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Biochemical-Genetic Characterization and Distribution of OXA-22, a Chromosomal and Inducible Class D β-Lactamase from *Ralstonia* (*Pseudomonas*) *pickettii*

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From genomic DNA of *Ralstonia pickettii* isolate PIC-1, a β-lactamase gene was cloned that encodes the oxacillinase OXA-22. It differs from known oxacillinases, being most closely related to OXA-9 (38% amino acid identity). The hydrolytic spectrum of OXA-22 is limited mostly to benzylpenicillin, cloxacillin, and restricted-spectrum cephalosporins. OXA-22-like genes were identified as single chromosomal copies in five other *R. pickettii* clinical isolates. The expression of OXA-22-like β-lactamases was inducible in *R. pickettii*.

Ralstonia pickettii is a nonfermenting gram-negative rod that is an occasional pathogen of nosocomial septicemia and tissue infections (10, 17, 20). In 1992, this organism was transferred from the genus *Pseudomonas* RNA homology group II to the genus *Burkholderia* (8) and recently to the novel genus *Ralstonia* (22).

The β-lactam resistance mechanism(s) of *R. pickettii* isolates is not known in detail. In this report, we describe the genetic and biochemical characterization of inducible oxacillinases that occur naturally in *R. pickettii* and that may explain part of its β-lactam resistance profile.

Bacterial strains, PFGE, plasmids, and conjugation assays. *R. pickettii* clinical isolates PIC-1, PIC-2, and PIC-3 were from the hospitals Bicêtre and Antoine Béclère (Paris area, France), *R. pickettii* reference strains CIP 103413 and CIP 74.22 were from the strain collection of the Pasteur Institute (Paris, France), and strain ATCC 27511 was from the American Type Culture Collection. These strains were identified by standard biochemical techniques (8).

Comparison of *R. pickettii* whole-cell DNAs was performed by a pulsed-field gel electrophoresis (PFGE) technique as previously reported (5, 14). PFGE of either *Xba*I- or *Spe*I-restricted DNAs of *R. pickettii* strains showed that they are not clonally related, except for *R. pickettii* strains PIC-1 and PIC-3 (data not shown).

Plasmid DNA extractions (3, 13, 14) failed to detect any plasmid in *R. pickettii* strains. Direct transfer (13, 14) of an amoxicillin resistance marker from *R. pickettii* strains to rifampin-resistant *E. coli* JM109 also failed.

Susceptibility testing. MICs of selected β-lactams were determined as described previously (14). *R. pickettii* isolates were resistant or had decreased susceptibility to aminopenicillins, ureidopenicillins, restricted-spectrum cephalosporins, ceftazidime, and aztreonam (Table 1), as previously reported (7). Addition of clavulanic acid and tazobactam did not significantly modify this resistance profile. Only *R. pickettii* CIP 103413 was more resistant to piperacillin, cephalothin, and cefepime (Table 1); tazobactam decreased the piperacillin MIC 16-fold.

Cloning and sequencing of the β-lactamase OXA-22 gene. Genomic DNA of *R. pickettii* PIC-1 was partially digested with *Sau*3AI and ligated into *Bam*HI-digested phagemid pBK-CMV as previously described (13). Only one *Escherichia coli* DH10B strain that contained recombinant plasmid pSC13 was obtained. Sequence analysis (3) of the 1.2-kb insert of pSC13 revealed an open reading frame (ORF) of 828 bp (data not shown). The G+C content of this ORF was 65%, which is within the range of G+C contents of *Ralstonia* genes (60.1 to 69.5%; GenBank database).

Within the deduced protein of this ORF (275 amino acids), an S-T-F-K tetrad was found at class D β-lactamase (DBL) (6)

TABLE 1. MICs of β-lactams for *R. pickettii* isolates, *E. coli* DH10B harboring recombinant plasmid pSC13, and reference strain *E. coli* DH10B

β-Lactam[a]	MIC (μg/ml)			
	R. pickettii PIC-1, PIC-2, PIC-3, ATCC 2751, CIP 74.22	*R. pickettii* CIP 103413	*E. coli* DH10B (pSC13)[b]	*E. coli* DH10B
Amoxicillin	8–64	128	64	4
Amoxicillin + CLA	8–64	128	8	4
Ticarcillin	8–64	256	64	4
Ticarcillin + CLA	8–64	256	16	4
Piperacillin	2–4	32	64	1
Piperacillin + TZB	0.5–1	2	32	1
Cephalothin	4–8	64	16	2
Cephalothin + CLA	4–8	64	8	2
Cefuroxime	8	8	16	0.5
Cefoxitin	1–4	4	4	1
Ceftazidime	4–16	16	0.5	0.5
Ceftazidime + CLA	4–16	16	0.5	0.5
Cefotaxime	0.5–2	2	0.12	0.12
Cefotaxime + CLA	0.5–2	2	0.12	0.12
Cefepime	0.5–1	8	0.25	0.03
Cefepime + CLA	0.5–1	4	0.12	0.03
Moxalactam	8–16	32	0.5	0.12
Aztreonam	128–256	256	1	0.25
Aztreonam + CLA	128–256	256	0.5	0.25
Imipenem	0.5–1	1	0.25	0.12

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

[b] *E. coli* DH10B harboring recombinant plasmid pSC13 produced the β-lactamase OXA-22.

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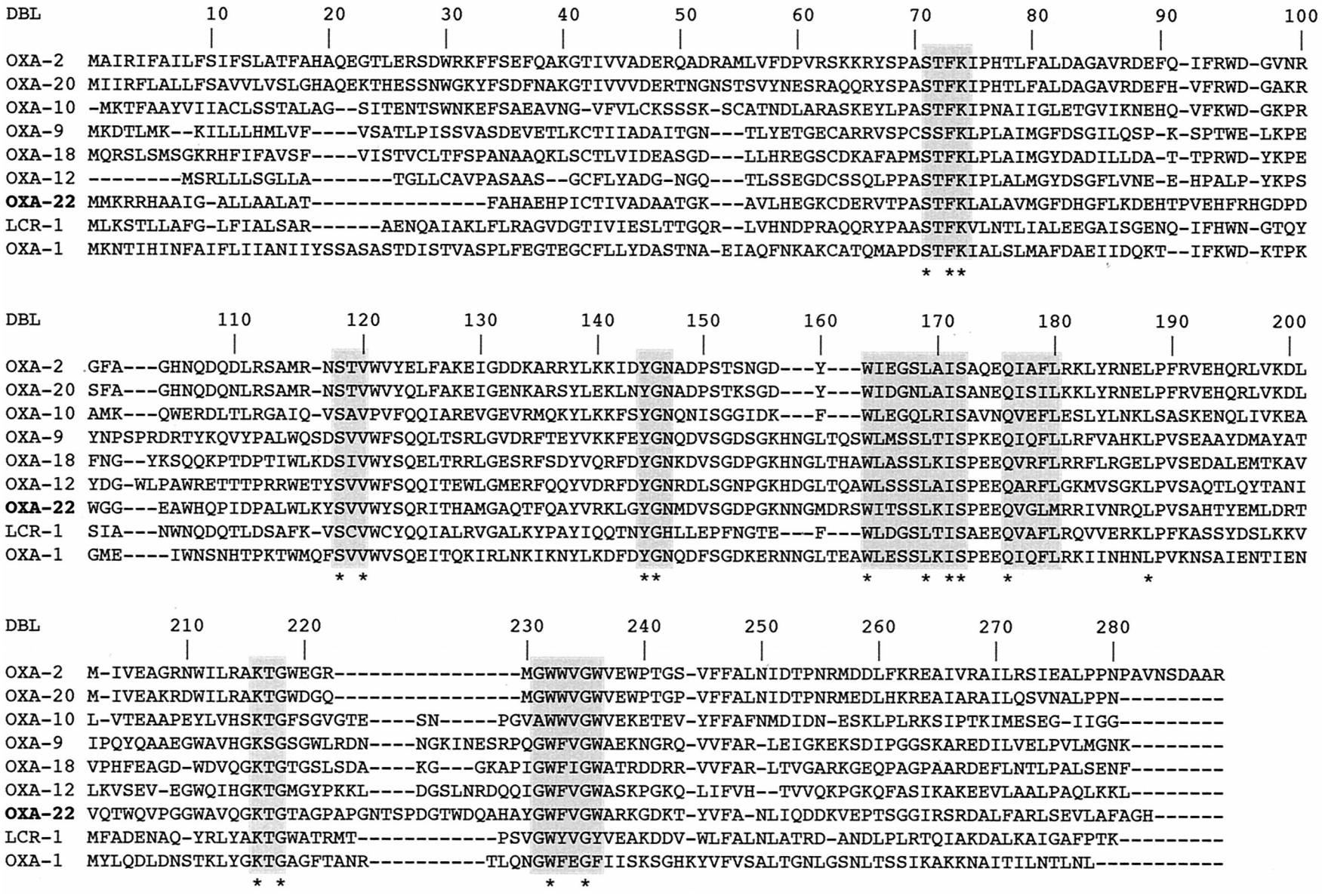

FIG. 1. Alignment of the OXA-22 amino acid sequence with those of the most closely related DBLs and some oxacillinases taken as representatives of each phylogenetic subgroup of DBLs (6, 10). The shaded boxes indicate regions conserved among DBLs, and stars indicate highly conserved residues. Dashes indicate gaps in the alignment.

TABLE 2. Steady-state kinetic parameters of the β-lactamase OXA-22

β-Lactam	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (μM^{-1} s^{-1})
Amoxicillin	0.1	6	0.016
Aztreonam	—[a]	—	—
Benzylpenicillin	0.1	<2	>0.04
Cephaloridine	5.5	10	0.55
Cephalothin	0.9	3	0.33
Cefotaxime	—	—	—
Cefoxitin	0.05	10	0.004
Ceftazidime	—	—	—
Cefuroxime	0.05	5	0.01
Cloxacillin	0.7	5	0.14
Oxacillin	—	—	—
Piperacillin	—	—	—
Ticarcillin	0.1	9	0.01

[a] —, not determinable (the initial hydrolytic rate [k_{cat}] was lower than 0.001 s^{-1}).

positions 71 to 75 (Fig. 1). Four structural elements characteristic of DBLs (2) were found in this novel enzyme; they were named OXA-22 (Y-G-N at DBL positions 144 to 146, W-X-E-X-X-L-X-I-S at DBL positions 164 to 172, Q-X-X-X-L at positions 176 to 180, and K-T-G at positions 216 to 218 (Fig. 1) (11). In addition, another stretch of amino acids, at DBL position 231 to 236, which is conserved in class D enzymes was also present (Fig. 1). OXA-22 had low amino acid identity with other Ambler DBLs, ranging from 19 to 38% for OXA-20 to OXA-9, respectively (12, 19). The highest percentages of identity were with OXA-9, OXA-18, and OXA-12, at 38, 37, and 34%, respectively (1, 6, 13, 19).

Biochemical properties of the OXA-22 β-lactamase. Cultures of *E. coli* DH10B(pSC13) were grown overnight at 37°C in 6 liters of Trypticase soy broth with amoxicillin at 30 μg/ml and kanamycin at 30 μg/ml. Unpurified extract of OXA-22 was obtained in 30 ml of sodium phosphate buffer as previously described (3). This extract was dialyzed in 20 mM H_2SO_4-Tris (pH 8) overnight at 4°C and then loaded onto a preequilibrated Q-Sepharose column (Amersham Pharmacia Biotech) in Tris buffer-H_2SO_4. The β-lactamase was eluted with a linear K_2SO_4 gradient (0 to 500 mM). The elution peak containing the highest β-lactamase activity was subsequently dialyzed overnight against 50 mM phosphate buffer, pH 7.0, prior to 10-fold concentration with a Centrisart-C30 microcentrifuge filter (Sartorius, Goettingen, Germany). Kinetic parameters were obtained as described previously (3) using a UV spectrophotometer with 100 μM cephalothin as the substrate for inhibition studies, and one unit of enzyme activity was defined (15) as the activity which hydrolyzed 1.0 μmol of cephalothin per min.

The specific activity of OXA-22 was 9.1 mU/mg, and its purification coefficient was 20-fold. OXA-22 showed its strongest hydrolytic activity against benzylpenicillin, cephalothin, cephaloridine, and cloxacillin (Table 2). The lack of oxacillin hydrolysis by OXA-22 was peculiar for an oxacillinase (4, 11). Further OXA-22 purification may help to detect oxacillin hydrolysis as described for LCR-1 (Y. Yang and K. Bush, Letter, Antimicrob. Agents Chemother. **39:**1209, 1995). AmpS, an oxacillinase related to OXA-12 (90% amino acid identity) from *Aeromonas jandaei*, and LCR-1 strongly hydrolyze oxacillin but not cloxacillin (9, 21; Yang and Bush; letter). Inhibition studies that measured 50% inhibitory concentrations (IC_{50}) (14) showed that OXA-22 activity was inhibited partially by clavulanic acid (IC_{50}, 1.2 μM) and less by tazobactam (IC_{50}, 6.5 μM). These results contrasted with those found for the other oxacillinases, for which the tazobactam inhibitory property is equal to or higher than that of clavulanic acid (4, 11). OXA-22 activity was inhibited by NaCl, as were other oxacillinases (IC_{50}, 80 mM) (4).

Analytical isoelectric focusing (IEF), performed as previously reported (14), revealed that a culture of *R. pickettii* PIC-1 gave two β-lactamase activities with pI values of 7.0 and 7.1, like *E. coli* DH10B(pSC13) cultures (data not shown). These pI values may correspond to proteolytic cleavage, partial unfolding, and/or monomer-dimer conversion of OXA-22. The relative molecular mass of OXA-22, determined with an *E. coli* DH10B(pSC13) culture as previously described (3), was 28 kDa.

OXA-22-like β-lactamases in *R. pickettii* isolates. IEF analysis revealed that the unpurified β-lactamase extract of each *R. pickettii* culture gave different pI values: for PIC-3 (as for *R. pickettii* PIC-1), 7.0 and 7.1; for *R. pickettii* PIC-2 and ATCC 27511, 7.4; for *R. pickettii* CIP 74.22, 7.5. An *R. pickettii* CIP 103413 culture gave two very different pI values of 6.8 and 7.5, likely corresponding to two β-lactamases.

PFGE of *Xba*I-restricted genomic DNAs of *R. pickettii* strains and reference strains *Pseudomonas putida* CIP 55.5, *Brevundimonas diminuta* CIP 63.27T, and *Comamonas acidovorans* 103685 (Pasteur Institute strain collection) produced a template used in a Southern transfer experiment (14, 18) with a PCR-obtained 622-bp internal fragment of bla_{OXA-22} as a labeled probe (3). bla_{OXA-22}-like genes were identified in all of the *R. pickettii* isolates but in none of related gram-negative species (data not shown). The hybridizing *Xba*I DNA fragment differed for each *R. pickettii* isolate, except for *R. pickettii* isolates PIC-1 and PIC-3. OXA-22-like genes were found at a single copy on a large DNA fragment (>ca. 250 kb), further underlining the chromosomal origin of these genes (data not shown).

Most of the OXA-22-like β-lactamase genes were PCR amplified (14) from *R. pickettii* genomic DNAs (622 out of 828 bp) as a result of the choice of internal PCR amplification primers of bla_{OXA-22} (OXA-22A, 5′-TTGCATGAAGGCAAGTGCG ACGAG-3′; OXA-22B, 5′-TCAACCTTGTCGTCCTGGAT C-3′). The deduced proteins had 96 to 100% amino acid identity with OXA-22. We identified OXA-22 in *R. pickettii* PIC-1 and PIC-3, OXA-22a in *R. pickettii* PIC-2 and ATCC 27511, and OXA-22b in *R. pickettii* CIP 7422 (Table 3). Amplification of an OXA-22-like gene from *R. pickettii* CIP 103413 failed, despite the use of other primer combinations and different experimental setups.

Induction studies. Induction studies with cefoxitin at 0.5 μg/ml as a β-lactam inducer (15) and 100 μM cephaloridine as the substrate identified induced β-lactamase expression in cultures of all of the *R. pickettii* isolates tested. The induction rates ranged from 25- to 64-fold depending on the strain. In induced cultures, no other β-lactamase activity appeared on an IEF gel, compared to noninduced cultures, thus confirming that induced β-lactamase activities corresponded to those of OXA-22-like enzymes. Regulation of oxacillinase expression is known

TABLE 3. Amino acid differences among OXA-22-like β-lactamases compared to OXA-22

β-Lactamase	Amino acid at position:							
	90	95	96	104	126	189	193	198
OXA-22	H	R	H	E	I	P	H	L
OXA-22a		K	R	D	V	S	R	V
OXA-22b	Y	K	R	D		S	R	V

[a] DBL amino acid numbering is shown (6).

for OXA-12 and AmpS from *A. jandaei*, which are chromosomally located and occur naturally (1, 9, 16, 21). Further work will be directed toward the identification of the regulatory system of OXA-22-like β-lactamases and its comparison with the two-component regulon identified for coordinated expression of β-lactamases of different Ambler classes of *A. jandaei* (1, 16).

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper will appear in the GenBank nucleotide database under accession no. AF064820.

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