OXA-28, an Extended-Spectrum Variant of OXA-10 β-Lactamase from *Pseudomonas aeruginosa* and Its Plasmid- and Integron-Located Gene

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*Pseudomonas aeruginosa* ED-1, isolated from a pulmonary brush of a patient hospitalized in a suburb of Paris, France, was resistant to ceftazidime and of intermediate susceptibility to ureidopenicillins and to cefotaxime. Cloning and expression of the β-lactamase gene content of this isolate in *Escherichia coli* DH10B identified a novel OXA-10 variant, OXA-28, with a pI value of 8.1 and a molecular mass of 29 kDa. It differed from OXA-10 by 10 amino acid changes and from OXA-13 and OXA-19 by 2 amino acid changes, including a glycine instead of tryptophan at position 164, which is likely involved in its resistance to ceftazidime. Like OXA-11, -14, -16, and -19 and as opposed to OXA-17, OXA-28 predominantly compromised ceftazidime and had only marginal effect on the MICs of aztreonam and cefotaxime in *P. aeruginosa*. Once expressed in *E. coli*, OXA-28 raised the MIC of ceftazidime to a much higher level than those of amoxicillin, cephalexin, and cefotaxime (128, 16, 8, and 4 μg/ml, respectively). OXA-28 β-lactamase had a broad spectrum of activity, including ceftazidime. Its activity was partially antagonized by clavulanic acid (50% inhibitory concentration, 10 μM) and NaCl addition. The *oxa28* gene cassette was inserted in the variable region of a class 1 integron, In57, immediately downstream of an amino 6′-N-acetyltransferase gene cassette, *aac(6′)Ib*. The structures of the integrons carrying either *oxa28*, *oxa13*, or *oxa19* gene cassettes were almost identical, suggesting that they may have derived from a common ancestor as a result of the common European origin of the *P. aeruginosa* isolates. In57 was located on a self-transferable plasmid of ca. 150 kb that was transferred from *P. aeruginosa* to *P. aeruginosa*.

Resistance to extended-spectrum cephalosporins in *Pseudomonas aeruginosa* is associated (i) mostly with overexpression of the chromosomally located AmpC β-lactamase, decreased uptake by porin alteration, and increased efflux (1); (ii) with clavulanic acid-inhibited Ambler class A extended-spectrum β-lactamases (ESBLs), such as TEM and SHV derivatives, PER-1, and VEB-1 (CEF-1) (23, 24, 28, 31, 37); and (iii) with β-lactamases (ESBLs), such as TEM and SHV derivatives, cefepime, cefpirome, aztreonam, and moxalactam. They are mostly point mutant derivatives of OXA-2 (OXA-15) or of OXA-10 (OXA-11, -13, -14, -15, -16, -17, and -19) (5–8, 12, 20–22, 29). These extended-spectrum oxacillinases possess a hydrolysis spectrum extended either to ceftazidime, and/or to cefotaxime, cefepime, cefpirome, aztreonam, and moxalactam. They are mostly point mutant derivatives of OXA-2 (OXA-15) or of OXA-10 (OXA-11, -13, -14, -15, -16, -17, and -19) (5–8, 12, 20–22), except for OXA-18 (29). The activity of these serine β-lactamases is not inhibited significantly by clavulanic acid, except for OXA-18 (29). There have been only rare reports of extended-spectrum oxacillinases, and these are almost exclusively from *P. aeruginosa* clinical isolates from Turkey (5–8, 12, 38). ARI-1 (OXA-23), has been identified from *Acinetobacter baumannii* in the United Kingdom; it possesses carbapenem-hydrolyzing activity and shares only 36% amino acid identity with OXA-10 (10). Additionally, laboratory-obtained mutants of the OXA-10 β-lactamase conferring resistance to ceftazidime in *P. aeruginosa* have been described (9).

Most of the oxacillinase genes are plasmid and integron located (11, 22). Integrons are genetic structures capable of capturing gene cassettes. Class 1 integrons, which are most commonly found in antibiotic-resistant clinical isolates, possess two conserved segments located on either side of the integrated genes (11). The 5′ conserved segment (5′-CS) includes a gene, *intI1*, encoding the integrase; *attI1*, the cassette integration site; and the promoter, *P*, (and sometimes a secondary *P* promoter) which is located within the integrase gene and is responsible for expression of the downstream-located and cassette-integrated genes (11). The 3′-conserved segment (3′-CS) most often includes, along with an open reading frame (ORF) of unknown function (*orf5*), the disinfectant (*qacED1*) and the sulfonamide (*sul1*) resistance determinants. The gene cassettes are discrete mobile units comprising a gene, usually an antibiotic resistance gene, and a recombination site that is recognized by the integrase (11). The cassette-associated recombination sites, known as 59-base elements, are located downstream of the inserted genes and are of variable length (35).

In this report, we describe the β-lactamase content and its genetic support of a *P. aeruginosa* isolate whose resistance to ceftazidime was partially reversed by addition of clavulanic acid and that was of intermediate susceptibility to ureidopenicillins and cefotaxime. This resistance pattern not typical of a class A ESBL and suggested the presence of an extended-spectrum oxacillinase.

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**RESULTS AND DISCUSSION**

Susceptibility testing, plasmid DNA analysis, and \( \beta \)-lactamases of *P. aeruginosa* ED-1. *P. aeruginosa* ED-1 was studied for its ceftazidime resistance, which was slightly inhibited by clavulanic acid addition and was associated with an intermediate susceptibility to uredopenicillins and cefotaxime (Table 1). Cefotaxime resistance in *P. aeruginosa* is most often caused by hyperproduction of the chromosomal class C cephalosporinase or by efflux or impermeability (1). These mechanisms raised the MICs of uredopenicillins and cefotaxime above that of ceftazidime (1). The resistance pattern observed in *P. aeruginosa* ED-1 resembled that of OXA-15, an extended-spectrum derivative of OXA-2 (7). Most of the extended-spectrum oxacillinases confer resistance to ceftazidime and to cefotaxime and are not inhibited significantly by clavulanic acid (6–8, 12, 20). *P. aeruginosa* ED-1 was also resistant to chloramphenicol, kanamycin, gentamicin, netilmicin, tobramycin, and sulfonamides according to the results of a disk diffusion antibiogram. A plasmid DNA preparation of *P. aeruginosa* ED-1 revealed a large plasmid, pGIR-1, of ca. 150 kb. This plasmid was transferred by conjugation from *P. aeruginosa* ED-1 to rifampin-resistant *E. coli* DH10B(pDG-1) at a frequency of 2 \( \times \) 10\(^{-7}\), and not to rifampin-resistant *E. coli* K-12 C600 (<1 \( \times \) 10\(^{-11}\)). The comparison of the \( \beta \)-lactam resistance profiles of *P. aeruginosa* ED-1 shown in Fig. 2B.
TABLE 1. MICs of β-lactams for the P. aeruginosa ED-1 clinical isolate, its transconjugant P. aeruginosa PU21(pGIR-1), reference strain P. aeruginosa PU21, E. coli DH10B(pDG-1), and reference strain E. coli DH10B

<table>
<thead>
<tr>
<th>β-Lactam</th>
<th>P. aeruginosa ED-1 (µg/ml)</th>
<th>P. aeruginosa PU21 (pGIR-1)²</th>
<th>P. aeruginosa PU21 (µg/ml)</th>
<th>E. coli DH10B (pDG-1)²</th>
<th>E. coli DH10B (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin</td>
<td>&gt;512</td>
<td>&gt;512</td>
<td>32</td>
<td>16</td>
<td>2</td>
</tr>
<tr>
<td>Ticarcillin</td>
<td>64</td>
<td>128</td>
<td>16</td>
<td>32</td>
<td>2</td>
</tr>
<tr>
<td>Ticarcillin plus CLA</td>
<td>64</td>
<td>128</td>
<td>16</td>
<td>32</td>
<td>2</td>
</tr>
<tr>
<td>Pipercillin</td>
<td>8</td>
<td>16</td>
<td>2</td>
<td>32</td>
<td>1</td>
</tr>
<tr>
<td>Pipercillin plus TZB</td>
<td>8</td>
<td>16</td>
<td>1</td>
<td>16</td>
<td>1</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>Cefoxolin</td>
<td>256</td>
<td>64</td>
<td>2</td>
<td>32</td>
<td>2</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>256</td>
<td>512</td>
<td>0.25</td>
<td>128</td>
<td>0.06</td>
</tr>
<tr>
<td>Ceftazidime plus CLA</td>
<td>128</td>
<td>64</td>
<td>0.25</td>
<td>64</td>
<td>0.06</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>16</td>
<td>16</td>
<td>2</td>
<td>4</td>
<td>0.12</td>
</tr>
<tr>
<td>Cefepime</td>
<td>32</td>
<td>16</td>
<td>0.06</td>
<td>0.5</td>
<td>&lt;0.06</td>
</tr>
<tr>
<td>Moxalactam</td>
<td>128</td>
<td>256</td>
<td>8</td>
<td>1</td>
<td>0.25</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>32</td>
<td>32</td>
<td>0.5</td>
<td>8</td>
<td>0.12</td>
</tr>
<tr>
<td>Imipenem</td>
<td>2</td>
<td>0.25</td>
<td>0.12</td>
<td>0.06</td>
<td>0.06</td>
</tr>
</tbody>
</table>

¹ CLA, clavulanic acid at a fixed concentration of 2 µg/ml; TZB, tazobactam at a fixed concentration of 4 µg/ml.
² P. aeruginosa PU21 harboring natural plasmid pGIR-1 and E. coli DH10B harboring recombinant plasmid pDG-1 produced the β-lactamase OXA-28.
TABLE 2. Amino acid differences among extended-spectrum oxacillinases compared to OXA-10

<table>
<thead>
<tr>
<th>Oxacillinase</th>
<th>Amino acid(s) at position(s):</th>
</tr>
</thead>
<tbody>
<tr>
<td>OXA-10</td>
<td>I G S D S-T-F-K Y-A-G-N W L</td>
</tr>
<tr>
<td>OXA-M102</td>
<td>S Y-A G-Y R-Y T-K-G E S E L</td>
</tr>
<tr>
<td>OXA-M103</td>
<td>S Y-A G-Y R-Y T-K-G E S E L</td>
</tr>
<tr>
<td>OXA-11</td>
<td>T S N S F G N A</td>
</tr>
<tr>
<td>OXA-14</td>
<td>T S N S F G N A</td>
</tr>
<tr>
<td>OXA-16</td>
<td>S T S N S F G N A</td>
</tr>
<tr>
<td>OXA-17</td>
<td>T S N S F G N A</td>
</tr>
<tr>
<td>OXA-13-1</td>
<td>T S N S F G N A</td>
</tr>
<tr>
<td>OXA-19</td>
<td>T S N S F G N A</td>
</tr>
<tr>
<td>OXA-28</td>
<td>T S N S F G N A</td>
</tr>
</tbody>
</table>

*The standard numbering scheme for class D enzymes (DBL numbering) is used (3). The amino acids in boldface may contribute to the extension of the substrate hydrolysis profile, leading to extended-spectrum derivatives.

b In vitro-obtained mutant (9).

determined. It may be that the OXA-28 enzyme was not correctly folded in *E. coli* and/or that it was unstable even in *P. aeruginosa*. Similarly, although OXA-16 confers resistance to ceftazidime, hydrolysis of ceftazidime could not be detected (8).

Studies of inhibition, as measured by IC<sub>50</sub>s, showed that OXA-28 activity was partially inhibited by clavulanic acid (10 μM), which is similar to what was found for other extended-spectrum OXA-10 derivatives (22). This IC<sub>50</sub> may explain the two-fold reduction of the MIC of ceftazidime for *P. aeruginosa* ED-1 and for its transconjugant as found for several OXA-10 derivatives (Table 1) (22). However, this IC<sub>50</sub> was much higher than that (0.08 μM) found for OXA-18 (29), the only known extended-spectrum oxacillinase strongly inhibited by clavulanic acid. OXA-28 activity was inhibited by NaCl, as for most oxacillinases (IC<sub>50</sub> 75 mM) (22).

The molecular mass of the mature β-lactamase from *E. coli* DH10B(pDG-1) was 29 kDa, which is close to the computer-calculated molecular mass of the mature protein (27.3 kDa).

Compared to the other extended-spectrum derivatives, OXA-28 was most closely related to OXA-13, the in vitro-obtained mutant OXA-13-1, and OXA-19 (two amino acid changes); these β-lactamases have similar pI values (8.0 to 8.1) (20, 21). OXA-13 conferred weak resistance to aztreonam, cefotaxime, and ceftazidime, while OXA-13-1 and OXA-19 are true ceftazidime-hydrolyzing extended-spectrum oxacillinases (20, 21). OXA-13, OXA-13-1, and OXA-19 activities are strongly inhibited by imipenem. A very slight synergy between imipenem and cefsulodin or ceftazidime was found for OXA-19 (20, 21). OXA-13 conferred weak resistance to aztreonam, while OXA-13-1 and OXA-19 are strongly inhibited by imipenem and cefsulodin or ceftazidime (20, 21). OXA-13 has been suggested to be involved in the extension of the hydrolysis profile, leading to extended-spectrum derivatives.

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To determine the amino acid changes in OXA-28, the DNA sequences surrounding the *bla* gene cassette were determined. The nucleotide sequence of the *bla* gene cassette was found to be identical to that of the *bla* gene cassette of *P. aeruginosa* ED-1 (Fig. 1). This result suggests that the changes in OXA-28 are likely to be the result of a single nucleotide change in the integron.

The standard numbering scheme for class D enzymes (DBL numbering) is used (3). The amino acids in boldface may contribute to the extension of the substrate hydrolysis profile, leading to extended-spectrum derivatives.

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An aspartic acid at DBL 167 instead of a glycine in OXA-10 and OXA-13 has been suggested to be involved in the extension of the hydrolysis spectrum of OXA-19 (Table 2). In OXA-28 this amino acid change was not found, but a glycine instead of tryptophan at DBL 164 (as for OXA-10 and OXA-13) was found (Table 2). This change, like the amino acid change at DBL167 in OXA-19, was within a conserved class D structural element (Fig. 1). A similar amino acid change (leucine instead of tryptophan) at the same position DBL 164 was found in the in vitro-obtained extended-spectrum oxacillinase OXA-M101 mutant, which is more closely related to OXA-10 than to OXA-13 (Table 2). Both OXA-M101 and OXA-28 hydrolyzed ceftazidime at a much higher level than cefotaxime and aztreonam (Table 1) (9). The role of glycine at DBL 164 in the extended hydrolysis spectrum of OXA-28 needs to be confirmed by site-directed mutagenesis experiments.

**Genetic environment of *bla*<sub>oxa-28</sub>**

Sequence analysis of the DNA sequences surrounding *bla*<sub>oxa-28</sub> revealed another antibiotic resistance gene immediately upstream of *bla*<sub>oxa-28</sub> (Fig. 1). It encoded an aminoglycoside acetyltransferase AAC(6')Ib enzyme (36) that shared 100% identity with AAC(6')Ib-9, which has been associated with OXA-19, considering only the amino acid sequence present within the cassette boundaries and starting with the first possible translation initiation codon after the recombinase core site (Fig. 1) (36). Thus, as expected, AAC(6')Ib conferred resistance to gentamicin and tobramycin in *P. aeruginosa* ED-1, *P. aeruginosa* PU21(pGIR-1), and *E. coli* DH10B(pDG-1) (data not shown).

FIG. 1. Nucleotide sequence of 2,254 bp of recombinant plasmid pDG-1 containing the *bla*<sub>oxa-28</sub> coding region and part of its integron, In57. The deduced amino acid sequence is designated in single-letter code below the nucleotide sequence. The slash indicates the putative cleavage site for the leader peptide for the mature OXA-28 β-lactamase. Underlined amino acid sequences are those conserved for the oxacillinase family and are numbered according to DBL numbering (3). The start codons of *intI1*, *bla*<sub>oxa-28</sub>, and *aac(6')Ib* are indicated by horizontal arrows, and their stop codons are indicated by asterisks. The –35 and –10 sequences of the promoters *P<sub>o</sub>*<sub>oxa</sub>, *P<sub>int</sub>*<sub>oxa</sub>, and *P<sub>ase</sub>*<sub>ase</sub> are underlined. The conserved core and inverse core sites located at the *oaxa28* and *aac(6')Ib* cassette boundaries are boxed and the composite 59-base elements are italicized. The additional base of the 59-base element of the *oaxa28* gene cassette as compared to the 59-base element of the *oaxa19* gene cassette is indicated by a square below the 59-base element of the *oaxa28* gene cassette. The cassette boundaries are indicated by vertical arrows. The left part of the *attI1* site is underlined with a dotted line.
Further upstream and downstream of the identified antibiotic resistance genes included in In57 was driven by two promoter sequences Pc (−35 region, TGGACA; −10 region, TAAGCT) and P2 (−35 region, TTGA TTA; −10 region, TACAGT), which both lay within the inte-grase-coding sequence (Fig. 1). Compared to the corresponding promoter regions for the blaOXA-19- and blaOXA-13- containing class 1 integrons, a three-guanosine insertion 119 bp downstream of promoter Pc (between the −35 and −10 regions of promoter P2) brought its spacing to an optimal 17 bp, resulting in a strong promoter. Thus, promoter P2 may be responsible for up to 90% of blaOXA-19 and blaOXA-28 gene expression (2, 19). The weak expression of blaOXA-28 in E. coli DH10B, as found for OXA-17, may reflect its second position within the variable region of In57. Indeed, the 59-base element of blaOXA-28 may generate stem-loop structures that may pro-vide attenuation of the downstream-located sequence and/or premature transcript stops. The overall structures of the integrons carrying blaOXA-13-, blaOXA-19-, and blaOXA-28 were similar, possibly as a result of the same geographical origin of the clinical isolates (Paris region, France). These structures differ from the absence of any reduced-spectrum blaOXA-2- derived from those carrying the extended-spectrumblaOXA-10 derivatives identified from Turkish isolates.

Identification of blaOXA-28 in an integron underlines the fact that most of the oxacillinase genes are class 1 integron located, regardless of the country in which the P. aeruginosa isolates had been identified (22). Among the extended-spectrum oxacillinase genes, the OXA-11, -13,-15, -16, -17, -18, and -19 genes are part of class 1 integrons. In addition, In57 is another example of the association of oxacillinase genes with amino-glycoside resistance genes in class 1 integrons, and this is for unknown reasons (11, 22, 32). The origin of the blaOXA-4 and blaOXA-10 extended-spectrum derivatives, including blaOXA-28, may not be Pseudomonas spp., considering that their G+C contents are close to those of Enterobacteriaceae genes. The only known extended-spectrum oxacillinase gene that may derive from Pseudomonas spp. is blaoxa-18 (G+C content of 62% [29]). This observation fits with the concept of integrons that may capture and express bacterial genes of various species (13).

A practical approach for detection and identification of OXA-10-derived ceftazidime-hydrolyzing ESBLs based on PCR ampli-fication followed by restriction digestion of the 720-bp amplicon has been proposed (38). However, this screening method does not allow differentiation between blaoxa-19, blaoxa-28, and blaoxa-13, although OXA-13 does not hydrolyze ceftazidime significantly.

Finally, this work underlines that ceftazidime resistance in P. aeruginosa that is weakly clavulanic acid inhibited could be due to ESBLs other than class A enzymes. This is the second report, after that of OXA-19, of a ceftazidime-resistant extended-spectrum OXA-10 derivative from a P. aeruginosa clinical isolate outside Turkey.

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REFERENCES


