ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Oct. 1997, p. 2188–2195 Vol. 41, No. 10
0066-4804/97/$04.00+0
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OXA-18, a Class D Clavulanic Acid-Inhibited Extended-Spectrum β-Lactamase from *Pseudomonas aeruginosa*

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Received 27 January 1997/Returned for modification 29 April 1997/Accepted 4 August 1997

Clinical isolate *Pseudomonas aeruginosa* Mus showed resistance both to extended-spectrum cephalosporins and to aztreonam. We detected a typical double-disk synergy image when ceftazidime or aztreonam was placed next to a clavulanic acid disk on an agar plate. This resistance phenotype suggested the presence of an extended-spectrum β-lactamase. Isoelectric focusing revealed that this strain produced three β-lactamases, of pI 5.5, 7.4, and 8.2. A 2.6-kb *Sau*3A fragment encoding the extended-spectrum β-lactamase of pI 5.5 was cloned from *P. aeruginosa* Mus genomic DNA. This enzyme, named OXA-18, had a relative molecular mass of 30.6 kDa. OXA-18 has a broad substrate profile, hydrolyzing amoxicillin, ticarcillin, cephalothin, ceftazidime, cefotaxime, and aztreonam, but not imipenem or cephamycins. Its activity was totally inhibited by clavulanic acid at 2 μg/ml. Hydrolysis constants of OXA-18 (V_{max}, K_m) confirmed the MIC results. Cloxacillin and oxacillin hydrolysis was noticeable with the partially purified OXA-18. The bla_{OXA-18} gene encodes a 275-amino-acid protein which has weak identity with all class D β-lactamases except OXA-9 and OXA-12 (45 and 42% amino acid identity, respectively). OXA-18 is likely to be chromosomally encoded since no plasmid was found in the strain and because attempts to transfer the resistance marker failed. OXA-18 is peculiar since it is a class D β-lactamase which confers high resistance to extended-spectrum cephalosporins and seems to have unique hydrolytic properties among non-class A enzymes.

The main mechanism of resistance to β-lactam antibiotics among gram-negative isolates is β-lactamase biosynthesis. β-Lactamases inactivate penicillins and cephalosporins by hydrolyzing the amide bond of the β-lactam ring. The numerous β-lactamase sequences allow them to be divided into four molecular classes according to their amino acid content, designated A to D (1). Resistance to extended-spectrum cephalosporins is usually observed in members of the family *Enterobacteriaceae*, with extended-spectrum variants from class A β-lactamases TEM-1, TEM-2, and SHV-1 (33). These plasmid-mediated extended-spectrum enzymes were first reported in *Klebsiella pneumoniae* and later in almost all other *Enterobacteriaceae*. These variants differ from their parent enzymes by only a few amino acid positions (<4) within their catalytic sites (33) but can hydrolyze broad-spectrum β-lactam antibiotics such as penicillins and cephalosporins, including oxyimino β-lactams (cefotaxime, ceftazidime, and aztreonam). However, they do not hydrolyze cephamycins (cefoxitin) or carbapenems (imipenem or meropenem) (33). Their activities are inhibited by clavulanic acid (2).

Plasmid-mediated β-lactamases are observed in *Pseudomonas aeruginosa* isolates in fewer than 2% of samples, according to multicenter surveys in the United Kingdom in 1982 and 1993 (20). However, TEM-1 and TEM-2 have been described for this species (34), in which they confer resistance to aminopenicillins, carboxy-penicillins, and ureido-penicillins. CARB- or PSE (for *Pseudomonas*-specific enzyme)-type β-lactamases (with the exception of PSE-2 [OXA-10], which is in fact an oxacillinase), also called carbenicillin-hydrolyzing enzymes, are primarily found in this bacterial species but have also been identified in the *Enterobacteriaceae*. PSE-1 is the most frequently found plasmid-mediated β-lactamase in *P. aeruginosa* (34).

The OXA (oxacillin-hydrolyzing)-type enzymes are frequently described for *P. aeruginosa*. They usually confer resistance to amoxicillin and cephalothin and possess high-level hydrolytic activity against cloxacillin, oxacillin, and methicillin. Their activities are poorly inhibited by clavulanic acid (2). All characterized oxacillin-hydrolyzing β-lactamases belong to Ambler class D (1) and thus possess an active-site serine, as do class A and C β-lactamases (17). Overall amino acid identity between class D and class A β-lactamases is about 16% (4). Ambler class D includes OXA-1 to OXA-17, as well as PSE-2 (OXA-10). While some present significant degrees of amino acid sequence identity (for example, OXA-1 and OXA-4; OXA-7, OXA-5 and OXA-10 (PSE-2); and OXA-2 and OXA-3), most of them have only low percentages (20 to 30%) of amino acid identity (37, 38). *P. aeruginosa*, like *Enterobacter* spp., *Citrobacter* spp., *Serratia* spp., or *Morganella* spp., may affect hydrolysis of extended-spectrum cephalosporins by producing elevated amounts of chromosomally encoded Ambler class C β-lactamases (20). Nevertheless, such cephalosporinase activity is not inhibited by clavulanic acid.

Seven extended-spectrum β-lactamases have been described for *P. aeruginosa*. Five of them are oxacillin-hydrolyzing enzymes, poorly inhibited by clavulanic acid: OXA-11 (12), OXA-14 (7), OXA-16 (9), and OXA-17 (8), which are derived from OXA-10 (PSE-2), and OXA-15, which is derived from OXA-2 (10). Among penicillinases found in *P. aeruginosa*,

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or phenotype	Source or reference
Strains		
E. coli JM109	*endA1 hsdR17 gyrA96* Δ(*lac proA*) *recAB1 relA supE44 thi* F′ (*lacI*^{q} *lacZ*ΔM15 *proAB*$^+$ *traD36*)	47
In vitro-obtained resistant *E. coli* JM109	Ciprofloxacin resistant	This study
E. coli NCTC 50192	154-, 66-, 48-, and 7-kb plasmids	7
P. aeruginosa Mus	The studied β-lactamase	This study
P. aeruginosa PU21	*ilv leu* Str^r Rif^r	12
In vitro-obtained resistant *P. aeruginosa* PU21	Ciprofloxacin resistant	This study
A. hydrophila 76-14	Wild-type phenotype	IPSC[a]
In vitro-obtained resistant *A. hydrophila* 76-14	Ciprofloxacin resistant	This study
Plasmids		
pBK-CMV phagemid	Neomycin and kanamycin resistant	Stratagene
pBR322	Recombinant plasmid containing 560-bp *Ssp*I-*Pst*I internal fragment of bla_{TEM-1}	40
pHUC37	Recombinant plasmid containing 435-bp *Pst*I-*Not*I internal fragment of bla_{SHV-3}	29
pPZ1	Recombinant plasmid containing 1.1-kb *Sna*BI internal fragment of bla_{PER-1}	31
pPL1	2.6-kb *Sau*3AI fragment from *P. aeruginosa* Mus cloned into pBK-CMV	This study

[a] IPSC, Institut Pasteur strain collection.

TEM-42 (26) is an extended-spectrum β-lactamase derived from TEM-2 by four point mutations. PER-1 (30, 31), on the other hand, is an extended-spectrum class A β-lactamase not derived from any other known enzymes. PER-1 activity is inhibited by clavulanic acid. Oxacillin-hydrolyzing extended-spectrum β-lactamases have been described only for isolated strains, whereas PER-1 seems to be endemic in Turkey, where it has been found in 14 different *P. aeruginosa* strains (6) as well as in *Salmonella typhimurium* (45).

Here, we describe a chromosomally encoded extended-spectrum β-lactamase from a clinical isolate of *P. aeruginosa*. We analyze the gene of this enzyme by cloning and sequencing and compare its sequence with those of other class D β-lactamase genes. We determine the enzymatic properties of the β-lactamase and attempt to characterize its genetic determinant. This new class D β-lactamase has moderate hydrolysis activity for oxacillin and higher activity against extended-spectrum cephalosporins. Its activity is inhibited by clavulanic acid, sulbactam, tazobactam, and imipenem.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this work are listed in Table 1. *P. aeruginosa* Mus was isolated in 1995 at Hôpital Saint-Antoine, Paris, France, from a biliar drain of a hospitalized patient from Sicily, Italy. Before strain isolation, the patient had received an empiric antibiotic treatment consisting of a combination of amoxicillin, ceftazidime, and fluconazole. The strain was identified with an API-20 NE gallery (Biomérieux, Marcy l'Etoile, France) and belonged to serogroup P:11 (antisera from Diagnostics Pasteur, Marnes-La-Coquette, France).

Antimicrobial agents and MIC determinations. The antimicrobial agents used in this study were obtained from standard laboratory powders and were used immediately after their solubilization. The agents and their sources were as follows: amoxicillin, clavulanic acid, cloxacillin, and ticarcillin, Smith-Kline French-Beecham (Nanterre, France); aztreonam and cefepime, Bristol-Myers Squibb (Paris La Défense, France); ceftazidime, Glaxo (Paris, France); cefamandole, cephalothin, and moxalactam, Eli Lilly (Saint-Cloud, France); piperacillin and tazobactam, Lederle (Oullins, France); sulbactam, Pfizer (Orsay, France); cefotaxime and cefpirome, Hoechst-Roussel (Paris, France); and cefoxitin and imipenem, Merck Sharp & Dohme-Chibret (Paris, France).

MICs were determined by an agar dilution technique on Mueller-Hinton agar (Diagnostics Pasteur) with a Steers multiple inoculator and an inoculum of 10^4 CFU. All plates were incubated at 37°C for 18 h. MICs of β-lactams were determined alone or in combination with a fixed concentration of 2 μg of clavulanic acid per ml.

Hybridization. DNA-DNA hybridizations were performed as described by Maniatis et al. (21). Three microliters of a culture of *P. aeruginosa* Mus was put on a nitrocellulose membrane (Hybond; Amersham, Les Ulis, France) lying on a Mueller-Hinton agar plate. The plate was incubated for 18 h at 37°C. The membrane was treated first with a 10% sodium dodecyl sulfate (SDS) solution (for bacterial lysis), second with a solution containing 0.5 N NaOH and 1.5 M NaCl (for DNA denaturation), and finally with a solution containing 1.5 N NaCl and 0.5 M Tris-HCl (pH 7.5) (for DNA neutralization). The membrane was washed with 1× SSC solution (0.15 M NaCl plus 0.015 M sodium citrate; Bioprobe Systems, Montreuil sous Bois, France) and dried at 80°C. Hybridizations were performed at 65°C.

The probes consisted of the 1.1-kb *Sna*BI fragment from recombinant plasmid pPZ1 for bla_{PER-1}, the 450-bp *Pst*I-*Not*I fragment from recombinant plasmid pHUC37 for bla_{SHV-3}, and the 560-bp *Ssp*I-*Pst*I fragment from plasmid pBR322 for bla_{TEM-1}. The DNA probe was labeled with [α-^{32}P]dATP with a random primer DNA labeling kit (Bio-Rad, Ivry sur Seine, France).

Plasmid content and mating-out assays. Plasmid DNA of *P. aeruginosa* Mus was prepared by four different methods as described by Danel et al. (6), Hansen and Olsen (14), Kado and Liu (18), and Takahashi and Nagano (42). Plasmid DNA was detected by electrophoresis on a 0.8% agarose gel (Life Technologies, Eragny, France) containing 0.25 μg of ethidium bromide (Pharmacia Biotech, Orsay, France) per ml. Standard sizes of plasmid DNAs were extracted from *Escherichia coli* NCTC 50192.

Direct transfer of resistance into in vitro-obtained ciprofloxacin-resistant *P. aeruginosa* PU21, *E. coli* JM109, or *Aeromonas hydrophila* 76-14 was attempted by liquid and solid mating-out assays at 30 and 37°C. Transconjugant selection was performed on Trypticase soy agar plates (Diagnostics Pasteur) containing ciprofloxacin (3 μg/ml) and either ceftazidime (10 μg/ml) or ticarcillin (150 μg/ml).

Cloning experiments and analysis of recombinant plasmids. Genomic DNA of *P. aeruginosa* Mus was extracted as described previously (21). Fragments from *Sau*3AI (Pharmacia Biotech) partially digested genomic DNA were ligated into the *Bam*HI (Pharmacia Biotech) site of phagemid pBK-CMV (Stratagene, La Jolla, Calif.). Ligation was performed at a 1:1 vector-to-insert ratio at a final concentration of 200 ng of DNA in a ligation mixture containing 1 U of T4 DNA ligase (Amersham) at 16°C for 18 h. Recombinant plasmids were transformed by electroporation (Bio-Rad gene pulser II) into *E. coli* JM109 electrocompetent cells (Bio-Rad). Antibiotic-resistant colonies were selected on Trypticase soy agar plates containing, per milliliter, 50 μg of amoxicillin, 5 μg of ceftazidime, or 30 μg of kanamycin.

Recombinant plasmid DNAs were obtained from 500-ml Trypticase soy broth cultures grown overnight with amoxicillin (100 μg/ml) at 37°C. The plasmid DNAs were prepared with Qiagen columns (Coger, Paris, France). Plasmid mapping was performed after double restriction analysis. Fragment sizes were estimated according to the molecular weight standard 1-kb DNA ladder (Life Technologies).

β-Lactamase preparation. Cultures were grown overnight at 37°C in 20 ml of Trypticase soy broth with amoxicillin, 100 μg/ml. Bacterial suspensions were disrupted by sonication (twice for 30 s at 20 Hz with a Vibra Cell 300 phospholyser; Bioblock, Illkirch, France) and centrifuged (30 min, 10,000 × *g*, 4°C). The supernatant containing the crude enzyme extracts was used for molecular mass determination and isoelectric focusing.

Isoelectric focusing. Crude β-lactamase extracts were subjected to analytical isoelectric focusing on a pH-3.5 to -9.5 ampholine polyacrylamide gel (Ampholin PAG plate; Pharmacia Biotech) for 90 min at a constant power of 1,500 V (50 mA, 30 W). The focused β-lactamases were detected by overlaying the gel with 1 mM nitrocefin (Glaxo, Paris, France) in 100 mM phosphate buffer (pH 7.0). The pI values were determined and compared to those from known β-lactamases, i.e., TEM-1, 5.4; TEM-2, 5.6; SHV-3, 7.0; SHV-5, 8.2 (2).

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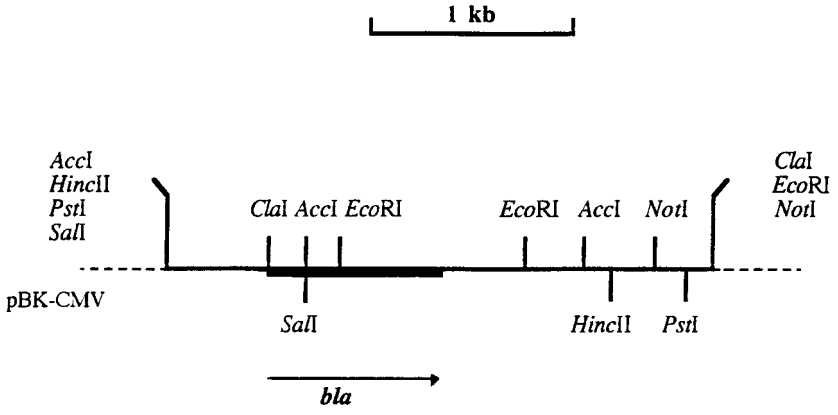

FIG. 1. Restriction endonuclease map of the recombinant plasmid pPL1, which codes for the extended-spectrum β-lactamase from the *P. aeruginosa* Mus clinical strain. The thin line represents the cloned insert from *P. aeruginosa* Mus, the broken lines indicate vector pBK-CMV, and the thick line represents the studied gene, with the arrow indicating its translational orientation.

β-Lactamase purification. Overnight cultures of *E. coli* JM109 harboring recombinant plasmid pPL1, grown at 37°C in Trypticase soy broth with amoxicillin, 100 μg/ml, were diluted 100-fold in the same medium, prewarmed at 37°C, and then incubated for 18 h at 37°C under vigourous shaking. Five hundred milliliters of culture was harvested by centrifugation, and the pellet was resuspended in 20 ml of 20 mM Tris-HCl buffer (pH 7.6). The resulting suspension was disrupted by sonication (four times for 2 min at 20 Hz) and was subsequently clarified by centrifugation at 20,000 × *g* for 15 min at 4°C. The residual nucleic acids in the supernatant were precipitated by 7% (vol/vol) spermin (Sigma, Saint Quentin Fallavier, France) for 15 min on ice. This suspension was ultracentrifuged at 35,000 × *g* for 30 min at 4°C. The supernatant was then dialyzed overnight in 20 mM Tris-HCl (pH 7.6). The β-lactamase was first purified by anion-exchange column chromatography (Q-Sepharose fast-flow; Pharmacia Biotech) equilibrated with the same buffer. The active fractions were then pooled and applied to a gel filtration column (Superose 12 HR 10/30; Pharmacia Biotech) equilibrated with the same buffer. At each step, fractions containing the enzyme were identified with nitrocefin hydrolysis and electrophoresis on an SDS–12% polyacrylamide gel. Finally, the purest fractions were pooled and stored at −20°C.

β-Lactamase activity and determination of kinetic constants. The β-lactamase activity present in the crude extracts of *E. coli* JM109 harboring recombinant plasmid pPL1 was assayed by UV spectrophotometry (Uvikon 940; Kontron Instruments, Paris, France) at 30°C in 100 mM phosphate buffer (pH 7.0) (23). The following wavelengths were used, according to Matagne et al. (23): for ampicillin and ticarcillin, 235 nm; for aztreonam, 318 nm; for benzylpenicillin, 232 nm; for cefotaxime, cefsulodin, and ceftazidime, 260 nm; for cefepime, 264 nm; for cefoxitin, 265 nm; for cephaloridin, 255 nm; for cephalothin, 262 nm; for cloxacillin, 230 nm; for imipenem, 297 nm; and for oxacillin, 263 nm. Antibiotic solutions were freshly prepared in 100 mM phosphate buffer (pH 7.0). Kinetic parameters were derived from the initial velocity obtained with four to six substrate concentrations. K_m values were determined according to the Eadie-Hofstee representation [$K_i = f(V_i/S)$, where V_i is the initial velocity and S is the substrate concentration]. V_{max} values were expressed relative to that of benzylpenicillin, which was set at 100.

The kinetic parameters (V_{max}, K_m) of oxacillin and cloxacillin (as recommended for group 2d β-lactamases [2]) were determined according to the same protocol with a partially purified β-lactamase.

Enzyme inhibition was studied with cephaloridine (100 μM) as the substrate. Inhibitor, at various concentrations, was preincubated with enzyme for 3 min at 30°C before addition of the substrate. The inhibitor concentration required to inhibit 50% of enzyme activity was determined graphically. Five potential inhibitors were tested: clavulanic acid, EDTA, imipenem, sulbactam, and tazobactam.

Determination of relative molecular mass. The relative molecular mass of the β-lactamase obtained from *E. coli* JM109 harboring recombinant plasmid pPL1 was estimated by SDS-polyacrylamide gel electrophoresis (PAGE) analysis. Crude extracts and marker proteins were boiled for 10 min in a 1% SDS–3% mercaptoethanol solution and then subjected to electrophoresis on a 12% gel (200 V for 4 h at room temperature). Renaturation of β-lactamase activity after denaturing electrophoresis was performed as described previously (22).

DNA sequencing and protein analysis. The 2.6-kb cloned DNA fragment from pPL1 was sequenced on both strands with an Applied Biosystems sequencer (model ABI 377). The nucleotide sequence and the deduced protein sequence were analyzed with the Genetics Computer Group software package (Biotechnology Center, University of Wisconsin—Madison, Madison, Wis.). Multiple sequence alignment of deduced peptide sequences was carried out with the Genetics Computer Group program Pileup, which uses a simplification of the progressive alignment method of Feng and Doolittle (11). Among the known class D β-lactamases, ten were compared to OXA-18: OXA-1 and OXA-7 from *E. coli* (32, 38); OXA-2 from *S. typhimurium* (5); OXA-5, OXA-10, OXA-11, OXA-15, and LCR-1 from *P. aeruginosa* (4, 10, 12, 15); OXA-9 from *K. pneumoniae* (44); and OXA-12 (Asb1) from *Aeromonas sobria* (35). A dendrogram was derived from the multiple sequence alignment by a parsimony method using the phylogeny package PAUP, version 3.0 (41).

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper will appear in the GenBank nucleotide database under accession no. U85514.

RESULTS

Preliminary hybridizations. The bla_{TEM-1}, bla_{SHV-3}, and bla_{PER-1} probes gave no positive signal, indicating that *P. aeruginosa* Mus might possess an extended-spectrum β-lactamase gene not structurally related to these β-lactamase genes (data not shown).

Cloning of the extended-spectrum β-lactamase gene. Total DNA from *P. aeruginosa* Mus was partially digested with restriction endonuclease *Sau*3AI and ligated to *Bam*HI-digested plasmid pBK-CMV. The ligation product was transformed into *E. coli* JM109 by electroporation. Recombinant strains were selected on Trypticase soy agar plates with either amoxicillin (50 μg/ml), ceftazidime (5 μg/ml), or kanamycin (30 μg/ml). About one hundred recombinant colonies expressing one of the following resistance phenotypes were obtained: (i) a high level of amoxicillin and ticarcillin resistance which was inhibited by clavulanic acid or (ii) additional resistance to ceftazidime, aztreonam, and cephalosporins, with a marked synergistic effect with clavulanic acid. The recombinant plasmids expressing the extended-spectrum β-lactamase resistance phenotype were extracted and analyzed. The insert sizes were estimated to be between 2.6 and 20 kb. A detailed restriction map was generated for the plasmid containing the 2.6-kb insert (pPL1) (Fig. 1).

Antibiotic susceptibility. The MICs of β-lactams revealed high resistance of *P. aeruginosa* Mus to penicillins, broad-spectrum cephalosporins, and extended-spectrum cephalosporins (Table 2). MICs of β-lactams for *E. coli* JM109 harboring recombinant plasmid pPL1 indicated resistance to penicillins and to extended-spectrum cephalosporins at various levels. MICs of aztreonam and ceftazidime were markedly higher than those of cefotaxime and cephalothin. The MIC of moxalactam was slightly increased, while those of cefoxitin and imipenem were not. All β-lactams tested, except imipenem and cefoxitin, had decreased MICs in the presence of clavulanic

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TABLE 2. MICs of β-lactams for *P. aeruginosa* Mus, *E. coli* JM109 harboring recombinant plasmid pPL1, and reference strain *E. coli* JM109

Antibiotic(s)	MIC (μg/ml) for:		
	P. aeruginosa Mus	*E. coli* JM109(pPL1)[a]	*E. coli* JM109
Amoxicillin	>512	32	2
Amoxicillin-Cla[b]	>512	4	2
Ticarcillin	256	128	2
Ticarcillin-Cla	64	8	1
Piperacillin	64	16	1
Piperacillin-Cla	32	1	0.5
Cephalothin	>1,024	8	4
Cephalothin-Cla	>512	4	2
Cefamandole	>512	4	1
Cefamandole-Cla	>512	1	1
Cefoxitin	>1,024	8	8
Cefoxitin-Cla	>512	8	8
Ceftazidime	128	64	0.25
Ceftazidime-Cla	8	0.5	0.25
Cefotaxime	128	2	0.06
Cefotaxime-Cla	8	0.06	0.06
Cefepime	16	1	0.06
Cefepime-Cla	4	0.03	0.06
Cefpirome	32	0.5	0.125
Cefpirome-Cla	8	0.06	0.06
Moxalactam	64	1	0.25
Moxalactam-Cla	64	0.25	0.125
Aztreonam	256	64	0.125
Aztreonam-Cla	16	0.25	0.06
Imipenem	8	0.06	0.06
Imipenem-Cla	8	0.06	0.06

[a] *E. coli* JM109 harboring recombinant plasmid pPL1 produced the extended-spectrum β-lactamase.
[b] Cla, clavulanic acid at a fixed concentration of 2 μg/ml.

acid. This result was more obvious in *E. coli* JM109 harboring pPL1 than in *P. aeruginosa* Mus.

Isoelectric focusing. Analytical isoelectric focusing revealed that *P. aeruginosa* Mus had three distinct β-lactamase activities, of pIs 5.5, 7.4, and 8.2 (data not shown). *E. coli* JM109 harboring recombinant plasmid pPL1 had only one β-lactamase activity, of pI 5.5. The *E. coli* JM109 recombinant strain expressing the highest level of amoxicillin and ticarcillin resistance had a β-lactamase of pI 7.4 (not studied in this work). The last band, of pI 8.2, in *P. aeruginosa* Mus crude extract was possibly an AmpC-type β-lactamase.

Molecular mass. The relative molecular mass of the cloned β-lactamase from *E. coli* JM109 harboring pPL1 was estimated to be 30.6 kDa (Fig. 2).

Sequence analysis of the *P. aeruginosa* β-lactamase gene. The 2.6-kb cloned DNA fragment was entirely sequenced on both strands. Analysis of this insert for coding regions revealed a sufficiently large open reading frame of 830 bp encoding a 275-amino-acid protein of approximately 30.6 kDa in size. The DNA sequence of this gene, along with some flanking sequences, is shown in Fig. 3. Within this protein, a serine-threonine-phenylalanine-lysine tetrad (S-T-F-K) was found at positions 65 to 68; it included the conserved serine and lysine residues characteristic of β-lactamases possessing a serine active site (17). Four structural elements characteristic of class D β-lactamases were found: tyrosine-glycine-asparagine (Y-G-N) at positions 140 to 142, Q-X-X-F-L at positions 171 to 175, E-X-X-L-X at positions 187 to 191, and lysine-threonine-arginine (K-T-G) at positions 210 to 212. A putative ATG initiation codon was found at nucleotides 150 to 152 (Fig. 3). The open reading frame was preceded by a −10 (54 to 59) region and a −35 (26 to 35) region consistent with a putative *P. aeruginosa* promoter (Fig. 3). This promoter may fit the consensus sequences YTGCTTR and RRNTGGGCAT and may thus belong to the *rpoN* promoter family of *P. aeruginosa* (36).

The overall GC content of this gene was 61.2%, which lies within the expected range of GC content (60.1 to 69.5%) for *P. aeruginosa* genes (except for pilin genes) (46). Moreover, the pattern of codon usage was typical of that of *P. aeruginosa* genes (data not shown). Usually, *P. aeruginosa* genes exhibit a strong bias for cytosine and guanine in the wobble position; NNC codons are used 54.5% of the time, NNG codons are used 34.2% of the time, NNT codons are used 6% of the time, and NNA codons are used 5.3% of the time. In the sequence here presented, the corresponding values were 42.4, 35.9, 13.4, and 8.33%, respectively. The translation stop codon (TAG), found at positions 975 to 977, corresponds to that usually found in *P. aeruginosa* genes.

Homology with other β-lactamases. The nucleotide sequence of this structural gene has about 25% amino acid identity with OXA-2, OXA-5, OXA-10 (PSE-2), OXA-7, OXA-11, and LCR-1. OXA-9 and OXA-12 were the two oxacillin-hydrolyzing β-lactamases with the highest percentages of identity (45 and 42%, respectively). The enzyme is a novel class D β-lactamase and thus was named OXA-18.

A dendrogram was constructed to relate OXA-18 to 10 other class D β-lactamases (Fig. 4). OXA-18 was mostly related to OXA-9 and OXA-12 and, to a lesser extent, to OXA-1 (33% amino acid identity).

OXA-18 β-lactamase activities. Kinetic parameters of the OXA-18 β-lactamase obtained with a crude extract of *E. coli* JM109 harboring recombinant plasmid pPL1 showed that the enzyme had strong activity against ticarcillin and extended-spectrum cephalosporins (Table 3). Surprisingly, both the V_{max} and the K_m values of cephalosporins and particularly ceftazidime were high. Aztreonam was highly hydrolyzed. Hydrolysis of imipenem and cefoxitin was not detected. Oxacillin and cloxacillin hydrolysis was too slow to be accurately determined with the OXA-18 crude extract. Therefore, an OXA-18 partially purified β-lactamase preparation was used for the determination of the kinetic parameters of these β-lactams (see results below).

The results of the determination of the minimum inhibitor concentration required to inhibit 50% of enzyme activity, with cephaloridine (100 μM) as the substrate, were as follows: clavulanic acid, 0.08 μM; imipenem, 0.01 μM; sulbactam, 0.56 μM; tazobactam, 0.13 μM. Clavulanic acid and imipenem were the best inhibitors. Activity of the enzyme was not inhibited by EDTA.

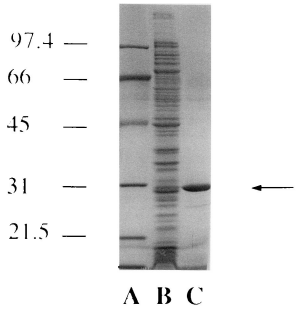

FIG. 2. SDS-PAGE. Lane A, protein size marker (in kilodaltons) as indicated on the left; lane B, crude extract of *E. coli* JM109 harboring pPL1, which produces extended-spectrum β-lactamase; lane C, partially purified OXA-18 β-lactamase (indicated by the arrow).

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```
1     AGACCAGTCACTGGCGAATGCATCACAACTGGGCAAAGGCTTGGGAACCAAGCTGGTTCGAGCACTGGTTGAGTTGCTGTTCAATGATCCCGAGGTCAGA   100
                                  -35                    -10
101   TATCATCGTTTGTCGGACCCGGCAAGCTGAGCTAGGACTTGGGACATCGATGCAACGGAGCCTGTCCATGAGCGGAAAAAGACATTTCATCTTTGCAGTA   200
                                                        M  Q  R  S  L  S  M  S  G  K  R  H  F  I  F  A  V    17
201   TCATTTGTTATTTCAACGGTTTGCCTTACGTTCTCCCCGGCAAATGCCGCACAAAAACTGTCCTGCACGCTTGTTATCGACGAGGCGAGCGGCGACCTGC   300
       S  F  V  I  S  T  V  C  L  T  F  S  P  A  N  A  A  Q  K  L  S  C  T  L  V  I  D  E  A  S  G  D  L    50
301   TGCACCGGGAAGGCAGTTGCGACAAGGCTTTTGCGCCGATGTCGACGTTCAAACTGCCTTTGGCCATCATGGGCTACGATGCCGATATCCTGCTCGACGC   400
      L  H  R  E  G  S  C  D  K  A  F  A  P  M  S  T  F  K  L  P  L  A  I  M  G  Y  D  A  D  I  L  L  D  A    84
401   CACCACGCCGCGCTGGGATTACAAGCCGGAATTCAACGGCTACAAATCGCAGCAGAAGCCGACCGATCCGACCATCTGGCTGAAGGATTCCATCGTCTGG   500
        T  T  P  R  W  D  Y  K  P  E  F  N  G  Y  K  S  Q  Q  K  P  T  D  P  T  I  W  L  K  D  S  I  V  W   117
501   TATTCGCAGGAGCTGACGCGCCGCCTCGGCGAAAGCCGCTTTTCCGATTACGTGCAGCGCTTCGATTACGGCAACAAGGATGTTTCCGGCGATCCCGGCA   600
       Y  S  Q  E  L  T  R  R  L  G  E  S  R  F  S  D  Y  V  Q  R  F  D  Y  G  N  K  D  V  S  G  D  P  G   150
601   AGCATAACGGCCTGACCCATGCCTGGCTCGCCTCGTCGCTGAAGATCTCGCCGGAGGAGCAGGTGCGTTTCCTGCGTCGTTTCCTGCGCGGCGAATTGCC   700
      K  H  N  G  L  T  H  A  W  L  A  S  S  L  K  I  S  P  E  E  Q  V  R  F  L  R  R  F  L  R  G  E  L  P   184
701   GGTCTCCGAGGACGCGTTGGAGATGACGAAAGCCGTCGTGCCGCATTTCGAGGCCGGCGATTGGGACGTGCAGGGCAAGACCGGCACCGGTTCGCTTTCC   800
       V  S  E  D  A  L  E  M  T  K  A  V  V  P  H  F  E  A  G  D  W  D  V  Q  G  K  T  G  T  G  S  L  S   217
801   GATGCCAAGGGCGGCAAGGCGCCGATCGGCTGGTTCATCGGCTGGGCGACACGCGACGACCGCCGCGTCGTCTTCGCCCGCCTAACGGTCGGGGCGAGGA   900
       D  A  K  G  G  K  A  P  I  G  W  F  I  G  W  A  T  R  D  D  R  R  V  V  F  A  R  L  T  V  G  A  R   250
901   AGGGCGAGCAGCCGGCCGGGCCCGCCGCTCGCGACGAGTTCCTCAACACCCTGCCGGCCCTGTCGGAAAACTTCTGACCAGTCAGGCCGTCCACTGTGTG  1000
      K  G  E  Q  P  A  G  P  A  A  R  D  E  F  L  N  T  L  P  A  L  S  E  N  F                            275
1001  ATGTCGTCGCGCTCGCCGATGAGCGCGAGACCATGGGCGATGGACAGCAATTCGCCGCCGCTTTCGATGCGGTCGGCATCGAAGCGCTGGGTGAACAGGC  1100
1101  GGCGCACCGCCGGCACGAAGGAGGTGCCGCCGGTCAGGAACACCTTGTCGATATCCCCCGCCTTGCTGTCGGTTTTTGCGAGGAT                1185
```

FIG. 3. Nucleotide sequence of the 1,185-bp fragment of pPL1 containing the β-lactamase coding region. The deduced amino acid sequence is shown in single-letter code below the nucleotide sequence. The putative promoter sequence is represented by −10 and −35 regions. The promoter sequences, the start and stop codons, and the five structural elements characteristic of class D β-lactamases are underlined.

β-Lactamase purification and assay. A partially purified β-lactamase was obtained in two steps (as described in Materials and Methods). The fraction with β-lactamase activity obtained from the gel filtration column was analyzed by SDS-PAGE. A major band at 30.6 kDa and some minor bands were observed (Fig. 2), and the protein's purity was estimated to be 90%. The final protein concentration was 7.65 mg/ml. With this β-lactamase preparation, cloxacillin was better hydrolyzed than oxacillin (Table 4). This last β-lactam had a particularly slow V_{max} (relative to that of benzylpenicillin) for an oxacillinase.

Plasmid DNA and transfer of resistance. No plasmid was detected in *P. aeruginosa* Mus. Direct mating-out experiments failed to transfer the β-lactam resistance marker into *P. aeruginosa* PU21, *E. coli* JM109, or *A. hydrophila* 76-14. Therefore, bla_{OXA-18} seems to be chromosomally located.

DISCUSSION

A clinical isolate of *P. aeruginosa* Mus presented an extended-spectrum resistance phenotype with a marked synergistic effect between clavulanic acid and ceftazidime or aztreonam. The β-lactamase gene coding for the observed phenotype was not derived from previously known extended-spectrum β-lactamases. The GC content, codon usage, and identified promoter suggested that it is very likely of a pseudomonad origin (36, 46). The deduced amino acid sequence belongs to Ambler class D β-lactamases, since an active-site serine and three other structural elements characteristic of class D β-lactamases (17) were found. Protein alignments with other class D β-lactamase amino acid sequences showed that this extended-spectrum β-lactamase had the greatest percentage of sequence identity with two restricted-spectrum oxacillinases, OXA-9 (45%) and OXA-12 (42%) (35, 44). Based on its genetic characteristics, we proposed to name this enzyme OXA-18. It is not a simple point mutation derivative from TEM-1 or SHV-3 enzymes, nor from any oxacillinase already described, as was

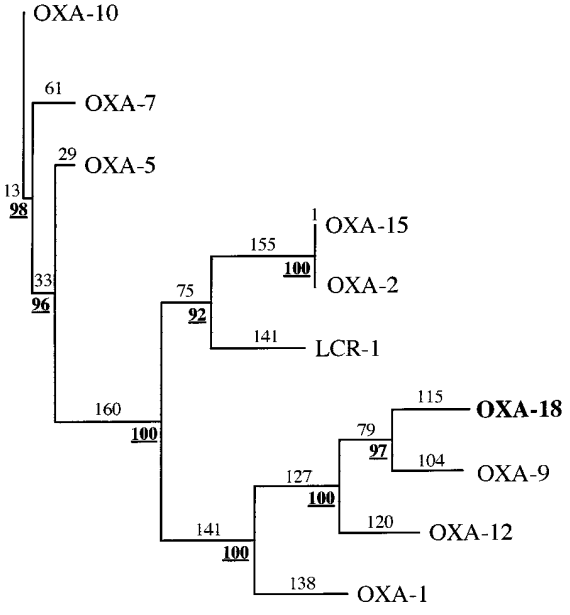

FIG. 4. Dendrogram of 10 class D β-lactamases according to parsimony (41). Branch lengths (lightface numbers) are to scale and proportional to the number of amino acid changes. The boldface numbers at branching points are the numbers of times a particular node was found in 100 bootstrap replications. Distances along the vertical axes have no significance.

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TABLE 3. Kinetic parameters of various β-lactam antibiotics for the OXA-18 β-lactamase

Substrate	V_{max}[a]	K_m (μM)	Ratio of V_{max}/K_m
Ampicillin	63	1.3	48.5
Aztreonam	63	3	21
Benzylpenicillin	100	2.5	40
Cefepime	160	64	2.5
Cefotaxime	470	23	20.5
Cefoxitin	<0.1[b]	ND[c]	ND
Cefsulodine	261	87	3
Ceftazidime	808	499	1.6
Cephaloridine	80	132	0.6
Cephalothin	81	26.5	3
Imipenem	<0.1	ND	ND
Ticarcillin	229	12	19

[a] V_{max} values are relative to that of benzylpenicillin, which was set at 100.
[b] <0.1, nondetectable hydrolysis.
[c] ND, not determined.

the case for OXA-17 (8), OXA-16 (9), OXA-14 (7), and OXA-11 (12), derived from OXA-10 (PSE-2), or OXA-15, derived from OXA-2 (10). OXA-14 is an intermediate between OXA-10 and OXA-11. It seems that an aspartate instead of a glycine at position 157 in the amino acid sequences of these two enzymes is critical for the extended-spectrum activity of OXA-10-related class D β-lactamases. It is suggested that the novel OXA-10 (PSE-2) variants have an altered three-dimensional structure; this may change the surface exposure of the charged group (12) and therefore may confer extended-spectrum cephalosporin resistance. The same replacement occurred in an extended-spectrum laboratory mutant of OXA-13 (25a, 27), which itself has 96% amino acid homology with OXA-10. Such a mutation is not found in the OXA-18 amino acid sequence. It is difficult to anticipate the critical positions in the OXA-18 amino acid sequence responsible for extended-spectrum cephalosporin hydrolysis and hydrolytic activity inhibition by clavulanic acid. Unfortunately, no crystal structure is yet available for a prototype class D β-lactamase, and computer-assisted modelling is limited by low homology between class D and class A β-lactamases.

OXA-18 confers high-level resistance to amoxicillin, ticarcillin, piperacillin, cefotaxime, ceftazidime, and aztreonam. Like TEM and SHV derivatives and oxacillinases (33), production of OXA-18 enzyme affects the MICs of neither cephamycin nor imipenem for *E. coli* JM109 harboring recombinant plasmids. Uncommonly for an extended-spectrum β-lactamase and for an oxacillin-hydrolyzing enzyme, the MIC of moxalactam is slightly increased for *E. coli* JM109 expressing OXA-18. Penicillin, cephalosporin, and aztreonam resistances are reversed by clavulanic acid at a concentration of 2 μg/ml. Such an inhibition has already been observed among oxacillin-hydrolyzing enzymes with OXA-12 (35), a restricted-spectrum oxacillinase described for *A. sobria* (an enzyme presenting one of the highest percentages of identity with OXA-18). Like OXA-12 and clavulanic acid-inhibited extended-spectrum β-lactamase (16, 35), OXA-18 activity is inhibited by tazobactam and sulbactam less efficiently than by clavulanic acid. Similar to a currently unnamed chromosomally encoded oxacillinase of pI 8.0 (28), OXA-18 activity is inhibited by imipenem. The mechanism of this inhibition is not well established, but the property of imipenem acting as an inhibitor has been already described for class A β-lactamases such as *Bacillus cereus* (24) and PER-1 (31).

From a biochemical point of view, the OXA-18 hydrolytic properties are atypical for a class D β-lactamase. OXA-18 hydrolyzes cloxacillin faster than benzylpenicillin. Nevertheless, unlike all functional group 2d β-lactamases except OXA-9 (2), OXA-18 hydrolyzes oxacillin slower than benzylpenicillin. Moreover, as is well known for carbenicillin-hydrolyzing β-lactamases but not for oxacillin-hydrolyzing enzyme, ticarcillin is hydrolyzed twofold faster than benzylpenicillin. OXA-18 is able to hydrolyze extended-spectrum cephalosporins and aztreonam. Among β-lactamases described for *P. aeruginosa* species, high V_{max} values for ceftazidime, cefotaxime, or aztreonam have only been reported with PER-1 class A β-lactamase (31). Among extended-spectrum class D β-lactamases in *P. aeruginosa*, OXA-11 (12) and OXA-14 (7), which are poorly inhibited by clavulanic acid, exhibit lower V_{max} values for the same β-lactams than those obtained with OXA-18.

The mechanism of bla_{OXA-18} gene insertion into *P. aeruginosa* is unknown. In *P. aeruginosa* Mus, no plasmid was found to carry the bla_{OXA-18} gene. Transposition has been extensively described for *P. aeruginosa* as a source of genetic plasticity. Most of the genes encoding oxacillin-hydrolyzing β-lactamase and carbenicillin-hydrolyzing β-lactamase isolated from *P. aeruginosa* have been described as parts of transposons, such as Tn*21* (19), Tn*3* (43), or Tn*7* (25). All of the oxacillinase genes identified so far, except that of OXA-11, are located on the variable region of integrons (13, 39). The inserted genes are flanked at their 5′ ends by the motif GTTPuPu and in their 3′ ends by an imperfect inverted repeat of 59 bp (3). None of these sequences have been found in the 2.6-kb cloned fragment, so bla_{OXA-18} may not be part of a cassette in an integron, as are the other known oxacillinase genes except that of OXA-11. These β-lactam resistance genes are usually associated with sulfonamide, aminoglycoside, and mercury resistance genes which may be present in *P. aeruginosa* Mus as suggested by phenotypic analysis of its antibiotic resistance pattern (data not shown). Some recombinant clones expressing extended-spectrum resistance phenotypes were resistant to tobramycin or streptomycin. Those recombinant clones may harbor inserts containing the bla_{OXA-18} gene associated with other resistance genes. Their study will be the purpose of further work.

In summary, this study describes the first extended-spectrum class D β-lactamase fully inhibited by clavulanic acid. This novel enzyme has peculiar properties for an oxacillin-hydrolyzing enzyme, such as (i) slow oxacillin hydrolysis compared to hydrolysis of benzylpenicillin or cloxacillin and (ii) high maximal velocity of ticarcillin hydrolysis. OXA-18 could be the first member of a novel subgroup, named 2d′, related to other group 2d β-lactamases but with extended-spectrum hydrolytic properties and full inhibition by clavulanic acid. Further work will evaluate the potential presence of a transposon containing the OXA-18 gene along with other resistance genes such as aminoglycoside or sulfonamide resistance genes. Furthermore, characterization of the penicillinase-type gene encoding a pI-7.4 β-lactamase also present in the same *P. aeruginosa* strain will be performed to determine if this β-lactamase and OXA-18 are derived from the same ancestor. From a thera-

TABLE 4. Kinetic parameters of oxacillin and cloxacillin β-lactams for partially purified OXA-18 β-lactamase

Substrate	V_{max}[a]	K_m (μM)	Ratio of V_{max}/K_m
Benzylpenicillin	100	1.6	62.5
Cloxacillin	150	9	16
Oxacillin	36	12	3

[a] V_{max} values are relative to benzylpenicillin, which was set at 100.

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peutic point of view, this study emphasizes the fact that other β-lactamases besides the common cephalosporinase found in *P. aeruginosa* may lead to failure of therapeutic regimens which include extended-spectrum cephalosporins. *P. aeruginosa* may, like the *Enterobacteriaceae*, constitute a reservoir of extended-spectrum β-lactamase genes since OXA-18 is, for this species, the third (after PER-1 and TEM-42 [31, 26]) described clavulanic acid-inhibited enzyme.

ACKNOWLEDGMENTS

We thank V. Jarlier, in whose laboratory part of this work was performed, W. Sougakoff, for precious advice, J. C. Petit, in whose laboratory *P. aeruginosa* Mus was isolated, and F. Danel, for confirmatory experiments.

L. N. Philippon was a recipient of a grant from the Fondation pour la Recherche Médicale, France. This work was financed by a grant from the Faculté de Médecine Paris-Sud, Université Paris XI, Le Kremlin-Bicêtre, France.

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