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Characterization of OXA-25, OXA-26, and OXA-27, Molecular Class D β-Lactamases Associated with Carbapenem Resistance in Clinical Isolates of *Acinetobacter baumannii*

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Carbapenem resistance in *Acinetobacter* spp. is increasingly being associated with OXA-type β-lactamases with weak hydrolytic activity against imipenem and meropenem. Such enzymes were characterized from *Acinetobacter* isolates collected in Belgium, Kuwait, Singapore, and Spain. The isolates from Spain and Belgium had novel class D β-lactamases that were active against carbapenems. These were designated OXA-25 and OXA-26, respectively, and had >98% amino acid homology with each other and with the OXA-24 enzyme recently described by others from an *Acinetobacter* isolate collected elsewhere in Spain. The isolate from Singapore had OXA-27 β-lactamase, another novel class D type with only 60% homology to OXA-24, -25, and -26, but with 99% homology to OXA-23 (ARI-1), described previously from an *Acinetobacter baumannii* isolate collected in Scotland. Sequence data were not obtained for the carbapenem-hydrolyzing OXA enzyme from the isolate from Kuwait; nevertheless, the enzyme was phenotypically similar to OXA-25 and -26. The enzymes OXA-23, -24, -25, -26, and -27 retained the STFK and SXV motifs typical of class D β-lactamases, but the YGN motif was altered to FGN. The KTG motif was retained by OXA-27 and -23 but was replaced by KSG in OXA-24, -25, and -26. OXA-25 and -26 enzymes were strongly active against oxacillin, but unusually for an OXA-type β-lactamase, OXA-27 had apparently weak activity, although measurement was complicated by biphasic kinetics. None of the new enzymes was transmissible to *Escherichia coli* recipients. Many *Acinetobacter* isolates are multiresistant to other antibiotics, and the emergence of class D enzymes with carbapenem-hydrolyzing activity is a disturbing development for antimicrobial chemotherapy.

Acinetobacter spp. are important opportunistic nosocomial pathogens and are particularly important in ventilator-associated pneumonias and in infections of burn wounds. *Acinetobacter baumannii* is the predominant species in clinical settings, and isolates are often multiresistant, complicating therapy (3). Carbapenems have become the drugs of choice for serious *Acinetobacter* infections in many centers and have retained better activity than other antimicrobials; nevertheless, there is a growing literature on carbapenem resistance. Some early reports described acinetobacters with β-lactamase-independent carbapenem resistance (6, 11, 23), but most recent reports describe β-lactamase-mediated resistance. The first known *A. baumannii* isolate with a carbapenem-hydrolyzing β-lactamase was collected in 1985 in Scotland, and its enzyme was initially designated ARI-1 (16, 19). Isolates with carbapenem-hydrolyzing β-lactamases subsequently have been reported from Argentina (2), Belgium (1), Brazil (S. F. Costa, J. Woodstock, J. Child, H. H. Calaffa, M. Gill, R. Wise, and A. S. Levin, Abstr. 36th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 1123, 1996), Cuba (17), France (12), Hong Kong (5a), Italy (7), Japan (21), Kuwait (1), Singapore (1), and Spain (1). A minority of these isolates, including the organisms from Cuba, Hong Kong, Italy, and Japan, have IMP-type metallo-β-lactamases (5a, 7), but most have zinc-independent β-lactamases, many apparently belonging to molecular class D. Examples already sequenced include ARI-1, now renamed OXA-23 (9), and OXA-24 (5). Other carbapenem-hydrolyzing β-lactamases from resistant *Acinetobacter* isolates have not yet been sequenced but have the strong oxacillinase activity characteristic of class D β-lactamases (2, 12). We describe the properties of four more OXA-type enzymes, extracted from carbapenem-resistant *A. baumannii* isolates collected in Belgium, Kuwait, Spain, and Singapore, and report the sequences of three of these four.

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MATERIALS AND METHODS

Bacterial strains. Carbapenem-resistant *Acinetobacter* spp. isolates were sought worldwide between 1995 and 1997 and were identified as described previously (1, 2). Isolates with carbapenem-hydrolyzing β-lactamases were received, inter alia, from Argentina, Belgium, Hong Kong, Kuwait, Singapore, and Spain. Biochemical properties were reported previously for the oxacillinase extracted from isolate BA HCT 15, which was collected in Argentina (2), and this organism was included here for genetic studies only, whereas both genetic and biochemical aspects were studied for representative resistant isolates from Belgium, Kuwait, Singapore, and Spain. As controls, we used 40 susceptible *Acinetobacter* isolates; these were collected in 1984, prior to the clinical use of carbapenems (14).

Antimicrobial agents. Antimicrobials were provided by suppliers as follows: ampicillin and clavulanate (SmithKline Beecham, Brentford, United Kingdom); aztreonam and cefepime (Bristol-Myers Squibb, Syracuse, N.Y.); penicillin G, cephaloridine, and cephalothin (Lilly, Basingstoke, United Kingdom); cefotaxime (Aventis, Uxbridge, United Kingdom); cefoxitin and imipenem (Merck Sharp and Dohme, Hoddesdon, United Kingdom); ceftazidime and cefuroxime (GlaxoWellcome, Stevenage, United Kingdom); ciprofloxacin (Bayer, Newbury, United Kingdom); meropenem (Zeneca, Macclesfield, United Kingdom); piperacillin and tazobactam (Wyeth, Taplow, United Kingdom); sulbactam (Pfizer, Sandwich, United Kingdom); and nitrocefin (BBL Microbiology Systems, Cockeysville, Md.).

Determination of MICs. MICs were determined on Iso-Sensitest agar (Oxoid, Basingstoke, United Kingdom) with inocula of ca. 10^4 CFU. The results were read as the lowest concentration of antibiotics at which no growth was visible

TABLE 1. Buffers used in ion exchange chromatography

Isolate and source	Ion-exchange column and buffer
04737, Belgium	CM-Sephadex[a] in 10 mM sodium phosphate, pH 6.6
A-15, Kuwait	A-Sephadex in 20 mM Tris HCl, pH 8.2 A-Sephadex in 20 mM Tris HCl, pH 7.8[b]
I-16, Singapore	CM-Sephadex in 50 mM malonic acid-malonate, pH 5.6
327009, Spain	CM-Sephadex in 10 mM sodium phosphate buffer, pH 6.8

[a] Sephadex was obtained from Pharmacia.
[b] Second purification step, performed after dialysis of the enzyme against 20 mM Tris HCl buffer, pH 7.8.

after overnight incubation at 37°C. *Pseudomonas aeruginosa* NCTC 10662 was used as a control.

Isoelectric focusing. Cell extracts were prepared as for the bioassays but in 0.01 M phosphate buffer, pH 7.0. Isoelectric focusing was run on polyacrylamide gels containing equal proportions of Resolyte pH 3.5 to 10 and Resolyte pH 4 to 8 (BDH, Poole, United Kingdom). The gels were electrophoresed at 11 to 14 W for 90 min, and β-lactamases were located with 0.5 mM nitrocefin.

Curing and transfer of resistance. Cured variants were sought by growing cultures overnight in nutrient broth containing ethidium bromide at 0.25 to 0.5 times the MIC and then replica plating onto Iso-Sensitest agar with and without imipenem at 2 or 10 μg/ml. Transfer of resistance to *Escherichia coli* K-12 J53-2 (*pro* Rif^r) was attempted by conjugation in broth and on agar (13). Transconjugants were selected on Diagnostic Sensitivity Test agar (Oxoid) containing imipenem (1 μg/ml) plus rifampin (250 μg/ml).

β-Lactamase fractionation. Logarithmic-phase cells were grown in 10-liter volumes of Nutrient Broth No. 2 (Oxoid), with shaking, and then harvested by centrifugation at 5,000 × *g* for 30 min at 37°C, washed twice in an appropriate buffer (Table 1), and resuspended in 25 ml of the same buffer. The resuspended cells were disrupted by three passes through a French pressure cell at 12,000 lb/in^2 (SLM Aminco, Urbana, Ill.). Debris was then removed by ultracentrifugation at 100,000 × *g* for 45 min at 4°C, and the supernatants were loaded onto anion or cation exchange columns (40 by 2.6 cm) (Table 1), which were equilibrated in the same buffer as the cells. These columns were washed in two or three volumes of the loading buffer and then eluted with the same buffer containing a linear gradient of 0 to 0.5 M NaCl. The nitrocefin-reactive fractions from the washing and gradient elution were individually subjected to isoelectric focusing and tested for their ability to hydrolyze imipenem. Those fractions showing imipenemase activity were retained at −20°C.

β-Lactamase kinetics and inhibition assays. β-Lactamase assays were performed by using spectrophotometry at 37°C in 0.1 M phosphate buffer (pH 7.0), using the wavelengths specified previously (13). V_{max} and the K_m values were calculated from Hanes plots of the initial velocity data. Inhibition assays were conducted under conditions (i) where the enzyme was incubated with the inhibitor for 10 min at 37°C before the addition of penicillin G as the substrate and (ii) where the enzyme was added to a mixture of the inhibitor and the substrate.

PCR amplification of carbapenemase genes. DNA was extracted from the isolates by vortexing and briefly microcentrifuging two colonies suspended in 100 μl of PCR-quality water. The extracted DNA was then screened by PCR for the presence of bla_{OXA-23}-related sequences using the primers 5′-GAT GTG TCA TAG TAT TCG TCG-3′ and 5′-TCA CAA CAA CTA AAA GCA CTG-3′ (based on GenBank accession number AF201828). The conditions comprised 1 cycle at 94°C for 5 min, followed by 30 cycles at 94°C for 25 s, 52°C for 40 s, and 72°C for 50 s and a final elongation at 72°C for 6 min. The DNA extracts also were screened for bla_{OXA-24}-related sequences with the primers 5′-GTA CTA ATC AAA GTT GTG AA-3′ and 5′-TTC CCC TAA CAT GAA TTT GT-3′ (5). The conditions comprised 1 cycle of denaturation at 94°C for 4 min, 30 cycles at 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min and then a final elongation at 72°C for 10 min. Each sample was amplified in triplicate to ensure that there was enough DNA for cloning. The amplified products were recovered with the Recovery DNA Purification Kit II (Hybaid, Teddington, United Kingdom), and the quantity of DNA yielded was calculated with a GeneQuant spectrophotometer (Pharmacia, Milton Keynes, United Kingdom).

Hybridization studies. bla_{OXA-25} and bla_{OXA-27} amplicons were obtained as described above from isolates 327009 (Spain) and I-16 (Singapore), respectively. These products were used in a second round of PCR, in which digoxigenin-11-dUTP (Roche, Lewes, United Kingdom) was added to the mixture to produce labeled probes. These were used to probe genomic DNA prepared by the method of Pitcher et al. (18). Briefly, 5-μg amounts of this DNA were digested with 10 U of *Eco*R1 (Roche), and the restriction fragments were separated by agarose gel electrophoresis and blotted onto nylon membranes. These blots were hybridized with the digoxigenin-labeled probes under conditions of high stringency by the method described elsewhere (24).

Cloning and sequencing of bla_{OXA} genes. The PCR products generated by PCR with primers to bla_{OXA-23} and bla_{OXA-24} (described above) were cloned into pCR2.1-TOPO (Invitrogen, Groningen, The Netherlands), and the recombinant plasmids were transformed into chemically competent cells of *E. coli* TOP10 (Invitrogen) by heat shock, as detailed in the supplier's instructions. Transformants were selected and subcultured on nutrient agar plates containing ampicillin (50 μg/ml). Recombinant plasmid DNA was isolated from secondary cultures and purified using the Wizard *plus* SV Miniprep DNA purification system (Promega, Southampton, United Kingdom).

Cycle sequencing of inserts was performed on both strands by using an ABI Prism Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer, Warrington, United Kingdom) together with M13 forward primer 5′-GTA AAA CGA CGG CCA G-3′ and the reverse primer 5′-CAG GAA ACA GCT ATG AC-3′ (kit primers, Invitrogen). The thermal conditions were 1 cycle of 95°C for 60 s followed by 25 cycles of 96°C for 30 s, 50°C for 15 s, and 60°C for 4 min. The ramp rate was 1°C/s throughout. The samples were processed on an ABI PRISM 310 Genetic Analyser (PE Biosystems, Warrington, United Kingdom), and the raw data were visualized with Chromas 1.45 (http://www.technelysium.com.au/chromas14x.html). The DNA sequences were then manipulated and evaluated with the GCG Wisconsin package (Version 10, UNIX), which was accessed via the Human Genome Mapping Project of the Medical Research Council of the United Kingdom. Protein sequences were aligned with CLUSTAL W (22).

Nucleotide sequence accession numbers. The nucleotide sequences reported here have been assigned the following GenBank accession numbers: bla_{OXA-25}, AF201826; bla_{OXA-26}, AF201827; and bla_{OXA-27}, AF201828.

RESULTS

Antibiotic susceptibility. Isolate A-15 (Kuwait) had low-level carbapenem resistance, with imipenem and meropenem MICs of 4 μg/ml; low-level resistance was seen also in isolate BA HCT 15 (Argentina), as reported previously (2). Isolates 04737 (Belgium), 327009 (Spain), and I-16 (Singapore) had higher levels of carbapenem resistance, with imipenem MICs of 16 to 64 μg/ml and meropenem MICs of 32 to 128 μg/ml (Table 2). All the carbapenem-resistant isolates were also broadly resistant to penicillins and cephalosporins, but the MICs of sulbactam never exceeded 8 μg/ml. Carbapenem MICs for the control isolates collected in 1984 were between 0.12 and 0.5 μg/ml.

TABLE 2. MICs for *Acinetobacter* isolates

Antibiotic	MIC (μg/ml) for the isolate (source)				MICs (μg/ml) for control isolates (n = 40) from 1984[a]		
	04737 (Belgium)	327009 (Spain)	I-16 (Singapore)	A-15 (Kuwait)	Range	MIC_{50}	MIC_{90}
Imipenem	64	64	16	4	0.12–0.5	0.12	0.25
Meropenem	>128	>128	32	8	0.12–0.5	0.25	1
Ceftazidime	>128	>128	>128	16	1–32	8	16
Cefotaxime	>128	>128	>128	64	0.25–128	16	64
Cefuroxime	>128	>128	>128	>128	0.5–128	32	128
Cefepime	8	>128	16	16	0.25–64	8	16
Cefoxitin	>128	>128	>128	>128	2–256	64	128
Ampicillin	>128	>128	>128	>128	4–256	32	256
Piperacillin	>128	>128	>128	>128	4–256	32	256
Aztreonam	128	>128	32	32	0.5–1	1	1
Sulbactam	8	8	8	8	0.5–32	8	32

[a] MIC_{50} and MIC_{90}, MICs at which 50 or 90% of the isolates were inhibited, respectively.

TABLE 3. β-Lactamase profiles of carbapenem-resistant isolates

Source	Isolate	β-Lactamase pI[a]
Belgium	04737	**7.9**, 8.0, 8.2
Kuwait	A-15	6.3, **7.0**, 8.0, 9.5
Spain	327009	7.6, **8.0**
Singapore	I-16	6.0, **6.8**

[a] pI values shown in bold are for enzymes purified and shown to have carbapenemase activity.

Isoelectric focusing. Electrofocusing revealed multiple β-lactamases in all the carbapenem-resistant isolates (Table 3). Multiple enzymes were also found in many control isolates; most of these latter organisms had β-lactamases with pIs of >9.0, but some additionally had enzymes with the pIs characteristic of TEM-1 and -2 (pI, 5.4 and 5.6).

Curing and transfer-of-resistance studies. Neither transfer nor curing of imipenem resistance was achieved for any carbapenem-resistant isolate, despite multiple attempts.

Fractionation of carbapenemases. Ion-exchange chromatography allowed fractionation of the individual β-lactamases produced by the isolates. In each instance, detectable carbapenemase activity fractionated with single enzyme species, as indicated in bold in Table 3. Yields were low, and further purification was not attempted. Relative V_{max} and K_m data for the enzymes from the isolates from Belgium (04737) and Spain (327009) were similar: V_{max} for both these enzymes was greater for oxacillin than for penicillin G, whereas rates for ampicillin, piperacillin, and carbenicillin were 21 to 76% of those for penicillin G (Table 4). Both these enzymes had V_{max} values for cephaloridine that were about 30% of those for penicillin G, were less active against cephalothin than cephaloridine, and had minimal activity (V_{max}, <1% of those for penicillin) against oxyimino-aminothiazolyl cephalosporins. Relative V_{max} values for imipenem were 2.4 to 3% of those of penicillin G; those for meropenem were six- to eightfold lower. The enzyme from isolate A-15 (Kuwait) was similar to those from isolates 04737 and 327009, except that it was 25-fold more active against cephaloridine and had a higher V_{max} (264% of that for penicillin G) for ampicillin, albeit with a low affinity (higher K_m).

The carbapenem-hydrolyzing enzyme from isolate I-16 (Singapore) had a different kinetic profile from those just outlined, being much more active against penicillin G than against any other substrate tested. This enzyme hydrolyzed aminopenicillins and oxacillin with relative V_{max} rates of 3 to 6% of that for penicillin G. Biphasic kinetics were observed for oxacillin. The enzyme achieved very slow hydrolysis of oxyimino-aminothiazolyl cephalosporins and carbapenems, with relative V_{max} values ≤1% of that for penicillin G. All four enzymes were inhibited, albeit weakly, by clavulanate and tazobactam, but not by EDTA (Table 5). NaCl was a weak inhibitor.

Cloning and sequencing of β-lactamase genes. PCR was performed with primers designed from the known sequences of bla_{OXA-23} and bla_{OXA-24}. Isolate I-16 (Singapore) gave a product only with primers to bla_{OXA-23}; isolates 327009 (Spain) and 04737 (Belgium) gave products with primers for bla_{OXA-24}. Isolates BA HCT 15 (Argentina) and A-15 (Kuwait) did not give products with either set of primers, although biochemical characterization indicated their carbapenem-hydrolyzing β-lactamases were OXA types (Table 4 and reference 2). PCR products from I-16 (Singapore), 04737 (Belgium), and 327009 (Spain) were cloned into pCR2.1-TOPO. Isoelectric focusing confirmed that the β-lactamases acquired by these transformants corresponded, in pI, to the carbapenem-hydrolyzing enzymes that had been fractionated and biochemically characterized.

The inserts were sequenced on both strands. The cloned 1,062-bp amplicon from isolate I-16 (Singapore) contained an open reading frame of 822 bp which encoded a product predicted to have 99% amino acid homology with the OXA-23 enzyme (Fig. 1). This new enzyme was designated OXA-27. As compared with bla_{OXA-23}, bla_{OXA-27} had a silent T→C change at nucleotide position 162, an A→G change at nucleotide 283, causing a threonine-to-alanine substitution at amino acid 95, and a T→A change at nucleotide 741, causing an asparagine-to-lysine substitution at residue 247. The cloned fragments from isolates 327009 (Spain) and 04737 (Belgium) were 1,023 bp in size, including reading frames of 825 bp. The peptide deduced for isolate 327009 had 98.5% amino acid homology to OXA-24 β-lactamase (Fig. 2). Nucleotide substitutions, compared with bla_{OXA-24}, were as follows: A→C at position 624, replacing isoleucine with leucine at amino acid 142; A→G at position 424, replacing serine with leucine at amino acid 268;

TABLE 4. Kinetic properties of OXA-25, OXA-26, and OXA-27 β-lactamases and the enzyme from isolate A-15

Substrate	Belgium 04737 (OXA-26)		Spain 327009 (OXA-25)		Singapore I-16 (OXA-27)		Kuwait A-15	
	V_{max} (%)	K_m (μM)	V_{max} (%)	K_m (μM)	V_{max} (%)	K_m (μM)	V_{max} (%)	K_m (μM)
Cephaloridine	27	640	33	590	6	3	710	440
Cephalothin	8	90	3	80	0.3	260	31	20
Cefotaxime	<0.003		0.2	35	0.2	0.1	<0.006	
Cefuroxime	0.04	7	0.4	105	1.0	2	0.3	40
Ceftazidime	0.1		0.01		0.0005		0.006	
Penicillin G	100	25	100	100	100	88	100	65
Ampicillin	55	15	21	21	6	3	264	260
Piperacillin	53	10	22	55	4	10	56	100
Carbenicillin	25	210	76	300	<0.0005		42	95
Oxacillin	500	580	440	840	3 and 0.4[a]	402 and 208[a]	600	211
Imipenem	2.4	3	3	11	0.1	20	3	20
Meropenem	0.4	3	0.4	12	0.04	15	0.006	
Aztreonam	<0.003				<0.0005		0.006	

[a] Initial and steady state values, respectively, in a biphasic hydrolysis.

TABLE 5. Inhibition of carbapenem-hydrolyzing β-lactamases from *Acinetobacter* isolates

Isolate	IC_{50} (μM)[a] for					
	Clavulanate		Tazobactam		EDTA	
	Not pre-inc[b]	Pre-inc[c]	Not pre-inc	Pre-inc	Not pre-inc	Pre-inc
327009-Spain	1.7	0.1	2.0	0.02	>2	>2
04737-Belgium	0.86	0.04	1.6	0.007	>2	>2
A-15-Kuwait	1.10	0.1	1.1	0.05	>2	>2
I-16-Singapore	1.2	0.65	2.5	0.025	>2	>2

[a] 50% inhibitory concentration.
[b] Not pre-inc, enzyme and inhibitor not preincubated together before addition of substrate.
[c] Pre-inc, enzyme and inhibitor preincubated for 10 min at 37°C before the addition of penicillin as the substrate.

and A→G at position 604, replacing lysine with glutamate at position 202. In addition, an extra glutamate residue was inserted between amino acids 199 and 200 (Fig. 2). This enzyme was designated OXA-25. The β-lactamase from isolate 04737 also had 98.5% homology with OXA-24 β-lactamase (Fig. 2). As with OXA-25, isoleucine 142 was replaced by leucine and there was insertion of glutamate between positions 199 and 200. In addition, a T→A mutation resulted in the replacement of serine 257 by threonine. This enzyme was designated OXA-26.

Gene hybridization. The amplicons from isolates I-16 (*bla*$_{OXA-27}$) and 327009 (*bla*$_{OXA-25}$) were labeled with digoxigenin and used to probe digested genomic DNA from the carbapenem-resistant *Acinetobacter* strains. Under conditions of high stringency, the *bla*$_{OXA-27}$ probe hybridized only with DNA from isolate I-16 (Singapore, *bla*$_{OXA-27}$), whereas the *bla*$_{OXA-25}$ probe hybridized with DNA from isolates 327009 (Spain, *bla*$_{OXA-25}$) and 04737 (Belgium, *bla*$_{OXA-26}$) but not with DNA from isolate I-16. Neither probe hybridized with DNA from isolates BA HCT 15 (Argentina) or A-15 (Kuwait).

DISCUSSION

Multiresistance has long been a problem in *A. baumannii*, and carbapenem resistance has begun to appear (3). Although carbapenem-resistant isolates remain rare, they have been found worldwide and have caused major local outbreaks (for examples, see reference 4 and E. T. S. Houang, N. W. S. Lo, A. F. B. Cheng, L. J. V. Piddock, and D. Livermore, Abstr. 40th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 1037, 2000).

Some carbapenem-resistant isolates have metallo-β-lactamases (7, 17; unpublished data), but a greater proportion have unusual OXA-type enzymes with weak activity against carbapenems. In the present paper we describe the properties of four such enzymes: OXA-25, from an isolate (327009) collected in Spain; OXA-26, from an isolate (04737) collected in Belgium; OXA-27, from an isolate (I-16) collected in Singapore; and an unsequenced enzyme from an isolate (A-15) collected in Kuwait. Other carbapenem-hydrolyzing class D enzymes recently sequenced from *Acinetobacter* species include OXA-23 (ARI-1), which is from an isolate collected in Scotland in 1985 (9, 16, 19), and OXA-24, which is from isolates collected in Spain (4, 5). Further unsequenced carbapenemases with the oxacillinase activity typical of class D enzymes include the enzymes from the *Acinetobacter* isolates BA HCT 15, collected in Argentina (2), and A-148, collected in France (12). Despite the phenotypic similarity of their products, PCR and hybridization both failed to demonstrate relationships between the carbapenemase determinants of isolates BA HCT 15 and A-15 and the genes for OXA-23, -24, -25, -26, and -27.

The carbapenem-hydrolyzing OXA enzymes sequenced so far form two clusters. The first cluster comprises OXA-23 and OXA-27 enzymes, with 99% amino acid homology; the second cluster includes OXA-24, OXA-25, and OXA-26 enzymes, which share 98% homology. Homology between these two clusters is only 60%, but they are more closely related to each

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                    *        20         *        40         *        60
oxa23 : MNKYFTCYVVASLFLSGCTVQHNLINETPSQIVQGHNQVIHQYFDEKNTSGVLVIQTDKK
oxa27 : ............................................................

                    *        80         *       100         *       120
oxa23 : INLYGNALSRANTEYVPASTFKMLNALIGLENQKTDINEIFKWKGEKRSFTAWEKDMTLG
oxa27 : ..................................A.........................

                    *       140         *       160         *       180
oxa23 : EAMKLSAVPVYQELARRIGLDLMQKEVKRIGFGNAEIGQQVDNFWLVGPLKVTPIQEVEF
oxa27 : ............................................................

                    *       200         *       220         *       240
oxa23 : VSQLAHTQLPFSEKVQANVKNMLLLEESNGYKIFGKTGWAMDIKPQVGWLTGWVEQPDGK
oxa27 : ............................................................

                    *       260         *
oxa23 : IVAFALNMEMRSEMPASIRNELLMKSLKQLNII
oxa27 : ......K..........................
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FIG. 1. Comparison of the amino acid sequences of OXA-23 and -27 enzymes. The motifs normally conserved among class D enzymes are shaded gray; the proposed signal peptide (9) is shaded black.

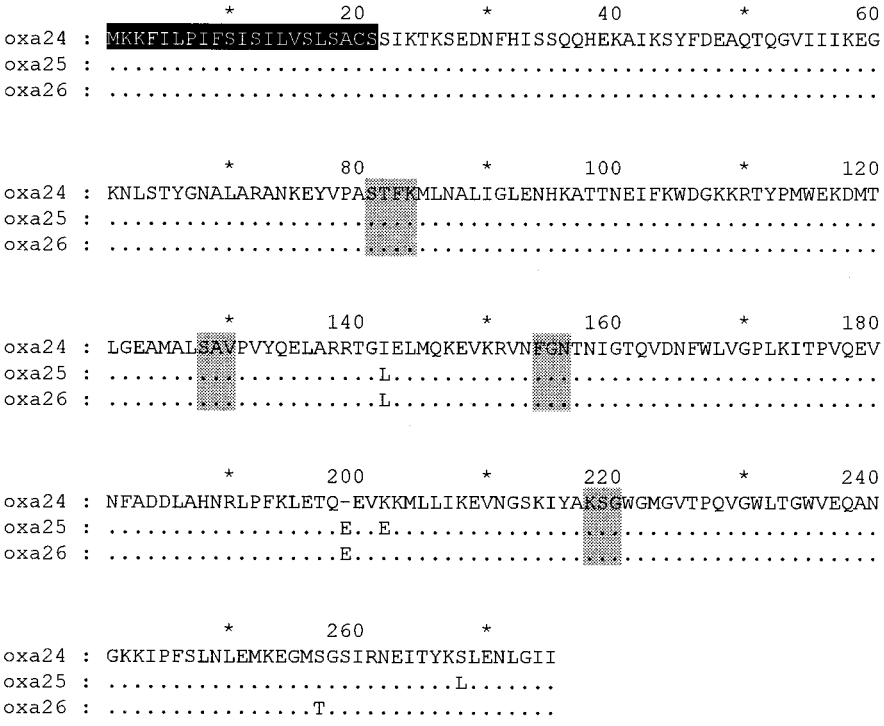

FIG. 2. Comparison of the amino acid sequences of OXA-24, -25, and -26 enzymes. The motifs normally conserved among class D enzymes are shown in gray. The predicted signal peptide (http://www.cbs.dtu.dk/services/SignalP/) is shaded black.

other than to any other OXA-type β-lactamases, none of which has significant carbapenem-hydrolyzing activity. The enzymes all retain the STFK tetrad, which is typical of class D β-lactamases (8, 9), at amino acids 81 to 84 (Fig. 1 and 2) and the SXV triplet at positions 126 to 128; however, the third conserved motif of class D β-lactamases—YGN at positions 154 to 156—is replaced by FGN in all five enzymes, and the final characteristic motif of OXA enzymes—KTG at positions 216 to 218—is retained in OXA-23 and -27 but is replaced by KSG in OXA-24 (positions 217 to 219) and OXA-25 and -26 (positions 218 to 220). Substitutions on the third amino acid of the YGN triplet occur in other OXA β-lactamases, including OXA-11 and LCR-1 (8), but no other OXA enzymes besides the carbapenem-hydrolyzing types have modifications to the first residue. The consistent replacement of tyrosine (Y) by phenylalanine (F) may therefore be significant, as suggested by Donald et al. (9). Moreover, this substitution also implies that the free hydroxyl group of the tyrosine does not play a fundamental role in the hydrolysis of the β-lactam ring, whereas such a fundamental role was proposed (15)—though disputed (10)—for tyrosine 150, which lies in the corresponding structural element of AmpC enzymes. The replacement of threonine (T) by serine (S) in the KTG motif seems unlikely to be significant, given the similarity of these amino acids.

In view of their sequence homology, it is unsurprising that OXA-25 and -26 enzymes had similar kinetic properties (Table 4) and isoelectric points (7.9 and 8.0). Only very limited kinetic data are available for their close relative, the OXA-24 enzyme (4), which was reported to have a pI of 9.0. The lower pIs of OXA-25 and -26 compared with that of OXA-24 are in keeping with the presence of additional glutamate residues in their structures.

OXA-27 had a pI of 6.8 compared with a reported value of 6.65 for OXA-23. This increase in pI accords with the Asn(247)→Lys substitution. From the limited kinetic data available, OXA-23 appears more active than OXA-27 against cephaloridine (V_{max}, 35.5% of that for penicillin G compared with 6% for OXA-27 enzyme), whereas imipenem-hydrolyzing activity was relatively weaker for OXA-23. Curiously, OXA-23 hydrolyzed oxacillin rapidly, relative to ampicillin (H. M. Donald, S. G. B. Amyes, and H. K. Young, Abstr. 39th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 1462, 1999), whereas OXA-27 had only weak activity against both these compounds compared with penicillin G.

Although enzymes OXA-23, -24, -25, -26, and -27 have only feeble carbapenemase activity, MICs of both imipenem and meropenem for the producer strains were consistently higher than those for control isolates collected before carbapenems entered use (Table 2) and also exceeded those for the generality of *Acinetobacter* isolates presently being encountered (≤0.25 μg/ml; data on file at PHLS). Nevertheless, it is not clear whether the enzymes are the sole cause of resistance and it remains possible that some of the source *Acinetobacter* isolates may have possessed secondary resistance mechanisms such as impermeability and up-regulated efflux. In this context it should be added that IMP-type metallo-β-lactamases, which have much greater carbapenemase activity than the present OXA types, often only confer significant carbapenem resistance in *P. aeruginosa* or *Klebsiella pneumoniae* strains that have other secondary mechanisms such as impermeability (20; also T.-H. Koh, D. M. Livermore, et al., unpublished observations). Bou et al. (4) noted reduced expression of 22- and 33-kDa outer membrane proteins in carbapenem-resistant *A. baumannii* with OXA-24 enzyme, and they suggested that

these might be porins and that their diminution might be a contributing factor to resistance. Although the present isolates studied here had other β-lactamases besides those purified (Table 3), these β-lactamases lacked discernible carbapenemase activity after fractionation.

There remains the question of the origin of these carbapenem-hydrolyzing class D β-lactamases. Three models might be envisaged. First, an *Acinetobacter* strain (or strains) may have acquired a parental enzyme gene which has since diversified by mutation. Secondly, related genes may have separately and repeatedly spread into *Acinetobacter* spp. from unknown source organisms. Thirdly, carbapenem-hydrolyzing OXA enzymes may have existed for a long period in a tiny subset of *Acinetobacter* strains which are now being selected. These hypotheses are not mutually exclusive. None can yet be proved, but several comments can be made on their likelihood. First, typing of the host strains for OXA-25, -26, and -27 by pulsed-field gel electrophoresis revealed no similarity (M. E. Kaufmann, personal communication). Secondly, the OXA-23/27 and OXA-24/25/26 enzyme clusters share only ca. 60% amino acid homology, and such wide divergence cannot have evolved rapidly. Thirdly, and in favor of the hypothesis of repeated gene escape, $bla_{\text{OXA-23}}$ has been found on transferable plasmids (19); nevertheless, most of the genes encoding these enzymes seem not to be readily transmissible.

Whatever their origins, carbapenem-hydrolyzing OXA enzymes from *Acinetobacter* spp. present an increasing concern. Their host strains often are broadly resistant to other β-lactam and non-β-lactam antibiotics. Some strains remain susceptible to penicillanic acid sulfones, but others do not; virtually all *Acinetobacter* isolates remain susceptible to polymyxins, but the therapeutic efficacy of these drugs is unreliable. The problems of multiresistance are compounded by the propensity of *A. baumannii* to cause outbreaks, with clonal strain spread among patients (3). The Spanish isolate 327009, which yielded OXA-25 enzyme, was among 28 isolates from an outbreak in Murcia, and the Belgian isolate 04737, with OXA-26 enzyme, represented a strain that was prevalent in Ghent in 1996. Similarly, OXA-24 was isolated from representatives of a strain prevalent at a hospital in Madrid during 1997 (4), and *Acinetobacter* strains with IMP metallo-β-lactamases were repeatedly isolated at a Hong Kong hospital from 1994 onwards (E. T. S. Houang, N. W. S. Lo, A. F. B. Cheng, L. J. V. Piddock, and D. Livermore, Abstr. 40th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 1037, 2000). This combination of clonality and multiresistance presents major challenges for chemotherapy and for infection control.

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