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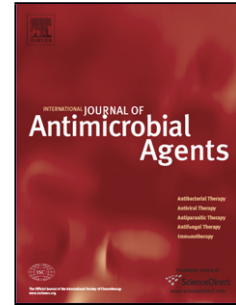
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1 **Genetic Regulation of the ramA Locus and its' Expression in Clinical Isolates**

2 ***Klebsiella pneumoniae***

3 **Running Title:** Genetic Regulation of ramA expression in *Klebsiella pneumoniae*

4

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Accepted Manuscript

27 **Abstract**

28 Tigecycline resistance has been attributed to ramA overexpression and subsequently acrA  
29 upregulation. The ramA locus, originally identified in Klebsiella pneumoniae has homologs in  
30 Enterobacter and Salmonella spp. In this study we identify in-silico that the ramR binding site is  
31 also present in Citrobacter spp and that Enterobacter, Citrobacter and Klebsiella spp. share key  
32 regulatory elements in the control of the romA-ramA locus. RACE mapping indicates that there  
33 are two promoters from which romA-ramA expression can be regulated in K. pneumoniae.  
34 Correspondingly electrophoretic binding studies clearly show that purified RamA and RamR  
35 proteins bind to both these promoters. Hence there appear to be two RamR binding sites within  
36 the Klebsiella romA-ramA locus. Like MarA, RamA binds the promoter region implying that it  
37 might be subject to autoregulation. In geographically distinct clinical isolates of K. pneumoniae,  
38 we have identified changes within ramR. Intriguingly the levels of romA and ramA expression  
39 were not uniformly affected by changes within the ramR gene thereby supporting our dual  
40 promoter finding. Furthermore, a subset of strains sustained no changes within the ramR gene but  
41 which still overexpressed the romA-ramA genes strongly suggesting that a secondary regulator  
42 may control ramA expression.

43

44 **Keywords:** Klebsiella pneumoniae, romA, ramA, ramR, AcrA, tigecycline

45

## 46 **1 Introduction**

47 *Klebsiella pneumoniae* is a major nosocomial pathogen that causes both community and hospital  
48 acquired infections. Strains with chromosomal and/or plasmidic resistance mechanisms coupled  
49 with efflux / influx related mutations are increasingly identified [1-3]. The continued  
50 antimicrobial challenge of *K. pneumoniae* has precipitated the emergence of clones harbouring a  
51 plethora of resistance mechanisms to clinically relevant antibiotics (e.g. fluoroquinolones, third  
52 generation cephalosporins and carbapenems) where emerging pan-drug resistant clones have left  
53 few therapeutic strategies available to combat this pathogen [4].

54  
55 Tigecycline is a new glycycline with substantial anti Gram-negative activity that has been  
56 introduced for the treatment of community acquired Gram-negative infections caused by ESBL  
57 producing *K. pneumoniae* and *Escherichia coli* [5] where a recent study has shown that  
58 tigecycline is effective against pan-drug resistant *K. pneumoniae* and *Enterobacter* spp.  
59 Tigecycline is able to evade the classical mechanisms that confer resistance to tetracycline and  
60 minocycline such as efflux Tet(A-E) and ribosomal protection conferred by Tet(M) [5]. However  
61 tigecycline appears vulnerable to efflux by chromosomally encoded efflux pumps [6-10].

62  
63 As such, studies into tigecycline resistance in members of *Enterobacteriaceae* have shown that it  
64 is mediated via the upregulation of efflux pumps which are controlled by certain regulatory loci  
65 [6-8, 10]. For instance, several studies demonstrate that tigecycline resistance results from the  
66 upregulation of AraC-family transcriptional regulators such as MarA or RamA which in turn are  
67 linked to increased expression of the *acrAB* efflux pump [6-7, 10-11]. This pump not only  
68 functions as a clinically relevant drug exporter but also has been demonstrated to significantly

69 affect the virulence potential of the bacterium [12-13]. The AcrAB efflux pump is locally  
70 controlled by the transcriptional repressor AcrR [14]. However, transcription factors such as  
71 RamA and MarA are able to override AcrR-mediated repression and upregulate the expression of  
72 the *acrAB* efflux pump [6-7, 10-11]. Accordingly strains that have wildtype AcrR and increased  
73 RamA or MarA expression are linked to increased *acrAB* levels [11]. Whether levels of AcrA  
74 upregulation in the absence of *acrR* mutations are as significant as those seen when *acrR* is  
75 mutated is not clear.

76  
77 RamA is a member of the AraC/XylS family and is closely related to the MarA and SoxS  
78 proteins [15]. The *ramA* locus is only found in *Klebsiella*, *Salmonella*, *Enterobacter* and  
79 *Citrobacter* spp where the genetic organization in *Salmonella* differs from the others due to the  
80 lack of the *romA* gene. Like MarA, increased *ramA* expression is linked to the upregulation of the  
81 *acrAB* efflux pump which confers a multidrug resistant phenotype (MDR) to a variety of  
82 different antibiotic classes [7, 10-11, 16]. This phenotype is mediated exclusively through the  
83 *acrAB* gene as *ramA* overexpression in an *acrAB*-deleted strain does not produce a similar  
84 phenotype [11].

85  
86 The expression of the *ramA* gene is controlled at a transcriptional level and as such studies in  
87 both *S. typhimurium* [17] and *K. pneumoniae* [16] have identified a *tetR*-like gene, called *ramR*,  
88 that lies upstream of the *ramA* gene and acts as its repressor (See Fig. 1A & 1B). Of note, the  
89 stop codon of the *ramR* gene overlaps the start codon of the gene *ybdF* implying that the  
90 regulation of these genes is very likely linked. Studies into both, *S. typhimurium* [17] and *K.*  
91 *pneumoniae* [16], have reported that *ramR* mutations are directly linked to *ramA* overexpression.

92 In both *Salmonella* and *Klebsiella*, bioinformatic analyses suggest that the RamR protein binds a  
93 palindromic sequence that is either overlapping or downstream of the -10 sequence of the *ramR*  
94 gene [16-17]. In *K. pneumoniae* and *Enterobacter* spp. the genomic organization of *ramR*, its  
95 corresponding palindromic binding sites and *ramA* is conserved when compared to *Salmonella*,  
96 although these bacteria also harbour the *romA* gene (See Figure 1A & 1B). Mutations within the  
97 *ramR* gene have been shown to result in *ramA* upregulation [16-17] however the effect of this  
98 derepression on the surrounding genes within the locus, i.e. *romA*, is not known.

99  
100 In studies involving clinical strains, *ramA* upregulation is not normally the sole mechanism of  
101 resistance but appears to act in combination with other mutations, for example, cefuroxime  
102 resistant isolates also showed a decrease in the levels of the outer membrane protein, OmpK35  
103 [18] and in fluoroquinolone resistant isolates the upregulation of the *ramA* gene was in  
104 association with target-specific mutations in the topoisomerase genes (e.g. *gyrA* and *parC*) and  
105 mutations within *acrR*, the repressor of the *acrAB* efflux pump [11]. Hence the aims of this study  
106 were, firstly to elucidate the genetic regulation of the *ramA* locus and secondly whether its  
107 overexpression is always linked to changes within the *ramR* gene.

108



## 109 **2 Materials and Methods**

### 110 *2.1 Clinical Isolates*

111 All isolates were identified as *K. pneumoniae* and have been isolated from intensive care wards in  
112 hospitals within Chile, Turkey and Pakistan from 2006-2008. Three strains from Singapore  
113 characterised in a previous study [11] and the original strains (Ecl8 and Ecl8Mdr1) described by  
114 George et al [15] were also included. Other strains used as controls in this study were: for the  
115 validation of the AcrA western blot analyses, AG100A (deleted for *acrA*) and AG100B (deleted  
116 for *acrR*, AcrA overexpresser); for the validation of the *ramA* rt-PCR TS67 (deleted for *ramA*)  
117 and TS68 (deleted for *ramR*) [19]. The mutants TS67 and TS68 were generated from *K.*  
118 *pneumoniae* Ecl8 using a modified protocol as described by Merlin et al [20].

119

### 120 *2.2 MIC testing*

121 The minimum inhibitory concentrations to tigecycline (gift from Wyeth Pharmaceuticals) for the  
122 clinical isolates were determined and interpreted according to the BSAC protocols [21]. Strains  
123 exhibiting  $\leq 1\text{mg/l}$  and  $> 2\text{mg/l}$  to tigecycline were classed as sensitive and resistant respectively.

124

### 125 *2.3 Mapping the transcriptional start sites of the ramA locus*

126 The transcriptional start site of the *ramA* locus was mapped according to the manufacturer's  
127 instructions using the 5' RACE system (Invitrogen, Carlsbad, California). Briefly, 0.5 $\mu\text{g}$  of RNA  
128 extracted at mid-log phase was reverse transcribed into first strand cDNA which was then TdT-  
129 tailed. Subsequently the TdT-tailed cDNA was amplified by PCR and assessed for positive PCR  
130 products. A second round of amplification using nested gene specific primers (Table 1) and

131 Abridged Universal Amplification Primer (AUAP) was performed prior to the resulting products  
132 being sub-cloned into pGEMT-easy (Promega, UK) for sequencing.

133

#### 134 *2.4 Gene Expression Analyses using RT-PCR*

135 In order to assess the *ramA* and *romA* levels of gene expression, RT-PCR was performed. RNA  
136 samples were extracted from the different clinical isolates at mid-log phase (OD<sub>600</sub> 0.5-0.8) using  
137 Trizol. Total RNA was DNaseI digested to ensure the removal of contaminating genomic DNA  
138 prior to cDNA synthesis. For cDNA synthesis, the Superscript Vilo cDNA synthesis kit  
139 (Invitrogen, Carlsbad, California, USA) was used. Briefly, 300ng of DNase I treated total RNA  
140 was converted to cDNA and used in PCRs with gene specific primers shown in Table 1. A 1 in  
141 10 cDNA dilution was used for the amplification of 16S gene. The resulting PCR products were  
142 subjected to electrophoresis on a 1.5% agarose gel. Densitometric analyses using the Multigauge  
143 FujiFilm software of the gel bands were performed and normalised to the 16S expression. The  
144 fold increase of *ramA* expression relative to the sensitive strain Ecl8 (non *ramA* expresser) is  
145 shown in Table 3A.

146

#### 147 *2.5 Electrophoretic Mobility Shift Assays (EMSA)*

148 The *romA* - *ramA* promoter regions were amplified and subjected to EMSA with both purified  
149 RamA and RamR proteins. Purified RamA and RamR proteins were extracted from recombinant  
150 pET constructs containing the *ramA* and *ramR* genes using the metal chelation chromatography  
151 on superflow nickel / nitrilotriacetate agarose (Qiagen). Briefly, end labelled (using [ $\gamma$ -<sup>32</sup>P] ATP,  
152 Perkin Elmer, Boston, USA) PCR products were incubated with increasing concentrations (200  
153 nM, 400 nM) of RamA or RamR in binding buffer (125mM Tris-Cl, 250mM KCl, 5mM DTT,

154 160ng salmon sperm DNA and 25% glycerol). The complexes were run on 5% native PAGE gels  
155 for 2.5 hours. The gel was then dried and exposed to the phosphor screen for image analysis. To  
156 confirm that the interactions between RamA or RamR and the promoter regions were specific,  
157 competition experiments with BSA as a negative control and cold promoter were also performed.

158

### 159 *2.6 Mutations within the Promoter Regions, ramR and acrR Genes*

160 Only for those strains where ramA expression was elevated, the ramR, acrR genes and pI /pII  
161 promoter regions were amplified using primers shown in Table 1. The two promoter regions pI  
162 and pII were also amplified using the relevant promoter primers also shown in Table 1. The BIG  
163 Dye reaction (Applied Biosystems) was set up prior to the products being sequenced at the  
164 Genomics Core Facility in Belfast City Hospital.

165

### 166 *2.7 Western Blot Analyses*

167 The levels of AcrA were determined by Western blot analyses as described previously in  
168 Schneiders *et al* [11]. Briefly, cultures were grown to mid-log phase ( $OD_{600} \sim 0.6-0.7$ ) prior to  
169 protein extraction using sonication. Fifteen micrograms of total protein was loaded onto a 10%  
170 NuPage gel (Invitrogen, Carlsbad, California) prior to transfer to a nitrocellulose membrane as  
171 per the manufacturer's instructions (Invitrogen). The blots were hybridised with the primary  
172 antibody anti-AcrA (Kind gift from H. Zgurskaya, University of Oklahoma, Norman, USA, 1:  
173 40,000) over night at 4°C. The secondary antibody IRDye-800 goat anti-rabbit IgG (Licor  
174 BioSciences, Nebraska, USA) was incubated with the blots for 1 hour at room temperature and  
175 the membranes were scanned using the Odyssey Infrared Imaging system which is based on near  
176 infrared fluorescence detection. The membrane is scanned in two channels at 700 and 800nm that

177 detects the green and red channels respectively. All images were quantified using the reading  
178 from the 800nm channel. Analysis of the band intensities to assess AcrA levels was performed  
179 using the Odyssey software v3 (Licor BioSciences, Nebraska, USA). The levels of the AcrA  
180 protein of all test strains were compared against the sensitive *K. pneumoniae* strain Ecl8 to report  
181 a relative fold increase in AcrA levels. Both AG100A and AG100B were also used as controls in  
182 the AcrA western blots.  
183

## 184 **3 Results**

### 185 *3.1 Minimum Inhibitory Concentration*

186 The MIC<sub>50</sub> and MIC<sub>90</sub> values of tigecycline, minocycline, tetracycline, ciprofloxacin and  
187 ceftazidime against the strains are shown in Table 2. Of the 157 strains tested, 24 were resistant  
188 to tigecycline based on the BSAC breakpoint criteria [21]. The MICs to tigecycline generally  
189 ranged from 0.25 - 4 µg/ml. The minocycline and tetracycline MIC<sub>90</sub>s were 128µg/ml and  
190 >128µg/ml respectively, however cross-resistance to tigecycline was not observed, consistent  
191 with previous survey data [22]. Tigecycline resistant isolates exhibited cross-resistance to  
192 ceftazidime and ciprofloxacin.

193

### 194 *3.2 Bioinformatic Analyses for RamR binding sites in K. pneumoniae (Accession No:*

195 *NC\_009648), Citrobacter koseri (Accession No: NC\_009648) and Enterobacter sp. 638*  
196 *(Accession No: NC\_009648)*

197 In *S. typhimurium*, ramR, has been shown to function as its cognate negative regulator [17].

198 Given the similarities in the genomic organization of the ramR and romA-ramA loci between

199 Klebsiella, Enterobacter, Citrobacter and Salmonella, we sought to confirm whether ramR

200 controlled the expression of the romA-ramA locus directly in Klebsiella and whether other

201 accessory binding sites would exist for RamR within the romA-ramA locus. Bioinformatic

202 analyses (using ClustalW: <http://www.ebi.ac.uk/Tools/clustalw2/index.html>) shows that the

203 palindromic binding sites identified for RamR in Salmonella spp. is conserved within the

204 intergenic region between the ramR and romA coding sequences of K. pneumoniae, Enterobacter

205 and Citrobacter spp. (Fig. 1A). There appeared to be no perfect palindromic site for RamR

206 upstream of the *ramA* gene. Thus we surmised that the regulation of the *romA-ramA* locus  
207 mediated by RamR must occur via the palindromic sequence that lies upstream of the *romA* gene.

208

### 209 3.4 RACE Mapping

210 The transcriptional start site of *ramA* as determined by RACE mapping is shown in Figure 1B.

211 The transcriptional start site (hereby called pII, Fig. 1B) for *ramA* was found upstream of the

212 *romA* stop codon which implies that *romA* and *ramA* are part of an operon and are likely co-

213 transcribed in *K. pneumoniae*. The transcriptional start site mapped corresponds with our

214 previous observation that two transcripts sized at ~0.6kb and ~0.9kb were obtained with northern

215 blotting. The larger transcript (~0.9kb) must correspond to the promoter that lies upstream of the

216 *romA* gene. The presence of the pII promoter also suggests that *ramA* expression can be

217 modulated independently of *romA*. Correspondingly, this differential regulation is evident in the

218 differing levels of transcription of the *romA* and *ramA* genes as shown in Fig. 3A.

219

### 220 3.5 Electrophoretic Mobility Shift Assays

221 Bioinformatic analyses indicates the presence of a putative but conserved palindromic binding

222 site for RamR within the pI region, however no such site could be observed for the pII promoter.

223 We also wondered whether RamA, like other similar regulators i.e. MarA, autoregulates itself by

224 binding to its own promoter region. The intergenic regions, designated pI and pII, bound both

225 purified RamR and RamA proteins (Fig. 2A). The interaction of the pI and pII promoter regions

226 with the RamA and RamR proteins were found to be specific, as no shifts were observed with the

227 BSA alone (Fig. 2A) and addition of cold promoter was able to reduce binding of the proteins to

228 the labelled probe (data not shown). Of note, the fragment of DNA (pII\*) that contained no

229 transcriptional signals was found not to bind either RamA or RamR (Fig. 2B). As indicated  
230 previously the pI promoter has a conserved RamR binding site which is required for RamR-  
231 mediated control. However the lack of a similar or identical RamR binding site (by bioinformatic  
232 analyses) indicates that the RamR-mediated regulation of the pII promoter may be mediated via a  
233 more degenerate palindromic binding site. Accordingly bioinformatic analyses (using the  
234 EMBOSS Pairwise Alignment Tool: <http://www.ebi.ac.uk/Tools/emboss/align/>) of the pII  
235 promoter region implies that there is a putative but degenerate palindromic site upstream of the  
236 *ramA* gene (Fig. 1A).

237

238 *3.6 DNA Mutations in the promoter regions pI and pII, ramR and acrR.*

239 *3.6.1 Mutations within the ramR gene and the levels of romA, ramA expression*

240 Of the 24 strains that were resistant or intermediately susceptible to tigecycline, only 10  
241 harboured mutations within the *ramR* gene. Changes within the *ramR* gene were found in both  
242 the DNA and ligand binding domains of the protein (Prediction based on PSIPREDv3 [23])  
243 suggesting that there are no mutational hotspots within *ramR* although the majority of changes  
244 were clustered within the ligand-binding domain. As previously suspected not all the changes  
245 observed within RamR resulted in *ramA* expression, for example changes H186N, A187E  
246 observed in TS202 and E41K in TS215 did not result in *ramA* overexpression. The change,  
247 I141T, was observed in four of the isolates tested (Ecl8, Ecl8Mdr1, TS262 and TS293) (Table  
248 3A). Since this change is also found in the sensitive *Klebsiella* strain Ecl8 it is likely that it is not  
249 associated with *ramA* overexpression. Additionally strains (TS173, TS221, TS257 and TS259)  
250 that did not harbour changes within the *ramR* gene (Table 3A) and/or the intergenic regions but  
251 still overexpressed *ramA* (Fig. 3A). Several strains that exhibited MICs of  $\geq 4$ mg/l were found to

252 overexpress ramA but not harbour any changes within the ramR gene. Interestingly, the levels of  
253 ramA expression between those strains that harboured changes within ramR were not higher than  
254 those strains that sustained no changes within the gene. Additionally transcriptional levels of the  
255 romA gene did not appear linked to ramA expression (Fig. 3A). Although increased expression of  
256 romA was generally associated with strains that harboured changes within the ramR gene (Fig.  
257 3A). Ideally, complementation studies with wildtype ramR would have been performed to  
258 confirm whether the ramR changes were directly associated with ramA overexpression. However  
259 this was not possible due to the multidrug resistance profiles of the different strains.

260

### 261 3.6.2 Promoter (*pI* and *pII*) Mutations

262 Unlike previous reports, the majority of the clinical isolates did not harbour any changes within  
263 the pI promoter. However the lack of promoter associated changes linked to increased ramA  
264 expression is not unique to these cluster of isolates as the original ramA overexpressing strain  
265 Ecl8MdrI (as reported by George et al.), also does not harbour any changes within the ramR  
266 gene, the palindrome or the -10 and -35 hexamers at the pI promoter. Only one strain TS170  
267 which sustained a change within the palindromic sequence (C → T, recognized by RamR) but  
268 harboured no changes within the ramR gene was found. Correspondingly TS170 exhibited a  
269 small increase in ramA expression (Fig. 3A). Several changes associated with the pII promoter  
270 region were located downstream of the second putative palindrome but upstream of the RACE  
271 mapped transcriptional start site of ramA (Fig. 1B). Three changes (206: T → C, 229: G → A,  
272 235: A → T) were consistently identified in four strains (S7, TS152, TS165 and TS261) (Table  
273 3B), all of which overexpressed ramA. Of these strains, only S7 and TS261 harboured no changes  
274 within the ramR gene (Table 3A).



275 3.6.3 Mutations within the *acrR* gene and levels of the AcrA protein

276 Nine out of the 27 strains tested sustained changes within the *acrR* gene. Of these changes only  
277 two (T5N, L214F) have been previously documented [11]. The other nucleotide substitution but  
278 not amino acid change, E147E, was found in a region previously shown to be implicated in AcrA  
279 overexpression. One new silent change (P155P CCC→CCT) was observed. Given that most of  
280 the changes observed were silent it is unlikely that they would have contributed significantly to  
281 the AcrA levels. Strains (TS165, TS173, TS257 and TS293) which significantly overexpress  
282 *ramA* appear not to produce greater levels of the AcrA protein (Fig. 3B).

283

284 **4. Discussion**

285 Recent studies have linked antibiotic resistance, particularly tigecycline resistance, to the  
286 increased expression of the *ramA* gene. In this study we identify in silico that key regulatory  
287 features of the *ramA* locus are conserved amongst *Klebsiella*, *Enterobacter*, *Citrobacter* and  
288 *Salmonella* spp.

289  
290 Our work also shows that both the RamR and RamA proteins bind the pI and pII promoters  
291 specifically implying that the presence of recognition sites for both proteins within these  
292 promoter regions. Like other orthologous systems such as MarRAB, SoxRS [24], the binding of  
293 purified RamA to the pI and pII promoters indicates that *ramA* autoregulates its own expression.  
294 Additionally RamR is appears to be able to bind regions that either contain a perfect palindrome  
295 or more degenerate palindromic sites (Fig. 1A & 2A). Other TetR-like regulators have been  
296 shown to regulate via palindromic binding sites that are identified as lower affinity binding sites  
297 due to key differences in the palindromic binding sequence.

298  
299 Our results show that the regulation of the *romA-ramA* locus is not entirely identical to that  
300 observed in *Salmonella* as there are two promoters within the *romA-ramA* locus. The presence of  
301 these two promoters supports the possibility that the *romA-ramA* genes can be regulated  
302 independently of each other or as part of an operon (Fig. 3A). Consistent with this genetic  
303 arrangement, changes were identified within the pI and putative pII promoter regions in the *ramA*  
304 overexpressing strains (Table 3B). Interestingly the levels of *romA* and *ramA* expression are not  
305 linked supporting our finding that two different promoters control the regulation of the *romA*-  
306 *ramA* locus (Fig. 3A).

307 We chose to extend our molecular findings by determining the regulation of *ramA* expression in  
308 clinical isolates that were resistant or intermediately susceptible to tigecycline. Our results  
309 demonstrate that in geographically diverse isolates of *K. pneumoniae*, 17 isolates exhibited  
310 tigecycline MICs of  $\geq 4$ mg/l (Table 3A). A notable example is the strain, TS 257, which exhibits a  
311 tigecycline MIC of 16mg/l, where no mutations were observed within the *ramR-romAramA* locus  
312 but still overexpressed the *ramA* gene. Two key observations arise from our data, the first where  
313 *ramA* is not always associated with *ramR* mediated derepression and secondly decreased  
314 tigecycline susceptibility is not always associated with *ramA* expression. We also did not observe  
315 any significant mutations within the promoter regions as has been observed previously [16-17].  
316 An interesting point is that the isolates in this study pre date the use of tigecycline in the relevant  
317 hospitals, which underscores the likelihood that most antibiotics have the propensity to select for  
318 changes that result in *ramA* overexpression, a similar situation that is noted for the *marRAB* and  
319 *soxRS* systems [24]. The lack of a direct association between *ramA* and *acrA* expression has been  
320 noted previously [25] and supports our data.

321  
322 The overexpression of transcriptional regulators such as RamA triggers the expression of a  
323 multitude of genes that confer pleiotrophic phenotypes ([26], Schneiders T. unpublished data).  
324 Given the varied bacterial response that is mounted with *ramA* overexpression, we should  
325 consider the broader implications of these intrinsic resistance mechanisms in the development  
326 and persistence of antimicrobial resistance.

327

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331

332

333

334 **Declarations**

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338 **Competing Interests:** No conflict of interests

339 **Ethical Approval:** Not applicable

340

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412

413



415 **Figure and Table Captions:**

416 **Table 1:** showing primer sequences used in this study

417 **Table 2:** MIC<sub>50</sub> and MIC<sub>90</sub> (µg/ml) values for 157 clinical isolates.

418 **Legend:** CIP = ciprofloxacin, CAZ = ceftazidime, TET = tetracycline, MIN = minocycline, TGC  
419 = tigecycline

420

421 **Figure 1A:** Alignment of the RamR palindromic binding sequence at the pI and pII promoter in  
422 *Salmonella*, *Klebsiella*, *Enterobacter* and *Citrobacter* spp.

423

424 **Figure 1B:** Regulatory Elements within the *romA-ramA* locus

425 The inverted palindromic sequences are in bold and indicated by arrows. The two putative  
426 promoters are depicted as pI and pII. The transcription start of *ramA* as mapped by 5' RACE is  
427 indicated by +1. The translation start site of both *romA* and *ramA* is indicated in bold and  
428 superscript. All promoter changes are in grey with those hypothesized to be associated with  
429 *ramA* overexpression in grey and with an asterisk.

430

431 **Table 3A:** Association between RamR and AcrR changes, *ramA* and Tigecycline MICs

432

433 **Table 3B:** Changes observed within the pI and pII promoter regions

434

435 **Figure 2:** EMSA of the promoter regions with purified RamA or RamR proteins.

436 **A.** Electrophoretic Mobility Shift Assay showing the binding of purified RamR and RamA to the  
437 pI and pII promoter regions. **B.** pII\* represents the region underlined (dashed lines) in Figure 1B.

438

439 **Figure 3A:** Barchart showing levels of *romA* and *ramA* expression in clinical strains.  
440 The levels of *romA* and *ramA* were normalised to *K. pneumoniae* Ecl8 (non-*ramA* expresser).  
441  
442 **Figure 3B:** Barchart showing levels of AcrA expression.  
443 Relative fold increases in the AcrA levels were quantified after comparisons with a wild type  
444 sensitive *K. pneumoniae* isolate Ecl8.  
445

446 Table 1

Primers	Sequence
ramAF	5'-AGCCTGGGGCGCTATATT 3'
ramAR	5'-GTGGTTCTCTTTGCGGTAGG 3'
romAF	5'-GAAGCGTAACCAGACGCTGT 3'
romAR	5'-CTGGTCATACTGCCCGTTCT 3'
ramRF	5' AACTGCAGTCGTCAAGACGATTTTCAATTTT 3'
ramRR	5' AAAAGTACTAGTGTTTCCGGCGTCATTAG 3'
acrRF	5' TTAAGCTGACAAGCTCTCCG 3'
acrRR	5' ACGTAACCTCTGTAAAGTCAT 3'
pIF	5' GGGCCAGTTTTCTGTT 3'
pIR	5' ATAGTATCAATCACCTGAGC 3'
pIIF	5' CTACTIONTTTTTCCTCACGCAG 3'
pIIR	5' CCCTGCGGCGCCTTACCA 3'
16SF	5' GTTACCCGCAGAAGAAGCAC 3'
16SR	5' CTACGCATTTACCGCTACA 3'
ramAR1	5' TTGCAGATGCCATTTCTGA 3'
ramAR2	5' TATCATCAATACGCAGCG 3'
ramAR3	5' GGGGTACCATAGTATCAATCACCTGAGC 3'

447

448

449 Table 2

	MIC <sub>50</sub>	MIC <sub>90</sub>
CIP	128	>128
CAZ	>128	>128
TET	>128	>128
MIN	32	128
TGC	1	2

450

451 Table 3A

Strain	RamR	AcrR	TGC MIC ( $\mu\text{g/ml}$ )	Origin
S7	No change	[11]	8	Singapore
S28	15nt $\Delta$ (558-573bp)	[11]	2	Singapore
S29	7nt insertion at nt position 561	[11]	16	Singapore
TS 152	T119P	No change	4	Turkey
TS 165	T119P	GAG440GAA ( E147E)	4	Turkey
TS 170	No change	No change	2	Turkey
TS 173	No change	No change	2	Turkey
TS 184	No change	No change	4	Turkey
TS 202	H186N, A187E	No change	2	Chile
TS 215	E41K	CCC465CCT (P155P)	4	Chile
TS 221	No change	GAG440GAA ( E147E)	2	Chile
TS 238	A19V	GAG440GAA ( E147E)	4	Pakistan
TS 240	No change	CCC465CCT (P155P)	4	Pakistan
TS 245	No change	TTG641TTT (L214F)	4	Pakistan
TS 248	No change	ACC14AAC (T5N)	4	Pakistan
TS 250	No change	GAG440GAA ( E147E)	4	Pakistan
TS 251	No change	No change	8	Pakistan
TS 257	No change	No change	16	Pakistan
TS 259	No change	No change	2	Pakistan
TS 261	No change	GAG440GAA ( E147E)	4	Pakistan
TS 262	I141T	No change	4	Pakistan
TS 267	No change	No change	4	Pakistan
TS 293	I141T	CCC465CCU (P155P)	4	Pakistan
TS 308	W94Stop	No change	4	Pakistan

452

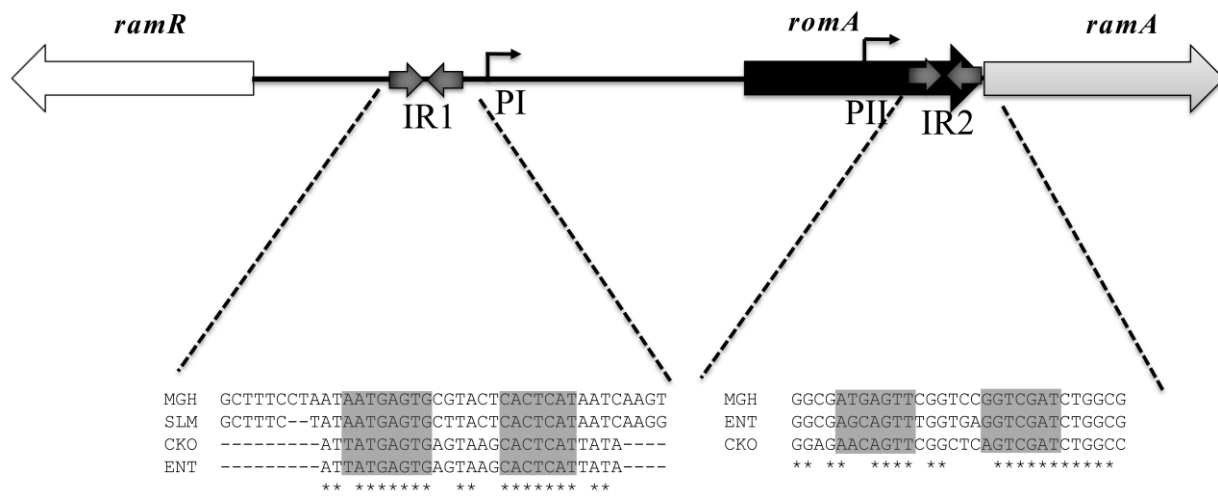
453 Table 3B

Strain	pI	pII
S7	t141c	<b>t206c</b>
S28	---	---
S29	---	---
TS 152	---	<b>t206c, g229a, a235t</b>
TS 165	---	
TS 170	t141c, c56t*	---
TS 243	t141c	---
TS 245	t141c	---
TS 248	t141c	---
TS 250	t141c	---
TS 251	t141c	---
TS 257	t141c	---
TS 261	t141c	<b>t206c, g229a, a235t</b>

454

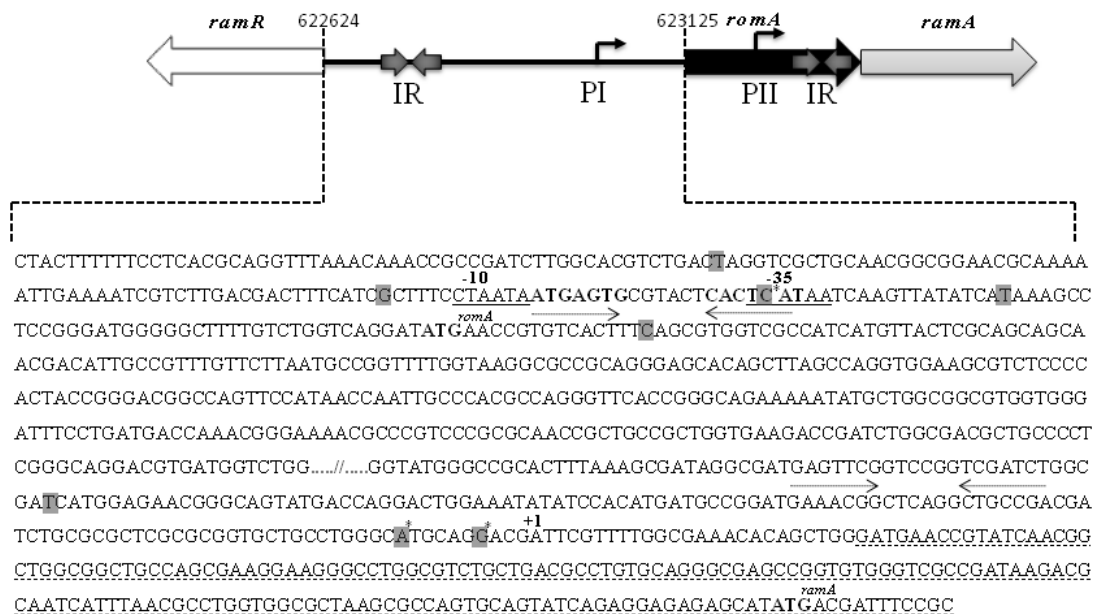
455 Figure 1

456 A)



457

458 B)

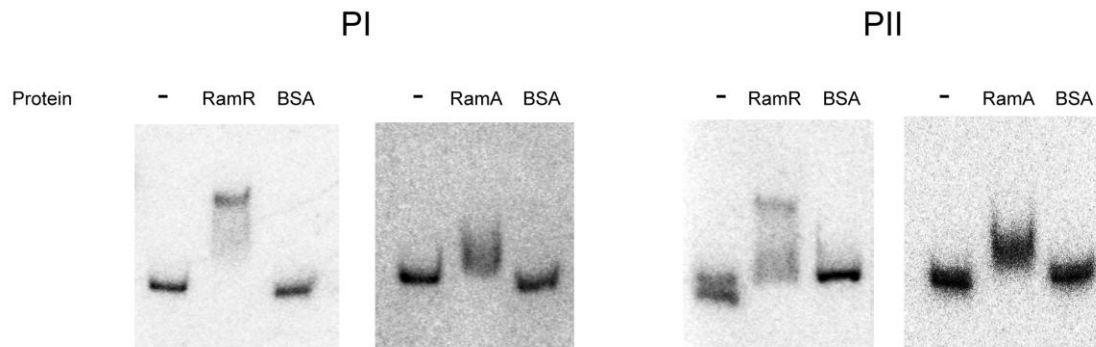


459  
 460 The inverted palindromic sequences are in bold and indicated by arrows. The two putative  
 461 promoters are depicted as pI and pII. The transcription start of *ramA* as mapped by 5' RACE is  
 462 indicated by +1. The translation start site of both *romA* and *ramA* is indicated in bold and  
 463 superscript. All promoter changes are in grey with those hypothesized to be associated with  
 464 *ramA* overexpression in grey and with an asterisk.

29

465 Figure 2

466 A) pI and pII promoter regions



467

468 B) pII\* promoter region

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476 A. Electrophoretic Mobility Shift Assay showing the binding of purified RamR and

477 RamA to the pI and pII promoter regions.

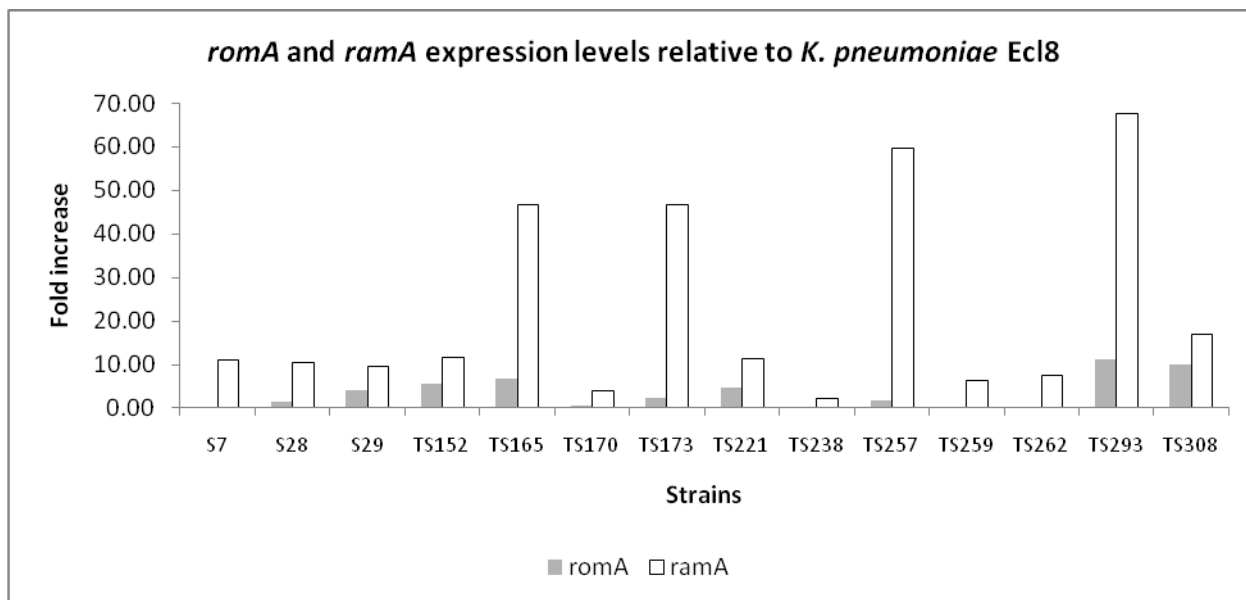
478 B. pII\* represents the region underlined (dashed lines) in Figure 1B.

479



480 Figure

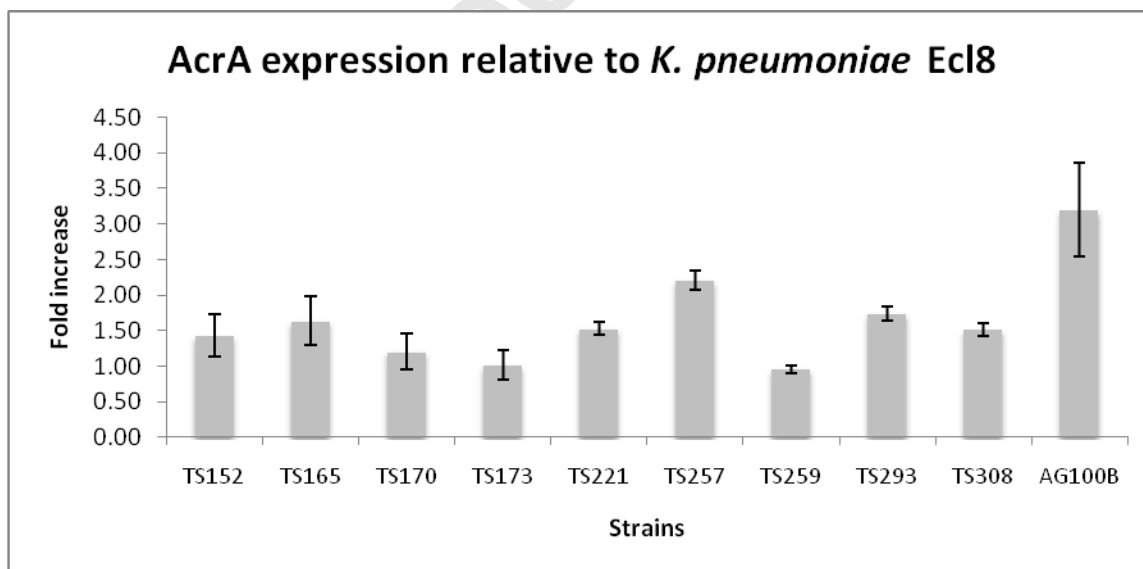
481 3A)



482

483

484 3B)



485

486

31

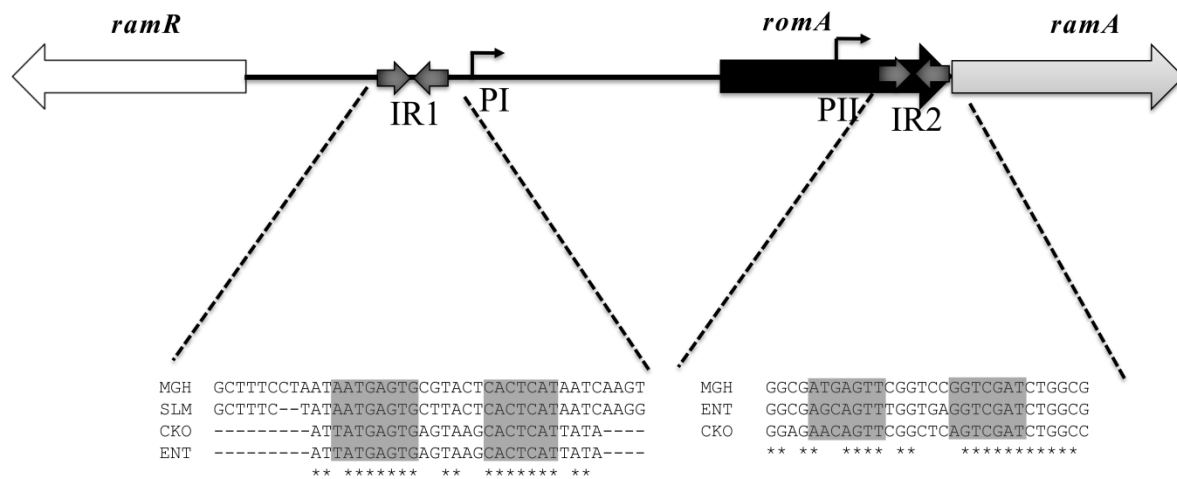


Figure 1A

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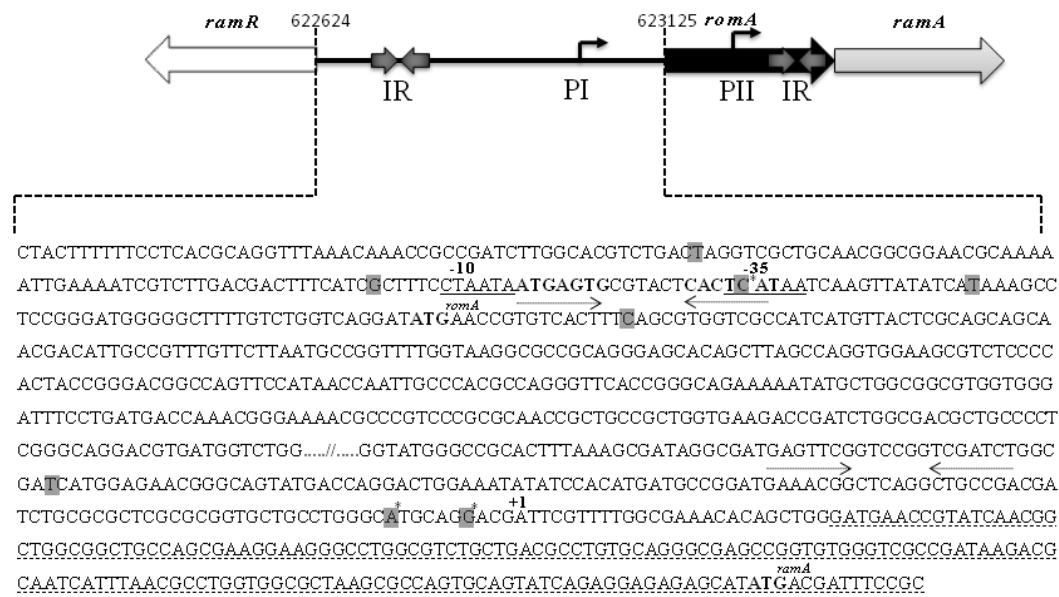
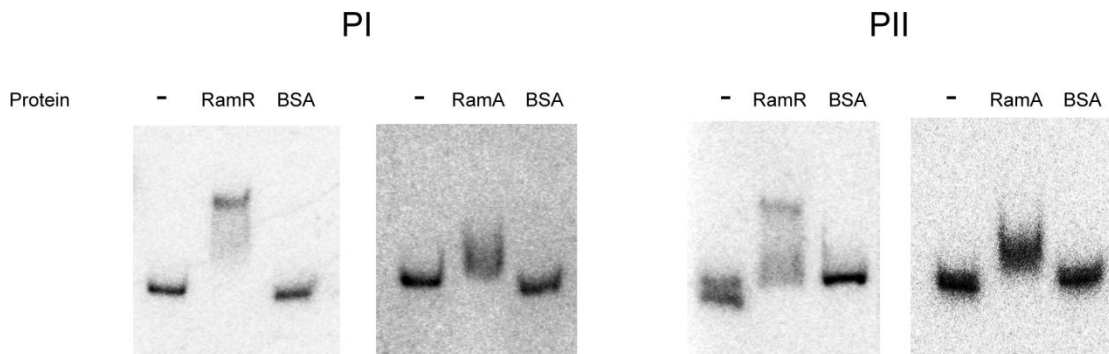


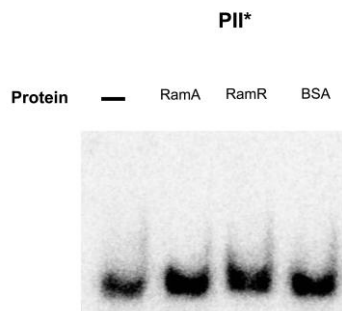
Figure 1B

Figure 2

A) pI and pII promoter regions



B) pII\* promoter region

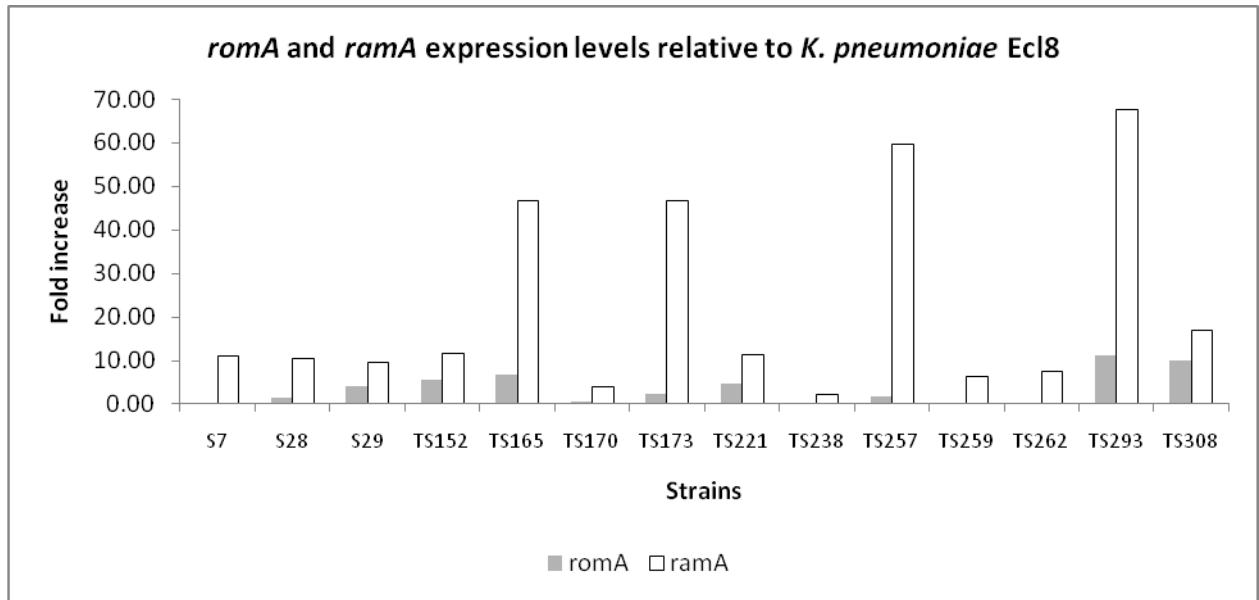


**A.** Electrophoretic Mobility Shift Assay showing the binding of purified RamR and RamA to the pI and pII promoter regions.

**B.** pII\* represents the region underlined (dashed lines) in Figure 1B.

1 Figure 3

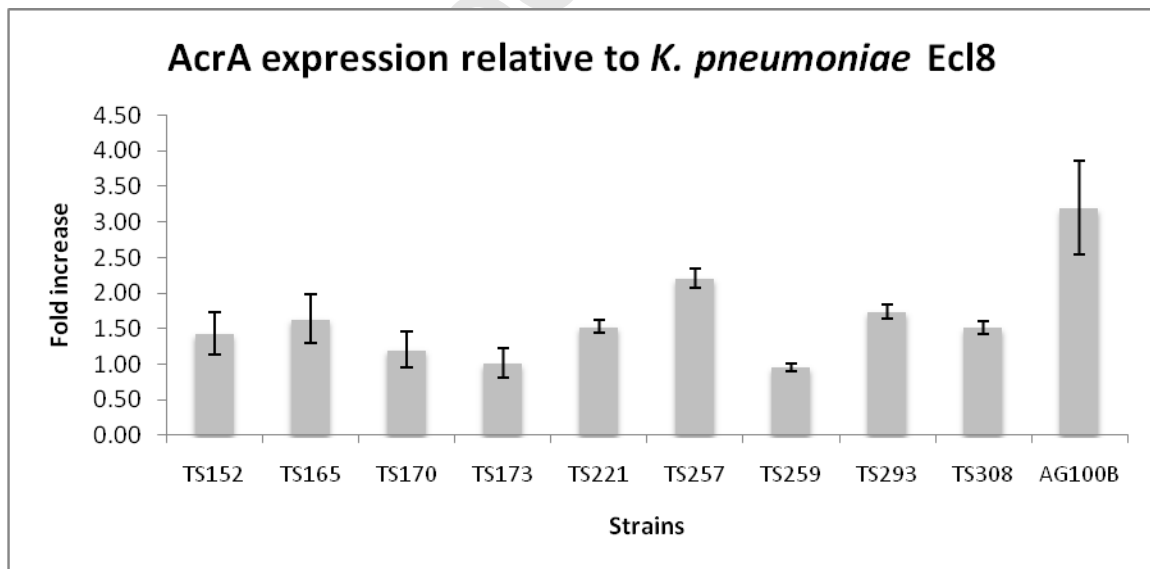
2 A)



3

4

5 B)



6

7

8