J Antimicrob Chemother 2016; **71**: 1820–1825
doi:10.1093/jac/dkw088 Advance Access publication 29 March 2016

Journal of Antimicrobial Chemotherapy

Comparative effects of overproducing the AraC-type transcriptional regulators MarA, SoxS, RarA and RamA on antimicrobial drug susceptibility in *Klebsiella pneumoniae*

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Received 17 February 2016; accepted 26 February 2016

Objectives: In *Klebsiella pneumoniae*, overproduction of RamA and RarA leads to increased MICs of various antibiotics; MarA and SoxS are predicted to perform a similar function. We have compared the relative effects of overproducing these four AraC-type regulators on envelope permeability (a combination of outer membrane permeability and efflux), efflux pump and porin production, and antibiotic susceptibility in *K. pneumoniae*.

Methods: Regulators were overproduced using a pBAD expression vector. Antibiotic susceptibility was measured using disc testing. Envelope permeability was estimated using a fluorescent dye accumulation assay. Porin and efflux pump production was quantified using proteomics and validated using real-time quantitative RT–PCR.

Results: Envelope permeability and antibiotic disc inhibition zone diameters both reduced during overproduction of RamA and to a lesser extent RarA or SoxS, but did not change following overproduction of MarA. These effects were associated with overproduction of the efflux pumps AcrAB (for RamA and SoxS) and OqxAB (for RamA and RarA) and the outer membrane protein TolC (for all regulators). Effects on porin production were strain specific.

Conclusions: RamA is the most potent regulator of antibiotic permeability in *K. pneumoniae*, followed by RarA then SoxS, with MarA having very little effect. This observed relative potency correlates well with the frequency at which these regulators are reportedly overproduced in clinical isolates.

Introduction

The ability of an antimicrobial drug to cross the Gram-negative bacterial envelope (referred to here as 'envelope permeability') is dictated by the rate of drug entry through outer membrane porins and the rate of expulsion by efflux pumps. Global regulatory systems exist to control envelope permeability in enteric bacteria. The paradigm is the Mar system, which has been widely studied in *Escherichia coli*. Loss-of-function mutations in the transcriptional repressor MarR confer multidrug resistance in *E. coli* via up-regulation of *marA*. MarA is an AraC-type transcriptional activator that stimulates transcription of a group of genes, the Mar regulon, including *acrAB* and *tolC*, that encode a tripartite efflux system. In addition, MarA causes decreased production of the OmpF porin in *E. coli* by activating the transcription of *micF*, an antisense RNA that binds to *ompF* mRNA, preventing protein synthesis.[1,2]

Klebsiella pneumoniae is a common cause of serious healthcare-associated infections, particularly in hospitals. Frequently, *K. pneumoniae* isolates are MDR, and reduced envelope permeability is at least partly responsible.[3–5] Overexpression of *marA* in *K. pneumoniae* MDR mutants has been seen in one study,[6] but overexpression of the *marA* homologue, *ramA*, is far more common in clinical isolates and frequently correlates with overexpression of the *acrAB* efflux pump operon.[7–11] Expression of *ramA* is repressed by RamR in *K. pneumoniae* in a manner analogous to the control of *marA* expression by MarR in *E. coli*.[12,13] Multidrug resistance in *K. pneumoniae* has occasionally also been associated with overexpression of two other *marA* homologues: *rarA*, which correlates with overexpression of the *oqxAB* efflux pump operon,[14–16] and *soxS*.[9]

The primary aim of the work presented below was to determine the relative effects that RamA, RarA, SoxS and MarA have on antimicrobial drug susceptibility, envelope permeability and efflux pump/porin production when overproduced in *K. pneumoniae* clinical isolates.

Materials and methods

Bacterial strains and antibiotic susceptibility testing

E. coli TOP10 (Invitrogen, Leek, The Netherlands) and the *K. pneumoniae* clinical isolates NCTC 5055 (a reference strain) and SM (a gift from Professor Alasdair MacGowan, Department of Microbiology, Southmead Hospital, Bristol, UK) and the otherwise isogenic pair ECL8 and ECL8Δ*ramR*[13] were used throughout. Disc susceptibility testing was performed according to CLSI methodology.[17]

Cloning K. pneumoniae regulator and porin genes and transformation of K. pneumoniae

K. pneumoniae NCTC 5055 genes were amplified by PCR using the primers listed in Table S1 (available as Supplementary data at *JAC* Online) and the method described previously.[18] The PCR amplicons were inserted into the pBAD-TOPO expression vector (Invitrogen) according to the manufacturer's instructions. A religated pBAD-TOPO derivative was used as a plasmid-only no-expression control. The pBAD vector adds a small N-terminal leader peptide to the expressed protein; this inhibited outer membrane targeting of porins (data not shown), so the DNA encoding this leader sequence was entirely deleted using NcoI digestion and re-ligation according to the manufacturer's instructions. Plasmids were used to transform *K. pneumoniae* NCTC 5055 or SM to ampicillin resistance (50 mg/L) using electroporation as standard for laboratory-strain *E. coli*.

Fluorescent Hoechst (H) 33342 dye accumulation assay

Each *K. pneumoniae* pBAD transformant was inoculated into a separate batch of 50 mL of CAMHB (Sigma) containing 50 mg/L ampicillin and arabinose [0.2% (w/v)] in a 250 mL foam-stoppered flask to an initial OD_{600} of ≈0.05. Cultures were incubated with shaking (160 rpm) until the OD_{600} had reached 0.5–0.7. Envelope permeability was estimated with an established fluorescent dye accumulation assay[19] with black flat-bottomed 96-well plates (Greiner Bio-one, Stonehouse, UK) and a Fluostar Optima (Aylesbury, UK) plate reader. H33342 (Sigma) was used at a final concentration of 2.5 μM (for strain SM) or 25 μM (for strain NCTC 5055).

Measurements of gene expression

Each pBAD transformant was inoculated into a separate batch of 50 mL of CAMHB (Sigma) [for outer membrane protein (OMP) analysis] or Nutrient Broth (Oxoid) (for total envelope proteomics or real-time quantitative RT–PCR) in a 250 mL foam-stoppered flask to an initial OD_{600} of ≈0.05. Cultures were incubated with shaking (160 rpm) for 3 h, when the OD_{600} had reached 0.5–0.7. Detailed methods for protein and RNA extraction and analysis are available as Supplementary data at *JAC* Online.

Results and discussion

RamA, and to a lesser extent RarA or SoxS, overproduction in K. pneumoniae reduces envelope permeability and antibiotic susceptibility, but MarA overproduction does not

In both test *K. pneumoniae* strains, inhibition zone diameter decreases were seen for 15/20 antimicrobials following RamA overproduction, resulting in intermediate resistance (according to CLSI criteria[20]) to cefuroxime and cefoxitin in the NCTC 5055 strain and full minocycline resistance in the SM strain. RamA overproduction from pBAD accurately reflected the phenotypic effect of 'natural' RamA overproduction from the chromosome because zone diameter changes in the *ramR* deletion strain ECL8Δ*ramR* compared with its *ramR*$^{+}$ parent, ECL8, were similar to those seen when overproducing RamA from pBAD using 0.2% (w/v) arabinose (Table 1 and Table S2).

RarA overproduction caused a smaller number and magnitude of zone diameter decreases than RamA overproduction in both strains. The effect of SoxS overproduction was similar to that of RarA in strain SM, though it had very little effect in strain NCTC 5055. Overproduction of MarA in both strains had almost no effect on inhibition zone diameters for the 20 test antimicrobial discs (Table 1 and Table S2).

An ∼40% reduction in the accumulation of H33342 (a measure of envelope permeability) by the RamA-overproducing transformant was observed relative to the plasmid-only control (set to 100%) in *K. pneumoniae* NCTC 5055 (Figure 1a). The reduction was even greater in the SM strain following overproduction of RamA (Figure S1A). In contrast, MarA overproduction had very little effect on envelope permeability and overproduction of RarA or SoxS had an intermediate effect in either strain (Figure 1a and Figure S1A). Thus, the relative effects of the four AraC-type regulators on envelope permeability and antibiotic susceptibility correlate.

Real-time quantitative RT–PCR confirmed similar levels of overexpression for each AraC-type regulator gene upon arabinose induction in liquid culture (Figure S2A). Overproduction of one AraC-type regulator did not significantly alter the expression of any of the other regulator genes [$P > 0.3$ for a *t*-test comparing transcript levels (C_T value) in the control versus overexpressing transformant]. This means that reciprocal effects on regulator gene expression (e.g. down-regulation of *ramA* expression from the chromosome when *marA* is overexpressed from pBAD) cannot explain why some regulators had a smaller phenotypic effect than others. A dose–response analysis of arabinose concentration confirmed that the observed differences between the effects of the regulators on antibiotic susceptibility are not due to differential protein production during the assay. Even when using 25 times more arabinose to induce the production of MarA, SoxS and RarA than to induce the production of RamA, this last regulator exerted by far the strongest effect on zone diameter. Furthermore, the effects of MarA, SoxS and RarA overproduction on zone diameter peaked at a lower arabinose concentration than the effect of RamA (Figure S2B).

Effect of RamA, RarA and SoxS overproduction on efflux pump and porin production

OMP profiles revealed that there is evidence for the overabundance of a protein of the expected molecular weight of the efflux pump OMP TolC only in RamA-overproducing NCTC 5055 (Figure 1b); for the SM strain, there is evidence for RamA, RarA and SoxS, but not MarA, inducing TolC overproduction (Figure S1B). Only two porin bands were observed in OMP extracts from *K. pneumoniae* NCTC 5055. By cloning and overproducing NCTC 5055 porin genes in *E. coli*, it was possible to confirm that OmpK35 (OmpF) is the non-detectable porin band in NCTC 5055 OMP preparations (Figure 1c). OmpK35 is visible in *K. pneumoniae* SM (though it is lower in abundance than OmpK36) and there is evidence for reduced production of OmpK35 in transformants overproducing RamA and RarA, but not MarA and SoxS (Figure S1B).

To give more quantitative data on efflux pump and porin protein production/gene expression following overproduction of the

Table 1. Disc susceptibility assay for *K. pneumoniae* NCTC 5055 transformants expressing regulator genes

Antibiotic (μg in disc)	pBAD (control) zone (mm)	pBAD(*marA*) zone (difference from control) (mm)	pBAD(*soxS*) zone (difference from control) (mm)	pBAD(*rarA*) zone (difference from control) (mm)	pBAD(*ramA*) zone (difference from control) (mm)	ECL8Δ*ramR* zone (difference from ECL8) (mm)
Amikacin (30)	24 (S)	NC	NC	NC	NC	NC (S)
Gentamicin (10)	23 (S)	NC	NC	NC	NC	−2 (S)
Tobramycin (10)	22 (S)	−2 (S)	NC	−2 (S)	NC	NC (S)
Cefoxitin (30)	23 (S)	NC	−2 (S)	−2 (S)	−6 (I)	−4 (S)
Cefuroxime (30)	22 (S)	NC	NC	−2 (S)	−5 (I)	−9 (I)
Ceftriaxone (30)	32 (S)	NC	NC	−2 (S)	−3 (S)	−7 (S)
Cefotaxime (30)	35 (S)	NC	−2 (S)	NC	−6 (S)	−4 (S)
Ceftazidime (30)	29 (S)	NC	−2 (S)	−2 (S)	−4 (S)	−5 (S)
Cefepime (30)	30 (S)	NC	NC	NC	−4 (S)	−3 (S)
Aztreonam (30)	34 (S)	−3 (S)	−2 (S)	−4 (S)	−6 (S)	−2 (S)
Imipenem (10)	29 (S)	NC	NC	NC	−2 (S)	NC (S)
Meropenem (10)	31 (S)	NC	NC	NC	NC	NC (S)
Doripenem (10)	30 (S)	NC	NC	NC	NC	NC (S)
Ciprofloxacin (5)	30 (S)	NC	NC	NC	−8 (S)	−3 (S)
Norfloxacin (10)	30 (S)	NC	−2 (S)	−3 (S)	−11 (S)	−9 (S)
Ofloxacin (5)	28 (S)	NC	NC	−3 (S)	−5 (S)	−9 (S)
Tigecycline (15)	21	NC	NC	−2	−4	−5
Minocycline (30)	21 (S)	NC	NC	NC	−4 (S)	−6 (S)
Chloramphenicol (30)	25 (S)	NC	NC	−3 (S)	−6 (S)	−9 (S)
Trimethoprim/sulfamethoxazole (1.25/23.75)	27 (S)	NC	NC	NC	−4 (S)	NC (S)

S, susceptible; I, intermediate resistant; NC, no change in zone diameter versus control.
Assays were performed using Mueller–Hinton agar with 0.2% (w/v) arabinose to stimulate expression of the cloned genes and 50 mg/L ampicillin to select for the pBAD plasmids. Otherwise, the assay was performed according to the CLSI protocol.[17]
Values reported are the means of at least three repetitions rounded to the nearest integer.
Mean changes <2 mm are reported as NC.
Susceptibility breakpoints are as set by the CLSI.[20]

regulators, total envelope proteomics and quantitative RT–PCR were performed. Normalized relative protein production/gene expression data in NCTC 5055 transformants overproducing RamA, RarA and SoxS versus the pBAD (control) transformant, set to 1 in each case, are recorded in Figure 1(d and e). MarA was excluded here due to the lack of phenotypic effect seen in previous experiments. In some cases, proteins were found to be below the level of detection in control cells, but they were identifiable in regulator-overproducing cells. Because of this binary result, it is not possible to quantify the fold change in their production; however, this must be considerable and therefore a nominal fold change of 20 is assigned for these proteins (Figure 1d).

As expected given its particularly strong effect on antibiotic susceptibility and envelope permeability, RamA overproduction had the greatest effect on efflux pump protein production (Figure 1d) and this effect is clearly exerted at the level of transcription (Figure 1e). OqxAB, which is particularly associated with quinolone efflux in enteric bacteria[21] and AcrAB, which is the most commonly identified efflux pump seen to be overproduced in MDR *K. pneumoniae* isolates,[6–15] were both overproduced >4-fold relative to control cells (Figure 1d). In addition, TolC was also overproduced >4-fold. Even though AcrAB and OqxAB are overproduced in the presence of RamA, the pump primarily responsible for multidrug resistance is likely to be AcrAB. We say this because OqxAB was ~100 times less abundant than AcrAB [0.7±0.43% (mean±95% CI, $n=3$)] in RamA-overproducing cells, according to the proteomic data. Proteomic analysis of Ecl8 versus Ecl8Δ*ramR* confirmed that AcrA, AcrB and TolC are overproduced when RamA is overproduced from the chromosome [3.34±0.83-fold, 4.25±0.47-fold and 3.06±0.33-fold, respectively (mean±SEM, $n=3$)].

Overproduction of RarA has previously been shown to control *oqxAB* and *acrAB* expression,[22] though in strain NCTC 5055 we could only see significant up-regulation of OqxAB (Figure 1d and e). This was, however, sufficient to explain the reduced susceptibility primarily to fluoroquinolones and tigecycline seen in this RarA-overproducing NCTC 5055 recombinant (Table 1). The effect of SoxS overproduction in NCTC 5055 was, as expected from the antibiotic susceptibility data (Table 1), less strong than RamA and RarA (Figure 1d and e). There was evidence for an increase in AcrAB production, though this was about a third of the effect of RamA. We could not detect the low-abundance pump OqxAB in the SoxS-overproducing recombinant, but the quantitative RT–PCR data confirmed that *oqxAB* is positively controlled by this regulator (Figure 1e).

Porin proteins previously shown to be involved in antibiotic entry were not down-regulated upon overproduction of any of the AraC-type regulators in *K. pneumoniae* NCTC 5055. For OmpK36, fold changes (mean±SEM, $n=3$) were 1.86±0.62 (SoxS), 0.78±0.11 (RarA) and 1.19±0.54 (RamA). For OmpK35, fold changes were 2.14±0.81 (SoxS), 1.60±0.25 (RarA) and

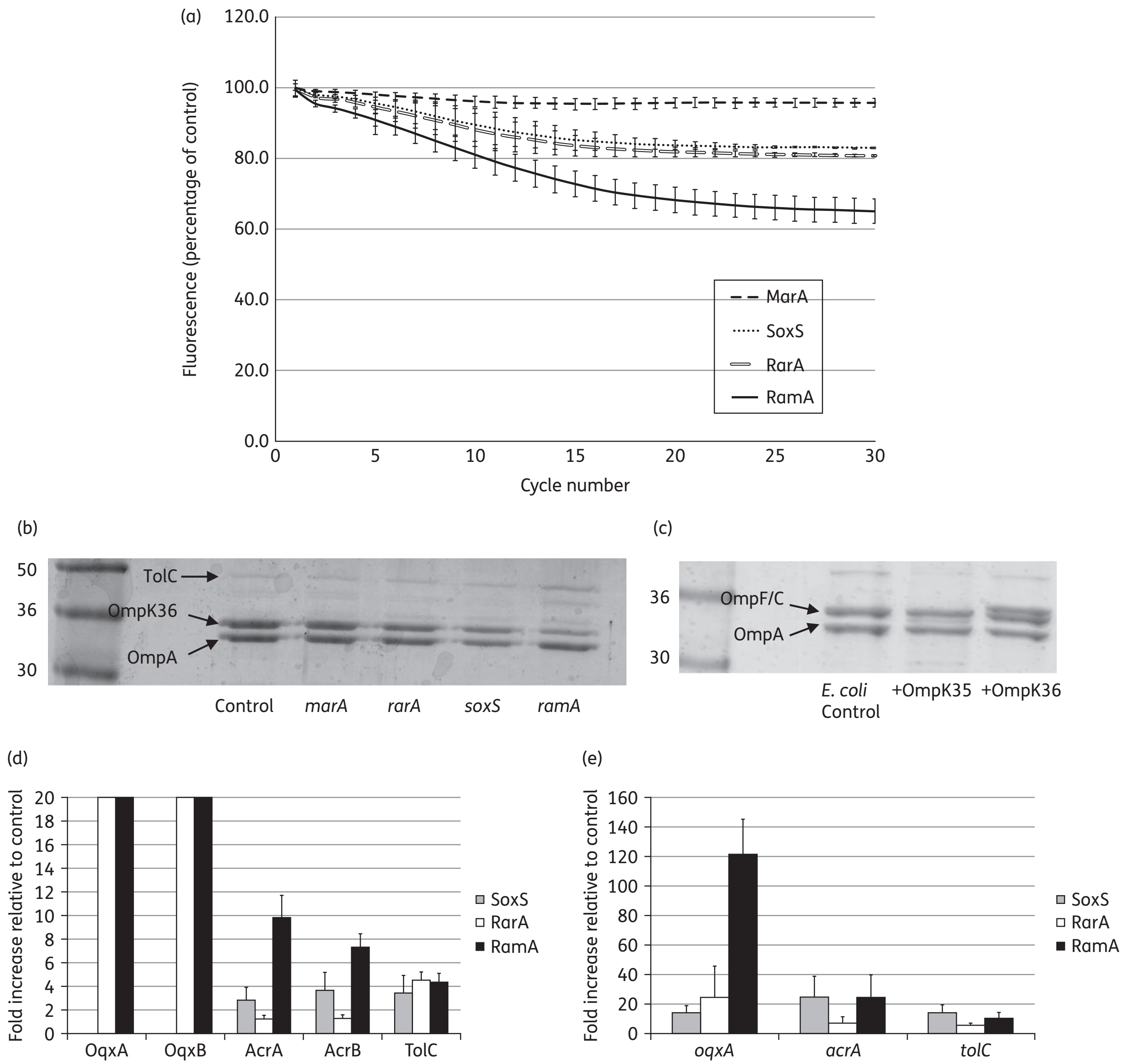

Figure 1. Effect of AraC-type regulator overproduction in *K. pneumoniae* NCTC 5055 on envelope permeability and gene expression. (a) The accumulation of H33342 dye by *K. pneumoniae* NCTC 5055 transformants overexpressing *ramA*, *rarA*, *soxS* or *marA* is represented as a percentage relative to accumulation in the plasmid-only control transformant (set to 100%) over a 30 cycle (45 min) incubation period. Each graph shows the mean data for four biological replicates with eight technical replicates in each, and error bars define the SEM. (b) OMPs were purified from *K. pneumoniae* NCTC 5055 transformants carrying the control plasmid or those overexpressing *ramA*, *rarA*, *soxS* or *marA*. The OmpK35 and OmpK36 porins were identified by reference to panel (c), which shows OMPs from *E. coli* TOP10 and transformants overexpressing *ompK35* or *ompK36* cloned from *K. pneumoniae* NCTC 5055. OmpA and TolC are marked by reference to their predicted molecular weight and relatively high abundance. All OMPs were separated using SDS–PAGE and stained as set out in the Materials and methods section. These data are representative of three biological replicates. (d) Fold change in the abundance of selected efflux pump proteins and (e) fold change in the efflux pump gene mRNA levels in *K. pneumoniae* NCTC 5055 transformants carrying the control plasmid (set to 1) or those overexpressing *ramA*, *rarA* or *soxS* growing in nutrient broth. Data are mean ± SEM; three biological replicates, with four technical replicates for the quantitative RT–PCR.

1.32 ± 0.22 (RamA). In contrast, in the Ecl8 strain, overproduction of RamA caused a repression of OmpK35 porin production (5.24 ± 1.34-fold down-regulated in Ecl8Δ*ramR* versus Ecl8), but no change in OmpK36 production (1.15 ± 0.05-fold). The qualitative OMP analysis (Figure 1b) led us to suggest that that OmpK35 is already down-regulated in NCTC 5055, and the ratio of OmpK36 to OmpK35 was calculated from the raw proteomic abundance data as 6.04 ± 1.28 to 1 in NCTC 5055 and

0.93 ± 0.04 to 1 in Ecl8. Accordingly, the repressive effect of RamA on production of OmpK35, apparent in Ecl8Δ*ramR* [and also in the SM strain (Figure S1B)] is masked in strain NCTC 5055 because OmpK35 production is already reduced by some other mechanism (Figure 1b). Importantly, however, the effect of RamA overproduction in Ecl8Δ*ramR* on antibiotic susceptibility was not significantly more than in NCTC 5055 (Table 1), so this additional effect of reducing OmpK35 levels does not appear to be phenotypically important.

Conclusions

Overall, the strength by which the four AraC-type regulators have been shown here to affect antibiotic susceptibility (RamA > RarA > SoxS > MarA) correlates well with previously published incidences of AraC-type regulator overproduction in clinical *K. pneumoniae* isolates and laboratory-selected mutants.[6–15] The strength of effect of these regulators appears to correlate with their ability to activate efflux pump gene expression; though the involvement of reducing OmpK35 production in some backgrounds cannot be excluded, it does not appear to be of major importance.

RamA overproduction in *K. pneumoniae* reduces susceptibility to a number of drug classes, including tetracyclines, quinolones and cephalosporins, but excluding aminoglycosides (Table 1 and Table S2). However, resistance based on clinical breakpoints[20] was only reached for minocycline, and only in the SM strain. Intermediate resistance to cefuroxime and cefoxitin was also seen, but only in the NCTC 5055 strain; no resistance was seen in Ecl8Δ*ramR*. Clearly, therefore, RamA is not a key antibiotic resistance determinant in *K. pneumoniae* on its own. It has recently been shown that the RamA regulon includes genes that contribute to other clinically relevant phenotypes, e.g. LPS biosynthesis.[23] This, rather than its impact on antibiotic resistance, might therefore explain why RamA overproduction is seen in clinical isolates.[6–13] However, it remains to be seen whether RamA-mediated efflux pump overproduction can enhance plasmid-mediated resistance or allow easier selection of MDR mutants with other additional regulatory or antimicrobial drug target site mutations. Indeed, the same must be said for the other regulators studied here and further work is necessary to investigate this possibility.

Acknowledgements

We are grateful to Lucy Pettman, who generated the *rarA* and *soxS* clones used in this study.

Funding

This work was supported in part by a grant to M. B. A. from the BSAC and University of Bristol internal funds. J.-C. J.-C. was funded by a postgraduate scholarship from CONACyT, Mexico. W. N. I. W. A. K. was funded by a postgraduate scholarship from the Malaysian Ministry of Education. M. S. T. was funded through the Wellcome Trust Clinical Veterinary Research Training Programme. J. B. was funded by a BSAC vacation scholarship.

Transparency declarations

None to declare.

Supplementary data

Supplementary methods, Tables S1 and S2 and Figures S1 and S2 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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