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Inactivation, or inhibition of AcrAB-TolC, increases resistance of carbapenemase-producing Enterobacteriaceae to carbapenems

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Running title: Heteroresistance of carbapenem-resistant Enterobacteriaceae

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Synopsis

Objectives: To study the contribution of the multi-drug resistance AcrAB-TolC efflux system and impact of the efflux inhibitor, PAβN, towards carbapenem resistance in carbapenemase-producing Enterobacteriaceae.

Methods: *Klebsiella pneumoniae*, *Escherichia coli*, *Salmonella enterica* serovar Typhimurium and their corresponding AcrAB-TolC mutants, each carrying carbapenemase carrying plasmids (pKpQIL-UK with bla_{KPC} and pNDM-HK with bla_{NDM}), were tested for their susceptibility to six β-lactam antibiotics according to the BSAC agar dilution method. MICs were also determined in the presence of efflux inhibitors. The susceptibility of ertapenem in the presence of 25 and 100 mg/L PAβN was also determined for 86 non-replicate clinical isolates of carbapenemase-producing Enterobacteriaceae with OXA-48-like (n=18), IMP (n=12), VIM (n=16), NDM (n=20) or KPC (n=20) enzymes. Outer membrane protein profiles were determined with SDS-PAGE.

Results: The carbapenemase producing AcrAB mutants of *K. pneumoniae* and *E. coli*, and TolC mutant of *S.* Typhimurium had elevated resistance to carbapenem antibiotics. In *S.* Typhimurium, the increase in carbapenem MIC correlated with the loss of OmpF. Sixty-two (72%) of the clinical isolates tested were also more resistant to ertapenem in the presence of PAβN. SDS-PAGE showed that the presence of PAβN affected outer membrane porin production, which was associated with the increased MIC values of ertapenem.

Conclusion: The decreased susceptibility to carbapenems of carbapenemase-producing Enterobacteriaceae in the absence of AcrAB or TolC and/or in the presence of an efflux inhibitor (e.g. PAβN) is likely due to the changes in porin expression (e.g. OmpF). Efflux inhibitors may not potentiate carbapenem activity, but rather could increase levels of resistance in carbapenemase-producing organisms.

Introduction

Antibiotic resistance is a major problem worldwide[1] and the lack of new efficacious antibiotics has severely limited the therapeutic options for treating bacterial infections.[2] Carbapenems are an important class of antibiotics, increasingly used as a last option in treating serious bacterial infections.[3] Use of these antibiotics has been threatened by the emergence and dissemination of carbapenemase enzymes which have a broad-spectrum hydrolytic profile. These carbapenemase enzymes include the *Klebsiella pneumoniae* carbapenemase (KPC), New Delhi metallo-β-lactamase (NDM), oxacillinase group β-lactamase (OXA), Verona integron-encoded metallo-β-lactamase (VIM) and imipenemase-type metallo-β-lactamase (IMP)[4]. Moreover, carbapenemase-producing Enterobacteriaceae (CPE) are often resistant to other classes of antibiotics, limiting the available therapeutic options and leading to therapy failure.[5]

Detection of CPE has traditionally been achieved using phenotypic tests that determine susceptibility to selected β-lactams; however, these tests can be unreliable. Some isolates of CPE are inhibited by carbapenem MIC values below recommended breakpoint concentrations and the clinical implications for treatment with carbapenems are debated. The CLSI carbapenem breakpoint concentrations were revised in 2010, where isolates previously classified as carbapenem-susceptible are now classified as carbapenem non-susceptible. However, phenotypic antimicrobial susceptibility testing does not always demonstrate concordance with carbapenemase activity.[6-8] EUCAST (http://www.eucast.org/) recommends very low cut-off points for identifying potential CPE. The heterogeneity in carbapenem MIC values observed in CPE may be associated with the diverse combinations of resistance mechanisms that can be present in each isolate.[9] The absence of outer membrane porin proteins such as the OmpK35 (OmpF homologue) and OmpK36 (OmpC homologue) have been shown to affect the susceptibility of carbapenemase-producing *K. pneumoniae* to carbapenems.[10, 11]

The resistance nodulation division (RND) efflux systems found amongst Gram-negative Enterobacteriaceae confer resistance to many antimicrobial compounds.[12] The AcrAB-TolC efflux system is the best-studied RND efflux system and it extrudes a wide variety of substrates including different classes of antibiotics. It has been well established that production of AcrAB-TolC is associated with resistance to some β-lactam antibiotics, including penicillins and cephalosporins.[12] Pages *et al.* have suggested that there is a synergistic effect between AcrAB-TolC and β-lactamase enzymes in conferring resistance to β-lactam antibiotics in *K. pneumoniae*.[13] They showed that various clinical isolates producing the chromosomal SHV-1 β-lactamase were more susceptible to piperacillin in the presence of tazobactam (a β-lactamase inhibitor) and PAβN (an efflux inhibitor). In the presence of tazobactam, the MIC values of piperacillin for the isolates decreased 4- to 8-fold. However, in the presence of both tazobactam and PAβN, the susceptibility of the isolates increased by a further 4-fold. The authors suggested that tazobactam was a substrate of the AcrAB-TolC efflux system, hence, inhibition of the efflux system by PAβN increased the periplasmic concentration of tazobactam, allowing a stronger protective effect of the β-lactamase inhibitor on the SHV-1 enzyme.[13] However, it has also been shown that inactivation of AcrAB-TolC in *E. coli* resulted in a 16-fold increase in susceptibility of the bacterium to piperacillin.[14] Therefore, it is also possible that the larger increase in susceptibility of the *K. pneumoniae* isolates to piperacillin in the presence of tazobactam and PAβN was a result of the combined loss of the functions of the SHV-1 β-lactamase and the efflux system. AcrAB-TolC has also been shown to play an important role in resistance to fluoroquinolones and triclosan by acting synergistically with other, specific resistance mechanisms for these agents.[15, 16] However, not much is known about the contribution of the AcrAB-TolC efflux system to carbapenem resistance and any possible synergy with carbapenemases.

In this study, we investigated whether a functional AcrAB-TolC efflux system is required for full elaboration of resistance towards a variety of clinically important carbapenems in carbapenemase-producing bacteria.

Materials and Methods

Bacterial strains, plasmid and growth conditions

All plasmids and bacterial strains used in this study are listed in Table 1. A range of control strains of different species of *Enterobacteriaceae* were used to allow comparisons of results in different species and to utilise panels of well characterised isogenic mutants lacking efflux pump genes. The rifampicin-resistant mutants of *K. pneumoniae* Ecl8, *K. pneumoniae* Ecl8 *acrAB*::*aph*, *E. coli* BW25113, BW25113 *acrB*::*aph*, BW25113 *tolC*::*aph* and *S.* Typhimurium 14028s were constructed as previously described using 100 mg/L rifampicin.[17] The Ecl8^{Rif} *tolC*::*aph* was generated by inactivating the *tolC* gene in the rifampicin-resistant Ecl8^{Rif} as previously described.[18] The *acrB*::*aph* (EG16566) and *tolC*::*aph* (EG16564) alleles were transduced with P22 into 14028s^{Rif} to generate isogenic efflux mutants. The plasmids were transferred into the various strains using filter-mating.[19] All strains constructed were verified by PCR and DNA sequencing as containing desired genes or mutations (Table 2).

Eighty-six non-replicate clinical isolates of CPE collected from referrals to the AMRHAI Reference Unit of PHE were studied. These included *Klebsiella oxytoca* (n = 4), *K. pneumoniae* (n = 25), *Enterobacter asburiae* (n = 1), *Enterobacter aerogenes* (n = 3), *Enterobacter cloacae* (n = 22), *Enterobacter gergoviae* (n = 2) and *E. coli* (n = 29). Each of these strains carried one of the major carbapenemase genes found in the UK i.e. bla_{NDM} (n = 20) and bla_{KPC} (n = 20), $bla_{OXA\text{-}48\ \text{–like}}$ (n = 18), bla_{VIM} (n = 16), or bla_{IMP} (n = 12).

Determination of antibiotic susceptibility

The minimum inhibitory concentration (MIC) of each antibiotic for the bacterial strains and clinical isolates was determined using the agar dilution method and interpretation as described by the BSAC.[20] Where efflux inhibitors were tested, the concentrations used were: phenylalanine-arginine-β-naphthylamide (PAβN; 25 mg/L); 3 µM CCCP; 1-(1-naphthylmethyl)-piperazine (NMP; 100 mg/L). All chemicals were obtained from Sigma-Aldrich, USA. The MICs were determined on at least two separate occasions.

Outer membrane protein (porin) extraction and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Bacterial outer membrane proteins were purified as previously described.[21] Briefly, an overnight culture of bacteria was incubated at 37°C until it reached an OD_{600} of 0.6. Cell pellets were obtained after centrifugation and washing in 50 mM sodium phosphate buffer pH 7.0, freezing overnight at -80°C. The thawed cell suspension sonicated for 30 seconds four times with 30-second intervals to avoid overheating and the cell lysates were centrifuged at 6,000 x *g* for 1 min at 4°C to discard larger cell debris. The supernatants were then centrifuged again at 12,000 x *g* for 30 min at 4°C and pellets re-suspended vigorously in 200 µL 2% sarcosyl and incubated at room temperature for 30 min. The samples were centrifuged again at 12,000 x *g* for 30 min at 10°C. All the supernatant was carefully removed to ensure most of the detergent containing solution had been removed. The protein pellet was resuspended in 100 µL of 50 mM sodium phosphate buffer pH 7.0 and centrifuged at 12,000 x *g* for 10 min at 4°C to remove residual detergent from the outer membrane protein preparations. The supernatant was discarded and the extracted outer membrane proteins were finally resuspended in 50 µL of 50 mM sodium phosphate buffer pH 7.0. The amount of total protein in each sample was quantified using the Bradford assay.

The extracted outer membrane proteins were separated by SDS-PAGE using 16 x 20 x 0.1 cm discontinuous gels (4% stacking gel and 10 % resolving gel). Sample loading buffer (Sigma-Aldrich) was added to the protein samples which were heated at 95°C for 10 min. A total of 2 µg of total protein was loaded per lane for separation. Once the protein samples had been loaded into the wells, electrophoresis was started using 100 volts (constant) until the samples migrated into the resolving gel where the voltage was increased to 200 volts. Gels were stained with PhastGel® Blue R (Sigma-Aldrich) for 1 hour with gentle shaking. Then, gels were de-stained using de-staining buffer [10% (v/v) methanol and 5% (v/v) acetic acid in water] overnight with gentle shaking. The de-stained gels were visualised and images taken a using G:Box Gel Documentation System (Syngene, Cambridge, UK).

Despite repeated attempts to improve the resolution of the *Salmonella* porin proteins on SDS PAGE, this proved challenging. Therefore, to confirm the identity of the protein bands mass spectrometry was carried out on the bands from gels containing 6 µg of total protein that had migrated to the molecular sizes typical of OmpC and OmpF. Gel slices were excised from the gel and processed at the Advanced Mass Spectrometry Facility of the University of Birmingham. Briefly, bands were subjected to digestion with trypsin and 10µL of extracted protein in 1% Formic Acid was then used to separate and analyse digested peptides using an Orbitrap Velos (Thermo Scientific). TurboSEQUEST software (Thermo Scientific) was used to analyse data and assign protein identities to samples.

Results

Inactivation of a gene encoding a component of the AcrAB-TolC efflux pump increases β-lactam resistance

There was no significant difference in the MICs of β-lactam antibiotics for the plasmid-free *K. pneumoniae* Ecl8 and its isogenic AcrAB and TolC mutants (Table 3). However, when pKpQIL-UK carrying bla_{KPC-2} was present, the AcrAB mutant strain (Ecl8^{Rif} pKPQIL-UK *acrAB*::*aph*) was 8- and 4-fold less susceptible than the parental strain (Ecl8^{Rif}) to ertapenem and meropenem, respectively. *E. coli* BW25113 *acrB*::*aph* carrying pKpQIL-UK also showed a 4-fold decrease in susceptibility to ertapenem. However, the *E. coli* BW25113 TolC mutant (without plasmid) showed a 4-fold increase in susceptibility to doripenem and biapenem (when compared to BW25113). This strain carrying pKpQIL-UK (BW25113 pKpQIL-UK *tolC::aph*) was also more susceptible to meropenem, doripenem, biapenem and ceftazidime than BW25113 containing pKpQIL-UK. In contrast to *E. coli*, *S.* Typhimurium 14028s *tolC::aph* carrying pKpQIL-UK was less susceptible to ertapenem, meropenem, doripenem and ceftazidime. With the exception of the *E. coli* TolC mutant, these data suggest that lack of AcrAB or TolC can increase the MICs of some carbapenems for some Enterobacteriaceae.

To determine whether increased expression of *acrAB* or *tolC* in *Salmonella* affected carbapenem MICs, pKpQIL-UK was introduced into *S.* Typhimurium 14028S *ramR*::*aph*. No differences in MIC values to those of 14028s were detected (data not shown).

Decreased susceptibility to β-lactam antibiotics in efflux pump mutants carrying β-lactamases is independent of the vector and β-lactamase gene

To investigate whether the observed decreases in carbapenem susceptibility in the pKpQIL-UK carrying efflux mutants were a specific feature of this plasmid or gene or a general phenomenon, two other clinically important plasmids (pNDM-HK and pCT) plus a laboratory vector, pUC18, were introduced into a series of isogenic mutants of *S.* Typhimurium 14028s lacking defined efflux components (Table 3). Each plasmid also carried a β-lactamase gene,

NDM-1, CTX-M-14 and TEM-1, respectively. In the presence of the NDM-1 carrying pNDM-HK, the *Salmonella* TolC mutant was again 4-fold less susceptible to ertapenem and meropenem. The presence of pCT in the TolC mutant also decreased its susceptibility to ceftazidime by 8-fold, but had no affect on the activity of carbapenems. To determine whether the data obtained with 14028s and mutants was strain specific, the MICs of antibiotics for *S.* Typhimurium SL1344 and its isogenic efflux mutants (Δ*acrA*, Δ*acrB* and Δ*tolC*) carrying a bla_{TEM-1} encoding plasmid (pUC18) were also determined. Except for ertapenem (4-fold less susceptible), no difference was observed in the MIC values for the various β-lactam antibiotics tested for the TolC mutant, a 4-fold decrease in susceptibility to biapenem was seen in the AcrAB mutant (Table 3). These data suggest that the reduced susceptibility to β-lactam antibiotics observed in *S.* Typhimurium lacking TolC or AcrB is not specific to the host strain, plasmid or β-lactamase gene it carries.

Efflux inhibitors reduce susceptibility to ertapenem

There are two hypotheses to explain the counter-intuitive observation of reduced susceptibility to β-lactams seen in the efflux mutants. The phenotype was due to (1) lack of AcrAB or TolC proteins due to inactivation of *acrAB* or *tolC,* or (2) a consequence of loss of efflux function. To explore the second hypothesis, three efflux inhibitor compounds (PAβN, CCCP and NMP) with different modes of action were investigated. Ertapenem and the *K. pneumoniae*, *E. coli* and *S.* Typhimurium strains carrying the pKpQIL-UK plasmid were used in these experiments. As shown in Table 4, both *Salmonella* and *E. coli* pKpQIL-UK carrying strains (wild-type, AcrB and TolC mutants) was less susceptible to ertapenem in the presence of both PAβN and NMP. The *E. coli* TolC mutant was also less susceptible to ertapenem in the presence of CCCP. No difference in susceptibility was observed in the presence of various efflux inhibitors for the *K. pneumoniae* Ecl8^{Rif} carrying the pKpQIL-UK plasmid. However, in the presence of PAβN, there was a 16- and 4-fold increase in susceptibility to ertapenem, respectively, for the isogenic *K. pneumoniae* Ecl8^{Rif} AcrAB and TolC mutants carrying pKpQIL-UK.

PAβN increases ertapenem resistance in clinical isolates of Enterobacteriaceae

As our data suggested that PAβN conferred increased resistance to some β-lactam antibiotics, it was hypothesised that the PAβN effect would be observed with clinical isolates of carbapenemase producing Enterobacteriaceae. In the presence of 25 mg/L PAβN, 42% (n = 36) of the panel of curated CPE isolates became 4-fold or more resistant to ertapenem (Table 5). A further 30% (n = 26) of the isolates showed a 2-fold increase in ertapenem MIC values. The remaining isolates (26%, n = 22) showed no change in MIC. When compared with the ertapenem MICs in the absence of the inhibitor, only two isolates were more susceptible to ertapenem in the presence of PAβN.

To investigate whether a higher concentration of PAβN would result in a larger number of clinical isolates showing greater resistance to ertapenem, 100 mg/L PAβN was used. Interestingly, at this concentration and compared with 25 mg/L PAβN, the number of isolates for which the ertapenem MIC increased 4-fold was actually reduced to seven (8.1%). A total of 26 (30.2%) and 33 (38.4%) isolates showed 2-fold increase or no changes in ertapenem MIC value, respectively. Twenty isolates (ca. 23%) showed more than a 2-fold increase in ertapenem susceptibility. The 36 isolates for which 25 mg/L PAβN conferred a 4-fold or more increase in the ertapenem MIC were affected differently when 100 mg/L PAβN was used (Table 6). At the higher PAβN concentration, only seven of the isolates were 4-fold less susceptible to ertapenem compared to when no PAβN was added. Of the remaining isolates, 23 were 2-fold less susceptible and 6 isolates had no change in ertapenem MIC value.

PAβN reduces outer membrane protein expression, which is associated with increased resistance to carbapenems

It was hypothesised that the PAβN effect on the MICs of ertapenem and the other carbapenems was due to altered expression of outer membrane proteins, possibly porin proteins. In the presence of the plasmid pKpQIL-UK, the increase in ertapenem MIC value was more apparent for a mutant lacking OmpF (8-fold increase) and an OmpC-OmpF double

mutant (16-fold increase) than an OmpC mutant (Table 4). When PAβN was present, this increase in carbapenem resistance was greater for the *S.* Typhimurium OmpC mutant (16-fold), than the OmpF and OmpCF mutants (4-fold) (Table 4). In the presence of NMP, a decrease in susceptibility was also observed in *S.* Typhimurium SL1344 and its isogenic OmpC mutant. These data suggest that OmpF plays a more important role than OmpC in the observed changes in susceptibility to ertapenem.

From the MIC data (Table 4), the loss of outer membrane proteins (OmpC and OmpF) was associated with the reduction in susceptibility of the *S.* Typhimurium strains towards ertapenem. Hence, it was hypothesised that the repression of outer membrane proteins (OmpC and OmpF) resulted in the reduced β-lactam antibiotic susceptibility in the *S.* Typhimurium TolC mutants. Therefore, the expression of outer membrane proteins of the *Salmonella* strains in the absence of efflux pump components and when treated with PAβN were investigated. However, no obvious differences were observed for the efflux pump mutants compared to the wildtype *S.* Typhimurium SL1344 SDS-PAGE (Figure 1).

As sixty-two Enterobacteriaceae clinical isolates had reduced susceptibility to ertapenem in the presence of PAβN (Table 5), it was hypothesised that PAβN altered the outer membrane and/or porin expression in these isolates, resulting in reduced susceptibility to ertapenem. Hence, SDS-PAGE of four isolates each of *E. coli*, Enterobacter spp and five isolates of *Klebsiella* which were less susceptible to ertapenem in the presence of 25 mg/L PAβN was carried out. The outer membrane protein profile of one isolate of each species for which the ertapenem MIC was not affected by the presence of PAβN was also determined. Isolates which showed an increase in ertapenem resistance in the presence of PAβN also had reduced expression of OmpF (or equivalent) when PAβN was added (Figure 2). *E. coli* 656, *K. pneumoniae* 664 and *E. asburiae* 278 which showed no differences in ertapenem MIC value when PAβN was added showed no OmpF (or its orthologue) changes. In conclusion, in the isolates for which PAβN increased ertapenem MIC values, the compound also altered porin expression.

Discussion

Previous studies have shown that in *E. coli* the AcrAB-TolC efflux system works synergistically with other mechanisms to confer a higher level of resistance to antibacterial compounds, such as triclosan and ciprofloxacin.[15, 16] As a consequence, inhibition of efflux or deletion of a component of AcrAB-TolC often increases susceptibility to antibiotics. However, data obtained from our study suggest that a functional AcrAB-TolC is not required for carbapenem resistance and that AcrAB-TolC does not act synergistically with carbapenemases. This finding corroborates a previous study which found no increase in expression of *acrB* mRNA transcripts among carbapenem-resistant isolates of *Klebsiella* spp. and *Enterobacter* spp.[22] Moreover, to date, there is no study that clearly associates carbapenems (in particular, ertapenem) as a substrate of the AcrAB-TolC efflux system or its orthologue in *P. aeruginosa*.

Counter-intuitively, the loss of TolC in *S.* Typhimurium carrying carbapenemase encoding plasmids was associated with an increase in the MICs of a variety of carbapenem antibiotics (ertapenem, meropenem and doripenem) and a cephalosporin (ceftazidime). Although as expected, the bla_{TEM-1}-encoding pUC18 plasmid did not confer a clinically significant level of resistance to ertapenem in the *Salmonella* TolC mutant (SL1344 ΔTolC pUC18), the fold increase in the ertapenem MIC (when compared with the SL1344 pUC18) was similar to those shown for strains containing the carbapenemase-encoding plasmids pKpQIL-UK and pNDM-HK. Taken together, these data suggest that the increase in β-lactam (especially carbapenem) resistance in the *Salmonella* TolC mutant was not an artefact of one vector, nor was it an effect of the specific carbapenemase or β-lactamase. This finding is similar to those reported for clinical isolates harbouring different carbapenemases and which showed heteroresistance towards various carbapenem antibiotics.[23-25] The addition of the efflux inhibitor, PAβN, increased the ertapenem MICs for the wild-type *S.* Typhimurium $14028s^{Rif}$ and its AcrAB mutant strain. The fold increase was similar to that observed for the *Salmonella* TolC mutant carrying the plasmids (in the absence of PAβN). Furthermore, 72 of

86 clinical isolates showed at least 2-fold increase in ertapenem resistance, in the presence of PAβN. A similar (8-fold) decrease in ertapenem susceptibility in the presence of 100 mg/L PAβN has been reported in an *E. coli* isolate.[26] This study showed that the effect of PAβN is concentration-dependent; with 25 mg/L giving an increase in MICs of antibiotics but a different impact upon MIC was seen with 100 mg/L. Others have investigated PAβN to examine the role of efflux in carbapenem resistance when a decrease in carbapenem MIC was observed in the presence of this efflux inhibitor.[26-28] It has been suggested that a low concentration of PAβN (20 μM ≈ 10.4 mg/L) inhibits efflux, whereas a higher concentration (0.1 mM ≈ 51.9 mg/L) enhances the rate of efflux of cephalosporins via AcrB[29]. PAβN has also been shown to have membrane-permeabilising effects.[30, 31] The permeabilising effect of PAβN may also explain the observation that most of the clinical isolates which showed a 4-fold increase in ertapenem MICs at 25 mg/L PAβN, did not show a significant increase when 100 mg/L of PAβN was used.

Mutation giving increased expression of a global regulator e.g. MarA or RamA gives increased expression of *acrAB* and *tolC* with concomitant repression of porin genes in *E. coli, K. pneumoniae* and *Salmonella*.[21, 32, 33] Disruption of the *acrB* or *tolC* gene in *Salmonella* is also associated with decreased expression of *ompF*.[34] RNA-sequencing of *S.* Typhimurium SL1344 after exposure to PAβN also showed decreased *ompF* mRNA transcript (Blair, JMA. & Piddock, LJV unpublished data). The ertapenem MICs for the porin mutants carrying pKpQIL-UK (KPC-2) were increased, with the greatest change seen after addition of PAβN seen for the OmpC mutant. Taken together, these data suggest that altered porin production was associated with the observed reduction in ertapenem susceptibility in the efflux mutant strains carrying the plasmids. This was supported by the SDS-PAGE gels, which showed a decrease in *Salmonella* porin expression with an increasing concentration of PAβN. Similar changes in outer membrane protein profile were observed with the clinical isolates of Enterobacteriaceae harbouring a variety of carbapenemases. These findings support a

previous study which showed OmpC and OmpF or their orthologues play a role in carbapenem resistance among *Enterobacter* spp. and *Klebsiella* spp.[22]

To investigate whether the decrease in carbapenem susceptibility was specific to PAβN, MICs of ertapenem were determined with two other efflux inhibitors (NMP and CCCP) for *S.* Typhimurium 14028s^{Rif} carrying the pKpQIL-UK plasmid. This carbapenem was chosen as it showed the largest change in susceptibility. Independent of the presence of a functional AcrAB-TolC efflux system, PAβN and NMP reduced the susceptibility of the *Salmonella* and *E. coli* strains harbouring the pKpQIL-UK plasmid. The regulation of porin expression is known to be complex and differs between species.[35, 36] This may explain the differences observed between the *K. pneumoniae*, *E. coli* and *S.* Typhimurium efflux mutants' susceptibility to the antibiotics tested. However, as distinguishing between OmpF and OmpC on SDS-PAGE was challenging with the *Salmonella* mutants it is possible that subtle differences in porin production were not detected.

It is well established that AcrAB-TolC contributes to inherent and acquired antibiotic resistance and that TolC forms the outer membrane channel of most MDR efflux pumps found in Enterobacteriaceae. Hence, this system and/or TolC alone have been suggested as a potential target for efflux inhibitors.[37] Our work has shown that addition of PAβN or loss of a component of a MDR efflux pump such as TolC increased resistance of bacteria against some antibiotics, which use outer membrane porins as entry routes into the bacterial cell. Hence, TolC may not be an ideal drug target as the loss of TolC or inhibition of efflux function may confer increased resistance to some β-lactams. Therefore, we recommend careful evaluation of new efflux inhibitors to ensure that there is no increased resistance to clinically important antibiotics in antibiotic resistant bacteria.

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Table 1. Strains created and plasmids used in this study

Name/Code	Description	Source
pKpQIL-UK	A *bla*$_{KPC-2}$ carrying pKpQIL plasmid isolated in the UK	This study
pNDM-HK	A *bla*$_{NDM-1}$ carrying plasmid isolated from Hong Kong	[38]
pCT	A *bla*$_{CTX-M-14}$ carrying plasmid isolated from scouring calves	[39]
pUC18	A multicopy cloning vector carrying *bla*$_{TEM-1}$	[40]
Ecl8	*K. pneumoniae* Ecl8	[41]
Ecl8^{Rif}	Rifampicin-resistant mutant of Ecl8, His537Leu	This study
Ecl8^{Rif} AcrAB	Rifampicin-resistant mutant of Ecl8 *acrAB*::*aph*[42], His537Leu	This study
Ecl8^{Rif} TolC	Ecl8^{Rif} with inactivated outer membrane protein channel (*tolC*::*aph*)	This study
Ecl8^{Rif} pKpQIL-UK	Ecl8^{Rif} transconjugant carrying pKpQIL-UK	This study
Ecl8^{Rif} AcrAB pKpQIL-UK	Ecl8 ^{Rif} *acrAB*::*aph* transconjugant carrying pKpQIL-UK	This study
Ecl8^{Rif} TolC pKpQIL-UK	Ecl8 ^{Rif} *tolC*::*aph* transconjugant carrying pKpQIL-UK	This study
BW25113^{Rif}	Rifampicin-resistant mutant of *E. coli* BW25113[43], Pro574Leu	This study
BW25113^{Rif} AcrB	Rifampicin-resistant mutant of *E. coli* BW25113 *acrB*::*aph* [43], Pro574Leu	This study
BW25113^{Rif} TolC	Rifampicin-resistant mutant of *E. coli* BW25113 *tolC*::*aph* [43], Pro574Leu	This study
BW25113^{Rif} pKPQIL-UK	BW25113^{Rif} transconjugant carrying pKpQIL-UK	This study
BW25113^{Rif} AcrB pKpQIL-UK	BW25113^{Rif} *acrB*::*aph* transconjugant carrying pKpQIL-UK	This study
BW25113^{Rif} TolC pKpQIL-UK	BW25113^{Rif} *tolC*::*aph* transconjugant carrying pKpQIL-UK	This study
14028s	*Salmonella* Typhimurium ATCC14028s	ATCC culture
EG16566	14028s *acrAB*::*cat*	[44]
EG16564	14028s *tolC*::*cat*	[44]
14028s^{Rif}	Rifampicin resistant mutant of *S.* Typhimurium ATCC14028s, Ser522Tyr	This study
14028s^{Rif} AcrAB	Transductant of *acrAB*::*cat* from EG16566 into 14028s^{Rif}	This study
14028s^{Rif} TolC	Transductant of *tolC*::*cat* from EG16564 into 14028s^{Rif}	This study
14028s^{Rif} pKpQIL-UK	14028s^{Rif} carrying the pKpQIL-UK plasmid	This study
14028s^{Rif} AcrAB/UK	14028s^{Rif} *acrAB*::*cat* carrying the pKpQIL-UK plasmid	This study
14028s^{Rif} TolC pKpQIL-UK	14028s^{Rif} *tolC*::*cat* carrying the pKpQIL-UK plasmid	This study
14028s^{Rif} pKpQIL-UK	14028s^{Rif} carrying the pNDM-HK plasmid	This study
14028s^{Rif} AcrAB pKpQIL-UK	14028s^{Rif} AcrAB carrying the pNDM-HK plasmid	This study
14028s^{Rif} TolC HK	14028s^{Rif} TolC carrying the pNDM-HK plasmid	This study
SL1344	*S.* Typhimurium strain SL1344	[45]
SL1344ΔAcrA	*S.* Typhimurium strain SL1344 with inactivated *acrA*	[46]
SL1344ΔAcrB	*S.* Typhimurium strain SL1344 with inactivated *acrB*	[47]
SL1344ΔTolC	*S.* Typhimurium strain SL1344 with inactivated *tolC*	[47]
SL1344 pUC18	Transformant of SL1344 with the pUC18 plasmid	This study
SL1344ΔAcrA pUC18	Transformant of SL1344ΔAcrA carrying pUC18 plasmid	This study
SL1344ΔAcrB pUC18	Transformant of SL1344ΔAcrB carrying pUC18 plasmid	This study
SL1344ΔTolC pUC18	Transformant of SL1344ΔTolC carrying pUC18 plasmid	This study

Table 2. Primers used in this study

Name	DNA sequence (5' to 3')	Description
KPCg-colpcrF	ATGTCACTGTATCGCCGTCT	To detect the presence of *bla*$_{KPC}$
KPCg-colpcrR	TAGACGGCCAACACAATAGG	
NDM1-colpcrF	TTGATGCTGAGCGGGTG	To detect the presence of *bla*$_{NDM}$
NDM1-colpcrR	CTGTCCTTGATCAGGCAGC	
KpTolC-KO-F	ATACCTATAACAATGGCTATCGCGACAGCA ACGGCATCAAGTGTAGGCTGGAGCTGCTTC	To inactivate *tolC* in *K. pneumoniae*
KpTolC-KO-R	TAATGTTCAGCTCGTTGATCAGGTAGTTGT AGCGCGCATTGGGAATTAGCCATGGTCCAT	
KpEcl8-TolC-F	TTTCACCCGCTTCAAT	To verify inactivation of *K. pneumoniae tolC*
KpEcl8-TolC-R	GGATTTTTCGAGCTGAAC	

Table 3. MICs of various antibiotics for efflux pump mutants carrying the pKpQIL-UK (KPC-2), pNDM-HK (NDM-1) or pUC18 plasmids.

Strains	Genotype	Plasmid Introduced	Antibiotics (mg/L)					
			ETP	IPM	MEM	DOR	BIA	CAZ
E. coli NCTC10418	Wild-type	-	0.015	0.25	0.03	0.03	0.06	0.25
***K. pneumoniae* Ecl8**								
Ecl8^{Rif}	Rif^{R}	-	0.015	0.12	0.015	0.03	0.25	0.03
Ecl8^{Rif}	*acrAB*::*aph*	-	0.015	0.12	0.03	0.03	0.25	0.06
Ecl8^{Rif}	*tolC*::*aph*		0.015	0.25	0.03	0.06	ND	0.06
Ecl8^{Rif}	Rif^{R}	pKpQIL-UK	4	4	1	2	4	16
Ecl8^{Rif}	*acrAB*::*aph*	pKpQIL-UK	**32**	8	**4**	4	8	16
Ecl8^{Rif}	*tolC*::*aph*	pKpQIL-UK	4	4	2	4	ND	1
***E. coli* BW25113**								
BW25113^{Rif}	Rif^{R}	-	0.015	0.25	0.03	0.06	0.06	0.06
BW25113^{Rif}	*acrB*::*aph*	-	0.015	0.5	0.03	0.06	0.06	0.06
BW25113^{Rif}	*tolC*::*aph*	-	0.008	0.12	0.015	0.015	0.015	0.03
BW25113^{Rif}	Rif^{R}	pKpQIL-UK	0.12	2	0.25	0.5	2	2
BW25113^{Rif}	*acrB*::*aph*	pKpQIL-UK	**0.5**	2	0.25	1	4	2
BW25113^{Rif}	*tolC*::*aph*	pKpQIL-UK	0.06	1	0.03	0.12	0.12	0.5
***S.* Typhimurium ATCC14028s**								
14028s	Wildtype	-	0.015	0.25	0.03	0.03	0.06	0.25
14028s^{Rif}	Rif^{R}	-	0.008	0.5	0.03	0.03	0.03	0.25
14028s^{Rif}	*acrAB*::*cat*	-	0.008	0.12	0.015	0.015	0.03	0.12
14028s^{Rif}	*tolC*::*cat*	**-**	0.03	0.25	0.03	0.03	0.06	0.5
14028s^{Rif}	Rif^{R}	pKpQIL-UK	2	4	1	1	4	8
14028s^{Rif}	*acrAB*::*cat*	pKpQIL-UK	2	4	2	1	4	8
14028s^{Rif}	*tolC*::*cat*	pKpQIL-UK	**16**	8	**8**	**4**	4	**32**
14028s^{Rif}	Rif^{R}	pNDM-HK	8	8	4	8	2	>512
14028s^{Rif}	*acrAB*::*cat*	pNDM-HK	8	8	4	4	2	>512
14028s^{Rif}	*tolC*::*cat*	pNDM-HK	**32**	16	**16**	16	1	>512
14028s^{Rif}	Rif^{R}	pCT	0.015	0.125	0.015	0.03	0.03	0.5
14028s^{Rif}	*acrAB*::*cat*	pCT	0.015	0.125	0.015	0.03	0.03	**2**
14028s^{Rif}	*tolC*::*cat*	pCT	0.03	0.25	0.015	0.03	0.03	**4**
***S.* Typhimurium SL1344**								
SL1344	Wildtype	-	0.03	0.5	0.06	0.125	0.06	2
SL1344	Δ*acrA*	pUC18	0.03	0.5	0.06	0.125	0.125	2
SL1344	Δ*acrB*	pUC18	0.03	0.5	0.06	0.125	**0.25**	2
SL1344	Δ*tolC*	pUC18	**0.12**	1	0.12	0.25	0.12	2

ETP; Ertapenem; IPM; Imipenem; MEM; meropenem; DOR; doripenem; BIA; biapenem; CAZ; ceftazidime; Bold font denotes significant increase in MIC values; A consistent ≥4-fold or more difference in MIC values between the MIC for the wildtype strain versus the mutant plasmid carrying strain is indicated with bold font, are considered significant: ND; Not determined.

Table 4. Ertapenem MICs in the presence of various efflux inhibitors for Enterobacteriaceae +/- a component of the AcrAB-TolC efflux pump +/- production of KPC-2.

Strains	Genotype	Plasmid Introduced	MIC of Ertapenem (mg/L)			
			-	+PABN	+CCCP	+NMP
E. coli NCTC10418	Wildtype	-	0.015	0.015	0.015	0.015
***K. pneumoniae* Ecl8**						
Ecl8^{Rif}	Rif^{R}	pKpQIL-UK	4	8	4	4
Ecl8^{Rif}	*acrAB*::*aph*	pKpQIL-UK	32*	***2***	32	32
Ecl8^{Rif}	*tolC*::*aph*	pKpQIL-UK	4	*1*	*1*	8
***E. coli* BW25113**						
BW25113^{Rif}	Rif^{R}	pKpQIL-UK	0.12	**1**	0.12	**0.5**
BW25113^{Rif}	*acrB*::*aph*	pKpQIL-UK	0.5*	**4**	1	**4**
BW25113^{Rif}	*tolC*::*aph*	pKpQIL-UK	0.06	**0.25**	**0.5**	**0.5**
***S.* Typhimurium ATCC14028s**						
14028s^{Rif}	Rif^{R}	pKpQIL-UK	2	**32**	2	**16**
14028s^{Rif}	*acrAB*::*cat*	pKpQIL-UK	1	**4**	2	**8**
14028s^{Rif}	*tolC*::*cat*	pKpQIL-UK	16	*0.008*	32	**64**
***S.* Typhimurium SL1344**						
SL1344^{Rif}	Rif^{R}	pKpQIL-UK	1	**8**	2	**8**
SL1344	*ompC*::*aph*	pKpQIL-UK	2	**32**	2	**8**
SL1344	*ompF*::*aph*	pKpQIL-UK	16*	32	16	16
SL1344	Δ*ompC* *ompF*::*aph*	pKpQIL-UK	32*	64	32	32

PAβN; phenylalanine-arginine-β-naphthylamide; CCCP; carbonyl cyanide m-chlorophenyl hydrazone; NMP: 1-(1-naphthylmethyl)-piperazine; Bold font denote significant increase in MIC value in the presence of an efflux inhibitor. Italic font denotes a significant decrease in MIC value in the presence of an efflux inhibitor compared with the same strain in the absence of inhibitor. Asterisks (*), indicate when the MIC of ertapenem for an efflux or porin mutant was increased compared with isogenic parent strain. The concentrations of PAβN, CCCP and NMP used were 25 mg/L, 3 µM and 100 mg/L, respectively.

Table 5. Comparison of the changes in ertapenem resistance between two PAβN concentrations on 86 carbapenem-resistant clinical isolates of Enterobacteriaceae

Changes in MIC	25 mg/L PAβN		100 mg/L PAβN	
	Number of isolates	Percentage (%)	Number of isolates	Percentage (%)
⩾4-fold Reduction	-	-	6	7.0
2-fold Reduction	2	2.3	14	16.3
No Change	22	25.6	33	38.4
2-fold Increase	26	30.2	26	30.2
⩾4-fold Increase	36[1]	41.9	7	8.1
Total	86	100.0	86	100.0

[1]The impact of 100 mg/L PAβN on these 36 isolates are also shown in Table 6.

The MIC values of ertapenem were determined for 86 non-replicate clinical isolates of various Enterobacteriaceae (*Klebsiella* spp., *E. coli* and *Enterobacter* spp.), each carrying one of the five major carbapenemase genes (bla_{KPC}, bla_{NDM}, bla_{VIM}, bla_{IMP} and bla_{OXA-48}) detected in the UK.

552 **Table 6.** The impact of 100 mg/L PAβN on the isolates which showed a ≥4-fold increase in
553 ertapenem MIC in the presence of 25 mg/L PAβN

Concentration	The number of isolates (%) with altered ertapenem MIC in the presence of PAβN[2]			Total number of isolates
	No Change	2-fold Increase	4-fold Increase	
100 mg/L	6 (16.7)	23 (63.9)	7 (19.4)	36

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555 [2]Change in ertapenem MIC is relative to the MIC of ertapenem in the absence of PAβN.

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Legends to Figures

Figure 1. Outer membrane protein profile of *Salmonella* Typhimurium SL1344 and its isogenic efflux pump mutants. Band identities were confirmed by mass spectrometry as OmpC, OmpD, OmpA and OmpF, respectively. L = PageRuler Plus Prestained Protein Ladder.

Figure 2. Panel A. Outer membrane proteins of *E. coli* clinical isolates in the presence and absence of 25 mg/L PAβN. **Panel B.** Outer membrane proteins of *Klebsiella* spp. clinical isolates in the presence and absence of 25 mg/L PaβN (marked by + and – signs, respectively). **Panel C.** Outer membrane proteins of *Enterobacter* spp. clinical isolates in the presence and absence of 25 mg/L PaβN (marked by + and – signs, respectively). Values above the lanes represent the MIC of ertapenem for each strain with or without PAβN. L = PageRuler Plus Prestained Protein Ladder. Arrows indicate porins lost upon PAβN exposure in strains where a decrease in carbapenem susceptibility was also seen (indicated by asterisks).