The Envelope Proteome Changes Driven by RamA Overproduction in *Klebsiella pneumoniae* that Enhance Acquired β-Lactam Resistance.

Juan-Carlos JIMÉNEZ-CASTELLANOS[1], Wan Ahmad Kamil WAN NUR ISMAH[1,2], Yuiko TAKEBAYASHI[1], Jacqueline FINDLAY[1], Thamarai SCHNEIDERS[3], Kate J. HEESOM[4] & Matthew B. AVISON[1,*]

[1]School of Cellular and Molecular Medicine, University of Bristol, Bristol, United Kingdom

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

[3]Division of Infection and Pathway Medicine, University of Edinburgh, Edinburgh, United Kingdom.

[4]University of Bristol Proteomics Facility, Bristol. United Kingdom.

[*]Corresponding author. Mailing address: School of Cellular and Molecular Medicine, University of Bristol, Biomedical Sciences Building, University Walk, Bristol, BS8 1TD, United Kingdom. Tel: +44(0)1173312035, Fax +44(0)1179287896. E-mail: bimba@bristol.ac.uk.

Running Title: RamA enhanced β-lactam resistance in *Klebsiella pneumoniae*

Abstract

OBJECTIVES

In *Klebsiella pneumoniae*, overproduction of RamA results in reduced envelope permeability and reduced antimicrobial susceptibility but clinically relevant resistance is rarely observed. Here we have tested whether RamA overproduction can enhance acquired β-lactam resistance mechanisms in *K. pneumoniae* and have defined the envelope protein abundance changes upon RamA overproduction during growth in low and high osmolarity media.

METHODS

Envelope permeability was estimated using a fluorescent dye accumulation assay. β-Lactam susceptibility was measured using disc testing. Total envelope protein production was quantified using LC-MS/MS proteomics and transcript levels quantified by Real Time RT-PCR.

RESULTS

RamA overproduction enhanced β-lactamase mediated β-lactam resistance, in some cases dramatically, without altering β-lactamase production. It increased production of efflux pumps and decreased OmpK35 porin production, though *micF* overexpression showed that OmpK35 reduction has little impact on envelope permeability. A survey of *K. pneumoniae* bloodstream isolates revealed *ramA* hyperexpression in 3 out of 4 carbapenemase producers, 1/21 CTX-M producers and 2/19 strains not carrying CTX-M or carbapenemases.

CONCLUSIONS

Whilst RamA is not a key mediator of antibiotic resistance in *K. pneumoniae* on its own, it is potentially important for enhancing the spectrum of acquired β-lactamase mediated β-lactam resistance. LC-MS/MS proteomics analysis has revealed that this enhancement is achieved predominantly through activation of efflux pump production.

Introduction

RamA is a global transcriptional activator [1] found in, amongst other Enterobacteriaceae: *Salmonella* spp.,[2] *Enterobacter* spp.[1,3] and *Klebsiella* spp.[1,4] but not *Escherichia coli*. Where characterised, RamA has revealed a function comparable to *E. coli* MarA.[1-5] In wild-type *Klebsiella pneumoniae*, at least during standard laboratory growth, RamA production is low because the transcriptional repressor, RamR, occludes the *ramA* promoter.[6] In some clinical isolates, RamA is overproduced due to de-repressing mutations in *ramR*.[7-10] RamA activates the transcription of a regulon including *oqxAB*, *acrAB* and *tolC*, encoding components of two tripartite antimicrobial efflux pumps.[4]

Overexpression of *ramA* in *K. pneumoniae* isolates that lack other resistance mechanisms increases MICs of a wide range of antimicrobials, including cephalosporins but not carbapenems. However, even overexpressing *ramA* >1000 fold only confers resistance to one or two antimicrobials and only in some isolates.[5] On its own, therefore, RamA is not a key resistance determinant in *K. pneumoniae*, but clinical isolates can carry acquired resistance mechanisms, particularly plasmid encoded β-lactamases.[11] Accordingly, the first aim of this work was to determine whether RamA overproduction can enhance the spectrum of resistance conferred by acquired β-lactamases. We also wanted to test how common *ramR* loss of function mutations are in *K. pneumoniae* clinical isolates having different β-lactam resistance phenotypes.

We were particularly keen to investigate whether reduced carbapenem susceptibility occurs in RamA overproducing *K. pneumoniae* having ESBL or AmpC type cephalosporinases. This was because RamA overproduction and porin loss both reduce envelope permeability, and

because porin loss has previously been shown to increase carbapenem MICs against *K. pneumoniae* isolates producing ESBLs or AmpC cephalosporinases.[12]

Enhancement of β-lactam MICs seen following OmpK35 porin loss in a cephalosporinase producing *K. pneumoniae* is reportedly minimised during growth in high osmolarity Muller Hinton media,[12] which is the medium of choice for most antibiotic susceptibility testing protocols,[13] but is maximised during growth in low osmolarity Nutrient media. This is because these media reportedly support different basal OmpK35 levels.[12] Accordingly, we also set out to define the envelope proteome changes stimulated by RamA overproduction in Muller Hinton broth (MHB) and Nutrient broth (NB) using Orbitrap LC-MS/MS. The aim was to identify common and growth medium-specific effects of RamA overproduction, and to confirm a previous report that basal OmpK35 levels are different in the two media.[12] Finally, we set out to define the contribution of RamA-mediated OmpK35 downregulation [4,5] to the overall effect of RamA overproduction on envelope permeability and antibiotic susceptibility in *K. pneumoniae*.

Materials and Methods

Bacterial strains and antibiotic susceptibility testing

E. coli TOP10 (Invitrogen, Leek, The Netherlands), 44 non-replicate *K. pneumoniae* human clinical isolates having various antimicrobial susceptibility profiles (provided by Dr Karen Bowker, Department of Microbiology, North Bristol NHS Trust), *K. pneumoniae* NCTC5055 transformants carrying pBAD(*ramA*) or a pBAD vector-only control plasmid [5] and the otherwise isogenic pair ECL8 and ECL8Δ*ramR* [4] were used throughout. Disc susceptibility testing was performed according to CLSI methodology [13] and interpreted using CLSI performance standards.[14]

Cloning plasmid-mediated β-lactamase genes and micF and sequencing ramR

Cloning *bla*$_{NDM-1}$ (with its IS*Aba125* promoter) into the cloning vector pSU18 [15] has previously been reported.[16] *K. pneumoniae micF* and the following additional β-lactamase genes were synthesised or amplified by PCR from the sources and using the primers listed in Table S1 and the PCR method previously described [17] in such a way as to include their native promoters: *bla*$_{IMP-1}$ and *bla*$_{VIM-1}$ (with hybrid and weak strength class 1 integron promoters, respectively),[18] *bla*$_{CTX-M1}$ and *bla*$_{CMY-4}$ (with IS*Ecp1* promoters), *bla*$_{KPC-3}$ (with IS*Kpn7* promoter) and *bla*$_{OXA-48}$ (with IS*1999* promoter). Some PCR amplicons were TA cloned into the pCR2.1-TOPO cloning vector (Invitrogen) according to the manufacturer's instructions. These pCR2.1 inserts, removed by restriction enzyme digestion, and other PCR amplicons directly cut with restriction enzymes, were ligated into the pSU18 [15] or pK18 [19] cloning vectors as illustrated in Table S1. Plasmids were used to transform *K. pneumoniae* isolates to chloramphenicol or kanamycin (30 mg/L) resistance for pSU18 and pK18, respectively, using electroporation as standard for lab strain *E. coli*. To sequence *ramR* from clinical isolates, the gene was amplified using PCR as previously [17] and sequenced; primers are recorded in Table S1. Sequence alignment and analysis were performed using the online ClustalOmega (http://www.ebi.ac.uk/Tools/msa/clustalo/) tool; the reference sequence used was *K. pneumoniae* Ecl8 (Accession Number HF536482.1).

Growth of cultures for all experiments

Each isolate or transformant was inoculated into a separate batch of 50 mL Cation Adjusted MHB (Sigma) or NB (Oxoid) containing appropriate selection in a 250 mL foam stoppered flask

114 to an initial Optical Density at 600 nm (OD_{600}) of ≈0.05. Cultures were incubated with shaking
115 (160 rpm) until the OD_{600} had reached 0.5-0.7.
116
117 *Fluorescent Hoechst (H) 33342 dye accumulation assay*
118 Envelope permeability was estimated as described previously [5] in bacteria grown in liquid
119 culture using an established fluorescent dye accumulation assay [20] with black flat-bottomed 96-
120 well plates (Greiner Bio-one, Stonehouse, UK) and a Fluostar Optima (Aylesbury, UK) plate
121 reader. H33342 (Sigma) was used at a final concentration of 2.5 µM.
122
123 *Characterisation of Envelope Proteomes and Real-Time RT-PCR*
124 Methods used for protein and RNA extraction and analysis of abundance were almost identical
125 to those used previously [5] and are presented in detail in Supplementary Material. For each LC-
126 MS/MS proteomics experiment, raw protein abundance data were collected for three biological
127 replicates of each growth condition. The significance of any observed difference between the
128 means of the triplicate abundance data for one protein in two different growth conditions was
129 calculated using a T-test comparing the raw abundance data as described in Supplementary
130 Material.
131
132 *β-Lactamase assays and multiplex PCR for β-lactamase gene carriage.*
133 Multiplex PCRs were performed for (i) carbapenemase genes encoding the IMP, VIM, OXA-48-
134 like, NDM and KPC enzymes,[21] though the NDM and OXA-48-like gene-identifying PCRs were
135 run separately and not as a multiplex with the rest (ii) genes encoding CTX-M groups 1, 2, 8, 9
136 and 25 [22] (iii) a bespoke multiplex for genes encoding TEM-1, SHV, OXA-1 and CMY. All

multiplex PCR primers and expected product sizes are recorded in Table S2. DNA template was prepared as previously described [17] and 1 µL of supernatant used in a final PCR reaction volume of 25 µL consisting of 12.5 µL REDTaq ReadyMix (Sigma) with 10 µM of each primer. PCR was performed using the following conditions: 94°C for 10 min, 35 cycles of amplification consisting of 94°C for 1 min, 52-56°C for 1 min, 72°C for 1 min 30 s and a final extension at 72°C for 10 min.

β-Lactamase assays were performed as follows: overnight cultures of bacteria were diluted to an optical density at 600 nm (OD_{600}) of 0.1 in MHB and grown at 37°C until the OD_{600} was 0.8 before cell extracts were prepared and levels of β-lactamase activity in cell extracts measured as described previously [23] using 100 µM nitrocefin as substrate. Linear gradients (ΔAU/min) were extrapolated and an extinction coefficient of 17,400 AU/M was used to calculate nitrocefin hydrolysing activity. The total protein concentration in each cell extract was quantified using the Bio-Rad protein assay reagent (Bio-Rad, Hemel Hempstead, United Kingdom) according to the manufacturer's instructions and used to calculate relative specific enzyme activity (pmol/min/µg) in each cell extract.

Results and Discussion

Enhancement of β-lactam resistance in K. pneumoniae by RamA in the presence of acquired β-lactamases

A library of seven clinically important mobile β-lactamase genes was created using pSU18, a low copy number vector.[15] Each gene was expressed from a promoter commonly seen in clinical isolates to give close to wild-type levels of β-lactamase production (see table S1). This

159 library was used to transform *K. pneumoniae* Ecl8 and Ecl8Δ*ramR*, and there was no significant
160 difference in β-lactamase production in Ecl8Δ*ramR* versus Ecl8 transformants (Table S3).

161 Ecl8 is susceptible to 17/18 tested β-lactams (Table 1). Overproduction of RamA (i.e.
162 Ecl8Δ*ramR*) reduced zone diameters ≥2 mm for 13/18 tested β-lactams, but clinically relevant
163 non-susceptibility [14] was not achieved for any of them (Table 1). The metallo-β-lactamases
164 (MBLs) NDM-1 and IMP-1 rendered Ecl8 non-susceptible to 17/18 and 14/18 β-lactams,
165 respectively, so the additional effect of RamA overproduction was difficult to see (Table 1).
166 Accordingly, $bla_{\text{VIM-1}}$ was cloned with a weak integron promoter and the resultant low-level
167 MBL production meant that Ecl8 remained susceptible to 12/18 test β-lactams. Here, the effect
168 of RamA overproduction was profound. Ecl8Δ*ramR* (VIM-1) was only susceptible to aztreonam
169 and cefotetan (Table 1).

170 RamA overproduction also affected β-lactam susceptibility in the presence of serine β-
171 lactamases (SBLs). There was a considerable zone diameter reduction for ertapenem (by 7 mm;
172 on the verge of being non-susceptible) in Ecl8Δ*ramR* (CTX-M1) versus Ecl8 (CTX-M1) but for the
173 other carbapenems, no change at all. In the case of the AmpC-type SBL, ertapenem non-
174 susceptibility was conferred in Ecl8Δ*ramR* (CMY-4), though the other carbapenems remained
175 equally active as against Ecl8 (CMY-4). In Ecl8 (KPC-3), only the cephamycins retained activity,
176 but even these were lost in Ecl8Δ*ramR* (KPC-3). In contrast, the effect of carrying OXA-48 was
177 relatively weak. When RamA was overproduced there was a slight reduction in inhibition zone
178 diameter, particularly for the carbapenems, but Ecl8Δ*ramR*(OXA-48) remained susceptible to
179 17/18 β-lactams (Table 1).
180

A survey of ramR function in clinical isolates carrying or not carrying cephalosporinases and carbapenemases.

Forty-four *K. pneumoniae* clinical isolates were randomly collected (Table S4). Twenty isolates carried a *ramR* allele encoding a Thr141Ile variant (in comparison with Ecl8) which has no impact on phenotype.[9] Two isolates encoded the Ala19Val RamR variant which is also functionally silent.[24] Seven other isolates encoded RamR protein variants and Real-Time RT-PCR was used to measure *ramA* transcript levels in in these seven isolates comparison with the *ramR* wild-type isolate AH. Six of the RamR variant isolates hyper-expressed *ramA*; the Ile106Ser variant was not associated with significantly higher *ramA* expression (Table S4).

Three multiplex PCRs were performed to categorise the β-lactamase genes present in each of the 44 clinical isolates. Four carried at least one carbapenemase gene, of which two also carried $bla_{CTX\text{-}M}$; 21 additional isolates carried $bla_{CTX\text{-}M}$. All CTX-M genes were of group 1. The remaining 19 isolates did not carry any cephalosporinase or carbapenemase gene (Table S4).

Finally, disc susceptibility testing was performed for eight β-lactams against the 44 clinical isolates. Using the sum of all the inhibition zone diameters to represent the combined β-lactam susceptibility for each isolate, we ranked the isolates (Table S4). Not surprisingly, the four carbapenemase positive isolates were the four least β-lactam susceptible. Interestingly, three out of four of these isolates also carry a *ramR* mutation and hyperexpress *ramA*. Only one of the 21 carbapenemase negative, CTX-M positive isolates hyperexpressed *ramA*, but this isolate (isolate T) was by far the least susceptible isolate in this group. As predicted from our transformation experiments, isolate T displayed reduced carbapenem susceptibility relative to the 20 isolates that have CTX-M but an intact *ramR*. However, this effect (Table S4) was greater

than that seen in the Ecl8/Ecl8Δ*ramR* transformants (Table 1), with resistance to ertapenem, and intermediate resistance to doripenem being observed in isolate T, together with reduced susceptibility to meropenem suggesting an additional as yet uncharacterised mechanism in isolate T. As seen with Ecl8Δ*ramR* (Table 1), *ramA* hyperexpression in the two clinical isolates (isolates N and AO) that lack any cephalosporinase or carbapenemase genes had minimal impact on β-lactam susceptibility (Table S4)

Envelope Proteome Changes Following RamA Overproduction in K. pneumoniae in NB and MHB and Impact on Envelope Permeability

We have recently shown that RamA overproduction in *K. pneumoniae* NCTC5055 using a pBAD expression plasmid increased AcrAB-TolC and OqxAB-TolC efflux pump production.[5] To obtain a more detailed understanding of the RamA regulon, we used Orbitrap LC-MS/MS proteomics. Table S5 shows summary data for the Ecl8/Ecl8Δ*ramR* pair during growth in MHB. It has previously been reported that low osmolarity media such as NB affect the levels of OmpK35 relative to growth in high osmolarity media such as MHB.[12] This observation is potentially important because OmpK35 levels might impact on envelope permeability and because antimicrobial susceptibility assays are generally performed using Muller-Hinton media. Accordingly, to see whether medium osmolarity affects the impact of RamA overproduction, we also determined envelope proteome changes in the Ecl8/Ecl8Δ*ramR* pair during growth in NB (Table S6).

Our LC-MS/MS methodology allowed identification and absolute quantification with a high degree of certainty (≥ 3 peptides identified) of 655 and 494 proteins in envelope preparations from cells grown in MHB and NB, respectively. Previous data from SDS-PAGE

analysis of outer membrane protein preparations have been interpreted as meaning that OmpK35 levels increase relative to OmpK36 levels upon shifting from MHB to NB,[12] and our LC-MS/MS data confirmed this finding. There is a shift in the OmpK36:OmpK35 ratio in Ecl8 from 1.7:1 (calculated from mean protein abundance data, p=0.013, n=3) during growth in MHB to 1.1:1 (p=0.45, n=3) during growth in NB. Our accurate absolute abundance data revealed that this change in OmpK36:OmpK35 ratio is achieved through downregulation of OmpK36 (2.2-fold, p=0.011, n=3) not upregulation of OmpK35 (0.7-fold, p=0.16, n=3) during growth in NB relative to growth in MHB.

Twenty-nine and 33 proteins were found to be ≥ 2-fold up or down regulated in Ecl8Δ*ramR* versus Ecl8 following application of our statistical significance cut-off (p<0.05 for a T-test comparing absolute protein abundance data, n=3) during growth in MHB and NB, respectively (Tables S5, S6). Of these, 12 proteins were similarly regulated in both media; 11 upregulated, with only OmpK35 downregulated (Table 2). Ten out of 11 proteins upregulated in Ecl8Δ*ramR* in both media were also upregulated in *K. pneumoniae* strain NCTC5055 following overproduction of RamA via the pBAD expression plasmid (Table S7). These 10 core upregulated proteins represent two efflux pumps (AcrAB and OqxAB, together with the outer membrane efflux protein TolC). The remaining proteins are poorly characterised and neither their precise role in RamA mediated reduced antimicrobial susceptibility, nor their direct control by RamA can be confirmed at this time.

Of the medium-specific impacts of RamA overproduction, two are striking. In MHB only, downregulation of several proteins encoded by the maltose transport operon occurs (Table S5). The LamB2 porin is amongst these. Interestingly, its loss by mutation has been implicated in reduced carbapenem entry in *K. pneumoniae*,[25,26] so it is conceivable that downregulation of

LamB2 might enhance the impact of RamA overproduction on carbapenem MICs during growth on Muller-Hinton agar, which is apparent in the presence of certain plasmid-mediated β-lactamases (Table 1, Table S4). In NB only, the efflux pump AcrEF is upregulated in Ecl8Δ*ramR* relative to Ecl8. Whilst this pump has not been specifically characterised in *K. pneumoniae*, its equivalent has a role in antimicrobial resistance in other enteric bacteria, and it is part of the *Salmonella* Typhimurium RamA regulon.[2] Also upregulated in NB only is the transporter complex proteins YrbCDEF (Table S6). RamA-mediated regulation of the *yrb* locus has previously been demonstrated using transcriptome analyses in both *K. pneumoniae* [4] and *S.* Typhimurium [2] and a potential RamA binding site has been proposed for the *yrb* locus in *K. pneumoniae*.[4] YrbCDEF from Ecl8 are >90% identical to the MlaCDEF proteins, part of the MlaABCDEF ABC transporter from *E. coli*, which has recently been shown to play an important role in retrograde phospholipid trafficking. The perturbation of the phospholipid content of the outer membrane is likely to result in a reduction in its sensitivity to chemical damage, and may also affect antimicrobial/cell affinity, reducing rate of entry.[27-29] The AcrEF and Yrb (Mla) proteins were also upregulated following RamA overproduction from the pBAD expression vector in *K. pneumoniae* NCTC, but LamB2 is not downregulated (Table S7). These NCTC5055 data were collected during growth in NB, which explains these findings, and confirms the medium dependence of the effects.

Despite these differences in envelope proteome seen during growth of Ecl8Δ*ramR* in MHB and NB, there is little impact of growth medium on RamA-mediated envelope permeability reduction. When measured using fluorescent dye accumulation, envelope permeability reduces by ≈75% in Ecl8Δ*ramR* versus Ecl8 during growth in MHB and ≈65% during growth in NB (Fig. 1A).

Overall, based on our test of significance, 51 proteins were differentially regulated in NCTC5055 carrying pBAD(*ramA*) versus the pBAD control transformant during growth in the presence of 0.2% w/v arabinose, which stimulates RamA overproduction (Table S7). This is more than the number of proteins differentially regulated in Ecl8Δ*ramR* versus Ecl8 (Tables S5, S6). However, according to Real Time RT-PCR analysis, there was 9200 +/- 390 fold (mean +/- SEM, *n*=3) more *ramA* transcript in NCTC5055::pBAD(*ramA*) than in NCTC5055::pBAD(control) when grown in the presence of 0.2% (w/v) arabinose, as shown previously.[5] This is dramatically more than that seen for the "natural" RamA overproducing mutant Ecl8Δ*ramR*, where there is 6.7 +/- 2.2 fold (mean +/- SEM, *n*=3) more *ramA* transcript than in Ecl8, and ≈100-fold more than even the most *ramA* overexpressing clinical isolate in our collection (Table S4). Hence the additional proteomic differences seen in NCTC:pBAD(*ramA*) are likely to be due to spurious occupation of regulatory binding sites by the greatly overproduced RamA, which has previously been reported in *Salmonella*.[2]

Role of micF in RamA Mediated Control of OmpK35 Levels.

There is clear downregulation of OmpK35 porin production following RamA overproduction in Ecl8 (Table 2). By analogy with the situation in *E. coli* following overproduction of MarA,[30] reduction of OmpK35 levels in *K. pneumoniae* is likely to be due to transcriptional upregulation of *micF* by RamA. To test this, we cloned the *micF* gene, with its own promoter into a high-copy vector pK18 [19] and used this recombinant to boost *micF* transcript levels *in trans* in Ecl8. LC-MS/MS confirmed that OmpK35 levels were downregulated (0.39 fold, *p*=0.005, *n*=3) in Ecl8(*micF*) compared with Ecl8(pK18) during growth in MHB; almost the same downregulation seen in Ecl8Δ*ramR* compared with Ecl8 during growth in MHB (0.43 fold, *p*=0.007, *n*=3).

However, dye accumulation assays unexpectedly revealed that steady-state accumulation of the fluorescent dye is approximately 20% more in Ecl8(*micF*) than in the Ecl8(pK18) control transformant, suggesting an increase in envelope permeability in the *micF* overexpressing recombinant (Fig 1B). Disc susceptibility testing confirmed that this is sufficient to increase antibiotic inhibition zones against Ecl8(*micF*) (Table S8).

We hypothesised that the reason for this increase in envelope permeability (Fig. 1B) despite OmpK35 levels being reduced is that *K. pneumoniae* responds by downregulating efflux pump production or upregulating porin production to balance envelope permeability. A reciprocal effect: downregulation of OmpK35 in *K. pneumoniae* having reduced AcrAB-TolC-mediated efflux has recently been reported.[31] In support of our hypothesis, Real Time RT-PCR showed that *acrA* levels are reduced in Ecl8(*micF*) versus Ecl8(pK18) (0.53 +/- 0.07-fold [mean +/- SEM, *n*=3]); LC/MS-MS confirmed that this led to a reduction in AcrA protein levels (0.62-fold, p=0.05, *n*=3). No porin proteins were seen to be upregulated in the proteomics data (not shown). In fact, OmpK36 and OmpA are downregulated (respectively, 0.57-fold, p=0.02, *n*=3 and 0.61-fold, p=0.03, *n*=3).

Interestingly, downregulation of *ramA* transcription was seen using Real Time RT-PCR in Ecl8(*micF*) compared with Ecl8(pK18) (0.09 +/- 0.04-fold [mean +/- SEM, *n*=3]). It is possible, therefore, that RamA downregulation may be responsible for the downregulation of AcrA seen in response to *micF*-mediated OmpK35 downregulation. If correct, this is suggestive of a feedback mechanism by which the cell can sense the balance of different factors affecting envelope permeability and controls RamA production as necessary.

Conclusions

RamA overproduction in *K. pneumoniae* has been associated with resistance to specific antimicrobials, e.g. tigecycline.[9,24] We conclude that RamA overproduction is predominantly mediated by efflux pump overproduction and can also be selected in isolates with acquired β-lactamases, which are very common in the clinic. The effect on carbapenem resistance in *K. pneumoniae* carrying a weakly expressed bla_{VIM-1} gene was particularly pronounced, and it was striking to see *ramA* hyperexpression in 3/4 carbapenemase producing bloodstream isolates from a collection (Table S4). It was also particularly concerning to see the generation of ertapenem resistance following loss of RamR repressor activity in combination with an AmpC-type enzyme in Ecl8 (Table 1) and in a *ramA* overexpressing clinical isolate carrying CTX-M (Table S4). There may be other ways by which RamA overproduction can be beneficial; for example, it may be a prerequisite for the selection of other resistance-causing mutation events such as target site mutations or those which cause porin loss.

Acknowledgements

We are grateful to Dr Aisha M. Al-Amri, who made the bla_{IMP-1} recombinant plasmid used in this study.

Funding

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

Transparency Declaration

None to declare – All authors.

References

1. George AM, Hall RM, Stokes HW. Multidrug resistance in *Klebsiella pneumoniae*: a novel gene, *ramA*, confers a multidrug resistance phenotype in *Escherichia coli*. *Microbiology* 1995; **141**:1909-20.
2. Bailey AM, Ivens A, Kingsley R *et al*. RamA, a member of the AraC/XylS family, influences both virulence and efflux in *Salmonella enterica* serovar Typhimurium. *J Bacteriol* 2010; **192**:1607-16.
3. Chollet R, Chevalier J, Bollet C *et al*. RamA is an alternate activator of the multidrug resistance cascade in *Enterobacter aerogenes*. *Antimicrob Agents Chemother* 2004; **48**:2518-23.
4. De Majumdar S, Yu J, Fookes M *et al*. Elucidation of the RamA regulon in *Klebsiella pneumoniae* reveals a role in LPS regulation. *PLoS Pathog* 2015; **11**:e1004627.
5. Jiménez-Castellanos JC, Wan Ahmad Kamil WN, Cheung CH *et al*. Comparative effects of overproducing the AraC-type transcriptional regulators MarA, SoxS, RarA and RamA on antimicrobial drug susceptibility in *Klebsiella pneumoniae*. *J Antimicrob Chemother* 2016; **71**:1820-5.
6. De Majumdar S, Yu J, Spencer J *et al*. Molecular basis of non-mutational derepression of *ramA* in *Klebsiella pneumoniae*. *J Antimicrob Chemother* 2014; **69**:2681-9.

7. Ruzin A, Immermann FW, Bradford PA. Real-time PCR and statistical analyses of *acrAB* and *ramA* expression in clinical isolates of *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* 2008; **52**:3430-2.
8. Bratu S, Landman D, George A *et al*. Correlation of the expression of *acrB* and the regulatory genes *marA*, *soxS* and *ramA* with antimicrobial resistance in clinical isolates of *Klebsiella pneumoniae* endemic to New York City. *J Antimicrob Chemother* 2009; **64**:278-83.
9. Hentschke M, Wolters M, Sobottka I *et al*. *ramR* mutations in clinical isolates of *Klebsiella pneumoniae* with reduced susceptibility to tigecycline. *Antimicrob Agents Chemother* 2010; **54**:2720-3.
10. Rosenblum R, Khan E, Gonzalez G *et al*. Genetic regulation of the *ramA* locus and its expression in clinical isolates of *Klebsiella pneumoniae*. *Int J Antimicrob Agents* 2011; **38**:39-45.
11. Schultsz C, Geerlings S. Plasmid-mediated resistance in Enterobacteriaceae: changing landscape and implications for therapy. *Drugs* 2012; **72**: 1-16.
12. Tsai YK, Fung CP, Lin JC *et al*. *Klebsiella pneumoniae* outer membrane porins OmpK35 and OmpK36 play roles in both antimicrobial resistance and virulence. *Antimicrob Agents Chemother* 2011; **55**:1485-93.
13. Clinical and Laboratory Standards Institute. *Performance Standards for Antimicrobial Disk Susceptibility Tests – Ninth Edition: Approved Standard M2-A9.* CLSI, Wayne, PA, USA, 2006.

14. Clinical and Laboratory Standards Institute. *Performance Standards for Antimicrobial Susceptibility Testing: Twenty-fourth Informational Supplement M100-S24.* CLSI, Wayne, PA, USA, 2014.
15. Bartolome B, Jubete Y, Martinez E *et al*. Construction and properties of a family of pACYC184-derived cloning vectors compatible with pBR322 and its derivatives. *Gene* 1991; **102**:75–78.
16. Brem J, Cain R, Cahill S *et al*. Structural basis of metallo-β-lactamase, serine-β-lactamase and penicillin-binding protein inhibition by cyclic boronates. *Nat Commun* 2016; **7**:12406.
17. Avison MB, von Heldreich CJ, Higgins CS *et al*. A TEM-2-β-lactamase encoded on an active Tn*1*-like transposon in the genome of a clinical isolate of *Stenotrophomonas maltophilia*. *J Antimicrob Chemother* 2000; **46**: 879-84.
18. Vinué L, Jové T, Torres C, Ploy MC. Diversity of class 1 integron gene cassette Pc promoter variants in clinical *Escherichia coli* strains and description of a new P2 promoter variant. *Int J Antimicrob Agents* 2011; **38**:526-9.
19. Pridmore, R. New and versatile cloning vectors with kanamycin resistance markers. *Gene* 1987; **56**:309–12.
20. Coldham NG, Webber M, Woodward MJ *et al*. A 96-well plate fluorescence assay for assessment of cellular permeability and active efflux in *Salmonella enterica* serovar Typhimurium and *Escherichia coli*. *J Antimicrob Chemother* 2010; **65**:1655-63.
21. Poirel L, Walsh TR, Cuvillier V, Nordmann P. Multiplex PCR for detection of acquired carbapenemase genes. Diagn Microbiol Infect Dis. 2011; **70**:119-23.

22. Woodford N, Fagan EJ, Ellington MJ. Multiplex PCR for rapid detection of genes encoding CTX-M extended-spectrum (beta)-lactamases. J Antimicrob Chemother. 2006 **57**:154-5.
23. Talfan A, Mounsey O, Charman M, *et al*. Involvement of mutation in *ampD* I, *mrcA*, and at least one additional gene in β-lactamase hyperproduction in *Stenotrophomonas maltophilia*. Antimicrob Agents Chemother. 2013 **57**:5486-91.
24. Chiu SK, Huang LY, Chen H, *et al.* Roles of *ramR* and *tet*(A) mutations in conferring tigecycline resistance in carbapenem-resistant *Klebsiella pneumoniae* clinical isolates. Antimicrob Agents Chemother. 2017 pii: AAC.00391-17. doi: 10.1128/AAC.00391-17.
25. García-Sureda L, Juan C, Doménech-Sánchez A *et al*. Role of *Klebsiella pneumoniae* LamB Porin in antimicrobial resistance. *Antimicrob Agents Chemother* 2011; **55**:1803-5.
26. Ruiz E, Ocampo-Sosa AA, Rezusta A *et al*. Acquisition of carbapenem resistance in multiresistant *Klebsiella pneumoniae* strains harbouring $bla_{\text{CTX-M-15}}$, *qnrS1* and *aac*(6')-Ib-cr genes. *J Med Microbiol* 2012; **61**:672-7.
27. Malinverni JC, Silhavy TJ. An ABC transport system that maintains lipid asymmetry in the gram-negative outer membrane. *Proc Natl Acad Sci U S A* 2009; **106**:8009-14.
28. Thong S, Ercan B, Torta F, *et al*. Defining key roles for auxiliary proteins in an ABC transporter that maintains bacterial outer membrane lipid asymmetry. *Elife*. 2016; **5**. pii: e19042.
29. Ekiert DC, Bhabha G, Isom GL, *et al*. Architectures of Lipid Transport Systems for the Bacterial Outer Membrane. *Cell* 2017; **169**:273-285.

30. Cohen SP, McMurry LM, Levy SB. *marA* locus causes decreased expression of OmpF porin in multiple-antibiotic-resistant (Mar) mutants of *Escherichia coli*. *J Bacteriol* 1988 ; **170**:5416-22.
31. Saw HT, Webber MA, Mushtaq S *et al*. Inactivation or inhibition of AcrAB-TolC increases resistance of carbapenemase-producing Enterobacteriaceae to carbapenems. *J Antimicrob Chemother* 2016; **71**:1510-9.

Table 1 MICs of β-lactams against *K. pneumoniae* Ecl8 and Ecl8Δ*ramR* transformants carrying clinically important β-lactamase genes.

	pSU18		IMP-1		NDM-1		VIM-1		CTX-M1		CMY-4		KPC-3		OXA-48	
Antibiotic (μg)	**Ecl8**	**Δ**	**Ecl8**	**Δ**	**Ecl8**	**Δ**	**Ecl8**	**Δ**	**Ecl8**	**Δ**	**Ecl8**	**Δ**	**Ecl8**	**Δ**	**Ecl8**	**Δ**
Ampicillin (10)	16	10	6	6	6	6	6	6	11	6	6	6	6	6	12	6
Piperacillin (100)	29	25	27	22	9	6	22	15	15	6	17	14	8	6	28	26
Piperacillin/ Tazobactam (110)	32	26	30	22	8	6	22	15	30	25	25	20	16	8	30	28
Cefotetan (30)	32	30	15	6	14	6	30	22	30	28	17	14	21	13	31	27
Cefoxitin (30)	30	23	6	6	6	6	22	15	28	23	11	6	23	10	28	20
Cefuroxime (30)	32	22	6	6	6	6	12	6	16	6	6	6	6	6	30	21
Cefotaxime (30)	40	32	20	12	6	6	22	15	15	6	15	12	19	10	39	32
Ceftriaxone (30)	38	33	19	14	6	6	25	16	16	6	15	10	13	6	35	32
Ceftazidime (30)	33	30	15	6	6	6	19	11	20	15	13	7	14	6	31	29
Cefoperazone (75)	34	29	19	15	10	6	22	15	17	6	22	20	11	8	30	26
Ceftizoxime (30)	40	35	20	15	6	6	25	19	30	15	18	16	20	11	40	33
Cefixime (5)	33	29	14	6	6	6	18	6	16	6	6	6	17	6	31	27
Cefepime (30)	36	36	25	20	18	10	26	20	22	15	37	33	19	11	35	31
Aztreonam (30)	40	38	40	37	40	38	40	37	19	15	18	15	12	6	38	33
Doripenem (10)	30	30	20	18	15	10	23	21	30	30	26	26	16	16	28	28
Ertapenem (10)	30	30	20	15	10	6	26	20	29	22	23	20	17	9	24	22
Imipenem (10)	30	30	16	16	14	10	19	19	30	30	30	30	14	11	26	25
Meropenem (10)	30	30	18	15	8	8	23	20	30	30	31	31	18	12	30	27

Assays were performed using Muller Hinton agar according to the CLSI protocol [13] for *K. pneumoniae* Ecl8 and Ecl8Δ*ramR* (Δ). Values reported are the means of three repetitions

rounded to the nearest integer. Zones confirming non-susceptibility are shaded grey. Susceptibility breakpoints are as set by the CLSI.[14]

Table 2. Significant Changes in Envelope Protein Abundance Seen in Ecl8Δ*ramR* versus Ecl8 During Growth in Both MHB and NB

Accession	Description	MHB		NB	
		p value	Fold Change	*p* value	Fold Change
A6T5M4	AcrB	0.038	4.87	0.044	6.21
A6T5M5	AcrA	0.002	3.88	0.002	3.38
A6T5Q5	Putative Outer Membrane Protein	<0.001	5.85	<0.001	>20
A6T6W3	Putative lipoprotein YbjP	0.001	5.30	0.002	7.01
A6T721	OmpK35	0.007	0.43	0.026	0.23
A6T7Z9	ABC Transporter SapF	<0.001	>20	<0.001	>20
A6T8F9	Heat shock protein HslJ	<0.001	>20	<0.001	>20
A6T9Y7	Putative uncharacterized protein YdhA	0.021	6.17	<0.001	>20
A6TCQ4	OqxA	<0.001	>20	<0.001	>20
A6TCQ5	OqxB	<0.001	>20	<0.001	>20
A6TCT2	Putative Uncharacterized Protein	0.012	4.06	<0.001	5.03
A6TE24	TolC	0.004	3.15	0.013	3.51

Methodology and details of data analysis are described in Supplementary Material. Where protein goes from being undetectable to being detectable in parent versus mutant, a nominal fold change of >20 and a p value of <0.001 have been applied, as described.[5]

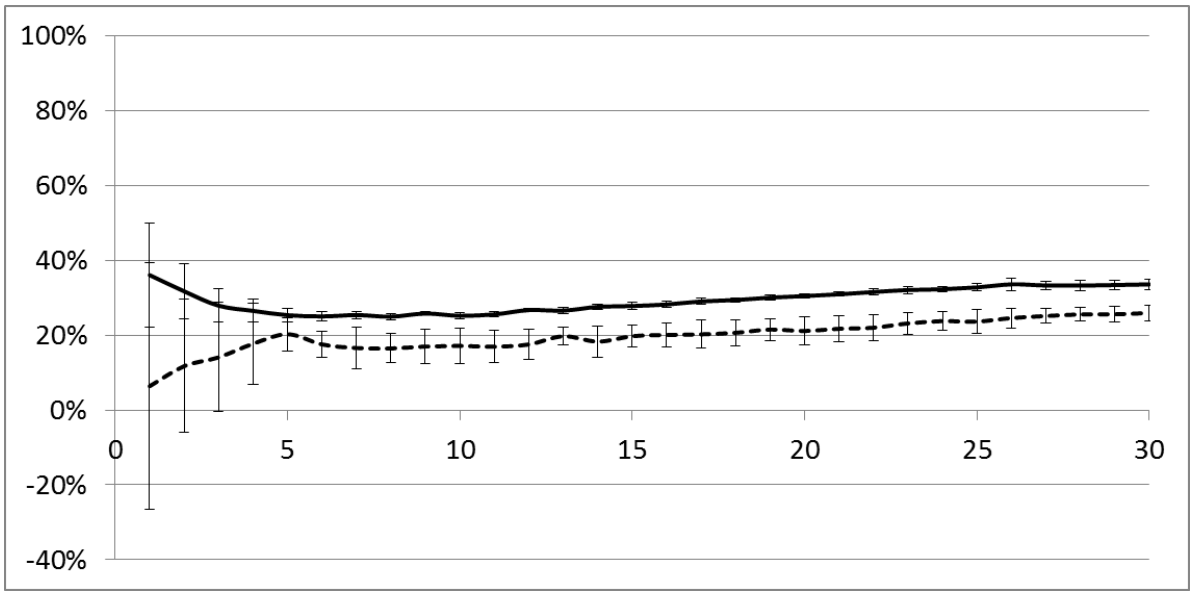
Figure Legends

Figure 1: Effect of RamA Overproduction or *micF* Overexpression in *K. pneumoniae* Ecl8 on Envelope Permeability in Different Growth Media.

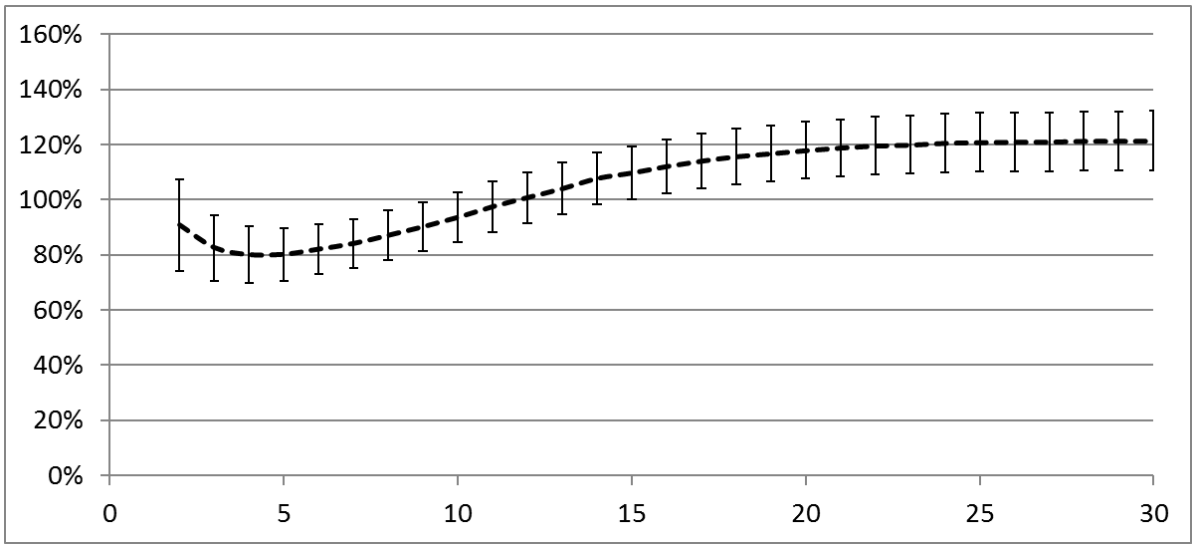
The accumulation of H33342 dye over a 30 cycle (45 minute) incubation period by (A) *K. pneumoniae* Ecl8Δ*ramR* compared with Ecl8 (set to 100%) grown in (solid line) NB and (dashed line) MHB. (B) *K. pneumoniae* Ecl8(*micF*) compared with Ecl8(control) (set to 100%) grown in MHB. Each line shows mean data for three biological replicates with 8 technical replicates in each, and error bars define the standard error of the mean (SEM).

Figure 1

A



B



480 **The Envelope Proteome Changes Driven by RamA Overproduction in *Klebsiella***

481 ***pneumoniae* that Enhance Acquired β-Lactam Resistance.**

482

483 **Supplementary Data**

SUPPLEMENTARY METHODS

Proteomic Analysis

Cells in cultures were pelleted by centrifugation (10 min, 4,000 × g, 4˚C) and resuspended in 20 mL of 30 mM Tris-HCl, pH 8 and broken by sonication using a cycle of 1 sec on, 0.5 sec off for 3 min at amplitude of 63% using a Sonics Vibracell VC-505TM (Sonics and Materials Inc., Newton, Connecticut, USA). The sonicated samples were centrifuged at 8,000 rpm (Sorval RC5B PLUS using an SS-34 rotor) for 15 min at 4˚C to pellet intact cells and large cell debris. The supernatant was removed and subjected to centrifugation at 20,000 rpm for 60 min at 4˚C using the above rotor to pellet total envelopes. To isolate total envelope proteins, this total envelope pellet was solubilised using 200 µL of 30 mM Tris-HCl pH 8 containing 0.5% (w/v) SDS. The protein concentration in each sample was quantified using Biorad Protein Assay Dye Reagent Concentrate according to the manufacturer's instructions. Proteins (5 µg/lane) were separated by SDS-PAGE using 11% acrylamide, 0.5% bis-acrylamide (Biorad) gels and a Biorad Min-Protein Tetracell chamber model 3000X1. Gels were resolved at 200 V until the dye front had moved approximately 1 cm into the separating gel. Proteins in all gels were stained with Instant Blue (Expedeon) for 20 min and de-stained in water.

The one centimeter of gel lane containing total envelope proteins was cut out and subjected to in-gel tryptic digestion using a ProGest automated digestion unit (Digilab UK). The resulting peptides were fractionated using an Ultimate 3000 nanoHPLC system in line with an LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific). In brief, peptides in 1% (v/v) formic acid were injected onto an Acclaim PepMap C18 nano-trap column (Thermo Scientific). After washing with 0.5% (v/v) acetonitrile plus 0.1% (v/v) formic acid, peptides were resolved on a

250 mm × 75 µm Acclaim PepMap C18 reverse phase analytical column (Thermo Scientific) over a 150 min organic gradient, using 7 gradient segments (1-6% solvent B over 1 min., 6-15% B over 58 min., 15-32% B over 58 min., 32-40% B over 3 min., 40-90% B over 1 min., held at 90% B for 6 min and then reduced to 1% B over 1 min.) with a flow rate of 300 nl.min−1. Solvent A was 0.1% formic acid and Solvent B was aqueous 80% acetonitrile in 0.1% formic acid. Peptides were ionized by nano-electrospray ionization at 2.1 kV using a stainless steel emitter with an internal diameter of 30 µm (Thermo Scientific) and a capillary temperature of 250°C. Tandem mass spectra were acquired using an LTQ-Orbitrap Velos mass spectrometer controlled by Xcalibur 2.1 software (Thermo Scientific) and operated in data-dependent acquisition mode. The Orbitrap was set to analyze the survey scans at 60,000 resolution (at m/z 400) in the mass range m/z 300 to 2000 and the top twenty multiply charged ions in each duty cycle selected for MS/MS in the LTQ linear ion trap. Charge state filtering, where unassigned precursor ions were not selected for fragmentation, and dynamic exclusion (repeat count, 1; repeat duration, 30 s; exclusion list size, 500) were used. Fragmentation conditions in the LTQ were as follows: normalized collision energy, 40%; activation q, 0.25; activation time 10 ms; and minimum ion selection intensity, 500 counts.

The raw data files were processed and quantified using Proteome Discoverer software v1.2 (Thermo Scientific) and searched against the UniProt K. pneumoniae strain ATCC 700721 / MGH 78578 database (5126 protein entries; UniProt accession 272620) using the SEQUEST (Ver. 28 Rev. 13) algorithm. Peptide precursor mass tolerance was set at 10 ppm, and MS/MS tolerance was set at 0.8 Da. Search criteria included carbamidomethylation of cysteine (+57.0214) as a fixed modification and oxidation of methionine (+15.9949) as a variable modification. Searches were performed with full tryptic digestion and a maximum of 1 missed cleavage was allowed.

The reverse database search option was enabled and all peptide data was filtered to satisfy false discovery rate (FDR) of 5 %. The Proteome Discoverer software generates a reverse "decoy" database from the same protein database used for the analysis and any peptides passing the initial filtering parameters that were derived from this decoy database are defined as false positive identifications. The minimum cross-correlation factor filter was readjusted for each individual charge state separately to optimally meet the predetermined target FDR of 5 % based on the number of random false positive matches from the reverse decoy database. Thus, each data set has its own passing parameters. Protein abundance measurements were calculated from peptide peak areas using the Top 3 method [S1] and proteins with fewer than three peptides identified were excluded.

Proteomic analysis was repeated three times for each test condition, each using a separate batch of cells. Data analysis was as follows: raw protein abundance data were uploaded into Microsoft Excel. A paired T-test was used to calculate the significance of any difference in protein abundance data in pooled data from the two test conditions; where protein abundance was below the level of detection the value was excluded and not set to zero. A p-value of <0.05 was considered significant. The fold change in abundance for each protein in two test conditions was calculated using the averages of absolute abundances for the three biological replicates for the two test conditions. All raw protein abundance data are provided in the attached proteomics data file.

Quantitative Reverse-Transcriptase PCR

RNA in cultures was stabilised using RNAprotect Bacteria Reagent (Qiagen, Crawley, UK) according to the manufacturer's instructions, before 1.5 mL of stabilised culture was

553 centrifugation for 5 min at 4000 rpm (Function Line Labofuge 400R centrifuge, Heraeus, Hanau,
554 Germany) to pellet cells. Following aspiration of the supernatant, 200 µL of TE buffer containing
555 lysozyme (50 mg/mL) was used to disrupt the pellet, and the mixture was left for 10 min at
556 room temperature, vortexing every 2 min before addition of 700 µL RLT buffer (Qiagen)
557 containing 150 mM β-mercaptoethanol. The mixture was transferred to a tube containing acid
558 washed silica lysing matrix B (MP Biochemicals, Eschwege, Germany) and cells were disrupted
559 in a Ribolyser (Hybaid, Basingstoke, UK) (setting 6.0; 2 cycles lasting 45 s each). Following
560 disruption, RNA was purified using a Qiagen RNeasy RNA purification kit, according to the
561 manufacturer's instructions. RNA from 1.5 mL of bacterial culture was dissolved in 50 µL RNase
562 free water, contaminating genomic DNA was digested using a TURBO DNA-free™ Kit (Ambion,
563 Foster City, CA, USA) following the manufacturer's instructions. The concentration of RNA in
564 each sample was measured using a NanoDrop ND-100 spectrophotometer (Labtech, UK). One
565 microgram of total RNA was converted into cDNA using qScript™ reverse transcriptase (Quanta
566 Biosciences, Gaithersburg, MD, USA) in a 20 µL reaction which included random hexamer
567 primers. The reaction was incubated for 5 min at 25°C, 30 min at 42°C and 5 min at 85°C. cDNA
568 samples were stored at -20°C until used as templates for gene-specific real-time qPCR. The
569 reference gene in each case was *rrsE*. Each real-time qPCR reaction was prepared using 100 ng
570 of cDNA (quantified by Nanodrop) as template in a 20 µL reaction with PowerUp™ SYBR Green
571 Master Mix (Applied Biosystems, Waltham, Massachusetts, USA) and 500 nM of each primer.
572 Primers are listed in the supplementary table of our previous paper.[S2] The amplification and
573 quantification of cDNA copies was performed using a StepOnePlus™ Real-Time PCR System
574 (Applied Biosystems). Samples were run as 4 technical replicates and 3 biological replicates
575 were used for each comparison, each from RNA purified from a separate batch of cells. The PCR

amplification cycles consisted of initial denaturation at 95°C for 2 min followed by 40 cycles of denaturation at 95°C for 2 s and annealing/extension at 60°C for 30 s. The ratio of target to reference cDNA in a sample of comparator B was calculated relative to that in a sample of comparator A according to the $\Delta\Delta C^T$ method.[S3] In each case, comparator A was set as the control.

SUPPLEMENTARY REFERENCES

1. Silva JC, Gorenstein MV, Li G-Z, et al. Absolute quantification of proteins by LCMSE: a virtue of parallel MS acquisition. *Mol Cell Proteomics* 2006; **5**: 144-156.
2. Jiménez-Castellanos JC, Wan Ahmad Kamil WN, Cheung CH, Tobin MS, Brown J, Isaac SG, Heesom KJ, Schneiders T, Avison MB. Comparative effects of overproducing the AraC-type transcriptional regulators MarA, SoxS, RarA and RamA on antimicrobial drug susceptibility in Klebsiella pneumoniae. *J Antimicrob Chemother* 2016; **71**:1820-5.
3. Pfaffl, MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 2001; **29**: e45.
4. Hentschke M, Wolters M, Sobottka I, Rohde H, Aepfelbacher M. *ramR* mutations in clinical isolates of *Klebsiella pneumoniae* with reduced susceptibility to tigecycline. *Antimicrob Agents Chemother*. 2010;**54**:2720-2723.
5. Poirel L, Walsh TR, Cuvillier V, Nordmann P. Multiplex PCR for detection of acquired carbapenemase genes. Diagn Microbiol Infect Dis. 2011; **70**:119-23.

6. Woodford N, Fagan EJ, Ellington MJ. Multiplex PCR for rapid detection of genes encoding CTX-M extended-spectrum (beta)-lactamases. J Antimicrob Chemother. 2006 **57**:154-5.

SUPPLEMENTARY TABLES

Table S1. Cloning strategies and PCR primers used.

Primer	Sequence (5' to 3')	Cloning strategy
bla$_{IMP-1}$ *F*	GCCTGTTCGGTTCG	PCR amplification from *Pseudomonas aeruginosa* clinical isolate 206-3105A clinical isolate (a gift from Dr Mark Toleman, Cardiff University). Sequence as in Genbank accession number AP012280.1. Amplicon TA-cloned into pCR2.1-TOPO and subcloned using EcoRI into pSU18.
bla $_{IMP-1}$ *R*	AGCGAAGTTGATATGTATTGTG	
bla$_{VIM-1}$ *F*	ACCCAGTGGACATAAGCCTG	PCR amplified from a *K. pneumoniae* clinical isolate (a gift from Dr Jim Spencer, University of Bristol). Sequence as in Accession number GQ422829.1. TA-cloned into pCR2.1-TOPO and subcloned using EcoRI into pSU18.
bla $_{VIM-1}$ *R*	TCTGCTACTCGGCGACTGAG	
bla$_{CTX-M-1}$ *F*	AATACTACCTTGCTTTCTGA	PCR amplification from *an E. coli* clinical isolate (a gift from Professor Peter Hawkey, University of Birmingham). Sequence as in Accession number AM003904. Amplicon TA-cloned into pCR2.1-TOPO and subcloned using EcoRI into pSU18.
bla$_{CTX-M-1}$ *R*	GGTGGCATAATTTTGAAGT	
bla$_{CMY-4}$ *F*	CTGCAGAATTCGCCCTTCATTGC AGCAAAGATGAAA	PCR amplification from a *K. pneumoniae* clinical isolate (a gift from Dr Jim Spencer, University of Bristol). Sequence as in Genbank accession number NG_036465.1. Amplicon digested with EcoRI (sites underlined in the primer sequence) and directly ligated into pSU18.
bla$_{CMY-4}$ *R*	TGTGCTGGAATTCGCCCTTGTT ATTTACCACGGTAACGC	
bla$_{KPC-3}$ *F*	ACCCTTGCCATCCCGTGTGC	PCR amplified from a *K. pneumoniae* clinical isolate (a gift from Dr Jim Spencer, University of Bristol). Sequence as in Genbank accession number EU176011.1. Amplicon TA-cloned into pCR2.1-TOPO and subcloned using EcoRI into pSU18.
bla$_{KPC-3}$ *R*	CGCCATCGTCAGTGCTCTAC	
bla$_{OXA-48}$ *F*	GAATTCGAGCAAACGATC	Gene synthesised as in Accession number NC_019154.1 with the addition of flanking EcoRI sites and ligated into vector pEX-A2. Sequence in previous column shows the ends of the synthesised sequence plus EcoRI sites (underlined). Subcloned into pSU18 using EcoRI.
bla$_{OXA-48}$ *F*	GAATTCGAATTCCCTAGCA	
micF F	CATATCTATAGCACTGAATGG	PCR amplified from *K. pneumoniae* Ecl8. Amplicon TA cloned

micF R	TAAAGAAGGGTAAAAAAAAGCG	into pCR2.1-TOPO and subcloned using EcoRI into pK18
ramR F	CTGCAGTGCCCGGTGAACCCTGGCGT	For PCR Sequencing of *ramR* in clinical isolates.[S4]
ramR R	CTGCAGATTTGCTGATTCAGCAGCGAC	

604

605

606 **Table S2. Multiplex PCR primers used in this study**

Primer	Sequence (5'-3')	Reference	Product size (bp)
IMP F	GGAATAGAGTGGCTTAACTCTC	(Poirel et al., 2011)[S5]	232
IMP R	GGTTTAACAAAACAACCACC		
VIM F	GATGGTGTTTGGTCGCATA	(Poirel et al., 2011)[S5]	390
VIM R	CGAATGCGCAGCACCAG		
OXA-48 F	GCGTGGTTAAGGATGAACAC	(Poirel et al., 2011)[S5]	438
OXA-48 R	CATCAAGTTCAACCCAACCG		
NDM F	GGTTTGGCGATCTGGTTTTC	(Poirel et al., 2011)[S5]	621
NDM R	CGGAATGGCTCATCACGATC		
KPC F	CGTCTAGTTCTGCTGTCTTG	(Poirel et al., 2011)[S5]	798
KPC R	CTTGTCATCCTTGTTAGGCG		
TEM F	CCGAAGAACGTTTTCCAATG	This study	249
TEM R	GTCCTCCGATCGTTGTCAGAA		
SHV F	CTTTCCCATGATGAGCACCT	This study	127
SHV R	GCGAGTAGTCCACCAGATCC		
OXA-1 F	TTATCTACAGCAGCGCCAGT	This study	451
OXA-1 R	AAGCTACTTTCGAGCCATGC		
CMY (G1) F	CGATCCGGTCACGAAATACT	This study	556
CMY (G1) R	CCAGCCTAATCCCTGGTACA		
CTX-M (G1) F	AAAAATCACTGCGCCAGTTC	(Woodford et al., 2006)[S6]	415
CTX-M (G1) R	AGCTTATTCATCGCCACGTT		
CTX-M (G2) F	CGACGCTACCCCTGCTATT	(Woodford et al., 2006)[S6]	552
CTX-M (G2) R	CCAGCGTCAGATTTTTCAGG		
CTX-M (G8) F	TCGCGTTAAGCGGATGATGC	(Woodford et al., 2006)[S6]	666
CTX-M (G8/25) R	AACCCACGATGTGGGTAGC		
CTX-M (G9) F	CAAAGAGAGTGCAACGGATG	(Woodford et al., 2006)[S6]	205
CTX-M (G9) R	ATTGGAAAGCGT TCATCACC		
CTX-M (G25) F	GCACGATGACATTCGGG	(Woodford et al., 2006)[S6]	327

607

608

609

610

611

612

613

Table S3. β-lactamase activity in Ecl8Δ*ramR* transformants versus Ecl8 transformants carrying the same β-lactamase gene.

	Ecl8		Ecl8Δ*ramR*	
	Mean	**SEM**	**Mean**	**SEM**
pSU18	7.56	4.53	8.70	8.14
pSU(IMP)	193.57	13.61	257.02	31.56
pSU(OXA)	111.50	4.05	89.80	6.59
pSU(CTX)	54.19	4.48	41.59	7.18
pSU(VIM)	227.49	34.16	142.35	5.62
pSU(CMY)	36.05	2.79	16.12	4.60
pSU(NDM)	51.80	6.13	52.93	6.71
pSU(KPC)	231.32	22.07	317.29	34.57

Table S4. Impact of β-lactamase carriage and *ramR* mutation on β-lactam susceptibility in *K. pneumoniae* clinical isolates

Strain	FOX	CXM	CTX	CAZ	FEP	ATM	IPM	MEM	DOR	ETP	Cephalosporinase/Carbapenemase	RamR	*ramA*
Y	6	6	6	6	6	6	10	6	6	6	CTX-M Group 1, OXA-48-like, NDM	Lys9Ile	12.7 +/- 2.5
X	6	6	10	6	7	6	10	6	6	6	KPC	Ala40Val	43.6 +/- 8.7
D	6	6	6	6	6	27	11	8	8	6	VIM		
C	7	6	6	14	9	8	16	10	11	6	CTX-M Group 1, OXA-48-like	Met184Val	4.3 +/- 1.0
T	8	6	6	11	6	6	25	23	21	17	CTX-M Group 1	Lys63 FS	45 +/- 6.3
E	24	6	6	6	8	6	28	30	28	24	CTX-M Group 1	Thr141Ile	
G	23	6	9	16	13	14	25	29	24	27	CTX-M Group 1		
AQ	23	6	10	15	19	13	26	27	27	23	CTX-M Group 1	Thr141Ile	
K	25	6	10*	18	18	17	25	29	26	27	CTX-M Group 1	Thr141Ile	
S	24	6	9	17	16	12	25	30	25	27	CTX-M Group 1	Thr141Ile	
AB	23	6	11	18	16	14	24	28	24	28	CTX-M Group 1		
AC	25	6	8	17	15	13	24	30	24	30	CTX-M Group 1	Thr141Ile	
W	24	6	10	16	17	10	26	29	27	28	CTX-M Group 1	Thr141Ile	
I	24	6	10*	19	19	17	27	30	27	29	CTX-M Group 1	Thr141Ile	
Q	25	6	10	18	17	14	25	30	26	27	CTX-M Group 1		
AA	25	6	11	18	17	15	26	28	26	29	CTX-M Group 1		
P	26	6	11	18	17	17	25	29	26	28	CTX-M Group 1	Thr141Ile	
O	25	6	12	18	19	17	25	29	26	28	CTX-M Group 1	Thr141Ile	
L	25	6	11	20	21	18	25	29	25	28	CTX-M Group 1	Thr141Ile	
B	25	6	13	20	18	17	24	29	27	30	CTX-M Group 1	Thr141Ile	
M	22	6	16	20	22	17	25	29	26	28	CTX-M Group 1	Thr141Ile	
R	27	6	12	19	21	18	26	30	26	27	CTX-M Group 1	Thr141Ile	
AD	26	6	11	21	19	19	27	29	25	30	CTX-M Group 1		
F	26	6	13	20	21	20	25	30	25	30	CTX-M Group 1		
J	25	6	14	23	21	22	25	30	27	27	CTX-M Group 1	Ala19Val	1.0 +/- 0.1
N	16	18	29	26	29	29	28	31	28	28		Arg44 FS	70.6 +/- 19.1
AE	23	21	27	29	31	31	25	25	25	25		Thr141Ile	0.8 +/- 0.1
Z	18	18	28	28	30	30	27	29	27	30		Thr141Ile	
AJ	23	22	28	29	31	32	27	24	27	25		Thr141Ile	
V	22	22	31	29	28	30	25	30	26	30			
AR	20	21	28	28	30	31	27	30	28	30		Thr141Ile	
U	21	20	30	27	31	32	29	30	28	29		Thr141Ile	
AI	24	23	31	27	30	32	25	30	27	28			
AG	23	22	31	26	30	33	26	31	28	29			
AN	25	25	34	27	29	31	23	31	25	30			
AF	23	24	30	28	31	34	26	29	27	29		Ala19Val	
AH	26	25	31	28	31	32	25	30	26	28			CONTROL
H	26	23	31	28	31	33	26	29	27	30		Thr141Ile	
A	26	21	32	29	32	33	26	28	27	31			
AP	24	23	32	30	32	33	27	30	27	29		Thr141Ile	
AK	25	27	33	29	31	33	25	30	25	30		Ile106Ser	0.7 +/- 0.1
AO	28	21	32	28	30	32	26	33	28	31		Thr50Ala	4.7 +/- 0.7
AM	23	24	34	29	32	33	26	32	27	31			
AL	26	26	34	29	32	33	26	31	26	30			

621 Abbreviations: FOX, cefoxitin; CXM, cefuroxime; CTX, cefotaxime; CAZ, ceftazidime; FEP,
622 cefepime; ATM, aztreonam; IPM, imipenem; MEM, meropenem; DOR, doripenem; ETP,
623 ertapenem.
624 Inhibition zone diameters were determined as per the CLSI methodology [13] and interpreted as
625 susceptible (blue), intermediate (green) or resistant (yellow) as per CLSI breakpoints.[14]
626 *ramA* expression levels measured are by reference to the control isolate AH.
627

Table S5 – Envelope proteome changes following overproduction of RamA in *K. pneumoniae* Ecl8 during growth in Muller-Hinton Broth.

Accession	Description	Ecl8 E1	Ecl8 E2	Ecl8 E3	Ecl8 Δ E1	Ecl8 Δ E2	Ecl8 Δ E3	T-Test	Fold Change
A6T4W5	DegP Serine Protease	2.30E+08	2.54E+08	2.44E+08	9.26E+08	4.59E+08	4.51E+08	0.039	2.52
A6T5M4	AcrB	1.38E+08	6.59E+08	5.71E+08	7.98E+08	3.17E+09	2.69E+09	0.038	4.87
A6T5M5	AcrA	2.96E+09	2.35E+09	2.33E+09	1.20E+10	7.66E+09	1.00E+10	0.002	3.88
A6T5Q5	Putative Outer Membrane Protein	1.08E+07			6.78E+07	4.26E+07	7.87E+07	<0.001	5.85
A6T5Y8	Mg2+ Transport ATPase	3.16E+07	5.03E+07	7.98E+07	9.69E+07	2.01E+08	2.05E+08	0.020	3.11
A6T6B0	Periplasmic Binding Protein GltI	6.47E+07	4.94E+07	2.01E+07				<0.001	0.05
A6T6D9	Potassium-transporting ATPase KdpA	2.19E+08	1.80E+08	4.06E+08				<0.001	0.05
A6T6K5	Putative cation transport protein		5.00E+06	9.22E+06		6.51E+07	7.52E+07	0.004	9.87
A6T6W3	Putative lipoprotein YbjP	8.94E+08	7.67E+08	6.96E+08	5.15E+09	3.41E+09	3.95E+09	0.001	5.30
A6T721	OmpK35	2.70E+10	2.14E+10	1.77E+10	1.17E+10	6.97E+09	9.95E+09	0.007	0.43
A6T7Z9	ABC Transporter SapF				1.38E+08	6.33E+07	7.30E+07	<0.001	20.00
A6T8F9	Heat shock protein HslJ				2.73E+08	1.87E+08	1.97E+08	<0.001	20.00
A6T9Y7	Uncharacterized protein YdhA	3.57E+07	3.03E+07	2.54E+07	2.94E+08	1.27E+08	1.43E+08	0.021	6.17
A6TAW2	Cell Volume Regulation Protein A	1.93E+07	5.96E+07	6.18E+07	1.43E+08	1.05E+08	7.99E+07	0.026	2.33
A6TB10	Putative Enzyme	3.00E+07	3.81E+07	1.57E+07				<0.001	0.05
A6TBM0	D-ala-D-alanine endopeptidase PbpG				2.93E+07	7.95E+07	2.11E+07	<0.001	20.00
A6TCA4	Putative permease PerM	1.62E+07	3.26E+07	6.06E+07		1.28E+08	1.58E+08	0.007	3.92
A6TCQ4	OqxA				7.10E+07	8.78E+07	6.32E+07	<0.001	20.00
A6TCQ5	OqxB				3.09E+07	6.46E+07	2.97E+07	<0.001	20.00
A6TCT2	Putative Uncharacterized Protein	3.49E+08	2.57E+08	2.44E+08	1.50E+09	6.85E+08	1.27E+09	0.012	4.06
A6TE24	TolC	3.33E+09	2.53E+09	2.88E+09	1.17E+10	7.49E+09	8.30E+09	0.004	3.15
A6TF45	Glycerol-3-Phosphate Dehydrogenase GlpD	2.00E+07	9.77E+07	8.46E+06	6.82E+08	3.69E+08	7.45E+08	0.005	14.23
A6TGU2	Maltose ABC transport protein MalG		2.48E+08	1.81E+08		4.83E+07	3.80E+07	0.018	0.20
A6TGU4	Maltose-Binding Protein MalE	6.95E+07	2.19E+08	2.32E+08	5.84E+07	5.02E+07	7.54E+07	0.050	0.35
A6TGU6	Maltoporin LamB2	5.39E+09	1.35E+10	1.42E+10	3.74E+09	3.86E+09	4.03E+09	0.032	0.35
A6TH80	Putative Periplasmic Protein	4.27E+07	2.53E+07	2.65E+07				<0.001	0.05
A6THB7	Peptidyl-Prolyl cis-trans Isomerase FklB	2.59E+08	2.26E+08	4.01E+08				<0.001	0.05
A6THB8	D-alanine/D-serine/glycine Transporter CycA		2.32E+08	2.46E+08	4.63E+07	5.82E+07	1.68E+08	0.030	0.38
A6THT5	Putative porin	9.11E+08	8.09E+08	6.17E+08	2.46E+09	1.49E+09	2.04E+09	0.007	2.56

Absolute protein abundance data are reported for three experiments (E1, E2 and E2), each from a different batch of cells of Ecl8 or Ecl8Δ*ramR* (Ecl8 Δ). Data are analysed as explained in Supplementary Methods, above. Proteins highlighted in Green are significantly regulated (up or down) during the growth conditions used to generate the data in Table S6. Proteins highlighted in blue are also significantly regulated during the growth conditions used to generate the data in Table S7.

Table S6 – Envelope proteome changes following overproduction of RamA in *K. pneumoniae* Ecl8 during growth in Nutrient Broth.

Accession	Description	Ecl8 E1	Ecl8 E2	Ecl8 E3	Ecl8 Δ E1	Ecl8 Δ E2	Ecl8 Δ E3	T-Test	Fold Change
A6T4P6	GMP reductase GuaC	1.41E+08	1.46E+07	8.29E+06	1.15E+08			<0.001	2.10
A6T4Q8	Dihydrolipoamide acetyltransferase AceF	1.69E+09			1.39E+09	1.54E+07	1.61E+07	<0.001	0.28
A6T5J2	ABC transporter MdlA	1.20E+07	1.21E+07	1.37E+07	4.44E+07	2.64E+07	2.31E+07	0.024	2.48
A6T5M4	AcrB	3.23E+08	1.42E+08	1.46E+08	2.20E+09	7.76E+08	8.12E+08	0.044	6.21
A6T5M5	AcrA	1.91E+09	9.54E+08	6.59E+08	4.20E+09	4.23E+09	3.45E+09	0.002	3.38
A6T5Q5	Putative outer membrane protein				5.99E+07	6.50E+07	4.62E+07	<0.001	20.00
A6T6Q4	ABC transporterer GlnP				3.92E+07	6.19E+07	5.86E+07	<0.001	20.00
A6T6W3	Putative lipoprotein YbjP	3.11E+08	4.07E+08	1.86E+08	1.68E+09	2.72E+09	1.94E+09	0.002	7.01
A6T721	OmpK35	2.48E+10	1.33E+10	9.85E+09	4.01E+09	3.45E+09	3.42E+09	0.026	0.23
A6T7Y0	Putative peptidase SohB	1.24E+08	5.59E+07	5.11E+07	1.51E+08	1.89E+08	1.39E+08	0.020	2.08
A6T7Z9	ABC Transporter SapF				4.45E+07	2.15E+07	1.75E+07	<0.001	20.00
A6T813	Putative enzyme YcjX				4.53E+07	5.68E+06	2.85E+06	<0.001	20.00
A6T8F9	Heat shock protein HslJ				4.83E+07	1.47E+08	4.82E+07	<0.001	20.00
A6T9P0	Nitrate reductase NarZ	1.10E+08	1.55E+08	3.92E+07	1.52E+08	2.07E+08	3.07E+08	0.050	2.19
A6T9W1	Glucose dehydrogenase				2.52E+07	7.99E+06	1.15E+07	<0.001	20.00
A6T9Y7	Putative uncharacterized protein YdhA				6.58E+07	1.03E+08	8.57E+07	<0.001	20.00
A6TAG5	Putative oxidase YdiJ		1.34E+07		5.38E+07	4.88E+07	2.09E+07	<0.001	3.06
A6TB64	Response regulator UvrY	1.26E+08			1.77E+08	7.77E+06	5.48E+06	<0.001	0.50
A6TBL5	Beta-D-glucoside glucohydrolase	1.58E+08			1.35E+08	1.15E+07	3.62E+07	<0.001	0.39
A6TC20	3-ketoacyl-CoA thiolase	4.97E+07	1.24E+07	1.06E+07				<0.001	0.05
A6TC59	Sulfate permease CysA		1.33E+07	1.22E+07		3.37E+07	3.39E+07	0.000	2.65
A6TCQ4	OqxA				1.15E+07	1.09E+07	1.56E+07	<0.001	20.00
A6TCQ5	OqxB				1.28E+07	1.64E+07	1.54E+07	<0.001	20.00
A6TCT2	Putative uncharacterized protein	5.93E+07	5.37E+07	7.11E+07	3.03E+08	3.27E+08	2.96E+08	<0.001	5.03
A6TE24	TolC	2.23E+09	1.20E+09	8.93E+08	6.81E+09	4.91E+09	3.44E+09	0.013	3.51
A6TEC8	Serine/threonine transporter SstT	4.48E+07	1.16E+07	1.86E+07			8.35E+06	<0.001	0.33
A6TEI6	Ribosome-binding factor A	6.73E+07	2.02E+07	1.70E+07	1.46E+08			<0.001	4.18
A6TEL1	Putative ABC Transporter YrbC		7.98E+06	2.00E+07	8.85E+07	5.39E+07	3.82E+07	0.050	4.30
A6TEL2	Putative ABC Transporter YrbD PE=4 SV=1 - [A6TEL2_KLEP7]	2.31E+08	2.97E+08	2.21E+08	6.21E+08	7.01E+08	6.00E+08	0.000	2.57
A6TEL3	Putative ABC Transporter YrbE		1.17E+07	1.42E+07		3.52E+07	4.21E+07	0.010	2.99
A6TEL4	Putative ABC Transporter YrbF		5.97E+07	6.79E+07	2.24E+08	1.51E+08	1.33E+08	0.031	2.65
A6TET0	AcrE		3.74E+08	2.73E+08		1.23E+09	1.29E+09	0.002	3.89
A6TET1	AcrF				2.58E+08	1.36E+08	1.34E+08	<0.001	20.00

Absolute protein abundance data are reported for three experiments (E1, E2 and E2), each from a different batch of cells of Ecl8 or Ecl8Δ*ramR* (Ecl8 Δ). Data are analysed as explained in Supplementary Methods, above. Proteins highlighted in Green are significantly regulated (up or down) during the growth conditions used to generate the data in Table S5. Proteins highlighted in orange are also significantly regulated during the growth conditions used to generate the data in Table S7.

Table S7 – Envelope proteome changes following overproduction of RamA in *K. pneumoniae* NCTC5055 using the pBAD expression vector during growth in Nutrient Broth.

Accession	Description	pBAD E1	pBAD E2	pBAD E3	RamA E1	RamA E2	RamA E3	T-Test	Fold Change
A6T4S4	Putative ATP transporter YadG	9.22E+07	1.04E+08	1.47E+08	6.21E+08	3.10E+08	4.80E+08	0.01	4.11
A6T4W5	Periplasmic serine protease DegP	1.36E+08	1.13E+08	3.83E+07	2.04E+08	2.02E+08	2.86E+08	0.01	2.41
A6T4X8	Membrane-associated protease YaeL	8.49E+07	1.75E+07	2.92E+07	1.15E+08			<0.001	2.61
A6T5A1	Uncharacterized protein YaiW			7.80E+06	9.83E+07	1.82E+07	3.86E+07	<0.001	6.64
A6T5M4	AcrB	1.12E+09	8.70E+07	9.87E+07	4.52E+09	9.67E+08	3.34E+09	0.04	6.74
A6T5M5	AcrA	1.86E+09	5.63E+08	4.27E+08	1.49E+10	4.92E+09	7.87E+09	0.03	9.73
A6T5Q5	Putative outer membrane protein				1.36E+08	2.25E+08	1.07E+08	<0.001	>20
A6T5S5	Lipid A biosynthesis protein				2.96E+08	6.11E+07	1.57E+08	<0.001	>20
A6T5S9	Lysine decarboxylase CadA	5.09E+07	1.45E+08	2.03E+07				<0.001	<0.05
A6T5Y2	Nitroreductase NfnB				5.85E+07	5.07E+07	2.99E+07	<0.001	>20
A6T5Y5	Putative regulatory protein				4.56E+08	1.93E+08	2.59E+08	<0.001	>20
A6T689	Lipoyl synthase LipA	1.36E+08	1.05E+08	2.10E+07	3.43E+08	3.89E+08		0.00	4.20
A6T694	Minor lipoprotein RlpA	1.46E+08	6.95E+07	4.97E+07	1.78E+08			<0.001	2.02
A6T6W2	Putative ABC transport protein ArtP				2.33E+08	1.08E+08	2.01E+08	<0.001	>20
A6T6W3	Putative lipoprotein YbjP	3.57E+08	1.96E+08	4.29E+08	8.42E+09	3.82E+09	9.74E+09	0.01	22.40
A6T746	Putative protein YmbA	1.24E+08	4.55E+07	8.41E+07	3.88E+08	1.77E+08	3.76E+08	0.02	3.71
A6T7Y0	Putative peptidase SohB	1.42E+08	4.68E+07	7.32E+07	5.98E+08	2.49E+08	5.11E+08	0.01	5.19
A6T8F7	Oxidoreductase YdbK	2.29E+07			8.86E+07	3.34E+08	6.50E+07	<0.001	7.08
A6T8F9	Heat shock protein HslJ				2.41E+09	8.45E+08	1.95E+09	<0.001	>20
A6T8N5	Putative protein YdgH	1.03E+08	1.25E+08	9.58E+07	3.31E+08	1.23E+08	2.73E+08	0.05	2.25
A6T961	Putative regulatory protein DeoR	1.00E+08	8.53E+07	1.44E+08	2.94E+07	6.08E+07		0.04	0.41
A6T963	Putative pentose kinase	8.11E+07	6.20E+07	7.69E+06				<0.001	<0.05
A6T9L3	Alcohol dehydrogenase AdhP		1.97E+07	1.32E+07	3.99E+07	5.44E+07	6.33E+07	0.01	3.20
A6T9Y7	Putative protein YdhA	1.65E+08	7.58E+07	4.97E+07	6.63E+08	1.73E+08	5.42E+08	0.04	4.75
A6TAP0	Glutamyl-tRNA reductase HemA	1.40E+08	1.93E+07	4.53E+07	1.71E+08			<0.001	2.51
A6TBQ3	Putative enzyme YeiR				3.75E+07	4.09E+07	3.43E+07	<0.001	>20
A6TC64	Putative uncharacterized protein YfeY			4.74E+07	2.31E+08		1.31E+08	<0.001	3.81
A6TCA4	Putative permease PerM				3.43E+08	5.25E+07	2.29E+08	<0.001	>20
A6TCI0	GTPase Era	8.89E+07	8.89E+07	6.94E+06	1.55E+08	1.37E+08		0.05	2.37
A6TCQ4	OqxA				7.75E+07	5.51E+07	8.69E+07	<0.001	>20
A6TCQ5	OqxB				1.50E+08	2.13E+07	9.34E+07	<0.001	>20
A6TCT2	Putative uncharacterized protein	1.87E+08	9.32E+07	6.25E+07	1.79E+09	7.68E+08	7.58E+08	0.02	9.67
A6TE24	TolC	1.63E+09	9.81E+08	1.45E+09	1.29E+10	3.17E+09	7.03E+09	0.04	5.67
A6TEC6	Putative dehydrogenase YgjR				6.10E+07	6.97E+07	6.35E+07	<0.001	>20
A6TEH9	Putative enzyme YhbW				1.04E+08	3.46E+08	3.18E+08	<0.001	>20
A6TEL0	Putative protein YrbB				2.14E+08	7.54E+07	1.38E+08	<0.001	>20
A6TEL1	Putative ABC transport protein YrbC	5.45E+07	3.55E+07	4.27E+07	1.02E+08	8.96E+07	1.04E+08	0.00	2.23
A6TEL2	Putative ABC transport protein YrbD	5.73E+08	2.29E+08	2.01E+08	3.31E+09	9.12E+08	2.08E+09	0.03	6.28
A6TEL3	Putative ABC transport protein YrbE	1.16E+08		5.29E+07	4.30E+08		4.39E+08	0.00	5.16
A6TEL4	Putative ABC transport protein YrbF	1.73E+08	6.02E+07	9.25E+07	7.47E+08	3.20E+08	6.00E+08	0.01	5.11
A6TET0	AcrE		1.04E+08		3.01E+09	3.94E+08	1.61E+09	<0.001	16.10

A6TET1	AcrF	1.82E+08	1.10E+08	2.21E+08	9.57E+08	2.81E+09	8.41E+08	0.05	8.99
A6TEZ2	Putative enzyme YheT			7.29E+06	5.19E+07	3.84E+07	6.11E+07	<0.001	6.92
A6TG29	Phosphate transport protein PstB	2.82E+07	3.56E+07	1.55E+07	8.19E+07	4.81E+07		0.04	2.46
A6TG98	Putative glycoprotein	1.55E+08	4.46E+07	9.57E+06				<0.001	<0.05
A6TH72	Succinate dehydrogenase FrdB	6.93E+07	1.22E+08	2.03E+08	5.38E+07	3.61E+07	2.29E+07	0.04	0.29
A6TH73	Fumarate reductase FrdA	1.20E+08	1.54E+08	3.21E+08	7.69E+07	5.78E+07	4.52E+07	0.05	0.30
A6THK0	Putative alcohol dehydrogenase YjgB				4.44E+08	3.93E+08	7.85E+07	<0.001	>20
A6THQ0	Putative uncharacterized protein			2.95E+07	6.08E+08		6.16E+07	<0.001	11.34
A6THX3	Replication protein DnaC	1.14E+07	8.25E+06	7.71E+06				<0.001	<0.05
A6TI57	ATPase with chaperone activity	7.63E+08	2.12E+08	2.93E+07				<0.001	<0.05

Absolute protein abundance data are reported for three experiments (E1, E2 and E2), each from a different batch of cells carrying the pBAD(control) or pBAD(RamA) plasmids.[5] Data are analysed as explained in Supplementary Methods, above. Proteins highlighted in Green are significantly regulated (up or down) during the growth conditions used to generate the data in Table S5 and S6. Proteins highlighted in blue are also significantly regulated during the growth conditions used to generate the data in Table S5 only. Proteins highlighted in orange are also significantly regulated during the growth conditions used to generate the data in Table S6 only.

Table S8. Disc susceptibility assay for *K. pneumoniae* Ecl8 transformants with and without *micF* overexpression

Antibiotic (µg in disc)	pK18(control) Zone (mm)	pK18(*micF*) [Difference from Control] (mm)
Amikacin (30)	25	+3
Gentamicin (10)	25	NC
Tobramycin (10)	24	+4
Cefoxitin (30)	26	+5
Cefuroxime (30)	30	NC
Ceftriaxone (30)	35	NC
Cefotaxime (30)	35	+5
Ceftazidime (30)	30	+8
Cefepime (30)	34	+6
Aztreonam (30)	40	NC
Imipenem (10)	30	+5
Meropenem (10)	30	+5
Doripenem (10)	30	+5
Ciprofloxacin (5)	36	+2
Norfloxacin (10)	32	+6
Ofloxacin (5)	30	+5
Tigecycline (15)	23	+2
Minocycline (30)	23	+2
Chloramphenicol (30)	28	-2
Trimethoprim/ Sulfamethoxazole (1.25/23.75)	28	+3

Assays were performed using Muller Hinton agar containing 30 mg/L kanamycin to select for the plasmid. Otherwise, the assay was performed according to the CLSI protocol.[13] Abbreviation used is NC: No Change in zone diameter versus control. Values reported are the means of at least three repetitions rounded to the nearest integer. Mean changes <2 mm are reported as NC.