ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Feb. 1982, p. 299–309
0066-4804/82/020299-11$02.00/0

Vol. 21, No. 2

Outer Membrane Permeability in *Pseudomonas aeruginosa*: Comparison of a Wild-type with an Antibiotic-Supersusceptible Mutant

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Received 29 May 1981/Accepted 28 October 1981

The *Pseudomonas aeruginosa* mutant Z61 has been shown to be highly supersusceptible to a wide range of antibiotics, including β-lactams, aminoglycosides, rifampin, tetracycline, and chloramphenicol (W. Zimmerman, Int. J. Clin. Pharmacol. Biopharm. 17:131–134, 1979). Spontaneous revertants were isolated, using gentamicin or carbenicillin as selective agents, and shown to have two patterns of susceptibility to a group of 12 antibiotics. Partial revertants had 2- to 10-fold greater resistance to these antibiotics than mutant Z61, whereas full revertants had antibiotic susceptibilities indistinguishable from those of the wild-type strain K799, from which mutant Z61 had been derived. Uptake of a chromogenic β-lactam nitrocefin was studied in both uninduced and induced cells of all strains by measuring the steady-state rate of nitrocefin hydrolysis by the inducible, periplasmic β-lactamase in both whole and broken cells. This demonstrated that outer membrane permeability decreased as antibiotic resistance increased in the series mutant Z61, partial revertants, wild type, and full revertants. The data were consistent with the idea of low outer membrane permeability being caused by a low proportion of open functional porins in the outer membrane as the reason for the high natural antibiotic resistance of wild-type *P. aeruginosa* strains. In addition, it was observed that levels of benzylpenicillin below the minimal inhibitory concentration for mutant Z61 failed to induce β-lactamase production. The possibility that this was related to the observed increase in outer membrane permeability is discussed. Preliminary evidence is presented that the pore-forming outer membrane porin protein F is not altered in mutant Z61.

The primary reason for the emergence of *Pseudomonas aeruginosa* as an important pathogen is its high natural resistance to a variety of antibiotics. A number of authors (23, 27, 28, 32) have suggested that this intrinsic antibiotic resistance mechanism results from the relative impermeability of *P. aeruginosa* cells to antibiotics, since other potential mechanisms have often been excluded. It would therefore seem reasonable to try to explain intrinsic resistance on the basis of the properties of the common permeability barrier which most antibiotics must cross to reach their sites of action, i.e., the outer membrane. We have approached this problem from two angles. First, since most commonly used antibiotics are hydrophilic (17), we have attempted to characterize the hydrophilic uptake pathway across the outer membrane of *P. aeruginosa*. It has been demonstrated in in vitro experiments that *P. aeruginosa* major outer membrane protein F forms large water-filled pores in model membrane systems (1a, 8). These pores have a cross-sectional area up to threefold larger than those of *Escherichia coli* porin proteins (1a) and a consequent exclusion limit for saccharides of approximately 6,000 daltons (8). Although porin protein F forms larger pores than *E. coli* porins (18) and is present in a higher copy number per cell (R. Hancock, unpublished data), the *P. aeruginosa* outer membrane apparently constitutes more of a barrier to antibiotic permeation. We have provided presumptive evidence (1a) that this can be simply explained on the basis of a relatively high proportion of nonfunctional porin protein F in the outer membrane of wild-type *P. aeruginosa* strains. The data presented below suggest that as few as 300 pores/cell may be open and functional at any given time.

A second approach is the study of the bio-

chemistry of mutants altered in outer membrane permeability toward antibiotics, and hence altered in antibiotic susceptibility. Antibiotic-supersusceptible mutants have been isolated in *P. aeruginosa*, including mutants supersusceptible to defined groups of β-lactam antibiotics (24, 27) or to aminoglycosides (15). Such mutants may have target alterations (19), reduced quantities of the inducible chromosomal β-lactamase (28, 34) or putative permeability alterations (15). To date, no biochemical characterization of this latter group has been performed. Recently, Zimmerman isolated a mutant supersusceptible to a range of antibiotics including most commonly used hydrophilic antibiotics (32). This mutant (strain K799/61, called here Z61) had normal penicillin-binding proteins (33), and preliminary evidence suggested that supersusceptibility involved, in part at least, enhanced permeability toward antibiotics. Since the mutant was isolated by using multiple mutagenesis steps, we have taken the approach of isolating revertants for comparative studies. Since *P. aeruginosa* has an inducible β-lactamase (28), we have characterized the activity and inducibility of this enzyme in wild-type, mutant, and revertant strains and used these data to clearly demonstrate an enhanced rate of β-lactam permeation across the mutant outer membranes. Furthermore, we provide evidence that the porin protein is not mutated in mutant Z61. In the accompanying paper (14), a detailed comparison of the mutant Z61 lipopolysaccharide (LPS) with the wild-type K799 LPS was performed, and the influence of LPS on porin is discussed.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Table 1 lists the *P. aeruginosa* strains used in this study. Media and growth conditions have been previously described (6).

Antibiotic susceptibility testing. Minimal inhibitory concentration (MIC) values were obtained for all strains and antibiotics to be tested. A multisyringe applicator similar to one previously used for phage susceptibility testing by Zierdt et al. (31) was used to deliver to each plate 24 separate drops of approximately 2-μl volume which contained an estimated 1,000 cells from fourfold-diluted overnight cultures. This allowed the simultaneous testing of 24 different cultures on a single plate.

Single-cell resistance levels were obtained by diluting an overnight culture 10^{-6} and then plating 0.1 ml onto proteose peptone no. 2 (PP2) agar plates containing various concentrations of antibiotics which ranged some degree lower than those used for MIC determinations.

Antibiotics and chemicals. Benzylpenicillin, ampicillin, polymyxin B sulfate, chloramphenicol, streptomycin sulfate, and tetracycline hydrochloride were purchased from Sigma Chemical Co. (St. Louis, Mo.). Tobramycin and moxalactam were a gift from Eli Lilly and Co. (Indianapolis, Ind.), carbenicillin was purchased from Ayerst Laboratories (Montreal, Canada), ticarcillin was a gift from Beecham Laboratories (Pointe Claire, Canada), gentamicin sulfate was a gift from Schering Co., Ltd. (Pointe Claire, Canada), and cefsulodin was kindly provided by Ciba Geigy AG (Basel, Switzerland). All other chemicals used were of the highest standard commercially available.

Phage susceptibility testing. All bacterial strains were tested for susceptibility to a large variety of *P. aeruginosa* phages (16). An additional deep rough-specific phage, ØPLS27 (K. Jarrell, Ph.D. thesis, Queen's University, Ontario, Canada, 1980), was also tested. The assays were conducted as described by Hancock and Reeves (11), using a multisyringe phage applicator.

β-Lactamase assays. β-Lactamase assays were performed by using the chromogenic cephalosporin nitrocefin (21), kindly provided by C. O'Callaghan (Glaxo Research Groups Ltd., Middlesex, England). Cells were grown to an optical density at 660 nm (OD_{660}) of 0.5 to 0.8 in PP2 broth, and then suspended to one-quarter volume in 50 mM sodium phosphate buffer, pH 7.0. One hundred microliters of cells was added to 0.65 ml of nitrocefin solution (0.25 mg/ml in phosphate buffer) in a semimicro cuvette, and the kinetics of nitrocefin hydrolysis was monitored at OD_{550} or OD_{490}, using a Perkin-Elmer (Coleman 124) dual-beam spectrophotometer coupled to a Sargent-Welch chart recorder. Assays were performed on whole and broken cells to give a measure of relative crypticity in the strains. Cells in phosphate buffer were broken once in a French pressure cell (American Instrument Co., Silver Spring, Md.) at 20,000 lb/in^2. Control experiments demonstrated that this method was up to threefold more efficient in releasing periplasmic β-lactamase than other methods, including 0.2 M $MgCl_2$ or 10 mM EDTA treatment. In our hands, sonication provided inconsistent release of β-lactamase. For measurements of induced β-lactamase, cells were grown for 2 h in PP2 broth containing 0.2 to 200 μg of benzylpenicillin per ml as an inducer and then prepared and assayed as above.

Whole-cell analyses. Analysis of whole-cell LPS content was performed by whole-cell fatty acid analysis, assuming that α-hydroxydodecanoic acid was 5% by weight of the LPS (14).

For analysis of hexose content, whole lyophilized cells were suspended by sonication in concentrated H_2SO_4 at 5 mg/ml, and hexose determinations were performed by both the phenol-H_2SO_4 method (1) and the method of Dische et al. (3), using glucose as a standard. Results obtained by the two methods were quite similar.

Analysis of whole-cell protein was done on 10-mg samples of lyophilized whole cells weighed into tubes to which 2 ml of 2% sodium dodecyl sulfate (SDS)–10 mM Tris, pH 6.8, was added. These were sonicated for two 15-s periods and then heated at 100°C for 10 min. Insoluble material was removed by centrifugation, and supernatant protein was assayed by the method of Sandermann and Strominger (25). Mg^{2+} content in the cell envelope was determined as described by Nicas and Hancock (16).

Cell fractionation and gel electrophoresis. Cell envelope and outer membrane purification and SDS-polyacrylamide gel electrophoresis were carried out as described previously (6). The presence of LPS was

TABLE 1. *P. aeruginosa* strains[a]

Strain	Derivation	Reference
K799	Pae K799/WT; prototroph	32
Z61	Mutant 61 isolated by mutagenesis of K799 and selection for antibiotic supersusceptibility; prototroph	32
H251, H252, H253	Spontaneous full revertants of Z61 isolated on PP2 agar containing carbenicillin (0.25–0.5 μg/ml); prototrophs	This study
H254, H255	Spontaneous partial revertants of Z61 isolated on PP2 agar containing carbenicillin (0.25–0.5 μg/ml); prototrophs	This study
H256, H257	Spontaneous partial revertants of Z61 isolated on PP2 agar containing gentamicin (0.4–0.7 μg/ml); prototrophs	This study
H258, H259	Mutant phenotype; selected as partial revertants (see H256 and H257) but re-reverted to Z61 antibiotic resistance phenotype; prototroph	This study
H103	*P. aeruginosa* PAO1 prototroph	10
AK43	*argC54 chl-2* E79^r	19

[a] Strain K799 and its antibiotic-supersusceptible mutant Z61 were a gift from W. Zimmermann. Cells with wild-type or partial resistance to antibiotics were isolated as spontaneous revertants of the supersusceptible mutant Z61. Strain Z61 was grown overnight in 1% PP2 medium and then subcultured into 10 ml of the same medium and grown to an OD_{600} of 1.0. The cells were then centrifuged, resuspended in 0.1 ml of PP2, and plated on medium containing carbenicillin or gentamicin at concentrations between the single-cell resistance levels for strain Z61 and K799.

detected by staining for carbohydrate, using the Schiff staining procedure (30). Densitometer scanning of gels stained for carbohydrate or protein was performed with a Quick Scan Jr. (Helena Laboratories Corp., Beaumont, Tex.) gel scanner. Sequential solubilization of outer membranes with Triton X-100, EDTA, and lysozyme was performed as described by Hancock et al. (9).

RESULTS

Isolation and antibiotic susceptibility of full and partial revertants of the antibiotic-supersusceptible mutant. Due to the complex phenotype of mutant Z61 and the number of mutagenesis steps involved in its isolation (32), we decided to isolate revertants so that we could specifically determine the number and nature of mutations in this mutant. Unfortunately, we were unable to transfer the wild-type genes into mutant Z61 by genetic techniques (conjugation or transduction), since the frequency of recombinants obtained was lower than the reversion frequency (approximately 10^{-7}/colony-forming units). Therefore, we selected for revertants directly by plating cells on nutrient medium containing either gentamicin or carbenicillin. Of 15 revertants selected for gentamicin resistance, 11 were shown to be partial revertants in that they had antibiotic resistance patterns intermediate between those of the wild-type strain K799 and its antibiotic-supersusceptible mutant, Z61 (see, for example, strains H256 and H257 in Table 2). The other four strains were as antibiotic susceptible as mutant Z61 (see strains H258 and H259 in Table 2). Presumably, these strains were unstable revertants which had then re-reverted to mutant phenotype on removal of the selective pressure. Of 23 revertants selected as carbenicillin resistant, 14 were partial revertants (e.g., H254 and H255; Table 2), and nine had fully reverted to wild type (e.g., H251, H252, and H253; Table 2).

Alterations in the antibiotic susceptibility patterns always involved all antibiotics tested in that the partial revertants showed intermediate resistance to all β-lactams, aminoglycosides, and other antibiotics tested, whereas the full revertants had wild-type levels of antibiotic resistances. Despite the complex procedure used to isolate mutant Z61, the single-step selection of full revertants which were phenotypically identical to wild-type K799 (see below) strongly suggested that there is a single major mutation in mutant Z61.

Mutant Z61 has been found to be between 20- and 2,000-fold more susceptible than the wild-type strain K799 to a total of 24 antibiotics tested both in this study and by Zimmermann (33; Table 2). These include six β-lactams which can be hydrolyzed by the inducible type Id β-lactamase of *P. aeruginosa*, eight β-lactams which are resistant to hydrolysis by this enzyme, three aminoglycosides, and essentially all other major antibiotics to which *P. aeruginosa* shows even slight susceptibility.

Use of nitrocefin in measuring outer membrane permeability. All *P. aeruginosa* strains have been shown to contain an inducible type Id β-lactamase (20, 23, 28). Since the apparent β-lactamase activity of whole cells of *P. aeruginosa* can be increased by EDTA and gentamicin, agents which disrupt the outer membrane permeability barrier (10, 12), we postulated that this enzyme was located in the periplasm and that

TABLE 2. MICs of a variety of antibiotics for parent and antibiotic-supersusceptible strains of *P. aeruginosa*

Strain	Phenotype[a]	MIC[b] (μg/ml)											
		β-Lactam						Aminoglycoside			Other		
		BP	AMP	TIC	CB	CF	MOX	GM	SM	TM	CM	PX	TC
H103	WT	>2,000	>500	20	25	2	20	8	20	0.8	>200	10	100
K799	WT	>2,000	>500	20	25	2	20	10	50	1	>200	20	100
H251, H252, H253	R	>2,000	>500	20	25	2	20	10	50	1	>200	10–20	100
H254	PR	10	5	0.5	0.5	0.3	5	1	5	0.5	>200	10	5
H255	PR	5	5	0.05	0.5	0.3	0.2	2	5	1	50	5	10
H256, H257	PR	10	5	0.5	0.5	0.3	0.2	2	5	0.5	50	5	50
Z61, H258, H259	M	1.0	0.5	0.05	0.05	0.1	0.2	0.5	2	0.5	25	5	2

[a] WT, Wild type (i.e., resembling strain K799); R, full revertant; PR, partial revertant; M, mutant phenotype (resembling strain Z61).

[b] BP, benzylpenicillin; AMP, ampicillin; TIC, ticarcillin; CB, carbenicillin; CF, cefsulodin; MOX, moxalactam; GM, gentamicin; SM, streptomycin; TM, tobramycin; CM, chloramphenicol; PX, polymyxin B; TC, tetracycline.

the outer membrane represented a significant barrier to antibiotic uptake in wild-type *P. aeruginosa*. As described below, these assumptions apparently were correct. In view of this, we decided to measure both the inducibility of the β-lactamase and the relationship between antibiotic susceptibility and outer membrane barrier properties. This study was made possible by the great sensitivity and simplicity of β-lactamase assays using the chromogenic β-lactam nitrocefin (21).

We wished to determine if nitrocefin was being taken up by the hydrophilic uptake pathway in our strains. Therefore, we examined the temperature dependence of nitrocefin permeation through the outer membrane by determining the equilibrium rates of nitrocefin diffusion (= rate of nitrocefin hydrolysis in whole cells at equilibrium [34]) at temperatures between 15 and 25°C. This allowed us to calculate Arrhenius activation energies, E_s, of 32, 29, and 33 kJ/mol for strain K799, strain K799 induced (see below), and mutant Z61. These rates were only slightly higher than those expected for free diffusion in water (approximately 24 kJ/mol) and lower than the value reported (18) for cephaloridine diffusion through the porin pores of *E. coli* (approximately 38 kJ/mol). In contrast, nafcillin uptake into deep rough mutants of *Salmonella typhimurium* has an E_s value to 250 kJ/mol (taken from the data of Nikaido [17]), and this uptake is assumed to occur via a hydrophobic mechanism, that is, through the lipid hydrocarbon core. Thus, we conclude that nitrocefin permeates the outer membrane through porin pores in whole cells of *P. aeruginosa*. The experiments below reflect this.

β-Lactamase activities and outer membrane permeability of K799 derivatives. It was shown that the β-lactamase levels of the wild-type strain K799 full revertants H251, H252, and H253 were increased 11- to 18-fold by growth of the cells for 2 h in the presence of benzylpenicillin (200 μg/ml) as an inducer. Induction had no apparent effect on the crypticity of these strains. Similar results were obtained for the wild-type strain H103 (Table 3), although in this case the fully induced level of β-lactamase was about 50 to 100% greater than that for strain K799. Similar levels of inducer could not be used for mutant Z61 and its partial revertant H257 since these strains had MICs for benzylpencillin of 1.0 to 10 μg/ml, respectively. The addition of benzylpenicillin to a concentration one-fifth of the MIC caused a 2.4-fold induction for H256 but was insufficient to induce mutant Z61.

We measured the activity of the β-lactamase in broken cells and in whole cells (Table 3) and used these results to calculate a crypticity value. It was apparent that the crypticity value varied with the antibiotic resistance phenotype such that wild-type and full revertant strains were the most cryptic and partial revertants were significantly less cryptic. The antibiotic-supersusceptible mutant strains H258, H259, and Z61 were not very cryptic at all, since the activities in whole cells were similar to those in broken cells. Although crypticity reflects the barrier function of the outer membrane, a number of researchers (26, 34) have demonstrated that a more quantitative result is provided by calculating the apparent periplasmic concentration of β-lactam (S_e) after the equilibrium rate of β-lactamase activity is reached. This calculation takes into account the kinetics of hydrolysis of nitrocefin in the periplasm and can be simply calculated from the Michaelis-Menten equation and V_I, the rate of hydrolysis in intact cells.

TABLE 3. β-Lactamase activities and nitrocefin permeation through the outer membrane of wild-type and antibiotic-supersusceptible strains of *P. aeruginosa*[a]

Inducer concn (μg/ml)	Strain	Phenotype	β-Lactamase activity (nmol of nitrocefin hydrolyzed/mg of cell dry weight per min)		Induction ratio	Crypticity (activity of broken cells/ activity of whole cells)	S_e/S_0 (calculated)
			Whole cells	French-pressed broken cells			
Uninduced	H103	WT	1.3	11.1		9.3	0.017
	K799	WT	1.4	9.6		6.9	0.024
	H251	R	0.92	9.4		10.2	0.015
	H255	PR	1.5	7.0		4.7	0.038
	H256	PR	2.1	7.8		3.7	0.053
	H258	M	3.6	5.5		1.5	0.284
	Z61	M	4.7	6.2		1.3	0.472
Induced							
200	H103	WT	10.1	229	21	22.7	0.006
200	K799	WT	13.0	128	13	9.8	0.016
200	H251	R	16.1	171	18	10.6	0.015
2	H256	PR	2.9	12.1	2.4	4.2	0.044
0.2	Z61	M	2.7	4.5	0.7	1.7	0.203

[a] The β-lactamase activities given are the means of three to five separate determinations. Similar data were obtained for strains H252 and H253 as for K799 and H251 above, for H254 and H257 as for strains H255 and H256 above, and for H259 as for H258 above. The ratio S_e (apparent periplasmic concentration of nitrocefin at steady state) to S_0 (concentration of nitrocefin added, i.e., 8×10^{-4} M) was calculated as described in the text. The abbreviations for the antibiotic susceptibility phenotypes are described in Table 1. Induced strains were grown for 2 h with the given concentration of benzylpenicillin as inducer. The induction ratio is the ratio of activities in broken cells of induced and uninduced cells.

The ratio of the calculated periplasmic concentration of a β-lactam antibiotic (S_e) to the medium concentration (S_0) has been shown to be inversely related to the MIC (26, 34). Thus, S_e/S_0 gives a direct measure of outer membrane permeability, and the ratio will approach 1.0 as the outer membrane becomes less of a permeation barrier, since such a ratio implies that the periplasmic concentration of antibiotic is the same as the medium concentration. The S_e/S_0 increased 20- to 40-fold such that mutants > partial revertants > wild type and full revertants (Table 3). This demonstrated conclusively that the mutant was altered in outer membrane permeability and that the full and partial revertants had correspondingly full and partial restoration of the barrier function of the outer membrane. Other measures of barrier function such as the diffusion parameter, *C*, of Zimmermann (33) and the permeability coefficient, *P* (= C divided by the total area of the outer membrane [18]) were shown to vary similarly; however, at the high concentrations of nitrocefin added to the cells (concentrations made necessary by the low activity of β-lactamase in uninduced cells), calculation of these parameters became quite inaccurate.

Characterization of the cell envelope protein composition. We characterized a range of cell components which had been previously shown to be altered in certain antibiotic-supersusceptible mutants (4, 5, 13, 15, 22, 29). There were no significant ($P > 0.5$ by Student's unpaired *t* test) alterations in the percentage dry weight of any of the cells in Table 1 which were hexose (3.56 ± 0.47%), phospholipids (7.8 ± 0.6%), or LPS (9.7 ± 1.2%). In addition, there were no differences ($P > 0.5$) in the Mg^{2+} content of the cell envelopes of the wild-type strain K799 (4.2 ± 0.43 μg/mg of cell dry weight) when compared with the full revertant H252 (4.04 ± 0.06), the partial revertant H257 (4.03 ± 0.04), or the mutant Z61 (3.97 ± 0.27).

In addition to these analyses, we found that all strains derived from K799 had equivalent protein concentrations per cell (56.3 ± 2.7% of the cell dry weight; $P > 0.5$ by Student's unpaired *t* test). Since the major outer membrane proteins are quite prominent even on SDS-polyacrylamide gel electrophoretograms of whole-cell proteins (16), we could directly compare the contents of the various major outer membrane proteins from gels loaded with equal amounts of whole-cell proteins. This enabled us to observe that the amounts of porin protein F and of other major outer membrane proteins D2, E, G, H1, and H2 were extremely similar. Similarly, the other 100 or so proteins visible in one-dimensional whole-cell protein gels were also apparently unchanged in amounts, although small changes might not be observed in this analysis. Comparison of cell envelope (Fig. 1, gels A to E) and of outer membrane (Fig. 1, gels F and G) or inner membrane proteins failed to reveal signifi-

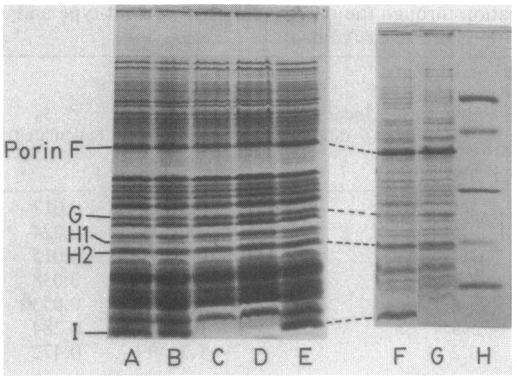

FIG. 1. SDS-polyacrylamide gel electrophoretograms of cell envelopes (A–E) and outer membranes (F and G) of strain K799 and its derivatives. The running gels used were 12% (wt/vol) acrylamide containing 0.07 M NaCl for the cell envelopes and 14% (wt/vol) acrylamide containing 0.07 NaCl for gels F through H. (A) Partial revertant H254; (B) partial revertant H257; (C) full revertant H251; (D and G) parent strain K799; (E and F) antibiotic-supersusceptible mutant Z61; (H) molecular weight standards bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400). The running positions of some of the major outer membrane proteins are indicated.

cant qualitative or quantitative differences in outer membrane protein patterns with the exception of the outer membrane lipoprotein I as described below. The slight differences in some major and minor outer membrane proteins occasionally seen in the purified outer membranes of the antibiotic-supersusceptible mutant Z61 were considered to be artifacts of outer membrane isolation, since SDS-polyacrylamide gel electrophoretograms of cell envelope proteins and whole-cell proteins did not have corresponding changes.

Since we had demonstrated above that mutant Z61 was apparently more permeable to hydrophilic antibiotics than the wild-type strain K799, we examined in detail the properties of porin protein F. This protein forms water-filled pores through lipid bilayers (1a), and, by analogy with enteric organisms (18), is probably involved in the permeation of hydrophilic antibiotics across the outer membrane. As noted above, there was no difference in the amounts of porin protein F in the whole-cell proteins and thus no difference in the outer membranes of the strains used in this study. In addition, the modifications of the mobility of porin-SDS complexes by varying the solubilization temperatures or by β-mercaptoethanol treatment (6) revealed no differences between the strains. Partial proteolysis of the porins and outer membrane proteins of the wild-type strain K799 and its antibiotic-supersusceptible mutant Z61, using both trypsin and pronase, showed no alterations in either protease susceptibility or protease digestion patterns (data not shown). To gain further information about the interrelationships of outer membrane proteins in strains K799 and mutant Z61, we subjected their outer membranes to the sequential solubilization techniques previously described (9). This led to the conclusion that in both strains, all of porin protein F and a portion of the major outer membrane lipoproteins H2 and I were noncovalently peptidoglycan associated as previously demonstrated for H103 (9). In this experiment, only one major difference between the two strains was observed. After four sequential solubilization steps in Triton X-100, EDTA, and lysozyme, alone or in combination, the amount of LPS resisting solubilization was far greater for the mutant strain Z61 (i.e., 18.6% of the initial LPS content was found in the residue) than for the wild-type strain K799 (i.e., 1.4%).

One consistent alteration was seen in the protein profiles of SDS-polyacrylamide gel electrophoretograms of whole-cell, cell envelope, and outer membrane proteins. The outer membrane lipoprotein I, which is one of the major cellular proteins, was apparently absent from the gel profiles of the wild-type strain K799 (Fig. 1, gels D and G) and the full revertants (Fig. 1, gel C) but was present in large amounts in the gels of Z61 (Fig. 1, gels E and F) and the partial revertants (Fig. 1, gels A and B). We had previously observed that the addition of Mg^{2+} during solubilization promoted the appearance of lipoprotein I in the gels of wild-type strains (9) and that rough, LPS-altered mutants showed patterns similar to that of the mutant Z61. Therefore, we postulated that the appearance of lipoprotein in the gel was related to the LPS, since this molecule would be solubilized from the outer membrane and might be expected to enter the gel with the detergent-protein complexes. Consistent with this, we observed that partially purified lipoprotein I, which had been separated from LPS, stained strongly in SDS-polyacrylamide gels (Fig. 2C). To locate the position of LPS in the gels, we stained gels for carbohydrate and subsequently stained the same gels for protein. In the absence of Mg^{2+}, the LPS of wild-type strain K799 appeared in a broad band with a mobility lower than that of partially purified lipoprotein I (Fig. 2D). Subsequent protein staining revealed a large band of purple-blue material coelectrophoresing with the LPS-carbohydrate band. This purple-blue staining band appeared only when Coomassie brilliant blue staining was performed after carbohydrate staining (cf. Fig. 1, gels D and G). Possibly the acid

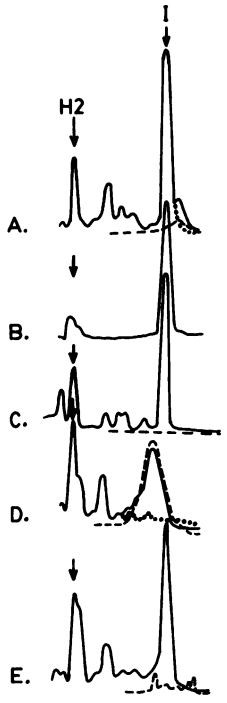

FIG. 2. Densitometry scans of SDS-polyacrylamide gel electrophoretograms of outer membranes stained for carbohydrate (- - -) and subsequently stained with Coomassie brilliant blue R250 for protein (——) or stained with Coomassie brilliant blue R250 without prior carbohydrate staining (. . . .). Outer membranes, containing 20 μg of outer membrane protein and approximately 0.23 μmol of LPS (the major carbohydrate-containing molecule in the outer membrane), were solubilized in 2% SDS, 5% β-mercaptoethanol, 62.5 mM Tris-hydrochloride (pH 6.8), and 10% glycerol with either EDTA or Mg^{2+} present as described below. Only that portion of the densitometry scans between the major outer membrane lipoproteins H2 (20,500 daltons) and I (9,000 daltons) is shown with the running position of the lipoproteins indicated by arrows. The two Coomassie brilliant blue-stained gels coincided except in regions of carbohydrate-staining reactions. Densitometry of the carbohydrate-stained gels was performed at a gain of 9.5, whereas protein-stained gels were scanned at a gain of 5.5; thus, these scans are not quantitatively comparable. (A) Mutant strain Z61 outer membrane solubilized in the presence of 2 mM EDTA. (B) Partially purified lipoprotein I of strain H103 (containing some protein H2 but no LPS) solubilized with 2 mM EDTA present. (C) LPS-deficient (rough) strain AK43 (19) solubilized with 40 mM EDTA present. No carbohydrate staining was observed. (D) Wild-type strain K799 solubilized in the presence of 2 mM EDTA. (E) Wild-type strain K799 solubilized in the presence of 20 mM Mg^{2+}. Gels A and D when stained for protein correspond to a portion of Fig. 1, gels F and G, respectively.

treatment involved in carbohydrate staining "revealed" the masked protein. These results could be explained if, in wild-type strains like K799, the LPS coelectrophoresed with and thereby masked the lipoprotein I. In direct agreement with this, Mg^{2+} treatment during solubilization prevented LPS from entering the gel, and lipoprotein I coelectrophoresed with partially purified lipoprotein I (Fig. 2E) and could be stained by Coomassie brilliant blue without pretreatment. Repeating these experiments with mutant Z61 demonstrated that significantly less carbohydrate-staining material had entered the gels and that this material ran with a mobility greater than that of the lipoprotein (Fig. 2A). The LPS was thus unable to mask or influence the electrophoretic mobility of lipoprotein I which now coelectrophoresed with partially purified lipoprotein and stained without pretreatment (Fig. 1, gels E and F; Fig. 2A). Similar results were obtained both in the presence and absence (i.e., after Mg^{2+} treatment) of LPS. We were unable to demonstrate carbohydrate-staining material in the gels of a rough LPS-deficient mutant AK43 and, as might be predicted, its lipoprotein I behaved like the partially purified lipoprotein I (Fig. 2C).

Phage susceptibilities. To gain further information regarding the state of the outer membrane, we screened our strains against a set of phages with a variety of partially characterized receptors (16). All strains tested were fully susceptible to the smooth-specific LPS phages F8, 109, 44, and E79, which plate only on strains with smooth LPS such as H103 but do not plate on LPS-altered, rough mutants like strain AK43 (13, 16). This implies that strain K799 and its derivatives have LPS compositions related to that of *P. aeruginosa* PAO1 and that none of these strains is a rough mutant. This was further indicated by the use of rough-specific phage PLS27, which plates only on rough, LPS-altered mutants such as AK43 but does not plate on wild-type smooth strains. This phage was unable to plate on strain K799 or its derivatives Z61 and H251-9. These strains were also resistant to the pilus-specific phages M6, B39, and 8, suggesting that they either were all pilus-deficient mutants or had pili chemically distinct from those of *P. aeruginosa* PAO1 strain H103. In addition, we studied 17 other phages with less well characterized receptors (16). Only three of these revealed consistent differences between the wild-type strain K799 and the mutant Z61. Phages 7, F116, and B6B all plated well on the antibiotic-supersusceptible mutant strains Z61, H258, and H259 and the partial revertants H254, H255, H256, and H257. In contrast, they plated poorly or not at all on the wild-type strain K799 and the full revertants H251, H252, and H253. Although

phages 7 and F116 (and possibly B6B also) are thought to have protein receptors (16), it is thought that this result reflects surface alterations rather than specific changes in the receptors related to antibiotic susceptibility. As evidence in favor of this view, strain H103, like mutant Z61, was fully susceptible to these phages, but had antibiotic resistance patterns resembling those of the wild-type strain K799 (Table 2).

DISCUSSION

There are three major possible mechanisms which might explain natural (non-R-plasmid mediated) antibiotic resistance in *P. aeruginosa*. One possibility involves an enzyme (e.g., β-lactamase) encoded by the cell which could modify the antibiotic molecule, thus altering or destroying its activity (23, 28). A second possibility is that the target of the antibiotic is altered in some way such that the antibiotic-binding affinity, and hence its effectiveness, is reduced. An example of this would be alterations in periplasmic penicillin-binding proteins (19). The third possibility is that *P. aeruginosa* is less permeable than, for example, enteric bacteria to antibiotics. Our studies of *P. aeruginosa* strain K799 and its mutant Z61 suggest that decreased permeability through the outer membrane is the primary mechanism of high natural antibiotic resistance in *P. aeruginosa*. In addition, physical and biochemical characterization of these strains has provided clues as to the possible mechanism of this lowered permeability to antibiotics in *P. aeruginosa* as compared with the enteric group of bacteria.

We isolated spontaneous revertants in order to obtain isogenic derivatives of the antibiotic-supersusceptible mutant Z61 with wild-type and intermediate antibiotic resistance levels. However, although these revertants provided a more valid basis of comparison with the mutant strain Z61, they provided conflicting evidence as to the exact nature of the mutation in strain Z61 leading to antibiotic supersusceptibility. The fact that single-step revertants to wild-type antibiotic susceptibility (e.g., strains H251, H252, and H253) could be isolated suggested that the antibiotic supersusceptibility of strain Z61 resulted from only one major mutation. Subsequent testing showed that these full revertants isolated were identical to K799 in all of the properties studied. Despite this, the five-step mutagenesis used to generate Z61 from K799 (32, 33) would be expected to generate multiple mutations. Our inability to generate a full revertant of strain Z61 by selection on gentamicin as well as the existence of partial revertants (suggesting a gradation of antibiotic resistance) seems to indicate that strain Z61 has either an additional secondary mutation (which might be phenotypically suppressed in the absence of the primary mutation) or a secondary adaptive alteration which might also be stabilized by the primary mutation. Despite this possibility, the clear correlation between antibiotic susceptibility (Table 2), outer membrane permeability (Table 3), and the biochemical alterations reported here and in the accompanying report (14) for the range of strains and revertants studied coupled to the demonstration of spontaneous revertants indistinguishable from the wild type allow valid comparisons to be made between mutant Z61 and the wild-type strain K799 or any of the full revertants.

One of the major findings of this study was that the inducibility of the β-lactamase correlated with the outer membrane permeability of the strains used. The apparent lack of inducibility of the β-lactamase in the mutant strain Z61 (Table 3) may well be an artifact caused by possession of a more permeable outer membrane. It is difficult to distinguish, using our experimental conditions, between an uninducible β-lactamase and the situation where the amount necessary for induction is greater than the amount which will inhibit the cell. As demonstrated by Nordström and Sykes (20), induction of β-lactamase in *P. aeruginosa* wild-type strains apparently takes 80 min at an inducer concentration of 200 μg of benzylpenicillin per ml. The time required for induction presumably reflects low-level exposure to β-lactam over this 80-min period. A combination of the low rate of permeation of benzylpenicillin across the outer membrane, the nonspecific binding of this β-lactam by cell surface and periplasmic components, and the hydrolysis of benzylpenicillin by the low level of β-lactamase in uninduced cells would result in only a small fraction of the external benzylpenicillin concentration being available to induce the β-lactamase. In contrast, the relatively high permeability of mutant Z61 outer membranes might prevent this period of low-level exposure, since an increase in the amount of penicillin in the periplasm, sufficient to saturate the β-lactamase and nonspecific binding sites, would quite rapidly result in binding of benzylpenicillin to its target penicillin-binding proteins and consequent killing of the cells. Two things argue in favor of this view. First, the amount of benzylpenicillin required to kill mutant Z61 (1 μg/ml; Table 2) could be hydrolyzed by the uninduced level of β-lactamase from 10^8 cells in 2 to 4 min (C. Soga and R. E. W. Hancock, unpublished data; Table 3). Thus, although this level might normally be sufficient for induction, in a highly permeable mutant the rapid influx of antibiotic might result in cellular inactivation before either hydrolysis of periplasmic β-lactam by the uninduced β-lactamase or β-lactamase induction

could occur. In contrast, in the wild-type strain, the escape of a few molecules of β-lactam from hydrolysis by the uninduced β-lactamase could, over time, result in induction of this β-lactamase. Second, a decrease in the permeability of outer membranes as seen for strain H256 (Table 3) allows for treatment with a level (i.e., 2 μg/ml) of inducer higher than the MIC for mutant Z61 and consequent slight induction of the β-lactamase (Table 3). Even so, this level of inducer was still 10-fold lower than the minimum external inducer concentration required to induce β-lactamase in wild-type strains (20).

It should be noted that even if the β-lactamase of the mutant has been altered such that it is no longer inducible, it is doubtful that such an alteration plays a major role in causing loss of resistance in this situation, where supersusceptibility of mutant Z61 to a wide range of non β-lactam antibiotics is involved (32; Table 2). The total loss of *P. aeruginosa* β-lactamase in studies by Ohmori et al. (22) or the loss of β-lactamase inducibility in studies by Zimmermann and Rosselet (34) did not result in loss of resistance to the extent occurring in mutant Z61. Furthermore, susceptibility to carbenicillin, a β-lactam which is apparently not hydrolyzed by the inducible β-lactamase of *P. aeruginosa*, was unaffected in the above β-lactamase mutants but was enhanced 500-fold in mutant Z61. When plasmid RP1 (Amp^r Tet^r Kan^r Neo^r), which carries β-lactamase type IIIa (23, 28), was transferred into mutant Z61, the resistance was increased toward carbenicillin (which is hydrolyzed by the plasmid-encoded β-lactamase) and tetracycline but not to gentamicin, for which resistance is not encoded on the plasmid (B. L. Angus and R. E. W. Hancock, unpublished data). Also, even with the addition of plasmid RP1 to mutant Z61, the resistance to tetracycline as measured by antibiotic disks was still less than that of the parent K799 without the plasmid. These lines of evidence further support the hypothesis that outer membrane impermeability is the major mechanism of resistance in K799. Studies by Zimmerman (33) showed that, compared with the parent strain K799, there were no alterations in the penicillin-binding proteins of mutant Z61 with respect to their penicillin-binding affinity, yet there was enhanced accessibility of β-lactams to the penicillin-binding proteins in the mutant strain. This is consistent with the enhanced outer membrane permeability as measured by β-lactamase assays (Table 3).

The general stability of the outer membrane in both the wild-type strain K799 and mutant Z61 appeared to be equal. The growth rates and amounts of Mg^{2+}, the major stabilizing cation in the cell envelope (2), were similar. Also, the amounts of β-lactamase excreted into the medium both before and after induction under the conditions used were similarly low for all strains studied (<0.5% of the total β-lactamase produced). In addition, during sequential solubilization in Tris–Triton X-100 followed by Tris–Triton X-100–EDTA, the susceptibility of the outer membrane to solubilization by these agents was similar with respect to outer membrane proteins and total mass, although the amount of LPS in the residue after treatment was greater in the mutant.

The above data show that neither enzymatic degradation of antibiotics, antibiotic target alterations, nor altered outer membrane stability can account for the differences in antibiotic susceptibility of wild-type *P. aeruginosa* strain K799 and its mutant Z61 and provide evidence to support low outer membrane permeability to antibiotics as the primary mechanism of generalized antibiotic resistance in wild-type *P. aeruginosa* strains. Correspondingly, only higher outer membrane permeability can adequately explain the antibiotic supersusceptibility of mutant Z61. The results presented here also suggest that no gross alterations in the cell envelope were responsible for higher outer membrane permeability.

Because most of the antibiotics, including nitrocefin, tested in this study are at least moderately hydrophilic (17), their main point of entry into the periplasm would be via the hydrophilic pores formed by the outer membrane porin protein (23). In view of this, outer membrane proteins were examined and compared in all strains by gel electrophoresis. There appeared to be no qualitative or quantitative differences in outer membrane protein F (porin) in the mutant as compared with the parent strain, K799. However, the antibiotic susceptibilities (Table 2) and nitrocefin hydrolysis experiments (Table 3) suggested that mutant Z61 was more permeable than strain K799. A possible explanation for this anomaly is that a large percentage of the pores of wild-type *P. aeruginosa* could be nonfunctional at any particular time. If this were so, it would also help to explain the fact that, although the *P. aeruginosa* outer membrane pores were shown to allow passage of molecules up to 6,000 daltons in molecular size (8), this organism is apparently less permeable to hydrophilic antibiotics than *E. coli*, with pores impermeable to molecules above 500 to 600 daltons (18).

From our data (Table 3) and using the method of Zimmermann and Rosselet (34), we could calculate a permeability parameter, C, for strains K799 and H103 in the order of 10^{-6}. An accurate value was difficult to obtain due to the high external concentrations of nitrocefin used, as required due to the low levels of β-lactamase

in uninduced strains. However, the C value for our wild-type *P. aeruginosa* strains was approximately two orders of magnitude lower than the values obtained by Zimmermann and Rosselet (34) in *E. coli* for other cephalosporins with equivalent partition coefficients in octanol-phosphate buffer. Similar differences between *E. coli* and *P. aeruginosa* have been measured by Benz and Hancock in model membrane systems (1a) and observed by H. Nikaido (personal communication). Diffusivity laws tell us that $C = \Delta \cdot dy \cdot dz$, where Δ is a coefficient of diffusion and $dy \cdot dz$ is the area through which the diffusing molecule, in this case nitrocefin, passes. Thus, since nitrocefin probably diffuses through the water-filled pore of porin protein F (1a, 8), then C must be proportional to the total area of the hydrophilic pores in the outer membrane. This would suggest that *P. aeruginosa* cells have an area of hydrophilic pores in their outer membrane approximately 100-fold less than that in *E. coli*. When corrected for the threefold-larger pore area of *P. aeruginosa* (1a), this implies that *P. aeruginosa* has only 1 in 300 pores in an open functional state at any given time. We can estimate the number of porin protein F molecules per cell by comparison with the number of molecules of protein D1 (2×10^4 to 4×10^4 molecules/cell; [7]) and LPS (2.4×10^6 molecules per cell assuming 1.5 mol of 2-hydroxydodecanoic acid/mol of LPS [14]). This suggests that there are about 1×10^5 to 3×10^5 molecules of porin per cell. Thus, if we assume *P. aeruginosa* porin to be a trimer like *E. coli* and *S. typhimurium* porins (18), then the number of open pores per cell may be as few as 100 to 300 in wild-type *P. aeruginosa* cells. Estimates of the C values for the antibiotic-supersusceptible mutant Z61 suggest that at least 5 to 10 times more pores may be in an open functional state in this strain. In agreement with this, preliminary studies with the purified porin protein F of strains K799 and Z61 incorporated into black lipid bilayers have demonstrated no large differences in pore properties but higher activity in current per unit weight of protein of Z61.

Although, as discussed above, there is apparently no alteration in the porin protein F of strain Z61 compared with strain K799, we have provided two pieces of suggestive evidence (the distribution of LPS on outer membrane fractionation and the influence of LPS on lipoprotein staining in SDS-polyacrylamide gels) for an LPS alteration. In the accompanying paper (14), a detailed comparison of LPS from the two strains is presented.

ACKNOWLEDGMENTS

We acknowledge financial assistance from the Canadian Cystic Fibrosis Foundation (to R.E.W.H.), the National Scientific and Engineering Research Council of Canada (to R.E.W.H. and A.K.), and the Medical Research Council of Canada (to A.K.).

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