiotic bacteriocins of gram-positive bacteria, such as pediocin, are also known to form pores or channels in cell membranes (10).

* Corresponding author. Mailing address: Department of Microbiology, University of Otago, P.O. Box 56, Dunedin, New Zealand. Phone: 64 3 479 7478. Fax: 64 3 479 8540. Electronic mail address: robin.simmonds@stonebow.otago.ac.nz.

All bacteria probably utilize cell wall lytic enzymes as part of their growth and repair mechanisms. When secreted extracellularly, such enzymes, bearing substrate or receptor-determining domains having specificity for the cell walls of only certain other bacteria, may be functionally indistinguishable from bacteriocins. Lysostaphin, a lytic molecule active against *Staphylococcus aureus*, is one such molecule. It has been suggested that lysostaphin may function catabolically to release nutrients from other staphylococci in the environment, with the producer cell being protected by a specific immunity protein (8, 18). The lytic effect of lysostaphin results from a direct attack on the integrity of the staphylococcal cell wall, specifically bringing about cleavage of the pentaglycine cross-link within the peptidoglycan macromolecule (4). The host specificity of lysostaphin appears to be determined by a substrate recognition domain in the C-terminal portion of the molecule (32).

We report in this article that the mode of action of zoocin A against sensitive streptococci is lytic, that lysis occurs as a direct result of the interaction of zoocin A with the cell, and that zoocin A and lysostaphin are both capable of cleaving the peptide substrate hexaglycine. We propose that streptococcal sensitivity to zoocin A may, in part, be a function of the type of peptidoglycan cross-link possessed by the different species.

MATERIALS AND METHODS

Media and chemicals. All commercial media used in this study were prepared as described in the manufacturers' specifications. The media used were Todd-Hewitt broth (THB) (Difco Laboratories, Detroit, Mich.) and Columbia agar base (GIBCO BRL, Life Technology Ltd., Paisley, United Kingdom). Blood agar was made by the addition of 5% (vol/vol) whole human blood to Columbia agar base. Lysostaphin, hexaglycine, triethylamine, 2,4,6-trinitrobenzene-1-sulfonic acid-3-hydrate (TNBS), sodium borate, and all chemicals used in the preparation of chemically defined medium were obtained from Sigma Chemical Co. (St. Louis, Mo.). All other chemicals and reagents were from BDH Chemicals (Port Fairy, Victoria, Australia).

Bacterial strains. The bacterial strains used in this study are listed in Table 1. Stock cultures of all strains were stored in skim milk at −70°C and when required were subcultured onto blood agar. Strains in regular use were maintained as plate cultures on blood agar and subcultured every 2 weeks in a 5% (vol/vol) CO_2-in-air atmosphere at 37°C.

Spectrum of inhibitory activity of the zoocin A producer *S. zooepidemicus* 4881. The deferred antagonism procedure, essentially as described by Tagg and Bannister (30), has been described previously (25) and was used to determine the sensitivity of the strains listed in Table 1 to zoocin A.

Titration assay to determine the inhibitory concentration of zoocin A. The

TABLE 1. Bacterial strains used in this study

Species	Strain designations (references)
S. zooepidemicus	4881 (zoocin A producer strain [24]), 8b, 8d, 8f, 8h, 8i, 8j, 8l, 9c, 9j (1)
S. gordonii	ATCC 10558, DL1, D105, G102, C219, 38, M5 (11)
S. mutans	MT703R, OMZ175, MT8148, 13M, UTB1, UTB2, NCTC 10449 (11)
S. oralis	H1 (31), C104, 34, ATCC 15914, J22 (11)
S. pyogenes	FF22, 71-679, 71-698, W-1 (30)
S. rattus	ATCC 19645, BHT (11), LG1 (27), IB, 6F71 (28)
Lactococcus spp.	T-21 (30), DRC1, DRC2, 223, ML8, 1816, C2[a]

[a] New Zealand Dairy Research Culture Collection.

method to determine the inhibitory concentration of zoocin A has been described previously (25). In brief, the inhibitory concentration of zoocin A was given as the reciprocal of the highest twofold dilution to give complete inhibition of a lawn of the specified indicator organism.

Estimation of protein concentration. The method used for estimating protein concentration was a modification of that used by Peterson (16). Standards were made by diluting bovine serum albumin in distilled water, purified with a Milli-Q system (Millipore Inc., Molsheim, France; MQ water), at a concentration between 0.2 and 0.8 mg/ml. Copper tartrate solution contained 0.019% cupric sulfate, 0.038% sodium potassium tartrate, and 4.81% sodium carbonate. Folin-Ciocalteu reagent was diluted to 50% (vol/vol) with MQ water. To 50 μl of each sample were added 50 μl of 1 M sodium hydroxide and 250 μl of copper tartrate solution. These contents were thoroughly mixed in small test tubes and incubated at room temperature for 10 min. Subsequently, 50 μl of diluted Folin-Ciocalteu reagent was added, the contents of each tube were thoroughly mixed, and the tubes were incubated for 20 min at room temperature; 100-μl volumes were then transferred to each of three wells of a flat-bottom microtiter plate, and the optical density at 690 nm (OD_{690}) was measured.

Preparation of zoocin A. Batch cultures (5 to 10 liters) of *S. zooepidemicus* 4881 were grown (36 h, pH 6.9-controlled in chemically defined medium [25]) with 0.5% maltose as the carbohydrate source and a 0.3% (vol/vol) inoculum of a chemically defined medium overnight starter culture. The cells were removed from the culture by centrifugation at 15,000 × *g* for 25 min, and the zoocin A was concentrated in the retentate (400 ml) with a 10,000-molecular-weight hollow-fiber cartridge filter (CD Medical Inc., Miami Lakes, Fla.). Zoocin A was recovered from the retentate by the addition of ammonium sulfate to 90% saturation, precipitation on ice overnight, and collection of the precipitate by centrifugation at 15,000 × *g* for 25 min and then dissolution of it in 100 ml of saline. This preparation was filtered through a 50,000-molecular-weight-cutoff ultrafiltration membrane (Diaflo; Amicon, Inc., Beverly, Mass.) until approximately 5 ml of retentate remained. Aliquots of 500 μl were loaded onto a Superose 12 (Pharmacia AB Biotechnology, Uppsala, Sweden) gel filtration column, equilibrated and run at 1 ml/min, in 25 mM ammonium acetate buffer (pH 6.0). One-milliliter fractions were collected and assayed for zoocin A activity. Active fractions were pooled, lyophilized, resuspended in saline, and stored at −20°C until required.

The purity of zoocin A was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli (13) using 12% discontinuous gels, a Mini-Protean II electrophoresis system (Bio-Rad, Richmond, Calif.), and the manufacturer's chemicals and protocols. Molecular weight markers were supplied by Pharmacia (Pharmacia LKB Biotechnology, Inc., Piscataway, N.J.). Following electrophoresis, protein bands were visualized by staining with brilliant blue R 250 (Sigma). All of the purified zoocin A used in this study was prepared as a single pool. Figure 1 shows the results obtained from running different quantities of this material in an SDS-PAGE gel. Some lanes were deliberately overloaded to detect possible contaminating proteins present in relatively low amounts. Further chromatographic separation (MonoS and ion exchange; Pharmacia) of this material produced a single chromatographic peak (A_{280}) and no apparent increase in purity by SDS-PAGE. Analysis of this material by mass spectrometry (matrix-assisted laser desorption ionization-time of flight; Lasermat 2000 mass analyzer; Finnigan Mat, San Jose, Calif.) yielded a single mass corresponding to the predicted mass of zoocin A. N-terminal amino acid sequencing of another batch of zoocin A, judged by SDS-PAGE to be of similar purity to that used in the present study, yielded a single peptide sequence (25).

Transmission electron microscopy of zoocin A-treated cells. *S. zooepidemicus* 4881, *Streptococcus mutans* 10449, and *S. pyogenes* FF22 were grown overnight in THB at 37°C. Cells were pelleted by sequential centrifugation of five 1-ml volumes of each culture in the same microcentrifuge tube. To each pellet were added 250 μl of zoocin A diluted in saline to approximately 80 μg/ml (titers of 0, 2, and 128 against *S. zooepidemicus* 4881, *S. mutans* 10449, and *S. pyogenes* FF22, respectively) and 250 μl of double-strength THB. The cells were then resuspended and the tubes were incubated in a water bath at 37°C for 2 h before pelleting and fixing were performed.

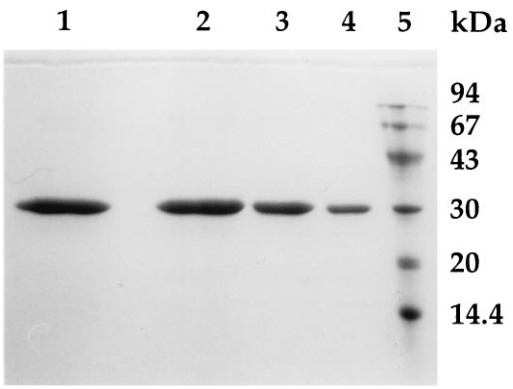

FIG. 1. SDS-PAGE of purified zoocin A. Lanes: 1, 2, 3, and 4, approximately 24, 16, 8, and 4 μg of zoocin A preparation, respectively; 5, molecular mass standards.

Fixing and embedding of cells for electron microscopy. The sectioning procedure was a modification of that used by Dajani et al. (5). The cell pellets were fixed for 45 min with 2% (vol/vol) glutaraldehyde and 0.025% calcium dichloride in 0.1 M cacodylate buffer (pH 6.5). After fixing, the cell pellet was washed twice (10 min per wash) with 0.1 M sodium cacodylate buffer (pH 6.5) containing 7.5% sucrose, care being taken to not disrupt the pellet. This was followed by the addition of 1% osmium tetroxide in 0.1 M cacodylate buffer, incubation for 45 min at ambient temperature, three 5-min washings of the cell pellet in 0.1 M cacodylate buffer, and staining in 0.5% uranyl acetate for 45 min at ambient temperature. The pellet was dehydrated in 70% (vol/vol) ethanol for 5 min before being transferred to a conical embedding capsule (BEEM Conical Embedding Capsules, Thuringowa, Australia). The pellet was further dehydrated by incubating for 10 min each in 95% ethanol, two changes of 100% ethanol, and two changes of propylene oxide. Infiltration of the pellet was achieved by sequentially filling the BEEM capsule with a mixture consisting of two parts propylene oxide and one part agar 100 resin for 2 h, a mixture consisting of one part propylene oxide and one part resin for 2 h, undiluted resin for 4 h, and undiluted resin and accelerator for 6 h.

Preparation and viewing of ultrathin sections. Ultrathin sections were cut with a glass knife by use of an ultratome (8800 Ultratome III; LKB-Produkter AB, Bromma, Sweden) and mounted on copper grids. The sections were stained for 20 min with uranyl acetate (a saturated solution in 50% ethanol) at 60°C, washed with MQ water, and dried on tissue paper. The grids were placed onto a drop of Sato lead citrate with the section side face downwards. After 5 min, the grids were washed with MQ water and dried on tissue paper. The grids were viewed with an Akashi electron microscope (EM-002; Akashi Seisakusho Ltd., Tokyo, Japan) at 65 kV.

Effect of Zoocin A on the viability and OD of *S. zooepidemicus* 4881, *S. pyogenes* FF22, and *S. mutans* 10449 cultures. Overnight THB cultures of *S. zooepidemicus* 4881, *S. pyogenes* FF22, and *S. mutans* 10449 were diluted with fresh THB to an OD_{550} of 0.5 in test tubes (12 by 1 cm), and 300 μl of each culture was inoculated into 4.2 ml of prewarmed (37°C) THB. When the OD_{550} reached 0.225, 160, 20, and 160 μg of zoocin A in 0.5 ml of saline were added to the *S. zooepidemicus* 4881 (final titer, 0), *S. pyogenes* FF22 (final titer, 8), and *S. mutans* 10449 (final titer, 1) cultures, respectively. An equivalent volume of MQ water was added to each control tube. At times 0, 45, 90, 120, 150, 180, 190, 200, 210, 240, 330, 445, and 1,285 min, the OD_{550} of each culture was measured with a spectrophotometer (Spectronic 20; Milton Roy Co., New York, N.Y.), 100-μl samples were taken and diluted 10^{-2}, 10^{-3}, and 10^{-4} in saline, and each dilution was spiral plated (Spiral System, Inc., Cincinnati, Ohio) on blood agar. After overnight incubation in air plus 5% CO_2, colonies were counted in sections containing more than 20 colonies.

Acetylation of *N*-acetylhexaglycine. The method used for acetylation of *N*-acetylhexaglycine was a modification of the procedure used by Kline et al. (12). Hexaglycine (1 g) was suspended in 100 ml of MQ water, stirred constantly, and dissolved by twice adding 0.78 ml of triethylamine followed by 0.26 ml of acetic anhydride. After 1 h and at hourly intervals for the next 3 h, 0.39 ml of triethylamine was added followed immediately by 0.26 ml of acetic anhydride. One hour after the final addition of acetic anhydride, the mixture was acidified to pH 2 by the dropwise addition of 1 M hydrochloric acid (HCl) and the resultant precipitate was collected by centrifugation at 27,000 × *g* for 15 min. The precipitate was washed by resuspending in 10 mM HCl, centrifuging as described above, and resuspending the pellet in 40 ml of MQ water. The precipitate was dissolved by the dropwise addition of 1 M sodium hydroxide, sufficient to attain a final pH of 8. This solution was filtered through a 0.2-μm-pore-size cellulose nitrate filter (Micro Filtration Systems, Dublin, Calif.), acidified to pH 2, and centrifuged as before to collect the precipitate. The precipitate was washed once with 1 mM HCl as described previously and dried for storage by lyophilization.

TABLE 2. Sensitivity of selected streptococcal species to zoocin A when tested by deferred antagonism and their cell wall peptidoglycan cross-links

Group tested[a]	Proportion[b] (%) of sensitive strains	Peptidoglycan cross-link[c]	Cell wall teichoic acid[d]
S. zooepidemicus	9/10[e] (90)	L-Ala_{2-3}	−
S. pyogenes	4/4 (100)	L-Ala_{2-3}	−
S. gordonii	7/7 (100)	L-Ala_{2-3}	−
S. mutans	7/7 (100)	L-Ala_{2-3}	−
S. rattus	0/5 (0)	L-Ala_{2-3}	+
Lactococcus spp.	0/7 (0)	D-Asp	+
S. oralis	0/5 (0)	Direct	−

[a] Individual strain designations are given in Table 1.
[b] Number of strains sensitive to zoocin A/number of strains tested.
[c] Data from references 22 and 23. Subscripts indicate the number of amino acids in cross-link.
[d] Data from references 7 and 23. −, absent; +, present.
[e] The single zoocin A-resistant *S. zooepidemicus* strain is the producer strain, 4881.

To ascertain whether all amine groups were acetylated, a 5 mM solution of *N*-acetylhexaglycine in 0.1 M sodium bicarbonate plus 5 mg of TNBS per ml was prepared. This was incubated at ambient temperature for 20 min in 1-ml cuvettes, and the OD_{420} was read. If the OD was greater than 0.05, the hexaglycine was reacetylated with one more addition of 0.39 ml of triethylamine and 0.26 ml of acetic anhydride.

Preparation of standard *N*-acetylhexaglycine stock and *N*-acetylhexaglycine substrate solution. A 100 mM *N*-acetylhexaglycine stock was prepared by suspending *N*-acetylhexaglycine in MQ water, dissolving by the dropwise addition of 0.1 M sodium hydroxide, and diluting in MQ water such that a 1:100 dilution of the solution had an OD_{214} of 0.235. The 100 mM *N*-acetylhexaglycine stock was stored at −20°C until used. The standard *N*-acetylhexaglycine substrate solution was made by diluting 100 mM *N*-acetylhexaglycine stock 1:10 in assay buffer. Assay buffer consisted of 5 mM trisodium citrate plus 1 mM disodium EDTA in 0.1 M sodium borate solution adjusted to pH 8.0 by the dropwise addition of 0.1 M sodium hydroxide.

Determination of endopeptidase activity by a colorimetric hexaglycine microassay. A stock solution of lysostaphin (0.2 mg/ml) was prepared in 20 mM sodium acetate buffer (pH 4.5) and stored at −20°C. Lysostaphin and zoocin A were prepared to an initial concentration of 80 μg/ml in 0.5% (vol/vol) Tween 20, and serial twofold dilutions were prepared in this medium over a concentration range of 0.04 to 40 μg/ml. Assay wells (flat-bottom microtiter trays; Nunc, Roskilde, Denmark) were prepared in quadruplicate and consisted of 50 μl of diluted zoocin A or lysostaphin mixed with 50 μl of standard *N*-acetylhexaglycine substrate solution. The microtiter plates were covered to prevent evaporation and incubated at 37°C for 90 min, and the presence of free amine groups was visualized by the addition of 10 μl of TNBS (3.25 mg/ml in 0.1 M sodium bicarbonate) to each well. After 20 min of incubation at ambient temperature, the reaction was stopped by the addition of 50 μl of 1 M sodium acetate to each well and the OD_{405} was read. Positive control wells contained twofold dilutions of glycine (from an initial concentration of 1.66 mg/ml) in 0.5% Tween 20. Negative control wells contained either lysostaphin or zoocin A to which was added 50 μl of MQ water instead of substrate solution or *N*-acetylhexaglycine substrate but with no zoocin A or lysostaphin.

RESULTS

Spectrum of inhibitory activity of the zoocin A producer *S. zooepidemicus* 4881. All *S. zooepidemicus* (other than the zoocin A producer strain itself), *S. pyogenes*, *Streptococcus gordonii*, and *S. mutans* strains tested proved sensitive to zoocin A. In contrast, all *Lactococcus*, *Streptococcus oralis*, and *S. rattus* strains were resistant (Table 2).

Electron microscopy of zoocin A-treated cells. Electron microscopic examination of zoocin A-treated *S. zooepidemicus* 4881 cells showed no observable differences from MQ water-treated *S. pyogenes* FF22 cells (Fig. 2A). Zoocin A-treated *S. pyogenes* FF22 cells (Fig. 2B) showed a loss of dark-staining cytoplasmic material into the surrounding extracellular space, the aggregation of cytoplasmic remains, and separation of cell membranes from cell walls. Various stages of cellular degeneration could be identified, from early stages showing large amounts of cytoplasmic material and bead-like aggregates to the almost complete loss of cytoplasmic structure with only a few aggregates remaining (Fig. 2C). This "ghosting" of cells appeared to occur rapidly, since relatively few cells were observed in intermediate stages. Most ghosted cells showed signs of cell wall damage, leading in its final stages to extrusion of the cytoplasmic membrane from the ruptured cell (Fig. 2D). Rupture of the cell wall appeared to occur most often at the point of septum formation (Fig. 2E). Zoocin A-treated *S. mutans* 10449 cells (Fig. 2F) also showed substantial differences from MQ water-treated cells. The changes observed in cellular structure appeared to be similar to those observed for zoocin A-treated *S. pyogenes* FF22 cells. The proportion of apparently intact to ghosted cells was different for zoocin A-treated *S. pyogenes* FF22 and *S. mutans* 10449 cells, being approximately 8 and 72%, respectively.

Effect of zoocin A on the viability and OD of *S. pyogenes* FF22, *S. mutans* 10449, and *S. zooepidemicus* 4881 cultures. The addition of zoocin A to mid-log-phase *S. pyogenes* FF22 cultures resulted in a rapid reduction in both viable count (Fig. 3A) and OD_{550} (Fig. 3B) when compared with that of comparable cultures that received MQ water. In contrast, the addition of zoocin A to mid-log-phase *S. mutans* 10449 cultures showed a significant decrease in viable count (Fig. 3C) only after 445 min of incubation with zoocin A and showed no significant decrease in OD (Fig. 3D) over the course of the experiment. The addition of zoocin A to mid-log-phase *S. zooepidemicus* 4881 cultures resulted in no significant difference in viable count or OD_{550} from those obtained in MQ water-treated control cultures.

Determination of endopeptidase activity by a colorimetric hexaglycine microassay. The OD_{405} increased with increasing enzyme concentration (Fig. 4). The dose response for lysostaphin was linear between enzyme concentrations of 5 and 40 μg/ml, giving a linear regression coefficient of 0.936 over this range. Over the same range of enzyme concentrations, the linear regression coefficient for zoocin A was 0.866, and over the range of 10 to 40 μg/ml, it was 0.919. Control wells containing enzyme but no substrate showed no significant change in absorbance (standard $deviation_{n-1}$ of 0.029 and 0.026 for those wells containing lysostaphin and zoocin A, respectively) over the range (0.04 to 40 μg/ml) of enzyme concentrations used, demonstrating that the rise in absorbance in the test wells was not due to the reaction of either lysostaphin or zoocin A with TNBS. Control wells containing *N*-acetylhexaglycine substrate and TNBS but no enzyme showed no significant change in absorbance (SD_{n-1} of 0.013) over the course of the experiment, demonstrating that the rise in absorbance in the test wells was not due to a reaction of the TNBS with the *N*-acetylhexaglycine substrate.

DISCUSSION

Zoocin A-treated *S. pyogenes* FF22 and *S. mutans* 10449 cells showed ghosting, the most commonly observed point of cell rupture being the region of septum formation. Similar ghosting and rupture at the point of septum formation have been reported for *Enterococcus faecalis* cells incubated in the presence of an autolysin (9). It may be that the point of septum formation is the most susceptible site for hydrolytic cleavage or that this is the weakest point in the structural integrity of the dividing cell. The considerably higher proportion of ghosted to intact cells observed in zoocin A-treated *S. pyogenes* FF22 cells compared with that observed in zoocin A-treated *S. mutans* 10449 cells is thought to reflect the relative sensitivities of these two strains to zoocin A rather than a difference in the action of zoocin A against these different species. The MIC of zoocin A for *S. mutans* 10449 was 64 times higher than that for *S. pyogenes* FF22. The MIC for *S. zooepidemicus* 4881 was not

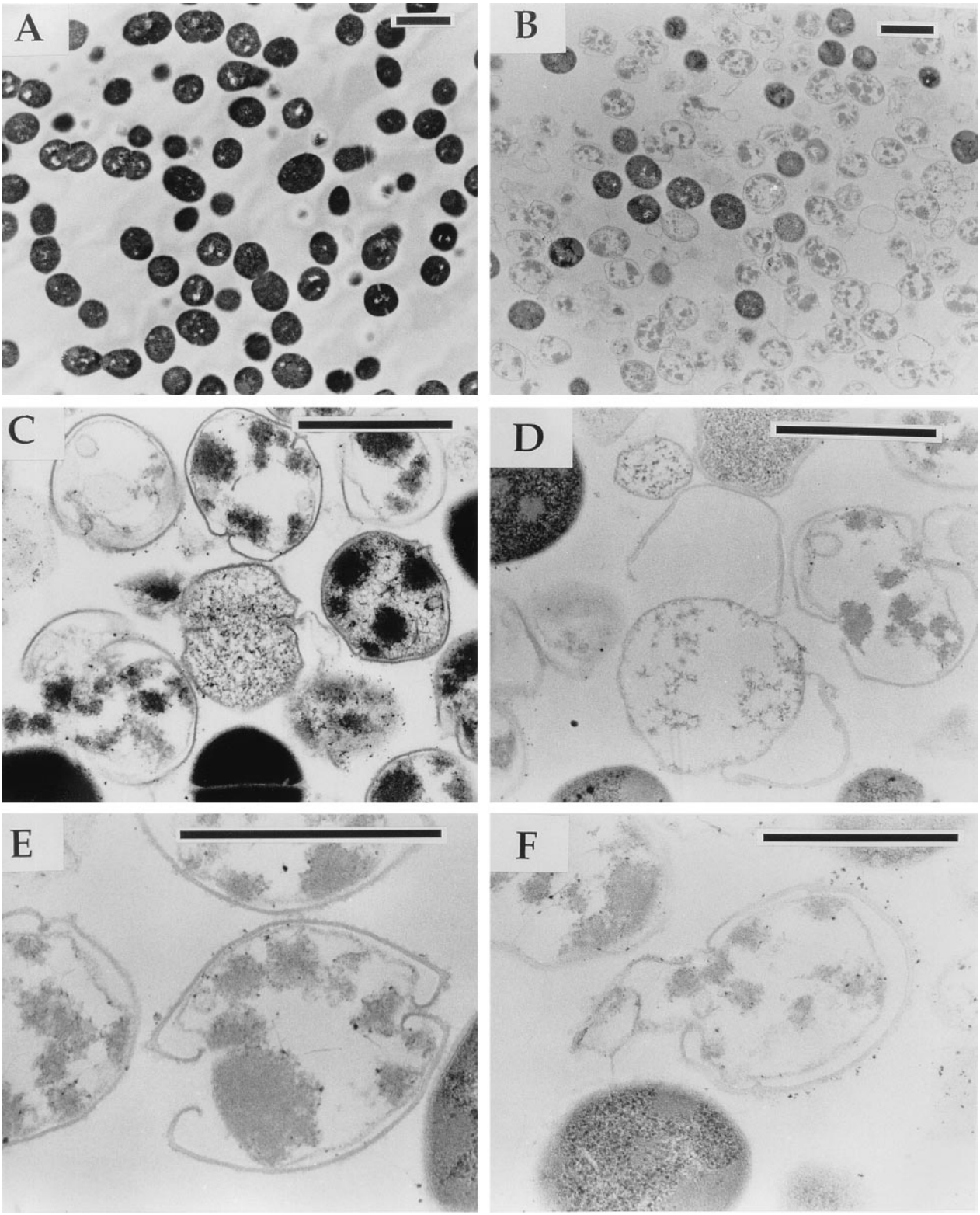

FIG. 2. Transmission electron microscopy of zoocin A-treated *S. pyogenes* FF22 and *S. mutans* 10449 cells. (A) *S. pyogenes* FF22 cells treated with MQ water; (B) *S. pyogenes* FF22 cells treated with zoocin A; (C) *S. pyogenes* FF22 cells treated with zoocin A illustrating the range of cellular disruption observed; (D) *S. pyogenes* FF22 cells treated with zoocin A showing complete extrusion of the cytoplasmic membrane from the ruptured cell; (E) *S. pyogenes* FF22 cells treated with zoocin A showing cell wall rupture at the point of septum formation; (F) *S. mutans* 10449 cells treated with zoocin A. Bars, 1 μm.

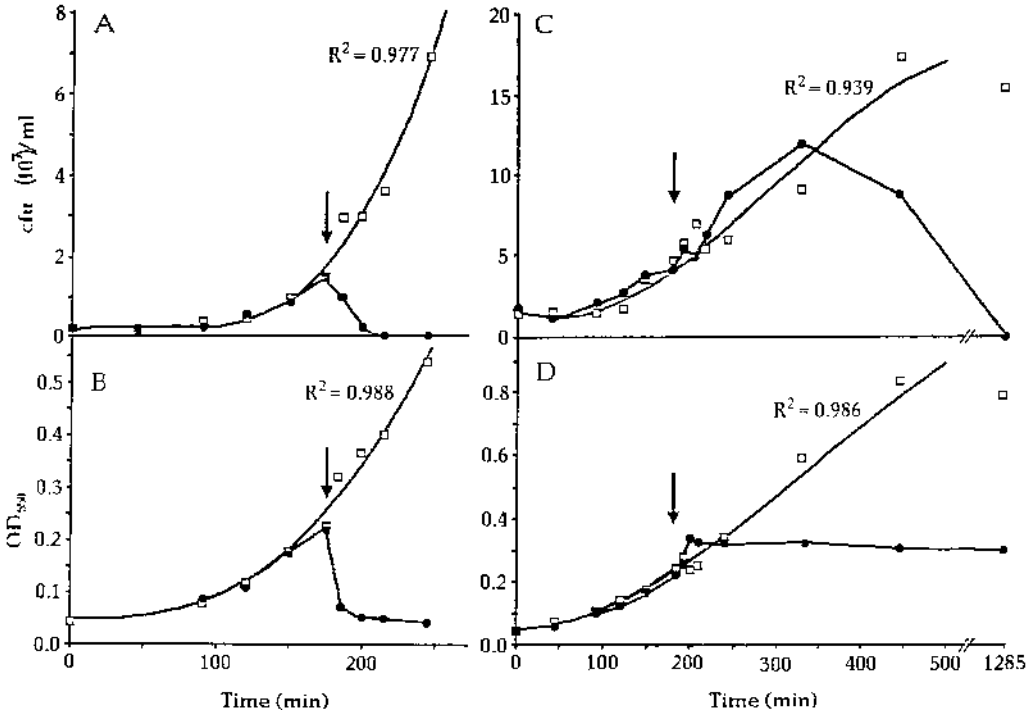

FIG. 3. Effect of zoocin A on the viability and OD of *S. pyogenes* FF22 and *S. mutans* 10449 cultures. (A and B) Effect of addition of zoocin A on the viable count (A) and OD_{550} (B) of *S. pyogenes* FF22 cultures. The values obtained for incubation times greater than 240 min have been omitted for the sake of clarity and do not differ significantly from those reported for 240 min. (C and D) Effect of addition of zoocin A on the viable count (C) and OD_{550} (D) of *S. mutans* 10449 cultures. The arrows show the time of zoocin A addition. R^2, regression coefficient for the best-fit third-order polynomial (Cricket Graph version 1.3; Cricket Software, Malvern, Pa.). Symbols: □, control (addition of MQ water); ●, test (addition of zoocin A).

determined, but previous studies have shown it to be not less than 4,000 times the MIC for *S. pyogenes* FF22 (25). The formation of ghosted cells alone does not necessarily imply that the cell wall is the primary site of cell damage. Pediocin AcH, which is thought to act primarily by forming pores or channels in cell membranes, can also cause ghost formation, and bacteria having differing susceptibilities to pediocin have been reported to give rise to a variety of morphological appearances, ranging from apparently normal to total ghosting (3).

Lysostaphin-treated *Staphylococcus aureus* cells show a rapid reduction in both viable count and OD (21). Zoocin A-treated *S. pyogenes* FF22 cultures showed an immediate and simultaneous loss of both OD and viability, suggesting that as with lysostaphin, lysis occurred as a direct result of zoocin A activity against the cell wall. In contrast, zoocin A-treated *S. mutans* 10449 cultures showed relatively little decrease in OD, even after prolonged incubation. However, the culture OD did stop rising within 10 min of the addition of zoocin A, and electron micrographs of *S. mutans* 10449 cells treated with a comparable concentration of zoocin A showed a number (28%) of ghosted or partially ghosted cells after 120 min of incubation. We believe the relative lack of effect of zoocin A against *S. mutans* 10449 cultures to be dose related and not to be indicative of an alternative mode of action.

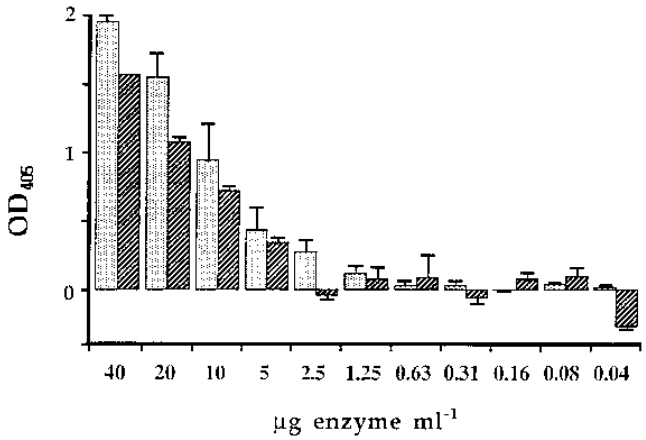

FIG. 4. Cleavage of hexaglycine substrate by lysostaphin and zoocin A. The OD_{405} reported is the mean value at each enzyme concentration less the mean of the corresponding negative control wells ± SD_{n-1} of the values for each enzyme concentration. Symbols: ▨, lysostaphin; ▨, zoocin A.

Peptidoglycan is insoluble as a result of the extensive cross-linking of the subunits during cell wall synthesis, and the hydrolysis of sufficient cross-links will bring about solubilization of the cell wall and consequent cell lysis (29). Several methods to detect the endopeptidase activity of lysostaphin have been developed, most of which measure the reduction in OD occurring as a result of solubilization of whole cells or cell wall extracts in the presence of the enzyme (14). However, difficulties in producing reproducible substrates, interference of extract components with the action of the enzyme, and the lack of standard controls make the interpretation of such assays difficult. Using N-terminally capped hexaglycine as the substrate and TNBS as the colorimetric reagent, Kline et al. developed a sensitive and reproducible microtiter assay for the quantitation of lysostaphin which avoids these difficulties (12). The authors further suggested that this assay could be modified to measure the activity of other endopeptidases. The application of this assay in the present study enabled the catalytic activity of zoocin A to be measured independently of its host specificity determinants. The linear dose-response relationship

of chromophore development to enzyme concentration shows that zoocin A, like lysostaphin, was capable of cleaving hexaglycine and, furthermore, that the specific activities of the two enzymes for this substrate were similar. This supports our previous suggestion that the conserved catalytic domains of zoocin A and lysostaphin imply a common mode of action (26).

S. zooepidemicus, *S. pyogenes*, *S. gordonii*, and *S. mutans* strains were sensitive to zoocin A, and all of these contain di- or tri-alanine cross-links in their peptidoglycan. In contrast, *S. oralis* and *Lactococcus* strains were resistant to the action of zoocin A and contain direct covalent and single D-Asp amino acid cross-links, respectively (22). It has been suggested that the presence of lysostaphin selects for mutants of *Staphylococcus staphylolyticus* having shorter peptide cross-links and a greater percentage of serine in their cross-links (19). We suggest that in the case of *S. oralis* and possibly in the case of *Lactococcus* strains, resistance occurs as a result of the inability of zoocin A to cleave the peptidoglycan cross-link. *S. rattus* strains are closely related to *S. mutans* strains, both having di- or tri-alanine cross-links in their peptidoglycan (23), and yet they differ completely in their sensitivity to zoocin A. This suggests that *S. rattus* at least must possess some novel means of defense against zoocin A. The presence of teichoic acid and lipoteichoic acid in staphylococcal cell walls has been shown to have a significant protective effect against the lantibiotic Pep5 (20), and the presence of exogenous lipoteichoic acid was shown to completely inhibit the binding of pediocin AcH to lactobacillus cells (3). It is interesting to note that of the strains tested in this study, only *S. rattus* and *Lactococcus* spp. possess teichoic acid in their cell walls. However, the possible role of teichoic or lipoteichoic acid in protecting cells from attack by zoocin A has not yet been examined experimentally. For most bacteriocins, including lysostaphin, the producer cell is protected from the effects of its own bacteriocin through the action of a specific immunity product (6). Since all of the *S. zooepidemicus* strains tested (other than strain 4881) are sensitive to zoocin A, it is not unreasonable to speculate that the zoocin A producer strain may also utilize a similar mechanism to protect itself from zoocin A. If this is the case, there may be at least three different mechanisms by which resistance to zoocin A may be effected, i.e., by lacking the specific cell surface determinants recognized by zoocin A (25, 26), by possessing a noncleavable peptidoglycan cross-link, or by producing a specific immunity product.

In summary, we suggest that the killing of streptococci by zoocin A occurs as a result of cleavage of the streptococcal peptidoglycan cross-links by zoocin A. This conclusion is supported by the observed effects of zoocin A on the cell wall of sensitive streptococci, its lack of ability to kill streptococci having direct covalently bonded peptidoglycan cross-links, its ability to enzymatically cleave hexaglycine, and the homology of the N-terminal region of zoocin A to that region of the lysostaphin molecule believed to contain the catalytically active sites (26).

ACKNOWLEDGMENTS

This work was supported by grants from the Otago Medical Research Foundation and the Health Research Council of New Zealand.

REFERENCES

1. **Barnham, M., G. Cole, A. Efstratiou, J. R. Tagg, and S. A. Skjold.** 1987. Characterization of *Streptococcus zooepidemicus* (Lancefield group C) from human and selected animal infections. Epidemiol. Infect. **98:**171–182.
2. **Benz, R., G. Jung, and H.-G. Sahl.** 1991. Mechanism of channel formation by lantibiotics in black lipid membranes, p. 359–372. *In* G. Jung and H.-G. Sahl (ed.), Nisin and novel lantibiotics. Escom Publishers, Leiden, The Netherlands.
3. **Bhunia, A. K., M. C. Johnson, B. Ray, and N. Kalchayanand.** 1991. Mode of action of pediocin AcH from *Pediococcus acidilactici* H on sensitive bacterial strains. J. Appl. Bacteriol. **70:**25–33.
4. **Browder, H. P., W. A. Zygmunt, J. R. Young, and P. A. Tavormina.** 1965. Lysostaphin: enzymatic mode of action. Biochem. Biophys. Res. Commun. **19:**383–389.
5. **Dajani, A. S., D. J. Law, R. O. Bollinger, and P. S. Ecklund.** 1976. Ultrastructural and biochemical alterations effected by viridin B, a bacteriocin of alpha-hemolytic streptococci. Infect. Immun. **14:**776–782.
6. **DeHart, H. P., H. E. Heath, L. S. Heath, P. A. LeBlanc, and G. L. Sloan.** 1995. The lysostaphin endopeptidase resistance gene (epr) specifies modification of peptidoglycan cross bridges in *Staphylococcus simulans* and *Staphylococcus aureus*. Appl. Environ. Microbiol. **61:**1475–1479.
7. **Deibel, R. M., and H. W. J. Seeley.** 1984. Streptococcus Rosenbach, p. 490–509. *In* R. E. Buchanan and N. E. Gibbons (ed.), Bergey's manual of determinative bacteriology, 8th ed. The Williams & Wilkins Co., Baltimore.
8. **Heath, H. E., L. S. Heath, J. D. Nitterauer, K. E. Rose, and G. L. Sloan.** 1989. Plasmid-encoded lysostaphin endopeptidase resistance of *Staphylococcus simulans* biovar *staphylolyticus*. Biochem. Biophys. Res. Commun. **160:**1106–1109.
9. **Higgins, M. L., H. M. Pooley, and G. D. Shockman.** 1970. Site of initiation of cellular autolysis in *Streptococcus faecalis* as seen by electron microscopy. J. Bacteriol. **103:**504–512.
10. **Jack, R. W., J. R. Tagg, and B. Ray.** 1995. Bacteriocins of gram-positive bacteria. Microbiol. Rev. **59:**171–200.
11. **James, S. M., and J. R. Tagg.** 1988. A search within the genera *Streptococcus*, *Enterococcus* and *Lactobacillus* for organisms inhibitory to mutans streptococci. Microb. Ecol. Health Dis. **1:**153–162.
12. **Kline, S. A., J. De la Harpe, and P. Blackburn.** 1994. A colorimetric microtitre plate assay for lysostaphin using a hexaglycine substrate. Anal. Biochem. **217:**329–331.
13. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
14. **Marova, I., and J. Kovar.** 1993. Spectrophotometric detection of bacteriolytic activity of diluted lysostaphin solutions. Folia Microbiol. **38:**153–158.
15. **Nomura, M.** 1967. Colicins and related bacteriocins. Annu. Rev. Microbiol. **21:**257–384.
16. **Peterson, G. L.** 1977. A simplification of the protein assay method of Lowry *et al.* which is more generally applicable. Anal. Biochem. **83:**346–356.
17. **Pugsley, A. P.** 1984. The ins and outs of colicins. II. Lethal action, immunity and ecological implications. Microbiol. Sci. **1:**203–205.
18. **Recsei, P. A., A. D. Gruss, and R. P. Novick.** 1987. Cloning, sequence, and expression of the lysostaphin gene from *Staphylococcus simulans*. Proc. Natl. Acad. Sci. USA **84:**1127–1131.
19. **Robinson, J. M., J. K. Hardman, and G. L. Sloan.** 1979. Relationship between lysostaphin endopeptidase production and cell wall composition in *Staphylococcus staphylolyticus*. J. Bacteriol. **137:**1158–1164.
20. **Sahl, H.-G., C. Hahn, and H. Brandis.** 1985. Interaction of the staphylococcin-like peptide Pep5 with cell walls and isolated cell wall components of gram-positive bacteria. Zentralbl. Bakteriol. Hyg. A **260:**197–205.
21. **Schindler, C. A., and V. T. Schuhardt.** 1964. Lysostaphin: a new bacteriolytic agent for the staphylococcus. Proc. Natl. Acad. Sci. USA **51:**414–421.
22. **Schleifer, K. H., and O. Kandler.** 1972. Peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriol. Rev. **36:**407–477.
23. **Schleifer, K. H., R. Kilpper-Balz, J. Kraus, and F. Gehring.** 1984. Relatedness and classification of *Streptococcus mutans* and "mutans-like" streptococci. J. Dent. Res. **63:**1047–1050.
24. **Schofield, C. R., and J. R. Tagg.** 1983. Bacteriocin-like activity of group B and group C streptococci of human and of animal origin. J. Hyg. **90:**7–18.
25. **Simmonds, R. S., J. Naidoo, C. L. Jones, and J. R. Tagg.** 1995. The streptococcal bacteriocin-like inhibitory substance, zoocin A, reduces the proportion of *Streptococcus mutans* in an artificial plaque. Microb. Ecol. Health Dis. **8:**281–292.
26. **Simmonds, R. S., W. Simpson, and J. R. Tagg.** Cloning and sequence analysis of *zoo A*, a *Streptococcus zooepidemicus* gene encoding a bacteriocin-like inhibitory substance having a domain structure similar to that of lysostaphin. Gene, in press.
27. **Skilton, C. J.** 1991. M.Sc. thesis. University of Otago, Dunedin, New Zealand.
28. **Skilton, C. J., and J. R. Tagg.** 1992. The production by *Streptococcus sanguis* of bacteriocin-like inhibitory substances (BLIS) with activity against *Streptococcus rattus*. Microb. Ecol. Health Dis. **5:**219–226.
29. **Strominger, J. L., and J. M. Ghuysen.** 1967. Mechanisms of enzymatic bacteriolysis. Science **156:**213–221.
30. **Tagg, J. R., and L. V. Bannister.** 1979. "Fingerprinting" β-haemolytic streptococci by their production of and sensitivity to bacteriocine-like inhibitors. J. Med. Microbiol. **12:**397–411.
31. **Tompkins, G. R.** 1986. Ph.D. thesis. University of Otago, Dunedin, New Zealand.
32. **Wang, X., B. J. Wilkinson, and R. K. Jayaswal.** 1991. Sequence analysis of a *Staphylococcus aureus* gene encoding a peptidoglycan hydrolase activity. Gene **102:**105–109.