Mechanisms of antibiotic resistance in *Pseudomonas aeruginosa*

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INTRODUCTION

Pseudomonas aeruginosa is a notoriously difficult organism to control with antibiotics or disinfectants[1]. Recent reports on the antibiotic sensitivity patterns of *P. aeruginosa* in the UK have highlighted the problem of antibiotic resistance in cystic fibrosis (CF) strains in comparison with other hospital isolates[2,3]. Table 1 summarizes the current position in which the resistance rates of *P. aeruginosa* strains from CF patients are all significantly higher than those from non-CF patients. Extensive use of these antibiotics to treat *P. aeruginosa* in CF has generated the selective pressure to encourage resistance development. Why is *P. aeruginosa* resistant to antibiotics and how can it become more resistant following exposure to antibiotics? Its general resistance is due to a combination of factors:

- It is intrinsically resistant to antimicrobial agents due to low permeability of its cell wall
- It has the genetic capacity to express a wide repertoire of resistance mechanisms
- It can become resistant through mutation in chromosomal genes which regulate resistance genes
- It can acquire additional resistance genes from other organisms via plasmids, transposons and bacteriophages.

An additional feature which contributes to the resistance of *P. aeruginosa* in CF is its mode of growth in the lungs. Aggregates of bacteria in the lung are surrounded by a layer of alginate polysaccharide. These microcolonies or biofilms are highly resistant to eradication by antibiotics, due to mechanisms which remain unclear.

P. aeruginosa is a highly adaptable organism. It can grow on a wide variety of substrates and alter its properties in response to changes in the environment. The determination of the entire genome sequence of *P. aeruginosa* and the application of powerful DNA array techniques to reveal microbial gene expression *in vivo* should provide us with a clearer insight into the mechanisms involved. It has a large genome containing 6.26 Mbp (encoding 5567 genes) compared to 4.64 Mbp (4279 genes) in *Eschericia coli* K12, 2.81 Mbp (2594 genes) in *Staphylococcus aureus* N315 and 1.83 Mbp (1714 genes) in *Haemophilus influenzae* Rd. An approximate calculation of the number of genes needed for cell growth and division in a minimal salts medium, including all enzymes needed for metabolism and structural proteins, is around 1500. *P. aeruginosa* therefore possesses considerable additional genetic capacity compared with other organisms. This explains its highly adaptable nature, including the ability to develop resistance where antibiotics are used extensively.

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Table 1 ***P. aeruginosa* resistance rates (%)**

Antibiotic	*Non-CF patients[2] (n=2067)*	*CF-patients[2] (n=127)*	*CF-patients[3] (n=282)*
Amikacin	3.9	36	–
Gentamicin	9.1	43	47
Tobramycin	–	–	24
Ciprofloxacin	7.3	24	16
Ceftazidime	1.7	14	32
Imipenem	6.7	31	–
Meropenem	3.5	11	–
Piperacillin	3.4	11	24
Piperacillin/tazobactam	2.4	9	–
Colomycin	–	–	5

MECHANISMS OF RESISTANCE

There are three basic mechanisms by which organisms resist the action of antimicrobial agents: restricted uptake and efflux; drug inactivation and changes in targets. The contribution of each of these to the resistance of *P. aeruginosa* in CF will be discussed.

Penetration of antibiotics through the cell envelope of *P. aeruginosa*

All of the major classes of antibiotics used to treat *P. aeruginosa* infections have to cross the cell wall to reach their targets (Figure 1). The aminoglycosides (gentamicin, tobramycin, amikacin) inhibit protein synthesis by binding to the 30S subunit of the ribosome. Quinolones (ciprofloxacin) bind to the A subunit of DNA gyrase, which maintains the ordered structure of the chromosome inside the cells. The β-lactams (e.g. piperacillin,

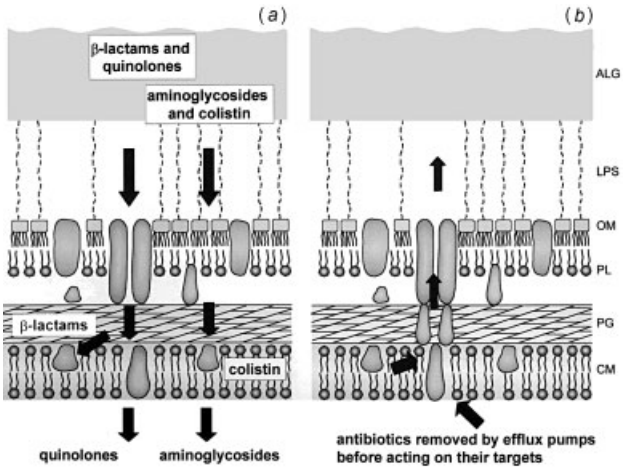

Figure 1 **Schematic representation of the arrangement of components in the cell wall of *P. aeruginosa*.** CM=cytoplasmic membrane; OM=outer membrane; PG=peptidoglycan; LPS=lipopolysaccharide; ALG=alginate. (*a*) The pathways for penetration of *β*-lactams and quinolones through porin channels in the OM. Aminoglycosides and colistin promote their own uptake by interacting with the LPS on the outer face of the OM. (*b*) How efflux systems reverse the diffusion of antibiotics across the OM. The efflux pumps comprise three components: an energy-dependent pump in the CM, a porin in the OM and an adapter protein joining the two membrane components. Antibiotics which have entered the cell are collected from the cytoplasm, the cytoplasmic membrane or the periplasm and expelled from the cells through the porin

ceftazidime, imipenem, meropenem and aztreonam) inhibit the peptidoglycan-assembling transpeptidases located on the outer face of the cytoplasmic membrane. Finally the polymyxins (colomycin, colistin) bind to phospholipids in the cytoplasmic membrane, destroying its barrier function. The innate resistance of *P. aeruginosa* to all classes of antibiotics has generally been attributed to the low permeability of its cell wall. Failure of antibiotics to accumulate within the organism is due to a combination of restricted permeability of the outer membrane and the efficient removal of antibiotic molecules that do penetrate by the action of efflux pumps.

Alginate as a barrier

A characteristic feature of many *P. aeruginosa* strains in cystic fibrosis is the production of a loosely associated layer of the anionic polysaccharide, alginate, which surrounds the cells and binds them together in aggregates. Although it has been shown that alginate can bind cationic antibiotics such as the aminoglycosides and restrict their diffusion[4], the effect on the overall sensitivity of mucoid *P. aeruginosa* is probably minimal. Indeed some mucoid isolates are fully sensitive to aminoglycosides[5].

The outer membrane as a barrier

The outer membrane of *P. aeruginosa* presents a significant barrier to the penetration of antibiotics, restricting the rate of penetration of small hydrophilic molecules and excluding larger molecules (Figure 1*a*). Small hydrophilic antibiotics such as the *β*-lactams and quinolones can only cross the outer membrane by passing through the aqueous channels provided by porin proteins. These are barrel-shaped molecules which span the outer membrane, usually associated as trimers (Figure 2). *P. aeruginosa* produces several different porins, oprF being the major species present in all strains[6]. Although mutants lacking oprF have been reported, loss of oprF has not been found to be a major cause of antibiotic resistance, presumably because such strains have restricted ability to take up hydrophilic nutrients. OprD is a specialized porin which has a specific role in the uptake of positively charged amino acids such as lysine. Loss of oprD is frequently associated with resistance to imipenem, which requires this porin to cross the outer membrane. Loss of the oprD porin increases the minimum inhibitory concentration from 1–2 to 8–32 mg/L and 17% rate of resistance has been reported during treatment[7]. Interestingly, meropenem is not affected by loss of oprD, indicating that the carbapenems have crossed the outer membrane by different channels.

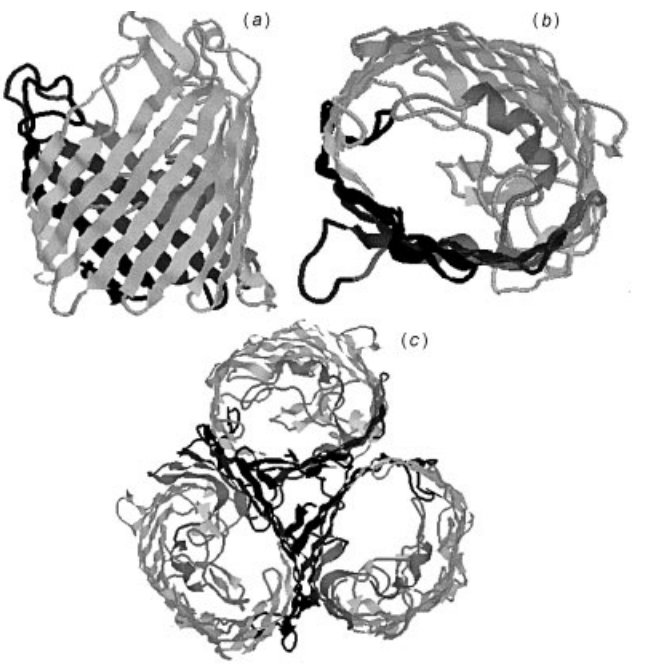

Figure 2 **General structure of a porin protein illustrating the membrane-spanning *β*-barrel structure in profile (*a*) and viewed from above (*b*) showing the aqueous channel through which hydrophilic antibiotics cross the outer membrane. Most porins form trimers in the outer membrane (*c*).** The structures shown are for the general ompF porin of *Escherichia coli* (Brookhaven Protein Databank entry 2omf). An animated model of the *P. aeruginosa* oprF porin can be viewed at [http://www.cmdr.ubc.ca/bobh/oprfmodel.htm]. For a comprehensive list of known *P. aeruginosa* outer membrane proteins, including porins see [http://www.cmdr.ubc.ca/bobh/ompknown.html]

The aminoglycosides and colistin do not cross the outer membrane via porin channels. Instead they promote their own uptake by binding to the lipopolysaccharide (LPS) on the outer face of the membrane. This destroys the permeability barrier of the outer membrane and allows the antibiotics to penetrate through the wall to the cytoplasmic membrane. The aminoglycosides are then actively transported into the cells where they interfere with protein synthesis at the ribosomes. Colistin exerts its bactericidal action through disruption of the cytoplasmic membrane. Resistance to aminoglycosides and colistin has been observed in laboratory strains of *P. aeruginosa* due to overexpression of an outer membrane protein, oprH, which protects the LPS from binding the antibiotics[8]. However, this form of resistance has not been encountered widely in clinical isolates.

The role of efflux systems in resistance

The multidrug efflux systems are composed of three protein components, an energy-dependent pump located in the cytoplasmic membrane, an outer membrane porin and a linker protein which couples the two membrane components together[9]. This tripartite arrangement forms an efficient extrusion system for toxic molecules present in the cytoplasm, the cytoplasmic membrane or the periplasm, i.e. the region between the outer and cytoplasmic membranes (Figure 1*b*). Four different antibiotic efflux systems have been described in *P. aeruginosa*: *mexAB-oprM*, *mexXY-oprM*, *mexCD-oprJ* and *mexEF-oprN*[10]. Their location on the genome is shown in Figure 3. All classes of antibiotics except the polymyxins are susceptible to extrusion by one or more of the efflux systems.

MexAB-oprM is responsible for extrusion of β-lactams, quinolones and a range of disinfectants. MexXY-oprM extrudes aminoglycosides and mexEF-oprN extrudes carbapenems and quinolones. The genes for the systems are present in all strains but they are not expressed at high levels. However, increased expression can result from mutation in regulatory genes such as *mexR*, which controls expression of the *mexAB-oprM* genes[11].

Inactivation and modification of antibiotics

All *P. aeruginosa* strains possess the *ampC* gene for the inducible chromosomal β-lactamase. However, induction alone probably does not account for resistance in CF strains. Instead, over-expression of the enzyme results from spontaneous mutation in the regulatory gene, *ampR*. This has occurred particularly where heavy reliance has been placed on ceftazidime therapy[12]. Although the enzyme is normally located in the periplasm, it has been detected in sputum during antipseudomonal treatment[13]. This extracellular enzyme is probably released from high-level

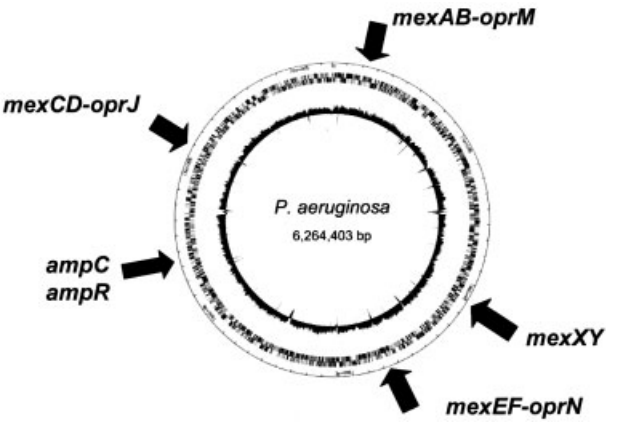

Figure 3 **Location of efflux and chromosomal β-lactamase genes on the genome of *P. aeruginosa* PAO1 (Pseudomonas Genome Project [http://www.pseudomonas.com]).** The genome is shown as a closed circle of 6.26 Mbp of DNA with the origin of replication at the top (outer circle). The positions of the 5567 known or putative genes are shown as shaded bands on their respective DNA strands on the middle circle. The inner circle represents an analysis of base sequences in the DNA, discontinuities indicate positions where foreign DNA sequences have been incorporated. The position of the genes encoding the multiple efflux (*mex*) components of four characterized pumps (*mexAB*, *mexCD*, *mexEF* and *mexXY*) are shown together with their respective porin (*opr*) components, *oprM* and *oprJ* and *oprN*. The position of the chromosomal β-lactamase gene (*ampC*) and its regulatory gene (*ampR*) is also shown.

producers in the lungs following lysis, it would protect low-level producers by reducing the local concentration of β-lactam antibiotic.

Over-production of the *ampC* β-lactamase poses a particular threat to cephalosporins. Other β-lactamases produced by *P. aeruginosa* include extended-spectrum plasmid-mediated enzymes (ESBLs) active against penicillins and cephalosporins[14]. Use of β-lactamase inhibitors (clavulanic acid with ticarillin and tazobactam with piperacillin) provides protection of these antibiotics against some of the plasmid-mediated enzymes, but not the *ampC* enzyme[15]. Inhibitor-resistant enzymes have also been reported[16] and their future appearance in *P. aeruginosa* strains from CF would threaten their effective use. Specific carbapenemases in *P. aeruginosa* are of two types, serine-based enzymes and metallo-enzymes (class D). Reports of plasmid-mediated carbapenemases in non-CF strains of *P. aeruginosa*[17] show that these enzymes have the potential to be transmitted to CF strains, especially under the selective pressure of widespread use of carbapenems.

Inactivation of aminoglycosides occurs through production of enzymes which transfer acetyl, phosphate or adenylyl groups to amino and hydroxyl substituents on the antibiotics. Prior to the recognition that aminoglycosides are susceptible to efflux, inactivation was regarded as the major mechanism of resistance for this group of antibiotics. The modifying enzymes use cytoplasmic cofactors (acetyl co-enzyme A or ATP) to supply the substituents added to the aminoglycosides so the modification process occurs

within the cytoplasm. The modifying enzymes are plasmid-mediated, consequently spontaneous mutations in cells during antibiotic treatment does not lead to over-expression of the enzymes, as seen with the chromosomal β-lactamases. Acquisition of the genes for the modifying enzymes would require transfer from strains bearing the plasmids. Currently treatment of *P. aeruginosa* infections in CF with aerosolized tobramycin does not appear to have resulted in increased resistance rates[18].

Changes in targets

This mechanism of resistance results from mutational changes in target enzymes which result in maintenance of their vital role in cell metabolism but resistance to the action of selective inhibition by antibiotics. In *P. aeruginosa* it is most commonly encountered with the quinolones through mutation in the *gyrA* gene encoding the A subunit of the target enzyme, DNA gyrase[19]. Together with active efflux this accounts for the current level of resistance seen in CF strains. Changes in the structure of the ribosome 30S subunit (the aminoglycoside target) influence streptomycin sensitivity but not that of the anti-pseudomonal aminoglycosides. Alteration in the penicillin-binding proteins of *P. aeruginosa* resulting in resistance to β-lactams has been reported but is not currently a major problem in CF strains[20].

Biofilms and resistance

In CF lung infections *P. aeruginosa* grows as aggregates of cells (microcolonies) encased in a protective alginate polysaccharide. This mode of growth also occurs on surfaces, where it is referred to as a biofilm. The characteristic property of all biofilms is their remarkable resistance to eradication by physical and biochemical treatments, including antibiotics[21]. Although this recalcitrance has been recognized for many years its biological basis has still not been thoroughly explained. Factors which might partly explain the resistance phenotype include the high bacterial cell density and physical exclusion of the antibiotic. Physiological changes might occur in cells within the biofilm involving a general stress response, in which key metabolic processes are shut down and protective mechanisms induced[22]. It is clear that cells in the biofilm, like free-living 'planktonic' cells, can sense the presence of other cells (quorum sensing) and alter their properties accordingly[23].

Finally, the population of cells within a biofilm is heterogeneous, containing fast- and slow-growing cells, some resistant through expression of inactivating enzymes and efflux pumps, others conspicuously not expressing such systems. The overall resistance is therefore dependent upon an interaction between the entire population of cells and therapy needs to be directed against a multicellular community[21].

Table 2 **Summary of resistance mechanisms of *P. aeruginosa* in cystic fibrosis**

Antibiotic	*Permeability and efflux*	*Inactivation*	*Changes in targets*
β-lactams	+++	+++	+
Aminoglycosides	++	++	–
Quinolones	+++	–	+++
Polymyxins	–	–	+

+++ most commonly encountered; ++ common; + reported but rare; – not reported

CONCLUSIONS AND IMPLICATIONS FOR THERAPY

Table 2 summarizes the contribution of different mechanisms to the current resistance levels of *P. aeruginosa* encountered in CF. Restricted permeability and efflux are common components of the resistance phenotype for β-lactams, aminoglycosides and quinolones and are essentially fundamental properties of the organism. The innate antibiotic resistance of *P. aeruginosa* results from the restricted permeability of the cell wall and is enhanced by the activity of efflux systems.

The occurrence of more specific mechanisms involving inactivation and changes in targets reflects the selective pressure resulting from heavy reliance on these agents in CF. For example, spontaneous mutations can increase the expression of chromosomal β-lactamase genes. These mutants will be selected under the pressure of antibiotic usage, especially where monotherapy is employed.

The increased recognition of the role of efflux systems in general antibiotic resistance has led to a search for efflux pump inhibitors as therapeutic adjuncts.

Similarly, an understanding of the complex interaction in biofilm communities may eventually lead to novel strategies for their control. However, *P. aeruginosa* has always proven to possess an answer to antibiotic therapy, the ominous size of its genome and current lack of knowledge of the function of many of its genes suggests that this will continue to be the case whatever new therapies are devised.

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