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Outer Membrane Protein H1 of *Pseudomonas aeruginosa*: Involvement in Adaptive and Mutational Resistance to Ethylenediaminetetraacetate, Polymyxin B, and Gentamicin

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It is well established that *Pseudomonas aeruginosa* cells grown in Mg^{2+}-deficient medium acquire nonmutational resistance to the chelator ethylenediaminetetraacetate and to the cationic antibiotic polymyxin B; this type of resistance can be reversed by transferring the cells to Mg^{2+}-sufficient medium for a few generations. Stable mutants resistant to polymyxin B were isolated and shown to have also gained ethylenediaminetetraacetate resistance. Both the mutants and strains grown on Mg^{2+}-deficient medium had greatly enhanced levels of outer membrane protein H1 when compared with the wild-type strain or with revertants grown in Mg^{2+}-sufficient medium. It was determined that in all strains and at all medium Mg^{2+} concentrations, the cell envelope Mg^{2+} concentration varied inversely with the amount of protein H1. In addition, the increase in protein H1 in the mutants was associated with an increase in resistance to another group of cationic antibiotics, the aminoglycosides, e.g., gentamicin. We propose that protein H1 acts by replacing Mg^{2+} at a site on the lipopolysaccharide which can otherwise be attacked by the cationic antibiotics or ethylenediaminetetraacetate.

In recent years considerable difficulty has been experienced in the treatment of *Pseudomonas aeruginosa* infections, due to the difference between in vitro and in vivo antibiotic susceptibilities (4, 6). Two possible causes are the development of adaptive (nonmutational) resistance in vivo (as demonstrated for certain antibiotics in vitro [1, 9, 18]) and the antagonism of antipseudomonal antibiotics, such as polymyxin B and aminoglycosides, by Mg^{2+} and Ca^{2+} (16, 23). Brown and Melling (1) first demonstrated that growth of sensitive *P. aeruginosa* in Mg^{2+}-limited medium resulted in the acquisition of resistance to polymyxin B and EDTA (see also reference 9). The latter observation was particularly interesting, since *P. aeruginosa* is unusually susceptible to lysis by EDTA (c.f. enteric bacteria [19]). The fact that the Ca^{2+}-specific chelator ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*-tetraacetate was relatively ineffective (13, 19), combined with the results for Mg^{2+}-limited cells, suggested a key role for Mg^{2+} in EDTA and polymyxin B lyses. Recently, the separation of outer and inner membranes of *P. aeruginosa* in the absence of EDTA was reported by Hancock and Nikaido (11). Six to eight major outer membrane proteins were shown to be present, depending on the growth conditions (10). We report here that Mg^{2+}-limited cells which have acquired resistance to polymyxin B and EDTA, as well as polymyxin B-resistant mutants, have greatly increased levels of outer membrane protein H1. We suggest that protein H1 replaces Mg^{2+} at a site on the lipopolysaccharide (LPS) and protects this site from EDTA and polymyxin B attacks.

MATERIALS AND METHODS

Media and growth conditions. The growth medium was BM2 minimal medium (9) containing 10 μM $FeSO_4$ and 20 mM succinate or 0.4% (wt/vol) glucose. Mg^{2+}-deficient medium contained 0.02 mM $MgSO_4$, whereas Mg^{2+}-sufficient medium contained 0.5 or 5 mM $MgSO_4$. Control experiments demonstrated that in these media protein H1 increased throughout the growth phase to a maximum in stationary phase. Cells grew at the same rate in both Mg^{2+}-sufficient and Mg^{2+}-deficient media, although the final yields of cells differed.

Strains and mutant isolation. Strains H181 and H185 were independently isolated from *P. aeruginosa* PAO1 strain H103 (10) after diethyl sulfate mutagenesis by selection on BM2 minimal agar plates (9) containing 10 μM $FeSO_4$, 20 mM potassium succinate, 0.5 mM $MgSO_4$, and 50 μg of polymyxin B sulfate per ml. The revertant H207 and five similar revertants of H181 and H185 were isolated from old cultures of H181 and H185 by screening for loss of polymyxin B resistance. The levels of resistance to polymyxin B on BM2 succinate agar plates were 0.8, 75, 75, and 0.8 μg/ml for H103, H181, H185, and H207, respectively.

Bacteriophages and aeroginocins. All methods used in the handling of bacteriophages were described previously (12). A pilus-deficient strain of *P. aerugi-*

nosa PAO1, AK1144, and an LPS-altered (rough) strain, AK43, obtained from A. Kropinski (Queen's University, Kingston, Canada), were used to characterize the phages used. Phages 2, 7, 21, 44, 68, 109, F8, M6, 1214, and C21 were obtained from T. L. Pitt (Public Health Laboratory, London, England), phage B39 was obtained from D. E. Bradley (Memorial University, St. John's, Canada); phages G101, F116, and D3c$^+$1$^-$ were obtained from T. Iijima (Institute for Fermentation, Osaka, Japan); and phage E79 was obtained from A. Kropinski. These phages were purified from single plaques, using H103 as a host strain. All other phages were isolated in our laboratory. Phages B9A, B9B, B9E, and B9F were independently isolated host range mutants of M6 able to plaque on AK1144. Four of the phages used, F8, 109, 44, and E79, were characterized as smooth LPS specific, since they failed to plaque on AK43 or other rough, LPS-altered strains and could be shown to adsorb to purified smooth LPS, in agreement with the results of Kropinski et al. (14). Three phages, M6, B39, and 8, were characterized as pilus specific by their inability to plate on pilus-deficient strains. Phages 7, 21, 68, B9A, B9B, C21, G101, and F116 plated well on LPS-altered and pilus (or flagellum)-deficient strains and failed to adsorb to LPS and thus appeared to have protein receptors. Phages 2, B5A, B6B, 1214, B7A, B9F, B7A, C7B, and D3c$^+$1$^-$, which also failed to absorb to LPS, plated well on pilus-deficient or wild-type strains but poorly on LPS-altered rough strains and may have had protein or LPS receptors.

The aeruginocins used were a *P. aeruginosa* typing set sent to us by A. Kropinski, in addition to the aeruginocins from strain H41 received from J. Govan (University of Edinburgh, Edinburgh, Scotland) and from strains PAF41 and PAH108 from B. Holloway (Monash University, Clayton, Victoria, Australia). The receptors of these aeruginocins are as yet uncharacterized, but they inhibit H103 and AK1144 equally well, although some only inhibit AK43 (but not H103).

Characterization of whole cell, cell envelope, and outer membrane proteins. For whole cell preparations, 18-h or mid-logarithmic-phase (grown to an optical density at 650 nm of 0.6) cultures were centrifuged down, and the cells were suspended in 2% sodium dodecyl sulfate–20 mM Tris-hydrochloride, pH 8.0. After treatment at 100°C for 10 min, residual cells were removed by centrifugation at 27,000 × *g* for 20 min. The resulting supernatant was sonicated (1 min, setting 5, Biosonik sonicator [Bronwill Scientific, Inc., Rochester, N.Y.]) to shear DNA and reduce viscosity, and the sample was applied directly to the gel. Outer membranes were prepared as described previously (10). To prepare cell envelopes, cells from 18-h or mid-logarithmic-phase cultures were collected by centrifugation, suspended in 10 mM sodium phosphate buffer (pH 7.4) containing 0.1 mg of pancreatic DNase I (Sigma Chemical Co., St. Louis, Mo.) per ml, and broken in a French Press at 14,000 lb/in^2. Whole cells were removed by centrifugation (1,000 × *g*, 10 min), and the resulting supernatant was diluted in the same buffer and centrifuged at 160,000 × *g* for 2 h. The cell envelope pellet was suspended in deionized water. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed, using the 14% acrylamide system containing 0.07 M NaCl in the running gel as previously described (10). Only in the presence of 0.07 M NaCl or with high acrylamide monomer concentrations are outer membrane proteins H1 and H2 separated (10).

Antibiotic and EDTA susceptibility testing. To test lysis by either polymyxin B or EDTA-Tris, cells were grown in BM2 succinate medium, containing the appropriate concentration of Mg^{2+}, until they reached an optical density at 650 nm of 0.6. They were then centrifuged down and suspended in either 75 μg of polymyxin B per ml in 30 mM sodium phosphate buffer (pH 7.4) or 10 mM EDTA–50 mM Tris-hydrochloride buffer (pH 8.5) and held at 37°C, and at regular intervals the optical density at 650 nm was read in a Perkin-Elmer model 124 spectrophotometer. To test killing by these agents, cells grown to an optical density at 650 nm of 0.6 in BM2 succinate medium with the desired Mg^{2+} levels were diluted 1,000-fold into 30 mM sodium phosphate buffer, pH 7.0, or 50 mM Tris-hydrochloride, pH 8.5. The cells were then incubated at 37°C, and 5 min after the addition of polymyxin B (final concentration, 75 μg/ml) or 10 mM EDTA, the cells were diluted and plated for viable counts on proteose peptone no. 2 agar. Antibiotic resistance was measured in 1-ml volumes of BM2 succinate medium with the stated Mg^{2+} levels. The inoculum used was approximately 10^5 cells of 18-h culture grown in medium identical to the test medium. The level of resistance was taken as the highest antibiotic concentration showing visible turbidity after 24 h at 37°C. Polymyxin B sulfate (8,000 U/mg) and colistin methane sulfonate (polymyxin E, 12,470 U/mg) were purchased from Sigma Chemical Co. Gentamicin sulfate was a gift from Schering Co. (Pointe Claire, Canada). Streptomycin sulfate and tetracycline hydrochloride were purchased from Sigma Chemical Co. Tobramycin was a gift from Eli Lilly & Co. (Indianapolis, Ind.). Carbenicillin (Pyopen) was purchased from Ayerst Laboratories (Montreal, Canada).

Other assays. The Mg^{2+} and Ca^{2+} levels of cell envelopes were obtained by atomic absorption spectroscopy with the kind help of S. Ma and D. H. Copp after extraction by the method of Kenward et al. (13). All other assays used have been described previously (11).

RESULTS

Resistance to polymyxin B and EDTA. Our wild-type *P. aeruginosa* PAO1 strain, H103, grown on Mg^{2+}-sufficient medium was susceptible to EDTA and polymyxin B killing (Table 1) and lysis (Fig. 1). In contrast, H103 grown under Mg^{2+}-deficient conditions was 70- to 700-fold more resistant to these agents (Table 1, Fig. 1), in agreement with previously published results (1, 9). This resistance could be reversed by culturing H103 on Mg^{2+}-sufficient medium for a few generations. Two polymyxin B-resistant mutants of H103, strains H181 and H185, were resistant to EDTA and polymyxin B killing (Table 1) and lysis (Fig. 1), irrespective of the me-

dium Mg^{2+} concentration. The mutant phenotype was stable for eight consecutive single-colony isolations on Mg^{2+}-sufficient medium. Six spontaneous revertants of H181 and H185 (selected for polymyxin susceptibility, e.g., strain H207) had regained all of the wild-type properties of strain H103 (see Fig. 1 and below). These data suggest that H181 and H185 each have a single mutation resulting in phenotypic alterations mimicking those of the Mg^{2+}-limited, nonmutationally resistant strain H103.

Outer membrane protein patterns. Outer membrane protein H1 was shown to be up to 24-fold increased in Mg^{2+}-limited H103 cells (Fig. 2, compare gel E with gel F; Table 2), whereas the protein G level was depressed 3-fold. Under these conditions, H1 was by far the major cellular protein as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of whole cell proteins. The polymyxin B-resistant mutants H181 and H185 had constitutively high levels of protein H1 (Fig. 2, gels B and C), which varied only twofold with changing Mg^{2+} concentrations in the medium (Table 2). The level of protein G was also depressed in both H181 and H185. Since other strains with greatly depressed levels of protein G (e.g., see reference 10) had no alteration in polymyxin B or EDTA resistance or in levels of protein H1, we conclude that the decrease in this protein was unrelated to the resistance observed in our adapted or mutant strains. Similarly, a variety of growth conditions (e.g., pyruvate as a carbon source, limiting NH_4^+) depressed levels of protein G without affecting protein H1 levels. We also observed an inner membrane protein of 75,000 daltons in all strains (e.g., Fig. 2, gel E) grown on Mg^{2+}-deficient medium but never in strains grown on Mg^{2+}-sufficient medium. Thus, we conclude that this protein also is unrelated to the resistance phenomena reported here. Although a slight decrease in the levels of porin protein F was sometimes observed in strains with high levels of protein H1, no correlation could be found between the levels of porin protein F and the reported resistances in a variety of strains or under various growth conditions. All six revertants had regained wild-type membrane protein patterns, indistinguishable from those of the wild-type strain, H103, at all Mg^{2+} concentrations.

Bacteriophages and bacteriocins have outer membrane receptors. Strains resistant to these agents often have outer membrane defects (e.g., see references 12 and 14). Thus, testing the susceptibility of strains to these agents is a useful

TABLE 1. *Resistance to killing by EDTA-Tris and polymyxin B tested in the absence of Mg^{2+}* [a]

Strain	Mg^{2+} during growth (mM)	Survivors (%)	
		EDTA-Tris	Polymyxin B
H103	0.02	65	15
	0.5	0.9	<0.02
H181	0.02	77	42
	0.5	76	22

[a] Strain H103 and its polymyxin B-resistant mutant H181 were grown in either Mg^{2+}-deficient (0.02 mM) or Mg^{2+}-sufficient (0.5 mM) medium to mid-logarithmic phase. They were then diluted 1,000-fold into Mg^{2+}-free buffer as described in the text. Five minutes after the addition of polymyxin B or EDTA, the cells were diluted and plated for viable counts.

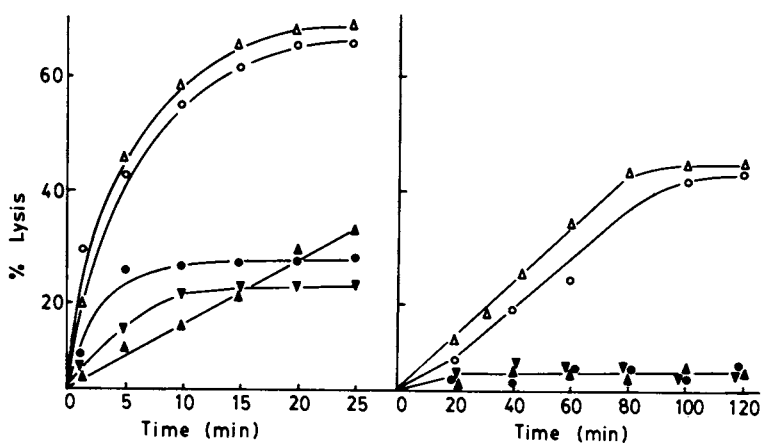

FIG. 1. *Effects of adaptation on Mg^{2+}-limited medium and of mutation to polymyxin B resistance on susceptibilities of cells to lysis by polymyxin B (B) and EDTA-Tris (A). Symbols: ▲, wild-type strain H103 grown in Mg^{2+}-limited (0.02 mM) medium; △, strain H103; ●, polymyxin B-resistant mutant H181; ▼, polymyxin B-resistant mutant H185; ○, revertant H207, grown in Mg^{2+}-sufficient (0.5 mM) medium.*

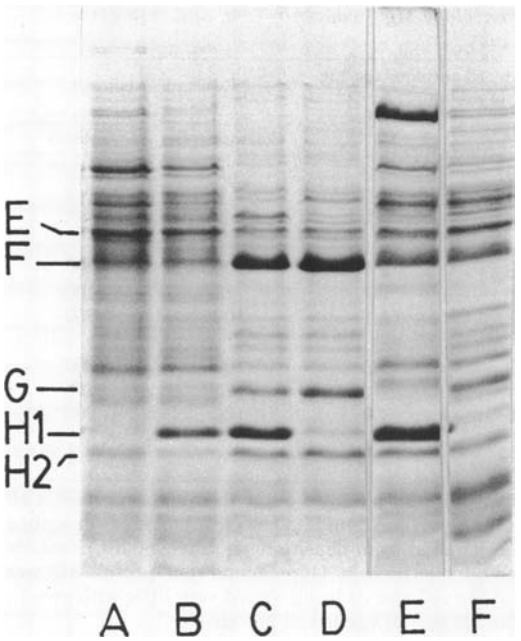

FIG. 2. *Effect of adaptation on Mg^{2+}-limited medium and of mutation to polymyxin B resistance on levels of protein H1. Gels: A and B, whole cell preparations of strains H103 and H185, respectively, grown with 5 mM Mg^{2+}; C and D, outer membrane preparations of strains H181 and H103, respectively, grown with 0.5 mM Mg^{2+}; E and F, cell envelopes of H103 grown with 0.02 and 5 mM Mg^{2+}, respectively. The high-molecular-weight protein seen in gel E but not gel F cell envelopes is an inner membrane protein of unknown function induced in either H103 or H181 grown in low Mg^{2+}.*

method of revealing outer membrane alterations which might otherwise elude biochemical detection. We found no differences in the susceptibilities of the polymyxin B-resistant mutants H181 and H185 to 24 phages and 22 aeroginocins when compared with the wild-type strain, H103. Since some of the phages were smooth LPS or pilus receptor specific, we concluded that H181 and H185 had smooth LPS and were piliated. Furthermore, it is unlikely that they had gross surface alterations. Although pilin has been shown to have a similar molecular weight to protein H1 (17), they are distinct proteins as determined in our sodium dodecyl sulfate-polyacrylamide gel electrophoresis system, using a pilin sample kindly provided by W. Parynchych (University of Alberta, Edmonton, Canada).

Resistance to polymyxins and cell envelope magnesium levels. The changes in the level of protein H1 in the outer membranes of strains H103, H181, H185, and H207 grown at various medium Mg^{2+} concentrations were reflected in part by the degrees of resistance to two polymyxin antibiotics (Table 2). Thus, a 7-fold increase in H1 levels between strains H103 and H181 grown in BM2 succinate medium containing 0.5 mM Mg^{2+} was associated with a 25-fold increase in polymyxin B and colistin resistances. Similarly, strain H103 adapted to growth on Mg^{2+}-deficient medium (0.02 mM Mg^{2+}) had 7-fold higher levels of protein H1 than did H103 grown on Mg^{2+}-sufficient medium (0.5 mM Mg^{2+}) and was 10-fold more resistant to the polymyxin antibiotics. It has been well established that high Mg^{2+} concentrations in the medium inhibit the action of polymyxin antibiotics (16). Thus, in the experiments described in Table 2, we observed an increase in polymyxin resistance in the presence to 5 mM Mg^{2+}, despite the reduced levels of protein H1. Since at this Mg^{2+} concentration there was a 60-fold (at 100 μg of polymyxin B per ml) to 1,000-fold (at 4 μg of polymyxin B per ml) molar excess of Mg^{2+} over polymyxin B, the results can be explained by competition of Mg^{2+} and the polymyxins for a site on the LPS normally occupied by Mg^{2+} (see Discussion). In agreement with this view, when cells were grown in 5 mM Mg^{2+} and then subjected to a polymyxin B killing assay in the absence of Mg^{2+} (such as described in Table 1), they were somewhat more susceptible to polymyxin B than were cells grown in 0.5 mM Mg^{2+} (data not shown). Competition between Mg^{2+} and the polymyxins also explains the 25-fold difference in the resistances of H181 or H185 grown and tested at 0.5 mM Mg^{2+} when compared with H103 grown at 0.02 mM Mg^{2+} despite similar protein H1 levels (Table 2). However, the resistances of these two strains grown at these Mg^{2+} concentrations were quite similar when measured in the absence of Mg^{2+} (Table 1). The changes in the levels of outer membrane protein H1 were also reflected by the Mg^{2+} concentration of the cell envelopes of strains H103, H181, and H185 grown at various medium Mg^{2+} concentrations. There was a reciprocal relationship between the level of Mg^{2+} in the cell envelope and the amount of protein H1 (Table 2). Since we used defined medium without added Ca^{2+}, the cell envelope Ca^{2+} levels were low and unrelated to protein H1 levels.

Susceptibility to aminoglycosides and other antibiotics. Since Mg^{2+} had been previously shown to antagonize gentamicin action (23), we tested our mutants for enhanced resistance to three representative aminoglycoside antibiotics, gentamicin, streptomycin, and tobramycin, in the presence of 0.5 mM Mg^{2+}. All resistance levels were consistently obtained in 6 to 10 independent determinations. Strains H181 and H185 were reproducibly fourfold more re-

TABLE 2. *Levels of outer membrane protein H1, cell envelope Mg^{2+} concentration, and resistance to polymyxins*

Strain	Mg^{2+} during growth (mM)	Outer membrane protein ratio (H1:H2)[a,b]	Cations in cell envelope (μg/mg of protein)[b,c]		Resistance[d] (μg/ml)	
			Mg^{2+}	Ca^{2+}	Polymyxin B	Colistin
H103	0.02	4.7	4.8	1.2	10	50
	0.5	0.7	17.1	0.9	1	4
	5.0	0.2	20.5	0.8	4	25
H181	0.02	6.3	3.8	1.4	100	200
	0.5	4.7	—[e]	—	25	100
	5.0	3.4	11.6	1.1	100	200
H185	0.02	6.4	2.9	1.3	100	200
	0.5	4.6	—	—	25	100
	5.0	2.9	12.6	1.0	100	200
H207	0.5	0.8	—	—	1	4

[a] Effect of various Mg^{2+} concentrations in the growth medium on levels of outer membrane protein. Cell envelopes were isolated and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described in the text. The ratios of protein H1 to protein H2 were calculated from densitometer tracings of Coomassie brilliant blue R250-stained gels loaded with a standard amount of protein. Outer membrane protein H2 was used as a reference, since it was one of the major proteins of the cell and its levels varied very little with growth conditions, as judged by sodium dodecyl sulfate-polyacrylamide gels of whole cell proteins.

[b] It was determined that the amount of protein per milligram (dry weight) of cell envelope and the amount of 2-keto-3-deoxyoctonate per milligram of protein varied less than 6% ($P > 0.05$ by Student's t test) under the growth conditions used. There was a linear reciprocal relationship between Mg^{2+} levels in the cell envelope and protein H1 levels (correlation coefficient r from linear regression of the above results = 0.983).

[c] The Mg^{2+} and Ca^{2+} levels were obtained by atomic absorption spectroscopy. Results are given as the amount per milligram of cell envelope protein.

[d] Cells (10^5 colony-forming units per ml) grown in either 0.02 or 0.5 mM Mg^{2+} were inoculated into the same medium containing various concentrations of polymyxin B or colistin (polymyxin E). In contrast to the results presented in Table 1, the stated Mg^{2+} concentration was present during the entire 24 h of incubation at 37°C. At the end of this period, the highest concentration of polymyxin allowing visible growth was taken as the resistance level.

[e] —, Not done.

sistant to gentamicin (i.e., resistant to 4 instead of 1 μg/ml) and streptomycin (resistant to 32 instead of 8 μg/ml) and twofold more resistant to tobramycin (resistant to 1 rather than 0.5 μg/ml) than was either strain H103 or strain H207. In contrast, all strains were equally susceptible to carbenicillin and tetracycline (resistant to 16 and 8 μg/ml, respectively).

DISCUSSION

Both polymyxin B and EDTA can interact with *P. aeruginosa* LPS (3, 15). An early consequence of polymyxin B and EDTA action is permeabilization of the outer membrane (20), and polymyxin B has been shown to cause blebbing of the outer monolayer of the outer membrane, which is the location of cellular LPS (8, 22). Recently, Schindler and Osborn (21) demonstrated that the 2-keto-3-deoxyoctonate–lipid A region of *Salmonella typhimurium* LPS has high-affinity binding sites for polymyxin B (K_d = 0.3 to 0.5 μM, an approximately 10-fold higher affinity than for polymyxin-phospholipid interactions) and for Mg^{2+} and Ca^{2+}. Earlier studies by Newton (16) demonstrated that Mg^{2+} and polymyxin B competed for a *P. aeruginosa* cellular site, which he postulated to be polyphosphate in nature. *P. aeruginosa* LPS has been demonstrated to have an especially high phosphate concentration (5) and may have at least 8 mol of phosphate per mol of LPS in the heptose–2-keto-3-deoxyoctonate region (A. Kropinski, personal communication). Since phosphoryl and phosphodiester groups are negatively charged at neutral pH, this high-phosphate region of the LPS would provide binding sites for Mg^{2+}, thus explaining the relatively high Mg^{2+} content of *P. aeruginosa* cell envelopes (2). The high Mg^{2+} content could in turn partially explain the high susceptibility of *P. aeruginosa* to EDTA and polymyxin B, since the former would remove Mg^{2+} from its LPS site, whereas the latter, being cationic, would compete with Mg^{2+} for this site.

In strains with high levels of outer membrane

protein H1, be it adaptively (e.g., Fig. 2, gel E) or mutationally (e.g., Fig. 2, gel F) induced, we could find no statistically significant alteration in the levels of 2-keto-3-deoxyoctonate (an LPS-specific sugar), but we did find decreased Mg^{2+} levels in the cell envelope. The fact that there appeared to be a linear reciprocal relationship between Mg^{2+} levels in the cell envelope and protein H1 levels (see Table 2, footnote *b*) suggests that protein H1 replaces Mg^{2+} at its LPS binding site in the outer membrane (e.g., via amino groups), thus rendering the cell EDTA resistant (Fig. 1, gel A; Table 1). At the same time, protein H1 would protect such LPS binding sites from highly cationic antibiotics like polymyxin B, colistin, and gentamicin. The inhibitory action of Mg^{2+} (and Ca^{2+}) on these antibiotics in cells with low levels of protein H1 could thus be partly explained by competition between Mg^{2+} and the antibiotics for this binding site. Since it required up to a 1,000-fold molar excess of Mg^{2+} to inhibit these antibiotics in cells with low H1 levels, we suggest that the Mg^{2+} binding site on LPS has a much higher affinity for the cationic antibiotics than for Mg^{2+}. Preliminary evidence that H1 might interact with the LPS will be presented elsewhere (R. E. W. Hancock, R. Irvin, J. W. Costerton, and A. M. Carey, manuscript in preparation).

The acquired polymyxin B resistance reported here differs from that reported by Gilleland and colleagues (7, 8). His strains, adapted to and grown with very high polymyxin B concentrations (750 μg/ml), are altered in colony morphology, growth rate, a number of outer membrane proteins, LPS content, and cytochrome content (7; T. Nicas and R. E. W. Hancock, unpublished data). In addition, Gilleland has concluded that the ultrastructural alterations of these strains differ from those of Mg^{2+}-limited cells (8). Thus, we cannot make valid comparisons of the two types of adapted strains.

Pechey and James (18) adapted a *P. aeruginosa* strain to growth on gentamicin and demonstrated that this strain had also acquired EDTA resistance. Culturing this strain in the absence of gentamicin resulted in reacquisition of wild-type gentamicin and EDTA susceptibilities. Thus, these adapted strains may well have changes related to the Mg^{2+}-limited cells reported here.

This paper provides a new possible molecular mechanism for non-mutationally acquired antibiotic resistance in *P. aeruginosa*. The acquisition of high levels of H1 results in resistance to two groups of therapeutically important antibiotics. We are currently studying the mode of induction of protein H1 to see if the cell only responds to Mg^{2+} limitation.

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