Absence, loss-of-function, or inhibition of \textit{Escherichia coli} AcrB does not increase expression of other efflux pump genes supporting the discovery of AcrB inhibitors as antibiotic adjuvants

Maria Laura Ciusa$^1$, Robert L. Marshall$^1$, Vito Ricci$^1$, Jack W. Stone$^1$ and Laura J. V. Piddock$^1$

$^1$Institute of Microbiology and Infection, College of Medical and Dental Sciences, University of Birmingham, Birmingham, UK

*Corresponding author. E-mail: l.j.v.piddock@bham.ac.uk

Received 11 August 2021; accepted 11 November 2021

Objectives: To determine whether expression of efflux pumps and antibiotic susceptibility are altered in \textit{Escherichia coli} in response to efflux inhibition.

Methods: The promoter regions of nine efflux pump genes (\textit{acrAB}, \textit{acrD}, \textit{acrEF}, \textit{emrAB}, \textit{macAB}, \textit{cusCFBA}, \textit{mdtK}, \textit{mdtABC}, \textit{mdfA}) were fused to \textit{gfp} in pMW82 and fluorescence from each reporter construct was used as a measure of the transcriptional response to conditions in which AcrB was inhibited, absent or made non-functional. Expression was also determined by RT-qPCR. Drug susceptibility of efflux pump mutants with missense mutations known or predicted to cause loss of function of the encoded efflux pump was investigated.

Results: Data from the GFP reporter constructs revealed that no increased expression of the tested efflux pump genes was observed when AcrB was absent, made non-functional, or inhibited by an efflux pump inhibitor/competitive substrate, such as PA$_B$N or chlorpromazine. This was confirmed by RT-qPCR for PA$_B$N and chlorpromazine; however, a small but significant increase in \textit{macB} gene expression was seen when \textit{acrB} is deleted. Efflux inhibitors only synergized with antibiotics in the presence of a functional AcrB. When AcrB was absent or non-functional, there was no impact on MICs when other efflux pumps were also made non-functional.

Conclusions: Absence, loss-of-function, or inhibition of \textit{E. coli} AcrB did not significantly increase expression of other efflux pump genes, which suggests there is no compensatory mechanism to overcome efflux inhibition and supports the discovery of inhibitors of AcrB as antibiotic adjuvants.

Introduction

The contribution of multidrug efflux to antibiotic resistance is well established and in Gram-negative bacteria the resistance nodulation-division (RND) family of transporters are reported to have the greatest effect on antibiotic susceptibility.\textsuperscript{1} \textit{Escherichia coli} has seven characterized RND transporters, of which AcrB, AcrD, AcrF, MdtB, MdtC and MdfF belong to the hydrophobic/amphiphilic efflux (HAE) subfamily, and CusA to the heavy metal efflux (HME) subfamily.\textsuperscript{2} A further 30 putative efflux pumps have been identified in \textit{E. coli} with some belonging to transporter families other than RND.\textsuperscript{3}

Overexpression of chromosomally encoded efflux pumps has been described as a mechanism of drug resistance in clinical and environmental isolates of multiple Gram-negative species.\textsuperscript{4–8} For this reason, MDR efflux systems are attractive targets for antimicrobial drug discovery as addition of efflux inhibitors to currently available antibiotics will extend their spectrum of activity and lengthen the period of effective use. However, the bacterial response to efflux inhibition and drug–inhibitor combinations still needs to be investigated.

Regulation of efflux pumps is complex and involves local as well as global regulators. The AcrAB-TolC system is constitutively expressed and altering the expression of the AcrB transporter has a profound effect on susceptibility to a wide variety of compounds.\textsuperscript{9} Most other efflux pumps are expressed at low levels under normal conditions or are synthesized de novo in response to a specific environmental stress.\textsuperscript{10} Nonetheless, overexpression of other pumps has been shown to confer a decrease in susceptibility to several toxic compounds.\textsuperscript{3} Several studies have shown that in the presence of AcrB, deletion of efflux genes has little to no impact on antibiotic susceptibility.\textsuperscript{11–14} High-level expression of efflux pumps can be achieved by mutation in the elements regulating their expression, or by the presence of their inducers.\textsuperscript{15–19} Increased expression of other efflux pumps in the absence of AcrB (or its homologue in other Gram-negative bacteria e.g. MexB) is hypothesized to be a compensatory effect due to the absence of...
the main RND efflux transporter gene of the species.\textsuperscript{4–8,15,17,19–22} However, in Salmonella enterica serovar Typhimurium this compensatory effect is not seen when AcrB is present but functionally inactivated.\textsuperscript{23}

In this study, we sought to determine whether chemical inhibition of AcrB impacts the expression of four RND (acrD, acrEF, mdtABC, cusCFBA), two MFS (emrAB, mdfA) one ABC (macAB) and one MATE (mdtK) efflux pump genes and drug susceptibility in E. coli. These pump genes were chosen as other studies indicated that, of 37 putative efflux pump-encoding open reading frames, these eight may influence susceptibility or tolerance to antibiotics and other toxic compounds.\textsuperscript{2,3,5,11,12,14} Two chemical inhibitors, phenylalanine-arginine-\textit{b}-naphthylamide (PA\textsubscript{N}) a well-studied efflux inhibitor, and chlorpromazine (CPZ), a phenothiazine compound, and an AcrAB competitive substrate were\textsuperscript{25–26} investigated.

Materials and methods

Bacterial strains and plasmids

All strains and plasmids used in this study are shown in Table 1. The method of Datsenko and Wanner\textsuperscript{27} was used to remove acrB from the chromosome of MG1655, with the acrB:apc cassette amplified from the Kcg collection strain JW054S1 using primers acrB, apsL_F (5'-GGTGTCAGGTTAAAGACGAAAGC-3'), and acrB, apsL_R (5'-GAGCTAAATACCGAGGAATGAATAAAG-3'); subsequent removal of the selection marker was also carried out as described by Datsenko and Wanner.\textsuperscript{27} The method of Kim et al.\textsuperscript{30} was used for construction of chromosomal missense mutations; the inserts were synthesized by GenScript, PCR-amplified from the supplied plasmids using Q5 polymerase (primers listed in Table S1), available as Supplementary data at JAC Online and purified using a gel extraction kit (Bn BioTech) according to the manufacturer’s instructions. For each experiment using these missense mutants, the presence of the mutation was confirmed by a PCR check from colony lyssates using the primers listed in Table S2. This was a necessary precaution as reversion to the wild-type sequence has been observed for an AcrB-inactivating mutation.\textsuperscript{24} Each forward primer has been used with the corresponding int-check reverse primer to selectively amplify only if the template has the WT or the mutated sequence. Cycling conditions were 95°C for 3 min, followed by 30 cycles of 95°C for 10 s and 72°C for 30 s (two-step).

Reporter plasmids were constructed by amplying the known or predicted regulatory region of the genes encoding nine \textit{E. coli} efflux pumps (AcrAB, AcrD, AcrEF, MdtABC, EmrAB, MacB, CusCFBA, MdtK, MdfA) using Q5 polymerase and the primers shown in Table S3, purified by gel extraction and cloned into the BamHI and XbaI restriction sites of pMW82.\textsuperscript{32} Each efflux pump gene promoter–GFP fusion was transformed into \textit{E. coli} MG1655, and its mutants, MG1655acrB D408A and MG1655 ΔacrB. Empty vector control strains were constructed by transforming the promoterless pMW82 plasmid into the same three strains. Bacterial strains were grown overnight at 37°C in Luria–Bertani broth, Lennox formulation (Sigma). PA\textsubscript{N} was supplied by Cambridge Bioscience, antibiotics and other toxic compounds including chlorpromazine were supplied by Sigma–Aldrich.

| Strain or plasmid | Description | Source or reference |
|-------------------|-------------|---------------------|
| MG1655            | \textit{Escherichia coli} K-12 derivative | This study |
| MG1655acrB        | MG1655 lacking the acrB gene | This study |
| MG1655acrB(D408A) | MG1655 with a missense mutation that inactivates AcrB | This study |
| MG1655acrD(D408A) | MG1655 with a missense mutation that inactivates AcrD | This study |
| MG1655acrF(D408A) | MG1655 with a missense mutation that inactivates AcrF | This study |
| MG1655macB(K47L)  | MG1655 with a missense mutation that inactivates MacB | This study |
| MG1655mdtK(D368A) | MG1655 with a missense mutation predicted to inactivate MdtK | This study |
| MG1655acrb(D408A) | MdtK(D368A) | This study |
| MG1655acrB(D408A)/AcrD(D408A) | AcrB(D408A) missense mutation introduced to MG1655 AcrD(D408A) | This study |
| MG1655acrB(D408A)/AcrF(D408A) | AcrF(D408A) missense mutation introduced to MG1655 AcrF(D408A) | This study |
| MG1655acrB(D408A)/MacB(K47L) | AcrB(D408A) missense mutation introduced to MG1655 MacB(K47L) | This study |
| MG1655acrB(D408A)/MdtK(D368A) | AcrB(D408A) missense mutation introduced to MG1655 MdtK(D368A) | This study |
| pMW82             | Plasmid without a promoter from which to express the encoded gfp | This study |
| pMW82-acrAbp      | pMW82 with the acrA promoter sequence regulating expression of gfp | This study |
| pMW82-acrDp       | pMW82 with the acrD promoter sequence regulating expression of gfp | This study |
| pMW82-acrEp       | pMW82 with the acrE promoter sequence regulating expression of gfp | This study |
| pMW82-cusCFBAp    | pMW82 with the cusC promoter sequence regulating expression of gfp | This study |
| pMW82-emrAbp      | pMW82 with a predicted emrA promoter sequence regulating expression of gfp | This study |
| pMW82-macAbp      | pMW82 with the macA promoter sequence regulating expression of gfp | This study |
| pMW82-mdfAp       | pMW82 with a predicted mdfA promoter sequence regulating expression of gfp | This study |
| pMW82-mdtABCp     | pMW82 with the mdtA promoter sequence regulating expression of gfp | This study |
| pMW82-mdtKp       | pMW82 with a predicted mdtK promoter sequence regulating expression of gfp | This study |
(absorbance at OD_{600} were measured every 3 min for 5 h on a FLUOstar Omega plate reader (BMG Labtech). To determine how efflux pumps in this study respond at the transcriptional level when AcrB is absent or made non-functional, overnight cultures were diluted 1/1000 and 100 mL per well used to inoculate assay plates. GFP fluorescence and OD_{600} were measured every 3 min for 15 h on a FLUOstar Omega plate reader (BMG Labtech). Two biological and three technical replicates of each culture were used in each assay. The blank corrected fluorescence value was divided by the OD_{600} value to give specific fluorescence (units of fluorescence per unit OD). The maximum specific fluorescence at any timepoint was used to compare the effect of each condition on each reporter.

**Antimicrobial susceptibility**

Susceptibility of each strain to each compound was tested using the method recommended by EUCAST. EUCAST guidelines were followed conforming to ISO 20776–1:2006. 

Antibiotics and efflux pump inhibitors (EPIs) were made up according to the manufacturer’s instructions. E. coli ATCC 25922 was used as the control strain.

**Statistical analysis**

Statistically significant differences in GFP reporter assays were identified with the Student’s t-test comparing the maximum fluorescence value achieved in a specific condition with the fluorescence value achieved in absence of that condition with values of P < 0.05 indicating significance.

Statistically significant differences in RT-qPCR assays were identified with the pairwise t-test comparing the expression in three conditions (no EPI, chlorpromazine 16 mg/L, and PAβN 32 mg/L) and in three genetic backgrounds (MG1655 WT, MG1655 acrB D408A mutant, and acrB deletion mutant) with values of P < 0.05 indicating significance. Expression of each gene was tested with a separate pairwise t-test, to analyse the chemical and genetic conditions both alone and in combination.

**Results**

**Except for AcrB, loss of function mutations in efflux pump genes had no effect on antimicrobial susceptibility**

In single efflux pump gene deletion mutants, only acrB had an effect upon antibiotic susceptibility. In previous publications, the acrB D408A missense mutation, causing AcrB to be expressed at normal levels but functionally inactivated, conferred increased susceptibility to AcrB substrates and had a different transcriptional

### Table 2. Primers for qPCR on efflux pump genes

| Primer name | Sequence (5’-3’) | Tm (°C) | Amplicon length (bp) |
|-------------|-----------------|---------|---------------------|
| acrB qPCR Fw | AAGAAGCTACCGCTAAGTTC | 57.3 | 107 |
| acrB qPCR Rv | AGTAGAACCACGGCAAAAGAA | 57.3 | |
| acrC qPCR Fw | TGGATTGTAGTAGAAGACG | 56.3 | 138 |
| acrD qPCR Rv | CAGCGACACACCAGGATAAC | 56.7 | |
| acrF qPCR Fw | AGGAGACCTATCCGGAAC | 57.3 | 130 |
| acrF qPCR Rv | CCTAAGGCCGACATACCAACAATA | 57.9 | |
| emrB qPCR Fw | TCTCATGGCGGGAATTAATCGA | 57.9 | 132 |
| emrB qPCR Rv | TAAACCCCTACCCGGAAT | 56.5 | |
| mdfA qPCR Fw | TGAAGAGATTCCTGCTGTCG | 57.3 | 152 |
| mdfA qPCR Rv | GCACAGTGCTCATGCTGTCG | 58.4 | |
| macB qPCR Fw | GCGGTCTTGAAGACTTGA | 55.2 | 106 |
| macC qPCR Fw | GTACGTGACACCGTATAA | 55.9 | |
| mdtA qPCR Fw | TTGAATGCGTCTACTTCC | 57.3 | 141 |
| mdtA qPCR Rv | CGACAGTGCTCATGCTGTCG | 57.3 | |
| mdtB qPCR Fw | ATGGACACGGAAAGACGCT | 57.3 | 142 |
| mdtB qPCR Rv | GCACAGTGCTCATGCTGTCG | 57.3 | |
| mdtC qPCR Fw | ATCCGGAACCCCTTCTCCC | 57.3 | 108 |
| mdtC qPCR Rv | GAAATCCCTGACCTTACC | 57.3 | |
| mdtK qPCR Fw | TAATGTTGTGGCTTCTCAATG | 55.2 | 128 |
| mdtK qPCR Rv | CATACGAAACCCGACAT | 55.3 | |
| 16S rRNA qPCR Fw | GTCAATACCCTGCACTG | 57.3 | 139 |
| 16S rRNA qPCR Rv | TCACTCTTCAGACCATCG | 57.3 | |

Primer sequences were designed based on the sequence of the E. coli K-12 complete genome (accession number NC_000913).
response to deletion of the same gene.\textsuperscript{23,32} In this study, single missense mutants were made in five other efflux pump genes in \textit{E. coli} to investigate the hypothesis that bacteria with pump gene deletions may be responding to loss of a large membrane protein and not loss of efflux. The susceptibility of these mutants with missense mutations known or predicted to cause loss of function of the encoded efflux pump was determined for known efflux pump substrates. Only mutational inactivation of AcrB conferred a change in susceptibility in \textit{E. coli} MG1655. To determine if the presence of AcrB masked the effect of loss of another efflux pump gene, double mutants were also constructed with loss of function of \textit{acrB} and another efflux pump gene. However, susceptibility of the double mutants to antibiotics was the same as for the strain with only the mutation in \textit{acrB}; mutation in the other efflux pumps caused no further effect (Table 3).

\textbf{Chemical inhibition of efflux only influenced the susceptibility profile in the presence of a functional AcrB}

To further test whether in \textit{E. coli} there is a contribution of efflux pumps other than AcrB in antibiotic susceptibility, the MICs of a selection of antibiotics and other toxic compounds were determined with wild-type MG1655, MG1655 \textit{ΔacrB} and the MG1655 strain producing the inactive D408A variant of AcrB alone and in the presence of 10 mg/L of efflux inhibitor (chlorpromazine and PAβN) (Table 4). In the WT, the presence of chlorpromazine did not show any effect on MIC values, while the presence of PAβN significantly decreased the MICs of chloramphenicol, erythromycin and novobiocin, which are substrates of AcrB. Except for the combination of PAβN with erythromycin (which reduced the erythromycin MIC from 8 to 1 mg/L in both the AcrB-deleted and functionally

\begin{table}[h!]
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\begin{tabular}{lcccccccccccc}
\hline
\textbf{MG1655 Genotype} & \textbf{CIP} & \textbf{NAL} & \textbf{KAN} & \textbf{CHL} & \textbf{TET} & \textbf{ERY} & \textbf{THZ} & \textbf{TFP} & \textbf{CPZ} & \textbf{AMI} & \textbf{DCA} & \textbf{SDS} & \textbf{EtBr} & \textbf{CCCP} \\
\hline
\textbf{WT} & 0.016 & 8 & 1 & 8 & 2 & 64 & 512 & 512 & 128 & 256 & >4096 & >1024 & 512 & 64 \\
\textbf{AcrB D408A} & \textit{<0.008} & 2 & 0.5 & 1 & 0.5 & 8 & 32 & 32 & 64 & 128 & 256 & 16 & 32 \\
\textbf{AcrD D408A} & 0.015 & 8 & 0.5 & 8 & 2 & 64 & 512 & 512 & 128 & 256 & >4096 & >1024 & 512 & 64 \\
\textbf{AcrE D408A} & 0.015 & 8 & 1 & 8 & 2 & 64 & 512 & 512 & 128 & 256 & >4096 & >1024 & 512 & 64 \\
\textbf{MacB K47L} & 0.015 & 4 & 1 & 8 & 2 & 64 & 512 & 512 & 128 & 256 & >4096 & >1024 & 512 & 64 \\
\textbf{MdtK D368A} & 0.015 & 8 & 1 & 8 & 2 & 64 & 512 & 512 & 128 & 256 & >4096 & >1024 & 512 & 64 \\
\textbf{AcrD D408A/AcrB D408A} & \textit{<0.008} & 2 & 0.5 & 1 & 0.5 & 8 & 32 & 32 & 64 & 128 & 256 & 16 & 32 \\
\textbf{AcrD D408A/AcrB D408A} & \textit{<0.008} & 2 & 0.5 & 1 & 0.5 & 8 & 32 & 32 & 64 & 128 & 256 & 16 & 32 \\
\textbf{MacB K47L/AcrB D408A} & \textit{<0.008} & 2 & 0.5 & 1 & 0.5 & 8 & 32 & 32 & 64 & 256 & 256 & 16 & 32 \\
\textbf{MdtK D368A/AcrB D408A} & \textit{<0.008} & 2 & 0.5 & 1 & 0.5 & 8 & 32 & 32 & 64 & 256 & 256 & 16 & 32 \\
\hline
\end{tabular}
\caption{Susceptibility of \textit{E. coli} strains with mutational inactivation of one or two efflux pumps against a selection of antibiotics and toxic agents}
\end{table}

Values shown in bold are significantly decreased compared with the wild-type strain. CIP, ciprofloxacin; NAL, nalidixic acid; KAN, kanamycin; CHL, chloramphenicol; TET, tetracycline; ERY, erythromycin; THZ, thioridazine; TFP, trifluoperazine; CPZ, chlorpromazine; AMI, amitryptiline; DCA, deoxycholic acid; SDS, sodium dodecyl sulfate; EtBr, ethidium bromide; CCCP, carbonyl cyanide \textit{m}-chlorophenyl hydrazine.

\begin{table}[h!]
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\begin{tabular}{lcccccccccc}
\hline
\textbf{Inhibitor} & \textbf{AcrB variant} & \textbf{CIP} & \textbf{NOR} & \textbf{TET} & \textbf{CHL} & \textbf{ERY} & \textbf{NOV} & \textbf{KAN} & \textbf{EtBr} & \textbf{ACR} \\
\hline
\textit{None} & \textbf{WT} & 0.015 & 0.06 & 1 & 8 & 64 & 256 & 1 & 512 & 64 \\
& \textit{deleted} & 0.004 & 0.03 & 1 & 8 & 4 & 1 & 8 & 8 \\
& \textit{D408A} & 0.004 & 0.03 & 0.5 & 1 & 8 & 4 & 1 & 8 & 8 \\
\textit{PAβN} & \textbf{WT} & 0.015 & 0.12 & 1 & \textit{8} & 64 & 2 & 512 & 128 \\
& \textit{deleted} & 0.004 & 0.03 & 0.25 & 1 & 1 & 2 & 1 & 8 & 8 \\
& \textit{D408A} & 0.004 & 0.03 & 0.25 & 1 & 1 & 2 & 1 & 4 & 4 \\
\textit{CPZ} & \textbf{WT} & 0.015 & 0.12 & 1 & 4 & 64 & 256 & 2 & 256 & 128 \\
& \textit{deleted} & 0.004 & 0.03 & 0.25 & 1 & 8 & 4 & 2 & 2 & 8 \\
& \textit{D408A} & 0.004 & 0.03 & 0.25 & 1 & 8 & 4 & 2 & 2 & 8 \\
\hline
\end{tabular}
\caption{Susceptibility of \textit{E. coli} MG1655 WT and AcrB mutants against a selection of agents, with and without the presence of 10 mg/L PAβN or chlorpromazine (CPZ)}
\end{table}

Bold font indicates statistically significant decreased value compared with in the absence of efflux inhibitor. Italic font indicates statistically significant decreased value compared to the wild-type strain in the same testing condition. CIP, ciprofloxacin; NOR, norfloxacin; TET, tetracycline; CHL, chloramphenicol; ERY, erythromycin; NOV, novobiocin; KAN, kanamycin; EtBr, ethidium bromide; ACR, acriflavine.
inactivated mutants), the antimicrobial activity of the tested antibiotics was unaffected by either chlorpromazine or PA in these mutants.

**Reporter construct functionality was confirmed by RT-qPCR**

GFP expression was measured for nine efflux pump gene transcriptional reporter plasmids (Table 2). The basal level of GFP expression was measured by a fluorescence assay and by RT-qPCR for each reporter in *E. coli* MG1655 during growth in minimal medium (MOPS). The basal level of fluorescence was very low for seven of the nine reporters with only pMW82acrABp and pMW82mdfAp showing fluorescence values significantly higher (*P* < 0.05) than those obtained with the corresponding empty vector (Figure 1a).

When we measured GFP fluorescence in conditions able to cause efflux pump induction or compensatory overexpression (Table S4), only three reporters responded: pMW82acrABp, pMW82acrEFp and pMW82cusCFBAp (Figure S1 and Figure S2). RT-qPCR analysis of the reporters confirmed they were all functional and expressing the reported genes at a higher level than the basal expression from the chromosome (Figure 1b and Table S6).

**Except for acrB, basal expression was low for the other eight efflux pump genes tested**

GFP expression from the reporter constructs was tested in MG1655 in which *acrB* had been deleted and a strain in which *acrB* had been mutated to give AcrB D408A. Loss of function or functional inactivation by missense mutation of *acrB* did not cause any...
A statistically significant increase in GFP fluorescence compared with WT acrB (Figure 2).

Data from RT-qPCR showed that the acrB gene (where present) was expressed 2 to 6-fold more than the other efflux genes (up to 10-fold in the case of the CusCFBA pump) confirming the overall low expression of the other efflux pumps shown in the GFP reporting assay in the three genetic backgrounds (MG1655 WT, MG1655 acrB D408A mutant, acrB deletion mutant) (Table 5).

Similar to the GFP fluorescence assay, data obtained from RT-qPCR showed that efflux pump expression was overall the same or even decreased when AcrB was absent or not functional, with the only exception being macB expression in the acrB deletion mutant, which was significantly increased 2.6-fold (Table 5). Expression of the acrF gene was also increased (1.6-fold) in the acrB deletion mutant and the mdfA gene showed a 1.4-fold increase in expression in the acrB D408A mutant, but those were not statistically significant, having P values > 0.05 (Table 5).

### Inhibition of AcrB has no significant effect on expression of efflux pump genes

In the presence of sub-inhibitory concentrations (1/8 MIC) of PAβN (32 mg/L) or chlorpromazine (16 mg/L), there was no increase in GFP expression from the reporter constructs compared with expression in the absence of EPI (Figure 3). The basal expression levels from efflux pump gene promoters were very low, and the subsequent amount of GFP produced provided fluorescence levels below the limit of detection of the GFP fluorescence assay for some of the constructs. Therefore, expression of the efflux pump genes in the presence of sub-inhibitory concentrations of chlorpromazine and PAβN was also quantified by RT-qPCR assay in the three background strains and compared with the expression of those genes in the absence of efflux inhibitor. No significant differences in expression were found in the presence of either of the compounds (Table 5).

### Discussion

Overcoming bacterial multidrug resistance by inhibiting efflux of antibiotics from the bacterial cell is an attractive prospect for bacterial infectious disease treatment. EPIs inhibit efflux pump activity leading to inactive drug transport. EPIs do not use the same

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**Figure 2.** Expression from transcriptional GFP reporters in acrB mutant backgrounds. Fold change in GFP fluorescence expressed from efflux pump gene promoter reporter constructs in E. coli MG1655 acrB D408 and ΔacrB mutant compared with expression in WT strain. Values are averages of three biological and two technical replicates for each reporter strain. Maximum specific fluorescence was seen at OD600nm 0.6. Student's t-test was performed comparing the maximum fluorescence value achieved in acrB mutants with the fluorescence value of the corresponding WT strain, with values of P < 0.05 indicating significance.

**Table 5.** Relative expression of efflux pump genes in the presence of chlorpromazine or PAβN

| Gene  | acrB WT | acrB D408A WT | ΔacrB WT |
|-------|---------|---------------|----------|
| Copy of transcript per copy of 16S rRNA | | | |
| No EPI | CPZ | PAβN | No EPI | CPZ | PAβN | No EPI | CPZ | PAβN |
| acrB | 0.10 | 0.10 | 0.11 | 0.10 | 0.09 | 0.11 | 0.00 | 0.00 | 0.00 |
| acrD | 0.05 | 0.04 | 0.04 | 0.04 | 0.03 | 0.04 | 0.05 | 0.04 | 0.04 |
| acrF | 0.03 | 0.02 | 0.02 | 0.01 | 0.01 | 0.01 | 0.04 | 0.03 | 0.03 |
| emrB | 0.06 | 0.05 | 0.05 | 0.03 | 0.03 | 0.03 | 0.01 | 0.01 | 0.01 |
| cusBA | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| macB | 0.02 | 0.02 | 0.03 | 0.01 | 0.01 | 0.02 | 0.05 | 0.04 | 0.05 |
| mdfA | 0.02 | 0.02 | 0.01 | 0.03 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 |
| mdtB | 0.05 | 0.04 | 0.04 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 |
| mdtC | 0.05 | 0.04 | 0.04 | 0.02 | 0.02 | 0.03 | 0.01 | 0.01 | 0.02 |
| mdk | 0.05 | 0.04 | 0.04 | 0.04 | 0.03 | 0.04 | 0.04 | 0.03 | 0.04 |

Values are averages of three biological and two technical replicates for each strain and condition. Gene transcription was measured at growth corresponding to OD600nm 0.6. For each of the genes, a pairwise t-test was performed for comparing the expression in the three conditions (no EPI, CPZ (16 mg/L) and PAβN (32 mg/L)) and in the three genetic backgrounds (MG1655 WT, MG1655 acrB D408A mutant, acrB deletion mutant) with values of P < 0.05 indicating significance. No significant differences were found in presence of EPIs. Bold font indicates statistically significantly increased expression of acrB mutant background versus WT. EPI, efflux inhibitor; CPZ, chlorpromazine; PAβN, phenyl-arginine-β-naphthylamide.
molecular target as antibiotics and can be used as adjuncts in combination with antibiotics to enhance their activity. Efflux inhibitors have been developed against the efflux pumps having the greatest effect on antibiotic susceptibility, which in E. coli is AcrB. One of the required characteristics of an EPI to make it usable in the clinical setting is to not lead to selection of resistance to its action. A compensatory increased expression of other efflux pumps, which are present in the cells as systems with redundant functions and that have been shown to have an overlapping range of substrates including antibiotics, might be a resistance mechanism to molecules inhibiting AcrAB.

The aim of this work was to investigate the molecular basis and consequences of efflux pump expression and to determine whether exposure to efflux inhibitors confers increased expression of other efflux pumps, which could undermine the development of efflux inhibitors as a drug discovery strategy. Except for acrB, basal expression of the other eight efflux pump genes tested was low. This is consistent with data previously described by Sulavik et al. who in 2001 showed that, except for AcrAB, MdfA and EmrE which confer drug resistance when overexpressed, the other pumps are poorly expressed under normal laboratory conditions.

Our data suggests that use of an AcrB (efflux) inhibitor will not lead to compensatory increases in expression of the eight investigated efflux pump genes in E. coli. Our data further showed that PAJN and chlorpromazine, which act as competitive substrates for AcrB and so act as inhibitors of antibiotic efflux, also caused no change in the transcriptional activity of the efflux pump genes. PAJN significantly decreased the MICs of chloramphenicol, erythromycin and novobiocin, which are substrates of AcrB, while chlorpromazine had no significant effect, as was observed in a previous study. Data comparing the activity of erythromycin against the wild-type, ΔacrB and AcrB(D408A) strains suggests that erythromycin is a substrate of AcrB. Therefore, these data suggest that erythromycin may be exported by other efflux pumps (which may be inhibited by PAJN), or it could be due to the membrane-permeabilizing effects of PAJN allowing erythromycin, a relatively large antibiotic, to gain better access to the bacterium.

There is conflicting evidence in the literature as to whether expression of other efflux pump genes is increased in response to the deletion of acrB in E. coli. As the susceptibility of eight efflux gene loss-of-function mutants was unchanged in our study, this suggests that in E. coli efflux pumps other than AcrB do not significantly contribute to inherent drug-resistance to the antibiotics and other antimicrobials and dyes investigated. Wang-Kan et al. showed that in Salmonella Typhimurium the acrB D408A mutation results in a strain expressing and producing a normal amount of AcrB protein but the protein is functional; in our study, introduction of the acrB D408A mutation in E. coli MG1655 did not induce overexpression of other RND efflux pumps. Furthermore, except for a 2.6-fold increase in expression of macB, no induction was observed when the acrB gene was deleted. Overall, our results in E. coli MG1655 correlate with data published by Sulavik et al. for E. coli W3110 and by Nishino et al. in an E. coli TG1 acrB deletion mutant.

Data published by Alon Cudkowicz and Schuldiner and Viveiros et al. for E. coli evolved in the presence of AcrB substrates, such as chloramphenicol and tetracycline, showed that efflux pump genes (including acrF, macB and mdfA) were overexpressed in their acrB deletion mutants (BW25113 and AG100, respectively), as a result of efflux pump up-regulation in the absence of the acrB gene, suggesting a compensatory role of these pumps in drug efflux. However, this compensation was related to a specific condition, and where the strain had evolved in presence of a pump substrate.

In conclusion, inhibition by chlorpromamine or PAJN, absence, or loss-of-function of E. coli AcrB did not significantly increase expression of other efflux pump genes suggesting there is no compensatory mechanism to overcome efflux inhibition. In the conditions tested, the other efflux pumps in E. coli provide no contribution to MDR, thus supporting the discovery of inhibitors of AcrAB as antibiotic adjuvants.

Acknowledgements
We thank Elizabeth Grimsey for reading and critical appraisal of this manuscript and Xuan Wang-Kan for construction of the E. coli AcrB D408A mutant.

Funding
This work was supported by a UK Medical Research Council grant (MR/P022596/1) awarded to Laura J.V. Piddock.

Transparency declarations
None to declare.

Supplementary data
Additional Methods, Tables S1 to S7 and Figures S1 to S3 are available as Supplementary data at JAC Online.
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