Resistance Mechanisms in *Pseudomonas aeruginosa* and Other Nonfermentative Gram-Negative Bacteria

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Nonfermentative gram-negative bacilli are still a major concern in compromised individuals. By far the most important of these organisms is *Pseudomonas aeruginosa,* although *Acinetobacter baumannii* (previously *Acinetobacter calcoaceticus*), *Stenotrophomonas maltophilia* (previously *Pseudomonas* and *Xanthomonas maltophilia*), and *Burkholderia cepacia* (previously *Pseudomonas cepacia*) are also of substantative concern because of their similar high intrinsic resistances to antibiotics. The basis for the high intrinsic resistance of these organisms is the low outer-membrane permeability of these species, coupled with secondary resistance mechanisms such as an inducible cephalosporinase or antibiotic efflux pumps, which take advantage of low outer-membrane permeability. Even a small change in antibiotic susceptibility of these organisms can result in an increase in the MIC of a drug to a level that is greater than the clinically achievable level. In this review, the major mechanisms of resistance observed in the laboratory and clinic are summarized.

Overview

Of the nonfermentative bacteria, only one, *Pseudomonas aeruginosa,* can be considered a major pathogen in developed countries. However, several others, including *Acinetobacter baumannii* (formerly *Acinetobacter calcoaceticus*), *Stenotrophomonas maltophilia,* and *Burkholderia cepacia* do cause serious infections that place hospitalized patients at serious risk largely because of the high intrinsic antibiotic resistances of these organisms (table 1) [1–8]. Therefore, this review will attempt to summarize those resistance mechanisms that have been studied in some detail in the laboratory.

To date, three classes of antibiotic resistance have been described: intrinsic resistance, acquired resistance, and genetic resistance. Intrinsic resistance comprises those mechanisms that exist in the average strain of a given species, irrespective of antibiotic exposure. Acquired resistance involves the induction of unstable resistance without any observable change in genotype because of exposure of a strain to a set of inducing conditions that can include antibiotic exposure. Such resistance will revert to full susceptibility when the inducing conditions are removed. Genetic resistance involves the stable acquisition of new genetic information, either through mutation of an existing gene product or control mechanism, or through acquisition of a drug resistance plasmid.

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Intrinsic Resistance

Outer Membrane Impermeability

The species best characterized with respect to the properties of its outer membrane is *P. aeruginosa.* My colleagues and I have recently reviewed the outer-membrane components [9, 10] and outer-membrane antibiotic uptake/exclusion mechanisms [1, 11]; thus only a brief overview is presented here. The outer membrane constitutes a semipermeable barrier to the uptake of antibiotics and substrate molecules. Because uptake of small hydrophilic molecules such as β-lactams is restricted to a small portion of the outer membrane (namely the water-filled channels of porin proteins), the outer membrane limits the movement of such molecules into the cell. This is true for all gram-negative bacteria, but is especially true in the case of *P. aeruginosa,* which has an overall outer-membrane permeability that is ~12–100-fold lower than for example, that of *E. coli* [12]. It is of interest that this is the case for all the nonfermentative bacteria that are considered herein (table 2) [2, 13–15]. What is more controversial is how this low outer-membrane permeability comes about.

There is reasonable (but disputed [16]) evidence that the major porin is OprF [17, 18] and that this porin is responsible for the large exclusion limit of the *P. aeruginosa* outer membrane. Despite its high copy number, OprF represents an inefficient uptake route for antibiotics (either because of heterogeneity in channel formation [19] or an inefficient channel architecture [17]), and thus other channels must be influential in the residual 25%–35% of nonspecific outer-membrane permeation that is not due to OprF. Overexpression studies with OprD have eliminated this porin as mediating passage of any antibiotic other than zwitterionic carbapenem β-lactams such as imipenem and meropenem [20]. OprC and OprE [16, 21] are anaerobic-inducible porins, although it is conceivable that low levels of these small-channel porins may be present in wild-type cells. However, *oprC oprD*

Table 1. MIC_{50} of selected nonfermenters.

Agent	*Pseudomonas aeruginosa*	*Burkholderia cepacia*	*Stenotrophomonas maltophilia*	*Acinetobacter baumannii*	*Escherichia coli*
Cephaloridine	>1,024	>1,024	>1,024	>1,024	4
Ceftazidime	24	2	>128	16	0.125
Cefotaxime	16	8	128	32	0.125
Cefpirome	2.5	8	64	2	0.04
Carbenicillin	32	>128	>128	12.5	4
Piperacillin	2	4	1,024	32	2
Aztreonam	2	. . .	. . .	4	0.03
Imipenem	2	3	16	0.5	0.25
Gentamicin	2	100	64	2	0.5
Tobramycin	0.5	64	32	1	0.5
Colistin	4	>64	8	2	1
Ciprofloxacin	0.2	6	1	0.5	0.025
Norfloxacin	0.8	25	32	8	0.1
Nalidixic Acid	1,024	. . .	16	. . .	4
Chloramphenic	128	32	8	128	8
Tetracycline	32	64	32	4	2
Co-trimoxazole	256	16	32	2	. . .

NOTE. Data (μg/mL) are from [1–8].

oprE triple mutants are normally susceptible to all antibiotics (except the carbapenems, because of OprD loss) [22]. Another possibility that we are currently examining, as the other antibiotic porin of *P. aeruginosa,* is OprB, which mediates nonspecific monosaccharide and disaccharide permeation [23]; alternatively, antibiotic uptake may occur directly through the bilayer via a nonporin route [11].

Of the other species, *B. cepacia* has as its porin the OpcPO complex or, more possibly, a predominant 36-kD protein, OpcP1, which is a component of this complex [13, 24], whereas proteins 1 and 2 of the *A. baumanii* outer membrane have been defined as the porins of this species [2]. All of these proteins have quite small channels. The porins of *S. maltophilia,* on the other hand, are proposed to be similar in size to those of *E. coli* but present in rather low copy numbers [15], explaining the low overall outer-membrane permeability of this species.

Specific channels are responsible for uptake of β-lactams that mimic the natural substrates of these channels. Thus, zwitterionic carbapenems such as imipenem and meropenem mimic dipeptides containing one basic amino acid [25]. Therefore, they use a specific porin protein, OprD, which contains a binding site for such amino acids and imipenem [20, 25]. Similarly, catechol β-lactams bind Fe^{3+} and in *E. coli* cross the outer membrane by promiscuous proteins which serve to scavenge iron bound to the degradation products of siderophore [26, 27]. Such proteins have not as yet been identified or characterized in the nonfermenters (see [27]).

Uptake across the outer membrane of polycationic antibiotics such as gentamicin, tobramycin, and colistin is mediated by an uptake system termed *self-promoted uptake* [11]. This system involves the interaction of the polycation with divalent cation binding sites that are on cell surface lipopolysaccharide (LPS) molecules and that normally stabilize the outer membrane. Since these polycationic antibiotics are much larger than the native divalent cations, they cause a disruption that permeabilizes the membrane to a variety of probes and presumably to

Table 2. Outer-membrane permeability of nonfermentative species.

Species	Relative outer-membrane permeability (%)	Porins	
		Major	Adjunct
Eschericia coli	100	OprF, OmpC	LamB, PhoE, OmpG, CE1248 porin
Pseudomonas aeruginosa	1–8	OprF	OprB, OprC, OprE, OprD
Burkholderia cepacia	11	OpcPO	OpcS
Stenotrophomonas maltophilia	3–5	. . .	. . .
Acinetobacter baumanii	1–3	45.5-kD	46.5-kD

the polycation itself. Based on their MICs to the polycations, we assume that all of the nonfermenters except *B. cepacia* have this uptake system. It is been clearly demonstrated that *B. cepacia* does not have a self-promoted uptake system [28] and is resistant to all polycations (and to EDTA). The reason is the low number of anionic sites on *B. cepacia* LPS [29], with only two phosphates per molecule (as compared with *P. aeruginosa* LPS, which has 12–18 phosphates). One of these phosphates, attached to the lipid A portion, is capped with 4-amino-4 deoxyarabinose, which presumably forms a salt bridge with an adjacent phosphate, such that it is unnecessary to have substantial divalent cation bridging to preserve outer membrane stability.

Efflux Pumps

It was originally believed that many gram-negative bacteria lacked a hydrophobic uptake pathway across their outer membranes [11, 12]. However, it is now believed that there might be appreciable trans-outer-membrane permeation of hydrophobic and amphipathic molecules [30] and that the major reason for resistance to such compounds is active efflux. This requires some additional study, since for example, the very hydrophobic fluorescent probe NPN was only taken up by *P. aeruginosa* when these cells were treated with a polycation such as an aminoglycoside to break down the outer-membrane permeability barrier [31]. The NPN taken up under these circumstances was rapidly effluxed unless an energy inhibitor was present. Despite these data, most amphipathic antibiotics are probably able to cross the outer membrane of *P. aeruginosa,* and at concentrations below the MIC, would be immediately exported. The recent breakthrough research of Poole and colleagues [32] has demonstrated that an efflux system, involving three proteins (MexA, MexB, and OprM) is critical for the intrinsic resistance of *P. aeruginosa.* Thus, a knock-out mutation in any of the genes encoding these proteins led to a fourfold to tenfold increase in susceptibility to quinolones, β-lactams (except imipenem), tetracycline, and chloramphenicol. The general profile of substrates for this efflux system involves compounds that are amphipathic with one or no positive charges. In contrast, β-lactams are negatively charged and generally quite hydrophilic. Thus, whether β-lactams are effluxed, or whether their supersusceptibility in strains lacking the pathway is due to some other secondary mechanism, is still rather controversial.

An efflux pump with homology to the above system is also a major contributor to intrinsic antibiotic resistance in *B. cepacia.* Thus, Burns et al. [33] have described a system involving the OpcK outer membrane protein in this species as being responsible for intrinsic resistance to tetracycline, chloramphenicol, and ciprofloxacin. No characterization of efflux proteins in the other species discussed herein has been performed.

Enzymes

With regards to β-lactamases, each of the species discussed herein contains an inducible, chromosomally encoded cephalosporinase [3, 14, 34–36]. The structural gene for this enzyme in *P. aeruginosa* has been cloned and sequenced, and it has been shown to be a typical class C, AmpC-type β-lactamase that is induced upon cell contact with sub-MIC levels of β-lactams, but especially imipenem (which is itself a very weak substrate). In addition, *S. maltophilia* has an inducible, chromosomally encoded class D β-lactamase [35, 37]. This β-lactamase, like others of its class, is able to efficiently hydrolyze imipenem and is a Zn^{2+}-metallo enzyme rather than a serine-β-lactamase like all other classes of β-lactamases.

P. aeruginosa also contains a chromosomally encoded kanamycin phosphotransferase [38]. However, it is normally expressed at quite low levels unless derepressed, possibly by mutation.

Relative Contributions to Intrinsic Resistance

The recent focus in the literature on the antibiotic efflux systems of *P. aeruginosa* could lead one to assume that efflux is the major factor involved in the high intrinsic antibiotic resistance of *P. aeruginosa.* This, however, is not the case, and indeed, efflux is a codeterminant of intrinsic resistance, together with the low permeability of the outer membrane. This can be clearly demonstrated with use of agents that overcome the permeability barrier of the outer membrane, such as polycations (see below) or by cloning in a large channel, such as a loop-5 deletion of OprD [39]. While it is true that low outer-membrane permeability is ineffectual without a secondary resistance mechanism such as efflux, it is this property that really distinguishes *P. aeruginosa* (and the other nonfermenters) from *E. coli,* for example, which also contains an efflux system involved in intrinsic resistance [12]. In the case of β-lactams, the secondary resistance mechanism that works together with low outer-membrane permeability is probably β-lactamase [11]. Both secondary mechanisms benefit from the slow exposure to antibiotics that is effected by the efficient semipermeable barrier created by the outer membrane.

Acquired Resistance

For *P. aeruginosa,* a major disparity has been observed between in vitro MICs and in vivo efficacy. Thus, despite achievement during therapy of antibiotic concentrations that should be therapeutically efficacious, cure is not achieved. Bryan [40] has discussed this phenomenon, which he termed *persistence,* in some detail. Because bacteria revert to full susceptibility when removed from the host, it is a very difficult phenomenon to study. However, we [41] and other investigators [42] have described systems in which it can be observed in vivo. In our

system, *P. aeruginosa* was placed in chambers implanted in murine peritoneums and treated therapeutically with a regimen that resulted in an intrachamber concentration of tobramycin of 3.8 μg/mL, which diffused in through the millipore filters that sealed the chambers.

Despite the fact that this concentration was four- to eightfold the in vitro MIC, killing of bacteria in the chambers was highly dependent on the growth phase of the bacteria. Thus, in the lag phase and early log phase, 6–8 logs killing were observed, while in the mid-to-late log phase, only 1–2 logs killing was achieved. Similar observations were made for ciprofloxacin therapy [42] (R. E. W. Hancock, unpublished observations). The basis for such acquired resistance was not entirely clear but could relate to the limited oxygen availability in the chambers at higher culture densities, since aminoglycosides require active electron transport for uptake.

Other potential causes of acquired resistance come from in vitro experiments. Thus, media composition, low-level derepression of β-lactamase, growth phase, growth rate, and other environmental factors are all prospective causes of acquired resistance [40].

Genetic Resistance

β-Lactams

In *P. aeruginosa,* β-lactam resistance is usually mediated by derepression of chromosomal β-lactamase [1]. This results in resistance to all of the moderately susceptible β-lactams and even the so-called β-lactamase-resistant β-lactams, with the exception of the zwitterionic carbapenems such as imipenem. This latter exception is because the carbapenems maximally induce β-lactamase at 0.5 $\times$ MIC, such that mutational derepression makes no difference. This mutational derepression of β-lactamase, caused by the *blaI* mutation, has been observed both in clinical isolates [4] and in experimental models of infection [43]. As described above, there is a profound synergy between the low outer-membrane permeability of this species and secondary resistance mechanisms such as high, derepressed β-lactamase levels. Therefore, even cephalosporins such as ceftazidime, which are extremely poorly hydrolyzed by the class-C β-lactamases, can have increases of MICs of $\geqslant$32-fold upon the mutational derepression of β-lactamase [4]. The newer, so-called fourth-generation cephalosporins (cefpirome, cefepime, and cefaclidine), are somewhat more effective under such circumstances, primarily because of their higher outer-membrane permeabilities and lower affinity for such β-lactamases [5]. However, *P. aeruginosa* can mutate, producing progressively higher MICs of such antibiotics because of increased levels of β-lactamase, resulting in MICs above the clinical range.

Other mechanisms that affect β-lactams are relatively rare. Permeability changes, for which the most obvious alteration is loss of the major porin OprF, have been observed in the laboratory and clinic [9] in the context of Mar (multiply antibiotic resistant) mutants and are discussed below. Such mutations cause rather modest increases in resistance and usually cannot be selected by β-lactams themselves. Target (penicillin-binding protein [PBP]) alterations have been observed in clinical, animal model, and selected laboratory isolates [1, 44] but are probably not common. For example, experimental studies have indicated that alteration of PBP3 [45] can result in increased resistance to many anti pseudomonal β-lactams.

A unique mechanism of resistance was observed in the laboratory for the catechol β-lactam BO-1341. In addition to mutants with derepressed β-lactamase, a mutant with a derepressed 84-kD outer-membrane protein was observed [46]. This is reminiscent of the changes observed with *tonB* mutants of *E. coli* (assuming that this 84-kD protein is an iron-regulated outer-membrane protein) [47], and it has been shown in *E. coli* that *tonB* mutants are deficient in uptake of such catechol β-lactams.

At least 13 plasmid-encoded β-lactamases have been described, including TEM-1, LCR-1, NPS-1, OXA-1, OXA-2, OXA-3, OXA-5, OXA-6, CARB-4, PSE-1 (the most common type in *P. aeruginosa*), PSE-2, PSE-3, and PSE-4 [35]. These β-lactamases are not a major factor in modern antimicrobial chemotherapy for *P. aeruginosa* infection, since they are relatively infrequent and tend to result in resistance to a restricted range of β-lactams (largely penicillins) that are relatively less frequently used for pseudomonas infections. One enzyme that has caused some concern is a plasmid-encoded carbapenemase observed in some Japanese *P. aeruginosa* isolates [35].

For imipenem and meropenem, the major resistance mechanism is loss of the specific porin OprD, which occurs in as many as 50% of *P. aeruginosa* infections treated for $>$1 week with imipenem [48]. Studies overexpressing OprD have indicated that OprD is very specific for these carbapenems and does not mediate passage of other β-lactams and quinolones [20]. Indeed, the observed cross-resistance to imipenem and fluoroquinolones that is coincident with a reduction in OprD levels [49] is probably due to a regulatory mutation, *nfxC* [50], that simultaneously influences an efflux system.

S. maltophilia is highly resistant to β-lactams (table 1) because of its two β-lactamases, and thus mutational resistance causes no further problems. *B. cepacia* does demonstrate susceptibility to a subset of β-lactams, but many isolates resistant to all known β-lactams, probably because of derepression of chromosomal β-lactamase, have been observed [14]. Similarly, *A. baumanii* can mutate to β-lactam resistance [3] through mutational overexpression of its class C β-lactamase. Another mechanism reported to occur in *B. cepacia* is reduced porin content [51], although the protein associated with this phenomenon, the 27-kD minor band of the OpcPO porin complex, has not been demonstrated convincingly to have porin activity.

Aminoglycosides

A broad range of aminoglycoside resistance mechanisms have been described for *P. aeruginosa* in the laboratory, but in most large studies of clinical outcome, frequencies of resistance of 5%–12% have been observed [1]. These resistance mechanisms fall into two classes. Acquisition of certain plasmids can lead to the production of enzymes that modify the aminoglycoside by variously acetylating, adenylylating, or phosphorylating the antibiotic molecule. This leads to high-level resistance but tends to be specific for given aminoglycosides. The actual effect of enzymatically modifying such drugs is reduced uptake and/or reduced ribosomal interaction. The predominant plasmid-encoded enzymes in *P. aeruginosa* are AAC(3)-I, AAC(3)-Ia, AAC(3)-II, AAC(6′)-I, AAC(6′)-II, and ANT(2″). In addition, this bacterium has an aminoglycoside resistance gene, *aphA,* in its chromosome, which can apparently be activated by mutation [38]. However, of the commercial aminoglycosides, the enzyme produced can modify only kanamycin, and this is not used therapeutically against *Pseudomonas.*

B. cepacia, as described above, is intrinsically resistant to aminoglycosides because of lack of outer-membrane uptake, whereas *S. maltophilia* is possibly resistant due to intrinsic modifying enzymes, since unlike *Burkholderia,* colistin resistance is not observed for this species. *A. baumannii* tends to be susceptible to most aminoglycosides but can acquire resistance by acquisition of the enzyme APH(3′)-VI, which can inactivate amikacin [6].

Another mechanism of resistance to aminoglycosides, resulting in lower-level resistance to all aminoglycosides, is decreased uptake. This results from alterations leading to reduced passage across the outer [52] or inner [53] membranes.

Quinolones

Quinolone-resistant mutants of *P. aeruginosa* fall into two classes, those resulting from target-site mutations in DNA gyrase [54], and those resulting from efflux mutations [32, 50]. When *P. aeruginosa* is exposed to step-wise selection with increasing levels of ciprofloxacin, mutations that cause an MIC change of less than 16-fold influence the susceptibility of only ciprofloxacin and other quinolones and are probably DNA gyrase mutations [55]. When MIC changes of more than 16-fold are selected, multiresistance is observed because of decreased permeability and/or increased efflux [55]. This latter class of Mar mutants is discussed below. The DNA gyrase mutations usually occur in the *gyrA* subunit affecting amino acids 83 or 87 [56]. However, a *gyrB* mutant was identified in a clinical isolate by complementation with the *E. coli gyrB* gene [54].

There have been few studies of quinolone resistance in other nonfermenters, although resistant isolates have been observed for all species. A putative *gyrA* mutant of *S. maltophilia* was selected by nalidixic acid in one study [7]. Other *S. maltophilia* mutants had increases in the amounts of outer-membrane proteins that were reminiscent of derepressed efflux mutants [7], and analagous mutants have been observed in *B. cepacia* [33].

Efflux and Multiple Antibiotic Resistance (Mar) Mutants

Quinolones can simultaneously select for mutants that are resistant to other classes of antibiotics, called Mar mutants. At least three mutational alterations have been observed in the laboratory to cause Mar mutations in *P. aeruginosa.* These are *nalB, nfxB,* and *nfxC* mutants, all of which cause changes in regulatory genes that lead to overexpression of efflux pumps in addition to other possible changes [50, 57]. Mutants in *nalB* result in derepression of the *mexAmexBoprM* operon, leading to resistance to quinolones, tetracycline, and chloramphenicol [50]. In addition, modest cross-resistance to certain β-lactams (but not imipenem) has been observed. However, it is uncertain as to whether the affected anionic β-lactams are actually substrates for this efflux pump, since other substrates tend to be positively charged (or neutral), amphipathic molecules.

It is possible that some other set of genes is affected simultaneously. Mutants in *nfxB* result in cross-resistance to all quinolones and certain β-lactams but slightly enhanced susceptibility to other β-lactams and aminoglycosides. Two alleles of *nfxB* are known that have differential susceptibility to tetracycline and chloramphenicol. In *nfxB* mutants, the *mexCmexDoprJ* efflux operon is derepressed. A third class of mutants in *nfxC* [50] overproduce an outer-membrane protein, OprN, which is the outer-membrane component of an efflux pathway, and underexpress the carbapenem-specific porin OprD. Thus, *nfxC* mutants are cross-resistant to quinolones, tetracycline, chloramphenicol, and imipenem [50]. As mentioned above, resistance to more than one class of antibiotics has also been observed in *S. maltophilia* [7] and *B. cepacia* [33], and in both cases, increased expression of an outer membrane protein of 50–55 kD was observed.

Approaches to Overcoming Resistance

There are several possible methods for overcoming resistance, including use of synergistic antibiotic combinations, addition of an antiresistance factor, and attacking the underlying disease. The first of these approaches, combination therapy, is commonly used clinically against infections due to *P. aeruginosa* [1]; for this approach a combination of an aminoglycoside (e.g., tobramycin) with a β-lactam (e.g., ticarcillin) is used. However, such treatments tend not to be appropriate for infection due to *B. cepacia* or *S. maltophilia* because of the high intrinsic resistance of these organisms to both classes of antibiotics. The second approach involves the application of an antibiotic with an agent (with no independent antimicrobial activity) that has been designed to overcome an antibiotic resistance mechanism. One example would be the application of ticarcillin with the β-lactamase inhib-

itor clavulanate, although this inhibitor has relatively little efficacy against class C β-lactamases. A far better β-lactamase inhibitor for this type of enzyme is BRL42715 [8], which is an effective class C inhibitor but which has not yet been used clinically. Another approach is to overcome outer-membrane permeability, and from this perspective, cationic peptide permeabilizers show great promise [58].

The third possibility is an attack on the underlying disease, since all of the nonfermenters are opportunistic pathogens with limited virulence, except in patients with severe underlying disease. Thus, since most healthy individuals do not develop infections caused by such organisms, a clear approach would be to boost nonspecific or specific defenses, for example, by the addition of cytokines to a patient's regimen [59, 60].

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