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Variability in carbapenemase activity of intrinsic OxaAb (OXA-51-like) beta-lactamase enzymes in *Acinetobacter baumannii*

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1 **Variability in carbapenemase activity of intrinsic OxaAb (OXA-51-like) beta-lactamase**

2 **enzymes in *Acinetobacter baumannii***

3

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17 **Running heading: Carbapenemase activity of intrinsic OxaAb**

ABSTRACT

Objectives

This study aimed to measure the variability in carbapenem susceptibility conferred by different OxaAb variants, characterise the molecular evolution of *oxaAb* and elucidate the contribution of OxaAb and other possible carbapenem resistance factors in the clinical isolates using WGS and LC-MS/MS.

Methods

Antimicrobial susceptibility tests were performed on ten clinical *A. baumannii* isolates. Carbapenem MICs were evaluated for all *oxaAb* variants cloned into *A. baumannii* CIP70.10 and BM4547, with and without their natural promoters. Molecular evolution analysis of the *oxaAb* variants was performed using FastTree and SplitsTree4. Resistance determinants were studied in the clinical isolates using WGS and LC-MS/MS.

Results

Only the OxaAb variants with I129L and L167V substitutions, OxaAb(82), OxaAb(83), OxaAb(107), and OxaAb(110) increased carbapenem MICs when expressed in susceptible *A. baumannii* backgrounds without an upstream IS element. Carbapenem resistance was conferred with the addition of their natural upstream IS*Aba1* promoter. LC-MS/MS analysis on the original clinical isolates confirmed overexpression of the four I129L and L167V variants. No other differences in expression levels of proteins commonly associated with carbapenem resistance were detected.

38 **Conclusions**

39 Elevated carbapenem MICs were observed by expression of OxaAb variants carrying clinically
40 prevalent substitutions I129L and L167V. To drive carbapenem resistance, these variants
41 required overexpression by their upstream IS*Aba1* promoter. This study clearly demonstrates
42 that a combination of IS-driven overexpression of *oxaAb* and the presence of particular amino
43 acid substitutions in the active site to improve carbapenem capture is key in conferring
44 carbapenem resistance in *A. baumannii* and other mechanisms are not required.

46 **INTRODUCTION**

47 Carbapenem-resistant *Acinetobacter baumannii* is a World Health Organisation (WHO) priority
48 level one pathogen, commonly associated with nosocomial infections in ICUs.[1,2] Once treatable
49 with broad-spectrum cephalosporins such as ceftazidime and cefepime, heavy usage of these
50 antibiotics has led to the reliance and subsequent resistance to last-resort carbapenem
51 treatment. *A. baumannii* are notorious for their genetic plasticity, enabling them to acquire
52 resistance genes from *Pseudomonas aeruginosa* and clinically relevant Enterobacterales such as
53 *Escherichia coli* and *Klebsiella pneumoniae*. Clinical *A. baumannii* have been reported to carry
54 multiple acquired β-lactamases from all four Class A-D molecular groups such as TEM, CARB,
55 PER, GES, VEB, CTX-M, IMP, VIM, NDM and OXA to varying frequencies, in addition to the
56 intrinsic AmpC (ADC) and OxaAb (OXA-51-like) enzymes.[3–6] Upregulation of some of these β-
57 lactamases by means of insertion sequences (IS) such as IS*Aba1* and IS*Aba125* have also driven
58 this resistance phenomenon.[7–9]

59 The main mechanism for carbapenem resistance in *A. baumannii* is carbapenem-hydrolysing
60 class D β-lactamases, most commonly Oxa23, Oxa40, OxaAb, Oxa58, Oxa143 and Oxa235
61 groups, frequently associated with IS elements.[10–15] Characterisation of clinical isolates has also
62 inferred the synergistic importance of the upregulation of multidrug efflux pumps (notably the
63 RND transporters AdeABC and AdeIJK) and the loss of certain porins (CarO, Omp33-36, OmpA
64 and OmpW).[16–22] However, the extent in which these proteins and their production levels play a
65 role in carbapenem resistance is not yet clear. In recent years there has been a concerted effort
66 to fill these gaps in our understanding of the factors contributing to carbapenem resistance
67 phenotypes in *A. baumannii* using WGS, whole transcriptome shotgun sequencing (RNA-Seq)

68 and proteomic approaches. However, these studies have not always been consistent with one
69 another - in some cases carbapenem-resistant strains were shown to overexpress efflux pumps
70 and downregulate porins[17,20], whereas in others, carbapenem resistance was associated with an
71 increase in porin abundance.[18] These inconsistencies demonstrate that our understanding of
72 the interplay between resistance mechanisms in *A. baumannii* remains incomplete.

73 OxaAb enzymes are intrinsic and by far the largest group of OXAs in *A. baumannii*, with 320
74 variants identified as of 03/06/2020.[23] When OxaAb variants are characterised in clinical
75 carbapenem resistant isolates worldwide, the presence of IS*AbaI* upstream is frequently noted
76 and this has led to the general acceptance that transcriptional upregulation of these enzymes
77 by upstream IS insertion, providing a strong promoter, can confer carbapenem resistance in the
78 absence of other β-lactamases. However, it is unclear whether only specific variants (e.g.
79 OxaAb(138) and OxaAb(82)[24,25]) confer this phenotype or if overproduction of all OxaAb types
80 can lead to carbapenem resistance. Studies from the last few years of the effect of specific
81 amino acid substitutions in OxaAb, for example at Ile-129, Leu-167 and Trp-222, have
82 demonstrated that this can alter the enzyme structure and significantly increase catalytic
83 activity with respect to the carbapenems.[26–29] However, the impact of such substitutions alone
84 on the antibiotic susceptibility of bacteria is unclear. Recent papers have highlighted clinical
85 isolates carrying IS*Aba1*/*oxaAb* genes that do not exhibit carbapenem resistance.[30,31] Nigro and
86 Hall also elude to differences in carbapenem MIC depending on the OxaAb variant and/or other
87 intrinsic factors in different backgrounds.[31] In order to address some of these stated unknowns
88 concerning carbapenem resistance in *A. baumannii*, this study aimed to i) measure the
89 variability in carbapenem MIC conferred by different OxaAb variants, ii) characterise the

90 molecular evolution of *oxaAb* and iii) elucidate the contribution of OxaAb and other possible

91 carbapenem resistance determinants in clinical isolates using WGS and LC-MS/MS proteomics.

93 **MATERIALS AND METHODS**

94 *Bacterial strains and antimicrobial susceptibility testing*

95 Ten clinical *A. baumannii* isolates were used in this study (**Table 1**). Imipenem and meropenem
96 MICs were previously characterised by Evans *et al* (except isolates B1 and A403), as well as the
97 identification of IS*AbaI* elements upstream of their respective *oxaAb* genes.[32] Recombinants
98 were made using the following strains: *E. coli* DH5α (Subcloning Efficiency DH5α Competent
99 cells, Invitrogen, United Kingdom), *A. baumannii* CIP70.10 and BM4547[33] (gifts from Laurent
100 Poirel, University of Fribourg). The presence of the spontaneous mutation P116L in *adeR* of
101 BM4547 responsible for increasing the AdeABC efflux pump expression was confirmed by PCR
102 and sequencing using primers R-am and R-av[33]. Disc susceptibility and MIC broth microdilution
103 tests were performed and interpreted according to CLSI guidelines.[34]

104 *WGS*

105 Genomes were sequenced by MicrobesNG on a HiSeq 2500 instrument (Illumina, San Diego, CA,
106 USA) as previously described.[35] Insertion Sequences (IS) were identified using ISFinder.[36]

107 *Proteome analysis via Orbitrap LC-MS/MS*

108 Total cell extractions of the clinical isolates (in three biological replicates) were prepared and 1
109 µg of each sample was analysed using an Orbitrap Velos mass spectrometer (Thermo Fisher
110 Scientific) and quantified using Proteome Discoverer software v1.4 (Thermo Fisher Scientific) as
111 outlined previously.[35] The raw data files were searched against the UniProt *A. baumannii* ACIBA
112 database (67,615 protein entries) and an in-house mobile resistance determinant database.[37]

113 Abundance values of each protein were converted to ratios relative to the average abundance
114 of 30S and 50S ribosomal proteins, for ease of comparison between isolates.

115 *Cloning and transformations*

116 The genes for the *oxaAb* variants encoding OxaAb(64), (65), (66), (69), (71), (82), (83), (107),
117 (110) and (111) were PCR amplified from clinical *A. baumannii* isolates (with additional NcoI and
118 XhoI sites introduced at the 5' and 3' ends respectively) using OXA-66-NcoI F, OXA-111-NcoI F
119 or OXA-71-NcoI F and OXA-66-XhoI R primers (**Table 2**) and TA cloned into the vector pGEM-T
120 Easy (Promega, United Kingdom). The inserts were confirmed by sequencing with the universal
121 T7 Promoter primer. For transformation into *E. coli* DH5α, *A. baumannii* CIP70.10 and BM4547,
122 the inserts were digested with NcoI and XhoI and ligated into pYMAb2, a pET-28a vector with
123 plasmid replicon fragments RepM and Ori from *A. baumannii* plasmid pMAC and an *oxa72*
124 promoter region subcloned from a clinical isolate with no presence of IS*Aba1* (a gift from Dr Te-
125 Li Chen, National Defense Medical Center, Taiwan).[38] For genes including their natural
126 upstream promoter regions, inserts were PCR amplified using OXA-51-like_XbaI F or
127 ISAba1_XbaI F and OXA-51-like_EcoRI R primers, digested with XbaI and EcoRI and ligated into
128 pUBYT,[37] a pYMAb2-derived vector with the *oxa72* promoter region deleted. All inserts were
129 confirmed by sequencing using the pYMAb2 Check primers (**Table 2**).

130 All plasmids were used to transform *E. coli* DH5α and *A. baumannii* CIP70.10 and BM4547
131 strains by electroporation. Transformants were selected with ampicillin (100 mg/L) and
132 ChromoMax IPTG/X-Gal (Fisher BioReagents, United Kingdom) for pGEM-T Easy recombinants
133 or kanamycin (50 mg/L) for pYMAb2 and pUBYT recombinants.

134 *Predicting the molecular evolution of OxaAb variants*

135 The nucleotide sequence of *oxaAb(66)* was used to query the NCBI nucleotide and genome
136 databases using BLAST, implemented in Geneious (https://www.geneious.com), and all
137 available *oxaAb* sequences were downloaded. The sequence for the gene of the naturally-
138 occurring OXA from *Acinetobacter calcoaceticus* (*oxa213*) was included as an outgroup.[39]
139 Duplicate sequences were removed and remaining sequences aligned. A maximum likelihood
140 phylogeny of the *oxaAb* genes was estimated using FastTree. Support for the resulting
141 phylogeny was estimated using 100 bootstraps. The Phi test was used to detect recombination
142 within the *oxaAb* alignment using SplitsTree4.[40] A translation of the nucleotide alignment was
143 used to identify all OXAs that were different from the consensus sequence at Ile-129 and Leu-
144 167 that have previously been described as being important for substrate specificity and
145 hydrolytic activity.[26,27,41]

147 **RESULTS AND DISCUSSION**

148 *Characterisation of β-lactam susceptibility in selected clinical isolates*

149 Ten clinical isolates[32] (**Table 1**) were chosen for encoding various OxaAb enzymes that are
150 representative of global clones (GC) 1 and 2 (OxaAb(69) and OxaAb(66) respectively). Some of
151 these isolates (A371, A404, A403 and A443) were also chosen for encoding variants containing
152 substitutions at sites considered important for substrate specificity (I129L in OxaAb(83) and
153 OxaAb(110), and L167V in OxaAb(82) and OxaAb(107) respectively). Others (A60, A37 and
154 A135) were chosen to represent sites where polymorphisms have arisen more than once across
155 the OxaAb phylogeny (E36V/D/K, with OxaAb(65) carrying the consensus Glu-36, and Q194P in
156 OxaAb(64) and OxaAb(111) respectively).[26,27,41]

157 β-lactam susceptibility results for the clinical isolates are shown in **Table 3**. All isolates were not
158 susceptible to ceftriaxone and cefotaxime except for A135. The four isolates encoding variants
159 with substitutions I129L and L167V (A404, A371, A443 and A403) were non-susceptible to all
160 tested antibiotics, including the carbapenems. WGS did not detect any other β-lactamases
161 known to confer carbapenem resistance.

162 **Table 1** summarises the carbapenem MICs and the designation of IS elements upstream of β-
163 lactamase genes based on the WGS data. Seven bla_{ampC} and four *oxaAb* genes had upstream IS
164 elements. IS-driven overproduction of AmpC and OxaAb enzymes was confirmed by analysis of
165 whole cell extracts of the clinical isolates and reference strain CIP70.10 in triplicate via LC-
166 MS/MS (**Figure 1a, 1b**). AmpC variants in CIP70.10, A90, B1 and A135 did not have an upstream

167 IS element and this was associated with enzyme levels below the level of detection. The AmpC
168 enzyme in A230 was the only variant with IS*Aba125* upstream and displayed the lowest
169 abundance amongst the seven variants with an IS element upstream. This implies that the
170 promoter in IS*Aba1* is stronger than in IS*Aba125*. Likewise, only the *oxaAb* genes in
171 carbapenem-resistant isolates A403, A371, A443 and A404 with upstream IS elements
172 produced detectable levels of enzyme. This confirms that without an IS element upstream of an
173 *oxaAb* gene, there is very little expression and hence, negligible contribution to intrinsic
174 resistance.

175 *OxaAb variants OxaAb(82), (83), (107), and (110) increase carbapenem MICs*

176 To determine whether substitutions at Ile-129 and Leu-167 in OxaAb contribute to the
177 observed carbapenem resistance in isolates A404, A371, A443 and A403, all *oxaAb* genes from
178 the 10 clinical isolates were cloned in the absence of their native promoter, all downstream of
179 the same Oxa24(72) promoter carried by pYMAb2. This was to exclude any confounding effects
180 on differential gene expression of upstream IS elements seen in the clinical isolates.

181 In an *A. baumannii* CIP70.10 background (representing a susceptible host), OxaAb variants with
182 a substitution at either Ile-129 or Leu-167 allowed for significantly increased carbapenem MICs
183 over the other OxaAb variants (t-test, meropenem: $p = 0.0196$, imipenem: $p = 0.0131$) (**Table 4**).
184 The same was true in the *A. baumannii* BM4547 background, which has increased *adeABC*
185 efflux pump gene expression[33] (t-test, meropenem: $p = 0.0239$, imipenem: $p = 0.0391$). Only
186 OxaAb(82) conferred meropenem resistance (8 mg/L) in both backgrounds. The presence of
187 OxaAb(107) and (83) increased meropenem MIC to an intermediate phenotype (4 mg/L) in

188 CIP70.10 and BM4547 respectively. While there appeared to be a slight increase in MIC in the
189 BM4547 background compared to the CIP10.10 background of the same magnitude observed in
190 previous studies[42], this was not statistically significant (t-test, meropenem: $p = 0.9209$,
191 imipenem: $p = 0.6887$). We therefore conclude that the Ile-129 or Leu-167 substitutions seen in
192 OxaAb(82), (83), (107) and (110), increase carbapenem MIC but not to the level of resistance
193 seen in the clinical isolates producing these variants. Furthermore, AdeABC overproduction is
194 not important for carbapenem MICs in strains producing these OxaAb variants.

195 *ISAbaI-driven expression of* oxaAb *only confers carbapenem resistance for certain* oxaAb
196 *variants*

197 The *oxaAb* variants were next cloned into pUBYT with their natural upstream promoter, to
198 identify if the presence of upstream IS elements can enhance MICs and confer carbapenem
199 resistance. Genes encoding three enzymes (OxaAb(82), (107), (110)) with changes at positions
200 Ile-129 or Leu-167, had natural promotors provided by IS*Aba1*, while the remaining *oxaAb*
201 genes had the native chromosomal promotor without the presence of an insertion sequence.
202 Transformation of OxaAb(83) was not achieved despite multiple attempts.

203 When expressed in CIP70.10, the carbapenem MICs against transformants encoding *oxaAb* with
204 an IS*Aba1* promotor increased to resistant levels seen in their parent clinical isolates (**Table 4**).
205 These were significantly higher than the MICs obtained for the other *oxaAb* variants (t-test,
206 meropenem – $p = 9.99\times10^{-12}$, imipenem – $p = 1.45\times10^{-4}$) where clinical resistance was not
207 reached. While there was an overall increase in meropenem MICs for all transformants under
208 the control of their native promoters compared to the pYMAb2 promoter (t-test, meropenem –

209 p = 0.0465, imipenem – p = 0.0817), significantly higher MICs were observed for the three
210 transformants encoding *oxaAb* with an IS*Aba1* promoter (t-test, meropenem – *p* = 0.0009,
211 imipenem – *p* = 0.0319). This demonstrates that the addition of IS*AbaI* upstream of *oxaAb*
212 variants encoding I129L or L167V substitutions confers carbapenem resistance in a recombinant
213 background without any other resistance determinants. When the *oxaAb* variants were cloned
214 into BM4547, the same pattern was also observed and there was no overall difference in the
215 MIC values between the CIP70.10 and BM4547 backgrounds (t-test, meropenem: p = 0.9700,
216 imipenem: p = 0.2391). Therefore, the increase in AdeABC efflux does not have a crucial role in
217 conferring carbapenem resistance in the context of these OxaAb variants.

218 It is worth noting that the recombinants with upstream IS*Aba1* were very difficult to obtain,
219 with extremely low transformation efficiency in both CIP70.10 and BM4547. Plasmid-mediated
220 carriage of these variants with upstream IS*Aba1* may be deleterious to the host's fitness and
221 may possibly be the reason for certain variants not being observed to be plasmid-borne in
222 nature.

223 *Predicting molecular evolution of oxaAb*

224 Given that OxaAb variants with specific amino acid polymorphisms at Ile-129 and Leu-167 have
225 been shown to confer carbapenem resistance in the presence of IS*Aba1*, it is reasonable to
226 hypothesise that these polymorphisms may have been selected for in the *A. baumannii*
227 population. To investigate the distribution of these two polymorphisms, a phylogenetic analysis
228 of all available *oxaAb* genes was conducted. Comparison of the *oxaAb* phylogeny with the
229 substitution patterns that result in amino acid changes at the two sites examined showed that

230 substitutions at these sites are likely to have occurred on multiple occasions (**Figure 2**). At
231 position 129, there are 4 alternative codons coding for 4 amino acid changes, suggesting at
232 least 4 independent mutations at this position. The phi test did not detect any significant
233 evidence for recombination within the *oxaAb* genes. Therefore, assuming there is no
234 recombination within these alleles, their distribution across the *oxaAb* phylogeny indicates that
235 mutations at position 129 have occurred on 10 occasions, as seen by alleles carrying the same
236 mutation being separated by alleles that do not share the mutation. Similarly, 3 amino acid
237 changes at position 167 are coded for by 4 different codons, with a phylogenetic distribution
238 suggesting independent mutations arising on 8 occasions. Overall, these data provide strong
239 evidence for selection for changes to the consensus sequence at these sites. Given that no
240 evidence for recombination within the *oxaAb* genes was detected, there are two possibilities
241 that may explain the distribution of polymorphisms: 1) all of the variants have evolved
242 independently and in some instances represent parallel evolution, and 2) there has been
243 recombination of entire *oxaAb* genes between strains, most likely by natural transformation.
244 The most conservative interpretation would be that each different codon only evolved once
245 and any occurrences of the same codon are due to common evolutionary descent or
246 recombination. While we did not detect evidence for recombination within the *oxaAb* genes
247 here, the possibility of between-strain recombination could be examined by a large whole
248 genome analysis, provided sufficient representation of the different *oxaAb* variants were
249 included. At the other extreme, the most liberal interpretation of the data is that each different
250 codon has evolved independently except where there is common evolutionary descent. The
251 relative contributions of independent mutation and recombination to the evolutionary genetics

252 of *oxaAb* remains to be determined; however, our experimental data does show that selection
253 for changes in these two sites do increase carbapenem MIC.

254 *Comparing carbapenem resistance signatures in clinical isolates by LC-MS/MS and WGS*

255 To determine if upregulation of OxaAb with substitutions in Ile-129 and Leu-167 is the only
256 mechanism of carbapenem resistance in the four resistant clinical isolates (A403, A371, A443
257 and A404), all 10 clinical isolates were analysed for the presence of proteins commonly
258 associated with carbapenem resistance that may be differentially produced in the resistant
259 isolates and from these, porins and efflux pumps were summarised in **Figure 1**.

260 *(i) Porins*

261 In *A. baumannii*, the major outer membrane protein associated with antimicrobial resistance is
262 a nonspecific slow porin OmpA.[43] It is generally accepted that OmpA is involved in the slow
263 diffusion of certain β-lactams across the membrane.[19,43,44] There were no changes in abundance
264 levels of OmpA in the clinical isolates (compared to CIP70.10) (**Figure 1c**).

265 In terms of porins associated with carbapenem susceptibility, abundance levels of CarO, OmpW
266 and OprD were compared. The disruption of CarO expression in MDR clinical *A. baumannii*
267 isolates by insertion sequences (such as IS*Aba1*, IS*Aba10*, IS*Aba125* and IS*Aba825*) has been
268 associated with reduced susceptibility to imipenem[16,45], although a reconstituted liposome
269 CarO system has been demonstrated not to transport imipenem.[46] CarO can be grouped into
270 two major isoforms CarOa and CarOb, with higher specificity for imipenem in the latter.[17] WGS
271 identified all isolates to have intact *carO* genes and no changes to the upstream promoter

272 sequences. A60 and B1 carry CarOa and all other isolates carry CarOb, except A37, A443 and
273 A135 which did not categorise in either groups. Abundance levels of CarO were similar across
274 all isolates except A60, suggesting that there is no critical association between production levels
275 or specific isoforms and carbapenem MIC in these clinical isolates (**Figure 1d**).

276 Loss of OmpW has been implicated with carbapenem resistance, although proteomics studies
277 have also observed increased levels of this porin in MDR isolates.[21] Another study observed that
278 deletion of *ompW* in carbapenem-susceptible *A. baumannii* ATCC 17978 did not affect
279 imipenem MIC.[47] No differences in abundance levels were observed (except A90), suggesting
280 that OmpW does not play a role in carbapenem resistance in these isolates (**Figure 1e**).

281 Decreased *oprD* expression has been associated with carbapenem resistance in clinical
282 isolates,[48–50] although subsequent knock-out experiments demonstrated no increase in
283 imipenem and meropenem susceptibilities.[51,52] More recent liposome model studies have
284 shown that OprD does uptake both carbapenems.[45] Here we observed no significant changes in
285 OprD abundance levels compared to CIP70.10 (**Figure 1f**).

286 *(ii) Efflux pumps*

287 Overexpression of RND efflux pumps AdeABC and AdeIJK have been associated with aiding
288 carbapenem resistance, although this was not the case in our BM4547 recombinants.[53–55] AdeB
289 was below the level of detection in CIP70.10 and A135, despite confirmation of the gene by
290 WGS. AdeC was not detected in CIP70.10, A37, A60, A230, A403 and A135 and the absence of
291 this gene was confirmed by WGS for CIP70.10, A37, A135 and shown to be truncated for A60.

292 Studies have shown that the *adeABC* operon is not always present in *A. baumannii* strains and
293 amongst the *adeRS-AB*-expressing strains, the outer membrane compartment gene *adeC* is not
294 always present.[33,56]

295 AdeI was not detectable in any of the samples processed despite WGS confirmation. This may
296 suggest (along with the non-detectable AdeBC mentioned above) that these proteins are not
297 expressed in abundance in these particular isolates or a more membrane-specific sample
298 preparation is required for better resolution of membrane proteins, although Yoon and
299 colleagues also reported AdeB to be undetectable in parent strain BM4587 by membrane
300 sample LC-MS/MS.[54,57]

301 There were no changes in abundance of AdeA, J and K in the carbapenem resistant isolates
302 compared to CIP70.10 (**Figure 1g-i**). However, there were higher levels in one or more of the
303 proteins in susceptible isolates A60, A230 and A90, with the former two having raised
304 meropenem MICs of 2 mg/L. This suggests that overexpression of these efflux pumps may play
305 a minor role in elevating MICs but the key driver of carbapenem resistance in the clinical
306 isolates under study is the upregulation of OxaAb variants with specific amino acid substitutions.

307 *(iii) Other proteins involved in membrane integrity*

308 Changes in expression levels of PBPs has been associated with carbapenem resistance, such as
309 the decrease in PBP2 expression levels[58] or increase in PBP1a and 5 in an imipenem-resistant
310 MDR strain in the presence of imipenem.[20] Four PBPs were identified in the LC-MS/MS data –

311 PBP1a, 2, 5 and 6 but no differences were observed between carbapenem susceptible and
312 resistant isolates.

313 *Concluding Remarks*

314 During the course of this study, other groups published work including clinical isolate
315 characterisation and structural studies that identified residues Trp-222, Ile-129, Pro-130 and
316 Leu-167 in the active site of OxaAb enzymes to contribute to weak carbapenem binding by
317 obstructing the active site from carbapenem interaction, and that substitutions at these sites
318 improve carbapenemase activity.[26,28,29] While none of the enzymes in this study had Trp-222 or
319 Pro-130 substitutions, this work confirms that OxaAb variants with I129L and L167V
320 substitutions do confer raised carbapenem MICs relative to wild-type genes when all are
321 expressed from the same promoter. When the expression of these enzymes with increased
322 carbapenemase activity are driven by the promoter within IS*Aba1*, this confers carbapenem
323 resistance. This was seen in recombinants lacking additional resistance proteins, and also in the
324 resistant clinical isolates, where no additional protein abundance changes predicted to
325 influence carbapenem MIC were observed in the LC-MS/MS data. Hence, we conclude that
326 overproduction of OxaAb variants with enhanced carbapenemase activity due to the
327 substitutions I129L and L167V is sufficient to confer carbapenem resistance in *A. baumannii*
328 with no additional mechanisms required.

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339 **TRANSPARENCY DECLARATIONS**

340 None for all authors.

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503

504 **Table 1. Selected WGS data and carbapenem MICs of clinical *A. baumannii* isolates.**

								AA		MIC (mg/L)	
***A. baumannii* ID**	**Geographic Location**	**OxaAb**	**GC**	**Other β-lactamases**	**IS*Aba1* OXA**	**IS*Aba1* ADC**	**IS*Aba125* ADC**	**129**	**167**	**IMP**	**MEM**
CIP70.10	France	(64)	-	ADC-50	-	-	-	I	L	0.125-0.25	0.125-0.5
A37	Singapore	(64)	-	ADC-174	-	+	-	I	L	0.5	0.5
A60	Argentina	(65)	-	ADC-5, TEM-1A, CARB-16	-	+	-	I	L	0.125	2
A230	United Kingdom	(66)	2	ADC-175, Oxa20	-	-	+	I	L	0.5	2
A90	United Kingdom	(69)	1	ADC-11, TEM-1D	-	-	-	I	L	0.125	0.25
B1	Unknown	(51)	-	ADC-180, Oxa10	-	-	-	I	L	1	1
A403	Taiwan	(82)	2	ADC-177, TEM-1D	+	+	-	I	V	**32**	**32**
A371	Czech Republic	(83)	2	ADC-30, TEM-1D	+	+	-	L	L	**16**	**32**
A443	Slovenia	(107)	1	ADC-176, TEM-1D	+	+	-	I	V	**16**	**16**
A404	Poland	(110)	1	ADC-178, TEM-1D	+	+	-	L	L	**8**	**16**
A135	Belgium	(111)	-	ADC-179	-	-	-	I	L	0.25	0.25

505

506 Resistant IPM and MEM MIC values (≥ 8 mg/L) in bold. IS*Aba1* and IS*Aba125* sequences were found upstream of *oxaAb* and bla_{ADC}
507 (bla_{ampC}) genes. GC, global clone; AA, amino acid present at positions 129 and 167, IPM, imipenem; MEM, meropenem.

508

509 **Table 2. Primers used in this study.**

Primer	Sequence (5'-3')
OXA-66-NcoI F	AAACCATGGATGAACATTAAAGCACTC
OXA-66-XhoI R	AAACTCGAGCTATAAAATACCTAATTGTTC
OXA-111-NcoI F	AAACCATGGATGAACATTAAAACACTC
OXA-71-NcoI F	AAACCATGGATGAACATTAAAGCCC
OXA-51-like_XbaI F	AAATCTAGAGTAAAACTTTATCTATCTCAA
ISAba1_XbaI F	AAATCTAGACTCTGTACACGACAAATT
OXA-51-like_EcoRI R	AAAGAATTCCTATAAAATACCTAATTGTTC
pYMAb2 Check F	TAACATGAATTTGCCATGG
pYMAb2 Check R	AGCTCGAATTCGGATCC

511 **Table 3. Disc susceptibility test results for selected β-lactams.**

***A. baumannii* ID**	**CRO**	**CTX**	**CAZ**	**FEP**	**IPM**	**MEM**	**DOR**
CIP70.10	I	I	S	S	S	S	S
A37	R	R	R	S	S	S	S
A60	R	R	I	R	S	S	S
A230	R	R	R	I	S	S	S
A90	I	R	S	S	S	S	S
B1	I	I	S	S	S	S	S
A403	R	R	R	R	R	R	R
A371	R	R	R	R	R	R	R
A443	R	R	R	R	R	R	R
A404	R	R	R	I	R	R	R
A135	S	I	S	S	S	S	S

512 R, resistant; I, intermediate; S, susceptible; CRO, ceftriaxone; CTX, cefotaxime; CAZ, ceftazidime;
513 FEP, cefepime; IPM, imipenem; MEM, meropenem; DOR, doripenem.

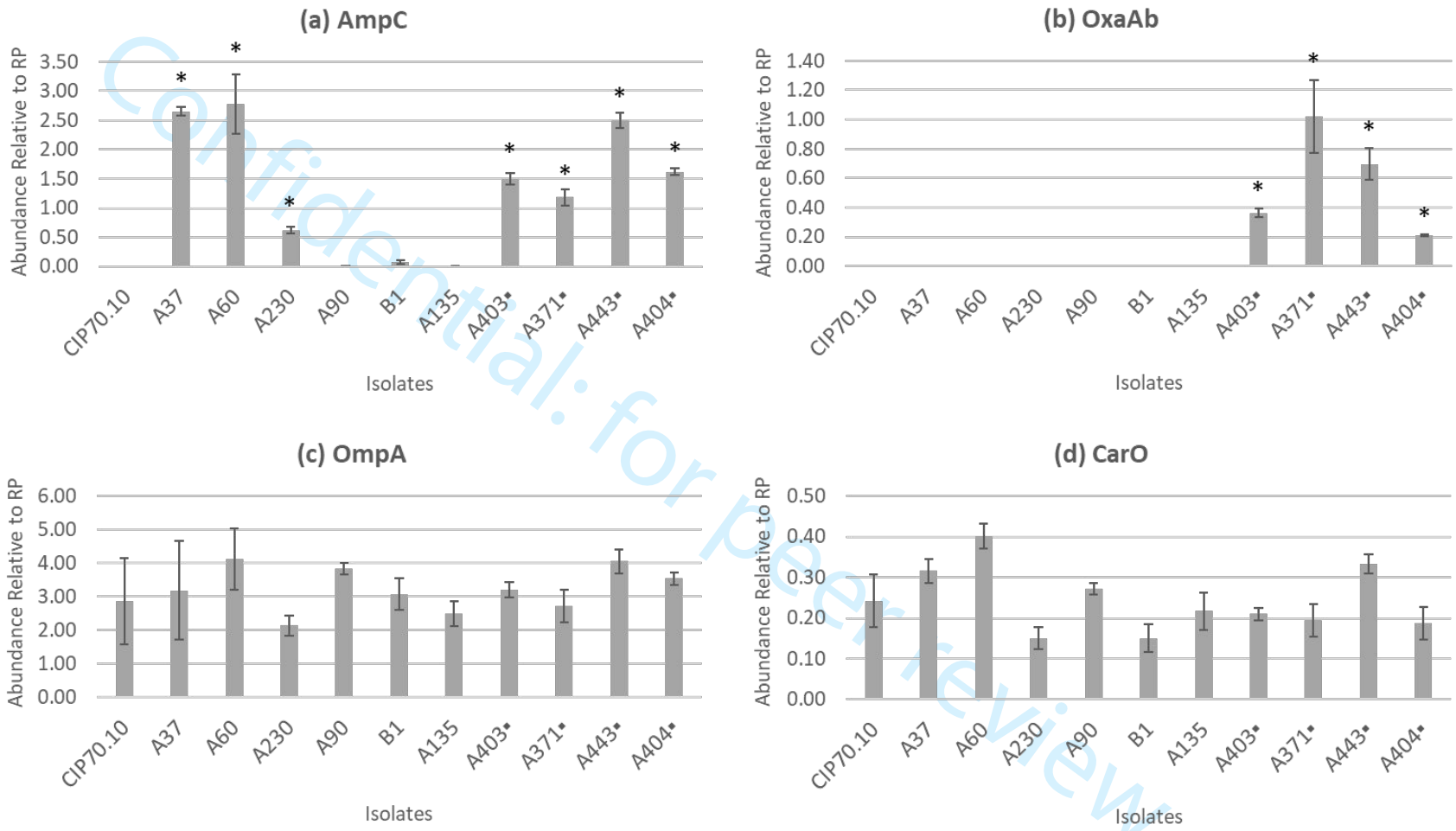
515 **Table 4. MIC (in mg/L) of recombinant *A. baumannii* strains carrying various OxaAb enzymes**
516 **± their natural upstream promoter regions.**

	pYMAb2		pUBYT	
Strain	**IPM**	**MEM**	**IPM**	**MEM**
CIP70.10 (No Vector)	0.125	0.125	0.125	0.125
Empty Vector	0.25	0.25	0.125	0.25
OxaAb(64)	0.125	0.5	0.5	**4**
OxaAb(65)	0.125	0.125	0.5	**4**
OxaAb(66)	0.25	0.25	0.25	**4**
OxaAb(69)	0.125	0.125	0.25	**4**
OxaAb(51)	0.06	0.25	0.25	**4**
OxaAb(82) ▪	2	**8**	**16**	**64**
OxaAb(83) ▪	0.5	2	-	-
OxaAb(107) ▪	2	**4**	**16**	**64**
OxaAb(110) ▪	0.5	2	**8**	**64**
OxaAb(111)	0.25	0.125-0.5	0.25	1
BM4547 (No Vector)	0.125	0.5	0.125	0.5
Empty Vector	0.125	0.5	0.125	0.25
OxaAb(64)	0.25	0.125-0.5	0.5	**4**
OxaAb(65)	0.125	0.5	0.5	**4**
OxaAb(66)	0.25	0.5	1	**8**
OxaAb(69)	0.125	0.5	0.5	2
OxaAb(51)	0.25	0.5	0.5	**4**
OxaAb(82) ▪	2	**8**	**32**	**> 64**
OxaAb(83) ▪	0.5	**4**	-	-
OxaAb(107) ▪	1	2	**64**	**> 64**
OxaAb(110) ▪	0.25	2	**32**	**> 64**
OxaAb(111)	0.06	0.125	0.125	**4**

517

518 IPM, imipenem; MEM, meropenem. Intermediate (4 mg/L) and resistant (≥ 8 mg/L) MIC values
519 in bold. "-" indicates strains were not tested. MIC values (n=6) that were variable are
520 represented by ranges. "▪" highlight OxaAb variants with substitutions in Ile-129 or Leu-167

521



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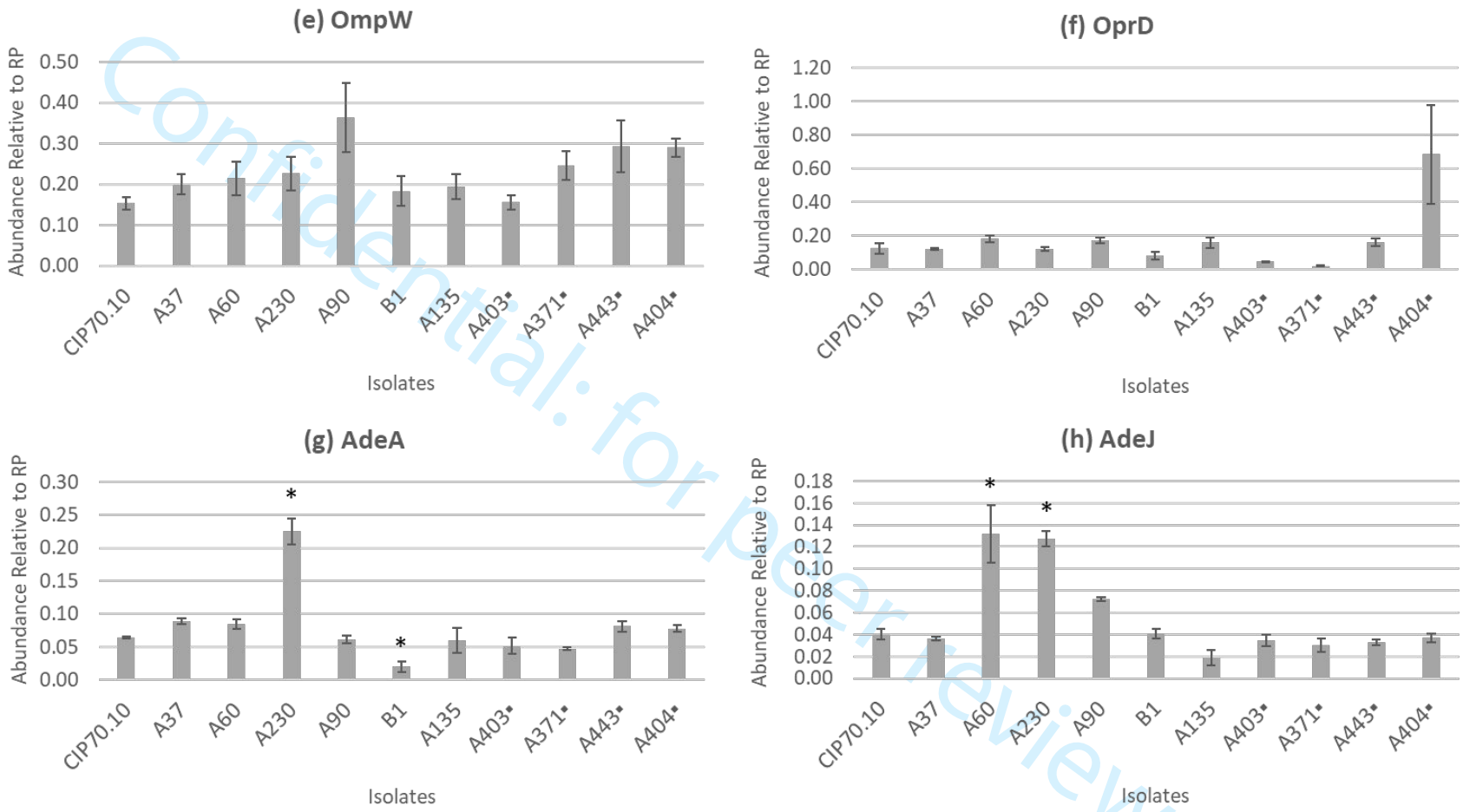

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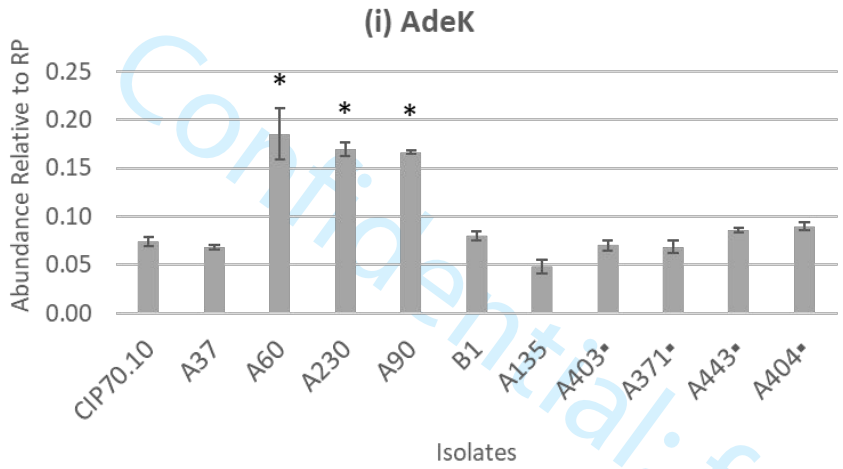

524

525 **Figure 1. Comparison of various resistance determinants by average abundance ratios relative to ribosomal protein (RP). (a)** AmpC,
526 **(b)** OxaAb, **(c)** OmpA, **(d)** CarO, **(e)** OmpW, **(f)** OprD, **(g)** AdeA, **(h)** AdeJ and **(i)** AdeK enzymes. Carbapenem resistant clinical isolates
527 are highlighted with "▪". The absolute abundance values of each protein of interest were divided by the average abundance values
528 of 30S and 50S ribosomal proteins and averaged to yield ratios with SEM error bars (n=3). Asterisks represent a significant difference
529 in abundance relative to CIP70.10, based on ≥ 2-fold difference and t-test ($p < 0.05$).

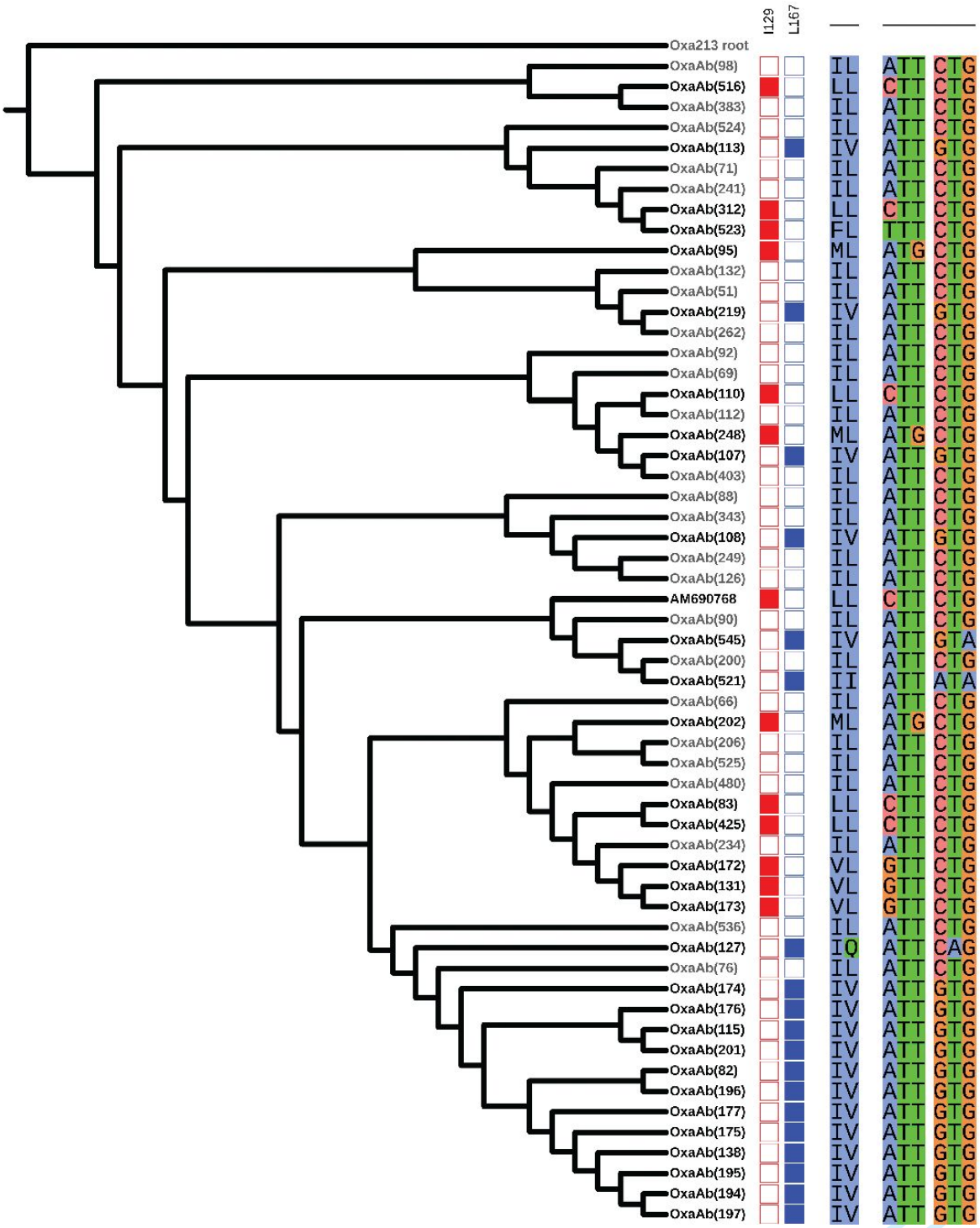

Figure 2. Cladogram of the nucleotide phylogeny of selected *oxaAb* sequences and their differences from consensus at amino acid positions 129 and 167. The phylogeny was drawn using FastTree with all available *oxaAb* sequences and rooted using *oxa213* from *Acinetobacter calcoaceticus* as an outgroup. The sequence labelled accession number AM690768 is an unnamed variant differing from OxaAb(90) by a single amino acid (at position 129). For clarity,

536 the majority of branches containing genes for OxaAb enzymes that do not have a change from
537 consensus at either position being examined have been hidden, with a minority retained to
538 provide context (shown in italic font). The boxes in the centre represent the amino acid
539 positions, with changes from consensus represented by a filled box. On the right are shown the
540 sequences of amino acids and the corresponding codons. The figure was drawn using iTOL[59].