

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, cephalothin, and cefotaxime (128, 16, 8, and 4 μ g/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 μ M) 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 Ambler class B wide-spectrum carbapenem-hydrolyzing β -lactamases (IMP-1, VIM-1, and VIM-2) (16, 30, 34) and extended-spectrum Ambler class D oxacillinases (5–8, 12, 20–22, 29). These extended-spectrum oxacillinases possess a hydrolysis spectrum extended either to ceftazidime, and/or to cefotaxime, cefepime, ceftipime, 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_c (and sometimes a secondary P_2 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 (*qacE Δ I*) and the sulfonamide (*sulI*) 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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MATERIALS AND METHODS

Bacterial strains and plasmids. The *P. aeruginosa* ED-1 clinical isolate was from a pulmonary brush of a patient hospitalized for nosocomial pneumonia at the Hôpital de Bicêtre (Le Kremlin-Bicêtre, France). This isolate was recovered while the patient was being treated with a combination of ceftazidime and amikacin. It was identified by the API-20 NE system (bioMérieux, Marcy-l'Etoile, France). In vitro-obtained rifampin-resistant *P. aeruginosa* PU21, in vitro obtained rifampin-resistant *Escherichia coli* K-12 C600, and *E. coli* DH10B were used as recipient strains for conjugation and cloning experiments (25, 29, 30). *E. coli* NCTC 50192, carrying plasmids of 154, 66, 38, and 7 kb, served as controls in a plasmid-sizing study (39). Plasmid pPCRScript-Cam (SK+) (Stratagene, Amsterdam, The Netherlands) carrying the chloramphenicol resistance marker was used for cloning experiments.

Antimicrobial agents and MIC determinations. The antimicrobial agents and their sources have been described elsewhere (29). Antibiotic-containing disks were used for detection of antibiotic susceptibility with Mueller-Hinton agar plates, disk diffusion assay (Sanofi-Diagnostics Pasteur, Marnes-La-Coquette, France), and double-disk synergy detection using either amoxicillin-clavulanic acid- or imipenem-containing disks and cefsulodin, ceftazidime, and aztreonam (21). MICs were determined by an agar dilution technique on Mueller-Hinton agar plates with a Steers multiple inoculator and an inoculum of 10^4 CFU per spot (29). Results of susceptibility testing were recorded according to the guidelines of the National Committee for Clinical Laboratory Standards after incubation at 37°C for 18 h (26).

Plasmid content, hybridizations, and mating-out assays. Plasmid DNAs from *P. aeruginosa* ED-1 and one of its transconjugants were extracted by two different methods, as described previously (29, 30) and with a plasmid DNA Maxi kit (Qiagen, Courtaboeuf, France). Plasmid DNAs were analyzed by electrophoresis on a 0.8% agarose gel containing 0.5 μ g of ethidium bromide for 16 h at 90 V and compared to standard sizes of plasmid DNAs of *E. coli* NCTC 50192. The gel was transferred to a nylon membrane (Hybond N⁺; Amersham Pharmacia Biotech, Orsay, France) by the Southern technique (33). The DNAs were then UV cross-linked (Stratalinker; Stratagene) for 2 min. The probe, made of a PCR-generated 720-bp internal fragment of *bla*_{OXA-10} (14, 38), was labeled with the ECL nonradioactive labeling and detection kit, based on a combination of enhanced chemiluminescence detection and random primer labeling of DNA (Amersham Pharmacia Biotech).

Direct transfer of the ceftazidime resistance marker into rifampin-resistant *E. coli* K-12 C600 or rifampin-resistant *P. aeruginosa* PU21 was attempted by liquid and solid mating-out assays at 37°C (30). Transconjugant selection was performed on Trypticase soy (TS) agar plates (Sanofi-Diagnostics Pasteur) containing ceftazidime (4 μ g/ml) and rifampin (200 μ g/ml).

Cloning experiments and analysis of recombinant plasmids. Whole-cell DNA of *P. aeruginosa* ED-1 was extracted as described previously (29). Since the oxacillinase genes are often integron located, a primer for detection of class 1 integrons (INT2F [5'-TCTCGGGTAAACATCAAGGCC-3']) located within the integrase gene of class 1 integrons and a 3'-CS primer located in the 3'-CS (24) were used to PCR amplify fragments, with whole-cell DNA of *P. aeruginosa* ED-1 used as template. The PCR products were ligated into the *SrfI* site of pPCRScript Cam (SK+) as recommended by the manufacturer (Stratagene). *E. coli* DH10B harboring recombinant plasmid DNAs was selected on chloramphenicol (30 μ g/ml)- and amoxicillin (15 μ g/ml)-containing TS agar plates and analyzed as described previously (29).

β -Lactamase purification and IEF. Cultures of *E. coli* DH10B harboring recombinant plasmid pDG-1 (see Results and Discussion) were grown overnight at 37°C in 6 liters of TS broth containing amoxicillin (15 μ g/ml) and chloramphenicol (30 μ g/ml), and a β -lactamase extract was obtained as described previously (30). β -Lactamase extracts were also obtained from 10-ml cultures of *P. aeruginosa* ED-1, *P. aeruginosa* PU21, and its transconjugant *P. aeruginosa* PU21(pGIR-1) suspended in 0.5 ml of 100 mM sodium phosphate buffer (pH 7.0).

The β -lactamase extract of the *E. coli* DH10B(pDG-1) culture was resuspended in 50 ml of 100 mM sodium phosphate buffer (pH 6.0) for further purification. The β -lactamase extract was loaded onto a pre-equilibrated S-Sepharose column (Amersham Pharmacia Biotech) in 100 mM sodium phosphate buffer (pH 6.0). The β -lactamase was eluted with K₂SO₄ at a concentration of 200 mM. The β -lactamase peak was concentrated using Centriscart-C30 spin columns (Sartorius, Goettingen, Germany) and loaded onto a 1.6- by 47-cm gel filtration column packed with Superdex 75 (Amersham Pharmacia Biotech) equilibrated with 50 mM phosphate buffer (pH 6.0) containing 200 mM K₂SO₄. The fractions containing the highest β -lactamase activity were pooled, subsequently dialyzed overnight against 100 mM phosphate buffer (pH 7.0), and

concentrated again using Centriscart-C30 columns. The β -lactamase activity was determined qualitatively using nitrocefin hydrolysis (Oxoid, Dardilly, France). The protein content was measured using the Bio-Rad DC protein assay, and the specific activities of the crude extract and of the purified β -lactamase from *E. coli* DH10B(pDG-1) were compared.

Enzyme preparations from cultures of *P. aeruginosa* ED-1, *P. aeruginosa* PU21, and its transconjugant *P. aeruginosa* PU21(pGIR-1) and the purified β -lactamase from *E. coli* DH10B(pDG-1) were subjected to analytical isoelectric focusing (IEF) as described previously (29, 30).

Kinetic measurements. Purified β -lactamase from a culture of *E. coli* DH10B(pDG-1) was used for determination of kinetic parameters (k_{cat} and K_m) which was performed at 30°C in 100 mM sodium phosphate (pH 7.0). The initial rates of hydrolysis were determined spectrophotometrically with an Amersham Pharmacia Biotech ULTROSPEC 2000 spectrophotometer, and the results were analyzed using the Swift II software (Amersham Pharmacia Biotech) (30). The 50% inhibitory concentration (IC₅₀) was determined as the clavulanate or NaCl concentration that reduced the hydrolysis rate of 100 μ M nitrocefin by 50% under conditions in which the enzyme was preincubated with various concentrations of inhibitor for 30 min at 30°C before addition of the substrate (30).

Determination of the β -lactamase molecular mass. The molecular mass of the purified β -lactamase from *E. coli* DH10B(pDG-1) was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis as described previously (29).

DNA sequencing and protein analysis. The cloned DNA fragment from recombinant plasmid pDG-1 was sequenced on both strands with an Applied Biosystems sequencer (model ABI 373). Additionally, the adjacent DNA sequences of the cloned DNA fragment were obtained by PCR amplification using laboratory-designed primers for class 1 integrons and whole-cell DNA of *P. aeruginosa* ED-1 as the template. The nucleotide and the deduced protein sequences were analyzed with software available over the Internet at the National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov>) and at Pedro's BioMolecular Research Tools website (http://www.fmi.ch/biology/research_tools.html), and the putative cleavage site of the signal sequence was identified as described previously (27) at the Center for Biological Sequence Analysis website (<http://genome.cbs.dtu.dk/services/SignalP/>). Multiple nucleotide and protein sequence alignments were carried out online using the program ClustalW, which is available over the Internet at the University of Cambridge website (<http://www2.cbi.ac.uk/clustalW/>).

Nucleotide sequence accession numbers. The nucleotide sequence data reported in this paper will appear in the EMBL/Genbank nucleotide sequence database under accession no. AF231133.

RESULTS AND DISCUSSION

Susceptibility testing, plasmid DNA analysis, and β -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 ureidopenicillins and cefotaxime (Table 1). Ceftazidime 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 ureidopenicillins 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 *P. aeruginosa* PU21 at a frequency of 2×10^{-7} and but not to rifampin-resistant *E. coli* K-12 C600 ($<1 \times 10^{-10}$). The comparison of the β -lactam resistance profiles of *P. aerugi-*

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

| β -Lactam ^a | MIC (μ g/ml) for: | | | | |
|------------------------------|---------------------------|---|---------------------------|---|----------------------|
| | <i>P. aeruginosa</i> ED-1 | <i>P. aeruginosa</i> PU21 (pGIR-1) ^b | <i>P. aeruginosa</i> PU21 | <i>E. coli</i> DH10B (pDG-1) ^b | <i>E. coli</i> DH10B |
| Amoxicillin | >512 | >512 | 32 | 16 | 2 |
| Ticarcillin | 64 | 128 | 16 | 32 | 2 |
| Ticarcillin plus CLA | 64 | 128 | 16 | 32 | 1 |
| Piperacillin | 8 | 16 | 2 | 32 | 1 |
| Piperacillin plus TZB | 8 | 16 | 1 | 16 | 1 |
| Cephalothin | >128 | >128 | >128 | 8 | 4 |
| Cefsulodin | 256 | 64 | 2 | 32 | 2 |
| Cefuroxime | >128 | >128 | >128 | 4 | 4 |
| Ceftazidime | 256 | 512 | 0.25 | 128 | 0.06 |
| Ceftazidime plus CLA | 128 | 64 | 0.25 | 64 | 0.06 |
| Cefotaxime | 16 | 16 | 2 | 4 | 0.12 |
| Cefepime | 32 | 16 | 0.06 | 0.5 | <0.06 |
| Moxalactam | 128 | 256 | 8 | 1 | 0.25 |
| Aztreonam | 32 | 32 | 0.5 | 8 | 0.12 |
| Imipenem | 2 | 0.25 | 0.12 | 0.06 | 0.06 |

^a CLA, clavulanic acid at a fixed concentration of 2 μ g/ml; TZB, tazobactam at a fixed concentration of 4 μ g/ml.

^b *P. aeruginosa* PU21 harboring natural plasmid pGIR-1 and *E. coli* DH10B harboring recombinant plasmid pDG-1 produced the β -lactamase OXA-28.

nosa ED-1 and its transconjugant indicated that the natural plasmid pGIR-1 conferred the same β -lactam resistance pattern as found in *P. aeruginosa* ED-1 (Table 1). It raised the MICs of cefsulodin, ceftazidime, and moxalactam and moderately raised those of aztreonam, cefepime, cefotaxime, piperacillin, and ticarcillin (Table 1). The large plasmid hybridized with an internal probe for *bla*_{OXA-10}, as found for several plasmid-mediated extended-spectrum oxacillinase genes (*bla*_{OXA-11}, *bla*_{OXA-14}, *bla*_{OXA-15}, and *bla*_{OXA-16}) (data not shown). Additionally, this plasmid conferred a transferable resistance to gentamicin, tobramycin, and sulfonamides.

Analytical IEF revealed that *P. aeruginosa* ED-1 had two β -lactamase activities with pI values of 8.1 and 8.5, while the transconjugant *P. aeruginosa* PU21(pGIR-1) had an additional β -lactamase with a pI of 8.1 as compared to *P. aeruginosa* PU21. Thus, the pI value of 8.1 corresponded to the identified OXA-10 derivative, and the pI value of 8.5 in *P. aeruginosa* ED-1 likely corresponded to an AmpC cephalosporinase (data not shown) (40).

Cloning and sequencing of the β -lactamase gene, identification of the β -lactamase, and its expression in *E. coli*. Several recombinant *E. coli* DH10B strains were obtained on amoxicillin- and chloramphenicol-containing TS agar plates after cloning of a ca. 2.3-kb PCR-amplified product using class 1 integron-specific primers and whole-cell DNA of *P. aeruginosa* ED-1 as a template. One of them (pDG-1) was retained for further sequence analysis. It encoded a β -lactamase in *E. coli* DH10B as assessed by the result of a preliminary nitrocefin hydrolysis test.

Sequence analysis revealed two ORFs, one of which was 801 bp and encoded a 266-amino-acid preprotein. The G+C content of this ORF was 41.2%, which did not lie within the expected range of the G+C content of *P. aeruginosa* genes (60.1 to 69) as found for several oxacillinase genes, such as the *bla*_{OXA-10} derivatives (22). No typical promoter sequences of *E. coli* or *P. aeruginosa* genes were found immediately upstream of this ORF. A computer-predicted signal peptide cleavage site was found by computer-assisted amino acid se-

quence analysis to be between alanine and serine at positions 19 and 20 (Fig. 1). Within the deduced protein of this ORF, a serine-threonine-phenylalanine-lysine tetrad (S-T-F-K) was found at positions DBL (numbering of class D β -lactamases) 70 to 73 (Fig. 1), it included the conserved serine and lysine amino acid residues characteristic of β -lactamases possessing a serine active site or penicillin-binding proteins (3, 4, 18). Four structural elements characteristic of class D β -lactamases were found: Y-G-N at DBL 144 to 146, W-X-E-X-X-L-X-I-S at DBL 164 to 172, Q-X-X-X-L at DBL 176 to 180, and K-T-G at positions 216 to 218 (Fig. 1) (3, 4, 18, 22). In addition to these boxes, another stretch of amino acids at DBL 231 to 236 which seems to be highly conserved within class D enzymes was also present (Fig. 1). Furthermore, the S-X-V triad at DBL 118 to 120 was proposed to be a better equivalent of the S-D-N motif of class A β -lactamases than the Y-G-N motif at DBL 144 to 146 (Fig. 1) (3). Compared to OXA-10, this novel oxacillinase, named OXA-28, possessed nine amino acid changes (Table 2).

MICs of β -lactams for *E. coli* DH10B(pDG-1) were higher for ceftazidime than for amoxicillin, ureidopenicillins, and cephalothin, which is not usually reported for oxacillinase-producing organisms (Table 1). IEF analysis revealed that *E. coli* DH10B(pDG-1) produced the same β -lactamase, with a pI of 8.1, as found for *P. aeruginosa* ED-1 and for its transconjugant.

Biochemical properties of OXA-28 β -lactamase and amino acid comparison. After purification from an *E. coli* DH10B(pDG-1) culture, the OXA-28 specific activity against nitrocefin was 560 mU \cdot mg of protein⁻¹ and its purification coefficient was 15-fold. This β -lactamase was poorly expressed in *E. coli*, although its gene was cloned onto a multicopy plasmid, and expression of this β -lactamase from *P. aeruginosa* cultures was also poor (data not shown). OXA-28 had activity against several β -lactam substrates, including nitrocefin, cloxacillin, benzylpenicillin, cefotaxime, and ceftazidime, but not piperacillin. The activity against the cephalosporin nitrocefin was significant ($k_{\text{cat}}/K_m = 160 \text{ nM}^{-1} \cdot \text{s}^{-1}$), as reported for OXA-2 (17). The k_{cat}/K_m values for the other β -lactams could not be precisely

-35

1 TCTCGGGTAACATCAAGGCCCGATCCTTGGAGCCCTTGCCTCCC-----//-----GAAGGCACGAACCCAGTGGACATAAGCCCTG

R V W H V Y A Q

P_c -10

446 TTCGGTTGCTAAGCTGTAATGCAAGTAGCGTATGCGCTCACGCAACTGGTCCAGAACCTTGACCGAACGCAGCGGTGGTAACGGCGCAGTGGCGGTTTTTC

E T R L S Y H L Y R I R E R L Q D L V K V S R L P P L P A T A T K

-35 P_i -10



int11

546 ATGGCTTGTTATGACTGTTTTTTTTGGGGTACAGTCTATGCCTCGGGCATCCAAGCAGCAAGCGCGTTACGCCGTGGGTTCGATGTTTTGATGTTATGGAGCA

M -10 P_{int} -35

646 GCAACGATGTTACGCAGCAGGGCAGTCGCCCTAAAACAAAGTTAGGATCACAAAGTACAGCATCGTGACCAACAGCAACGATTCCGTCACACTGCGCCT

M T N S N D S V T L R L

→ aac(6')-Ib

746 CATGACTGAGCATGACCTTCGGATGCTCTATGAGTGGCTAAATCGATCTCATATCGTCGAGTGGTGGGGCGGAGAAGAAGCACGCCCGACACTTGCCTGAC

M T E H D L A M L Y E W L N R S H I V E W W G G E E A R P T L A D

846 GTACAGGAACAGTACTTGCCAAGCGTTTTAGCGCAAGAGTCCGTCCTCCATACATTGCAATGCTGAATGGAGAGCCGATTGGGTATGCCAGTCGTACG

V Q E Q Y L P S V L A Q E S V T P Y I A M L N G E P I G Y A Q S Y

946 TTGCTCTTGGAAAGCGGGGACGGATGTTGGGAAGAAGAAACCGATCCAGGAGTACGCGGAATAGACCAGTCACTGGCGAATGCATCAAACTGGGCAAAGG

V A L G S G D G W W E E E T D P G V R G I D Q S L A N A S Q L G K G

1046 CTTGGGAACCAAGCTGTTTCGAGCTCTGTTGAGTGTCTGTTCAATGATCCCGAGGTCACCAAGATCCAAACGGACCCGTCGCCGAGCAACTTGCAGAGCG

L G T K L V R A L V E L L F N D P E V T K I Q T D P S P S N L R A

1146 ATCCGATGCTACGAGAAAGCGGGTTTTGAGAGGCAAGGTACCCTAACCCAGATGGTCCAGCCGTGTACATGGTTCAAACACGCCAGGCATTCGAGC

I R C Y E K A G F E R Q G T V T T P D G P A V Y M V Q T R Q A F E

1246 GAACACGCAGTGTGCTTCCATCGAGGGGACGTCGAAGGGCTGGCGCCCTTGGCCGCCCTCATGTCAAACGTTAGGCAACCAAGAAGGTGCC

R T R S D A *

→ bla_{OXA-28}

1346 ATGAAAACATTTGCCGCATATGTAATTACTGCGTGTCTTTCAAGTACGGCATTAGCTAGTTCAATTACAGAAAATACGCTCTTGGAAACAAAGAGTTCTCTG

M K T F A A Y V I T A C L S S T A L A / S S I T E N T S W N K E F S

1446 CCGAAGCCGTCATGTTGTTTTCGTTGCTTTGTAAGTACGAGTAAATCCTGCGCTACCAATAACTTAGCTCGTGCATCAAAGGAATATCTCCAGCATC

A E A V N G V F V L C K S S S K S C A T N N L A R A S K E Y L P A S

1546 AACATTTAAGATCCCAACGCAATTATCGGCCCTAGAAACTGGTGTCTATAAAGAATGAGCATCAGGTTTTCAAATGGGACGGAAAGCCAAGAGCCATGAAA

T F K I P N A I I G L E T G V I K N E H Q V F K W D G K P R A M K

73

1646 CAATGGGAAAGAGACTTGAGCTTAAGAGGGGCAATACAAGTTTCAGCGGTTCCCGTATTTCAACAAATCGCCAGAGAAGTTGGCGAAGTAAGAATGCAGA

Q W E R D L S L R G A I Q V S A V P V F Q Q I A R E V G E V R M Q

118 120

1746 AATACCTTAAAAAATTTTCATATGGCAACCAGAATATCAGTGGTGGCATTGACAAATTCGGGTTGGAGGGTCAGCTAAGAATTTCCGCAGTTAATCAAGT

K Y L K K F S Y G N Q N I S G G I D K F G L E G O L R I S A V N O V

144 146

164

172

176

1846 GGAGTTCTAGAGTCTCTAATTTTTAAATAAATGTCAGCATCAAAGAAAATCAGCTAATAGTAAAAGAGGCTTTGGTAACGGAGGCTGCGCCTGAATAT

E F L E S L F L N K L S A S K E N Q L I V K E A L V T E A A P E Y

180

1946 CTTGTGCATTCAAAACTGGTTTTTCTGGTGTGGGAACTGAGTCAAATCCTGGTGTGCGATGGTGGGTTGGTTGGGTTGAGAAGGGAACAGAGGTTTACT

L V H S K T G F S G V G T E S N P G V A W W V G W V E K G T E V Y

216 218

231

236

2046 TTTTCGCCTTTAACATGGATATAGACAACGAAAATAAGTTGCCGCTAAGAAAATCCATTCCACCACAAAATCATGGCAAGTGAGGGCATCATTGGTGGCTA

F F A F N M D I D N E N K L P L R K S I P T K I M A S E G I I G G *

2146 ACAATTGCTGCAGGGCGGACGGCCCTGACGGGCCGGCCTGAGCTCAAACGTTAGATTCACCTAAGCACATAATGCTCACAGCCAACTATCAGGTCA



2246 AGTCTGCTT 2254

TABLE 2. Amino acid differences among extended-spectrum oxacillinases compared to OXA-10

| Oxacillinase | Amino acid(s) at position(s) ^a : | | | | | | | | | | | | | | | | | | |
|-----------------------|---|----|----|----|---------|----------|----|-----|-----|----------|----------|----------|-----|-----|---------|-----|-----|-----|--|
| | 10 | 20 | 28 | 58 | 70-73 | 76 | 92 | 110 | 127 | 144-146 | 164 | 167 | 184 | 208 | 216-218 | 240 | 258 | 272 | |
| OXA-10 | I | G | S | D | S-T-F-K | N | V | T | A | Y-G-N | W | G | Y | A | K-T-G | E | S | E | |
| OXA-M101 ^b | | | | | | | | | | | L | | | | | | | | |
| OXA-M102 ^b | | | | | | | | | S | | | D | | | | | | | |
| OXA-M103 ^b | | | | | | | | | | K | | | | | | | | | |
| OXA-11 | | | | | | | | | | S | | D | | | | | | | |
| OXA-14 | | | | | | | | | | | | D | | | | | | | |
| OXA-16 | | | | | | | | | T | | | D | | | | | | | |
| OXA-17 | | | | | | S | | | | | | | | | | | | | |
| OXA-13 | T | S | | N | | S | | S | | | | | F | | | G | N | A | |
| OXA-13-1 ^b | T | S | | N | | S | | S | | | | D | F | | | G | N | A | |
| OXA-19 | T | S | | N | | | | S | | | | D | F | | | G | N | A | |
| OXA-28 | T | S | | N | | | | S | | | G | | F | | | G | N | A | |

^a 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 IC₅₀s, 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 IC₅₀ may explain the twofold 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 IC₅₀ 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 (IC₅₀, 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-28 according to double-disk diffusion assay with either *P. aeruginosa* ED-1, *P. aeruginosa* PU21(pGIR-1), or *E. coli*

DH10B(pDG-1) cultures (data not shown). Thus, this inhibition test could not be a valuable detection test for OXA-28.

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*_{OXA-28}. Sequence analysis of the DNA sequences surrounding *bla*_{OXA-28} revealed another antibiotic resistance gene immediately upstream of *bla*_{OXA-28} (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 recombination 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*_{OXA-28} 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*_{OXA-28}, 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 P₃, P₂, and P_{int} are underlined. The conserved core and inverse core sites located at the *oxa28* 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 *oxa28* gene cassette as compared to the 59-base element of the *oxa19* gene cassette is indicated by a square below the 59-base element of the *oxa28* 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, sequencing revealed the following class 1 integron features: (i) a 5'-CS containing a class 1 integrase gene with its own promoter (P_{int}), (ii) an *attI1* recombination site, and (iii) a 3'-CS containing *qacEΔ1* (Fig. 1). The *bla*_{OXA-28} gene cassette had a core site (5'-GTTAGCC-3'), an inverse core site (5'-GGCTAAC-3'), and a 59-base element made of 72 bp downstream of this gene that had an additional base compared to the 59-base element found in the *oxa-19* cassette (20). The core site, inverse core site, and 59-base element of the *aac(6')Ib* gene cassette were identical to those found for the *aac(6')Ib* gene cassette (Fig. 1) (15, 25).

The expression of the antibiotic resistance genes included in In57 was driven by two promoter sequences P_c (−35 region, TGGACA; −10 region, TAAGCT) and P_2 (−35 region, TTG TTA; −10 region, TACAGT), which both lay within the integrase-coding sequence (Fig. 1). Compared to the corresponding promoter regions for the *bla*_{OXA-19} and *bla*_{OXA-13} containing class 1 integrons, a three-guanosine insertion 119 bp downstream of promoter P_c (between the −35 and −10 regions of promoter P_2) brought its spacing to an optimal 17 bp, resulting in a strong promoter. Thus, promoter P_2 may be responsible for up to 90% of *aac(6')Ib* and *bla*_{OXA-28} gene expression (2, 19). The weak expression of *bla*_{OXA-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 *aac(6')Ib* may generate stem-loop structures that may provide attenuation of the downstream-located sequence and/or premature transcript stops. The overall structures of the integrons carrying *bla*_{OXA-13}, *bla*_{OXA-19}, and *bla*_{OXA-28} were similar, possibly as a result of the same geographical origin of the clinical isolates (Paris region, France). These structures differ from those carrying the extended-spectrum *bla*_{OXA-10} derivatives identified from Turkish isolates.

Identification of *bla*_{OXA-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 aminoglycoside resistance genes in class 1 integrons, and this is for unknown reasons (11, 22, 32). The origin of the *bla*_{OXA-2} and *bla*_{OXA-10} extended-spectrum derivatives, including *bla*_{OXA-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 *bla*_{OXA-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 amplification followed by restriction digestion of the 720-bp amplicon has been proposed (38). However, this screening method does not allow differentiation between *bla*_{OXA-19}, *bla*_{OXA-28}, and *bla*_{OXA-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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