

Gentamicin Interaction with *Pseudomonas aeruginosa* Cell Envelope

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Gentamicin, an aminoglycoside antibiotic known to inhibit protein synthesis, had a detrimental effect on the integrity of the cell wall of *Pseudomonas aeruginosa* ATCC 9027 (a susceptible strain) as shown by electron microscopy using negative-staining, thin-sectioning, and freeze-fracture techniques. The disruption occurred in a sequential manner, moving from the outer membrane to the inner membrane, and could result in lysis of the cell. During this process the outer membrane lost 34% of its total protein and 30% of its lipopolysaccharide (measured as 2-keto-3-deoxyoctonate) upon exposure to 25 µg of gentamicin per ml for 15 min. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the outer membrane proteins showed altered banding patterns after exposure to gentamicin. Atomic absorption spectrophotometry revealed a decrease in magnesium and calcium content (18 and 38%, respectively) in the cell envelopes after gentamicin treatment. It is proposed that gentamicin displaces essential metal cations within the outer membrane, consequently destabilizing and extracting organic constituents. Small transient holes are thereby produced which make the outer membrane more permeable to the antibiotic and which expose the protoplast to high concentrations of gentamicin. This membrane effect may contribute to the effects of protein synthesis inhibition during the killing process.

The accepted mechanism of action of the aminoglycoside antibiotics on sensitive strains of *Pseudomonas aeruginosa* requires that the antibiotic gain access to the cytoplasm by an energy-requiring process (2). Once inside, it is believed that these drugs work at the ribosomal level by causing either misreading or suppression of protein synthesis (4). There are three mechanisms by which bacteria may circumvent the action of aminoglycoside antibiotics. First, organisms may have altered ribosomes to which the antibiotics cannot bind. Because of the rarity of this occurrence, such organisms are not clinically significant (8). A second mechanism, which is of major clinical importance, is the inactivation of aminoglycosides by plasmid-coded enzymes (4). A third mechanism involves protection against antibiotic penetration by a permeability barrier (26). Studies with hypersensitive mutants have indicated that this resistance is at a structural level in the organism (23).

Permeability resistance may be enhanced in normally sensitive strains of *P. aeruginosa* by the presence of divalent cations. Studies originally undertaken to improve the testing methods for gentamicin resistance in *P. aeruginosa* have shown that concentrations of calcium and magnesium in the medium greatly influence susceptibility of *P. aeruginosa* to antibiotics (31); some strains of *P. aeruginosa* are more resistant to aminoglycosides, polymyxins, and colistin in the presence of increased amounts of magnesium or calcium. This phenomenon is a particular property of *P. aeruginosa* and is not due to modification of the antibiotics by divalent cations (31). The divalent cation-modified resistance of *P. aeruginosa* is believed to be a property of the outer membrane (OM) and has been variously described as a function of the proteins, lipopolysaccharides (LPS), or protein-LPS complexes of this membrane (3, 6, 9).

The uptake of gentamicin into *P. aeruginosa* cells has been recognized as consisting of three stages, first an electrostatic interaction with the exterior of the cell and then two uptake stages which require an energized membrane and which can be disrupted by electron transport inhibitors (3).

Our studies on the interaction of gentamicin with the cell

envelope of *P. aeruginosa* and its mode of penetration into the cell show that the antibiotic, at MIC or higher levels, is a major perturbant of the OM. This helps explain the mechanism of its penetration across the cell envelope.

MATERIALS AND METHODS

Microorganism and culture conditions. Stock cultures of *P. aeruginosa* ATCC 9027 were maintained at 30°C on Pseudomonas P (Difco) agar slants. The microorganism was grown in shake-flask culture at 37°C in the liquid minimal salts medium (pH 6.8) used by Bauchop and Elsdon (1) with the MgSO₄ · 7H₂O content raised to 1.6 mM. All cultures were harvested in mid- to late exponential growth phase and washed free of residual medium by centrifugation.

Sample preparation for electron microscopy. The MIC of the antibiotic on *P. aeruginosa* was 5 µM of gentamicin per ml. Two milliliters of a cell suspension in 100 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (Sigma Chemical Co.) was added to 50 ml of 0, 5, 25, or 50 µg of gentamicin (Sigma Chemical Co.) per ml in the presence or absence of 1 mM KCN or NaN₃ (Fisher) in 100 mM HEPES (pH 6.8). The KCN or NaN₃ was added at the same time as gentamicin to produce a 1 mM concentration. Because each of these reagents stops cellular energy production, their addition ensured the absence of active gentamicin uptake, the cessation of growth, and the absence of envelope repair. The suspension of cells and antibiotic (final optical density at 600 nm = 1.0) was incubated at 22°C with frequent mixing for 0, 5, 10, 15, and 30 min, and after each time period a 5-ml sample was taken from the reaction vessel and washed once with 100 mM HEPES (pH 6.8) containing 0.1% glutaraldehyde.

(i) **Negative staining.** Copper grids which had been coated with carbon and Formvar (Taab Laboratories, Reading, England) were touched to drops of prepared cells. The grids were then stained by the method of Horne and Ronchetti (18) using ammonium molybdate and uranyl acetate.

(ii) **Thin sectioning.** Glutaraldehyde (5%) and osmium tetroxide (1%) fixed cells were dehydrated in an ethanol series (25, 50, 80, 95, and 100%) and embedded in Epon 812. Thin sections were then cut using a Reichert Ultramicrotome

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OM U4 Ultracut. Sections were collected on copper grids (carbon and Formvar coated) and poststained with uranyl acetate and lead citrate.

(iii) **Freeze-fractured replicas.** Cell preparations, with and without 20% glycerol as a cryoprotectant, were mounted on brass planchets and quickly frozen to -196°C by immersion in liquid Freon 22. Samples were then transferred to the precooled specimen stub (-120°C) of a Balzers High Vacuum Freeze Etch Unit BA 360M and cleaved at -100°C under a vacuum of 1×10^{-6} Torr. The freshly cleaved surface was shadowed with platinum and coated with carbon, and the replicas were cleaned with concentrated H_2SO_4 and 5% hypochlorite and mounted on copper grids.

(iv) **Electron microscopy.** All samples were examined with a Philips EM300 transmission electron microscope operating at 60 kV and equipped with a liquid nitrogen cold trap.

Measurement of gentamicin binding. The binding of gentamicin to whole cells was measured by monitoring the amount of radioactivity associated with the cells after exposure for 1.0, 2.5, 5.0, 10.0, 15.0, 20.0, or 30.0 min to medium containing 25 μg of [^{14}C]gentamicin per ml. Harvested mid-exponential growth phase cells were added to fresh growth medium containing [^{14}C]gentamicin at a final cell concentration of 0.8 mg of cells (dry weight) per ml. After each exposure, a 10-ml portion of cells was filtered through a 0.45- μm membrane filter (Millipore Corp., Bedford, Mass.). The filters were then washed with 10 ml of 3% NaCl, dried, and suspended in Aquasol (New England Nuclear Corp., Boston, Mass.), and the associated radioactivity was determined. In a separate experiment the cell envelope binding of gentamicin was measured. A reduced quantity of [^{14}C]gentamicin was used to bring the binding of the antibiotic down to the level of whole-cell binding. Actively growing cells were exposed to 10 μg of [^{14}C]gentamicin per ml for 15 min in growth medium. Samples were taken for liquid scintillation counting after the whole cells were washed with buffer and after the envelope fraction was isolated by French pressure cell breakage (see below).

OM preparation. A total of 30 liters, in 3-liter batches, of *P. aeruginosa* cells was grown to mid-exponential growth phase in minimal salts medium. The cells were harvested by centrifugation and washed twice with 100 mM HEPES (pH 6.8). The washed pellet was either incubated in 100 mM HEPES for 15 min at room temperature (control) or incubated in 25 μg of gentamicin per ml in 100 mM HEPES at room temperature for 15 min (treated). Each of these preparations was then processed as follows. (i) The cells were washed twice by centrifugation at room temperature in 100 mM HEPES (pH 6.8). (ii) They were then suspended in 100 μg of RNase (type III-A, Sigma Chemical Co.) per ml–50 μg of DNase (beef pancreatic DNase type I, Sigma Chemical Co.) per ml–1 mg of lysozyme (Sigma Chemical Co.) per ml–10 μg of MgCl_2 per ml and kept on ice. (iii) The suspended cells were passed twice through a cold French pressure cell operating at 18,000 lb/in². (iv) The preparation was then centrifuged to remove whole cells, and the supernatant was centrifuged at 30,000 $\times g$ for 45 min at 4°C to pellet envelope fragments. (v) These fragments were washed five times by centrifugation to remove contaminating cytoplasmic material and then were lyophilized. The lyophilized material was then separated into inner membrane (IM) and OM material by the method of Hancock and Nikaido (16) using sucrose density gradient centrifugation. After extensive dialysis against distilled, deionized water, the material was lyophilized.

Biochemical assays. A Unicam SP-500 Series 2 spectropho-

tometer was used for all colorimetric assays. Protein contents were determined by the method of Markwell et al. (22). 2-Keto-3-deoxyoctonate content was measured by the method of Karkhanis et al. (20) and was used as a measure of LPS (30).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The method of Lugtenberg et al. (21) with modifications by Hancock and Carey (15) was used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Atomic absorption spectrophotometry. A Perkin-Elmer model 2380 Atomic Absorption Spectrophotometer with an HGA-400 graphite furnace was used to determine the magnesium and calcium content of the OM before and after treatment with gentamicin. OM material (1 mg/ml) was suspended in distilled, deionized water ($R = 5$ megaohm) and then diluted to a metal concentration which fit within the linear range of magnesium or calcium standard curves. Standard curves were constructed using anhydrous MgCl_2 and CaCl_2 dissolved in water at concentrations of 0.2 to 1.0 nM.

RESULTS

Electron microscopy. Negative stains of *P. aeruginosa* whole mounts indicated that the presence of gentamicin at all of the employed concentrations caused some change in cell wall organization. Although the MIC (5 $\mu\text{g}/\text{ml}$) caused the same structural response to the envelope, higher concentrations increased the frequency of the envelope defect and made observation easier. For this reason, an antibiotic concentration of 25 $\mu\text{g}/\text{ml}$ was most frequently used. Comparison of the control cells (Fig. 1A) with the treated cells (Fig. 1B) showed a great increase in the occurrence of membrane blebs on the cell surface. At a concentration of 25 μg of gentamicin per ml the blebs were visible after approximately 1 min of antibiotic incubation. Longer exposures, of up to 30 min, caused many of the cells to lyse.

Thin-sectioned material extended the negative-staining observations (Fig. 2): a sequential process of cell envelope disruption was seen after addition of gentamicin. First, numerous blebs were seen on the cell surface, which appeared to be OM material (Fig. 2B). Second, holes in the OM became visible (Fig. 2C). Immediately underneath the OM the peptidoglycan appeared to be disrupting, as was the underlying plasma membrane (Fig. 2D). Eventually some cells lysed. The entire process could be followed more easily in the presence of 1 mM sodium azide or 1 mM potassium cyanide which inhibited further growth (as measured by increase in optical density at 600 nm in the absence of antibiotic) and the repair of the envelope (Fig. 3).

To substantiate the thin-section results without the use of chemical fixatives such as glutaraldehyde, untreated and gentamicin-treated *P. aeruginosa* cells were rapidly frozen and freeze-fractured. The treated cells, exposed to 25 μg of gentamicin per ml for 15 min, showed significantly altered OM fracture (OMF) surfaces as compared with the control cells (Fig. 4 through 6). The outside surface of the cell wall became wrinkled after gentamicin treatment. Fractures through the OM leaflet exposed the concave OMF and convex OMF surfaces of the hydrophobic interior of the OM. When compared with those of the control cells, both the gentamicin-treated concave and convex OMF faces showed significant structural differences (Fig. 5 and 6). The homogeneous arrangement of the particles exposed in this plane of cleavage in the controls changed to a disorganized arrangement after gentamicin treatment. This reflects the molecular organization of the outer surface of the OM in gentamicin-treated samples. The plasma membrane had also

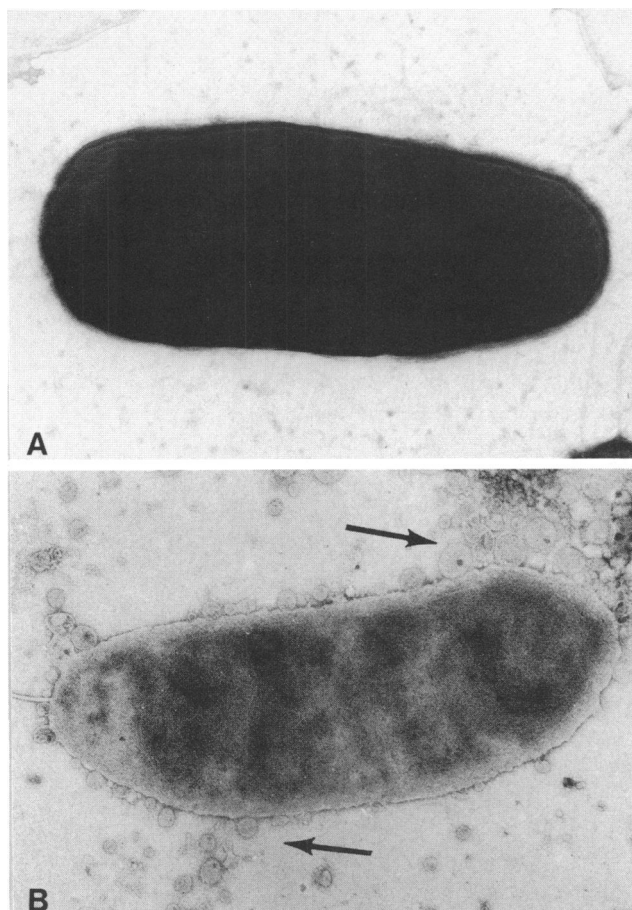


FIG. 1. Negatively stained whole-cell mounts of *P. aeruginosa*. (A) Control cell; (B) cell treated with 25 µg of gentamicin per ml for 15 min.

gentamicin-treated samples. The plasma membrane had also been fractured during the freeze-fracturing process. This membrane appeared intact, but there was either a reorganization, or a loss, of the large aggregates of intramembranous particles on the convex fracture face of the plasma membrane (Fig. 4).

Binding of gentamicin to whole cells. Since gentamicin was obviously affecting the intrinsic structure of the OM of *P. aeruginosa*, the magnitude of gentamicin binding to whole cells was measured. For our strain, the binding showed three phases (Fig. 7) which were similar to those observed in *P. aeruginosa* 280, as reported by Bryan and van den Elzen (2). The initial phase was essentially instantaneous, complete within a few seconds (or before we could measure it), and was presumably due to the binding of the antibiotic to the cell envelope. This was followed by a linear 10-min period of slow uptake. The third stage consisted of a rapid uptake period which began to decrease, or plateau, after several minutes. As with *P. aeruginosa* 280, KCN or NaN_3 inhibited second-stage uptake (data not shown), which is evidence that the final two stages are energy driven and is comparable to the results of Bryan and van den Elzen (2). [^{14}C]gentamicin binding experiments were also undertaken to determine whether gentamicin was either actually interacting with and binding to the cell envelope, simply passing through the cell envelope into the cytoplasm, or remaining in the external milieu. After 15 min of incubation there was no substan-

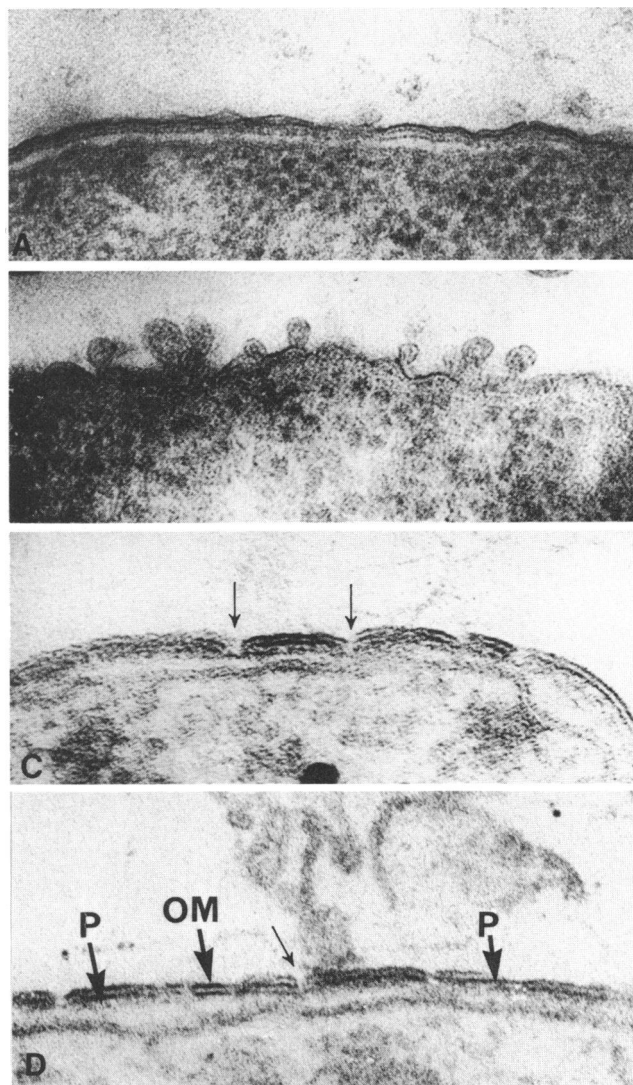


FIG. 2. Thin-sectioned *P. aeruginosa* cells poststained with uranyl acetate and lead citrate. (A) control cell; (B) cell exposed to 25 µg of gentamicin per ml for 2 min; (C) cell exposed to 25 µg of gentamicin per ml for 10 min; (D) cell exposed to 25 µg of gentamicin per ml for 15 min. Small arrows indicate holes in OM and peptidoglycan layer (P).

tial difference in the amount of radioactivity associated with whole cells (23.4 dpm/µg of protein) or cell envelopes (30.0 dpm/µg of protein) as measured on a per-gram-of-protein basis. The cells and cell envelope samples in the assay that included 1 mM KCN had consistently lower amounts of [^{14}C]gentamicin associated with them than those without KCN (whole cells, 16.7 dpm/µg of protein; envelopes, 18.5 dpm/µg of protein).

Biochemical analysis. Isolated *P. aeruginosa* OM was analyzed for protein and 2-keto-3-deoxyoctonate content before and after gentamicin treatment to determine whether a particular component of the cell wall was being lost during the treatment. A decrease in total protein of 34% and a decrease in 2-keto-3-deoxyoctonate of 30% were noted, indicating that both OM components were equally affected (Table 1).

OM samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to determine whether

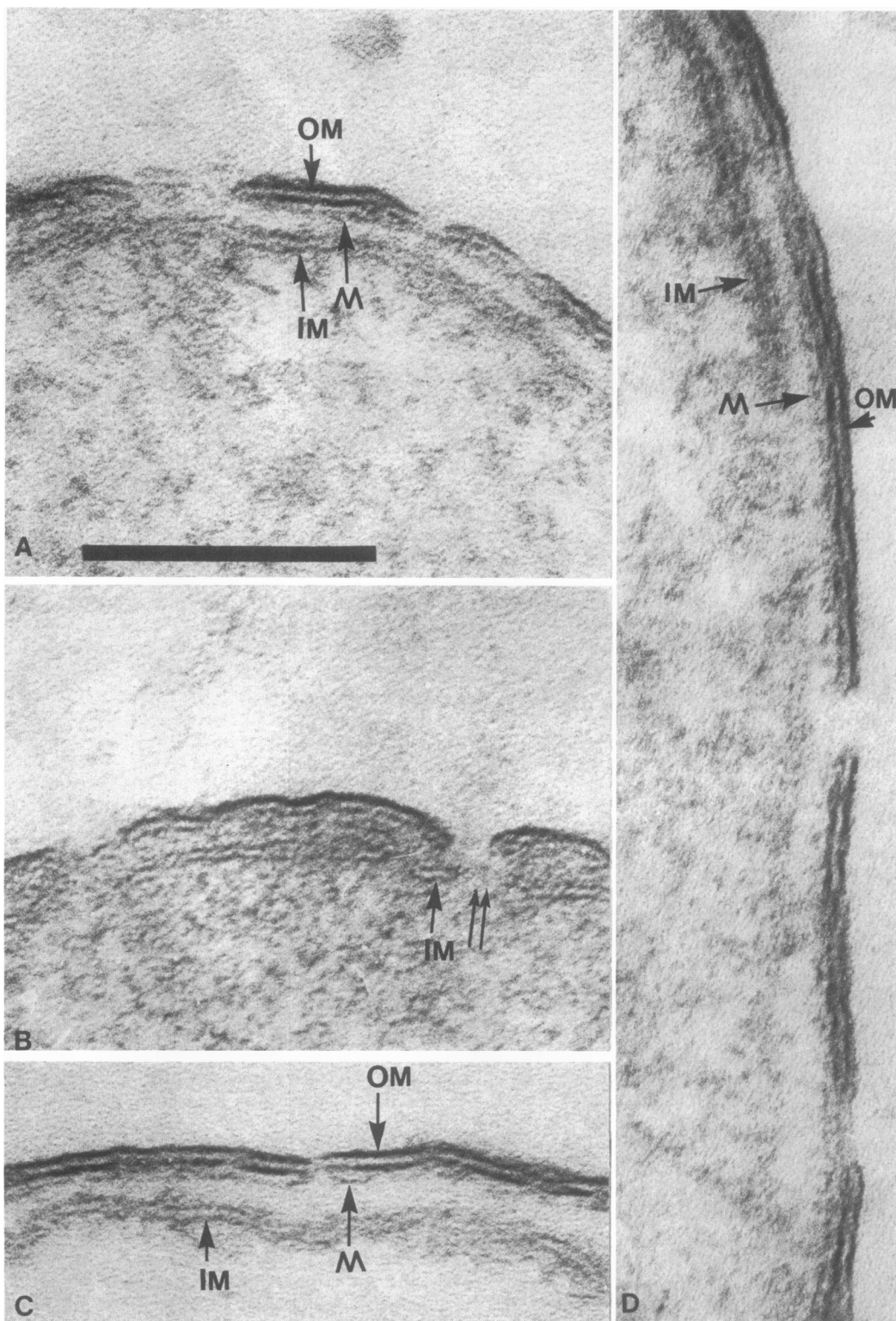


FIG. 3. Thin-sectioned *P. aeruginosa* cells treated with 1 mM potassium cyanide or 1 mM sodium azide in addition to 25 μ g of gentamicin per ml. (A) holes in OM after 15 min of antibiotic exposure in the presence of azide; (B) holes in both the OM and IM after 30 min of exposure to antibiotic in the presence of cyanide (double arrows); (C) visible holes in the OM, peptidoglycan (M), and IM after 30 min of exposure to antibiotic; (D) holes in the OM and the IM, with extensive degradation of the peptidoglycan (M).

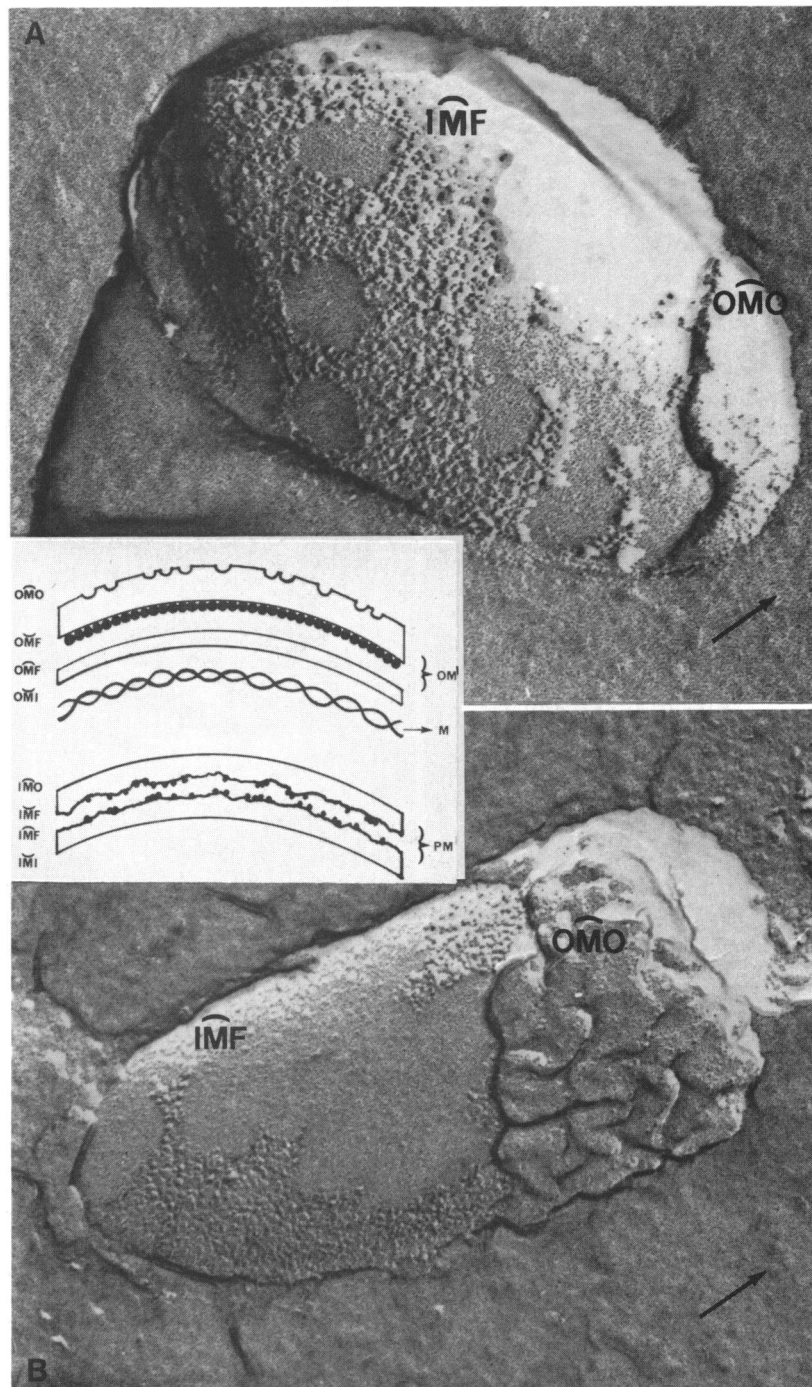


FIG. 4. Freeze-fractured replicas of *P. aeruginosa* cells showing the outer surface of the OM. Inset: Schematic representation of the fracture planes of the IM and OM of a gram-negative bacterium. Micrographs are labeled with symbols corresponding to those on the diagram. (A) Control cell; (B) cell exposed to 25 μ g of gentamicin per ml for 15 min.

specific proteins were removed from the envelope as a consequence of gentamicin exposure. The protein profile of the gentamicin-treated sample had changed as compared with the control sample. There was a protein band in the untreated control adjacent to the 20,100-molecular-weight marker; in the gentamicin-treated sample, this protein band was about one-half the thickness of that in the untreated sample, and an additional thin protein band was positioned just below it (Fig. 8). In addition, gel scans indicated that

there was a consistent decrease in the amount of protein F in the gentamicin-treated sample relative to the amounts of proteins D1, D2, and E (data not shown).

As magnesium and calcium have been shown to significantly affect the activity of aminoglycosides (25, 31), OM samples from untreated and gentamicin-treated cells were assayed for magnesium and calcium content. It was found that levels of both elements were substantially reduced in the OM of gentamicin-treated cells (Table 1). After a 15-min

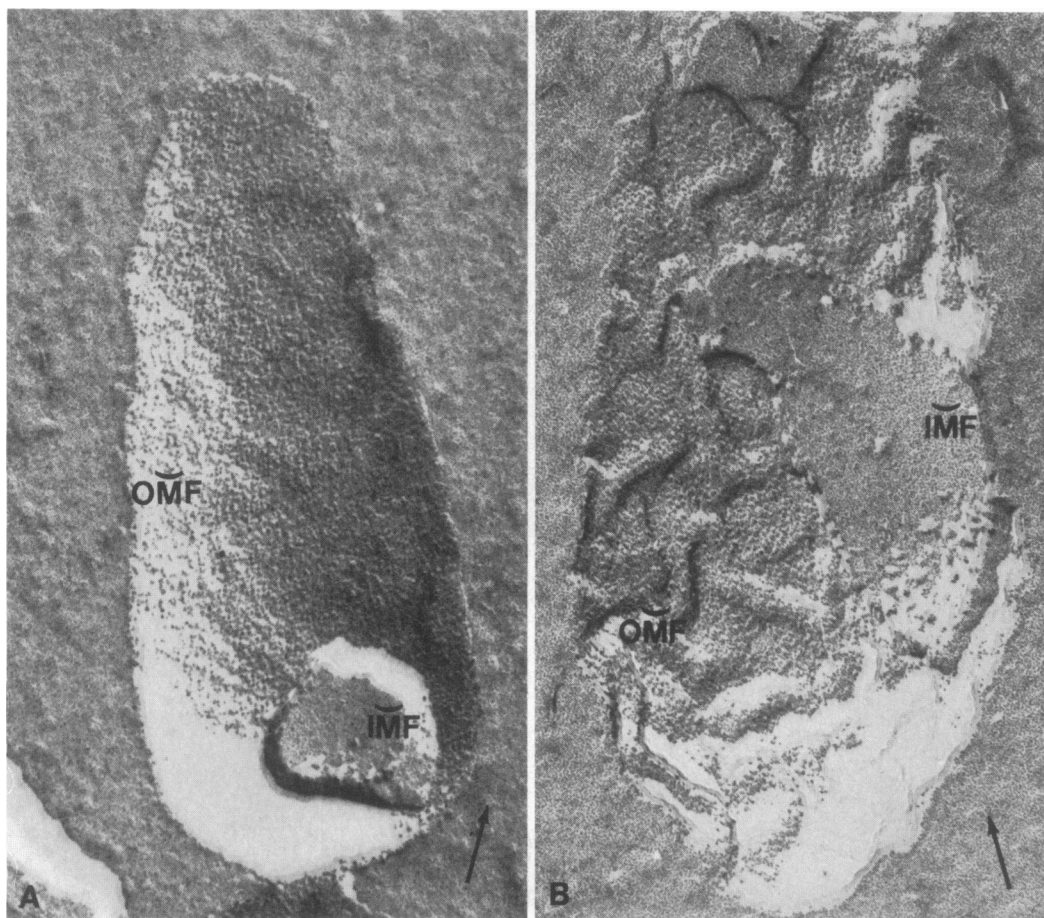


FIG. 5. Freeze-fractured replicas of *P. aeruginosa* showing the concave inner surface of the OM. (A) Control cell; (B) cell exposed to 25 μ g of gentamicin per ml for 15 min.

exposure to gentamicin, the magnesium content of the OM decreased by 18% while the calcium content decreased by 38%.

DISCUSSION

Freeze-fracturing is a process which requires no chemical fixatives and is therefore free of possible deformations caused by these chemicals. This factor and the rapid freezing of the specimen ensure a specimen as unaltered as possible during processing. In this sense, freeze-fracturing images lend conclusive support to the data obtained from negative stains and thin-sectioned material. The freeze-fractured replicas of *P. aeruginosa* indicated that gentamicin induced a disruption of the cell wall, most predominantly seen in the OM. These images (Fig. 4 to 6) showed the OM to be wrinkled and undergoing bleb formation. The wrinkling effect was limited to the OM and, therefore, is not a result of shrinking of the entire cell due to osmotic or freezing effects. There was also a change in the particle distribution within the hydrophobic plane of the IM. These IM particles typically appeared to pool together under the freezing conditions used for freeze-fracturing (reference 29 and Fig. 4A). In the gentamicin-treated cells the aparticle regions had grown in size (Fig. 4B). Since gentamicin caused disorganization of the OM and since the IM is a more loosely knit membrane, it was expected that some disruption would occur in the IM. However, the significance of the disturbance observed in the IM has not been studied further.

The envelope perturbation seen with freeze-fracturing was substantiated by both the negatively stained and thin-sectioned samples. A definite temporal sequence of envelope disruption was evident upon exposure of the cells to gentamicin. First, there was blebbing of OM membranous material away from the cell. This was followed by the appearance of holes in the OM, then in the peptidoglycan, and finally in the IM. Some cell lysis followed this sequence of events.

Some membrane blebbing can be a common occurrence during the growth of *P. aeruginosa*; however, gentamicin treatment elevated the blebbing far above normal amounts. Gentamicin-induced blebbing could readily be distinguished from background levels by electron microscopy (cf. Fig. 1A and B). The release of OM material was the first visual indication that gentamicin had come into contact with the cell. Similar blebs were seen when *Escherichia coli* K-12 was treated with gentamicin (unpublished data) or when *E. coli* B was treated with dideoxykanamycin A and streptomycin (19). In the latter case, it was found that blebbing did not occur if the cells were preincubated with chloramphenicol, tetracycline, or erythromycin, suggesting that active protein synthesis was a prerequisite for blebbing to occur (17, 19). In our work, blebbing still occurred in the presence of NaN_3 or KCN; it is unlikely that protein synthesis was continuing under these conditions, although we did not monitor this aspect. Other reagents which cause excessive membrane blebbing are EDTA (5, 27) and polymyxin (11, 12, 28). EDTA removes divalent cations from the membrane, which

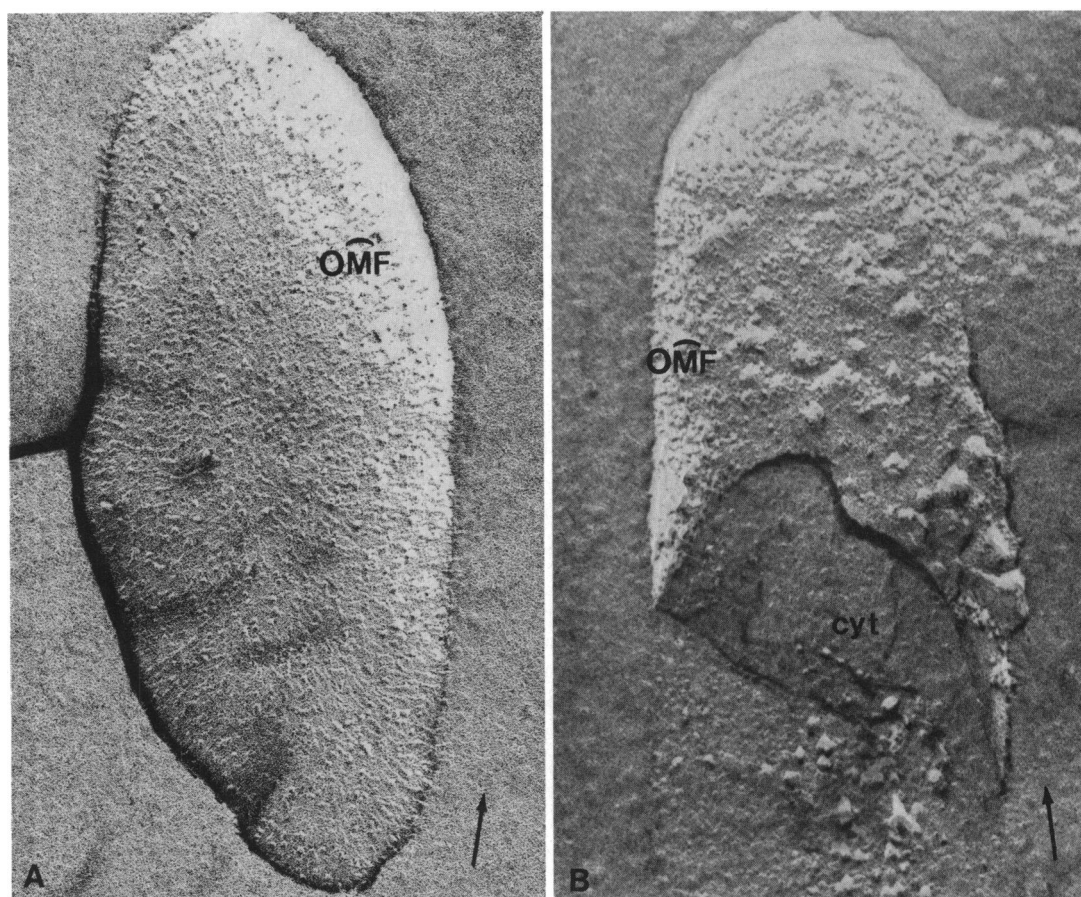


FIG. 6. Freeze-fractured replicas of *P. aeruginosa* showing the convex fracture plane of the OM (inner half of the OM leaflet). (A) Control cell; (B) cell exposed to 25 µg of gentamicin per ml for 15 min. cyt, Cytoplasm.

causes concomitant release of both LPS and protein in *P. aeruginosa* (10). Polymyxin also appears to displace magnesium and calcium, and it has been proposed that divalent cations, bound at a phosphate site on the LPS molecule, are required for OM stability (7, 24, 25). Since gentamicin appears to displace calcium and magnesium in the OM of our system, it must destabilize the membrane in a manner similar to that for EDTA and polymyxin. Indeed, biochemical

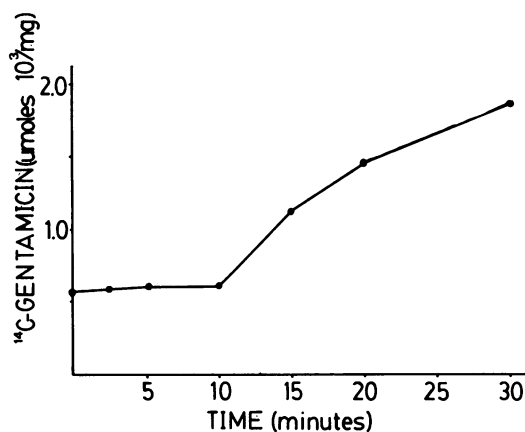


FIG. 7. Rate of association of 25 µg of [¹⁴C]gentamicin per ml to whole cells.

analyses of the OM which remained associated with the cell showed that protein and LPS had been extracted during gentamicin treatment. Once the OM was destabilized this material was released as small vesicles and was the conditional result of the removal of constituent Mg²⁺ and Ca²⁺.

The second physical stage of the gentamicin-cell wall interaction involved the appearance of distinct holes in the OM. In growing cells we imagine the holes to be transient, since continued synthesis of new OM material would reneal the membrane lesion; for a short time, however, the OM barrier would be breached. This would happen in a continuous fashion and would result in an increased penetration of gentamicin to the protoplast. Due to their transient nature the holes were easier to locate in thin sections of cells which were treated with KCN or NaN₃; these cells cannot replace lost OM material because the membrane repair mechanisms have been inactivated. Similar OM holes have been ob-

TABLE 1. Biochemical analysis of OM material untreated and treated with 25 µg of gentamicin per ml for 15 min^a

Sample	Protein (µg/ml [dry wt])	KDO ^b (nmol/mg [dry wt])	Calcium (µmol/100 mg [dry wt])	Magnesium (µmol/100 mg [dry wt])
Control	0.48 ± 0.021	138.1 ± 2.4	2.7 ± 0.23	7.6 ± 0.10
Treated	0.38 ± 0.019	97.1 ± 6.2	1.7 ± 0.17	6.2 ± 0.21

^a All data are significant at a 95% confidence level by Student's *t* test.

^b KDO, 2-Keto-3-deoxyoctonate.

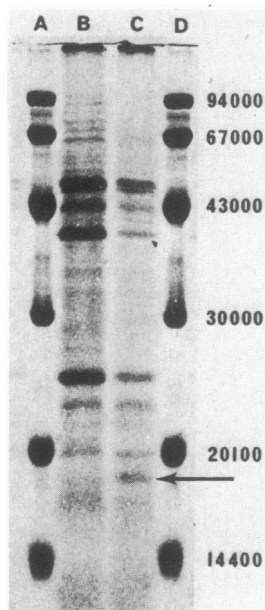


FIG. 8. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the OM of *P. aeruginosa* before (lane B) and after (lane C) 15 min of exposure to 25 µg of gentamicin per ml. Lanes A and D contain molecular weight markers.

served in clinical isolates of *P. aeruginosa* which had been exposed to gentamicin, dideoxykanamycin, or tobramycin (19).

The third stage involves the appearance of holes in the peptidoglycan. These, because of the thinness of the peptidoglycan layer, are difficult to ascertain, but when the specimen geometry was correct, they were seen in all gentamicin preparations and seemed to occur directly beneath the OM holes. They rapidly lead to IM holes and complete lysis. The mechanism of hole generation in the peptidoglycan is not readily understood. Possibly the stringent control on peptidoglycan autolysis is mediated by the state of the OM. If extension of the OM and peptidoglycan during cell growth were coregulated, a disruption of one layer could lead to deregulation in the other.

After the appearance of holes in the peptidoglycan, holes began to appear in the immediately underlying IM. Since the IM of gram-negative bacteria is so fragile in comparison with the OM, it is easy to see how this membrane could also destabilize in the presence of gentamicin upon the perturbation of the outer layers normally protecting it. Once holes appear in the IM, the cells lyse.

Seeing the loss of a significant proportion of the protein in the OM on exposure to gentamicin, we were surprised by the appearance of a new lower-molecular-weight band in the sodium dodecyl sulfate gels (Fig. 8). The most likely explanation for the appearance of this protein is that it is a degradation product induced by OM disruption. Ideally, a cleaved protein should produce two new bands in a gel pattern, but the smaller portion could have run off the gel. Treatment of isolated OM with gentamicin did not produce this new band.

Traditionally, gentamicin has been considered to be an antibiotic which inhibits protein synthesis by binding to the 30S ribosomal subunit. Our results, in agreement with others (see references 13 and 14 for reviews), invoke an OM destabilization mechanism to increase the penetration of the

antibiotic to the protoplast in addition to the effect at the level of protein synthesis. We feel that the sequential disruption of the cell envelope on exposure to gentamicin may also contribute to cell death.

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LITERATURE CITED

1. Bauchop, T., and S. R. Elsdon. 1960. Growth of microorganisms in relation to their energy supply. *J. Gen. Microbiol.* **23**: 457-469.
2. Bryan, L. E., and H. M. van den Elzen. 1975. Gentamicin accumulation by sensitive strains of *Escherichia coli* and *Pseudomonas aeruginosa*. *J. Antibiot.* **28**:696-703.
3. Bryan, L. E., and H. M. van den Elzen. 1977. Effects of membrane-energy mutations and cations on streptomycin and gentamicin accumulation by bacteria: a model for entry of streptomycin and gentamicin in susceptible and resistant bacteria. *Antimicrob. Agents Chemother.* **12**:163-177.
4. Brzezinska, M., R. Benveniste, J. Davies, P. J. L. Daniels, and J. Weinstein. 1972. Gentamicin resistance in strains of *Pseudomonas aeruginosa* mediated by enzymatic N-acetylation of the deoxystreptoamine moiety. *Biochemistry* **11**:761-765.
5. Cheng, K.-J., J. M. Ingram, and J. W. Costerton. 1970. Release of alkaline phosphatase from cells of *Pseudomonas aeruginosa* by manipulation of cation concentration. *J. Bacteriol.* **104**: 748-753.
6. Day, D. F. 1980. Gentamicin-lipopolysaccharide interactions in *Pseudomonas aeruginosa*. *Curr. Microbiol.* **4**:277-281.
7. Deguchi, T., A. Ishii, and M. Tanaka. 1978. Binding of aminoglycoside antibiotics to acidic mucopolysaccharides. *J. Antibiot.* **31**:150-155.
8. Garrod, L. P., H. P. Lambert, and F. O'Grady. 1981. *Antibiotics and chemotherapy*, 5th ed., p. 115-154. Churchill Livingstone, London.
9. Gilbert, P., and M. R. W. Brown. 1978. Influence of growth rate and nutrient limitations on the gross cellular composition of *Pseudomonas aeruginosa* and its resistance to 3- and 4-chlorophenol. *J. Bacteriol.* **133**:1066-1072.
10. Gilleland, H. E., Jr., J. Stinnett, I. Roth, and R. G. Eagon. 1973. Freeze-etch study of *Pseudomonas aeruginosa*: localization within the cell wall of an ethylenediaminetetraacetate-extractable component. *J. Bacteriol.* **113**:417-432.
11. Gilleland, H. E., Jr., and L. B. Farley. 1982. Adaptive resistance to polymyxin in *Pseudomonas aeruginosa* due to an outer membrane impermeability mechanism. *Can. J. Microbiol.* **28**:830-840.
12. Gilleland, H. E., Jr., and R. G. E. Murray. 1976. Ultrastructural study of polymyxin-resistant isolates of *Pseudomonas aeruginosa*. *J. Bacteriol.* **125**:267-281.
13. Hancock, R. E. W. 1981. Aminoglycoside uptake and mode of action—with special reference to streptomycin and gentamicin. I. Antagonists and mutants. *J. Antimicrob. Chemother.* **8**:249-276.
14. Hancock, R. E. W. 1981. Aminoglycoside uptake and mode of action—with special reference to streptomycin and gentamicin. II. Effects of aminoglycosides on cells. *J. Antimicrob. Chemother.* **8**:429-445.
15. Hancock, R. E. W., and A. M. Carey. 1979. Outer membrane of *Pseudomonas aeruginosa*: heat- and 2-mercaptoethanol-modifiable proteins. *J. Bacteriol.* **140**:902-910.
16. Hancock, R. E. W., and H. Nikaido. 1978. Outer membrane of gram-negative bacteria. XIX. Isolation from *Pseudomonas aeruginosa* PAO1 and use in reconstitution and definition of the

- permeability barrier. *J. Bacteriol.* **136**:381-390.
17. Hancock, R. E. W., V. Raffle, and T. Nicas. 1981. Involvement of the outer membrane in gentamicin and streptomycin uptake and killing in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* **19**:777-785.
 18. Horne, R. W., and I. P. Ronchetti. 1974. A negative staining-carbon film technique for studying viruses in the electron microscope. I. Preparative procedures for examining icosahedral and filamentous viruses. *J. Ultrastruct. Res.* **47**:361-383.
 19. Iida, K., and M. Koike. 1974. Cell wall alteration in gram-negative bacteria by aminoglycoside antibiotics. *Antimicrob. Agents Chemother.* **5**:95-97.
 20. Karkhanis, Y. D., J. Y. Zeltner, J. J. Jackson, and D. J. Carlo. 1978. A new and improved microassay to determine 2-keto-3-deoxyoctonate in the lipopolysaccharide of gram-negative bacteria. *Anal. Biochem.* **85**:595-599.
 21. Lugtenberg, B., J. Meijers, R. Peters, P. van der Hock, and L. van Alphen. 1975. Electrophoretic resolution of the major outer membrane proteins of *Escherichia coli* K-12 into 4 bands. *FEBS Lett.* **58**:254-258.
 22. Markwell, M. K., S. M. Hassm, L. L. Bieber, and N. E. Tolbert. 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal. Biochem.* **87**:206-210.
 23. Mills, B. J., and B. W. Holloway. 1976. Mutants of *Pseudomonas aeruginosa* that show specific hypersensitivity to aminoglycosides. *Antimicrob. Agents Chemother.* **10**:411-416.
 24. Nicas, T. I., and R. E. W. Hancock. 1983. Alteration of susceptibility to EDTA, polymyxin B, and gentamicin in *Pseudomonas aeruginosa* by divalent cation regulation of outer membrane protein H1. *J. Gen. Microbiol.* **129**:509-517.
 25. Peterson, A. A., R. E. W. Hancock, and E. J. McGroarty. 1985. Binding of polycationic antibiotics and polyamines to lipopolysaccharides of *Pseudomonas aeruginosa*. *J. Bacteriol.* **164**:1256-1261.
 26. Price, K. E., J. C. Godfrey, and H. Kawaguchi. 1974. Effect of structural modifications on the biological properties of aminoglycoside antibiotics containing 2-deoxystreptamine. *Adv. Appl. Microbiol.* **18**:191-307.
 27. Rogers, S. W., H. E. Gilleland, and R. G. Eagon. 1969. Characterization of a protein-lipopolsaccharide complex released from cell walls of *Pseudomonas aeruginosa* by ethylenediaminetetraacetic acid. *Can. J. Microbiol.* **15**:743-748.
 28. Schindler, P. R. G., and M. Teuber. 1975. Action of polymyxin B on bacterial membranes: morphological changes in the cytoplasm and in the outer membrane of *Salmonella typhimurium* and *Escherichia coli* B. *Antimicrob. Agents Chemother.* **8**:95-104.
 29. Sleytr, U. B., and A. W. Robards. 1982. Understanding the artifact problems in freeze-fracture replication: a review. *J. Microsc.* **126**:101-122.
 30. Weissbach, A., and J. Hurwitz. 1959. The formation of 2-keto-3-deoxyheptonic acid in extracts of *Escherichia coli* B. I. Identification. *J. Biol. Chem.* **234**:704-709.
 31. Zimelis, V. M., and G. G. Jackson. 1973. Activity of aminoglycoside antibiotics against *Pseudomonas aeruginosa*: specificity and site of calcium and magnesium antagonism. *J. Infect. Dis.* **127**:663-669.