Wan Ahmad Kamil, W. N. I., Takebayashi, Y., Findlay, J., Heesom, K., & Avison, M. (2018). Impact of OqxR loss of function on the envelope proteome of Klebsiella pneumoniae and susceptibility to antimicrobials. *Journal of Antimicrobial Chemotherapy*, *73*(11), 2990-2996. [dky293]. https://doi.org/10.1093/jac/dky293

Peer reviewed version

Link to published version (if available):
10.1093/jac/dky293

Link to publication record in Explore Bristol Research
PDF-document

This is the author accepted manuscript (AAM). The final published version (version of record) is available online via OUP at https://academic.oup.com/jac/advance-article/doi/10.1093/jac/dky293/5058072#119097015. Please refer to any applicable terms of use of the publisher.

University of Bristol - Explore Bristol Research

General rights

This document is made available in accordance with publisher policies. Please cite only the published version using the reference above. Full terms of use are available: http://www.bristol.ac.uk/red/research-policy/pure/user-guides/ebr-terms/

Impact of OqxR loss of function on the envelope proteome of *Klebsiella pneumoniae* and susceptibility to antimicrobials.

Wan Ahmad Kamil WAN NUR ISMAH,[1,2] Yuiko TAKEBAYASHI,[1] Jacqueline FINDLAY,[1] Kate J. HEESOM,[3] and Matthew B. AVISON[1*].

[1]School of Cellular & Molecular Medicine, University of Bristol, Bristol. UK

[2]Faculty of Biotechnology & Biomolecular Sciences, Universiti Putra Malaysia, Selangor Darul Ehsan, Malaysia

[3]Bristol Proteomics Facility, University of Bristol, UK

***Correspondence: Matthew B. Avison, School of Cellular & Molecular Medicine, Biomedical Sciences Building, University Walk, Bristol. BS81TD. UK. bimba@bris.ac.uk. +44(0)1173312063.**

Running Title: OqxR loss in *K. pneumoniae*

17 **Abstract**

18 **OBJECTIVES**

19 **In *Klebsiella pneumoniae*, loss of function mutations in the transcriptional repressors RamR**
20 **and OqxR both have an impact on the production of efflux pumps and porins relevant to**
21 **antimicrobial efflux/entry. Our aim was to define, in an otherwise isogenic background, the**
22 **relative effects of OqxR and RamR loss of function mutations on envelope protein**
23 **production, envelope permeability, and antimicrobial susceptibility. We have also**
24 **investigated the clinical relevance of an OqxR loss of function mutation, particularly in the**
25 **context of β-lactam susceptibility.**

26 **METHODS**

27 **Envelope permeability was estimated using a fluorescent dye accumulation assay.**
28 **Antimicrobial susceptibility was measured using disc testing. Total envelope protein**
29 **production was quantified using LC-MS/MS proteomics, and quantitative RT-PCR was used**
30 **to measure transcript levels.**

31 **RESULTS**

32 **Loss of RamR or OqxR reduced envelope permeability in *K. pneumoniae* by 45-55% relative**
33 **to wild-type. RamR loss activated AcrAB efflux pump production ≈5-fold, and this reduced**
34 **β-lactam susceptibility, conferring ertapenem non-susceptibility even in the absence of a**
35 **carbapenemase. In contrast, OqxR loss specifically activated OqxAB efflux pump production**
36 **>10,000-fold. This reduced fluoroquinolone susceptibility but had little impact on β-lactam**
37 **susceptibility even in the presence of a β-lactamase.**

38 **CONCLUSIONS**

39 **Whilst OqxR loss and RamR loss are both seen in *K. pneumoniae* clinical isolates, only RamR**
40 **loss significantly stimulates AcrAB efflux pump production. This means that only RamR**
41 **mutants have significantly reduced β-lactamase mediated β-lactam susceptibility and**
42 **therefore represent a greater clinical threat.**

43 **Introduction**

44 Multiple envelope proteins contribute to antimicrobial susceptibility and resistance in
45 *Klebsiella pneumoniae*. Two major porins, OmpK35 and OmpK36 are important for drug
46 entry,[1–4] and the OmpK35/36 ratio is affected by osmolarity.[5] Drug efflux is mainly catalysed
47 by the Resistance Nodulation Cell Division (RND)-type pump, AcrB, working with AcrA and
48 TolC as a tripartite system.[6–8] AcrAB production is controlled by the transcriptional repressor
49 AcrR and is upregulated following overproduction of the AraC-type transcriptional activator
50 RamA.[6,9,10] RamA overproduction also boosts transcription of *micF*, encoding an antisense
51 RNA, which downregulates OmpK35 porin production, and RamA upregulates a second RND-
52 type efflux pump, OqxAB.[10] RamA hyper-production occurs in *ramR* loss of function mutants
53 because RamR is a transcriptional repressor of *ramA*.[9-11] Loss of function mutations in the
54 transcriptional repressor OqxR also activate *oqxAB* transcription, as well as transcription of
55 *rarA*, which encodes a RamA orthologue.[11,12] To add to this complexity, increased efflux pump
56 and/or reduced porin production can confer an additive effect in combination with plasmid
57 mediated antimicrobial resistance proteins or target site mutations. This has been
58 demonstrated for the complex interplay of factors that influence ciprofloxacin susceptibility,[13]
59 and in the generation of potentially important and perhaps clinically under-reported
60 carbapenemase negative carbapenem resistance phenotypes.[10,14]

61 We have previously characterise the interplay between factors that confer RamA mediated
62 multi-drug resistance in *K. pneumoniae*.[10] The aim of the work reported here was to analyse
63 the importance of *oqxR* loss of function mutation in multi-drug resistance, and to identify the
64 resistance proteins involved.

65

66 **Materials and Methods**

67 <u>*Bacterial strains and susceptibility testing*</u>

68 *Four K. pneumoniae* strains were used in the study. Ecl8,[15] plus *in vitro* selected loss of
69 function mutants in *oqxR* (Tyr109STOP) or *ramR* (Thr124Pro)[13] and finally, Ecl8*ΔramR* [16]
70 additionally carrying a *gyrA* QRDR (Ser83Phe) and an *oqxR* loss of function mutation
71 (Arg93Cys).[13] *E. coli* XL10-Gold (Stratagene) was used for cloning. Disc susceptibility testing

72 was performed according to CLSI methodology [17] and interpreted using CLSI performance
73 standards.[18]

74 *Cloning genes, transformation and complementation studies*

75 The wild-type *oqxR* and *ramR* genes of *K. pneumoniae* Ecl8 were amplified by PCR using the
76 primers listed in **Table S1**. Each PCR amplicon was TA cloned into pCR2.1-TOPO (Invitrogen)
77 according to the manufacturer's instructions. The inserts were removed using EcoRI (for *oqxR*)
78 or HindIII plus XbaI (for *ramR*) and ligated into pK18.[19] The *rarA* gene was PCR amplified from
79 Ecl8 using primers listed in **Table S1** and directly ligated into pBAD322K [20] after cutting with
80 NcoI and XbaI. Cloning of plasmid-mediated β-lactamase genes $bla_{\text{CTX-M-1}}$ and $bla_{\text{CMY-4}}$ (with
81 their native promoters) using the vector pSU18 [10] and later sub-cloning into the vector pUBYT
82 has previously been reported.[14] Recombinant plasmids were used to transform *K.*
83 *pneumoniae* strains to kanamycin (30 mg/L for pBAD322 and 50mg/L for pUBYT) using
84 electroporation. All strains and plasmids are recorded in **Table S2**.

85 *Quantitative analysis of envelope proteome via Orbitrap LC-MS/MS and quantitative RT-PCR*

86 Each mutant and transformant was cultured in 50 mL Cation Adjusted Mueller-Hinton broth
87 (MHB) (Sigma) with appropriate antibiotic selection. Cultures were incubated with shaking
88 (160 rpm) at 37°C until the optical density at 600 nm reached 0.5-0.7. Total cellular RNA was
89 purified and qRT-PCR was performed as previously described,[10,21] using the primers listed in
90 **Table S1**. Total envelope proteomics was performed as previously described.[10,21] For each LC-
91 MS/MS proteomics experiment, raw protein abundance data were collected for three
92 biological replicates of each test condition. A paired T-test was used to calculate the
93 significance of any difference in protein abundance in pooled data from the two test
94 conditions. A *p*-value of <0.05 was considered significant. The fold change in abundance for
95 each protein in the two test conditions was calculated using the averages of absolute
96 abundances for the three biological replicates for the two test conditions.

97 *Fluorescent Hoechst (H) 33342 dye accumulation assay*

98 Envelope permeability was estimated as described previously [21] in bacteria grown in MHB
99 using an established fluorescent dye accumulation assay.[22] The assay was performed using a

100 black, flat-bottomed 96-well plates (Greiner Bio-one, Stonehouse, UK) and a Fluostar Optima
101 (Aylesbury, UK) plate reader. H33342 dye (Sigma) was used at a final concentration of 2.5 µM.

102

103 **Results and Discussion**

104 *Relative impact of OqxR and RamR loss of function mutations on antimicrobial susceptibility*
105 *and envelope permeability*

106 We have recently reported *in vitro* selection of *oqxR* and *ramR* loss of function mutants from
107 the wild-type *K. pneumoniae* strain Ecl8.[13] **Table 1** reports the relative impacts of these
108 mutations on inhibition zone diameter for a range of antimicrobials of different classes. As
109 expected, the *ramR* point mutant phenotype was very similar to the profile seen for an
110 Ecl8Δ*ramR* mutant previously studied.[10] The *oqxR* mutation had a larger effect on
111 fluoroquinolone and chloramphenicol susceptibility than the *ramR* mutation (68 mm versus
112 47 mm combined zone diameter reduction across five agents); the *ramR* mutation had a
113 larger impact on cephalosporin susceptibility than the *oqxR* mutation (69 mm versus 46 mm
114 combined zone diameter reduction across 10 agents). Both mutations conferred minocycline
115 non-susceptibility though neither had any effect on the zone diameters around carbapenem
116 or aminoglycoside discs.

117 Overall envelope permeability (a combination of outer membrane permeability and efflux) in
118 the two mutants was similar. The steady state accumulation of a fluorescent dye was ≈45%
119 and ≈55% of wild-type for the *ramR* and *oqxR* mutants, respectively (**Fig. 1**).

120 *Efflux pump and porin protein abundance changes in oqxR and ramR mutants*

121 We used Orbitrap LC-MS/MS proteomics to define the impact of the *ramR* and *oqxR*
122 mutations on the production of porins and efflux pumps previously implicated in
123 antimicrobial drug resistance in *K. pneumoniae*. The OqxA and OqxB efflux pump proteins
124 were found to be below the detection level in wild-type cells, but they were detectable in
125 both the *ramR* and *oqxR* mutants, with upregulation being almost two orders of magnitude
126 greater in the latter (**Fig. 2c,d**). By contrast, upregulation of AcrA and AcrB efflux pump
127 proteins was only observed in the *ramR* mutant (**Fig. 2a,b**) with similar fold increases to those
128 reported recently for an Ecl8Δ*ramR* mutant (4.79- versus 3.88-fold for AcrA and 3.56- versus

129 4.87-fold for AcrB).[10] Whist it has not been confirmed experimentally in *K. pneumoniae*, TolC
130 is likely to work as the outer membrane protein for both OqxAB and AcrAB. TolC levels were
131 significantly upregulated in both *oqxR* and *ramR* mutants relative to wild type (**Fig. 2e**).
132 Downregulation of the OmpK35 porin was equally observed in both mutants: 0.43-fold,
133 p=0.005 (*oqxR* mutant), 0.41-fold p=0.005 (*ramR* mutant) (**Fig. 2f**). Significant changes in
134 abundance of the OmpK36 porin were not observed in either mutant (**Fig. 2g)**. A list of
135 proteins for which abundance was significantly changed (≥2-fold, p<0.05) in the *oqxR* or *ramR*
136 mutant relative to Ecl8 during growth in MHB are shown in **Table S3** and **Table S4**. Ten of
137 eleven proteins previously shown to be significantly up-regulated ≥2-fold upon deletion of
138 *ramR* in Ecl8, irrespective of growth conditions,[10] were also regulated in the *ramR* point
139 mutant; the only protein significantly down-regulated ≥2-fold in Ecl8Δ*ramR* and the Ecl8 *ramR*
140 point mutant was OmpK35 (**Table S4**). Twelve of 23 proteins differentially regulated in the
141 *oqxR* loss of function mutant (**Table S3**) during growth in MHB were also differentially
142 regulated in the *ramR* mutant (**Table S4**) when grown in the same conditions.

143 To confirm the relative impacts of the *oqxR* and *ramR* mutations on efflux pump and porin
144 production, we took a previously selected *oqxR*/*ramR* double mutant,[13] and individually
145 complemented either *oqxR* or *ramR in trans*. The double mutant produced levels of AcrAB
146 similar to the *ramR* mutant and levels of OqxAB similar the *oqxR* mutant (**Fig. 3 a,b,c,d**; **Fig. 2**
147 **a,b,c,d**). We found TolC or OmpK35 production to be maximally altered in the *oqxR* or *ramR*
148 single mutants because protein levels were similar in the *oqxR*/*ramR* double mutant to levels
149 in the *oqxR* or *ramR* single mutants (**Fig. 3 e,f,g; Fig. 2 e,f,g**). However, the additive effect of
150 the two regulatory mutations on AcrB/OqxB efflux pump protein production was enough to
151 further reduce envelope permeability to ≈25% of wild-type, according to the fluorescent dye
152 accumulation assay (**Fig. S1**). This is because, in protein abundance terms, AcrB and OqxB are
153 limiting (**Fig. 2**). Complementation of either *ramR* or *oqxR* in the double mutant increased
154 envelope permeability to ≈55% of wild-type (**Fig. S1**), the level of permeability seen in the
155 single mutants (**Fig. 1**), confirming that there are no other significant factors involved.
156 Likewise, complementation of the double mutant with either *oqxR* or *ramR* resulted in a
157 recombinant that produced efflux pump and porin proteins as expected given the remaining
158 mutation (**Fig. 3 a,b,c,d**; **Fig. 2 a,b,c,d**).

159 *Impact of RarA overproduction in the oqxR mutant*

160 We have shown that *oqxR* loss has a large impact on OqxAB but no significant (p>0.05) impact
161 on AcrAB abundance (**Fig. 2**). This would fit with OqxR being a local transcriptional repressor
162 of *oqxAB*, as previously demonstrated.[11,12] However, loss of *oqxR* significantly upregulates
163 TolC and reduces levels of OmpK35 (**Fig. 2**). It has previously been shown that OqxR controls
164 transcription of *rarA*,[12] encoding a RamA-like global transcriptional activator.[23] Accordingly,
165 we hypothesised that the wider effects of *oqxR* loss in Ecl8 were caused by overexpression of
166 *rarA*. qRT-PCR revealed that *rarA* expression is indeed upregulated (417 $\pm$ 127-fold) in the
167 *oqxR* mutant. Expression of *acrA* does not significantly change (1.4 $\pm$ 0.1-fold) in the *oqxR*
168 mutant, as predicted from the proteomics (**Fig. 2**).

169 In order to dissect out the implications of RarA overproduction on the proteomic changes
170 seen in the *oqxR* mutant, we cloned *rarA* without its promoter into pBAD322K [20] and set about
171 overexpressing it by using arabinose induction. qRT-PCR confirmed *rarA* expression in
172 Ecl8(pBAD322K:*rarA*) in the presence of 0.02% w/v arabinose was very similar (415 $\pm$ 87.2 fold
173 more than control) to that seen in the *oqxR* mutant. Key efflux and porin protein production
174 levels following *rarA* overexpression at this level **(Fig. S2)** were very similar to those seen in
175 the *ramR* loss of function mutant **(Fig. 2)** in which *ramA*, a close homologue of *rarA*, is
176 overexpressed.[10]

177 Based on the phenotype of the Ecl8 *oqxR* mutant (**Fig. 2**), the very different impact of *rarA*
178 overexpression on protein levels in Ecl8 (**Fig. S2**) was surprising. Whilst *rarA* expression was
179 the same in the *oqxR* loss of function mutant and in the *rarA* over-expressing, *oqxR* wild-type
180 recombinant (see above), increased AcrAB production was only seen in the *rarA* over-
181 expressing, *oqxR* wild-type recombinant (**Fig 2, S2**). The positive impact of *rarA*
182 overexpression on *acrAB* expression in an *oqxR* wild-type background has also been reported
183 by others.[12] We hypothesise that RarA preferentially binds to the *oqxAB* promoter, so when
184 OqxR is not present – in the *oqxR* mutant – the overproduced RarA predominantly drives
185 *oqxAB* expression, and there is little impact on *acrAB* expression. However, when RarA is
186 overproduced in a background when OqxR is located as a repressor on the *oqxAB* promoter
187 (an artificial situation unlikely ever to be seen in a clinical isolate) RarA binds at less
188 preferential sites, including the *acrAB* promoter.

189 *Impact of OqxR loss of function in combination with plasmid mediated cephalosporinases on*
190 *carbapenem susceptiblity*

191 We have recently shown that *ramR* loss of function enhances the impact of carrying the serine
192 active site cephalosporinases *bla*$_{CTX-M-1}$ and *bla*$_{CMY-4}$, with particularly worrying effects on
193 carbapenem susceptibility, including causing ertapenem resistance in some cases.[10,14] To see
194 whether OqxR loss has a similar impact, plasmids encoding *bla*$_{CTX-M-1}$ or *bla*$_{CMY-4}$, were used to
195 transform our Ecl8 *oqxR* point mutant. There was a very slight impact on ertapenem
196 susceptibility in comparison with Ecl8 transformants (**Table S5**), but much less than that seen
197 previously in an Ecl8Δ*ramR* mutant.[10] This probably reflects the relatively low impact of *oqxR*
198 loss on β-lactam susceptibility (**Table 1**), because of its negligible impact on AcrAB efflux pump
199 production (**Fig. 2**) and the fact that OqxAB is not a noted β-lactam efflux pump.[24]

200 PCR sequencing was used to identify *oqxR* mutations in a collection of 44 *K. pneumoniae*
201 clinical isolates where *ramR* sequence and β-lactamase complement had already been
202 determined.[10] All *oqxR* mutants were confirmed to hyper-produce OqxAB using LC-MS/MS
203 proteomics of whole cell extracts as previously described.[13] As expected from our work with
204 Ecl8 transformants (**Table S5**), there was no impact of the *oqxR* loss of function mutation on
205 carbapenem susceptibility in isolates M and AD, which carry *bla*$_{CTX-M-15}$, though, as previously
206 noted, loss of *ramR* causes ertapenem resistance in *bla*$_{CTX-M-15}$ carrying isolate T (**Table S6**).[10]

207 *Conclusion*

208 It is known that *oqxR* loss of function causes *oqxAB* over-expression, and this is associated
209 with tigecycline resistance.[12,25,26] It is also known that *rarA* is overexpressed, and that when
210 overproduced in a recombinant form, RarA can have a wide range of effects on the cell,
211 including upregulation of *acrAB* and *tolC* transcription.[21,23] However, our use of proteomics
212 has allowed the first analysis of the relative impact of these events on the abundance of efflux
213 pump and porin proteins in *K. pneumoniae* and has allowed us to define the relative
214 importance of *oqxR* and *ramR* loss of function mutations, which can both be found in clinical
215 *K. pneumoniae* isolates.[13] Loss of OqxR has a dramatic effect on OqxAB efflux pump
216 production, with the absolute abundance of OqxB in an *oqxR* mutant being similar to that of
217 AcrB (≈2 x10^9 units) (**Fig. 2**). The impact of this >10,000-fold upregulation of OqxAB on
218 antimicrobial susceptibility is rather modest, however (**Table 1**). There is strong effect on
219 fluoroquinolone susceptibility, conferring ciprofloxacin resistance in the presence of other
220 mechanisms.[13] OqxAB overproduction also causes chloramphenicol, minocycline and,
221 reportedly, tigecycline resistance (**Table 1**),[25,26] but its overall impact on β-lactam

222 susceptibility is low, even in the presence of a plasmid mediated cephalosporinase (**Table 1,**
223 **S5, S6**). This limits the clinical importance of *oqxR* loss relative to *ramR* loss and is likely to be
224 because there is a negligible effect of *oqxR* loss on AcrAB production (**Fig. 2**). Indeed, when
225 AcrAB is overproduced only ≈5-fold more than wild-type levels, as seen in a *ramR* mutant, this
226 confers ertapenem resistance in the presence of a cephalosporinase.[10] Our work here also
227 adds support to our hypothesis that OmpK35 downregulation is not particularly important for
228 reducing β-lactam susceptiblity,[10] since OqxR loss and RamR loss both cause a similar
229 downregulation of OmpK35 (**Fig. 2**) but very different effects on β-lactam susceptibility (**Table**
230 **1, S5, S6**).

231 We were slightly surprised that *oqxR* loss does not cause AcrAB upregulation, because it has
232 previously been shown that *oqxR* loss causes *rarA* over-expression,[12] and that RarA
233 overproduction from a recombinant system in an *oqxR* wild-type background causes AcrAB
234 upregulation.[12,23] Whilst we were able to reproduce these previous findings (**Fig. S2**), the key
235 here is that RarA overproduction in an OqxR wild-type background, as used previously to
236 define the *rarA* regulon [23] is not relevant to what happens in an *oqxR* loss of function mutant.
237 The impact of RarA overproduction is less broad in an *oqxR* mutant because it predominantly
238 acts as a positive feed-forward mechanism to dramatically activate *oqxAB* transcription,
239 explaining the >10,000-fold upregulation of OqxAB production seen (**Fig. 2**) rather than taking
240 on a highly pleotropic role. Nonetheless, RarA overproduction does have additional effects in
241 the cell, even when overproduced due to *oqxR* loss, and many of the genes controlled are also
242 controlled by RamA (**Table S3**).

243

244 **Funding**

245 This work was funded by grant MR/N013646/1 to M.B.A. and K.J.H. and grant NE/N01961X/1
246 to MBA from the Antimicrobial Resistance Cross Council Initiative supported by the seven
247 research councils. W. A. K. W. N. I. was funded by a postgraduate scholarship from the
248 Malaysian Ministry of Education.

249

250 **Transparency Declaration**

251 None to declare – All authors.

252

253 **References**

254 1. Albertí S, Rodríquez-Quiñones F, Schirmer T, *et al*. A porin from *Klebsiella pneumoniae*:
255 sequence homology, three-dimensional model, and complement binding. *Infect Immun*
256 1995; **63**: 903–10.

257 2. Doménech-Sánchez A, Martínez-Martínez L, Hernández-Allés S, *et al*. Role of *Klebsiella*
258 *pneumoniae* OmpK35 porin in antimicrobial resistance. *Antimicrob Agents Chemother* 2003;
259 **47**: 3332–5.

260 3. Sugawara E, Kojima S, Nikaido H. *Klebsiella pneumoniae* Major Porins OmpK35 and
261 OmpK36 Allow More Efficient Diffusion of β-Lactams than Their *Escherichia coli* Homologs
262 OmpF and OmpC DiRita VJ, ed. *J Bacteriol* 2016; **198**: 3200–8.

263 4. Hernández-Allés S, Albertí S, Rubires X, *et al*. Isolation of FC3-11, a bacteriophage specific
264 for the *Klebsiella pneumoniae* porin OmpK36, and its use for the isolation of porin-deficient
265 mutants. *Can J Microbiol* 1995; **41**: 399–406.

266 5. Tsai Y-K, Fung C-P, Lin J-C, *et al*. *Klebsiella pneumoniae* outer membrane porins OmpK35
267 and OmpK36 play roles in both antimicrobial resistance and virulence. *Antimicrob Agents*
268 *Chemother* 2011; **55**: 1485–93.

269 6. Padilla E, Llobet E, Doménech-Sánchez A, *et al*. *Klebsiella pneumoniae* AcrAB efflux pump
270 contributes to antimicrobial resistance and virulence. *Antimicrob Agents Chemother* 2010;
271 **54**: 177–83.

272 7. Nikaido H. Multidrug efflux pumps of gram-negative bacteria. *J Bacteriol* 1996; **178**: 5853–
273 9.

274 8. Nikaido H, Zgurskaya HI. AcrAB and related multidrug efflux pumps of *Escherichia coli*. *J*
275 *Mol Microbiol Biotechnol* 2001; **3**: 215–8.

276 9. Schneiders T, Amyes SGB, Levy SB. Role of AcrR and RamA in Fluoroquinolone Resistance
277 in Clinical *Klebsiella pneumoniae* Isolates from Singapore. *Antimicrob Agents Chemother*
278 2003; **47**: 2831–7.

279 10. Jiménez-Castellanos J-C, Wan Nur Ismah WAK, Takebayashi Y, *et al*. Envelope proteome
280 changes driven by RamA overproduction in *Klebsiella pneumoniae* that enhance acquired β-
281 lactam resistance. *J Antimicrob Chemother* 2018; **73**: 88-94.

282 11. Bialek-Davenet S, Lavigne J-P, Guyot K, *et al*. Differential contribution of AcrAB and
283 OqxAB efflux pumps to multidrug resistance and virulence in *Klebsiella pneumoniae*. *J*
284 *Antimicrob Chemother* 2015; **70**: 81–8.

285 12. Veleba M, Higgins PG, Gonzalez G, *et al*. Characterization of RarA, a novel AraC family
286 multidrug resistance regulator in *Klebsiella pneumoniae*. *Antimicrob Agents Chemother*
287 2012; **56**: 4450–8.

288 13. Wan Nur Ismah WAK, Takebayashi Y, Findlay J, *et al*. Prediction of fluoroquinolone
289 susceptibility directly from whole genome sequence data using liquid chromatography-
290 tandem mass spectrometry to identify mutant genotypes. *Antimicrob Agents Chemother*
291 2018; **62**: e01814-17

292 14. Takebayashi Y, Wan Nur Ismah WAK, Findlay J, *et al*. Prediction Of Cephalosporin And
293 Carbapenem Susceptibility In Multi-Drug Resistant Gram-Negative Bacteria Using Liquid
294 Chromatography-Tandem Mass Spectrometry. *BioRxiv* 2017: 138594.

295 15. George AM, Hall RM, Stokes HW. Multidrug resistance in *Klebsiella pneumoniae*: a novel
296 gene, *ramA*, confers a multidrug resistance phenotype in *Escherichia coli*. *Microbiology*
297 1995; **141**: 1909–20.

298 16. De Majumdar S, Yu J, Spencer J, *et al*. Molecular basis of non-mutational derepression of
299 *ramA* in *Klebsiella pneumoniae*. *J Antimicrob Chemother* 2014; **69**: 2681–9.

300 17. Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial
301 Disk Susceptibility Tests – Ninth Edition: Approved Standard M2-A9. CLSI, Wayne, PA, USA.
302 2006.

303 18. Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial
304 Susceptibility Testing: Twenty-fourth Informational Supplement M100-S24. CLSI, Wayne, PA,
305 USA. 2014.

306 19. Pridmore RD. New and versatile cloning vectors with kanamycin-resistance marker. *Gene*

307 1987; 56:309-12.

308 20. Cronan JE. A family of arabinose-inducible *Escherichia coli* expression vectors having
309 pBR322 copy control. *Plasmid* 2006; **55**: 152–7.

310 21. Jiménez-Castellanos J-C, Wan Ahmad Kamil WNI, Cheung CHP, *et al*. Comparative effects
311 of overproducing the AraC-type transcriptional regulators MarA, SoxS, RarA and RamA on
312 antimicrobial drug susceptibility in *Klebsiella pneumoniae*. *J Antimicrob Chemother* 2016; **71**:
313 1820-5.

314 22. Coldham NG, Webber M, Woodward MJ, *et al*. A 96-well plate fluorescence assay for
315 assessment of cellular permeability and active efflux in *Salmonella enterica* serovar
316 Typhimurium and *Escherichia coli*. *J Antimicrob Chemother* 2010; **65**: 1655–63.

317 23. De Majumdar S, Veleba M, Finn S, *et al*. Elucidating the regulon of multidrug resistance
318 regulator RarA in *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* 2013; **57**: 1603–9.

319 24. Rodríguez-Martínez JM, Díaz de Alba P, Briales A, *et al*. Contribution of OqxAB efflux
320 pumps to quinolone resistance in extended-spectrum-β-lactamase-producing *Klebsiella*
321 *pneumoniae*. *J Antimicrob Chemother* 2012; **68**: 68–73.

322 25. Zhong X, Xu H, Chen D, *et al*. First Emergence of acrAB and oqxAB Mediated Tigecycline
323 Resistance in Clinical Isolates of *Klebsiella pneumoniae* Pre-Dating the Use of Tigecycline in a
324 Chinese Hospital Zhang Q, ed. *PLoS One* 2014; **9**: e115185.

325 26. Sheng Z-K, Hu F, Wang W, *et al*. Mechanisms of Tigecycline Resistance among *Klebsiella*
326 *pneumoniae* Clinical Isolates. *Antimicrob Agents Chemother* 2014; **58**: 6982-5.

327 **Table 1: Disc susceptibility testing for *K. pneumoniae* Ecl8 and its *ramR* and *oqxR* mutants**

Antibiotic disc (µg in disc)	Diameter of growth inhibition zone (mm) around antibiotic disc, or difference in zone diameter relative to control		
	Ecl8	***ramR***	***oqxR***
Ciprofloxacin (5)	37	-12	-15
Norfloxacin (10)	33	-9	-14
Ofloxacin (5)	31	-9	-11
Levofloxacin (5)	33	-8	-10
Chloramphenicol (30)	29	-9	-18
Cefotaxime (30)	37	-7	-4
Ceftizoxime (30)	36	-4	-2
Cephalothin (30)	25	-8	-5
Cefoxitin (30)	33	-13	-11
Cefotetan (30)	29	-3	NC
Cefuroxime (30)	30	-11	-9
Ceftazidime (30)	35	-7	-5
Ceftriaxone (30)	35	-6	-5
Cefoperazone (75)	32	-7	-5
Cefepime (30)	32	-3	NC
Aztreonam (30)	35	-3	-3
Imipenem (10)	27	NC	NC
Meropenem (10)	30	NC	NC
Doripenem (10)	27	NC	NC
Ertapenem (10)	29	NC	NC
Minocycline (30)	22	-10	-7
Tetracycline (30)	26	-6	-5
Tigecycline (15)	20	-5	-3
Amikacin (30)	25	NC	NC
Gentamicin (10)	23	NC	NC
Tobramycin (10)	23	NC	NC
Kanamycin (30)	23	NC	NC

328

329 Values reported are the means of three repetitions rounded to the nearest integer. Zone
330 diameters are reported for Ecl8; difference from the Ecl8 values are reported for the *ramR* or
331 *oqxR* loss of function mutants. NC means no change in zone diameter relative to Ecl8. Shading
332 represents non-susceptibility (resistance or intermediate resistance) according to breakpoints
333 set by the CLSI.[18]

334

Figure Legends

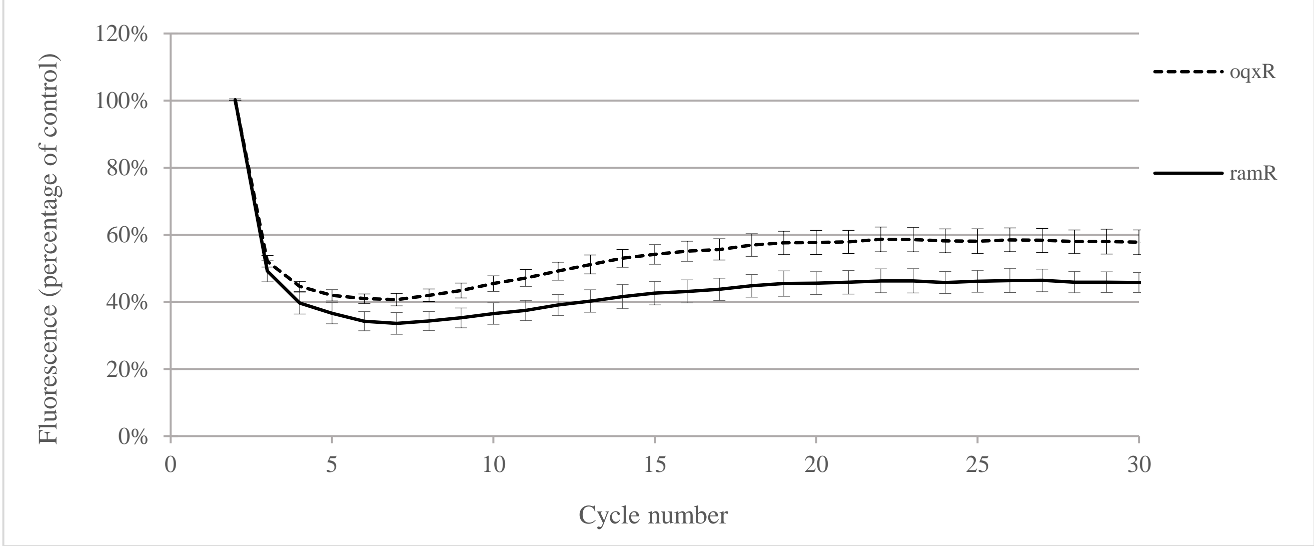
Figure 1: The accumulation of H33342 dye over a 30 cycle (45 minute) incubation period by *K. pneumoniae oqxR* and *ramR* mutants relative to Ecl8 wild-type. In each case, fluorescence of mutant cells incubated with the dye is presented relative to wild type Ecl8 cells (set to 100%) after each cycle. Each line shows mean data for three biological replicates with 8 technical replicates in each. Error bars define the standard error of the mean.

Figure 2: Abundance of key envelope proteins in *K. pneumoniae* Ecl8 wild-type and the *oqxR* and *ramR* mutants measured using LC-MS/MS proteomics. Values reported are absolute abundance values ($x10^{-7}$, arbitrary units) recorded by the instrument used. Data are means (n=3 biological replicates) error bars are standard error of the mean. Stars (*) above a bar indicate a significant difference in abundance relative to control based on the following rules: fold difference in abundance is $\geq$ 2 and $p<0.05$ for a T-test comparing absolute protein abundance data, n=3.

Figure 3: Envelope proteome changes in complemented mutants; Δ*ramR*+*oqxR*::pk18(*oqxR*) and Δ*ram*R+*oqxR*::pk18(*ramR*) in comparison with plasmid-only control. Values reported are absolute abundance values ($x10^{-7}$, arbitrary units) recorded by the instrument used. Data are means (n=3 biological replicates) error bars are standard error of the mean. Stars (*) above a bar indicate a significant difference in abundance relative to control based on the following rules: fold difference in abundance is $\geq$ 2 and $p<0.05$ for a T-test comparing absolute protein abundance data, n=3.

361 **Figure 1**

362



363

364

365

366

367

368

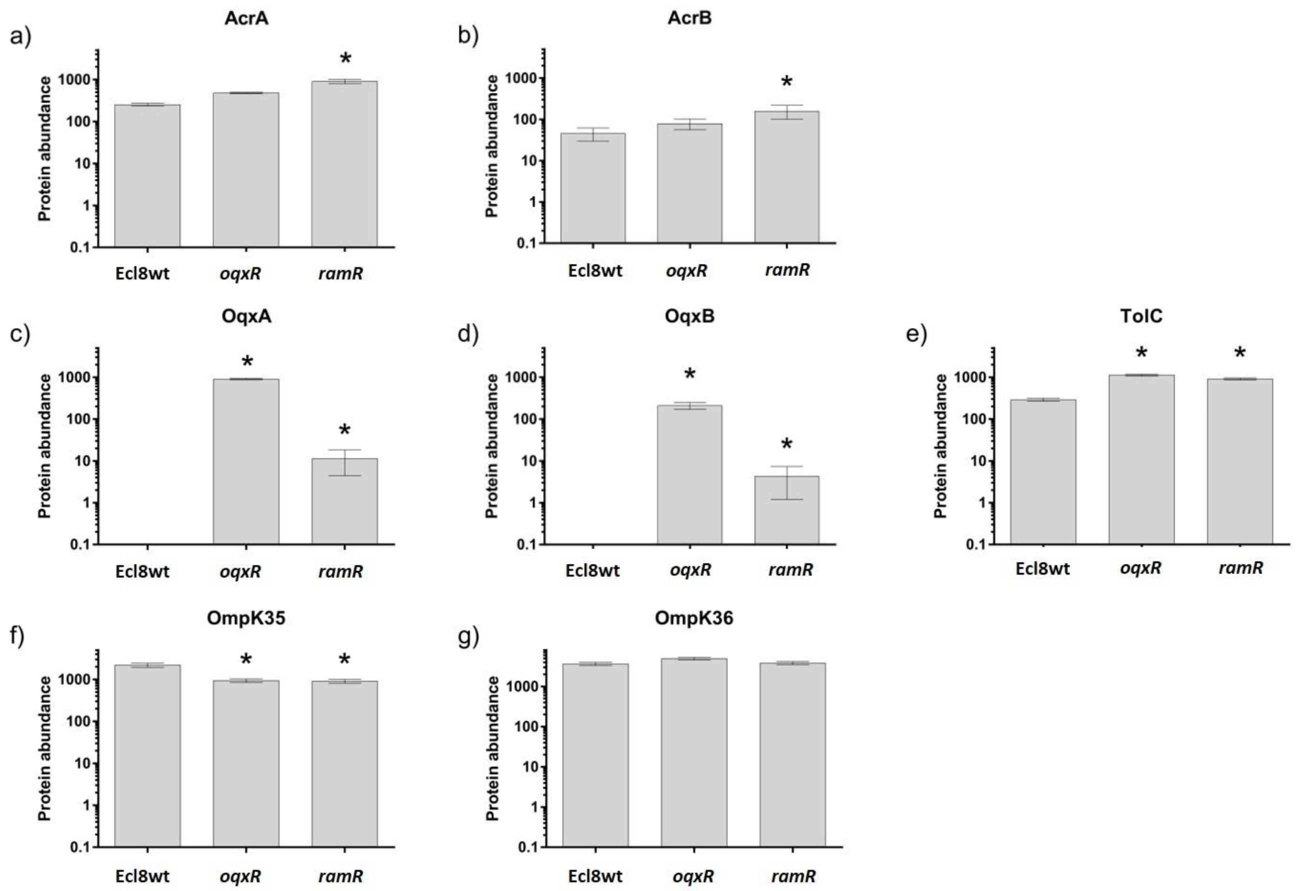
369

370

371

372

373 **Figure 2.**

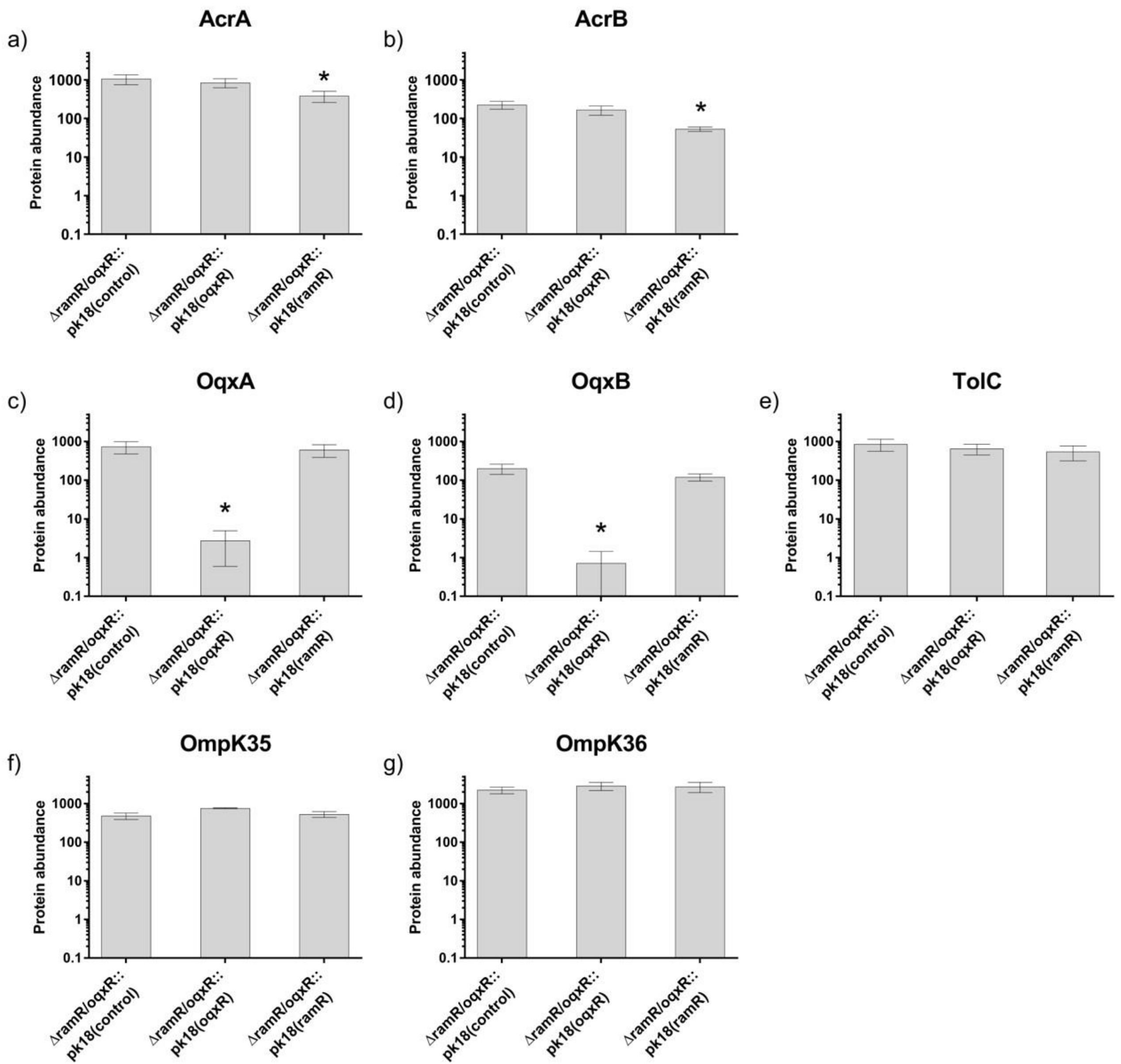


374

375

376 **Figure 3.**

377



378