

## Novel OXA-10-Derived Extended-Spectrum $\beta$ -Lactamases Selected In Vivo or In Vitro

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A clinical isolate of *Pseudomonas aeruginosa*, PAe191, was found to be highly resistant to all anti-*Pseudomonas*  $\beta$ -lactam antibiotics (except imipenem) and resistant also to aminoglycosides. It produced a  $\beta$ -lactamase (with an apparent pI of 7.6) which was not inhibited by clavulanic acid. Cloning and characterization of the  $\beta$ -lactamase gene showed that it coded for a novel extended-spectrum OXA-10 variant, called OXA-19, which differed from OXA-10 by nine amino acids and from OXA-13 by two, i.e., Asn in position 73 (Asn73) instead of Ser and Asp157 instead of Gly. Asparagine in position 157 is implicated in resistance to ceftazidime, while the amino acid in position 73, in this variant, seems to condition the level of resistance to penicillins. The *oxa19* gene was found to be inserted, in a typical integron structure, immediately downstream from an *aac(6')-Ib* gene coding for an aminoglycoside acetyltransferase variant, which was called AAC(6')-Ib<sub>9</sub>.

A major mechanism of resistance of *Pseudomonas aeruginosa* to cephalosporins used in clinical practice, such as cefsulodin or ceftazidime, is the overproduction of its cephalosporinase, resulting from depression of the *ampC* transcription inhibition pathway (14). Alternatively, resistance to the broad-spectrum cephalosporins, including ceftazidime and cefepime, may result from the production of an extended-spectrum  $\beta$ -lactamase (ESBL). In *P. aeruginosa*, as opposed to members of the family *Enterobacteriaceae*, production of TEM- and SHV-type ESBLs (3) seems to be rare (22), and other extended-spectrum enzymes, such as PER-1 (23), OXA-2-, or OXA-10-derived ESBLs (8–10), and a clavulanic acid-susceptible class D enzyme, OXA-18 (24), have been described for clinical strains of this species. The enzymes of molecular class D (13) comprise those of group 2d of the functional classification scheme described by Bush et al. (3) and are characterized by high relative rates of oxacillin hydrolysis and generally low susceptibility to inhibition by clavulanic acid (3, 18). Like the other active-site serine  $\beta$ -lactamases, the class D enzymes have at least three conserved amino acid motifs, which are shown in Fig. 1 for the OXA-10 derivatives.

High-resolution crystallography studies of several class A penicillinases (34) have generated a basis for understanding the molecular mechanisms underlying the substrate profile extensions secondary to a large array of point mutations in TEM- or SHV-type ESBLs (16, 26). For the class D enzymes, no such studies have been reported, and only two amino acid changes have been implicated in the extension of the substrate profiles to ceftazidime, i.e., Gly157 to Asp in the OXA-10-derived OXA-11 (10) and OXA-14 (8) and Asp150 to Gly in the OXA-2-derived OXA-15 (9). These positions are, respectively, 14 and 2 amino acids downstream from the YGN triad in the OXA-10 and OXA-2 derivatives (6, 8–10, 13) (Fig. 1). Here, we describe a novel ESBL, OXA-19, from a clinical *P. aerugi-*

*nosa* isolate, containing nine altered amino acids with respect to the non-ESBL OXA-10 (13) and two altered amino acids with respect to the non-ESBL OXA-13 (20), both of which appear to contribute to the level of resistance to anti-*Pseudomonas* penicillins and cephalosporins in *Escherichia coli* and *P. aeruginosa*.

### MATERIALS AND METHODS

**Strains and plasmids.** The strains and plasmids used are summarized in Table 1. *P. aeruginosa* PAe191 was isolated in 1991 at the Saint-Louis Hospital, Paris, France. This strain was highly resistant to ceftazidime (MIC, 512  $\mu$ g/ml). Strain PAO38(pAZ310) was selected on ceftazidime (16  $\mu$ g/ml) from strain PAO38 (pAZ309), which was described previously (20). Strains were grown in Mueller-Hinton (MH) medium at 37°C.

**MIC determinations and antibiotics.** MICs on MH agar containing serially twofold-diluted antibiotics were determined. Plates inoculated with a Steers-type inoculator and ca. 10<sup>4</sup> CFU per spot were incubated at 37°C for 18 h. The MICs of  $\beta$ -lactams were determined alone or in combination with imipenem (0.25  $\mu$ g/ml), clavulanic acid (2  $\mu$ g/ml), or tazobactam (4  $\mu$ g/ml). MIC determinations were repeated twice, with identical results. Antibiotics were provided from the following suppliers: ampicillin, aztreonam, and amikacin, Bristol Myers Squibb; ceftazidime, Glaxo Group Research, Ltd.; cefotaxime, Hoechst Roussel Pharmaceuticals Ltd.; piperacillin and tazobactam, Lederle Laboratories; imipenem, Merck Sharp and Dohme-Chibret; gentamicin, Schering Plough; clavulanic acid and ticarcillin, SmithKline Beecham; and cefsulodin, Takeda Laboratories.

**$\beta$ -Lactamase preparation.** Crude enzyme (S100) extracts were prepared as previously described (20). The supernatants were used immediately for the

	10	49	67	73	141	157	205	259											
OXA-10	I	G	S	K-S	D	STFK	N	V	T	YGN	G	Y	A	KTG	ET	S	E		
OXA-11										S									
OXA-14											D								
OXA-7	T	S	F	L	A	N			I	S			F	-			GA	N	A
OXA-13	T	S				N			S	S			F				G	N	A
OXA-13-1	T	S				N			S	S			D	F			G	N	A
OXA-19	T	S				N				S			D	F			G	N	A

FIG. 1. Amino acid differences between the  $\beta$ -lactamases of the OXA-10 group. Amino acid numbering is according to Huovinen et al. (13); the conserved motifs typical for class D enzymes are boxed. The amino acids shown in boldface contribute to the substrate profile, with asparagine in position 157 leading to extended-spectrum variants. The amino acids of OXA-10 that are not numbered are G20, S27, S50, D55, V89, T107, Y174, A197, E229, T230, S245, and E259; -, absence of an amino acid.

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TABLE 1. Strains and plasmids

Strain or plasmid	Genotype or description	Resistance phenotype <sup>a</sup>	Reference or source
<b>Strain</b>			
PAe191	Clinical strain of <i>P. aeruginosa</i>	Ti Ca Ak Gm Su Hg	This work
DH5 $\alpha$	<i>E. coli</i> ; F'/endA1 hsdR17 ( $r_k^- m_k^+$ ) <i>supE44 thi-1 recA1 gyrA</i> (Nal <sup>r</sup> ) <i>relA1 D(lacIZYA-argF)U169 deoR</i> [f80dlacD(lacZ)M15]	Nal	New England Biolabs
PAO38Rif	Rifampin-resistant derivative of <i>P. aeruginosa</i> PAO38	Ri (MIC, >500 $\mu$ g/ml)	20
<b>Plasmid</b>			
pHSS6	2.3-kb plasmid vector	Km	32
pK18	2.7-kb plasmid vector	Km	25
pNJR3-2	28-kb <i>E. coli</i> - <i>P. aeruginosa</i> shuttle vector	Tc	28
pAZ305	23-kb <i>EcoRI</i> fragment obtained from PAe191 total DNA, cloned into pHSS6; <i>oxa19</i>	Km Ti Ca Gm <sup>b</sup>	This work
pAZ316	4.0-kb <i>HindIII</i> fragment from pAZ305 cloned into <i>HindIII</i> -digested pNJR3-2; <i>oxa19</i>	Tc Ti Ca Gm <sup>b</sup>	This work
pAZ327	1,097-bp <i>NarI-HindIII</i> fragment from pAZ316 cloned into <i>AccI-HindIII</i> -digested pK18; <i>oxa19</i>	Km Ti Ca	This work
pAZ309	pNJR3-2 derivative encoding <i>oxa13</i>	Tc Ti Ak	20
pAZ310	In vitro-selected derivative of pAZ309 selected on ceftazidime; <i>oxa13-1</i>	Tc Ti Ca Ak	This work
pAZ326	1,097-bp <i>NarI-HindIII</i> fragment from pAZ310 cloned into <i>AccI-HindIII</i> -digested pK18; <i>oxa13-1</i>	Km Ti Ca	This work

<sup>a</sup> Ti, ticarcilline; Ca, ceftazidime; Km, kanamicin; Ak, amikacin; Gm, gentamicin; Nal, nalidixic acid; Ri, rifampin; Su, sulfamide; Tc, tetracycline; Hg, mercuric ions.

<sup>b</sup> Gm phenotype conferred by AAC(6')-Ib<sub>5</sub> with Ser at position 119.

determination of kinetic parameters and isoelectric points. The protein concentrations were measured by the technique described by Bradford (2), and the hydrolysis rates in phosphate buffer (50 mM, pH 7.0) were determined spectrophotometrically at 30°C with a model 550S double-beam spectrophotometer (Perkin-Elmer), with ampicillin and ceftazidime as the substrates. One unit of  $\beta$ -lactamase was defined as the amount hydrolyzing 1  $\mu$ mol of substrate per min.

**IEF.** Isoelectric focusing (IEF) of S100 extracts was for 2 h, with a mini-IEF cell 111 (Bio-Rad) and a gradient made up of two-thirds polyampholytes with a pH range of 3 to 9 and one-third of polyampholytes with a pH range of 2 to 11 (Serva). Extracts from OXA-10-, SHV-1-, and SHV-5-producing strains were used as standards for pIs of 6.1, 7.6, and 8.2, respectively.  $\beta$ -Lactamases were revealed by overlay with nitrocefin (1 mg/ml) in phosphate buffer (50 mM, pH 7.0).

**Cloning experiments and DNA sequencing.** Standard DNA methodology (1) was used to clone two ca. 4.0-kb *HindIII* DNA fragments carrying either the in vitro-selected ESBL gene *oxa13-1* or the in vivo-selected gene *oxa19* (Table 1) (20) into the pNJR3-2 shuttle vector, allowing the expression of both genes in *P. aeruginosa* PAO38. These fragments were also cloned into M13mp18 and M13mp19 phage vectors and were partially sequenced as described previously (20). The sequenced stretches which have been determined for *oxa19* and *oxa13-1* and their adjacent integron regions are represented schematically in Fig. 1. The -35 sequences of the promoter of the *aac(6')-Ib*, *oxa13-1* cistron were taken to be identical with that of the original *oxa13*-containing integron (20). For

expression of the two ESBL genes in an isogenic *E. coli* background, both genes were cloned on a *NarI-HindIII* fragment into the vector pK18.

**Nucleotide sequence accession numbers.** The nucleotide sequences for *oxa19* [including *aac(6')-Ib*] and *oxa13-1* have been deposited in GenBank under accession numbers AF043381 and AF043558, respectively.

## RESULTS AND DISCUSSION

**Resistance phenotypes of PAe191 and PAO38(pAZ316).** PAe191 was resistant to all anti-*Pseudomonas*  $\beta$ -lactam antibiotics tested, except imipenem, and also to aminoglycosides, sulfonamides, and mercuric ions. Ticarcillin, piperacillin, and ceftazidime were not protected when associated with a  $\beta$ -lactamase inhibitor, such as clavulanic acid or tazobactam. However, the high-level resistance of PAe191 to ceftazidime was reversed by imipenem (Table 2). The protective effect of imipenem on otherwise hydrolyzed  $\beta$ -lactam compounds in *P. aeruginosa* has been previously observed in the presence of OXA-10-related enzymes (20). In order to characterize the

TABLE 2. MICs of different  $\beta$ -lactams for PAe191 and OXA13-1, OXA-19- and OXA-13-producing transformants

Antibiotic + inhibitor <sup>a</sup>	MIC ( $\mu$ g/ml) for the following strains (plasmid/enzyme):							
	PAe191	PAO38 (pAZ310/OXA13-1)	PAO38 (pAZ316/OXA-19)	PAO38 (pAZ309/OXA-13) <sup>b</sup>	PAO38 (pNJR-32)	DH5 $\alpha$ (pAZ326/OXA13-1)	DH5 $\alpha$ (pAZ327/OXA19)	DH5 $\alpha$ (pK18)
Ampicillin	>512	>512	>512	— <sup>c</sup>	>512	8	64	2
Ticarcillin	512	64	256	256	16	8	64	1
+ CLA	512	64	256	256	16	8	32	1
Piperacilin	128	32	64	32	2	2	8	0.5
+ TAZ	128	16	64	32	2	0.5	1	0.5
Cefotaxime	128	32	32	16	16	—	—	—
Ceftazidime	512	256	256	2	2	8	16	0.12
+ CLA	256	128	128	2	16	2	2	0.06
+ IMI	32	16	32	16	16	—	—	—
Aztreonam	64	8	16	8	2	—	—	—
Cefsulodin	64	64	64	32	2	—	—	—
Imipenem	1	1	1	1	1	—	—	—
Amikacin	32	16	8	16	4	—	—	—
Gentamicin	256	4	64	—	4	—	—	—

<sup>a</sup> CLA, clavulanic acid (2  $\mu$ g/ml); TAZ, tazobactam (4  $\mu$ g/ml); IMI, imipenem (0.25  $\mu$ g/ml).

<sup>b</sup> Data are from reference 20.

<sup>c</sup> —, not determined.

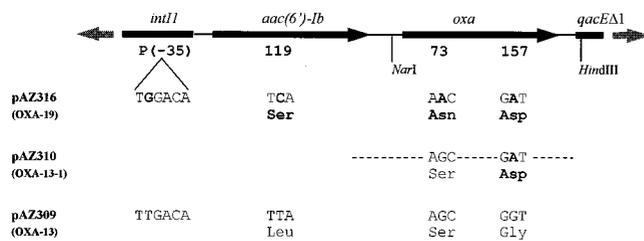


FIG. 2. Structure of the integron encoding the β-lactamases OXA-19 and OXA-13-1. Nucleotide sequences are identical in both clusters, except for three differences in the -35 sequences of the promoter and in the *aac(6')-Ib* and the *oxa* genes. Amino acid numbering is from Tran Van Nhieu and Collatz (36) and Huovinen et al. (13), respectively. Arrows represent the following open reading frames: *intI1*, for integrase; *aac(6')-Ib*, for the aminoglycoside acetyltransferases genes *aac(6')-Ib<sub>10</sub>* on pAZ310 (20) and *aac(6')-Ib<sub>9</sub>* on pAZ316; *oxa*, for the OXA variants indicated on the left; and *qacEΔ1*, for the typically truncated quaternary ammonium resistance-conferring gene associated with the 3' conserved segment of integrons. The *intI1* and *qacEΔ1* genes have been only partially sequenced here (indicated in boldface). Data for pAZ309 are from reference 20, and data for pAZ310 and pAZ316 are from the present study. Boldface, differences with respect to the OXA-13-encoding plasmid pAZ309; dashed line, the *oxa13-1* region that has been sequenced.

β-lactamase responsible for this phenotype and the nucleotide sequences controlling its production, a ca. 4.0-kb *HindIII* fragment from PAe191 DNA was cloned into pNJR3-2, yielding pAZ316 (Table 1). PAO38(pAZ316) showed the same β-lactam resistance pattern as that of PAe191, and a MIC of gentamicin greater than that of amikacin was observed. Both PAe191 (see Fig. 3, lane 3) and PAO38(pAZ316) (data not shown) produced only one β-lactamase, with an apparent pI ( $pI_{app}$ ) of 7.6, which was then likely to be an ESBL. No plasmid was found in the clinical strain PAe191 despite the use of techniques (15, 35) allowing the extraction of large plasmids, such as the OXA-1-encoding plasmid RGN238 (12).

**Nucleotide sequence of the gene cluster encoding aminoglycoside and β-lactam resistance in PAe191.** Sequence analysis of a ca. 2-kb fragment from pAZ316 revealed the presence of two resistance genes, each flanked by a recombinational element at its 3' extremity, which is typical of those found in the resistance gene cassettes in integrons (11, 30, 33). Only four mutations (Fig. 1) were observed in comparison with the sequence of a very similar integron-borne cluster described previously (20). The first was in the -35 sequence of the promoter P1, located within the integrase gene *intI1*, which controls the expression of the resistance genes (33). The three other mutations were in the resistance genes themselves. The gene immediately downstream from *intI1* encoded an AAC(6')-Ib variant, called AAC(6')-Ib<sub>9</sub>, with a Leu-to-Ser change at position 119 with respect to the otherwise identical AAC(6')-Ib protein encoded by pAZ301 (20) and to other typically amikacin resistance-conferring variants (36). This amino acid change has been associated previously with a shift from amikacin to gentamicin resistance in vitro after site-directed mutagenesis (27) and in clinical isolates of *Pseudomonas fluorescens* (17) and *Enterobacteriaceae* (4). The second resistance gene had two nucleotide differences accounting for two amino acid differences in comparison with OXA-13 (Fig. 1 and 2) (20), with Asn73 instead of Ser and Asp157 instead of Gly. This novel variant was named OXA-19. As already mentioned, Asp157 has been associated previously with the extension of the β-lactam resistance spectrum to ceftazidime (8, 10, 21).

**Selection in vitro and characterization of an ESBL variant of OXA-13.** To determine whether a derivative of OXA-13 conferring the resistance phenotype of OXA-19 in *P. aeruginosa* could be generated by a point mutation, a spontaneous mutant

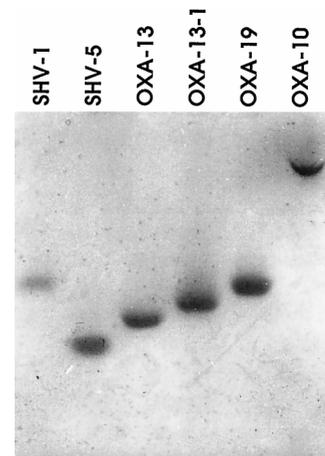


FIG. 3. Isoelectric focusing of extended-spectrum OXA-10-related β-lactamases. Enzymes SHV-1 ( $pI_{app}$ , 7.6), SHV-5 ( $pI_{app}$ , 8.2), OXA-13 ( $pI_{app}$ , 8 [18]), and OXA-10 ( $pI_{app}$ , 6.1) were used as pI standards. The two novel β-lactamases, OXA-13-1 and OXA-19, had estimated  $pI_{app}$ s of 7.8 and 7.6, respectively. β-Lactamase activity was revealed by overlaying the gel with nitrocefin.

of PAO38(pAZ309), which was called PAO38(pAZ310), was selected on ceftazidime (Table 1). An OXA variant of  $pI_{app}$  7.8 was obtained (Fig. 3). It differed from OXA-13 by only a Gly157-to-Asp change and was named OXA-13-1. Although the production of this enzyme in PAO38(pAZ310) led to a resistance pattern similar to that of OXA-19 in PAO38(pAZ316) (Table 2), the level of resistance of PAO38(pAZ316) to some of the β-lactams tested, especially ticarcillin and piperacillin, appeared to be higher than that of PAO38(pAZ310). In repeated assays, the MIC of ampicillin for a pAZ316-containing *E. coli* strain was also found to be four times as high as that for the same strain containing pAZ310 (data not shown). These observations could be related to a ca. 30-times-higher specific activity of OXA-19 against ampicillin, in comparison with OXA-13-1 (Table 3), while the specific activities of both enzymes against ceftazidime were similar.

**Effect of Asn or Ser in position 73 on the level of resistance to penicillins in OXA-19 and OXA-13-1 producers.** Two of the three nucleotide differences that distinguish the *aac(6')-Ib*, *oxa* clusters of pAZ316 and pAZ310 (Fig. 2) could be involved in modulating the levels of penicillin resistance, the difference in the -35 promoter sequence and that concerning amino acid position 73 (Fig. 2). It eliminated the likely difference in promoter strength (the -35 sequence TTGACA [Fig. 2] being known to occur in promoters stronger than TGGACA in *E. coli* [19]) and the possible influence of secondary-structure variations in the so-called 59-bp element (5) on *oxa* expression.

TABLE 3. Specific activities of OXA-13-1 and OXA-19 from *P. aeruginosa* and *E. coli* producers

Substrate (50 mM)	Sp act (mU/mg)			
	PAO38		DH5α	
	pAZ316/ OXA-19	pAZ310/ OXA-13-1	pAZ327/ OXA-19	pAZ326/ OXA-13-1
Ampicillin	999	28	826	30
Ceftazidime	0.9	1.0	1.5	1.8

Table 2 shows the  $\beta$ -lactam resistance patterns of *E. coli* DH5 $\alpha$  harboring pAZ326 or pAZ327. In these constructs, the higher MICs of penicillins for the OXA-19-producing strain correlated with a higher specific activity of OXA-19 against ampicillin in comparison with that of OXA-13-1 (Table 3). Thus, it is conceivable that Asn in position 73 conditions the level of resistance to  $\beta$ -lactams, and to penicillins in particular, also for *P. aeruginosa* PAO38(pAZ316) and, hence, the clinical strain PAe191.

An asparagine is generally found at position 73 (numbering of Huovinen et al [13]) in OXA variants of the OXA-10 branch (13, 29), whether they are penicillinases, such as OXA-5 (5) or OXA-7 (31), or of the extended spectrum, such as OXA-11 (10), OXA-14 (8), or OXA-16 (7). Thus, OXA-13 seems to be a rather rare variant with respect to the amino acid in position 73. Although the effect of a serine at this position is not known in OXA-10 derivatives with a glycine in position 157 which have a penicillinase profile, its association with aspartate at position 157 in the ESBL OXA-13-1 seems to result in a relatively low level of resistance to penicillins and a relatively low level of penicillinase activity in the producing strains.

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