Ultrastructural Study of Polymyxin-Resistant Isolates of *Pseudomonas aeruginosa*

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Upon exposure to 6,000 U of polymyxin B sulfate per ml, cells of the polymyxin-sensitive PAO 1 strain of *Pseudomonas aeruginosa* displayed in thin sections long projections arising from the outer membrane of the cell wall and extensive cytoplasmic degradation with accumulation of cytoplasmic membrane infoldings. Polymyxin-resistant isolates derived from the PAO 1 strain, however, grew well in the presence of 6,000 U of polymyxin per ml and exhibited none of these effects, having instead the appearance of a typically healthy cell. Freeze-etching of cells of the sensitive strain grown in basal medium without polymyxin revealed a concave cell wall layer studded with numerous particles. Freeze-etching of cells of the resistant isolates grown in basal medium containing 6,000 U of polymyxin per ml revealed a concave cell wall layer (i.e., the outer half of the outer membrane) in which most of these particles were absent. Thus, acquisition of resistance to polymyxin was correlated with an alteration in the architecture of the outer membrane. When the resistant isolates were grown in the basal medium lacking polymyxin and then freeze-etched, the particle distribution in the concave cell wall layer resembled that of the sensitive parent strain. The cells had regained sensitivity to polymyxin upon suspension in medium containing 6,000 U/ml as determined by their failure to grow and by internal damages seen in thin sections. These cells also had acquired increased sensitivity to ethylenediaminetetraacetate, whereas the polymyxin-resistant cells grown in the presence of polymyxin were resistant to lysis by ethylenediaminetetraacetate. The polymyxin-resistant isolates were not stable mutants but instead represented an adaptive response to the presence of polymyxin in the medium.

There has been considerable interest about both how polymyxin exerts its antimicrobial action on *Pseudomonas aeruginosa* and how resistance to polymyxin is acquired. Polymyxin is thought to interact with phospholipids in the cell envelope (30), causing a disorganization of the cytoplasmic membrane that results in disruption of the osmotic equilibrium of the cell and leakage of the cell contents (21, 24, 30). Koike et al. (15) have shown that treatment of *P. aeruginosa* with polymyxin caused projections to appear on the cell wall and damaged the cytoplasmic membrane with part of the cytoplasmic contents extruding through cracks in the membrane.

Studies of polymyxin-resistant cells have been mainly chemical in nature and without reference to an ultrastructural basis for resistance. An increase in cell wall lipid content appears to be a consistent finding, in resistance not only to polymyxin (6) but to other agents such as a quaternary ammonium compound (2) and carbenicillin (31). Hamilton (11) has advanced the hypothesis that resistance to membrane-active agents is due to the agent not penetrating through the cell wall to reach the sensitive site on the cytoplasmic membrane.

The present study was undertaken to seek an ultrastructural basis for polymyxin resistance in *P. aeruginosa*. If Hamilton's hypothesis is correct, one might expect to see an alteration in the outer membrane of the cell wall correlated with decreased permeability to the antibiotic.

**MATERIALS AND METHODS**

**Selection of polymyxin-resistant isolates.** The parent strain of *P. aeruginosa* used was the PAO 1 strain (12) obtained from B. W. Holloway (Department of Genetics, Monash University, Clayton, Victoria, Australia). The minimal inhibitory concentration of this strain was 32 U of polymyxin per ml. Three milliliters of a logarithmically growing culture in basal medium was added to 40 ml of basal medium.
containing 300 U of polymyxin per ml in 125-ml Erlenmeyer flasks. The flasks were incubated at 30 C with shaking on a water bath equipped with a reciprocal shaker. Flasks exhibiting growth of the organism within 36 h were then used to inoculate new medium containing 3,000 U of polymyxin per ml. Flasks showing growth of the organism in 3,000 U/ml were used to inoculate flasks containing medium with 6,000 U of polymyxin per ml. Upon growth of the organism in medium containing 6,000 U of polymyxin per ml, a loopful of culture from five flasks was streaked onto agar plates and isolated colonies were picked from each plate. Two isolates derived in this manner were chosen for ultrastructural analysis. These isolates will be referred to hereafter as isolates A and B. These resistant isolates were maintained on medium containing 6,000 U of polymyxin per ml. A determination of the minimal inhibitory concentration of the two isolates was made.

Growth conditions. All cells were grown in a basal medium (BM) previously used as "basal medium" in a fragmentary study of P. aeruginosa (9). This medium contained: 0.03 M glucose, 0.04 M K_2HPO_4, 0.022 M KH_2PO_4, 0.007 M (NH_4)_2SO_4, and 0.005 M MgSO_4. The pH was 7.0. The polymyxin-containing medium consisted of this basal medium plus 6,000 U of polymyxin B sulfate per ml (BM + 6,000). The polymyxin B sulfate was obtained from Sigma Chemical Co. (St. Louis, Mo.).

The cells were routinely grown in 125-ml Erlenmeyer flasks containing 40 ml of medium with shaking on a reciprocal shaker in a water bath at 30 C. A starter culture was always used to inoculate a fresh flask of medium to give an initial absorbance at 600 nm (A_{600}) of approximately 0.05. These cells were used in experiments after they had reached mid- to late logarithmic growth phase.

Determination of growth characteristics in the basal media. A 300-ml side arm Erlenmeyer flask containing 20 ml of medium was inoculated with a starter culture to give an initial A_{600} reading of 0.03 to 0.06, and A_{600} readings were taken hourly for 20 h. The ability of the PAO 1 strain and of two resistant isolates to grow in BM and in BM + 6,000 was monitored in this manner.

Thin-sectioning procedures. Initial fixation was accomplished by the addition of a 1/10 volume of fixative solution containing 5% acrolein, 0.4% glutaraldehyde, and 0.05 M sodium cacodylate buffer (pH 7.5) to the shaking culture. After 3 to 5 min the cells were harvested by centrifugation at 5,000 x g for 10 min at room temperature (RT; 22 C) and suspended in 1 to 2 ml of the full-strength fixative solution for a minimum of 3 h at RT. The cells were then washed in buffer, postfixed with osmium tetroxide and uranyl acetate, dehydrated, and embedded in Epon, and sections were cut and post-stained with uranyl acetate and lead citrate as previously described (8).

The following preparations were thin-sectioned according to this standard procedure: the PAO 1 strain growing in BM; the PAO 1 strain after growth in BM, harvesting by centrifugation, and resuspension in BM + 6,000 for 15 min, 30 min, 1 h, 1.5 h, 2 h, and 3 h; resistant isolate A growing in BM + 6,000; isolate A after growth in BM, harvesting by centrifugation, and resuspension in BM + 6,000 for 1 and 3 h; isolate A after subjection to cold shock and osmotic-cold shock procedures as described below; resistant isolate B after growth in BM + 6,000; and isolate B after growth in BM, harvesting by centrifugation, and resuspension in BM + 6,000 for 1 and 3 h.

Cells of strain PAO 1 both growing in BM and after resuspension in BM + 6,000 for 1 and 2 h were also fixed, using 1% osmium tetroxide or 2% crotonaldehyde-2% glutaraldehyde as the initial fixative.

Freeze-etch procedures. Freeze-etching was performed as described previously (32). After growth in BM, the PAO 1 strain was freeze-etched using cryoprotection by suspension of the cells in 30% glycerol for 1 h at RT before freezing. This strain was also freeze-etched both with and without glycerol cryoprotection after growth in BM followed by suspension in BM + 6,000 for 1 or 2 h. Both isolates A and B were freeze-etched with cryoprotection with glycerol after growth in BM and in BM + 6,000. In addition, isolate A was freeze-etched without cryoprotection after growth in BM + 6,000.

Cold shock and osmotic-cold shock procedures. Isolate A was grown in BM + 6,000 until mid-logarithmic growth phase, and then 20-ml samples of culture were harvested by centrifugation. For the cold shock procedure, the pellets were resuspended in 20 ml of either ice-cold BM or ice-cold BM + 6,000. For the osmotic-cold shock procedure, the pellets were resuspended in 20 ml of 25% sucrose and held for 20 min at RT, then harvested by centrifugation, and resuspended in 20 ml of either ice-cold BM or ice-cold BM + 6,000. The cells were incubated in the shocking fluid for 30 min at 30 C after the shock and were then fixed for thin-sectioning as described above. In addition, growth in the medium after shock was monitored at A_{600}. Isolate A growing in the BM + 6,000 used for the resuspension medium and the PAO 1 strain grown in BM and resuspended in ice-cold BM + 6,000 were run as controls.

Determination of sensitivity to EDTA. PAO 1 cells grown in BM and cells of isolate A and B grown in both BM and BM + 6,000 were tested for sensitivity to lysis by ethylenediaminetetraacetae (EDTA) by incubation in a system of 3 ml (total volume) containing a final concentration of 5 mM EDTA and 33 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, pH 9.0. Cells suspended in distilled water were added to give the complete system an initial A_{600} reading of 0.55 to 0.60. Incubation was at RT, and cell lysis was measured as a drop in A_{600} readings.

Negative staining procedure. Two drops of 1% ammonium molybdate at pH 7.0 were mixed with 2 drops of culture of PAO 1 cells after growth in BM and then resuspension in BM + 6,000 for 15 min at 30 C. A thin film of the mixture was picked up with an inoculating loop and broken over an electron microscope grid that had been covered with Formvar and carbon. The excess fluid was absorbed with bibulous
isolating A had a doubling time of 2.0 h in BM and 2.2 h in BM + 6,000 for this same growth phase. The PAO 1 cells were sensitive to the polymyxin in the BM + 6,000 and failed to grow within 20 h of incubation. Isolate B grew with characteristics similar to isolate A in both media.

Observations of thin-sectioned preparations. Under the conditions of fixation used, the cells of the PAO 1 strain were well preserved, with the cytoplasm having the appearance of a normal healthy cell (Fig. 2A). The fixation caused the outer membrane of the cell wall to lose material in the form of small blebs. Treatment of the sensitive PAO 1 cells with 6,000 U of polymyxin per ml rapidly caused a loss in viable cell counts from an initial 10^8 colony-forming units/ml to 10^4 colony-forming units/ml after 15 min of incubation (Gilleland and Murray, unpublished observations). The treated cells were altered ultrastructurally, with the cytoplasm appearing clumped and the outer membrane layer displaying long projections (Fig. 2B). In addition, the treated PAO 1 cells exhibited extensive intrusion of the cytoplasmic membrane (Fig. 3 and 4), often taking on the appearance of myelin figures. These cytoplasmic membrane infoldings appeared as early as 15 min after suspension in the BM + 6,000, increased in size and frequency at 1 or 2 h of exposure, and then became more difficult to find at 3 h, when most of the cells had undergone extensive lysis.

The use of 1% osmium tetroxide or 2% crotonaldehyde-2% glutaraldehyde as the initial fixative revealed findings similar to those seen with initial fixation by acrolein-glutaraldehyde. In particular, the outer membrane of PAO 1 cells grown in BM displayed small blebs in both instances, whereas the polymyxin-treated cells had long projections of outer membrane and extensive cytoplasmic damage with cytoplasmic membrane intrusion.

The polymyxin-resistant isolates A and B both appeared healthy upon growth in BM + 6,000, with no cytoplasmic clumping, none of the extensive cytoplasmic membrane ingrowth, and no blebbing of the outer membrane layer. A typical cell of isolate A grown in BM + 6,000 is shown in Fig. 5. Note that the outer membrane did not bleb even to the extent of the PAO 1 control cell shown in Fig. 2A.

Freeze-etch observations. The PAO 1 strain exhibited a concave cell wall layer studded with particles embedded in or lying upon an underlying smooth layer (Fig. 6A) upon freeze-etching with glycerol cryoprotection after

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RESULTS

Selection of resistant isolates. At each step-up to an increased concentration of polymyxin in the BM, growth was obtained within 24 to 36 h in a majority of the inoculated flasks. Thus, the resistant isolates capable of growth in 6,000 U of polymyxin per ml in BM were easily obtained. It is worthy of note, however, that when the same procedure had been tried previously using nutrient broth, none of the flasks containing 300 U of polymyxin per ml exhibited growth within 72 h despite repeated attempts. Therefore, it would appear that the emergence of polymyxin-resistant cells is inhibited in nutrient broth but not in BM containing glucose as the sole source of carbon and energy.

The minimal inhibitory concentration of isolate A was 7,000 U of polymyxin per ml, and that of isolate B was 10,000 U/ml.

Growth characteristics in the basal media. Isolate A grew well in both BM and BM + 6,000, growing at a rate and to a cell concentration in both media that compared favorably with that of the PAO 1 strain in BM (Fig. 1). The PAO 1 strain had a doubling time of 1.8 h in early logarithmic growth phase, whereas
FIG. 2. *P. aeruginosa* PAO 1. (A) Typical healthy cell after growth in BM. Note the small blebs which arise from the outer membrane (arrows). (B) Cell fixed after suspension in BM + 6,000 for 30 min. The cytoplasm appears clumped, and numerous long projections of outer membrane are seen. The horizontal bar in all figures represents 100 nm. In all figures showing thin-sectioned cells, initial fixation was with acrolein-glutaraldehyde.
growth in BM. This appearance for this outer membrane fracture surface agrees with that seen previously in \textit{P. aeruginosa} strain OSU 64 cells grown in trypsic soy broth (10) and in basal medium (9).

The resistant isolates both exhibited the same alteration of this cell wall layer, and an example (isolate A) is shown in Fig. 6B. A loss of the majority of the particles in this layer is readily apparent. Freeze-etching of isolate A cells without glycerol after growth in BM + 6,000 did not reveal any alterations of the outer surface of the cell wall; i.e., no additional layers that might have afforded protection against polymyxin were found. The only alteration in the cell envelope discernible was the loss of particles from the concave cell wall layer, which corresponds to the inner surface of the outer track of the outer membrane (10, 26).

Freeze-etch of PAO 1-sensitive cells after exposure to BM + 6,000 for 1 to 2 h revealed that the particles in the concave cell wall layer were not removed from this layer by exposure to polymyxin (Fig. 7A). The treated cells were found to contain cytoplasmic membrane infoldings (Fig. 7B and C, compare with Fig. 4D) similar to those seen in thin-sectioned cells. However, there was no evidence of the extensive projections from the outer membrane seen in thin-sectioned cells found in treated cells freeze-etched either with (Fig. 7A-C) or without glycerol. Likewise, negative staining of PAO 1 cells exposed to BM + 6,000 for 15 min showed cytoplasmic membrane ingrowth (Fig. 7D) but failed to reveal outer membrane projections.

**Effects of cold shock and osmotic-cold shock procedure on polymyxin-resistant cells.** The cells of isolate A were both ultrastructurally altered (Fig. 8B and D) and inhibited in their growth (Fig. 9) by cold shock or osmotic-cold shock using BM + 6,000. However, the use of cold shock or osmotic-cold shock with BM did not lead to these ultrastructural alterations (Fig. 8A and C) or to as great an inhibition of growth (Fig. 9). The controls for this experiment were as expected, with isolate A growing in the BM + 6,000 without any shock procedure having no ultrastructural alterations and PAO 1 cells cold shocked with BM + 6,000 undergoing the same sort of alterations as did isolate A (Fig. 8B and D). It appeared that cold shock and osmotic-cold shock overcame the resistance to polymyxin that the isolate A cells possessed normally after growth in BM + 6,000.

**Effects of growth of the resistant isolates in BM.** When both isolates A and B were transferred from growth in BM + 6,000 to growth in BM, they underwent an alteration in the concave cell wall fracture surface as seen in freeze-etched cells. This cell wall layer once again became studded with particles (Fig. 10A) quite similar to those of the sensitive PAO 1
cells (Fig. 6A). This indicated a reversion of the outer membrane architecture to that of the sensitive cell.

Suspension of the formerly resistant cells into BM + 6,000 caused ultrastructural alterations such as were found in sensitive cells (Fig. 10B). When growth was monitored by $A_{600}$ readings upon shift back to BM + 6,000 from BM, there was no increase in readings within the 27-h period in which readings were taken. These observations indicated that resistance to polymyxin was lost upon growth in the absence of polymyxin.

**Lysis by EDTA-Tris.** The control cells of the PAO 1 strain rapidly lysed in the presence of EDTA-Tris (Fig. 11A). The response of the cells of isolates A and B varied according to which medium the cells were grown in. Cells
of isolate B grown in BM + 6,000 were relatively resistant to EDTA-Tris, whereas growth in BM caused the cells to exhibit markedly increased sensitivity to EDTA-Tris (Fig. 11A). Cells of isolate A followed the same trend as isolate B, but the cells grown in BM, although more sensitive than those grown in BM + 6,000, were still significantly resistant to lysis by EDTA-Tris (Fig. 11B). Only upon extension of the time of incubation did the increased sensitivity of the cells of isolate A grown in BM over those grown in BM + 6,000 become apparent.

**DISCUSSION**

Chemical studies (21, 24, 30) have previously indicated that polymyxin acts on sensitive cells by a rapid attachment to cytoplasmic membrane sites, causing loss of the osmotic barrier followed by autolysis due to breakdown of cytoplasmic constituents like ribosomes. A previous ultrastructural study by Koike et al. (15) on the effect of polymyxin on *P. aeruginosa* showed cytoplasmic extrusion through breaks in the cytoplasmic membrane and projections formed from the cell wall layers. Wahn et al. (33) showed high concentrations of polymyxin-produced protuberances from the cell surface and rapid cytoplasmic destruction in *Escherichia coli*. Our thin-sectional observations on the anatomic effects of polymyxin on sensitive cells of *P. aeruginosa* PAO 1 were similar in that we also found projections from the outer membrane and extensive cytoplasmic breakdown (Fig. 2B). In addition, the high concentration of polymyxin caused extensive ingrowth (Fig. 3) and infoldings (Fig. 4) of the cytoplasmic membrane, which supports the concept of polymyxin affecting sensitive sites on the cytoplasmic membrane. The long projections of outer membrane found in thin sections of polymyxin-treated sensitive cells were not found in either freeze-etched (Fig. 7A–C) or negatively stained cells (Fig. 7D). This suggested that these projections are not due directly to the action of polymyxin on the outer membrane but rather that polymyxin interacts with the outer membrane in such a way that subsequent fixation or dehydration produces the projections. These projections were found if either osmium tetroxide or crotonaldehyde-glutaraldehyde was used as the initial fixative in place of acrolein-glutaraldehyde. It should be noted that PAO 1 cells grown in BM displayed small outer membrane blebs, indicating a tendency of the outer membrane to produce such projections even in the absence of polymyxin under the conditions of fixation and embedding used. The infoldings of cytoplasmic membrane were seen in freeze-
Fig. 6. Freeze-etching using glycerol cryoprotection. (A) The PAO 1 strain grown in BM. The concave cell wall layer, which corresponds to the inner surface of the outer track of the outer membrane, is studded with numerous particles embedded in or resting upon an underlying smooth layer. (B) The polymyxin-resistant isolate A grown in BM + 6,000. Note that the concave cell wall layer has lost most of the particles, indicating that an alteration in the outer membrane architecture has occurred. The arrowhead in the upper left corner in these and all following freeze-etch micrographs represents the direction from which the metal was evaporated in the production of the replica.
Fig. 7. *P. aeruginosa* PAO 1 after exposure to polymyxin. (A) Cell freeze-etched with glycerol showing the concave cell wall layer after 2 h of suspension in BM + 6,000. The number of particles in this layer has not been reduced. (B) Cross-fractured cell after 2 h in BM + 6,000 which reveals a membranous infolding similar to that seen in thin section in Fig. 4D. (C) Cross-fractured cell after 1 h of exposure to BM + 6,000. Cytoplasmic membrane infoldings are seen. Note that no evidence of the long projections of outer membrane seen in thin-sectioned cells can be found in these freeze-etched cells (A–C). (D) Cell negatively stained with ammonium molybdate after 15 min of exposure to BM + 6,000. A large cytoplasmic membrane invagination can be seen, but no cell wall blebbing is apparent.
FIG. 8. Isolate A grown in BM + 6,000 and subjected to various shock procedures. (A) Cold shocked with ice-cold BM. No evidence of cytoplasmic damage or membrane ingrowth is found. (B) Cold shocked with ice-cold BM + 6,000. Cytoplasmic membrane infolding and layering is seen (arrows). Compare with Fig. 4C. (C) Osmotically cold shocked with ice-cold BM after suspension in 25% sucrose for 20 min at RT. No evidence of cytoplasmic membrane ingrowth is found. (D) Osmotically cold shocked with ice-cold BM + 6,000 after suspension in 25% sucrose for 20 min at RT. Extensive cytoplasmic membrane infolding has occurred.
Fig. 9. Growth of isolate A after various shock procedures. The cells were grown in BM + 6,000, harvested by centrifugation, and then shocked. Symbols: •, cells cold shocked by suspension in ice-cold BM; ◇, cells cold shocked by suspension in ice-cold BM + 6,000; ■, cells osmotically cold shocked by suspension in sucrose followed by suspension in ice-cold BM; □, cells osmotically cold shocked by suspension in sucrose followed by suspension in ice-cold BM + 6,000.

etched (Fig. 7B and C) and negatively stained (Fig. 7D) cells, as well as in thin-sectioned cells after initial fixation with acrolein-glutaraldehyde, osmium tetroxide, or crotonaldehyde-glutaraldehyde. The appearance of these structures seemed to be directly related to treatment with polymyxin. Thus, our thin-sectional data seemed to confirm that polymyxin interacts with both the outer membrane and the cytoplasmic membrane and causes cytoplasmic clumping.

The resistant isolates did not undergo any ultrastructural damage when grown in the presence of 6,000 U of polymyxin per ml (Fig. 5). There was a definite lack of outer membrane blebbing, which would indicate that the outer membrane of the resistant cells must differ from the sensitive-cell outer membrane. Freeze-etching of the resistant cell showed this to indeed be the case (Fig. 6B). The outer membrane lacked most of the particles found in the concave cell wall fracture of sensitive cells (Fig. 6A). The loss of particles did not result from extraction of the particles from the outer membrane by polymyxin as shown by there being no reduction in particle number in sensitive cells upon treatment with polymyxin (Fig. 7A). These particles have previously been shown by Gilleland et al. (10) to be extractable by EDTA-Tris and have been shown to consist of protein and lipopolysaccharide (LPS) (22, 27). Their loss from the outer membrane would thus be expected to be reflected in a proportional increase in total lipid, a decrease in LPS, and a decrease in protein in the cell wall. It has been a consistent finding that resistant-cell envelopes have more lipid than sensitive-cell envelopes upon acquisition of resistance not only to polymyxin (6) but also to a quaternary ammonium compound (2) and to carbenicillin (31). Furthermore, Brown and Wood (7) have suggested that their chemical findings indicated that polymyxin-resistant cells of P. aeruginosa might have less LPS. Barrett and Asscher (3) suggested that the development of habituated carbenicillin resistance in P. aeruginosa is due to a change in type or amount, or both, of cell wall LPS, while alterations in LPS also have been implicated in the resistance of E. coli to ampicillin (4, 19). Koike and Iida (14) concluded that polymyxin exerts its effect on the sensitive gram-negative cell wall by disorganizing the LPS that results in the formation of the outer membrane projections. More recently, Louvatmaa et al. (J. Ultrastruct. Res. 50:391, 1975) have suggested that polymyxin-resistant mutants of Salmonella have altered LPS and a specific change in the structure of the outer membrane. The permeability of the outer membrane to antibiotics, lysozyme, and certain other low-molecular-weight compounds has been shown to be influenced by alterations in LPS in Salmonella typhimurium (23) and E. coli (28, 29). Recently, alterations in the LPS have been shown to be accompanied by a concomitant decrease in the amount of outer membrane protein in both S. typhimurium (1) and E. coli (16, 25). These various chemical studies indicate the importance of LPS in outer membrane permeability and suggest that outer membrane proteins also may play an important role. Thus, the ultrastructural alteration we observed in the structure of the outer membrane appears to be consistent with previous chemical findings.

Inouye (13) recently presented a model in which hydrophilic pores were formed through the outer membrane by lipoprotein complexes.
FIG. 10. Isolate A. (A) Cell freeze-etched with glycerol cryoprotection after growth in BM. The concave cell wall layer once again is studded with numerous particles similar to that of the sensitive PAO 1 strain (compare with Fig. 6A). (B) Cells after growth in BM followed by suspension in BM + 6,000 for 3 h. The cells are now sensitive to the action of polymyxin as evidenced by cytoplasmic membrane infoldings and cytoplasmic damage similar to that found in the sensitive PAO 1 cells in BM + 6,000 (see Fig. 4).
as a possible explanation of the permeability of the outer membrane. Although the particles in the concave cell wall layer have not been correlated with lipoprotein complexes, the idea that loss of protein particles from the outer membrane might result in decreased permeability, possibly on the basis of the loss of pores through the lipid bilayer, is appealing. This decreased permeability of the outer membrane could act to exclude polymyxin from still sensitive sites on the cytoplasmic membrane as suggested by Hamilton (11). Another possibility would be for the cytoplasmic membrane to also be altered so that it became insensitive to polymyxin. To test the hypothesis of Hamilton, resistant cells were subjected to cold shock and osmotic-cold shock procedures. Cold-shock procedures have been shown to result in greater than 95% loss of intracellularly accumulated substrates in P. aeruginosa (18) as well as in E. coli (17). Thus the low temperature seems to cause the cell to become permeable to solutes. This increase in permeability is not fully understood but is thought to result from a phase transition in the membrane lipids (17). The cold shocked cells would be expected to be transiently more permeable to the polymyxin. If the cytoplasmic membrane still possesses sensitive sites, growth of the cells should be subsequently inhibited and ultrastructural damage should be observable. Such was found to be the case. The cells were both inhibited in growth (Fig. 9) and underwent ultrastructural alterations (Fig. 8) identical to those in sensitive cells. We believe these observations support the hypothesis of Hamilton that the impermeable outer membrane is shielding still sensitive cytoplasmic membrane sites in resistant cells.

It should be pointed out that there are other possible explanations for the results seen in our cold shock experiments. For example, since osmotic-cold shock procedures have been shown to release periplasmic enzymes (20), sensitivity to polymyxin after the shock procedures could be attributed to a loss of an inducible enzyme that protected the resistant cell by alteration of the polymyxin molecule. Such an explanation, however, would not explain the ultrastructural alteration of the outer membrane of the resistant cells, nor would it account for the resistance to EDTA that these cells exhibit.

After growth of the resistant isolates in BM, the concave cell wall surface had regained the appearance of the sensitive cells; i.e., it was studded with numerous particles (Fig. 10A). This change in outer membrane architecture would predict a loss in resistance to polymyxin. This was found to be the case since the cells failed to grow when shifted to BM + 6,000, and thin sections of cells after shift to BM + 6,000 revealed ultrastructural alterations (Fig. 10B) as found in sensitive cells. It would appear that resistance in these two isolates was not the result of a stable genetic mutation but resulted from an adaptive alteration in the architecture of the outer membrane layer of the cell wall in the presence of polymyxin which was lost upon growth in medium lacking polymyxin.

As mentioned previously, the particles in the concave cell wall layer have been shown to be a site of an EDTA-Tris-extractable component of the cell wall (10) corresponding to a protein-LPS complex (22, 27). Thus, loss of these EDTA-Tris-sensitive particles would predict that the resultant cell wall should be resistant to EDTA-Tris. The results shown in Fig. 11 show this to be correct. The polymyxin-resistant isolates are both markedly resistant to lysis by EDTA-Tris as compared with the parent strain. Furthermore, upon the acquisition of numerous...
particles in the outer membrane layer after growth of the isolates in BM, one would predict that the cells should now have become sensitive to EDTA-Tris lysis. This was also found to be the case (Fig. 11). Thus, the sensitivity or resistance of the cells to lysis by EDTA-Tris correlated quite well with the ultrastructural architecture exhibited by the outer membrane layer of the cell wall.

Brown and Melling (5) have shown that upon Mg\textsuperscript{2+} limitation, cells of *P. aeruginosa* acquired resistance to both polymyxin and EDTA. Gilleland et al. (9) showed that such Mg\textsuperscript{2+}-limited cells had an altered outer membrane ultrastructure. It is of interest that the polymyxin-resistant cells in this study that grow well in non-Mg\textsuperscript{2+}-limited medium containing polymyxin are also resistant to EDTA and have an ultrastructurally altered cell wall. It must be pointed out, however, that, although the same cell wall layer is altered in both instances, the alteration is not identical since the Mg\textsuperscript{2+}-limited cells had a concave cell wall layer that appeared disorganized and crowded with particles (9).

This study has shown that acquisition of resistance to polymyxin is correlated with an architectural change in the outer membrane cell wall layer that can be monitored by freeze-etching. This alteration appears to decrease the permeability of the outer membrane, affording protection to still sensitive sites on the cytoplasmic membrane by the exclusion of polymyxin from these sites. This alteration also results in an EDTA-Tris-resistant cell envelope as would be predicted from loss of the EDTA-Tris-extractable particles. Growth of the resistant isolates in BM without polymyxin results in a return to normal outer membrane architecture, with the expected loss of resistance to both polymyxin and EDTA-Tris. It is worthy of note that in this study the susceptibility of the cell to both polymyxin and EDTA-Tris can be predicted from the outer membrane architecture upon freeze-etching.

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LITERATURE CITED


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