REVIEW 32
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Susceptibility and Resistance of *Pseudomonas aeruginosa* to Antimicrobial Agents

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1. INTRODUCTION

The treatment of *Pseudomonas aeruginosa* infections is a constant challenge for physicians. A combination of features such as ability to survive and spread in hostile environments, multiple virulence determinants, and intrinsic resistance to commonly used antibiotics and disinfectants makes this bacterium a major life-threatening pathogen. Particularly susceptible are those patients with altered host defenses or those who are debilitated by cystic fibrosis (CF), major surgery, traumatic wounds, severe burns, or neoplastic diseases. Indeed, *P. aeruginosa* is the third most common cause of nosocomial infections after *Escherichia coli* and *Staphylococcus aureus*, accounting for approximately 10% of all hospital-acquired infections. *P. aeruginosa* strains are not susceptible to many of the conventionally used antibiotics (Table I). This high intrinsic resistance has been ascribed to low outer-membrane permeability, due to certain structural features that distinguish *P. aeruginosa* from other common gram-negative bacteria (see section 2). In addition, *P. aeruginosa* may develop several mechanisms of resistance to oppose the action of antibiotics,

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TABLE I
Comparison of *P. aeruginosa* and *E. coli* Susceptibilities to Antibiotics

Antibiotic class	Representative antibiotics	MIC (μg/ml)[a]	
		E. coli	*P. aeruginosa*
β-lactams			
Aminopenicillins	Ampicillin	4	>128
α Carboxypenicillins	Carbenicillin/Ticarcillin	4	32–64
Acylureirodopenicillins	Piperacillin	2	2
	Azlocillin	16	4
Carbapenems	Imipenem	0.25	2
Cephems	Cefoperazone	0.1	4
	Cefsulodin	64	2
	Cefotaxime	0.03	32
	Ceftazidime	0.12	2
	Cefepime	0.04	2.5
Monobactams	Aztreonam	0.03	2–8
Aminoglycosides	Gentamicin	0.5	2
	Tobramycin	0.5	0.5
	Amikacin	2	4
Polymyxins	Colistin	1	4
Quinolones	Ciprofloxacin	0.025	0.1
Others	Tetracycline	2	32
	Chloramphenicol	4	64
	Erythromycin	64	200

[a]Normal MIC of strains lacking resistance transfer plasmids, derepressed chromosomal β-lactamase or increased intrinsic resistance due to porin alterations.

including modifications of the outer-membrane entry pathways, production of antibiotic-inactivating enzymes, and alterations of target-structures. The main antibiotics showing antipseudomonal activity belong to the β-lactam and aminoglycoside families. However, recently, fluoroquinolones such as ciprofloxacin have also proved to be useful against *P. aeruginosa*. Due to space limitations, no attempt will be made here to present an exhausting analysis of the complexity and the numerous facets of antibiotic action against *P. aeruginosa*. Instead emphasis will be placed on the main determinants of antibiotic activity and bacterial resistance for the above commonly used antibiotics.

2. β-LACTAMS

2.1. Antipseudomonal β-Lactams

During the last two decades, rigorous efforts of the pharmaceutical industry to improve on the properties of the first penicillin derivatives have led to a profusion of new β-lactams. Considering the diversity of their chemical structure, the traditional distinction between penicillins and cephalosporins has become inappropriate for the classification of these new products. In this review we have adopted the nomenclature suggested by Brown[1] which is based on chemical structure. The only classes of β-lactams discussed here will be those presenting consistent, therapeutically relevant antipseudomonal activity.

The first commercially available β-lactam to show activity against *P. aeruginosa* was carbenicillin. Another α-carboxypenicillin, ticarcillin, was from two to four times more active, allowing administration of lower doses and, accordingly, reduced platelet dysfunction and hypokalemia.[2] Despite this breakthrough in antibiotherapy, the clinical use of these two compounds against *P. aeruginosa* was limited by their moderate activity and high sensitivity to β-lactamase inactivation (Table I). Consequently, the emergence of resistant strains was rapid and led to frequent therapeutic failures. Subsequently, newer penams, with an extended spectrum of activity, were developed. The minimum inhibitory concentration (MIC) of the acylureidopenicillins, mezlocillin, piperacillin and azlocillin against *P. aeruginosa* were 20-fold lower than those of α-carboxypenicillins. However, these antibiotics still remained susceptible to hydrolysis by β-lactamases. This problem seems to have been overcome by development of a new generation of penams such as alpacillin and foramidocillin. Like the α-carboxypenicillins, ureidopenicillins show excellent synergy with aminoglycosides against *P. aeruginosa*.

Another class of β-lactams exhibiting excellent activity against *P. aeruginosa* are the carbapenems. These molecules have a double bond at the 2–3 position and a carbon atom instead of sulfur in the penem ring. While preserving the efficacy of penams against gram-positive bacteria, these substitutions extend the spectrum of activity to a level unmatched by any other class of antibiotic. The only commercially available compound of this group is imipenem, a semi-synthetic derivative of thienamycin. Its wide range of activity includes *Psedomonas* spp. with the exception of *P. maltophilia* and *P. cepacia*.

The name cephem refers to all the cephalosporins and cephamycins. The latter differ from cephalosporins by the presence of a methoxy moiety in the 7α position. None of the cephamycins shows good antipseudomonal activity and therefore, they will not be discussed here. The main cephems that are active against *P. aeruginosa* are the so-called third- and fourth-generation cephalosporins. Cefotaxime and ceftriaxone were the first (third-generation) cephalosporins to exhibit moderate antipseudomonal activity. However, cefoperazone, and especially ceftazidime, are the only third-generation agents with excellent activity against *P. aeruginosa*. New investigational products such as cefepime, cefpirome, E-1040, and BO-1341, called fourth-generation cephalosporins because they are all characterized by a quaternary nitrogen moiety at the 3 position, have proved to be promising antipseudomonal agents.[4–6] Cefsulodin, a cephem that, like piperacillin, has a piperazine side chain, is a unique cephalosporin in that its activity is limited to *P. aeruginosa*.

Azthreonam, a monobactam containing also the aminothiazole oxime side chain of the third- and fourth-generation cephalosporins, is exclusively active against gram-negative bacteria.[7] However, its efficacy against *P. aeruginosa* is moderate and clearly lower than that of the best antipseudomonal cephalosporins (Table I).

Although most of the newer cephalosporins also show *in vitro* synergy with aminoglycosides, physicians prefer to combine an aminoglycoside with a carboxy- or ureidopenicillin because this combination is potentially less nephrotoxic and less expensive.[8]

2.2. Determinants of Efficacy

The efficacy of each β-lactam molecule depends on its ability to access and subsequently inhibit essential inner membrane-bound enzymes, the penicillin-binding-proteins (PBP), which are necessary for the biosynthesis of peptidoglycan.[9] In gram-positive bacteria, β-lactams have relatively free access to these enzymes. However, in gram-negative microorganisms, the outer membrane is a major obstacle any antibiotic must overcome before reaching its target(s) (Fig. 1). In the case of β-lactams, once in the periplasm they must also resist the hydrolytic inactivation of β-lactamases. Thus, the efficacy of β-lactams in gram-negative bacteria results from the interplay of at least three factors: the rate of permeation across the outer membrane, the kinetic parameters of the β-lactamase–β-lactam interaction, and the affinity of the PBP for the β-lactam (Fig. 1). Zimmermann and Rosselet proposed a model to describe this complex interplay.[10] These authors postulated that the penetration of β-lactams into bacterial cells equilibrates at a steady state in which the rate of diffusion of β-lactams through the outer membrane is balanced by the hydrolysis of β-lactamase in the periplasmic space. These events can be described by the Fick's first law of diffusion and the Michaelis-Menten equation, respectively, in the following equation

$$V = C\cdot(S_o - S_p) = Vmax\cdot S_p/S_p + Km \qquad (1)$$

in which V = the rate of hydrolysis by intact cells, C = outer membrane permeability constant specific for the given microorganism and β-lactam, $Vmax$ and Km are the Michaelis constants, S_o = the external concentration of the antibiotic, and S_p = the periplasmic concentration of the β-lactam.

2.2.1. Outer Membrane Permeability

The structure of the outer membrane of *P. aeruginosa* (Fig. 1) and its role in resistance to β-lactam antibiotics have been documented extensively in recent reviews.[11–14] Briefly, the outer membrane of gram-negative bacteria consists of an asymmetric lipid bilayer in which proteins are associated with an external leaflet of lipopolysaccharide (LPS) molecules and an internal phospholipid monolayer[15]. The outer membrane is the structure that controls the trafficking of nutrients and metabolites between the bacterium and its environment. Because LPS molecules are negatively charged, and tightly bound to each other by cross-bridging with Mg^{2+} cations, the outer membrane acts as a permeability barrier for hydrophobic compounds.[11] To gain access to its internal targets, any antibiotic molecule must cross the outer membrane. A large number of hydrophobic or amphiphilic antibiotics such as macrolides, rifamycins, lincosamides, fusidic acid, and novobiocin are thought to be inactive against many gram-negative pathogens because of their slow penetration through the outer membrane.[11] In *P. aeruginosa*, two main pathways allow antipseudomonal antibiotics to enter into the cell.[11] Polycationic antibiotics such as aminoglycosides and polymyxin have been shown to promote their own uptake through the outer membrane by destroying the stabilizing effect of Mg^{2+}–crossbridging of the adjacent LPS molecules (see section 3.2). In contrast, β-lactams are thought to penetrate to the periplasm through channel-forming proteins, named porins. Several lines of evidence have shown that most porins form nonspecific water-filled pores, which act as a sieving barrier allowing the free passage of molecules with a molecular size lower than the exclusion limit.[15]

Protein F or OprF,[13] the major outer membrane protein of *P. aeruginosa*, has been suggested as the entry pathway for β-lactam antibiotics.[12–14] In addition, OprF plays an important role in outer membrane stability and bacterial cell shape.[16,17] However, as discussed in several recent reviews,[12,14,18] the role of this protein is currently a source of controversy. Several authors[11–13] have produced a variety of evidence that OprF functions as a porin with a large channel, although only a small proportion of the total OprF molecules per cell seem to form such channels (with the bulk of OprF molecules forming small, antibiotic-impermeable channels).[12] Conversely, Nakae and collaborators, using the same liposome swelling methods as used by one of the authors above,[11] have suggested OprF does not function as a porin.[17,19] The same authors, using the same methodology, *i.e.* the liposome swelling assay, proposed OprC, OprE and OprD function as general porins and as the major routes of entry for β-lactams in *P. aeruginosa*.[19] However, the data produced by this laboratory have been criticized for lack of internal consistency.[13,14] Recently, Bellido *et al.*[19a] cloned the *E. coli* raffinose into wild-type and OprF-deficient *P. aeruginosa* strains to allow the study of polysaccharides' penetration through the outer membrane. Their results strongly suggest that OprF is the most prominent porin for compounds larger than disaccharides in *P. aeruginosa*.

Protein D2 (OprD) has been shown to be a saturable, substrate-specific channel for imipenem and basic amino acids.[20] This selectivity for imipenem over the other β-lactams seems due to the presence in OprD channels of a specific binding site for positively charged penems and carbapenems. Other, investigational β-lactam antibiotics seem also to take advantage of specific transport systems across the gram-negative bacterial outer membrane. For example, the catechol-cephalosporins are able to chelate ferric iron and

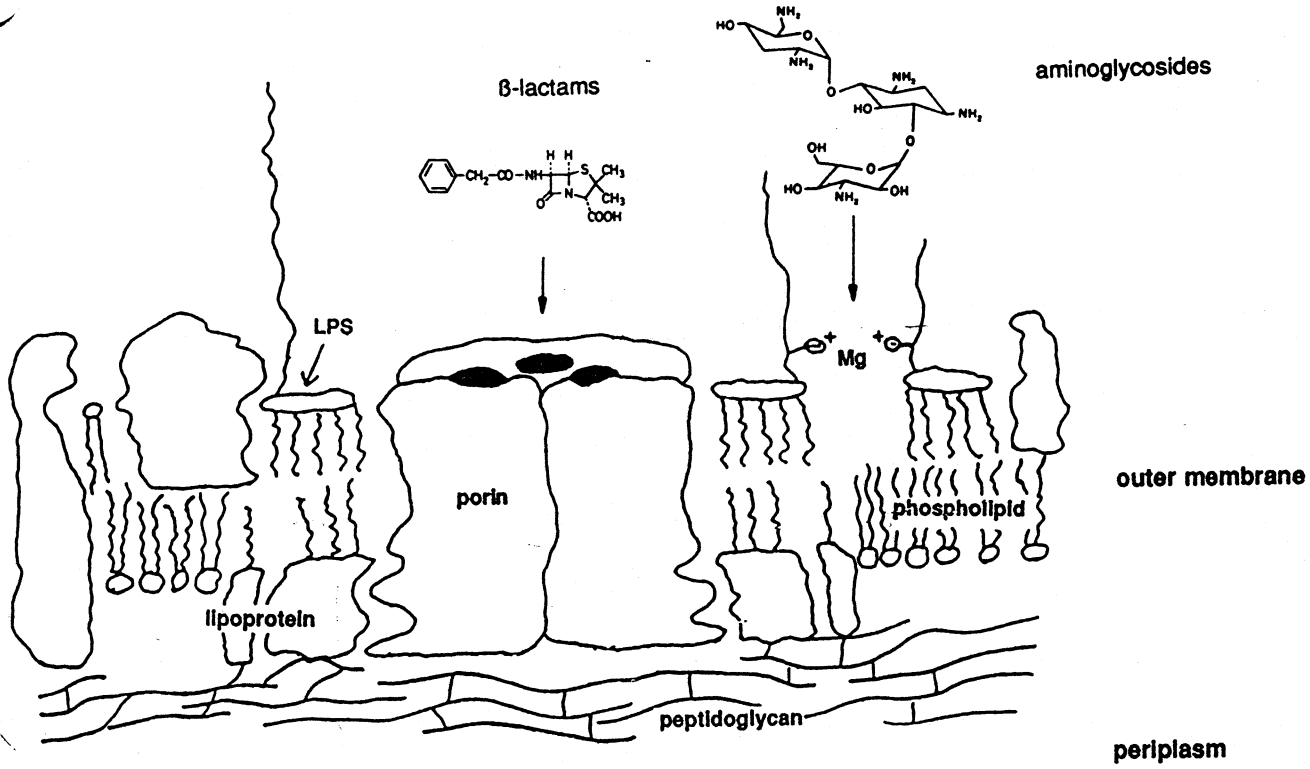


FIGURE 1. Schematic representation of the cell envelope showing the two major antibiotic uptake pathways through the outer membrane of *P. aeruginosa*, namely the porin pathway (left arrow) for β-lactams and the self-promoted uptake pathway (right arrow) for aminoglycosides. The asymmetric outer membrane consist of an external leaflet of lipopolysaccharide (LPS) molecules and an internal phospholipid monolayer. The β-lactam targets are located in the cytoplasmic membrane layer which is orientated towards the periplasm. Aminoglycosides have to cross this inner membrane to reach their cytoplasmic targets.

penetrate the *P. aeruginosa* outer membrane through the siderophore-scavenger pathways.[21]

Outer membrane permeability of β-lactams in intact *P. aeruginosa* cells have been measured by the Zimmermann and Rosselet method.[11] Results for easily β-lactamase-hydrolysed β-lactams such as nitrocefin[22], cephacetrile, and cephaloridine[23] showed outer membrane permeability coefficients to be 12 to 500-fold lower than the values obtained for *E. coli*. Data for the newer β-lactams are not available from intact *P. aeruginosa* cells because of several methodological limitations.[24] Indeed, the Zimmermann and Rosselet method can only be applied if the β-lactam is a good substrate for the β-lactamase. Most of the recently developed β-lactams exhibit too high a stability to β-lactamase hydrolysis for their outer membrane permeability to be accurately determined under the same experimental conditions. Another source of difficulty in applying the Zimmermann and Rosselet method to *P. aeruginosa* has been illustrated by the work of Hewinson *et al.*[25] These authors found that the permeability coefficient of cephalosporin C varied according to external antibiotic concentration, whereas Fick's law states that it should be a constant under normal conditions. Such a puzzling effect likely may be ascribed to the presence of outer membrane-bound β-lactamase that considerably limits the applicability of Zimmermann and Rosselet method to *P. aeruginosa*.[14,24] The study of the diffusion of β-lactams through porin-reconstituted vesicles is also difficult because of the problem of controlling charge and counter-ion effects that obscure actual permeation rates of the β-lactam studied and because the low total permeability of *P. aeruginosa* porins limits assay sensitivity. However, this method has been applied in *P. aeruginosa* to newer zwitterionic β-lactams such as the fourth-generation cephalosporins.[26] These molecules penetrate the outer membrane at rates about 300-fold lower than *E. coli* and 10-fold lower than *E. cloacae*. Results with these latter two bacteria also suggest that their outer membranes are much more permeable to zwitterionic compounds than to negatively charged β-lactams. However, the extrapolation of these findings to *P. aeruginosa* does not necessarily follow because the outer membrane of *P. aeruginosa* seems to be relatively indifferent to the physicochemical properties of the β-lactams,[11,44] a result that is predictable given the proposed large channel size of the major porin.

2.2.2. β-*Lactamase*

P. aeruginosa strains can produce both chromosomal and plasmid-mediated β-lactamases. Sabath *et al.*[27] first reported in 1965 the discovery of an inducible chromosomal cephalosporinase. Since then this enzyme has been reported in all wild-type strains of *P. aeruginosa* and has been further classified as Class Id in the scheme of Richmond and Sykes.[28] The amino-acid sequence around the active site ser of *P. aeruginosa* cephalosporinase showed a

high degree of homology with the chromosomal β-lactamase of *Enterobacteriaceae*.[29] Therefore, this enzyme belongs to the molecular class C in the scheme of Amber, extended by Knott-Hunziker.[29] Based on analytical isoelectric focusing studies, *P. aeruginosa* seems to produce four distinct subgroups of cephalosporinase corresponding to four alkaline pIs.[30] Several β-lactams are capable of inducing the *P. aeruginosa* cephalosporinase. This property diminishes the efficacy of inducing β-lactams. For example, it has been reported that self-induced β-lactamase production decreased imipenem susceptibility of *P. aeruginosa*, although insufficiently to elevated MIC beyond the clinical range.[31,36] Constitutive high levels of cephalosporinase may also be observed in *P. aeruginosa* after a double mutation that results in a stably derepressed β-lactamase.[29] Consequently, the bacteria become resistant to multiple β-lactams.

In addition to the chromosomal cephalosporinase, at least 15 plasmid-borne β-lactamases have been isolated from *P. aeruginosa*.[29] These enzymes include broad-spectrum β-lactamases (TEM-1, TEM-2, SHV-1, HMS-1, LCR-1, NPS-1), oxallinases (OXA-2, OXA-3, OXA-4, OXA-5, OXA-6), and carbenicillinases (CARB-3, CARB-4, PSE-1, PSE-2, PSE-3, PSE-4).

New β-lactams such as the third-generation cephalosporins, monobactams and carbapenems were thought to completely resist β-lactamase inactivation since several authors were unable to detect hydrolysis with β-lactamase crude extracts[32]. Nevertheless, cephalosporinases exhibited high affinity for all of these compounds. To explain the resistance to new β-lactams in β-lactamase-overproducing gram-negative strains, Then and Angerhn[32] proposed the "trapping" model, involving non-hydrolytic binding of β-lactams to β-lactamase. However, several authors have pointed out that this concept was mistakenly based on inappropriate measurements of β-lactamase hydrolysis.[14,33,42] Two newer antibiotics, *i.e.*, cefpirome and cefepime, remain effective against β-lactamase-overproducing gram-negative strains that are resistant to third-generation cephalosporins. The reason of this efficacy has been ascribed to high β-lactamase stability and low affinity for the enzyme, although higher rates of outer membrane permeability are also involved.[26,33]

2.2.3. *Penicillin-Binding Proteins (PBP)*

P. aeruginosa has been shown to possess a PBP pattern equivalent to that of *E. coli*.[34] Seven PBP have been described with molecular weights ranging from 118,000 to 350,000 Da. However, the functions of these enzymes are not as well known as in *E. coli*. Thus, only PBP-5 has been described as a D-alanine carboxypeptidase.[35] Some PBP of *P. aeruginosa* also may not be totally similar to their *E. coli* counterparts. For example, a modified PBP-4 was associated with a clinical case of resistance to imipenem whereas the equivalent protein in *E. coli* is a non-essential carboxypeptidase.[36] Inhibition of *aeruginosa*

PBP by β-lactams results in morphological changes comparable to those observed in *E. coli*. However, differences in affinity are also noticeable (Table II). For example, low concentrations of sulfocephalosporins such as cefsulodin inhibit *P. aeruginosa* PBP-3, whereas its *E. coli* counterpart is insensitive. Further, in *P. aeruginosa*, to a greater extent than in *E. coli*, the affinity of PBP for given β-lactams seems not to be correlated with their efficacy (cf. Tables I and II). This is probably due to the role played by other factors in *P. aeruginosa* such as low outer-membrane permeability and inducible β-lactamase.

Recently, a possible correlation between the affinity of β-lactamase and the affinity of PBP for the new β-lactams has been suggested in *E. cloacae*.[33] We feel that this finding probably can be extrapolated to other bacteria and might have considerable implications in the design of new β-lactam molecules.

2.3. Resistance

As mentioned before, *P. aeruginosa* is intrinsically resistant to a wide range of β-lactams. Low outer-membrane permeability by itself cannot account for this resistance because even slower-penetrating β-lactams equilibrate their external and periplasmic concentrations within 1 minute.[37,38] Therefore, an additional mechanism must be associated with low outer-membrane permeability to achieve high intrinsic resistance.[37,38] This mechanism may involve the inducible type-Id chromosomal cephalosporinase in the case of easily hydrolyzed β-lactams or those that are strong inducers of β-lactamase.[39]

Initially susceptible strains can develop resistance during β-lactam therapy. In some cases, this can involve acquisition of a plasmid-borne β-lactamase. A recent survey has revealed emergence of resistance and therapeutic failure may occur in approximately 15% of patients suffering from *P. aeruginosa* infections and treated with one of the new β-lactams.[40] The mechanisms responsible for the emergence of resistance in *P. aeruginosa* may involve overproduction of chromosomal cephalosporinase, diminished outer membrane permeability, and modified PBP affinity. The first one has been incriminated in the emergence of resistance during ceftazidime therapy in CF patients[41] and in experimental *P. aeruginosa* endocarditis in rabbits.[42] It is likely that *in vivo* selection of stably derepressed β-lactamase-producing *P. aeruginosa* strains can account in most cases for the rapid emergence of β-lactam resistance in CF patients.[43] Fortunately, the future use of newer agents such as fourth-generation cephalosporins, which are effective even against such derepressed strains, could considerably decrease the risk of emergence of resistance in chronic infections. Indeed, a recent study has shown that spontaneous *in vitro* mutation to cefepime-resistance is extremely low in *P. aeruginosa*.[44] Emergence of resistance to β-lactams has also been linked to *P. aeruginosa* outer-membrane modifications. Godfrey *et al.* observed a correlation between β-lactam resistance and changes in LPS structure[45] or in OprF structure.[46] However, the role of the latter in β-lactam resistance is a source of controversy (see section 2.2.1.). Outer membrane protein D2 (OprD) of *P. aeruginosa* has been unequivocally associated with the emergence of resistance to one class of β-lactams. Several authors have reported the emergence of resistant strains during imipenem therapy.[36,47,48] Interestingly, this resistance affects only imipenem and not other β-lactams and is associated with the loss of outer-membrane porin protein OprD. This is probably because OprD contains a specific binding site for imipenem and its loss substantially reduces uptake across the outer membrane (see section 2.2.1.)

Several studies have associated resistance to β-lactams with modified PBP in different clinical isolates of *P. aeruginosa*.[36,49,50] However, it seems that the emergence of clinical resistance due to changes in PBP affinity is rare in gram-negative bacteria. This is consistent with the idea that these alterations may impair the viability of cells.[36] However, with the clinical use of newer β-lactams, such as the fourth-generation cephalosporins, showing high β-lactamase resistance and rapid uptake through the gram-negative outer membrane, there is the possibility that bacteria will develop such resistance mechanisms more commonly in the future.

TABLE II
Affinity of β-Lactams for the Essential PBP[a] of *P. aeruginosa* and *E. coli*[51,52]

	I_{50} for PBP (βg/ml)							
	P. aeruginosa				*E. coli*			
β-lactam	1A	1B	2	3	1A	1B	2	3
Ampicillin	*0.25*	0.9	0.5	1.3	1.4	3.9	*0.7*	*0.9*
Carbenicillin	*0.07*[b]	0.5	0.3	*0.1*	*2.1*	5.0	4.0	*2.1*
Azlocillin	0.1	0.5	ND[c]	*0.02*	0.8	1.6	0.4	*0.05*
Cefsulodin	19	2	>250	*0.3*	*0.5*	3.7	>250	>250
Cefoperazone	1.0	2.0	>200	*<0.02*	0.5	1.5	0.9	*<0.02*
Cefotaxime	0.04	0.2	ND	*0.01*	0.02	0.4	4.0	*0.01*
Ceftazidime	0.8	6.0	25	*0.1*	0.9	3.4	240	*0.06*
Imipenem	0.3	0.6	<0.6	4.0	0.2	0.6	*<0.1*	9.8
Aztreonam	8.4	2.6	ND	*0.04*	1.7	310	>500	*0.17*

[a] Three other PBP (PBP4, 5 and 6) are not included here since they were shown to be nonessential in *E. coli*.
[b] Numbers in italics represent I_{50} values for the PBP with the highest affinity.
[c] ND = not determined.

3. AMINOGLYCOSIDES

3.1. Determinants of Efficacy

Aminoglycosides remain one of the most valuable tools possessed by physicians to combat serious gram-negative infections. They are especially effective in treatment of urinary tract infections due to their high excretion in

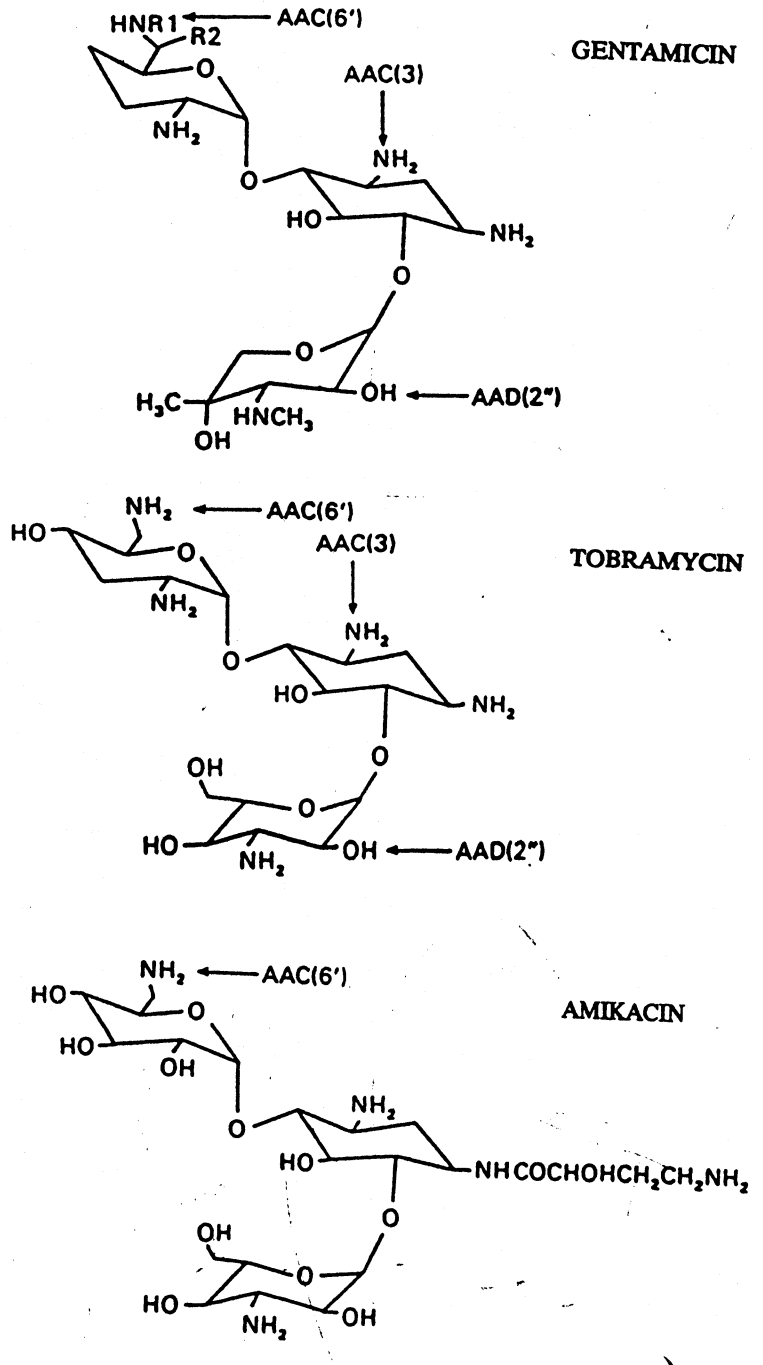
urine, however their major use against life-threatening *P. aeruginosa* infections is in combination with an anti-pseudomonal penicillin or cephalosporin.[53] One severe limitation in combating infections in specific tissues is their limited and at times unpredictable penetration.[54] Attempts to overcome this problem in *P. aeruginosa* infections of the lungs of CF patients include aerosolization and inhalation therapy[55] and use of doses up to 10 times the normal human dose.[55] Antagonism by divalent cations (see section 3.2.) and polyanions can also decrease aminoglycoside effectiveness.

A major factor that limits the usage of aminoglycosides is their strong reputation as toxic antibiotics. The word "reputation" is used advisedly because the frequency of aminoglycoside nephrotoxicity varies from 2–10%. Futhermore it appears to be dose-related and somewhat restricted to specific patient groups, especially those who are elderly and debilitated.[54] Nephrotoxicity is usually mild and reversible, although aminoglycoside ototoxicity, which occurs with a similar frequency, is irreversible.

Of the many known aminoglycosides, only gentamicin, amikacin, and tobramycin (Fig. 2) are commonly used against *P. aeruginosa* infections. The MIC_{90} of gentamicin and tobramycin for *P. aeruginosa* is approximately 6 μg/ml, which is close to the achievable peak serum levels at the usual human dose (5 mg/kg/day).[54] For this reason, these antibiotics are usually combined with an anti-pseudomonal penicillin.[56] In contrast, the peak serum concentration of amikacin following the standard intravenous dose is approximately 25–35 μg/ml, or nearly double the MIC_{90} for *P. aeruginosa* (16 μg/ml). Despite this, amikacin is usually also combined with anti-pseudomonal penicillin (*e.g.* ticarcillin) with which it is often synergistic and most effective.[53]

Forty-six years have elapsed since the first reported isolation of the aminoglycoside streptomycin, and numerous prominent scientists have attempted to uncover details of the mode of action and uptake of aminoglycosides.[51,58–60] However, the precise molecular details of the mode of uptake or action are still not known and we refer the reader to the above review articles. In general terms, aminoglycoside action against susceptible *P. aeruginosa* can be described as follows: Cationic aminoglycosides initially interact electrostatically with the negatively charged cell surface, followed by disruption of the outer membrane and self-promoted uptake across the outer membrane. This is followed by two energized uptake steps across the outer membrane, the slow-phase EDPI and the rapid-phase EDPII. Killing of cells apparently

FIGURE 2. Structure of the major anti-*Pseudomonas* aminoglycosides and the points of action of the aminoglycoside-modifying agents found in *Pseudomonas*. Abbreviations: AAC = acetylating enzyme; AAD = adenylating enzyme (the number in brackets represents the carbon at which the enzyme acts: *N.B.* there are three types of AAC(6′) and two types of AAC(3)(with differing activity spectra); R1 and R2 in the gentamicin structure each represent H or CH_3 depending on which of the three forms of gentamicin (C1, C1a or C2) present as a mixture in commercial gentamicin preparations, is represented.



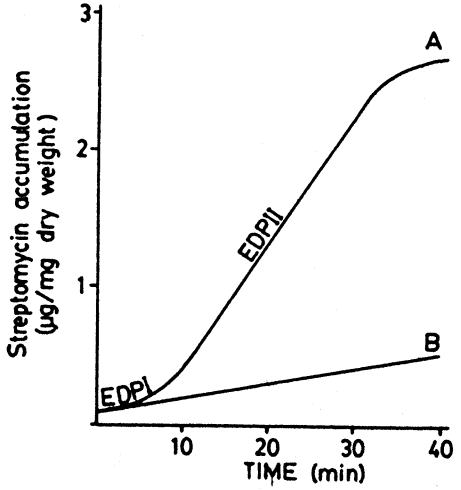


FIGURE 3. Kinetics of aminoglycoside uptake in *Pseudomonas aeruginosa* wild-type, susceptible strains (curve A) and strains resistant to aminoglycosides by virtue of possession of an R-plasmid specifying an aminoglycoside-modifying enzyme (curve B). Data are shown for streptomycin, but similar kinetics are seen with tobramycin. EDPI, EDPII = energy-dependent phases I and II, respectively.

occurs at or near the time of initiation of EDPII (Fig. 3).[59] The actual mechanism of killing remains unknown and recently has been proposed to involve either inhibition of the initiation of DNA replication[59,62] or creation of membrane channels by induced errors during protein synthesis.[61] Nevertheless, it is apparent that the ability of aminoglycosides to interact with ribosomes is involved since, *e.g.*, *rpsL* mutants altered in ribosomal protein S12 do not initiate EDPII and are resistant to streptomycin (which has a high affinity binding site on S12). Because such mutants occur at low frequency in the laboratory, and are apparently not found in the clinic,[63] we will not discuss further the interaction of aminoglycosides with ribosomes. Instead we will concentrate on those stages of aminoglycoside interaction with cells that can be altered to cause clinically significant resistance.

3.2. Outer Membrane Permeation

It is now well-established that aminoglycosides cross the outer membrane, using the self-promoted uptake mechanism in *P. aeruginosa* (see ref. 64 for review and original references). Thus laboratory mutants with a defect in this pathway (due to overproduction of an outer-membrane protein OprH = H1, which apparently blocks the pathway) are resistant to killing by aminoglycosides. Such mutants have no alteration in the porin pathway. Conversely,

a *tolA* mutant, which demonstrated enhanced outer membrane interaction with aminoglycosides, was supersusceptible to aminoglycosides.

The self-promoted uptake pathway involves sites in the outer membrane at which divalent cations (usually Mg^{2+} or Ca^{2+}) crossbridge adjacent lipopolysaccharide molecules on the surface of the bacterium (Fig. 1). Such sites have been shown to be important in maintaining the barrier function of the outer membrane. These sites have an affinity that is at least two orders of magnitude higher for polycationic aminoglycosides than for these divalent cations. Thus aminoglycosides can interact with such sites in a cooperative fashion to competitively displace the divalent cations, as shown in studies using fluorescent or spin label probes. Due to the bulky nature and higher positive charge of aminoglycosides relative to the displaced cations, the packing order of the LPS in the outer membrane is disturbed, as assessed using spin-label probes, and this can be observed electron microscopically as morphologial distortions (*e.g.*, blebs, cracks) at higher concentrations. This results in an increase in outer-membrane permeability toward at least three different probes: a chromogenic β-lactam nitrocefin; a hydrophobic fluorophor NPN; and lysozyme, an enzyme that digests the peptidoglycan. Such increased outer-membrane permeability is dependent upon the aminoglycoside concentration in a sigmoidal fashion and is blocked by excess divalent cations, suggesting that it is due to cooperative interactions at divalent cation binding sites. Consistent with this, LPS from the aminoglycoside supersusceptible *tolA* mutant binds aminoglycosides better than does wild-type LPS, and the *tolA* mutant is more susceptible to the effect of aminoglycosides on permeability. Furthermore, the relative ability of eight aminoglycosides to make permeable the outer membrane was found to be related to their MIC. For these reasons we have proposed[58,64] that aminoglycosides make permeable the outer membrane to other molecules of aminoglycoside, *i.e.* promote their own uptake.

3.3. Cytoplasmic Membrane Penetration

Aminoglycosides cross the cytoplasmic membrane of *P. aeruginosa* by two energy-dependent steps, the slow-EDPI and accelerated-EDPII phases. Bryan and collaborators[57,65] showed that both steps had similar features in both *P. aeruginosa* and *E. coli*, *i.e.* inhibition by divalent cations, uncouplers, and inhibitors of electron transport. In addition, streptomycin-resistant *rpsL* mutants of both bacteria did not initiate the EDPII phase of streptomycin uptake. Thus we can assume that aminoglycosides use similar pathways of energized uptake in both bacteria. Whereas the precise mechanism is unclear even in *E. coli* where it has been better studied, several features seem evident. First, aminoglycoside uptake in EDPII is kinetically unusual in that it is irreversible. Second, the major driving force for aminoglycoside uptake appears to be the electrical potential gradient (which is orier interior

negative) across the cytoplasmic membrane,[24,25,27] and uptake requires a threshold value of this potential. Third, there is an apparent requirement for electron transport independent of this electrical potential gradient. Bryan and Kwam[57] have proposed that this reflects the use, by aminoglycosides, of oxidized respiratory quinones as carriers.

3.4. Plasmid-Mediated Resistance

Plasmid-mediated resistance to aminoglycosides has been discussed in several excellent reviews[66,67] and we concern ourselves here with only those mechanisms that influence the widely-used anti-pseudomonal aminoglycosides gentamicin, tobramycin, and amikacin. The incidence of plasmid-mediated resistance is difficult to assess due to the existence in some cases of significant levels of non-plasmid-mediated low-level resistance.[66,68] In addition, there are large differences in reported incidences from study to study, possibly due to local outbreaks and/or poor antibiotic management.[69] Nevertheless the incidence of plasmid-mediated resistance usually ranges between 5 and 12% of all isolates.[69–71]

Plasmid-mediated aminoglycoside resistance in *P. aeruginosa* is often due to aminoglycoside-modifying enzymes. Such enzymes can transfer phosphate, acetyl, or nucleotidyl groups to specific sites on the aminoglycosides.[66,67] The sites of action of enzymes that specifically affect MIC for gentamicin, tobramycin, and/or amikacin in *Pseudomonas* are shown in Fig. 2. Other enzymes are specified in *P. aeruginosa* but do not affect resistance to these aminoglycosides.[72] In addition, a chromosomal gene can be amplified by mutation to result in aminoglycoside resistance although not to the above commonly used aminoglycosides.[73] Aminoglycoside-modifying enzymes apparently are located in the periplasm[74] and interact with aminoglycosides to reduce affinity for ribosomes and prevent the onset of EDPII.

3.5. Clinical Resistance Development

Three large published studies (Table III) have indicated that the frequency of gentamicin resistance among *P. aeruginosa* isolates is *ca.* 5–10%,[69,70] whereas a task force study from 13 centers worldwide indicated levels of

TABLE III
Aminoglycoside Resistance in *Pseudomonas aeruginosa*[69,70]

Date of survey	Location	Number of isolates examined	% isolates resistant to gentamicin
1981	Switzerland	2235	5.9
1984	U.K.	1866	5.5
19[illegible]4	Netherlands	2635	10.1

resistance from 9–20%.[73] However, higher resistance levels were observed in some centers.[67,69,71] There are two major causes of resistance. These include high-level enzymatic resistance, as described above, and so-called permeability or non-enzymatic resistance. The former high-level resistance is specific (Fig. 2); Thus often it can be overcome by merely changing the aminoglycoside administered. However, nonenzymatic resistance, although it causes lower level resistance, is still clinically significant and is of substantial concern because it results in cross-resistance to all aminoglycosides.[67,68] Either mechanism can predominate in a given center for unknown reasons.[67–69] It should be noted, however, that aminoglycoside resistance apparently can be reduced when a β-lactam is co-administered.[75]

Nonenzymatic (impermeability-mediated) resistance has been studied by Bryan and colleagues in some detail. It was found to be associated with diminished energized uptake of streptomycin[76] and gentamicin[74] but no evidence was found for resistant ribosomes, enzymatic inactivation, slower growth rate, or R factors. It was shown in one isolate that impermeability-type resistance was associated with a smooth-to-rough transition in the LPS (*i.e.* loss of O antigen).[77] On the basis of the known mechanism of uptake of aminoglycosides across the *P. aeruginosa* outer membrane, we propose that this LPS alteration decreases the affinity of LPS for aminoglycosides, resulting in lower levels of uptake due to reduced self-promoted uptake across the outer membrane. Such resistance has not been modeled in the laboratory. When serial subcultures of *P. aeruginosa* are exposed to increasing levels of aminoglycosides, isolates are obtained with high levels of resistance to all aminoglycosides, markedly slower growth rates, reduced virulence in mice, and acquisition of undefined growth requirements.[78] Some of these properties were shown by a mutant isolated by Bryan *et al.*[79] This mutant proved to have a decrease in the activity of cytochrome C_{552} a component of nitrate reductase. This in turn led to a specific alteration in energized aminoglycoside transport. Although such a resistance mechanism has not been shown *in vivo* it seems to fit with the proposals of Bryan with regard to phenotypic adaptation (persistence) in the CF lung.[63]

Animal models have been used to demonstrate another type of resistance, adaptive resistance.[80–82] In these studies it was shown that inoculum and time of initiation of treatment after infection substantially influenced the ability of aminoglycosides to inhibit the growth of *P. aeruginosa*. A delay in the initiation of aminoglycoside therapy of as little as 2 hr, had an enormous effect on the rate of recovery of organisms.[81] Such resistance was phenotypic rather than due to mutation and reversed *in vitro*.[81,82] One potential cause was oxygen limitation, since the presence of *Pseudomonas in vivo* at a localized infection site led to decreased oxygen tension, a factor that increased the MIC *in vitro* 16-fold.[82] Such a phenotypic resistance mechanism could result in the dissociation of *in vivo* and *in vitro* antibiotic susceptibility of *P. aeruginosa*, a phenomenon observed previously.[63,80] Similar consideratic[illegible] may apply to the observed high resistance of *P. aeruginosa* associated wit[illegible] theter mate-

rials.[83] Reduced oxygen tensions within the thick adherent biofilm and/or reduced metabolic capacity could in this instance explain the observed aminoglycoside resistance (and consequently the failures of antibiotic chemotherapy in catheter-associated urinary tract infections).

4. QUINOLONES

4.1. Determinants of Efficacy

One of the major hopes for chemotherapy of *P. aeruginosa* infections in recent years has been the introduction of fluoroquinolones. Although a large variety of related antibiotics have been introduced, we will largely restrict our discussion here to ciprofloxacin, which remains the quinolone with the lowest MIC for *P. aeruginosa* (Table I); as the first such compound introduced into clinical practice, it is the most-used in therapy. Despite the low MIC^{90} for *P. aeruginosa in vitro* (*ca.* 0.25–1 μg/ml), therapeutic results have sometimes been disappointing, in large part due to resistance development.[84–89] Nevertheless, in CF patients, despite a rarity of bacteriological cures and high incidence of resistance development, there is often an improvement in lung function with ciprofloxacin therapy.[87,88] Combinations of ciprofloxacin with other agents usually has an additive, rather than synergistic effect,[90] and resistance to ciprofloxacin can develop in CF patients.

The probable target of ciprofloxacin, as with other quinolones, appears to be the A subunit of DNA gyrase. Thus the concentration of ciprofloxacin causing 50% inhibition of DNA synthesis in *P. aeruginosa* made permeable by EDTA[91] equates well with the MIC, whereas *gyr A* mutants with alterations in DNA gyrase subunit A (Table IV) have eight-fold or greater increases in MIC. However, the binding site still remains a source of controversy. Whether quinolones bind directly to DNA gyrase or to the tertiary DNA gyrase–DNA complex remains to be determined.[95] The involvement of SOS DNA repair response in quinolone action is also under active investigation.[95]

A point of major concern is whether MIC performed *in vitro* on normal media accurately reflect *in vivo* susceptibility. Similar concerns were previously expressed for aminoglycoside antibiotics. For example, it has been demonstrated that cation supplementation,[91,92] in addition to low pH,[92] increased the MIC of ciprofloxacin and other quinolones about two- to eight-fold depending on the medium. This might substantially increase the MIC for clinical isolates to a level outside the clinically achievable range.

4.2. Uptake Across the Outer Membrane

Several studies have shown that the outer membrane of *P. aeruginosa* is a significant barrier to quinolone uptake. In particular, Kubesch *et al.*[93] demonstrated that the outer membrane permeabilizer polymyxin B nonapeptide decreased the MIC, for *P. aeruginosa* strains, of ciprofloxacin, norfloxacin and ofloxacin by two- to 40-fold whereas nalidixic-acid MIC were decreased more than 100-fold. In addition, Bedard *et al.*[91] demonstrated that the permeabilizer EDTA lowered the ciprofloxacin concentration required to observe inhibition of DNA synthesis by five-fold. Our own data has shown that overexpression of outer-membrane protein OprH in the regulatory mutant strain H181 or from the cloned gene decreased quinolone MIC eight to 16-fold when compared with the isogenic wild-type strain H103 (R.E.W. Hancock and M. Bains, unpublished data). In contrast to these data, there is considerably less evidence that the outer membrane of wild-type *E. coli* strains forms a substantial barrier to fluoroquinolones.[91,94] Although, the IC_{50} of ciprofloxacin for *E. coli* NT525[95] and *P. aeruginosa* MP-001[96] DNA gyrase in supercoiling assays is similar, the MIC are 100-fold higher for *P. aeruginosa*, demonstrating the importance of low permeability in this species. Also *gyr A* mutants of these strains have similar IC_{50} values but MIC that are 100-fold different.[95,96]

The mechanism of uptake of fluoroquinolones across the outer membrane of *P. aeruginosa* remains a mystery. Several mechanisms have been suggested. These include self-promoted uptake (divalent cations antagonize killing and uptake).[91,93,97] However, our own studies (unpublished data) have shown that quinolones cause none of the increases in outer-membrane per-

TABLE IV
Genetically Mapped Quinolone-Resistant Mutants of *P. aeruginosa* Strain PAO[96,104,105]

Mutation	Genetic Map Position (min)	Altered Phenotype	Times increase in MIC relative to wild-type[a]						
			CIP	NOR	NAL	CB	CAP	GM	IMP
gyr A (= *nalA,cfxA nfxA)*	39	DNA gyrase subunit A	8×	8×	64×	1×	1×	1×	ND
nalB (= *cfxB)*	20	Transport; OM protein over-produced	8×	4×	8×	4×	8×	0.5×	1×
nfxB	0	Transport; OM protein over-produced	8×	16×	2×	0.25×	1×	0.25×	0.5×
nfxC	46	Transport; OM altered	16×	32×	16×	0.5×	>2×	0.25×	8×

[a]CIP = ciprofloxacin; NOR = norfloxacin; NAL = nalidixic acid; CB = carbanicillin; CAP = chloramphenicol; GM = gentamicin; IMP = imipenem.

meability that are symptomatic of interaction of aminoglycosides with the outer membrane, whereas outer-membrane mutants, resistant to aminoglycosides by virtue of OprH overproduction,[64] are supersusceptible to fluoroquinolones. Other researchers have suggested that *P. aeruginosa* outer-membrane protein OprF is responsible for fluoroquinolone uptake, based on the fact that clinical resistant mutants lacking OprF have been demonstrated in some studies.[98–100] This has been contradicted by Chamberland *et al.*[101] who showed no correlation between quinolone resistance and OprF deficiency in one clinical isolate and its derivatives, whereas Woodruff and Hancock[102] concluded that OprF-deficient strains had such severe structural defects that they did not permit definitive conclusions to be made regarding antibiotic permeation. Nevertheless, it seems possible that OprF deficiency does give rise to fluoroquinolone resistance *in vivo*; otherwise it is difficult to understand why fluoroquinolone therapy should give rise to such mutants. This scenario would be appropriate if we assume that *in vitro* experiments do not accurately reflect *in vivo* conditions and susceptibility; one problem with clinical mutants is that one often observes multiple alterations.[100] Thus the association between any one alteration and quinolone resistance should rely on a series of isogenic strains. In this regard, the relationship (or lack of relationship) between LPS phenotype and ciprofloxacin resistance[101,103] requires further study. Three other genetically defined ciprofloxacin-resistant mutants, *nalB*, *nfxB* and *nfxC*, have been ascribed to outer membrane alterations[96,103,104] (Table IV). In the former two, which map separately, outer-membrane proteins with different molecular weights (51kDa and 54 kDa) have been reported to be overproduced.[96,104] However, no formal proof exists that this overproduction of an outer-membrane protein directly results in the observed decrease in quinolone uptake and quinolone resistance. For example, a regulatory mutation giving rise to ciprofloxacin resistance and overexpression of a 60-kDa GroEL-like protein has been observed in *E. coli*.[106] Other authors have demonstrated alterations in minor outer-membrane proteins that correlate with ciprofloxacin resistance[105,107,108]; one of these, *nfxC*, is well characterized genetically.[105] These authors have suggested the involvement of outer-membrane proteins OprG and a 40-kDa minor outer-membrane protein,[107] OprC, OprD, and OprE[108] or underexpression of OprD (and OprG) but overexpression of a 50-kDa outer-membrane protein.[105] These alterations are not consistent either with one another or with the other mutants described above. Thus we conclude that current data is insufficient to define the mechanism of uptake of quinolones across the outer membrane. If the critical alterations are indeed in the outer membrane, a major possibility would be the involvement of LPS in quinolone uptake since (1) LPS structure can be altered by numerous individual gene mutations; and (2) LPS mutants of *P. aeruginosa* show alterations in outer-membrane protein profiles.[109]

4.3. Assays of Quinolone Uptake

The literature on quinolone uptake both in *P. aeruginosa* and *E. coli* is remarkably inconsistent[e.g.91,94,105,110] Examples include: the substantial backgrounds (*i.e.* zero time values although these can be partly suppressed by increasingly vigorous washing); frequent lack of observed time kinetics; variable effects of CCCP and other inhibitors; differences in uptake conditions required to show transport defects in ciprofloxacin-resistant mutants; lack of correlation between whole-cell inhibitory concentrations in intact cells and MIC in *P. aeruginosa* (cf. *E. coli*); inconsistencies between concentrations resulting in DNA-gyrase inhibition and MIC in *E. coli*,[95] suggesting concentrative uptake, and the linearity of uptake levels as a function of ciprofloxacin concentration, that is more consistent with simple or facilitated diffusion; disparities in one study between Mg^{2+} effects on MIC and lack of Mg^{2+} inhibition of uptake; lack of correlation in *P. aeruginosa* cf. *E. coli* of MIC with measured rates of uptake. Detailed studies have been performed in *P. aeruginosa* by Bedard *et al.*[91] and in *E. coli* by Diver *et al.*[94] However, we do not feel that it has been clearly demonstrated that productive ciprofloxacin uptake (leading to internalization and target inhibition) has been demonstrated or measured. Clearly this awaits the isolation of strains with mutations specifically influencing trans-cytoplasmic membrane uptake. Nevertheless, it is difficult to see how hydrophilic molecules such as quinolones could be taken up by simple diffusion across the cytoplasmic membrane, as suggested by some authors.

4.4. Mechanisms of Resistance

The common occurrence of quinolone resistance during clinical therapy of *P. aeruginosa* infections by quinolones, has led to a large number of *in vivo* studies attempting to define resistance mechanisms. These are summarized in Table V, where we have attempted to correlate findings with the genetically well-characterized mutants. The most complete study was recently presented by Yoshida *et al.*[100] who used the *E. coli gyrA* or *gyrB* genes in an attempt to complement mutants with alterations in these genes in *P. aeruginosa* quinolone-resistant mutants isolated after clinical therapy. Among 17 resistant clinical strains, 12 bore a *gyrA* mutation and one a *gyrB* mutation. At least two of the *gyrA*-altered mutant strains and the *gyrB* mutant apparently had other underlying mutations.

Whereas many well-defined mutants exist, it is not uncommon for resistant isolates to revert, post-therapy, to susceptibility levels similar to or at least closer to those of pre-therapy isolates.[63,101] Two explanations are possible. One is that such resistant isolates (termed persisters by Bryan[63]) have phenotypic adaptations (as opposed to mutations) that allow them to resist

TABLE V
Analysis of Other Quinolone-Resistant Mutants of *Pseudomonas aeruginosa*

Isolation[a]	Selecting quinolone[b]	Tentative identification[c]	Other properties	Ref.
Clinic	CIP	*gyrA*[d]	rough LPS	110
Clinic	–[e]	*gyrA*[d,f]	–	100
Clinic	CIP	*gyrA*[d,f]	OprF–	101
Clinc	ENOX	*gyrA*[f]	OprF–	98
Clinic	–[e]	*gyrB*[d,f]	–	100
Animals	PER	*gyrA*[d]	rough LPS	107
Animals	VAR	*gyrA*	—	111
Animals	PER/CIP	*nfxC*[f]	–	112
In vitro	NAL/ENOX	*gyrA*[d]	–	100
In vitro	CIP/NOR	*gyrA*	–	113
In vitro	CIP	*gyrA*	–	103
In vitro	CIP	*nalB*[f]	rough LPS	103
In vitro	NAL	*nalB*	–	100
In vitro	CIP	*nalB*	= Qr2	107
In vitro	CIP	*nfxC*	= Qr1; OprG reduced; 40 K minus	207
In vitro	ENOX	*nfxB*[f]	–	100

[a]Clinic = isolates arising during clinical therapy; animals = isolate arising during experimental therapy of animals; *In vitro* = laboratory selected isolates.
[b]Abbreviations as per Table IV; PER = perfloxacin; ENOX = enoxacin; VAR = various quinolones used.
[c]Identification based on MIC and phenotypic descriptions in ref. 63, 70, 71, and Table IV.
[d]Confirmed genetically or by DNA gyrase assays.
[e]Not described.
[f]One or two MIC differences for non-quinolone antibiotics observed.

killing by the quinolone. Withdrawal of the adaptive pressure causes the organism to return to the pre-therapy state. An alternative explanation is that these are true genetic mutants that, upon therapeutic failure and subsequent discontinuation of the therapy, could revert. The revertant would then overgrow the mutant as long as it could grow more rapidly *in vivo* (as suggested by Bryan[63]). The high frequency of selection of quinolone-resistant mutants *in vitro* (10^{-5} to 10^{-7} in two studies[99,111]) and an expected similar frequency of back mutations, make this a distinct possibility.

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