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OXA-60, a Chromosomal, Inducible, and Imipenem-Hydrolyzing Class D β-Lactamase from *Ralstonia pickettii*

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A chromosomally encoded oxacillinase, OXA-22, had been characterized from *Ralstonia pickettii* PIC-1 that did not explain by itself the resistance profile of this strain to β-lactams. Thus, further analysis of the genetic background of this species led to the identification of another oxacillinase, OXA-60, that was expressed only after β-lactam induction. This chromosomally encoded oxacillinase shared 19% amino acid identity with OXA-22. It has a narrow-spectrum hydrolysis profile that includes imipenem. OXA-60-like enzymes were identified in several *R. pickettii* strains. Gene inactivation and induction studies of the bla_{OXA-60} and bla_{OXA-22} genes in *R. pickettii* identified the relative contribution of each oxacillinase to the resistance profile of *R. pickettii* to β-lactams.

Ralstonia pickettii is a nonfermenting gram-negative rod that is isolated from water, soil, plants, fruits, and vegetables (13). It is rarely involved in nosocomial septicemia and tissue infections (7, 42). Most of the infections are traced to contamination of parenteral fluids or of medical equipment (8, 10, 20, 38). In 1992, this species was transferred from the genus *Pseudomonas* RNA homology group II to the genus *Burkholderia* (13) and subsequently in 1995 to the novel genus *Ralstonia* (44).

Analysis of the β-lactamase content of *R. pickettii* showed that it contained a chromosomally located and inducible oxacillinase, OXA-22 (31). The oxacillin-hydrolyzing β-lactamases (oxacillinases) belong to the Ambler class D of β-lactamases (19). They usually hydrolyze oxacillin, methicillin, and cloxacillin better than benzylpenicillin, and their activity is inhibited by NaCl (6). Whereas most of the oxacillinases are plasmid mediated, several chromosomally encoded oxacillinases have been reported (2, 16, 17, 35, 37) or identified in silico in the genomes of *Agrobacterium tumefaciens* (15), *Mesorhizobium loti* (21), *Bradyrhizobium japonicum* (22), "*Cyanobacterium anabaena*" (23), and *Ralstonia solanacearum* (39).

OXA-22 is a narrow-spectrum oxacillinase that could not explain by itself the entire resistance profile of *R. pickettii* (31). Thus, further characterization of the β-lactamase content of *R. pickettii* was conducted that led to the discovery of a second chromosomally encoded and inducible oxacillinase, OXA-60. Both oxacillinases, OXA-22 and OXA-60, are regulated and widespread in *R. pickettii*. The contribution of both oxacillinases to the overall resistance profile has been assessed also.

MATERIALS AND METHODS

Bacterial strains and plasmids. *R. pickettii* clinical isolates PIC-1, PIC-2 and PIC-3 have previously described (31). *R. pickettii* reference strains (ATCC 27511, CIP 103413, and CIP 74.22), *R. solanacearum* (CIP 104762^T), *R. basilensis* (CIP 106792^T), *R. eutropha* (CIP 104763^T), *R. gilardii* (CIP 105966^T), and *R. paucula* (CIP 105943^T) were from the Pasteur Institute strain collection (CIP, Paris, France). *Escherichia coli* DH10B and rifampin-resistant *E. coli* JM109 were used as hosts for cloning and conjugation experiments. The kanamycin-resistant pBKCMV and pPCRBluntII-TOPO plasmids (Invitrogen/Life Technologies, Cergy-Pontoise, France), the chloramphenicol-resistant plasmid pPCRScript-Cam (Stratagene, Amsterdam, The Netherlands) were used as cloning vectors. The tetracycline-resistant plasmid pACYC184 (New England Biolabs/Ozyme, Saint-Quentin-en-Yvelines, France) was used as a suicide vector for gene inactivation in *R. pickettii* PIC-1 strain since its P15A *oriE* origin of replication restricts its host range to *E. coli* and a few other enterobacterial species (12). The plasmid pET9a was used as an expression vector (Stratagene). Bacterial cultures were grown in Trypticase soy broth (TSB) at 37°C for 18 h unless otherwise indicated.

Antimicrobial agents and MIC determinations. The antimicrobial agents and their sources have been described elsewhere (33). MICs were determined by an agar dilution technique on Mueller-Hinton agar (Sanofi-Diagnostics Pasteur, Marnes-La-Coquette, France) plates with a Steers multiple inoculator and an inoculum of 10^4 CFU per spot (33). The results of susceptibility testing were recorded according to the guidelines of the National Committee for Clinical Laboratory standards (29).

Plasmid extraction and conjugation assays. Extraction of natural plasmid DNAs from *R. pickettii* isolates were attempted as described by Kieser et al. (24). Recombinant plasmid DNA was prepared by using Qiagen Maxi columns (Coger, Paris, France).

Direct transfer of the amoxicillin resistance marker into rifampin-resistant *E. coli* JM109 obtained in vitro was attempted by liquid and solid conjugation assays at 30 and 37°C. Transconjugants were selected on Trypticase soy agar (TSA) plates (Sanofi-Diagnostics Pasteur) containing amoxicillin (50 μg/ml) and rifampin (100 μg/ml).

Cloning experiments and PCR experiments. All enzymes for DNA manipulations were used according to the recommendations of the supplier (Amersham Biosciences, Orsay, France). Unless specified otherwise, standard molecular techniques were used (40). For each PCR experiment, 500 ng of total DNA was used in a standard PCR mixture supplemented with 10% dimethyl sulfoxide (40). PCR amplifications of the bla_{OXA-60}-like genes were performed with the internal primers OXA-60C and OXA-60D (Table 1).

Partially Sau3AI-restricted whole-cell DNA was cloned into BamHI-restricted vector pBKCMV as previously described (33). *E. coli* harboring recombinant plasmids was selected onto amoxicillin (30 μg/ml) and kanamycin (30 μg/ml)-containing TSA plates. The recombinant plasmid, pC1, with the smallest Sau3AI insert, was retained for further analysis. The recombinant plasmid pC2 was obtained by ligation of SacII-digested genomic DNA from *R. pickettii* PIC-1 in the SacII-restricted plasmid pPCRScript-Cam, followed by amoxicillin-chloramphenicol selection (33). Recombinant plasmids were transformed by electroporation (Bio-Rad Gene Pulser II; Bio-Rad, Ivry-sur-Seine, France) into *E. coli* DH10B electrocompetent cells (Invitrogen/Life Technologies).

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TABLE 1. Nucleotide sequences of primers used for amplification and sequence analysis

Primer[a]	Position	Sequence (5′→3′)	Reference or GenBank no.
OXA-60 A	2757–2782	AAAGGAGTTGTCTCATGCTGTCTCG	AF525303
OXA-60 B	3605–3579	AACCTACAGGCGCGCGTCTCACGGTG	AF525303
OXA-60 C	2789–2813	AAGACCCTCGCGTTTGCCGTGGTGG	AF525303
OXA-60 D	3552–3529	AACTGCTTGCCCAGCGGAATGCGC	AF525303
OXA-60 NdeI	2757–2782	AAAGGAGTTGTCATATGCTGTCTCG[b]	AF525303
OXA-60 BamHI	3605–3579	AACCTACAGGATCCCGTCTCACGGTG	AF525303
OXA-60 NcoI	2789–2813	AAGACCCTCGCGTTTGCCATGGTGG	AF525303
OXA-60 EcoRI	3552–3529	AACTGCTTGCCCAGCGGAATTCGC	AF525303
OXA-60 GSP1	3020–2997	GATCAGGCTGTTCGGAATCTTGAA	AF525303
OXA-60 GSP2	2961–2942	CCGGATCGACGACATAGGTA	AF525303
OXA-22 RBS	1–21	GATCGATGATGAAACGCCGC	AF064820
OXA-22 BAC	804–784	TTCCGACAGGCGAGCAAACAG	AF064820
OXA-22 NcoI	114–137	AAGGCCGTCTTCCATGGAGGCAAG	AF064820
OXA-22 EcoRI	733–713	AGGTTGGCGAATTCGTAGGTC	AF064820
184NcoI	3923–3944	TCGCCTTGCGTATAATATTTGC	12
184EcoRI	23–6	TGATGAATGCTCATCCGG	12
577f23	559–577	GCGTACCTTTTGTATAATG	43
1622r23	1622–1605	CACCTGTGTCGGTTTGSG	43

[a] Expected sizes of PCR products for primer combinations: OXA-60 A (or OXA-60 NdeI) and OXA-60 B (or OXA-60 BamHI), 848 bp; OXA-60 C (or OXA-60 NcoI) and OXA-60 D (or OXA-60 EcoRI), 763 bp; OXA-22 RBS and OXA-22 BAC, 804 bp; and OXA-22 NcoI and OXA-22 EcoRI, 619 bp.
[b] Restriction sites located in primers are underlined.

Recombinant plasmid, pET-OXA-60, used for OXA-60 overexpression, was constructed as follows: a 848-bp PCR generated fragment by using primers containing the NdeI/BamHI restriction site (Table 1) was cloned into pPCRBluntII-TOPO plasmid (Invitrogen/Life Technologies) according to the manufacturer's instructions, resulting in plasmid pTOPO-OXA-60. The insert of the latter plasmid was removed with NdeI-BamHI and cloned into NdeI/BamHI-restricted pET9a expression vector (Stratagene).

Internal fragments of the bla_{OXA-60} and bla_{OXA-22} genes were amplified by PCR with primers containing NcoI and EcoRI restriction sites (Table 1). The amplified fragments (726 and 595 bp for the bla_{OXA-60} and bla_{OXA-22} genes, respectively) were cloned in *E. coli* DH10B into NcoI- and EcoRI-digested tetracycline-resistant plasmid pACYC184 resulting in the recombinant plasmids pΔOXA-60 (4,673 bp) and pΔOXA-22 (4,542 bp), respectively.

Gene inactivation. Mutant strains deficient in the bla_{OXA-60} and bla_{OXA-22} genes were constructed by homologous recombination as follows. Recombinant plasmids pΔOXA-60 and pΔOXA-22 were transferred into wild-type *R. pickettii* strain PIC-1 by electroporation, and strains in which the plasmid integrated into the bla_{OXA-60} or $bla_{OXA-22,}$ genes, respectively, by a single recombination event were selected for their tetracycline resistance marker (50 μg/ml). Genomic DNA from 10 tetracycline-resistant strains was prepared and disruption of the genomic copy of the bla_{OXA-60} or bla_{OXA-22} genes was confirmed by PCR. The lack of replication ability of the plasmid pACYC184 in *R. pickettii* was checked by PCR with the primers 184NcoI and 184EcoRI (Table 1) located on the plasmid on each side of the inserted truncated oxacillinase gene.

Southern transfer and pulsed-field gel electrophoresis analyses. Genomic DNA of *R. pickettii* PIC-1 was digested either with SacII, PstI, SalI, AccI or EagI restriction enzymes and genomic DNAs from several strains of different *Ralstonia* species were digested with AccI restriction enzyme overnight at 37°C. The DNA fragments were resolved on a 1% agarose gel prior to their transfer onto a Hybond-N^+ membrane (Amersham Pharmacia Biotech, Orsay, France). Blots were probed with 763- and 619-bp internal PCR fragments specific for the bla_{OXA-60} and bla_{OXA-22} genes, respectively. The probes were labeled by using the ECL labeling kit according to the manufacturer's recommendations (Amersham Pharmacia Biotech, Orsay, France).

To search for a chromosomal location of the β-lactamase genes, the whole-cell DNA of *R. pickettii* was restricted with I-CeuI restriction enzyme (New England Biolabs/Ozyme) as previously described (25, 36). After a Southern transfer (40), DNAs were hybridized with PCR-generated probes specific to the 23S rRNA (with primers 577f23 and 1622r23), bla_{OXA-22} (with primers OXA-22 NcoI and OXA-22 EcoRI), and bla_{OXA-60} (with primers OXA-60 NcoI and OXA-60 EcoRI) (Table 1) genes.

DNA sequencing and protein analysis. PCR-generated fragments, purified by using QiaQuick PCR purification spin columns, and the inserts of the recombinant plasmids were sequenced on both strands on an ABI 3100 automated sequencer (Applied Biosystems, Les Ulis, France). The nucleotide and the deduced protein sequences were analyzed with software available on the internet at the National Center of Biotechnology Information website (http://www.ncbi.nlm.nih.gov). Multiple nucleotide and protein sequence alignments were carried out online by using the program CLUSTAL W available online at the University of Cambridge (http://www2.cbi.ac.uk/clustalW/).

GenBank accession numbers and references for the bla_{OXA} genes can be found at the website http://www.lahey.org/studies/other.asp except for LCR-1 (9, 27) and OXA-50 (14) from *Pseudomonas aeruginosa*, OXA-58 (AY570763) from *A. baumannii*, all2480 from "*Cyanobacterium anabaena*" (23), bll5360 from *B. japonicum* (22), mll0916 from *M. loti* (21), agrC1700p from *A. tumefaciens* (15), and Rsp0030 from *R. solanacearum* (39). Dendrograms were derived from the multiple sequence alignment by a parsimony method by using the phylogeny package PAUP (Phylogenetic Analysis Using Parsimony) version 3.0 (41).

Mapping the bla_{OXA-60} transcription start site. Reverse transcription and rapid amplification of cDNA ends (RACE) were performed with the 5′RACE system version 2.0 (Invitrogen/Life Technologies). A total of 5 μg of total RNAs extracted from imipenem-induced culture of *R. pickettii* PIC-1 (Qiagen RNeasy Maxi Kit) and the OXA-60GSP1 and OXA-60GSP2 antisense bla_{OXA-60} gene-specific primers were used to determine the transcription initiation site of the bla_{OXA-60} gene.

IEF analysis and induction studies. Isoelectric focusing (IEF) was performed with a pH 4 to 6.5 Ampholine polyacrylamide gel (Amersham Pharmacia Biotech) for 2.5 h at 25 W, 25 mA, and 2,000 V in a flatbed apparatus with culture extracts of *R. pickettii* isolates and *E. coli* DH10B harboring plasmids pC1 and pC2.

Inducibility of the β-lactamase content from each *R. pickettii* culture was tested in TSB at 37°C by using the induction protocol with imipenem (1 μg/ml) as β-lactam inducer as described previously (34). The β-lactamase activity was defined as 1 U of enzyme that hydrolyzed 1 μmol of nitrocefin per min. The total protein content was measured with bovine albumin as the standard (DC Protein Assay Kit Bio-Rad).

β-Lactamase purification. Induction of an exponentially growing culture of *E. coli* BL21(DE3) (pET-OXA-60) with 0.4 mM IPTG (isopropyl-β-D-thiogalactopyranoside) was performed at 37°C for 5 h in TSB. One liter of this culture was pelleted and resuspended in 30 ml of 20 mM Tris-H_2SO_4 buffer (pH 8.5). The protein extracts obtained were purified as described previously (14) with some modifications. Briefly, culture extracts were subjected to several purification steps, including ion-exchange chromatography with Q-Sepharose columns, first with a 20 mM Tris-HCl buffer (pH 8.5) and then a 20 mM BisTris buffer (pH 6.5). Elution of the β-lactamase was performed with a linear K_2SO_4 gradient (0 to 500 mM). Peaks of β-lactamase activities were pooled and dialyzed with 50 mM phosphate buffer (pH 7.0). The protein content was measured by the Bio-Rad DC protein assay. The protein purification rate and the relative molecular mass of OXA-60 β-lactamase were estimated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis analysis (33). The signal peptide cleavage site was

TABLE 2. MICs of β-lactams for *R. pickettii* PIC-1 and its isogenic strains, *E. coli* DH10B harboring recombinant plasmids pC1 and pSC13, and reference strain *E. coli* DH10B

β-Lactam(s)[a]	MICs of β-lactams for:					
	R. pickettii			*E. coli*		
	PIC-1	PIC-1 (ΔOXA-60)	PIC-1 (ΔOXA-22)	DH10B(pC1)[b]	DH10B(pSC13)	DH10B
Amoxicillin	64	2	32	256	64	4
Amoxicillin + CLA	64	0.25	32	256	8	4
Ticarcillin	64	<0.06	4	256	64	4
Ticarcillin + CLA	64	<0.06	4	256	16	4
Piperacillin	2	<0.06	<0.06	2	64	1
Piperacillin + TZB	2	<0.06	<0.06	2	32	1
Cephalothin	4	4	0.25	2	16	2
Cefuroxime	0.5	0.12	0.06	4	16	0.5
Cefoxitin	1	0.25	<0.06	4	4	1
Ceftazidime	0.5	0.5	0.25	0.5	0.5	0.5
Cefotaxime	0.5	<0.06	<0.06	<0.06	0.12	0.12
Cefepime	2	0.06	0.06	<0.06	0.25	<0.06
Moxalactam	8	4	1	<0.06	0.5	0.12
Aztreonam	128	128	128	0.12	1	0.25
Imipenem	0.5	0.25	0.5	0.5	0.5	0.12
Meropenem	2	0.5	2	<0.06	<0.06	<0.06

[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 pC1 produced β-lactamase OXA-60, whereas *E. coli* DH10B harboring recombinant plasmid pSC13 produced β-lactamase OXA-22 (31).

identified as described previously (14). Briefly, the purified protein was transferred from a SDS-polyacrylamide gel electrophoresis onto a polyvinylidene difluoride (PVDF) membrane (Problott; Applied Biosystems) by passive absorption. Subsequently, the membrane was washed in 10% methanol with vortexing. N-terminal Edman sequencing was performed on an Applied Biosystems Procise 494HT sequencer with the reagents and by the methods recommended by the manufacturer.

Kinetic studies. Purified β-lactamase was used for determination of kinetic parameters (k_{cat} and K_m) performed at 30°C in 100 mM Tris-H_2SO_4–300 mM K_2SO_4 (pH 7.0) (32). The initial rates of hydrolysis of β-lactams were determined with a UV spectrophotometer as previously described (28). The 50% percent inhibitory concentration (IC_{50}) was determined as the clavulanate, tazobactam, or sulbactam concentration that reduced the hydrolysis rate of 100 μM concentrations of nitrocefin by 50% under conditions in which the enzyme was preincubated with various concentrations of inhibitor for 3 min at 30°C before addition of the substrate (28). The effect of carbon dioxide on the modulation of enzymatic properties of OXA-60 was investigated by adding $NaHCO_3$ to the reaction buffer at a 10 mM final concentration. The k_{cat} and K_m values were determined for appropriate substrates in the presence or absence of bicarbonate (26, 32).

Nucleotide sequence accession number. The nucleotide sequence data reported in the present study have been added to the EMBL/GenBank nucleotide database under accession numbers AF525303, AY662675, AY664504, AY664505, and AY664506.

RESULTS AND DISCUSSION

Susceptibility testing and IEF analysis. MICs of β-lactams for *R. pickettii* PIC-1 showed resistance or decreased susceptibility to aminopenicillins, carboxypenicillins, narrow-spectrum cephalosporins, moxalactam, and aztreonam as previously reported (Table 2) (31). Addition of clavulanic acid and tazobactam did not modify significantly the resistance pattern (Table 2). The presence of the bla_{OXA-22} gene in *R. pickettii* PIC-1 could only partially explain the resistance profile (31).

Induction experiments with cultures of *R. pickettii* PIC-1 with imipenem as β-lactam inducer and IEF analysis revealed a single β-lactamase with a pI of 7.1 corresponding to OXA-22 (31) in noninduced cultures and two distinct β-lactamase bands of pI 7.1 and 5.1 (faint band) in induced cultures (data not shown). This result indicated the likely presence of a second β-lactamase.

Cloning and sequencing of the β-lactamase gene. Shotgun cloning with partially Sau3AI-restricted genomic DNA yielded several recombinant *E. coli* clones. Of 10 tested clones, all had an oxacillinase phenotype that differed from that of *E. coli* DH10B recombinant clones harboring pSC13 with bla_{OXA-22} (31). These clones contained inserts ranging from 1.6 to 2.5 kb. The smallest recombinant plasmid pC1 was retained for further analysis. The β-lactamase expressed by *E. coli* DH10B (pC1) conferred resistance to amino- and ureidopenicillins unchanged after clavulanic acid addition (Table 2). MIC of imipenem for *E. coli* DH10B (pC1) was 1 μg/ml, a fourfold-higher value than that of *E. coli* DH10B (pSC13) expressing OXA-22, indicating that the β-lactamase carried by pC1 may be able to hydrolyze imipenem.

E. coli DH10B(pC1) produced a β-lactamase with a pI of 5.1 that corresponded to one of the two pI values obtained for culture extracts of *R. pickettii* PIC-1 after imipenem induction.

DNA sequence analysis of the 1,629-bp insert of pC1 revealed an open reading frame (ORF) of 816 bp encoding a 271-amino-acid preprotein, OXA-60, of relative molecular mass of 27.7 kDa (Fig. 1). The G+C content of this ORF was 64.6%, which is within the expected range of the G+C content of *Ralstonia* spp. genes (63 to 70%) (44). The two oxacillinase genes of *R. pickettii* PIC-1 shared a weak nucleotide identity (<19%). Using 5′RACE PCR experiments, the site of initiation of transcription of the bla_{OXA-60} gene was mapped in the genome of *R. pickettii* PIC-1. The nucleotide sequence of the 5′RACE PCR product showed that transcription starts at the cytosine located 55 bp upstream of the bla_{OXA-60} gene translational start site (Fig. 1). Upstream of this transcriptional start site, a −35 promoter sequence, TGGCCG, was found, separated by 17 bp from a −10 promoter sequence, TACGAT (Fig. 1b).

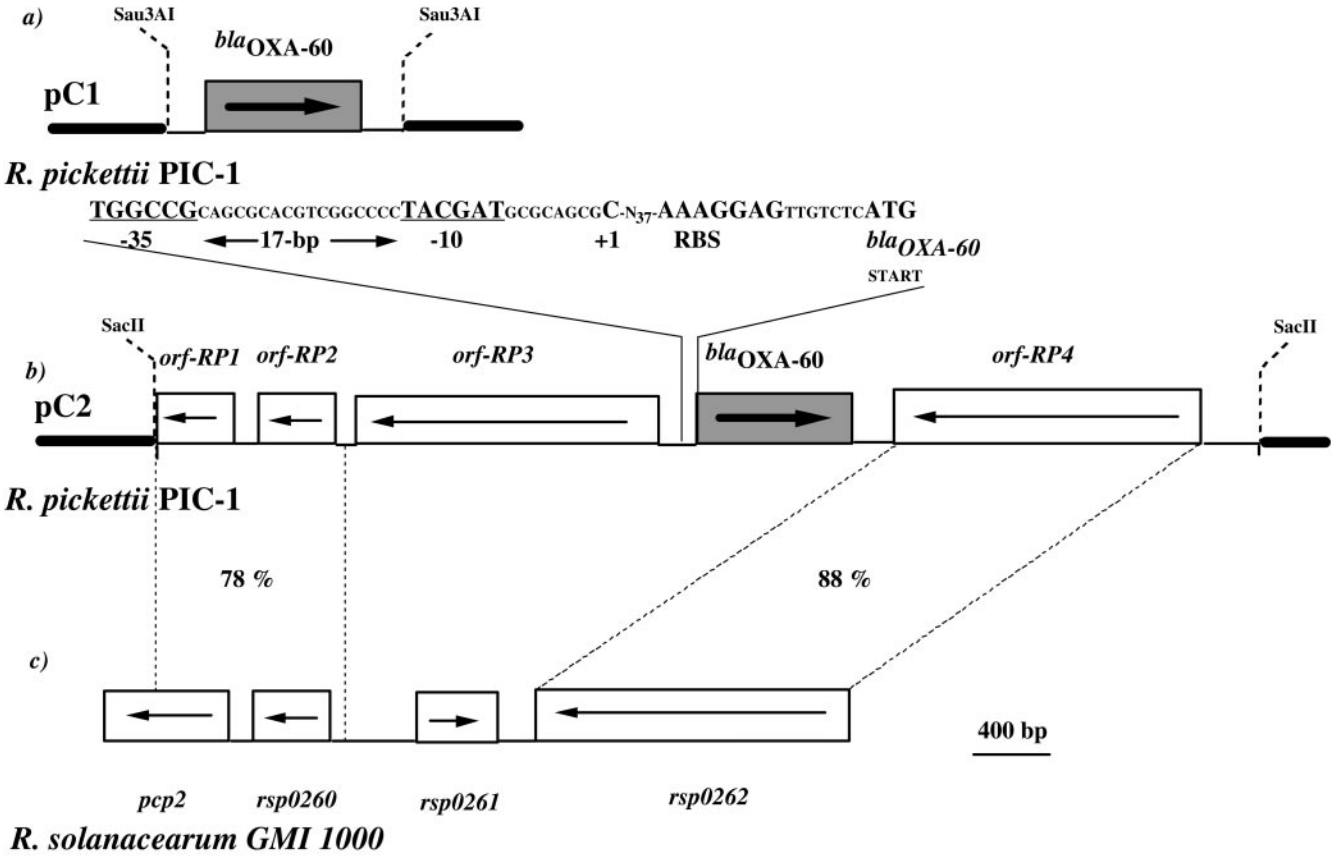

FIG. 1. Schematic representation of recombinant plasmids containing the bla_{OXA-60} gene, pC1 (a), and pC2 (b). The thin lines represent the cloned inserts from *R. pickettii* PIC-1, whereas the thick lines indicate the vector sequence. The structure of the promoter of the bla_{OXA-60} gene, the conserved regions (−35, −10, and +1) for RNA polymerase, and the ribosome-binding site (RBS) are represented in panel b. The coding regions (*orf-RP1* to *orf-RP4* and bla_{OXA-60}) are represented as boxes, with arrows indicating their transcription direction. (c) Chromosomally encoded genes of *R. solanacearum* GMI 1000 (39) sharing nucleotide identity with several coding regions of *R. pickettii* PIC-1.

Sequence analysis of β-lactamase OXA-60. The 271-amino-acid protein OXA-60 contained two conserved motifs of serine β-lactamase S-T-F-K and K-T-G at positions 70 to 73 and positions 216 to 218 (class D β-lactamase [DBL] numbering) (6, 9, 27) (Fig. 2). A typical motif Y-G-N of oxacillinases was found at DBL positions 144 to 146, as opposed to other oxacillinases with carbapenem-hydrolyzing properties identified in *A. baumannii* (OXA-23 to OXA-27 and OXA-40) that possessed an F-G-N motif at these positions (1, 4, 11, 16). As described previously, the phenylalanine residue at DBL position 144 is likely not critical for imipenem hydrolysis (16). Furthermore, the S-X-V triad at DBL position 118 to 120 was proposed to be a better equivalent of the S-D-N motif of class A β-lactamase than the Y-G-N motif at positions 144 to 146 (Fig. 2) (27).

The common motif Q-X-X-X-L of oxacillinases was not found at DBL position 176 to 180 in OXA-60, but it was replaced by the motif E-A-R-F-T (glutamate-alanine-arginine-phenylalanine-threonine). Interestingly, this motif is also absent in sequences of other oxacillinases OXA-23, OXA-24, OXA-27, and OXA-40 (1, 4, 11, 16).

The N-terminal amino acid sequencing of the mature protein revealed the cleavage site for the leader peptide between the alanine and the glutamic acid between residues 17 and 18 (HA-EL) (Fig. 2).

β-Lactamase OXA-60 shared 46% amino acid identity with the naturally occurring oxacillinase OXA-50 from *P. aeruginosa* (14), 37% identity with the putative β-lactamase bll5360 from *B. japonicum* (22), 36% with OXA-55 from *Shewanella algae* (17), 34% with OXA-5 from *P. aeruginosa*, and OXA-48 from *K. pneumoniae* (36), 33% with OXA-54 from *S. oneidensis* (35), and 31% with OXA-27 from *A. baumannii* (1). Surprisingly, OXA-60 shared only 19% amino acid identity with OXA-22, suggesting that they did not derive from a common ancestor. A phylogenetic tree based on amino acid sequence identity (Fig. 3) showed that OXA-60 could not be included into any of the five defined groups of oxacillinases. Interestingly, OXA-60 shared the highest amino acid identity with several oxacillinases known to hydrolyze imipenem (OXA-27, OXA-48, OXA-50, OXA-54, and OXA-55) (1, 14, 17, 35, 36).

Biochemical properties of OXA-60. After purification from *E. coli* BL21(DE3)/(pET-OXA-60) extract, specific activity of β-lactamase OXA-60 against benzylpenicillin was 637 U/mg of protein, and its purity was estimated to be >95% by SDS-polyacrylamide gel electrophoresis analysis where a single pro-

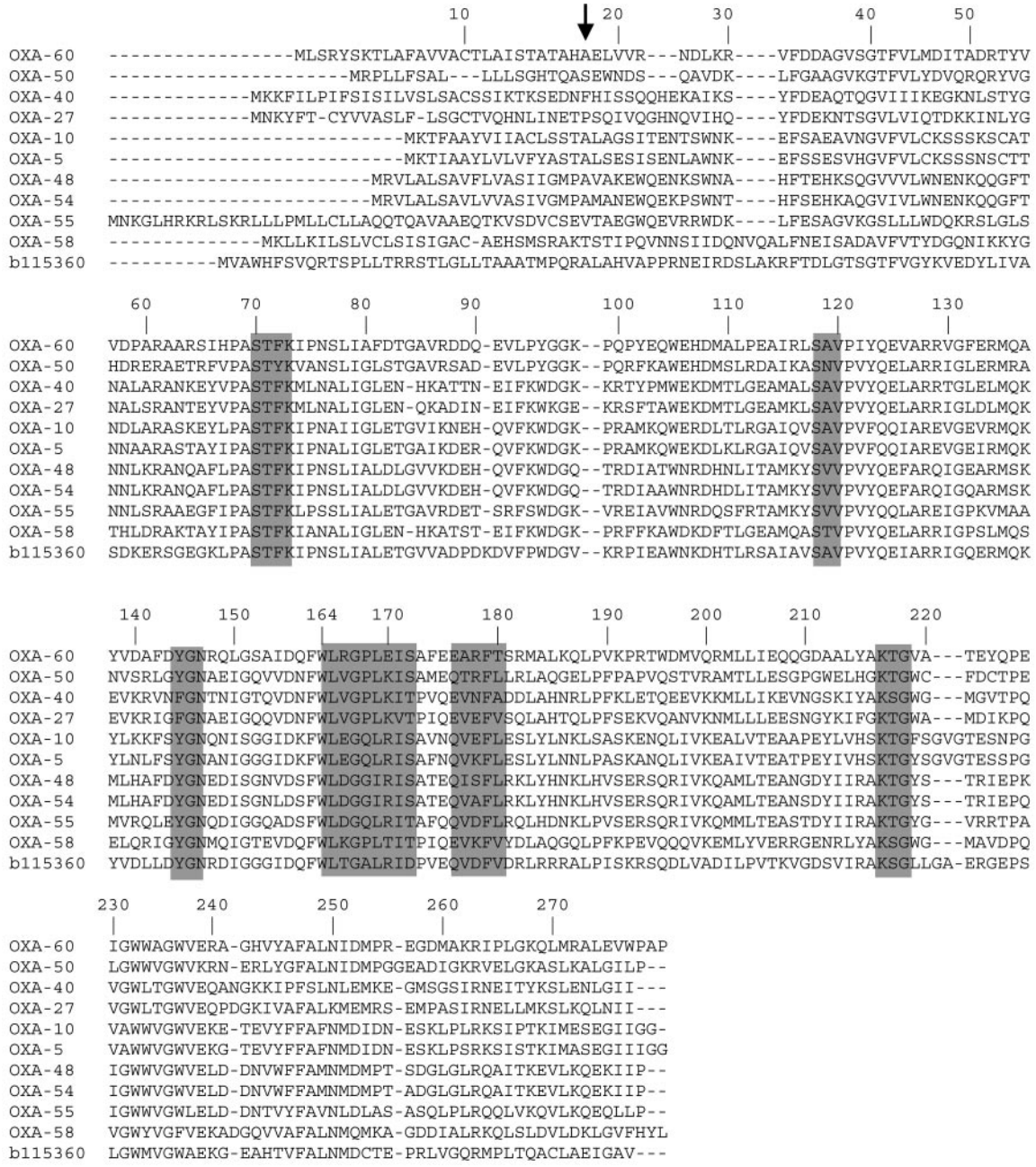

FIG. 2. Amino acid sequence comparison of oxacillinase OXA-60 with the sequences of oxacillinases OXA-50 from *P. aeruginosa* PAO1 (14); OXA-40, OXA-27, and OXA-58 from *A. baumannii* (1, 16; GenBank number AY570763); OXA-5 and OXA-10 from *P. aeruginosa*; OXA-48 from *K. pneumoniae*; OXA-54 from *S. oneidensis* (35); OXA-55 from *S. algae* (17); and bll5360 from *B. japonicum* (22). Boxes indicate conserved residues for oxacillinases. Numbering of β-lactamases is according to DBL (9). The arrow indicates the cleavage site for the leader peptide of OXA-60.

tein of ca. 27.7 kDa was seen after Coomassie blue staining (data not shown).

Kinetic parameters of purified β-lactamase OXA-60 showed that it had a hydrolysis profile that includes amoxicillin, benzylpenicillin, ticarcillin, and imipenem (Table 3). The very weak catalytic efficiency (k_{cat}/K_m) of OXA-60 for most β-lactams resulted from a low affinity (high K_m values) for these substrates. The highest affinity was found for imipenem with a K_m value of 2 μM. Biphasic kinetics were seen for amoxicillin, benzypenicillin, cephaloridine, oxacillin, and piperacillin. For these substrates, k_{cat} and K_m were determined in the steady-state part of the kinetics (the second part of the curve). Since carbon dioxide may influence the kinetics of oxacillinases (26, 32), hydrolysis parameters were determined also with $NaHCO_3$. Carbon dioxide did not transform the hydrolysis biphasic curve to a linear curve for any of these substrates. The addition of 10 mM $NaHCO_3$ did not significantly modify the catalytic efficiency of OXA-60 except for imipenem (data not shown). However, a lower affinity of OXA-60 for imipenem was observed in the presence of $NaHCO_3$, thus explaining

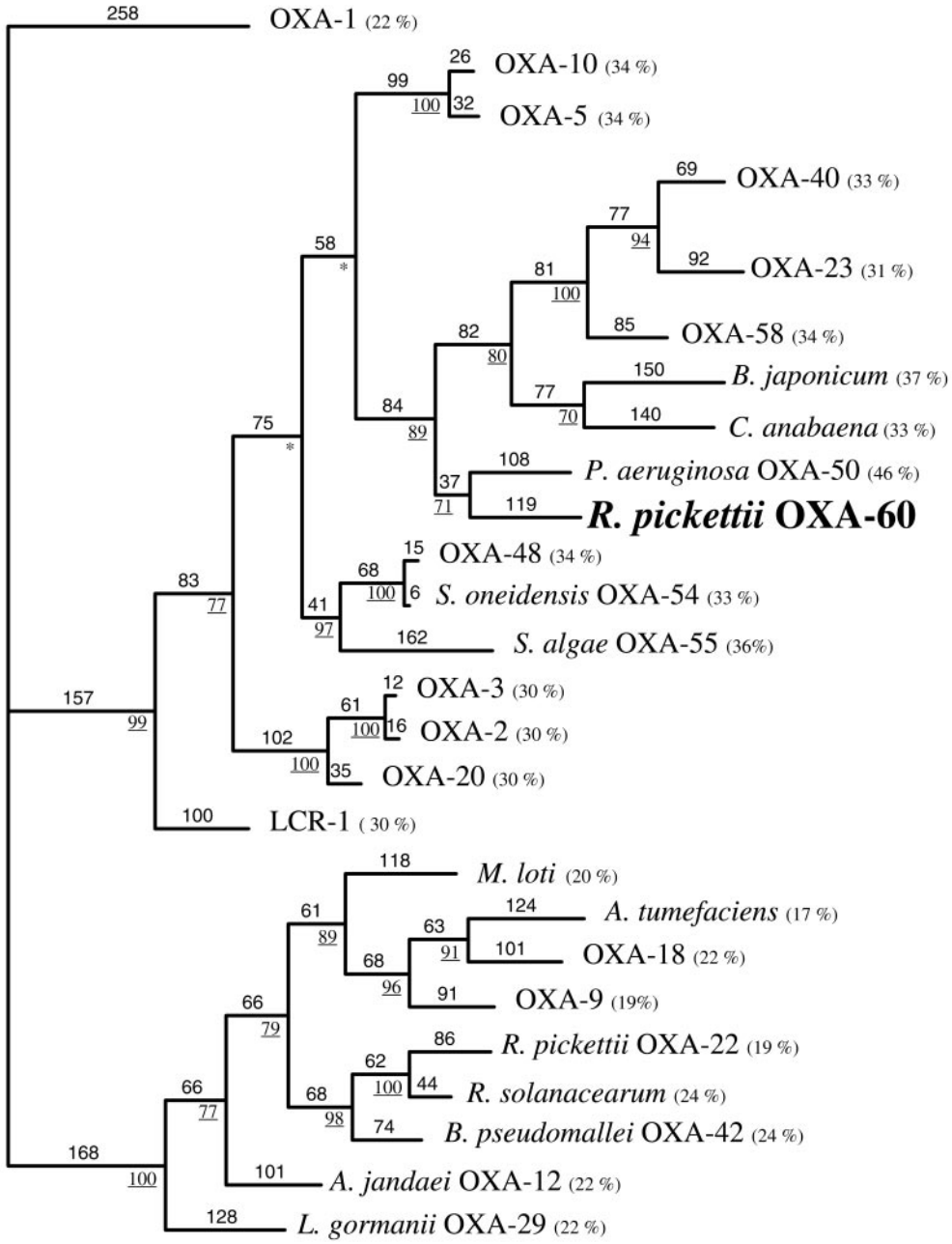

FIG. 3. Dendrogram obtained for 26 oxacillinases as determined by parsimony analysis (41). Branch lengths are drawn to scale and are proportional to the number of amino acid changes. The number of changes is indicated above each branch, and the percentage values at branching points (underlined) refer to the number of times a particular node was found in 100 bootstrap replications (the asterisks indicate uncertainty about nodes with bootstrap values of <50%). The distance along the vertical axis has no significance. Numbers in parentheses indicate percentage of amino acid identity with the β-lactamase OXA-60. Bacterial species names correspond to oxacillinases that occur naturally in those species.

why the k_{cat}/K_m ratio with or without $NaHCO_3$ remained unchanged for this substrate (data not shown). Similar results were obtained with OXA-50 from *P. aeruginosa*, the β-lactamase that shared the highest sequence identity with OXA-60 (14). Hydrolysis of oxacillin and cloxacillin were also detected at a low level as for other carbapenem-hydrolyzing oxacillinases (6, 27). Oxacillin was better hydrolyzed by OXA-60 than cloxacillin, although the enzyme had a weaker affinity for oxacillin ($K_m > 2$ mM). Hydrolysis of expanded-spectrum cephalosporins were not measurable except for ceftazidime. No hydrolysis of cephalothin was observed for OXA-60, whereas it was the best cephalosporin substrate for OXA-22 (31). Interestingly, OXA-60 hydrolyzed imipenem, and the catalytic activity of OXA-60 is more robust than that of other carbapenem-hydrolyzing oxacillinases, including OXA-54 of *S. oneidensis* (1, 4, 11, 16, 17, 35). Nevertheless, the ability of OXA-60 to hydrolyze imipenem was similar to that of OXA-54 (k_{cat}/K_m values for imipenem being, respectively, 260 and 250 mM^{-1} s^{-1}).

Inhibition studies as measured by IC_{50} values showed that

TABLE 3. Steady-state kinetic parameters of the β-lactamase OXA-60 and comparison of parameters obtained for β-lactamase OXA-22 (31)[a]

β-Lactam	OXA-60			OXA-22 (k_{cat}/K_m [mM^{-1} s^{-1}])
	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (mM^{-1} s^{-1})	
Benzylpenicillin	420	40	11,350	>40
Amoxicillin	60	300	190	16
Piperacillin	>300[b]	>2,000	<150	–
Ticarcillin	200	400	520	10
Nitrocefin	500	380	1,400	ND
Oxacillin	>130	>2,000	<65	–
Cloxacillin	70	1,500	50	140
Cephaloridine	>0.5	>1,000	<0.5	550
Cephalothin	–	–	–	330
Cefotaxime	–	–	–	–
Cefoxitin	–	–	–	4
Ceftazidime	2	1,000	2	–
Cefuroxime	–	–	–	10
Aztreonam	–	–	–	–
Imipenem	0.5	2	260	–
Meropenem	–	–	–	–

[a] Data are means of three independent experiments. Standard deviations were within 10% of the means. –, not determinable or no detectable hydrolysis (<0.01 s^{-1}); ND, not done.
[b] Data were determined for the corresponding K_m value (2,000 or 1,000 μM).

OXA-60 activity was weakly inhibited by clavulanic acid (450 μM), tazobactam (73 μM), and sulbactam (320 μM). This result corresponded to that found for other oxacillinases for which the tazobactam inhibitory property is equal to or higher than that of clavulanic acid (6, 27). OXA-60 activity was inhibited by NaCl as for most of the oxacillinases (IC_{50} = 23 mM) (6).

Genetic environment of *bla*$_{OXA-60}$. Recombinant plasmid pC2 was obtained by ligation of SacII-digested DNA of *R. pickettii* PIC-1 in plasmid pPCRScriptCam (Fig. 1). The flanking sequences of *bla*$_{OXA-60}$ were then analyzed by sequencing the 6,183-bp insert of pC2. Several ORFs were found that shared identity with chromosomally encoded genes of *R. solanacearum* (39) and *Chromobacterium violaceum* (5). An ORF located 192-bp upstream of the ATG codon of *bla*$_{OXA-60}$ (*orf-RP3*) and divergently transcribed encoded a 519-amino-acid protein that had 35% identity with an hypothetical protein CV3151, from *C. violaceum*. *orf-RP2* that was located 1,876 bp upstream of the ATG codon of *bla*$_{OXA-60}$ and divergently transcribed, encoded a 140-amino-acid protein that had 79% identity with Rs0260, a putative transcription regulator from *R. solanacearum* and further upstream (*orf-RP1*), a sequence that contained the 5′ end of a gene encoding a putative pyrrolidone carboxylate peptidase (PCP2) (63% of the gene) from *R. solanacearum*. Both genes shared 81 and 84% nucleotide identity with *rsp0260* and *pcp2*, respectively (39). The 3′ end of another ORF was identified 657 bp downstream of the *bla*$_{OXA-60}$ gene. Its deduced sequence shared 91% amino acid identity with Rs0262, a putative transmembrane protein of unknown function of *R. solanacearum* (39).

Although high-molecular-weight plasmids have been detected in *R. pickettii* PIC-1 (31), conjugation assays with *R. pickettii* PIC-1 as donor and rifampin-resistant *E. coli* JM109 as a recipient strain failed to transfer a β-lactam resistance marker. The location of the *bla*$_{OXA-60}$ gene was determined more precisely with the endonuclease I-CeuI technique. Three DNA fragments (3,700, 140, and 150 kb) were generated from whole-cell DNA of *R. pickettii* PIC-1. The probe for the rRNA genes hybridized with all DNA fragments. Hybridization of restricted DNA of *R. pickettii* PIC-1 with the *bla*$_{OXA-60}$-specific and with the *bla*$_{OXA-22}$-specific probe gave a single signal corresponding to the 3,700-kb fragment, indicating the chromosomal location for both oxacillinase genes. In contrast to most of the oxacillinase genes, the *bla*$_{OXA-60}$-like genes were not located within class 1 integrons since no core site or inverse core site was found surrounding the coding sequences of these genes (data not shown). No sequence coding for a putative AmpR regulator was found immediately upstream of the *bla*$_{OXA-60}$ gene.

Distribution of OXA-60-like genes in *R. pickettii* isolates. Analysis of the PFGE patterns of the XbaI-restricted DNAs of several *R. pickettii* clinical isolates and reference strains showed that these strains were not epidemiologically related except for *R. pickettii* PIC-1 and PIC-3 that differed only by a few XbaI bands (31). Analytical IEF performed on a pH 4 to 6.5 Ampholine polyacrylamide gel revealed pI values of additional β-lactamases in extracts from imipenem-induced culture extracts of *R. pickettii.* An identical β-lactamase with a pI of 5.1 was detected in *R. pickettii* PIC-1 and PIC-3, a band of pI 5.2 was found in *R. pickettii* PIC-2 and ATCC 27511, and a band of pI 5.3 was found in *R. pickettii* CIP 103413 and CIP 74.22 (data not shown). These pI values corresponded to *bla*$_{OXA-60}$-like genes that gave positive signals by PCR and that were sequenced. The deduced proteins shared 91 to 100% amino acid identity. The genetic variability of OXA-60-like sequences in *R. pickettii* was slight, as found for OXA-22-like sequences (Table 4) (31). Southern blot analyses showed that the *bla*$_{OXA-22}$ and *bla*$_{OXA-60}$ genes did not hybridize to the same SacII, PstI, SalI-, AccI-, or EagI-restricted DNA fragments (data not shown). In addition, Southern blot hybridization of DNA fragments performed with strains of other *Ralstonia* species

TABLE 4. Amino acid substitutions for OXA-60-like β-lactamases

Strain	β-Lactamase	Amino acid at position[a]:																pI
		31	49	65	103	132	143	153	200	229	234	243	244	264	272	275	277	
PIC-1	OXA-60	V	T	S	E	F	D	A	Q	E	V	H	V	I	R	E	W	5.1
PIC-3	OXA-60a																V	5.1
PIC-2	OXA-60b			R														5.2
ATCC 2751	OXA-60b			R														5.2
CIP 103413	OXA-60c		S	H	K			V	H	Q	I	R	I		Q	A		5.3
CIP 7422	OXA-60d	A	G	R	Q	L	E	V						V	Q	A		5.3

[a] Numbering is according DBL (class D β-lactamase numbering) (9).

TABLE 5. β-Lactamase activity of *R. pickettii* PIC-1 and isogenic mutants

Substrate	Strain	Relevant genotype	β-Lactamase activity (mU/mg of protein) ± SD[a]	
			Basal	Induced[b]
Nitrocefin	PIC-1	*oxa-22*$^+$ *oxa-60*$^+$	<1	250 ± 4
	PIC-1ΔOXA-22	*oxa-60*$^+$	<1	88 ± 16
	PIC-1ΔOXA-60	*oxa-22*$^+$	<1	232 ± 33
Piperacillin	PIC-1	*oxa-22*$^+$ *oxa-60*$^+$	<1	33 ± 4
	PIC-1ΔOXA-22	*oxa-60*$^+$	<1	17 ± 1
	PIC-1ΔOXA-60	*oxa-22*$^+$	<1	2.4 ± 0.5
Cephalothin	PIC-1	*oxa-22*$^+$ *oxa-60*$^+$	<1	2.6 ± 0.5
	PIC-1ΔOXA-22	*oxa-60*$^+$	<1	<1
	PIC-1ΔOXA-60	*oxa-22*$^+$	<1	7.3 ± 1

[a] The results shown are the means ± the standard deviations of results of three separated experiments.
[b] Imipenem (1 μg/ml) was used as the β-lactam inducer.

(*R. solanacearum*, *R. basilensis*, *R. eutropha*, *R. gilardii*, and *R. paucula*) by using each of the oxacillinase probes showed that bla_{OXA-60}-like genes and bla_{OXA-22}-like genes were not found, underlining the specificity of these oxacillinase genes for *R. pickettii.* These oxacillinase genes may be useful tools for species identification.

Gene inactivation and induction studies. Expression of β-lactamases OXA-22 and OXA-60 were studied in *R. pickettii* PIC-1, in *R. pickettii* PIC-1ΔOXA-60, and in *R. pickettii* PIC-1ΔOXA-22, respectively (Table 5). Truncated bla_{OXA-60} and bla_{OXA-22} genes were cloned into pACYC184 and introduced into *R. pickettii* PIC-1 by electroporation, leading to the disruption of the genomic copy of either the bla_{OXA-60} or bla_{OXA-22} genes. Inactivation of each oxacillinase gene resulted in a higher susceptibility to β-lactams (Table 2). Indeed, inactivation of the bla_{OXA-60} gene resulted in a 4- to 16-fold decrease of MICs of β-lactams for *R. pickettii* PIC-1ΔOXA-60, except for cephalothin, aztreonam, and moxalactam (Table 2). This suggests that OXA-22 has a minor contribution in resistance to β-lactams of *R. pickettii* PIC-1ΔOXA-60. Inactivation of the bla_{OXA-22} gene resulted in a decrease of MICs of cephalothin and moxalactam (Table 2). The MICs of piperacillin, cefotaxime, cefoxitin, cefuroxime, and cefepime were decreased in bla_{OXA-60}- and bla_{OXA-22}-deficient strains, indicating that both β-lactamases contribute to the resistance of *R. pickettii* to these substrates. Interestingly, the MICs of imipenem remained unchanged for bla_{OXA-22}-deficient *R. pickettii* PIC-1, suggesting that OXA-60 alone played a role in reduced susceptibility to imipenem. Finally, these data showed that none of these oxacillinases contributed to resistance to aztreonam, which may result from another mechanism of resistance (e.g., penicillin-binding protein affinity).

IEF analysis confirmed that OXA-60 biosynthesis was detected only after induction in the OXA-22-deficient strain *R. pickettii* PIC-1, whereas OXA-22 biosynthesis was detected with or without induction in the OXA-60-deficient strain *R. pickettii* PIC-1 (data not shown).

Kinetic parameters indicated that no hydrolysis of cephalothin was observed for OXA-60, whereas it was the best cephalosporin substrate for OXA-22 (31), and that hydrolysis of piperacillin by OXA-60 occurred at a high level, whereas hydrolysis of piperacillin was not detectable for OXA-22.

Hydrolysis of piperacillin and cephalothin was used to identify the remaining β-lactamase produced by each deficient *R. pickettii* PIC-1 strain. Hydrolysis of nitrocefin was used to estimate the overall β-lactamase activity in the culture extracts. The specific activity of crude β-lactamase extracts of cultures of *R. pickettii* PIC-1, *R. pickettii* PIC-1ΔOXA-22, and *R. pickettii* PIC-1ΔOXA-60 with or without induction was measured (Table 5). Hydrolysis of nitrocefin indicated that the level of β-lactamase activity of the parental strain and of both OXA-60- and OXA-22-deficient *R. pickettii* PIC-1 strains was low and inducible, thus confirming that both oxacillinases produced were regulated (Table 5). Nevertheless, *R. pickettii* PIC-1ΔOXA-22 that produces only OXA-60 had a threefold-lower β-lactamase expression after induction than the parental strain or than the *R. pickettii* PIC-1ΔOXA-60 strain. The absence of cephalothin hydrolysis by *R. pickettii* PIC-1ΔOXA-22 that produced only OXA-60 corresponded to the MICs (Table 2) and the kinetic parameters determined for OXA-60 (Table 3). *R. pickettii* PIC-1ΔOXA-60 that produced only OXA-22 showed a level of β-lactamase activity similar to that of the parental strain and threefold higher as indicated by cephalothin hydrolysis. Kinetic parameters of OXA-22 could not be precisely determined for piperacillin, although MICs of *R. pickettii* PIC-1ΔOXA-22 showed that OXA-22 was responsible for part of the resistance of *R. pickettii* to piperacillin. The specific activity of crude β-lactamase extracts of cultures of *R. pickettii* PIC-1ΔOXA-60 showed that OXA-22 accounted only for 7% of the hydrolysis of piperacillin (Table 5).

Regulation of oxacillinase gene expression has been documented for OXA-12 from *Aeromonas* spp. that is chromosomally located (2, 18, 37). In *Aeromonas hydrophila*, expression of three β-lactamase genes is regulated by a two-component system regulator response, the activity of which depends on the phosphorylation state (30). This transcriptional control involves at least one direct repeat of a promoter-proximal DNA sequence motif, TTCAC, a "blr-tag" or "cre-tag" (3). None of these motifs have been found near the promoter of the bla_{OXA-60} gene. Furthermore, no LysR-type transcriptional regulator has been found surrounding the bla_{OXA-60} gene that could regulate the transcription of this gene (34).

The present study provides several interesting results. (i) Two nonrelated oxacillinases may be found naturally in a single bacterial species, further underlining the diversity of oxacillinases. (ii) Two oxacillinase genes may be regulated. (iii) Finally, another oxacillinase with carbapenem-hydrolyzing property has been characterized.

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