

Structural and biochemical features of OXA-517: a Carbapenem and expanded-spectrum Cephalosporin hydrolyzing OXA-48 variant

Laura Dabos, Joanna Raczynska, Pierre Bogaerts, Agustin Zavala, Delphine Girlich, Remy Bonnin, Laurent Dortet, Aurélie Peyrat, Pascal Retailleau,

Bogdan Iorga, et al.

► To cite this version:

Laura Dabos, Joanna Raczynska, Pierre Bogaerts, Agustin Zavala, Delphine Girlich, et al.. Structural and biochemical features of OXA-517: a Carbapenem and expanded-spectrum Cephalosporin hydrolyzing OXA-48 variant. Antimicrobial Agents and Chemotherapy, 2023, 67 (2), pp.e0109522. 10.1128/aac.01095-22 . hal-03966994

HAL Id: hal-03966994 https://hal.science/hal-03966994

Submitted on 1 Feb 2023

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers. L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

1	
2	Structural and biochemical features of OXA-517: a carbapenem and expanded-spectrum
3	cephalosporin hydrolyzing OXA-48 variant.
4	
5	Laura Dabos ^{1,2} , Joanna E. Raczynska ³ , Pierre Bogaerts ⁵ , Agustin Zavala ⁶ , Delphine Girlich ^{1,2} , Remy A.
6	Bonnin ^{1,2} , Laurent Dortet ^{1,2,4} , Aurélie Peyrat ¹ , Pascal Retailleau ⁶ , Bogdan I. Iorga ⁶ , Mariusz
7	Jaskólski ^{3,7} , Youri Glupczynski ⁵ , Thierry Naas ^{1,2,4*}
8	
9	¹ Team "Resist" UMR1184, INSERM, CEA, University Paris-Saclay, LabEx LERMIT, Faculty of
10	Medicine, Le Kremlin-Bicêtre, France.
11	² French National Reference Center for Antibiotic Resistance: Carbapenemase-producing
12	Enterobacterales, Le Kremlin-Bicêtre, France:
13	³ Center for Biocrystallographic Research, Institute of Bioorganic Chemistry, Polish Academy of
14	Sciences, Poznan, Poland.
15	⁴ Department of Bacteriology-Hygiene, Bicêtre Hospital, APHP Paris-Saclay, Le Kremlin-Bicêtre,
16	France
17	⁵ Laboratory of clinical microbiology, National reference center for monitoring antimicrobial
18	resistance in Gram-negative bacteria, CHU UCL Namur, Yvoir, Belgium.
19	⁶ Université Paris-Saclay, CNRS UPR 2301, Institut de Chimie des Substances Naturelles, Labex
20	LERMIT, Gif-sur-Yvette, France.
21	⁷ Department of Crystallography, Faculty of Chemistry, A. Mickiewicz University, Poznan, Poland.
22	Abstract: 203; Manuscript: 6474
23	Tables: 3; Figures: 6
24	Supplemental material: Clinical case, and 2 Figures
25	Running title: OXA-517, an extended-spectrum OXA-48-variant
26	Keywords: oxacillinase, extended-spectrum β -lactamase, carbapenem, cephalosporin, hydrolysis,
27	antibiotic resistance
28	* Corresponding author's address: Service de Bactériologie-Virologie, Hôpital de Bicêtre, 78 rue du
29	Général Leclerc 94275 Le Kremlin-Bicêtre Cedex, France. Phone: +33 1 45 21 29 86. Fax: +33 1 45
30	21 63 40. E-mail: thierry.naas@aphp.fr

31 Abstract (203)

32 OXA-48-producing Enterobacterales have now widely disseminated throughout the world. Several 33 variants have now been reported, differing by just a few amino-acid substitutions or deletions, 34 mostly in the region of the loop β 5- β 6. As OXA-48 hydrolyzes carbapenems but lacks significant 35 expanded-spectrum cephalosporin (ESC) hydrolytic activity, ESCs were suggested as a therapeutic 36 option. Here, we have characterized OXA-517, a natural variant of OXA-48- with an Arg214Lys 37 substitution and a deletion of Ile215 and Glu216 in the β 5- β 6 loop, capable of hydrolyzing at the same time ESC and carbapenems. MICs values of *E. coli* expressing *bla*_{OXA-517} gene revealed 38 39 reduced susceptibility to carbapenems (similarly to OXA-48) and resistance to ESCs. Steady state 40 kinetic parameters revealed high catalytic efficiencies for ESCs and carbapenems. The bla_{OXA-517} 41 gene was located on a ca. 31-kb plasmid identical to the prototypical IncL bla_{OXA-48}-carrying plasmid 42 except for an IS1R-mediated deletion of 30.7-kb in the tra operon. The crystal structure of OXA-43 517, determined to 1.86 Å resolution, revealed an expanded active site as compared to that of OXA-48, which allows for accommodation of the bulky ceftazidime substrate. Our work illustrates 44 45 the remarkable propensity of OXA-48-like carbapenemases to evolve through mutation/deletion in 46 the β 5- β 6 loop to extend its hydrolysis profile to encompass most β -lactam substrates.

48 Introduction

49 Antimicrobial resistance is the most alarming problem that is emerging in infectious 50 diseases. B-Lactams, due to their safety, reliable killing properties and clinical efficacy, are among 51 the most frequently prescribed antibiotics used to treat bacterial infections. However, their utility 52 is being threatened by the worldwide proliferation of β -lactamases (BLs) with broad hydrolytic 53 capabilities (1). Currently, BL-mediated resistance does not spare even the most powerful β-54 lactams (i.e. carbapenems), whose activity is challenged by carbapenemases (1,2). OXA-48, a 55 Carbapenem-Hydrolyzing class D β -Lactamase (CHDL) initially identified in a carbapenem-resistant 56 Klebsiella pneumoniae isolate from Turkey in 2001, has now spread globally in Enterobacterales 57 (3,4). Several variants have since been reported, differing by a few amino acid substitutions or 58 deletions (5). For a complete list of variants see the Beta-Lactamase DataBase (6; 59 http://bldb.eu/BLDB.php?class=D#OXA). OXA-48 hydrolyzes penicillins at a high level, 60 carbapenems at a low level and lacks significant hydrolytic activity towards expanded-spectrum 61 cephalosporin (ESC) (5). While some OXA-48-variants with single amino acid substitutions have 62 similar hydrolytic profiles as OXA-48, others, such as OXA-163 or OXA-405, carry a four-amino-acid 63 deletion that results in the loss of carbapenemase activity and a gain of the ability to hydrolyze 64 expanded-spectrum cephalosporins (5, 7-9). A novel OXA-48-variant, OXA-438, with two AA deletions and four AA changes in the $\beta 5-\beta 6$ loop, has been described with increased ability to 65 66 hydrolyze carbapenems and decreased inactivation of oxyimino-cephalosporins compared to OXA-67 163 (9,10). Finally, Dabos et al. has shown by introducing one to six amino-acids deletions in the 68 β 5- β 6 loop in both directions, that loss of carbapenem-hydrolysis and gain of cephalosporin hydrolysis occurred after four-amino acid deletions (11). 69

The hydrolysis of β-lactam antibiotics by OXA-48 and other class D β-lactamases (DBLs)
 proceeds via acylation and deacylation of the active-site serine and involves a carboxylated lysine

as the general base (12,13). This reversible lysine modification, which is essential for DBL-activity, is proposed to be a spontaneous reaction, facilitated by the hydrophobic environment of the active site, which favors the unprotonated state of the lysine, and on the availability of CO_2 (12,14,15).

76 Despite a remarkable sequence divergence with other DBLs, the tertiary structure of OXA-48 is very similar to that of other DBLs (16,17). One notorious difference is that the β 5 and β 6 77 78 strands, located close to the substrate binding site, are overall shorter in OXA-48 than in other 79 DBLs. (16,17). Consequently, the β 5– β 6 loop has a different length and orientation from that 80 found in other DBLs. The observed conformation of the loop in OXA-48 is promoted by a salt 81 bridge of Arg214 with the Ω -loop Asp159 residue (16,18). The β 5– β 6 loop extends into the outer 82 portion of the active site crevice and defines a rather hydrophilic cavity filled with several water 83 molecules, one of which will perform the nucleophilic attack on the acyl-enzyme intermediate in 84 the deacylation step (16,18).

85 In OXA-163 or OXA-405, OXA-48-like enzymes that hydrolyze expanded-spectrum 86 cephalosporins instead of carbapenems, the β 5- β 6 loop is shorter due to a 4 AA deletion that 87 results in an expanded active-site cavity compared to that of OXA-48 (8,19). Arg214 is within the 88 four-amino acid deletion, which eliminates one boundary of the active site and elongates the 89 groove. As Arg214 has been suggested to form electrostatic interactions with carbapenem 90 substrates to facilitate their hydrolysis, this could explain the low activity of OXA-163 and OXA-405 91 toward carbapenems (8,18). Additionally, this expansion of the active site of OXA-163 and OXA-92 405 is consistent with the ability of the enzyme to accommodate a larger substrate such as 93 ceftazidime (8,19). The hypothesis that Arg214 contributes to the inability of OXA-48 to 94 accommodate ceftazidime is additionally supported by the observation that a shorter and

95 uncharged side chain at position 214 (Arg214Ser substitution) results in an increased ceftazidime
96 hydrolysis activity by OXA-232, an OXA-48-like enzyme (18).

97 The aim of the present study was to characterize OXA-517, a novel OXA-48-variant capable
98 of hydrolyzing carbapenems and expanded-spectrum cephalosporins, identified in a clinical *K*.
99 *pneumoniae* isolate recovered in Belgium.

100

101 Results

102 Clinical case.

103 K. pneumoniae 1219 clinical isolate (Kp 1219) was recovered from culture of a sputum 104 specimen of 81-year-old woman hospitalized in a Belgium hospital in September 2015 105 (Supplementary material). Antimicrobial therapy was initiated with temocillin and rapidly switched 106 to piperacillin because of rapid alteration of the status and development of a severe septic shock 107 with acute renal failure. The patient was admitted to the intensive care unit where she received several antimicrobial agents including meropenem followed by cefepime, colistin and tigecycline. 108 109 After four weeks, Kp 1219 isolate was repeatedly isolated over the period from throat, sputum, 110 tracheobronchial aspirates, and BAL (bronchoalveolar lavage) specimens of the patient.

111 Antimicrobial susceptibility testing of *Kp* 1219 and carbapenemase confirmation tests.

Using broth microdilution (Sensititre), *Kp* 1219 was resistant to amoxicillin and piperacillin (Table 1), expanded spectrum cephalosporins, and carbapenems, except for meropenem where we observed intermediate susceptibility (Table 1). In addition, the strain was resistant to colistin.

Biochemical tests based on imipenem hydrolysis (Carba NP and BYG Carba tests [20,21]) gave positive results. Although the resistance phenotype was compatible with the production of an OXA-48-like carbapenemase enzyme, the immunochromatographic OXA-48 K-SeT[®] assay (Coris

118 BioConcept) yielded a negative result. On the other hand, OXA-163 K-SeT[®], that specifically 119 detects the OXA-163 enzyme, and NG-Test Carba5 (NG Biotech) assays were positive for OXA-163 120 and OXA-48, respectively. Other commercially-available tests based on carbapenem-hydrolysis 121 such as Maldi-TOF (Star BL, Brucker) and ß-Carba (BioRad) (22,23) or on phenotypic approaches 122 such as CIM or rCIM (24,25) clearly identified this isolate as Carbapenemase-Producing 123 Enterobacterales (CPE). Similarly, with an inoculum of 10³ CFU, K. pneumoniae 1219 strain grew in ChromID® ESBL (BioMérieux) and on the CARBA side of ChromID® CARBA SMART (BioMérieux), 124 125 but not on the OXA-48 side.

PCR and sequencing revealed the presence of a novel bla_{0XA-48} allele, designated $bla_{0XA-517}$ gene. $bla_{0XA-517}$ gene differed from the bla_{0XA-48} gene by a 6-bp deletion, which included 2 nucleotides of the codon coding for Arg214, the entire codon coding for Ile215 and one nucleotide of the codon coding for Glu216. With this deletion, a novel codon was created coding for a Lys214, while the Ile215 and Glu216 were deleted (Figure 1).

131

132 Genomic features of K. pneumoniae 1219

WGS sequencing of *Kp* 1219 revealed a genome of 5,339,000 bp with a mean coverage of 90.7 X. The Multi Locus Sequence Typing (MLST) *of Kp* 1219 according to MLST 1.8 software (26) (https://cge.cbs.dtu.dk/services/,) revealed ST-188 (3-1-1-3-4-28-39), an ST type rarely described in hospital settings associated with carbapenemase or ESBL genes (27,28).

The resistome was analyzed by searching acquired genes and point mutations involved in resistance (29). The naturally occurring bla_{SHV-37} gene, the acquired bla_{TEM-1} gene and the $bla_{OXA-517}$ carbapenemase gene, were identified in this isolate. *Kp* 1219 also contained an *aph(3')-lc* gene conferring resistance to kanamycin and neomycin (30), *str*A and *str*B conferring resistance to streptomycin (31), *sul*1, *sul*2 genes conferring resistance to sulphonamides (32) and the 142 dihydrofolate reductase gene (*dfr*A14) conferring resistance to trimethoprim (33). In addition, 143 *oqxA* and *oqxB* genes were also present in the isolate, conferring reduced susceptibility to 144 fluoroquinolones (MIC of 0.5 μ g/ml to ciprofloxacin) (34). *FosA* gene conferring resistance to 145 fosfomycin was also present in the genome (35).

According to PlasmidFinder (36), different plasmid replication origins belonging to the incompatibility groups, IncFIB, IncX3, ColRNAI, IncL and IncFII were identified. The $bla_{OXA-517}$ gene was carried by the IncL-type plasmid as described previously for the $bla_{OXA-48-like}$ carbapenemase genes, except for $bla_{OXA-181}$ and $bla_{OXA-232}$ genes (37-39).

150

151 Genetic support and environment of *bla*OXA-517 gene

152 The bla_{OXA-517} gene was located on a Tn1999 composite transposon (Fig. 2A), as described 153 for bla_{OXA-48} gene (4). The pOXA-517 plasmid was identical to the prototypical pOXA-48 plasmid of 154 61,881-bp (38; accession number JN626286) except for a 31,488-bp deletion from nucleotide 155 31,191 to 21 according to the pOXA-48 plasmid numbering. This deletion includes ssb gene, mobC 156 and mobA genes; nikB and nikA genes, all the Locus Tra, and part of excA genes that were replaced 157 by an insertion sequence IS1R (Fig 2B). Transfer of the β -lactam resistance marker into E. coli J53 158 by mating-out experiments failed with pOXA-517, confirming that this deletion impacted the transfer ability of pOXA-517. Electroporation of this plasmid into E. coli TOP10 yielded 159 160 transformants resistant to β -lactams including carbapenems.

161

162 Phenotypical characterization of OXA-517 β-lactamase

163 The comparison of antibiotic susceptibility profiles conferred by OXA-48, OXA-163, and 164 OXA-517 was performed by cloning the corresponding genes into pCR-Blunt II-Topo kit (Invitrogen) 165 and expressing them in *E. coli* TOP10 (Table 1). Unlike pTOPO-OXA-48, pTOPO-OXA-163 and 166 pTOPO-OXA-517 conferred similar resistance phenotype for ceftazidime (MIC 16 μ g/mL and 4 167 μ g/mL, respectively), conferred decreased susceptibility to aztreonam and an increased 168 susceptibility to temocillin (32 μ g/mL). At the same time, pTOPO-OXA-517 conferred decreased 169 susceptibility to carbapenems similarly to pTOPO-OXA-48, with MIC value for imipenem of 0.38 170 μ g/mL (Table 1).

171

172 Biochemical properties of OXA-517

173 Steady-state kinetics parameters were determined to compare the catalytic properties of 174 OXA-517 with those of OXA-48 and OXA-163. OXA-517 was purified in one step pseudo-affinity 175 chromatography using an NTA-Ni column. OXA-163 and OXA-48 were previously purified by 176 Oueslati et al. (5) and Docquier et al. (16) and the kinetic parameters determined previously for 177 these enzymes were used for comparison. The catalytic efficiency of OXA-517 for ceftazidime was overall close to that observed for OXA-163 (2 mM⁻¹s⁻¹ and 3 mM⁻¹s⁻¹ respectively). Conversely, 178 179 OXA-48 was not capable of hydrolyzing ceftazidime (Table 2). OXA-517 had a catalytic activity for 180 imipenem \sim 250-fold higher in comparison with the one of OXA-163 and \sim 25-fold lower than that 181 of OXA-48 (Table 2). These results are in agreement with the obtained MIC values. OXA-517 had poor affinity for temocillin, with $K_m \sim 15$ -fold higher than that for OXA-48. Another notable 182 difference with OXA-48 and with OXA-163 concerns the behavior of OXA-517 with penicillins. The 183 addition of $NaHCO_3$ as a source of CO_2 did not change the profile of hydrolysis of these penicillins. 184 185 The catalytic efficiency of OXA-517 was \sim 9-fold lower than that of OXA-48 but \sim 4-fold higher than 186 that of OXA-163. These results correlate with the differences in enzymatic turnover, while the $K_{\rm m}$ 187 values are similar for all three enzymes (Table 2). Unlike OXA-48, OXA-517 was capable of hydrolyzing aztreonam, with a catalytic efficiency similar to the one of OXA-163 (3.1 mM⁻¹ s⁻¹ and 188 $2.0 \text{ mM}^{-1} \text{ s}^{-1}$, respectively). 189

190 Inhibition studies by determining the IC_{50} values indicated that OXA-517 was more 191 potently inhibited by clavulanic acid (14 μ M) and tazobactam (2 μ M) than OXA-48 (30 μ M and 20 192 μ M, respectively). However, the inhibition efficiency with NaCl was lower for OXA-517 as 193 compared to OXA-48 (400mM and 35mM respectively).

194

195 Crystal structure of OXA-517

Four OXA-517 protein molecules are present in the asymmetric unit of the crystal, and 196 they form two non-crystallographic dimers related by pseudo translation. Each dimer has a 197 198 two-fold rotational symmetry, with the symmetry axis approximately parallel to the [110] 199 direction. The electron density maps are of good quality with all four polypeptide chains clearly 200 defined. Each subunit possesses a two-domain structure with a helix bundle domain and an α/β 201 domain with a central seven-stranded antiparallel β -sheet, as described for OXA-48 (16) (Fig. 3). 202 Compared to OXA-48, OXA-517 carries an Ile215-Glu216 deletion coupled with an Arg214Lys 203 mutation in the functionally important β 5- β 6 loop. In OXA-517 the loop is comprised of residues 204 Thr213, Lys214, Pro217 and Lys218 (residue numbering corresponding to OXA-48). All the 205 backbone atoms within the loop are visible in the electron density map but are not well ordered as 206 indicated by their high atomic displacement parameters (Fig. 3, Fig. 4). The side chain of Lys214 is 207 not visible in the electron density in any of the protein molecules. The peptide bond between 208 Lys214 and Pro217 has a cis configuration as observed in OXA-48. A neighboring loop (residues 209 241-246), connecting the β 7 strand with helix α 10, shows a similarly high mobility, with high 210 average B values (Fig. 3).

The four protein molecules are nearly identical with rmsd values between 0.05 and 0.32 Å
 for their Cα superpositions. The largest deviations between the subunits are visible at the

213 N-terminus and within the loop between helices α 3 and α 4, which participates in crystal-lattice 214 interactions.

215

216 Dimer interface

217 The OXA-517 dimer interface is the same as in OXA-48. In each of the two dimers a sulfate 218 ion is bound between two Arg206 residues contributed by the dimer-forming subunits (Fig. 4A, B). Refining the sulfate ions at full occupancy resulted in negative difference density peaks (Fig. 4C) 219 220 and poor electron density fit criteria. Refinement at 0.5 occupancy gives good fit to the electron 221 density but results in the appearance of a localized positive difference density peak in the position 222 right in the middle between the two arginine residues (Fig. 4D). Modeling a half-occupancy 223 chloride ion in this position resulted in clear electron density maps and realistic temperature 224 factors for both partially occupied anions (Fig. 4B). In the structures of OXA-48 (PDB id 4s2p) and 225 OXA-163 (4s2l) that we used for comparisons, a water molecule was modeled in this position. 226 However, in both cases the water had an unrealistically low temperature factors and we noted 227 positive difference density peaks. Refinement of a chloride ion gave good electron density fit with 228 no peaks in the difference map and B factors like those of the environment. We conclude that in 229 those two structures, a chloride ion is also bound at each dimer interface. The Arg206 residue that 230 interacts with the bound anions originates at the amino end of the β 5 strand, opposite to the β 5β6 loop. 231

232

233 The active site

The active site is located at the interface of the two domains, between helices $\alpha 3$, $\alpha 5$ and $\alpha 6$, and the $\beta 5$ strand. All the active site residues are well defined in the electron density map, including the carboxylated Lys73 (KCX) residue. The Oy atom of the catalytically important Ser70

237 nucleophile exhibits considerably higher B factor than its surroundings, except in chain B. The full 238 hydrogen bond network within the active site area is shown in Fig. S1. The Oy atom of Ser70 239 interacts with KCX73, with the hydroxyl group of Ser118 and with a water molecule bound 240 between the amide NH groups of Ser70 and Tyr211 (the oxyanion hole). This water molecule also 241 interacts with the main-chain carbonyl of Tyr211. A sulfate ion is bound in the active site in all the 242 four protein subunits and forms an ionic interaction with Arg250. The orientation of this anion is different in each of the subunits. The sulfate ion forms hydrogen bonds with the Oy1 atom of 243 244 Thr209 and water in all chains, and with the Nζ atom of Lys208 in chains A, B and C. Only one 245 water position within the active site is conserved between the four protein subunits.

Similarly, as in OXA-48, the walls of the active site cavity are lined by the Leu158 side chain and by residues in the β 5 strand, Tyr211-Ser212-Thr213. The groove ends with a hydrophilic cap formed by Oy1_Thr213, N_Thr213, Oy_Ser212 and O_Leu158.

249

250 Comparison with OXA-48 and OXA-163

251 The molecular structure of OXA-517 aligns well with that of OXA-48 (PDB id 4s2p) with an average rmsd of 0.50 Å for 238 superposed C α atoms, and with OXA-163 (4s2l) with an average 252 253 rmsd of 0.28 Å for 237 Ca atoms (Fig. 5A). The total number of Ca atoms in the aligned chains was 254 equal to 244 for OXA-517, 238 for OXA-163 and 243 for OXA-48. The largest differences between 255 these structures are found in the β 5- β 6 loop, where each protein has a slightly different sequence. The seven-residue loop (with the sequence ²¹²STRIEPK) in OXA-48 is replaced by a shorter 256 sequence (²¹²STKPK) in OXA-517, and shorter yet in OXA-163 (²¹²DTK). In OXA-48, Arg214 forms a 257 258 salt bridge with Asp159. This arginine is replaced by Lys214 in OXA-517 but a similar interaction is 259 not observed in the structure of OXA-517. The side chain of Lys214 itself is not visible in the 260 electron density but the loop is considerably shorter and there is a 3.8 Å distance between the Ca

261 atoms of Arg214 and Lys214 in the superposed structures of OXA-48 and OXA-517. An interaction 262 between Lys214 and Asp159 would not be possible without significant conformational changes in 263 either the β 5- β 6 or the Ω loop. A common feature of the β 5- β 6 loop in OXA-48 and OXA-517 is a 264 cis peptide bond preceding Pro217 and a similar backbone conformation of this residue, even 265 though it has a different position and orientation (Fig. 5B). The side chain of Thr213 has a different 266 orientation in the two structures with the Oy1 atom in OXA-517 pointing more 'down' towards the 267 bottom of the binding cleft (Fig. 5 C, D). The fragment of the loop with the most pronounced 268 differences between these two structures is between Thr213 and Lys218. Before and after this 269 region the backbone atoms align well with each other. This contrasts with the structure of OXA-270 163, where the main chain atoms on the side of the β 5 strand only converge with the other two 271 structures at the N atom of Asp212 (Fig. 5B, E). In OXA-517 and OXA-48 the carbonyl O atom of 272 Ser212 forms a hydrogen bond with N_Ile219. The Asp212-Thr213 peptide bond in OXA-163 has a 273 different conformation and there is no hydrogen bond with N_Ile219. The side chain of Asp212 in 274 this protein folds towards the loop and interacts with N Lys218. Also, the side chain of Tyr211 has 275 a different position in OXA-163 compared with the other structures but the electron density for 276 this residue is weak. The relevant details of these comparisons are illustrated in Fig. 5B-E.

277

278 Comparison with OXA-48 P68A mutant in complex with ceftazidime

No naturally occurring OXA-48-like enzyme has been crystallized with ceftazidime, but Frohlich et al (40) report the structure of ceftazidime in complex with the OXA-48 P68A mutant, obtained by directed evolution *in vitro* (PDB id 6q5f). In this complex they observe ceftazidime with its carboxypropyloxiimino substituent placed between Thr213 from the β 5- β 6 loop and Leu158 from the Ω loop, and the aminothiazole ring pointing outwards the active site cavity, towards the solvent. When aligned onto OXA-517, RMSD between 223 pruned atom pairs is 0.388 angstroms.

285 Main differences are observed in the conformation of the β 5- β 6 loop, as explained previously, as 286 well as in the Ω loop, which seems to be highly disordered between Asp148 and Ile162 in the 287 ceftazidime-bound structure and therefore not modelled. When aligning this structure on the 288 OXA-48 native structure (PDB id 4s2p), numerous severe clashes with the β Carbon of Thr213 and 289 the whole Arg214 residue occur, as well as complete superposition with Leu158 on the Ω loop (Fig. 290 6A). Frohlich et al. propose that ceftazidime can bind in their OXA-48 mutant because of the 291 increased flexibility of its loops due to the P68A mutation (40). Instead, when aligned on the β 5- β 6 292 loop of OXA-517, only two clashes occur, with Thr213 atoms Oy1 and Cy2, which might be avoided 293 by rotation of this sidechain (Fig. 6B). Regarding Leu158 on the Ω loop, severe clashes are 294 observed here as well (Fig. 6B), which may suggest that the same mechanism proposed by Frohlich 295 et al might be at play here (40). The β 5- β 6 loop mutations may render the Ω loop more flexible in 296 OXA-517 (see below), possibly allowing ceftazidime to bind.

297 Comparison with OXA-48 and OXA-163 in complex with imipenem

298 Two OXA-48 structures in complex with imipenem (PDB id 6p97 by Smith et al. (41) and 299 PDB id 7kh9 by Stojanoski et al. (42)) may also be used for comparison with OXA-517. Both 300 structures show relevant catalytic site residues mostly acquiring the same conformation, as does 301 the core and R2 substituent of imipenem. Only the R1 tail, which shows signs of flexibility in both 302 cases, adopts a different orientation in these two structures. When aligning OXA-517 to these 303 structures, RMSD between 238 pruned atom pairs is 0.366 angstroms for 6p97 and 0.423 304 angstroms for 7kh9. The main difference is in the orientation of the β 5- β 6 loop, as shown for the 305 OXA-48 apo structure, and most residues around the binding pocket adopt virtually the same 306 conformation (Fig. S2). It was proposed that, similar to other class D carbapenemases, OXA-48 is 307 capable of hydrolyzing carbapenems such as imipenem because of a hydrophobic barrier formed 308 by Leu158 on the Ω loop and Val120 on the α 4- α 5 loop that protects the carboxylated Lys73 from 309 exposure to bulk solvent, while still allowing inside the active site a water molecule that will be 310 activated to attack the acyl-enzyme complex (41). These residues adopt the same orientations in 311 the active site cavity of OXA-517, the highest displacement being observed for Leu158 (0.7 Å) 312 towards the carbapenem R2 substituent (Fig. S2). Leu158 maintains nevertheless the same 313 distance to Val120 as in the other structures. It was also proposed that tight binding of 314 carbapenems in the active site cavity may play a role in restraining them in a catalytically 315 competent conformation, as suggested by the fact that non-carbapenemase OXA-163 showcases a 316 wider catalytic pocket which binds imipenem more loosely (42). As described before, the β 5- β 6 317 loop mutations in OXA-517 seem to cause this loop to move farther from the Ω loop, closer to the 318 β 7- α 10 loop, and allow Tyr211 to shift 0.8-1.3 Å (Fig. S2), which would slightly broaden the active 319 site. In OXA-48, the β 5- β 6 loop interacts tightly with the Ω loop and subjacent Leu67-Ala69 320 sequence (Fig. 7A): Tyr211 and Ser212 maintain multiple contacts with Ala69; Ser212, Arg214 and 321 Ile215 all interact with Leu67, while the latter two also contact Leu158 and Asp159 on the Ω loop. 322 In OXA-163, however, with the β 5- β 6 loop deletions and the Ser212Asp mutation, most of these 323 interactions are lost, and only one contact is observed, between Asp212 C β and Ala69 (Fig. 7C). In 324 contrast, in OXA-517, some of the interactions between the β 5- β 6 and Ω loops are indirectly 325 maintained via the Leu67-Ala69 stretch: similar to OXA-48, Tyr211 and Ser212 maintain almost all 326 their contacts to Ala69 and Leu67, while the latter is now also contacted by the backbone of Lys214 (Fig. 7B). The Leu67-Pro68-Ala69 stretch, in turn, packs tightly with residues 157-162 of the 327 328 Ω loop, as illustrated for the OXA-48 structure (Fig. 7D). It is also possible that less contacts 329 between the β5 strand and Ala69 may influence the flexibility of Ser70 and the stability of the acyl-330 enzyme complex in a catalytically competent conformation. Therefore, this active site cavity 331 broadening and maintenance of indirect interactions between the β 5- β 6 and Ω loops may explain 332 why, unlike OXA-48, OXA-517 has acquired the capacity to hydrolyze ceftazidime, albeit at the cost

of a diminished affinity for imipenem. On the other hand, the turnover rate of OXA-517 forimipenem is closer to that of OXA-48 than of OXA-163.

335

336 Discussion

337 In this study, we have characterized a novel OXA-48 variant, OXA-517, recovered from a 338 clinical isolate of *K. pneumoniae* from Belgium. OXA-517 hydrolyzes both ESC and carbapenems. 339 OXA-163 and OXA-405 have a four-residue deletion in the region from Tyr-211 to Pro-217 in the 340 β5-β6 loop and hydrolyze ESC but not carbapenems (8,19), whereas the OXA-48-like variants that possess carbapenemase activity have only substitutions of some residues in the \$5-\$6 loop region 341 342 but no deletions. It is worth noting that contrary to what was observed for the OXA-48 variants 343 OXA-163 and OXA-405, OXA-517 displays a deletion of only two AA residues in the β 5- β 6 loop and 344 this is sufficient to confer resistance to expanded-spectrum cephalosporins as well as 345 carbapenems. This is yet another fact that supports the hypothesis that substrate specificity of 346 class D oxacillinases with respect to CHDL resides, at least in part, in the length and amino acid 347 composition of loop β 5- β 6 (16, 18, 19, 43). OXA-438, another OXA-48 variant with two AA deletions 348 (Thr213, Arg214) in the β 5- β 6 loop but with additional 4 AA substitutions (Ser212Gly, Ile215Tyr, 349 Glu216Asp, Pro217Thr), has the same length of loop as OXA-517 but with different hydrolytic 350 properties: comparable imipenem hydrolysis, 10-fold higher cefotaxime hydrolysis, and no 351 detectable ceftazidime hydrolysis as compared to OXA-48 (10).

Temocillin has been proposed as a marker for the detection of OXA-48-like carbapenemase-producing Enterobacterales (44). Indeed, a diameter <12 mm for temocillin on disk diffusion antibiograms suggests the presence of OXA-48-producers with high specificity (90%). OXA-517 producing *K. pneumoniae* was not able to grow on the OXA-48 side of chromID[®] CARBA SMART (BioMérieux) even though the diameter around temocillin was 12mm (data not shown),

357 corresponding to a MIC value of 192 μ g/mL. Thus OXA-517, similarly to OXA-244 or OXA-232, 358 challenges the role of temocillin as a screening marker of OXA-48-like enzymes with true 359 carbapenemase activity (45,46).

360 The $bla_{0XA-517}$ gene was identified on a plasmid with the backbone like the IncL bla_{0XA-48} -361 bearing plasmid (pOXA-48) (38), with the difference of a deletion of ca. 31 kb including ssb gene, 362 mobC and mobA genes, nikB and nikA genes, all Locus Tra and part of excA gene. In the pOXA-517 363 plasmid, this region was replaced by the insertion sequence IS1R. Since the deleted region includes 364 several genes involved in the mobilization and conjugative process of plasmids, we assume that 365 this genetic rearrangement has led to the abrogation of the conjugative properties of the pOXA-366 517 plasmid as compared to pOXA-48 and, indeed, pOXA-517 was not found to be self-367 conjugative. Consequently, we might hypothesize that the genetic rearrangement observed in the 368 pOXA-517 plasmid could limit its dissemination.

369 Considering the ability of OXA-517 to hydrolyze expanded-spectrum cephalosporins, our 370 findings are consistent with the conclusions drawn for OXA-163 that a larger active site cavity 371 allows for the binding and hydrolysis of the bulky ceftazidime substrate (19). The expansion of the 372 cavity results from shortening of the β 5- β 6 loop and the elimination of arginine at position 214. 373 Arg214 in OXA-48 forms a salt bridge with Asp159 and occupies part of the binding cavity (16,18). 374 Any movement of this residue to free up more space for substrate binding would involve breaking 375 this ionic interaction. In OXA-517, position 214 is occupied by a lysine residue which does not form 376 a similar contact. As the β 5- β 6 loop in OXA-517 is by two residues shorter, there is a 3.8 Å distance 377 between the C α atom positions of residue 214 in OXA-517 and in OXA-48, and a similar salt bridge 378 interaction between Lys214 and Asp159 would require a drastic conformational change. The larger 379 size of the cavity is also attributed to the length of the β 5- β 6 loop, which in OXA-517 and OXA-163

is shorter than in OXA-48. Finally, as proposed by Frohlich et al. for the P68A OXA-48 mutant (40), it is also possible that in OXA-517, with its mutated β 5- β 6 loop and broken interactions with the Ω loop, the latter becomes now more flexible and allow for more space in the active site cavity to bind ceftazidime.

384 On the other hand, the structural basis for carbapenemase activity is elusive. Regarding 385 affinity, it is possible that the differences between the OXA-48 and OXA-517 sequences cause the 386 latter to showcase a looser cavity. For example, the lle102 is slightly farther away from Tyr211, as 387 illustrated in Fig. S2. This can also be observed when comparing the apo OXA-48 structure with the 388 apo OXA-517 structure (data not shown). However, the side chain of this latter residue is not well 389 defined in the electron density and exhibits high mobility, as indicated by the high temperature 390 factors in the crystal structure of OXA-517, as well as in OXA-163 (pdb id 4s2l) and OXA-48 (4s2p). 391 Therefore, the observed differences in the conformation of this residue might not be significant. 392 Perhaps the native OXA-48 structure is fairly optimal for the binding of carbapenems, and any 393 alteration, such as those observed in OXA-517 and OXA-163, are enough to alter altogether the 394 size of the cavity and diminish its affinity for them. It is possible that the acylation reaction for 395 carbapenem antibiotics proceeds in the same way for these three OXA enzymes, and only the 396 deacylation step is considerably slower for OXA-163, possibly due to its larger β5-β6 loop 397 modification having deeper consequences in its catalytic cycle dynamics. The kinetic data reported 398 for OXA-163 (19) show that a reduced catalytic efficiency for carbapenems, compared to OXA-48, 399 is mainly caused by a decrease in the turnover rate, with unchanged K_m . However, other literature 400 data show that there is both an increase in the K_M value and a decrease in k_{cat} for the tested 401 carbapenem compounds (5). The creation of an unusually stable carbapenem acyl-enzyme 402 intermediate was described for OXA-10 (43). It was also suggested that some orientations of the

403 hydroxyethyl group may restrict access of the hydrolytic water molecule to the covalent404 intermediate, and thus prevent deacylation.

405 A possible explanation for the differences in carbapenemase activity of these three 406 enzymes is that the β 5- β 6 loop undergoes conformational changes and moves closer to the 407 substrate, thus influencing its conformation during the hydrolysis reaction. Such a view has been 408 already proposed by Docquier et al. (16). In our OXA-517 crystal structure all the loop residues 409 have high temperature factors, suggesting high conformational freedom of this structural element, 410 in agreement with its proposed long-range movements. The cis-Pro217 residue that is present in 411 both OXA-517 and OXA-48, but not in OXA-163, enforces a specific backbone conformation that 412 cannot be adopted by any other residue. This may be an important aspect of the activity of these 413 enzymes towards carbapenems. Indeed, the sequence comparisons presented by De Luca et al. 414 (43) revealed the presence of a conserved PxxG sequence motif (residues 217-220 in OXA-48 415 numbering) in all class D carbapenemases, but not in other OXA-type enzymes. Further structural 416 and biochemical data would be needed to confirm the importance of the *cis*-Pro217 residue. 417 Another possibility, as depicted in Fig. 7, is that the β 5- β 6 loop in OXA-517, relatively more similar 418 to that of OXA-48 than that of OXA-163, allows it to conserve some of the dynamic properties of 419 the native OXA-48 catalytic machinery, thus allowing for the turnover rate for imipenem to remain 420 similar too.

Our crystal structure of OXA-517 shows that the dimer interface is stabilized by a chloride or/and sulfate anions bound between two Arg206 residues from the neighboring subunits. In OXA-10, the structurally equivalent His203 residue forms part of a metal ion binding site (47). Interestingly, it has been shown that supplementation of the buffer with some transition metal ions shifts the equilibrium in solution towards the dimeric form of this enzyme, which in turn

increases its activity. An attractive hypothesis suggests itself, that the evolutionary advantage of
OXA-48-like proteins compared to OXA-10 is that their dimer formation is independent of metal
cations and is instead stabilized by the abundantly present Cl⁻ ions.

Finally, our work illustrates the possibility for OXA-48 to extend its substrate specificity to include ESC by point mutations/deletions. This finding may have clinical consequences, as the increased use of ESC to treat infections caused by OXA-48-producing isolates, remaining susceptible to ESC, may lead to selection of OXA-163-like or OXA-517-like variants.

433

434 Materials and methods

435 Bacterial strains

Klebsiella pneumoniae 1219 was identified with matrix-assisted laser desorption
ionization-time of flight (MALDI-TOF) mass spectrometry (MALDI, Biotyper CA system, Bruker
Daltonics, Billerica, MA, USA). *Escherichia coli* TOP10 (Invitrogen, Saint-Aubin, France) and *E. coli*BL21 (DE3) (Novagen, VWR International, Fontenay-sous-Bois, France) were used for cloning
experiments. Azide-resistant *E. coli* J53 was used for conjugation assays. *K. pneumoniae* 11978 and *K. pneumoniae* 6299 were used as reference strains for OXA-48 and OXA-163 cloning experiments,
respectively (4,7).

443

444 Antimicrobial agents, susceptibility testing, and microbiological techniques

Antimicrobial susceptibilities were determined by disk diffusion and Minimal Inhibitory Concentration (MICs) and interpreted as previously described (48). *Kp* 1219 was plated in different chromogenic medias: chromID[®] ESBL and chromID[®] CARBA SMART (BioMérieux).

448

449 Biochemical and immunochromatographic detection of carbapenemase-producing 450 *Enterobacterales*

451 Carbapenemase activity was investigated using the Carba NP test (20), RAPIDEC® CARBA
452 NP (BioMérieux), β-CARBA[™] Test (Bio-Rad) (23), Maldi-TOF (Star BL, Brucker, Bremen, Germany)
453 (22), BYG test (21), or using phenotypic approaches such as CIM or rCIM (24,25) as previously
454 described. The expression of a carbapenemase was investigated using OXA-48 K-SeT[®] and OXA455 163 K-SeT[®] immunochromatographic assay (Coris BioConcept, Gembloux, Belgium) and NG-Test
456 Carba 5 (NG Biotech, Guipry, France) as recommended by the manufacturers.

457 PCR, cloning experiments, and DNA sequencing

458 Whole-cell DNAs of K. pneumoniae 1219 and K. pneumoniae 11978 isolates were extracted 459 using the QIAamp DNA mini kit (Qiagen, Courtaboeuf, France) and were used as templates for PCR 460 as previously described (48). The amplicons obtained were then cloned into the pCR®-Blunt II-461 TOPO[®] plasmid (Invitrogen, Illkirch, France), as previously described (48). The recombinant 462 pTOPO-OXA plasmids were electroporated into E. coli TOP10 cells; and selected on kanamycin (50 463 μ g/ml) containing plates. The *bla*_{OXA-517} gene fragment corresponding to the mature β -lactamase 464 was cloned into the expression vector pET41b (+) (Novagen, VWR International, Fontenay-sous-465 Bois, France) as previously described (48). The recombinant plasmid pET41-OXA-517 was 466 transformed into the chemically competent E. coli strain BL21 (DE3) (Novagen).

Recombinant plasmids were extracted using the Qiagen miniprep kit and both strands of the 467 468 inserts were sequenced using M13 and T7 primers, with an automatic sequencer (ABI Prism 3100; 469 Applied Biosystems, Les Ulis, France). The nucleotide sequences were analyzed using software 470 available at the National Center for Biotechnology Information website 471 (http://www.ncbi.nlm.nih.gov).

472 Whole genome sequencing (WGS)

473 Total DNA was extracted from colonies using the Ultraclean Microbial DNA Isolation Kit 474 (MO BIO Laboratories, Carlsbad, CA, US) following the manufacturer's instructions. The DNA 475 concentration and purity were controlled by a Qubit® 2.0 Fluorometer using the dsDNA HS and/or 476 BR assay kit (Life technologies, Carlsbad, CA, US). The DNA library was prepared using the Nextera 477 XT-v3 kit (Illumina, San Diego, CA, US) as previously described (48). De novo assembly was 478 performed by CLC Genomics Workbench v 9.5.3 (Qiagen) after quality trimming (Qs \ge 20) with 479 word size 34. The acquired antimicrobial resistance genes were identified by uploading the assembled genomes to the Resfinder server v2.1 (http://cge.cbs.dtu.dk/services/ResFinder-2.1) 480 481 (29). The Multi Locus Sequence Typing (MLST) and the identification of the different plasmids were also obtained by uploading the assembled genomes to the MLST 1.8 (26) and PlasmidFinder 482 483 1.3 (36) servers, respectively, available at https://cge.cbs.dtu.dk/services/.

484

485 Plasmid characterization and conjugation assay

486 Plasmid DNA of the clinical isolates Kp 1219 and K. pneumoniae 11978 were extracted 487 using the Kieser method (49). The Kieser extract from both K. pneumoniae isolates were used to 488 transform E. coli TOP10 strain by electroporation. The electroporants were plated on a TSA plate 489 containing 100 μ g/ml ampicillin. The presence $bla_{0XA-517}$ gene was sought by PCR as previously 490 described (53). From the transformants harboring the *bla*_{OXA-517} gene, plasmid DNA extraction was 491 done using the Kieser's method and subsequently analyzed on 0.7% agarose gel stained with 492 ethidium bromide. Plasmids of ca. 154, 66, 48, and 7 kb of E. coli NCTC 50192 were used as 493 plasmid size markers (50).

494 Quantitative filter mating-out assay was performed as previously described (48), and 495 transconjugants were selected with 100 μ g/ml ampicillin and 100 μ g/ml azide. Transfer

496 frequencies were calculated by dividing the number of transconjugants by the number of donor497 cells.

498

499 **β-Lactamase purification**

500 An overnight culture of *E. coli* strain BL21 (DE3) harboring pET41b-OXA-517 was used to 501 inoculate 2 L of LB broth containing 50 µg/mL kanamycin. Expression of OXA-517 was induced overnight at 25°C with 0.2 mM IPTG, as previously described (48). Cultures were centrifuged at 502 6000 g for 15 min and the pellets were resuspended in 10 mL of Buffer A (20 mM phosphate 503 504 buffer, 175 mM potassium sulfate (K_2SO_4), 40 mM imidazol, pH 7.4). The cells were disrupted by 505 sonication and bacterial debris was removed by two consecutive centrifugation steps. OXA-517 506 was purified in one step pseudo-affinity chromatography using an NTA-Ni column (GE Healthcare, 507 Les Ulis, France) as previously described (48). Protein purity was estimated by SDS-PAGE, pure 508 fractions were pooled and dialyzed against 20 mM Hepes, 50 mM K₂SO₄ buffer (pH 7.0) and 509 concentrated using Vivaspin[®] columns (GE Healthcare, Freiburg, Germany). Protein concentration 510 was determined by Bradford Protein assay (Bio-Rad) (51). OXA-163 and OXA-48 were previously 511 purified by Oueslati et al. (5) and Doquier et al. (16).

512

513 Steady-state kinetic parameters

514 Kinetic parameters of purified OXA-517 were determined at 30°C in 100 mM sodium 515 phosphate buffer (pH 7.0). The k_{cat} and K_m values were determined by analyzing hydrolysis of β -516 lactams under initial-rate conditions with an ULTROSPEC 2000 model UV spectrophotometer (GE 517 Healthcare). Kinetic parameters were calculated using the Eadie–Hoffstee linearization of the 518 Michaelis–Menten equation, as previously described (54). The β -lactams were purchased from 519 Sigma–Aldrich (Saint-Quentin-Fallavier, France). Comparative activation of OXA-517 by CO₂ was

520 conducted by monitoring the rate of hydrolysis of penicillins in the same buffer supplemented 521 with 50 mM sodium hydrogen carbonate (NaHCO₃) as the source of CO₂ (15,18). OXA-163 and 522 OXA-48 kinetic parameters, used in the comparison, were previously determined by Oueslati *et al.* 523 (5) and Doquier et al. (16).

524

525 Crystallization, X-ray data collection, structure refinement and analysis

A 27 mg/ml protein solution was used for crystallization screening with the NeXtal 526 527 crystallization kit (Qiagen). Single crystals of OXA-517 grew in 0.1 M MES pH 6.5 and 20% (w/v) 528 PEG10000. The diffraction data were collected at the SOLEIL PROXIMA II beamline equipped with 529 an Eiger detector (Table 3). The images were processed and scaled with the XDS package (52,53) 530 producing intensities merged in the P21 space group as output. The intensities were then 531 converted to structure factor amplitudes with Truncate (54) from the CCP4 program suite (55). An 532 inspection of the Patterson map, calculated using the FFT algorithm implemented in fftbig (56) 533 from the CCP4 package, revealed a large non-origin peak at 0.5, 0, 0.5, indicating translational 534 pseudosymmetry. The structure was solved by molecular replacement using Phaser (57) and 535 taking advantage of the detected pseudo translation. The starting model was a single subunit of 536 OXA-48 (PDB id 4s2p). The calculations gave a single solution with a Z-score of 15.4 for the translation function and a final LLG of 2405. No twinning was found by the Phaser twin detection 537 algorithm (58), which corrects for translational NCS. An alternative solution, shifted by 0.25, 0, 538 539 0.25, was also tested but it gave significantly higher R factors. The Achesym server (59) was used 540 to standardize the placement of the model inside the unit cell. Maximum-likelihood refinement 541 was carried out in Refmac5 (60), while model rebuilding and analysis was done in Coot (61). For 542 validation of the final model, we used Molprobity (62) and the PDB validation server (63). The SSM

algorithm implemented in *Coot* was used for 3D superpositions of different models. Figures weregenerated with *Pymol* (64).

545 For comparisons of our structure with other models, we downloaded their coordinates 546 and, when available, the corresponding electron density maps from the Uppsala Electron Density 547 Server (*EDS*) (65). For a few PDB models, we applied some minor adjustments to their coordinates 548 to improve the electron density fit or stereochemical parameters. We used the *CCP4* package for 549 conversions between file formats and *Truncate* to convert the intensities to structure factor 550 amplitudes, if necessary. For structure comparison, UCSF ChimeraX software was used (66).

551

552 Nucleotide sequence and PDB accession numbers

The nucleotide sequence of the *bla*_{OXA-517} gene has been submitted to the EMBL/Genbank nucleotide sequence database under the accession number KU878974. The nucleotide sequence of plasmid pOXA-517 has been submitted also, under the accession number KY200950. The Whole Genome Shotgun of Kp1219 has been deposited at the DDBJ/ENA/GenBank under the accession code NXNM00000000. The version described in this paper is version NXNM01000000. Atomic coordinates and structure factors for the crystal structure of OXA-517 have been deposited with the Protein Data Bank (PDB) with the accession number 6HB8.

560

561 Acknowledgments

We acknowledge SOLEIL for provision of synchrotron radiation facilities (proposal ID BAG20170782) in using PROXIMA beamlines. This work has also benefited from the I2BC crystallization platform, supported by FRISBI ANR-10-INSB-05-01. This work was supported by the Assistance Publique – Hôpitaux de Paris (AP-HP), the University Paris-Saclay, the Laboratory of

- 566 Excellence in Research on Medication and Innovative Therapeutics (LERMIT) supported by a grant
- 567 from the French National Research Agency [ANR-10-LABX-33], the Joint Programming Initiative on
- 568 Antimicrobial Resistance (JPIAMR) DesInMBL [ANR-14-JAMR-002], and DIM Malinf, Ile de France,
- 569 for LD's PhD fellowship.
- 570 Conflict of interest
- 571 None to declare

572 References

- Bonomo RA. 2017. β-Lactamases: A Focus on Current Challenges. Cold Spring Harb
 Perspect Med 7:a025239.
- Bonomo RA, Burd EM, Conly J, Limbago BM, Poirel L, Segre JA, Westblade LF. 2018.
 Carbapenemase-Producing Organisms: A Global Scourge. Clin Infect Dis 66:1290-1297.
- 5773. Poirel L, Heritier C, Tolun V, Nordmann P. 2004. Emergence of oxacillinase-mediated578resistance to imipenem in *Klebsiella pneumoniae*. Antimicrob Agents Chemother 48:15–
- 579 22.
- Aubert D, Naas T, Héritier C, Poirel L, Nordmann P. 2006. Functional characterization of
 IS1999, an IS4 family element involved in mobilization and expression of beta-lactam
 resistance genes. J Bacteriol 188:6506-6514.
- 583 5. Oueslati S, Nordmann P, Poirel L. 2015. Heterogeneous hydrolytic features for OXA-48-like
 584 β-lactamases. J Antimicrob Chemother 70:1059-63.
- 585 6. Naas T, Oueslati S, Bonnin RA, Dabos ML., Zavala A, Dortet L, Retailleau P, Iorga BI. 2017.
 586 Beta-Lactamase DataBase (BLDB) Structure and Function. J Enz Inh Med Chem 32:917-
- 587 919.

- Poirel L, Castanheira M, Carrer A, Rodriguez CP, Jones RN, Smayevsky J, Nordmann P.
 2011. OXA-163, an OXA-48-related class D β-lactamase with extended activity toward
 expanded-spectrum cephalosporins. Antimicrob Agents Chemother 55:2546–2551.
- S. Oueslati S, Retailleau P, Marchini L, Dortet L, Bonnin RA, Iorga BI, Naas T. 2019.
 Biochemical and Structural Characterization of OXA-405, an OXA-48 Variant with
 Extended-Spectrum β-Lactamase Activity. Microorganisms 8:24.
- Dabos L, Oueslati S, Bernabeu S, Bonnin RA, Dortet L, Naas T. 2022. To Be or Not to Be an
 OXA-48 Carbapenemase. Microorganisms 10:258.
- 596 10. De Belder D, Ghiglione B, Pasteran F, de Mendieta JM, Corso A, Curto L, Di Bella A, Gutkind
- 597 G, Gomez SA, Power P. 2020. Comparative Kinetic Analysis of OXA-438 with Related OXA-
- 598 48-Type Carbapenem-Hydrolyzing Class D beta-Lactamases. ACS Infect Dis 6:3026-3033.
- 599 11. Dabos L, Bonnin R, Dortet L, Iorga B, Naas, T. 2016. Biochemical and structural
 600 investigation of the role of beta5-beta6 loop in substrate specificity of OXA-48–like
 601 enzymes. ECCMID Amsterdam, The Netherlands
- 602 12. Golemi D, Maveyraud L, Vakulenko S, Samama JP, Mobashery S. 2001. Critical involvement
- of a carbamylated lysine in catalytic function of class D β-lactamases. Proc Natl Acad Sci
 USA 98:14280–14285.
- Li J, Cross JB, Vreven T, Meroueh SO, Mobashery S, Schlegel HB .2005. Lysine carboxylation
 in proteins: OXA-10 β-lactamase. Proteins 61: 246–257.
- 607 14. Leonard DA, Bonomo RA, Powers RA .2013. Class D β-lactamases: A reappraisal after five
 608 decades. Acc Chem Res 46: 2407–2415.
- 609 15. Baurin S, Vercheval L, Bouillenne F, Falzone C, Brans A, Jacquamet L, Ferrer JL, Sauvage E,
 610 Dehareng D, Frere JM, Charlier P, Galleni M, Kerff F. 2009. Critical role of tryptophan 154
- for the activity and stability of class D β -lactamases. Biochemistry 48:11252–11263.

- 612 16. Docquier JD, Calderone V, De Luca F, Benvenuti M, Giuliani F, Bellucci L, Tafi A, Nordmann
 613 P, Botta M, Rossolini GM, Mangani S .2009. Crystal structure of the OXA-48 β-lactamase
 614 reveals mechanistic diversity among class D carbapenemases. Chem Biol 16:540–547.
- 615 17. Dabos L, Zavala A, Bonnin RA, Beckstein O, Retailleau P, Iorga BI, Naas T. 2020. Substrate
 616 Specificity of OXA-48 after β5-β6 Loop Replacement. ACS Infect Dis 6:1032-1043.
- 617 18. Oueslati S, Retailleau P, Marchini L, Berthault C, Dortet L, Bonnin RA, Iorga BI, Naas T.
 618 2020. Role of Arginine 214 in the Substrate Specificity of OXA-48. Antimicrob Agents
 619 Chemother 64:e02329-19.
- 620 19. Stojanoski V, Chow DC, Fryszczyn B, Hu L, Nordmann P, Poirel L, Sankaran B, Prasad BV,
- Palzkill T. 2015. Structural basis for different substrate profiles of two closely related class
 D β-lactamases and their inhibition by halogens. Biochemistry 21:3370-3380.
- 20. Dortet L, Agathine A, Naas T, Cuzon G, Poirel L, Nordmann P. 2015. Evaluation of the
 RAPIDEC[®] CARBA NP, the Rapid CARB Screen[®] and the Carba NP test for biochemical
 detection of carbapenemase-producing Enterobacteriaceae. J Antimicrob Chemother
 70:3014-22.
- Bogaerts P, Oueslati S, Meunier D, Nonhoff C, Yunus S, Massart M, Denis O, Woodford N,
 Hopkins KL, Naas T, Dortet L, Huang TD, Glupczynski Y. 2017. Multicentre evaluation of the
 BYG Carba v2.0 test, a simplified electrochemical assay for the rapid laboratory detection
 of carbapenemase-producing *Enterobacteriaceae*. Sci Rep 7:9937.
- 22. Dortet L, Tandé D, de Briel D, Bernabeu S, Lasserre C, Gregorowicz G, Jousset AB, Naas T. 631 632 2018. MALDI-TOF for the rapid detection of carbapenemase-producing 633 Enterobacteriaceae: comparison of the commercialized MBT STAR®-Carba IVD Kit with two 634 in-house MALDI-TOF techniques and the RAPIDEC® CARBA NP. J Antimicrob Chemother 635 73:2352-2359.

- 636 23. Bernabeu S, Dortet L, Naas T. 2017. Evaluation of the β-CARBA[™] test, a colorimetric test
 637 for the rapid detection of carbapenemase activity in Gram-negative bacilli. J Antimicrob
 638 Chemother 72:1646-1658.
- 639 24. Gauthier L, Bonnin RA, Dortet L, Naas T. 2017. Retrospective and prospective evaluation of
 640 the Carbapenem inactivation method for the detection of carbapenemase-producing
 641 Enterobacteriaceae. PLoS One 12:e0170769.
- 642 25. Muntean MM, Muntean AA, Gauthier L, Creton E, Cotellon G, Popa MI, Bonnin RA, Naas T.
 643 2018. Evaluation of the rapid carbapenem inactivation method (rCIM): a phenotypic
 644 screening test for carbapenemase-producing Enterobacteriaceae. J Antimicrob Chemother
 645 73:900-908.
- 26. Larsen MV, Cosentino S, Rasmussen S, Friis C, Hasman H, Marvig RL, Jelsbak L, SicheritzPontén T, Ussery DW, Aarestrup FM, Lund O. 2012. Multilocus sequence typing of totalgenome-sequenced bacteria. J Clin Microbiol 50:1355-61.
- 649 27. Ewers C, Stamm I, Pfeifer Y, Wieler LH, Kopp PA, Schønning K, Prenger-Berninghoff E,
- 650 Scheufen S, Stolle I, Günther S, Bethe A. 2014. Clonal spread of highly successful ST15-CTX-
- 651 M-15 *Klebsiella pneumoniae* in companion animals and horses. J Antimicrob Chemother 652 69:2676-80.
- 28. Zhou K, Lokate M, Deurenberg RH, Tepper M, Arends JP, Raangs EG, Lo-Ten-Foe J,
 Grundmann H, Rossen JW, Friedrich AW. 2016. Use of whole-genome sequencing to trace,
 control and characterize the regional expansion of extended-spectrum β-lactamase
 producing ST15 *Klebsiella pneumoniae*. Sci Rep 6:20840.
- 657 29. Zankari E, Hasman H, Cosentino S, Vestergaard M, Rasmussen S, Lund O, Aarestrup FM,
- 658 Larsen MV. 2012. Identification of acquired antimicrobial resistance genes. J Antimicrob
- 659 *Chemother* 67:2640-264.

- 30. Ramirez MS, Tolmasky ME. 2010. Aminoglycoside Modifying Enzymes. Drug Resist Updat
 13:151–171.
- 662 31. Sundin GW. 2002. Distinct recent lineages of the strA- strB streptomycin-resistance genes
 663 in clinical and environmental bacteria. Curr Microbiol 45: 63-9.
- 32. Skold O. 2000. Sulfonamide resistance: mechanisms and trends. Drug Resist Updat 3:155160.
- 33. Bossé JT, Li Y, Walker S, Atherton T, Fernandez Crespo R, Williamson SM, Rogers J,
 Chaudhuri RR, Weinert LA, Oshota O, Holden MT, Maskell DJ, Tucker AW, Wren BW,
 Rycroft AN, Langford PR BRaDP1T Consortium. 2015. Identification of dfrA14 in two
 distinct plasmids conferring trimethoprim resistance in Actinobacillus pleuropneumoniae.
 J Antimicrob Chemother 70:2217-22.
- 671 34. Rodríguez-Martínez JM, Díaz de Alba P, Briales A, Machuca J, Lossa M, Fernández-Cuenca
 672 F, Rodríguez Baño J, Martínez-Martínez L, Pascual Á. 2013. Contribution of OqxAB efflux
 673 pumps to quinolone resistance in extended-spectrum-β-lactamase-producing *Klebsiella*674 *pneumoniae*. J Antimicrob Chemother 68:68-73.
- 675 35. Fillgrove KL, Pakhomova S, Newcomer ME, Armstrong RN. 2003. Mechanistic diversity of
 676 fosfomycin resistance in pathogenic microorganisms. J Am Chem Soc 125:15730-15731.
- 677 36. Carattoli A, Zankari E, Garcia-Fernandez A, Voldby Larsen M, Lund O, Villa L, Møller
 678 Aarestrup F, Hasman H. 2014. *In silico* detection and typing of plasmids using
 679 PlasmidFinder and plasmid multilocus sequence typing. Antimicrob Agents Chemother 58:
 680 3895-903.
- 37. Carattoli A, Seiffert SN, Schwendener S, Perreten V, Endimiani A. 2015. Differentiation of
 IncL and IncM Plasmids Associated with the Spread of Clinically Relevant Antimicrobial
 Resistance. PLoS One 10: e0123063.

684	38. Poirel L, Bonnin RA, Nordmann P. 2012. Genetic features of the widespread plasmid
685	coding for the carbapenemase OXA-48. Antimicrob Agents Chemother 56: 559–562.
686	39. Potron A, Rondinaud E, Poirel L, Belmonte O, Boyer S, Camiade S, Nordmann P. 2013.
687	Genetic and biochemical characterisation of OXA-232, a carbapenem-hydrolysing class D
688	β-lactamase from <i>Enterobacteriaceae</i> . Int J Antimicrob Agents 41: 325–329.
689	40. Fröhlich C, Sørum V, Thomassen AM, Johnsen PJ, Leiros HS, Samuelsen Ø. 2019. OXA-48-
690	Mediated Ceftazidime-Avibactam Resistance Is Associated with Evolutionary Trade-Offs.
691	mSphere 4: e00024-19.
692	41. Smith CA, Stewart NK, Toth M, Vakulenko SB. 2019. Structural Insights into the Mechanism
693	of Carbapenemase Activity of the OXA-48 β -Lactamase. Antimicrob Agents Chemother 63:
694	e01202-19.
695	42. Stojanoski V, Hu L, Sankaran B, Wang F, Tao P, Prasad BVV, Palzkill T. 2021. Mechanistic
696	Basis of OXA-48-like β -Lactamases' Hydrolysis of Carbapenems. ACS Infect Dis 7: 445-460.
697	43. De Luca F, Benvenuti M, Carboni F, Pozzi C, Rossolini GM, Mangani S, Docquier JD. 2011.
698	Evolution to carbapenem-hydrolyzing activity in noncarbapenemase class D β -lactamase
699	OXA-10 by rational protein design. Proc Natl Acad Sci USA 108:18424–18429.
700	44. Huang TD, Poirel L, Bogaerts P, Berhin C, Nordmann P, Glupczynski Y. 2014. Temocillin and
701	piperacillin/tazobactam resistance by disc diffusion as antimicrobial surrogate markers for
702	the detection of carbapenemase-producing Enterobacteriaceae in geographical areas with
703	a high prevalence of OXA-48 producers. J Antimicrob Chemother 69:445-50.
704	45. Rima M, Emeraud C, Bonnin RA, Gonzalez C, Dortet L, Iorga BI, Oueslati S, Naas T. 2021.
705	Biochemical characterization of OXA-244, an emerging OXA-48 variant with reduced eta -
706	lactam hydrolytic activity. J Antimicrob Chemother 76:2024-2028.

- 707 46. Emeraud C, Biez L, Girlich D, Jousset AB, Naas T, Bonnin RA, Dortet L. Screening of OXA-244
 708 producers, a difficult-to-detect and emerging OXA-48 variant? J Antimicrob Chemother
 709 75:2120-2123.
- 710 47. Paetzel M, Danel F, de Castro L, Mosimann SC, Page, MG, Strynadka NC. 2000. Crystal
 711 Structure of the Class D Beta-Lactamase OXA-10. Nat Struct Biol 7:918–925.
- 48. Dabos L, Bogaerts P, Bonnin RA, Zavala A, Sacré P, Iorga B, Huang DT, Glupczynski Y, Naas T.
- 713 2018. Genetic and Biochemical characterization of OXA-519, a novel OXA-48-like β714 lactamase. Antimicrob Agents Chemother 62: e00469-18.
- 715 49. Kieser T. 1984. Factors affecting the isolation of CCC DNA from *Streptomyces lividans* and
 716 *Escherichia coli*. Plasmid 12:19–36.
- 50. Naas T, Sougakoff W, Casetta A, Nordmann P. 1998. Molecular characterization of OXA-20,
- a novel class D β-lactamase, and its integron from *Pseudomonas aeruginosa*. Antimicrob
 Agents Chemother 42:2074-2083.
- 51. Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram
 quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–
- 722 254.
- 52. Kabsch W. 2010. XDS. *Acta Crystallogr D Biol Crystallogr*. 66: 125-32.
- 53. Krug M, Weiss MS, Heinemann U, Mueller U .2012. XDSAPP: a graphical user interface for
 the convenient processing of diffraction data using XDS. J Appl Cryst 45:568-572.
- 726 54. French GS, Wilson KS. 1978. Acta. Cryst A 34:517.
- 55. Winn MD, Ballard CC, Cowtan KD, Dodson EJ, Emsley P, Evans PR, Keegan RM, Krissinel EB,
- 728 Leslie AG, McCoy A, McNicholas SJ, Murshudov GN, Pannu NS, Potterton EA, Powell HR,
- 729 Read RJ, Vagin A, Wilson KS. 2011. Overview of the CCP4 suite and current developments.
- Acta Crystallogr D Biol Crystallogr 67: 235-242.

- 56. Ten Eyck LF .1973. Crystallographic fast Fourier transforms. *Acta Cryst*. A29: 183-191.
- 57. McCoy AJ, Grosse-Kunstleve RW, Adams PD, Winn MD, Storoni LC, Read RJ. 2007. Phaser
 crystallographic software. J Appl Crystallogr 40:658-674.
- 734 58. Read RJ, Adams PD, McCoy AJ. 2013. Intensity statistics in the presence of translational
 735 noncrystallographic symmetry. Acta Crystallogr D Biol Crystallogr 69:176-83.
- 59. Kowiel M, Jaskolski M, Dauter Z .2014. ACHESYM: an algorithm and server for standardized
 placement of macromolecular models in the unit cell. Acta Crystallogr D Biol Crystallogr
 70:3290-3298.
- 739 60. Murshudov GN, Skubák P, Lebedev AA, Pannu NS, Steiner RA, Nicholls RA, Winn MD, Long
- F, Vagin AA. 2011. REFMAC5 for the refinement of macromolecular crystal structures. Acta
 Cryst D 67:355-367.
- Features and development of Coot. *Acta Crystallogr D Biol Crystallogr*. 66: 486-501.
- 62. Chen VB, Arendall WB, Headd JJ, Keedy DA, Immormino RM, Kapral GJ, Murray LW,
 Richardson JS, Richardson DC. 2009. MolProbity: all-atom structure validation for
 macromolecular crystallography. Acta Crystallogr D Biol Crystallogr 66:12-21.
- 63. Berman HM, Westbrook J, Feng Z, Gilliland G, Bhat TN, Weissig H, Shindyalov IN, Bourne
 PE. 2000. The Protein Data Bank. Nucleic Acids Res 28:235-242.
- 64. DeLano WL .2014. The PyMOL Molecular Graphics System, version 1.7.0.0, Schrodinger,
 LLC, New York.
- 65. Kleywegt GJ, Harris MR, Zou JY, Taylor TC, Wählby A, Jones TA. 2004. The Uppsala Electron Density Server. Acta Crystallogr D Biol Crystallogr 60:2240-2249.
- 753 66. Goddard TD, Huang CC, Meng EC, Pettersen EF, Couch GS, Morris JH, Ferrin TE. 2018. UCSF
- 754 ChimeraX: Meeting modern challenges in visualization and analysis. Protein Sci. 27: 14-25.

Table 1. MICs of β-lactam antibiotics for *K. pneumoniae* 1219, *E. coli* TOP 10 (pOXA-517), *E. coli* TOP
10 (pTOPO-OXA-48), *E. coli* TOP 10 (pTOPO-OXA-517), *E. coli* TOP 10 (pTOPO-OXA-405), *E. coli* TOP
10 (pTOPO-OXA-163), and *E. coli* TOP 10.

758

			MIC (µg	g/mL)			
Antibiotic	Kp 1219	pOXA-517 ^ª	pTOPO-OXA-48	pTOPO-OXA-517 ^t	° pTOPO-OXA-163	E. coli TOP10	
Amoxicillin	>256	>256	>256	>256	>256	2	
Amoxicillin + CLA ^c	192	64	192	64	96	2	
Piperacillin	>256	>256	128	>256	>256	1.5	
Piperacillin + TZB ^d	96	96	12	96	32	1	
Cefotaxime	>32	2	0.094	0.38	3	0.06	
Ceftazidime	48	16	0.19	4	16	0.12	
Ceftazidime+Avi ^e	1	1	0.5	0.5	ND	0.25	
Cefepime	12	1	0.047	0.25	0.5	0.023	
Imipenem	8	0.75	0.38	0.38	0.25	0.25	
Meropenem	3	0.125	0.047	0.047	0.023	0.016	
Ertapenem	3	0.125	0.047	0.047	0.032	0.003	
Doripenem	3	0.094	0.094	0.094	0.023	0.06	
Temocillin	192	192	>1024	32	32	4	
Aztreonam	4	2	0.094	0.75	2	0.047	

759

760 ^a natural plasmid carrying OXA-517

761 ^b recombinant pTOPO plasmids carrying OXA-48, OXA-517 and OXA-163

762 ^cCLA, clavulanic acid (2 μ g/mL)

763 ^d TZB, tazobactam (4 μ g/mL)

764 ^e AVI: Avibactam (4 μ g/mL)

Table 2. Kinetic parameters of OXA-48, OXA-517 and OXA-163, with representative β-lactam

767 substrates.

	<i>K</i> _M (μM)			$k_{\rm cat} ({\rm s}^{-1})$			$k_{\rm cat}/K_{\rm M}~({\rm mM}^{-1}{\rm s}^{-1})$		
Substrate	OXA-48	OXA-163	OXA-517	OXA-48	OXA-163	OXA-517	OXA-48	OXA-163	OXA-517
Ampicillin	395	315	226	955	23	58	2418	70	256
Oxacillin	95	90	48	130	34	30	1368	370	629
Temocillin	45	N.H.	729	0.30	N.H.	0.24	7	N.D.	0.32
Cefalotin	195	10	>1000	44	3	>59	226	300	38
Cefoxitin	>200	N.D	>1000	>0.05	N.D.	>6	0.26	N.D.	0.38
Ceftazidime	N.H.	>1000	>1000	N.H.	8	>20	N.D.	3	2
Cefotaxime	>900	45	>1000	>9	10	>22	10	230	16
Cefepime	>550	N.D.	>1000	>0.60	N.D.	>18	1.1	N.D.	11
Imipenem	13	520	411	5	0.03	6	369	0.06	14
Meropenem	11	>2000	>1000	0.07	0.10	>0.9	6	0.03	0.89
Ertapenem	100	130	>1000	0.13	0.05	>2.6	1.3	0.30	0.63
Aztreonam	N.H.	230	319	N.H.	0.50	1	N.D.	2	3.1

768

769 N.D., not determined. N.H., no detectable hydrolysis was observed with concentrations of substrate and

enzymes up to 500 μM and 400 nM, respectively. Measurements were done in triplicate, and SD was within
 10%

772 Data for OXA-48 are from Docquier *et al* (16). Data for OXA-163 are from Oueslati *et al* (5).

Table 3. X-ray data collection and structure refinement statistics

Data collection and processing	
Wavelength [Å]	0.98010
Resolution [Å]	46.37-1.86 (1.91-1.86)*
Space group	<i>P</i> 2 ₁
Cell parameters [Å, °]	50.05/125.74/83.03/94.6
R _{meas} [%]	15.6 (125.3)
<i σi=""></i>	6.57 (1.03)
CC _{1/2} [%]	99.5 (34.7)
Completeness [%]	98.5 (91.9)
No. of unique reflections	84532 (13554)
Redundancy	3.3 (3.0)
Refinement	
R _{work} /R _{free} [%]	17.0/21.9
Rmsd bond lengths [Å]	0.012
Rmsd bond angles [°]	1.37
No. of protein molecules in ASU	4
No. of modeled amino acid residues	979
No. of all/protein/water/other atoms	8981/8072/738/185
Wilson B factor [Å ²]	24.2
Average B factor [Å ²] protein/water/other solvent/all	30.9/39.0/43.0/31.6
Ramachandran favored/allowed [%]	98/2
PDB code	6hb8

Data collection and processing

*Values in parentheses correspond to the highest resolution shell

778 Figure legends

Figure 1. (A) Nucleotide alignment of the β5-β6 loop region of the bla_{OXA-48} and $bla_{OXA-517}$ genes (small letters). The corresponding amino-acid sequences of OXA-48 (3) and OXA-517 are shown in capital letters. **(B)** Alignment of the amino acid sequences of OXA-48 (3), OXA-163 (7) and OXA-517. For OXA-405 (8) and OXA-438 (10) only the β5-β6 loop is indicated. Asterisks indicate identical residues with OXA-48 AA sequence. Dashes indicate deleted amino acid residues. Amino acid motifs that are well conserved among class D β-lactamases are highlighted in gray. The β5-β6 loop sequence is boxed.

Figure 2. (A) Plasmid structures and genetic environments. Schematic representation of the genetic environments of the bla_{0XA-48} (a), $bla_{0XA-405}$ (b), $bla_{0XA-517}$ (c), $bla_{0XA-163}$ (d), $bla_{0XA-247}$ (e), $bla_{0XA-181}$ (f), $bla_{0XA-232}$ (g), and $bla_{0XA-204}$ (h) genes. The Tn1999 composite is formed by two copies of the IS1999 insertion sequence, bracketing a fragment containing the bla_{0XA-48} , $bla_{0XA-405}$ and $bla_{0XA-517}$ genes. (B) Comparison of the major structural features of the plasmid pOXA-517 from *K*. *pneumoniae* 1219 with the prototype IncL bla_{0XA-48} plasmid pOXA-48 (GenBank accession number JN626286) and pOXA-405. Common structures are highlighted in gray.

Figure 3. Cartoon model of a single subunit of OXA-517. The secondary structure elements are labeled according to (16). The model is colored by the B factor of the C α atoms and the bar shows the color range in Å².

Figure 4. (A) An OXA-517 dimer shown as a cartoon model with two Arg206 residues and the bound sulfate ion shown as sticks. (**B**) A zoom-in view on the dimer interface with the partially occupied chloride (green sphere) and sulfate (red and yellow sticks) ions. Two equivalent arginine residues from the complementary subunits interact with the ions. This view is rotated 90° upwards compared to A. (**C**) The sulfate ion when modeled at full occupancy, in the 2mFo-DFc (blue,

contoured at 1.5σ) and mFo-DFc (+/-3.0σ, green/red) electron density maps, showing negative
difference density. (D) The sulfate modeled at 0.5 occupancy with the electron density maps
contoured at the same level as in C. A positive difference density peak is well visible, finally
modeled as partial-occupancy Cl⁻ anion (green sphere in B).

Figure 5. (A) Alignment of the structures of OXA-517 (orange), OXA-48 (green) and OXA-163 (gray) showing variation in the length of the β 5- β 6 loop. (B) A close-up view of the backbone conformation of the loop with visible *cis*-peptide bonds preceding Pro217. Also visible is the difference in backbone conformation between OXA-163 (gray) and the other structures at Asp212. The distance between the C α atoms of Arg214 (OXA-48) and Lys214 (OXA-517) is 3.8 Å. (C)-(E) Detailed views of the binding site in OXA-517 (C), OXA-48 (D) and OXA-163 (E) with key residues rendered as sticks. All three figures have the same orientation.

Figure 6. (A) Superposition of the OXA-48 mutant P68A acyl-enzyme complex with ceftazidime structure (PDB id 6q5f, cyan) onto the native OXA-48 structure (PDB id 4s2p, green). Clashes shown as purple thick dashed lines. (B) Superposition of the OXA-48 mutant P68A acyl-enzyme complex with ceftazidime structure (PDB id 6q5f, cyan) onto the OXA-517 structure (orange). Clashes shown as purple thick dashed lines. All panels correspond to superposed structures viewed from the same distance and angle.

Figure 7. (A-C) Contacts between the β5-β6-loop residues and the residues previous to SER-70 αhelix or the Ω-loop residues in OXA-48 (PDB id 4s2p) (A), OXA-517 (PDB id 6hb8) (B) and OXA-163 (PDB id 7khz) (C). (D) Contacts between the residues before SER-70 α-helix and the Ω-loop residues in OXA-48 (PDB id 4s2p). Contacts are shown as purple thin dashed lines. Panels A, B, C correspond to superposed structures viewed from the same distance and angle.

Α				210					
				Ī				220	
	OXA-48	I	r a k	ΤG	(ST	RI	E P _K	I G	
	bla	atto	aaactaaa				aaccta.	agattaga	
	DIA _{OXA-48}	alle	yyyctaac	actygat	actegat	layaatt	yaactta	ayattyyt	,
	bla _{OXA-517}	atte	gggctaaa	actggat	actcgac	cta	-aacctaa	agattggc	
		****	******	* * * * * * * [·]	*******	* *	******	******	
	OXA-51/	I	R A K	ΤG,	/ S T	К	<u>Р</u> К	I G	
В									
	1	10	20	30	40	50	60	70	80
	I			Ι			I	<u> </u>	
OXA-48	MRVLALS	AVFLVAS	IIGMPAVAKEW	QENKSWNAHI	TEHKSQGVV	VLWNENKQQGF	TNNLKRANQA	VELPASTEKIP1	ISLIA
OXA-163	******	******	*********	* * * * * * * * * *	*******	*********	* * * * * * * * * *	**********	****
OXA-517	* * * * * * *	******	* * * * * * * * * * * *	* * * * * * * * * * *	******	* * * * * * * * * * * *	*******	*********	****
	81	90	100	110	120	130	140	150	160
	I	I	I	Ι	I	I	Ι		
OXA-48	LDLGVVK	DEHQVFK	WDGQTRDIATW	NRDHNLITAN	IKYSVVPVYQI	EFARQIGEARM	ISKMLHAFDYG	NEDISGNVDSF	FWLDG
OXA-163	******	* * * * * * * *	********	* * * * * * * * * *	******	* * * * * * * * * * *	*******	*********	****
OXA-517	* * * * * * *	******	**********	*******	***	* * * * * * * * * * *	*******	* * * * * * * * * * * *	****
	161	170	180	190	200	210	220	230	240
	<u> </u>	<u> </u>		Ι			I	Ι	
OXA-48	GIRISAT	EQISFLRI	KLYHNKLHVSE	RSQRIVKQAN	ILTEANGDYI	IRAKTG¥STRI	EPKIGWWVGW	IVELDDNVWFFI	AMNMD
OXA-163	* * * * * * *	******	* * * * * * * * * * *	* * * * * * * * * *	******	******D*	*******	**********	****
OXA-517	*****	******	* * * * * * * * * * *	* * * * * * * * * *	*******	*** <mark>***</mark> ***K-	-********	**********	****
OXA-438						****GY	'DT***		
OXA-405						***			
	0.4.1	050	0.60			β5-β6 L	оор		
	241	250	260						
0377 40									
OXA = 48	MPTSDGL	GLKQAIT.	KEVLKQEKIIF						
OXA-103	******	******	*****						
ULA-DI/									















Supplementary material

Structural and biochemical features of OXA-517: a carbapenem and expanded-

spectrum cephalosporin hydrolyzing OXA-48 variant.

Laura Dabos^{1,2}, Joanna E. Raczynska³, Pierre Bogaerts⁵, Agustin Zavala⁶, Delphine Girlich^{1,2}, Remy A. Bonnin^{1,2}, Laurent Dortet^{1,2}, Aurélie Peyrat¹, Pascal Retailleau⁶, Bogdan I. Iorga⁶, Mariusz Jaskólski^{3,7}, Youri Glupczynski⁵, Thierry Naas^{1,2,4*}

¹ Team "Resist" UMR1184 "Immunology of Viral, Auto-immune, Hematological and Bacterial diseases (IMVA-HB), INSERM, University Paris-Saclay, LabEx LERMIT, Faculty of Medicine, Le Kremlin-Bicêtre, France.

² French National Reference Center for Antibiotic Resistance: Carbapenemase-producing Enterobacteriaceae, Le Kremlin-Bicêtre, France:

³ Center for Biocrystallographic Research, Institute of Bioorganic Chemistry, Polish Academy of Sciences, Poznan, Poland.

⁴ Department of Bacteriology-Hygiene, Bicêtre Hospital, APHP Paris-Saclay, Le Kremlin-Bicêtre, France

⁵ Laboratory of clinical microbiology, National reference center for monitoring antimicrobial resistance in Gram-negative bacteria, CHU UCL Namur, Yvoir, Belgium.

⁶ Université Paris-Saclay, CNRS UPR 2301, Institut de Chimie des Substances Naturelles, Labex LERMIT, Gif-sur-Yvette, France.

⁷ Department of Crystallography, Faculty of Chemistry, A. Mickiewicz University, Poznan, Poland.

Clinical case Figure S1 and S2

Clinical Case

K. pneumoniae 1219 clinical isolate (Kp 1219) was recovered from culture of a sputum specimen of 81-year-old women hospitalized in a Belgium hospital for rapid alteration of general status, fever (39°C) and lumbar pain shown to be associated with a hydro-ureteronephrosis due to an obstructive renal stone for which stenting of the left ureteral orifice was performed in the operative room on the next day. Blood and urine cultures obtained on admission revealed the presence of a Proteus mirabilis (wild-type susceptible to all antibiotics). Antimicrobial therapy was initiated with temocillin (2 g/IV bid) and rapidly switched to piperacillin 4 g/IV gid because of rapid alteration of the status and development of a severe septic shock (thrombocytopenia, 3.000 platelets/mm³) with acute renal failure (estimated GFR (glomerular filtration rate) of 21 ml/min). The patient had to be admitted to the intensive care unit where she received high dose of vasopressor drugs (norepinephrine 1 µg/kg/min) and was intubated and mechanically ventilated. Her stay was complicated by the development of a right lower lobe ventilatorassociated pneumonia episode for which she was treated with several antimicrobial agents including meropenem (1g/IV tid) followed by cefepime (2g/IV tid), colistin (4.5 million units/bid after a loading dose of 9 million units) and tigecycline (100 mg/IV bid). She then developed an acute infectious enterocolitis with active gross bleeding due to Clostridium difficile for which she was treated by metronidazole (500 mg tid) and vancomycin (250 mg qid) per os. The patient was nursed in single isolation room during the remaining duration of her ICU stay. After four weeks, she was transferred to another unit for palliative care and eventually died shortly after Kp 1219 isolate was repeatedly isolated over the period from throat, sputum, tracheobronchial aspirates and BAL (bronchoalveolar lavage) specimens of the patient.



Figure S1. The active site of OXA-517 with residues implicated in substrate binding and the hydrolysis reaction shown as sticks. The only water molecule (small red sphere) in the active site that is preserved in all four protein subunits in the crystal is bound in the oxyanion hole. 2mFo-DFc electron density (contoured at 1 σ) is shown for the carboxylated lysine residue (Kcx73), the sulfate ion and the residues in loop β 5- β 6.



Figure S2. Superposition of OXA-48 acyl-enzyme complex with imipenem structure (PDB id 6p97, pink) onto the OXA-517 structure (PDB id 6hB8, orange). Residues surrounding imipenem are shown as thick sticks. Imipenem is shown as thin sticks, to allow a clearer view of surrounding residues potentially blocked by it.