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 administered 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 (carbencillin, ticarcillin), ureidopenicillins (azlocillin, piperacillin), some third generation cephalosporins (ceftazidime, cefotulodine, ceferazone), 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 (cefepine and cepirome) 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). 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 [enzymatic 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 (Langaa 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-anhydromurampeptides, 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-1-alanine amidase, which hydrolyses 1,6-anhydromurampeptides, 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 (Langaa 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 carbencillin hydrolysing β-lactamases.** Four
carbencillin 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, uroseidopenicillins 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
and CARB-4 (Bush et al., 1995). Their substrate profile includes
carboxypenicillins, uroseidopenicillins and cefsulodine.
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CARB-3 are closely related (they differ by just 1 or 2
amino acids), but they are only 86.3% homologous with
and CARB-4 (Bush et al., 1995). The bla\textsubscript{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 cepafepime, cepafepime and aztreonam, and
100% susceptibility towards cefazidime 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 uroseidopenicillins, but also to extended-spectrum
cephalosporins (ceftazidime, cepafepime, cepafepime) and
aztreonam (Weldhagen et al., 2003). They show low
affinity to carbenapenems. 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 affinity 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 hydro-
lyses 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 (Poiret
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; Poiret 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 aztreo-
nam (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, chloramphe-
col and sulfonamides were encoded by a 180 kb plasmid
transferred by conjugation into *E. coli* HB101. The multiplcity 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 a
widespread dissemination of bla\textsubscript{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; Claeyts 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\textsubscript{VEB-like} genes (93%)
in ceftazidime-resistant clinical isolates of *P. aeruginosa* in
the University Hospital in Thailand. During that study, a
new bla\textsubscript{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
(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. Production of this β-lactamase determines resistance to ceftazidime and other oxyiminocephalosporins, and is inhibited by imipenem, clavulanic acid and tazobactam.

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

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Location of encoding gene</th>
<th>Initial isolation</th>
<th>Other geographical regions of isolation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEM-4</td>
<td>P, C</td>
<td>1996 France</td>
<td>Thailand</td>
<td>Dubois et al. (2002a, 2005)</td>
</tr>
<tr>
<td>TEM-21</td>
<td>C</td>
<td>1997 France</td>
<td></td>
<td>Marchand et al. (2000)</td>
</tr>
<tr>
<td>TEM-42</td>
<td>P</td>
<td>1992 France</td>
<td></td>
<td>David et al. (2008)</td>
</tr>
<tr>
<td>VEB-1</td>
<td>C, P, I</td>
<td>1998 France</td>
<td>Thailand, India, China, Bulgaria</td>
<td>Poirel et al. (2001c)</td>
</tr>
<tr>
<td>VEB-1a</td>
<td>C, I</td>
<td>1999 Kuwait</td>
<td>India</td>
<td>Girlich et al. (2002)</td>
</tr>
<tr>
<td>VEB-2</td>
<td>C, I</td>
<td>1999 Thailand</td>
<td>Turkey, Italy, Belgium, Poland</td>
<td>Poirel et al. (2005a), Labuschagne et al. (2008)</td>
</tr>
<tr>
<td>PER-1</td>
<td>C</td>
<td>1991 France</td>
<td></td>
<td>Poirel et al. (2005b)</td>
</tr>
<tr>
<td>GES-1</td>
<td>P, I</td>
<td>1999 France</td>
<td>Brazil</td>
<td>Dubois et al. (2002b), Castanheira et al. (2004a)</td>
</tr>
<tr>
<td>GES-9</td>
<td>P, I</td>
<td>2004 France</td>
<td></td>
<td>Poirel et al. (2005b)</td>
</tr>
<tr>
<td>BEL-1</td>
<td>C, I</td>
<td>2004 Belgium</td>
<td></td>
<td>Poirel et al. (2005c), Bogaerts et al. (2007)</td>
</tr>
</tbody>
</table>

C, Chromosomal; I, integron borne; P, plasmid borne.
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*<sub>BEL-1</sub>, is a part of a class 1 integron, In120, 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 cefazidime (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). Cefazidime 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 cefazidime 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 cepfempe, in contrast to third generation cepfazidime (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 cefazidime (Bradford, 2001).

OXA-18 enzyme is encoded by the chromosomal *bla*<sub>OXA-18</sub> 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, cefotolin, cefazidime, 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 Blagu 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*<sub>OXA-45</sub> 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 $\text{Zn}^{2+}$ in their active centre (Nordmann & Guibert, 1998). They belong to molecular class B (Bush et al., 1999). 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_{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 Int31 integron. Recently, IMP-1 MBL was reported among carbapenem-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_{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_{IMP-9} was found in China (Xiong et al., 2006), and bla_{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 carbapenemase, 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_{VIM-1} is a part of a gene cassette inserted in the In70 class 1 integron, which carries the following genes: the integrase-encoding gene, bla_{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_{VIM-2} was located on a 45 kb plasmid that, in addition, conferred resistance to sulfonamides. Also, bla_{VIM-2} was the only gene cassette located within the variable region of a novel class 1 integron, In56 (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_{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; Tsakis 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; Peña et al., 2007), Germany (Henrichreise 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_{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_{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_{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*

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

ATM, Aztreonam; AZL, azlocillin; CAR, carbenicillin; CAZ, ceftazidime; CLV, clavulanic acid; CPO, cepiroxim; FEP, cepime; IMP, imipenem; MEM, meroopenem; PIP, pipercillin; 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*<sub>SPM</sub>-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*<sub>m</sub> values (Walsh et al., 2005). Zavascki et al. (2000) reported the first nosocomial infection caused by *P. aeruginosa* producing carbapenem-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*<sub>GIM</sub>-1 can be located on a plasmid (22 kb plasmid) and an integron (takes first
position on a 6 kb class 1 integron, In77, which also includes aacA4, aadA1 gene cassettes and blaOXA-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 cephalosporins (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* 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).
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 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\(^\text{′}\))-II (determines resistance to gentamicin, tobramycin and netilmicin), AAC(3)-I (resist-

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

<table>
<thead>
<tr>
<th>Cytoplasmic membrane pump</th>
<th>Periplasmic linker</th>
<th>Outer membrane channel</th>
<th>Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>MexB</td>
<td>MexA</td>
<td>OprM</td>
<td>Quinolones, macrolides, tetracyclines, lincomycin, chloramphenicol, novobiocin, β-lactams except imipenem</td>
</tr>
<tr>
<td>MexD</td>
<td>MexC</td>
<td>OprI</td>
<td>Quinolones, macrolides, tetracyclines, lincomycin, chloramphenicol, novobiocin, penicillins except carbencillin and sulbenicillin, cefepime, cefpirome, meropenem</td>
</tr>
<tr>
<td>MexF</td>
<td>MexE</td>
<td>OprN</td>
<td>Fluoroquinolones, carbapenems</td>
</tr>
<tr>
<td>MexY</td>
<td>MexX</td>
<td>OprM</td>
<td>Quinolones, macrolides, tetracyclines, lincomycin, chloramphenicol, aminoglycosides, penicillins except carbencillin and sulbenicillin, cefepime, cefpirome, meropenem</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Overexpression of:</th>
<th>Mutation site</th>
<th>Impact on resistance to antimicrobial agents with antipseudomonal activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>MexA–MexB–OprM</td>
<td>mcrR, PA3721; and PA3719; PA3574</td>
<td>r/R r/R r/R r/R r/R – r – –</td>
</tr>
<tr>
<td>MexC–MexD–OprJ</td>
<td>nfxB</td>
<td>r/R r/R r/R r/R r/R – r – –</td>
</tr>
<tr>
<td>MexE–MexF–OprN</td>
<td>mcrT</td>
<td>r/R r/R r/R r/R r/R r r – –</td>
</tr>
<tr>
<td>MexX–MexY–OprM</td>
<td>mcrZ</td>
<td>r/R r/R r/R r/R r/R – r r/R –</td>
</tr>
</tbody>
</table>

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 Tn5041 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...
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 & Ohyda, 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.

References


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