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Chemical Alterations in Cell Envelopes of Polymyxin-Resistant *Pseudomonas aeruginosa* Isolates

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Cell envelopes from *Pseudomonas aeruginosa* strains resistant to polymyxin were compared with the cell envelopes from polymyxin-sensitive strains as to their content of total protein, carbohydrate, and 2-keto-3-deoxyoctonate and as to their protein composition as determined by slab polyacrylamide gel electrophoresis. The cell envelopes of the polymyxin-resistant strains had reduced amounts of lipopolysaccharide, as indicated by a reduction in both carbohydrate and 2-keto-3-deoxyoctonate concentrations, and a greatly altered protein composition as shown by polyacrylamide gel electrophoresis. There was a quantitative increase in total cell envelope protein in these strains. However, those protein bands identified as being major outer membrane proteins upon polyacrylamide gel electrophoresis of separated outer and cytoplasmic membranes were reduced greatly in concentration in the polymyxin-resistant cell envelopes. Thus, it appears that polymyxin resistance in these strains is associated with the alteration of the outer membrane through a loss of lipopolysaccharide and outer membrane proteins.

Polymyxin exerts its antimicrobial action on gram-negative bacteria by binding to lipopolysaccharide (LPS) and phospholipids in the outer membrane (OM) (1, 21, 29, 34, 37, 38), penetrating through the cell wall layers, and then interacting with the phospholipids in the cytoplasmic membrane (1, 34, 37), thus disrupting the osmotic equilibrium of the cell and causing the leakage of cell contents (24, 31, 36). Ultrastructural studies of *Pseudomonas aeruginosa* treated with polymyxin have supported this mechanism of action (8, 17).

Resistance to membrane-active agents such as polymyxin has been suggested by Hamilton (13) to be due to the agent not penetrating through the cell wall to reach the sensitive sites on the cytoplasmic membrane. In a previous ultrastructural study of polymyxin-resistant isolates of *P. aeruginosa* (8), it was shown by freeze-etching that the OM was altered in architecture upon the acquisition of the antibiotic resistance. The number of particles on the concave fracture surface of the OM, which corresponds to the underneath side of the outer half of the lipid bilayer, was greatly reduced in the resistant isolates (8) as compared with the number of particles present on the similar fracture surface of polymyxin-sensitive cells. The loss of the particles from the OM appeared to be associated with a decrease in permeability to polymyxin so that sensitive sites on the cytoplasmic membrane were protected (8). These particles have been identified as OM protein-LPS complexes in *P. aeruginosa* (7, 10, 28, 33), with the complex reported to be 60% protein, 30% LPS, and 10% loosely bound lipid (27). Recently, these particles have been identified as protein-LPS complexes in *Escherichia coli* as well (39, 42). Thus, based on the loss of these particles from the OM, one would have predicted that polymyxin-resistant isolates should show a loss of OM protein and LPS.

The present study was undertaken to determine whether the cell envelopes of polymyxin-resistant strains of *P. aeruginosa* are altered chemically with a reduction in OM proteins and LPS, as predicted by the freeze-etch ultrastructural study. The association of resistance to polymyxin with a reduction both in LPS and in the major OM proteins was observed.

MATERIALS AND METHODS

Bacterial strains. The strains of *P. aeruginosa* used in this study are given in Table 1. The original source, the year in which it was obtained, and the minimal inhibitory concentration (43) of polymyxin B are given for each strain. Those strains inhibited by 50 U/ml are considered polymyxin sensitive, whereas those strains having a minimal inhibitory concentration of 100 U/ml or greater are considered polymyxin resistant. The strains comprise three different types of

TABLE 1. *P. aeruginosa strains examined*

Strain designation	Minimal inhibitory concentration of polymyxin B (U)	Original source and year obtained
PAO1	50	B. W. Holloway, 1974
Isolate A	>6,000	H. E. Gilleland, Jr., 1975
Isolate A-reverted	100	H. E. Gilleland, Jr., 1978
BR-1	50	M. R. W. Brown, 1976
BR-6	200	M. R. W. Brown, 1976
BR-9	800	M. R. W. Brown, 1976
BR-6000	>6,000	H. E. Gilleland, Jr., 1978
HG-1	50	Louisiana State University Medical Center clinical laboratory, 1977
HG-2	50	Louisiana State University Medical Center clinical laboratory, 1977
HG-3	>6,000	Louisiana State University Medical Center clinical laboratory, 1977
HG-4	>6,000	S. Roman, Piedmont Hospital laboratory, Atlanta, Ga., 1977

isolates. The first system includes the polymyxin-sensitive PAO1 strain and the polymyxin-resistant isolate A which were used in the previous ultrastructural study (8). Isolate A was derived from the PAO1 wild-type strain. However, isolate A is not a stable genetic mutant but represents an adaptive response to the presence of polymyxin in the growth media. Upon growth in medium lacking polymyxin, isolate A reverts to polymyxin sensitivity. Similar adaptive resistance to polymyxin has been reported for *E. coli* (11) and *Proteus* strains (32). Brown's isolates (strains BR-6 and BR-9) are stable genetic mutants derived from the wild-type strain BR-1 and representing increasing levels of resistance (2). These strains were obtained from M. R. W. Brown (University of Aston in Birmingham, Birmingham, England). The last four strains listed in Table 1 are clinical isolates whose genetic backgrounds are not known. HG-1 and HG-2 were determined to be polymyxin sensitive, whereas HG-3 and HG-4 were determined to be polymyxin resistant upon initial isolation and antibiotic sensitivity testing by the hospital laboratories.

Growth conditions. All cells were grown in a basal medium previously used as "basal medium 2" in an ultrastructural study of *P. aeruginosa* (9). This medium contained the following at 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 0.005 M $MgSO_4$. The polymyxin-containing media consisted of this basal medium plus the appropriate concentration of polymyxin B sulfate per milliliter. The polymyxin B sulfate was kindly provided by Pfizer Inc., New York.

The cells were routinely grown in 4-liter Erlenmeyer flasks containing 2 liters of medium with vigorous stirring in an incubator at 30°C. A starter culture always was used to inoculate the fresh flask of medium to give an initial absorbance at 660 nm of approximately 0.05. The cells were harvested for the production of cell envelopes after they had reached the mid- to late-logarithmic growth phase.

The following strains were grown in basal medium without polymyxin before cell envelope production: PAO1, isolate A-reverted, BR-1, HG-1, and HG-2. Strains isolate A, BR-6000, HG-3, and HG-4 were grown in basal medium containing 6,000 U of polymyxin B per ml. BR-6 was grown in basal medium with 125 U of polymyxin B per ml, and BR-9 was grown in basal medium with 500 U of polymyxin B per ml.

Cell envelope production. Cell envelopes were prepared by the procedure of Stinnett et al. (33), using cells harvested from 8 liters of medium.

Separation of cytoplasmic membrane and OM. The OM was separated from the cytoplasmic membrane by the technique of Hancock and Nikaido (14).

Chemical analyses. Total protein was estimated by the Hartree modification (15) of the Lowry method with bovine serum albumin as the standard. Carbohydrate was determined by the anthrone method (30) with glucose as the standard. 2-Keto-3-deoxyoctonate (KDO) was estimated in samples after hydrolysis at 100°C for 8 min in 0.25 N H_2SO_4 by the method of Dröge et al. (4). Authentic KDO (Sigma Chemical Co., St. Louis, Mo.) was used as the standard. Succinate dehydrogenase was assayed as described by Kasahara and Anraku (16), and D-lactate dehydrogenase was assayed as described by Futai (6).

Statistics. The statistical significance of the values obtained in the chemical analyses was determined by performing the Student *t* test on the means, employing the two-tailed *t* table (3).

PAGE procedures. For polyacrylamide gel electrophoresis (PAGE), proteins were solubilized from the cell envelopes by heating at 100°C for 2 min in the sample buffer comprised of 0.0312 M Tris-hydrochloride, pH 6.8, with 2% sodium dodecyl sulfate (SDS), 10% glycerol, 5% 2-mercaptoethanol, and 10% urea. Slab gel electrophoresis was performed with a Studier gel apparatus (model 220, Bio-Rad Laboratories, Richmond, Calif.). The discontinuous SDS method employed was the Lugtenberg et al. modification(19) of the Laemmli procedure (18), utilizing gels of 15% acrylamide. A 125-μg sample of cell envelope, which contained approximately 60 to 65 μg of protein, was added to each slot of the gel apparatus in a volume of 100 μl. The following proteins (with corresponding molecular weights) were used as standards: albumin, 68,000; ovalbumin, 43,000; lactic dehydrogenase, 36,000; and trypsin, 23,000. Electrophoresis was performed at room temperature, using a constant current of 30 mA. Gels were stained by the four-step method of Fairbanks et al. (5).

Molecular weight calculations. The apparent molecular weights of the protein bands of interest were calculated by the method of Weber and Osborn (44), using relative electrophoretic mobilities.

RESULTS

Chemical analyses. The polymyxin-resistant strains in general showed an increase in cell

envelope total protein and a decrease in both cell envelope carbohydrate and KDO content (Table 2). Isolate A, which is derived from the wild-type PAO1 strain, has acquired as an adaptive response to the presence of polymyxin in the growth medium the ability to grow in medium containing 6,000 U of polymyxin per ml. The cell envelopes of this isolate showed a significant increase in protein, a significant decrease in carbohydrate, and a significant decrease in KDO as compared with these cell envelope components in the PAO1 polymyxin-sensitive strain. When isolate A reverted to greater sensitivity to polymyxin upon growth in medium lacking polymyxin, there was no statistically significant difference between the cell envelope concentrations of protein, carbohydrate, or KDO as compared with the wild-type PAO1 strain. Thus, the emergence of polymyxin resistance appeared to be associated with chemical alterations of the cell envelope, with these alterations being lost when the cell reverted to increased sensitivity to polymyxin.

The stable polymyxin-resistant mutants of Brown (2) showed a similar pattern of cell envelope alterations. Although the BR-6 strain with resistance to 200 U of polymyxin per ml did not show a significant increase in cell envelope total protein, both strains BR-9 and BR-6000 did have a significant increase in cell envelope total protein. The cell envelopes of all three strains (BR-6, BR-9, and BR-6000) showed a significant decrease in KDO, whereas the envelopes of BR-6000 also had a significant decrease in carbohydrate.

The clinical strains also followed the same pattern of cell envelope alterations as was seen in the PAO1-isolate A system. A direct comparison of the clinical isolates resistant to polymyxin with clinical isolates sensitive to polymyxin is complicated by the fact that the sensitive strains do not represent the wild-type strain from which the resistant strains were derived. Instead, the clinical isolates are all of unknown genetic backgrounds. Nevertheless, the cell envelopes from both clinical isolates resistant to polymyxin (HG-3 and HG-4) showed a significantly increased concentration of protein and a significantly lower content of both carbohydrate and KDO when compared with cell envelopes from the HG-2 polymyxin-sensitive clinical isolate. When compared with the HG-1 sensitive clinical isolate, cell envelopes from the HG-3 and HG-4 resistant clinical isolates did not show a statistically significantly increased amount of protein, but they did show a significantly lower content of both carbohydrate and KDO.

PAGE of the cell envelope. The cell envelope from the PAO1 polymyxin-sensitive strain contained a number of protein bands upon PAGE (Fig. 1). Ten bands of particular interest are labeled in Fig. 1, and the calculated molecular weight of each is given. It should be noted that the band labeled as protein 6 does not appear to represent a single protein species. Instead, this band is resolved in Fig. 2 and 3 into

TABLE 2. *Chemical analyses of cell envelopes of the P. aeruginosa strains*[a]

STRAIN	TOTAL PROTEIN			CARBOHYDRATE			KDO		
	MEAN[b]	PERCENT CHANGE[c]	P VALUE[d]	MEAN	PERCENT CHANGE	P VALUE	MEAN	PER CENT CHANGE	P VALUE
PAO 1	50.7 (±2.3)	—	—	3.94 (±0.48)	—	—	1.24 (±0.06)	—	—
Isolate A	56.5 (±2.2)	+11.44	<.001	3.28 (±0.12)	-16.75	<.05	0.96 (±0.11)	-22.58	<.001
Isolate A-Reverted	48.1 (±2.3)	- 5.13	>.05	4.08 (±0.10)	+ 3.55	>.50	1.18 (±0.09)	- 4.84	>.20
BR-1	52.9 (±2.9)	—	—	3.02 (±0.40)	—	—	1.03 (±0.08)	—	—
BR-6	53.5 (±3.5)	+ 1.13	>.70	3.12 (±0.33)	+ 3.31	>.70	0.86 (±0.10)	-16.50	<.02
BR-9	57.4 (±3.2)	+ 8.51	<.05	2.93 (±0.55)	- 2.98	>.70	0.68 (±0.03)	-33.98	<.001
BR-6000	56.4 (±1.8)	+ 6.62	<.05	2.28 (±0.10)	-24.50	<.02	0.74 (±0.09)	-28.16	<.001
HG-1	48.9 (±4.2)	—	—	4.78 (±0.26)	—	—	1.24 (±0.11)	—	—
HG-2	46.5 (±3.4)	—	—	6.98 (±0.43)	—	—	1.30 (±0.08)	—	—
HG-3	52.3 (±5.2)	+6.95, +12.47	>.20, <.05	3.62 (±0.48)	-24.26, -47.85	<.01, <.001	1.01 (±0.05)	-18.55, -22.31	<.01, <.001
HG-4	53.3 (±3.7)	+8.99, +14.62	>.05, <.01	3.58 (±0.92)	-25.10, -48.71	<.05, <.001	1.06 (±0.05)	-14.52, -18.46	<.01, <.001

[a] Results expressed as dry weight percentages.

[b] Mean with standard deviation given in parentheses. The numbers of determinations made for protein, carbohydrate, and KDO were six, four, and five, respectively.

[c] For each group of strains, this value represents the percent increase (+) or decrease (−) of the resistant strain compared with the sensitive wild-type strain of that group. In the clinical isolate group, the first value compares the resistant strain with the HG-1 sensitive strain, and the second value compares it with the HG-2 sensitive strain.

[d] Value determined by performing the Student *t* test on the means, employing the two-tailed *t* table. *P* values of <0.05 and lower were considered statistically significant.

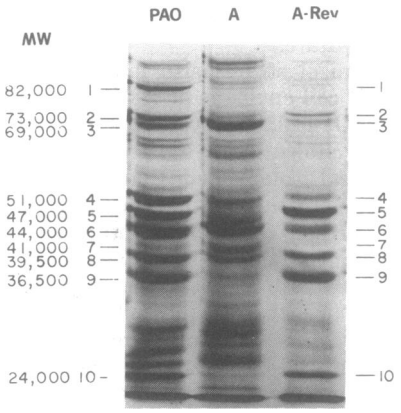

FIG. 1. *SDS-PAGE of cell envelopes of P. aeruginosa. PAO, Polymyxin-sensitive PAO1 strain; A, polymyxin-resistant isolate A; A-Rev, isolate A strain after reversion to greater polymyxin sensitivity. Ten protein bands (1 through 10) of interest are labeled, with their apparent molecular weights (MW) given on the left. Note that isolate A had losses in proteins 1, 2, 4, 5, 9, and 10, with increased concentrations of proteins 3 and 7. Of particular interest is the regaining of proteins 5, 9, and 10 by isolate A-reverted upon regaining polymyxin sensitivity.*

doublet bands. Comparison of the PAGE protein pattern of the polymyxin-resistant isolate A with that of the PAO1 strain reveals that the emergence of resistance to polymyxin is associated with alterations in the concentrations of a number of proteins in the cell envelope. Isolate A had a significant loss in concentration of proteins 1, 2, 4, 5, 9, and 10. Proteins 3 and 7, on the other hand, appeared to be increased significantly in concentration. In association with reversion to greater polymyxin sensitivity, the cell envelope of the isolate A-reverted strain had regained many of the characteristics of the parent PAO1 strain. Specifically, the concentration of protein 2 was increased and the concentration of protein 3 was reduced, restoring an appearance more like that of the PAO1 strain than like that of isolate A for these bands. The concentrations of proteins 5, 9, and 10 were increased, whereas protein 7 was lost. The concentrations of proteins 1 and 4 remained essentially the same as found in the cell envelopes of isolate A.

The Brown strains revealed findings similar to those observed with the PAO1-isolate A strains. As shown in Fig. 2, the pattern of protein bands seen in the wild-type BR-1 strain agreed quite well with the pattern found in the PAO1 strain. Only protein band 1 was missing from the BR-1 strain out of the 10 bands of interest seen in PAO1. The cell envelopes from the polymyxin-resistant strains BR-6, BR-9, and BR-6000 showed protein alterations similar to those detected in isolate A. The concentrations of proteins 2, 4, 5, 9, and 10 were reduced, whereas proteins 3 and 7 were increased in concentration. Thus, there was excellent agreement in the protein alterations found in these two different groups of bacterial strains upon PAGE analysis.

The protein patterns of the cell envelopes of the polymyxin-sensitive clinical isolates HG-1

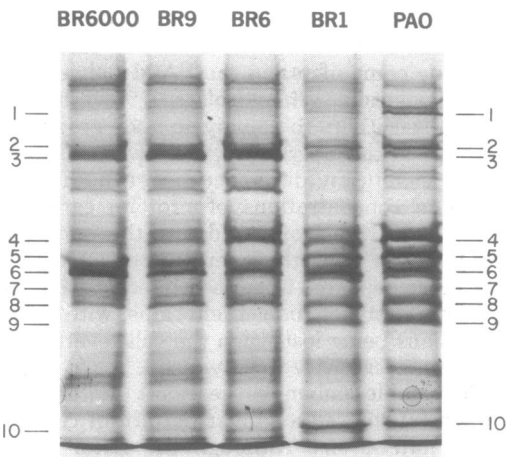

FIG. 2. *SDS-PAGE of cell envelopes of P. aeruginosa. PAO, Polymyxin-sensitive PAO1 strain; BR1, polymyxin-sensitive Brown wild-type strain; BR6, Brown mutant resistant to 200 U of polymyxin per ml; BR9, Brown mutant resistant to 800 U of polymyxin per ml; BR6000, Brown mutant resistant to 6,000 U of polymyxin per ml. Note particularly the loss of proteins 5, 9, and 10 in the polymyxin-resistant strains.*

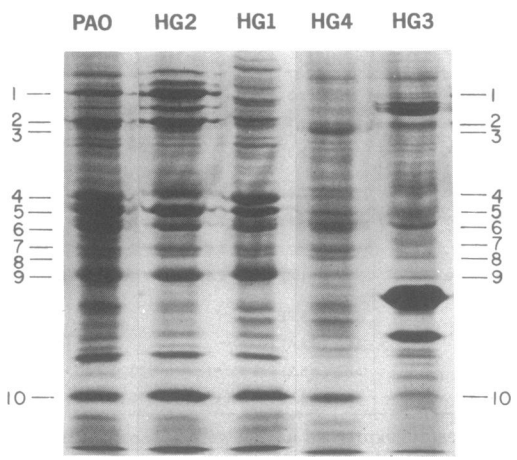

FIG. 3. *SDS-PAGE of cell envelopes of P. aeruginosa. PAO, polymyxin-sensitive PAO1 strain; HG1 and HG2, polymyxin-sensitive clinical isolates; HG3 and HG4, polymyxin-resistant clinical isolates. Note the loss of the major protein bands in both of the resistant clinical isolates.*

and HG-2 showed remarkable agreement with the PAGE protein pattern of the polymyxin-sensitive PAO1 strain (Fig. 3). On the other hand, the polymyxin-resistant clinical isolates HG-3 and HG-4 both had losses in proteins 4, 5, 6, 9, and 10 as compared with the polymyxin-sensitive strains HG-1 and HG-2. In addition, both HG-3 and HG-4 appeared to have lost protein 1. However, the polymyxin-sensitive HG-1 strain had a similar loss of protein 1. Likewise, the alterations in concentrations of proteins 2 and 3 did not show a consistent pattern in these two polymyxin-resistant clinical isolates. Thus, the alterations in proteins 1, 2, and 3 were not associated in a clear-cut manner with polymyxin resistance in these clinical isolates, whereas the losses in proteins 4, 5, 6, 9, and 10 were.

It is worth noting that polymyxin-resistant strains in all three groups of isolates appeared to have increased concentrations of some protein bands. For instance, both isolate A and isolate BR-6000 had increased concentrations of proteins 3 and 7, whereas HG-3 had greatly increased concentrations of two protein bands in the 30,000-molecular-weight range on the gel below the location of protein 9. In addition to these more obviously increased bands, a number of the less concentrated bands in the gel appeared to be increased. These increases could account for the increase in total protein in the cell envelopes from polymyxin-resistant strains, even though the concentration of the major protein bands was reduced.

Identification of OM protein bands. Separation of the OM from the cytoplasmic membrane by the method of Hancock and Nikaido (14) yielded four components after the second sucrose gradient centrifugation. This was in agreement with the results obtained by Hancock and Nikaido (14), with the four components corresponding to their cytoplasmic membrane, intermediate membrane, and two OM bands. These four components were analyzed by SDS-PAGE (Fig. 4). The cytoplasmic membrane (IM in Fig. 4) contained a large number of minor protein bands, with no band appearing prominent on the gel. The intermediate component (M in Fig. 4) appeared to be a mixture of both proteins prominent in the OM and proteins which appeared to be cytoplasmic membrane proteins. The OM components (OM1 and OM2 in Fig. 4) contained nine protein bands in increased concentrations, with the proteins labeled as proteins 9 (36,500 molecular weight), 10 (24,000 molecular weight), and 5 (47,000 molecular weight) judged to be "major" OM proteins.

The separation of the OM from the cytoplasmic membrane appeared to be successful not only on the basis of the PAGE protein patterns closely matching those reported by Hancock and Nikaido but also on the basis of the analysis of the KDO content and enzymatic activities of the four membrane components (Table 3).

DISCUSSION

The polymyxin-resistant strains showed the expected reduction in LPS, as evidenced by an average loss in KDO content of approximately 24.4%. Upon SDS-PAGE (Fig. 1, 2, and 3) all of

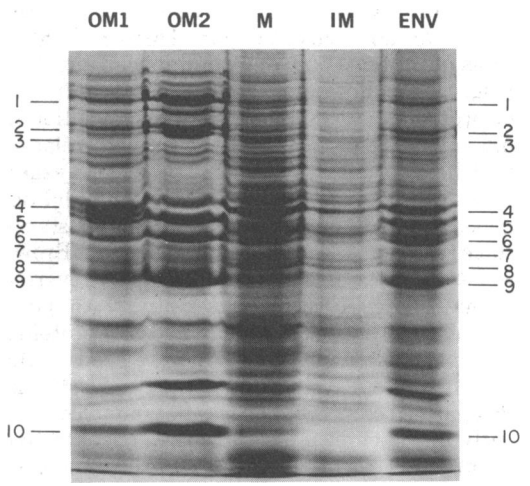

FIG. 4. *SDS-PAGE of the separated membrane fractions of P. aeruginosa. ENV, Whole cell envelope; IM, cytoplasmic membrane fraction; M, intermediate fraction; OM1 and OM2, OM fractions. Note that the predominant protein bands are located in the OM fractions. Proteins 5, 9, and 10 appear to be major OM proteins.*

TABLE 3. *Analyses of the isolated membrane components*

Envelope component[a]	Enzymatic activity		KDO[d]
	LDH[b]	SDH[c]	
IM	16.3	41.9	0.14
M	3.8	13.0	0.77
OM2	1.4	10.4	1.04
OM1	1.2	2.8	1.45
OM2/IM ratio	0.09	0.25	7.43
OM1/IM ratio	0.07	0.07	10.36

[a] IM, Cytoplasmic membrane; M, intermediate component; OM1 and OM2, OM components.

[b] LDH, Lactate dehydrogense. Activity is expressed as micromoles of dimethylthiazolyl-diphenyltetrazolium bromide reduced per minute per milligram of protein.

[c] SDH, Succinate dehydrogenase. Activity is expressed as nanomoles of dichloroindolephenol reduced per minute per milligram of protein.

[d] Amount expressed as dry weight percentage.

the polymyxin-resistant strains revealed a loss in protein bands which were subsequently shown (Fig. 4) to be OM proteins. The major OM proteins, which were judged to be proteins 9 (36,500 molecular weight), 10 (24,000 molecular weight), and 5 (47,000 molecular weight), were greatly reduced in all of the polymyxin-resistant strains. Thus, polymyxin resistance was associated with the expected reduction of OM protein and LPS in this study. The association of the loss of the OM major proteins with the acquisition of polymyxin resistance was strengthened by the observation that when isolate A reverted to greater sensitivity, it regained high concentrations of proteins 5, 9, and 10 in its cell envelope (Fig. 1). Furthermore, the loss in concentration of proteins 5, 9, and 10 upon the acquisition of polymyxin resistance was the only correlation that held for all three systems of strains examined. For instance, the loss of protein 1 did not appear to be essential for polymyxin resistance since protein 1 was not found in the Brown isolates nor was it regained in isolate A-reverted when isolate A regained sensitivity to polymyxin. Likewise, even though a loss of protein 2 and an increase in protein 3 were seen in the PAO1-isolate A system and in the Brown strains, this pattern of alterations was not found consistently in the clinical isolates. The concentration of protein 4 was not regained in the isolate A-reverted strain, nor did it appear to be reduced in the BR-6 polymyxin-resistant strain. The concentration of protein 6 was unchanged in the PAO1-isolate A system and in the Brown strains, and the concentration of protein 7 was not increased in the clinical isolates resistant to polymyxin. Thus, the loss in concentration of proteins 5, 9, and 10 appears to be the most likely of all the alterations seen upon SDS-PAGE analysis responsible for resistance to polymyxin.

The loss of OM major proteins may be correlated with the acquisition of polymyxin resistance through the loss of pores through the OM which one or more of these proteins would normally produce. Several OM proteins have been shown to play a role in pore formation in the OM of *E. coli* (22, 40, 41) and *Salmonella typhimurium* (23). If polymyxin utilizes a pore to penetrate the OM, loss of the pore protein would result in a loss of permeability through the OM. This would result in protection of the still-sensitive polymyxin target sites on the cytoplasmic membrane by an exclusion mechanism as has been previously postulated (8, 13). The idea of polymyxin resistance being due to the loss of protein pores is made more attractive by the previous finding in *Proteus mirabilis* that the cell envelope lipids were not the important determinants of sensitivity and resistance to polymyxin B (35). In addition, the finding by Hancock and Nikaido (14) that the exclusion limit of the OM of *P. aeruginosa* for saccharides is substantially larger (3,000 to 9,000 daltons) than that of the enteric bacteria (500 to 600 daltons) makes the possibility of the polymyxin molecule (1,200 daltons) being able to penetrate the OM of *P. aeruginosa* through a pore more tenable. A likely candidate for the pore protein which might be involved would be protein 9 of 36,500 apparent molecular weight, as the porins in enteric gram-negative bacteria have been found to be around 35,000 to 40,000 molecular weight.

The ability of gram-negative bacteria to alter the protein composition and architecture of the OM in response to its environment is truly remarkable. In this study, *P. aeruginosa* appears to have altered its OM both ultrastructurally (7, 8) and chemically to gain resistance to the antibiotic polymyxin B. *Neisseria gonorrhoeae* has also been found to alter its OM protein composition in association with antibiotic resistance (12). Both *S. typhimurium* (25) and *E. coli* K-12 (20, 26) also are known to be capable of varying the OM protein composition. This ability to alter the protein composition of the OM in response to the environment is undoubtedly of great survival value to the bacterium.

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