CELLULAR AND MOLECULAR EVALUATION OF FLUOROQUINOLONE RESISTANCE IN *Pseudomonas aeruginosa*

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CELLULAR AND MOLECULAR EVALUATION OF FLUOROQUINOLONE RESISTANCE IN *Pseudomonas aeruginosa*

BY

GEORGE G. ZHANEL

A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

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ABSTRACT

We undertook a detailed comprehensive study of Pseudomonas aeruginosa made resistant to fluoroquinolones by in vitro serial passage on agar containing increasing concentrations of fluoroquinolones. Our experimental hypothesis was that very high levels of fluoroquinolone resistance would be associated with both DNA gyrase and permeability changes. In addition, we believed that these permeability changes would lead to a multiple antibiotic resistant (Mar) phenotype. We compared these laboratory derived fluoroquinolone-resistant mutants with clinical fluoroquinolone resistant isolates obtained from patients on fluoroquinolone therapy.

At fluoroquinolone concentrations up to 4-32 fold the wild-type MIC, fluoroquinolone-resistant laboratory mutants demonstrated (relative to wild-type) normal morphology, growth rates and adherence, resistance solely to fluoroquinolones, no change in fluoroquinolone uptake, and no change in LPS or outer-membrane protein (Opr) profiles. Clinical fluoroquinolone resistant isolates demonstrated no change in their biological characteristics and represented gyrA mutants.

At fluoroquinolone concentrations greater than 4-32 fold the wild-type MIC (achieved by continued passage on fluoroquinolone containing agar) fluoroquinolone-resistant laboratory isolates demonstrated altered morphology (rounding), a Mar phenotype, reduced fluoroquinolone uptake and altered outer-membrane proteins (reductions in 25KDa and 38KDa Opr as well as several Opr's in the 43KDa-66KDa region).

Complementation of both categories of laboratory mutants with wild-type E. coli gyrA reduced the level of fluoroquinolone resistance approximately 8-32 fold suggesting that the high-level mutants displayed both gyrA and non-gyrA mutations. Complementation of mutants with pRW5 (Opr F expression vector) demonstrated that Opr F has a dual role, structural, and as a porin involved in antibiotic uptake.

We conclude that laboratory-derived fluoroquinolone resistant mutants represented gyrA mutants up until 4-32x the MIC. Continued serial passage on fluoroquinolone containing agar to higher MIC's led to a multiple-antibiotic-resistant (Mar) phenotype that appeared to display both gyrA and permeability mutations. Clinical fluoroquinolone-resistant mutants represented gyrA mutants.

INTRODUCTION

Fluoroquinolones such as ciprofloxacin are broad spectrum, rapidly bactericidal antibiotics. These agents represent a class of antibiotics that even when administered orally attain high enough serum and tissue concentrations to be used in the treatment of moderate to severe infections caused by gram-negative bacilli, such as Pseudomonas aeruginosa. However, the effectiveness of these agents in the treatment of P. aeruginosa infections is being compromised by the increasing incidence of fluoroquinolone-resistant P. aeruginosa. We therefore, undertook a comprehensive study to better understand the characteristics and mechanisms of fluoroquinolone-resistance in P. aeruginosa. Strains of P. aeruginosa were made resistant to fluroquinolones by in vitro serial passage on agar containing increasing concentrations of fluoroquinolones. Our experimental hypothesis was that resistance to very high concentrations of fluoroquinolones would be associated with both DNA gyrase and permeability changes. In addition, we believed that these permeability changes would lead to a multiple antibiotic resistant (Mar) phenotype. We compared the characteristics of and mechanisms of fluoroquinolone resistance in our laboratory-derived fluoroquinolone-resistant isolates with clinical fluoroquinolone-resistant isolates obtained from patients who had received fluoroquinolone therapy.

Clinical fluoroquinolone-resistant isolates demonstrated resistance to fluoroquinolones but unchanged susceptibility to

other antibiotics. These data were consistent with fluoroquinolone uptake experiments that suggested no difference in uptake between fluoroquinolone-sensitive (wild-type) and clinical fluoroquinolone-resistant isolates. In addition, morphology, virulence as assessed by adherence, LPS and outer membrane protein (Opr) analysis demonstrated no differences between clinical fluoroquinolone resistant isolates compared to their fluoroquinolone-sensitive pairs. Complementation studies with wild-type E. coli gyrase (which is dominant over its mutant allele) conferred fluoroquinolone-susceptibility to all fluoroquinolone-resistant clinical isolates demonstrating that they were gyrA mutants.

The major part of this thesis involved the creation of laboratory-derived fluoroquinolone resistant mutants of P. aeruginosa by serially passaging wild-type organisms on agar containing increasing concentrations of fluoroquinolone. We hypothesized that continued passage on fluoroquinolone agar would lead to a Mar phenotype. Our laboratory-derived fluoroquinolone resistant mutants had both DNA gyrase changes (as evidenced by the partial restoration of fluoroquinolone susceptibility upon complementation with E. coli gyrA) and permeability alterations. In passaging our isolates on fluoroquinolone containing agar, none of these properties (altered morphology and growth, reduced fluoroquinolone uptake, reduced adherence, multiple-antibiotic-resistance and outer-membrane protein changes) occurred until individual strains exhibited 4-32 fold increases in MIC to

fluoroquinolones occurred. As an example, strain 4047 derivatives appeared to be DNA gyrase mutants when they had ciprofloxacin MICs ≤8μg/ml, as evidenced by complementation with E. coli gyrA. Whether the DNA gyrase mutations at ciprofloxacin MICs of 2μg/ml, 4μg/ml and 8μg/ml (wild-type MIC 1μg/ml) represented 1 or more mutations in gyrA is unclear. With continued serial passage, when strain 4047 derivatives achieved ciprofloxacin MICs of 16μg/ml, they demonstrated a Mar phenotype with resistance not only to fluoroquinolones but also to chemically unrelated classes of antibiotics including beta-lactams, chloramphenicol and tetracycline. Evidence is presented here that the mutation(s) conferring higher levels of fluoroquinolone resistance were associated with altered morphology, reduced adherence, reduced fluoroquinolone uptake, alterations in outer-membrane proteins and a Mar phenotype.

The importance of Opr F in serving both a structural role (involved in cell morphology) as well as porin role involved in antibiotic uptake was investigated. Complementation of fluoroquinolone resistant mutants with pRW5 (Opr F expression vector) restored wild-type bacillary morphology on rounded cells. In addition, upon complementing our laboratory derived fluoroquinolone-resistant mutants with pRW5 and testing for antibiotic susceptibility, we observed that OprF served only a minor role in fluoroquinolone uptake and resistance. It did, however, act as a porin involved in beta-lactam, chloramphenicol and tetracycline uptake. Therefore, these data suggest that OprF in fact does have a dual role, one

structural and a second as a porin involved in antibiotic uptake.

The reduced signals of OprF in immunoblots (using monoclonal antibody to OprF) led to sequencing of the gene (oprF). All laboratory derived fluoroquinolone resistant mutants possessed deletions of an alanine at position 19 of the 24 amino acid signal peptide. We believe that this deleted alanine serves as a critical residue for cleavage by signal peptidases.

LITERATURE REVIEW

1. History and Properties of Fluoroquinolones

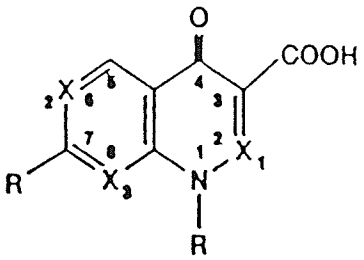
The quinolone era began in 1962 with the description by Lesher and colleagues (1) of the 1,8-naphthyridine derivatives (eg. nalidixic acid), a new class of chemotherapeutic agents. Although nalidixic acid was a bactericidal agent active against gram-negative organisms, it lacked activity against Pseudomonas and did not inhibit any of the tested gram-positive micro-organisms. In addition, its poor pharmacokinetics resulted in low serum concentrations, precluding use for infections other than of the urinary tract. Nalidixic acid frequently caused adverse reactions (gastrointestinal, dermatologic and neurologic) [2,3]. A major problem with nalidixic acid was bacterial resistance, even with increasing dosage (4). Resistance was not carried on extrachromosomal plasmids (R-factors) and therefore was not transferable among organisms in the bowel (5).

Various analogues of nalidixic acid were subsequently synthesized including oxolinic acid, cinoxacin, pipemidic acid, rosoxacin and flumequine. Some of these agents demonstrated greater activity than nalidixic acid against gram-negative organisms, but none was a significant advance in the treatment of clinical infections (3,6). It was not until the early 1980's, when a 7-piperazine and 6-fluorine were added to the molecule creating the fluorinated piperazinyl quinolones also called 4-quinolones, carboxyquinolones, quinolone carboxylic acid and fluoroquinolones, that a potent

broad spectrum class of antibiotics was created (7-9).

a) Structure. The chemical structure of fluoroquinolones differs from the older analogs such as nalidixic acid in two common features, the presence of a fluorine atom at position 6, and a piperazinyl substitution at position 7 of the quinolone nucleus (Figure 1).

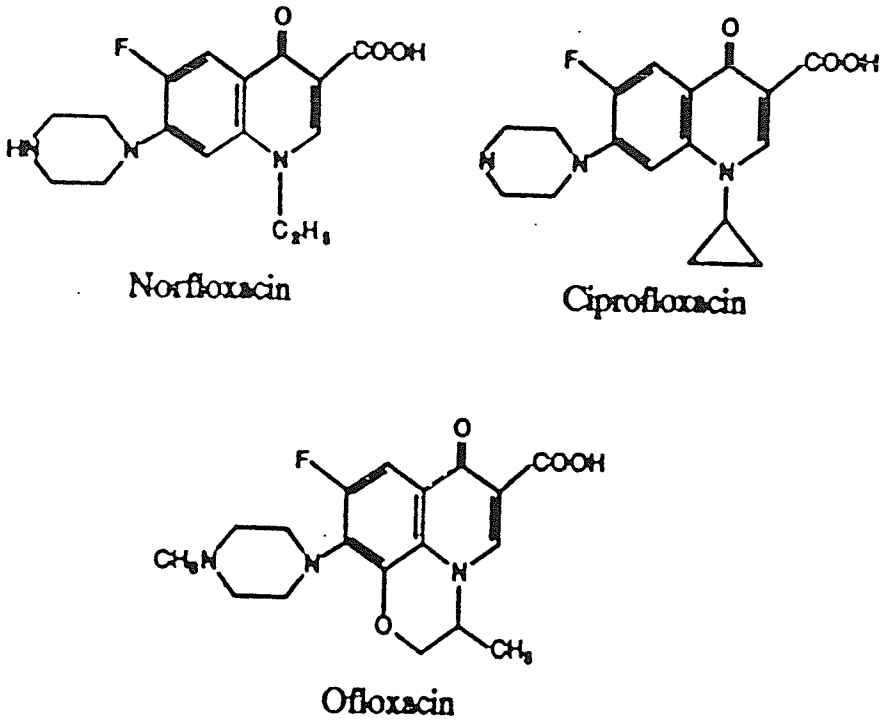
Figure 1. The Basic Fluoroquinolone Structure



(adapted from reference 6)

The newer fluoroquinolones differ among themselves principally in the nature of substituents attached to the nitrogen at the 1 position and carbon at the 7 position (9). Figure 2 depicts three of the fluoroquinolones marketed in the United States and Canada (6,8). This figure in no way includes all of the fluoroquinolones marketed or under study worldwide (some 10,000 compounds) [10]. Ciprofloxacin is commonly considered the prototypical fluoroquinolone and will be primarily used in these studies.

Figure 2. Fluoroquinolones Marketed in Canada and the United States



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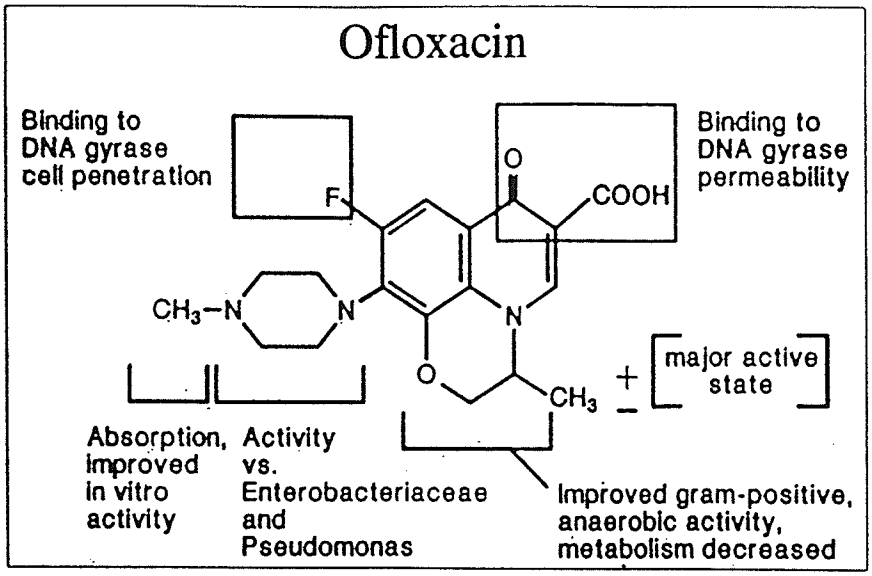
The fluoroquinolones are small molecules with molecular weights between 300 and 500 daltons. These compounds are zwitterions and exhibit different solubility characteristics with changes in pH. The hydrophobicity of these agents varies from very hydrophobic agents like ciprofloxacin, enoxacin and norfloxacin to very hydrophilic agents like sparfloxacin (11).

Knowledge of the structure/activity relationship of fluoroquinolones aids in understanding the mechanism of action and mechanisms of resistance. For a fluoroquinolone to be effective, it must be adequately absorbed, systemically distributed, penetrate the bacterial cell wall and bind to its

target, DNA/DNA gyrase (discussed in section 4). Figure 3 describes the general structure/activity relationship for ofloxacin which is representative of fluoroquinolones (6).

Figure 3. Structure Activity Relationships of Fluoroquinolones



(adapted from reference 10)

Changes at N-1 greatly affect activity against gram-positive cocci. The carboxyl group at C-3 and the ketone at C-4 represent critical elements for appropriate binding to the enzyme DNA gyrase (10). A halogen at C-6 is also thought to be involved in binding to DNA gyrase, but also greatly increases activity against gram-negative bacilli compared to nalidixic acid (12). A ring structure such as piperazine at C-7 affords the greatest activity against gram-negative bacilli and especially Pseudomonas aeruginosa (13). In addition, it appears that the group at C-7 is important for

interacting with DNA gyrase (14). As will be discussed in section 4, knowledge of fluoroquinolone structure activity relationships is essential for understanding the cooperative fluoroquinolone/DNA/DNA gyrase binding model which explains how fluoroquinolones act against bacterial cells.

b) Antibacterial Activity. The fluoroquinolones are broad spectrum, bactericidal agents active against gram-positive and gram-negative organisms (Table 1) [6-9,12,13,16-22]. These agents are very potent against gram-negative bacilli such as Escherichia coli, Proteus spp., Klebsiella spp. and Pseudomonas aeruginosa (Table 1). The minimum bactericidal concentrations (MBCs) are usually the same or one to two tube dilutions above the minimum inhibitory concentraction (MIC) for sensitive strains. The in vitro activity of fluoroquinolones is reduced by anaerobic conditions, acidic pH (eg. in urine) and high concentrations of divalent cations (9,23). The addition of human serum has little to no effect on the activity of fluoroquinolones, probably due to the low percentage of protein binding of these agents (24). Fluoroquinolones when combined with other antibiotics usually show additive or indifferent effects (9,20).

Antagonistic interactions seldom occur in vitro, although combinations with rifampin against strains of S. aureus or with nitrofurantoin against gram-negative bacilli can exhibit antagonistic effects. Synergy is most predictable with

Table 1 In-Vitro Activity of Selected Fluoroquinolones*

Bacterial Species	MIC_{90} (μg/ml) Ciprofloxacin	Norfloxacin	Ofloxacin	Pefloxacin
Gram-positive				
Staphylococcus aureus	1.0	6.3	0.4	0.5
Staphylococcus epidermidis	0.25	3.1	0.8	0.5
Streptococcus pneumoniae	2.0	16	2.0	8.0
Enterococcus faecalis	2.0	8.0	4.0	4.0
Corynebacterium JK	1.0	4.0	1.0	2.0
Gram-negative				
Escherichia coli	0.06	0.12	0.12	0.25
Klebsiella pneumoniae	0.12	0.5	0.2	1.0
Enterobacter cloacae	0.06	0.4	0.1	0.1
Proteus mirabilis	0.06	0.5	0.2	0.5
Proteus vulgaris	0.06	0.4	1.6	2.0
Salmonella spp.	0.06	0.06	0.06	0.12

Table 1 (cont'd)

Serratia spp.	0.25	3.1	1.6	1.0
Pseudomonas aeruginosa	0.5	2.0	2.0	2.0
Haemophilus influenzae	0.015	0.06	0.03	0.06
Legionella pneumophila	0.02	2.0	0.1	0.25
Anaerobic				
Peptococcus spp.	2.0	64	4.0	16
Clostridium difficile	8.0	128	16	1.0
Bacteroides fragilis	8.0	128	8.0	16
Other				
Mycoplasma pneumoniae	2.0	12	2.0	4.0
Chlamydia trachomatis	1.6	25	0.8	2.0
Ureaplasma urealyticum	2.0	32	1.6	8.0
Mycobacterium tuberculosis	0.5	8.0	1.3	8.0

* Adapted from references 6-9, 12, 13, 16-22.

combinations of fluoroquinolones and ß-lactam against Enterobacteriaceae and P. aeruginosa (25). Synergy also occurs, although infrequently, against these pathogens with fluoroquinolone-aminoglycoside combinations. In general, in vitro results of fluoroquinolones with other antibiotics cannot be predicted and must be tested individually. The clinical relevance of in vitro synergy studies remains unknown.

c) Pharmacokinetics. Fluoroquinolone pharmacokinetics are considerably more complex than other classes of antibiotics such as aminoglycosides or penicillins. All available agents are rapidly absorbed with peak concentrations occurring within 2 hours (Table 2). Norfloxacin has the lowest bioavailability and resultant serum concentrations, limiting its use to urinary tract and gastrointestinal infections (26). An increase in dose usually results in linear increases in serum concentrations with usual therapeutic doses; however, higher doses may not produce significantly higher serum levels with some agents, such as norfloxacin (9).

All fluoroquinolones demonstrate rapid and extensive tissue distribution. Volumes of distribution exceed total body water and range from 1.5-3 L/kg. Protein binding is less than 50% for all agents. Fluoroquinolones attain high concentrations in prostate, bone, sputum, bile, kidney, seminal fluid and many other tissues and biological fluids except the cerebrospinal fluid (27).

Fluoroquinolones are eliminated by a combination of

Table 2. Pharmacokinetics of Selected Fluoroquinolones[a]

Drug	Oral Dose (mg)	Bioavailability (%)	C_{max} (mg/L)	Half-life (hrs)	Oral Dose Excreted Unchanged in Urine (%)
Ciprofloxacin	500	70	2.5	4.0	40
Enoxacin	400	90	2.5	4.5	52
Fleroxacin	400	95	4.0	10	60
Lomefloxacin	400	90	2.5	8.0	70
Norfloxacin	400	55	1.5	3.5	30
Ofloxacin	300	95	4.0	6.0	85
Pefloxacin	400	92	4.0	11	9.0

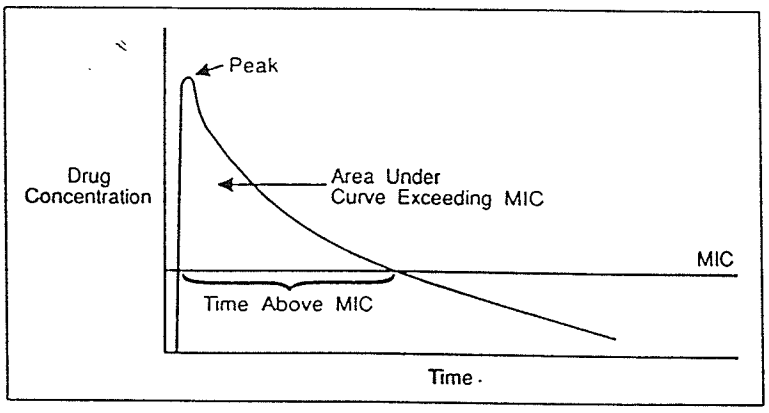
[a]Average values from single-dose studies in subjects with normal renal function.

Data adapted from references 8, 9, 15, 22.

excretion of unchanged drug in the urine, hepatic metabolism followed by urine excretion and secretion into the bile and later excretion in the feces.

d) Pharmacodynamics. Fluoroquinolones display rapid and dose-dependent bacterial killing (28). The pharmacokinetic parameter that best correlates with fluoroquinolone killing is the area under the curve (AUC) exceeding the MIC (AUC/MIC) [Figure 4].

Figure 4. Fluoroquinolone Pharmacokinetic Parameters Correlating With Bacterial Killing



(adapted from reference 28)

Fluoroquinolones also demonstrate a prolonged dose-dependent postantibiotic effect (PAE) [28]. The PAE is defined as the recovery phase of bacterial growth, after antibiotic exposure and subsequent antibiotic removal. This PAE may also be prolonged in biological fluids such as human serum and cerebrospinal fluid (24,29). Finally, fluoroquinolones

display extensive antibacterial activity even at concentrations below the MIC (sub-MIC) (28).

2. Prevalence and Incidence of Fluoroquinolone-Resistance in P. aeruginosa

Fluoroquinolones represent the first available class of oral antibiotics that are bactericidal against P. aeruginosa. Thus, for the first time, a class of antibiotics is available that can attain high enough serum and tissue concentrations after oral dosage allowing treatment for moderate to severe infections caused by gram-negative bacilli, like P. aeruginosa. However, resistance of gram-negative bacilli, especially P. aeruginosa, to fluoroquinolones is becoming a significant problem (30). Thornsberry et al. (30) tested 154,689 isolates (128 species or groups) from 26 institutions in 21 states and reported that for P. aeruginosa, 12.3% were either resistant or only moderately-sensitive to fluoroquinolones. Individual reports of P. aeruginosa resistant to fluoroquinolones are common (31-33). Wolfson and Hooper have recently described conditions where fluoroquinolone-resistant P. aeruginosa and other bacteria are likely to be found (8) [Table 3].

Table 3. Clinical Settings of Fluoroquinolone Resistance

Site/Settings	% of infections where resistance may develop during treatment
Cystic fibrosis	10-15
Colonization states	27
Malignancy prophylaxis	15
Complicated UTI	10
Bone and Joint Infections	15
Skin and Soft Tissue Infections	13

As can be seen, patients with severe underlying disease (cystic fibrosis, malignancy, etc.) those with structural alterations (foreign-body infections, complicated urinary tract infections, and burn and surgical wound infections), difficult-to-heal infections (infected decubitus ulcers or diabetic foot ulcerations), or who are otherwise colonized are most likely to harbor fluoroquinolone-resistant organisms (8). Since many of these infections occur most frequently in patients in tertiary-care institutions, it is not surprising that the highest prevalence of fluoroquinolone-resistant P. aeruginosa is found there (30). Fluoroquinolone-resistant P. aeruginosa is not simply a problem in North America, but a significant problem in Central and South America as well as Europe (34).

More worrisome than the prevalence of fluoroquinolone-resistant P. aeruginosa is the observation that the incidence is increasing dramatically (35,36). Podzol et al. (35) evaluated the incidence of fluoroquinolone-resistant P. aeruginosa over a 6 month period when fluoroquinolone usage

(doses/month) was increasing. The prevalence of P. aeruginosa resistant to fluoroquinolones was 7.4% in study period 1 (June-October 1992) and increased to 13.5% during study period 2 (December 1992-March 1993). Coronado et al. (36) used the database of the national nosocomial infections surveillance system to analyze ciprofloxacin resistance in P. aeruginosa associated with nosocomial infections during 1989-1992. Of the 8,448 isolates analyzed, 5% were resistant to ciprofloxacin. A logistic regression model controlling for site of infection, time period, intensive care unit, and teaching affiliation of the hospital demonstrated a 57% increase in P. aeruginosa resistant to ciprofloxacin during 1991-1992. From these data it is clear that not only is the prevalence of fluoroquinolone-resistant P. aeruginosa high, but the incidence of resistance is dramatically increasing. This has occurred despite the fact that these agents have been clinically available for a relatively short time (less than 10 years).

3. Pseudomonas aeruginosa as a Pathogen

Pseudomonas aeruginosa is an oxidase-positive, obligate aerobic gram-negative bacilli that metabolize carbohydrates via the 2-keto-deoxyglyconate (Entner-Doudoroff) pathway rather than by glycolysis (37). Because glucose is metabolized in the presence of oxygen, the metabolic pathway is not called glycolytic or anaerobic or fermentative, instead P. aeruginosa is classified as a non-fermenter (38,39). These

organisms are motile, have polar, usually monotrichous flagella and a high GC content of their DNA. On solid media they demonstrate distinct colonial morphology, growth at 42°C, and a characteristic grapelike odor of aminoacetophenone (38). On blood agar plates, P. aeruginosa form flat spreading colonies with irregular edges. Strong hemolysis is noticeable on blood agar plates, especially upon longer incubation. P. aeruginosa produce a water-soluble, fluorescent, green-yellow pigment called pyoverdin and a second non-fluorescent, blue, phenazine pigment termed pyocyanin (37). The combination of the yellow and blue pigments produces the characteristic green color associated with most P. aeruginosa strains (39).

Pseudomonas aeruginosa is widely distributed in soil, water, sewage, the mammalian gut and plants, and is frequently isolated from infusion fluids, disinfectants, cosmetics and foodstuffs (38). P. aeruginosa rarely causes disease in healthy individuals, but is an important pathogen in patients with immunodeficiencies or malignancies (40,41). Subjects with cystic fibrosis invariably eventually become colonized in their respiratory tract by highly mucoid variants (42-44). Those undergoing long-term indwelling bladder catheterization, invasive surgical procedures and severe trauma, especially burn patients, are also at risk of P. aeruginosa infection. P. aeruginosa produces a variety of potential virulence factors including alginate, hemolysin, fibrinolysin, lipase, esterase, elastase, lecithinase, DNase, pyoverdin, endotoxin, exotoxin, phospholipase, fimbriae, which contribute to

pathogenicity (45-52).

Pseudomonas aeruginosa is an important nosocomial pathogen (41). In tertiary care institutions that deal extensively with immunocompromised patients, burn patients and subjects who have indwelling catheters (eg. elderly, paraplegic etc.), infections caused by P. aeruginosa are very common (up to 25%) [41,53]. Immunocompromised subjects with bacteremia due to P. aeruginosa have a mortality ranging from 40-93% (41). As a nosocomial pathogen, P. aeruginosa causes the highest mortality of any bacterium (53).

The majority of P. aeruginosa are resistant to commonly used oral antibiotics for two reasons. First, the outer-membrane consisting mainly of lipopolysaccharide and outer-membrane proteins prevents antibiotics entry into the periplasmic space (53). Secondly, P. aeruginosa possesses many plasmid and chromosomally encoded antibiotic resistance elements including beta-lactamases, aminoglycoside-modifying enzymes, efflux systems for tetracycline and acetylation enzymes for chloramphenicol (38,53). Thus, P. aeruginosa is a very important nosocomial pathogen because of its ubiquitous nature within hospitals, its cause of high morbidity and mortality in infected patients, and because of its intrinsic resistance to many chemically unrelated classes of antibiotics.

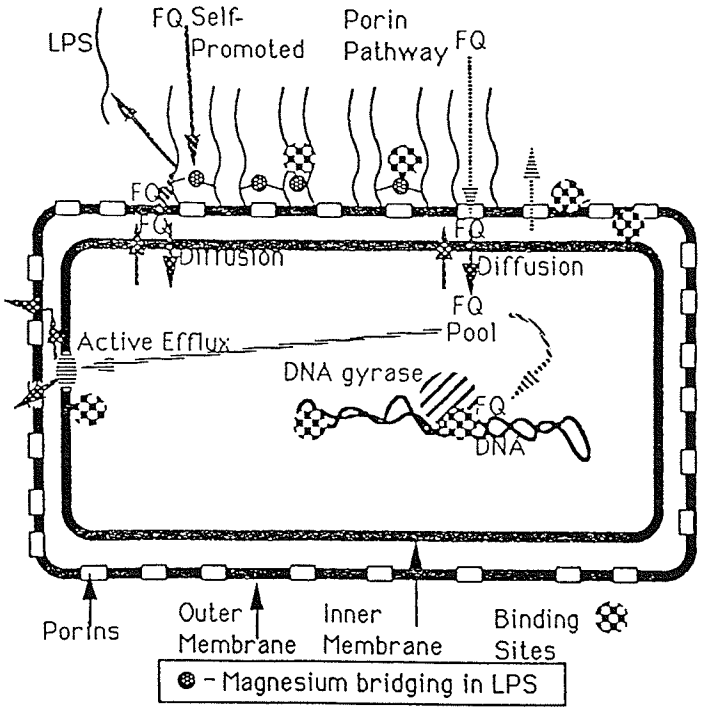
4. Mechanism of Action of Fluoroquinolones

a) Fluoroquinolone uptake into bacteria. The uptake of

antibiotics into bacterial cells is an important factor in determining their activity and antibacterial spectrum. The majority of research into the uptake of fluoroquinolones has been performed in gram-negative bacilli, principally E. coli (54-60).

In gram-negative bacteria, like E. coli, the first well characterized cellular barrier for quinolones is the outer membrane. It is generally thought that there are three routes by which antibiotics can penetrate this structure. These are the porin pathway, the hydrophobic pathway and the self-promoted route used by cationic compounds (Figure 5) [55].

Figure 5. Uptake of Fluoroquinolones Into Gram-Negative Bacilli



(adapted from reference 55)

Before any of these routes are possible, the hydrophilic layer of the polysaccharide side chains of the lipopolysaccharide (LPS) is encountered in so-called smooth bacteria. For the hydrophilic quinolones with partition coefficients of 0.03 (determined in n-octanol-0.1M phosphate buffer, pH 7.2) or less (cinoxacin, ciprofloxacin, enoxacin, norfloxacin, and pipemidic acid) the LPS is not a barrier (61). Quinolones with partition coefficients of 0.08-8.92 (fleroxacin, ofloxacin, pefloxacin, nalidixic acid) demonstrate reduced activity in smooth strains and somewhat increased activity in rough strains (55,61,62).

The role of outer-membrane proteins (OMP) in fluoroquinolone uptake has been investigated with the use of antibiotic resistant strains deficient in one or more of their OMPs (61,63,64). These results have shown that in E. coli the loss of OMP F is associated with significantly reduced uptake of fluoroquinolones with consequent reduced susceptibility (increased MICs). Reduced quinolone uptake in the setting of reduced or lost OMP F along the relative hydrophilic nature of these compounds has led to the conclusion the fluoroquinolones cross the outer membrane of gram-negative bacteria by passive diffusion using porins (water-filled channels) [57,65]. It is not presently clear which OMP is important for passage of fluoroquinolones into P. aeruginosa. The exact mechanism of fluoroquinolone passage across the cytoplasmic (inner) membrane of gram-negative bacteria is presently unknown (66,67). Most data suggest that fluoroquinolones cross the

cytoplasmic membrane by passive diffusion (energy independent) [66,67]. Some investigators have suggested the passage of these hydrophilic antibiotics across the hydrophobic phospholipid bilayer involves specific transport proteins that facilitate the passage of drug into the cytoplasm (67). It is difficult to directly compare the results of these studies, as vastly different methodologies were used.

Uptake of fluroquinolones into gram-negative bacteria is rapid, reaching a plateau or steady state within minutes. When 10 mg/l of quinolone is used, a steady state is reached after 60-80 sec in Enterobacteriaceae with steady state concentrations between 50-120 ng quinolone/mg dry weight of cells (54). P. aeruginosa and K. pneumoniae accumulate quinolones more slowly than other species, reaching steady state at around 6 min (55,59). P. aeruginosa attains a lower steady state fluoroquinolone concentration. Uptake is non-saturable in E. coli and P. aeruginosa (66,68), indicating that uptake is not mediated by a saturable carrier protein and probably takes place by simple diffusion through non specific protein channels. No competition for uptake between enoxacin or ciprofloxacin has been observed (66).

Magnesium and calcium ions decrease fluoroquinolone uptake (66). In addition, reduced temperature and acid pH decreases uptake (23,58).

Recent data suggest that gram-negative bacilli including E. coli and Pseudomonas aeruginosa have an energy-dependent efflux system located in the cytoplasmic membrane, which can

actively pump fluoroquinolones out of the cell (55,59,69). Treatment of whole cells or spheroplasts with metabolic inhibitors like carbonyl cyanide m-chlorophenyl hydrazone (CCCP) or dinitrophenol (DNP) doubles the concentration of fluoroquinolone inside E. coli (67). If everted (inside-out) vesicles are treated with CCCP, fluoroquinolone uptake is greatly reduced.

It has been proposed that fluoroquinolone uptake into gram-negative bacilli such as E. coli and P. aeruginosa occurs by a similar model (66): (a) fluoroquinolones passively diffuse through outer membrane porins; (b) transport through the cytoplasmic membrane is also by diffusion, although some evidence suggests coupling with the proton motive force; (c) there is an active efflux system that pumps fluoroquinolones across the cytoplasmic membrane into the periplasmic space (54).

b) Fluoroquinolone interaction with DNA/DNA Gyrase. Once fluoroquinolones enter the bacterial cell they interact with a DNA/DNA gyrase complex which is thought to inhibit both DNA and RNA polymerase passage along DNA (70). Whether this complex is the lesion responsible for cell death is presently unclear (70,71). In this section, the function of DNA gyrase (topoisomerase II) will be discussed as well as how fluoroquinolones interact with DNA gyrase and the DNA/DNA gyrase complex.

The double-helix structure of DNA encodes genetic information, allows recombination and mutation, and serves as

a template for semiconservative replication and transcription (62,72,73). This configuration of the DNA molecule leads to four major problems (62). One problem arises from the condensed state of DNA within the cell. In E. coli, the chromosome is a circular DNA molecule 1,100 μm long present in a cell only 1 to 2 μm long (72,73). This DNA molecule, despite its 1,000-fold-condensed state, must be able to replicate, segregate into daughter chromosomes, and allow transcription of individual genes without becoming entangled.

The helical nature of the DNA duplex leads to the second problem. With each turn of the helix, which occurs on the average every 10.4 bp, two single strands are wrapped around each other. In the E. coli chromosome, which contains approximately 4 million bp, strands are intertwined about 400,000 times, generating a linking number of 400,000 (Linking number [L] = Twist [T] - Writhe [W]) [73]. A swivel mechanism to permit untwisting of the DNA during strand separation was proposed approximately 30 years ago (62).

For procaryotes, a third problem exists because negative supercoils are present in bacterial DNA (74,75). These negative supercoils result in bacterial DNA that contains slightly less than one helical turn for each 10.4 bp and therefore has a linking number lower than that of eucaryotic DNA. This slightly underwound state of intracellular bacterial DNA is thought to facilitate strand separation required for DNA replication and initiation of transcription. Negative supercoils are energetically unfavorable, and

therefore an energy-consuming process within the bacterial cell is needed for their generation (62).

A fourth problem in DNA topology occurs during transcription of certain genes by RNA polymerase. For constrained segments of DNA, tracking of RNA polymerase along the helical DNA template generates positive DNA supercoils ahead of and negative supercoils behind the enzyme (75). If this supercoiling problem is not somehow resolved, accumulation of supercoils of opposite polarities in these domains would likely limit the efficiency of transcription. It is believed that all four of these topological problems of DNA are resolved by a class of enzymes called topoisomerases.

Several excellent reviews have described how the problems of DNA entanglement, strand unwinding and supercoiling are solved by topoisomerases (62,74-78). Topoisomerases are enzymes that alter the number of times one single strand of a DNA duplex winds around its complementary strand. Said another way, topoisomerases selectively alter the linking number of a double-stranded DNA molecule. DNA molecules that differ only in linking numbers are called topological isomers or topoisomers (62). DNA topoisomerases have been isolated from, viral, procaryotic and eucaryotic sources (78). Topoisomerases are categorized into three groups: (a) type I topoisomerases (include type I and type III in procaryotes); (b) type II topoisomerases (includes DNA gyrase and topoisomerase IV in procaryotes); and (c) special topoisomerases such as enzymes catalysing transposition or

integration into and excision of bacteriophage DNA from the bacterial chromosome (62,74-78). The majority of research into bacterial topoisomerases has been performed with E. coli (74-78). Table 4 describes the topoisomerases studied in E. coli.

Type I topoisomerases (topoisomerase I and III) transiently break one strand of a double helix and pass through another single strand. Topoisomerase I was discovered in E. coli, but is found in both procaryotes and eucaryotes (77). This enzyme catalyzes the removal of negative supercoils from DNA in the absence of ATP (77). Within bacteria, topoisomerase I along with DNA gyrase

Table 4. Topoisomerases Characterized in E. coli

Topoisomerase	Type	Subunit(s)	Gene(s)	Location(min)
I	I	TopA	topA	28
II (DNA gyrase)	II	GyrA	gyrA	48
		GyrB	gyrB	83
III	I	TopB	topB	39
IV	II	ParC	parC	65
		ParE	parE	65

modified from reference 62.

regulates the level of negative supercoiling of intracellular DNA and is required for the transcription of certain operons

(79). Deletion mutants of topA (Table 4) are viable but require compensatory mutations in the genes coding for DNA gyrase (80). A direct role for the enzyme in transcription probably occurs as accumulation of negative supercoils in plasmid pBR322 DNA occurs in topA mutants (62).

Topoisomerase III, also indentified in E. coli is able to remove (relax) negative but not positively supercoiled DNA without a requirement for ATP (75). The exact role of topoisomerase III within the cell is unknown, but it is clear that topB is not an essential gene (62). The finding that topB mutants display an increased frequency of spontaneous DNA deletions has suggested that it may have a role in DNA recombination (81).

Topoisomerase IV enzymes catalyze ATP-dependent relaxation of negatively and positively supercoiled DNAs and unknotting of unnicked duplex DNA. Although the above property is similar to DNA gyrase and hence topoisomerase IV is classified as a type II enzyme, unlike DNA gyrase it has demonstrated no DNA supercoiling activity (62). The exact in vivo role of type IV topoisomerases are not known, however, conditional lethal mutants of parC and parE (loci coding for topoisomerase IV) indicate that these are essential genes (82). The products of parC and parE demonstrate considerable homology to the products of gyrA and gyrB (which code for DNA gyrase), respectively, and complementation studies using gyrA and gyrB restore function in parC and parE mutants (82).

The intracellular target of the fluoroquinolones is

topoisomerase II also known as DNA gyrase (70). DNA gyrase was first discovered in 1976 by Gellert et al. (83), while attempting to establish the Escherichia coli host factors required for bacteriophage site-specific integration. DNA gyrase is encoded by genes previously identified as genetic loci determining resistance to either nalidixic acid or coumermycin (nalA and cou). These loci are now referred to as gyrA and gyrB and are located at 48 minutes and 83 minutes, respectively, on the standard E. coli k-12 chromosomal map (Table 4). DNA gyrase has been cloned in E. coli, K. pneumoniae, S. aureus, Neisseria gonorrrhoeae, Bacillus subtilis, Campylobacter jejuni (78,84-88) [Table 5]. It should be mentioned that to the best of our knowledge neither gyrA nor gyrB in Pseudomonas aeruginosa have been cloned and sequenced, although gyrA has successfully been amplified using consensus primers based on areas of homology between known gyrA sequences (90). In the majority of organisms, gyrA and gyrB are found contiguously within the genome (Table 5). Some exceptions occur, as with E. coli, where the loci are separated by 1.5×10^6 bp (78). The synthesis of gyrase is controlled at the level of DNA supercoiling within the cell (91). Agents that block gyrase activity, and thus decrease the level of intracellular supercoiling, can increase the in vivo rates of synthesis of gyrA and gyrB up to ten-fold.

Based upon sequencing data, gyrA encodes a protein of 875 amino acids (M_r 97,000), while gyrB encodes a protein of 804 amino acids (M_r 90,000) [Table 5]. The molecular mass values

Table 5. DNA Gyrase From Different Organisms

Organism	Gene	Gene length (bp)	Amino Acids in protein	Protein Size (kDa)
E. coli	gyrA	2625	875	97
	gyrB	2412	804	90
K. pneumoniae	gyrA	2628	876	97
	gyrB	---	---	---
S. aureus	gyrA	2667	889	100
	gyrB			
N. gonorrhoeae	gyrA	---	---	---
	gyrB	2313	771	86
B. subtilis	gyrA	2623	771	86
	gyrB	1914	821	92
C. jejuni	gyrA	2589	863	97
	gyrB	---	---	---

modified from reference 78.

are in close agreement with those predicted from SDS-polyacrylamide electrophoresis (78). The holoenzyme is an A_2B_2 complex (M_r 374 kDa). All activities of the enzyme appear to require both subunits, but certain domains mediate differentfunctions. Most research into DNA gyrase has been performed in E. coli. Table 6 describes properties of E. coli DNA gyrase.

GyrA mediates DNA strand breakage and reunion with the tyrosine residue at position 122 (Tyr-122), forming a transient phosphotyrosine linkage with the broken DNA strand. GyrB mediates the ATPase activity of the enzyme. Studies using fragments of GyrA and GyrB have also suggested functional subdomains of these polypeptides. For GyrA, a 59-kDa amino-terminal (N-terminal) tryptic fragment complexed with GyrB was sufficient to support weak DNA supercoiling activity. Addition of the carboxy-terminal (C-terminal) 33-kDa GyrA fragment improved enzyme efficiency and was thought to stabilize the complex (Figure 6) [91].

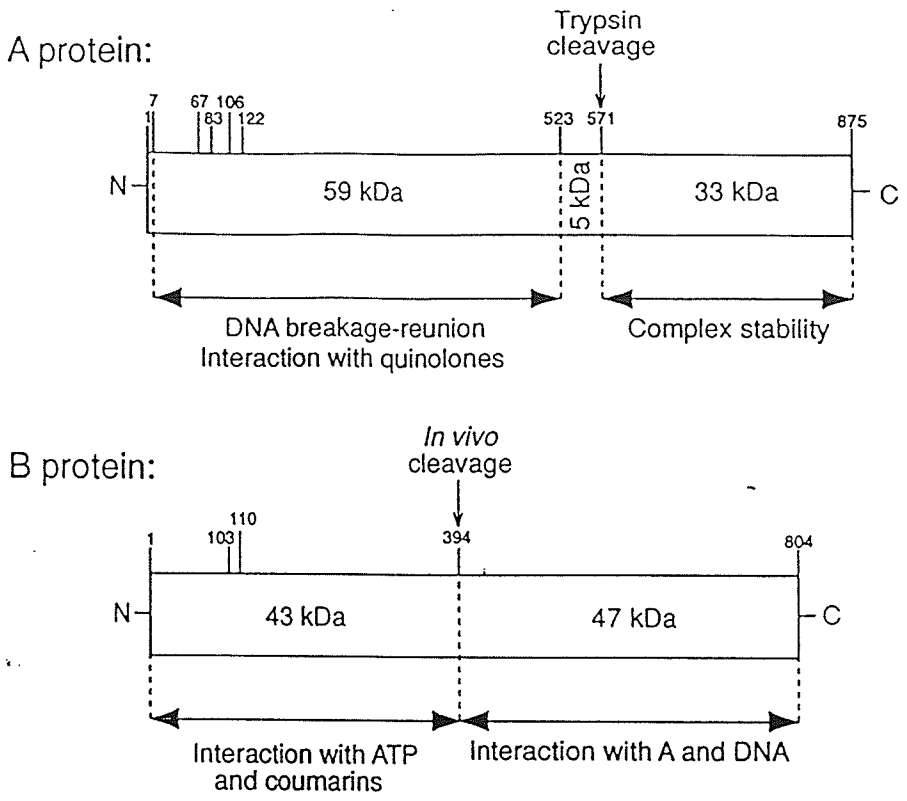
For GyrB, a 47-kDa C-terminal fragment complexed with GyrA supports DNA relaxation but not supercoiling or ATP hydrolysis. The N-terminal domain of GyrB (amino acids 2 through 220) is now know to contain the ATP-binding site, according to X-ray analysis of the crystal structure of an N-terminal fragment of GyrB with an ATP analogue (92). This information is consistent with earlier studies in which lysines at position 103 and 110 were selectively labelled with a reactive ATP analogue (93).

Table 6. Properties of E. coli DNA Gyrase

Characteristic	A Protein	B Protein
Gene	gyrA (2625 bp, formerly nalA)	gyrB (2412 bp, formerly cou)
Mol. wt	96,887 (875 amino acids)	89,893 (804 amino acids)
pI	4.5 - 5.5	~6
Major role	Breakage and reunion of DNA	ATP ase activity
Drug interactions	Target of fluoroquinolones (eg. ciprofloxacin)	Target of coumerins (eg. coumermycin A_1, novobiocin)

modified from reference 78.

Figure 6. Domain Organization of E. coli DNA Gyrase



(adapted from reference 78)

DNA gyrase performs a number of topological interconversions of DNA molecules. Most notable is its capability of relaxing negatively supercoiled DNA in the absence of ATP, as well as catenating and decatenating two duplex DNA circles (94), and resolving a topologically knotted single DNA duplex. DNA gyrase is needed during DNA replication for initiation, fork propagation and termination; for transcription of certain operons; and for aspects of DNA repair, recombination and transposition. Involvement of initiation and fork propagation likely represents facilitation of strand unwinding and the need for negative supertwists for proper binding of proteins to the double helix. The role in

termination likely represents the need for decatenating activity, because partially segregated nucleoids have been shown to accumulate in dividing bacteria in which DNA gyrase has been inhibited (60). The various reactions performed by DNA gyrase are outlined in Table 7 (60,78).

Although DNA gyrase has not been cloned and sequenced in P. aeruginosa, the enzyme has been purified from the PAO strain (95). The holoenzyme is approximately 400 kDa in size and is inhibited by fluoroquinolones. The properties of DNA gyrase from P. aeruginosa are very similar to that of E. coli (96).

DNA gyrase is the primary target of fluoroquinolones. The most compelling evidence in support of this is the existence of single-point mutations in the gyrase genes which confer high levels of resistance to fluoroquinolones (95-95). The supercoiling activity of purified DNA gyrase containing an A subunit isolated from quinolone-resistant gyrA mutants is more resistant to fluoroquinolones than DNA gyrase isolated from isogenic quinolone-susceptible strains (97). Table 7 describes the various DNA gyrase functions that are antagonized by fluoroquinolones.

An important additional effect of quinolones on DNA gyrase is site-specific cleavage of DNA by the enzyme in the presence of detergent (sodium dodecyl sulfate) and a protease (proteinase K) [98]. In this reaction, staggered single-strand breaks are introduced four base-pairs apart on a DNA duplex, and the gyrase A subunit is covalently attached to the 5′ single-stranded ends at the cleavage site. The DNA is

Table 7. Reactions Performed by DNA Gyrase

Reaction	Subunits Req'd	ATP Req'd	Quinolones[a]	Coumarins[a]
Supercoiling	A,B	Yes	Yes	Yes
Relaxation				
-ve supercoils	A,B	No	Yes	No
+ve supercoils	A,B	Yes	Yes	?
Catanation	A,B	Yes	Yes	Yes
Decatanation	A,B	Yes	Yes	?
Unknotting	A,B	Yes	Yes	Yes
DNA cleavage	A,B	No	No	No
ATPase	B	Yes	No	Yes

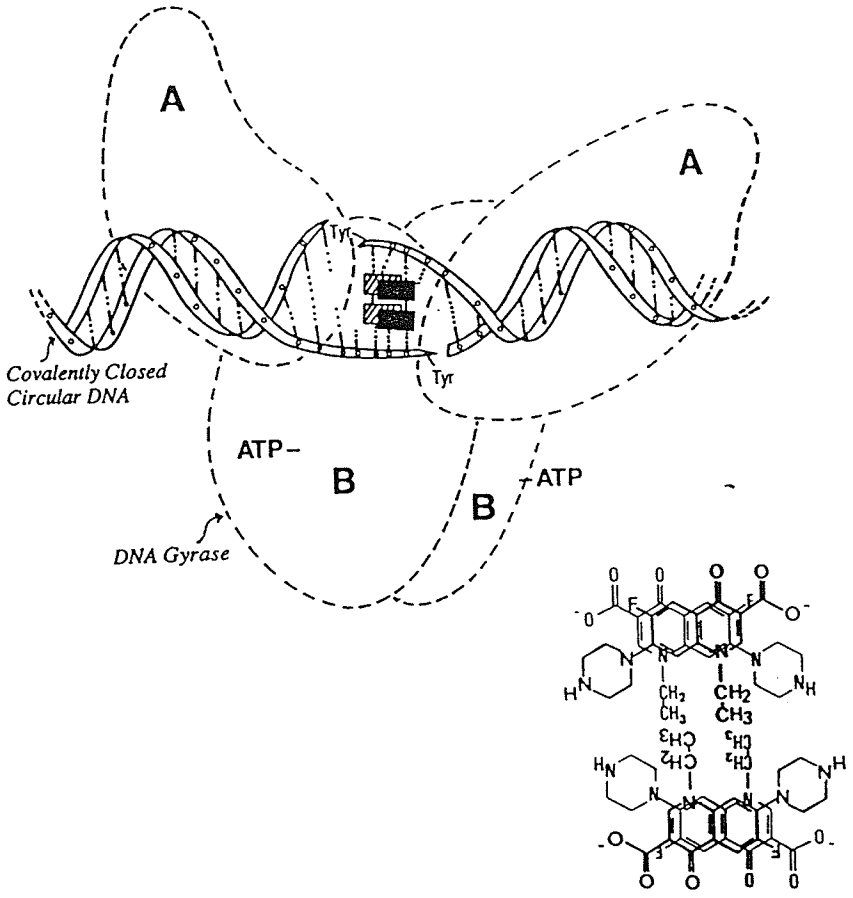
Modified from references 60 and 78.

[a]Inhibited by

covalently linked at tyrosine 122 of the A subunit (99). The cleavage reaction is stoichiometric (noncatalytic) and likely represents blocking of the gyrase-mediated ligation of the DNA strand breaks. Cleavage occurs at DNA sites that broadly define a consensus sequence and are present within approximately 150 base-pair segment of DNA bound by enzyme. Repetitive extragenic palindromic (REP) sequences, which are present between 100 and 200 times in the E. coli chromosome, are potent DNA gyrase binding sites in vitro and may be sites of action for the maintenance of intracellular supertwisting (100).

Recent data have conclusively shown that fluoroquinolones bind to a DNA gyrase/DNA complex (but not to either alone) as the intracellular target of these antibiotics (101). Fluoroquinolone, DNA gyrase/DNA binding studies (102,103) along with x-ray cyrstallography of nalidixic acid (104) suggest a cooperative quinolone-DNA binding model for inhibition of DNA gyrase by quinolones (105). In this model, binding of DNA gyrase to double-stranded DNA in the presence of ATP results in a local region of strand melting, which generates a pocket of single-stranded DNA to which four drug molecules bind cooperatively and interfere with enzymatic activity (Figure 7).

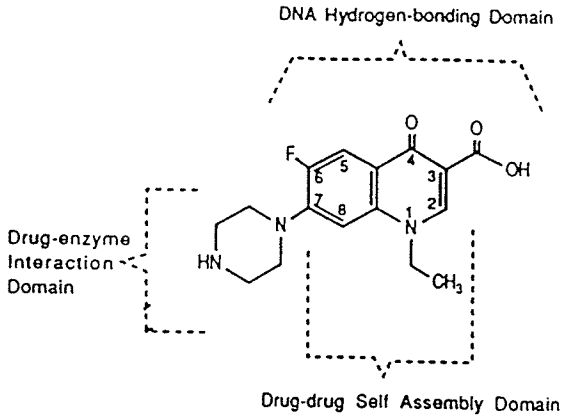
Figure 7. Fluoroquinolone DNA/DNA Gyrase Binding Method



(adapted from reference 105)

This model has been extended to describe the functional domains on the fluoroquinolone molecule (Figure 8) [105]. The proposed model suggests three functional domains of the quinolone molecule: the DNA-binding domain, the drug self-association domain, and the drug-enzyme interaction domain [105].

Figure 8. Functional Domains of Fluoroquinolones



(adapted from reference 105)

Fluoroquinolones demonstrate 100-1000 fold decreased potency against eucaryotic topoisomerase II as compared to procaryotic DNA gyrase (106,107), which correlates well with the fact that they produce little toxicity against mammalian cells. This selective action against procaryotic versus eucaryotic topoisomerases is not due to poor drug penetration, because fluoroquinolones concentrate within mammalian cells (108). Recently developed quinolone derivatives (CP-67,015, CI-934) have demonstrated activity against both procaryotic as well as eucaryotic topoisomerase II indicating the potential among some congeners for decreased selectivity (106).

c) Fluoroquinolone effects on bacteria. Fluoroquinolones inhibit DNA synthesis and ultimately have a bactericidal effect (109,110). The potency in inhibiting DNA synthesis correlates well with antibacterial activity. It is clear,

however, that their ability to inhibit DNA synthesis is not sufficient to explain bacterial killing (70). Although poorly understood, it appears that new RNA and protein synthesis are required for killing, based upon experiments showing that rifampin and chloramphenicol eliminate or diminish bactericidal activity (109).

Fluoroquinolone treatment of bacteria produces many cellular changes secondary to inhibition of DNA gyrase (110). Fluoroquinolones are potent inducers of SOS response (SOS DNA repair system) leading to filamentation (110). They also activate certain heat shock proteins. Fluoroquinolone treatment of E. coli leads to loss of cell membrane integrity, leakage of cytoplasmic contents and ultimately, cell death (111). Finally, exposure of bacterial cells to fluoroquinolones results in single and double-stranded breakage of DNA and partial degradation of the bacterial chromosome. Thus it is clear that fluoroquinolones produce a whole host of cellular changes in bacteria. How these ultimately lead to cell death is unclear.

5. Fluoroquinolone Resistance in E. coli

Most research into the mechanisms of fluoroquinolone resistance has been performed with E. coli, so this organism will be briefly discussed. Plasmid-mediated resistance has, to this date, not been identified for fluoroquinolones. The following discussion is restricted to identified chromosomal mutations.

The frequency of selection of chromosomal mutants in the

laboratory depends on the particular quinolone, the drug concentration used for selection, and the particular bacterium. Selection frequencies differ greatly among quinolones, with nalidixic acid (10^{-8}) selecting resistant mutants 100-1,000 fold more frequently than with ciprofloxacin, norfloxacin or ofloxacin (10^{-9} - 10^{-11}) [112,113]. As the concentration increases above the MIC, the number of resistant mutants identified in the bacterial population decreases (114). Resistant mutants of Pseudomonas aeruginosa are selected much more frequently than E. coli (76). Currently, for any bacterium studied, two mechanisms of resistance to quinolones have been identified: alterations in the target DNA gyrase, and alterations that decrease drug permeability.

a) Alterations in DNA Gyrase. Based on a combination of genetic mapping and DNA sequencing studies, mutations in gyrA (coding for the GyrA protein) lead to fluoroquinolone resistance (115-119). The largest amount of information on changes in protein structure responsible for quinolone resistance has been developed through nucleotide sequencing of resistant gyrA mutants of E. coli. These changes cluster in the amino-terminal portion of the polypeptide near tyrosine 122. This amino acid that is covalently linked to DNA following exposure of gyrase/DNA complexes to fluoroquinolones and protein denaturants, and is presumed to be the active site (Table 8) [115]. Of note, alterations at position 83, are

Table 8. Mutations in E. coli GyrA Associated With Quinolone Resistance

Amino Acid Number	Amino Acid Change	Increase in MIC (fold) Nalidixic Acid	Ciprofloxacin
67	Ala → Ser	8	4
81	Gly → Cys	16	8
	Gly → Asp		
83	Ser → Leu	128	32
	Ser → Trp	128	32
	Ser → Ala	20	10
84	Ala → Pro	8	8
87	Asp → Asp	64	16
	Asp → Val	64	16
106	Gln → His	4	4
	Gln → Arg	2.5	10

Modified from references 60, 62, 115-119.

very common with both laboratory and clinical isolates. In all of these isolates a polar residue (serine) was replaced by a non-polar bulky residue (leucine or tryptophan) [Table 8].

The effects of these amino acid changes on the secondary and tertiary structures of gyrase A protein and the means by which these changes confer resistance are not yet known, although the simplest explanation is that these alterations affect the affinities of quinolones for the DNA gyrase-DNA complex. An alternative possibility, based on the data of Shen and associates suggesting that quinolones bind cooperatively to single-stranded-DNA pockets created by DNA gyrase (103,105, 120), is that the resistant DNA gyrase blocks access of the quinolones to the pocket or produces a pocket in which quinolone binding is decreased without alteration in drug binding to the protein itself.

Rarely, and almost exclusively with nalidixic acid, mutations associated with quinolone resistance have been localized to gyrB (60,62,121). These changes have occurred in residues 426 and 447. Proof of a direct or indirect interaction of quinolones with Gyr B awaits further study.

b) Changes in permeability. Several non-gyrase resistance loci selected with fluoroquinolones have been mapped in E. coli and include nfxB, cfxB, norB and norC (122,123). All of these mutations result in a reduction of porin Omp F with concommitant reduction in fluoroquinolone uptake (122,123). Studies with Omp F mutants demonstrate reduced fluoroquinolone uptake and two-fold increases in MICs (57-59). It is believed

that the resistance does not achieve higher increments due to compensatory increases in Omp C porin (67).

Although reductions in OmpF contribute to resistance of hydrophilic quinolones in E. coli, factors in addition to changes in porin pathways are also involved. The details of the mechanism(s) of quinolone resistance in the nfxB, norB, and cfxB mutants are complex and not fully defined. The level of resistance in these mutants is two- to fourfold greater than that in OmpF mutants suggesting that other factors are contributing. In addition, although nfxB (and cfxB) mutants at steady state accumulate less [^{3}H]norfloxacin than do wild-type cells (123), by a factor similar to the increment in resistance between mutant and wild-type cells, the reduction in the initial rate of drug accumulation is insufficient to account for a reduced steady-state level of drug in mutant cells whose mass doubles every 40 min.

Energy is an additional factor that appears to be necessary for the reduced norfloxacin accumulation found in nfxB, cfxB, as was first recognized for wild-type and OmpF E. coli (67,123). Energy inhibitors such as dinitrophenol, carbonyl cyanide m-chlorophenylhydrazone (CCCP), and azide all produce increases in [^{3}H]norfloxacin accumulation and abolish the differences between mutant and wild-type cells (123). The demonstration of energy-dependent and saturable accumulation of [^{3}H]norfloxacin in everted (inside-out) inner membrane vesicles from wild-type cells suggested that there is at the inner membrane (even in wild-type E. coli) an energy-requiring

transporter mediating norfloxacin efflux (67). For such an efflux mechanism to contribute to resistance, however, the drug must be transported across both the inner and the outer membranes, perhaps at zones of adherence between the two membranes. No mutants that affect the putative efflux carrier have been identified, however, and direct assessment of the role of such a carrier in the resistance phenotype is not yet possible. NorA, a candidate fluoroquinolone efflux protein, has, however, been identified in S. aureus (124,125).

A recently cloned and sequenced locus, marA (multiple-antibiotic-resistance) appears to not only confer resistance to fluoroquinolones but also to multiple other antibiotics (126,127). In Mar mutants, OmpF expression is reduced leading to reduction in fluoroquinolone uptake. However, many questions regarding marA have arisen, including how are Mar mutants created, how is marA regulated and what is the biological function of marA.

Thus, fluoroquinolone resistance in E. coli may occur by either alteration in DNA gyrase or reduced drug permeability. Alterations in DNA gyrase occur almost exclusively in the N-terminal portion of GyrA. Permeability alterations result in reduced uptake and/or increased efflux of fluoroquinolone from the cell.

6. Fluoroquinolone Resistance in P. aeruginosa

Fluoroquinolone resistance in P. aeruginosa has been associated with modification of DNA gyrase and/or alteration in outer-membrane permeability. Both mechanisms will be

discussed.

a) Alterations in DNA Gyrase. To the best of our knowledge the genes coding for DNA gyrase in P. aeruginosa have not been cloned or sequenced. The enzyme, however, has been purified from the PAO strain (95). The holoenzyme is approximately 400kDa and is inhibited by fluoroquinolones. Unlike E. coli, where specific mutations in gyrA or gyrB leading to quinolone resistance can be identified, mutations in P. aeruginosa DNA gyrase causing fluoroquinolone resistance are assessed indirectly. The most common method is by demonstrating reduced fluoroquinolone sensitivity to inhibition of DNA supercoiling by fluoroquinolone-resistant isolates (128-130). The second indirect way is by demonstrating reduced inhibition of DNA synthesis by fluoroquinolone-resistant isolates upon exposure to fluoroquinolones (130-132). More definitive evidence that fluoroquinolone-resistant mutations in DNA gyrase may be due to alterations in gyrA comes from complementation studies expressing wild-type gyrA from E. coli in fluoroquinolone-resistant P. aeruginosa and restoring fluoroquinolone susceptibility (133-134). Several clinical fluoroquinolone resistant isolates have been proven by the above methods to be DNA gyrase mutants (128,130,131,132,135-140). Fluoroquinolone-resistant clinical isolates are cross-resistant to all fluoroquinolones, but remain susceptible to chemically unrelated antibiotics such as beta-lactams and aminoglycosides. Laboratory derived fluoroquinolone resistant isolates have been derived either by selecting spontaneous

mutants through plating on fluoroquinolone containing media (130,133,140-142) or by insertional mutagenesis with fluoroquinolone selection (131). Many of these isolates have been indirectly proven to be DNA gyrase mutants (130,133,140,143). As with the clinical fluoroquinolone resistant isolates, laboratory derived fluoroquinolone resistant isolates which have been proven to be DNA gyrase mutants are resistant to fluoroquinolones only and thus sensitive to chemically unrelated antibiotics.

b) Changes in Permeability

Both clinical and laboratory derived fluoroquinolone resistant isolates may be permeability mutants instead of DNA gyrase mutants. Permeability mutants may be resistant to fluoroquinolones (130,135,136,138,140,143-146) or demonstrate multiple antibiotic resistance including various beta-lactams, chloramphenicol and tetracycline (136,139,144,145). Generally, permeability mutants are susceptible to imipenem and aminoglycosides. Outer-membrane changes that have been associated with fluoroquinolone resistant permeability mutants include a new 54kDa outer-membrane protein (Opr) [142,143], relative reductions in OprF (26), loss of a 40kDa band (131) diminished or absent 31-32kDa band (135), reduced Opr D_1 and Opr H_1 (137), loss of Opr G (25.5kDa) [131], and LPS changes (131,136,142). Permeability mutations leading to a multiple antibiotic resistant (Mar) phenotype demonstrate the following outer membrane changes: alterations in OprF (139,146,147), reduced 22 and 35kDa bands 9136), increase in several bands in

the 38-48kDa region (136,139), reduced Opr D_1 and H_1 (137), and LPS changes (137). Finally, examples of clinical and laboratory derived fluoroquinolone resistance isolates possessing both DNA gyrase changes and altered permeability have been reported (136,139,144,145).

Thus, the available literature suggests that fluoroquinolone resistant P. aeruginosa may contain DNA gyrase and/or permeability changes and are resistant to fluroquinolones but susceptible to chemically unrelated antibiotics. We, therefore, undertook a comprehensive study of P. aeruginosa made resistant to fluoroquinolones by in vitro serial passage on agar containing increasing concentrations of fluoroquinolones. Our experimental hypothesis was that very high levels of fluoroquinolone resistance would be associated with both DNA gyrase and permeability changes. In addition, we believed that these permeability changes would lead to a multiple antibiotic resistant (Mar) phenotype. We compared the characteristics and mechanisms of fluoroquinolone resistance in our laboratory-derived fluoroquinolone-resistant isolates with clinical fluoroquinolone-resistant isolates obtained from patients on fluoroquinolone therapy.

MATERIALS AND METHODS

1. List of Strains and Plasmids

The bacterial strains and plasmids used or constructed in this study are listed in Table 9. Clinical isolates of *P. aeruginosa* resistant to fluoroquinolones (Clin R) were obtained from patients with urinary infection receiving fluoroquinolone therapy (strains 3623 and 4190) (46). In addition, a matched pair (strains 3818 and 3818r respectively) of isolates was obtained from one patient before fluoroquinolone therapy (sensitive to fluoroquinolones) and six days after start of fluoroquinolone therapy (resistant to fluoroquinolones). Laboratory-derived fluoroquinolone mutants (Lab R) were created by serially passaging wild-type fluoroquinolone-sensitive *P. aeruginosa* (3854,4047,4048) on fluoroquinolone containing BHI (brain-heart infusion) agar containing increasing concentrations of fluoroquinolone. Laboratory-derived fluoroquinolone resistant mutants included 3854r, 4047r, and 4048r.

2. Media and Buffers

The various media and buffers are listed in Appendix A (Section H). Specific media buffers or reagents appear in each section.

3. Antibiotics Used

The following antimicrobial agents were obtained from the indicated sources: ciprofloxacin (Miles Laboratory, Rexdale,

Table 9. Bacterial Strains and Plasmids Used in This Study

Strain or plasmid	Genotype or phenotype	Reference or source
E. coli		
S17-1[a]	Pro$^-$ Res$^-$ Mod$^+$ Tp^r Sm^r	Robillard (134)
P. aeruginosa		
3623	FQ resistant	Clinical isolate
4190	FQ resistant	Clinical isolate
3818	FQ sensitive	Clinical isolate
3818r	FQ resistant	Clinical isolate
3854	FQ sensitive	Clinical isolate
3854r	FQ resistant	This laboratory
4047	FQ sensitive	Clinical isolate
4047r	FQ resistant	This laboratory
4048	FQ sensitive	Clinical isolate
4048r	FQ resistant	This laboratory
PA02	ser-3	Holloway collection[b]
PA04701	cfxAZ-ser-3	Robillard (134)
Plasmids		
pLA2917	Cloning vector Km^r Tc^r	Robillard (134)
pNJR3-2	Gyrase A clone (pLA2917)	Robillard (134)
pRW5	OprF Ap^r	Hancock collection[c]

[a] S17-1 is a mobilizing E. coli strain which carries the transfer genes of the broad-host-range incompatibility group P-type plasmid RP4 integrated in its chromosome. This strain can transfer any plasmid containig a P-type Mob site to any gram-negative bacterium.

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

[c] Robert Hancock, Department of Microbiology, University of British Columbia, Vancouver, British Columbia

Ontario, Canada); cefotaxime (Hoechst-Roussel Canada Inc., Montreal, Quebec, Canada); cefoxitin, norfloxacin and imipenem (Merck Sharp and Dohme, Rayway, New Jersey, USA); chloramphenicol (Parke-Davis, Ann Arbor, Michigan); gentamicin (Schering, Pointe-Claire, Quebec, Canada); piperacillin (Cyanamid Canada, Baie d'Urfe, Quebec, Canada); tobramycin (Eli Lilly and Co. Canada, Scarborough, Ontario, Canada). Pefloxacin and [^{14}C]-Pefloxacin (23.6mCi/mmol) was provided as a generous gift from R. Phillips, Rhone-Poulenc Pharma, Montreal, Quebec, Canada.

4. Method of Mutant Selection

To create high-level fluoroquinolone-resistant isolates of P. aeruginosa, wild-type fluoroquinolone-sensitive P. aeruginosa (3854,4047,4048) were serially passaged on fluoroquinolone containing BHI (brain-heart infusion) agar with increasing concentrations of fluoroquinolone. Laboratory-derived fluoroquinolone resistant mutants included 3854r, 4047r and 4048r.

Lab R P. aeruginosa and Clin R P. aeruginosa strains were subsequently assessed for stability of fluoroquinolone resistance by passaging on antimicrobial free BHI agar for 40 passages.

5. Isolation of Genomic DNA

To confirm that wild-type and fluoroquinolone-resistant organisms were isogenic strains (eg. 4047 and 4047r),

genotypic analysis was performed using restriction fragment length polymorphism. This technique involves isolating genomic DNA, performing restriction enzyme digestion, southern blotting and probing with a strain specific probe.

Isolation of chromosomal DNA from P. aeruginosa was performed using the following protocol (148):

1. Harvest bacteria from overnight broth culture (250ml BHI)
2. Resuspend in 20ml 20mM TRIS-HCL (pH 7.5) 100mM NaCl and 1mM EDTA
3. Add 100-150μg lysozyme and incubate at 37°C for 30 min
4. Add 1ml Triton X-100 (20%) and 20μg proteinase K; incubate 37°C for 30 min
5. Add 20g CsCl and 0.8ml EtBr (10mg/ml)
6. Centrifuge to equilibrium in fixed angle rotar (175,000 x g, 18-24 hours)
7. Collect chromosomal band, add sterile water to 5ml
8. Extract with isoamyl alcohol until clear
9. Remove isoamyl alcohol, decant into cortex tubes and precipitate DNA with cold 100% EtOH
10. Recover DNA, spool if possible. Centrifugation may be required to pellet DNA if not readily visible
11. Wash in 70% EtOH x2
12. Air dry
13. Resuspend in 20μl of T10E1 (allow 1-2 hours for DNA to dissolve)

6. Agarose Gel Electrophoresis

Agarose gel electrophoresis was performed according to the following protocol (149).

1. tape both sides of tray
2. position appropriate comb in top of tray
3. add dissolved agarose to tray (heated until dissolved)
   - 1% gel (0.6g agarose in 60ml of 0.5% TBE)
   - add 3μl of Ethidium Bromide
4. allow to harden (approximately 30 minutes)
5. remove tape and comb
6. put in gel box
7. add 2.3L of 0.5% TBE buffer to gel box
8. load DNA samples with volumes appropriate for comb (always put kb ladder)
9. runs anode (-ve) to cathode (+ve) or "run to red"
10. run at 100 volts for 2-3 hours

* All DNA samples were digested to completion with BamH1, Hind III or EcoR1.

7. Southern Blotting

Southern blotting was performed according to the following protocol (150):

1. Expose the gel to UV light for 15 min for >1Kb and above, 5 min for <1Kb. Alternatively soak gel in 2N HCl
2. Soak gel 3x 20 min in 0.5N NaOH/1.5M NaCl; rinse

gel briefly in distilled H_2O

3. Soak gel next in 0.5M TRIS pH 7.5/1.5M NaCl 2x 20 min; rinse gel briefly in distilled H_2O

4. Set up transfer in 10x SSC

   a) Pour pool of SSC into glass dish, place glass plate across dish.

   b) Lay 3MM paper across the plate and into the pool of SSC on each side. Soak with SSC.

   c) Cut 3 pieces of 3MM paper to size of gel, soak with SSC and place on top of plate/3MM paper. Press out air bubbles.

   d) Place treated gel, bottom side up in the centre of the 3MM paper. **NO BUBBLES.**

   e) Cut Genescreen membrane to fit gel, wet with SSC and place on top of gel, smoothing out bubbles.

   f) Surround membrane with even edged pieces of parafilm, draping over soaked 3MM.

   g) Place 3 more cut pieces of 3MM paper on top of membrane, soak with SSC and place large stack of paper towels on top. Compress with large book on top for 16-72 hours.

   h) With membrane still moist from transfer, UV crosslink for 3 min.

   i) Soak in 2x SSC to reduce salt content.

5. Bake membrane at 80°C for 2 hours in vacuum oven.

6. Seal membrane in plastic bag.

0.5N NaOH/1.5M NaCl	0.5M TRIS/1.5M NaCl
10g NaOH	125ml 2M TRIS pH 7.5
43.8g NaCl	43.8g NaCl
QS to 500ml water	QS to 500ml water

8. Restriction Fragment Length Polymorphism (RFLP)

Laboratory isolates 3854 and 3854r, 4047 and 4047r, and 4048 and 4048r, and clinical isolates 3818 and 3818r were verified as isogenic strains using southern hybridization with an epidemiological DNA probe (PAK 1.2Kb Hind III pilin DNA fragment) [Ogle et al. (151) method with modification]. The Hind III pilin DNA fragment was labelled using the random primer labelling technique:

1. Use the T7 Quick Prime Kit (Biobar).
2. Make DNA to concentration of 10ng/μl.
3. Denature 25mg of DNA by heating for 2-3 minutes at 95-100°C.
4. After denaturation perform all reactions on ice.
5. Add the following to a clean sterile microfuge tube
   - denatured DNA 2.5μl
   - reagent mix 10μl
   - [α^{32}P] dCTP (3000 Ci/mmol) 5μl (50μCi)
   - DSW qs 49μl
6. Add 1μl T7 DNA polymerase.
7. Mix and centrifuge briefly (10 seconds).
8. Incubate at 37°C for 5-15 minutes.
9. Add entire contents to hybridization bag.

After the probe was random primer labelled, southern blots were probed using the following protocol:

1. Genescreen® placed in plastic bag.
   Add 10-15ml hybridization buffer.
   Remove all air, seal bag (double bag) and place in shaking waterbath at 40°C for 6 hours.
2. Pour out buffer and replace with fresh buffer.
   Add α^{32}P probe 50μl.
   Remove air, seal and double bag, incubate for 18 hours at 42°C.
3. Carefully remove genescreen and place in plastic container.
   Wash with 2x SSC/0.1% SDS, 1 hour, 50°C.
   Repeat wash.
4. Wrap genescreen in saran wrap and tape to cardboard backing.
   Place genescreen and intensifying screen in autoradiograph cassette.
5. In darkroom, place x-ray film between genescreen and intensifying screen.
   Expose overnight at -80°C.

The hybridization buffer used was 50% (vol/vol) formamide, 1M sodium chloride, 10% dextran sulfate and 1% SDS (151).

9. Gram Staining

Both gram stain and electron microscopy were used to

assess the morphology of wild-type and fluoroquinolone-resistant organisms.

Gram staining was performed according to the following protocol (152):

1. Put a loop of water on a slide along with a small sample of a colony, mix and heat fix.
2. Put slide on staining rack, specimen side up.
3. Flood slide with crystal violet. Leave on for 1 minute.
4. Wash slide with running water.
5. Flood slide with Gram's iodine. Leave 1 minute.
6. Wash slide with running water.
7. Decolorize with acetone until purple stops running from slide (2-3 seconds).
8. Wash slide with running water.
9. Flood slide with diluted carbol fuchsin. Leave 30 seconds.
10. Wash slide with running water.
11. Blot off excess water with a paper towel and allow to dry completely.
12. Examine under the microscope using the 100X objective with oil.

10. Electron Microscopy

Electron microscopy was performed according to the negative staining method (153):

1. Remove an aliquot of cells in suspension at

designated times.

2. Pellet cells by gentle centrifugation (1-5000g for 5 minutes)
3. Draw off and discard the supernatant.
4. Add one drop (25-50μl) of 0.1M Sodium cacodylate buffer pH 7.2 and resuspend the pellet. Depending on the pellet size the resuspension can be in as little as 10μl. Ideally you want an opalescent or even turbid suspension.
5. Mount the cells by floating the suspension on a 200 mesh Hex Formvar coated grid for about 1 minute. Hold the grid in self-closing forceps during this step.
6. Draw off the suspension with a pasteur pipette.
7. Remove the excess suspension by touching the edge of the grid with the freshly torn edge of a piece of filter paper.
8. Stain the preparation by floating one drop (10-15μl) 8.25 Ammonium Molybdate pH 6.0 (0.2μm filtered) on the specimen for about 1 minute.
9. Remove all excess stain by touching the edge of the grid with a freshly torn edge of a piece of filter paper.
10. View in the EM.

11. <u>Adherence Assays</u>

To assess one measure of bacterial virulence (ie.

adherence), two adherence assays were used, the voided uroepithelial cell assay and the Vero cell assay. Both wild-type and fluoroquinolone-resistant isolates were assessed in adherence assays. The voided uroepithelial cell assay was performed as outlined (154):

a) Voided Uroepithelial Cell Assay

1. First morning urine voids were obtained from young, healthy, nonbacteriuric women.
2. Urine was centrifuged at 3,000 rpm for 10 minutes to collect voided uroepithelial cells.
3. Cells were washed with PBS and resuspended in PBS at a concentration approximately 10^5 cells/ml.
4. Bacterial strains grown to logarithmic phase in MHB were resuspended in PBS to a concentration approximately 10^8 cfu/ml.
5. One milliliter (10^8 cfu) of bacteria was added to 1ml (10^5) of uroepithelial cells and incubated on a titer plate shaker at 120 rpm at 37°C for 30 minutes.
6. Following incubation, uroepithelial cells were washed 3 times with 10ml of Hepes-Hanks to remove nonadherent bacteria.
7. Trypan blue was added to the suspension to allow exclusion of nonviable stained cells.
8. The number of bacteria adhering to each of 40 randomly chosen uroepithelial cells was determined by light microscopy.

Bacterial adherence using Vero cell technique was performed as follows (155):

b) Vero Cell Assay

1. Vero cells were obtained from ATCC (Rockville, MD, USA).
2. The cell line was maintained in Corning tissue culture flasks with minimal essential medium supplemented with 10% fetal calf serum.
3. Cell cultures were kept at 37°C in a humidified atmosphere containing 5% CO_2 and subcultured every 4-5 days.
4. Vero cell monolayers were prepared in multiwell dishes (24 wells/plate) with $5x10^5$ cells used as an inoculum for each monolayer.
5. Monolayers were confluent after 20 hours.
6. Confluent monolayers were washed with Hepes Hanks (NaCl 8.0g/L, KCl 0.4g/L, $CaCl_2$ 0.14g/L, $MgSO_4$ $7H_2O$ 0.2g/L, Na_2PO_4 0.05g/L, KH_2PO_4 0.06g/L, $NaHCO_3$ 0.35g/L, phenol red 0.01g/L and Hepes 2.6g/L) adjusted to pH 7.4 with 1N NaOH before use.
7. Bacterial suspensions (5ml) were grown statically for 18 hours at 37°C with 10μCi of [^{3}H]-leucine.
8. After incubation, cells were centrifuged at 3,000 rpm x 10 minutes, washed x3 with PBS and adjusted to a concentration of 10^8 cfu/ml.
9. The adherence assay was performed as described by Vosbeck et al. (156).

10. Bacterial suspensions were added to monolayers and incubated for 30 minutes.
11. After incubation aliquots of the solubilized mixture were passed through a filtration manifold (membrane pore size 0.45) and radioactivity counted in a liquid scintillation counter.
12. Results were calculated as a percentage of bacterial radioactivity bound to monolayers and normalized to a bacterial concentration of 10^8/ml.
13. All assays were performed in triplicate.

12. Antibiotic Susceptibility Testing

MIC's were determined by the macrodilution broth method using doubling dilutions prepared in 1ml of Mueller-Hinton broth [5]. Cation (25mg of $CaCl_2$ per liter, 12.5mg of $MgSO_4$ liter)-supplemented Mueller-Hinton broth (pH 7.2-7.4; Difco Laboratories, Detroit, Mich., USA) was used for MIC determinations. The inoculum was adjusted from an exponential-phase culture to yield an initial concentration of approximately $5x10^5$ colony-forming units (CFU ml). After 16-20 h of incubation at 35°C, the MIC was determined as the lowest concentration that inhibited growth. MIC's were performed in duplicate on separate occasions.

13. Fluoroquinolone Uptake

A modified method of Chamberlain et al. (131) was employed for the determination of fluoroquinolone accumulation

in P. aeruginosa. This method results in exponential phase cultures containing approximately 10^8 cfu/ml.

1. 1-2 colonies from a 24 hour old blood agar plate are used to inoculate 50ml Iso-Sensitest broth and incubate overnight for 16-18 hours at 37°C.
2. 0.5ml of the overnight culture was transferred to 50ml fresh Iso-Sensitest broth and allowed to incubate at 37°C for 2.5 hours in a shaking water bath.
3. A 10ml aliquot was centrifuged at 4000 x g for 10 minutes, washed with PBS and the resulting pellet resuspended in 10ml fresh Iso-Sensitest broth.
4. The culture was allowed to equilibrate for 10 minutes at 37°C.
5. A 50µl aliquot was removed for determination of the viable cell count and an additional 50µl removed for determination of total cell protein.
6. The reaction was initiated by addition of ^{14}C-fluoroquinolone.
7. At timed intervals, a 0.5ml aliquot was removed and immediately diluted in 10ml PBS at 7°C.
8. The sample was filtered through a 0.45µm pore, 25mm diameter nylon membrane filter (Micron Separations Inc., Westbora, MA) using a vacuum filtration manifold (Millipore Corp, Bedford, MA). Just prior to filtration, filters were presoaked in PBS.
9. Filters were washed with 20ml PBS, removed from the

manifold, dried at 60°C for 1 hour and placed in 10ml Cytoscint scintillation cocktail (ICN, Cost Mesa, CA) for counting in a LKB Rackbeta 1217 counter.

10. Cell associated radioactivity was determined after correction for non-specific binding of the radiolabel to filters in the absence of bacterial cells.

11. Total cell protein was determined by the method of Lowry et al. (158).

For fluoroquinolone uptake studies, carbonyl cyanide m-chlorophenylhydrazone (CCCP) was obtained from Sigma Chemical Co., St. Louis, Missouri. CCCP was added at a concentration of 50μm 10 minutes after addition of radiolabelled antibiotic (159). The concentration of CCCP was determined not to be bactericidal over the course of the assay.

14. Lipopolysaccharide Analysis

Since fluoroquinolone uptake is affected by the outer-membrane, outer-membrane components (lipopolysaccharide and outer-membrane proteins) were analyzed from both wild-type and fluoroquinolone-resistant isolates.

a) Isolation. Lipopolysaccharide (LPS) was isolated by the method of Darveau and Hancock (160).

1. Grow 1 litre of bacterial cell in an appropriate media at 37°C until an O.D. (600 nm) of 0.6-0.8.

2. Harvest cells at 7,000 rpm for 15 minutes.

Lyophilize. (Note: 1 litre of wet bacterial cells equivalent to 500mg of dried bacterial cells.)

3. Resuspend 500mg dried bacterial cells in 15ml of 10 mM Tris-HCl, pH 8.0, 2mM $MgCl_2$.
4. Add DNase (100μg/ml) and RNase (25μg/ml).
5. French press the cell suspension twice at 15,000 psi.
6. Sonicate for two 30s bursts at a probe intensity of 75 (Vibracell® , Sonics and Materials Inc., Danbury, CT).
7. Add DNase and RNase to final concentrations of 200μg/ml and 50μg/ml respectively.
8. Incubate the suspension at 37°C for 1 hour.
9. Add 5ml of 0.5M EDTA (ED_4SS)/10 mM Tris pH 8.0; 2.5ml of 20% SDS/10 mM Tris, pH 8.0 and 2.5ml of 10 mM Tris-HCl, pH 8.0 to give a final volume of 25ml, final pH approximately 9.5.
10. Vortex and centrifuge at 50,000 g for 30 minutes at 20°C to (25.5 K RPM on 70 Ti) remove peptidoglycan
11. Save supernatant. Add pronase to give a final concentration of 200μg/ml.
12. Incubate at 37°C with constant shaking overnight.
13. Add two volumes of 0.375 M $MgCl_2$/95% EtOH. Mix and cool to 0°C in -20°C refrigerator.
14. After the sample has cooled to 0°C, centrifuge at 12,000 g for 15 (13 K RPM on 70 Ti) minutes at 0-4°C.

15. Resuspend pellet in 25ml of 0.1 M EDTA (ED_4SS), 2% SDS, 10 mM Tris-HCl, pH 8.0.
16. Sonicate at a probe intensity of 75 for two 30 s bursts.
17. Incubate the solution at 85°C for 30 minutes. Cool to room temperature. Bring pH to 9.5 by addition of 4N NaOH.
18. Add pronase to give a final concentration of 25μg/ml. Incubate at 37°C overnight with constant shaking.
19. Add two volumes of 0.375 M $MgCl_2$/95% EtOH and cool solution to 0°C as before.
20. Centrifuge at 12,000 g for 15 minutes at 0-4%. (13K RPM on 70 Ti).
21. Resuspend pellet in 15ml of 10 mM Tris-HCl, pH 8.0. Sonicate at a probe intensity of 75 for two 30 s bursts.
22. Centrifuge at 1000 rpm for 5 minutes to remove insoluble Mg/EDTA complexes.
23. Add $MgCl_2$ to give a final concentration of 25 mM (35.7 mg $MgCl_2$ into 15ml). Centrifuge at 200,000 g for two hours. (51K RPM on 70 Ti).
24. Resuspend pellet in distilled water.
25. Store preparations at -70°C.

After isolation LPS was run on SDS-PAGE.

b) Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS PAGE). Polyacrylamide gels were used to separate LPS

according to molecular weight. The discontinuous buffer system of Laemli (161) was used with all reagents being of electrophoresis purity grade (Bio-Rad). Gel concentrations ranged from 10-15% using the Mini Protean II (Bio-Rad) with gel dimensions of 8cm x 10cm x 0.75mm. The gels were run in 0.025 M TRIS/0.192 glycine M/0.1% SDS at 150 volts until the tracking dye reached the bottom of the gel. The gels were prepared as listed in following Table 10.

After running LPS gels they were silver stained as follows (162):

c) Silver Staining of Polyacrylamide Gels.

1. Wear gloves.
2. Following a PAGE run, the gel should be rinsed X3 with H_2O.
3. Immerse gel in 100ml of 50%/10%/40%-methanol/acetic acid/H_2O for 15 minutes with gentle shaking.
4. Pour off and add 100ml of 5%/7%/88%-methanol/acetic acid/H_2O for 15 minutes with shaking.
5. Pour off and add 100ml of 10% glutaraldehyde for 15 minutes with shaking.
6. Pour off and rinse gel X3 with H_2O.
7. Wash gel for 15 minutes with H_2O or let sit in H_2O overnight.
8. Pour off H_2O and add 100ml of dithiothreitol solution (5μg/ml in H_2O) for 15 minutes with shaking.

Table 10. Preparation of SDS-PAGE Gels

Small Gel (0.75mm)

Separating Gel

		18%	15%	12%	10%	7.5%
	DH_2O	0.9ml	1.9ml	2.9ml	3.6ml	4.4ml
(B)	1.5 M Tris	2.5ml	2.5ml	2.5ml	2.5ml	2.5ml
(D)	10% SDS	0.1ml	0.1ml	0.1ml	0.1ml	0.1ml
(A)	Acrylamide	6.0ml	5.0ml	4.0ml	3.33ml	2.5ml
	Temed	0.005ml	0.005ml	0.005ml	0.005ml	0.005ml
	1% Persulfate	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml

* 1% Persulfate

- 15mg in 1.5ml DH_2O
- 20mg in 2.0ml DH_2O

Stacking Gel

	DH_2O	3.05ml
(C)	0.5 M Tris	1.25ml
(D)	SDS	0.05ml
(A)	Acrylamide	0.65ml
	Temed	0.005ml
	1% Persulfate	0.25ml

9. Pour off and add 100ml of silver nitrate solution(1mg/ml in H_2O) for 15 minutes with shaking.
10. Pour off and rinse gel X1 with H_2O then wash in H_2O for 15 minutes X2 (to remove unbound silver nitrate).
11. Pour off, add 50ml of developer (250ml of 3% sodium carbonate [7.5g], 125μl of 37% formaldehyde in H_2O) to rinse gel.
12. Pour off, repeat.
13. Pour off, add 100ml of developer and develop with shaking until the desired level of staining is attained.
14. Pour off developer and rinse gel X3 with H_2O.
15. Wash gel in H_2O for 15 minutes with shaking.
16. Gel is either photographed or dried for permanent record.

50/10/40		5/7/88	
methanol	250ml	methanol	50ml
acetic acid	50ml	acetic acid	35ml
H_2O	200ml	H_2O	415ml
	500ml		500ml

15. Outer-membrane Protein (Opr) Analysis

Outer-membrane proteins of P. aeruginosa were isolated by the method of Hancock and Nikaido (163).

a) Opr isolation.

1. From fresh plate, grow 2-3 colonies in 20ml of TSB

at 37°C overnight.

2. Dilute in 2L of TSB and grow overnight with vigorous shaking.
3. Centrifuge at 5,000 rpm x 20 minutes.
4. Wash pellet with 40ml of 30 mM Tris, ph 8.0.
5. Centrifuge at 10,000 rpm x 15 minutes (can freeze pellet at this stage for several days if desired).
6. Suspend in 20ml 20% sucrose in 50 mM Tris-0.2 mM DTT pH 7.9.
7. Pass through french press x 2.
8. Add 2ml lysozyme (1mg/ml) and leave in cold room on ice 10 minutes.
9. Add 1μl/ml of PMSF (phenylmethyl sulfonyl fluoride) 100 mM.
10. Sonicate x 3 for 20 seconds at power 7.
11. Centrifuge at 15,000 rpm for 30 minutes - take supernatant and discard pellet.
12. Centrifuge supernatant at 33,000 rpm (70 Ti) for 1 hour (discard supernatant).
13. Suspend pellet in 2ml Tris DTT, mix with glass rod.
14. Add 2ml of 2% sarcosyl and let sit at room temperature for 30 minutes - mix with glass rod.
15. Centrifuge at 33,000 rpm (70 Ti) x 1 hour.
16. Resuspend pellet in 1ml Tris DTT - mix with glass rod.
17. Freeze at -70°C.

b) Protein Determination (Opr). Outer-membrane protein determination was performed based on the assay designed by Bradford (164) [termed the Biorad method]. The unknown would be diluted to give a number of samples with a protein content between 1 and 25μg/ml. 800μl of this would be added to 200μl of Dye Reagent Concentrate (Bio-Rad), vortexed and allowed to stand at least 5 minutes. The OD (595nm) would be used against a reagent blank with no protein. A standard curve would be produced in the same way using bovine serum albumin.

c) SDS-PAGE of Opr. Outer membrane proteins (50μg of protein) were suspended in electrophoretic buffer (2% SDS, 10% glycerol, 0.5 M Tris-hydrochloride pH 6.8, 0.002% bromophenol blue with 5% 2-mercaptoethanol). Samples were heated to 100°C for 4 minutes and electrophoresed in 15% discontinuous sodium dodecyl sulfate-polyacrylamide gels by the method of Laemli and Favre (161). Running time was approximately 1 hour at 150 volts (time for tracking dye to reach the bottom). Estimation of molecular weights was done using the molecular weight standards lysozyme (14,400), soybean trypsin inhibitor (21,500), carbonic anhydrase (31,000), ovalbumin (45,000), bovine serum albumin (66,200), and phosphorylase B (92,500) supplied by BioRad. After running, the gels were either stained with Coomasie blue to facilitate visualization or electroblotted (see below) to transfer the protein to a nitrocellulose membrane.

d) Coomasie Blue Protein Staining.

1. After PAGE run, the gel should be rinsed X3 with water.
2. Add 100ml of Coomasie stain to gel and shake for 30 minutes.
3. Pour off stain back into main Coomasie blue bottle with filtration.
4. Add 100ml of 7% acetic acid to gel and shake for 1 hour.
5. Pour off, add 100ml of 7% acetic acid to gel and shake overnight.

Coomassie Brilliant Blue (0.05%) in 25% 2-Propanol/10% acetic acid.

CBB R250	0.2g
2-propanol	100ml
Glacial acetic acid	40ml
DSW	260ml

16. Western Blotting

This procedure involves the transfer of proteins separated by SDS-PAGE from the gel matrix onto a nitrocellulose membrane (NCM) allowing them to be reacted with monoclonal antibodies (MAbs). A variation of the original method of Towbin et al. (165) was used in our laboratory as follows:

Biorad SDS minigel apparatus and Biorad minigel blotting apparatus were used.

1. Two identical protein gels are run, one is stained and the other used for blotting. Lane 1 is the prestained low MW standard (dilute 1-2 with DSW and load 10μl).

2. Electrophoretic blot transfer:

   a) Prepare 1X blotting buffer:

25 mM Tris	12.11 grams
192 mM glycine	57.6 grams
20% v/v methanol	800 ml

   Make to 3 litres with distilled water. Store at 4°C. If the buffer is made fresh, cool approximately 400ml at 4°C.

   b) Cut nitrocellulose paper (S&S NC pure nitrocellulose nucleic acid and protein transfer membrane - 0.45μm pore size, from Canlab) to the size of the gel. Label with a soft pencil to orient the membrane. Soak nitrocellulose paper in blotting buffer for approximately 20 minutes. All air bubbles must be removed from underneath the paper.

   c) Cut Whatman filter (3mm) paper into 4" by 3" (you will need 4/gel). (Filter paper should be slightly larger than the gel). Soak all four pieces, along with "scotch brite" pads (2/gel) in blotting buffer.

   d) Once the gel has finished running, remove and soak 5 minutes in blotting buffer for 5

minutes (this should remove most of the SDS). Fill blotting chamber to just above grids with blotting buffer.

e) To assemble the apparatus:

On Black side (cathode):

scrotch brite pad

2 filter papers

gel (face side down)

nitrocellulose paper (remove bubbles)

2 filter papers

scotch brite pad

Bring the clear side over and close the sandwich.

f) Place vertically between parallel electrodes in the transfer-blot cell, containing cold blotting buffer. The nitrocellulose is always placed towards the positive electrode while the gel is towards the negative electrode. The grey panel of the holder must be facing the grey electrode panel.

g) Place the apparatus on a magnetic stirrer, turn power to 30 V (approximately 0.25 amps). Run overnight at room temperature.

h) Blot is then removed and can either be used or stored in plastic wrap and placed in -20°C.

3. Primary antibody: MA 4-10 (G-10)-5μl

Secondary antibody: goat antimouse IgG HRP-2μl

a) Block the nitrocellulose membrane with 10ml of blocking buffer (PBS 40ml with 1% skim milk) for 1 hour on a shaker at room temperature.

b) Pour off blocking buffer and add 10ml (wash buffer -PBS with 0.05% Tween 20) with 1% skim milk containing primary monoclonal antibody (5μl = 1:2000 dilution). Incubate on shaker overnight at room temperature or 2 hours at 37°C.

c) Wash with wash buffer (add 10ml and leave on shaker for 10 minutes at room temperature) pour off and add fresh wash buffer and repeat four times.

d) Add 10ml of secondary antibody diluted (1:5000 - 2μl in 10ml) in PBS with 1% skim milk. Incubate on shaker for 2 hours at 37°C.

e) Wash membrane 3X with wash buffer for 5 minutes each.

f) Stain blot with (mix just prior to use)

- 25ml PBS
- 10μl H_2O_2 3%
- 500μl Cobalt Cl 1% in H_2O
- 12.5mg of DAB (diaminobenzidine) [or 1ml of 25X conc].

Colour develops quickly, rinse with DSW to terminate reaction. Blot with paper towel.

17. Complementation With E. coli gyrA

To assess whether fluoroquinolone resistance was partially or wholly due to mutations in gyrA, we used a complementation strategy employing a vector that had gyrA cloned into it (pNJR3-2). Plasmids, pLA2917 or pNJR3-2 from E. coli S17-1 were introduced into P. aeruginosa by conjugation by the method of Robillard (134). E. coli S17-1 contains chromosomal tra genes that are capable of mobilizing pLA2917 (cloning vector) or pNJR3-2 (cloning vector with E. coli gyrA). For matings, E. coli S17-1 donor strains were grown in ML broth overnight at 32°C with aeration. Donor strains were grown in 5μg/ml of tetracycline. Donor culture (0.5ml) was added to 0.5ml of recipient culture in a microcentrifuge tube and centrifuged. The pellet (mating mixture) was suspended in 50μl of 0.15M NaCl and plated onto ML agar. During 5 hours of incubation at 35°C, mating and expression occurred. Three millilitres of 0.15M NaCl was added to the agar plate and the mating mixture was harvested and plated onto ML agar containing 200μg/ml of tetracycline. For laboratory-derived fluoroquinolone-resistant P. aeruginosa (3854r, 4047r and 4048r) recipients, 1024μg/ml of tetracycline was added to Pseudomonas isolation agar (Difco). P. aeruginosa recipient strains that inherited pLA2917 or pNJR3-2 grew in the presence of tetracycline while plasmid-containing E. coli donor strains could not. Transconjugants were purified on selection media. The MIC's of several fluoroquinolones were determined for each strain in the

presence or absence of each plasmid. The MIC's for strains containing plasmid were performed (in duplicate) in the presence of 200μg/ml of tetracycline.

18. Complementation With OprF Expression Vector

To assess the role of OprF in the structural morphology of P. aeruginosa and its potential role as a porin responsible for the uptake of antibiotics including fluoroquinolones, we employed an expression vector pRW5 (166). This construct expresses functional OprF in P. aeruginosa to wild-type levels (166). E. coli DH5α containing pRW5 was maintained on ampicillin (50-75μg/ml) LB agar. pRW5 was isolated according to the following protocol (167):

a) Large Scale Preparation of Plasmid DNA

Plasmid amplification:

1. Incubate bacteria harboring plasmid for 19-24 hours at 37°C in 5ml LB broth containing appropriate antibiotic (100mg/L amp).
2. Inoculate 500ml LB broth (100mg/L amp) with 1ml of overnight culture and incubate at 37°C with vigorous shaking to an OD (600nm) of 0.6-0.8.
3. Add chloramphenicol to make a final conc. of 25μg/ml; incubate overnight.

Harvesting and lysis of bacteria (lysis by alkali):

Prepare the following solutions prior to DNA isolation:

Solution I

50 mM glucose	2.5ml 2M glucose
25 mM Tris-HCI (pH 8.0)	2.5ml 1M Tris
10 mM EDTA	2.0ml 0.5M EDTA
	93ml H_2O

When required, add lysozyme (5mg/ml) to an aliquot

Solution II

(100ml)

0.2N NaOH	0.8g NaOH
1% SDS	5ml 20% SDS

Prepare fresh just prior to DNA prep

Solution III

60ml KOAc

11.5ml Glacial acetic acid

28.5ml H_2O

Store on ice just prior to use. Final solution is pH 4.8, 3M K+ and 5M Acetate.

1. Divide 500ml broth into two aliquots and spin 20 minutes at 5K (Beckman J2-HS rotor).
2. Resuspend pellet from each aliquot in 5ml solution I containing lysozyme (5mg/ml).
3. Transfer to polyallomer tubes and let stand at room temperature for 5 min.
4. Add 10ml solution II to each tube, shake and store on ice for 10 minutes.
5. Add 7.5ml solution III to each tube, shake and store on ice for another 10 minutes.

6. Spin 30 minutes, 15K, 4°C (JA-20 rotor).
7. Transfer supernatant from each tube to another polyallomer tube.
8. Add 0.6 vol isopropyl alcohol and let stand 15 minutes at room temperature. A precipitate should be visible.
9. Centrifuge 30 minutes, 15.5K, room temperature.
10. Resuspend pellet from each tube in 10ml T10E1. Combine to make 20ml.

Isolation of Plasmid DNA (CsCl/EtBr gradients)

1. To 20ml suspension add:

   21.0g CsCl

   1250μl EtBr

   625μl Sarkosyl (10% solution)

2. Seal in 30ml ultracentrifuge tube and spin 18-24 hours, 48K, room temperature (Ti70 rotor).
3. Extract plasmid band (bottom) and bring volume up to 5ml with H_2O.
4. Add V/V isoamyl alcohol, remove organic layer until both phases clear, repeat.
5. Precipitate plasmid DNA with 2 vol 100% EtOH and store at -20°C 30 minutes.
6. Recover DNA by centrifugation at 8K, 30 minutes, 4°C.
7. Resuspend pellet in 5ml H_2O, 1/10 vol NaOAc (3M), and 2 vol EtOH.
8. Incubate overnight -20°C, in AM centrifuge at 8000

rpm x 15 minutes, air dry, dissolve in 1ml T10E1.

After isolation of PRW5, this expression vector was transformed into E. coli C441. E. coli C441 was chosen as this strain would subsequently mobilize pRW5 into recipient P. aeruginosa using conjugation. Transformation of E. coli C441 with pRW5 was achieved as follows (168):

b) $CaCl_2$ Transformation of E. coli

1. Grow cells overnight in 5ml LB broth (10g tryptone, 5g yeast extract, 5g NaCl per litre).
2. Inoculate into fresh LB broth (50ml will yield 1ml of competent cells; 0.1ml/transformation) at a 1:200 dilution (OD 550 should be 0.05 or less). Grow at 37°C with vigorous shaking in a large flask to facilitate aeration.
3. When cells reach an OD 550 of 0.2 to 0.4 put them on ice for 10 minutes.
4. Centrifuge the cells at 10,000 rpm at 4°C for 5 minutes in a cold, sterile tube in the SS34 rotor. Discard the supernatant and resuspend the cells in half volume of sterile, ice-cold (4°C) 0.1 M $CaCl_2$. Leave the cells on ice for 20 to 40 minutes.
5. Centrifuge the cells as above. Gently resuspend the cells in 1/50 of the original culture volume in cold, sterile 0.1 M $CaCl_2$. Leave the cells on ice for 1 to 40 hours (24 hours is optimal for many common strains, but will kill sick ones). The cells are now competent for transformation.

6. Aliquot 0.1ml of cells to a prechilled Eppendorf tube and add the DNA in 10μl or less volume. Mix the tube gently before aliquoting the cells as they tend to settle at the bottom of the tube. Leave the cells on ice for 20-40 minutes to allow the DNA to adhere to the cells.
7. Heat shock the cells in a 42°C water bath for 45 to 60 seconds and return to ice for 1 minute.
8. Add 1.2ml of LB broth to the tube, mix and place the Eppendorf tube in a small glass test tube and secure with parafilm. Put the tube on a tube roller at 37°C for 1 hour.
9. Plate 0.1ml of the transformed cells on selective plates (LB agar with ampicillin 50-70μg/ml). Centrifuge the remaining cells in a microfuge for 20 seconds and pour off all media except the last drop (i.e. do not shake out the tube). Resuspend the pellet in the last drop and plate on a selective plate. If many transformants are anticipated, plate a 1/10 dilution of the original tube. Incubate the cells overnight at 37°C, and screen.

After transformation of pRW5 into E. coli C441 using appropriate selection, E. coli C441 containing pRW5 was mated with fluoroquinolone-sensitive (3854, 4047, 4048) and fluoroquinolone-resistant (3854r, 4047r, 4048r) P. aeruginosa. The following method was used (169):

c) Biparental mating.

1. Overnight cultures of the following strains using approprite antibiotics were made:
   a) E. coli C441 containing helper plasmid and pRW5, 30°C
   b) P. aeruginosa recipient strain, 42°C.

   (Ensure 42°C water bath, as this temperature is required to shut off RE modifying mechanism)
2. Place 0.1ml of B in 2ml of LB and add 0.1ml of A.
3. Filter the mixture using a 0.45μm Nalgene filter unit. Remove the filter with sterile forceps and place filter side up on an LB agar. Incubate overnight at 30°C.
4. Resuspend the bacteria in 2ml of sterile saline (.9% NaCl). Make a 1:100 dilution of this suspension in saline. Spread 100μl of the undiluted & diluted samples on selection plates (Pseudomonas isolation agar with carbenicillin 500μg/ml). Incubate for 1-2 days at 37°C.
5. Restreak colonies on selection plates to ensure purity and presence of the antibiotic marker.

19. Amplification of OprF Using PCR

To identify the mutational site(s) in OprF leading to reduced OprF expression, OprF was amplified using polymerase chain reaction (PCR) and sequenced (170).

Primers. Oligonucleotide primers were synthesized and gel purified by Physiology DNA Synthesis Laboratory, University of Manitoba. One 20-base (primer 1) and one 21-base (primer 2) oligonucleotide (see below), based on sequences initially reported were used as primers for the initial PCR amplification.

Primer 1

antisense 5′ GCG ACC GAA ACA TAG TGG GG 3′

Primer 2

sense 5′ CCC TGA GCG CCT GAC GAG CGG 3′

Additional primers, primers 3-5 (antisense) and primers 6-8 (sense) were used for DNA sequencing.

Primer 3

antisense 5′ CGA TCG GCT ACT TCC TGA CCG 3′

Primer 4

antisense 5′ GCG TGA CAA CGG TCA CCA GGG 3′

Primer 5

antisense 5′ CCA CCG TTG AAG GTC ATA CCG 3′

Primer 6

sense 5′ GTT GTA AGC GTC GGT ACC GAC 3′

Primer 7

sense 5′ GGC GTC AAC GGT GAC GTT GGC 3′

Primer 8

sense 5′ CTG ACG GCC TTG GCT GTC GCT 3′

PRC Amplification. Standard precautions were taken to avoid contamination of specimens and reaction mixtures. PCR was done in a total volume of 100μl. The final mixture

contained primer (100 pmoles of each), 0.2mM dNTPs, PCR buffer (10mM TRIS-HCl, pH 8.3, 50mM KCl, 15mM $MgCl_2$, 0.01% gelatin, and 2.5 units of Taq polymerase (Perkin-Elmer, Roche). The PCR reaction contained 0.3μg of DNA. Samples were heated at 94°C for 2 minutes, then subjected to 30 amplification cycles. A typical cycle included denaturation (94°C for 30 seconds), annealing (64°C for 30 seconds), and extension (72°C for 1 minute) in a thermal cycler (Gene Amp PCR System 9600 - Perkin Elmer, Norwalk, CT). After the last cycle, all reaction mixtures were incubated for a further 10 minutes at 72°C to ensure the extension step was complete.

20. Electroelution of Amplification Products

After the PCR run, the products were purified by the following protocol (171):

Purification of PCR products

1. After PCR run put sample in 1.5ml sterile microfuge tube.
2. Add 100μl phenol/chloroform (bottom layer)
3. Shake x 10 seconds.
4. Centrifuge x 10 seconds (in microfuge).
5. Remove top layer (aqueous layer with DNA) put into fresh 0.5ml sterile microfuge tube.
6. Add 100μl $CHCl_3$.
7. Shake x 10 seconds.
8. Centrifuge x 10 seconds (in microfuge).
9. Remove top layer put into sterile 0.5ml microfuge

tube (~100μl).

10. Add 10μl of NaOAC 3M to 100μl sample.
11. Add 250μl cold 100% ethanol.
12. Leave overnight in -20°C freezer.
13. Centrifuge for 15 minutes after overnight.
14. Remove supernatant carefully and discard.
15. Dry DNA in bottom of microfuge tube by putting in hood for 15 minutes.
16. Resuspend in 50μl of T10E1.
17. Run 2-5μl of sample on 1% agarose gel to quantify DNA.

The predicted 1257-bp product was visualized by ethidium bromide staining of 1% agarose gels after electrophoresis. The appropriate DNA band was excised and electroeluted as described:

Electroelution of DNA

1. Under UV slice appropriate band out of agarose gel and place in sterile microfuge (1.5ml) tube.
2. Add 1L of 0.5x TBE to electroeluter.
3. Fill collection tubes with 100μl 3M NaOAc-bromophenol blue.
4. Put gel slices into well.
5. Run at 90 volts for approximately 30 minutes (assess when all DNA gone with handheld UV lamp).
6. Recover DNA in NaOAc-bromophenol blue (in 0.5ml microfuge tube) and add 2 volumes of ice cold 100% ethanol.

7. Leave overnight in freezer at -20°C.
8. Carefully remove ethanol and air dry tube in hood.
9. Wash with 200μl of 70% ethanol.
10. Centrifuge for 1 minute.
11. Remove ethanol and air dry in hood.
12. Resuspend DNA in 15μl of DSW and use for DNA sequencing.

21. DNA Sequencing (Exo⁻Pfu Method)

The amplified products dissolved in distilled H_2O were used for DNA sequencing. The sequencing strategy employed the DNA cycle sequencing method (Cyclist Exo⁻ Pfu DNA Sequencing Kit - Stratagene) [172]. The prereaction mix consisted of DNA template 200 fmol, 1 pmol of primer, 4μl of 10x sequencing buffer, 10μCi of ^{35}S, 1μl of Exo⁻ Pfu DNA polymerase, qs to 26μl with DSW and 4μl of DMSO. 7μl of the prereaction mixture were then added to each of four tubes containing 3μl of termination mixes A, C, G and T. The tubes were then incubated at 95°C for 5 minutes followed by 30 cycles of 95°C for 30 seconds (denaturation), 62°C for 30 seconds (annealing) and 72°C for 60 seconds (extension). The exact annealing temperature varied with the oligonucleotide used. After the run 5μl of stop solution was added to each tube. Samples were frozen at -20°C. Each sample (2-4μl) was denatured (2-5 minutes at 95°C) before being loaded onto either a 4% or 8% polyacrylamide sequencing gel. Exact voltage and duration of electrophoresis varied from run to run. The gels were fixed

(7.5% methanol/7.5% acetic acid), dried and subjected to autoradiography.

RESULTS

1. Susceptibility

The antibiotic susceptibilities of the laboratory derived fluoroquinolone-resistant isolates are listed in Table 11. Fluoroqouinolone-resistant isolates (3854r, 4047r and 4048r) not only exhibitied high level fluoroquinolone-resistance (ciprofloxacin MIC of 1024μg/ml) but exhibited multiple-antibiotic-resistance (Table 11). This multiple-antibiotic resistance included various antibiotic classes including beta-lactams, tetracycline and chloramphenicol. Two-fold increases in MIC's occurred with imipenem, while aminoglycoside susceptibilities either did not change or increased two-fold (Table 11).

The Mar phenotype did not occur until strains 3854, 4047 and 4048 exhibited 4, 16, 32 increases in MIC's to fluoroquinolones, respectively. Thereafter, these isolates demonstrated resistance to fluoroquinolones and other antibiotic classes (Table 11).

Figures 9-11 describe the susceptibilities of laboratory-derived fluoroquinolone isolates at various levels of fluoro-quinolone resistance. Figure 9 describes the antbiotic susceptibility of strain 4047 at various levels of fluoro-quinolone resistance (ciprofloxacin MIC 1μg/ml, 4μg/ml, 16μg/ml, 64μg/ml and 256μg/ml, respectively). When the ciprofloxacin MIC was ≤8μg/ml, these phenotypes demonstrated unchanged susceptibilities to various beta-lactams (e.g. cefoxitin, cefotaxime, piperacillin) as well as other

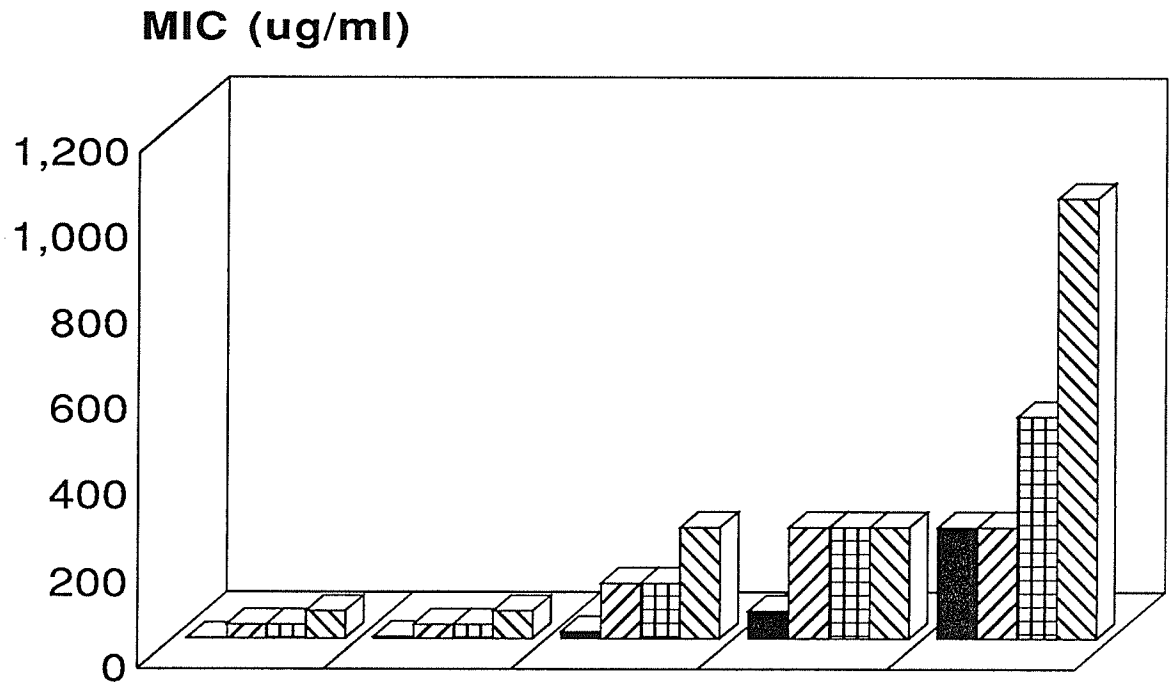
antibiotics such as tetracycline and chloramphenicol. Once strain 4047 displayed a ciprofloxacin MIC of 16μg/ml, it demonstrated a Mar phenotype (Figure 9).

Table 11. Susceptibilities of Wild-Type Laboratory Derived Fluoroquinolone-Resistant Strains of P. aeruginosa

	MIC (μg/ml)					
Antimicrobial	3854	3854r	4047	4047r	4048	4048r
Fluoroquinolones						
Ciprofloxacin	0.25	1024	1.0	1024	0.125	1024
Norfloxacin	0.5	2048	2.0	2048	0.5	2048
Pefloxacin	2.0	4096	4.0	2048	1.0	4096
ß-lactams						
Cefoxitin	64	512	32	256	64	1024
Cefotaxime	32	256	32	512	32	256
Piperacillin	16	128	16	256	16	128
Imipenem	2.0	4.0	4.0	8.0	2.0	4.0
Aminoglycosides						
Gentamicin	4.0	8.0	4.0	4.0	4.0	8.0
Tobramycin	1.0	2.0	1.0	2.0	2.0	4.0
Others						
Chloramphenicol	32	256	32	512	64	512
Tetracycline	32	1024	64	1024	32	512

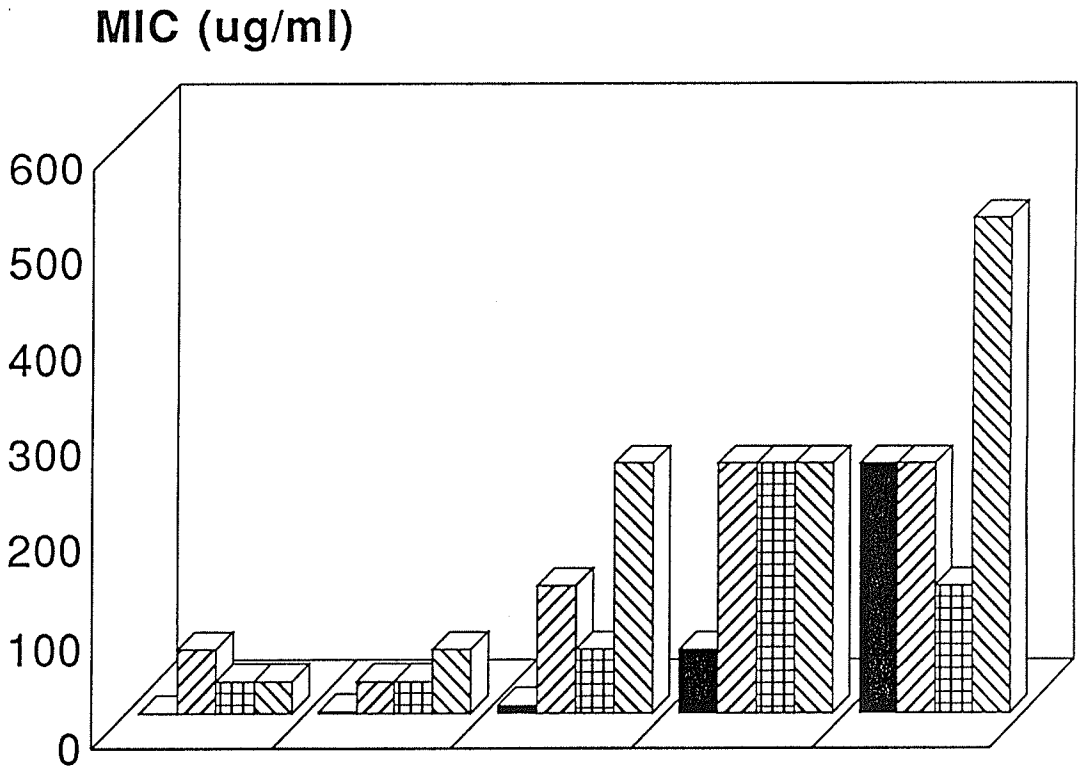
Figure 9

SUSCEPTIBILITY OF STRAIN 4047 AT VARIOUS LEVELS OF FQ RESISTANCE



	1	4	16	64	256
Ciprofloxacin	1	4	16	64	256
Cefoxitin	32	32	128	256	256
Chloramphenicol	32	32	128	256	512
Tetracycline	64	64	256	256	1,024

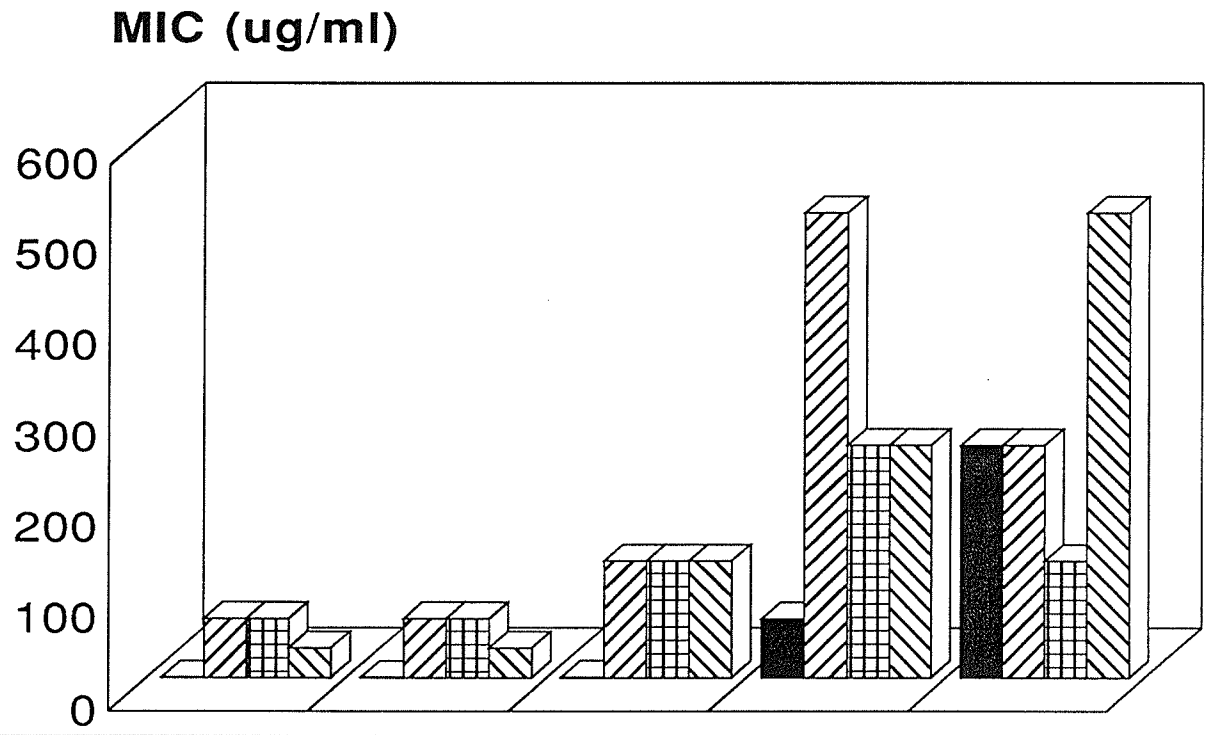
Figure 10 *SUSCEPTIBILITY OF STRAIN 3854 AT VARIOUS LEVELS OF FQ RESISTANCE*



Ciprofloxacin	0.25	2	8	64	256
Cefoxitin	64	32	128	256	256
Chloramphenicol	32	32	64	256	128
Tetracycline	32	64	256	256	512

Figure 11

SUSCEPTIBILITY OF STRAIN 4048 AT VARIOUS LEVELS OF FQ RESISTANCE



Ciprofloxacin	0.125	0.25	0.5	64	256
Cefoxitin	64	64	128	512	256
Chloramphenicol	64	64	128	256	128
Tetracycline	32	32	128	256	512

The antibiotic susceptibilities of clinical fluoroquinolone resistant isolates (3623 and 4190) are depicted in Table 12. Resistance to all fluoroquinolones was observed. The paired isolates (3818 and 3818r) differed only in fluoroquinolone susceptibility.

Table 12. Susceptibilities of clinical fluoroquinolone-resistant P. aeruginosa

	MIC (μg/ml)			
Antimicrobial	3623	4190	3818	3818r
Fluoroquinolones				
Ciprofloxacin	8.0	4.0	0.5	8.0
Norfloxacin	16	16.0	1.0	16.0
Pefloxacin	32	32.0	2.0	32.0
ß-lactams				
Cefoxitin	64	64	64	64
Cefotaxime	64	64	32	32
Piperacillin	16	8	8	8
Imipenem	2.0	2.0	2.0	2.0
Aminoglycosides				
Gentamicin	4.0	8.0	4.0	4.0
Tobramycin	1.0	1.0	1.0	1.0
Others				
Chloramphenicol	32	32	32	32
Tetracycline	64	32	32	32

2. Stability of Fluoroquinolone Resistance

Both clinical fluoroquinolone-resistant isolates (3623, 3818r and 4190) and laboratory-derived fluoroquinolone-resistant mutants (3854r, 4047r, 4048r) were passaged 40 times on

antibiotic free BHI-agar. Laboratory-derived fluoroquinolone resistant isolates demonstrated a reduction in the level of resistance on the first few passages, then plateaued at high-level fluoroquinolone-resistance (MIC 16-256μg/ml) [Figure 12]. All clinical isolates maintained fluoroquinolone resistance over repeated in vitro passages (Figure 13).

Figure 12

STABILITY OF LAB MUTANTS

(Susceptibility to Ciprofloxacin)

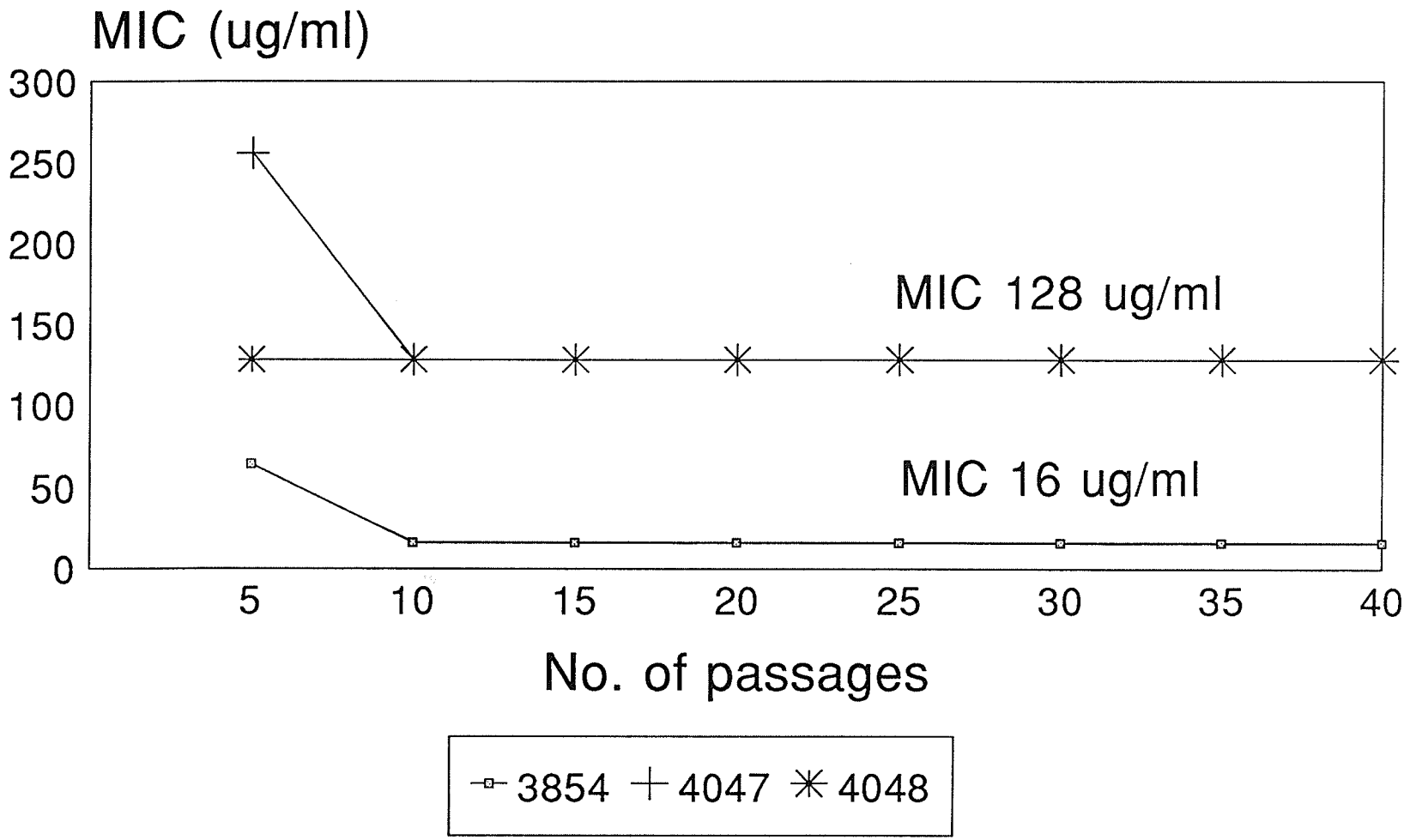
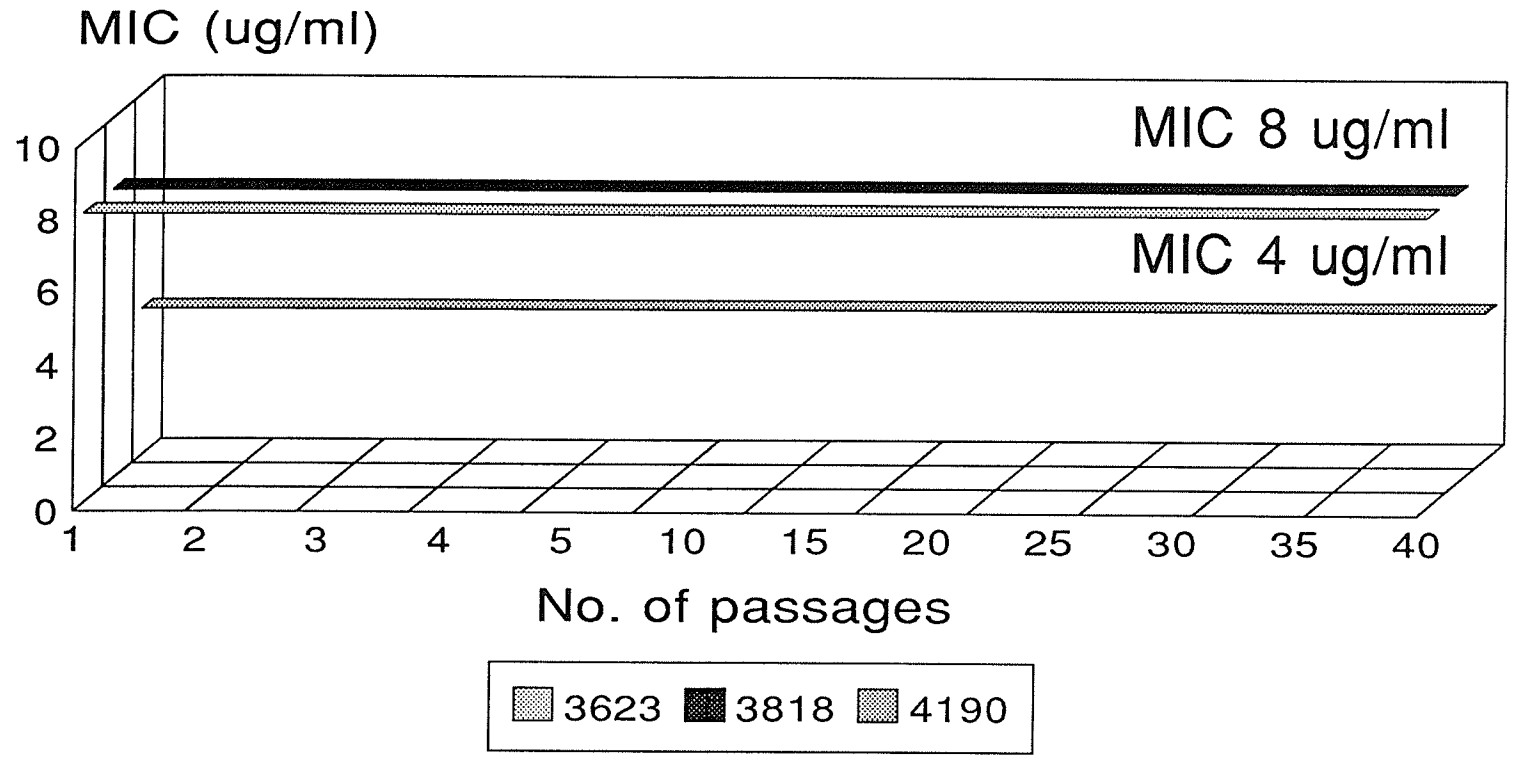


Figure 13

STABILITY OF CLINICAL MUTANTS

(Susceptibility to Ciprofloxacin)



3. Growth and Morphology

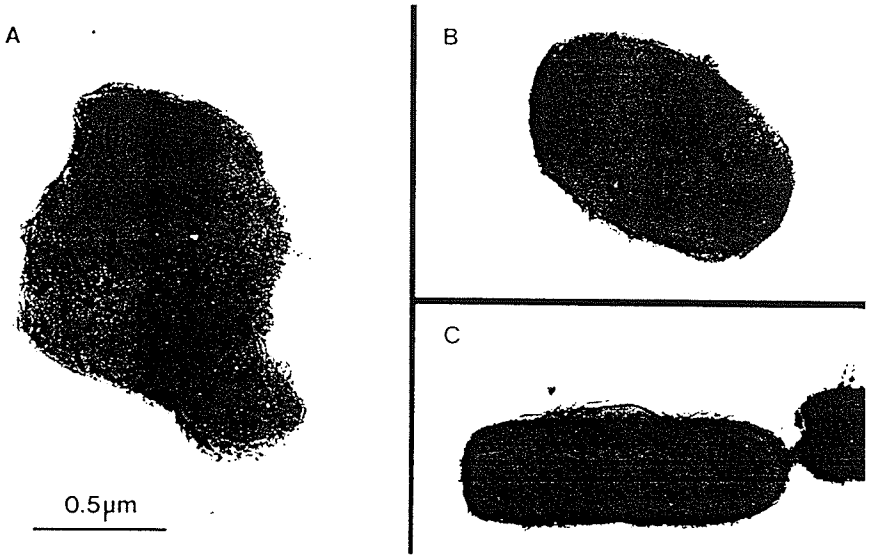
All wild type strains (3854, 4047, 4048 and 3818) and clinical fluoroquinolone-resistant isolates (3623, 3818r, 4190) grew quickly with doubling times in BHI-broth of approximately 30 minutes. Laboratory-derived fluoroquinolone-resistant mutants (3854r, 4047r, 4048r) grew slower than wild-type isolates with doubling times ranging from approximately 40 minutes to 1.5 hours.

Morphological analysis performed by light microscopy showed that all wild-type isolates (3854, 4047, 4048 and 3818) and clinical fluoroquinolone-resistant isolates (3623, 3818r and 4190) demonstrated good stain uptake and typical gram-negative bacillary morphology. However, laboratory-derived fluoroquinolone-resistant mutants had poor stain uptake, cells lost their rod-like shape, and appeared rounded or coccal.

Electron microscopy demonstrated that all wild-type (3854, 4047, 4048 and 3818) and clinical fluoroquinolone resistant isolates displayed characteristic gram-negative bacillary morphology (Figure 14C). Laboratory-derived fluoroquinolone-resistant mutants,however, displayed loss of rod-like shape and appeared rounded or coccal (Figure 14A and B). Many cells showed large bleb formation in the outer membrane (Figure 14A). These gross morphological changes in the laboratory derived fluoroquinolone-resistant mutants began to appear after these organisms demonstrated at least 4-32 fold increases in MICs to fluoroquinolones. For example, isoalte 4047 (MIC 1.0μg/ml with ciprofloxacin) began to show these morphological changes once

the ciprofloxacin MIC attained 16μg/ml.

Figure 14. Electron Micrograph of Fluoroquinolone Sensitive and Fluoroquinolone Resistant P. aeruginosa



A and B Fluoroquinolone-resistant P. aeruginosa (strain 4047r)

C Wild-type fluoroquinolone-sensitive P. aeruginosa (strain 4047)

4. Fluoroquinolone Uptake

The occurence not only of fluoroquinolone-resistance but also resistance to various antibiotic groups of unrelated chemical classes in laboratory-derived fluoroquinolone-resistant

mutants suggested altered membrane permeability. We therefore studied the uptake of pefloxacin in laboratory-derived isolates. Compared to their respective parental strains (3854, 4047 and 4048), laboratory derived fluoroquinolone-resistant isolates (3854r, 4047r and 4048r) demonstrated significantly reduced uptake of pefloxacin ($p<0.05$ t-test) [Table 13]. In contrast, pefloxacin uptake with clinical fluoroquinolone-resistant isolates was similar to fluoroquinolone-susceptible strains (Table 13). Figure 15 compares the uptake of 14C-Pefloxacin in a clinical fluoroquinlone resistant mutant (3818r) to a laboratory-derived fluoroquinolone resistant mutant (4047r) both compared to a wild-type fluoroquinolone sensitive organism. Significantly reduced uptake was observed in the laboratory derived isolates, whereas no difference was observed between wild-type and fluoroquinolone-resistant clinical isolates. Reductions in fluoroquinolone uptake did not occur until after 4-32 fold increases in the MIC's to fluoroquinolones occurred. Figure 16 depicts the uptake of pefloxacin into strain 4047 at various levels of fluoroquinolone resistance. At ciprofloxacin MICs $\leq 8\mu g/ml$ no changes in pefloxacin uptake were noted. However, at ciprofloxacin MICs $\geq 16\mu g/ml$ reductions in fluoroquinolone uptake occurred. For strain 3854 no changes in pefloxacin uptake was noted until the ciprofloxacin MIC attained $8\mu g/ml$ (Figure 17). Finally, for strain 4048, reduction in fluoroquinolone uptake were observed at an MIC of ciprofloxacin of $0.5\mu g/ml$ (Figure 18).

Table 13. Uptake of ^{14}C-Pefloxacin Into Clinical and Laboratory Fluoroquinolone-Resistant *P. aeruginosa*

	14C-Pefloxacin Uptake[a]	
Strain	- CCCP[b]	+ CCCP[c]
Clinical Isolates		
3623	3.2 ± 0.4	4.0 ± 0.4
3818	2.9 ± 0.3	3.7 ± 0.3
3818r	3.0 ± 0.4	3.8 ± 0.5
4190	4.1 ± 0.5	5.5 ± 0.5
Laboratory Isolates		
3854	2.4 ± 0.3*	3.2 ± 0.4
3854r[d]	1.2 ± 0.4*	1.6 ± 0.3
4047	3.3 ± 0.3**	4.3 ± 0.5
4047r[d]	2.2 ± 0.2**	2.9 ± 0.4
4048	4.3 ± 0.6***	5.7 ± 0.5
4048r[d]	3.1 ± 0.3***	4.1 ± 0.4

[a] Uptake recorded as ng pefloxacin/mg total cell protein at 30 minutes

[b] Without CCCP

[c] With CCCP

[d] Ciprofloxacin MIC 1024μg/ml

* $p<0.05$ t-test (with or without CCCP)

** $p<0.05$ t-test (with or without CCCP)

*** $p<0.05$ t-test (with or without CCCP)

Figure 15

FLUOROQUINOLONE UPTAKE IN P.aeruginosa

(14C Pefloxacin)

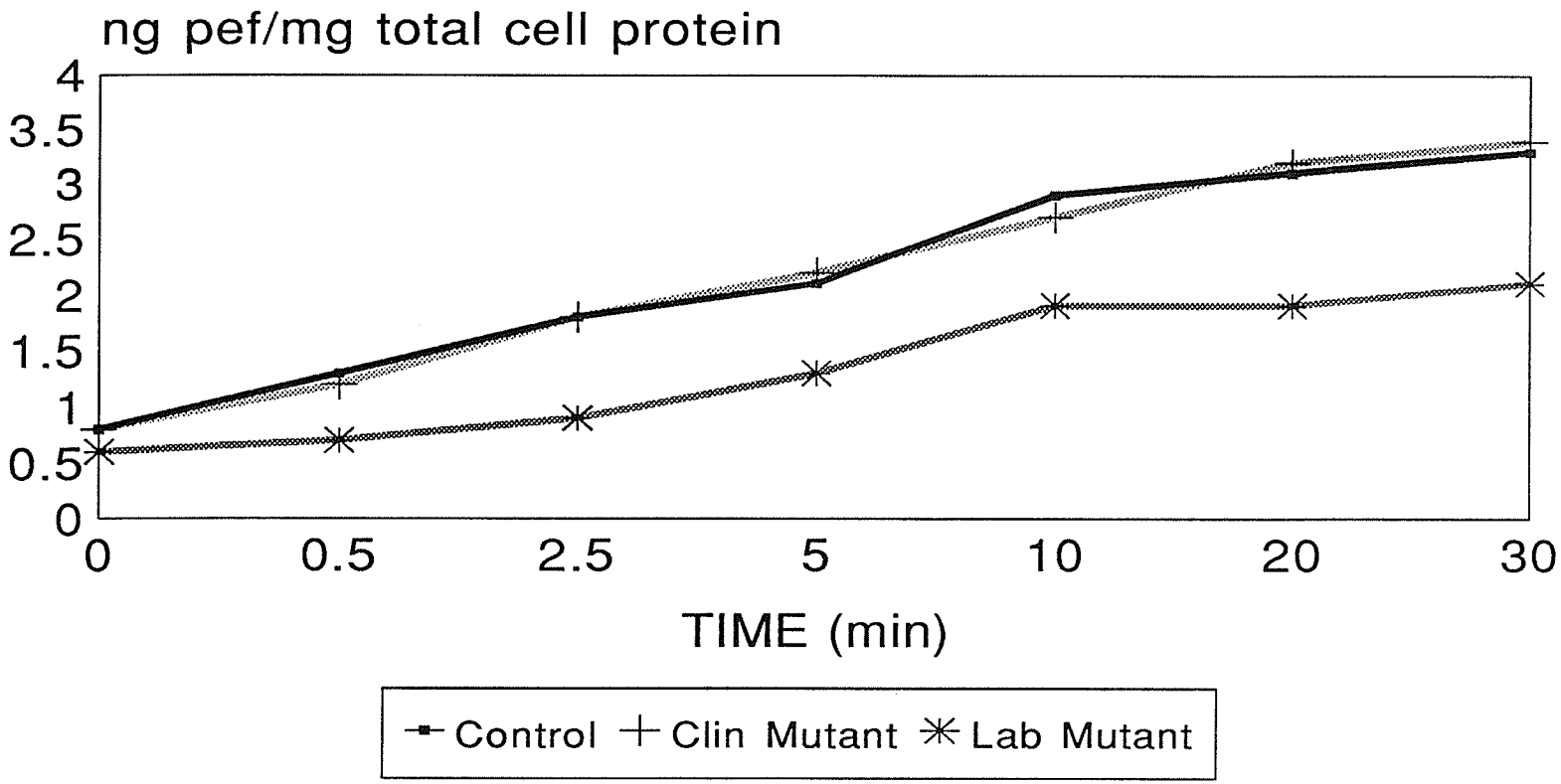


Figure 16

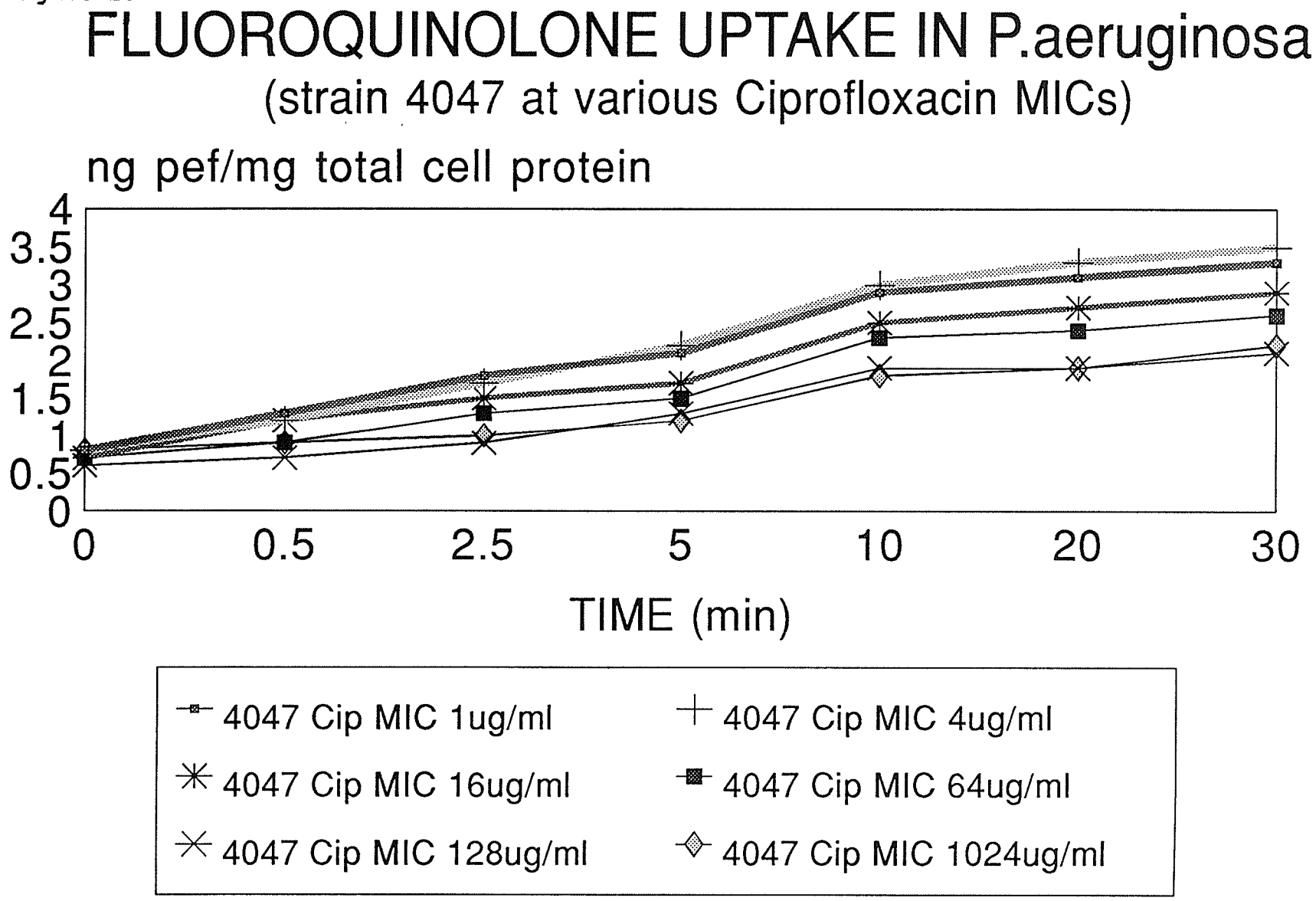
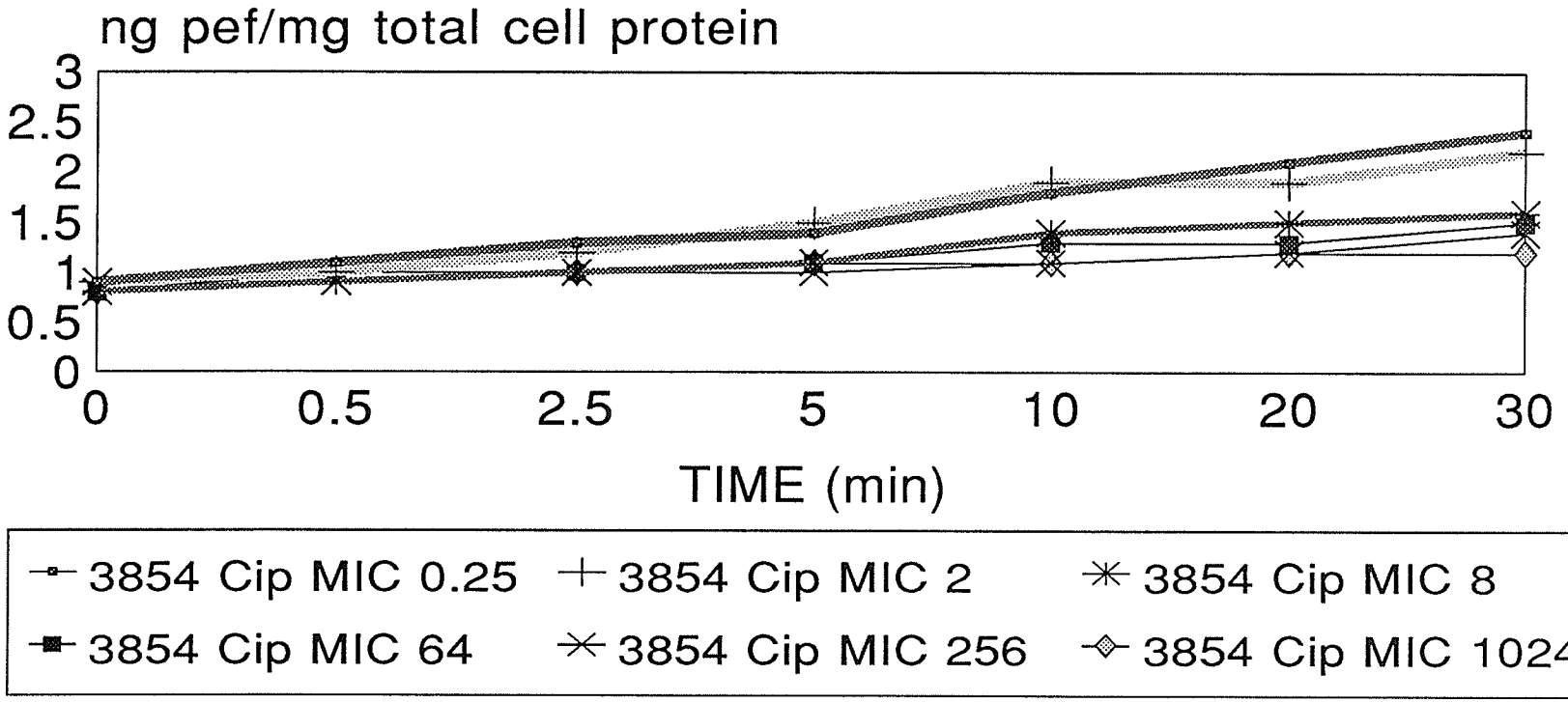

FLUOROQUINOLONE UPTAKE IN P.aeruginosa
(strain 4047 at various Ciprofloxacin MICs)
ng pef/mg total cell protein
4
3.5
3
2.5
2
1.5
1
0.5
0
0
0.5
2.5
5
10
20
30
TIME (min)
4047 Cip MIC 1ug/ml
4047 Cip MIC 4ug/ml
4047 Cip MIC 16ug/ml
4047 Cip MIC 64ug/ml
4047 Cip MIC 128ug/ml
4047 Cip MIC 1024ug/ml

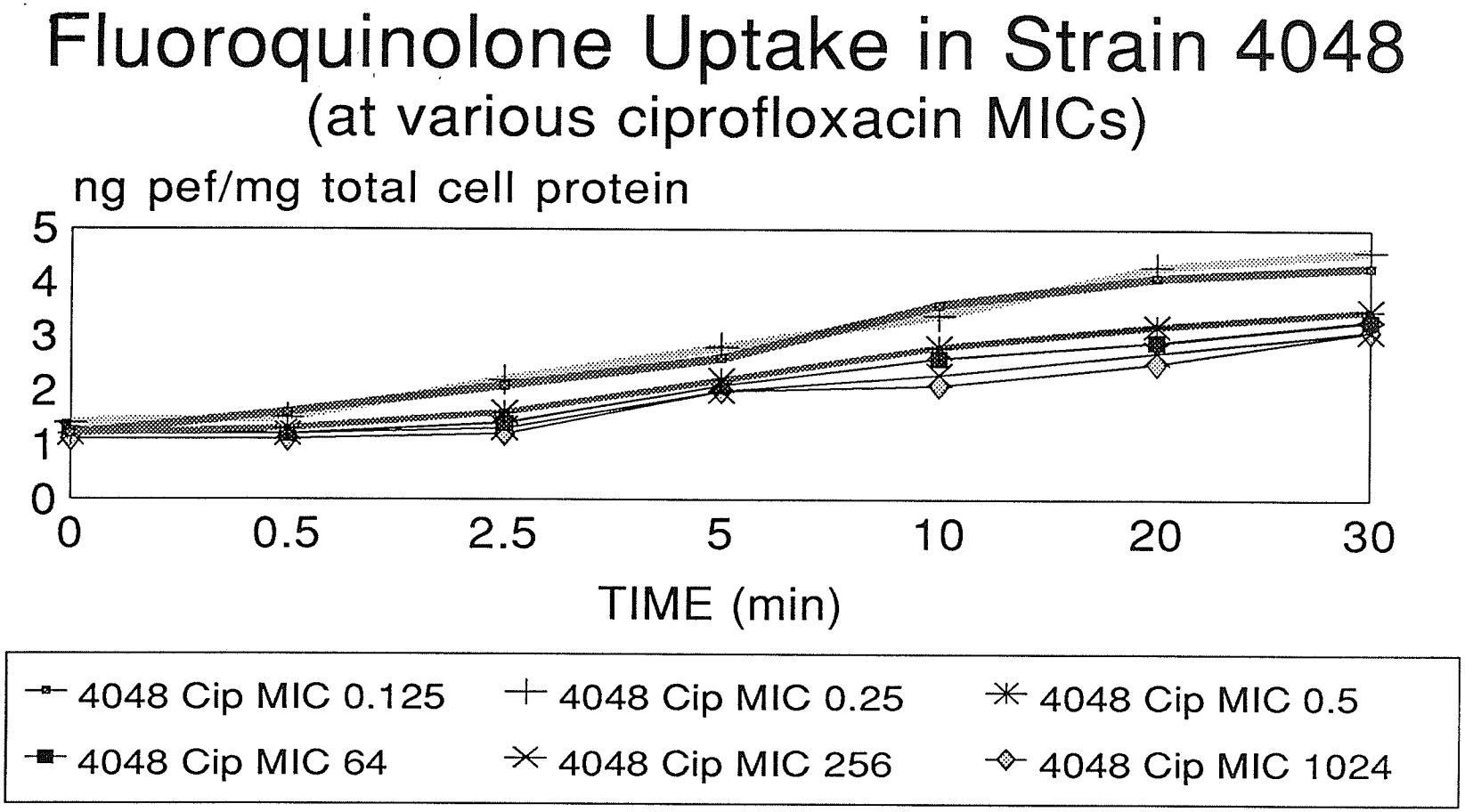
Figure 17

Fluoroquinolone Uptake in Strain 3854
(at various ciprofloxacin MICs)



* 14C-Pefloxacin

Figure 18



* 14C-Pefloxacin

Addition of the energy inhibitor CCCP enchanced intracellular pefloxacin concentrations by 30-40% after 30 minutes for all strains whether fluoroquinolone-sensitive or resistant, suggesting that P. aeruginosa possesses an active efflux system for fluoroquinolones (Table 13). In addition, these data suggest that this fluoroquinolone efflux system is intact in fluoroquinolone-resistant isolates.

5. Bacterial Adherence

To determine whether the acquisition of fluoroquinolone-resistance was associated with altered bacterial virulence, we assessed the ability of fluoroquinolone-sensitive and fluoroquinolone-resistant P. aeruginosa to adhere in vitro. Adherence is only one of many virulence factors, but was chosen as a representative membrane-associated virulence factor. Two assays were performed, the voided uroepithelial cell assay and the Vero (African green monkey kidney) cell assay. Both assays produced similar results.

No significant difference in bacterial adherence was observed between clinical fluoroquinolone sensitive (3818) and clinical fluoroquinolone-resistant (3818r) isolates using either the voided uroepithelial cell assay or the Vero cell assay ($p>0.05$) [Table 14].

Table 14. Adherence of Clinical FR Isolates Using the Voided Uroepithelial Cell Assay (VUC)[a] or the Vero Cell Assay (VC)[b]

Strain	Adherence	
	VUC	VC
3818	12.5 ± 10.1	100
3818r	13.6 ± 9.5	107 ± 11
3623	49.0 ± 21.1	135 ± 24
4190	47.9 ± 22.1	143 ± 22

[a] VUC = Voided uroepithelial cell assay; numbers represent the mean number of bacteria adhering to each of 40 uroepithelial cells

[b] VC = Vero cell assay; numbers represent mean number of cells adhering as a percentage of 3818 (serving as control) [mean ± S.D. of 3 assays]

Compared to their parental wild-types, laboratory-derived fluoroquinolone-resistant isolates demonstrated significantly ($p<0.05$) reduced adherence (Table 15) using both assays. These data suggest that clinical fluoroquinolone-resistant isolates and wild-type showed unchanged adherence, while in laboratory derived fluoroquinolone-resistant isolates adherence was reduced.

Table 15. Adherence of Laboratory FR Isolates Using the Voided Uroepithelial Cell Assay (VUC)[a] or the Vero Cell Assay (VC)[b]

Strain	Adherence	
	VUC	VC (%)
3854	96.7 ± 36.71*	100*
3854r	50.1 ± 16.2*	62 ± 11*
4047	98.1 ± 40.6**	100**
4047r	43.4 ± 19.5**	51.4 ± 13**
4048	68.2 ± 18.2***	100***
4048r	42.1 ± 16.2***	73.1 ± 14***

[a] VUC = Voided uroepithelial cell assay; numbers represent the mean number of bacteria adhering to each of 40 uroepithelial cells

[b] VC = Vero cell assay; numbers represent mean number of cells adhering as a percentage of the respective parental type (e.g. 4047 and 4047r) [mean ± S.D. of 3 assays]

* $p<0.05$ t-test

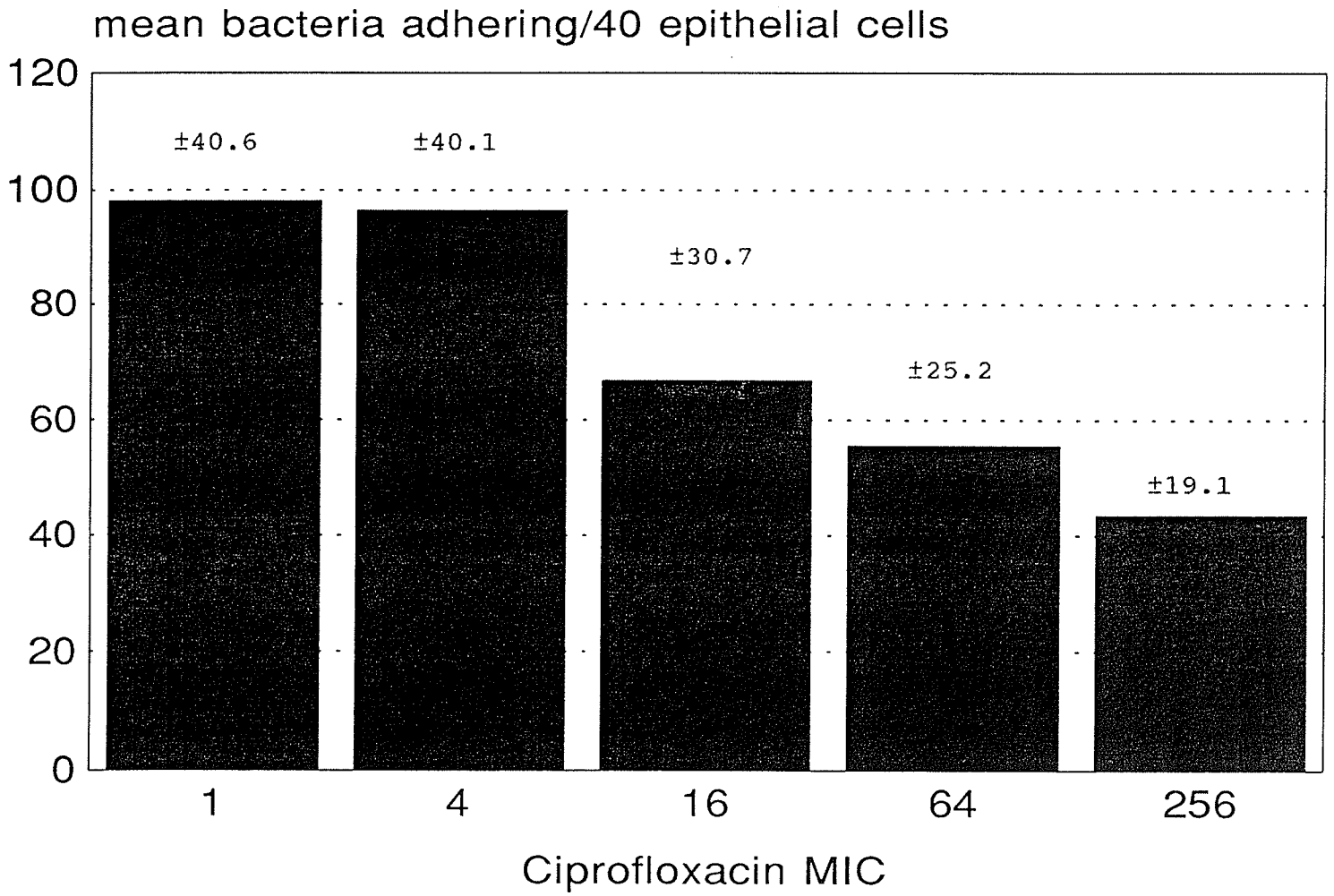
** $p<0.05$ t-test

*** $p<0.05$ t-test

Figure 19 describes the adherence (voided uroepithelial cell assay) of strain 4047 at various levels of fluoroquinolone resistance. As with the data on antibiotic susceptibility, morphology and fluoroquinolone uptake, no significant ($p<0.05$) difference in adherence was noted until the ciprofloxacin MICs attained ≥16μg/ml, thereafter, adherence was reduced ($p>0.05$) compared to wild type. Similar data were obtained for all strains.

Figure 19

Adherence of Strain 4047 at Various levels of Fluoroquinolone Resistance

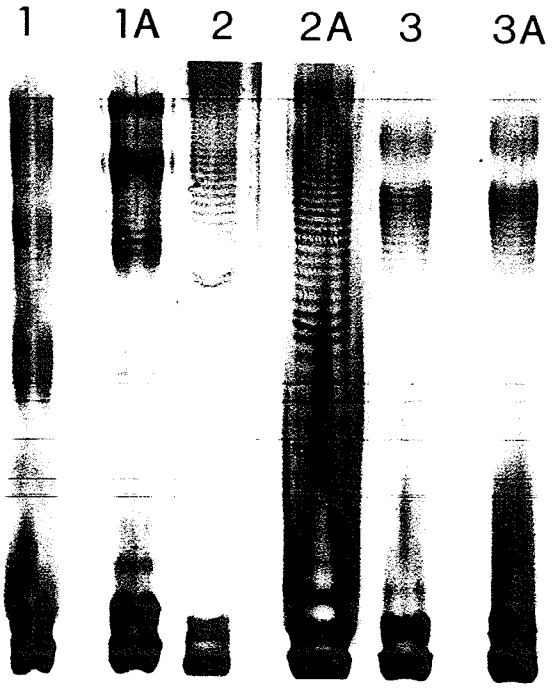


6. Lipopolysaccharide (LPS) Analysis

Since alterations in antibiotic susceptibility (including uptake), morphology and adherence can be affected by the outer-membrane, we investigated lipopolysaccharide (LPS) and outer-membrane proteins (Opr) from both clinical and laboratory-derived fluoroquinolone-resistant isolates.

Analysis of purified LPS electrophoresed on SDS-PAGE gel demonstrated no differences between the parental strain (3818) and the clinical fluoroquinolone-resistant isogenic pair 3838r. The LPS profiles of the parental fluoroquinolone sensitive (3854, 4047 and 4048) and laboratory-derived fluoroquinolone resistant (3854r, 4047r, 4048r all had ciprofloxacin MIC's of 1024μg/ml) isolates were also unchanged (Figure 20).

Figure 20. Lipopolysaccharide (LPS) Analysis in Laboratory Derived Fluoroquinolone Resistant P. aeruginosa

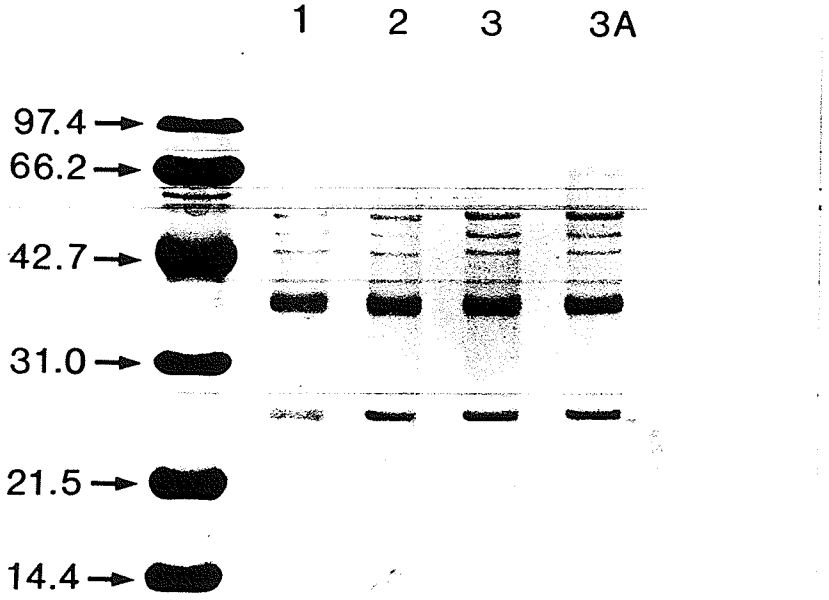


1 - 3854, 1A - 3854r, 2 - 4047, 2A - 4047r, 3 - 4048, 3A - 4048r

7. Outer-Membrane Protein (Opr) Profiles

Outer membrane protein profiles of clinical fluoroquinolone isolates (3623, 4190) and parental strain 3818 along with its clinical fluoroquinolone-resistant isogenic pair (3818r) were similar (Figure 21).

Figure 21. Outer-Membrane Protein (Opr) Profiles of Clinical Fluoroquinolone-Resistant P. aeruginosa[a]



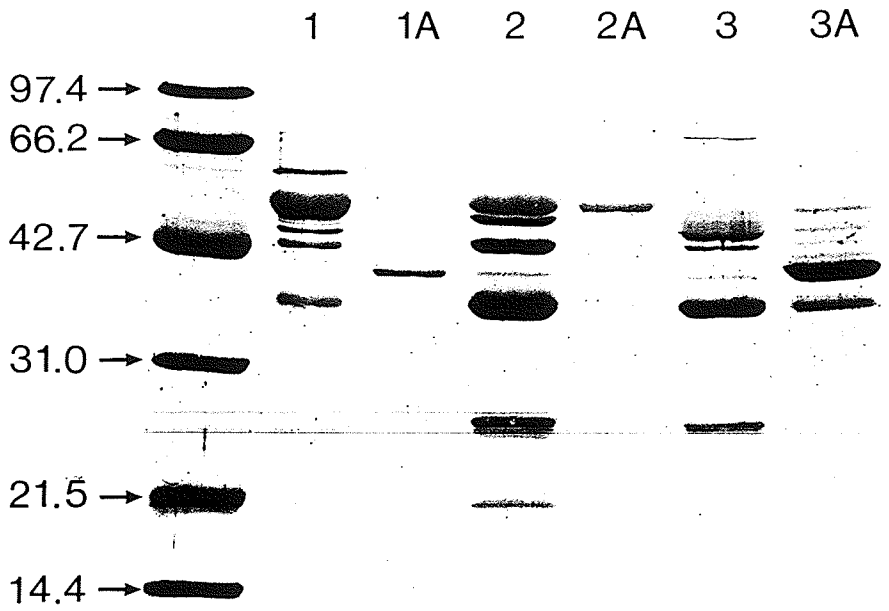
Lane 1 - 3623, 2 - 4190, 3 - 3818, 4 - 3818r

[a] = Numbers of the left indicate molecular weights (10^3) of standard proteins

[b] = Purified outer-membrane proteins (50μg/lane)

Outer-membrane protein profiles of fluoroquinolone-sensitive (3854, 4047 and 4048) and their laboratory-derived fluoroquinolone-resistant pairs (3854r, 4047r and 4048r) were considerably different (Figure 22).

Figure 22. Outer-Membrane Protein (Opr) Profiles of Laboratory-Derived Fluoroquinolone Resistant P. aeruginosa[a]



Lane 1 - 3854, 2 - 3854r, 3 - 4047, 4 - 4047r, 5 - 4048, 6 - 4048r

[a] = Numbers on the left indicate the molecular weights (10^3) of standard proteins

[b] = Purified outer-membrane proteins (50μg/lane)

Laboratory-derived fluoroquinolone-resistant isolates demonstrated reduced intensity of bands in the 25 KDa and 38 KDa

region. In addition, several bands with molecular weights ranging from 43 KDa-66 KDa showed reduced intensity in the lab-derived fluoroquinolone resistant isolates. One new band was visualized in strains 3854r and 4048r (Figure 22). This outer membrane protein had an aproximate molecular weight of 40 KDa (Figure 22). As with antibiotic susceptibility, morphological analysis, fluoroquinolone uptake and adherence studies, changes in outer-membrane proteins did not occur until after the laboratory-derived isolates demonstrated 4-32 fold increases in MIC's to fluoroquinolones. For example, with strain 4047, changes in outer membrane proteins did not occur until the MIC to ciprofloxacin became ≥16μg/ml.

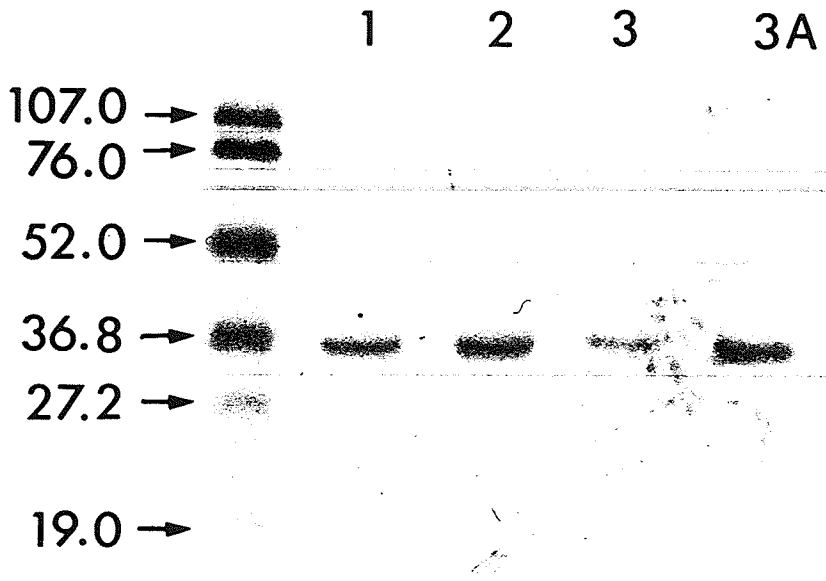
8. Immunoblots of Outer-Membrane Protein F (OprF)

We were particularly interested in OprF, as this Opr likely serves a dual function, one of a structural role affecting cell morphology and one of a porin and thus affecting antibiotic uptake. Immunoblots using monoclonal antibody (MA 4-10 or G 10) detected OprF with strong signals from parental fluoroquinolone-sensitive and clinical fluoroquinolone-resistant isolates (Figure 23). It should be noted that monoclonal MA 4-10 is very sensitive and specific in detection of Opr F from P. aeruginosa (166).

Use of an Opr F specific monoclonal antibody (MA 4-10 or G10) either failed to detect Opr F (3854r) or demonstrated a reduced signal (4047r and 4048r) in laboratory derived fluoroquinolone resistant isolates compared to their

fluoroquinolone-sensitive isogenic pairs (Figure 24). The new band (mw - 40 KDa in 3854r and 4048r) did not give a positive signal with Opr F specific monoclonal antibody suggesting that it was either not a modified form of Opr F, or that if it was an altered form of Opr F, it had lost the epitope for the monoclonal antibody.

Figure 23. Immunoblot Using Opr F Specific Monoclonal Antibody in Clinical FR Isolates

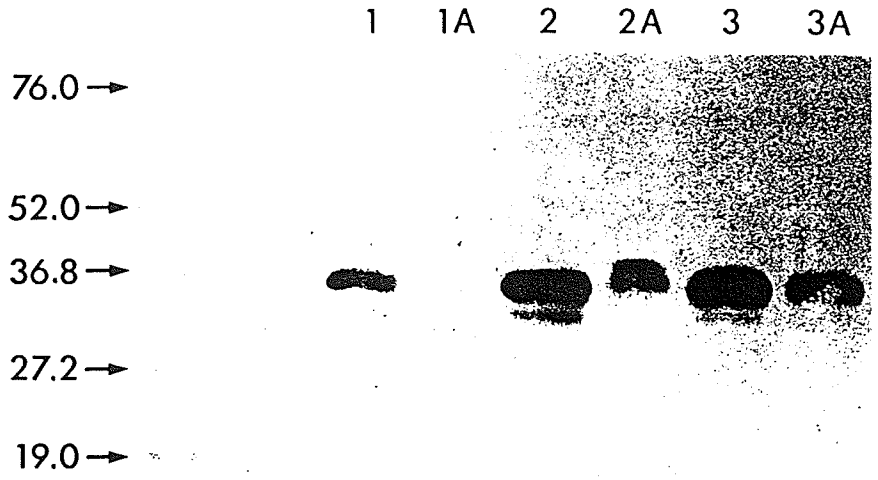


Lane 1 - 3623, 2 - 4190, 3 - 3818, 3A - 3818r

[a] = Numbers on the left indicate the prestained molecular weights ($x10^3$) of standard proteins

[b] = Purified outer-membrane proteins

Figure 24. Immunoblot Using Opr F Specific Monoclonal Antibody in Laboratory Derived PR Isolates[a]



Lane 1 - 3854, 2 - 3854r, 3 - 4047r, 4 - 4047r, 5 - 4048, 6 - 4048r

[a] = Numbers on the left indicate the prestained molecular weight (10^3) of standard proteins

[b] = Purified outer-membrane proteins

9. Complementation With E. coli gyrA

Plasmids pLA2917 and pNJR3-2 were introduced via conjugal mating into the gyrase A+ strain PA02, a known gyrase A mutant PA04701, as well as fluoroquinolone sensitive and fluoroquinolone-resistant isolates. The fluoroquinolone MIC's for these strains are presented in Tables 16 and 17. As expected, vector pLA2917 had no effect on PA02, PA04701 or any other isolate. Also, as expected pNJR3-2 had no effect on PA02 but did confer fluoroquinolone susceptibility on PA04701. Plasmid pNJR3-2 conferred fluoroquinolone susceptibility to all clinical fluoroquinolone-resistant isolates (3623, 3818r, 4190) [Table 16].

Table 16. Expression of E. coli gyrA in gyrA+ and gyrA Clinical P. aeruginosa Strains

Strain and plasmid	Ciprofloxacin	Norfloxacin	Pefloxacin
PA02 (no plasmid)	0.25	1.0	1.0
PA02 (pLA2917)	0.25	1.0	1.0
PA02 (pNJR3-2)	0.25	1.0	1.0
PA04701 (no plasmid)	2.0	8.0	16.0
PA04701 (pLA2917)	2.0	8.0	16.0
PA04701 (pNJR3-2)	0.25	1.0	1.0
3623 (no plasmid)	8.0	16.0	32.0
3623 (pLA2917)	8.0	8.0	32.0
3623 (pNJR3-2)	0.5	1.0	1.0

Table 16 (cont'd)

	MIC (μg/ml)		
Strain and plasmid	Ciprofloxacin	Norfloxacin	Pefloxacin
3818 (no plasmid)	0.5	1.0	1.0
3818 (pLA2917)	0.25	1.0	2.0
3818 (pNJR3-2)	0.5	1.0	2.0
3818r (no plasmid)	8.0	16.0	32.0
3818r (pLA2917)	8.0	16.0	32.0
3818r (pNJR3-2)	0.5	1.0	1.0
4190 (no plasmid)	4.0	16.0	32.0
4190 (pLA2917)	4.0	16.0	16.0
4190 (pNJR3-2)	0.5	1.0	2.0

Plasmid pNJR3-2 reduced the level of fluoroquinolone resistance approximately 8-32 fold with laboratory-derived fluoroquinolone-resistant isolates (Table 17). This is consistent with mutations in gyrA contributing to but not fully explaining resistance. Thus non-gyrA mutations must also occur in these strains.

Table 17. Expression of E. coli gyrA in gyrA+ and gyrA Laboratory P. aeruginosa Strains

	MIC (μg/ml)		
Strain and plasmid	Ciprofloxacin	Norfloxacin	Pefloxacin
PA02 (no plasmid)	0.25	1.0	1.0
PA02 (pLA2917)	0.25	1.0	1.0
PA02 (pNJR3-2)	0.25	1.0	1.0
PA04701 (no plasmid)	2.0	8.0	16.0
PA04701 (pLA2917)	2.0	8.0	16.0
PA04701 (pNJR3-2)	0.25	1.0	1.0
3854 (no plasmid)	0.25	0.5	2.0
3854 (pLA2917)	0.25	0.5	2.0
3854 (pNJR3-2)	0.25	0.5	2.0
3854r (no plasmid)	1024	2048	4096
3854r (pLA2917)	1024	2048	4096
3854r (pNJR3-2)	32	64	64
4047 (no plasmid)	1.0	2.0	2.0
4047 (pLA2917)	1.0	1.0	4.0
4047 (pNJR3-2)	1.0	2.0	4.0
4047r (no plasmid)	1024	1024	2048
4047r (pLA2917)	512	2048	2048
4047r (pNJR3-2)	64	64	128

Table 17 (cont'd)

	MIC (μg/ml)		
Strain and plasmid	Ciprofloxacin	Norfloxacin	Pefloxacin
4048 (no plasmid)	0.125	0.5	1.0
4048 (pLA2917)	0.125	1.0	2.0
4048 (pNJR3-2)	0.25	1.0	1.0
4048r (no plasmid)	1024	2048	4096
4048r (pLA2917)	512	1024	4096
4048r (pNJR3-2)	128	256	512

Figures 25-27 describe the results of complementing strains 3854, 4047 and 4048 at different levels of ciprofloxacin resistance with E. coli gyrA. For strain 3854, at ciprofloxacin MICs ≤4μg/ml gyrA complementation restored the MIC to wild-type levels (Figure 25). Once the MIC attained ≥8μg/ml, gyrA complementation only partially restored susceptibility, suggesting both gyrA and non-gyrA mutation. Figure 26 describes the ciprofloxacin susceptibility of strain 4047 both before and after complementation with E. coli gyrA. At ciprofloxacin MICs of ≤8μg/ml, complementation with E. coli gyrA restored ciprofloxacin susceptibility, suggesting gyrA mutation. At ciprofloxacin MICs ≥16μg/ml, complementation with E. coli gyrA restored only partial susceptibility suggesting both gyrA and non-gyrA mutation. For strain 4048, gyrA complementation restored wild-type susceptibility at ciprofloxacin MICs ≤0.25μg/ml suggesting gyrA mutation. At ciprofloxacin MICs

Figure 25

Complementation of P.aeruginosa with E.coli gyrA+

(strain 3854 at various Ciprofloxacin MICs)

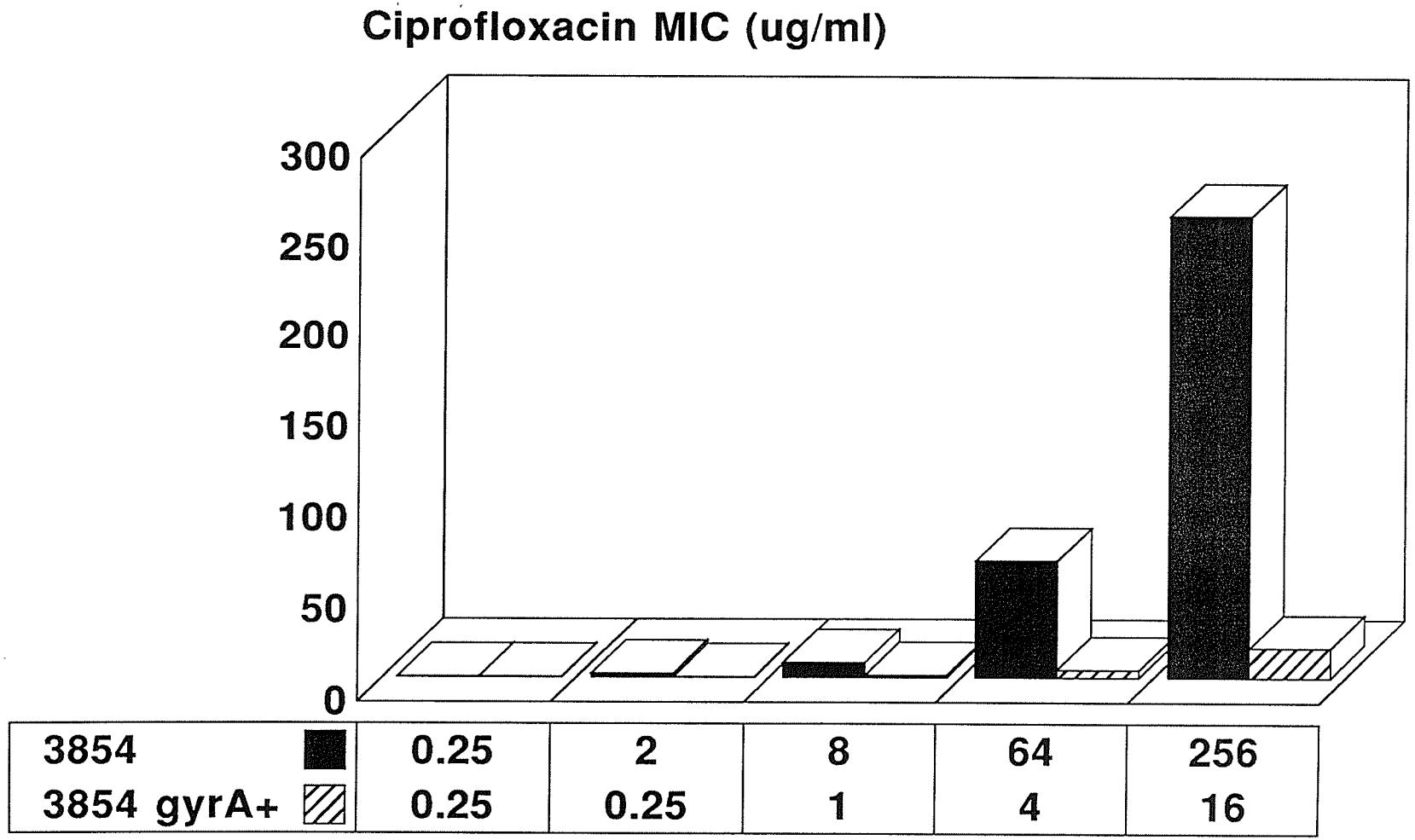


Figure 26

Complementation of P.aeruginosa with E.coli gyrA+

(strain 4047 at various Ciprofloxacin MICs)

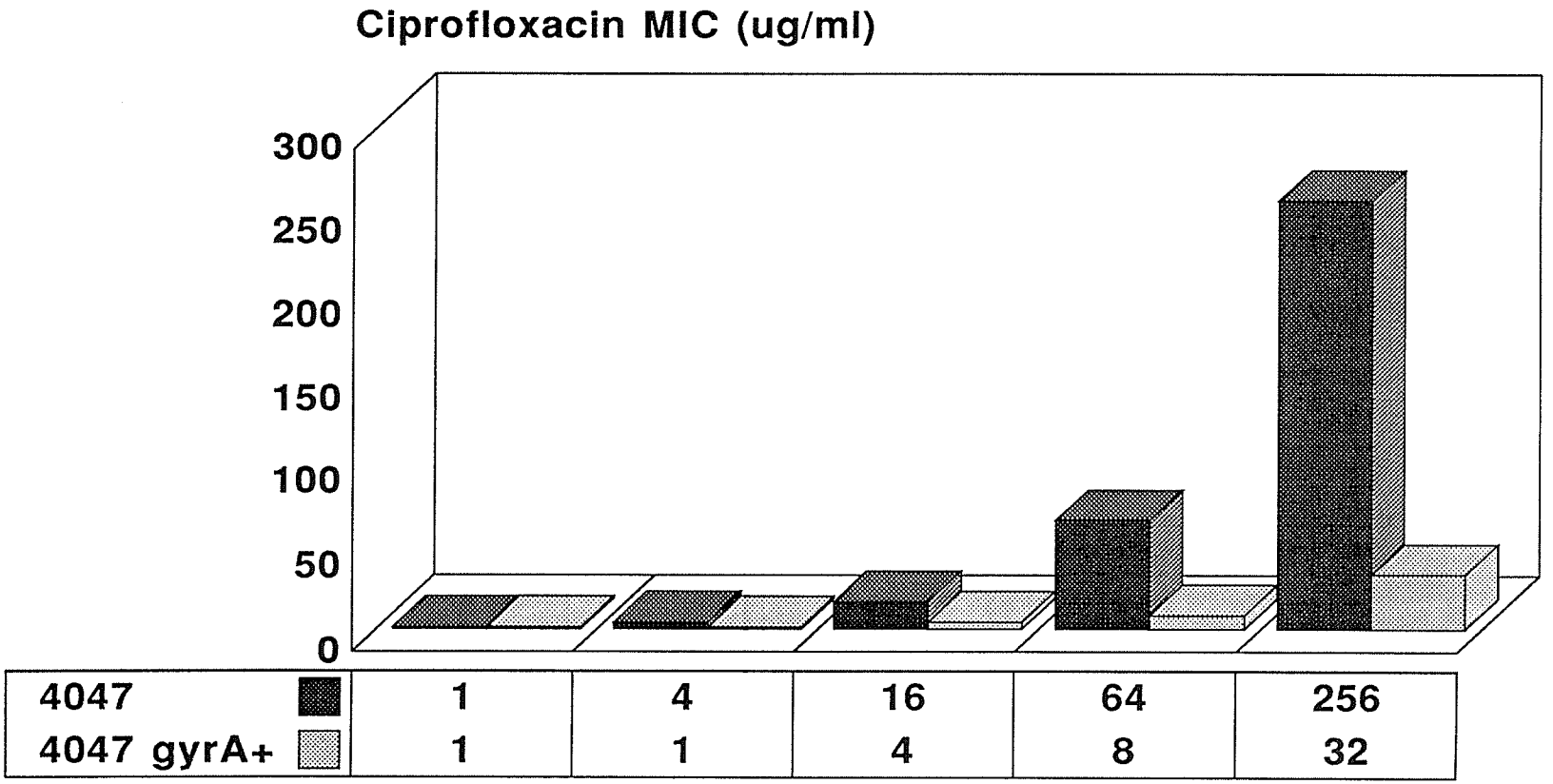
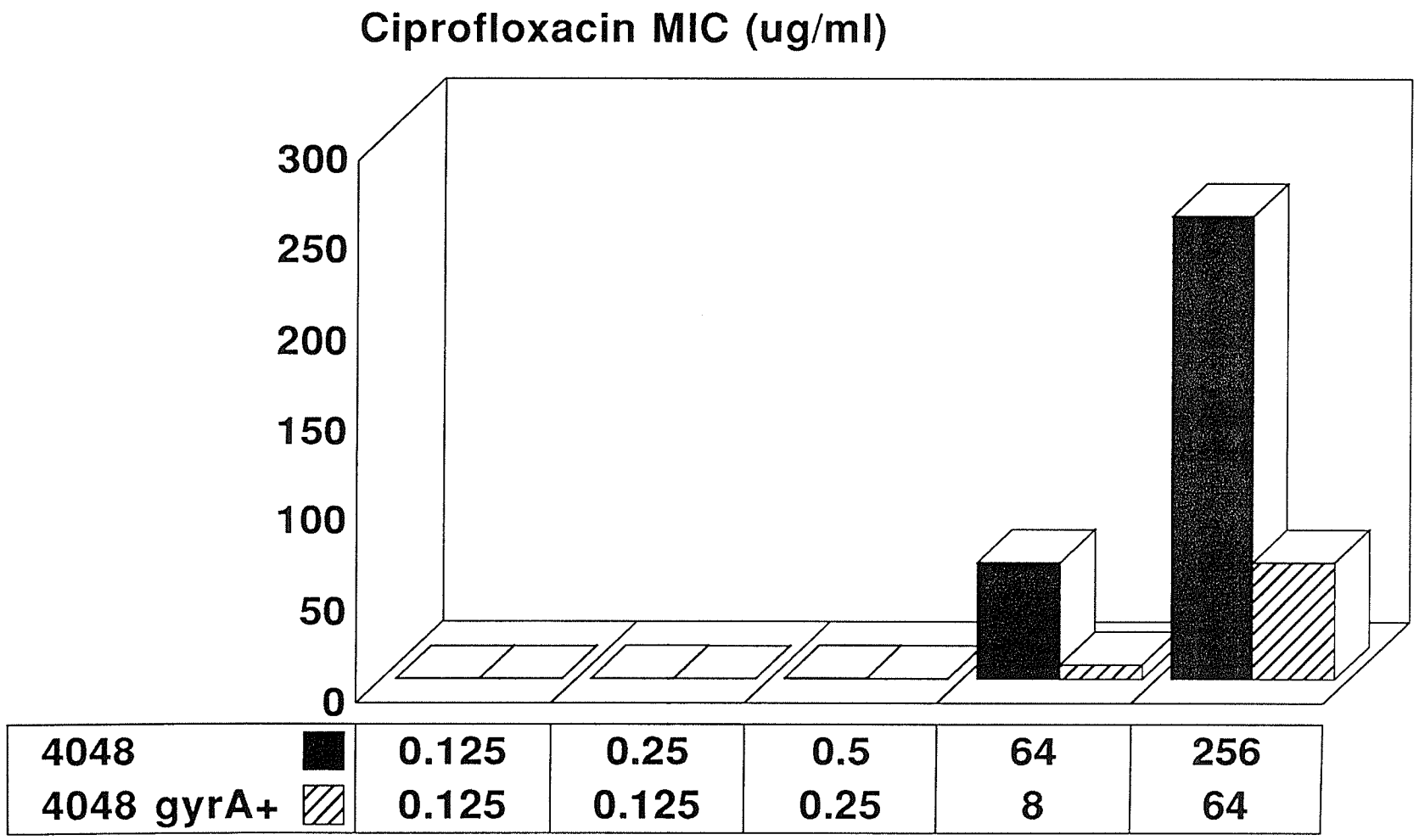


Figure 27

Complementation of P.aeruginosa with E.coli gyrA+

(strain 4048 at various Ciprofloxacin MICs)



≥0.5μg/ml both gyrA and non-gyrA mutations occurred (Figure 27).

These data suggest that at low level resistant MICs, laboratory derived fluoroquinolone-resistant strains initially represented gyrA mutants. However, at higher MICs non-gyrA mutation(s) must also be contributing to resistance.

10. Complementation with Opr F

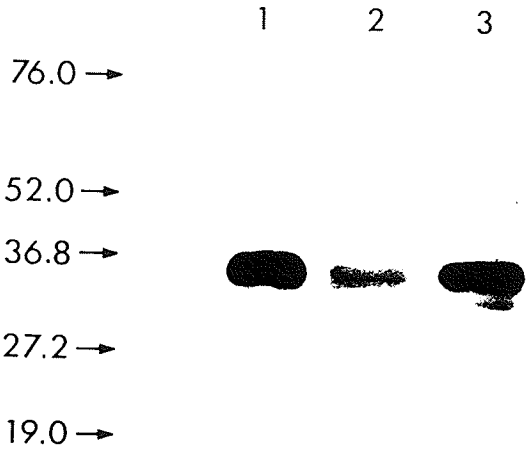
We hypothesized that the abnormal morphology (coccal shape and large outer-membrane blebs) of the laboratory-derived fluoroquinolone-resistant isolates was due to a reduction in outer-membrane protein F (Opr F). In addition, we believed that Opr F functioned as a porin and thus allowed the entry of antibiotics such as fluoroquinolones, beta-lactams and/or tetracycline and chloramphenicol. To verify that these hypotheses were true, we inserted a plasmid, pRW5, into fluoroquinolone resistant laboratory isolates and tested for function. As previously mentioned plasmid pRW5 is an expression vector that has Opr F (from wild-type P. aeruginosa) cloned into it (Table 9) [166,167]. This construct expresses functional Opr F in P. aeruginosa to wild-type levels (166).

Figure 28 displays an immunoblot (probed with a monoclonal to Opr F) of laboratory-derived fluoroquinolone resistant mutants and the same strains complemented with pRW5. pRW5 consistently restored Opr F to wild-type levels.

Antibiotic susceptibilities (Table 18) demonstrated 0-2 fold reduction in MICs of fluoroquinolones when fluoroquinolone resistant strains (3854r, 4047r, 4048r) were complemented with

pRW5. MICs to beta-lactams, chloramphenicol, and tetracyclines were reduced by only 2-4 fold in strains complemented with pRW5. The susceptibilities of imipenem and aminoglycosides where generally unaffected.

Figure 28. Immunoblot of Laboratory Derived Fluoroquinolone-Resistant Isolate 4047r With and Without pRW5[a]



Lane 1 - 4047, 2 - 4047r, 3 - 4047r (pRW5)

[a] = Numbers on the left indicate the prestained molecular weight (10^3) of standard proteins

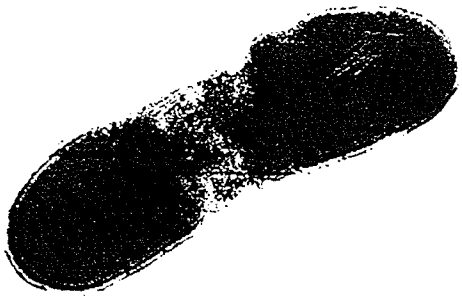
Table 18. Susceptibilities of Laboratory Derived Fluoroquinolone-Resistant P. aeruginosa With and Without Opr F

	MIC (μg/ml) Isolates								
Antibiotic	3854	3854r	3854 OprF	4047	4047r	4047 OprF	4048	4048r	4048 OprF
Fluoroquinolones									
Ciprofloxacin	0.25	1024	512	1.0	1024	1024	0.125	1024	512
Norfloxacin	0.5	2048	2048	2.0	2048	1024	0.5	2048	1024
Pefloxacin	2.0	4096	2048	4.0	2048	1024	1.0	4096	2048
ß-lactams									
Cefoxitin	64	512	128	32	256	64	64	1024	256
Cefotaxime	32	256	128	32	512	256	32	256	64
Piperacillin	16	128	32	16	256	128	16	128	64
Imipenem	2.0	4.0	4.0	4.0	8.0	8.0	2.0	4.0	4.0
Aminoglycosides									
Gentamicin	4.0	8.0	4.0	4.0	4.0	4.0	4.0	8.0	8.0
Tobramycin	1.0	2.0	2.0	1.0	2.0	1.0	2.0	2.0	1.0
Others									
Chloramphenicol	32	256	64	32	512	128	64	512	128
Tetracycline	32	1024	256	64	1024	512	32	512	128

Figures 29-31 display the uptake of fluoroquinolones into laboratory-derived fluoroquinolone mutants complemented with pRW5. All three isolates demonstrated no changes in uptake with complementation.

These data suggest that restoring OprF has little influence on uptake of fluoroquinolones, and Opr F by itself is not responsible for uptake. However, it appears to have a small role in the uptake of beta-lactams, chloramphenicol and tetracycline. Outer membrane protein F also appears to be important in maintaining cell shape as evidenced by the restoration of the bacillary morphology in fluoroquinolone-resistant isolates containing pRW5 (Figure 32). Collectively these data suggest that OprF is a dual function outer-membrane protein responsible for structure and antibiotic uptake, but absence of Opr F is not sufficient to explain resistance.

Figure 32. Influence of pRW5 on Morphology (Strain 4047r)



EM at magnification of 27,500X

Figure 29

Influence of pRW5 on Fluoroquinolone Uptake

(14C Pefloxacin - Strain 3854)

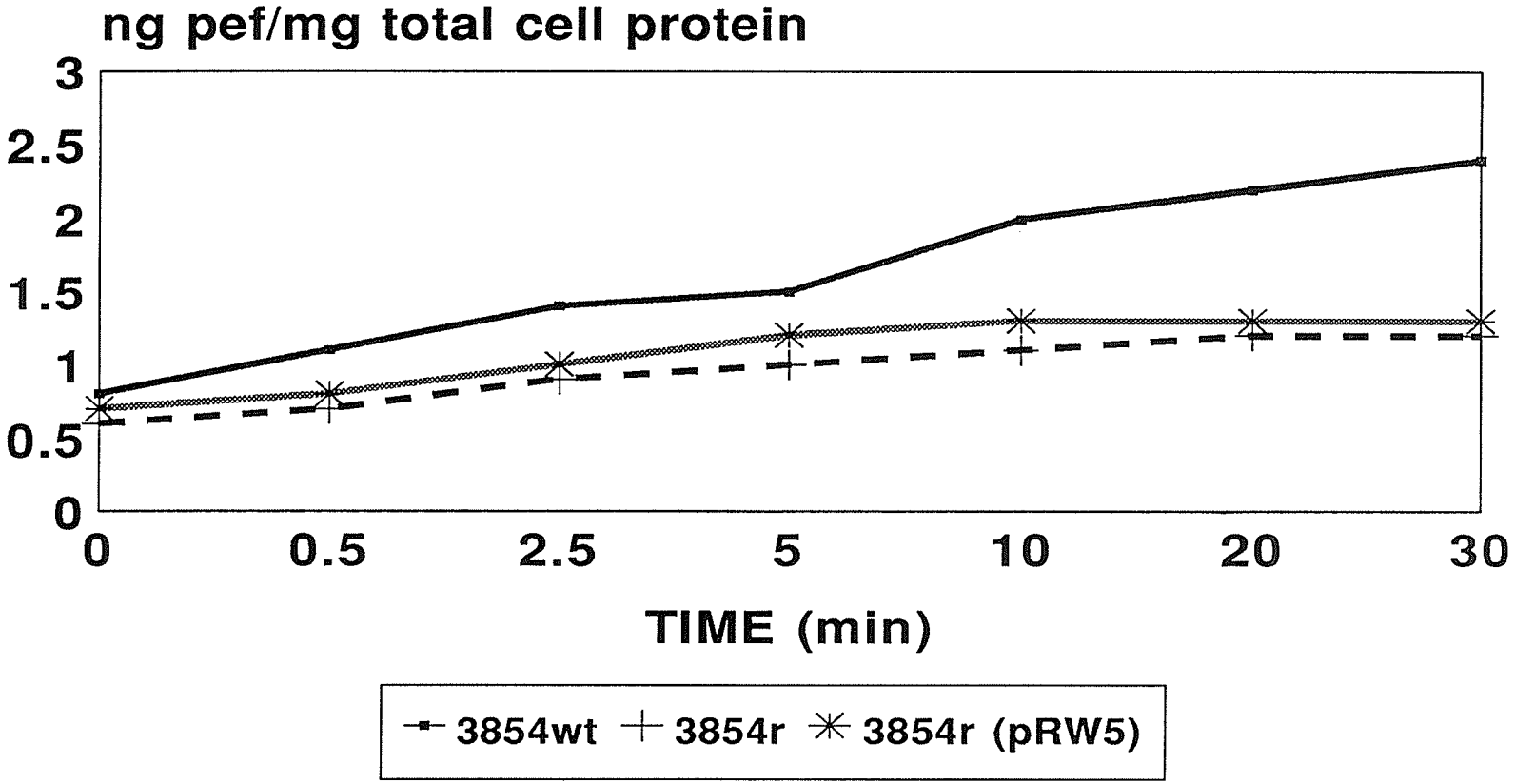


Figure 30

Influence of pRW5 on Fluoroquinolone Uptake

(14C Pefloxacin - Strain 4047)

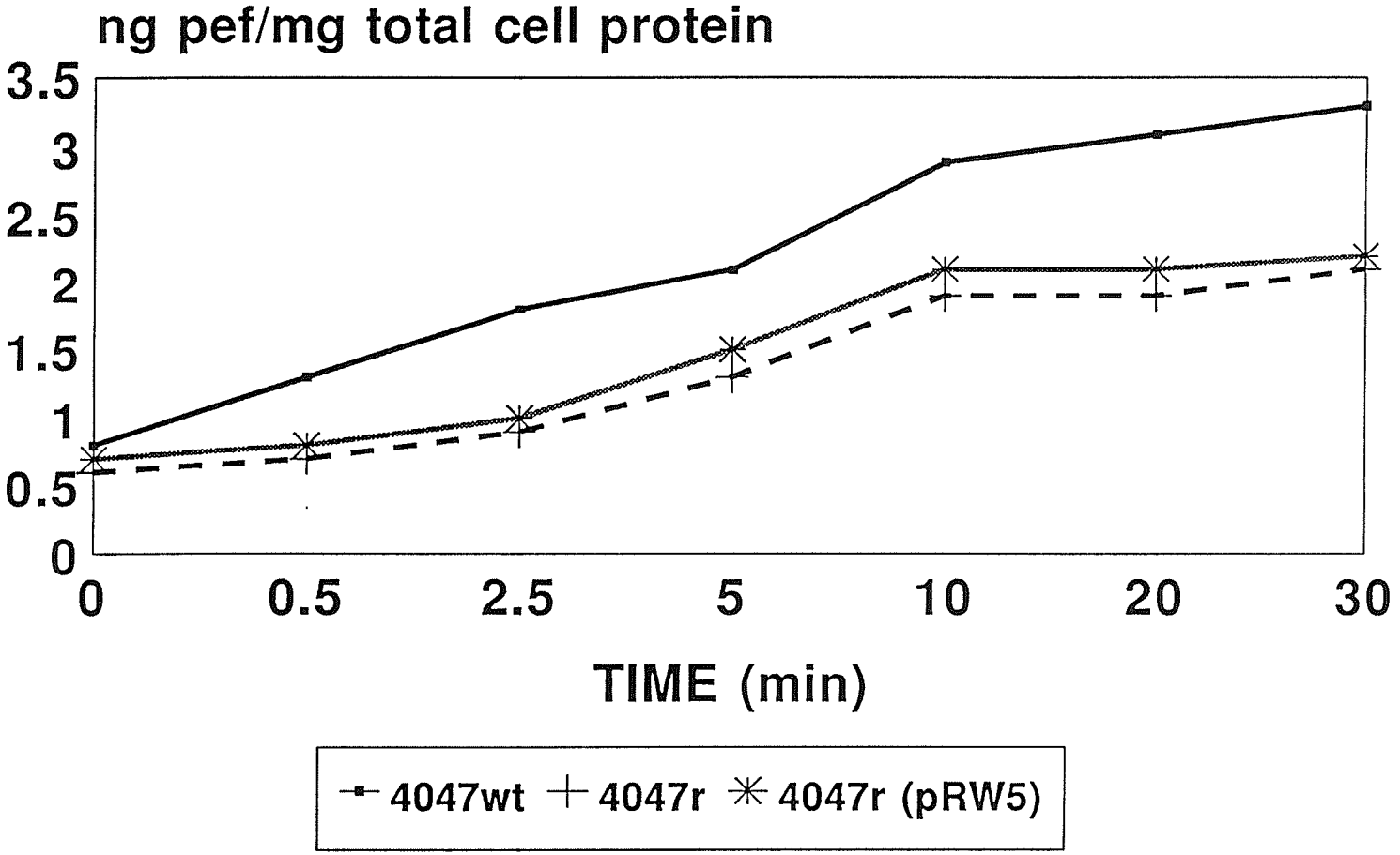
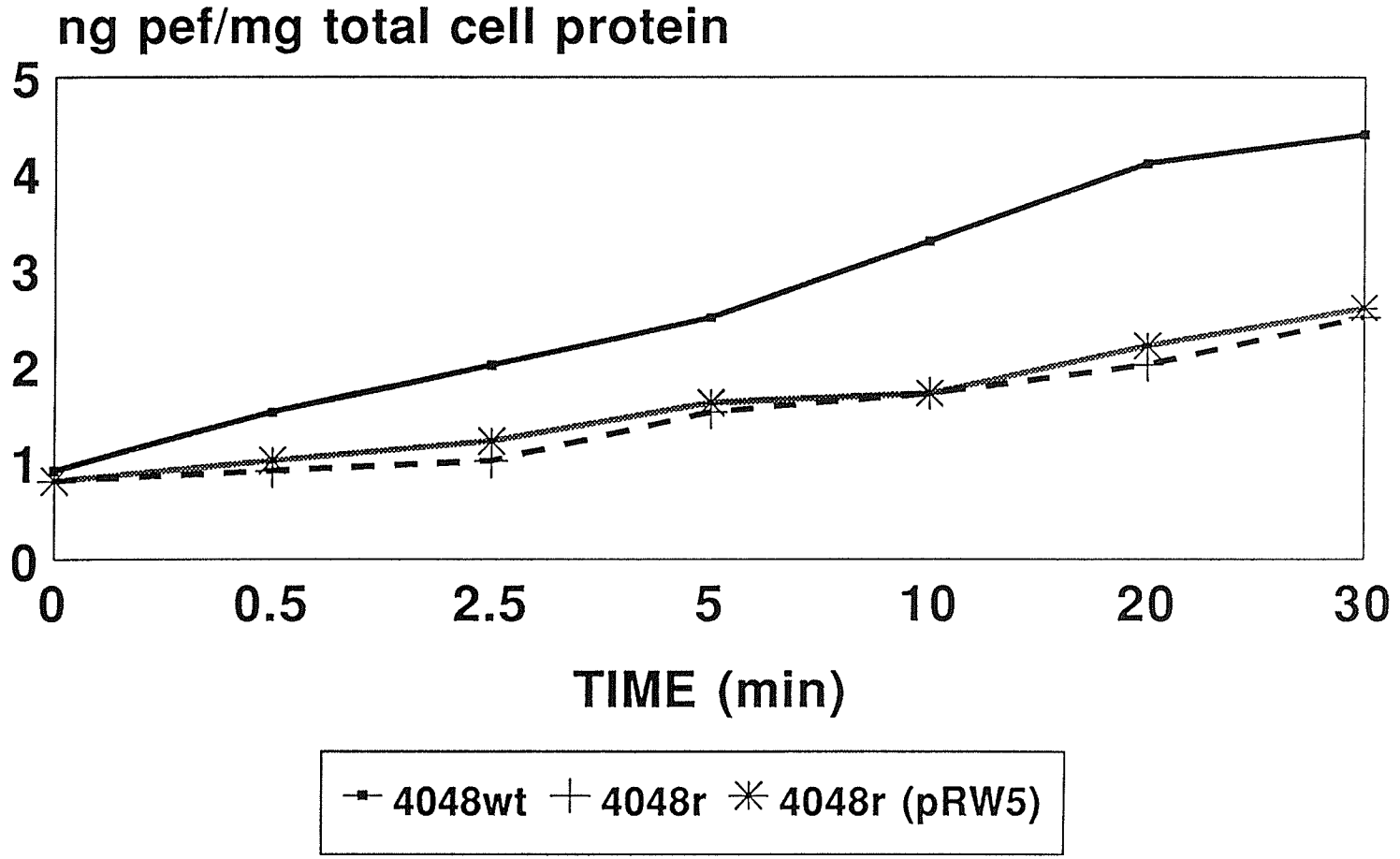


Figure 31

Influence of pRW5 on Fluoroquinolone Uptake

(14C Pefloxacin - Strain 4048)



11. DNA Sequence Analysis of oprF

Based upon the published sequence (173a), primers were designed (4 for the antisense and 4 for the sense strands) to sequence oprF from resistant strains using cycle sequencing (172). The DNA sequences are listed in Table 19. No changes were found in either the promoter region (TTG TCT and TAA ACT) or in the open reading frame of the mature peptide in any of the fluoroquinolone resistant mutants. All fluoroquinolone resistant mutants, however, contained mutation(s) in the 24 amino acid signal peptide. The signal peptide contains on initiation methionine, followed by two positively charged lysine residues. There is a hydrophobic core of approximately 13 residues followed by the sequence Ala-Ser-Ala which is thought to be a potential cleavage site for a procaryotic signal peptidase (173b). In all three laboratory derived fluoroquinolone-resistant isolates the Ala (residue 19 of signal peptide) was deleted.

Table 19. DNA Sequence of OprF

Strain														
3854	-128	GCG	ACC	GAA	ACA	TAG	TTG	GGT	AAA	TAT	TGT	CTC	TCT	ATG
3854r		---	---	---	---	---	---	---	---	---	---	---	---	---
4047		---	---	---	---	---	---	---	---	---	---	---	---	---
4047r		---	---	---	---	---	---	---	---	---	---	---	---	---
4048		---	---	---	---	---	---	---	---	---	---	---	---	---
4048r		---	---	---	---	---	---	---	---	---	---	---	---	---

Strain																
3854	-84	CGG	GAA	GTT	CTG	ATA	AAC	TTG	CCA	CCC	AAG	TTG	TGC	GGC	TGA	TTG	TTG
3854r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4047		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4047r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4048		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4048r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---

Table 19 (cont'd)

Strain																
3854	-36	GAC	AAC	TAA	CTG	ACC	ATC	AAG	ATG	GGG	ATT	TAA	CGG	ATG	AAA	CTG	AAG
3854r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4047		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4047r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4048		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4048r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---

Strain													met	lys	leu	lys
3854	13	AAC	ACC	TTA	GGC	GTT	GTC	ATC	GGC	TCG	CTG	GTT	GCC	GCT	TCG	GCA	ATG
3854r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4047		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4047r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4048		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4048r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---

Table 19 (cont'd)

Strain		asn	thr	leu	gly	val	val	ile	gly	ser	leu	val	ala	ala	ser	ala	met
3854	61	AAC	GCC	TTC	GCC	CAG	GGC	CAG	AAC	TCG	GTA	GAG	ATC	GAA	GCC	TTC	GGC
3854r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	XXX	---
4047		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4047r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	XXX	---
4048		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4048r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	XXX	---

Strain		asn	ala	phe	ala	gln	gly	gln	asn	ser	val	glu	ile	glu	ala	phe	gly
3854	109	AAG	CGC	TAC	TTC	ACC	GAC	AGC	GTT	CGC	AAC	ATG	AAG	AAC	GCT	GAC	CTG
3854r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4047		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4047r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4048		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4048r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---

Table 19 (cont'd)

Strain		lys	arg	tyr	phe	thr	asp	ser	val	arg	asn	met	lys	asn	ala	asp	leu
3854	157	TAC	GGC	GGC	TCG	ATC	GGC	TAC	TTC	CTG	ACC	GAC	GAC	GTC	GAG	CTG	GCT
3854r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4047		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4047r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4048		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4048r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---

Strain		tyr	gly	gly	ser	ile	gly	tyr	phe	leu	thr	asp	asp	val	glu	leu	ala
3854	205	CTG	TCC	TAC	GGT	GAG	TAC	CAC	GAT	GTT	CGT	GGC	ACC	TAC	GAA	ACC	GGC
3854r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4047		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4047r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4048		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4048r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---

Table 19 (cont'd)

Strain		leu	ser	tyr	gly	glu	tyr	his	asp	val	arg	gly	thr	tyr	glu	thr	gly
3854	253	AAC	AAG	AAG	GTC	CAT	GGC	AAC	CTG	ACC	TCC	CTG	GAC	GCC	ATC	TAC	CAC
3854r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4047		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4047r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4048		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4048r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---

Strain		asn	lys	lys	val	his	gly	asn	leu	thr	ser	leu	asp	ala	ile	tyr	his
3854	301	TTC	GGT	ACC	CCG	GGC	GTA	GGT	CTG	CGT	CCG	TAC	GTG	TCG	GCT	GGT	CTG
3854r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4047		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4047r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4048		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4048r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---

Table 19 (cont'd)

Strain		phe	gly	thr	pro	gly	val	gly	leu	arg	pro	tyr	val	ser	ala	gly	leu
3854	349	GCT	CAC	CAG	AAC	ATC	ACC	AAC	ATC	AAC	AGC	GAC	AGC	CAA	GGC	CGT	CAG
3854r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4047		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4047r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4048		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4048r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---

Strain		ala	his	gln	asn	ile	thr	asn	ile	asn	ser	asp	ser	gln	gly	arg	gln
3854	397	CAG	ATG	ACC	ATG	GCC	AAC	ATC	GGC	GCT	GGT	CTG	AAG	TAC	TAC	TTC	ACC
3854r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4047		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4047r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4048		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4048r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---

Table 19 (cont'd)

Strain		gln	met	thr	met	ala	asn	ile	gly	ala	gly	leu	lys	try	tyr	phe	thr
3854	445	GAG	AAC	TTC	TTC	GCC	AAG	GCC	AGC	CTC	GAC	GGC	CAG	TAC	GGC	CTG	GAG
3854r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4047		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4047r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4048		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4048r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---

Strain		glu	asn	phe	phe	ala	lys	ala	ser	leu	asp	gly	gln	tyr	gly	leu	glu
3854	493	AAG	CGT	GAC	AAC	GGT	CAC	CAG	GGT	GAG	TGG	ATG	GCT	GGC	CTG	GGC	GTC
3854r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4047		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4047r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4048		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4048r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---

Table 19 (cont'd)

Strain		lys	arg	asp	asn	gly	his	gln	gly	glu	trp	met	ala	gly	leu	gly	val
3854	541	GGC	TTC	AAC	TTC	GGT	GGT	TCG	AAA	GCC	GCT	CCG	GCT	CCG	GAA	CCG	GTT
3854r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4047		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4047r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4048		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4048r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---

Strain		gly	phe	asn	phe	gly	gly	ser	lys	ala	ala	pro	ala	pro	glu	pro	val
3854	589	GCC	GAC	GTT	TGC	TCC	GAC	TCC	GAC	AAC	GAC	GGC	GRC	TGC	GAC	AAC	GTC
3854r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4047		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4047r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4048		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4048r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---

Table 19 (cont'd)

Strain		ala	asp	val	cys	ser	asp	ser	asp	asn	asp	gly	val	cys	asp	asn	val
3854	637	GAC	AAG	TGC	CCG	GAC	ACC	CCG	GCC	AAC	GTC	ACC	GTT	GAC	GCC	AAC	GGC
3854r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4047		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4047r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4048		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4048r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---

Strain		asp	lys	cys	pro	asp	thr	pro	ala	asn	val	thr	val	asp	ala	asn	gly
3854	685	TGC	CCG	GCT	GTC	GCC	GAA	GTC	GTA	CGC	GTA	CAG	CTG	GAC	GTG	AAG	TTC
3854r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4047		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4047r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4048		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4048r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---

Table 19 (cont'd)

Strain		cys	pro	ala	val	ala	glu	val	val	arg	val	gln	leu	asp	val	lys	phe
3854	733	GAC	TTC	GAC	AAG	TCC	AAG	GTC	AAA	GAG	AAC	AGC	TAC	GCT	GAC	ATC	AAG
3854r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4047		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4047r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4048		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4048r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---

Strain		asp	phe	asp	lys	ser	lys	val	lys	glu	asn	ser	tyr	ala	asp	ile	lys
3854	781	AAC	CTG	GCC	GAC	TTC	ATG	AAG	CAG	TAC	CCG	TCC	ACT	TCC	ACC	ACC	GTT
3854r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4047		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4047r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4048		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4048r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---

Table 19 (cont'd)

Strain		asn	leu	ala	asp	phe	met	lys	gln	tyr	pro	ser	thr	ser	thr	thr	val
3854	829	GAA	GGT	CAT	ACC	GAC	TCC	GTC	GGT	ACC	GAC	GCT	TAC	AAC	CAG	AAG	CTG
3854r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4047		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4047r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4048		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4048r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---

Strain		glu	gly	his	thr	asp	ser	val	gly	thr	asp	ala	tyr	asn	gln	lys	leu
3854	877	TCC	GAG	CGT	CGT	GCC	AAC	GCC	GTT	CGT	GAC	GTA	CTG	GTC	AAC	GAG	TAC
3854r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4047		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4047r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4048		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4048r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---

Table 19 (cont'd)

Strain		ser	glu	arg	arg	ala	asn	ala	val	arg	asp	val	leu	val	asn	glu	tyr
3854	925	GGT	GTG	GAA	GGT	GGT	CGC	GTG	AAC	GCT	GTC	GGT	TAC	GGC	GAG	TCC	CGC
3854r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4047		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4047r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4048		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4048r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---

Strain		gly	val	glu	gly	gly	arg	val	asn	ala	val	gly	tyr	gly	glu	ser	arg
3854	973	CCG	GTT	GCC	GAC	AAC	GCC	ACC	GCT	GAA	GGC	CGC	GCT	ATC	AAC	CGT	CGC
3854r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4047		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4047r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4048		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4048r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---

Table 19 (cont'd)

Strain		pro	val	ala	asp	asn	ala	thr	ala	glu	gly	arg	ala	ile	asn	arg	arg
3854	1021	GTT	GAA	GCC	GAA	GTA	GAA	GCC	GAA	GCC	AAG	TAA	TCG	GCT	GAG	CCT	TCA
3854r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4047		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4047r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4048		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4048r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---

Strain		val	glu	ala	glu	val	glu	ala	glu	ala	lys	***					
3854	1069	AAG	AAA	AAC	CGG	CCC	AGG	CCG	GGT	TTT	TCT	TTG	CCT	GGA	AAA	AGA	CCG
3854r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4047		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4047r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4048		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4048r		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---

Table 19 (cont'd)

Strain		
3854	1117	CTC GTC AGG CGC TCA GGG
3854r		--- --- --- --- --- ---
4047		--- --- --- --- --- ---
4047r		--- --- --- --- --- ---
4048		--- --- --- --- --- ---
4048r		--- --- --- --- --- ---

X deletion of nucleotide

DISCUSSION

1. Fluoroquinolone Resistance in P. aeruginosa

Our experimental hypothesis was that very high concentrations of fluoroquinolone resistance would be associated with both DNA gyrase and permeability changes. In addition, we believed that these permeability changes would be associated with a multiple antibiotic resistant (Mar) phenotype. We compared the characteristics of and mechanisms of fluoroquinolone resistance in our laboratory-derived fluoroquinolone-resistant isolates with clinical fluoroquinolone-resistant isolates obtained from patients receiving fluoroquinolone therapy.

2. Clinical Fluoroquinolone Resistant Isolates

Results with clinical fluoroquinolone-resistant isolates demonstrated resistance to fluoroquinolones but unchanged susceptibility to other antibiotics (Table 12). These data are consistent with fluoroquinolone uptake experiments which suggested no difference in uptake between fluoroquinolone-sensitive (wild-type) and clinical fluoroquinolone-resistant isolates (Figure 15). In addition, morphology, virulence assessed by adherence, LPS, and outer-membrane protein analysis demonstrated no differences between clinical fluoroquinolone resistant isolates compared to their fluoroquinolone-sensitive pairs (Figure 21). Complementation studies with wild-type E. coli gyrase (which is dominant over its mutant allele) conferred fluoroquinolone-susceptibility to

all fluoroquinolone-resistant clinical isolates demonstrating that they were gyrA mutants.

It is not suprising that the clinical fluoroquinolone-resistant isolates represented simple gyrA mutants, as all isolates were obtained from patients on fluoroquinolones for a short duration (approximately 7 days). Although no sequence data are available to identify the specific sites of mutation, one could speculate that these are minor amino acid changes near the N-terminal region of gyrA which reduce fluoroquinolone binding to the A subunit of DNA gyrase (173b).

In clinical fluoroquinolone-resistant isolates the stability of fluoroquinolone resistance has been studied (135, 136). Daikos et al. (135) reported that after 5-15 passages on drug-free medium, all 3 of their fluoroquinolone-resistant isolates reverted to full fluoroquinolone susceptibility. Isolates with outer-membrane changes required longer time to revert to full susceptibility. Diver et al. (136) studied the generation of in-vitro revertants of resistant clinical isolates after 40 passages on antimicrobial-free media and described the development of 3 distinct classes of fluoroquinolone-resistance. Class 1 organisms returned to full fluoroquinolone susceptibility. Class 2 isolates remained unchanged, while class 3 isolates demonstrated partial reversion to fluoroquinolone susceptibility. All our clinical fluoroquinolone resistant isolates maintained their resistance to fluoroquinolones after 40 passages on antibiotic-free media. That is, they likely represented class

2 fluoroquinolone-resistant isolates as per the Diver et al. (136) classification.

Several published studies are available assessing the characteristics and possible mutation sites of clinical fluoroquinolone-resistant P. aeruginosa (128,130-132,135-137,139-140,144-146). DNA gyrase alterations have been shown to be responsbile for fluoroquinolone-resistance in several cases (128,130-132,135-137,139-140,145). DNA gyrase mutants of P. aeruginosa display similar growth rates and morphology as wild-type organisms. In addition, they display the same antibiotic susceptibility profile (except for resistance to fluoroquinolones) and similar fluoroquinolone uptake profiles as wild-type organisms. To our knowledge the virulence of clinical fluoroquinolone resistant isolates has not been studied. The outer-membrane (LPS and Opr's) of clinical DNA gyrase mutants is unchanged compared to wild-type isolates. Our clinical fluoroquinolone resistant isolates represented typical gyrA mutants with similar antibiotic susceptibility profile, growth rate, morphology, virulence, fluoroquinolone uptake and outer-membrane profile as wild-type organisms.

Permeability mutants have also been reported with clinical fluoroquinolone-resistant isolates (130,135,136,138,144-146). Permeability mutants selected by fluoroquinolones may demonstrate resistance to fluoroquinolones alone (131,132,135,136), or exhibit multiple-antibiotic-resistance (Mar), including many beta-lactams, chloramphenicol and tetracycline (136,139,144,145). Generally

speaking, these permeability mutants remain sensitive to imipenem and aminoglycosides, however, occasionally fluoroquinolone derived permeability mutants have been characterized with resistance to imipenem (138,147). Outer-membrane changes selected by fluoroquinolones and possibly responsible for fluoroquinolone resistance include LPS alterations (131,136) reduced Opr D1 and Opr H1 (137) and diminished or absent 31-32 KDa band (135), while multiple-antibiotic-resistance include alterations in Opr F (139,146,147), reduced 22 and 35 KDa bands (136), increase in several bands in 38-48 KDa region (136,139), reduced Opr D1 and H1 (137), and LPS changes (137). Finally, examples of isolates possessing both DNA gyrase changes and altered permeability have been reported (131,136,138,139). Our clinical isolates did not demonstrate any permeability changes as evidenced by similar morphology, fluoroquinolone uptake, and outer membrane profile compared with wild-type isolates.

3. Laboratory Derived Fluoroquinolone Resistant Mutants

Numerous studies have been published assessing the characteristics and possible sites of mutation in laboratory derived fluoroquinolone-resistant P. aeruginosa (130,131,133, 140-143). Fluoroquinolone-resistant laboratory isolates were derived either by selecting spontaneous mutants through plating on fluoroquinolone containing media (130,133,140-142) or by isertional mutagenesis with fluoroquinolone selection

(131). These studies characterized isolates as being DNA gyrase mutations (130,133,140,143), permeability mutants (131,139,173) or both (142). DNA gyrase mutants demonstrated normal morphology and growth rates, resistance only to fluoroquinolones, no change in fluoroquinolone uptake and an unchanged outer-membrane compared to wild-type. Laboratory-derived permeability mutants selected by fluoroquinolones demonstrate resistance to fluoroquinolones (130,140,143) or multiple-antibiotic-resistance including several beta-lactams, chloramphenicol and tetracycline. In addition, a recently characterized nfxC permeability mutant demonstrated resistance to fluoroquinolones, imipenem and chloramphenicol and hypersusceptibility to beta-lactams and aminoglycosides (141). The majority of these permeability mutants were susceptible to aminoglycosides, although exceptions were observed (142).

Outer-membrane changes that have been associated with laboratory-derived fluoroquinolone resistant permeability mutants include a new 54KDa OMP (141,143), relative reduction in Opr F (142), loss of Opr G (25.5kd) (131), loss of a 40KDa band (131), and LPS changes (142).

The major work of this thesis involved the creation and characterization of laboratory-derived fluoroquinolone resistant mutants of P. aeruginosa by serially passaging wild-type organisms on agar containing increasing concentrations of fluoroquinolone. We hypothesized that continued passage on fluoroquinolone agar would lead to a Mar phenotype. Our laboratory-derived fluoroquinolone resistant mutants had both

DNA gyrase changes (as evidenced by the partial restoration of fluoroquinolone susceptibility upon complementation with E. coli gyrA) and permeability alterations. In passaging our isolates on fluoroquinolone containing agar, none of these properties (altered morphology and growth, reduced fluoroquinolone uptake, reduced adherence, multiple-antibiotic-resistance and outer-membrane protein changes) occurred until 4-32 fold increases in MIC to fluoroquinolones were achieved. As an example, strain 4047 derivatives appeared to be DNA gyrase mutants when they had ciprofloxacin MICs ≤8μg/ml, as evidenced by complementation with E. coli gyrA (Figure 26). Whether the DNA gyrase mutations at ciprofloxacin MICs of 2μg/ml, 4μg/ml and 8μg/ml (wild-type MIC 1μg/ml) represent 1 or more mutations is unclear. With continued serial passage, when strain 4047 derivatives achieved ciprofloxacin MICs of 16μg/ml, they demonstrated a Mar phenotype with resistance not only to fluoroquinolones but also to chemically unrelated classes of antibiotics including beta-lactams, chloramphenicol and tetracycline (Figure 9). Evidence is presented here that the mutations(s) conferring higher levels of fluoroquinolone resistance were associated with altered morphology, reduced adherence, reduced fluoroquinolone uptake, alterations in outer-membrane proteins and a Mar phenotype.

4. Mar Phenotype

Piddock et al. (139) have recently described a post-

therapy enoxacin-resistant P. aeruginosa mutant with very similar biological characteristics to our fluoroquinolone derived Mar mutants. Their post-therapy enoxacin-resistant sputum isolate demonstrated a Mar phenotype with increased resistance to fluoroquinolones but also beta-lactams (cefsulodin, cefotaxime, ceftazidime, carbenicillin, mezlocillin), chloramphenicol and tetracycline. The isolate remained susceptible to aminoglycosides and imipenem. This Mar phenotype grew more slowly than its pre-therapy isolate. Outer-membrane proteins were altered, with OprF totally absent as confirmed by immunoblots using monoclonal antibody. These investigators suggested that since the acquisition of fluoroquinolone resistance was associated with the development of a Mar phenotype, these multiple phenotypic changes were genetically linked (139). We believe that a similar scenario may have occurred in our fluoroquinolone derived Mar mutants. That is, initial fluoroquinolone exposure led to the development of fluoroquinolone-resistant mutants. Continued serial fluoroquinolone exposure led to genetically linked changes resulting in a Mar phenotype. A summary below describes the resulting characteristics after correction of mutations (eg. gyrA or OprF) in laboratory derived fluoroquinolone-resistant strains:

Complementation with: opr F gene	Complementation with: gyr A gene	Resultant characteristics:
No	No	Quinolone resistance Mar phenotype Defective shape
Yes	No	Quinolone resistance Partial Mar phenotype Normal shape
No	Yes	Partial Quinolone resistance Mar phenotype Defective shape

These genetically linked changes may have resulted in reduced Opr F, which lead to reduced antibiotic uptake and a Mar phenotype.

We believe that exposure to fluoroquinolones resulting in not only fluoroquinolone resistance but also multiple antibiotic resistance may occur in one of two ways (37): (a) either the resistant mutants contain an altered regulatory locus that influences several other genes involved in antibiotic resistance; or (b) a single dominant mutation which stabilizes other mutations and upon reversion of this dominant mutation leads to the loss of the effects of the other mutations. An example of the former is marA in E. coli where mutations in the putative regulatory locus affect the expression of other loci in the marRAB operon (127). marA is a recently identified chromosomal locus in E. coli, which when activated leads to a Mar phenotype. This locus has been identified as an operon, the marRAB operon (127). In this operon, MarO is hypothesized to be the putative

promoter/operator, marR the putative repressor, marA the putative positive transcriptional regulator and marB, function unknown. Although presently unclear, it appears that exposure to antibiotics (possible fluoroquinolones) selects out organisms that have mutations in marO and/or marR. The resulting lack of repression on marA results in increased marA expression. MarA subsequently, directly or indirectly increases the expression of micF (an antisense RNA) which hybridized with OmpF-MRNA resulting in instability of OmpF-MRNA and reduced OmpF. Decreased OmpF is partially but not totally responsible for the Mar phenotype.

5. Outer Membrane Protein F (OprF)

The observation of reduced signals of OprF in our laboratory derived fluoroquinolone isolates in protein gels (Figure 22) and immunoblots using a monoclonal to OprF (Figure 24) led us to hypothesize that changes in OprF may be responsible for the gross morphological alterations in these Mar phenotypes. This hypothesis was based on the observations that Opr F has important structural functions in the outer membrane (174) and forms strong noncovalent interactions with both peptidoglycan and LPS (173,175). Thus, alterations in Opr F may cause detachment of the outer membrane from peptidoglycan resulting in protrusion and bleb formation (173). DNA sequencing studies have revealed highest homology between Opr F of P. aeruginosa and Omp A (major structural protein) of E. coli. The importance of Opr F in serving a

structural role was confirmed when laboratory derived fluoroquinolone mutants complemented with pRW5 (OprF expression vector) regained wild-type bacillary morphology (Figure 32).

Upon sequencing the promoter region, the signal peptide and the open reading frame of OprF, all laboratory derived fluoroquinolone resistant mutants displayed the same mutation in the signal peptide (Table 19). All laboratory mutants possessed the deletion of an alanine at position 19 of the 24 amino acid signal peptide. It is possible that this deleted alanine serves as a critical residue for cleavage by signal peptidases (176). If inefficient cleavage of the signal peptide occurs, then localization of the mature protein into the outer-membrane would be reduced. Even though all laboratory derived fluoroquinolone resistant strains displayed the same deletion at position 19, they demonstrated different immunoblots. Reasons why this may occur include the varying stability of OprF once translated. Different concentrations of endogenous proteases may lead to different levels of functional OprF.

Regarding OprF's role in fluoroquinolone uptake and the development of a Mar phenotype, the literature is divided. Presently, data exist suggesting that OprF is a porin that may lead to antibiotic resistance (146,177,178) and also that OprF is not a porin and is not responsible for antibiotic resistance (132,179). Upon complementing our laboratory derived fluoroquinolone-resistant mutants with pRW5 and

testing for antibiotic susceptibility, we observed that OprF had only a very minor role to play in fluoroquinolone uptake and resistance. It did, however, act as a porin for beta-lactam, chloramphenicol and tetracycline uptake. Therefore, these data suggest that OprF in fact does have a dual role, one structural, and second as a porin for antibiotic uptake. It should, however, be mentioned that several other outer-membrane proteins in the 43-66 KDa range were altered in these mutants. Whether these proteins are also involved in antibiotic uptake and the development of a Mar phenotype is unclear.

The observation of the development of a Mar phenotype after serial exposure to fluoroquinolones is important. As previously discussed, the Mar phenotype has been described in clinical isolates (139). P. aeruginosa infections treated with a fluoroquinolone could not only develop resistance to fluoroquinolone but also to other antibiotics. Thus, the original sensitive isolate after prolonged fluoroquinolone therapy, may develop a Mar phenotype resistant to various chemically unrelated antibiotics. A patient treated with an agent such as a fluoroquinolone could result in an isolate with altered permeability to beta-lactams, tetracycline, chloramphenicol and a Mar phenotype. This phenotype may or may not be sensitive to aminoglycosides or carbapenems.

Although a locus analogous to the recently identified mar locus in E. coli (127), has not yet been cloned in P. aeruginosa, we believe that such an operon exists. This

operon once activated leads to the development of a Mar phenotype (127). Whether the putative mar locus exists in P. aeruginosa and whether it is regulated directly or indirectly by fluoroquinolone requires study.

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LIST OF PUBLICATIONS PERTAINING TO THESIS

a) ABSTRACTS

1. Zhanel GG, Nicolle LE, Kim SO, Davidson RJ, Hoban DJ: Comparison of voided uroepithelial cell and vero cell assays in measuring the effect of ciprofloxacin of gentamicin on adherence of Pseudomonas aeruginosa. American Society for Microbiology (ASM), New Orleans, Louisiana, 1991.
2. Zhanel GG, Karlowsky JA, Davidson RJ, Hoban DJ, Nicolle LE: Acquisition of fluoroquinolone resistance in Pseudomonas aeruginosa is associated with reduced expression of pili and altered outer-membrane protein and lipopolysaccharide profiles. American Society for Microbiology (ASM), Atlanta, Georgia, 1993.
3. Zhanel GG, Karlowsky JA, Davidson RJ, Hoban DJ, Nicolle LE: Comparison of clinical and laboratory derived fluoroquinolone resistant mutants. Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC), New Orleans, Louisiana, 1993.
4. Zhanel GG, Karlowsky JA, Davidson RJ, Hoban DJ, Hancock REW, Nicolle LE: The role of Opr F of Pseudomonas aeruginosa on fluoroquinolone (FQ) resistance and multiple antibiotic resistance (Mar). Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC), Orlando, Florida, 1994.
5. Zhanel GG, Karlowsky JA, Davidson RJ, Hoban DJ, Nicolle LE: Development of multiple antibiotic resistant (Mar)

mutants of Pseudomonas aeruginosa after exposure to fluoroquinolones. Conjoint Meeting on Infectious Diseases, Montreal, Quebec, 1994.

b) PUBLICATIONS

1. Zhanel GG, Nicolle LE: Effects of subinhibitory antimicrobial concentrations on bacterial adherence in the urinary tract. Journal of Antimicrobial Chemotherapy 1992; 29:617-627.

2. Zhanel GG, Crampton J, Kim S, Nicolle LE, Davidson RJ, Hoban DJ: Antimicrobial activity of subinhibitory concentrations of fluoroquinolones against Pseudomonas aeruginosa as determined by the killing curve method and the postantibiotic effect (PAE). International Journal of Experimental and Clinical Chemotherapy 1992; 38:388-394.

3. Zhanel GG, Nicolle LE, Kim SO, Davidson RJ, Hoban DJ: Effect of subinhibitory concentrations (sub-MIC's) of ciprofloxacin and gentamicin on the adherence of Pseudomonas aeruginosa to Vero cells and voided uroepithelial cells. International Journal of Experimental and Clinical Chemotherapy 1993; 39:105-111.

4. Zhanel GG, Karlowsky JA, Saunders MH, Davidson RJ, Hoban DJ, Hancock REW, McLean I, Nicolle LE: Development of multiple antibiotic resistant (Mar) mutants of Pseudomonas aeruginosa after serial exposure to fluoroquinolone. Antimicrobial Agents and Chemotherapy 1994 (submitted).

APPENDIX A

A: **MEDIA**

1. Mueller-Hinton broth was used for susceptibility testing
2. Iso-Sensitest broth was used to grow organisms for uptake studies
3. Brain Heart Infusion broth was used to grow organisms prior to DNA extraction

B: **BUFFERS**

1. Phosphate buffered saline

   a) stock solution:

Na_2HPO_4	27.4g
NaH_2PO_4	7.87g
H_2O	QS to 1000ml

   b) working solution:

NaCl	8.5g
stock solution	40ml
H_2O	QS to 1000ml
filter sterilize	

2. T10E1

Tris-HCl	1.57g
EDTA	0.37g
H_2O	QS to 1000ml
pH 8.0	

3. 20x SSC

NaCl	175.3g
Sodium Citrate	88.2g
H_2O	QS to 1000ml
pH 7.0	

4. 5x TBE buffer

Tris base	121.1g
Boric acid	61.8g
EDTA	7.4g
H_2O	QS to 1000ml
pH 8.3	

LIST OF SUPPLIERS

1. Baxter-Canlab, Mississauga, Ontario, Canada
2. Beckman Instruments, Toronto, Ontario, Canada
3. Biorad Laboratories, Mississauga, Ontario, Canada
4. Boehringer Mannheim, Dorval, Quebec, Canada
5. Canadawide Scientific, Ottawa, Ontario, Canada
6. Fisher Scientific, Ottawa, Ontario, Canada
7. Gibco Canada, Burlington, Ontario, Canada
8. NEN Research Products, Mississauga, Ontario, Canada
9. Pharmacia Canada, Dorval, Quebec, Canada
10. Sigma Chemical Company, St. Louis, Missouri, USA