Contribution of the efflux pump AcrAB-ToIC to the tolerance of chlorhexidine and other biocides in *Klebsiella* spp.

Matthew E. Wand¹*, Elizabeth M. Darby², Jessica M. A. Blair² and J. Mark Sutton¹

**INTRODUCTION**

Infection prevention is critical to combat the rise of multi-drug-resistant (MDR) bacterial infections. One component of infection prevention is the use of disinfectants and antiseptics (biocides) to prevent the colonization and transmission of pathogens. There is, however, concern that the increase in use of these biocides could lead to increased bacterial tolerance to biocides and/or cross-resistance to frontline antibiotics. This is because many potential biocide resistance mechanisms, such as upregulation of specific bacterial efflux pumps, are common antibiotic resistance determinants, and biocide tolerance genes are carried on...
antimicrobial resistance (AMR) plasmids [1]. When both Salmonella enterica and Stenotrophomonas maltophilia were exposed to the biocide triclosan, increased resistance to certain antibiotics was shown through upregulation of particular efflux pumps [2, 3]. In Gram-negative bacteria probably the most clinically relevant efflux pumps are members of the resistance–nodulation–division (RND) family, which recognize a broad range of substrates, including antibiotics and biocides [4]. This family includes the well-characterized members MexAB-OprM from Pseudomonas aeruginosa, AdeABC in Acinetobacter baumannii and the Enterobacteriaceae MDR efflux pump AcrAB-TolC [5–7].

AcrAB-TolC is a tripartite RND efflux pump comprising an outer membrane channel TolC, the inner membrane transporter AcrB and the periplasmic membrane fusion protein AcrA. In Escherichia coli the expression of acrAB is primarily controlled by MarA [8, 9], with marA overexpression generating increased resistance to multiple antibiotics, disinfectant pine oils and triclosan [10, 11], but in S. enterica and Klebsiella pneumoniae the major regulator is RamA [12]. The pump is also regulated by a variety of other different factors, including SoxS and the TetR family transcriptional repressors, AcrR and EnvR.

K. pneumoniae is an important opportunistic pathogen that is prominent in causing respiratory and urinary tract infections. A few high-risk sequence types (STs), e.g. ST258, ST11, ST512, ST14 and ST101, are responsible for the global dissemination of carbapenemases and other multi-drug resistance determinants [13–15]. In K. pneumoniae several efflux pumps have been linked to tolerance to various biocides, including MdfA, MdtK and Acel [16]. For chlorhexidine, increased tolerance was shown in strains with an upregulated major facilitator superfamily (MFS) efflux pump SmvA, which was due to mutations in the adjacent repressor (SmvR) [17]. Strains lacking SmvA also have increased chlorhexidine susceptibility and SmvA has additionally been linked to tolerance in Klebsiella to another cationic biocide, octenidine [18]. Other potential mechanisms of increased chlorhexidine tolerance include efflux pumps such as CepA (FieF) [19, 20] and EmrAB (KpnGH) [21]. Efflux is linked to increased biocidal tolerance and these strains often have upregulated MDR efflux pumps, such as AcrAB-TolC. However, little is known about the contribution of these individual efflux pumps to biocidal tolerance. Mutations and insertions/deletions in ramR (the repressor of RamA) have been shown to increase the expression of ramA by preventing RamR binding to the ramA promoter, which caused an increase in the expression of acrAB-TolC in K. pneumoniae [22, 23], resulting in an MDR phenotype [24]. Exposure to triclosan and benzalkonium chloride in K. pneumoniae frequently yielded ramA-overexpressing mutants [25], whilst mutations in RamR were identified in S. enterica following exposure to several biocides [26]. Therefore, multiple efflux pumps may contribute towards increased biocidal tolerance.

The aim of these experiments was to study the relative contribution of AcrAB-TolC to the tolerance to chlorhexidine and other biocides in Klebsiella using already generated chlorhexidine adapted mutants to understand which efflux pump is the primary resistance mechanism. We utilized SmvAR as a comparator since we have previously shown that this pump is important for chlorhexidine tolerance but contributes little to antibiotic resistance. Therefore, changes in AcrAB-TolC are potentially more clinically relevant due to an associated increase in antibiotic resistance. Strains with upregulated acrAB-tolC are also isolated more regularly in the clinic.

This study shows that AcrAB-TolC has an important role in the tolerance of many biocides and that specific changes in the sequence of the main regulators of AcrAB-TolC, ramAR and acrR affect susceptibility to chlorhexidine and other cationic biocides. Although the study mainly focuses on AcrAB-TolC, we cannot ignore the importance of other efflux pumps, namely SmvAR. This study shows that the response to biocides in Klebsiella is multifaceted and the contribution of each individual efflux pump is likely to be biocide and strain dependent.

**METHODS**

**Bacterial strains and culture conditions**

All Klebsiella strains were grown in tryptic soy broth (TSB) with aeration or on tryptic soy agar at 37°C unless stated otherwise. The strains chosen include a mixture of clinical isolates, primarily isolated post-2015, from the UK. The majority are carbapenem-resistant K. pneumoniae ST258 isolates. Whole-genome sequences were available for all strains used in this study. Important strain characteristics, including antibiotic resistance profiles generated by RES-FINDER, are listed in Table S1 (available in the online version of this article). Transposon mutants from K. pneumoniae MKP103 are also listed in Table S3 and were described previously [27]. Transposon mutants KP02744 (MKP103ΔacrA), KP02740 (ΔacrB), KP02746 (ΔacrR), KP03203 (ΔramA) and KP03197 (ΔramR) were whole-genome sequenced and mapped against the parental MKP103 strain to confirm that the only mutations that were found in each strain were the respective transposon insertions. K. pneumoniae transposon mutants were adapted to chlorhexidine using a stepwise method. Cultures were initially grown in subinhibitory concentrations of chlorhexidine (one-quarter the MIC level) and then every 2 days passages into double the previous chlorhexidine concentration until a concentration of eight times the initial MIC was reached. Cultures were subsequently passaged on agar plates 10 times in the absence of chlorhexidine selection. Ethidium bromide dye uptake assays were performed as previously described [28]. Briefly, strains were cultured to mid-log phase (OD600 0.4) in the presence of sub-MIC chlorhexidine levels (8 and 4 mg l⁻¹) in TSB. Cells were then pelleted at 3500 r.p.m. for 10 min and resuspended in 0.02 M potassium phosphate buffer (pH 7.0) with 1 mM MgCl₂. Cells were adjusted to OD600 0.2 and 190 μl was loaded into a flat-bottomed, black, 96-well plate (Greiner Bio-one, Stonehouse,
Table 1. Susceptibility of chlorhexidine-adapted *Klebsiella* strains to various antibiotics (a) and biocides (b)

| (a)                | CIP | LVX | NOR | MXF | NAL | FOX | CAZ | CTX | AZM | DOX | CHL | GEN | TOB | CST | TGC |
|--------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 6 WT               | 0.06| 0.125| 0.25| 0.5 | 16  | 4   | 0.25| 0.06| 32  | 4   | 8   | 2   | 4   | 0.5 | 1   |
| 6 CHD              | 0.5 | 1   | 2   | 2   | 64  | 64  | 2   | 0.5 | >64 | 32  | 64  | 2   | 4   | 0.5 | 2–4 |
| CFI_080_KPC2 WT    | 0.03| 0.125| 0.25| 0.25| 8   | 64  | >64 | >64 | 64  | 8–16| 4   | 8   | 16  | 0.5 | 1   |
| CFI_080_KPC2 CHD   | 0.125| 0.5 | 1   | 1   | 16  | >64 | >64 | >64 | >64 | 32  | 16  | 8   | 8–16| 0.5 | 2–4 |

| (b)                | ALX | CET | DQC | TRC | CPC | HDPCM | CHD | CTAB | DDAB | OCT | BAC | BEC | Eth |
|--------------------|-----|-----|-----|-----|-----|--------|-----|------|------|-----|-----|-----|-----|
| 6 WT               | 4   | 0.0009| 256| 0.25| 4   | 4   | 32   | 16   | 8    | 4   | 8   | 32  | 6.25 |
| 6 CHD              | 4   | 0.007 | 256| 1   | 64  | 64   | 128  | 128  | 16   | 4   | 32  | 64  | 6.25 |
| CFI_080_KPC2 WT    | 2   | 0.003 | 128| 0.125| 8   | 8   | 16   | 16   | 8    | 4   | 16  | 32  | 6.25 |
| CFI_080_KPC2 CHD   | 2   | 0.03 | 128| 1   | 16  | 16   | 64   | 128  | 16   | 4   | 16  | 32  | 6.25 |

MIC values (mg l⁻¹) except where indicated are shown for the antibiotics ciprofloxacin (CIP), levofloxacin (LVX), norfloxacin (NOR), moxifloxacin (MXF), naladixic acid (NAL), cefoxatin (FOX), ceftazidime (CAZ), cefotaxime (CTX), aztreonam (AZM), doxycycline (DOX), chloramphenicol (CHL), tobramycin (TOB), colistin (CST) and tigecycline (TGC) (1A), and for the biocides alexidine dihydrochloride (ALX), cetrimide (CET) (%), dequalinium chloride hydrate (DQC), triclosan (TRC), cetylpyridinium chloride (CPC), hexadecylpyridinium chloride monohydrate (HDPCM), chlorhexidine digluconate (CHD), cetyltrimethylamonium bromide (CTAB), didecyldimethylammonium bromide (DDAB), octenidine hydrochloride (OCT), benzalkonium chloride (BAC), benzethonium chloride (BEC) and ethanol (Eth) (%). Values in bold indicate an increase of ≥fourfold in MIC levels for the chlorhexidine adapted mutants (strain CHD) versus the wild-type (WT).
UK). Subsequently, 10 µl of ethidium bromide (50 mg l\(^{-1}\)) was added to each well. The accumulation of ethidium bromide was quantified using a FLUOstar Omega plate reader (BMG Labtech) where fluorescence was measured using excitation and emission filters at 544 and 590 nm, respectively, and a gain multiplier of 1460 for 2 h.

**Whole-genome sequencing (WGS)**

*K. pneumoniae* genomic DNA was prepared using a Wizard Genomic DNA purification kit (Promega). Whole-genome sequencing of chlorhexidine-exposed isolates was performed by PHE-GSDU (Public Health England Genomic Services and Development Unit) on an Illumina HiSeq 2500 with paired-end read lengths of 150 bp. All sequencing analyses were performed using PHE Galaxy [29]. FastQ files were quality trimmed using Trimmomatic and reads from chlorhexidine-exposed isolates were mapped to their respective pre-exposure chromosomal sequence using BWA0.7.5. Bam files were generated using Samtools and VCF files were constructed using GATK2 Unified Genotyper 0.0.7. They were further filtered to identify high-confidence SNPs using the following criteria: mapping quality, >30; genotype quality, >40; variant ratio, >0.9; read depth, >10. BAM files were visualized in Integrative Genomics Viewer (IGV) version 2.3.55 (Broad Institute). All sequences have been deposited with the National Center for Biotechnology Information (NCBI) under the Bioproject ID PRJNA777533.

**Determination of MIC and MBC**

The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of various antibiotics and disinfectants/antiseptics for bacterial isolates were determined using a standard broth microdilution method detailed by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) with a starting inoculum of 1×10\(^{5}\) c.f.u. ml\(^{-1}\), except that 96-well polypropylene plates (Griener Bio-One Ltd, Stonehouse, UK) were used instead of polystyrene plates to test colistin.
| Strain | ALX | DDAB | BEC | BAC | CHD | DQC | OCT | HDPCM | TRC | Eth | CET | CPC | CTAB | RamR | RamA | AcrR |
|--------|-----|-------|-----|-----|-----|-----|-----|-------|-----|-----|-----|-----|------|-------|-------|------|
| NCTC 13438 | 4   | 4     | 32  | 16  | 32  | 256 | 2–4 | 8     | 1   | 6.25| 0.0015 | 16 | 32 | A37V | N14Y | –    |
| 46704   | 4   | 4     | 32  | 16  | 16  | 256 | 4   | 8     | 0.5 | 6.25| 0.0015 | 16 | 32 | –    | –    | –    |
| CFI_131_KPC2 | 2   | 2     | 32  | 8   | 16  | 256 | 4   | 4     | 1   | 3.125| 0.0007 | 8  | 16 | Absent| Absent| –    |
| CFI_141_KPC3 | 4   | 4     | 32  | 16  | 16  | 256 | 2   | 8     | 0.5 | 3.125| 0.003 | 16 | 32 | –    | –    | –    |
| CFI_147_KPC2 | 2   | 2     | 64  | 16  | 16  | 256 | 2   | 4     | 0.5 | 3.125| 0.0007 | 8  | 8  | L54F | –    | –    |
| MKP103 | 4   | 8–16  | 32  | 16–32 | 64–128 | 256 | 4   | 16    | 4–8 | 6.25| 0.003–0.007 | 16 | 32–64 | G42V | –    | –    |

MIC values (mg l⁻¹) except where indicated are shown for the biocides alexidine dihydrochloride (ALX), didecyldimethylammonium bromide (DDAB), benzethonium chloride (BEC), benzalkonium chloride (BAC), chlorhexidine digluconate (CHD), dequalinium chloride hydrate (DQC), octenidine hydrochloride (OCT), hexadecylpyridinium chloride monohydrate (HDPCM), triclosan (TRC), ethanol (Eth) (%), cetrimide (CET) (%), cetylpyridinium chloride (CPC) and cetyltrimethylammonium bromide (CTAB). For other genes that have been implicated as regulators of AcrAB-TolC, including marAR, soxRS, rob, sdiA, fis and envR, the sequence for all ST258 strains was identical except for a premature stop codon in soxR (Q97STOP) in strain CFI_141_KPC3.
The optical density at 600 nm (OD 600 ) was measured after 20 h of static incubation at 37 °C, and the MIC was defined as the lowest concentration of antibiotic/disinfectant at which no bacterial growth was observed. MBCs were measured by plating out onto TSA plates 10 µl of MIC dilutions from and including the MIC level and the subsequent three further higher biocide concentrations (where applicable). The efflux pump inhibitors phenylalanine-arginine β-naphthylamide (PaβN) and carbonyl cyanide 3-chlorophenylhydrazone (CCCP) were added at concentrations of 25 and 10 mg l⁻¹ respectively.

Real-time PCR
Overnight cultures grown in TSB were back-diluted to an OD 600 of 0.1 in TSB and grown for a further 1 h. Cultures were back-diluted to an OD 600 of 0.25 in TSB alone (unexposed) or TSB containing sub-MIC (8 mg l⁻¹) or lethal (128 mg l⁻¹) concentrations of chlorhexidine and incubated for 30 min with shaking at 37 °C. Cultures were then harvested using RNA protect (Qiagen) and RNA was extracted using the RNeasy minikit (Qiagen) according to the manufacturer’s instructions. cDNA was synthesized, and real-time PCR was carried out and analysed as previously described [17] using K. pneumoniae infB, gapA and rpoB as internal control genes. Primers used have already been described [17] except for acrA primers (KPacrA665R3  TCATTGCTCGACTGGGTGAC; KPacrA586F3  CAGAATGGTCAAACGACCGC) and ramA primers (KPramA332R3  CGACTGTGGTTCTCTTTGCG; KPramA260F3  AGACCTTTACCCGCGTCTTC).

Statistical analysis
Real-time PCR data were analysed for significance using Student’s unpaired t-test. For significance, P values <0.0001 ****, 0.001–0.0001 ***, 0.01–0.001 **, 0.05–0.01 * and ≥0.05 non-significant were used.

RESULTS AND DISCUSSION
Chlorhexidine-adapted Klebsiella strains with RamR mutations have increased resistance to several antibiotics
Chlorhexidine-exposed Klebsiella strains had shown mutations in ramR previously [18] and to understand the contribution of AcrAB-TolC, these mutants were analysed for their change in antibiotic and biocide tolerance. Strain 6 CHD [which contains mutations in both smvR (Del nucleotides 48–54) and ramR (E7STOP)] and Klebsiella oxytoca strain CFI_080_KPC2 CHD (which contains a deletion in ramR) both showed decreased susceptibility to several antibiotics and biocides (Table 1a and b). Many of these antibiotics are known substrates for acrAB-TolC, such as the fluoroquinolones and doxycycline. SmvAR has been shown to have a negligible effect on antibiotic susceptibility and is more likely a selective pump for certain cationic compounds [18]. Therefore, the change in antibiotic MICs observed in strain 6 CHD are likely to be because of mutations in ramR. For certain biocides (Table 1b) strain 6 CHD showed a larger fold increase in biocide resistance – e.g. CPC, HDPCM and cetrimide (up to 16-fold) – than CFI_080_KPC2 CHD (2–8-fold) when compared to their respective wild-types. This potentially indicates that the presence of mutations in regulators of both AcrAB-TolC and SmvA has a cumulative effect on tolerance to these biocides and that they are substrates for both pumps.

Exposure to chlorhexidine causes upregulation of acrAB
Early log-phase K. pneumoniae strain MGH 78578 was exposed to sub-lethal and lethal levels of the cationic biocides chlorhexidine and octenidine. This included a biocide (chlorhexidine) where an increase in MIC was observed in strains containing ramR mutations following chlorhexidine adaptation, and a biocide (octenidine) where no increase in MIC was observed. Increased expression of acrA (approximately 2.7-fold for sub-lethal and 2-fold for lethal concentrations of both biocides) and its regulator ramA (14.7-fold for sub-lethal and 4.7-fold for lethal concentrations of both biocides) was shown (Fig. S1). This showed that Klebsiella responds to the presence of both sub-lethal and lethal concentrations of chlorhexidine and octenidine through increased expression of acrAB-TolC, but that these increased expression levels do not necessarily correlate with a change in MIC. The

Table 4. Expression levels (fold change) for genes in K. pneumoniae ST258 strains relative to strain 46704. Significance is indicated

|          | acrA  | ramA  | smvA  | smvR  |
|----------|-------|-------|-------|-------|
| NCTC 13438 | 0.691 | 3.204 | 1.067 | 0.542 |
| 46704     | 1.000 | 1.000 | 1.000 | 1.000 |
| CFI_131_KPC2 | 0.427 | 0.000 | 0.427 | 0.734 |
| CFI_141_KPC3 | 0.912 | 0.733 | 0.492 | 0.655 |
| CFI_147_KPC2 | 4.639* | 15.554** | 1.514 | 1.109 |
| MKP103    | 8.159* | 20.329** | 1.143 | 1.385 |
Table 5. The effect of the efflux pump inhibitors PaβN and CCCP on biocide susceptibility in chlorhexidine-adapted strains and their respective wild-types. Numbers highlighted in bold indicate a ≥fourfold change in MIC relative to no EPI (alone).

|          | CHD          | CET          | CPC          | HDPCM         |
|----------|--------------|--------------|--------------|---------------|
|          | Alone +PaβN/N +CCCP | Alone +PaβN/N +CCCP | Alone +PaβN/N +CCCP | Alone +PaβN/N +CCCP |
| 6 CHD    | 8–16         | 8–16         | 1            | 0.0007        | ≤0.0003        | 0.0007        | 4            | 4            | 16           | 4            | 2            | 4            |
| CFI_080_KPC2 | 4–8         | 8–16         | 1            | 0.0007        | ≤0.0003        | 0.007         | 8            | 2            | 32           | 16           | 2            | 32           |
| CFI_080_KPC2 CHD | 6 16 | 64 64 | 2 0.007 | ≤0.0003 | 0.015 | 32 4 | 64 64 | 4 | 64 64 |
| MKP103      | 128 128 | 1 0.007 | ≤0.0003 | 0.015 | 32 4 | 64 64 | 16 4 | 64 64 |

Antimicrobials tested included chlorhexidine digluconate (CHD), CET, cetylpyridinium chloride (CPC), hexadecylpyridinium chloride monohydrate (HDPCM), cetyltrimethylammonium bromide (CTAB), chloramphenicol (CHL) and ciprofloxacin (CIP). All values in mg l⁻¹ except for CET whose values represent % of active ingredient.
changes in the expression levels of \(acrA\) and \(ramA\) was not as large a fold increase as shown for \(smvA\) [18], but this may be due to a lower \(smvA\) basal level.

Since exposure to sub-lethal concentrations of chlorhexidine led to higher transcript levels of \(acrAB\)-\(TolC\), it was hypothesized that this increased expression could lead to elevated MICs for several antibiotics. \(K.\ pneumoniae\) strains were challenged with antibiotics known to be substrates of the AcrAB-TolC pumps in the presence of sub-lethal levels of chlorhexidine (4 and 8 mg l\(^{-1}\)). However, the results showed that the effect of chlorhexidine with the antimicrobial was additive; the presence of chlorhexidine led to decreased MIC values for all antibiotics tested, which were further decreased as the chlorhexidine concentration increased (Table S2). This showed that chlorhexidine is working in synergy with the antibiotics, probably through permeation of the bacterial membrane, an observation previously described with another cationic biocide alexidine [30]. Experiments using fluorescent dyes, which are often used to study efflux, showed increased accumulation of the dye in the presence of sub-inhibitory concentrations of chlorhexidine (data not shown). The presence of sub-lethal levels of chlorhexidine is likely to induce an increased stress response, which possibly masks the effect of upregulation in \(acrAB\)-\(tolC\) through \(ramA\). It has been shown that the rate of induction of \(acrA\) is dependent upon the rate of stress introduction [31]. However, in \(Salmonella\) the expression of \(ramA\) was found to be unchanged when bacteria were challenged with several antibiotic substrates of AcrAB-TolC [32]. Therefore, the response of \(ramA\) and \(acrA\) after challenge with cationic biocides is condition and species specific but does not necessarily indicate a role in chlorhexidine tolerance.

**Transposon mutants in \(acrAB\) show increased susceptibility to biocides**

One strain in our collection, NCTC 7427, is an ST86 strain and contains a premature stop codon in \(acrB\) and thus an inactive AcrAB-TolC pump. Comparison with the only other ST86 strain in our collection, KPUK02, showed that susceptibility to several biocides, including chlorhexidine, triclosan, CTAB and benzalkonium chloride was increased (2-fold) in NCTC 7427 (Table 2). This shows that for ST86 strains AcrAB-TolC is an important component for tolerance to several biocides, such as chlorhexidine, benzalkonium chloride and triclosan, but not for others, e.g. silver nitrate, glutaraldehyde and sodium hypochlorite. Comparison of the genomes of NCTC 7427 and KPUK02 showed that NCTC 7427 contained identical DNA sequences for all the other major efflux pumps, such as \(oqxAB\), \(emrAB\), \(cepA\) and \(smvA\), and their regulators, again suggesting that the changes seen are due to the presence/absence of AcrAB. The only exception is that NCTC 7427 lacked a homologue to KpnEF, which is thought to have some activity against certain biocides [21].

Transposon mutants in \(acrAB\) and specific regulators known to affect \(acrAB\) expression in \(K.\ pneumoniae\) strain ST258 strain MKP103 were also analysed for their tolerance to several biocides (Table S3). Mutants in the efflux pump \(acrAB\) reduced the MIC for chlorhexidine (8–16-fold), DQC (8-fold), triclosan (2–4-fold) and several others (2-fold). This agrees with the reduced MKP103 were also analysed for their tolerance to several biocides (Table S3). Mutants in the efflux pump \(acrAB\) strain ST258 strain Transposon mutants in \(acrAB\) and specific regulators known to affect expression in \(acrAB\) to KpnEF, which is thought to have some activity against certain biocides [21].

**Sequence variation within RamR and AcrR leads to increased expression of \(acrAB\) and may contribute towards increased biocide tolerance**

The genome sequence of MKP103 was analysed for a possible explanation for the constant \(acrAB\) upregulation. This revealed a unique amino acid change (G42V) in RamR relative to other ST258 strains in our collection. It is well known that changes in RamR regulate the expression levels of \(acrAB\) [33–35]. Therefore, it is plausible that this change in strain MKP103 leads to constant upregulation of \(acrAB\), and removal of the repressors \(ramR\) and \(acrR\) would have minimal effect on antimicrobial tolerance. Sequence analysis of all ST258 strains in our collection showed other strains with unique changes in RamR and RamA but not in other potential AcrAB-TolC regulators.

Six ST258 strains were analysed for their susceptibility to biocides. Strain MKP103 had increased MICs (often fourfold) to DDAB, chlorhexidine, HDPCM, triclosan and cetrimide when compared to the other strains (Table 3). This increase in tolerance could be due to elevated expression of \(acrAB\)-\(TolC\). To investigate this the basal expression levels of \(acrA\) and \(ramA\) for all ST258 strains were measured. For comparison and due to their importance in biocide tolerance, the levels of \(smvAR\) were also investigated, although there were no sequence differences for these genes within the ST258 strains. The results showed that MKP103 did indeed have elevated expression levels of \(acrA\) and \(ramA\) relative to other ST258 strains (Table 4). Although not to the same level as MKP103, strain CFL\_147\_KPC-2 also had elevated \(acrA\) and \(ramA\) expression. This strain contains the unique mutation L54F in RamR and shows that both mutations in RamR (G42V and L54F) cause derepression of \(ramA\) that in turn leads to increased expression of \(acrAB\)-\(TolC\). However, only in MKP103 does this change result in elevated MIC values for biocides. For \(smvAR\) expression levels, no significant difference was observed between all strains.
Since the rate of efflux does not always correlate with baseline MIC/MBC values [36, 37], we attempted to measure the impact of chlorhexidine-mediated acrAB-tolC expression on ethidium bromide dye accumulation after challenge with chlorhexidine for the ST258 K. pneumoniae strains. Unfortunately, despite repeated attempts, we were unable to gain reproducible data, probably because chlorhexidine acts as a membrane permeabilizer.

**Chlorhexidine and other biocide tolerance levels in Klebsiella are dependent on multiple efflux pumps**

To further attempt to decipher the importance of the individual efflux pumps SmvA and AcrAB-TolC, MKP103 transposon mutants in ramA, ramR and smvA were adapted to chlorhexidine in a stepwise manner. This was to generate strains that had different levels of expression of acrAB-TolC and smvA efflux pumps. Adaptation of KP02744 (ΔacrA) and KP02740 (ΔacrB) to chlorhexidine was attempted, but despite repeated efforts we were unsuccessful. Exposure of strains KP03202 (ΔramA), KP03197 (ΔramR) and the parental MKP103 to chlorhexidine selected for mutations in smvR, but no mutations in acrAB or its regulators were detected in KP05925 (ΔsmvA) (Table S5). This is probably due to the already upregulated acrAB-TolC expression levels, meaning that additional mutations would have minimal effect. Comparison of the MIC values for various biocides for the chlorhexidine adapted mutations concluded that again a cumulative effect was seen, particularly for chlorhexidine (fourfold increase) in strains that had mutations in smvR and already upregulated acrAB-TolC (Table S6). Strain MKP103ΔsmvA CHD showed no increase in biocide MIC values, except for chlorhexidine (two–fourfold). That it was not possible to generate adapted mutants in strains KP02744 (ΔacrA) and KP02740 (ΔacrB) supports an important role for AcrAB-TolC in the export of chlorhexidine in MKP103.

To further aim to separate the role of AcrAB-TolC and SmvA in biocide tolerance, the efflux pump inhibitors PaβN and CCCP were employed on selected Klebsiella strains, including those with upregulated acrAB-TolC. PaβN is a competitive inhibitor of AcrAB-TolC [38] but should not affect the MFS pump SmvA. CCCP has been shown to enhance the efficacy of chlorhexidine as well as colistin in Klebsiella [17] and is an uncoupler of the proton motive force. This should theoretically affect both RND and MFS pumps, but will also have pleiotropic effects on other aspects of membrane function [39]. The addition of CCCP was only effective for chlorhexidine, whilst the presence of PaβN resulted in reduced MIC values for CET, CPC, HDPCM and CTAB, as well as the antibiotics CHL and CIP, which are known to be subject to efflux by AcrAB-TolC (Table 5). This indicated that AcrAB-TolC is a major efflux pump in Klebsiella for those biocides, but that its role in efflux of chlorhexidine is either fully complemented by SmvA or it is not inhibited by PaβN. PaβN acts as a competitive inhibitor in competition with antimicrobials in the AcrAB-TolC binding pocket [40] and therefore the binding affinity for chlorhexidine to AcrAB-TolC may be higher relative to PaβN and thus chlorhexidine is able to outcompete PaβN, rendering it ineffective. Chlorhexidine and PaβN may also interact with different amino acids in AcrAB. This has been shown for PaβN and tetracycline, where the binding pockets for each chemical do not overlap [41]. Studies have shown that the addition of CCCP did not affect sensitivity to carbapenems or tigecycline in Enterobacteriaceae [42] but these antibiotics have been shown to be subject to efflux by AcrAB-TolC in Klebsiella [43, 44]. Having previously shown that SmvA is an important efflux pump for several cationic biocides in Klebsiella [18], it was perhaps surprising that the addition of CCCP had no effect on susceptibility to the biocides tested. One solution is that CCCP has no effect on SmvA, and that the effect with chlorhexidine is nothing to do with SmvA. Another well-studied MFS efflux pump, EmrAB, has been shown to efflux CCCP [45], and therefore the major effect of CCCP might instead be to act directly on the cell membrane, which is the site of action for chlorhexidine. This potentially shows that chlorhexidine has a different mechanism of action from other cationic biocides, resulting in different resistance mechanisms.

**CONCLUSION**

This study provides evidence that AcrAB-TolC is an important efflux pump in K. pneumoniae for specific biocides. Adaption to chlorhexidine, although predominantly driven by mutations in smvAR, can result in mutations in the acrAB regulator ramR, which leads to decreased susceptibility to several antibiotics. This study shows an important role for both SmvA and AcrAB-TolC in the efflux of biocides in Klebsiella, with each pump likely to efflux multiple biocides, including chlorhexidine. Exposure to chlorhexidine can result in a decrease in susceptibility to many antibiotics through ramR mutations and, therefore, whilst adaptation to chlorhexidine is more likely to result in changes to SmvAR in a laboratory setting, within the clinic, strains with ramR mutations are more problematic due to potential cross-resistance to antibiotics.

**Funding information**

This project was funded by UK Health Security Agency grant project 111743. The views expressed are those of the authors and not necessarily those of the funding body.

**Conflicts of interest**

The authors declare that there are no conflicts of interest.
References

1. Poole K. Efflux pumps as antimicrobial resistance mechanisms. *Ann Med* 2007;39:162–176.

2. Karatzas KAG, Webber MA, Jorgensen F, Woodward MJ, Piddock LJV, et al. Prolonged treatment of *Salmonella enterica* serovar Typhimurium with commercial disinfectants selects for multiple antibiotic resistance, increased efflux and reduced invasiveness. *J Antimicrob Chemother* 2007;60:947–955.

3. Sanchez P, Moreno E, Martinez JL. The biocide triclosan selects *Stenotrophomonas maltophilia* mutants that overproduce the SmoDEF multidrug efflux pump. *Antimicrob Agents Chemother* 2005;49:781–782.

4. Colclough AL, Alav I, Whittle EE, Pugh HL, Darby EM, et al. RND efflux pumps in Gram-negative bacteria; regulation, structure and role in antibiotic resistance. *Future Microbiol* 2020;15:143–157.

5. Masuda N, Sakagawa E, Ohya S, Gotoh N, Tsujimoto H, et al. Substrate specificities of MexAB-OprM, MexCD-OprJ, and MexXY-OprM efflux pumps in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 2000;44:3322–3327.

6. Ruzin A, Keeney D, Bradford PA. AdeABC multidrug efflux pump is associated with decreased susceptibility to ticarcylene in *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex. *J Antimicrob Chemother* 2007;59:1001–1004.

7. Du D, Wang Z, James NR, Voss JE, Klimont E, et al. Structure of the AcrAB-TolC multidrug efflux pump. *Nature* 2014;509:512–515.

8. Weston N, Sharma P, Ricci V, Piddock LJV. Regulation of the AcrAB-TolC efflux pump in *Enterobacteriaceae*. *Res Microbiol* 2018;169:425–431.

9. Okusu H, Ma D, Nkaido H. AcrAB efflux pump plays a major role in the antibiotic resistance phenotype of *Escherichia coli* multiple-antibiotic-resistance (Mar) mutants. *J Bacteriol* 1996;178:306–308.

10. Moken MC, McMurry LM, Levy SB. Selection of multiple-antibiotic-resistant (mar) mutants of *Escherichia coli* by using the disinfectant pine oil: roles of the mar and acrAB loci. *Antimicrob Agents Chemother* 1997;41:2770–2772.

11. McMurry LM, Oethinger M, Levy SB. Overexpression of marA, soxS, or acrAB produces resistance to triclosan in laboratory and clinical strains of *Escherichia coli*. *FEMS Microbiol Lett* 1998;16:305–309.

12. Ricci V, Piddock LJV. Only for substrate antibiotics are a functional AcrAB-TolC efflux pump and RamA required to select multidrug-resistant *Salmonella Typhimurium*. *J Antimicrob Chemother* 2009;64:654–657.

13. Roe CC, Vazquez AJ, Esposito EP, Zarilli R, Sahl JW. Diversity, virulence, and antimicrobial resistance in isolates from the newly emerging *Klebsiella pneumoniae* ST101 lineage. *Front Microbiol* 2019;10:542.

14. Chen L, Mathema B, Pitout JJD, DeLeo FR, Kreiswirth BN. Epidemic *Klebsiella pneumoniae* ST258 is a hybrid strain. *mBio* 2014;5:e01355–14.

15. Giske CG, Fröding I, Hasan CM, Turlet-Rogacka A, Toleman M, et al. Diverse sequence types of *Klebsiella pneumoniae* contribute to the dissemination of blaNDM-1 in India, Sweden, and the United Kingdom. *Antimicrob Agents Chemother* 2012;56:2735–2738.

16. Slipski CJ, Zhanel GG, Bay DC. Biocide selective TolC-independent efflux pumps in *Enterobacteriaceae*. *J Membr Biol* 2018;251:15–33.

17. Wand ME, Bock LJ, Bonney LC, Sutton JM. Mechanisms of increased resistance to chlorhexidine and cross-resistance to colistin following exposure of *Klebsiella pneumoniae* clinical isolates to chlorhexidine. *Antimicrob Agents Chemother* 2017;61:e01162–16.

18. Wand ME, Jamshidi S, Bock LJ, Rahman KM, Sutton JM. SmA is an important efflux pump for cationic biocides in *Klebsiella pneumoniae* and other *Enterobacteriaceae*. *Sci Rep* 2019;9:1344.

19. Fang CT, Chen HC, Chuang YP, Chang SC, Wang JT. Cloning of a cation efflux pump gene associated with chlorhexidine resistance in *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* 2002;46:2024–2028.

20. Wand ME, Baker KS, Benthall G, McGregor H, McCowen JW, et al. Characterization of pre-antibiotic era *Klebsiella pneumoniae* isolates with respect to antibiotic/disinfectant susceptibility and virulence in *Galleria mellonella*. *Antimicrob Agents Chemother* 2015;59:3966–3972.

21. Srivivasan VB, Singh BB, Priyadarshi N, Chauhan NK, Rajamohan G. Role of novel multidrug efflux pump involved in drug resistance in *Klebsiella pneumoniae*. *PLoS One* 2014;9:e96288.

22. Bialek-Davenet S, Leflon-Guibout V, Tran Minh O, Marcon E, Moraes R, et al. Complete deletion of the ramR gene in an in vitro-selected mutant of *Klebsiella pneumoniae* overexpressing the AcrAB efflux pump. *Antimicrob Agents Chemother* 2013;57:672–673.

23. 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.

24. Schneider T, Amyes SGB, Levy SB. Role of AcrR and ramA in fluoroquinolone resistance in clinical *Klebsiella pneumoniae* isolates from Singapore. *Antimicrob Agents Chemother* 2003;47:2831–2837.

25. Curiao T, Marchi E, Yiti C, Oggioni MR, Baquero F, et al. Polymorphic variation in susceptibility and metabolism of triclosan-resistant mutants of *Escherichia coli* and *Klebsiella pneumoniae* clinical strains obtained after exposure to biocides and antibiotics. *Antimicrob Agents Chemother* 2015;59:3431–3432.

26. Webber MA, Whitehead RN, Mount M, Loman NJ, Pallen MJ, et al. Parallel evolutionary pathways to antibiotic resistance selected by biocide exposure. *J Antimicrob Chemother* 2015;70:2241–2248.

27. Ramage B, Erolin R, Held K, Gasper J, Weiss E, et al. Comprehensive arrayed transposon mutant library of *Klebsiella pneumoniae* outbreak strain KPNH1. *J Bacteriol* 2017;199:20.

28. Blair JMA, Piddock LJV. How to measure export via bacterial multidrug resistance efflux pumps. *mBio* 2016;7:e00840-16.

29. Afgan E, Baker D, Batut B, van den Beek M, Bouvier D, et al. The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2018 update. *Nucleic Acids Res* 2018;46:W537–W544.

30. Hind CK, Dowson CG, Sutton JM, Jackson T, Clifford M, et al. Evaluation of a library of FDA-approved drugs for their ability to potentiate antibiotics against multidrug-resistant gram-negative pathogens. *Antimicrob Agents Chemother* 2019;63:e00769-19.

31. Langevin AM, Dunlop MJ. Stress induction rate alters the benefit of AcrAB-TolC efflux pumps. *J Bacteriol* 2018;200:e00525-17.

32. Lawler AJ, Ricci V, Busby SJW, Piddock LJV. Genetic inactivation of acrAB or inhibition of efflux induces expression of ramA. *J Antimicrob Chemother* 2013;68:1551–1557.

33. Bailey AM, Paulsen IT, Piddock LJV. RamA confers multidrug resistance in *Salmonella enterica* via increased expression of acrB, which is inhibited by chlorpromazine. *Antimicrob Agents Chemother* 2008;52:3604–3611.

34. Fàbrega A, Ballesté-Delpierre C, Vila J. Differential impact of ramA mutations on both ramA transcription and decreased antibiotic susceptibility in *Salmonella Typhimurium*. *J Antimicrob Chemother* 2016;71:617–624.

35. Grimsey EM, Weston N, Ricci V, Stone JW, Piddock LJV. Overexpression of RamA, which regulates production of the multidrug resistance efflux pump AcrAB-TolC, increases mutation rate and influences drug resistance phenotype. *Antimicrob Agents Chemother* 2020;64:e02460-19.

36. Lim SP, Nkaido H. Kinetic parameters of efflux of penicillins by the AcrAB-TolC efflux system. *Antimicrob Agents Chemother* 2010;54:1800–1806.

37. Nkaido H, Pagès J-M. Broad-specificity efflux pumps and their role in multidrug resistance of Gram-negative bacteria. *FEMS Microbiol Rev* 2012;36:340–363.

38. Kinana AD, Vargiu AV, May T, Nkaido H. Aminoacyl-β-naphthalamides as substrates and modulators of AcrB multidrug efflux pump. *Proc Natl Acad Sci USA* 2016;113:1405–1410.
39. Strahl H, Hamoen LW. Membrane potential is important for bacterial cell division. *Proc Natl Acad Sci USA* 2010; 107:12281–12286.

40. Opperman TJ, Nguyen ST. Recent advances toward a molecular mechanism of efflux pump inhibition. *Front Microbiol* 2015; 6:421.

41. Jamshidi S, Sutton JM, Rahman KM. Mapping the dynamic functions and structural features of AcrB efflux pump transporter using accelerated molecular dynamics simulations. *Sci Rep* 2018; 8:10470.

42. Osei Sekyere J, Amoako DG. Carbonyl cyanide m-chlorophenylhydrazine (CCCP) reverses resistance to colistin, but not to carbapenems and tigecycline in multidrug-resistant enterobacteriaceae. *Front Microbiol* 2017; 8:228.

43. Saw HTH, Webber MA, Mushtaq S, Woodford N, Piddock LJV. Inactivation or inhibition of AcrAB-TolC increases resistance of carbapenemase-producing Enterobacteriaceae to carbapenems. *J Antimicrob Chemother* 2016; 71:1510–1519.

44. Sheng Z-K, Hu F, Wang W, Guo Q, Chen Z, et al. Mechanisms of tigecycline resistance among *Klebsiella pneumoniae* clinical isolates. *Antimicrob Agents Chemother* 2014; 58:6982–6985.

45. Griffith JM, Basting PJ, Bischof KM, Wrona EP, Kunka KS, et al. Experimental evolution of *Escherichia coli* K-12 in the Presence of Proton Motive Force (PMF) uncoupler carbonyl cyanide m-chlorophenylhydrazone selects for mutations affecting PMF-driven drug efflux pumps. *Appl Environ Microbiol* 2019; 85:e02792-18.

---

**Five reasons to publish your next article with a Microbiology Society journal**

1. The Microbiology Society is a not-for-profit organization.
2. We offer fast and rigorous peer review – average time to first decision is 4–6 weeks.
3. Our journals have a global readership with subscriptions held in research institutions around the world.
4. 80% of our authors rate our submission process as ‘excellent’ or ‘very good’.
5. Your article will be published on an interactive journal platform with advanced metrics.

Find out more and submit your article at microbiologyresearch.org.