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Genetic and Functional Analysis of the Chromosome-Encoded Carbapenem-Hydrolyzing Oxacillinase OXA-40 of *Acinetobacter baumannii*

Claire Héritier, Laurent Poirel, Daniel Aubert, and Patrice Nordmann*

Service de Bactériologie-Virologie, Hôpital de Bicêtre, Assistance Publique/Hôpitaux de Paris, Faculté de Médecine Paris-Sud, 94275 Le Kremlin-Bicêtre, France

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Clinical isolate *Acinetobacter baumannii* CLA-1 was resistant to a series of antibiotic molecules, including carbapenems. Cloning and expression of the β-lactamase gene content of this isolate in *Escherichia coli* DH10B identified a chromosome-encoded oxacillinase, OXA-40, that differed by one or two amino acid changes from OXA-24, -25, and -26 and an AmpC-type cephalosporinase. The OXA-40 β-lactamase had a mainly narrow-spectrum hydrolytic profile, but it included ceftazidime and imipenem. Its activity was resistant to inhibition by clavulanic acid, tazobactam, sulbactam, and, like most of the other carbapenem-hydrolyzing oxacillinases, NaCl. OXA-40 had an FGN triad replacing a YGN motif at class D β-lactamase (DBL) positions 144 to 146. Site-directed DNA mutagenesis leading to a Phe-to-Tyr change at DBL position 144 in OXA-40 gave a mutant enzyme with increased hydrolytic activity against most β-lactams, including imipenem. Conversely, with a gene encoding the narrow-spectrum oxacillinase OXA-1 as the template, a nucleotide substitution leading to a Tyr-to-Phe change in the YGN motif of OXA-1 gave a mutant enzyme with decreased hydrolytic activity without an increase in carbapenem-hydrolyzing activity. Thus, the Phe residue in the FGN motif was not associated with carbapenem-hydrolyzing activity by itself but instead was associated with weak overall hydrolytic activity. Finally, this Phe residue in OXA-40 explained resistance to inhibition by NaCl whereas a Tyr residue in motif YGN was related to susceptibility to NaCl.

Acinetobacter baumannii is an important cause of nosocomial infections such as pneumonia, septicemia, urinary tract infections, and wound infections (6). In many cases, carbapenems have become the drugs of choice for treatment of infections due to multiple-drug-resistant *A. baumannii* strains (6). Nevertheless, there are growing reports of carbapenem resistance in this species. The early reports described *A. baumannii* isolates with β-lactamase-independent carbapenem resistance (13, 16, 30), but the most recent reports have described β-lactamase-mediated resistance (1–3, 8, 10, 15, 22, 25, 26, 28, 31). In rare cases, IMP-like and VIM-like metallo-β-lactamases (Ambler class B) have been described in Southeast Asian and Italian isolates of the species (1, 12, 25, 26, 31).

Five oxacillinases (Ambler class D) with carbapenem-hydrolyzing activity have been characterized recently in that species, several strains of which were responsible for nosocomial outbreaks (3, 8, 10, 15, 22). OXA-23 (also named ARI-1 [15, 22, 28]) and OXA-27 (3), have 99% amino acid identity, whereas they have only 60% identity with a second group of oxacillinases consisting of OXA-24, -25, and -26, which differ by a few amino acid substitutions (3, 10).

We have characterized the genetics and biochemistry of the carbapenem-hydrolyzing oxacillinase OXA-40, which belongs to the subgroup of oxacillinases containing OXA-24, -25, and -26. Like the other carbapenem-hydrolyzing oxacillinases, OXA-40 has an FGN motif in place of the classical YGN motif of oxacillinases (14, 19). To analyze the role of this Phe residue in carbapenem-hydrolyzing activity and in resistance to NaCl, we performed a series of site-directed mutagenesis experiments.

(This work was previously presented in part [L. Poirel, C. Héritier, and P. Nordmann, 12th Eur. Congr. Clin. Microbiol. Infect. Dis., abstr. O384, 2002].)

MATERIALS AND METHODS

Bacterial strains and plasmids. *A. baumannii* clinical isolate CLA-1 was from a pulmonary brush of a Portuguese patient hospitalized for septic shock at the Bicêtre hospital (Le Kremlin-Bicêtre, France). He had been transferred from the Mirandelo hospital (near Porto, Portugal), where he had been hospitalized previously in an intensive care unit for a cranial injury. This strain was identified by the API 20NE system (bioMérieux, Marcy l'Etoile, France). *Escherichia coli* reference strain DH10B and plasmid pBK-CMV (carrying a kanamycin resistance marker) (Stratagene, Amsterdam, The Netherlands) were used for cloning and site-directed mutagenesis experiments. *Salmonella enterica* serotype Typhimurium strain RGN238 is a reference strain that produces the oxacillinase OXA-1 (gift from R. Labia) (21). Strains *A. baumannii* CIP7034^T (Institut Pasteur strain collection) and *E. coli* K-12 (gift from P. Plesiat) were used as reference strains for endonuclease I-*Ceu*I digestion.

Antimicrobial agents and MIC determinations. The antimicrobial agents used and their sources have been referenced elsewhere (23). 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) (www.sfm.fr). MICs were determined by an agar dilution technique as previously reported (23), and results were interpreted in accordance with the guidelines of the NCCLS (20).

Cloning experiments. Whole-cell DNA of *A. baumannii* CLA-1 was extracted as previously described (23). Since the oxacillinase genes are often located in integrons (19), primers for detection of class 1 integrons (primer INT2F, located within the integrase gene of class 1 integrons, and a primer [3′-CS] located in the 3′ conserved segment [24]) were used to generate fragments by PCR with whole-cell DNA of *A. baumannii* CLA-1 as the template. A cloning experiment

* Corresponding author. Mailing address: Service de Bactériologie-Virologie, Hôpital de Bicêtre, 78 rue du Général Leclerc, 94275 Le Kremlin-Bicêtre Cédex, 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. Primers used in this study

Primer	Sequence (5′ to 3′)[a]	Reference
F144Y	GCGGGTTAATTATGGAAATACAAATATTGGAACACAGGTCG	This study
F144Yrev	CGACCTGTGTTCCAATATTTGTATTTCCATAATTAACCCGC	This study
PreOXA1A	AGCCGTTAAAATTAAGCCC	4
PreOXA1B	CTTGATTGAAGGGTTGGGCG	4
Y144F	CTCAAGGATTTTGATTTTGGAAATCAAGACTTCTTCGG	This study
Y144Frev	CCAAAATCAAAATCCTTGAGATAATTCTTG	This study
A	AGAGTTTGATCHTGGYTYAGA	5
B	ACGGYTACCTTGTTACGACTTC	5
Oxa-imp1	GCAAATAMAGAATATGTSCC	This study
Oxa-imp2	CTCMACCCARCCRGTCAACC	This study

[a] H is A, T, or C; Y is C or T; S is G or C; M is A or C; R is A or G.

was then performed with *Bam*HI-digested whole-cell DNA of *A. baumannii* CLA-1 and *Bam*HI-restricted plasmid pBK-CMV, followed by expression in *E. coli* DH10B, as described previously (24). Antibiograms obtained by disk diffusion were performed with *E. coli* DH10B strains harboring recombinant plasmids, and the sizes of the plasmid inserts were determined by restriction analysis (27). Recombinant plasmids pOXA-40 and pABC-1 were retained for further analysis.

Site-directed mutagenesis. Since the identified oxacillinase contained an FGN triad in place of the more common YGN at class D β-lactamase (DBL) positions 144 to 146 (14), a site-directed mutagenesis protocol was used as described by the manufacturer (Quick Change site-directed mutagenesis kit; Stratagene) for a nucleotide that substituted a Phe residue for the Tyr residue at DBL position 144. Recombinant plasmid pOXA-40 was used as the template with primers F144Y and F144Yrev to generate recombinant plasmid pOXA-40-Tyr (Table 1).

Conversely, the effects of a nucleotide change leading to the change from a Tyr residue to a Phe residue at DBL position 144 were investigated by using as the template the gene encoding OXA-1, a reference narrow-spectrum oxacillinase that does not possess significant carbapenem-hydrolyzing activity (19). The bla_{OXA-1} gene was PCR amplified with primers PreOXA1A and PreOXA1B (Table 1) from bla_{OXA-1}-containing whole-cell DNA of *S. enterica* serotype Typhimurium. This 911-bp fragment, encompassing the bla_{OXA-1} gene, was cloned into the *Sma*I site of plasmid pBK-CMV, giving recombinant plasmid pOXA-1. Plasmid pOXA-1 was then used as a template with primers Y144F and Y144Frev (Table 1), giving rise to recombinant pOXA-1-Phe.

DNA sequencing and protein analysis. The nucleotide changes induced by site-directed mutagenesis experiments were checked by sequence analysis. Both strands of the cloned DNA fragments from recombinant plasmids were sequenced with an Applied Biosystems sequencer (ABI 373). 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* CLA-1 was attempted by two different methods (24). To search for a possible chromosomal location of the oxacillinase gene, we used the endonuclease I-*Ceu*I (New England Biolabs, Ozyme, Saint-Quentin-en-Yvelines, France), which digests a 26-bp sequence in *rrn* genes for the 23S large-subunit rRNA (18). After digestion, separation of the resulting fragments by pulsed-field gel electrophoresis was performed with a CHEF-DRII apparatus (29). The sizes of endonuclease I-*Ceu*I-generated fragments were determined by comparison with those of *E. coli* K-12 (18).

After transfer onto a nylon membrane (Hybond N^+; Amersham Pharmacia Biotech, Orsay, France) by the Southern technique (28), endonuclease I-*Ceu*I-generated DNA fragments of *A. baumannii* CLA-1 and CIP7034^T were UV cross-linked (Stratalinker; Stratagene) for 2 min. They were hybridized with two different probes: a 1,504-bp PCR-generated probe for 16S and 23S rRNA genes (Roche Diagnostics, Meylan, France) (primers A and B [Table 1]) and a 495-bp PCR-generated probe (primers Oxa-imp1 and Oxa-imp2 [Table 1]) for detection of the genes encoding carbapenem-hydrolyzing oxacillinases. Labeling and revelation were performed as previously reported (24).

β-Lactamase purification. Cultures of *E. coli* DH10B harboring recombinant plasmids pOXA-40 and pOXA-40-Tyr were grown overnight at 37°C in 4 liters of Trypticase soy (TS) broth containing amoxicillin (100 μg/ml) and kanamycin (30 μg/ml). Protein extracts were then obtained as previously detailed (24). These extracts were subjected to purification steps including ion-exchange chromatography with Q-Sepharose followed by S-Sepharose columns as previously described (23). Elution of the β-lactamase activities was realized with NaCl at a concentration of 50 mM. The peaks of β-lactamase activity were concentrated by using Centrisart-C30 spin columns (Sartorius, Göttingen, Germany) and dialyzed with 100 mM phosphate buffer (pH 7.0).

Kinetic parameters. Purified β-lactamases were used for kinetic measurements performed at 30°C in 100 mM sodium phosphate (pH 7.0) (24). k_{cat} and K_m values were determined by analyzing β-lactam hydrolysis under initial-rate conditions with a UV spectrophotometer as previously described (24).

The 50% inhibitory concentrations (IC_{50}s) of clavulanic acid, tazobactam, sulbactam, and NaCl were determined (24).Various concentrations of these inhibitors were preincubated with purified enzymes for 3 min at 30°C to determine the concentrations that decreased the rate of hydrolysis of 100 μmol of cephalothin by 50%.

Specific activities of protein extracts and of purified β-lactamases from *E. coli* DH10B harboring pOXA-40 and pOXA-40-Tyr were obtained as described previously (4) with 100 μM cephalothin as the substrate. The degree of protein purification was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis (27). The protein content was measured by the Bio-Rad DC protein assay. β-Lactamase specific activities for several β-lactams were also determined with extracts of *E. coli* DH10B harboring plasmids pOXA-40, pOXA-40-Tyr, pOXA-1, and pOXA-1-Phe. In those cases, overnight cultures were obtained in 10 ml of TS broth containing amoxicillin (100 μg/ml) and kanamycin (30 μg/ml) and extracts were obtained as previously described (24).

IEF analysis. Isoelectric focusing (IEF) analysis was performed with an ampholine polyacrylamide gel (pH 3.5 to 9.5) as described previously (24) with extracts of *A. baumannii* CLA-1 and *E. coli* DH10B strains harboring recombinant plasmids and with purified β-lactamases OXA-40 and OXA-40-Tyr.

Nucleotide sequence accession number. The nucleotide sequence reported in this paper has been submitted to the EMBL/GenBank nucleotide sequence database and assigned accession no AF509241.

RESULTS

Antibiotic susceptibility and β-lactamases of *A. baumannii* CLA-1. Isolate CLA-1 had high levels of resistance to penicillins, cephalosporins, and carbapenems (Table 2). Addition of clavulanic acid and tazobactam did not significantly decrease the MICs of ticarcillin and piperacillin (Table 2), indicating that a Bush group 2b or 2be β-lactamase was not likely to be solely involved (11). A preliminary experiment showed that the *A. baumannii* CLA-1 extract had significant imipenem-hydrolyzing activity (50 mU/mg of protein). Antibiotic susceptibility testing showed that *A. baumannii* CLA-1 was also resistant to gentamicin, tobramycin, netilmicin, fluoroquinolones, sulfon-

TABLE 2. MICs of β-lactams for *A. baumannii* clinical isolate CLA-1; *E. coli* DH10B harboring recombinant plasmids pOXA-40, pOXA-40-Tyr, pOXA-1, and pOXA-1-Phe; and *E. coli* reference strain DH10B

β-Lactam(s)[a]	MIC (μg/ml)					
	A. baumannii CLA-1 (OXA-40)	*E. coli* DH10B (pOXA-40) (OXA-40)	*E. coli* DH10B (pOXA-40-Tyr) (OXA-40-Tyr)	*E. coli* DH10B (pOXA-1) (OXA-1)	*E. coli* DH10B (pOXA-1-Phe) (OXA-1-Phe)	*E. coli* DH10B
Amoxicillin	>512	>512	>512	>512	>512	4
Amoxicillin + CLA	>512	>512	>512	128	128	4
Ticarcillin	>512	>512	>512	>512	>512	4
Ticarcillin + CLA	>512	>512	>512	256	256	4
Piperacillin	>512	256	>512	128	16	1
Piperacillin + TZB	>512	128	>512	128	16	1
Cephalothin	>512	8	32	8	4	2
Cefuroxime	>512	8	16	8	4	2
Ceftazidime	>512	0.5	0.06	0.25	0.12	0.06
Cefotaxime	>512	0.12	0.12	0.12	0.12	0.12
Cefepime	64	0.25	0.5	0.5	0.12	0.06
Cefpirome	256	0.25	0.25	1	0.25	0.06
Moxalactam	256	0.5	0.5	0.12	0.12	0.06
Aztreonam	128	0.12	0.06	0.12	0.12	0.12
Imipenem	256	2	4	0.5	0.25	0.06
Meropenem	256	0.5	0.5	0.12	0.12	0.06

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

amides, and tetracycline and susceptible to amikacin, dibekacin, kanamycin, rifampin, and colistin (data not shown). An extract of *A. baumannii* CLA-1 subjected to IEF analysis gave two β-lactamases with pIs of 8.6 and 9.4 (data not shown).

Cloning and sequencing of bla_{OXA-40} and surrounding sequences and β-lactamase expression in *E. coli*. PCR amplification with primers annealing at the ends of class 1 integrons and whole-cell DNA of *A. baumannii* CLA-1 as the template failed. Recombinant plasmids were obtained after cloning of *Bam*HI-restricted DNA of *A. baumannii* CLA-1 into pBK-CMV and expression in *E. coli*. Antibiotic susceptibility testing revealed that these *E. coli* clones expressed either (i) a β-lactamase whose activity included imipenem resistance and which was resistant to inhibitors or (ii) a cephalosporinase-type enzyme (data not shown). Recombinant plasmids pOXA-40 and pABC-1 were isolated from clones with each of these antibiotic resistance phenotypes that contained 2.5- and 2-kb inserts, respectively.

DNA sequence analysis of the 2.5-kb insert of pOXA-40 identified an 828-bp open reading frame (ORF), named bla_{OXA-40}, encoding a 275-amino-acid protein (Fig. 1). The G+C content of this ORF was 35%, which is not within the expected range of the G+C content of *A. baumannii* genes (39 to 47%) (6). Within the deduced protein encoded by this ORF, a serine-threonine-phenylalanine-lysine tetrad (S-T-F-K) was found at positions 70 to 73 (DBL numbering [14]) (Fig. 1). Two out of the four structural elements characteristic of DBLs were found: W-X-X-X-X-L-X-I-X at DBL positions 164 to 172 and Q-X-X-X-X at DBL positions 176 to 180 (Fig. 1) (19). The third element, K-T-G at positions 216 to 218, was replaced by a K-S-G motif as in OXA-24, -25, and -26 but not as in OXA-23 and -27. The fourth structural element, YGN (DBL

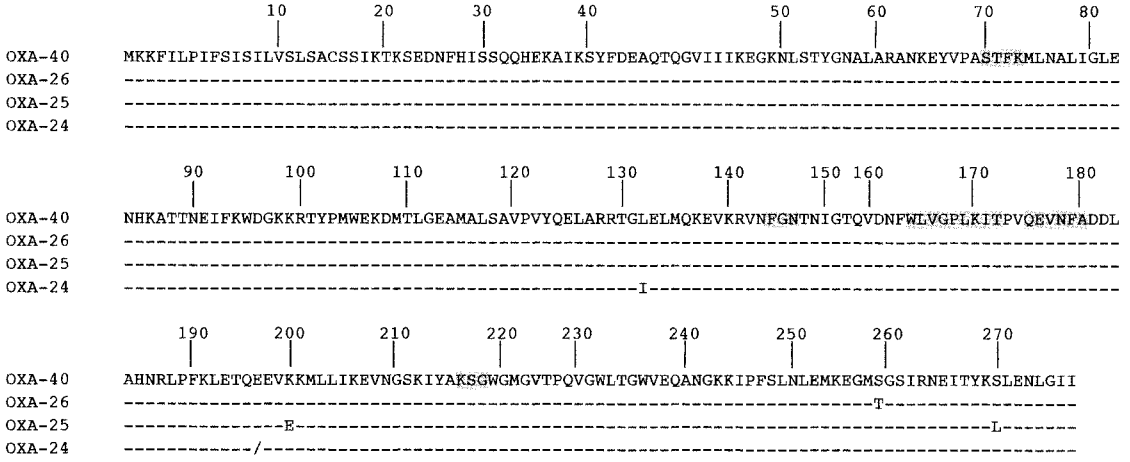

FIG. 1. Comparison of the amino acid sequence of OXA-40 to those of the most closely related oxacillinases, OXA-24, -25, and -26 (3, 10). Dashes indicate identical amino acids. The numbering is in accordance with the DBL numbering system (14). The motifs usually conserved among DBLs are shaded.

TABLE 3. Kinetic parameters of purified β-lactamase OXA-40 and mutant enzyme OXA-40-Tyr[a]

Antibiotic	OXA-40			OXA-40-Tyr		
	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($nM^{-1} \cdot s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($nM^{-1} \cdot s^{-1}$)
Benzylpenicillin	5	23	220	30	9	3,000
Ampicillin	5	220	20	40	180	220
Ticarcillin	1	60	20	10	65	170
Piperacillin	1	23	50	15	40	350
Cephalothin	3	72	50	5	120	30
Cephaloridine	5	1,000	5	15	950	15
Cefotaxime	ND[b]	—[c]	—	ND	—	—
Ceftazidime	20	2,500	10	ND	—	—
Cefepime	ND	—	—	2	3,000	1
Cefpirome	ND	—	—	1	620	2
Aztreonam	ND	—	—	ND	—	—
Imipenem	0.1	6.5	15	0.5	1	360
Meropenem	ND	—	—	—	—	—
Oxacillin	2	876	3	6	155	40
Cloxacillin	ND	—	—	35	780	50

[a] Data are the means of three independent experiments. Standard deviations were within 10% of the means.
[b] ND, no detectable hydrolysis (<0.0.01 s^{-1}).
[c] —, not determinable.

positions 144 to 146), found in most oxacillinases was replaced by an FGN motif as in all of the carbapenem-hydrolyzing oxacillinases. OXA-40 had one or two amino acid substitutions compared to OXA-24, -25, and -26 (Fig. 1).

Analysis of the DNA sequences surrounding the bla_{OXA-40} gene revealed 100% identity with 20 and 40 bp of the DNA sequences upstream and downstream of bla_{OXA-25} and bla_{OXA-26}, respectively, whereas the further 40-bp sequences located downstream were different. Additionally, further upstream of the bla_{OXA-40} gene, there was a 500-bp sequence that was identical to that encoding part of the 16S rRNA gene of *A. baumannii*, suggesting the chromosomal location of bla_{OXA-40}.

Sequence analysis of the insert of pABC-1 that conferred a cephalosporinase phenotype identified an ORF that encodes a protein that has a one-amino-acid substitution compared to the sequence of the only known AmpC-type enzyme of *A. baumannii* AbRYC52763/97(data not shown) (9), thus identifying the second β-lactamase gene from *A. baumannii* CLA-1.

IEF analysis revealed that *E. coli* DH10B(pOXA-40) produces a β-lactamase with a pI of 8.6, like that found in extracts of *A. baumannii* CLA-1, a pI value higher than that of β-lactamases OXA-24, -25, and -26 (3, 10). In addition, extracts of *E. coli* DH10B(pABC-1) contained a β-lactamase with a pI value of 9.4 that was identical to one of those found in extracts of *A. baumannii* CLA-1 and to that of the AmpC enzyme of *A. baumannii* AbRYC52763/97 (9).

MICs of β-lactams for *E. coli* DH10B(pOXA-40) showed resistance to penicillins not antagonized by clavulanic acid and tazobactam addition and a slightly decreased susceptibility to carbapenems (Table 2). A similar pattern of resistance to β-lactams had been found for *E. coli* expressing OXA-23 and OXA-24 (7, 10). (MICs for *E. coli* expressing OXA-25, -26, -27 have not been published [3].)

Genetic location of bla_{OXA-40} Plasmid extraction of *A. baumannii* CLA-1 failed. However, extraction of plasmid DNA from *Acinetobacter* strains may be difficult. Thus, the location of bla_{OXA-40} was determined by using the endonuclease I-*Ceu*I technique. We generated five fragments from restricted DNAs of *A. baumannii* CLA-1, six from the *A. baumannii* CIP7034^T reference strain (2, 200, 500, 150, 60, and 40 Mb), and seven from the *E. coli* K-12 reference strain (data not shown). The DNA probe for rRNA genes hybridized with the endonuclease I-*Ceu*I-generated fragments, except for the 2,200-Mb fragment. The bla_{OXA-40} probe hybridized only with the 500-Mb fragment of strain CLA-1, indicating the chromosomal location of this gene (data not shown).

Biochemical properties of β-lactamase OXA-40. After purification from extracts of *E. coli* DH10B(pOXA-40), the specific activity of OXA-40 against cephalothin was 2 U/mg of protein and its purification factor was 37-fold. This relatively weak activity is similar to those of many purified oxacillinases (4, 19, 23, 24). Protein purity was estimated to be >90% (data not shown). OXA-40 has a narrow-spectrum hydrolytic profile including mostly penicillins (Table 3). Hydrolysis of oxacillin was detected as for OXA-25 and -26 (3), whereas it was not detected for OXA-24 (10). Hydrolysis of imipenem was low in level, whereas hydrolysis of meropenem was not detected. Hydrolysis of meropenem has been found for OXA-24, -25, and -26, although at a level lower than that of imipenem (3, 10). Additionally, OXA-40 retained some hydrolytic activity against ceftazidime (Table 3).

Studies of activity inhibition, as measured by determination of IC_{50}s, showed that OXA-40 was weakly inhibited by clavulanic acid (300 μM), tazobactam (180 μM), and sulbactam (190 μM), as found for most of the oxacillinases (17, 19). These IC_{50}s were higher than those found for partially purified oxacillinases with carbapenem-hydrolyzing activity (3, 10), which were 6- to 100-fold lower, depending on the β-lactam inhibitors. OXA-40 activity was not inhibited by NaCl (IC_{50}, 3 M) whereas NaCl is a good inhibitor of most oxacillinases (11, 17, 19).

Site-directed mutagenesis leading to a Tyr or a Phe residue at DBL position 144 and consequences for MICs and β-lactamase activity. The role of the Phe residue at DBL position 144 of OXA-40 was first investigated by generating plasmid pOXA-40-Tyr, which contained the exact same insert as

TABLE 4. Specific activities of oxacillinases OXA-40, OXA-40-Tyr, OXA-1, and OXA-1-Phe from extracts of *E. coli* DH10B harboring recombinant plasmids

Substrate	Sp act (mU/mg of protein)[a]			
	OXA-40	OXA-40-Tyr	OXA-1	OXA-1-Phe
Benzylpenicillin	120	1,400	550	ND[b]
Amoxicillin	20	100	850	ND
Piperacillin	30	350	850	ND
Cephalothin	10	30	35	15
Imipenem	10	35	1.5	1.5

[a] Standard deviations were within 20% of the means (means of three independent determinations are shown).
[b] ND, not determinable (nonlinear hydrolysis curve).

pOXA-40 except for a T-to-A change leading to the Phe-to-Tyr change at DBL position 144 of the encoded β-lactamase. Comparison of the MICs of β-lactams for *E. coli* DH10B(pOXA-40-Tyr) to those for *E. coli* DH10B(pOXA-40) showed similar resistance profiles, except for a decrease in the ceftazidime MIC for *E. coli* DH10B(pOXA-40-Tyr) (Table 2). The levels of imipenem resistance of the two strains were similar (Table 2).

The specific activities for several β-lactams were determined with extracts of *E. coli* DH10B harboring pOXA-40 and pOXA-40-Tyr. For all substrates, specific activities for extracts of *E. coli* DH10B(pOXA-40-Tyr) were higher than those for *E. coli* DH10B(pOXA-40) (Table 4).

To compare further the hydrolytic activity of OXA-40-Tyr to that of OXA-40, purification of OXA-40-Tyr was also performed. The specific activity of OXA-40-Tyr against cephalothin was 5.3 U/mg of protein, and its purification factor was 79-fold. Protein purity was estimated to be >90% (data not shown). The catalytic efficacy of OXA-40-Tyr was higher than that of OXA-40 for all substrates except cephalothin and ceftazidime (Table 3). The catalytic efficacy of OXA-40-Tyr for imipenem was 24-fold higher than that of OXA-40 because of both higher hydrolytic activity and greater affinity (Table 3). Kinetic parameters for cloxacillin could be determined for OXA-40-Tyr, whereas no values could be obtained for OXA-40 (Table 3). Inhibition of β-lactamase activities showed that the IC_{50}s of tazobactam and sulbactam for OXA-40-Tyr and OXA-40 were similar whereas the IC_{50} of clavulanic acid for OXA-40-Tyr (1 mM) was threefold greater than that for OXA-40. The Phe-to-Tyr substitution in OXA-40-Tyr compared to OXA-40 restored its susceptibility to NaCl inhibition (IC_{50}, 40 mM).

To evaluate further the role of the Phe residue at DBL position 144 in conferring carbapenem-hydrolyzing activity, we performed a Tyr-to-Phe residue change at DBL position 144 of the amino acid sequence of OXA-1, an oxacillinase that does not possess significant carbapenem-hydrolyzing activity (19). A site-directed mutagenesis experiment with pOXA-1, containing the bla_{OXA-1} gene, as the template was then performed to obtain recombinant plasmid pOXA-1-Phe, which encodes OXA-1-Phe with a Tyr-to-Phe substitution at DBL position 144. Specific activity of extracts of *E. coli* DH10B(pOXA-1) for the β-lactams studied was detected, although that for imipenem was feeble (Table 4). The specific activity of extracts of *E. coli* DH10B(pOXA-1-Phe) for cephalothin was lower than that of extracts of *E. coli* DH10B(pOXA-1) (Table 4). The Tyr-to-Phe substitution at DBL position 144 led to a decrease in the overall hydrolytic activity of OXA-1-Phe, mirroring the results obtained with OXA-40 and OXA-40-Tyr. Extracts of *E. coli* DH10B producing OXA-1 and OXA-1-Phe gave the same low-level activity against imipenem (Table 4). Inhibition of β-lactamase activities showed that the IC_{50}s of NaCl for extracts of *E. coli* DH10B expressing OXA-1 and OXA-1-Phe were 50 and 2,000 mM, respectively, mirroring the IC_{50}s of NaCl for OXA-40-Tyr and OXA-40, respectively.

DISCUSSION

This work characterizes the genetics and biochemistry of a carbapenem-hydrolyzing oxacillinase, OXA-40, produced by a multiple-drug-resistant *A. baumannii* clinical isolate from Portugal. OXA-40 had a few amino acid changes compared to OXA-24, -25, and -26, belonging to the same subgroup of carbapenem-hydrolyzing oxacillinases. While this work was in progress, others (F. Lopez-Otsoa, L. Gallego, K. Towner, L. Tysall, D. Livermore, and N. Woodford, 12th Eur. Congr. Clin. Microbiol. Infect. Dis., abstr. P1442, 2002) reported the same β-lactamase from *A. baumannii* isolates from Bilbao (northwestern Spain), and OXA-24 to -26 were from Spain (Madrid) and Belgium (Ghent) (3, 10). Thus, a subgroup of carbapenem-hydrolyzing oxacillinases may have spread in *A. baumannii*, at least in Europe.

The chromosomal location of the bla_{OXA-40} gene has been determined clearly as it has been suggested for the genes encoding OXA-24, -25, and -26 (3, 10). The DNA sequences located immediately up- and downstream of the oxacillinase genes encoding OXA-25, -26, -40 were the same, suggesting a common origin of these β-lactamase genes, whereas a clonal relationship was not identified for the OXA-24- and OXA-25-producing *A. baumannii* isolates (3). The mechanism of transfer of these oxacillinase genes remains unknown, since none of these genes has been found to be bracketed by class 1 integron or insertion sequences.

The two groups of carbapenem-hydrolyzing oxacillinases (OXA-23 and -27 on the one hand and OXA-24, -25, -26, and -40 on the other) have an FGN triad instead of the common YGN motif at DBL positions 144 to 146. Contrary to expected results (3, 10, 15), a Phe-to-Tyr substitution at DBL position 144 in OXA-40 gave a mutant enzyme, OXA-40-Tyr, that had stronger hydrolytic activity against most β-lactams, including imipenem. Additionally, a Tyr-to-Phe substitution at DBL position 144 in the narrow-spectrum oxacillinase OXA-1 did not provide additional carbapenem-hydrolyzing activity but instead gave a mutant enzyme with weaker overall hydrolytic activity. These experiments indicated that the Phe residue at DBL position 144 is not, by itself, responsible for carbapenem-hydrolyzing activity and that this residue, in the place of a Tyr residue, confers weak overall hydrolytic activity on carbapenem-hydrolyzing oxacillinases. The weak activity of these enzymes may explain why they have remained undetected for a long time in carbapenem-resistant *A. baumannii* isolates and why carbapenem resistance in *A. baumannii* may result from combined antibiotic resistance mechanisms (1, 8).

Resistance to inhibition by NaCl was also found in OXA-25 and -26 (3), whereas susceptibility to inhibition by NaCl was

surprisingly reported for the structurally related oxacillinase OXA-24 (10). We have shown that resistance to inhibition and, conversely, susceptibility to inhibition by NaCl are related to the presence of Phe and Tyr residues at DBL position 144, respectively. Substitution of a Tyr residue for a more neutral Phe residue may prevent NaCl from binding the active site of oxacillinases, which involves at least the amino acid residues at DBL positions 144 to 146 (19).

Finally, whereas detection of carbapenem-hydrolyzing oxacillinases is difficult in multidrug-resistant *A. baumannii* isolates, it remains to be determined if these strains are a reservoir of carbapenem-hydrolyzing genes spreading to other gram-negative rods. In that respect, OXA-23 has been recently identified in *Proteus mirabilis* isolates (7).

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