Adaptive resistance to aminoglycoside antibiotics in *Pseudomonas aeruginosa*

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Summary. Aminoglycoside-resistant variants of *Pseudomonas aeruginosa* strain PAO1 were readily selected by culturing the organism in medium containing increasing concentrations of gentamicin, tobramycin or amikacin until the strains were growing in a concentration of drug 128-fold greater than the minimal inhibitory concentration for the sensitive parent strain. These resistant strains exhibited characteristics previously associated with the impermeability type of resistance mechanism, i.e., they grew more slowly than the parent strain, the resistance was unstable in the absence of the antibiotic, and adaptation to one of the antibiotics conferred cross-resistance to other aminoglycosides. The adapted strains grew, with minimal morphological alterations, in concentrations of the various aminoglycosides that normally produced cell envelope damage, misshapen and filamentous cell formation, and cell lysis in the sensitive strain. Neither protein H1 nor phospholipid alterations appear to play a significant role in adaptive resistance to aminoglycoside antibiotics in this model system. The acquisition of adaptive resistance to the aminoglycoside antibiotics did not confer resistance to polymyxin B, another cationic antibiotic which is thought to share binding sites within the outer membrane with the aminoglycosides.

Introduction

Resistance to the cationic polymyxin and aminoglycoside antibiotics remains an incompletely understood phenomenon. When grown in the presence of one of these antibiotics, a population of gram-negative enteric rods or pseudomonads may exhibit increased resistance to the antibiotic through an unstable “impermeability mechanism”. This resistance is readily lost when the strain is grown in the absence of the antibiotic. We have previously studied adaptive resistance to polymyxin B in a model system with *Pseudomonas aeruginosa* (Gilleland and Murray, 1976). The adaptation to increased polymyxin resistance involves both ultrastructural (Gilleland and Murray, 1976) and various chemical (Gilleland and Lyle, 1979; Conrad and Gilleland, 1981; Champlin *et al.*, 1983) alterations of the outer membrane (OM) resulting in the prevention of polymyxin reaching the still sensitive cytoplasmic membrane (Gilleland and Farley, 1982). The chemical alterations comprised various

lipid alterations, including a decrease in the phospholipid content in the readily extractable lipid (REL) with a reduction in phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) content (Conrad and Gilleland, 1981), accompanied by the appearance of a large amount of free fatty acids (Champlin *et al.*, 1983). We (Conrad and Gilleland, 1981; Champlin *et al.*, 1983; Gilleland *et al.*, 1984; Gilleland, 1988) and others (Kubesch *et al.*, 1987) have proposed that changes in both the lipopolysaccharide (LPS) and the phospholipid composition of the OM might reduce the number of binding sites for polymyxin and render the OM more resistant to polymyxin. Kubesch *et al.* (1987) identified that PG, the major anionic phospholipid in the cell envelope, plays a critical role for the action of polymyxin on bacterial membranes.

The aminoglycoside antibiotics are a much more clinically important group of cationic antibiotics than are the polymyxins. It has been proposed (Hancock, 1981, 1986; Abdel-Sayed *et al.*, 1982; Eagon, 1984; Taber *et al.*, 1987) that the aminoglycoside antibiotics penetrate through the OM via a self-promoted pathway mediated by binding to

cation-binding sites within the OM. Both the polymyxins and the aminoglycosides are thought to bind to the same sites within the OM, and Hancock and co-workers (Nicas and Hancock, 1980, 1983; Hancock, 1981; Hancock *et al.*, 1981) have proposed that induction of OM-protein H1 in *P. aeruginosa* confers resistance to both the polymyxins and the aminoglycosides by protecting the binding site on LPS from attack. However, the role of the H1 protein in conferring resistance to these antibiotics has been disputed (Gilleland and Conrad, 1982; Said *et al.*, 1987). Various investigators (Pechey and James, 1974; Dimitracopoulos *et al.*, 1979; Al-Asadi *et al.*, 1981; Gerber and Craig, 1982; Gerber *et al.*, 1982; Bayer *et al.*, 1987) have reported the isolation of aminoglycoside-resistant strains that exhibit the impermeability mechanism of resistance. This mechanism of resistance to the aminoglycoside antibiotics has been associated with alterations in LPS (Bryan *et al.*, 1984; Galbraith *et al.*, 1984; Clark *et al.*, 1986). In this study we examined strains of *P. aeruginosa* made adaptively resistant to gentamicin, tobramycin and amikacin for the presence of alterations in their content of OM proteins and cell-envelope phospholipids. In particular, we examined whether the PG content of these aminoglycoside-resistant strains was reduced in a similar manner to the polymyxin-resistant strains.

Materials and methods

Bacterial strains

The aminoglycoside-resistant strains were all selected from the PAO1 parent strain of *P. aeruginosa*, which was susceptible to MICs of gentamicin 1 mg/L, tobramycin 0.5 mg/L, and amikacin 4 mg/L. A resistant variant to each aminoglycoside was selected by stepwise growth of strain PAO1 in the presence of increasing concentrations of antibiotic until the strain was growing in the presence of an antibiotic concentration 128-fold greater than the MIC for the parent PAO1 strain. Thus, the gentamicin-resistant strain (PAO1-Gen^r) was obtained by adding 3 ml of a logarithmic-phase culture of strain PAO1 in basal medium (BM) after adjusting the absorbance at 660 nm (A_{660}) to 0.3, corresponding to a cell count of $(5-7.5) \times 10^8$ cfu/ml, to 50 ml of BM containing gentamicin 1 mg/L in a 125-ml Erlenmeyer flask. After aerobic incubation at 30°C for 36 h, this culture was diluted with BM to give an A_{660} of 0.3, and 3 ml was added to 50 ml of BM containing gentamicin 2 mg/L, followed by incubation of the flask at 30°C with shaking for 24 h. This stepwise transfer into media containing two-fold increases in the concentration of gentamicin was continued until the organism was growing in a gentamicin concentration of 128 mg/L. A loopful of culture from this

flask was streaked on basal medium agar plates containing gentamicin 100 mg/L, and an isolated colony was picked as the PAO1-Gen^r strain. Similar adaptation procedures were used to obtain a tobramycin-resistant (PAO1-Tob^r) and an amikacin-resistant (PAO1-Ami^r) strain able to grow in the presence of tobramycin 64 mg/L and amikacin 512 mg/L respectively.

Growth media

The PAO1 strain was grown in a basal medium (BM) previously described as "basal medium 2" (Gilleland *et al.*, 1974). This medium contained the following ingredients adjusted to pH 7.0: 0.03 M glucose, 0.04 M K_2HPO_4 , 0.022 M KH_2PO_4 , 0.007 M $(NH_4)_2SO_4$, and 0.0005 M $MgSO_4$. The aminoglycoside-resistant strains were grown in BM containing gentamicin 100 mg/L for the PAO1-Gen^r strain, tobramycin 50 mg/L for the PAO1-Tob^r strain, and amikacin 500 mg/L for the PAO1-Ami^r strain.

Cells for experiments were routinely grown at 30°C with aeration either in 125-ml Erlenmeyer flasks containing 50 ml of the appropriate medium or in 4-L Erlenmeyer flasks containing 2 L of medium.

Determination of growth characteristics

The growth characteristics for the PAO1 strain and each of the three aminoglycoside-resistant strains were determined as follows. A 300-ml side arm Erlenmeyer flask containing 20 ml of BM plus the appropriate concentration of antibiotic was seeded from a logarithmic-phase starter culture to give an initial A_{660} reading of 0.05. The flasks were incubated at 30°C in a shaking water bath, and A_{660} readings were taken at 1-2 h intervals for 22 h.

Reversion of the aminoglycoside-resistant strains

The ability of the PAO1-Gen^r, PAO1-Tob^r, and PAO1-Ami^r strains to revert to antibiotic susceptibility was assessed as follows. An inoculum of 0.5 ml from a logarithmic culture growing in BM plus the appropriate antibiotic was used to inoculate a flask containing 50 ml of BM without antibiotic for each strain. After incubation for 24 h at 30°C with shaking, 0.5 ml was transferred to a second flask containing 50 ml of BM without antibiotic and again incubated for 24 h. These cells were used as inocula for MIC determinations (see below) and for cultures for cell envelope preparation. Data regarding reverted strains were obtained after growth of the strains for three successive transfers in antibiotic-free BM.

Determination of minimal inhibitory concentrations of various antibiotics

The MICs of various antibiotics were determined for the parent PAO1 strain, the three aminoglycoside-resistant strains, and the three reverted strains as described by Gilleland and Farley (1982). The antibiotics

tested included gentamicin sulphate from Schering Corp. (Kenilworth, NY), tobramycin sulphate (Nebcin) and streptomycin sulphate from Eli Lilly and Co. (Indianapolis, IN), amikacin from Bristol Laboratories (Syracuse, NY), and polymyxin B sulphate from Pfizer, Inc. (New York, NY).

Electronmicroscopy of thin-sectioned preparations

All strains were prepared and fixed with glutaraldehyde and OsO₄, with poststaining of sections with uranyl acetate and lead citrate as described by Gilleland and Farley (1982).

Analysis of cell-envelope proteins

Cell envelopes were prepared from the PAO1 strain, the three aminoglycoside-resistant strains, and the reverted strains according to the procedure of Stinnett *et al.* (1973). Proteins were extracted from these cell envelopes and analysed by slab sodium dodecylsulphate-polyacrylamide gel electrophoresis as described by Gilleland and Lyle (1979).

Lipid analysis

The PAO1 strain grown in BM and the three aminoglycoside-resistant strains grown in BM containing the appropriate antibiotic were used for lipid analysis. The readily extractable lipid (REL) fraction was obtained by chloroform:methanol (2:1) extraction of freeze-dried whole cells by the Conrad *et al.* (1979) modification of the method of Folch *et al.* (1957). The cellular dry weight percentage of RELs was measured gravimetrically (Conrad and Gilleland, 1981). The phosphate content of REL fractions was measured by the method of Ames and Dubin (1960). Lipids in the REL fractions were resolved by thin-layer chromatography on Adsorbosil-Plus 1 silica gel plates (Alltech Associates, Inc., Deerfield, IL). Chromatograms were developed to a distance of 10 cm in chloroform:methanol:acetic acid:water (85:15:10:3.5) (Hanna *et al.*, 1984), air dried, and developed to a distance of 14 cm in hexane:diethyl ether (4:1). Separated lipids were visualised by spraying with potassium dichromate-saturated 50% H₂SO₄ and charring at 150°C for 30 min. RELs were tentatively identified by cochromatography with authentic reference lipids (Sigma Chemical Co., St Louis, MO) which included diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), palmitic acid, and oleic acid. The ratios of individual lipids were determined by scanning densitometry as previously described (Conrad and Gilleland, 1981).

Statistical analysis

The statistical significance of the results of the lipid analysis was determined by Student's *t* test on the means, with (two-tailed) *p*-values of 0.05 and lower considered significant.

Results

Characteristics of the aminoglycoside-resistant strains

Strains resistant to each of the three aminoglycoside antibiotics were readily obtained but they grew more slowly than did the parent strain (PAO1) and attained a lower cell density in cultures. In the absence of antibiotic, the sensitive PAO1 strain grew in BM with a doubling time of 2.6 h to reach a final A₆₆₀ reading of 1.2 within an incubation period of 16 h. The PAO1-Gen^r strain grew in BM plus gentamicin 100 mg/L with a doubling time of 3 h to reach a final A₆₆₀ reading of 1.0 within 20 h. The PAO1-Tob^r strain grew in BM containing tobramycin 50 mg/L with a doubling time of 3.9 h to reach a final A₆₆₀ reading of 0.75 within 18 h. The PAO1-Ami^r strain grew in BM plus amikacin 500 mg/L with a doubling time of 5.3 h to reach a final A₆₆₀ reading of 0.68 within 22 h.

Adaptive resistance from growth in the presence of one of the aminoglycoside antibiotics conferred cross-resistance to the other three aminoglycosides tested (table I). The adaptive resistance was unstable; growth of the resistant strains for three transfers in BM without antibiotic resulted in all three strains reverting to antibiotic susceptibility (table I).

Electronmicroscopy of thin-sectioned preparations

The unadapted PAO1 strain could not survive and grow, even with prolonged incubation, in BM containing gentamicin 100 mg/L, tobramycin 50 mg/L, or amikacin 500 mg/L. Exposure of PAO1 cells to gentamicin 100 mg/L resulted in a progression of cellular damage as shown in fig. 1. After exposure to gentamicin for 30 min (fig. 1A), numerous small blebs of the OM were seen, with an occasional larger ballooning out of the membrane. After exposure for 1 h (fig. 1B), more filamentous cells were found, and the cells also became more misshapen. The cell population became even more misshapen and filamentous after exposure for 2 h (fig. 1C), and lysis of cells began to occur. By 4 h, most of the cells were in various stages of lysis (fig. 1D). Exposure to tobramycin 50 mg/L had a similar effect on the PAO1 cells; blebbing of the OM and misshapen cells were prominent findings (fig. 2A). Lesions and infoldings of the cytoplasmic membrane were more extensive in cells exposed to tobramycin (fig. 2C) than in those exposed to gentamicin. Extensive cell lysis occurred in tobramycin-exposed cells by 4 h, with

Table I. MIC's of aminoglycoside antibiotics and polymyxin B for the *P. aeruginosa* strains

Strain	Antibiotic MIC (mg/L)				
	Gentamicin	Tobramycin	Amikacin	Streptomycin	Polymyxin B
PAO1	1	0.5	4	32	8
PAO1-Gen ^r	128	64.0	512	512	16
PAO1-Tob ^r	128	64.0	256	512	8
PAO1-Ami ^r	64	32.0	512	512	8
PAO1-Gen ^r -reverted	4	2.0	16	64	ND
PAO1-Tob ^r -reverted	1	2.0	8	32	ND
PAO1-Ami ^r -reverted	2	2.0	16	64	ND

ND = not determined.

separation of the OM from the cytoplasmic membrane (fig. 2D) frequently preceding lysis. Amikacin produced effects in the PAO1 cells (fig. 2B) similar to those produced by the other two aminoglycosides. However, the cells were not as misshapen nor was the cytoplasm as darkly staining within the first hour as with gentamicin or tobramycin.

The resistant strains were capable of survival and growth in the presence of the concentrations of aminoglycoside antibiotics which produced extensive cell damage and lysis in the PAO1 strain. These strains did not show the ballooning and separation of the OM layer, the formation of grossly misshapen and filamentous cells, the cytoplasmic membrane infoldings, and the cell lysis produced in the PAO1 strain by these concentrations of antibiotics. These resistant strains appeared remarkably healthy. However, in comparison to the PAO1 strain grown in BM, growth in the presence of the antibiotic did result in slightly misshapen cells.

Analysis of OM proteins

In all the resistant strains there was a reduction in a number of outer membrane protein bands in comparison to the sensitive PAO1 strain and the reverted strains (fig. 3). All three resistant strains demonstrated a reduced content of several of the bands in the 73–69-Kda range. Most obviously associated with loss and regain in all three strains was the 69-Kda band, which was reduced in all three strains and regained to wild-type concentrations in all three reverted strains. Protein F (porin, 36 Kda) was reduced in all three strains, as was protein H1 (21 Kda). No band was increased in the resistant strains relative to the PAO1 and reverted strains.

Lipid analysis

Separation of REL fraction lipids by thin-layer chromatography revealed no apparent alteration of lipid composition in any of the aminoglycoside-resistant strains compared with the parental PAO1 strain. Quantitation of individual lipids by densitometry (table II) confirmed that there were no consistently significant differences between the content of the various lipids in the PAO1 strain and in the three resistant strains. The only statistically significant differences between the PAO1 strain and the resistant strains were an increase in free fatty acids in the PAO1-Ami^r strain and an increase in DPG in the PAO1-Tob^r strain (table II). Although the PG content was slightly reduced in all three resistant strains (reduction of 7.4% in PAO1-Gen^r, 12% in PAO1-Tob^r, and 10.7% in PAO1-Ami^r) compared to the PAO1 strain, the magnitude of the reduction did not approach the 74–90% reduction in PG content previously reported (Conrad and Gilleland, 1981; Champlin *et al.*, 1983) for the polymyxin-resistant *P. aeruginosa* strains. Neither the cell percentage of REL nor the phospholipid proportion of the REL (as measured by determining μ mol of phosphorus per mg of REL) differed significantly between the PAO1 and the resistant strains (table II). Thus, the adaptation to aminoglycoside resistance did not result in any significant alteration in lipid content in the REL.

Discussion

In our in-vitro model of resistance to aminoglycoside antibiotics, resistant strains were readily obtained for each of the three antibiotics used. All three resistant strains exhibited characteristics associated with the impermeability mechanism of resistance. These characteristics included slower

Fig. 1. *P. aeruginosa* strain PAO1 after exposure to gentamicin 100 mg/L. (A) Cells fixed after exposure for 30 min; note blebbing of the OM and misshapen cells. (B) Cells fixed after exposure for 1 h; note the cells have become more misshapen and filamentous. (C) Cells fixed after exposure for 2 h; cell lysis has become more frequent. (D) Cells fixed after exposure for 4 h; extensive cell lysis has occurred. Bars = 200 nm.

Fig. 2. *P. aeruginosa* strain PAO1 after exposure to aminoglycoside antibiotics. (A) Cells fixed after exposure to tobramycin 50 mg/L for 1 h; note the OM blebs and misshapen cell. (B) Cells fixed after exposure to amikacin 500 mg/L for 1 h; note the filamentous cell with a large OM bleb. (C) Cell fixed after exposure to tobramycin 50 mg/L for 2 h; note the infolding of the cytoplasmic membrane (arrow)—such lesions in the cytoplasmic membrane were frequently encountered in tobramycin-treated cells. (D) Cell fixed after exposure to tobramycin 50 mg/L for 4 h; note the separation of the OM from the cytoplasmic membrane, which was commonly seen before cell lysis. Bar = 200 nm.

growth than the parent strain (Gilleland and Murray, 1976; Eagon, 1984) and cross-resistance to all four aminoglycosides tested (Pechey and James, 1974; Al-Asadi *et al.*, 1981; Shannon and Phillips, 1982). This resistance was unstable and strains readily reverted to susceptibility during several transfers in the absence of antibiotic (Pechey and James, 1974; Gilleland and Murray, 1976; Dimitra-

copoulos *et al.*, 1979; Bayer *et al.*, 1987). Furthermore, we were unable to detect aminoglycoside-inactivating enzymes in any of the three resistant strains by techniques that did allow demonstration of such an enzyme from a positive control strain (data not shown). Therefore, we conclude that our resistant strains were resistant by an impermeability mechanism.

Fig. 3. SDS-PAGE of cell envelope proteins of the various *P. aeruginosa* strains. Lane 1, the susceptible PAO1 strain; lane 2, the gentamicin-resistant PAO1-Gen^r strain; lane 3, PAO1-Gen^r reverted to susceptibility; lane 4, the tobramycin-resistant PAO1-Tobr^r strain; lane 5, PAO1-Tobr^r reverted to susceptibility; lane 6, the amikacin-resistant PAO1-Ami^r strain; lane 7, PAO1-Ami^r reverted to susceptibility; lane 8, the polymyxin B-resistant isolate A strain; lane 9, molecular weight standards of 66, 44, 36 and 24 Kda. On the left, the location of a 69-Kda band (arrow), protein F (labelled F), and protein H1 (labelled H) is indicated. Note that the resistant strains (lanes 2, 4, 6, and 8) do not have an increased content of protein H1. Reductions in various protein bands, including protein F and those in the 69–73-Kda range, are evident.

The precise role played by the impermeability type of resistance to aminoglycoside antibiotics in clinical medicine remains undetermined. Since this type of resistance is generally unstable in the absence of the antibiotic, strains possessing impermeable-type resistance in the presence of the

antibiotic in the patient would lose the resistance upon routine culturing for isolation in the clinical laboratory and would appear to be susceptible to the antibiotic upon routine testing of antibiotic susceptibilities in the clinical laboratory (Gerber *et al.*, 1982). Emergence of such aminoglycoside-

Table II. Analysis of REL fractions from the *P. aeruginosa* strains

Strain	REL Component						REL, mean per- centage of dry cell weight†§ (SD)	Mean Pi content in µmol/mg of REL§ (SD)
	Mean percentage composition† (SD)							
	Total un- known	FFA	DPG	PE	PG			
PAO1	12.24 (2.40)	1.10 (0.49)	14.78 (2.33)	49.76 (3.13)	22.14 (2.02)	11.75 (0.65)	1.18 (0.15)	
PAO1-Gen ^r	11.26 (2.28)	1.30 (0.40)	16.77 (3.56)	50.69 (3.37)	20.51 (2.81)	11.94 (0.48)	1.23 (0.13)	
PAO1-Tob ^r	10.44 (2.91)	1.42 (0.51)	19.11 (3.55)*	50.69 (3.68)	19.49 (2.80)	12.42 (0.23)	1.18 (0.14)	
PAO1-Ami ^r	12.22 (2.21)	1.89 (0.40)*	13.74 (2.14)	52.38 (3.45)	19.78 (2.47)	11.61 (0.04)	1.13 (0.13)	

† Seven to eight determinations were used for each value.

‡ Three determinations were used for each value.

§ Eight to ten determinations were used for each value.

* Value is statistically significantly different from the equivalent value for the PAO1 strain as determined by Student's *t* test on the means, employing the two-tailed *t* table.

resistant strains has been shown to occur not only *in vitro* but also in animal models (Gerber *et al.*, 1982; Bayer *et al.*, 1987) and in human patients (Bryan *et al.*, 1976; Mawer and Greenwood, 1977; Bryan *et al.*, 1984; Olson *et al.*, 1985). The emergence of resistance occurs more frequently with *P. aeruginosa* than with the Enterobacteriaceae (Gerber and Craig, 1982; Nilsson *et al.*, 1987) and is thought to play a role in the difficulty encountered in treating infections caused by *P. aeruginosa* with aminoglycoside therapy (Olson *et al.*, 1985). Clinical strains displaying the impermeability type of resistance have low-level aminoglycoside resistance, which some authors (Gerber and Craig, 1982; Gerber *et al.*, 1982) attribute to the antibiotic selection of resistant subpopulations already existing in the generally susceptible population. However, it appears that a pre-existing resistant subpopulation is not necessary for the emergence of adaptive resistance, because newly resistant subpopulations also appear (Olson *et al.*, 1985). Indeed, in-vitro models of resistance development to high-level resistance, after several steps, show strains growing in antibiotic concentrations which would normally kill a population of the original parent strain. We believe that the emergence of more resistant subpopulations, which are not found in the initial cell population, plays a role in in-vitro models and in-vivo clinical situations.

In this study the three resistant strains were grown routinely in the presence of aminoglycosides in concentrations corresponding to $100 \times$ MIC for the parent PAO1 strain, with only quite minor aberrations in cell morphology. These minor ultrastructural abnormalities in cell shape may indicate that exclusion of the antibiotic by the impermeability mechanism is not absolute but that only small quantities are able to penetrate. However, the resistant cells are protected against extensive ultrastructural alterations (misshapen and filamentous cells, figs. 1, 2A, and 2B) which these antibiotic concentrations produce in the PAO1 strain. Others (Nishino and Nakazawa, 1975; Martin and Beveridge, 1986) have reported that aminoglycoside antibiotics cause breaks or holes in the cell envelope layers of susceptible cells, as well as causing blebbing or outpouching of the OM (Iida and Koike, 1974; Nishino and Nakazawa, 1975; Martin and Beveridge, 1986). We found similar effects in the PAO1 strain treated with each of the aminoglycoside antibiotics. In addition to the OM and cytoplasmic membrane alterations noted, the antibiotics all produced alterations in cell morphology, with extensive cell lysis occurring after exposure for 2–4 h. Marked morphological changes with a

loss of cell shape and the occurrence of rounded cells leading to lytic death of *P. aeruginosa* upon exposure to aminoglycoside antibiotics was described previously by Nishino and Nakazawa (1975) and by Lorian and Atkinson (1986). We believe these morphological changes and cell lysis result from indirect effects on cell metabolic processes consequent upon inhibition of protein synthesis by the binding of the aminoglycoside to the 30S ribosomal subunit. The resistant strains described here would be protected from these effects by exclusion of the antibiotic from entry into the cytoplasm in sufficient quantity to disrupt cell metabolism.

In this study we examined the role played by two different cell-envelope components in the adaptive impermeability type of resistance to aminoglycoside antibiotics. An increase in OM protein H1 has been proposed (Nicas and Hancock, 1980, 1983; Hancock, 1981; Hancock *et al.*, 1981) to mediate resistance to aminoglycosides, polymyxin B, and EDTA. However, since no increase in OM protein H1 was evident in any of the three resistant strains (fig. 3), we could find no evidence that this protein plays any role in adaptive resistance to aminoglycosides in our model system. Several of the other OM proteins were reduced in all three adapted strains. However, since the aminoglycoside antibiotics are believed not to use OM porins for entry but to cross the OM by a self-promoted pathway involving binding to cationic binding sites within the OM (Abdel-Sayed *et al.*, 1982; Hancock, 1986), the possible significance of the reduced content of these OM proteins is uncertain. However, the reduction in several bands in the 73–69-Kda range is noteworthy since Morris and Brown (1988) recently reported that subinhibitory concentrations of the aminoglycosides inhibit the production of iron-chelating siderophores. Certain of these high molecular weight OM proteins may be iron-regulated proteins that are reduced in the adapted strains. The second cell envelope component we examined for a possible role in adaptive aminoglycoside resistance was the composition of the REL fraction. Alterations in the phospholipid composition, particularly the loss of PG (Kubesch *et al.*, 1987), have been reported to be associated with adaptive resistance to another cationic antibiotic polymyxin B (Conrad and Gilleland, 1981; Champ-*lin et al.*, 1982). However, no consistent alteration in REL lipid content could be demonstrated in the three aminoglycoside-resistant strains (table II), and we conclude that lipid alterations such as those reported for adaptive resistance to polymyxin B play no significant role in this resistance to the

aminoglycosides. LPS alterations have been reported in strains exhibiting the impermeability type of resistance to aminoglycoside antibiotics in in-vitro models (Dimitracopoulos *et al.*, 1979; Galbraith *et al.*, 1984) and clinical isolates (Bryan *et al.*, 1984; Clarke *et al.*, 1986). Alterations in LPS thus remain the most likely basis for adaptive resistance to the aminoglycosides.

It has been proposed (Brown, 1975; Hancock, 1981; Nicas and Hancock, 1980, 1983) that EDTA and the cationic aminoglycoside and polymyxin antibiotics all act at cationic binding sites on the LPS so that various conditions (growth in Mg^{2+} -deficient medium, increase in OM protein H1, etc.) confer resistance to all three agents. This implies that these agents share common binding require-

ments within the OM of *P. aeruginosa*. However, since resistance to polymyxin B (MIC raised from 6 mg/L for the PAO1 strain to 5000 mg/L for isolate A) results in only low-level (2-fold increase in MIC) resistance to aminoglycoside antibiotics (Gilleland and Farley, 1982) and an 128-fold increase in MIC for aminoglycoside antibiotics results in no significant increase in resistance to polymyxin B (this study), we conclude that these two groups of cationic antibiotics must have, within the OM, certain binding requirements that are unique to each antibiotic group. The observation that lipid alterations appear to play an important role in resistance to polymyxin B but appear to play no significant role in adaptive resistance to aminoglycosides is consistent with this suggestion.

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