ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 1989, p. 1921–1926
0066-4804/89/111921-06$02.00/0

Vol. 33, No. 11

Factors Influencing the Accumulation of Ciprofloxacin in *Pseudomonas aeruginosa*

ROGER A. CELESK* AND NORMAN J. ROBILLARD

Pharmaceutical Division, Miles Inc., West Haven, Connecticut 06516

Received 7 April 1989/Accepted 9 August 1989

Ciprofloxacin accumulation in *Pseudomonas aeruginosa* was measured by a bioassay. Drug accumulation in strain PAO2 was compared with that of three spontaneous ciprofloxacin-resistant mutants selected with 0.5 μg of ciprofloxacin per ml. PAO4701 *cfxA2* contains a mutation in the *gyrA* gene, PAO4742 *cfxB5* may represent a permeability mutant based on pleiotropic drug resistance, and PAO4700 *cfxA1 cfxB1* contains both types of mutations. In all strains, drug accumulation was similar, reaching steady state during the first minute of exposure. Drug accumulation was unsaturable over a range of 5 to 80 μg/ml, suggesting that ciprofloxacin accumulates by diffusion in *P. aeruginosa*. Although all four strains accumulated two- to sevenfold more ciprofloxacin in the presence of the inhibitor carbonyl cyanide *m*-chlorophenylhydrazone, the *cfxB* mutants accumulated two- to fourfold less drug than either PAO2 or the *cfxA2* mutant. Polyacrylamide gel analysis revealed a protein common to *cfxB* mutants only, while all strains had similar lipopolysaccharide profiles. The results suggest that ciprofloxacin accumulation in *P. aeruginosa* is a complex phenomenon that may be affected by both an energy-dependent drug efflux process and outer envelope composition.

Ciprofloxacin is a highly active carboxy-quinolone antimicrobial agent that is therapeutically effective against infections caused by either gram-positive or gram-negative bacteria including *Pseudomonas aeruginosa*. The bactericidal activity of the drug has been attributed to the inhibition of DNA gyrase (21), an enzyme active in the replication, repair, and transcription of bacterial DNA (11). In gram-negative organisms, chromosomal mutations affecting DNA gyrase cause reduced susceptibility to quinolones (3, 19, 21–23, 35). However, quinolones must penetrate the cell envelope before they can act on their intracellular target. Recent investigations have suggested that the hydrophilic quinolones including ciprofloxacin permeate the outer membranes of gram-negative bacteria through water-filled channels created by porin proteins (21). The principal porin in *P. aeruginosa*, porin F, produces channel openings that are substantially smaller than those of other gram-negative bacteria (15, 40). This confers intrinsically lower permeability to hydrophilic antibiotics, which may account for the relatively lower susceptibility of *P. aeruginosa* to quinolones. We and other investigators have observed that chromosomal mutations which are associated with pleiotropic drug resistance and altered permeability further enhance quinolone resistance in *P. aeruginosa* (8, 19, 35). One such mutation is *cfxB*, which recently was described by this laboratory (35).

This study examines ciprofloxacin accumulation in *P. aeruginosa* and the nature of *cfxB* mutations in strain PAO2. We provide evidence that (i) ciprofloxacin accumulates in *P. aeruginosa* by diffusion; (ii) disruption of membrane potential or electron transport or both promotes drug accumulation; (iii) drug accumulation in *cfxB* mutants is less than in other strains in the presence of the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP); and (iv) *cfxB* mutants uniformly differ from other strains in outer membrane protein (OMP) composition.

(This paper was presented in part at the 88th Annual Meeting of the American Society for Microbiology [R. A. Celesk and N. J. Robillard, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, A112, p. 19].)

MATERIALS AND METHODS

Bacterial strains and bacteriophage. *P. aeruginosa* strains used in this study are listed in Table 1. Transducing phage F116L was obtained from Paul Phibbs, East Carolina School of Medicine, Greenville, N.C.

Bacterial growth media. ML broth and agar were prepared as reported previously (30). Cation-supplemented Mueller-Hinton broth and Mueller-Hinton agar were prepared as directed in National Committee for Clinical Laboratory Standards standard M-7A (31). Basal salts medium (BSM) has been described previously (35).

Chemicals. Silicon oils DC550 and DC556 were obtained from Dow Corning Corp. (Midland, Mich.). The bicinchoninic acid reagent kit for protein determination was obtained from Pierce Chemical Co. (Rockford, Ill.). All other chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Antibiotics. Antibiotics were obtained as follows: chloramphenicol, novobiocin, and tetracycline, Sigma; ciprofloxacin, Pharmaceutical Division, Miles Inc., West Haven, Conn.; ofloxacin, Ortho Diagnostics, Inc., Raritan, N.J.; enoxacin, Warner-Lambert Co., Ann Arbor, Mich.; norfloxacin, Merck & Co., Inc., Rahway, N.J.; nalidixic acid, Sterling Winthrop, Rensselaer, N.Y.; and carbenicillin, Beecham Laboratories, Bristol, Tenn.

Isolation of ciprofloxacin-resistant mutants. The procedure for selecting ciprofloxacin-resistant strains of *P. aeruginosa* has been described previously (35). Briefly, cells were grown overnight in 50 ml of ML broth at 35°C with aeration. After cells were harvested by centrifugation and suspended in 1 ml of fresh ML broth, samples of 0.1 ml were plated on ML agar plates containing 0.5 μg of ciprofloxacin per ml. The ciprofloxacin-resistant mutants were screened in BSM agar for resistance to 800 μg of novobiocin per ml. Low-level novobiocin resistance is indicative of *cfxB* mutants. Four of the mutants (PAO4739, PAO4740, PAO4741, and PAO4742) were chosen for further study.

* Corresponding author.

TABLE 1. *P. aeruginosa* strains used in this study

Strain	Genotype	Reference or derivation
PAO2	*ser-3*	Holloway collection[a]
PAO4	*pyrB52 arg-47*	Holloway collection
PAO660	*phe-2*	Holloway collection
PAO969	*proC130*	Rella and Haas (34)
PAO6005	*proC130 nalB8*	Rella and Haas (34)
PAO4700	*cfxA1 cfxB1 ser-3*	Robillard and Scarpa (35)
PAO4701	*cfxA2 ser-3*	Robillard and Scarpa (35)
PAO4739	*cfxA6 cfxB2 ser-3*	This study
PAO4740	*cfxA7 cfxB3 ser-3*	This study
PAO4741	*cfxB4 ser-3*	This study
PAO4742	*cfxB5 ser-3*	This study

[a] Bruce Holloway, Department of Genetics, Monash University, Clayton, Victoria, Australia.

Genetic mapping. With ML agar as the enriched medium, generalized transducing phage F116L (25) was propagated in ciprofloxacin-novobiocin-resistant mutants as previously reported (7, 10). Phage lysates were stored at 4°C. Transductions were performed by published methods (33, 36) and with a multiplicity of infection of 1.

Susceptibility testing. Standard microdilution susceptibility tests with either cation-supplemented Mueller-Hinton broth or Mueller-Hinton agar were performed for all drugs except novobiocin (31). The MIC of novobiocin was determined by macrodilution with BSM broth supplemented with glucose and appropriate amino acids.

Accumulation assays. Overnight BSM cell cultures were diluted 1:20 in fresh BSM and grown until the late log phase with aeration at 35°C. Cells were harvested by centrifugation and resuspended to a turbidity of 260 Klett units in cold BSM. Cell volumes of 5 ml were added to 50-ml flasks and temperature equilibrated at 37°C for 5 min in a shaking water bath. When used, the inhibitor CCCP was added just before the temperature equilibration period. The desired concentration of drug in each assay was obtained by adding 25 to 50 μl of a ciprofloxacin stock solution at time zero. At appropriate time intervals, 1-ml samples were removed to 1.5-ml microcentrifuge tubes containing 0.5 ml of silicon oil prepared as described previously (24). To separate the cells from extracellular ciprofloxacin, the samples were centrifuged in a microcentrifuge at 15,000 × *g* for 3 min. Most cells entered the oil phase in less than 30 s. Samples were frozen at −70°C overnight. A heated scalpel was used to cut off the bottom of each microcentrifuge tube below the water-oil interface. Tube bottoms were inverted over absorbent paper to remove residual oil from the cell pellets. These cell pellets were suspended in 0.2 ml of phosphate-buffered saline (pH 7.2) and transferred to new 1.5-ml microcentrifuge tubes. Samples were boiled for 7 min to extract intracellular drug as previously described (17) followed by centrifugation at 15,000 × *g* for 5 min to remove cell debris. Ciprofloxacin levels in the supernatants were determined by an agar well diffusion bioassay with *Klebsiella pneumoniae* ATCC 10031 as the assay organism (13).

Membrane protein isolation. Late-log-phase cells grown with aeration at 35°C in BSM were diluted 1:50 into 2-liter flasks containing 400 ml of fresh BSM. Cultures were incubated overnight with aeration at 35°C. The cells were harvested by centrifugation and washed in 0.01 M phosphate buffer (pH 7.0). Cell lysates containing OMPs were produced by a modification of the method of Ames (1) as described below. Cells were suspended in cold 0.0625 M Tris hydrochloride (pH 6.8) and maintained over ice during disruption by sonic oscillation with the maximum output setting for the microprobe of a Virtis Virsonic 300. The cells were subjected to 10 bursts of 30 s each with a 30-s pause between bursts. Debris from the lysate was removed by centrifugation at 6,000 × *g* for 20 min in a Sorvall SS34 rotor. Resultant supernatants were centrifuged in a Beckman Ti70 rotor at 111,000 × *g* for 35 min at 10°C. OMPs contained in the pellets were purified by a modified method of Sawai et al. (37) which included an overnight incubation of OMPs in 2% *N*-lauroylsarcosine, sodium salt. The purified OMPs were suspended in 0.01 M phosphate buffer (pH 7.0). Protein content was estimated by the method of Smith et al. (38).

LPS preparation. Lipopolysaccharides (LPS) were partially purified from whole-cell lysates by the method of Hitchcock and Brown (20). After growth in BSM with aeration at 35°C, cells were harvested in the late log phase, washed in Dulbecco phosphate-buffered saline (pH 7.2), and suspended to a density of 300 Klett units. For each strain, a 1.5-ml sample was centrifuged in a microcentrifuge tube and the supernatant was discarded. Cell pellets were suspended in 50 μl of a lysing buffer containing 2% sodium lauryl sulfate (SDS), 4% 2-mercaptoethanol, 10% glycerol, and 0.1 mg of bromphenol blue per ml in 1 M Tris hydrochloride (pH 6.8). The lysate was boiled for 10 min. Contaminating protein in LPS samples was digested with proteinase K at 60°C for 1 h.

Electrophoresis. The isolated OMPs were separated by SDS-polyacrylamide gel electrophoresis with the Laemmli buffer system (27). The acrylamide concentrations for the stacking and separating gels were 4 and 10%, respectively. Gels were fixed and stained overnight in a solution containing 50% methanol, 10% acetic acid, and 0.125% Coomassie blue R-250.

Samples of partially purified LPS were separated on 4 and 15% acrylamide stacking and separating gels, respectively, containing 2% SDS and 4 M urea. A constant current of 15 mA was applied until the dye front had moved 10 to 11 cm. The method of Tsai and Frasch (39) as modified by Hancock et al. (16) was used for fixation and silver staining of the LPS gels.

RESULTS

Characterization of *cfxB* mutants. Of 250 ciprofloxacin-resistant mutants selected on 0.5 μg of ciprofloxacin per ml, approximately one-third exhibited cross resistance to novobiocin, suggesting the presence of a *cfxB* mutation. For four strains, PAO4739, PAO4740, PAO4741, and PAO4742, F116L-mediated transduction was used to determine the location of ciprofloxacin resistance mutations on the PAO2 chromosome. Phage grown in each strain were used to transduce PAO4, PAO969, and PAO660. Ciprofloxacin resistance in all four strains was cotransducible at frequencies ranging from 18 to 30% (Table 2) for *pyrB52* and 4 to 6% for *proC130*, which is consistent with the location of *cfxB*. Ciprofloxacin resistance in PAO4739 and PAO4740 also was cotransducible with *phe-2* at frequencies from 72 to 75%, indicating that these strains are *cfxA cfxB* double mutants. The mutations in PAO4739 and PAO4740 were designated *cfxA6 cfxB2* and *cfxA7 cfxB3*, respectively, while the mutations in PAO4741 and PAO4742 were designated *cfxB4* and *cfxB5*, respectively. PAO4700 *cfxA1 cfxB1* and PAO4701 *cfxA2* were characterized previously (35).

MICs of antibiotics. Both the *cfxA* and *cfxB* mutants of PAO2 were eightfold less susceptible to ciprofloxacin and four- to eightfold less susceptible to the other newer quinolones tested in comparison with the wild type (Table 3). *cfxA*

TABLE 2. Cotransduction of ciprofloxacin-resistant mutations

Donor and recipient strains	Selected marker	Unselected marker	No. of cotransductants/ total (%)[a]
PAO4739			
PAO4	*pyrB52*	Nal[b]	25/100 (25)
PAO969	*proC130*	Nal	9/250 (4)
PAO660	*phe-2*	Cip[c]	36/50 (72)
PAO4740			
PAO4	*pyrB52*	Nal	30/100 (30)
PAO969	*proC130*	Nal	16/250 (6)
pAO660	*phe-2*	Cip	75/100 (75)
PAO4741			
PAO4	*pyrB52*	Nal	24/100 (24)
PAO969	*proC130*	Nal	15/250 (6)
PAO660	*phe-2*	Cip	0/100 (0)
PAO4742			
PAO4	*pyrB52*	Nal	18/100 (18)
PAO969	*proC130*	Nal	12/250 (5)
PAO660	*phe-2*	Cip	1/100 (1)

[a] The fractions represent the number of transductants carrying the unselected marker over the total number of transductants tested. The values in parentheses are percent cotransduction, obtained by multiplying the fraction by 100.

[b] ML agar containing 500 μg of nalidixic acid per ml was used to score inheritance of the *cfxB* marker.

[c] ML agar containing 0.5 μg of ciprofloxacin per ml was used to score inheritance of the *cfxA* marker.

cfxB double mutants were 32- to 64-fold less susceptible to quinolones than PAO2. While all strains harboring the *cfxA* mutation were at least 64-fold more resistant to nalidixic acid than wild type (Table 3), *cfxB* single mutants were only eightfold more resistant. Also, strains containing *cfxB* mutations were two- to fourfold less susceptible to novobiocin, tetracycline, chloramphenicol, and carbenicillin, while the *cfxA* mutation did not affect susceptibility to these drugs (Table 4). The pleiotropic drug resistance associated with *cfxB* mutations is consistent with the drug resistance pattern previously reported for a *cfxB* mutant (35).

Ciprofloxacin accumulation. The concentrations of ciprofloxacin accumulated by PAO2, PAO4700, PAO4701, and PAO4742 over a 20-min time course are displayed in Fig. 1. Drug accumulation reached saturation within the first minute of exposure, and equilibrium was maintained during the observation period. Ciprofloxacin accumulation was proportional to the extracellular drug concentration, and all strains accumulated similar amounts. To determine the saturability of accumulation, cells were exposed to 5 to 80 μg of ciprofloxacin per ml for 1 min and the accumulated drug was measured. Ciprofloxacin accumulation was unsaturable over this range, suggesting that drug accumulates in these strains by diffusion.

TABLE 3. Susceptibility of ciprofloxacin-resistant mutants of *P. aeruginosa* PAO2 to quinolones

Strain or mutant	MIC (μg/ml)				
	Ciprofloxacin	Norfloxacin	Ofloxacin	Enoxacin	Nalidixic acid
PAO2 (wild type)	0.25	1.0	2.0	1.0	50[a]
PAO4700 (*cfxA1 cfxB1*)	16.0	32.0	>32.0	32.0	>3,200[a]
PAO4701 (*cfxA2*)	2.0	8.0	8.0	8.0	3,200[a]
PAO4739 (*cfxA6 cfxB2*)	16.0	32.0	>32.0	>32.0	>3,200
PAO4740 (*cfxA7 cfxB3*)	16.0	32.0	>32.0	>32.0	>3,200
PAO4741 (*cfxB4*)	2.0	4.0	8.0	4.0	400
PAO4742 (*cfxB5*)	2.0	4.0	8.0	4.0	400

[a] Reported previously (35).

TABLE 4. Susceptibility of ciprofloxacin-resistant mutants of *P. aeruginosa* PAO2 to nonquinolone antibiotics

Strain or mutant	MIC (μg/ml)			
	Novobiocin	Tetracycline	Chloramphenicol	Carbenicillin
PAO2 (wild type)	300	50	150	50[a]
PAO4700 (*cfxA1 cfxB1*)	1,200	100	>300	200[a]
PAO4701 (*cfxA2*)	300	50	150	25[a]
PAO4739 (*cfxA6 cfxB2*)	1,200	125	>300	200
PAO4740 (*cfxA7 cfxB3*)	2,400	150	>300	200
PAO4741 (*cfxB4*)	1,200	100	>300	200
PAO4742 (*cfxB5*)	1,200	100	>300	200

[a] Reported previously (35).

Effect of CCCP. To test whether ciprofloxacin accumulation was affected by inhibition of energy metabolism or perturbation of membrane potential, we monitored accumulation in the presence of CCCP. A comparison of ciprofloxacin accumulation with and without CCCP is presented in Table 5. Although all strains accumulated substantially higher levels of drug in the presence of 0.25 mM CCCP, the inhibitor promoted up to fourfold-greater accumulation of ciprofloxacin in PAO2 and the *cfxA* mutant than in the *cfxB* mutants.

Cell envelope composition. To further characterize the *cfxB* mutants, we compared the composition of their cell enve-

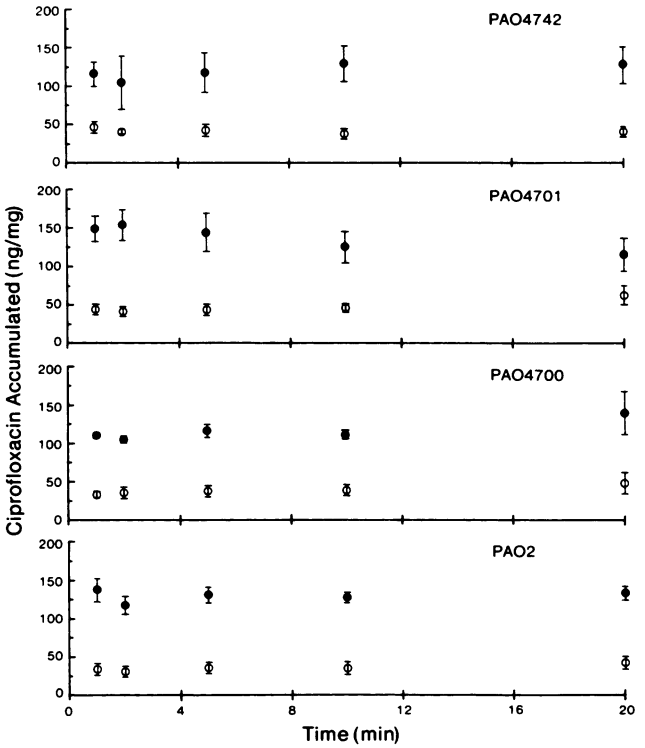

FIG. 1. Accumulation of ciprofloxacin by *P. aeruginosa*. At time zero, ciprofloxacin was added to a concentration of 10 μg/ml (○) or 40 μg/ml (●). Each point represents the mean of a minimum of three separate determinations. Bars, ±1 standard error.

TABLE 5. Influence of CCCP on the accumulation of ciprofloxacin by *P. aeruginosa* PAO2 and mutants exposed to 40 μg of ciprofloxacin per ml for 5 min

Inhibitor	Concn (M)	Ciprofloxacin accumulated (ng/mg)[a]			
		PAO2 (wild type)	PAO4700 (*cfxA1 cfxB1*)	PAO4701 (*cfxA2*)	PAO4742 (*cfxB5*)
None		182	156	182	171
CCCP	2.5×10^{-4}	1,070	398	1,276	317

[a] Each value is the mean of a minimum of three separate determinations.

lopes with those of PAO2 and PAO4701 *cfxA2*. Figure 2 presents the profiles produced by electrophoretic separation of purified OMPs from each strain. All the strains had similar amounts of the 34-kilodalton (kDa) major porin F, as well as other OMPs characteristic of *P. aeruginosa*. However, the *cfxB* mutants had an additional protein band with a molecular mass of approximately 51 kDa. This protein also was observed in OMP profiles from PAO4739, PAO4740, PAO4741, and the *nalB* mutant, PAO6005 (gel not shown). Additionally, the LPS profile of PAO2 was compared with those of PAO4700, PAO4701, and PAO4742. *cfxA* and *cfxB* mutants had LPS profiles identical to that of PAO2 (Fig. 3).

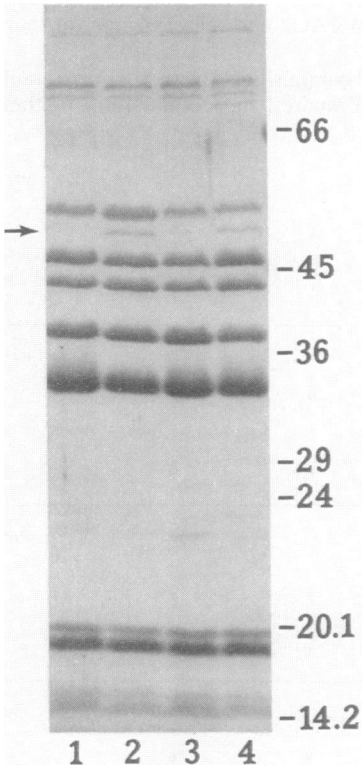

FIG. 2. SDS-polyacrylamide electrophoretograms of OMPs of *P. aeruginosa* PAO2 and mutants. Lanes: 1, PAO2 (wild type); 2, PAO4700 (*cfxA1 cfxB1*); 3, PAO4701 (*cfxA2*); 4, PAO4742 (*cfxB5*). A protein band apparent only in OMP preparations from *cfxB* mutants (arrow) is shown migrating at approximately 51 kDa. Molecular size standards were bovine albumin (66 kDa), egg albumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), bovine pancreatic trypsinogen (24 kDa), soybean trypsin inhibitor (20.1 kDa), and bovine lactalbumin (14.2 kDa).

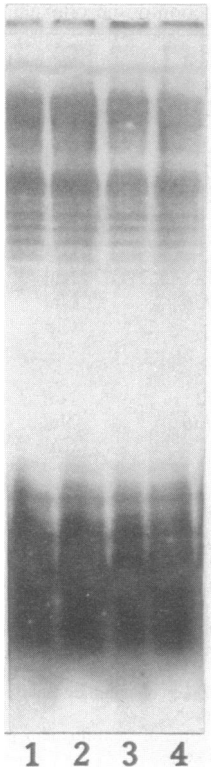

FIG. 3. SDS-urea polyacrylamide electrophoretograms of partially purified LPS obtained from *P. aeruginosa* PAO2 and mutants. Lanes: 1, PAO4742 (*cfxB5*); 2, PAO4701 (*cfxA2*); 3, PAO4700 (*cfxA1 cfxB1*); 4, PAO2 (wild type).

For each strain, the population of O-polysaccharide side chains which appear in a ladderlike arrangement in the upper portion of the gel and the core material at the bottom of the gel stained with similar intensity. This is consistent with the appearance of smooth-type LPS from *P. aeruginosa* (5).

DISCUSSION

P. aeruginosa is intrinsically less susceptible than other bacteria to hydrophilic antibiotics, owing to significantly slower diffusion of drugs across the outer membrane (2, 14, 32, 41). This phenomenon is believed to be the result of narrower porin channels in *P. aeruginosa* relative to other organisms (40). Mutations which affect outer membrane porin proteins or LPS structure further reduce drug permeability. These mutations have been associated with resistance to norfloxacin (8, 19, 22), as well as β-lactams (6, 12, 26), aminoglycosides (5), and other antibiotics (26). Recent work, however, suggests that energy-dependent efflux across the inner membrane also contributes to antibiotic resistance in bacteria. McMurry et al. (29) associated resistance to tetracyclines in *Escherichia coli* with an energy-dependent efflux system. More recently, Cohen et al. (9) demonstrated energy-dependent efflux of norfloxacin in *E. coli* and Chamberland et al. (8) suggested that norfloxacin efflux occurs in *P. aeruginosa*. Our study provides evidence that the accumulation of ciprofloxacin by *P. aeruginosa* involves diffusion and suggests the presence of an energy-dependent efflux system.

We isolated pleiotropic drug-resistant mutants of PAO2 which are resistant to quinolones as well as novobiocin,

carbenicillin, tetracycline, and chloramphenicol. The mutation responsible for this phenotype is *cfxB*. In addition, some of the mutants contained a *cfxA* mutation. This class of mutation has been shown to reside in the gyrase A gene (35). PAO2 and the drug-resistant mutants were compared with respect to ciprofloxacin accumulation, the effect of CCCP on drug accumulation, and the composition of the outer membrane. All strains were similar in rapidly accumulating steady-state levels of ciprofloxacin that were proportional to the extracellular drug concentration. The accumulation of drug in *P. aeruginosa* PAO2 exposed to 10 μg of ciprofloxacin per ml was similar to norfloxacin accumulation in PAO4009 reported by Hirai et al. (19) but approximately fivefold lower than the norfloxacin accumulation in PAO1 observed by Chamberland et al. (8). Accumulation of ciprofloxacin in *P. aeruginosa* was 2- to 10-fold lower than levels of norfloxacin or enoxacin accumulated by *E. coli* or *Citrobacter freundii* (3, 4, 18).

Ciprofloxacin accumulation was unsaturable over a wide range (5 to 80 μg/ml) of concentrations, suggesting that ciprofloxacin accumulates in *P. aeruginosa* by diffusion. Similarly, Bedard et al. (4) reported that enoxacin permeated *E. coli* by diffusion. If diffusion was the only process occurring, ciprofloxacin uptake should not be influenced by an inhibitor of active transport. However, in the presence of CCCP, all strains took up two- to sevenfold more drug. A similar observation reported by Chamberland et al. (8) showed increased norfloxacin accumulation by *P. aeruginosa* in the presence of CCCP. While this suggests that *P. aeruginosa* expends energy to expel ciprofloxacin, energy-dependent uptake of drug by everted membrane vesicles must be demonstrated to confirm this possibility. One would not expect diffusion kinetics for ciprofloxacin accumulation in cells capable of active efflux unless the efflux system was saturated at the lowest concentration of drug tested. Therefore, it would be necessary to determine drug accumulation at much lower concentrations of ciprofloxacin than those used in this study to detect an efflux without using a transport inhibitor. Further, detecting small differences in uptake may be difficult if cells bind significant amounts of ciprofloxacin. Bedard et al. (4) determined that norfloxacin accumulation by heat-killed *E. coli* was as much as 80% of the level accumulated in viable cells.

In the presence of CCCP, *cfxB* mutants accumulated as much as fourfold less ciprofloxacin than PAO2 and PAO4701. The difference in drug accumulation was correlated with the appearance of a 51-kDa protein in the outer membrane of *cfxB* mutants. Hirai et al. (19) previously reported a new 54-kDa OMP in a norfloxacin-resistant mutant (*nfxB*) of *P. aeruginosa*. However, the *nfxB* mutation was linked with *ilvBC*, which is located 20 min from *cfxB* on the *P. aeruginosa* chromosome. Unless the expression of these OMPs is regulated by at least two different genes, they are probably different proteins. Legakis et al. (28) reported cell envelope alterations in two quinolone-resistant mutants of clinical isolates of *P. aeruginosa*. Both mutants had altered LPS content and were cross resistant to carbenicillin and aminoglycosides. However, only one mutant had a higher amount of a 54-kDa OMP than the parent strain. This suggests that the LPS alteration in these mutants is responsible for the nonquinolone cross resistance observed by Legakis et al. (28). In our *cfxB* mutants, neither LPS alteration nor cross resistance to aminoglycosides was evident, but a 51-kDa OMP not seen in the wild type or *cfxA* mutants was observed. Therefore, the cause of altered ciprofloxacin accumulation in *cfxB* mutants appears to be related to the protein composition of the outer membrane.

The function of the 51-kDa OMP in *cfxB* mutants has not been established. One possibility suggested by the pleiotropic drug resistance of these mutants is that the protein reduces permeability. However, reduced ciprofloxacin accumulation by *cfxB* mutants was evident only in the presence of CCCP and, to a lesser extent, 2,4-dinitrophenol (data not shown). The inconsistency in these observations might be resolved if the 51-kDa protein binds ciprofloxacin, since bound drug could mask reduced permeability. This seems unlikely since we did not observe saturability for ciprofloxacin accumulation. At present, we are unable to establish to what extent either specific or nonspecific ciprofloxacin binding contributes to the total drug accumulated by viable cells of *P. aeruginosa*.

It is interesting that ciprofloxacin accumulation by *cfxB* mutants was lower than that by PAO2 in the presence of CCCP or 2,4-dinitrophenol. One interpretation of this observation is that active efflux of drug from the cytoplasm of *P. aeruginosa* obscures the effect of reduced outer membrane permeability on drug accumulation in whole cells. However, we cannot discount the possibility that these inhibitors differentially alter the membrane structure in *cfxB* mutants, causing reduced ciprofloxacin accumulation. Inhibiting active drug efflux across the inner membrane could allow cytoplasmic accumulation to become a function of the ciprofloxacin level in the periplasmic space alone. Our observations suggest that the *cfxB* mutation reduces outer membrane permeability, thereby lowering the amount of ciprofloxacin in the periplasmic space and ultimately in the cytoplasm. The magnitude of this reduction apparently is considerably less when active efflux occurs. Detection of this difference in the absence of CCCP will require a more sensitive assay than the bioassay used in this investigation.

ACKNOWLEDGMENTS

We thank Holly Godin, Janet Herrington, Denise Rasimas, and Barbara Thurberg for doing the antimicrobial susceptibility tests and bioassays. We also thank Janice Walsh for typing the manuscript.

LITERATURE CITED

1. **Ames, G. F.-L.** 1974. Resolution of bacterial proteins by polyacrylamide gel electrophoresis on slabs. J. Biol. Chem. **249:** 634–644.
2. **Angus, B. L., A. M. Carey, D. A. Caron, A. M. B. Kropinski, and R. E. W. Hancock.** 1982. Outer membrane permeability in *Pseudomonas aeruginosa*: comparison of a wild-type with an antibiotic-supersusceptible mutant. Antimicrob. Agents Chemother. **21:**299–309.
3. **Aoyama, H., K. Sato, T. Fujii, K. Fugimaki, M. Inoue, and S. Mitsuhashi.** 1988. Purification of *Citrobacter freundii* DNA gyrase and inhibition by quinolones. Antimicrob. Agents Chemother. **32:**104–109.
4. **Bedard, J., S. Wong, and L. E. Bryan.** 1987. Accumulation of enoxacin by *Escherichia coli* and *Bacillus subtilis*. Antimicrob. Agents Chemother. **31:**1348–1354.
5. **Bryan, L. E., K. O'Hara, and S. Wong.** 1984. Lipopolysaccharide changes in impermeability-type aminoglycoside resistance in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **26:**250–255.
6. **Buscher, K.-H., W. Cullmann, W. Dick, and W. Opferkuch.** 1987. Imipenem resistance in *Pseudomonas aeruginosa* resulting from diminished expression of an outer membrane protein. Antimicrob. Agents Chemother. **31:**703–708.
7. **Calhoun, D. H., and T. W. Feary.** 1968. Transductional analysis of *Pseudomonas aeruginosa* methionineless auxotrophs. J. Bacteriol. **97:**210–216.

8. **Chamberland, S., A. S. Bayer, T. Schollaardt, S. A. Wong, and L. E. Bryan.** 1989. Characterization of mechanisms of quinolone resistance in *Pseudomonas aeruginosa* strains isolated in vitro and in vivo during experimental endocarditis. Antimicrob. Agents Chemother. **33**:624–634.
9. **Cohen, S. P., D. C. Hooper, J. S. Wolfson, K. S. Souza, L. M. McMurry, and S. B. Levy.** 1988. Endogenous active efflux of norfloxacin in susceptible *Escherichia coli*. Antimicrob. Agents Chemother. **32**:1187–1191.
10. **Feary, T. W., B. Williams, D. H. Calhoun, and T. A. Walker.** 1969. An analysis of arginine requiring mutants in *Pseudomonas aeruginosa*. Genetics **62**:673–686.
11. **Gellert, M.** 1981. DNA topoisomerases. Annu. Rev. Biochem. **50**:879–910.
12. **Godfrey, A. J., L. Hatelid, and L. E. Bryan.** 1984. Correlation between lipopolysaccharide structure and permeability resistance in β-lactam-resistant *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **26**:181–186.
13. **Gonzalez, M. A., A. H. Moranchel, S. Duran, A. Pichardo, J. L. Magana, B. Painter, A. Forrest, and G. L. Drusano.** 1985. Multiple-dose pharmacokinetics of ciprofloxacin administered intravenously to normal volunteers. Antimicrob. Agents Chemother. **28**:235–239.
14. **Hancock, R. E. W.** 1986. Intrinsic antibiotic resistance of *Pseudomonas aeruginosa*. J. Antimicrob. Chemother. **18**:653–659.
15. **Hancock, R. E. W., and A. M. Carey.** 1979. Outer membrane of *Pseudomonas aeruginosa*: heat- and 2-mercaptoethanol-modifiable proteins. J. Bacteriol. **140**:902–910.
16. **Hancock, R. E. W., L. M. Mutharia, L. Chan, R. P. Darveau, D. P. Speert, and G. B. Pier.** 1983. *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis: a class of serum-sensitive, nontypable strains deficient in lipopolysaccharide O side chains. Infect. Immun. **42**:170–177.
17. **Hirai, K., H. Aoyama, T. Irikura, S. Iyobe, and S. Mitsuhashi.** 1986. Differences in susceptibility to quinolones of outer membrane mutants of *Salmonella typhimurium* and *Escherichia coli*. Antimicrob. Agents Chemother. **29**:535–538.
18. **Hirai, K., H. Aoyama, S. Suzue, T. Irikura, S. Iyobe, and S. Mitsuhashi.** 1986. Isolation and characterization of norfloxacin-resistant mutants of *Escherichia coli* K-12. Antimicrob. Agents Chemother. **30**:248–253.
19. **Hirai, K., S. Suzue, T. Irikura, S. Iyobe, and S. Mitsuhashi.** 1987. Mutations producing resistance to norfloxacin in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **31**:582–586.
20. **Hitchcock, P. J., and T. M. Brown.** 1983. Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. J. Bacteriol. **154**:269–277.
21. **Hooper, D. C., J. S. Wolfson, E. Y. Ng, and M. N. Swartz.** 1987. Mechanisms of action and resistance to ciprofloxacin. Am. J. Med. **82(4A)**:12–20.
22. **Hooper, D. C., J. S. Wolfson, K. S. Souza, C. Tung, G. L. McHugh, and M. N. Swartz.** 1986. Genetic and biochemical characterization of norfloxacin resistance in *Escherichia coli*. Antimicrob. Agents Chemother. **29**:639–644.
23. **Inoue, V., K. Sato, T. Fujii, K. Hirai, M. Inoue, S. Iyobe, and S. Mitsuhashi.** 1987. Some properties of subunits of DNA gyrase from *Pseudomonas aeruginosa* PAO1 and its nalidixic acid-resistant mutant. J. Bacteriol. **169**:2322–2325.
24. **Johnson, J. D., W. L. Hand, J. B. Francis, N. King-Thompson, and R. W. Corwin.** 1980. Antibiotic uptake by alveolar macrophages. J. Lab. Clin. Med. **95**:429–439.
25. **Krishnapillai, V.** 1971. A novel transducing phage. Its role in recognition of a possible new host-controlled modification system in *Pseudomonas aeruginosa*. Mol. Gen. Genet. **114**:134–143.
26. **Kropinski, A. M. B., L. Chan, and F. H. Milazzo.** 1978. Susceptibility of lipopolysaccharide-defective mutants of *Pseudomonas aeruginosa* strain PAO to dyes, detergents, and antibiotics. Antimicrob. Agents Chemother. **13**:494–499.
27. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
28. **Legakis, N. J., L. S. Tzouvelekis, A. Makris, and H. Kotsifaki.** 1989. Outer membrane alterations in multiresistant mutants of *Pseudomonas aeruginosa* selected by ciprofloxacin. Antimicrob. Agents Chemother. **33**:124–127.
29. **McMurry, L. M., D. A. Aronson, and S. B. Levy.** 1983. Susceptible *Escherichia coli* cells can actively excrete tetracyclines. Antimicrob. Agents Chemother. **24**:544–551.
30. **Morrison, T. G., and M. H. Malamy.** 1970. Comparison of F factors and R factors: existence of independent regulation groups in F factors. J. Bacteriol. **103**:81–88.
31. **National Committee for Clinical Laboratory Standards.** 1985. Approved standard M7-A. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. National Committee for Clinical Laboratory Standards, Villanova, Pa.
32. **Nicas, T. I., and R. E. W. Hancock.** 1983. *Pseudomonas aeruginosa* outer membrane: isolation of a porin protein F-deficient mutant. J. Bacteriol. **153**:281–285.
33. **Phibbs, P. V., Jr., T. W. Feary, and W. T. Blevins.** 1974. Pyruvate carboxylase deficiency in pleiotropic carbohydrate-negative mutant strains of *Pseudomonas aeruginosa*. J. Bacteriol. **118**:999–1009.
34. **Rella, M., and D. Haas.** 1982. Resistance of *Pseudomonas aeruginosa* PAO to nalidixic acid and low levels of β-lactam antibiotics: mapping of chromosomal genes. Antimicrob. Agents Chemother. **22**:242–249.
35. **Robillard, N. J., and A. L. Scarpa.** 1988. Genetic and physiological characterization of ciprofloxacin resistance in *Pseudomonas aeruginosa* PAO. Antimicrob. Agents Chemother. **32**:535–539.
36. **Roehl, R. A., and P. V. Phibbs, Jr.** 1982. Characterization and genetic mapping of fructose phosphotransferase mutations in *Pseudomonas aeruginosa*. J. Bacteriol. **149**:897–905.
37. **Sawai, T., R. Hiruma, N. Kawana, M. Kaneko, F. Taniyasu, and A. Inami.** 1982. Outer membrane permeation of β-lactam antibiotics in *Escherichia coli*, *Proteus mirabilis*, and *Enterobacter cloacae*. Antimicrob. Agents Chemother. **22**:585–592.
38. **Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk.** 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. **150**:76–85.
39. **Tsai, C.-M., and C. E. Frasch.** 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal. Biochem. **119**:115–119.
40. **Woodruff, W. A., T. R. Parr, Jr., R. E. W. Hancock, L. F. Hanne, T. I. Nicas, and B. H. Iglewski.** 1986. Expression in *E. coli* and function of *Pseudomonas aeruginosa* outer membrane porin protein F. J. Bacteriol. **167**:473–479.
41. **Yoshimura, F., and H. Nikaido.** 1982. Permeability of *Pseudomonas aeruginosa* outer membrane to hydrophilic solutes. J. Bacteriol. **152**:636–642.