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1 **Role of Amino-Acid 159 in carbapenem and temocillin hydrolysis of OXA-933, a novel**
2 **OXA-48 variant**

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23 **Abstract**

24 OXA-48 has rapidly disseminated worldwide and became one of the most common
25 carbapenemase in many countries with more than 45 variants reported with in some cases
26 significant differences in their hydrolysis profiles. The R214 residue, located in the β 5- β 6 loop,
27 is crucial for the carbapenemase activity, as it stabilizes carbapenems in the active site and
28 maintains the shape of the active site through interactions with D159. In this study, we have
29 characterized a novel variant of OXA-48, OXA-933 with a single D159N change. To evaluate
30 the importance of this residue, point mutations were generated (D159A, D159G, D159K,
31 D159W), kinetic parameters of OXA-933, OXA-48 D159G, and OXA-48 D159K were
32 determined and compared to those of OXA-48 and OXA-244. The *bla*_{OXA-933} gene was borne on
33 Tn2208, a 2696-bp composite transposon made of two *IS1* elements surrounded by 9-bp target
34 site duplications and inserted into a non self-transmissible plasmid pOXA-933 of 7872-bp in
35 size. MIC values of *E. coli* expressing *bla*_{OXA-933} gene or of its point mutant derivatives were
36 lower for carbapenems (except for D159G) as compared to those expressing *bla*_{OXA-48} gene.
37 Steady state kinetic parameters revealed lower catalytic efficiencies for expanded spectrum
38 cephalosporins and carbapenems. A detailed structural analysis confirmed the crucial role of
39 D159 in shaping the active site of OXA-48 enzymes by interacting with R214. Our work further
40 illustrates the remarkable propensity of OXA-48-like carbapenemases to evolve through
41 mutations at positions outside the β 5- β 6 loop, but interacting with key residues of it.

42

43 **Introduction**

44 Carbapenemase producing Enterobacterales constitute a serious health issue limiting the
45 therapeutic options for gram-negative bacterial infections and increasing the morbidity and
46 mortality rates among patients (1). Carbapenemases belong to three molecular classes of β -
47 lactamases (1,2). Class A and D correspond to serine-active β -lactamases and class B enzymes
48 are metallo- β -lactamases that require two Zn^{2+} ions to coordinate a water molecule in the active
49 site for hydrolysis (1,3). Among Carbapenem-Hydrolyzing class D β -Lactamase (CHDL), OXA-
50 48, initially described in 2004, in a carbapenem-resistant *Klebsiella pneumoniae* isolate from
51 Turkey in 2001, has now spread globally in Enterobacterales, to become the most common
52 carbapenemase in many countries (4-6). OXA-48 hydrolyzes penicillins at a high level,
53 carbapenems at a low level and lacks significant hydrolytic activity towards expanded-spectrum
54 cephalosporin (ESC) (6-7). More than 45 variants have since been reported, differing by a few
55 amino acid substitutions or deletions (Beta-Lactamase DataBase
56 <http://bldb.eu/BLDB.php?class=D#OXA>; 8). While some OXA-48-variants with single amino
57 acid substitutions have similar hydrolytic profiles as OXA-48, others, especially those with
58 changes in the β 5- β 6 loop located close to the substrate binding site, which represents 22/45 of
59 described variants, have significant changes in the hydrolytic spectrum of β -lactams (7,8). This is
60 the case for variants with a change at position R214, such as OXA-244 (R214G) and OXA-232
61 (R214S) that have decreased carbapenemase activity (7,9), but also for variants with a four-
62 amino-acid deletion inside the loop such as OXA-163 or OXA-405, which results in the loss of
63 carbapenemase activity and a gain of the ability to hydrolyze oxyimino-cephalosporins (10,11).
64 Finally, other variants such as OXA-438, OXA-517 and OXA-793 with two AA deletions in the

65 β 5– β 6 loop, have been described with the ability to hydrolyze carbapenems and oxyimino-
66 cephalosporins equally well (12-14).

67 The overall 3D structure of OXA-48 is very similar to that of other class D beta-
68 lactamases (DBLs) (15,16), with however one main difference, e.g. an overall shorter the β 5 and
69 β 6 strands (15,16). Consequently, the β 5– β 6 loop has a different orientation from that found in
70 other DBLs. The observed conformation of the loop in OXA-48 is promoted by a salt bridge of
71 R214 with the Ω -loop D159 residue, which itself is stabilized by S155 (15-18). In 16 out the 22
72 OXA-48 variants with changes in the β 5- β 6 loop, the R214 is changed to S (n=3), G (n=3), N
73 (n=1), D (n=3), K (n=1), E (n=2), and G (n=3) (8). In 5 out of the 6 remaining, R214 is deleted
74 and only in one case, in OXA-438, the R214 is still present, but two amino acids 215-216 have
75 been deleted (12). Changes at position R214 have a drastic impact on the hydrolysis of
76 carbapenems because of the loss of the salt bridge with D159 (18). The β 5– β 6 loop extends into
77 the outer portion of the active site crevice and defines a rather hydrophilic cavity filled with
78 several water molecules, one of which will perform the nucleophilic attack on the acyl-enzyme
79 intermediate in the deacylation step (15,17).

80 In this study, we have characterized a novel natural OXA-48 variant, OXA-933 with a
81 D159N change. To evaluate the impact of this AA change and to further characterize the role of
82 this position and its interaction with R214, susceptibility testing, steady state kinetic parameters
83 together with a detailed structural analysis were performed for OXA-933 (OXA-48-D159N), and
84 several in vitro generated OXA-48 point mutant derivatives (D159A, D159G, D159K, D159W).
85 In addition, the genetic support of this novel OXA-48 variant was determined.

86

87 **Results**

88 ***K. pneumoniae* 241B9 clinical isolate, antimicrobial susceptibility testing of and**
89 **carbapenemase confirmation tests**

90 The clinical *K. pneumoniae* 241B9 strain was recovered from an urinary tract infection of
91 an hospitalized woman in a long term care facility in 2019. Disk diffusion antibiogram revealed
92 that *K. pneumoniae* 241B9 was resistant as expected to amoxicillin, ticarcillin, and piperacillin,
93 but also to amoxicillin/clavulanic acid, ticarcillin/clavulanic acid and piperacillin/tazobactam,
94 likely suggesting the presence of an OXA-type acquired β -lactamase. In addition, the isolate was
95 also resistant to temocillin, but with a zone diameter of 13 mm, which could be compatible with
96 an OXA-48 type variant. *K. pneumoniae* 241B9 remained susceptible to all other β -lactams
97 tested, including carbapenems. The zone diameter of ertapenem was slightly reduced (24 mm)
98 and that of meropenem (28 mm) was at the EUCAST recommended screening cut-off of >0.125
99 $\mu\text{g/ml}$ (zone diameter <28 mm). MIC testing using E-tests revealed an MIC of $0.5 \mu\text{g/ml}$ and
100 $0.032 \mu\text{g/ml}$ for ertapenem and meropenem, respectively. The presence of a carbapenem-
101 hydrolyzing enzyme was further searched using the Carba NP, a home-made biochemical test
102 based on imipenem hydrolysis (19), and the immunochromatographic NG-Test Carba5 assay
103 (NG Biotech, Guipry, France; (20). While Carba NP test was inconsistently positive, the NG-
104 Test Carba5 was repeatedly positive for OXA-48-like enzyme.

105 Disc diffusion antibiogram revealed further that *K. pneumoniae* 241B9 was susceptible to
106 all the other tested antibiotic families e.g. aminoglycosides, fluoroquinolones, chloramphenicol,
107 tigecyclines, and colistin (confirmed by MIC testing [$\text{MIC}=0.5 \mu\text{g/ml}$]), except to rifampin.

108 As *K. pneumoniae* 241B9 had a reduced susceptibility toward carbapenems and
109 temocillin, we tested its ability to grow on standard chromogenic and selective media, such as
110 ChromID® CARBA SMART. Using an inoculum of 10^3 CFU, *K. pneumoniae* 241B9 strain

111 grew on both sides (Carba and OXA-48) of the ChromID® CARBA SMART plate
112 (BioMérieux).

113

114 **Genomic features of *K. pneumoniae* 241B9**

115 WGS sequencing of *K. pneumoniae* 241B9 revealed a genome of 5,339,000-bp with a
116 mean coverage of 90.7 X. The Multi locus sequence typing (MLST) of *K. pneumoniae* 241B9
117 according to MLST 1.8 software (21), revealed ST-1079 (2-5-1-1-9-1-19), an ST rarely
118 described and with only 10 occurrences in the *Klebsiella* Pasteur MLST database over the last 15
119 years from Argentina, United Kingdom, France and Australia
120 (<https://bigsd.bpasteur.fr/klebsiella/>).

121 Acquired resistance genes and point mutations involved in resistance were analyzed using
122 ResFinder (22). The naturally occurring *bla*_{SHV-1} gene, and a variant of *bla*_{OXA-48} carbapenemase
123 gene with a G₄₇₅ to A mutation that results in D159N AA change, named *bla*_{OXA-933} gene, were
124 identified in this isolate. In addition, *oqx*A and *oqx*B genes were also present in the isolate, likely
125 at the origin of reduced susceptibility to fluoroquinolones (MIC of 0.5 µg/ml to ciprofloxacin) as
126 no chromosomal mutation was present in the QRDR region. *fos*A gene conferring resistance to
127 fosfomycin was also present in the genome (23). In addition, point mutations were found in
128 *ompK36* and *ompK37*, but none has been described to be involved in reduced susceptibility to
129 third generation cephalosporins. According to PlasmidFinder (24), no plasmid replication origin
130 could be evidenced.

131 **Genetic support and environment of *bla*_{OXA-933} gene**

132 The *bla*_{OXA-933} gene was located on Tn2208, a 2696-bp composite transposon made of
133 two *IS1* elements inserted with a 9-bp target site duplication into a non-self-transmissible

134 plasmid resulting in plasmid pOXA-933 of 7872-bp in size. This plasmid differed only by 5
135 nucleotides (including the G₄₇₅ to A mutation that resulted in D159N AA change in OXA-933)
136 from plasmid pMTY17816 expressing OXA-48 in an MDR *K. pneumoniae* clinical isolate
137 TUM17816 from Vietnam (AP019554) or from plasmid pIPCEC31_3 expressing OXA-48 in an
138 *E. coli* IPCEC31 isolate from Japan (AP026786), 19 nucleotides from plasmid p53_E-OXA48
139 expressing OXA-48 in an *E. coli* 53 isolate from Switzerland (CP048364.1), and 24 nucleotides
140 from plasmid pMTY17823_OXA48 isolated in *K. pneumoniae* TUM17823 from Japan
141 (AP019557). Electroporation of this plasmid into *E. coli* TOP10 yielded transformants resistant
142 to β -lactams and with reduced susceptibility to carbapenems (Table 1).

143 **Phenotypical characterization of OXA-933 β -lactamase and comparison with *in vitro***
144 **generated point mutant derivatives**

145 MICs, Carba NP test and immunochromatographic assays were performed on *E. coli*
146 TOP10 harboring pTOPO-OXA-48, pTOPO-OXA-933 (D159N), and pTOPO-OXA-48-D159-
147 A,-G,-K, and -W (Table 1). All the OXA-48 variants exhibited lower MICs values for penicillins
148 and carbapenems as compared to OXA-48. In contrast, no change of MIC values for ceftazidime
149 and cefotaxime was observed between OXA-48, OXA-933 and the four mutants. CarbaNP test
150 revealed an inconsistent and weak positive result with the clinical strain but was negative for all
151 the mutants except for D159G suggesting that OXA933 and OXA-48-D159G exhibited a weak
152 imipenem hydrolysis. Finally, NG-test Carba5, an immunochromatographic assay used for
153 detection of the most prevalent carbapenemases such as OXA-48, was positive for the clinical *K.*
154 *pneumoniae* 249B1 strain, and for *E. coli* harboring pTOPO-OXA-48, pTOPO-OXA-933
155 (D159N), pTOPO-OXA-48-D159-A,-G, and -K, but not for pTOPO-OXA-48-D159W. Together,
156 the lack of carbapenem-hydrolyzing activity and the absence of detection with specific

157 antibodies suggest that the aspartate replacement with the bulky and hydrophobic tryptophan
158 induced a significant modification of the 3D structure of the enzyme.

159 MICs assays were also performed in an OmpC/OmpF porin-deficient *E. coli* HB4 strain
160 harboring the natural pOXA-933, and pTOPO-OXA-933 (Table 1). With the natural plasmid
161 pOXA-933, the *E. coli* HB4 was resistant to all β -lactams tested, while *E. coli* HB4 pTOPO-
162 OXA-933, that contains only the open reading frame of OXA-933, only reduced susceptibilities
163 are observed, as transcription and translation are in suboptimal configuration.

164

165 **Biochemical properties of OXA-933**

166 Steady-state kinetic parameters were determined to compare the catalytic properties of
167 OXA-933 and two in vitro generated mutants (OXA-48-D159G and OXA-48-D159K) with those
168 of OXA-48 and OXA-244 (Table 3).

169 The catalytic efficiency (k_{cat}/K_m) of OXA-933 regarding ampicillin hydrolysis was 5.8-
170 fold lower ($0.413 \text{ mM}^{-1} \text{ s}^{-1}$) than that of OXA-48 ($2.400 \text{ mM}^{-1} \text{ s}^{-1}$) (Table 3), comparable to
171 OXA-244 ($0.549 \text{ mM}^{-1} \text{ s}^{-1}$). In addition, hydrolysis of temocillin by OXA-933 revealed a K_m
172 and a k_{cat} 3-fold higher and 4-fold higher, respectively, thus resulting in a 2-fold higher catalytic
173 efficiency as compared with OXA-48. This result is very different from that of OXA-244, where
174 a 20-fold decrease was observed (9). Cefalotin is hydrolyzed with a 20-fold lower k_{cat}/K_m as
175 compared with the value for OXA-48. Oxyimino cephalosporins, such as cefotaxime, are weakly
176 hydrolyzed, and the k_{cat}/K_m was comparable with that of OXA-48. For ceftazidime, a bulkier
177 oxyimino cephalosporin, no hydrolysis was observed even with $1.92 \mu\text{M}$ purified enzyme and up
178 to $500 \mu\text{M}$ substrate (data not shown), as shown previously for OXA-48 (4,7).

179 Carbapenems were among the preferred OXA-48 substrates (7), with the highest catalytic
180 efficiency (k_{cat}/K_m), observed for the hydrolysis of imipenem ($0.37 \cdot 10^3 \text{ mM}^{-1}\text{s}^{-1}$) (7). OXA-933
181 has k_{cat}/K_m values 3-fold, 20-fold and 4-fold lower for imipenem, meropenem and ertapenem
182 hydrolysis, respectively, as compared with OXA-48. These values are similar to those observed
183 for OXA-244, with k_{cat}/K_m values 6-fold, 14-fold, and 10-fold lower for imipenem, meropenem
184 and ertapenem hydrolysis, respectively, as compared with OXA-48.

185 These reduced catalytic efficiencies for carbapenems in OXA-933 were mainly due to a
186 reduction in the turnover number. Interestingly, as observed for OXA-232, the k_{cat}/K_m of
187 ertapenem for OXA-933 was 480-fold lower than that of imipenem, but only slightly affected as
188 compared with the value for OXA-48 (1 versus $0.26 \text{ mM}^{-1} \text{ s}^{-1}$). Overall, the catalytic
189 efficiencies of OXA-48-D159G and OXA-48-D159K are similar to those of OXA-933.

190 Determination of IC₅₀s showed that OXA-933 and OXA-48 were similarly inhibited by
191 clavulanic acid ($4.55 \mu\text{M}$ and $28.5 \mu\text{M}$, respectively), tazobactam ($4.23 \mu\text{M}$ and $20 \mu\text{M}$,
192 respectively) and avibactam ($6 \mu\text{M}$ and $2 \mu\text{M}$, respectively).

193
194 **Molecular modelling.** An *in-silico* study was performed to identify the structural
195 determinants that could explain the experimentally determined differences between the
196 hydrolytic profiles of OXA-933 and its variants in comparison with OXA-48. The three-
197 dimensional structures of OXA-48, OXA-244 (i.e. OXA-48-R214G), OXA-933 (i.e. OXA-48-
198 D159N), OXA-48-D159G and OXA-48-D159K were modelled, showing different
199 conformations of key residues of the active site, and compared with the docking conformation of
200 temocillin in the active site of OXA-48 (Figures 2 and S1). In all complexes, temocillin
201 establishes hydrogen bonds with the backbone of Y211 and with the side chain of T209, and a

202 strong ionic bridge with R250. In the OXA-48 structure, R214 interacts with temocillin through a
203 strong ionic interaction and with D159 through a hydrogen bond. A hydrogen bond is also
204 observed between D159 and S155 (Figure 2a). The mutation R214G in OXA-244 compared with
205 OXA-48 induces a destabilization of the interaction with temocillin, which shows a 8-fold lower
206 affinity (Figure 2b). In OXA-933, the OD1 atom of N159 is already engaged in the hydrogen
207 bond with S155 and no hydrogen bond is possible between the ND2 atom of N159 and the side
208 chain of R214. Therefore, R214 would change the conformation to make simultaneously a
209 hydrogen bond with T213 and an ionic bridge with temocillin (Figure 2c). These interactions
210 restore almost completely the affinity of the protein for temocillin. In the absence of possible
211 interactions between R214 and the residue in position 159, the same conformational change is
212 also observed for the OXA-48-D159G and OXA-48-D159K mutants (Figure 2d,e), with similar
213 effects on the affinity for temocillin. All these results are in perfect agreement with the
214 experimental data (Table 3).

215 ***Discussion***

216 Since the first description of an Enterobacteriales producing OXA-48 (4) in 2004, a rapid
217 dissemination has been observed, and OXA-48 producers became endemic in many countries
218 worldwide (6). OXA-48 variants such as OXA-232, OXA-244, OXA-484 with changes at
219 position R214 located in the β 5- β 6 loop display reduced hydrolytic activity especially of
220 carbapenems and temocillin as compared to OXA-48 (7,9,13,15,16,18). This reduction is related
221 to the absence of a salt bridge between R214 and D159 that stabilizes the shape of the active site
222 in OXA-48 (13,15).

223 *K pneumoniae* isolate 241B9 expresses a D159N OXA-48 variant that gave an
224 inconsistently positive Carba NP test results, suggesting imipenem-hydrolysis at the limit of

225 detection of the assay. These observations were in favor of the hypothesis that the D159 plays
226 also a major role in the hydrolytic profile of OXA-48 by establishing a salt bridge with R214 to
227 stabilize the active site for carbapenem-hydrolysis (13). To validate this hypothesis, we
228 generated mono-mutants of OXA-48 (D159A, D159G, D159K and D159W) and analyzed MICs
229 for β -lactams and kinetic parameters for OXA-933, and for OXA-48-D159G and D159K. These
230 mutations were chosen regarding their intrinsic properties: alanine and glycine as hydrophobic
231 aliphatic small AA, tryptophan a bulky hydrophobic aromatic AA and lysine a polar positively
232 charged AA. Thus, in comparison with OXA-48 we observed that substitution of the D159 led to
233 an increase of susceptibility toward all the β -lactams. The D159W substitution has almost
234 completely inactivated the β -lactamase (data not shown).

235 CarbaNP test revealing carbapenemase activity was evaluated on several *E. coli* TOP10
236 isolates expressing OXA-48, OXA-244, OXA-933 and point mutant derivatives of position D159
237 from pTOPO plasmids. Except for pTOPO-OXA-48 and TOP10-pTOPO-OXA-48-D159G, the
238 Carba NP test remained repeatedly negative suggesting carbapenem-hydrolytic activity below
239 the detection limit of the assay. All these OXA-48-variants were sufficiently expressed, as the
240 lateral flow immunoassay NG-Test Carba5 yielded positive signals in less than 2 minutes, except
241 for TOP10-pTOPO-OXA-48-D159W, suggesting that replacement with this bulky AA has
242 substantially modified the structure of the protein, at least the part that is recognized by one of
243 the antibodies included in the test.

244 The clinical *K. pneumoniae* 24B1 strain, *E. coli* Top10 pOXA-933 and *E. coli* Top10
245 pTOPO-OXA-933 had different MIC values, likely due to species specific expression and
246 impermeability problems, but all had reduced MICs as compared to OXA-48. These reductions
247 were comparable to OXA-244 (Table 1). The MICs of the various mutants were similar to those

248 of OXA-933, confirming a loss of ability to hydrolyze carbapenems (Table 1). Even though
249 temocillin MICs were much lower for OXA-933 and the generated mutants as compared to
250 OXA-48, these results could not be confirmed through steady state kinetics determination.
251 Similar discrepancies between MIC for temocillin and its hydrolysis have already been
252 observed for other OXA-48-like variants (7,9,13).

253 Differences in catalytic efficiencies were due to changes in both K_m and k_{cat} . The steady-
254 state kinetics of OXA-933, which differs by D159N from OXA-48, revealed that the OXA-933
255 active site can still accommodate carbapenem and temocillin substrates, but with decreased
256 catalytic efficiencies as compared with OXA-48. Kinetic parameters of the OXA-48-D159G and
257 D159K variants show overall decreased catalytic efficiencies (k_{cat}/K_m) for ampicillin, cephalotin,
258 imipenem and meropenem, similar to OXA-933. The most significant difference was observed
259 for meropenem, with a decrease of 15 to 20-fold. This result is a consequence of at least a 10-
260 fold decrease of affinity. Regarding imipenem, a weak decrease of catalytic efficiency was
261 noticed mostly due to a diminution of the turnover (k_{cat}). Thus, these results tend to show the
262 importance of residue D159 for the hydrolysis of carbapenems. We have shown previously
263 (9,18), that R214 could interact also with the substrate and D159. This interaction could stabilize
264 R214 in the right position and thus stabilize the small substrates as carbapenems in the active
265 site. S155 would also be a residue of a particular interest since it directly interacts with D159 and
266 could be the target for further studies. Overall, OXA-933 and OXA-244, even though differing
267 by the AA changes (R214G for OXA-244), have similar catalytic efficiency values (Table 3),
268 further supporting the importance of the salt bridge established between these two residues. The
269 IC50s values for clavulanic acid, tazobactam and avibactam of OXA-933, being similar to those
270 of OXA-48, suggest that D159N is not involved in inhibition by the tested inhibitors (7,10, 25).

271 The structural analysis of the active sites of these mutants (Figure S1) and their
272 interaction with temocillin (Figure 2) show important changes in the conformation and in the size
273 of the active site induced by these mutations. In OXA-244, the active site is enlarged as a
274 consequence of the mutation R214G and the flexibility of this region is likely to be increased in
275 the absence of the salt bridge R214-D159. This mutation also destabilizes the interaction of
276 temocillin, leading to a significant decrease of affinity. In OXA-933, although the mutation
277 D159N looks minor at the first sight, it also breaks the salt bridge R214-D159, allowing the
278 R214 to change the conformation and to interact with T213 through a hydrogen bond and with
279 temocillin through an ionic bridge. The conformational change and interactions are also observed
280 in the OXA-48-D159G and OXA-48-D159K mutants, leading to an important remodeling of the
281 active site surface in this region and to the reinforcement of the interaction with temocillin.
282 Overall, these changes in the conformation of the active site and in the interaction with
283 temocillin are in agreement with the kinetic data determined experimentally (Table 3).

284 Most OXA-48-like variants are associated with different *Tn1999* variants inserted on
285 IncL plasmids, but some such as OXA-181 and OXA-232 are associated with *ISEcp1*,
286 *Tn2013* on ColE2, and IncX3 types of plasmids (6,26). The pOXA-933 natural plasmid of 7872-
287 bp in size was closely related (5-24 nucleotide differences) to plasmids carrying OXA-48 from
288 an *K pneumoniae* clinical isolate TUM17816 from Vietnam (AP019554), an *E. coli* IPCEC31
289 isolate from Japan (AP026786), an *E. coli* 53 isolate from Switzerland (CP048364), and *K.*
290 *pneumoniae* TUM17823 from Japan (AP019557), suggesting that this type of plasmid may have
291 already spread on several continents and in different species. *IS1*-based composite transposons
292 have the tendency to transpose at high frequency in Enterobacterales and could thus promote
293 even further the spread of OXA-48 type enzymes, even beyond Enterobacterales, in a similar

294 manner to Tn9 or Tn1681, two highly dispersed composite transposons carrying the *cat* gene,
295 conferring chloramphenicol resistance and a heat stable toxin, respectively (27,28).

296 ***Conclusions***

297 This study characterized a new variant of OXA-48, OXA-933 with a D159N amino acid
298 change located outside of the β 5- β 6 loop but involved in active site stabilization through salt
299 bridges with R214 and S155. According to Dabos *et al.* OXA-933, even though with reduced
300 activity towards carbapenems, has still enough carbapenemase activity to be considered as a true
301 carbapenemase, especially when expressed in bacteria with impaired outer membrane
302 permeability (30). Nevertheless, this reduction in hydrolytic activities, especially for
303 carbapenems and temocillin, results in detection problems using hydrolysis tests, such as Carba
304 NP, and growth on selective medias, such as ChromID CarbaSmart biplates (bioMérieux). The
305 use LFIA, such as NG-Test CARBA 5 may be an interesting tool to detect these OXA-48-like
306 variants. The detection from rectal swab samples may still be possible using chromogenic
307 selective media for *K. pneumoniae* isolates, but the situation may be different with *E. coli*
308 isolates, as observed with OXA-244-producers (9, 31). Even though OXA-933 hydrolyses
309 carbapenems only weakly, its transfer into a porin-deficient genetic background may yield high
310 level of carbapenem-resistance (Table 4). Finally, OXA-933 is present on a novel composite
311 transposon made of two *ISI* elements, Tn2208, inserted on a high copy number plasmid further
312 illustrating the genetic variability associated with OXA-48 carbapenemases.

313

314 ***Materials and methods***

315 ***Bacterial strains***

316 *Klebsiella pneumoniae* 241B9 clinical isolate was identified using MALDI-TOF (MALDI
317 Biotyper, Bruker Daltonics, Hamburg, Germany). *Escherichia coli* TOP10 (Invitrogen, Saint-
318 Aubin, France) and *E. coli* BL21 (DE3) (Novagen, VWR International, Fontenay-sous-Bois,
319 France) were used for cloning and over expression experiments, respectively (13).

320 ***Susceptibility testing and carbapenemase detection***

321 Antimicrobial susceptibilities were determined by disk diffusion technique on Mueller-Hinton
322 agar (Bio-Rad, Marnes-La-Coquette, France) and Minimal inhibitory concentration (MIC) values
323 were determined using the Etest technique (BioMérieux, Paris, France). Susceptibility results
324 were interpreted according to the EUCAST breakpoints, updated in 2022
325 (<http://www.eucast.org>). Detection of a carbapenemase activity were carried out with the Carba
326 NP test as previously described (19). The Lateral flow immunoassay NG-Test[®] CARBA-5 (NG
327 Biotech, Guipry, France) was used to detect members of the five main carbapenemases families
328 (i.e., KPC-, NDM-, VIM-, IMP-, and OXA-48-like enzymes) according to the manufacturer's
329 instructions (20).

330 **Whole genome sequencing**

331 Total DNA was extracted from colonies using the Ultraclean Microbial DNA Isolation
332 Kit (MO BIO Laboratories, Carlsbad, CA, US) following the manufacturer's instructions. The
333 DNA concentration and purity were controlled by a Qubit[®] 2.0 Fluorometer using the dsDNA
334 HS and/or BR assay kit (Life technologies, Carlsbad, CA, US). The library was prepared using
335 the NEBNext Ultra II FS DNA Library Prep Kit for Illumina (NEB, France) according to the
336 manufacturer's instructions. Whole genome sequencing was performed on an Illumina NextSeq
337 500 instrument (Illumina). De novo assembly was performed by CLC genomics 10.2 program
338 (Qiagen, Les Ulis, France), and the genomes were analyzed online using software available at

339 the Center for Genomic Epidemiology-CGE (<https://cge.food.dtu.dk/services/ResFinder/>). The
340 acquired antimicrobial resistance genes were identified by uploading the assembled genomes to
341 the Resfinder server v2.1 (<http://cge.cbs.dtu.dk/services/ResFinder-2.1>) (22). The Multi Locus
342 Sequence Typing (MLST) and the identification of the different plasmids were also obtained by
343 uploading the assembled genomes to the MLST 1.8 (21) and PlasmidFinder 1.3 (24) servers,
344 respectively, available at <https://cge.cbs.dtu.dk/services/>.

345 **Plasmid characterization**

346 Plasmid DNA of the clinical isolate *K. pneumoniae* 241B9 was extracted using the Kieser
347 method (32) and electroporated into *E. coli* TOP10 strain by electroporation (GenePulser,
348 Biorad, Marne-La-Coquette, France) as previously described (13). Plasmids of parental *K.*
349 *pneumoniae* 241B9 isolate and transformants were analyzed on 0.7% agarose gel stained with
350 ethidium bromide. Plasmids of ca. 154, 66, 48, and 7 kb of *E. coli* NCTC 50192 were used as
351 plasmid size markers (13).

352 **Site directed mutagenesis**

353 Site-directed mutagenesis was done using the QuikChange II site-directed mutagenesis kit
354 (Agilent Technologies, Les Ulis, France) as recommended the manufacturer's protocol, and the
355 pTOPO-*bla*_{OXA-48} plasmid as a template (18). Specific primers used for the different mutations,
356 were designed using the QuikChange Primer Design software (Agilent Technologies) (Table
357 S1): Plasmids pTOPO-OXA-48-D159G, pTOPO-OXA-48-D159A, pTOPO-OXA-48-D159K
358 and pTOPO-OXA-48-D159W were then electroporated into *E. coli* TOP10 and the presence of
359 the mutation was confirmed by Sanger sequencing using an ABI Prism 3100 sequencer (Applied
360 Biosystems, Les Ulis, France) as previously described (7).

361 **PCR and cloning experiments**

362 Total DNAs of *K. pneumoniae* 241B9 isolate extracted using the QIAamp DNA mini kit
363 (Qiagen, Courtaboeuf, France) was used as templates for PCR amplification of *bla*_{OXA-933} gene
364 using the Phusion High-Fidelity DNA Polymerase (ThermoFischer Scientific, Villebon-sur-
365 Yvette, France) and OXA-48A and OXA-48B primers (Table S1). The amplicon obtained was
366 then cloned into the pCR®-Blunt II-TOPO® plasmid (Invitrogen, Illkirch, France), downstream
367 of the pLac promoter, in the same orientation, resulting in pOXA-933 as previously described
368 (18). The *bla*_{OXA-933}, *bla*_{OXA-48-D159G}, and *bla*_{OXA-48-D159K} gene fragments were amplified using
369 primers INF-48 F and INF-48 R (Table S1) corresponding to the mature β-lactamase and cloned
370 into the expression vector pET41b (+) (Novagen, VWR International, Fontenay-sous-Bois,
371 France) as previously described (18). The recombinant plasmid pET41-OXA-933, pET41-OXA-
372 48-D159G, and pET41-OXA-48-D159K were then transformed into the chemically competent *E.*
373 *coli* strain BL21 (DE3) (Novagen).

374 Recombinant plasmids were verified by Sanger on an automated ABI Prism 3100 sequencer
375 (Applied Biosystems, Les Ulis, France) as previously described (18). Nucleotide sequences were
376 analyzed using software available at the National Center for Biotechnology Information website
377 (<https://www.ncbi.nlm.nih.gov>).

378 **β-Lactamase production and purification**

379 Overnight cultures of *E. coli* BL21(DE3) harboring recombinant plasmid pET41b-OXA-
380 933, pET41b-OXA-48-D159G and pET41b-OXA-48-D159K were used to inoculate 2 liters of
381 LB medium broth containing 50 µg/ml of kanamycin. Bacteria were grown at 37 °C until OD₆₀₀
382 of 0.6 is reached and expression of the OXA-48 mutants was induced overnight at 25 °C with 0.2
383 mM IPTG. Purification of OXA-933, OXA-48-D159G and OXA-48-D159K was done as
384 previously described (7,13). Protein purity was estimated by SDS-PAGE, pure fractions were

385 pooled and dialyzed against 100 mM sodium phosphate, 50 mM K₂SO₄ (pH 7.0) and
386 concentrated using Vivaspin® 10 kDa columns (GE Healthcare, Freiburg, Germany). Protein
387 concentration was determined by Bradford Protein assay (Bio-Rad) (13).

388 **Steady-state kinetic parameters**

389 The k_{cat} and K_{m} values of purified OXA-933, OXA-48-D159K and OXA-48-D159G were
390 determined at 30°C in 100 mM sodium phosphate buffer (pH 7.0) as previously described (7,13).
391 The β -lactams were purchased from Sigma–Aldrich (Saint-Quentin-Fallavier, France).
392 Comparative activation of OXA-933 by CO₂ was conducted by monitoring the rate of hydrolysis
393 of penicillins in the same buffer supplemented with 50 mM sodium hydrogen carbonate
394 (NaHCO₃) as the source of CO₂ (7,9). OXA-48 and OXA-244 kinetic parameters, used in the
395 comparison, were previously determined by Docquier et al. and Rima et al., respectively (9,15).

396 **Molecular modelling**

397 UCSF Chimera package (33) was used for structural analysis and image plotting. The three-
398 dimensional structures were generated by homology modeling using MODELLER version 10.3
399 (34) and the structure 6PQI (35) as template, or directly from sequence using AlphaFold (36),
400 through a local implementation of ColabFold (37). Three-dimensional structures of the β -lactam
401 ligands were generated using the Corina (version 3.60) program (Molecular Networks GmbH,
402 Erlangen, Germany). Molecular docking calculations were performed using the Gold program
403 (Cambridge Crystallographic Data Centre, Cambridge, UK) (38) and the GoldScore scoring
404 function. The binding site, defined as a 20-Å-radius sphere, was centred on the OG oxygen atom
405 of Ser70. All other parameters had default values.

406 **Nucleotide sequence accession numbers**

407 The nucleotide sequence of the *bla*_{OXA-933} gene and pOXA-933 have been submitted to the
408 EMBL/Genbank nucleotide sequence database under the accession number MW073108 and
409 OQ915017, respectively. The Whole Genome Sequencing data of *K. pneumoniae* 241B9 has
410 been deposited at the DDBJ/ENA/GenBank under the accession code JARYTJ000000000.

411

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417 **Conflicts of interest**

418 None to disclose.

419 **References**

- 420 1. Bonomo RA, Burd EM, Conly J, Limbago BM, Poirel L, Segre JA, Westblade LF. 2018.
421 Carbapenemase-Producing Organisms: A Global Scourge. *Clin Infect Dis* 66:1290-1297.
- 422 2. Hammoudi Halat D, Ayoub Moubareck C. 2020. The Current Burden of Carbapenemases:
423 Review of Significant Properties and Dissemination among Gram-Negative Bacteria.
424 *Antibiotics* (Basel, Switzerland). 9:186
- 425 3. Queenan AM, Bush K. Carbapenemases: the versatile beta-lactamases. 2007. *Clin*
426 *Microbiol Rev.* 20: 440-58.
- 427 4. Poirel L, Héritier C, Tolün V, Nordmann P. 2004. Emergence of oxacillinase-mediated
428 resistance to imipenem in *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* 48: 15-
429 22.

- 430 5. Aubert D, Naas T, Héritier C, Poirel L, Nordmann P. 2006. Functional characterization of
431 IS1999, an IS4 family element involved in mobilization and expression of beta-lactam
432 resistance genes. *J Bacteriol* 188: 6506-14.
- 433 6. Pitout JDD, Peirano G, Kock MM, Strydom KA, Matsumura Y. 2019. The Global
434 Ascendency of OXA-48-Type Carbapenemases. *Clin Microbiol Rev* 33:e00102-19.
- 435 7. Oueslati S, Nordmann P, Poirel L. 2015. Heterogeneous hydrolytic features for OXA-48-
436 like β -lactamases. *J Antimicrob Chemother* 70: 1059-63.
- 437 8. Naas T, Oueslati S, Bonnin RA, Dabos ML., Zavala A, Dortet L, Retailleau P, Iorga BI.
438 2017. Beta-Lactamase DataBase (BLDB) - Structure and Function. *J Enz Inh Med Chem*
439 32:917-919.
- 440 9. Rima M, Emeraud C, Bonnin RA, Gonzalez C, Dortet L, Iorga BI, Oueslati S, Naas T.
441 2021. Biochemical characterization of OXA-244, an emerging OXA-48 variant with
442 reduced β -lactam hydrolytic activity. *J Antimicrob Chemother* 76:2024-2028.
- 443 10. Oueslati S, Retailleau P, Marchini L, Dortet L, Bonnin RA, Iorga BI, Naas T. 2019.
444 Biochemical and Structural Characterization of OXA-405, an OXA-48 Variant with
445 Extended-Spectrum β -Lactamase Activity. *Microorganisms* 8:24.
- 446 11. Poirel L, Castanheira M, Carrër A, Rodriguez CP, Jones RN, Smayevsky J, Nordmann P.
447 2011. OXA-163, an OXA-48-related class D β -lactamase with extended activity toward
448 expanded-spectrum cephalosporins. *Antimicrob Agents Chemother* 55: 2546-51.
- 449 12. De Belder D, Ghiglione B, Pasteran F, de Mendieta JM, Corso A, Curto L, Di Bella A,
450 Gutkind G, Gomez SA, Power P. 2020. Comparative Kinetic Analysis of OXA-438 with
451 Related OXA-48-Type Carbapenem-Hydrolyzing Class D β -Lactamases. *ACS Infect Dis*
452 6:3026-33.

- 453 13. Dabos L, Raczynska JE, Bogaerts P, Zavala A, Girlich D, Bonnin RA, Dortet L, Peyrat A,
454 Retailleau P, Iorga BI, Jaskólski M, Glupczynski Y, Naas T. 2023. Structural and
455 Biochemical Features of OXA-517: a Carbapenem and Expanded-Spectrum Cephalosporin
456 Hydrolyzing OXA-48 Variant. *Antimicrob Agents Chemother* 67:e0109522.
- 457 14. Dabos L, Bonnin RA, Dortet L, Naas T. 2021. OXA-793, a carbapenem, expanded-
458 spectrum cephalosporin and aztreonam hydrolysing OXA-48 variant. 2023. O2981 ePoster,
459 ECCMID Online.
- 460 15. Docquier JD, Calderone V, De Luca F, Benvenuti M, Giuliani F, Bellucci L, Tafi A,
461 Nordmann P, Botta M, Rossolini GM, Mangani S. 2009. Crystal structure of the OXA-48
462 beta-lactamase reveals mechanistic diversity among class D carbapenemases. *Chem Biol*
463 16:540-7.
- 464 16. Dabos L, Zavala A, Bonnin RA, Beckstein O, Retailleau P, Iorga BI, Naas T. 2020.
465 Substrate Specificity of OXA-48 after β 5- β 6 Loop Replacement. *ACS Infect Dis* 6:1032-
466 43.
- 467 17. Smith CA, Stewart NK, Toth M, Vakulenko SB. 2019. Structural Insights into the
468 Mechanism of Carbapenemase Activity of the OXA-48 β -Lactamase. *Antimicrob Agents*
469 *Chemother* 63:e01202-19.
- 470 18. Oueslati S, Retailleau P, Marchini L, Berthault C, Dortet L, Bonnin RA, Iorga BI, Naas T.
471 2020. Role of Arginine 214 in the Substrate Specificity of OXA-48. *Antimicrob Agents*
472 *Chemother* 64:e02329-19.
- 473 19. Dortet L, Agathine A, Naas T, Cuzon G, Poirel L, Nordmann P. 2015. Evaluation of the
474 RAPIDEC® CARBA NP, the Rapid CARB Screen® and the Carba NP test for

- 475 biochemical detection of carbapenemase-producing Enterobacteriaceae. *J Antimicrob*
476 *Chemother* 70:3014-22.
- 477 20. Boutal H, Vogel A, Bernabeu S, Devilliers K, Creton E, Cotellon G, Plaisance M, Oueslati
478 S, Dortet L, Jousset A, Simon S, Naas T, Volland H. 2018. A multiplex lateral flow
479 immunoassay for the rapid identification of NDM-, KPC-, IMP- and VIM-type and OXA-
480 48-like carbapenemase-producing Enterobacteriaceae. *J Antimicrob Chemother* 73: 909-
481 15.
- 482 21. Larsen MV, Cosentino S, Rasmussen S, Friis C, Hasman H, Marvig RL, Jelsbak L,
483 Sicheritz-Pontén T, Ussery DW, Aarestrup FM, Lund O. 2012. Multilocus sequence typing
484 of total-genome-sequenced bacteria. *J Clin Microbiol* 50: 1355-61.
- 485 22. Bortolaia V, Ruppé E, Roberts MC, Schwarz S, Cattoir V, Philippon A, Allesoe RL,
486 Rebelo AR, Florensa AR, Fagelhauer L, Chakraborty T, Neumann B, Werner G, Bender
487 JK, Stingl K, Nguyen M, Coppens J, Xavier BB, Malhotra-Kumar S, Westh H, Pinholt M,
488 Anjum MF, Duggett NA, Kempf I, Nykjaer S, Oikola S, Wiczorek K, Amaro A,
489 Clemente L, Mossong J, Losch S, Ragimbeau C, Lund O, Aarestrup FM. 2020. Resfinder
490 4.0 for predictions of phenotypes from genotypes. *J Antimicrob Chemother* 75: 3491-500.
- 491 23. Ito R, Mustapha MM, Tomich AD, Callaghan JD, McElheny CL, Mettus RT, Shanks
492 RMQ, Sluis-Cremer N, Doi Y. 2017. Widespread Fosfomycin Resistance in Gram-
493 Negative Bacteria Attributable to the Chromosomal fosA Gene. *mBio* 8:e00749-17.
- 494 24. Carattoli A, Zankari E, García-Fernández A, Voldby Larsen M, Lund O, Villa L, Møller
495 Aarestrup F, Hasman H. 2014. In silico detection and typing of plasmids using
496 PlasmidFinder and plasmid multilocus sequence typing. *Antimicrob Agents Chemother*
497 58:3895-903.

- 498 25. Fröhlich C, Sørum V, Thomassen AM, Johnsen PJ, Leiros HKS, Samuelsen O. 2019.
499 OXA-48-Mediated Ceftazidime-Avibactam Resistance Is Associated with Evolutionary
500 Trade-Offs. *MSphere*; 4: e00024-19.
- 501 26. Poirel L, Bonnin RA, Nordmann P. 2012. Genetic features of the widespread plasmid
502 coding for the carbapenemase OXA-48. *Antimicrob Agents Chemother* 56: 559-62.
- 503 27. Matthews PR, Cameron FH, Stewart PR. 1983. Occurrence of chloramphenicol
504 acetyltransferase and Tn9 among chloramphenicol-resistant enteric bacteria from humans
505 and animals. *J Antimicrob Chemother* 11:535-42.
- 506 28. Zsolt Fekete P, Schneider G, Olasz F, Blum-Oehler G, Hacker JH, Nagy B. 2003.
507 Detection of a plasmid-encoded pathogenicity island in F18+ enterotoxigenic and
508 verotoxigenic *Escherichia coli* from weaned pigs. *Int J Med Microbiol* 293:287-98.
- 509 29. Dabos L, Bogaerts P, Bonnin RA, Zavala A, Sacré P, Iorga BI, Huang DT, Glupczynski Y,
510 Naas T. 2018. Genetic and Biochemical Characterization of OXA-519, a Novel OXA-48-
511 Like β -Lactamase. *Antimicrob Agents Chemother* 62:e00469-18.
- 512 30. Dabos L, Oueslati S, Bernabeu S, Bonnin RA, Dortet L, Naas T. 2022. To Be or Not to Be
513 an OXA-48 Carbapenemase. *Microorganisms* 10:25829.
- 514 31. Emeraud C, Biez L, Girlich D, Jousset AB, Naas T, Bonnin RA, Dortet L. 2020. Screening
515 of OXA-244 producers, a difficult-to-detect and emerging OXA-48 variant? *J Antimicrob*
516 *Chemother* 75: 2120-2123.29.
- 517 32. Kieser T. 1984. Factors affecting the isolation of CCC DNA from *Streptomyces lividans*
518 and *Escherichia coli*. *Plasmid* 12:19–36.

- 519 33. Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, Ferrin TE.
520 2004. UCSF Chimera--a visualization system for exploratory research and analysis. *J*
521 *Comp Chem* 25: 1605-12.
- 522 34. Verdonk ML, Cole JC, Hartshorn MJ, Murray CW, Taylor RD. 2003. Improved protein-
523 ligand docking using GOLD. *Proteins*. 52: 609-23.
- 524 35. Sali A, Blundell TL. 1993. Comparative protein modelling by satisfaction of spatial
525 restraints. *J Mol Biol* 234: 779-815.
- 526 36. Jumper J, Evans R, Pritzel A, Green T, Figurnov M, Ronneberger O, Tunyasuvunakool K,
527 Bates R, Žídek A, Potapenko A, Bridgland A, Meyer C, Kohl SAA, Ballard AJ, Cowie A,
528 Romera-Paredes B, Nikolov S, Jain R, Adler J, Back T, Petersen S, Reiman D, Clancy E,
529 Zielinski M, Steinegger M, Pacholska M, Berghammer T, Bodenstein S, Silver D, Vinyals
530 O, Senior AW, Kavukcuoglu K, Kohli P, Hassabis D. 2021. Highly accurate protein
531 structure prediction with AlphaFold. *Nature*. 596: 583-9.
- 532 37. Mirdita M, Schütze K, Moriwaki Y, Heo L, Ovchinnikov S, Steinegger M. 2022.
533 ColabFold: making protein folding accessible to all. *Nat Meth* 19: 679-82.
- 534 38. Verdonk ML, Cole JC, Hartshorn MJ, Murray CW, Taylor RD. 2003. Improved protein-
535 ligand docking using GOLD. *Proteins Struct Funct Bioinform* 52:609–623.

536

537 Table 1: CarbaNP assay, NG test Carba5 results and MICs of β -lactams for *K. pneumoniae* 241B9 clinical strain, *E. coli* HB4 with the
538 natural plasmid pOXA-933, with pTOPO-OXA-933, *E. coli* HB4 alone and *E. coli* TOP10 expressing OXA-48, OXA-933, OXA-48-
539 D159K, OXA-48-D159G, OXA-48-D159W and OXA-48-D159A. ^a; OXA-244; ^b; OXA-933.

	MIC ($\mu\text{g/mL}$)											
	<i>K. pneumoniae</i> 241B9	<i>E. coli</i> HB4			<i>E. coli</i> TOP10							
	OXA-933 SHV-1	pOXA- 933	pTOPO- OXA-933	-	pTOPO- OXA-48	pTOPO- OXA-48- R214G ^a	pTOPO- OXA48- D159N ^b	pTOPO- OXA-48- D159K	pTOPO- OXA-48- D159G	pTOPO- OXA-48- D159W	pTOPO- OXA-48- D159A	
Amoxicillin	>256	>256	>256	24	>256	>256	12	12	24	4	12	2
Piperacillin	128	>256	6	4		>256						
Ticarcillin/clavulanic acid	>256	>256	>256	12								
Temocillin	128				>256	16	12	12	12	12	4	4
Ceftazidime	<0.25	2	0.5	0.5	0.19	0.19	0.125	0.19	0.19	0.19	0.19	0.12
Cefotaxime	<0.25				0.06	0.047	0.06	0.06	0.06	0.06	0.06	0.06
Cefepime	0.094	8	0.5	0.5		0.064						
Imipenem	0.25	>32	0.16	0.12	0.75	0.25	0.25	0.25	0.25	0.25	0.25	0.25
				5								
Meropenem	0.016	32	0.25	0.25	0.25	0.016	0.032	0.016	0.023	0.016	0.016	0.016
Ertapenem	0.5	>256	32	1	0.25	0.064	0.003	0.003	0.003	0.003	0.003	0.003
CarbaNP test	+/-	-	-	-	+	+/-	-	-	+	-	-	-
NG-Test CARBA 5	OXA	OXA	OXA	-	OXA	OXA	OXA	OXA	OXA	-	OXA	-

540

541 Table 2: Steady-state kinetic parameters for hydrolysis of β -lactam substrates by OXA-933, OXA-D159G, OXA-D159K, OXA-244
 542 and OXA-48.

Enzyme	K_m (μM)					k_{cat} (s^{-1})					k_{cat}/K_m ($\text{mM}^{-1}\cdot\text{s}^{-1}$)				
	OXA-48 ^a	OXA-48 R214G ^b	OXA-48 D159N ^c	OXA-48 D159G	OXA-48 D159K	OXA-48	OXA-48 R214G ^a	OXA-48 D159N ^b	OXA-48 D159G	OXA-48 D159K	OXA-48	OXA-48 R214G ^a	OXA-48 D159N ^b	OXA-48 D159G	OXA-48 D159K
Benzylpenicillin	NA	450 ± 40	88 ± 9	64 ± 48	48 ± 5	NA	72 ± 8	126 ± 6	6.2 ± 4	69 ± 17	NA	163 \pm 30	1450 \pm 199	133 \pm 14	1445 \pm 256
Ampicillin	400 ± 160	657 ± 103	392 ± 103	ND	ND	955	373 ± 158	152 ± 32	ND	ND	2400	549 ± 130	413 ± 147	ND	ND
Temocillin	45 ± 20	364 ± 20	125 ± 9	86 ± 90	35 ± 6	0.3	0.11 ± 0.02	1.4 ± 0.03	0.74 ± 0.9	0.6 ± 0.03	6	0.31 ± 0.05	11 ± 0.8	7 \pm 2	19 ± 3.6
Cephalothin	195 ± 30	88 ± 30	788 ± 33	85 ± 19	152 ± 75	44	3.3 ± 0.8	7.4 ± 1.6	2.4 ± 0.4	3.8 ± 1.3	225	39 ± 4	10 ± 2.4	29 ± 7	29 ± 18
Cefotaxime	>900	>1000	321 ± 72	248 ± 155	104 ± 24	>900	ND	11 ± 8	1.2 ± 0.34	0.9 ± 0.2	10	ND	32 ± 21	6 ± 3	9 ± 1.4
Imipenem	13 ± 0.2	0.5 ± 0.2	22 ± 7	48 ± 9	5 ± 0.4	5	0.02 ± 0.001	1.8 ± 1.6	2.5 ± 0.4	0.6 ± 0.12	370	38 ± 10	125 ± 35	52 ± 1.5	120 ± 16
Meropenem	10 ± 4	23 ± 4	160 ± 46	193 ± 104	103 ± 10	0.07	0.02 ± 0.001	0.05 ± 0.002	0.08 ± 0.03	0.04 ± 0.002	6	0.8 ± 0.2	0.3 ± 0.09	0.4 ± 0.1	0.4 ± 0.02
Ertapenem	100 ± 0.3	22.9 ± 0.3	200 ± 67	154 ± 110	150 ± 28	0.13	0.02 ± 0.01	0.05 ± 0.007	0.074 ± 0.02	0.11 ± 0.06	1	0.1 ± 0.03	0.26 ± 0.06	0.6 ± 0.2	0.75 ± 0.4

543 ^a OXA-48 values were retrieved from Docquier *et al.* (15); ^b data for OXA-244 were from Rima *et al.* (9); ^c correspond to OXA-933;
 544 ^d: NA, not available; ^e: ND, not determined. Data are the mean of three independent experiments.
 545

546 **Legends to figures**

547 Figure 1: Map of the natural plasmid pOXA-933 carrying *bla*_{OXA-933} gene located on Tn2208.

548

549 Figure 2: Docking conformation of temocillin (non-covalent binding) on OXA-48 (**a**), OXA-244

550 (**b**), OXA-933 (**c**), OXA-48-D159G (**d**) and OXA-48-D159K (**e**). The residue Ser70 is colored in

551 green. The black strings represent hydrogen bonds.

552

553

554

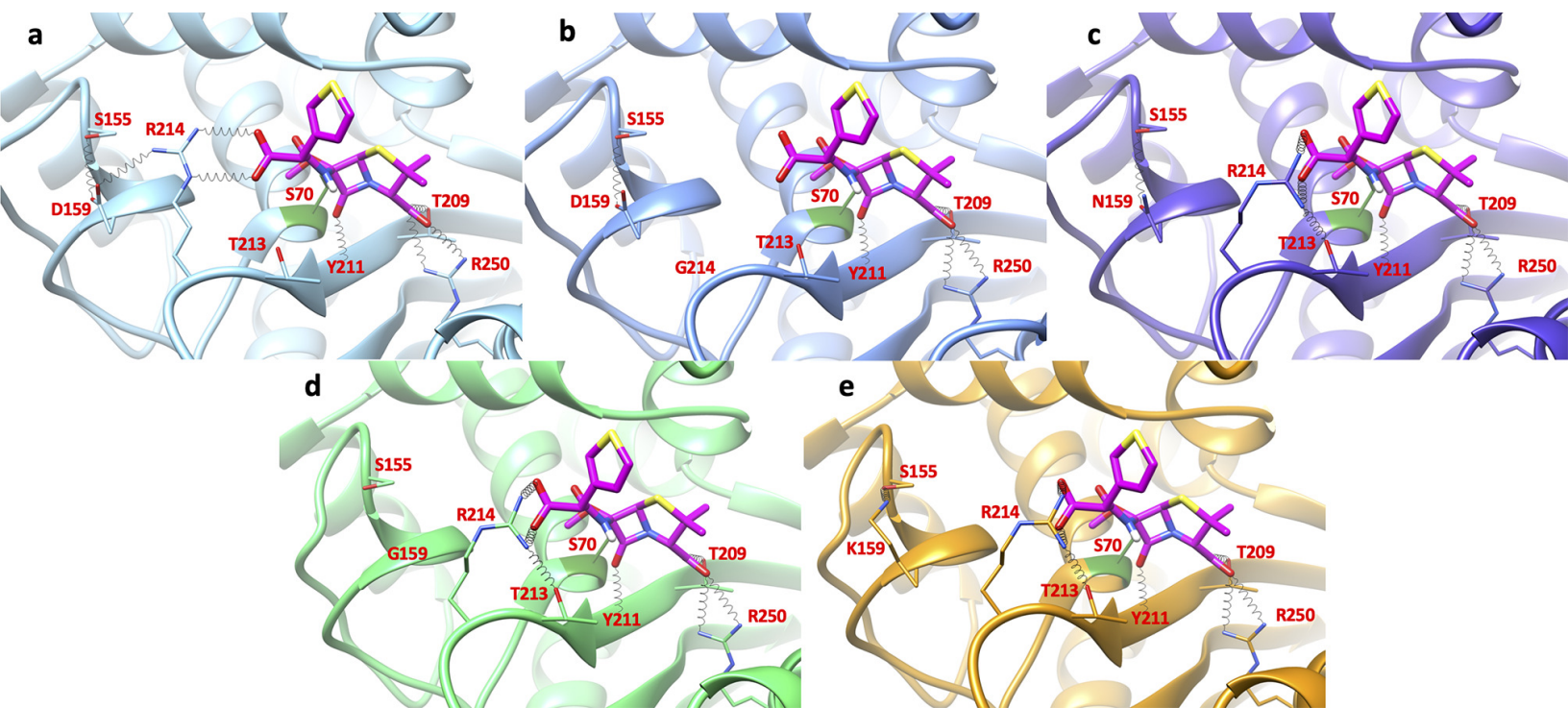


Table S1 : Primers used in this study
for site directed mutagenesis

D159Aa	5'- CGAAATTCGAATACCACCGGCGAGCCAGAAACTGTCTACA-3'
D159Ab	5'- TGTAGACAGTTTCTGGCTCGCCGGTGGTATTTCGAATTTTCG-3'
D159Ka	5'- GAAATTCGAATACCACCCTTGAGCCAGAAACTGTCTA-3'
D159Kb	5'- TAGACAGTTTCTGGCTCAAGGGTGGTATTTCGAATTTTC-3'
D159Wa	5'- GGCCGAAATTCGAATACCACCCAGAGCCAGAAACTGTCTACATT-3'
D159Wb	5'- AATGTAGACAGTTTCTGGCTCTGGGGTGGTATTTCGAATTTTCGGCC-3'
D159Ga	5'- CGAAATTCGAATACCACCGCCGAGCCAGAAACTGTCTACA-3'
D159Gb	5'- TGTAGACAGTTTCTGGCTCGGCGGTGGTATTTCGAATTTTCG-3'

For OXA-48 cloning into pTOPO

OXA-48A	5'-TTGGTGGCATCGATTATCGG-3'
OXA-48B	5'-GAGCACTTCTTTTGTGATGGC-3'

For OXA-48-cloning in pET41b

INF-48 F	5'-AAGGAGATATACATATGGTAGCAAAGGAATGGCAAG-3'
INF-48 R	5'-GGTGGTGGTGCTCGAAGGGAATAATTTTTCTGTTTGAG-3'

Legend to Figure S1: Docking conformation of temocillin (non-covalent binding) on OXA-48 (a), OXA-244 (b), OXA-933 (c), OXA-48-D159G (d) and OXA-48-D159K (e), in surface representation. The surface of residues in positions 155, 159, 213 and 214 is partially transparent to show the conformation of the corresponding residues, represented as sticks. The surface of Ser70 is colored in green. The black strings represent hydrogen bonds.

