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Genetic and Biochemical Characterization of OXA-519, a Novel OXA-48-Like β-Lactamase

Laura Dabos,[a,b] Pierre Bogaerts,[c,d] Remy A. Bonnin,[a,b,e] Agustin Zavala,[f] Pierre Sacré,[c,d] Bogdan I. Iorga,[f] Daniel T. Huang,[c,d] Youri Glupczynski,[c,d] Thierry Naas[a,b,e,g]

[a]EA7361 "Structure, dynamic, function and expression of broad spectrum β-lactamases," LabEx Lermit, Faculty of Medicine, Université Paris Sud, Université Paris Saclay, Le Kremlin-Bicêtre, France

[b]Evolution and Ecology of Resistance to Antibiotics Unit, Institut Pasteur—APHP, Université Paris Sud, Paris, France

[c]Belgian National Reference Laboratory for Monitoring of Antimicrobial Resistance in Gram-Negative Bacteria, CHU UCL Namur asbl, Yvoir, Belgium

[d]Institut de Recherche Expérimentale et Clinique (IREC), Pôle Mont-Godinne (MONT), Université catholique de Louvain, Yvoir, Belgium

[e]Associated French National Reference Center for Antibiotic Resistance: Carbapenemase-producing Enterobacteriaceae, Le Kremlin-Bicêtre, France

[f]Institut de Chimie des Substances Naturelles, CNRS UPR 2301, Gif-sur-Yvette, France

[g]Bacteriology-Hygiene unit, Assistance Publique/Hôpitaux de Paris, Bicêtre Hospital, Le Kremlin-Bicêtre, France

ABSTRACT A multidrug-resistant *Klebsiella pneumoniae* 1210 isolate with reduced carbapenem susceptibility revealed the presence of a novel plasmid-encoded $bla_{OXA-48-like}$ gene, named $bla_{OXA-519}$. The 60.7-kb plasmid (pOXA-519) was similar to the IncL-OXA-48 prototypical plasmid except for a ca. 2-kb deletion due to an IS*1R* insertion. OXA-519 differed from OXA-48 by a Val120Leu substitution, which resulted in an overall reduced β-lactam-hydrolysis profile, except those for ertapenem and meropenem, which were increased. Thus, detection of OXA-519 producers using biochemical tests that monitor imipenem hydrolysis will be difficult.

KEYWORDS carbapenemase, mutant, steady-state kinetics, OXA-48 like, detection

Class D β-lactamases (DBLs), or OXA-type β-lactamases (OXAs), form a very diverse family of enzymes (1–3), the diversity of which is reflected at both genetic and biochemical levels. OXA-48, the main carbapenem-hydrolyzing class D β-lactamase (CHDL) encountered in *Enterobacteriaceae* in many countries around the Mediterranean area, was initially identified from a carbapenem-resistant *Klebsiella pneumoniae* isolate from Turkey in 2001 (4, 5). Although OXA-48 hydrolyzes penicillins at a high level and carbapenems at a low level, it shows very weak or no activity against expanded-spectrum cephalosporins (6). Along with the spread of OXA-48, several variants have been reported that differ from OXA-48 by up to 5 amino acid substitutions or deletions (http://bldb.eu/BLDB.php?class=D#OXA) (7). The aim of this study was to characterize OXA-519, a novel OXA-48-like β-lactamase detected in a clinical *K. pneumoniae* isolate recovered in Belgium in 2015.

K. pneumoniae 1210 was recovered from a urine sample of an 87-year-old patient who had not been hospitalized in the last 12 months. The urine displayed hyperleukocyturia, and the culture grew $>10^5$ CFU/ml of *K. pneumoniae*, which was identified using matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (MALDI Biotyper; Bruker Daltonics, Illkirch, France). Disk diffusion antibiotic susceptibility testing was done and interpreted according to the EUCAST guidelines (http://www.eucast.org). The isolate *K. pneumoniae* 1210 was resistant to penicillins and expanded-spectrum cephalosporins, was intermediate to ertapenem, and

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Address correspondence to Thierry Naas, thierry.naas@aphp.fr.

TABLE 1 MICs of β-lactams for *K. pneumoniae* and *E. coli* strains[a]

Antibiotic	MIC (mg/liter) against:										
		E. coli TOP10					*E. coli* HB4				
	K. pneumoniae 1210	pNOXA-519	pR-OXA-48	pR-OXA-519	pR-OXA-163	*E. coli* TOP10	pR-OXA-48	pR-OXA-519	pR-OXA-163[c]	pN-OXA-519	*E. coli* HB4
Amoxicillin	>256	>256	>256	>256	>256	2	>256	>256	>256	>256	16
Amoxicillin + CLA[b]	48	32	192	32	96	2	>256	32		>256	8
Cefotaxime	>32	0.047	0.094	0.047	3	0.06	8	0.75	128	1.5	1
Ceftazidime	48	0.125	0.19	0.125	16	0.12	1	0.5	256	1	0.75
Cefepime	16	0.023	0.047	0.023	0.5	0.023	12	0.75		1	0.75
Imipenem	0.25	0.25	0.38	0.25	0.25	0.25	>32	1.5	0.5	>32	0.125
Meropenem	0.75	0.19	0.047	0.19	0.023	0.016	>32	>32	4	>32	0.38
Ertapenem	2	0.125	0.047	0.125	0.032	0.003	>32	>32	32	>32	0.75
Temocillin	>1,024	>1,024	>1,024	>1,024	32	4	>1,024	>1,024	64	>1,024	24
Aztreonam	>256	0.047	0.047	0.047	2	0.047	0.5	0.38		0.5	0.38

[a]*K. pneumoniae* 1210, *E.coli* TOP10 pTOPO OXA-48, *E. coli* TOP10 pTOPO-519, *E. coli* TOP 10 pTOPO-163, *E. coli* TOP10, *E. coli* HB4 pOXA-48, *E. coli* HB4 pOXA-519, and *E. coli* HB4.
[b]CLA, clavulanic acid (2 mg/liter).
[c]Values from Oueslati et al. (15).

remained susceptible to meropenem and imipenem (Table 1). The Carba NP test, β-Carba test (Bio-Rad, Marnes-La-Coquette, France), Bogaerts-Yunus-Glupczynski (BYG) Carba test, and Maldi-TOF Star-BL assay (Brucker Daltonics) were performed as previously described (9–12). Carba NP and BYG Carba tests gave negative test results while β-Carba and Star BL gave weak but reproducible positive results. OXA-48 K-SeT (Coris BioConcept, Gembloux, Belgium) and the NG Carba 5 (NG Biotech, Rennes, France) immunochromatographic assays revealed a positive OXA-48 band (13, 14). PCR experiments and subsequent sequencing, as previously described (15), revealed the presence of a novel $bla_{\text{OXA-48-like}}$ gene, designated the $bla_{\text{OXA-519}}$ gene, which showed a single nucleotide change resulting in a single amino acid Val to Leu substitution at position 120, according to DBL numbering (3, 16, 17).

The $bla_{\text{OXA-48}}$, $bla_{\text{OXA-163}}$, and $bla_{\text{OXA-519}}$ genes were amplified using preOXA-48A (5′-TATATTGCATTAAGCAAGGG-3′) and cloning-OXA-48B (5′-AAAAGGATCCCTAGGGAA TAATTTTTTCCTGTTTGAGCA-3′) primers and were subsequently cloned into the pCR-Blunt II-TOPO plasmid (Invitrogen, Illkirch, France), resulting in recombinant pR-OXA-48, pR-OXA-163, and pR-OXA-519 plasmids, respectively. OXA-519 conferred similar, but reduced resistance profiles on *Escherichia coli* TOP10 compared to those conferred by OXA-48, consisting of susceptibility to expanded-spectrum cephalosporins, resistance to temocillin and piperacillin-tazobactam, and decreased susceptibility to imipenem (MIC, 0.25 μg/μl). OXA-519 presented slightly higher MICs for meropenem and ertapenem compared to those presented by OXA-48 (~4-fold and ~3-fold, respectively) (Table 1), and it was more inhibited by clavulanic acid compared to OXA-48 and OXA-163 (Table 1). In order to see whether these enzymes may lead to carbapenem resistance in bacterial hosts with impaired outer-membrane permeability, pR-OXA-48, pR-OXA-163, and pR-OXA-519 plasmids were transformed into *E. coli* HB4 lacking the major porins OmpF and OmpC (15). In *E. coli* HB4, all three enzymes presented increased MICs for all of the β-lactams tested, including the carbapenems. For OXA-519, MIC values increased by more than ~256-fold for ertapenem and meropenem, thus resulting in resistance to these two molecules, but only by 6-fold for imipenem (Table 1), resulting in a MIC of 1.5 μg/μl, which is still in the susceptibility range.

The $bla_{\text{OXA-519}}$ gene was cloned into the expression vector pET41b (+) (Novagen, VWR International, Fontenay-sous-Bois, France) using the PCR-generated fragment with primers INF-OXA-48Fw (5′-AAGGAGATATACATATGGTAGCAAAGGAATGGCAAG-3′) and INF-OXA-48Rv (5′-GGTGGTGGTGCTCGAAGGGAATAATTTTTTCCTGTTTGAG-3′), and the NEBuilder HiFi DNA assembly cloning kit (New England BioLabs Inc., United Kingdom), following the manufacturer's instructions. Recombinant plasmid pET41-OXA-519 was electroporated into *E. coli* strain BL21(DE3) and expressed at 25°C with 0.2 mM IPTG

TABLE 2 Steady-state kinetic parameters of β-lactamases OXA-48, OXA-519, and OXA-163

	Kinetic parameter[a]								
	K_m (μM)			k_{cat} (s^{-1})			k_{cat}/K_m (mM^{-1}/s^{-1})		
Substrate	OXA-48	OXA-519	OXA-163	OXA-48	OXA-519	OXA-163	OXA-48	OXA-519	OXA-163
Ampicillin	395	776	315	955	131	23	2,418	169	70
Piperacillin	410	109	ND	75	36	ND	180	328	ND
Oxacillin	95	338	90	130	8	34	1368	23	370
Temocillin	45	>1,000	NH	0.30	>12	NH	6.6	8.8	ND
Cephalothin	195	>1,000	10	44	>13	3.0	226	3.5	300
Cefoxitin	>200	>1,000	ND	>0.05	>4	ND	0.26	1.7	ND
Ceftazidime	NH	373	>1,000	NH	0.02	8	ND	0.04	3
Cefotaxime	>900	>1,000	45	>9	>1.3	10	10	0.39	230
Cefepime	>550	>1,000	ND	>0.60	>1.3	ND	1.1	0.31	ND
Imipenem	13	982	520	4.8	2.1	0.03	369	2.1	0.06
Meropenem	11	358	>2,000	0.07	3.4	0.10	6.2	9.5	0.03
Ertapenem	100	83	130	0.13	1.1	0.05	1.3	13	0.30

[a]NH, hydrolysis could not be detected with concentrations of substrate and enzymes up to 1,000 mM and 400 nM, respectively; ND, not determined.

(isopropyl-β-D-thiogalactopyranoside) as inducer. OXA-519 was purified by one-step pseudo-affinity chromatography, using a nitrilotriacetic acid (NTA)-nickel column (GE Healthcare, Freiburg, Germany). Kinetic parameters were determined as previously reported (15). The lowest catalytic efficiencies were observed for cephalosporins. Interestingly, and unlike for OXA-48, hydrolysis of ceftazidime could be detected for OXA-519, although at lower level than that for OXA-163. With respect to carbapenems, OXA-519 had an increased catalytic efficiency for ertapenem (10-fold) and meropenem (2-fold), but the k_{cat}/K_m value for imipenem was 176-fold smaller than that of OXA-48 (Table 1). Unlike OXA-48, which is not inhibited by clavulanic acid and is well inhibited by NaCl (50% inhibitory concentration [IC_{50}], 7 mM) (4), OXA-519 was significantly inhibited by clavulanic acid (IC_{50}, 83 μM) and weakly inhibited by NaCl (IC_{50}, 200 mM). This inhibition profile is similar to that of OXA-163, which has IC_{50} values of 13.4 μM and 270 mM for clavulanic acid and NaCl, respectively (4, 15).

An analysis of all OXA-48-like sequences available in the Beta-Lactamase DataBase (7) showed that position 120 is very conserved within this family. The mutated residue in position 120 is situated at the bottom of the binding site (data not shown), in the close vicinity of the active Ser70 and the β5-β6 loop, and thus it is likely involved in the β-lactamase activity of OXA-48 (6, 18, 19). In OXA-51, the chromosomal CHDL of *Acinetobacter baumannii* that hydrolyzes carbapenems less efficiently than other CHDLs, position 120 is occupied by Ile instead of Val (20). The carbon δ of this Ile would cause a steric clash with the hydroxyethyl group of carbapenems, leading to an increase in the K_m values of OXA-51 (8, 21). Likewise, the bulkier side chain of Leu120 in OXA-519, compared to that of Val120 in OXA-48, hampers the approach of β-lactam substrate, resulting in a decrease of the substrate affinity. This is in agreement with higher K_m values determined for OXA-519 compared with those for OXA-48 (Table 2).

The whole genome of *K. pneumoniae* 1210 was sequenced using Illumina technology, as previously reported (22). The genome was 5,549,801 bp in size, with a mean coverage of 57×. Multilocus sequence typing (MLST) of *K. pneumoniae* 1210, determined using MLST 1.8 software (23), revealed a novel ST, which was assigned the novel allelic profile ST2728 (1-1-211-1-1-1-1) by the *K. pneumoniae* MLST database (http://bigsdb.web.pasteur.fr). This sequence type is a single-locus variant (SLV) of ST15 (1-1-1-1-1-1-1-1), a pandemic clone widely described in association with carbapenemases or ESBLs and sometimes involved in outbreaks (24–26). The acquired resistance genes were identified using ResFinder server v2.1 (http://cge.cbs.dtu.dk/services/ResFinder-2.1) (27). *K. pneumoniae* 1210 presented four β-lactamases genes, including the naturally occurring bla_{SHV-28}, the acquired bla_{OXA-1}, the $bla_{CTX-M-15}$ ESBL, and the $bla_{OXA-519}$ carbapenemase genes. The bla_{OXA-1} gene, the *aac(6′)-Ib-cr* gene conferring resistance to kanamycin, tobramycin, and amikacin and decreased susceptibility to fluoroquinolones (28), and the *catB4* gene conferring chloramphenicol resistance were

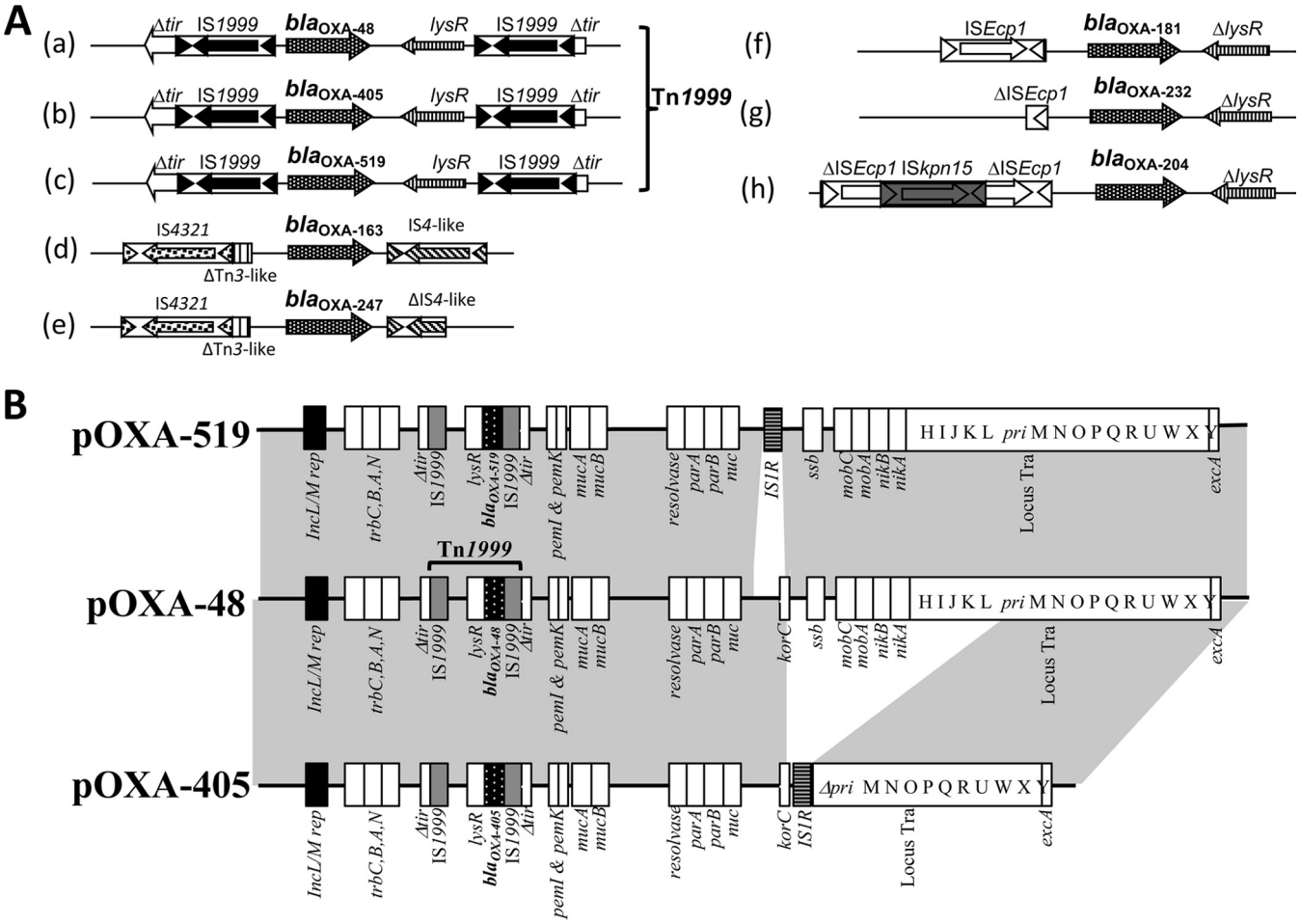

FIG 1 (A) Schematic representation of the genetic environment of $bla_{OXA-48-like}$ β-lactamases. Bla_{OXA-48} (a), $bla_{OXA-405}$ (b), $bla_{OXA-519}$ (c), $bla_{OXA-163}$(d), $bla_{OXA-247}$ (e), $bla_{OXA-181}$ (f), $bla_{OXA-232}$ (g), and $bla_{OXA-204}$ (h) genes. The Tn*1999* composite is formed by two copies of insertion sequence IS*1999* bracketing a fragment containing the bla_{OXA-48}, $bla_{OXA-405}$, and $bla_{OXA-519}$ genes. (B) Major structural features of the plasmids pNOXA-519 from *K. pneumoniae* 1210 with the prototypical IncL bla_{OXA-48} plasmid (pOXA-48) (GenBank accession number JN626286) and pOXA-405. Common structures are highlighted in gray.

part of a class 1 integron, as previously described (29). A *fosA*-like gene involved in the decreased susceptibility to fosfomycin in *K. pneumoniae* was also evidenced. In addition, GyrA topoisomerase exhibited several substitutions (S83F and D87A) that are known to confer high-level resistance to fluoroquinolones in Gram-negative rods (30). However, the *gyrB*, *parC*, and *parE* genes did not display any mutation in the quinolone resistance-determining region (QRDR) (data not shown). Finally, the *ompK35* gene coding for the OMPK35 porin was disrupted by the insertion sequence belonging to the IS*1* family, while the *ompK36* gene was not altered.

The $bla_{OXA-519}$ gene was located on a Tn*1999* composite transposon, unlike the $bla_{OXA-163}$ or $bla_{OXA-247}$ genes, which are associated with IS*4321* and IS*4* elements, and the $bla_{OXA-181}$, $bla_{OXA-204}$, and $bla_{OXA-232}$ genes, which are associated with the IS*Ecp1* element (Fig. 1A). Three different plasmid-replication origins belonging to the incompatibility groups, IncFIB, IncL, and IncFII(K) were identified using PlasmidFinder 1.3 (https://cge.cbs.dtu.dk/services/). The $bla_{OXA-519}$ gene was carried by an IncL-type plasmid of ca. 60 kb, similar to the prototypical pOXA-48 plasmid (GenBank accession number JN626286) but differing by a 1,986-bp deletion encompassing the *korC* gene (31–33) (Fig. 1B). Conjugation assays, performed as previously described (34), revealed a high conjugation frequency of pN-OXA-519 ($1.05 \times 10^{-1} \pm 0.003$), which was similar to that of pOXA-48 ($1.13 \times 10^{-1} \pm 0.002$), suggesting that the deletion has no impact on the conjugation efficiency.

We report here a novel OXA-48-like β-lactamase, OXA-519, which presents reduced activity toward imipenem compared to that of OXA-48. It is important to stress that, even though OXA-519 has reduced imipenem-hydrolytic activity compared to that of OXA-48, it has increased meropenem and ertapenem catalytic efficiency, and when expressed in a porin-deficient strain, the MICs rose significantly in the resistance range. Of note, OXA-519 may spread silently, since conventional biochemical tests based on carbapenem hydrolysis failed to detect this variant. The mutation Val120Leu is located at the bottom of the active site cavity of the protein. The bulkier side chain of Leu in OXA-519 compared with that of Val in OXA-48 induces a decrease in substrate affinity. Considering the high transfer frequency of pOXA-519, which is similar to that of pOXA-48, the risk of dispersion of this gene in the gut of patients may result in high-level carbapenem resistance, even though the initial isolate may be susceptible.

Accession number(s). The nucleotide sequences of the $bla_{OXA-519}$ gene and of its natural plasmid pOXA-519 have been submitted to the EMBL/GenBank nucleotide sequence database under the accession numbers KX349732 and KY215945, respectively. This whole-genome shotgun sequence of strain *K. pneumoniae* 1210 has been deposited at DDBJ/ENA/GenBank under the accession number PCGD00000000. The version described in this paper is PCGD01000000.

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We declare no competing interests.

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