Carbapenem-resistance mechanisms of multidrug-resistant \textit{Pseudomonas aeruginosa}

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Clonal dissemination of multidrug-resistant \textit{Pseudomonas aeruginosa} (MDRPA) is a major concern worldwide. The aim of this study was to explore the mechanisms leading to the carbapenem resistance of an MDRPA clone. Isolates were obtained from a surgical wound, sputum, urine and a blood culture. Pulsed-field gel electrophoresis (PFGE) showed high genomic homogeneity of these isolates and confirmed the circulation of an endemic clone belonging to serotype O4. Outer membrane protein (OMP) bands were visualized by SDS-PAGE, meropenem accumulation was measured in a bioassay and integrons were detected by PCR. Efflux pumps were studied for several antimicrobial agents and synergic combinations thereof in the presence or absence of both carbonyl cyanide \textit{m}-chlorophenylhydrazone (CCCP) and Phe-Arg-\textit{β}-naphthylamide (PA\textit{β}N) at final concentrations of 10 and 40 mg l\(^{-1}\), respectively. On OMP electrophoretic profiles, MDRPA showed a reduction of outer membrane porin D (OprD) and PCR demonstrated the presence of a class 1 integron with a cassette encoding aminoglycoside adenyltransferase B (\textit{aadB}). Meropenem accumulation was slightly higher in bacilli than in the filamentous cells that formed in the presence of antibiotics. Overexpression of the efflux pump MexAB-OprM and a functional MexXY-OprM were detected in all isolates.

\textbf{INTRODUCTION}

\textit{Pseudomonas aeruginosa} frequently causes infections that develop in wound and burn patients and in the severe respiratory tract infections commonly seen in cystic fibrosis patients. In many countries, a major cause of concern is the increasing prevalence of infections caused by isolates of multidrug-resistant \textit{P. aeruginosa} (MDRPA). Controlling the spread of MDRPA, enlarging the therapeutic arsenal used to fight these infections and reducing the mortality of infected individuals (Woodford \textit{et al.}, 2011; Cabot \textit{et al.}, 2012) are currently important goals of microbiological surveillance. However, a serious challenge to these efforts is that some of the classic antimicrobial drugs as well as several of the newly available drugs are no longer effective against MDRPA since many strains are resistant (Kanj & Kanafani, 2011).

The high level of antibiotic resistance in \textit{P. aeruginosa} involves several mechanisms, including the overexpression of active efflux systems, the production of modifying enzymes, a decrease in outer membrane (OM) permeability and structural alterations of topoisomerases II and IV, involved in quinolone resistance (Strateva & Yordanov, 2009).

Carbapenems such as meropenem and imipenem are potent broad-spectrum antimicrobial agents used to treat MDRPA infections. These antibiotics bind to critical penicillin-binding proteins, thereby disrupting growth and structural integrity of the bacterial cell wall. However, the resistance of non-fermenting Gram-negative bacteria, including \textit{P. aeruginosa}, to imipenem and meropenem is increasing (Nicolau, 2008). Among the mechanisms implicated in resistance are diminished cell-wall permeability, the overexpression of intrinsic efflux systems and the production of carbapenem-hydrolysing \textit{β}-lactamases.

In \textit{P. aeruginosa}, one of the main mechanisms of carbapenem resistance is a reduction of OM permeability.
through alterations in or decreased production of outer membrane porin D (OprD). This porin allows the cellular entry of carbapenems, and especially of imipenem (Farra et al., 2008).

Imipenem has no effect on the shape of *P. aeruginosa* whereas meropenem induces alterations that give rise to filamentous cells. Previous *in vitro* studies showed that these morphological changes depend on the concentration of meropenem and the antibiotic exposure period (Trautmann et al., 1998).

Horii et al. (1999) demonstrated that meropenem leads to the release of greater amounts of endotoxin than induced by other carbapenems. In *P. aeruginosa*, resistance to carbapenems, other β-lactam antibiotics and aminoglycosides is strongly related to the production of several enzymes, especially β-lactamases, carbencillin hydrolysing β-lactamases, extended-spectrum β-lactamases (ESBL), class D β-lactamases (oxacillinases), metallo-β-lactamases (MBLs) carbapenemases and aminoglycoside-modifying enzymes (Stratova & Yordanov, 2009). In MDRPA, some of these antibiotic-resistance genes are clustered in a cassette carried by a class 1 integron (Llanes et al., 2006). Nonetheless, mechanisms other than low OM permeability, β-lactamases and genetic elements are needed to explain the intrinsic and acquired resistance of *P. aeruginosa*. For example, the elevated expression of genes encoding multidrug efflux pumps frequently produces high levels of antibiotic resistance in Gram-negative bacteria (Nikaido, 1998). Studies with clinical isolates, including epidemic clones, support the role of these pumps in multidrug resistance (Morita et al., 2012). Indeed, in *P. aeruginosa* ten such pumps (excluding metal cation transporters) belonging to the resistance–nodulation–division (RND) family have been identified (Woodford et al., 2011; Falagas et al., 2006). Accordingly, a new molecular approach to block efflux and thereby restore drug susceptibility in resistant clinical strains is via efflux pump inhibitors (EPIs). The recently discovered inhibitor Phe-Arg-β-naphthylamide (PAfN), which is highly effective against *P. aeruginosa* efflux mechanisms, reverses fluoroquinolone resistance and has potent broad-spectrum activity (Pagès et al., 2005).

Given the multiple routes of bacterial resistance, the aim of the present study was to examine some of the resistance mechanisms used by MDRPA isolates derived from a clone endemic to our hospital.

**METHODS**

**Bacteria, media, antibiotics and EPIs.** MDRPA isolates 459, 133, 162 and 527 (serotype O4), belonging to a clone endemic to Hospital del Mar (Barcelona) and isolated, respectively, from sputum, a surgical wound, urine and a blood culture between 2005 and 2008 were used to explore mechanisms leading to carbapenem resistance. The clone was confirmed by pulsed-field gel electrophoresis (PFGE) of a *speI* DNA digest using the method described by Maslow et al. (1993), with some modifications. Strain *P. aeruginosa* ATCC 27853 served as the control in electrophoresis and in susceptibility testing.

Cefazidine, ciprofloxacin, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and Pa/N were purchased from Sigma Chemical Co. Meropenem was from Astra Zeneca UK Ltd, imipenem from Merck and tobramycin from Fagron Iberica. All bacteriological culture media were from Scharlab.

**Drug susceptibility testing.** Susceptibility to the antimicrobial agents was determined using the broth microdilution method in Mueller–Hinton broth, as recommended by the CLSI (CLSI, 2010), and expressed as MIC. The breakpoints were those recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2011).

**OM isolation.** OMs were isolated from 100 ml of stationary-phase cultures. Harvested bacteria were suspended in 30 ml of Ringer 1:4, centrifuged at 6000 g at room temperature for 15 min and suspended in 30 ml of 10 mM HEPES (pH 7.4). The suspended cells were disrupted at a pressure of 30 kPa (Constant Cell Disruption Systems) followed by sedimentation at 3000 g for 15 min at 21 °C to remove any remaining whole cells. Whole membranes were harvested by centrifugation at 60 000 g for 90 min at 21 °C. The pellet, containing the OMs, was suspended in 2 ml of 1% sodium lauryl sarcosinate in 10 mM HEPES (pH 7.4), incubated at room temperature for 20 min and centrifuged at 60 000 g for 60 min (Puig et al., 1993).

Proteins contained in the resulting supernatant were resolved by SDS-PAGE following a modification of the Laemmli method (Laemmli, 1970). Sizes were determined using a molecular marker from Bio-Rad.

**Bioassay of meropenem accumulation.** Accumulation of the antibiotic was measured in a turbidimetric assay based on the method of Rodriguez et al. (2004), performed as follows: 1 day before the assay, 20 ml of Mueller–Hinton medium in a 20 mm × 200 mm glass tube was inoculated with a loop of *E. coli* ATCC 25922 from a 24 h stock culture and incubated for 16–18 h at 37 °C. The inoculum was prepared by transferring 13 ml of this overnight culture to 1 l of assay broth. One millilitre of each working standard solution (concentrations range from 0.01 to 25 mg meropenem l−1) and 1 ml of each sample dilution was inoculated into each of two sterile glass tubes. One to two tubes containing 1 ml of phosphate buffer pH 4.5 were included as controls. Two tubes containing 1 ml of a 7% phenol solution were used as blanks. Nine millilitres of inoculum was then added to each tube; they were then incubated in a water bath at 37 ± 0.2 °C for 2–4 h. Bacterial growth was stopped by the addition of 1 ml of a 7% phenol solution. Absorbances at 550 nm were measured in tubes containing the highest to the lowest concentration of working solution. The sample dilution readings were then translated into the equivalent levels of the working standards. A standard curve was prepared of log absorbance versus concentration using the mean turbidity of each pair of tubes. Total protein was measured using the Pierce BCA protein assay kit (Thermoscientific).

**Detection and analysis of class 1 integrons.** DNA for integron detection was extracted using a modification of the method described by Lévesque et al. (1995). Briefly, 200 μl of a bacterial culture grown in 4 ml of brain heart infusion/10% glycerol at 30 °C overnight was added to 800 μl of distilled water and boiled for 10 min. The cell suspension was centrifuged at 12 000 g for 2 min, with the supernatant used for PCR analysis. PCR was performed in 100 μl volumes containing 30 μl of template DNA and 70 μl of PCR mix [2.5 pmol oligonucleotides, 1 U *Taq* DNA polymerase, 200 μM deoxynucleoside triphosphate, 3 mM MgCl2, 10 mM Tris/Cl (pH 8), 50 mM KCl, 0.001% gelatin, distilled water; Fermentas] and the appropriate primers (Invitrogen). The three-step reaction was performed in a thermal cycler as follows: 1 min of denaturation at 94 °C, 1 min of annealing at 55 °C and 5 min of extension at 72 °C for a total of 35 cycles. All completed reactions were maintained at
The PCR products were subjected to electrophoresis (85 V for 2 h) in a 0.7 % agarose gel and then visualized by staining the gel with ethidium bromide.

DNA sequencing and submission. The MinElute PCR purification kit (Qiagen) was used to purify the PCR products representing integron variable regions (IVRs). The purified amplicons were sequenced using the ABI PRISM BigDye Terminator version 3.1 cycle sequencing ready reaction kit and the ABI PRISM 3700 DNA analyser (Applied Biosystems).

Sequences were compared using BLAST software, available online (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Efflux-pump-mediated fluoroquinolone resistance. To establish the prevalence of efflux-pump-mediated fluoroquinolone resistance in the MDRPA strains, MICs were determined in assays performed in 96-well microtitre plates. The twofold standard broth microdilution method, using Mueller–Hinton broth and carried out with or without a fixed concentration of the efflux inhibitor PAßN, was employed in these experiments (Kriengkauykiat et al., 2005). Ciprofloxacin was tested at 11 concentrations (128–0.125 mg l⁻¹) and PAßN at 40 mg l⁻¹. A confirmation test was also performed.

To measure active efflux under different environmental conditions, parallel growth inhibition assays using the EPIs PAßN and CCCP were carried out according to Fenosa et al. (2009).

Detection of a functioning MexXY-OprM efflux pump. To detect the presence of a functioning MexXY-OprM efflux pump in the MDRPA strains, the MIC of the aminoglycoside tobramycin was determined in 96-well microtitre plates. The twofold standard broth microdilution method, using Mueller–Hinton broth, in the absence and presence of 40 mg l⁻¹ PAßN, was used (Mao et al., 2001).

Effect of PAßN on antimicrobial activity. A chequerboard titration assay was carried out to assess the interaction of ceftazidime or meropenem with tobramycin (0–128 mg l⁻¹), alone and in combination with 40 mg l⁻¹ PAßN.

RESULTS

Drug susceptibility testing

MIC data for the ATCC 27853 and MDRPA isolates for several antimicrobial agents are shown in Table 1.

Electrophoresis of outer membrane proteins (OMPs)

Fig. 1 shows the electrophoretic profiles of OMPs from the MDRPA strains and from ATCC 27853. The only apparent difference was a relative reduction in the OprD (46 kDa) band in the MDRPA strains.

Bioassay of meropenem accumulation

In the presence of a high meropenem concentration (4 × MIC), the normally coccobacillic P. aeruginosa was able to survive as long undivided filaments. When antibiotic pressure was withdrawn, the cells returned to their original shape (Fig. 2). Meropenem accumulation was 15 % higher in the normal cells than in the filamentous cells.

Detection of class 1 integrons

Amplifications from all MDRPA endemic clones yielded a 750 bp PCR product (Fig. 3a). The class 1 integrons mapped to chromosomal regions of variable lengths, consistent with the presence of a mobile genetic element that could be transferred between bacterial species.

Table 1. MICs (mg l⁻¹) of ATCC 27853 and MDRPA strains determined by a microdilution method

<table>
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<tr>
<th>Strain</th>
<th>Drug</th>
<th>Family</th>
<th>MIC (mg l⁻¹)</th>
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<td>459</td>
</tr>
<tr>
<td></td>
<td>Imipenem</td>
<td>Carbapenem</td>
<td>8</td>
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<tr>
<td></td>
<td>Meropenem</td>
<td>Carbapenem</td>
<td>8</td>
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<td></td>
<td>Ciprofloxacin</td>
<td>Fluoroquinolone</td>
<td>64</td>
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<tr>
<td></td>
<td>Tobramycin</td>
<td>Aminoglycoside</td>
<td>32</td>
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<tr>
<td></td>
<td>Ceftazidime</td>
<td>Cephalosporin</td>
<td>128</td>
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4 °C. The PCR products were subjected to electrophoresis (85 V for 2 h) in a 0.7 % agarose gel and then visualized by staining the gel with ethidium bromide.

DNA sequencing and submission. The MinElute PCR purification kit (Qiagen) was used to purify the PCR products representing integron variable regions (IVRs). The purified amplicons were sequenced using the ABI PRISM BigDye Terminator version 3.1 cycle sequencing ready reaction kit and the ABI PRISM 3700 DNA analyser (Applied Biosystems).

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RESULTS

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MIC data for the ATCC 27853 and MDRPA isolates for several antimicrobial agents are shown in Table 1.
from these strains were identical and carried only one resistance gene, for aminoglycoside adenyltransferase B [aadB, also called \textit{ant(2')-Ia}], contained in a cassette. The enzyme encoded by this gene is involved in aminoglycoside resistance (Fig. 3b), specifically to gentamicin, kanamycin and tobramycin.

**Efflux-pump-mediated fluoroquinolone resistance**

All MDRPA strains had a ciprofloxacin MIC of 64 mg l$^{-1}$ and were thus interpreted as resistant. The efflux pump overexpression phenotype was defined based on at least a twofold decrease in the ciprofloxacin MIC when determined in the presence of PAβN and was observed in all MDRPA strains (Fig. 4).

In the presence of PAβN (40 mg l$^{-1}$), efflux inhibition was detected for all of the tested antibiotics except tobramycin, a PAβN antagonist. By contrast, CCCP (10 mg l$^{-1}$ concentration), despite its apparent efflux-inhibiting activity, caused bacterial death, even in the absence of antibiotics, indicating its toxicity for \textit{P. aeruginosa} (Fig. 5a, b).

**Detection of a functioning MexXY-OprM efflux pump**

Although all of the MDRPA strains had a high tobramycin MIC, in the presence of 40 mg l$^{-1}$ PAβN their sensitivity to this antibiotic increased, as evidenced by a significant increase in the MIC (Fig. 6).
Effect of PAβN on antimicrobial activity

The addition of PAβN resulted in only negligible reductions in the ceftazidime and meropenem MICs, in contrast to the antagonistic effects of tobramycin and ceftazidime or tobramycin and meropenem regardless of the β-lactam concentration (Table 2).

DISCUSSION

The *P. aeruginosa* strains examined in this study were resistant to all of the antimicrobials tested (Table 1), including carbapenems, and were therefore considered as MDRPA, i.e. resistant to at least three different classes of antimicrobial agents, mainly aminoglycosides, carbapenems, antipseudomonal penicillins, quinolones and cephalosporins (Falagas et al., 2006). The increasing emergence of MDRPA strains is a cause for concern as it compromises the therapeutic options for treating infections with these bacteria. At the same time, it highlights the importance of developing alternative approaches, such as the use of drug combinations or EPIs, and of identifying novel antipseudomonal agents (Lomovskaya & Watkins, 2001).

The homogeneity of the PFGE profiles of the tested isolates confirmed the existence of an endemic *P. aeruginosa* clone in our hospital. A similar scenario was described by Koutsogiannou et al. (2013), who also reported the clonal...
dissemination of MDRPA in a university hospital. Indeed, the inter-hospital spread of MDRPA clones in Spanish hospitals other than ours was recently determined, emphasizing the need for measures to prevent the clinical dissemination of these isolates (Cabot et al., 2012).

We observed that the survival of *P. aeruginosa* in the presence of high concentrations of meropenem (4× MIC) coincided with a change in its shape, to long undivided filaments. When antibiotic pressure was withdrawn the bacteria returned to their normal shape. According to these findings, meropenem accumulation in *P. aeruginosa* seems to differ depending on cell shape. Both Trautman et al. (1998) and Horii et al. (1999) described the meropenem-related induction of filamentous forms in *P. aeruginosa* and suggested that these morphological changes depended on the length of exposure to the antibiotic and on the growth phase. Several studies have focused on the relationship between morphology and the amount of endotoxin released by *P. aeruginosa* exposed to carbapenems, but none has examined meropenem accumulation as a function of bacterial morphology. The bioassay used in this study, designed to measure meropenem accumulation, was able to detect small differences in antibiotic concentrations, which allowed us to identify the slightly greater accumulation of meropenem in normally shaped bacilli than in those that became filamentous in the presence of an antibiotic concentration fourfold higher than the MIC. However a more precise interpretation of these data awaits further studies. Meropenem has a high affinity for penicillin-binding protein 3, a bacterial protein involved in the final steps of cell-wall synthesis. During bacterial

**Table 2.** MICs of synergic combinations of antibiotic with and without PAβN

Meropenem and ceftazidime were tested at concentrations between 0.125 and 256 mg l⁻¹.

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<tr>
<th>Fixed concentration (mg l⁻¹)</th>
<th>Tobramycin</th>
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<td>459 Tob + PAβN</td>
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<td>133 Tob + PAβN</td>
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<td>Fixed concentration (mg l⁻¹)</td>
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division, the inhibition of penicillin-binding protein 3 prevents the separation of daughter bacteria (Weiss et al., 1999) and leads to the formation of long filaments. These altered cells have a non-functional penicillin-binding protein 3 but are able to survive for relatively long periods, and under appropriate conditions resume their original morphotype. Thus, morphological changes may at least partially explain the failure of antibiotic treatments in infected patients, since exposed cells can survive as long rods and serve as the origin of recidivisms when their ‘normal’ morphology is restored.

The reduction of OprD seen in all MDRPA isolates compared to the wild-type strain (ATCC 27853) may also account for the decreased susceptibility of the isolates to imipenem and meropenem. However, in vivo quantitative determinations of the actual role of the OM and porins in the presence of antibiotics are difficult, since both the physiological state of a bacterium and OMP expression depend strongly on the environmental conditions. Studies have reported that downregulation of the oprD gene and inactivation of the OprD protein contribute, respectively, to imipenem resistance and to a reduction in meropenem susceptibility (Veiga-Crespo et al., 2011; Rodriguez-Martinez et al., 2009). In view of these studies, intermediate susceptibility or resistance to imipenem can be ascribed to OprD inactivation alone whereas more complex mechanisms apparently underlie meropenem resistance, including the overexpression of AmpC and of several efflux pumps. Meropenem activity is clearly affected by two of these pumps, MexAB-OprM and MexXY-OprM (Poole, 2004). Ikonomidis et al. (2008) found that in populations of P. aeruginosa exhibiting carbapenem heterogeneity, efflux is upregulated following an increase in mexB and mexY genes transcripts and reduced expression of OprD (46 kDa). We found that these heterogeneous strains also produce the oxacillin-hydrolysing enzymes OXA-1 and OXA-2, which contribute to carbapenem resistance (unpublished data).

Based on our experiments using the inhibitor PAβN, the phenotype of the MDRPA isolates was found to include both the overexpression of MexAB-OprM and a functioning MexXY-OprM efflux pump. However, the unexpected results obtained with CCCP rule out the use of this inhibitor in studies of P. aeruginosa and thus perhaps in other strictly respiratory bacteria. Both structural changes in target enzymes and active efflux are relevant mechanisms leading to fluoroquinolone resistance in P. aeruginosa (Nakajima et al., 2002). Similarly, MexXY-OprM overexpression has been related to carbapenem resistance. Mao et al. (2001) reported that PAβN antagonizes aminoglycoside activity only in strains containing a functioning MexXY-OprM, with antagonism greater in strains expressing high levels of this efflux pump. The negligible reductions in the meropenem and ceftazidime MICs of our MDRPA strains in the presence of PAβN is understandable because MexAB is an efficient pump for both antibiotics (Drusano et al., 2009). Many reports have suggested the convenience of ‘rescuing’ traditional antibiotics to devise synergistic combinations of antimicrobials that could be used to treat infections by multidrug-resistant micro-organisms. Although combinations of tobramycin and either ceftazidime or meropenem were shown to be synergistic (Blumer et al., 2005), their efficacy in our MDRPA strains with respect to efflux pump overexpression could not be determined because of the remarkable antagonism of tobramycin by PAβN irrespective of the antibiotic concentration.

Furthermore, we found two mutations in the studied strains, one in parC and the other in gyrA (unpublished data). These results correlate with the observation that quinolone resistance in Enterobacteriaceae is generally caused by a cooperative effect between mutations in the gyrA and parC genes and efflux pumps, mostly acrAB (Ikonomidis et al., 2008).

Nonetheless, P. aeruginosa resistance is complicated since many mechanisms are involved, including integrons and gene cassettes. Class 1 integrons are a common presence in MDRPA, since among all isolates reported thus far those resistant to less than three agents are intI1-negative, whereas isolates resistant to more than two agents are intI1-positive (Nemec et al., 2010). All of our MDRPA strains had a class 1 integron containing the gene cassette array aadB, associated with resistance to kanamycin, gentamicin and tobramycin (Azucena & Mobashery, 2001). This type of integron was found previously in some clinical P. aeruginosa strains obtained from Bellvitge Hospital, Barcelona, and studied in our laboratory (Ruiz-Martinez et al., 2011). However, this integron has been described not only in Spain but also in hospitals in Iran, where among MDRPA isolates from five hospitals 78% had an integron containing aadB. Amplifications of the IVRs of class 1 integrons from these isolates confirmed the high prevalence of class 1 integrons with a limited diversity of gene cassette arrays, including aadB, aadA6–orfD and blaOXA10–aacA4 (Shahcheraghi et al., 2010).

Syrmis et al. (2008) also determined a high prevalence of the class 1 integron-associated aadB gene cassette in P. aeruginosa, isolated from a population of cystic fibrosis patients in Australia. However, some of the strains carrying the aadB gene were nonetheless tobramycin sensitive. Thus, the position of the aadB gene alone does not confer tobramycin resistance. In Brazil, 106 P. aeruginosa clinical isolates were analysed and none of the class 1 integrons detected included the aadB gene cassette (Fonseca et al., 2005). Other studies showed that some class 1 integrons with more than one resistance gene also contained aadB. For instance, many MDRPA isolates (serotype O4) from Forth Valley, UK, detected a high prevalence of class 1 integrons with a limited diversity of gene cassette arrays, including aadB, aadA6–orfD and blaOXA10–aacA4 (Shahcheraghi et al., 2010).

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Although integron-associated gene cassettes harboured by \textit{P. aeruginosa} can vary among different geographical locations (Syrmis \textit{et al.}, 2008), the clonal dissemination of MDRPA strains remains an important problem, with effective measures required to prevent their transmission. Multidrug-resistant bacteria serve as hosts not only of integrons but also of other genetic elements, such as transposons and plasmids, involved in the development of antibiotic-resistance phenotypes. Consequently, bacteria containing these elements are extremely effective vehicles for their broad dissemination (Woodford \textit{et al.}, 2011).

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