ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Jan. 2005, p. 202–208 Vol. 49, No. 1
0066-4804/05/$08.00+0 doi:10.1128/AAC.49.1.202–208.2005

OXA-58, a Novel Class D β-Lactamase Involved in Resistance to Carbapenems in *Acinetobacter baumannii*

Laurent Poirel,[1] Sophie Marqué,[1] Claire Héritier,[1] Christine Segonds,[2] Gérard Chabanon,[2] and Patrice Nordmann[1]*

Service de Bactériologie-Virologie, Université Paris XI, Hôpital de Bicêtre, Assistance Publique/Hôpitaux de Paris, Faculté de Médecine Paris-Sud, Le Kremlin-Bicêtre,[1] and Laboratoire de Bactériologie-Hygiène, CHU Rangeuil, Toulouse,[2] France

Received 1 April 2004/Returned for modification 6 July 2004/Accepted 11 September 2004

A carbapenem-resistant *Acinetobacter baumannii* strain was isolated in Toulouse, France, in 2003. Cloning and expression in *Escherichia coli* identified the carbapenem-hydrolyzing β-lactamase OXA-58, which is weakly related (less than 50% amino acid identity) to other oxacillinases. It hydrolyzed penicillins, oxacillin, and imipenem but not expanded-spectrum cephalosporins. The bla_{OXA-58} gene was located on a ca. 30-kb non-self-transferable plasmid. After electrotransformation in the *A. baumannii* CIP7010^T reference strain, it conferred reduced susceptibility to carbapenems. The bla_{OXA-58} gene was bracketed by two novel IS*Aba3*-like insertion elements. This study describes a newly characterized β-lactamase that may contribute to carbapenem resistance in *A. baumannii*.

Carbapenem resistance in *Acinetobacter baumannii* is observed increasingly in nosocomial isolates, especially in isolates recovered from intensive care units (4). This resistance phenotype is often associated with multidrug resistance, leading to limited choices for treating *A. baumannii* infections. This bacterial species naturally produces a chromosomally encoded cephalosporinase (6) that may be overexpressed due to insertion of IS*Aba1*, which brings promoter sequences necessary for high-level expression of this β-lactamase (12, 36). Carbapenem resistance may be due to a reduced permeability related to porin deficiency and to modification of penicillin-binding protein affinity (10, 17, 39), but recent reports showed that β-lactamase-mediated carbapenem resistance is the most common mechanism (29). Only a few instances of metallo-β-lactamase have been described for *A. baumannii* (9, 15, 33, 37, 40), but identification of several Ambler class D β-lactamases (oxacillinases) has been reported recently (23).

Six oxacillinases with carbapenem-hydrolyzing activity have been sequenced from *A. baumannii*, and these isolates were responsible for nosocomial outbreaks in several cases (5, 14). OXA-23 (also named ARI-1) (16, 25) and OXA-27 (2) have 99% amino acid identity, whereas they share 60% identity with a second group of oxacillinases consisting of OXA-24, -25, -26, and -40, which differ by a few amino acid substitutions (2, 7, 18). In addition, OXA-48 was characterized recently from a *Klebsiella pneumoniae* clinical isolate that hydrolyzed imipenem to a significant extent (32). This plasmid-mediated Ambler class D β-lactamase likely originated from *Shewanella* spp., since it shared 92% amino acid identity with the naturally occurring β-lactamase OXA-54 from *Shewanella oneidensis* (31).

In this study, we have characterized a novel imipenem-hydrolyzing oxacillinase identified in *A. baumannii*.

MATERIALS AND METHODS

Bacterial strains and plasmids. *A. baumannii* clinical isolate MAD was isolated in 2003. It was identified with the API20NE system (bioMérieux, Marcy-l'Etoile, France). *Escherichia coli* reference strain DH10B and plasmid pBK-CMV (Stratagene, Amsterdam, The Netherlands) were used for cloning experiments. *A. baumannii* CIP7010^T (Pasteur Institute, Paris, France) and *E. coli* DH10B were used in transformation and conjugation experiments.

Antimicrobial agents and MIC determinations. The antimicrobial agents and their sources have been referenced elsewhere (26). Antibiotic-containing disks were used for detection of antibiotic susceptibility with Mueller-Hinton agar plates and a disk diffusion assay (Sanofi Diagnostics Pasteur, Marnes-La-Coquette, France). MICs were determined by an agar dilution technique as previously reported (28), and results were interpreted according to the guidelines of the National Committee for Clinical Laboratory Standards (24).

Cloning experiments. Whole-cell DNA of *A. baumannii* MAD was extracted as previously described (26). Cloning experiments were performed with partially Sau3AI-digested DNA of *A. baumannii* MAD and BamHI-restricted plasmid pBK-CMV, followed by expression of recombinant plasmids in *E. coli* DH10B, as described previously (28). Antibiograms were obtained with *E. coli* DH10B harboring recombinant plasmids, and sizes of the plasmid inserts were determined by restriction analysis (34). Recombinant plasmid pOXA-58 was retained for further analysis.

DNA sequencing and protein analysis. Both strands of the cloned DNA fragments were sequenced with an Applied Biosystems sequencer (ABI 3100). The nucleotide and 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).

Plasmid analysis and hybridizations. Extraction of plasmid DNA from *A. baumannii* MAD and from electroporants was attempted by using the Kieser method (20). A Southern transfer was performed on a nylon membrane (Hybond N$^+$; Amersham Pharmacia Biotech, Orsay, France), as described previously (27). The plasmid extract of *A. baumannii* MAD was also used for transformation experiments with a Gene Pulser II electroporator (Bio-Rad, Ivry-sur-Seine, France) and the *A. baumannii* CIP7010^T and *E. coli* DH10B reference strains. Electroporation products were selected on ticarcillin (50 μg/ml)-containing plates.

Mating-out assays were attempted in liquid and solid media with *A. baumannii* MAD as the donor and *A. baumannii* CIP7010^T as the recipient strain. Transconjugants were selected on MH plates containing ticarcillin (100 μg/ml).

The membrane was hybridized with a probe specific for the bla_{OXA-58} gene made of a 528-bp PCR-generated fragment (primers OXA-58A [5′-CGATCAG AATGTTCAAGCGC-3′] and OXA-58B [5′-ACGATTCTCCCCTCTGCGC-

* Corresponding author. Mailing address: Service de Bactériologie-Virologie, Hôpital de Bicêtre, 78 rue du Général Leclerc, 94275 Le Kremlin-Bicêtre Cedex, France. Phone: 33-1-45-21-36-32. Fax: 33-1-45-21-63-40. E-mail: nordmann.patrice@bct.ap-hop-paris.fr.

TABLE 1. MICs of β-lactams for the *A. baumannii* MAD clinical isolate, *E. coli* DH10B harboring recombinant plasmid pOXA-58, *A. baumannii* CIP7010^T harboring natural plasmid pMAD, and the *A. baumannii* CIP7010^T and *E. coli* DH10B reference strains

β-Lactam(s)[a]	MIC (μg/ml) for:				
	A. baumannii MAD	*A. baumannii* CIP7010^T (pMAD)	*A. baumannii* CIP7010^T	*E. coli* DH10B (pOXA-58)	*E. coli* DH10B
Amoxicillin	>512	>512	>512	>512	4
Amoxicillin + CLA	>512	>512	512	128	4
Ticarcillin	>512	>512	4	>512	4
Ticarcillin + CLA	>512	>512	4	256	4
Piperacillin	256	256	4	8	1
Piperacillin + TZB	256	128	4	8	1
Cephalothin	>512	>512	>512	8	2
Cefuroxime	>512	256	256	4	2
Ceftazidime	128	2	2	0.12	0.06
Cefotaxime	32	8	8	0.12	0.12
Cefepime	256	1	1	0.12	0.06
Cefpirome	256	8	2	0.25	0.06
Moxalactam	512	256	32	0.12	0.06
Aztreonam	32	64	64	0.12	0.12
Imipenem	32	2	0.25	0.5	0.06
Meropenem	>64	2	0.25	0.5	0.06

[a] CLA, clavulanic acid at a fixed concentration of 2 μg/ml; TZB, tazobactam at a fixed concentration of 4 μg/ml.

3′]). Southern hybridization was performed with the ECL nonradioactive labeling and detection kit as described by the manufacturer (Amersham Pharmacia Biotech).

IEF analysis. Isoelectric focusing (IEF) analysis was performed with an ampholine polyacrylamide gel (pH 3.5 to 9.5) as described previously (28) with culture extracts of *A. baumannii* MAD and *E. coli* DH10B harboring recombinant plasmid pOXA-58.

β-Lactamase purification. A culture of *E. coli* DH10B harboring recombinant plasmid pOXA-58 that produced OXA-58 was grown overnight at 37°C in 4 liters of Trypticase soy broth containing 100 μg of amoxicillin per ml and 30 μg of kanamycin per ml. The protein extracts obtained were purified as described previously (30). After sonication, the crude extract was treated with DNase and ultracentrifuged at 100,000 × *g*, and then the extracts were filtered through a 0.45-μm-pore-size filter and subjected to further purification steps, including ion-exchange chromatography with Q-Sepharose and 20 mM Tris-HCl buffer (pH 8). The β-lactamase was recovered in the flowthrough, and this partially purified extract was ultrafiltered with a Sartorius (Göttingen, Germany) instrument. The extract was subsequently dialyzed in 20 mM diethanolamine (pH 9.3) and loaded again on the Q-Sepharose column equilibrated with the same buffer. The β-lactamase was retained on the column, and elution was performed with a K_2SO_4 gradient to prevent any inhibition by NaCl. Finally, the fractions containing the highest β-lactamase activity were dialyzed against 100 mM phosphate buffer (pH 7.0). Their purity was estimated by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Kinetic studies. Purified β-lactamase was used for kinetic measurements performed at 30°C in 100 mM sodium phosphate (pH 7.0) (28). The k_{cat} and K_m values were determined by analyzing β-lactam hydrolysis under initial-rate conditions with a UV spectrophotometer, as previously described (28). When biphasic hydrolysis was observed, reaction rates were measured at the steady state. The 50% inhibitory concentrations (IC_{50}) of clavulanic acid, tazobactam, sulbactam, and NaCl were determined as described previously (28). Specific activities of protein extracts and purified β-lactamase from cultures of *E. coli* DH10B(pOXA-58) were determined as described previously (3). The protein content was determined by using the Bio-Rad DC protein assay. β-Lactamase specific activity for imipenem was also determined with culture extracts of 10 ml of TS broth of *A. baumannii* MAD by using UV spectrophotometry and imipenem as described previously (3). One unit of enzyme activity was defined as the activity that hydrolyzed one micromole of imipenem per minute per milligram of protein.

Nucleotide sequence accession number. The nucleotide sequence reported in this paper has been submitted to the EMBL/GenBank nucleotide sequence database under accession number AY570763.

RESULTS

Origin of the *A. baumannii* MAD isolate and preliminary antibiotic susceptibility testing. *A. baumannii* MAD was isolated in August 2003 at the Rangueil University hospital (Toulouse, France) from a skin burn infection of a 24-year-old female. This patient had been treated with imipenem and amikacin. Antibiotic susceptibility testing revealed that this isolate was resistant to most tested β-lactams and was of intermediate susceptibility to aztreonam and imipenem (Table 1). Addition of clavulanic acid and tazobactam did not reduce the MICs of ticarcillin and piperacillin (Table 1). *A. baumannii* MAD was also resistant to fluoroquinolones, chloramphenicol, trimethoprim, and aminoglycosides and was susceptible to sulfonamides, tetracycline, and rifampin (data not shown). Culture extracts of *A. baumannii* MAD analyzed by IEF gave two β-lactamases with pIs of 7.2 and 9.4, the latter corresponding to the naturally produced AmpC of *A. baumannii* (data not shown). To determine whether resistance to carbapenems was at least partially β-lactamase mediated, a preliminary hydrolysis experiment was performed with culture extract of *A. baumannii* MAD and revealed a significant imipenem hydrolysis activity equal to that of OXA-40-producing *A. baumannii* CLA-1 (4.6 mU/mg of protein) (18).

Cloning and sequencing of the β-lactamase. Cloning of Sau3AI-restricted whole-cell DNA of *A. baumannii* MAD into pBK-CMV, followed by expression in *E. coli* DH10B gave *E. coli* DH10B(pOXA-58), expressing an oxacillinase resistance phenotype (pI, 7.2) with a carbapenem-hydrolyzing activity; the phenotype consisted of resistance to most penicillins that was not antagonized by clavulanic acid and a reduced susceptibility to imipenem (Table 1).

DNA sequence analysis of plasmid pOXA-58 identified a 843-bp open reading frame (ORF) for bla_{OXA-58}, encoding a 280-amino-acid protein (Fig. 1). Within the deduced protein encoded by this ORF, a serine-threonine-phenylalanine-lysine

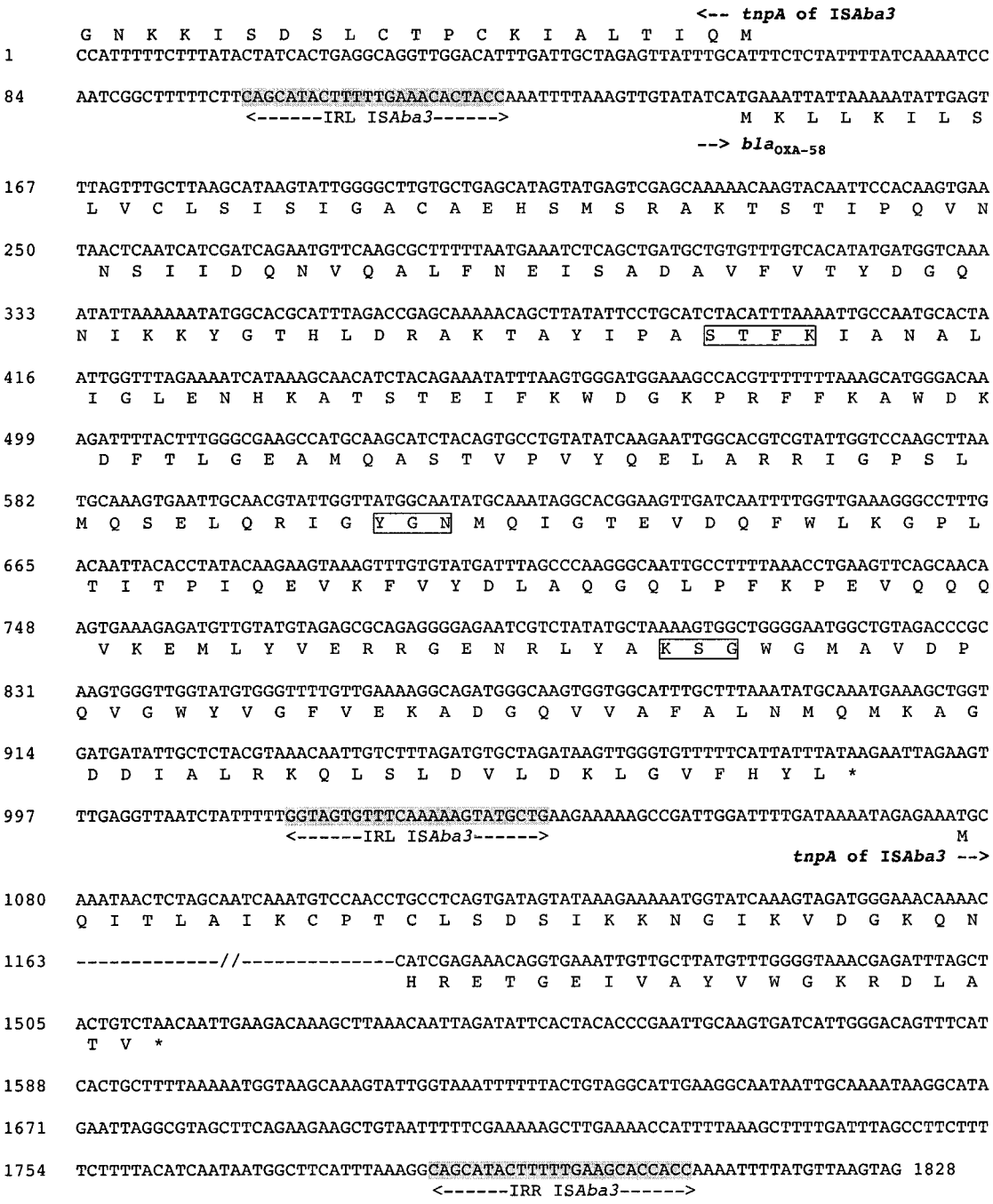

FIG. 1. Nucleotide sequence of a 1,828-bp fragment of recombinant plasmid pOXA-58 containing the bla_{OXA-58} gene. The deduced amino acid sequence is designated in single-letter code below the nucleotide sequence. The start codons of the ORFs are indicated by horizontal arrows. The left inverted repeats (IRL) of IS*Aba3* are shaded in grey. The *tnpA* transposase gene of IS*Aba3* is indicated. Asterisks indicates stop codons.

tetrad (STFK) was found at positions DBL (Ambler class D β-lactamase numbering [13]) 70 to 73. A KSG element (positions 216 to 218) was found, as observed in the carbapenem-hydrolyzing β-lactamases OXA-24, -25, -26, and -40, whereas a KTG motif is present in the carbapenem-hydrolyzing oxacillinases OXA-23, -27, -48, -54, and -55 and in most of the class D β-lactamases without carbapenemase activity (13, 21, 23) (Fig. 2). The oxacillinase structural element YGN at positions DBL 144 to 146 was not replaced in OXA-58 by an FGN motif, in contrast to what is found in sequences of the carbapenem-hydrolyzing oxacillinases identified in *A. baumannii* (18). OXA-58 was weakly related to other oxacillinases sharing, 48 and 47% amino acid identity with OXA-23 and OXA-24, respectively, which were taken as representatives of the two carbapenem-hydrolyzing oxacillinase subgroups of *A. baumannii* (7, 16). In addition, OXA-58 shared 35, 33, and 18% amino

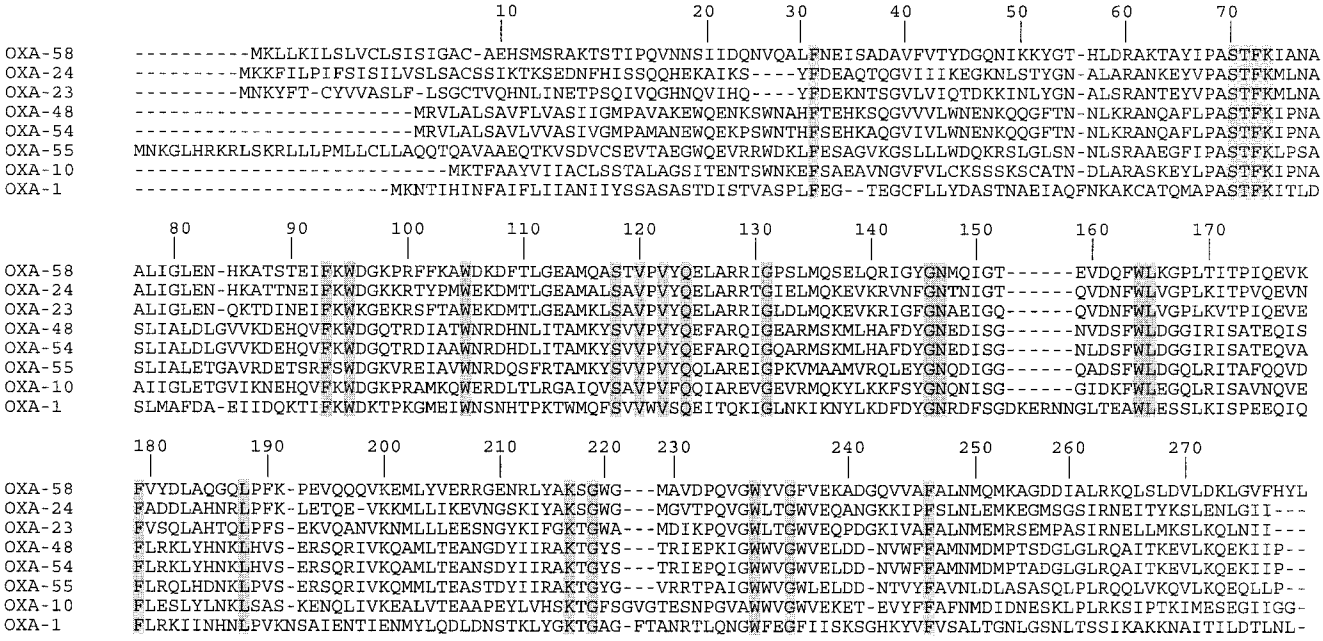

FIG. 2. Comparison of the amino acid sequence of OXA-58 with those of OXA-23, -24, -54, and -55, which possess carbapenem-hydrolyzing activity, and with those of reference oxacillinases OXA-1 and OXA-10. The conserved residues for oxacillinases are shaded. Numbering of β-lactamases is according to DBL numbering (13).

acid identity with OXA-5, OXA-10, and OXA-1, respectively, and 32% amino acid identity with the naturally occurring carbapenem-hydrolyzing oxacillinases OXA-54 and OXA-55 from *S. oneidensis* and *S.*, respectively (19, 31) (Fig. 3).

Genetic location of bla_{OXA-58}. Plasmid extraction of *A. baumannii* MAD revealed a ca. 30-kb plasmid that was successfully electroporated in *A. baumannii* CIP7010^T and not in the *E. coli* DH10B reference strains. Thus, plasmid pMAD was likely not able to replicate in *E. coli*. The *A. baumannii* CIP7010^T(pMAD) transformant had a β-lactam resistance pattern consistent with the expression of OXA-58, conferring resistance to ticarcillin and a reduced susceptibility to imipenem and meropenem (Table 1). No other additional antibiotic resistance markers were observed in *A. baumannii* CIP7010^T(pMAD) transformants. Mating-out assays using *A. baumannii* MAD or *A. baumannii* CIP7010^T (pMAD) as the donor and rifampin-resistant *A. baumannii* CIP7010^T failed.

Downstream of the plasmid-carried bla_{OXA-58} gene, a novel insertion sequence (IS), IS*Aba3*, was identified. It was 800 bp long and possessed a 145-amino-acid putative transposase

TABLE 2. Kinetic parameters of purified β-lactamase OXA-58 from *A. baumannii* MAD compared to those of OXA-40 from *A. baumannii* CLA-1[a]

Substrate	OXA-58				OXA-40[b]			
	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)	Relative k_{cat}/K_m	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)	Relative k_{cat}/K_m
Benzylpenicillin	5.5	50	110	100	5	23	220	100
Ampicillin	1	130	8	7	5	220	20	9
Ticarcillin	1	240	4	8	1	60	20	9
Piperacillin	2.5	50	50	48	1	23	50	23
Cephalothin	0.1	150	1	1	3	72	50	23
Cefotaxime	ND[c]	—[d]	—	—	ND	—	—	—
Ceftazidime	ND	—	—	—	20	2,500	10	4.5
Cefepime	ND	—	—	—	ND	—	—	—
Cefpirome	0.1	200	0.5	0.5	ND	—	—	—
Oxacillin	1.5	70	2	2	2	876	3	0.1
Aztreonam	ND	—	—	—	ND	—	—	—
Imipenem	0.1	7.5	13.5	13	0.1	6.5	15	7
Meropenem	<0.01	0.075[e]	<0.15	<0.7	ND	—	—	—

[a] Data are means from three independent experiments. Standard deviations were within 10% of the geometric means.
[b] Data are from reference 18.
[c] ND, no detectable hydrolysis (<0.01 s^{-1}).
[d] —, not determinable
[e] The K_i was determined with benzylpenicillin as the substrate.

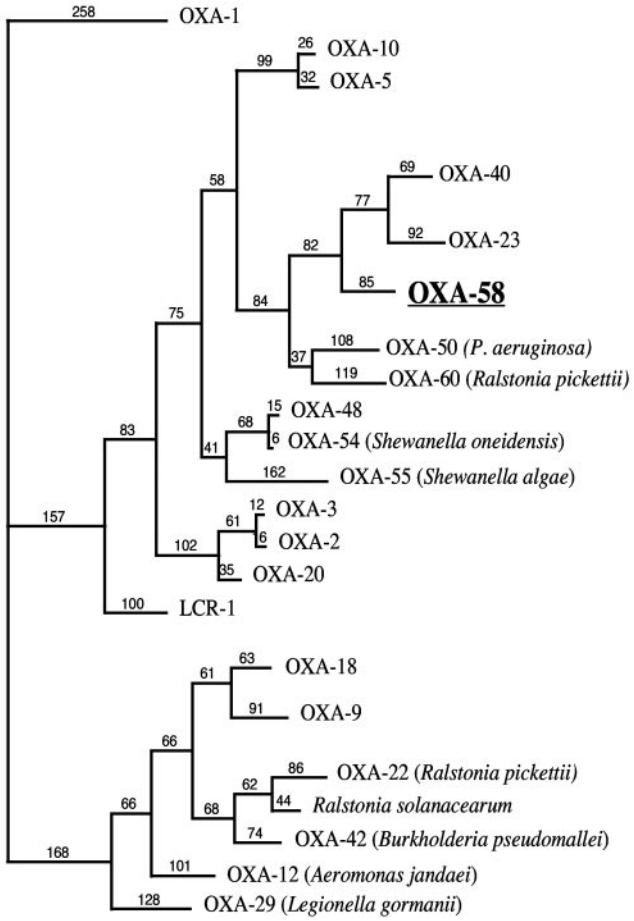

FIG. 3. Dendrogram obtained for representative natural and acquired Ambler class D β-lactamases by parsimony analysis. The origins of the naturally occurring oxacillinases are indicated. The alignment used for tree calculation was performed with ClustalW followed by minor adjustments. Branch lengths are drawn to scale and are proportional to the number of amino acid changes. The distance along the vertical axis has no significance.

weakly related to other transposase sequences, sharing 44% amino acid identity (56 amino acids) to part of the orfB transposase of IS*1*. In contrast to IS*1*, only one open reading frame is present in IS*Aba3*. The perfect inverted repeats were 21 bp in length (Fig. 1). Immediately upstream of the bla_{OXA-58} gene, a similar IS*Aba3* element was present. The left inverted repeat of IS*Aba3* was identified 17 bp upstream of the start codon of the bla_{OXA-58} gene, and the 125-bp-long sequence of this IS revealed 100% nucleotide identity with IS*Aba3*. Thus, bla_{OXA-58} likely is bracketed by two very similar IS elements. This structure may correspond to a composite transposon including the two IS*Aba3* elements.

Biochemical properties of β-lactamase OXA-58. After purification from extracts of *E. coli* DH10B(pOXA-58), the specific activity of OXA-58 against benzylpenicillin was 2.3 U per mg of protein and its purification factor was 70-fold. This weak β-lactamase activity is similar to those of many purified oxacillinases (3, 26, 28). Protein purity was estimated to be >95% by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis (data not shown). OXA-58 has a narrow-spectrum hydrolysis profile, including mostly penicillins (Table 2). Biphasic hydrolyses were obtained only for ampicillin, ticarcillin, and imipenem. Hydrolysis of imipenem was low, whereas hydrolysis of meropenem was not detected, although the MICs of both carbapenems were increased eightfold. However, determination of the K_i for meropenem revealed a strong affinity (0.075 μM) of OXA-58 for this substrate. In general, catalytic activities of OXA-58 were similar to those of OXA-40, which was taken as reference for an oxacillinase with carbapenem-hydrolyzing activity (Table 2). Nevertheless, the relative k_{cat}/K_m compared to that of benzylpenicillin showed that OXA-58 hydrolyzed imipenem twice as much as OXA-40 did. Additionally, OXA-58 had some hydrolytic activity against cefpirome, whereas activity against ceftazidime, cefotaxime, and cefepime remained undetectable (Table 2).

Studies of activity inhibition, as measured by determination of IC_{50}, showed that OXA-58 was weakly inhibited by clavulanic acid (310 μM), tazobactam (60 μM), and sulbactam (2.5 mM), as found for most of the oxacillinases (21, 23). These IC_{50}s were similar to those found for OXA-40 (18), except for sulbactam, which inhibits OXA-58 more than 10 times less. OXA-58 activity was well inhibited by NaCl (IC_{50}, 12 mM).

DISCUSSION

In our preliminary experiments we were not able to identify known oxacillinase or metallo-β-lactamase genes in a carbapenem-resistant *A. baumannii* strain. Thus, we have cloned and characterized a novel carbapenem-hydrolyzing oxacillinase, OXA-58, that constitutes a novel group of oxacillinases. The β-lactamase OXA-58 hydrolyzed penicillins and imipenem significantly, sparing most expanded-spectrum cephalosporins. The significant k_{cat}/K_m values obtained for imipenem and meropenem are related more to a good affinity for these substrates than to a strong hydrolysis. Interestingly, the decrease of susceptibility to carbapenems conferred by OXA-58 in *A. baumannii* is significant, and in view of the kinetic data, it is likely the result of a competitive inhibition toward these molecules. It is likely, from comparison of the β-lactam resistance phenotypes of *A. baumannii* MAD and its transformant, that additional mechanisms are responsible for the high level of resistance to imipenem.

OXA-58 possesses the classical YGN motif in positions DBL 144 to 146 of oxacillinases instead of an FGN motif as found in all of the carbapenem-hydrolyzing oxacillinases identified in *A. baumannii* (18, 23). This confirms that a Phe residue in position DBL144 is not required by itself for providing carbapenem hydrolytic activity (18). Nevertheless, we have not identified the critical residue(s) involved in the carbapenem hydrolysis property of OXA-58. The inhibition of OXA-58 by NaCl confirmed that this property is related to a Tyr residue in position DBL 144, whereas the other carbapenem-hydrolyzing oxacillinases that have a Phe residue at this position are resistant to inhibition by NaCl (18). This discrepancy can be a useful tool for screening of OXA-58 from a crude extract of any carbapenem-resistant *A. baumannii* strain, since this is currently the only β-lactamase inhibited by NaCl that is able to hydrolyze imipenem in that species.

This is the second report of a tight association between IS elements and an oxacillinase gene, after that of IS*1999* and

bla$_{OXA-48}$ in *K. pneumoniae* (32), which raises again the question of the origin of these oxacillinase genes and their mobilization process. As observed for the other carbapenem-hydrolyzing oxacillinase genes, *bla*$_{OXA-58}$ was not present in the form of a gene cassette in a class 1 integron, a situation that contrasts to that found for most of the oxacillinase genes. The IS element located upstream of *bla*$_{OXA-58}$ likely provided promoter sequences responsible for its expression. Further experiments are in progress to determine the putative role of this IS element in *bla*$_{OXA-58}$ expression.

This study constitutes the second description of a plasmid-encoded carbapenem-hydrolyzing oxacillinase in *A. baumannii*, after that of OXA-23 and ARI-2 (8, 35). A plasmid location of that gene may enhance the spread of the carbapenem resistance marker in *A. baumannii*. A prevalence study of spread of similar oxacillinase genes among European isolates would be interesting, especially in southern Europe, where imipenem-resistant *A. baumannii* strains seem to be increasingly identified (1, 11, 22, 38).

ACKNOWLEDGMENTS

This work was funded by a grant from the Ministère de l'Education Nationale et de la Recherche (UPRES-EA3539), Université Paris XI, France, and by the European Community (6th PCRD, LSHM-CT-2003-503-335). L.P. is a researcher from the INSERM, France.

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