Carbapenems as inhibitors of OXA-13, a novel, integron-encoded β -lactamase in *Pseudomonas aeruginosa*

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A clinical *Pseudomonas aeruginosa* strain, PAe391, was found to be resistant to a number of antibiotics including ticarcillin, piperacillin, cefsulodin and amikacin, and a disk diffusion assay showed evidence of pronounced synergy between imipenem and various β -lactam antibiotics. Cloning and nucleotide sequence analysis revealed the dicistronic arrangement of an *aac(6')-Ib* variant and a novel *bla*_{OXA}-type gene between the *intI* and *qacE Δ 1* genes typical of integrons. In PAe391, this integron was apparently chromosome-borne. The β -lactamase, named OXA-13, displayed nine amino acid changes with respect to OXA-10: I in position 10 of OXA-10 to T (I10T), G20S, D55N, N73S, T107S, Y174F, E229G, S245N and E259A. OXA-13 ($pl_{app} = 8.0$) showed poor catalytic activity against penicillins as well as cephalosporins, but was efficient in hydrolysing some penicillinase-resistant β -lactams, such as cefotaxime and aztreonam. It was efficiently inhibited by imipenem ($K_{iapp} = 11$ nM), and formed a stable complex. While the K_{iapp} value of meropenem was similar (16 nM), the corresponding complex was less stable.

Keywords: β -lactam resistance, oxacillinase, carbapenem, integron, *Pseudomonas aeruginosa*

INTRODUCTION

Production of β -lactamases is the most frequent cause of resistance to β -lactam antibiotics in Gram-negative bacteria (Livermore, 1995). *Pseudomonas aeruginosa* produces a chromosome-encoded cephalosporinase which renders it naturally resistant to ampicillin and cephalothin. This may be one reason why secondary (or 'acquired') β -lactamases are more rarely observed in this species than in the *Enterobacteriaceae*. In *P. aeruginosa*, secondary β -lactamases are mostly plasmid-encoded CARB-, PSE- or OXA-, rather than TEM- or SHV-type enzymes. As a result, the β -lactam susceptibility of strains producing secondary β -lactamases is not consistently restored by the commercially available β -lactamase inhibitors, such as clavulanic acid, sulbactam and tazobactam, which are potent inhibitors of

group 2b and 2c, but not of group 2d enzymes (Bush *et al.*, 1995).

Carbenicillinase and oxacillinase genes are frequently carried by integrons (Lafond *et al.*, 1989; Stokes & Hall, 1989; Hall & Collis, 1995) or related genetic structures, which explains why the β -lactam resistance is associated with resistance to various other antibiotics, including aminoglycosides. Indeed, several genes encoding β -lactam, aminoglycoside, chloramphenicol or trimethoprim resistance may be inserted in the form of cassettes into an ancestral integron, or one that contains one or more resistance gene cassettes, by site-specific integrase-dependent recombination (Grinsted *et al.*, 1990; Stokes & Hall, 1989). The sequential integration of cassettes may then yield a great variety of resistance gene arrangements (Lafond *et al.*, 1989; Lévesque *et al.*, 1995).

Here we describe an oxacillinase variant, OXA-13 (formerly called OXA-12; Mugnier *et al.*, 1994), which is efficiently inhibited by imipenem. This β -lactamase was produced by a clinical *P. aeruginosa* strain, in association with an aminoglycoside 6'-N-acetyltransferase variant, AAC(6')-Ib₁₀. Both the *aac(6')-Ib* and the

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The GenBank/EMBL/DBJ accession number for the sequence reported in this paper is U59183.

*bla*_{OXA} genes were localized, in that order, between the 5'- and the 3'-conserved segments of a *sulI*-type integron.

METHODS

Bacterial strains, plasmids and growth conditions. The bacterial strains and plasmids used are listed in Table 1. *P. aeruginosa* PAe391 was isolated in 1990 at the Saint-Joseph Hospital, Paris, from the sputum of a patient suffering from chronic bronchitis. It was resistant to several anti-*Pseudomonas* β -lactam antibiotics such as ticarcillin, piperacillin and cefsulodin, whether combined with commercially available β -lactamase inhibitors or not, and also to gentamicin, netilmicin, tobramycin, amikacin, isepamicin, sulfonamides and mercuric ions. Bacterial strains were grown in Mueller-Hinton (MH) or Brain Heart Infusion (BHI) media at 37 °C.

Determination of MICs. This was done on MH agar containing serially twofold-diluted antibiotics. Inocula were adjusted to approximately 10^4 c.f.u. per spot and applied with a Steers-type inoculator. Plates were incubated at 37 °C for 18 h. The MICs of β -lactam antibiotics were determined alone or in the presence of clavulanic acid or tazobactam at $4 \mu\text{g ml}^{-1}$. Imipenem was also used as an inhibitor, at $0.03 \mu\text{g ml}^{-1}$ with *Escherichia coli* and $0.25 \mu\text{g ml}^{-1}$ with *P. aeruginosa* strains, i.e., at one-quarter of its respective MIC. MICs were determined twice, with identical results.

Antimicrobial agents were provided as follows: ampicillin, aztreonam and oxacillin, Bristol Myers Squibb; ceftazidime and cephaloridine, Glaxo Group Research; cefotaxime, Hoechst Roussel Pharmaceuticals; piperacillin and tazobac-

tam, Wyeth-Lederle Laboratories; imipenem, Merck Sharp and Dohme; cefoperazone, Pfizer Laboratories; amoxicillin, clavulanic acid and ticarcillin, SmithKline Beecham; benzylpenicillin, Spécia Rhône-Poulenc Rorer Laboratories; cefsulodin, Takeda Laboratories; meropenem, Zeneca Pharmaceuticals.

Plasmid isolation, conjugation, cloning and transformation experiments. Several methods were used to extract plasmid DNA from PAe391 (Kado & Liu, 1981; Takahashi & Nagano, 1984) and recombinant plasmids from *E. coli* (Birnboim & Doly, 1979). Attempts to transfer the β -lactam resistance marker into HB101 and PAO38Rif (Table 1) were made, using mating procedures in solid and liquid media (Thompson, 1986; Robillard, 1990). Plasmids pNJR32 and pAZ309 were transferred to PAO38 from *E. coli* S17.1.

For the cloning of the β -lactam resistance marker, 1 μg of total DNA from PAe391 was digested for 5 h with *EcoRI* and then ligated into the similarly digested vector pHSS6. *E. coli* HB101 was transformed with pHSS6 and its β -lactam-resistance-conferring derivatives, pAZ301 and pAZ304 (Table 1), by electroporation (Bio-Rad gene pulser). Transformants were selected on MH agar plates containing ampicillin ($100 \mu\text{g ml}^{-1}$).

Preparation, purification and N-terminal sequence analysis of OXA-13. Crude enzyme extracts were prepared from cultures grown at 37 °C under vigorous shaking to an OD₆₅₀ of approximately 0.8. Cells were harvested by centrifugation and disrupted by sonolysis in sodium phosphate buffer (10 mM, pH 7.0). Lysates were centrifuged for 15 min at 4000 g and then for 45 min at 100 000 g. The supernatants

Table 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or description	Resistance phenotype*	Reference or source
<i>P. aeruginosa</i> strains			
PAe391	Clinical strain	Ti Im Ak Ge Su Hg	This study
PAO38	β -lactam- and aminoglycoside-susceptible strain		Pemberton & Holloway (1972)
PAO38Rif	High-level rifampin-resistant derivative of PAO38 (MIC > 500 $\mu\text{g ml}^{-1}$)	Ri	This study
<i>E. coli</i> strains			
HB101	F ⁻ <i>D(gpt-proA)62 LeuB6 supE44 ara-14 galK2 lacY1 D(mcrC-mrr) rpsL20(StrR) xyl-5 mtl-1 recA13</i>	Sm	New England Biolabs
S17.1	Pro ⁻ Res ⁻ Mod ⁺	Tp Sm	Robillard (1990)
Plasmids			
pHSS6	2.3 kb plasmid vector	Km	Seifert <i>et al.</i> (1986)
pAZ505	pBR322 carrying <i>aac(6')</i> -Ib	Ti Ak	Tran Van Nhieu & Collatz (1987)
pAZ301	7.3 kb <i>EcoRI</i> fragment obtained by digestion of PAe391 total DNA, cloned into pHSS6	Km Ti Ak Su	This study
pAZ304	2.7 kb <i>BamHI</i> - <i>PstI</i> fragment of pAZ301 cloned into the <i>BamHI</i> - <i>PstI</i> site of pHSS6	Km Ti Ak	This study
pNJR32	28 kb <i>E. coli</i> - <i>P. aeruginosa</i> shuttle vector	Tc	Robillard (1990)
pAZ309	4.0 kb <i>HindIII</i> fragment of pAZ301 cloned into the <i>HindIII</i> site of pNJR32	Tc Ti Ak	This study

* Resistance to: Ak, amikacin; Ge, gentamicin; Hg, mercuric ions; Im, imipenem; Km, kanamycin; Ri, rifampin; Sm, streptomycin; Su, sulfonamide; Tc, tetracycline; Ti, ticarcillin; Tp, trimethoprim.

Fig. 1. Restriction map of the *aac(6)-lb₁₀*- and *bla_{OXA-13}*-encoding 7.3 kb region of PAe391. The portion sequenced is indicated by the thick line; small arrows represent the newly synthesized primers used. The restriction endonuclease sites were determined on the isolated fragment (data not shown).

CCGTGGAAC	GGATGAAGC	ACGAACCCG	<u>TTGACATAAG</u>	CCTGTTCCGGT	<u>TCGTAACCTG</u>	TAATGCAAGT	70
H F	R I F A	R V W	N V Y	A Q E T	R L S	Y H L	
AGCGTATCG	CTCAGCGAAC	TGGTCCAGAA	CCTTGACCGA	ACGCAGCGGT	GGTAACGGCG	CAGTGGCGGT	140
Y R I R	E R L	Q D L	V K V S	R L P	P L P	A T A T	
<i>int</i>							
TTTCATGGCT	TGTTATGACT	GTTTTTTTGT	ACAGTCTATG	CCTCGGGCAT	CCAAGCAGCA	AGCGCGTTAC	210
K M							
GCGGTGGTC	GATGTTTGAT	GTTATGGAGC	AGCAACGATG	TTACGCAGCA	GCAGTCGCC	<u>TAAACAAAG</u>	280
<u>TTTGGCATCA</u>	CAAAGTACAG	CATCGTGACC	AACAGCAACG	ATTCGCTCAC	ACTGCGCCTC	ATGACTGAGC	350
L G I T	K Y S	I V T	N S N D	S V T	L R L	M T E H	
ATGACCTTGC	GATGCTCTAT	GAGTGGCTAA	ATCGATCTCA	TATCGTCGAG	TGCTGGGGCG	GAGAAGAAGC	420
D L A	M L Y	E W L N	R S H	T V E	W W G	E E A	
ACGCCGACAC	CTTGCTGACG	TACAGGAACA	GTACTTGCCA	AGCGTTTTAT	CGCAAGAGTC	CGTCACTCCA	490
R P T	L A D V	Q E Q	Y L P	S V L A	Q E S	V T P	
TACATTGCAA	TGCTGAATGG	AGAGCCGATT	GGGTATGCC	AGTCGTACGT	TGCTCTTGA	AGCGGGGAGC	560
Y I A M	L N G	E P I	G Y A Q	S Y V	A L G	S G D G	
GATGGTGGGA	AGAAGAAACC	GATCCAGGAG	TACCGGAAT	AGACCACTTA	CTGGCGAATG	CATCACAACT	630
W W E	E E T	D P G V	R G I	D Q L	L A N A	S Q L	
GGGCAAGGC	TTGGGAACCA	AGCTGGTTCG	AGCTCTGGTT	GAGTTGCTGT	TCAATGATCC	CGAGGTCAAC	700
G K G	L G T K	L V R	A L V	E L L F	N D P	E V T	
AAGATCCAAA	CGGACCCGTC	GCCGAGCAAC	TTGCGAGCGA	TCCGATGCTA	CGAGAAAGCG	GGGTTTGAGA	770
K I Q T	D P S	P S N	L R A I	R C Y	E K A	G F E R	
GGCAAGGTAC	CGTAACACC	CCAGATGGTC	CATGGTTCAA	ACACGCCAGG	CATTCTGAGC		840
Q G T	V T T	P D G P	A V Y	M V Q	T R Q A	F E R	
AACACGCAGT	GATGCTTAAC	CCTTCCATCG	AGGGGGACGT	CGAAGGGCTG	CGGCCCTTGA	CGGCCCTTCA	910
T R S	D A						
<u>TTGCAACAGT</u>	<u>TTGCCACCAA</u>	<u>GAAGGTGCCA</u>	<u>TGAAACATT</u>	<u>TGCCGCATAT</u>	<u>GTAATTACTG</u>	<u>CGTGTCTTTC</u>	980
AAGTACGGCA	TTAGCTAGTT	CAATTACAGA	AAATACGTCT	TGGAACAAAG	AGTTCTCTCT	CGAAGCCGTC	1050
s t a	l a	i t e	n t s	w n k e	f s a	e a v	
AATGGTGT	TCGTCTTTG	TAAAGATAGC	AGTAAATCCT	CGCTACCAA	TAACTTAGCT	CGTGCATCAA	1120
N G V F	V L C	K S S	C	A T T	L A R	A S K	
AGGAATATCT	TCCAGCATCA	ACATTTAAGA	TCGCCAGCGC	AATTATCGCG	CTAGAAACTG	GTGTCTATAA	1190
E V L	P A S	T F K I	P A	T T G	L E T	G	
GAATGAGCAT	CAGTCTTTCA	AATGGACGG	AAAGCGAAGA	GCCATGAAC	AATGGGAAG	AGACTTTGAGC	1260
N E H	Q V F K	W D G	K P R	A M K Q	W E R	D L S	
TTAAGAGGG	CAATACAAGT	TTGACCGGTT	CCCGTATTTC	AACAATCCG	CAGAGAAGTT	GGCGAAGTAA	1330
L R G A	I Q V	S A V	P V F Q	Q I A	R E V	G E V R	
GAATGCAGAA	ATACCTTAAA	AAATTTTCAT	ATGGCAACCA	GAATATCAGT	GGTGGCATTG	ACAAATTCGT	1400
M O K	Y L K	K F S Y	G N Q	N I S	G G I D	K F W	
GTGCGAGGT	CAGCTAAGAA	TTTCCGAGT	TAATCAAGTG	GAGTTTCTAG	AGTCTCTATT	TTTAAATAAA	1470
L E G	Q L R I	S A V	N Q V	E F L E	S L F	L N K	
TTGTCAGCAT	CAAAAGAAAA	TCAGCTAATA	GTAAGAGAGG	CTTTGGTAAC	GGAGGCTGCG	CCTGAATATC	1540
L S A S	K E N	L I	V K E A	L V T	E A P	E Y L	
TTGTGCATTC	AAAAACTGGT	TTTCTGGTG	TGGGAATCGA	GTCAAATCCT	GGTGTCCGAT	GGTGGGTTTG	1610
V H S	K T G	F S G V	G T E	S N P	G V A W	W V G	
TTGGGTTGAG	AAGGCAACAG	AGGTTTACTT	TTTCGCCTTT	AACATGGATA	TAGACAAAGA	AAATAAGTTG	1680
W V E	K	V Y F	F A P	N M D I	D N E	N K L	
CCGCTAAGAA	AATCATTCC	CACCAAAATC	ATGCCAAGTG	AGGGCATCAT	TGTTGGCTAA	CAATTCTGCTG	1750
P L R K	S I P	T K I	M S E	G I T	G		
<u>CAGCGCGAC</u>	<u>GGCCCTGACG</u>	<u>GCCGCGGCT</u>	<u>GAGCTCAAAC</u>	<u>GTTAGATGCA</u>	<u>CTAAGCAGAT</u>	<u>AATTGCTCAC</u>	1820
AGCCAAACTA	TCAGGTCAAG	TCTGCTTTTA	TTATTTTAA	CGGTGCATAA	TAAGCCCTAC	ACAAATTGGG	1890
AGATATATCA	TGAAAGGCTT	GCTTTTCTT	CTTATCGCAA	TAGTTGGCGA	AGTAATCGCA	ACATCCGCAT	1960
N H	M K G W	L F L	V I A I	V G E	V I A	T S A L	
TAAATCTAG	CG						1972
K S S							

Fig. 2. Nucleotide sequence of the *aac(6)-lb₁₀*, *bla_{OXA-13}* region carried by pAZ304. The 5' conserved integrator segment ends at nucleotide 282, and the 3' conserved segment begins at nucleotide 1790. Putative promoter sequences (−10, −35) and ribosome-binding sites (RBS) are underlined. Recombination sites are boxed. Two 12 bp inverted repeat sequences are underlined with broken arrows. Partial or complete deduced amino acid sequences are given for the integrase, AAC(6)-lb₁₀, OXA-13, and the *qacEΔ1*-encoded quaternary ammonium resistance marker. The amino acids given in lower case letters represent the signal peptide of OXA-13, the letters in italics the amino acids determined by Edman degradation, and the letters in bold the conserved amino acid of class D enzymes (Huovinen et al., 1988). OXA-13 displayed nine amino acid changes with respect to OXA-10: I in position 10 of OXA-10 to T (I10T), G20S, D55N, N73S, T107S, Y174F, E229G, S245N and E259A. The corresponding positions are circled.

(S100) were used for isoelectric focusing experiments and for assaying the stability of β -lactamase:inhibitor complexes.

OXA-13 was partially purified from a crude extract of HB101(pAZ304) using the preparative isoelectric focusing cell Rotofor (Bio-Rad). The fraction containing the highest β -lactamase activity was used for the determination of kinetic parameters. This fraction was subjected to SDS-PAGE (Laemmli, 1970) and the β -lactamase concentration was estimated by comparative densitometry with BSA as standard. For N-terminal sequence analysis, OXA-13 was transferred to

a PVDF membrane (Millipore), stained with Coomassie blue and cut out, and its N-terminal amino acid sequence determined on a model 473 protein sequencer (Applied Biosystems). Phenylthiohydantoin derivatives of the eight N-terminal amino acids were separated and identified by on-line reverse phase-HPLC with an RP-18 column (Brownlee Labs, Applied Biosystems). This analysis was carried out in the laboratory of L. Camoin (ICGM, Paris, France).

Isoelectric focusing. S100 preparations were analysed by isoelectric focusing using a mini IEF cell III (Bio-Rad) and a

Table 2. MICs of β -lactam antibiotics and amikacin for PAe391 and transformants

Antibiotic	Inhibitor*	MIC ($\mu\text{g ml}^{-1}$)				
		PAe391	PAO38 (pAZ309)	HB101 (pAZ304)	PAO38 (pNJR32)	HB101 (pHSS6)
Amoxicillin	—	> 512	> 512	> 512	> 512	8
	Cla	> 512	> 512	512	> 512	4
	Imi	> 512	> 512	32	> 512	4
Ticarcillin	—	128	256	> 512	8	4
	Cla	64	256	256	16	2
	Imi	8	64	16	64	2
Piperacillin	—	32	32	64	2	1
	Taz	32	32	32	2	1
Cefoperazone	—	128	128	32	2	0.12
Cefotaxime	—	8	16	2	8	0.06
Ceftazidime	—	2	2	1	1	0.12
	Cla	2	2	0.12	2	0.12
	Imi	4	16	0.12	16	0.12
Aztreonam	—	4	8	4	2	0.12
	Cla	2	4	0.25	2	0.06
	Imi	2	8	0.12	16	0.06
Cefsulodin	—	16	32	ND	1	ND
	Imi	2	16	ND	8	ND
Meropenem	—	0.5	2	0.03	0.5	0.015
	Imi	0.12	0.5	0.015	1	0.015
Imipenem	—	4	1	0.12	1	0.12
Amikacin	—	16	16	64	4	8

ND, Not determined (natural low susceptibility to cefsulodin).

* Cla, clavulanic acid ($2 \mu\text{g ml}^{-1}$); Imi, imipenem ($0.03 \mu\text{g ml}^{-1}$ for *E. coli* strains and $0.25 \mu\text{g ml}^{-1}$ for *P. aeruginosa* strains); Taz, tazobactam ($4 \mu\text{g ml}^{-1}$); —, none.

gradient made up of one-half polyampholytes (Serva) of pH range 2–11 and one-half of pH range 6–9. Extracts of OXA-10-, SHV-1- and SHV-5-producing strains were used as standards for pIs of 6.1, 7.6 and 8.2, respectively. β -Lactamases were revealed by overlay with nitrocefin (0.1 mg ml^{-1}) in phosphate buffer (50 mM, pH 7.0).

Enzyme assays. Kinetic parameters were determined spectrophotometrically in phosphate buffer (50 mM, pH 7.0) at 30°C with a model 550S double beam spectrophotometer (Perkin Elmer). Relative V_{max} and K_m values of substrates were determined by computerized linear regression analysis of Eadie–Hofstee plots. K_{app} values of inhibitors were deduced from Dixon plots. When burst or lag phenomena were observed during the initial phase of hydrolysis, the subsequent steady state rates were used for the determination of the kinetic constants.

Estimation of the stability of OXA-13:inhibitor complexes. An S100 sample of OXA-13 was incubated for 30 min at room temperature in phosphate buffer (50 mM, pH 7.0) in the presence of $70 \mu\text{M}$ imipenem or meropenem. Free inhibitor was separated from the OXA-13:inhibitor complex during isoelectric focusing (see above), or by gel filtration on a $1 \times 30 \text{ cm}$ Trisacryl/GF05 column (IBF-LKB), equilibrated with phosphate buffer (50 mM, pH 7.0) at 4°C . The column fraction with the highest absorbance at 280 nm was used for

the subsequent steps. A second S100 sample of identical volume and concentration, incubated in the absence of inhibitor, was processed in exactly the same way as a control. The experimental and control column fractions of practically identical OD_{280} (less than 8% variation) were diluted 18-fold in phosphate buffer (50 mM, pH 7.0) and kept at 30°C . The enzymic activity was determined in 900 μl aliquots at regular time intervals with cephaloridine ($100 \mu\text{M}$) as the substrate. The relative hydrolytic activity in the extract preincubated with inhibitor was expressed with respect to the activity in the control, which was set at 1 at each time point. The stability of the complexes was estimated from two independent experiments.

DNA sequencing. The 3.0 kb *Bam*HI–*Hind*III fragment of pAZ301 (Fig. 1) was cloned into the *Bam*HI–*Hind*III site of phages M13mp18 and M13mp19. Two clones of each single-stranded recombinant phage were partially sequenced by the dideoxy chain-termination method using the T7 sequencing kit (Pharmacia). The universal primer and custom synthesized primers were used. When putative compressions were observed after the sequencing of one strand, the sequence of the complementary strand was determined. Since compressions are frequently encountered during DNA sequencing of integron-associated recombination sites (Hall *et al.*, 1991), the nucleotide sequences from positions 850 to 930 and 1730 to

1800 (Fig. 2) were determined on both strands using deaza-nucleotides and a thermal cycle DNA sequencing system (*fmol* DNA sequencing system, Promega).

RESULTS

The clinical strain PAe391 was moderately resistant to ticarcillin, piperacillin, cefoperazone and cefsulodin, and showed reduced susceptibility to aztreonam and imipenem (Table 2). It was also resistant to amikacin, tobramycin, isepamicin, netilmicin and gentamicin (data not shown), compatible with the production of an AAC(6')-I and an AAC(3)-II (Shaw *et al.*, 1993), and to sulfonamides (Table 1). PAe391 was peculiar in that it appeared susceptible to the synergistic activity of imipenem on one hand, and ticarcillin, cefsulodin, aztreonam or meropenem on the other, when tested in a standard disk diffusion assay (for cefsulodin and meropenem, see Fig. 3).

Transfer and cloning of the resistance genes of PAe391

Despite the use of two techniques and repeated attempts, no resistance marker was found to be transferable by conjugation, either to *P. aeruginosa* PAO38Rif or to *E. coli* HB101. No plasmid was detected in PAe391, even when techniques appropriate for the preparation of large plasmids (≥ 100 kb) were used.

Expression of β -lactam resistance was observed in *E. coli* HB101 after transformation with recombinant plasmids which were obtained by cloning of apparently chromosomal, 7.3, 2.7 and 4 kb fragments from PAe391 into pHSS6, yielding pAZ301 and pAZ304, and into pNJR32, yielding pAZ309, respectively (Table 1). Plasmid pAZ301 coded for β -lactam, amikacin and sulfonamide resistance, while pAZ304 and pAZ309 coded for β -lactam and aminoglycoside resistance only.

Fig. 3. Disk diffusion assay of PAe391 with imipenem (I), meropenem (M) and cefsulodin (C). The extensions of the circular zones of growth inhibition around M and C are indicative of enzyme inhibition by imipenem.

β -Lactam resistance phenotypes

As shown in Table 2, the β -lactam resistance levels of PAe391 and PAO38(pAZ309) were almost identical and overall very similar to those of *E. coli* HB101(pAZ304). The increases in MICs for the resistant transformants, when compared to their homologues carrying the cloning vector only, were obvious with ticarcillin, piperacillin and cefoperazone, and more subtle with aztreonam, cefotaxime and ceftazidime. The increases observed with these two groups of antibiotics were more pronounced in *E. coli*. They ranged, respectively, from 16- to 64-, and 2- to 4-fold for *P. aeruginosa*, and from 64- to 256-, and 8- to 32-fold for *E. coli*. The MIC of cefsulodin increased 32-fold for *P. aeruginosa*.

Clavulanic acid, and even more so imipenem, when used in association with other β -lactam antibiotics against the resistant transformants, restored various degrees of the β -lactam susceptibility that was displayed by the isogenic recipients. This phenomenon was somewhat masked in *P. aeruginosa*, particularly in PAO38(pAZ309), due to the well-known inducibility, by imipenem, of the chromosome-encoded cephalosporinase in this species (Livermore & Yang, 1987). Surprisingly, and as observed in Fig. 3, there was also some synergistic effect between the two carbapenems, meropenem and imipenem, which was not masked in *P. aeruginosa* (Table 2), most likely because meropenem is not hydrolysed by the chromosomal enzyme, even after induction (Livermore, 1991).

The decreased susceptibility of PAe391 to imipenem was attributed to the decrease in production of an outer-membrane protein of approximately 45 kDa (Fig. 4), possibly OprD2 (Trias & Nikaido, 1990).

Identification and sequence determination of the β -lactam and aminoglycoside resistance genes of PAe391

The nucleotide sequence of a 1972 bp portion of the 3 kb *Bam*HI–*Hind*III fragment of pAZ301 was determined (Figs 1, 2). Analysis of the nucleotide and deduced amino acid sequences revealed the presence of two resistance genes in the same orientation, encoding an aminoglycoside 6'-N-acetyltransferase and an oxacillinase. Both genes were carried by an integron-related structure (Stokes & Hall, 1989). The sequenced portion included at its extremities the 5' conserved integron segment of Tn21 (nucleotides 1–282), encoding the integrase *IntI* (Ouellette & Roy, 1987), a central section containing the two antibiotic resistance genes, and the 3' conserved segment (nucleotides 1791–1972) containing part of the *qacEA1* gene (Paulsen *et al.*, 1993).

Three recombination site sequences, also called 59 bp elements (Cameron *et al.*, 1986; Hall *et al.*, 1991), hot spots (hs, Schmidt *et al.*, 1989) or recombination hot spots (RHS, Francia *et al.*, 1993), were found (Fig. 2). The hs2 sequence was typical of the 3' extremity of the 5' conserved segment. The 67 bp sequence downstream

Fig. 4. Outer-membrane protein (OMP) profiles of *P. aeruginosa* strains. OMPs were prepared as described by Lee *et al.* (1992) but stained with Coomassie blue. Lanes: 2, PAe MDK1395-S (MIC of imipenem: $2 \mu\text{g ml}^{-1}$); 3, PAe MDK1395-R (MIC of imipenem: $32 \mu\text{g ml}^{-1}$) (for a description of the two PAe strains labelled MDK, see Zhou *et al.*, 1993); 4, PAe191 (clinical isolate; MIC of imipenem: $1 \mu\text{g ml}^{-1}$); 5, PAe391. The arrow indicates the position of the presumed OprD2 protein. Molecular mass markers (in kDa) are shown in lane 1.

Fig. 5. Immunoblot of AAC(6')-Ib₁₀ (lane 1) and AAC(6')-Ib (200 amino acids, lane 2) produced by HB101(pAZ505). The positions of molecular mass markers (in kDa) are indicated on the left.

from the aminoglycoside resistance gene was similar to corresponding sequences found adjacent to other AAC(6')-Ib genes (Tran Van Nhieu & Collatz, 1987; Galimand *et al.*, 1993), but less so to the 59 bp consensus sequence (Hall *et al.*, 1991), while the 61 bp sequence downstream from the OXA-13 gene was similar to the corresponding sequence of OXA-7 (Scoulica *et al.*, 1995)

Fig. 6. Isoelectric focusing of OXA-13. Equal amounts of soluble proteins (S100) from HB101(pAZ304) were focused, without (lane 2), or after preincubation with imipenem (lane 3), or meropenem (lane 4), or meropenem (lane 5). β -Lactamase activity was revealed by overlay with nitrocefin. Isoelectric point standards were SHV-1 (pI 7.6, lane 1) and SHV-5 (pI 8.2, lane 2).

and also to the 59 bp consensus sequence (Hall *et al.*, 1991).

The nucleotide sequence of the *aac(6')-Ib* gene was most similar to that carried on plasmid pAZ007 (Tran Van Nhieu & Collatz, 1987), with the exception of the 5' terminus, raising the question of the identity of the translation initiation codon. Using immunoblotting with anti-AAC(6')-Ib antibodies as described previously (Tran Van Nhieu *et al.*, 1992), PAe391 was found to produce a cross-reactive protein with a slightly lower mobility than that of the pAZ007-encoded AAC(6')-Ib, which is 200 amino acids in length (Fig. 5). The only start codon preceded by a putative ribosome-binding site and consistent with a protein longer than 200 amino acids appeared to be the A248TG codon (Fig. 2), the same codon that was previously determined experimentally to initiate translation of a different acetyltransferase gene (Wohlleben *et al.*, 1989). The resulting AAC variant of 203 amino acids with a computed molecular mass of 22.6 kDa was named AAC(6')-Ib₁₀.

The second ORF coded for a protein of 266 amino acids of the OXA-type β -lactamases, showing 97% amino acid identity with both OXA-10 (Huovinen *et al.*, 1988) and OXA-7 (Scoulica *et al.*, 1995). It was a novel variant of this family and was named OXA-13. N-terminal analysis of the mature protein yielded the sequence Ser-Ser-Ile-Thr-Glu-Asn-Thr-Ser-Trp. The deduced signal peptide was 19 amino acids in length and the computed molecular mass of the mature protein 27.4 kDa.

Properties of the β -lactamase OXA-13

Isoelectric focusing of an S100 preparation from HB101-(pAZ304) revealed the production of a β -lactamase with a pI_{app} of 8.0 (Fig. 6, lane 3). Non-induced PAe391 produced this same OXA-13 enzyme, but after induction at mid-exponential-phase with imipenem at $5 \mu\text{g ml}^{-1}$, no OXA-13 activity was apparent, and only the in-

Table 3. Kinetic constants of OXA-13

	Substrate*						
	Cri	Pen G	Amp	Oxa	Ctx	Atm	Caz†
Relative $V_{\max}$ (%)	100‡	69	80	213	119	4	—
K_m (μ M)	615	65	62	71	691	104	—
Relative $V_{\max}/K_m$ (%)	100§	530	640	1840	105	24	—

* Hydrolysis of: Cri, cephaloridine; Pen G, penicillin G; Amp, ampicillin; Oxa, oxacillin; Ctx, cefotaxime; Atm, aztreonam; Caz, ceftazidime.

† Hydrolysis of ceftazidime was detected, but kinetic parameters were not measurable.

‡ Corresponds to 11.4 mmol s^{-1} .

§ Corresponds to 7 s^{-1} .

ducible cephalosporinase, with a pI_{app} of 8.2, was observed after isoelectric focusing (data not shown). The presence of OXA-13 was similarly masked when the S100 proteins from HB101(pAZ304) were incubated with imipenem prior to isoelectric focusing (Fig. 6, lane 4). Masking of the OXA-13 activity under these conditions was much less evident with meropenem (Fig. 6, lane 5).

Kinetic constants for OXA-13 produced by HB101(pAZ304) are shown in Table 3. The K_m values of the three penicillins tested were similar, (60–70 μ M), and approximately ten times lower than those of cephaloridine and cefotaxime. The highest relative $V_{\max}/K_m$ ratio was observed for oxacillin, which is in agreement with the molecular features of OXA-13, a class D β -lactamase closely related to OXA-10 (Ledent *et al.*, 1993). OXA-13 was more efficient as a penicillinase than as a cephalosporinase, with rather high K_m values for those cephalosporins for which they could be determined, but was able to hydrolyse cefotaxime and ceftazidime as well as aztreonam. This should explain the increase in the MICs of these antibiotics for the enzyme-producing *E. coli* (Table 2). Kinetic constants could not, however, be determined for ceftazidime, possibly because the K_m of this compound was much higher than the substrate concentrations that could be used under our experimental conditions.

In a disk diffusion test, imipenem showed inhibitor activity against OXA-13, as evidenced by increased zones of growth inhibition around disks containing β -lactams that can be hydrolysed by the enzyme, such as cefsulodin, and to some degree apparently also meropenem (Fig. 3). Meropenem and clavulanic acid displayed no inhibitor activity in this test (data not shown). The differences in inhibitor activities of the carbapenems could not be attributed to differences in affinity, since the K_{app} values of imipenem and meropenem, 11 and 16 nM, respectively, were found to be similar, while that of clavulanic acid was 54 μ M.

The stability of the OXA-13:carbapenem complexes was estimated by determining the kinetics of enzyme

Fig. 7. OXA-13 reactivation kinetics. Enzyme:inhibitor complexes were isolated by gel filtration after preincubation of an S100 sample from HB101(pAZ304) in the presence of 70 μ M of imipenem (Δ) or meropenem ($\bullet$) at 30 °C. A control sample was preincubated in the absence of inhibitor, but otherwise treated identically. Enzyme activity was measured with cephaloridine as the substrate and the activity in the control ($58\text{--}72 \text{ nmol min}^{-1} \text{ mg}^{-1}$ at time zero) set at 1 at each time point. For details see Methods.

reactivation (Fig. 7). Under the conditions used, the rate of hydrolysis of cephaloridine was completely stable for 8 h. It decreased to approximately 70% after 18 h and to approximately 50% after 24 h. In agreement with the observation shown in Fig. 6, imipenem formed a very stable complex. About 30% of the enzyme remained inhibited after 24 h at 30 °C, following a biphasic course of recovery of enzyme activity with an initial rapid reactivation over 2 h, and a much slower reactivation thereafter (Fig. 7). By contrast, the OXA-13:meropenem complex was less stable, with 100% of the β -lactamase activity regained after 18 h at a steady rate throughout. The apparent half-lives of the respective OXA-carba-

penem complexes were estimated to be approximately 12 and 5 h.

DISCUSSION

The susceptibility pattern of the clinical *P. aeruginosa* strain PAe391, with resistance to β -lactam antibiotics, aminoglycosides, sulfonamides and mercuric ions, raised the possibility that the resistance genes were carried by a Tn21-related transposable element, and nucleotide sequence analysis revealed that novel variants of an aminoglycoside acetyltransferase gene, *aac(6')-Ib₁₀*, and of a β -lactamase gene, *bla_{OXA-13}*, were inserted in the form of cassettes in an integron structure. This integron appeared to be chromosome-borne, an apparently uncommon situation (Stokes & Hall, 1989; Francia & Garcia Lobo, 1996). Although its complete sequence has not been determined, it is likely that it also encodes the sulfonamide resistance determinant which was present in the cloned DNA fragment of pAZ301, a fragment with integron-typical endonuclease restriction sites (Fig. 1). The responsible gene, *sull*, is normally found in the 3' conserved integron segment (Bissonnette & Roy, 1992; Stokes & Hall, 1989). The arrangement of an *aac(6')-Ib* and a *bla_{OXA}* gene within an integron does not seem to have been reported previously. However, in an arrangement somewhat similar to that observed on the cloned fragment of pAZ301, a segment of approximately 600 bp separated an *aac(6')-Ib* from the *bla_{OXA-3}* gene (Sanschagrín *et al.*, 1995), incompatible with the length of the recombination site sequences typically found between the gene cassettes in integrons (Hall *et al.*, 1991).

Comparative analysis by immunoblotting of the

AAC(6')-Ib produced by PAe391 revealed that it was slightly larger than the prototype enzyme of 200 amino acids (Fig. 5). Considering the genomic context of *aac(6')-Ib* in PAe391, it appeared that the translation initiation codon was localized in the 5' conserved segment of the integron, upstream from the recombination site named *hs2* by Schmidt *et al.* (1989). An ATG codon preceded by a ribosome-binding site has been identified in that location in two aminoglycoside acetyltransferase genes, *aac(3)-Ia* (Wohlleben *et al.*, 1989; Tenover *et al.*, 1989) and *aac(6')-Ib* (Mabilat *et al.*, 1992), which carry a 19 bp duplication adjacent to the recombination site (Fig. 8). It is likely that the same initiation codon is functional in PAe391, although the N-terminal sequence of its AAC(6')-Ib₁₀, encoded by the 5' conserved integron segment, is different from that of the other two acetyltransferases. One explanation for this difference could be a 21 bp in-frame deletion covering part of the 19 bp duplication (Fig. 8). The N-terminal sequence of AAC(6')-Ib₁₀ in PAe391 might alternatively result from the deletion of two G residues (positions 58 and 59 in Fig. 8) in an integron lacking the 19 bp duplication. A different deletion affecting the 19 bp duplication has been observed in the integron carried by pAT127, leaving no start codon in the proper reading frame in that region and resulting in the synthesis of an acetyltransferase thought to be shorter by approximately 30 amino acids (Galimand *et al.*, 1993). Beyond the tenth amino acid, AAC(6')-Ib₁₀ was identical to the variant initially described (Tran Van Nhieu & Collatz, 1987) and those referred to in Fig. 8, except for the two penultimate residues (cf. Fig. 2).

The second resistance gene cassette in the integron of PAe391 coded for an oxacillin-hydrolysing β -lactamase,

Fig. 8. Deduced N-terminal AAC(6')-Ib sequences. The start codon on pCFF04 (Mabilat *et al.*, 1992; positions 25–27 in this figure) was localized in the first of two 19 bp direct repeat sequences at the 3' extremity of the 5' conserved integron segment of Tn21 by analogy with that determined previously for the *aac(3)-Ia* gene (Wohlleben *et al.*, 1989). The corresponding codon on pAZ301 would remain in-frame after a 21 bp deletion, but would be eliminated by a 19 bp deletion on pAT127 described by Galimand *et al.* (1993). Open rectangles, putative ribosome-binding sites; filled rectangles, start codons.

which was named OXA-13. OXA-type enzymes, less closely related to one another than the TEM- and SHV-type enzymes (Jacoby, 1994), have been assigned to four groups (Sanschagrin *et al.*, 1995). OXA-13 is closest to the OXA-10-related group. It differs from OXA-7 (Scoulica *et al.*, 1995) and OXA-10 (formerly PSE-2; Huovinen *et al.*, 1988) in seven and eight amino acids, respectively, and from the extended-spectrum, ceftazidime-resistance-conferring β -lactamases of this group, OXA-11 (Hall *et al.*, 1993), OXA-14 (Danel *et al.*, 1995), OXA-13-1 (formerly OXA-13; Mugnier *et al.*, 1994) and OXA-19 (P. Mugnier, I. Casin, & E. Collatz, unpublished results), essentially in that it does not display the Gly-to-Asp change in position 157. Determination of the kinetic parameters of OXA-13 showed that it is a broad-spectrum β -lactamase only moderately efficient against cephalosporins and aztreonam. This is in agreement with the moderate increase in the MICs of most β -lactams for PAe391. The property of hydrolysing the more recent penicillinase-resistant β -lactam antibiotics has previously been observed with OXA-10 (Bush *et al.*, 1995; Hall *et al.*, 1993; Ledent *et al.*, 1993), which has 97% amino acid identity with OXA-13.

OXA-13 was efficiently inhibited by imipenem. This was suggested by the synergistic effect of the combination of imipenem with other β -lactam compounds, as seen from the MIC data (Table 2) and in the disk diffusion assay (Fig. 3). A similar effect was noted with OXA-10- and OXA-7-producing strains (data not shown). Used at a 64-fold lower concentration, imipenem was as efficient as clavulanic acid, for which a K_{iapp} of 54 μ M was found, in reducing the MICs of ceftazidime and aztreonam for *E. coli*. A stable association between OXA-13 and imipenem was substantiated by the isoelectric focusing results (Fig. 6, lane 4) and by the observation of an OXA-13:imipenem complex which dissociated only partially after as much as 24 h (Fig. 7). With the K_{iapp} values of imipenem and meropenem being similar, the apparent difference in stability of the OXA-13:imipenem and OXA-13:meropenem complexes (Fig. 7) is not easily explained. One possible explanation could be the existence of a branched pathway of substrate inactivation with a very low (and difficult to quantify) proportion of the enzyme involved in the hydrolysis branch. Using an undiluted enzyme preparation we indeed observed some steady state hydrolysis of meropenem, which appeared to be three- to fourfold greater than that of imipenem, which was marginal (data not shown).

Imipenem has been known for several years to act as an inhibitor of β -lactamases, including chromosome-encoded cephalosporinases of Gram-negative bacteria (Hashizume *et al.*, 1984; Monks & Waley, 1988; Nouda *et al.*, 1992), TEM-type enzymes (Neu & Labthavikul, 1982; Nouda *et al.*, 1992) and oxacillinases (Toda *et al.*, 1980). The inhibition of OXA-13 with imipenem seemed particularly stable, and more so than with meropenem (Figs 6, 7), although their apparent K_i values were similar.

Interest in imipenem as a β -lactamase inhibitor in *P. aeruginosa* is limited by its powerful activity as an inducer of the chromosome-encoded cephalosporinase (Livermore & Yang, 1987). This limitation might be less important in strains such as PAe391 which do not synthesize the porin D2 responsible for preferential diffusion of imipenem (Trias & Nikaido, 1990). Thus, imipenem, when used as an inhibitor, decreased the MICs of all β -lactams tested to levels four- to eightfold lower in PAe391 than in PAO38(pAZ309) (Table 2). The ability of OXA-10-related enzymes, whether of extended spectrum or not, to hydrolyse antibiotics such as aztreonam, cefotaxime or cefsulodin, in conjunction with its susceptibility to inhibition by imipenem, should aid in the detection of such enzymes in *P. aeruginosa* and in their differentiation from other OXA-type enzymes and from class A β -lactamases.

ACKNOWLEDGEMENTS

This work was supported by grants from the Institut National de la Santé et de la Recherche Médicale (CRE 93063 and CRI 95061). We are grateful to A. Philippon for helpful discussions, to A. Coutrot for help with the immunoblotting experiments, to L. Gutmann for the gift of strain S17.1 and plasmid pNJR32, to G. Paul for the gift of OXA-10- and OXA-7-producing strains, and to C. Harcour for secretarial assistance.

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Received 23 July 1997; revised 13 November 1997; accepted
21 November 1997.