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Review

Pseudomonas aeruginosa – a phenomenon of bacterial resistance

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Pseudomonas aeruginosa is one of the leading nosocomial pathogens worldwide. Nosocomial infections caused by this organism are often hard to treat because of both the intrinsic resistance of the species (it has constitutive expression of AmpC β-lactamase and efflux pumps, combined with a low permeability of the outer membrane), and its remarkable ability to acquire further resistance mechanisms to multiple groups of antimicrobial agents, including β-lactams, aminoglycosides and fluoroquinolones. *P. aeruginosa* represents a phenomenon of bacterial resistance, since practically all known mechanisms of antimicrobial resistance can be seen in it: derepression of chromosomal AmpC cephalosporinase; production of plasmid or integron-mediated β-lactamases from different molecular classes (carbenicillinases and extended-spectrum β-lactamases belonging to class A, class D oxacillinases and class B carbapenem-hydrolysing enzymes); diminished outer membrane permeability (loss of OprD proteins); overexpression of active efflux systems with wide substrate profiles; synthesis of aminoglycoside-modifying enzymes (phosphoryltransferases, acetyltransferases and adenylyltransferases); and structural alterations of topoisomerases II and IV determining quinolone resistance. Worryingly, these mechanisms are often present simultaneously, thereby conferring multiresistant phenotypes. This review describes the known resistance mechanisms in *P. aeruginosa* to the most frequently administrated antipseudomonal antibiotics: β-lactams, aminoglycosides and fluoroquinolones.

Introduction

Pseudomonas aeruginosa is responsible for 10–15 % of the nosocomial infections worldwide (Blanc *et al.*, 1998). Often these infections are hard to treat due to the natural resistance of the species, as well as to its remarkable ability of acquiring further mechanisms of resistance to multiple groups of antimicrobial agents. *P. aeruginosa* represents a phenomenon of antibiotic resistance, and demonstrates practically all known enzymic and mutational mechanisms of bacterial resistance (Pechere & Kohler, 1999). Often these mechanisms exist simultaneously, thus conferring combined resistance to many strains (McGowan, 2006). This review describes the resistance mechanisms to the most frequently administrated antipseudomonal antibiotics: β-lactams, aminoglycosides and fluoroquinolones.

Mechanisms of resistance to β-lactams

P. aeruginosa is intrinsically resistant to many structurally unrelated antimicrobial agents (Mesaros *et al.*, 2007) because of the low permeability of its outer membrane (1/100 of the permeability of *E. coli* outer membrane) (Livermore, 1984), the constitutive expression of various efflux pumps with wide substrate specificity (Livermore, 2001) and the naturally occurring chromosomal AmpC β-lactamase (also known as cephalosporinase) (Nordmann & Guibert, 1998). The natural resistance of the species relates to the following β-lactams: penicillin G; aminopenicillins, including those combined with β-lactamase inhibitors; first and second generation cephalosporins. *P. aeruginosa* easily acquires additional resistance mechanisms, which leads to serious therapeutic problems.

The susceptible *P. aeruginosa* phenotype (the so called wild-type) includes susceptibility to carboxypenicillins (carbenicillin, ticarcillin), ureidopenicillins (azlocillin, piperacillin), some third generation cephalosporins (ceftazidime, cefsulodine, cefoperazone), all the fourth generation cephalosporins, the monobactam aztreonam, and the carbapenems imipenem and meropenem (Pechere & Kohler, 1999). There are several basic resistance phenotypes. (i) Often called 'intrinsic resistance to carbenicillin', this phenotype is characterized by a fourfold to eightfold increase of MIC for most of the β-lactams, including meropenem but not imipenem. No production of chromosomal AmpC β-lactamase above the basic level is found. This phenotype includes resistance to non-β-lactam antibiotics like quinolones, trimethoprim, tetracycline and chloramphenicol. The cause for the rise in MIC is the low outer membrane permeability combined with activation or derepression of efflux systems (Pechere & Kohler, 1999).

(ii) The second phenotype affects resistance to all β-lactams except cephems (cefepime and cefpirome) and carbapenems. The extent of the change is antibiotic-dependent, and is caused by derepression of the AmpC β-lactamase (Livermore, 1995). (iii) In the third phenotype, resistance to penicillins (in particular ticarcillin, azlocillin and piperacillin) is affected more than resistance to cephalosporins, resulting from production of OXA-type β-lactamases (Pechere & Kohler, 1999). These narrow-spectrum oxacillinases determine resistance to carboxypenicillins and ureidopenicillins, but not to extended-spectrum cephalosporins, aztreonam and moxalactam (Bert *et al.*, 2002). (iv) The fourth phenotype is characterized by increased MICs to carbapenems. Resistance to other β-lactams is not affected because strains exhibiting this phenotype have a decreased level of OprD, a carbapenem-specific porin (Livermore, 2001).

Other resistance phenotypes are determined mainly by the production of plasmid- or integron-encoded extended-spectrum β-lactamases (ESBLs) from different molecular classes. In *P. aeruginosa* all possible mechanisms determining resistance to β-lactam antibiotics [enzymic inactivation, active efflux, changes in outer membrane permeability and synthesis of penicillin-binding proteins (PBPs) with lower affinity to β-lactams] may exist simultaneously or in various combinations.

Resistance to β-lactams due to β-lactamase production

Enzyme production is the major mechanism of acquired resistance to β-lactam antibiotics in *P. aeruginosa*. Penicilloyl-serine transferases (usually referred to as β-lactamases) rupture the amide bond of the β-lactam ring, thus the obtained products lack antibacterial activity (Sykes & Mattew, 1976). Molecular classification of β-lactamases is based on the nucleotide and amino acid sequences in these enzymes (Ambler, 1980). To date, four classes are recognized (A–D), correlating with the functional classification defined by enzyme substrate and inhibitor profiles (Bush *et al.*, 1995). Classes A, C and D act through a serine-based mechanism, whereas class B or metallo-β-lactamases (MBLs) need zinc for their action. A significant number of β-lactamases of all four molecular classes are found in *P. aeruginosa*, including ESBLs of classes A, B and D.

AmpC β-lactamase. *P. aeruginosa* is naturally susceptible to carboxypenicillins, ceftazidime and aztreonam; however, it can acquire resistance to third generation cephalosporins. The most frequent mechanism by which this occurs is through the constitutive hyperproduction of AmpC β-lactamase (so called stable derepression) (Bagge *et al.*, 2002). Like some species of the *Enterobacteriaceae* family (*Enterobacter* spp., *Serratia marcescens*, *Citrobacter freundii*, *Morganella morganii* and *Yersinia enterocolitica*), *P. aeruginosa* produces an inducible chromosome-encoded AmpC β-lactamase (cephalosporinase) that belongs to molecular class C, based on Ambler and the first functional group according to Bush (Bush *et al.*, 1995). Usually the enzyme is produced in low quantities ('low-level' expression) and determines resistance to aminopenicillins and most of the early cephalosporins (Langaee *et al.*, 2000). However, chromosomal cephalosporinase production in *P. aeruginosa* may increase from 100 to 1000 times in the presence of inducing β-lactams (especially imipenem) (Bagge *et al.*, 2002). AmpC cephalosporinase activity is not inhibited by β-lactamase inhibitors used in clinical practice, for example clavulanic acid, sulbactam and tazobactam (Nordmann & Guibert, 1998).

AmpC β-lactamase is encoded by the *ampC* gene (Lodge *et al.*, 1993). Mechanisms regulating *ampC* expression have been studied in detail for *Enterobacter cloacae*. Similar mechanisms regulate the expression of the enzyme in *P. aeruginosa*. Several genes are involved in *ampC* induction – a process that is intimately linked to peptidoglycan recycling (Normark, 1995). Of the genes involved, *ampR*, is contiguous to *ampC* but divergently transcribed, and it encodes a positive transcriptional regulator that is a member of the LysR family (AmpR). This regulator is necessary for the β-lactamase induction (Lodge *et al.*, 1993). AmpR transcriptional regulatory activity is related to peptidoglycan processing (Jacobs *et al.*, 1994). The second gene, *ampG*, encodes a transmembrane protein that acts as a permease for 1,6-anhydromurapeptides, which are considered to be the signal molecules involved in *ampC* induction (Dietz *et al.*, 1997). The third gene, *ampD*, encodes a cytosolic *N*-acetyl-anhydromuramyl-L-alanine amidase, which hydrolyses 1,6-anhydromurapeptides, acting as a repressor of *ampC* expression (Höltje *et al.*, 1994). Mutational inactivation of *ampD* in *P. aeruginosa* PAO1 leads to partially derepressed expression of AmpC β-lactamase (Langaee *et al.*, 2000). The fourth gene, *ampE*, forms the bicistronic *ampDE* operon and encodes a cytoplasmic membrane protein that is thought to act as a sensory transducer molecule necessary for induction (Honore *et al.*, 1989). Recently, Juan *et al.* (2006) demonstrated that *ampC* expression is co-ordinately repressed by three AmpD homologues, including the previously described protein AmpD plus two additional proteins designated AmpDh2 and AmpDh3. The three AmpD homologues are responsible for a stepwise *ampC* upregulation mechanism ultimately leading to constitutive hyperexpression of the chromosomal cephalosporinase and high-level antipseudomonal β-lactam resistance, as shown by the analysis of the three single *ampD* mutants, the three double *ampD* mutants and the triple *ampD* mutant. This analysis was achieved by a three-step escalating mechanism generating four expression states: basal-level inducible expression (wild-type), moderate-level hyperinducible expression with increased antipseudomonal β-lactam resistance (*ampD* mutant), high-level hyperinducible expression with high-level β-lactam resistance (*ampD ampDh3* double mutant) and very high-level (more than 1000-fold compared to the wild-type) derepressed expres-

sion (triple mutant). Unlike enterobacteria, *P. aeruginosa* have not yet been found to contain plasmid-mediated cephalosporinases, although some of the plasmid-encoded cephalosporinases demonstrate a remarkably similar structure to that of the pseudomonal AmpC β-lactamase.

Class A carbenicillin hydrolysing β-lactamases. Four carbenicillin hydrolysing β-lactamases of *Pseudomonas*-specific enzyme (PSE) type were found in *P. aeruginosa*: PSE-1 (CARB-2), PSE-4 (CARB-1), CARB-3 and CARB-4 (Bert *et al.*, 2002). Their substrate profile includes carboxypenicillins, ureidopenicillins and cefsulodine. These enzymes belong to molecular class A and functional group 2c (Bush *et al.*, 1995). PSE-1, PSE-4 and CARB-3 are closely related (they differ by just 1 or 2 amino acids), but they are only 86.3 % homologous with CARB-4 (Sanschagrin *et al.*, 1998). The $bla_{\text{CARB-4}}$ gene is likely to have been acquired from other bacterial species, as the mol% G+C in this gene is 39.1 % unlike the mol% G+C of genes that are typical for *P. aeruginosa*, which is 67 %. Carbenicillinase producers show variable susceptibility to cefepime, cefpirome and aztreonam, and 100 % susceptibility towards ceftazidime and carbapenems.

Class A ESBLs. Unlike PSEs, ESBLs of molecular class A and functional group 2b' (Bush *et al.*, 1995) lead to the development of resistance not only to carboxypenicillins and ureidopenicillins, but also to extended-spectrum cephalosporins (ceftazidime, cefepime, cefpirome) and aztreonam (Weldhagen *et al.*, 2003). They show low affinity to carbapenems. Their *in vitro* activity is inhibited by clavulanic acid and tazobactam (Nordmann & Guibert, 1998). Discovery of class A ESBLs in clinical isolates of *P. aeruginosa* occurred after 1990. Apart from the TEM and SHV types of enzyme that are well known in the *Enterobacteriaceae* family, in *P. aeruginosa* other enzymes that were identified are PER (mostly in clinical isolates from Turkey), VEB (from South-East Asia, France and Bulgaria), GES/IBC (France, Greece and South Africa) and BEL types (Table 1). These six types have low identity at the genetic level, and yet they have similar hydrolysis profiles.

SHV-2a was originally detected in France (in 1995) (Naas *et al.*, 1999a), and later in Thailand and Poland (Chanawong *et al.*, 2001). This enzyme vigorously hydrolyses fourth generation cephalosporins (Weldhagen *et al.*, 2003). SHV-5 and SHV-12 producing *P. aeruginosa* strains were also found in Thailand (Chanawong *et al.*, 2001). Afterwards, SHV-5 and SHV-12 ESBLs were identified in clinical *P. aeruginosa* isolates from Greece as well (Poirel *et al.*, 2004a; Neonakis *et al.*, 2003). SHV-5 determines high level of resistance to ceftazidime and monobactams.

Between 1992 and 1998 in France, *P. aeruginosa* strains producing the following TEM enzymes were consecutively isolated: TEM-42, TEM-4, TEM-21 and TEM-24 (Mugnier *et al.*, 1996; Poirel *et al.*, 1999; Dubois *et al.*, 2002a; Marchandin *et al.*, 2000). The hydrolytic spectrum of TEM enzymes in *P. aeruginosa* is similar to that of the classical ESBLs in *Enterobacteriaceae* and includes: narrow-spectrum penicillins, extended-spectrum cephalosporins and aztreonam (Weldhagen *et al.*, 2003).

It is likely that the genes for the TEM- and SHV-type ESBLs in *P. aeruginosa* originate from *Enterobacteriaceae*, from which genes are passed by gene transfer. This has been shown for the sequence of TEM-24 (Marchandin *et al.*, 2000) and the downstream-located chromosomal DNA sequences of *P. aeruginosa* RP-1, producing SHV-2a, which were found to be identical to those reported to be plasmid encoded in a *Klebsiella pneumoniae* isolate (Naas *et al.*, 1999a). Several *P. aeruginosa* strains, including respiratory and urinary isolates producing TEM-24 ESBL, were isolated from a long-term-hospitalized woman (Marchandin *et al.*, 2000). TEM-24-producing isolates of *Enterobacter aerogenes* recovered from wound, venous catheter and faeces, and TEM-24-producing wound *E. coli* isolate were cultured from the same patient. TEM-24 and the resistance markers for aminoglycosides, chloramphenicol and sulfonamides were encoded by a 180 kb plasmid transferred by conjugation into *E. coli* HB101. The multiplicity of TEM-24-producing bacteria recovered from the same patient strongly suggests the *in vivo* horizontal transfer of this plasmid-mediated ESBL from *Enterobacteriaceae* to *P. aeruginosa*.

PER-1 was the first identified and fully characterized ESBL in *P. aeruginosa*. It was found in 1991 in France in an isolate from the urine culture of a Turkish citizen (Nordmann & Naas, 1994) and was chromosome encoded. Later, plasmid encoded PER-1 enzymes were reported as well (Nordmann & Guibert, 1998). Currently, there is widespread dissemination of $bla_{\text{PER-1}}$ among nosocomial *P. aeruginosa* isolates in Turkey (Vahaboglu *et al.*, 2001; Kolayli *et al.*, 2005). Other geographical regions where PER-1 producing *P. aeruginosa* strains were isolated were Italy, Belgium and Poland (Luzzaro *et al.*, 2001; Pagani *et al.*, 2004; Claeys *et al.*, 2000; Empel *et al.*, 2007). PER-1 exhibits the substrate profile typical of classical ESBLs. It is moderately inhibited by β-lactamase inhibitors and imipenem (Weldhagen *et al.*, 2003).

Another type of molecular class A ESBLs are the VEB enzymes. The first isolation of a VEB-1 β-lactamase was in 1998 in France (Naas *et al.*, 1999b); later Girlich *et al.* (2002) found a high prevalence of $bla_{\text{VEB-like}}$ genes (93 %) in ceftazidime-resistant clinical isolates of *P. aeruginosa* in the University Hospital in Thailand. During that study, a new $bla_{\text{VEB-2}}$ gene was identified. VEB-2 differed from VEB-1 by just a single amino acid outside the active centre of the enzyme. In 2007 high dissemination (56.8 %) of VEB-1 ESBL among ceftazidime-resistant nosocomial *P. aeruginosa* isolates from Bulgaria was reported (Strateva *et al.*, 2007). The substrate profile of VEB enzymes was identical with that of PER-1 (Weldhagen *et al.*, 2003).

At the very end of the 20th century, a novel family of ESBLs was described, referred to as Guiana extended spectrum

Table 1. Epidemiology of molecular class A ESBLs produced by *P. aeruginosa*

Enzyme	Location of encoding gene	Initial isolation		Other geographical regions of isolation	Reference
		Year	Country		
SHV-2a	C, P	1995	France	Thailand, Poland	Naas *et al.* (1999a), Chanawong *et al.* (2001)
SHV-5	P	1994–1996	Thailand	Greece	Chanawong *et al.* (2001), Poirel *et al.* (2004a)
SHV-12	C	1994–1996	Thailand	Greece	Chanawong *et al.* (2001), Neonakis *et al.* (2003)
TEM-4	P, C	1996	France		Poirel *et al.* (1999)
TEM-21	C	1997	France		Dubois *et al.* (2002a, 2005)
TEM-24	P	1998	France		Marchandin *et al.* (2000)
TEM-42	P	1992	France		Mugnier *et al.* (1996)
TEM-116	Unknown	2004–2006	France		David *et al.* (2008)
VEB-1	C, P, I	1998	France	Thailand, India, China, Bulgaria	Naas *et al.* (1999b), Girlich *et al.* (2002), Strateva *et al.* (2007)
VEB-1a	C, I	1999	Kuwait	India	Poirel *et al.* (2001c), Aubert *et al.* (2004)
VEB-1b	C, I	1999	Kuwait		Poirel *et al.* (2001c)
VEB-2	C, I	1999	Thailand		Girlich *et al.* (2002)
PER-1	C	1991	France	Turkey, Italy, Belgium, Poland	Nordmann & Naas (1994), Vahaboglu *et al.* (1997), Luzzaro *et al.* (2001), Pagani *et al.* (2004), Claeys *et al.* (2000), Empel *et al.* (2007)
GES-1	P, I	1999	France	Brazil	Dubois *et al.* (2002b), Castanheira *et al.* (2004a)
GES-2	P, I	2000	South Africa		Poirel *et al.* (2002b), Weldhagen & Prinsloo (2004)
GES-5	P, I	2004	France	South Africa	Poirel *et al.* (2005a), Labuschagne *et al.* (2008)
GES-9	P, I	2004	France		Poirel *et al.* (2005b)
IBC-2 (GES-8)	C, I	1998	Greece		Mavroidi *et al.* (2001)
BEL-1	C, I	2004	Belgium		Poirel *et al.* (2005c), Bogaerts *et al.* (2007)

C, Chromosomal; I, integron borne; P, plasmid borne.

(GES), named after the country of origin of the first isolate, French Guiana. GES-1 and GES-2 were found in France and Brazil (Dubois *et al.*, 2002b; Castanheira *et al.*, 2004a), and South Africa (Poirel *et al.*, 2002b), respectively. GES-1 has an unusually low level of catalytic activity, low affinity to the most of the substrates, and an unusual inhibition profile that includes clavulanic acid and imipenem. Unlike most of class A ESBLs, GES-1 has strong affinity to the second generation cephalosporin cefoxitin (Weldhagen *et al.*, 2003). GES-2 (discovered in 2000) possesses carbapenemase activity (Weldhagen & Prinsloo, 2004). This enzyme originated through a point mutation of GES-1. Like GES-1, GES-2 β-lactamase has cysteine residues in positions 69 and 238 that may form a disulphide bridge, which explains the imipenem-binding properties. GES-2 demonstrates 100 times higher catalytic activity towards imipenem than GES-1; but this is still a much lower activity than that of the metallo-enzymes of molecular class B (Nordmann & Poirel, 2002). New variants of GES-1 enzyme have been reported: GES-5 (Poirel *et al.*, 2005a) and GES-9 (Poirel *et al.*, 2005b). In comparison with GES-1 identified in 1999 (in France) the newly discovered GES-5 hydrolyses penicillins to a greater extent, as well as extended-spectrum cephalosporins and aztreonam. It is distinguished from GES-1 by a Gly242Ser amino acid substitution. Enzyme activity is suppressed by clavulanic acid, tazobactam and imipenem. The $bla_{\text{GES-5}}$ gene is located on a class 1 integron simultaneously with $aacA_4$, encoding resistance to aminoglycosides. GES-9 enzyme also differs from GES-1 by a single amino acid substitution (Gly243Ser). It is inactivated by clavulanic acid and imipenem and hydrolyses aztreonam. The $bla_{\text{GES-9}}$ gene encoding it is localized within a class 1 integron containing two copies of an insertion sequence from the IS*1111* family.

IBC-2 (now called GES-8) ESBL was discovered in a clinical *P. aeruginosa* isolate in Greece (Mavroidi *et al.*, 2001). It is a variant of GES-1 with a single substitution (Ala125Leu) compared to GES-1. Production of this β-lactamase determines resistance to ceftazidime and other oxyiminocephalosporins, and is inhibited by imipenem, clavulanic acid and tazobactam.

Recently, in Belgium, a discovery of a new type of ESBL of Ambler class A was made – BEL-1. The new ESBL was identified in a clinical strain of *P. aeruginosa* isolated at a hospital in Flanders, Belgium (Poirel *et al.*, 2005c). The enzyme had a hydrolysis profile comprising extended-spectrum cephalosporins and aztreonam, and its activity was suppressed by clavulanic acid, tazobactam, cefoxitin, moxalactam and imipenem. The encoding gene, $bla_{\text{BEL-1}}$, is a part of a class 1 integron, In*120*, localized in a chromosome transposon that also contains three other gene cassettes. Later on, between May and November 2006, BEL-1-producing *P. aeruginosa* isolates were discovered in several hospitals located in different geographical areas of Belgium (Bogaerts *et al.*, 2007).

Dissemination of class A ESBL-encoding genes plays an important role in antibiotic resistance dissemination, and may limit the possibilities for the choice of antibiotic regimen in the treatment of life-threatening infections caused by *P. aeruginosa*. Plasmids and integrons are important factors for this dissemination. In this regard, plasmid localization was proven for most of the genes encoding TEM and SHV enzymes in *P. aeruginosa* (Chanawong *et al.*, 2001; Mugnier *et al.*, 1996). Whereas genes encoding β-lactamases of Ambler class B (metalloenzymes) and Ambler class D (oxacillinases) were usually located in class 1 integrons, genes encoding VEB- and GES-type enzymes were the only class A ESBL-encoding genes that are associated with these genetic determinants (Girlich *et al.*, 2002; Poirel *et al.*, 2002b). Localization of some genes on transposons provides an additional route for the mobilization of antimicrobial-resistance genes, and this fact can explain the simultaneous localization of the same ESBL-encoding genes on plasmids, as well as on the chromosome of *P. aeruginosa* (Weldhagen *et al.*, 2003).

Class D β-lactamases (oxacillinases). Oxacillinases (OXA type enzymes) belong to molecular class D and functional group 2d (Bush *et al.*, 1995). Classical OXA enzymes (OXA-1, OXA-2, OXA-10) determine resistance to carboxypenicillins and ureidopenicillins but not to ceftazidime (Bert *et al.*, 2002). Resistance to ticarcillin and piperacillin resulting from production of OXA-2 enzymes is lower than the resistance that develops when OXA-10 and OXA-1 oxacillinases are produced (Bert *et al.*, 2003). Ceftazidime hydrolysing extended-spectrum oxacillinases have the greatest clinical importance. Their hydrolysis spectrum also includes: cefotaxime, cefepime, cefpirome, aztreonam and moxalactam (Bradford, 2001). With the exception of OXA-18, the activity of these enzymes is not suppressed by β-lactamase inhibitors (clavulanic acid and tazobactam). This fact hampers their identification by routine laboratory practices (Naas & Nordmann, 1999). Sanschagrin *et al.* (1995) described five different groups of oxacillinases in *P. aeruginosa*. OXA group I integrates OXA-5, OXA-7, OXA-10 and its derivates (OXA-11, OXA-14, OXA-16 and OXA-17), and OXA-13 and its derivates (OXA-19 and OXA-28) (Couture *et al.*, 1992; Scoulica *et al.*, 1995; Mugnier *et al.*, 1998a, b; Hall *et al.*, 1993; Danel *et al.*, 1995, 1998, 1999; Poirel *et al.*, 2001a). In the last few years OXA-13 and its derivates (OXA-19 and OXA-28) were defined as an OXA-10-related subgroup (Bert *et al.*, 2002). OXA-11, OXA-14 and OXA-19 affect mostly ceftazidime activity (Aubert *et al.*, 2001) while OXA-17 attacks mainly cefotaxime (Danel *et al.*, 1999). Generally, extended-spectrum variants of OXA-10 determine low-level resistance to fourth generation cephalosporin cefepime, in contrast to third generation ceftazidime (which is 'high level') (Aubert *et al.*, 2001). OXA group II includes OXA-2, OXA-3, OXA-15 and OXA-20 (Sanschagrin *et al.*, 1995; Dale *et al.*, 1985; Danel *et al.*, 1997; Naas *et al.*, 1998). OXA-15 is an extended-spectrum variant of OXA-2 β-lactamase (Danel *et al.*, 1997). Recently, Poirel *et al.* (2002a) found one more derivate of OXA-2 (OXA-32) that is an ESBL. The OXA group III includes OXA-1 and its derivates – OXA-4, OXA-30 and OXA-31 (Aubert *et al.*, 2001). The OXA group IV comprises just a single enzyme – OXA-9; OXA group V is represented by LCR-1 (Couture *et al.*, 1992; Sanschagrin *et al.*, 1995).

Apart from OXA-15 and OXA-32, the rest of the extended-spectrum oxacillinases derive from OXA-10 β-lactamase. Most of the class D ESBLs were found in clinical isolates from Turkey (Bradford, 2001). It is known that all extended-spectrum variants of OXA-10 have one of the following two amino acid substitutions: Ser73Asn or Gly157Asp. The latter determines high-level resistance to ceftazidime (Bradford, 2001).

OXA-18 enzyme is encoded by the chromosomal $bla_{\text{OXA-18}}$ gene and has low amino acid identity with the other class D oxacillinases produced by *P. aeruginosa* (the highest identity is with OXA-9 and OXA-12 – 45 and 42 %, respectively) (Philippon *et al.*, 1997). This enzyme does not belong to any of the groups introduced by Sanschagrin *et al.* (1995). Its hydrolytic properties are like these of class A ESBLs – it affects amoxicillin, ticarcillin, cefalotin, ceftazidime, cefotaxime and aztreonam, but not imipenem. OXA-18 activity is totally inhibited by clavulanic acid. Recently, at the National Centre of Bone Marrow Transplantations in Tunisia the first outbreak in the world of a nosocomial infection (1998–2000) caused by OXA-18-producing *P. aeruginosa* strains was reported (Kalai Blagui *et al.*, 2007).

In 2003, a new class D ESBL – OXA-45 – was identified in a multidrug-resistant clinical *P. aeruginosa* isolate from Texas, USA. Its substrate profile was similar to that of OXA-18, and clavulanic acid inhibited its activity. The enzyme revealed highest amino acid identity with OXA-18 (65.9 %) and OXA-9 (42.8 %). $bla_{\text{OXA-45}}$ is located on a 24 kb plasmid (Toleman *et al.*, 2003).

Most of the extended-spectrum oxacillinases are encoded by plasmid- or integron-located genes (Nordmann & Guibert, 1998), and this contributes to their easy dissemination and to the increased prevalence of class D

ESBLs, producing *P. aeruginosa* isolates throughout Europe.

Class B MBLs. Another group of ESBLs occurring in *P. aeruginosa* are the carbapenem-hydrolysing enzymes, which are also known as carbapenemases or MBLs due to the presence of Zn^{2+} in their active centre (Nordmann & Guibert, 1998). They belong to molecular class B (Bush *et al.*, 1995). Carbapenemase production determines resistance to all β-lactams including the carbapenems imipenem and meropenem. Only the monobactam aztreonam is not influenced by the hydrolytic features of MBLs. The activity of class B carbapenem hydrolysing enzymes is not inhibited by clavulanic acid and tazobactam, but is suppressed by bivalent ionic chelators, e.g. EDTA (Nordmann & Poirel, 2002). IMP, VIM, SPM and GIM type MBLs were identified in *P. aeruginosa* (Table 2).

The first carbapenemase proven in *P. aeruginosa* was IMP-1. It was found in Japan in a large-scale study of carbapenem-resistant clinical isolates during 1992–1994 (Senda *et al.*, 1996). A total of 11 % of the strains studied harboured $bla_{\text{IMP-1}}$. The gene was localized to a large plasmid (36 kb) and found to be part of a gene cassette within a class 1 In*31* integron. Recently, IMP-1 MBL was reported among carbepenem-resistant *P. aeruginosa* isolated in two hospitals in Singapore (Koh *et al.*, 2004).

From 2000 until 2001 other IMP variants of MBLs were found in various Gram-negative bacteria worldwide. $bla_{\text{IMP-7}}$ were identified among *P. aeruginosa* clinical isolates in Canada (Gibb *et al.*, 2002; Parkins *et al.*, 2007) and Singapore (Koh *et al.*, 2004), and $bla_{\text{IMP-9}}$ was found in China (Xiong *et al.*, 2006), and $bla_{\text{IMP-13}}$ in Italy (Pagani *et al.*, 2005). In 2002, IMP-16 MBL was found in a *P. aeruginosa* strain from Brazil (Mendes *et al.*, 2004a). Its encoding gene is chromosomal and is located in a class 1 integron that also carries genes for aminoglycoside-modifying enzymes. Currently, the most recent IMP MBL (IMP-18) was found in a *P. aeruginosa* clinical isolate in the USA (Hanson *et al.*, 2006).

VIM-1 carbapenamase, found in a nosocomial *P. aeruginosa* strain isolated at the Verona University Hospital, Italy, in 1997, is the first representative of a new family of acquired MBLs (Lauretti *et al.*, 1999). Although VIM-1 shows less than 30 % amino acid identity to IMP enzymes, it has the same extended spectrum of hydrolysis (Nordmann & Poirel, 2002). Like bla_{IMP} genes, $bla_{\text{VIM-1}}$ is a part of a gene cassette inserted in the In*70* class 1 integron, which carries the following genes: the integrase-encoding gene, $bla_{\text{VIM-1,}}$ and the aminoglycoside resistance encoding gene, *aacA4* (Riccio *et al.*, 2001). In 2003–2004 a new nosocomial infection outbreak was registered in two departments of the same Italian hospital. It was caused by VIM-1 producers of *P. aeruginosa* (Mazzariol *et al.*, 2005a). In 2004–2005 Corvec *et al.* (2006) detected four *P. aeruginosa* clinical isolates producing VIM-1 from different French hospitals.

VIM-2 was originally identified in a *P. aeruginosa* bloodstream isolate from a patient with neutropenia in Marseille (South France) (Poirel *et al.*, 2000). It was closely related to VIM-1 MBL reported from Italian *P. aeruginosa* clinical isolates (90 % amino acid identity). The $bla_{\text{VIM-2}}$ was located on a 45 kb plasmid that, in addition, conferred resistance to sulfonamides. Also, $bla_{\text{VIM-2}}$ was the only gene cassette located within the variable region of a novel class 1 integron, In*56* (Poirel *et al.*, 2000). Two clonally unrelated *P. aeruginosa* clinical strains expressing VIM-2 enzyme were isolated in 1997 and 1998 from patients hospitalized in a suburb of Paris (Poirel *et al.*, 2001b). In both isolates, the $bla_{\text{VIM-2}}$ cassette was part of a class 1 integron that also encoded aminoglycoside-modifying enzymes (AMEs): AAC(6′)-29a and AAC(6′)-29b. These aminoglycoside acetyltransferases (AACs) conferred resistance to amikacin, isepamicin, kanamycin and tobramycin, but not to gentamicin, netilmicin and sisomicin. A retrospective epidemiological study in the Marseille hospital where the first VIM-2 producer was isolated found 20 more genetically indistinguishable *P. aeruginosa* isolates producing VIM-2 from several departments during 1996–1998 (Nordmann & Poirel, 2002). At the same time, VIM-1 and VIM-2-positive *P. aeruginosa* were reported as causes for numerous nosocomial infections in Italy and Greece (Cornaglia *et al.*, 2000; Lagatolla *et al.*, 2004; Tsakris *et al.*, 2000; Mavroidi *et al.*, 2000). Besides these VIM-2 metalloenzymes were found in *P. aeruginosa* clinical isolates in Spain (Prats *et al.*, 2002; Peňa *et al.*, 2007), Germany (Henrichfreise *et al.*, 2005), Portugal (Pena *et al.*, 2005), Poland (Patzer *et al.*, 2005), Russia (Toleman *et al.*, 2007a), Ireland (Walsh & Rogers, 2007), Turkey (Yakupogullari *et al.*, 2008), Venezuela (Mendes *et al.*, 2004b), Korea (Lee *et al.*, 2002), Japan (Yatsuyanagi *et al.*, 2004), Saudi Arabia (Guerin *et al.*, 2005), China (Yu *et al.*, 2006), India (Toleman *et al.*, 2007b), the USA (Lolans *et al.*, 2005), Columbia (Villegas *et al.*, 2006) and Canada (Parkins *et al.*, 2007), i.e. in the territories of four continents. In *P. aeruginosa*, VIM-2 is now the most widespread MBL that is associated with the localization of its encoding gene. The $bla_{\text{VIM-2}}$ allele was found to be carried on mobile elements known as gene cassettes. They are inserted into class 1 integrons (Poirel *et al.*, 2000, 2001b; Yu *et al.*, 2006). Integron-located resistance genes provide them with an increased potential for expression and dissemination. Several class 1 integrons have been found in transposons (Yu *et al.*, 2006), which enables the integrons to be transposed. This increases the threat of the $bla_{\text{VIM-2}}$ gene being disseminated among diverse genera of bacteria.

VIM-3 metalloenzyme was identified in a *P. aeruginosa* isolate in Taiwan (Yan *et al.*, 2001). VIM-3 differs from VIM-2 by two amino acid substitutions and $bla_{\text{VIM-3}}$ is a chromosomal gene. The following discoveries of VIM-type MBLs in *P. aeruginosa* isolates were made: VIM-4 in Greece (Pournaras *et al.*, 2002), Hungary (Libisch *et al.*, 2004), Poland (Patzer *et al.*, 2004) and Sweden (Giske *et al.*, 2003);

Table 2. Molecular class B MBLs found in *P. aeruginosa*

Enzyme	Geographical dissemination	Location of encoding gene	Impact on β-lactam antibiotics						Inhibition by	
			CAR TIC	PIP AZL	CAZ	FEP CPO	ATM	IMP MEM	CLV	TAZ
IMP-type		Integrons in plasmid or chromosome	R	R	R	R	S	r/R	No	No
IMP-1	Japan									
IMP-7	Singapore									
IMP-9	Canada, Singapore									
IMP-13	China									
IMP-16	Italy									
IMP-18	Brazil, USA									
VIM-type		Integrons in plasmid or chromosome	R	R	R	R	S	r/R	No	No
VIM-1	Italy, France, Greece									
VIM-2	France, Italy, Greece, Spain, Germany, Portugal, Poland, Russia, Ireland, Turkey, Venezuela, Korea, Japan, China, Saudi Arabia, India, USA, Columbia, Canada									
VIM-3	Taiwan									
VIM-4	Greece, Hungary, Poland, Sweden									
VIM-5	Turkey									
VIM-7	USA									
VIM-8	Columbia									
VIM-11	Argentina, Italy									
VIM-13	Spain									
VIM-15	Bulgaria									
VIM-16	Germany									
SPM-1	Brazil	Plasmid borne	R	R	R	R	S/R	r/R	No	No
GIM-1	Germany	Plasmid and integron borne	R	R	R	R	S	R	No	No

ATM, Aztreonam; AZL, azlocillin; CAR, carbenicillin; CAZ, ceftazidime; CLV, clavulanic acid; CPO, cefpirome; FEP, cefepime; IMP, imipenem; MEM, meropenem; PIP, piperacillin; r, reduced susceptibility; R, resistance; S, susceptibility; TAZ, tazobactam; TIC, ticarcillin.

VIM-5 in Turkey (Bahar *et al.*, 2004); VIM-7 in the USA (Toleman *et al.*, 2004); VIM-8 in Columbia (Crespo *et al.*, 2004); VIM-11 in Argentina (Pasteran *et al.*, 2005) and Italy (Mazzariol *et al.*, 2005b); VIM-13 in Spain (Juan *et al.*, 2008); VIM-15 in Bulgaria (Schneider *et al.*, 2008); and VIM-16 in Germany (Schneider *et al.*, 2008).

In 2002 Toleman *et al.* (2002) detected a plasmid bla_{SPM-1} gene determining production of a new Ambler type class B MBL – SPM-1 – in a clinical *P. aeruginosa* isolate from Brazil. This enzyme is significantly distinct from IMP and VIM types MBLs (it has just 35.5 % amino acid identity with IMP-1), and is considered to be a representative of a new subfamily of class B MBLs (Poirel *et al.*, 2004b). SPM-1 has a significantly higher molecular mass due to a unique loop containing 23 amino acid residues, which is not present in IMP and VIM-metalloenzymes. Generally, this carbapenemase binds cephalosporins more tightly than penicillins, which results in relatively large K_m values (Walsh *et al.*, 2005). Zavascki *et al.* (2000) reported the first nosocomial infection caused by *P. aeruginosa* producing carbepenem-resistant SPM-1 strains at the University Hospital in Porto Allegre, South Brazil (Zavascki *et al.*, 2005).

In 2002, Castanheira *et al.* (2004b) found a new MBL subclass – GIM-1 – in five multidrug-resistant *P. aeruginosa* strains isolated from different patients at a medical centre in Düsseldorf, Germany. The GIM-1 enzyme contains 250 amino acid residues and has a pI of 5.4. In respect to the amino acid identity with currently known molecular class B carbapenemases it differs from IMP, VIM and SPM-1 by 39–43, 28–31 and 28 %, respectively. GIM-1 does not hydrolyse aztreonam and serine-β-lactamase inhibitors. bla_{GIM-1} can be located on a plasmid (22 kb plasmid) and an integron (takes first

position on a 6 kb class 1 integron, In*77*, which also includes *aacA4*, *aadA1* gene cassettes and bla_{OXA-2}).

Resistance to β-lactams due to active efflux

Generally, *P. aeruginosa* clinical isolates are less susceptible than *Enterobacteriaceae* to most classes of antimicrobials. For a long time the main reason for this natural resistance was considered to be the low outer membrane permeability due to the presence of proteins with high molecular mass – about 50 kDa (Livermore, 1984). According to modern concepts, these proteins (OprM, OprJ, OprN) act as components of active efflux systems with wide substrate specificity. Thus, the inherent resistance level of *P. aeruginosa* is to a great extent determined by the interplay between low membrane permeability and efflux of antimicrobial agents (Livermore, 2001). Active efflux is an important non-enzymic mechanism of β-lactam resistance in *P. aeruginosa*. Efflux also contributes to the development of multiple resistances to all strategic antipseudomonal antibiotics and is mediated by four genetically different three-component efflux systems that belong to the resistance–nodulation–division (RND) family (Livermore, 2001, 2002): MexA–MexB–OprM, MexC–MexD–OprJ, MexE–MexF–OprN and MexX–MexY–OprM. The structure of these efflux systems is shown in Table 3. The first component is a protein located in the cytoplasmic membrane (MexB, MexD, MexF and MexY) that operates as an energy-dependent pump with wide substrate specificity. The second component is a gated outer membrane protein (OprM, OprJ, OprN and OprM). The third protein (MexA, MexC, MexE and MexX) is located in the periplasmic space and links the other two (Livermore, 2002).

MexA–MexB–OprM and MexX–MexY–OprM efflux systems participate simultaneously in natural and acquired antimicrobial-resistance mechanisms of *P. aeruginosa*, while MexC–MexD–OprJ and MexE–MexF–OprN act only in acquired resistance (Llanes *et al.*, 2004; Poole *et al.*, 1996; Kohler *et al.*, 1999). Substrate specificities of the active three-component efflux systems operating in *P. aeruginosa* are also presented in Table 3. The substrate profiles include various classes of antimicrobials (Masuda *et al.*, 2000a).

MexA–MexB–OprM overproduction often occurs in clinical isolates of *P. aeruginosa* and usually it is a result of increased transcription of the *mexA–mexB–oprM* operon due to mutations in the chromosomal gene encoding the MexR repressor protein, i.e. mutations at the *mexR* locus (Saito *et al.*, 1999). *nalB* mutants are characterized by increased MICs and corresponding clinical resistance to most of the β-lactams (penicillins, cephalosporins, monobactams, meropenem to some extent, but not imipenem), quinolones, tetracyclines, chloramphenicol and trimethoprim (Livermore, 2001). They can be selected *in vitro* or during treatment with fluoroquinolones, penicillins or cephalosporins (Ziha-Zarifi *et al.*, 1999). There are also other mutants called *nalC* that have intact *mexR* genes (Srikumar *et al.*, 2000). *nalC* mutants originate from the wild-type *P. aeruginosa* PAO1 and are characterized by a mutation in the *PA3721* gene (Cao *et al.*, 2004). The protein encoded by this gene is a repressor of a two-gene operon; its function is unclear and its overexpression in *nalC* mutants leads to overproduction of the MexAB–OprM efflux system. Recently, *nalD* mutants were found. They have a mutation in the *PA3574* gene that leads to MexA–MexB–OprM overexpression (Sobel *et al.*, 2005). Masuda & Ohya (1992) were the first to report that MexA–MexB–OprM overexpression in *P. aeruginosa* determines decreased susceptibility to meropenem, but does not affect the activity of the other carbapenems – imipenem and panipenem (compared to wild-type *P. aeruginosa*). This is due to the different molecular structure of carbapenems – meropenem has a hydrophobic side-chain at the second position, which makes it a substrate for this efflux system, while imipenem and panipenem are not substrates as their side-chains are strongly charged and hydrophilic.

The *mexC–mexD–oprJ* operon cannot be expressed constitutively, but is overexpressed in *P. aeruginosa* mutants possessing mutations in the *nfxB* gene, which encodes a transcriptional repressor (Poole *et al.*, 1996). This efflux system predominantly exports extended-spectrum cephems (cefepime and cefpirome) from the bacterial cell, as well as quinolones, macrolides, tetracycline and chloramphenicol (Li *et al.*, 2000).

The third known efflux operon, *mexE–mexF–oprN*, determines resistance to quinolones, chloramphenicol and trimethoprim, and is overexpressed by the so called *nfxC P. aeruginosa* mutants (having a mutation at the *mexT* locus) (Kohler *et al.*, 1999). *nfxC* mutants also show cross-resistance towards carbapenems (predominantly imipenem) as these have decreased expression of OprD outer membrane proteins. Unlike the rest of the efflux operons, *mexE–mexF–oprN* is subject to positive regulation by MexT protein, which belongs to the LysR family of transcriptional activators (Kohler *et al.*, 1999; Li *et al.*, 2000).

Masuda *et al.* (2000b) found that MexX and MexY proteins export aminoglycosides, tetracycline and erythromycin from bacterial cells, and cooperate closely with spontaneously expressed OprM outer membrane proteins; thus taking part in the so called 'intrinsic resistance' of *P. aeruginosa* to antimicrobial agents. Like MexAB–OprM, MexXY proteins may be constitutively overproduced due to mutations in the *mexZ* repressor gene, which is located nearby and transcribed independently from the *mexXY* operon (Llanes *et al.*, 2004; Vogne *et al.*, 2004). Upregulation of MexXY–OprM affects aminoglycosides and fluoroquinolones (Mao *et al.*, 2001).

Overexpression of efflux systems with wide substrate profiles is an important mutational mechanism in *P. aeruginosa*. Its impact on the resistance to antipseudomonal antibiotics (β-lactams, fluoroquinolones, aminoglycosides and polymyxin B) is summarized in Table 4 (Livermore, 2002).

Table 3. Structure and substrate specificity of the three-component active efflux systems in *P. aeruginosa*

Cytoplasmic membrane pump	Periplasmic linker	Outer membrane channel	Substrate
MexB	MexA	OprM	Quinolones, macrolides, tetracyclines, lincomycin, chloramphenicol, novobiocin, β-lactams except imipenem
MexD	MexC	OprJ	Quinolones, macrolides, tetracyclines, lincomycin, chloramphenicol, novobiocin, penicillins except carbenicillin and sulbenicillin, cefepime, cefpirome, meropenem
MexF	MexE	OprN	Fluoroquinolones, carbapenems
MexY	MexX	OprM	Quinolones, macrolides, tetracyclines, lincomycin, chloramphenicol, aminoglycosides, penicillins except carbenicillin and sulbenicillin, cefepime, cefpirome, meropenem

Resistance to β-lactams due to altered outer membrane permeability

Many of the imipenem-resistant *P. aeruginosa* clinical isolates are characterized by a deficiency of OprD (referred to as D2 porins) (Pechere & Kohler, 1999). OprD proteins form specific channels promoting the entry of basic amino acids and carbapenems, but no other β-lactam antibiotics (Livermore, 2001). In comparison with imipenem, meropenem cell influx is less affected by OprD deficiency. While imipenem MICs of the *oprD* mutants are within the range 8–32 mg l^{-1}, MICs for meropenem are 2–4 mg l^{-1} (Pechere & Kohler, 1999; Pai *et al.*, 2001). Loss of OprD determines resistance to carbapenems only in cases of expressed chromosomal AmpC β-lactamase, and this demonstrates the close cooperation between these two mechanisms (Livermore, 1992). Selection of resistant *P. aeruginosa* strains during imipenem treatment is a much more frequent phenomenon than the rise of ceftazidime-, piperacillin- or ciprofloxacin-resistant mutants (Livermore, 2001).

Resistance to β-lactams due to an altered target

The rarest mechanism of resistance to β-lactams in *P. aeruginosa* involves modification of the target site – PBPs. Altered PBP-4s with low affinity were reported after imipenem treatment, as well as after administration of high doses of piperacillin in patients suffering from cystic fibrosis. There are reports of reduced susceptibility to β-lactams in *P. aeruginosa* strains with overproduction of PBP-3s (Pechere & Kohler, 1999).

Mechanisms of resistance to aminoglycosides

Several groups of aminoglycoside resistance mechanisms are known: enzyme modification (major), low outer membrane permeability, active efflux and, rarely, target modification (Vakulenko & Mobashery, 2003; Poole, 2005; Magnet & Blanchard, 2005).

AMEs

AMEs attach a phosphate, adenyl or acetyl radical to the antibiotic molecule, and thus they decrease the binding affinity of the modified antibiotics to the target in the bacterial cell (30S ribosomal subunit) (Llano-Sotelo *et al.*, 2002). AMEs are plasmid encoded and are divided into three classes: aminoglycoside phosphoryltransferases (APHs), aminoglycoside adenylyltransferases (also known as nucleotidyltransferases) (AADs or ANTs) and aminoglycoside acetyltransferases (AACs) (Vakulenko & Mobashery, 2003). Most frequently *P. aeruginosa* expresses the following AMEs: AAC(6′)-II (determines resistance to gentamicin, tobramycin and netilmicin), AAC(3)-I (resist-

Table 4. Impact of overexpression of the active efflux systems on the resistance to antipseudomonal antibiotics

Overexpression of:	Mutation site	Impact on resistance to antimicrobial agents with antipseudomonal activity								
		Fq	CAR TIC	PIP AZL	CAZ ATM	FEP CPO	IMP	MEM	Agl	PB
MexA–MexB–OprM	*mexR*; *PA3721* and *PA3719*; *PA3574*	r/R	R	r/R	r/R	r/R	–	r	–	–
MexC–MexD–OprJ	*nfxB*	r/R	r/R	r/R	r/R	R	–	r	–	–
MexE–MexF–OprN	*mexT*	r/R	r/R	r/R	r/R	r/R	r	r	–	–
MexX–MexY–OprM	*mexZ*	r/R	r/R	r/R	r/R	r/R	–	r	r/R	–

Agl, Aminoglycosides; ATM, aztreonam; AZL, azlocillin; CAR, carbenicillin; CAZ, ceftazidime; CPO, cefpirome; FEP, cefepime; Fq, fluoroquinolones; IMP, imipenem; MEM, meropenem; PB, polymyxin B; PIP, piperacillin; r, reduced susceptibility; R, resistance; TIC, ticarcillin.

ance to gentamicin), AAC(3)-II (resistance to gentamicin, tobramycin and netilmicin), AAC(6′)-I (resistance to tobramycin, netilmicin and amikacin) and ANT(2′)-I (resistance to gentamicin and tobramycin) (Miller *et al.*, 1997).

Impermeability

Aminoglycoside resistance that is independent from AMEs is characterized by resistance to all aminoglycosides, and often associated with reduced aminoglycoside accumulation (Bryan *et al.*, 1976). This resistance is attributed to a reduced uptake due to diminished outer membrane permeability and is typically referred to as impermeability resistance. Numerous studies have highlighted the significance of impermeability resistance in aminoglycoside-resistant clinical isolates, particularly in cystic fibrosis isolates in which it is often the most common aminoglycoside resistance mechanism (MacLeod *et al.*, 2000).

Active efflux

Active aminoglycoside efflux is a relatively rare resistance mechanism that is due to MexXY proteins operating simultaneously with OprM (Masuda *et al.*, 2000a; Vogne *et al.*, 2004), as well as with some other outer membrane proteins – OpmB, OpmG, OpmI (Jo *et al.*, 2003) – thus forming three-component active efflux systems.

Target modification

Methylation of 16S rRNA has recently emerged as a new mechanism of resistance against aminoglycosides among Gram-negative pathogens belonging to the family *Enterobacteriaceae* and glucose-nonfermentative microbes, including *P. aeruginosa* and *Acinetobacter* species (Doi & Arakawa, 2007). This event is mediated by a newly recognized group of 16S rRNA methylases, which share modest similarity to those produced by aminoglycoside-producing actinomycetes. The responsible genes are usually located on transposons within transferable plasmids, which provides them with the potential to spread horizontally, and may partially explain the worldwide distribution of this novel resistance mechanism.

The first 16S rRNA methylase, called RmtA, was reported in an aminoglycoside-resistant *P. aeruginosa* clinical isolate from Japan, in 2003 (Yokoyama *et al.*, 2003). The enzyme was found to confer a high-level resistance to all parenterally administered aminoglycosides, including amikacin, tobramycin, isepamicin, kanamycin, arbekacin and gentamicin (MICs >1024 mg l^{-1}). The structural gene of RmtA was associated with a genetic element that resembled a mercury-resistance transposon Tn*5041* on a transferable plasmid (Yamane *et al.*, 2004). The mol% G+C of *rmtA* was 55 %, suggesting its origin from some mol% G+C rich microbes, including actinomycetes.

RmtD, a novel 16S rRNA methylase, was identified in a panresistant *P. aeruginosa* strain isolated in 2005 from an in-patient in Brazil (Doi *et al.*, 2006). The methylase accounts for a high-level resistance to all 4,6-disubstituted deoxystreptamine aminoglycosides, such as amikacin, tobramycin, and gentamicin. RmtD shares a moderate degree of identity with RmtA. β-Lactam resistance of the studied isolate is conferred by the production of the MBL SPM-1.

Non-enzymic mechanisms involved in the gradual development of aminoglycoside resistance

Recently, El'Garch *et al.* (2007) examined the interplay and cumulative effects of different non-enzymic mechanisms engineered in a reference strain *P. aeruginosa* PAO1. Their data revealed that the accumulation of mutants leads to a gradual increase in the resistance to aminoglycosides, as seen in cystic fibrosis patients (Hurley *et al.*, 1995; MacLeod *et al.*, 2000). Four genes of *P. aeruginosa* (namely, *galU*, *nuoG*, *mexZ* and *rplY*) have been shown to be involved in the gradual increase in MICs of aminoglycosides (El'Garch *et al.*, 2007).

P. aeruginosa galU is required for synthesis of a complete LPS core and its inactivation results in the production of truncated (rough) LPS molecules lacking both A- and B-band polysaccharides in *P. aeruginosa* (Dean & Goldberg, 2002). Loss of the A- and B-band LPS was reported to impair the antibacterial activity of aminoglycosides by compromising their binding to the cell surface (Kadurugamuwa *et al.*, 1993).

Abolished *nuoG* expression promotes the disruption of the *nuoABDEFGHIJKLMN* operon that codes for proton-translocating type I NADH oxidoreductase (El'Garch *et al.*, 2007). Inactivation of the enzymic complex that significantly contributes to proton electrochemical gradient impairs membrane energetics and thereby the uptake of aminoglycosides (Taber *et al.*, 1987).

Inactivation of the repressor gene *mexZ* results in increased expression of the *mexY* gene and constitutive overproduction of the multidrug transporter MexY (Vogne *et al.*, 2004; El'Garch *et al.*, 2007). Suppression of the gene *rplY*, which encodes for ribosomal protein L25, results in both moderate upregulation of the efflux system MexXY–OprM and hypersusceptibility to β-lactam antibiotics (El'Garch *et al.*, 2007).

Disruption of the described genes individually led to increased aminoglycoside resistance (in the region of twofold). Construction of double, triple and quadruple mutants demonstrated cumulative effects of the different mechanisms on aminoglycoside resistance, with MICs increasing from 16- to 64-fold in the quadruple mutant compared to the wild-type *P. aeruginosa* PAO1 strain. Altogether, these results illustrate how *P. aeruginosa* may gradually develop high resistance to these antibiotics via

non-enzymic mechanisms, as seen in cystic fibrosis patients (El'Garch *et al.*, 2007).

Mechanisms of resistance to fluoroquinolones

Two major mechanisms lead to fluoroquinolone resistance in *P. aeruginosa*: structural changes in target enzymes and active efflux (Hooper, 2001). Modification of the primary target for fluoroquinolones (DNA gyrase, also known as topoisomerase II) occurs by point mutations in *gyrA*/*gyrB* genes within the QRDR (quinolone-resistant-determinative region) motif, which is considered as the enzyme's active site. As a result of these mutations, the amino acid sequence of A and B subunits alters, which leads to synthesis of modified topoisomerase II with low binding affinity to quinolone molecules. Modifications of a secondary target (topoisomerase IV) occur as a result of point mutations in *parC* and *parE* genes encoding ParC and ParE enzyme subunits, respectively.

As described above, four well known genetically different efflux systems were identified in *P. aeruginosa*: MexAB–OprM, MexCD–OprJ, MexEF–OprN and MexXY–OprM. While each pump has a preferential set of antimicrobial agent substrates, the fluoroquinolones are universal substrates for each of them (Masuda *et al.*, 2000a). A new member of the tripartite multidrug efflux pumps, MexV (membrane fusion protein)–MexW (RND-type membrane protein)–OprM, was found in *P. aeruginosa* in 2003 (Li *et al.*, 2003). It confers resistance to fluoroquinolones, tetracycline, chloramphenicol, erythromycin, ethidium bromide and acriflavine. High-level fluoroquinolone resistance in *P. aeruginosa* is attributable to the interplay of the efflux pump systems and mutations of the genes encoding DNA gyrase and topoisomerase IV (Nakajima *et al.*, 2002; Wang *et al.*, 2007).

According to a number of studies, quinolones may select multidrug-resistant phenotypes *in vitro*, as well as *in vivo* (Masuda & Ohya, 1992; Kohler *et al.*, 1997). The most common causes for their appearance are the following mutations: *nalB*, *nfxB* and *nfxC* leading, respectively, to overexpression of MexA–MexB–OprM, MexC–MexD–OprJ and MexE–MexF–OprN (Table 4). The new fluoroquinolones select predominantly *nfxB P. aeruginosa* mutants, while older quinolones select for *nfxC* or *nalB* mutants (Kohler *et al.*, 1997).

Conclusion

P. aeruginosa is a uniquely problematic nosocomial pathogen because of the following: the species' natural resistance to many drug classes; its ability to acquire resistance (via mutations) against all relevant treatments; its high resistance rates worldwide; and frequent implication in severe infections. Multidrug resistance (MDR) is common and increasing. A number of strains have now been identified that exhibit resistance to essentially all reliable antipseudomonal antibiotics. This problem grows with the incidence of integrons that carry gene cassettes encoding both carbapenemases and AACs.

MDR in *P. aeruginosa* makes treatment of infections caused by this organism both difficult and expensive. Improved methods for antimicrobial susceptibility testing are needed, including detection of emerging strains producing ESBLs and MBLs. Clinical studies are needed to identify risk factors for MDR development, as well as to determine the most efficacious antimicrobial regimens and duration of therapy to maximize successful outcomes in the treatment of severe infections due to multiresistant *P. aeruginosa*. Prudent antimicrobial policies combined with good infection control practices worldwide could guarantee a limitation in the development and spread of resistance to β-lactams, aminoglycosides and fluoroquinolones, ensuring that these agents will maintain their place in the therapy of *P. aeruginosa* infections.

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