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Evidence for Two Distinct Mechanisms of Resistance to Polymyxin B in *Pseudomonas aeruginosa*

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***Pseudomonas aeruginosa* H181 and H185 are resistant to initial exposure to polymyxin B and continue to grow in its presence. Growth of the strains in the presence of 50 U of polymyxin B per ml was characterized by a doubling time of 120 min, whereas the doubling time in the absence of polymyxin was 60 min. Growth for two generations in the presence of polymyxin caused a 23 to 31% increase in lipopolysaccharide content. In addition, a marked increase in susceptibility to the detergents sodium deoxycholate, Triton X-100, and sodium dodecyl sulfate was observed. The resistant mutants had a small but significant reduction in their levels of dodecanoic acid as compared with the parent strain; however, this was the only consistent alteration observed in levels of fatty acids or readily extractable lipids. Polymyxin was fluorescently labeled by coupling to 1-dimethylaminonapthalene-5-sulfonyl chloride (dansyl chloride). Growth of strains H181 and H185 in the presence of dansylated polymyxin resulted in a stable association between the fluorescent antibiotic and the outer membrane. We postulate that these alterations are part of an adaptive response by the strains to the presence of polymyxin in the growth medium and reflect a resistance mechanism distinct from the mechanism affording polymyxin B resistance when these strains are initially exposed to the antibiotic.**

There are at least two ways in which *Pseudomonas aeruginosa* can become resistant to the membrane-active antibiotic polymyxin B. One way results from stepwise adaptation to the presence of polymyxin in growth medium. Resistance acquired in this manner is unstable, and adapted isolates revert to polymyxin susceptibility upon growth in polymyxin-free medium (11). The detailed mechanism of adaptive resistance is not known; however, extensive examination of adapted isolates has demonstrated a number of cell surface alterations in these strains. These include ultrastructural and chemical changes (6, 10, 11, 14, 15), alterations of the outer membrane (13) including a reduction of lipopolysaccharide (LPS) (14), reduced levels of specific outer membrane proteins (14), reductions in cell envelope Mg^{2+} and Ca^{2+} contents (6), and lipid alterations (6). This work has led to the proposal of two possible mechanisms which could account for adaptive polymyxin resistance (6). The first postulated mechanism involves the loss of outer membrane porin proteins, which have been proposed by Gilleland and co-workers (11, 14) to be involved in the penetration of polymyxin B. Another mechanism postulates a reduction in binding of polymyxin to the cell envelope as a result of changes in lipid and LPS composition (6).

A second way that *P. aeruginosa* can become resistant to polymyxin results from genetic mutation. Unlike adaptative polymyxin resistance, mutational polymyxin resistance is inheritable and is characterized by alterations in the outer membrane which are distinct from those found in polymyxin-adapted strains (25, 26). When these mutated strains are grown in the absence of polymyxin, the major outer membrane alterations observed are a 24-fold increase in the levels of outer membrane protein H1 and a concomitant decrease in Mg^{2+} content of the cell envelope (25). This form of resistance does not involve porin proteins, and in addition, these strains are resistant to EDTA and gentamicin (25, 26).

The aim of this study was to further characterize the molecular mechanism of mutational and adaptive polymyxin resistance in *P. aeruginosa*. Evidence presented here indicates that polymyxin-resistant mutants, when grown in the presence of polymyxin, display outer membrane alterations in addition to those described previously (6, 10, 11, 13–15). These additional alterations may be an adaptive response to polymyxin.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. *P. aeruginosa* PAO1 H103 and its derivatives H181 and H185 have been previously described (25). Strains H181 and H185 were isolated as polymyxin-resistant mutants and have been previously shown to exhibit EDTA resistance and low-level aminoglycoside resistance (25). Cells were grown in BM2 minimal medium (17) containing 0.1 mM $FeSO_4$, 0.5 mM $MgSO_4$, and 0.4% (wt/vol) glucose. Overnight cultures were inoculated into fresh medium at an optical density at 600 nm (OD_{600}) of 0.1 and grown at 37°C with vigorous aeration to an OD_{600} of 0.4 to 0.8. When required, 50 U of polymyxin B sulfate (Sigma Chemical Co., St. Louis, Mo.) per ml (6 μg/ml) was added to the medium before inoculation with an overnight culture grown in the absence of polymyxin.

Characterization of outer membrane proteins and LPS isolation. Separation of outer and inner membranes and characterization of membrane proteins by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was accomplished as described previously (19, 20). LPS was isolated as described by Darveau and Hancock (8).

Lipid and fatty acid analysis. Readily extractable lipids were isolated by a chloroform methanol extraction procedure as described by Folch et al. (10). Cells grown in BM2 medium to an OD_{600} of 0.6 were centrifuged, and the pellets were suspended to one-third the original volume in 10 mM Tris-hydrochloride (pH 7.4). The suspended cells were frozen immediately and lyophilized to dryness. A 20-mg portion of lyophilized cells was used for each determination. Extracted lipids were separated by thin-layer chromatography as described by Fine and Sprecker (9). Samples were spotted onto Silica Gel G plates saturated with boric acid and developed in chloroform-methanol-water-ammonium hydroxide (120:75:6:2). Lipids were visualized by iodine stain-

TABLE 1. Fatty acid composition of polymyxin-resistant strains of *P. aeruginosa* grown in the presence and absence of polymyxin B

Strain	Amt of polymyxin (U/ml) in growth medium	Concn of the following fatty acid (nmol/mg [dry wt] of cells)[a]:							
		30H C10	C12	20H C12	30H C12	16:1	16:0	18:1	18:0
H103	0	8.9 ± 5.2	8.4 ± 0.6	8.6 ± 1.8	5.5 ± 0.7	32.4 ± 5.9	88.5 ± 16.0	81.5 ± 16.0	6.7 ± 3.8
H181	0	10.7 ± 3.0	6.0 ± 1.4[b]	8.1 ± 1.8	5.0 ± 1.2	28.4 ± 4.0	79.8 ± 17.7	71.8 ± 12.0	5.2 ± 4.0
H181	50	14.3 ± 2.5[c]	6.2 ± 1.1	10.5 ± 3.2	8.0 ± 2.8[c]	22.11 ± 2.5[c]	67.3 ± 12.1	71.6 ± 10.9	6.1 ± 7.1
H185	0	12.8 ± 2.7	4.9 ± 0.6[b]	8.9 ± 1.5	5.0 ± 0.6	29.2 ± 5.0	73.4 ± 19.7	70.9 ± 9.4	7.9 ± 3.9
H185	50	16.0 ± 5.8	5.1 ± 1.2	11.0 ± 3.2	6.8 ± 1.2[c]	24.9 ± 6.3	76.8 ± 28.4	69.8 ± 19.8	8.2 ± 1.9

[a] Four to eight independent determinations of fatty acid composition were made. Abbreviations: 30H C10, 3-hydroxydecanoic acid; C12, dodecanoic acid; 3OH C12, 2-hydroxydodecanoic acid; 16:1, hexadecenoic acid; 16:0, hexadecanoic acid; 18:1, octadecenoic acid; and 18:0, octadecanoic acid. *P* values were determined by using the Student *t* test for unpaired data, employing the two-tailed *t* table. With the exception of those results indicated, no significant difference ($P < 0.2$) were observed.

[b] Significantly different ($P < 0.05$) than corresponding value in strain H103.

[c] Significantly different ($P < 0.05$) than corresponding value in the same strain grown in the absence of polymyxin.

ing (21) and identified by comparison with authentic standard phospholipids (Sigma). After staining, spots were scraped off, and the phosphate content of each was determined (1). Phosphate amounts were converted to moles of phospholipid values, assuming that 1 mol of phosphatidylethanolamine, phosphophatidylglycerol, or the unidentified phospholipid contained 1 mol of phosphate and that 1 mol of diphosphatidylglycerol contained 2 mol of phosphate.

Whole-cell fatty acid analysis was performed essentially as described by Kropinski et al. (23). Lyophilized cells (10 mg) were added to 1 ml of 2 M methanolic hydrochloride containing 400 nmol of pentadecanoic acid per ml as an internal standard, and the suspension was sonicated and heated at 100°C for 20 h in a sealed ampoule. The solution was then neutralized with 0.5 N NaOH. The methylesters were analyzed with a Vista 4600 gas-liquid chromatography (Varian, Palo Alto, Calif.) equipped with a 10-m glass column containing SP2100 DOH resin (Supelco). The following program was used: initial temperature, 140°C; final temperature, 210°C; programmed at 4°C/min for 10 min. Helium was used as a carrier gas and maintained at a flow rate of 20 ml/min. Peaks were integrated with a Vista 401 chromatography data system (Varian). The recovery of methylated hydroxyl fatty acids by this method is nearly quantitative, as discussed by Gmeiner and Schlecht (18).

Dansyl-polymyxin experiments. Dansyl-polymyxin was prepared as previously described (28) and quantitated by dinitrophenylation (2). The fluorescence from whole cells of *P. aeruginosa* and LPS treated with dansyl-polymyxin was measured with a Perkin-Elmer 650-10S fluorescence spectrophotometer set with an excitation wavelength of 340 nm and an emission wavelength of 485 nm. Whole cells treated with dansyl-polymyxin were examined with a Zeiss microscope (standard RA, with a condenser for fluorescence microscopy) containing a halogen lamp and suitable filters for emission of dansyl-polymyxin at 485 nm.

Detergent and antibiotic susceptibility. To monitor the susceptibility to detergents in liquid cultures of strains H185 and H181 grown in the presence or absence of polymyxin, the following procedure was used. Cells from a logarithmically growing culture (OD_{600} of 0.4 to 0.5) were either centrifuged and suspended in an equal volume of water and washed twice in BM-2 glucose medium or left untreated before placing in a cuvette and adding detergent to a final concentration of 1.0%. Cell lysis was monitored by following the decrease in OD_{600}. To determine the susceptibility to detergents of the polymyxin-resistant strains on plates, 0.05 ml of a logarithmically growing culture (grown in the absence of polymyxin) was spread plated onto a BM2-glucose agar plate containing polymyxin B as indicated. Sterile filter disks (6-mm diameter) were placed on the plate, and 15 μl of a 10% sodium deoxycholate solution, a 20% Triton X-100 solution, or a 1-mg/ml gentamicin solution was added to the disk. After overnight growth, the diameter of the inhibition zone was recorded. The MIC of polymyxin B was determined by the broth dilution method as described previously (25).

TABLE 2. LPS and phospholipid fatty acid content of *P. aeruginosa* H181 and H185 grown in the presence and absence of polymyxin

Strain	Amt of polymyxin (U/ml) in growth medium	Concn of the following type of fatty acid (nmol/mg [dry wt]):		Ratio of LPS to phospholipid fatty acids
		LPS[a]	Phospholipid[b]	
H103	0	31.4	209.1	0.15
H181	0	29.8	185.1	0.16
H181	50	39.0	167.1	0.23
H185	0	31.7	181.4	0.17
H185	50	39.0	179.7	0.22

[a] Calculated by summing amounts of 30H C10, C12, 20H C12, and 30H C12 from Table 1.

[b] Calculated by summing amounts of 16:1, 16:0, 18:1, and 18:0 from Table 1.

RESULTS

Growth, protein H1 levels, and MICs of polymyxin for inhibition of polymyxin-resistant strains. Strains H181 and H185 grown in BM2-glucose medium in the absence of polymyxin had doubling times of 60 min. Strain H103 had a similar growth rate. When grown in BM2-glucose medium containing 50 U of polymyxin per ml, strains H181 and H185 had a much slower doubling time (120 min) despite the fact that the level of polymyxin used was less than 10% of the MIC reported to inhibit these strains (25). This suggested that at the level used, the cells were being partly affected by polymyxin. In agreement with this, we could demonstrate that after growth in these concentrations of polymyxin B for two generations, the outer membrane protein H1 levels of strain H181 decreased ca. 10- to 20-fold judged by densitometry of SDS-polyacrylamide gels loaded with equal amounts of outer membrane proteins, as shown also by Gilleland and Conrad (13).

We have previously postulated that protein H1 was responsible for polymyxin resistance in strains H181 and H185 (25, 26). Therefore, we measured the average MIC of polymyxin for inhibition of strains H181 and H185 grown in the absence or presence of polymyxin on glucose minimal medium. Similar data were obtained for both strains, although substantial day-to-day variation was observed. When

TABLE 3. Readily extractable lipid composition of polymyxin-resistant strains of *P. aeruginosa* grown in the presence and absence of polymyxin

Strain	Amt of polymyxin (U/ml) in growth medium	% of the following lipid[a]:			
		Diphosphatidyl-glycerol	Phosphatidyl-ethanolamine	Unidentified phospholipid	Phosphatidyl-glycerol
H103	0	15.1 ± 1.9	59.7 ± 3.5	17.6 ± 2.3	7.7 ± 1.6
H181	0	15.2 ± 2.0	59.6 ± 3.9	17.3 ± 3.1	7.6 ± 2.4
H181	50	16.7 ± 1.5	56.7 ± 6.2	17.0 ± 1.65	7.6 ± 2.5
H185	0	13.6 ± 1.2	60.7 ± 7.1	17.1 ± 3.7	8.9 ± 3.2
H185	50	19.8 ± 1.85[b]	55.5 ± 1.2	17.7 ± 3.3	6.9 ± 1.6

[a] Values represent the means of six determinations with standard deviations. *P* values were determined by using the Student *t* test for unpaired data, employing the two-tailed *t* table.

[b] Significantly different ($P < 0.05$) than the corresponding value in the same strain grown in the absence of polymyxin.

grown in the presence of polymyxin, strains H181 and H185 required for inhibition an average MIC of 102 μg/ml (13 determinations); when grown in the absence of polymyxin, the average MIC was 46 μg/ml (14 determinations). Thus despite the apparent differences in protein H1 levels, similar MICs were observed whether or not the strains were grown in the presence of polymyxin. In contrast, these strains were far more resistant than the parent strain H103, which required for inhibition an MIC of 3.4 μg/ml (seven determinations). Therefore, we considered the possibility that our protein H1-overproducing mutants H181 and H185, when grown in the presence of polymyxin, underwent an adaptive alteration such that the mechanism of polymyxin resistance after this adaptation was different from the genetic resistance caused by the presence of protein H1.

Fatty acid composition and LPS content. Analysis of fatty acids by gas-liquid chromatography revealed some differences among strains H103, H181, and H185 grown in the presence and absence of polymyxin. Amounts of the LPS-specific fatty acid dodecanoic acid were significantly lower in both of the polymyxin-resistant strains grown in the absence of polymyxin when compared with strain H103 (Table 1). Growth of strain H181 in the presence of polymyxin resulted in a significant increase in the levels of 3-hydroxydecanoic acid and 3-hydroxydodecanoic acid as well as a decrease in the amount of hexadecanoic acid. The growth of strain H185 in the presence of polymyxin (as compared with growth in the absence of polymyxin) resulted in a significant increase only in levels of 3-hydroxydodecanoic acid.

P. aeruginosa strains commonly contain four fatty acids, which are characteristic of the lipid A region of LPS. These include dodecanoic acid, 3-hydroxydecanoic acid, and 2- and 3-hydroxydodecanoic acids. In addition, lipid A contains a small quantity of hexadecanoic acid (23), the majority of which is present as phospholipid fatty acids. Measurement of LPS fatty acids therefore provides an accurate method of quantitating LPS levels in whole cells (18, 23). This method of LPS quantitation in whole cells is superior to the measurement of 2-keto-3-deoxyoctanate amounts, since a number of compounds in cells may interfere with the 2-keto-3-deoxyoctanate assay (8, 28) and because fractionation of cells and corresponding losses of LPS are avoided.

Since polymyxin B has been shown to bind at or near the lipid A region of LPS (2), this quantitation of moles of LPS per cell would give the number of potential binding sites for polymyxin B. Analysis of the LPS content (moles per cell) of the polymyxin-resistant strains revealed a 23 to 31% higher content of LPS in the strains grown in the presence of polymyxin as compared with growth in the absence of polymyxin (Table 2). When the resistant strains were grown in the absence of polymyxin, virtually no change in LPS fatty acid content was observed when compared with strain H103. The increase in LPS fatty acids of strains H181 and H185 was not accompanied by an increase in phospholipid fatty acid components, resulting in higher ratios of LPS to phospholipid fatty acid when the polymyxin-resistant strains were grown in the presence of polymyxin (Table 2). These data indicated that exposure to polymyxin for only two generations resulted in an increased LPS content of strains H181 and H185.

Lipid composition. In an earlier study, Gilleland and Conrad (13) examined the lipid composition in *P. aeruginosa* H181 and H185. In that study, lipids were separated by thin-layer chromatography, identified by charring, and quantitated by densitometer scanning. Although we attempted to repeat their published procedures, the data we obtained differed in two major respects, i.e., the mobility of the lipid spots and the number of observed lipid species. Therefore, we sought to confirm their lipid analysis by using a slightly different procedure. The primary differences in our procedure were (i) use of a different solvent system to separate lipids on thin-layer chromatography plates, (ii) the identification of lipids by iodine staining, and (iii) the quantitation of phospholipids by phosphate determination of iodine-stained areas.

The major phospholipids identified in our analysis included phosphatidylethanolamine, diphosphatidylglycerol, and phosphatidylglycerol. In addition, we observed a phospholipid which comigrated in our solvent system with phosphatidylcholine, but which was shown by Hancock and Meadows (19) to differ from authentic phosphatidylcholine. No other phospholipids or neutral lipids were observed, even after charring. Our analysis did not reveal significant differences in lipid composition among strains H103, H181, and H185 (Table 3). Comparison of the lipids of strain H185 grown in the presence or absence of 50 U of polymyxin B per ml revealed a small but statistically significant increase in diphosphatidylglycerol content when the strain was grown in the presence of polymyxin (Table 3). This change was not observed in strain H181 grown in the presence of polymyxin. Total phospholipid fatty acids levels were slightly lower in the polymyxin-resistant stains (Table 2), indicating a small decrease in phospholipid content in these strains.

Incorporation of dansyl-polymyxin into the outer membrane. Two possible hypotheses could explain the changes in LPS content after the growth of strain H181 in polymyxin. First, the cell could become totally refractory to polymyxin such that no polymyxin could interact with or be taken up by the outer membrane. Alternatively, the polymyxin could interact with the outer membrane, but further uptake of polymyxin might be blocked. In either case, the alteration in

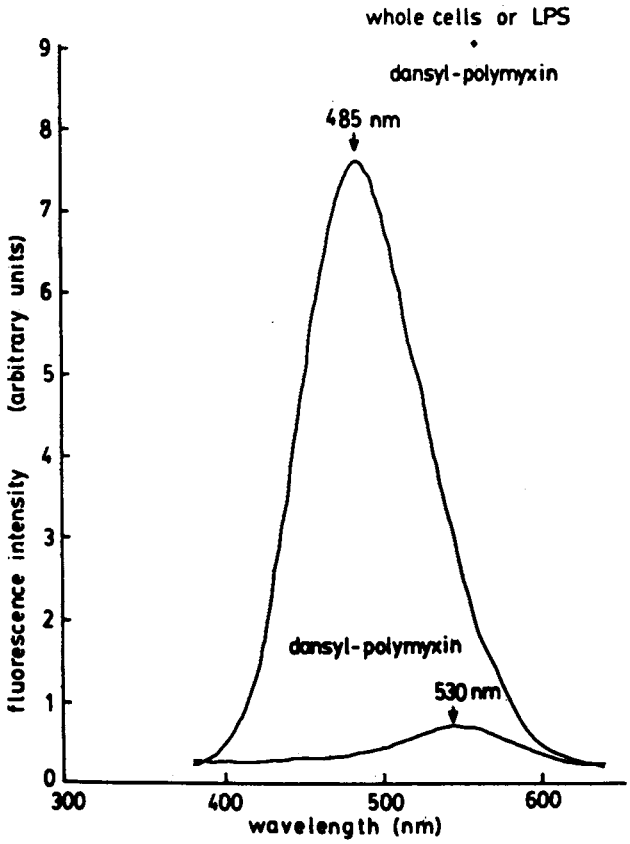

FIG. 1. Fluorescence emission spectra of dansyl-polymyxin (0.2 μg/ml) in 5 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 7.0) and a mixture of dansyl-polymyxin and purified *P. aeruginosa* H103 LPS (0.5 μg/ml) or whole cells (10^7 cells per ml). Superimposable spectra were obtained by using either LPS or whole cells with dansyl-polymyxin. Only background fluorescence was observed from purified outer membranes alone.

LPS content might be a manifestation of the adaptive response to the presence of polymyxin.

To differentiate these two possibilities, we tagged polymyxin such that we could follow its fate in intact *P. aeruginosa* cells. A fluorescent derivative of polymyxin was prepared by coupling 1-dimethylaminonaphthalene-5-sulfonyl chloride (dansyl chloride) to polymyxin sulfate as described by Schindler and Teuber (27). It was shown to be homologous and free of dansyl chloride and unmodified polymyxin by thin-layer chromatography. Interaction of dansyl-polymyxin with *Salmonella typhimurium* LPS results in enhanced fluorescence of the fluorescent antibiotic (27). We confirmed that our dansyl-polymyxin formed a stable complex with purified *P. aeruginosa* LPS or with intact *P. aeruginosa* H181 cells, resulting in a characteristic blue shift in the emission spectrum and an enhanced relative fluorescence intensity (Fig. 1).

When strain H181 was grown in the presence of dansyl-polymyxin and examined with a fluorescence microscope, individual cells exhibited strong fluorescence surrounding the cell, suggesting that the cells had bound dansyl-polymyxin to their outer membranes (Fig. 2). The fluorescently tagged polymyxin remained surface bound even after repeated washing. To confirm that the dansyl-polymyxin was bound to the cell surface (outer) membrane, the cells were disrupted, and the inner and outer membranes were separated by sucrose density centrifugation. In these experiments, most of the dansyl-polymyxin was associated with outer membranes. It was, however, dissociated from outer membrane components by SDS treatment, showing that the fluorescent antibiotic had not become covalently bound to outer membranes (data not shown). These results indicate that dansyl-polymyxin forms a stable complex with the outer membrane of strain H181. The spectral characteristics of dansyl-polymyxin after isolation of outer membranes from cells grown in polymyxin were identical to those of dansyl-polymyxin mixed with intact strain H181 cells or LPS.

Susceptibility to detergents. Polymyxin B is known to act synergistically with detergents to promote cell lysis (4, 5). It has been suggested that polymyxin-induced damage to the outer membrane facilitates the passage of the detergent through the outer membrane (3). We examined the action of detergents on the resistant strains grown in the presence of polymyxin to determine whether polymyxin was still able to act synergistically with detergents and cause cell lysis. In one type of experiment, susceptibility to detergents in the presence of polymyxin was tested on plates, and other experiments examined susceptibility to detergents in liquid medium. The results of these experiments demonstrated that the polymyxin-resistant strains grown in the presence of polymyxin showed enhanced susceptibility to detergents even when cells were washed free of polymyxin in the medium (Table 4). Gentamicin susceptibility increased only slightly under the same conditions, indicating the specificity of the detergent effects. These results suggest that the polymyxin tightly bound to the outer membrane is capable of acting synergistically with detergents.

DISCUSSION

The results of this paper suggest at least two distinct mechanisms of polymyxin resistance in *P. aeruginosa*, in contrast to the previous suggestion of Gilleland and Conrad (13) that the phenotypic alterations of cells grown in the presence of polymyxin (adaptively resistant) and of our polymyxin-resistant mutants (genetically resistant) were

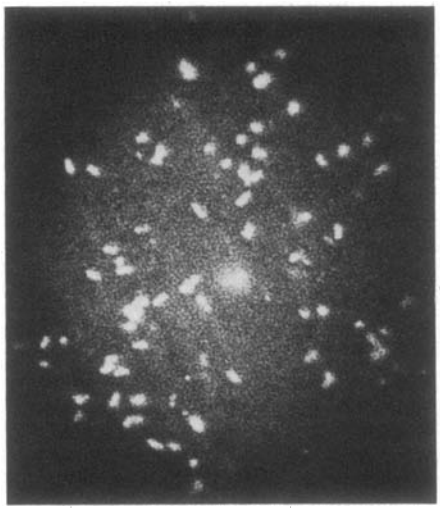

FIG. 2. *P. aeruginosa* H181 grown in the presence of 50 U of dansyl-polymyxin per ml. Cells from a logarithmically growing culture (OD_{600}, 0.6) were centrifuged, and the pellet was suspended to 1/20 of the original volume. Wet mounts prepared from the cell suspension were examined with a Zeiss microscope equipped for fluorescence microscopy.

TABLE 4. Susceptibility of polymyxin-resistant strains to detergents

Strain	Amt of polymyxin B (U/ml) in growth medium	% of lysis decrease after 20 s[a]		Diam of inhibition zone (mm)[b]		
		SDS	Deoxycholate	Deoxycholate	Triton X-100	Gentamicin
H103	0	11.7 ± 6.2	8.8 ± 2.1	0	0	12
H181	0	8.9 ± 3.4	9.6 ± 4.2	0	0	10
	50	86.5 ± 7.1	18.8 ± 5.1	15	9	12
H185	0	5.8 ± 5.0	6.7 ± 1.1	0	0	13
	50	81.5 ± 3.9	15.1 ± 2.5	22	12	15

[a] Cell lysis was followed by observing the decrease in OD_{600}. Lysis values were obtained with cells removed directly from culture flasks. Similar lysis values were obtained when the experiment was performed with cells that had been centrifuged and suspended in an equal volume of water or that had been washed twice in BM2-glucose medium.

[b] Inhibition zone was measured from the edge of the disk to the edge of the inhibition zone.

similar. Our data favor the hypothesis that strains H181 and H185 were resistant to polymyxin because of their high content of protein H1 (which displaces Mg^{2+} from the outer membrane). Growth of these strains in polymyxin causes a further adaptation such that the polymyxin resistance of these adapted cells occurred by a mechanism independent of protein H1.

Adaptation to polymyxin has been examined in detail by Gilleland et al. (13–16). In contrast to the stable mutants used in this study, their strains were adapted stepwise to growth in the presence of 6,000 U of polymyxin per ml and reverted to polymyxin susceptibility when grown in the absence of polymyxin. They proposed two alternative models to account for adaptive polymyxin resistance (7). Gilleland and Conrad reported that the strains used in this study, H181 and H185, share a number of characteristics, besides polymyxin resistance, with their stepwise-adapted strains (13). However, our characterization of strains H181 and H185 has produced some results which argue against the conclusions of Gilleland and Conrad. For example, our quantitation of individual lipids from strains H103, H181, and H185 grown in the absence of polymyxin did not reveal any significant differences in lipid composition, whereas Gilleland and Conrad (13) reported a significant decrease in phosphatidylglycerol content in both strains H181 and H185 as well as other strain-specific alterations. We also observed a reduction in total phospholipid fatty acid composition in strains H181 and H185 (Table 1) and that growth in the presence of polymyxin caused an increase in the diphosphatidylglycerol content of strain H185 (Table 2). No other significant lipid alterations were seen under these conditions. Because the increase in diphosphatidylglycerol occurred in only one of the polymyxin strains, it is not likely that this alteration is an important part of the resistance mechanism of these strains when grown in the presence of polymyxin. It is possible that the reduetion of total phospholipid fatty acid is a result of replacement of phospholipids by the outer membrane protein H1 when the strains are grown in the absence of polymyxin (25) or by LPS when the strains are grown in the presence of antibiotic (Table 2).

In contrast to our studies, Gilleland and Conrad (7, 13) have reported that the growth of strains H181 and H185 in the presence of polymyxin resulted in a substantial decrease in the amounts of phosphatidylethanolamine in both H181 and H185, an increase in diphosphatidylglycerol levels in strain H181, and an increase in an unidentified neutral lipid in both strains. In addition, their studies did not identify the phospholipid comigrating with but distinct from phosphatidylcholine (as identified by both ourselves and others [19]). A partial explanation for the discrepancies found between our analysis and those of Gilleland and Conrad is the methodology used to quantitate lipids. In our study, lipids on thin-layer chromatography plates were identified by iodine staining, the stained areas were scraped off, and the phosphate content of each spot was determined, whereas in the Gilleland and Conrad studies, lipids on the thin-layer chromatography plates were identified by charring and quantitated by densitometer scanning (7, 13). Kates (22) has suggested that the former method of analysis provides a more accurate quantitation of lipids. We also used a different solvent system in our thin-layer chromatography studies. Our failure to observe the unidentified lipid seen by Gilleland and Conrad (13) (recently identified as free fatty acids arising from phospholipase action [6]) could have a variety of explanations. For example, the free fatty acids seen by Gilleland and Conrad could represent a late adaptation in growth in polymyxin (although these authors did not state the amount of time that they exposed strains H181 and H185 to polymyxin). Since strains H181 and H185 were exposed to polymyxin for only two generations of logarithmic-phase growth in our experiments, it is possible that there was insufficient time to induce phospholipase activity. Alternatively, the free fatty acids observed by Gilleland and Conrad may have resulted from phospholipase activity stimulated by polymyxin (23) after harvesting the cells. Regardless, phospholipase action cannot entirely explain polymyxin resistance in these strains, since mutants lacking this enzyme have unaltered polymyxin susceptibility (29).

In an earlier study, Nicas and Hancock (25) demonstrated that the outer membrane protein H1 was overproduced 24-fold in strains H181 and H185 when grown in the absence of polymyxin. In addition, the amount of Mg^{2+} in the cell envelope was decreased, thus leading to the hypothesis that protein H1 replaces Mg^{2+} at a site on LPS and protects the site from polymyxin attack. A strong correlation between H1 production and resistance to polymyxin B, gentamicin, and EDTA was also demonstrated (26). Work by Gilleland and Conrad (13) has shown that protein H1 levels do not increase when strains H181 and H185 are grown in the presence of polymyxin B. Our own unpublished data confirmed this result. Gilleland and Conrad interpreted this finding to mean that H1 per se was not responsible for polymyxin resistance in these strains. Their results, however, do not rule out the role of protein H1 in polymyxin resistance. The genetic alteration in strains H181 and H185 (as opposed to adaptive alteration) allows the strains to resist attack by moderate levels of polymyxin (25). This is demonstrated by the fact that in the first 5 min of exposure to 75 μg of polymyxin per ml, there is a 1,000-fold difference in the amount of killing of the polymyxin-resistant strains and the wild-type strain H103 (25). This substantial difference in killing occurs despite the fact that MICs of polymyxin for inhibition of the

polymyxin-resistant strains and strain H103 differ only 10-fold (24). We believe that the decrease in protein H1 levels in strains H181 and H185 when grown in the presence of polymyxin is a manifestation of the subsequent adaptive response to polymyxin. In addition to a decrease in protein H1, the results presented here demonstrate that the growth of strains H181 and H185 in the presence of polymyxin B results in a significantly slower growth rate, stable binding of polymyxin to the outer membrane, an increase in LPS content, and enhanced susceptibility to detergents. Interestingly, growth of *P. aeruginosa* PAO1 and H103 in the presence of sub-MIC levels of polymyxin B or dansyl-polymyxin caused similar effects (R. A. Moore and R. E. W. Hancock, unpublished data). This suggested that adaptation to polymyxin resistance (caused by exposure to sub-MIC levels of polymyxin in both H181 [this paper] and *P. aeruginosa* PAO1 [16]) involved a similar mechanism.

Although mechanistically unresolved, it is clear that adaptation to polymyxin in *P. aeruginosa* is a complicated phenomenon which results in a number of cell surface alterations. It is apparent, however, that at least two mechanisms can account for polymyxin resistance. The first of these, as described by Conrad and Gilleland (7), results from stepwise adaptation in increasing amounts of polymyxin and is characterized by a variety of outer membrane alterations (7, 12, 15, 16). Interestingly, such adaptation to polymyxin resistance in *P. aeruginosa* has only been reported in basal glucose medium and not in complex medium, such as nutrient broth (16). The second mechanism is mutational resistance (which can be mimicked by growth of cells on divalent cation-deficient media [26]) and is characterized by increased levels of the outer membrane protein H1, a decrease in Mg^{2+} content of the cell envelope, and increased resistance to polymyxin, EDTA, and aminoglycosides (25). Mutants of this type apparently undergo further alterations of the outer membrane when grown in the presence of polymyxin, which may be a result of stepwise adaptation as described above. Further study is needed to elucidate the detailed molecular basis of polymyxin resistance of both mechanisms.

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