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A putative RND-type efflux pump, H239_3064, contributes to colistin resistance through CrrB in *Klebsiella pneumoniae*

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Background: Colistin is one of the last-resort antibiotics used to treat carbapenem-resistant *Klebsiella pneumoniae* infection. Our previous studies indicated that clinical strains encoding CrrB with amino acid substitutions exhibited higher colistin resistance (MICs ≥512 mg/L) than did colistin-resistant strains encoding mutant MgrB, PmrB or PhoQ.

Objectives: CrrAB may regulate another unknown mechanism(s) contributing to colistin resistance, besides modifications of LPS with 4-amino-4-deoxy-L-arabinose and phosphoethanolamine.

Methods: To identify these potential unknown mechanism(s), a transposon mutant library of A4528 *crrB*(N141I) was constructed. Loci that might contribute to colistin resistance and were regulated by *crrB* were confirmed by deletion and complementation experiments.

Results: Screening of 2976 transposon mutants identified 47 mutants in which the MICs of colistin were significantly decreased compared with that for the parent. Besides *crrAB*, *crrC* and *pmrHFIJKLM* operons, these 47 transposon insertion mutants included another 13 loci. Notably, transcript levels of one of these insertion targets, *H239_3064* (encoding a putative RND-type efflux pump), were significantly increased in A4528 *crrB*(N141I) compared with the A4528 parent strain. Deletion of *H239_3064* in the A4528 *crrB*(N141I) background resulted in an 8-fold decrease in the MIC of colistin; complementation of the deletion mutant with *H239_3064* restored resistance to colistin. Susceptibilities of A4528-derived strains to other antibiotics were also tested. Mutations of *crrB* resulted in decreased susceptibility to tetracycline and tigecycline, and deletion of *H239_3064* in A4528 *crrB*(N141I) attenuated this phenomenon.

Conclusions: This study demonstrated that missense mutations of *K. pneumoniae crrB* lead to increased expression of *H239_3064*, leading in turn to decreased susceptibility to colistin, tetracycline and tigecycline.

Introduction

Colistin is one of the last-resort antibiotics reserved for use in treating carbapenem-resistant *Klebsiella pneumoniae* (CRKP) infection.[1] According to a recent report, 17% of CRKP isolates are resistant to colistin.[2] The WHO has also indicated that *K. pneumoniae* is one of the priority pathogens for which new antibiotics need to be developed, since multidrug-resistant *K. pneumoniae* has been detected throughout the world. Therefore, investigation of colistin resistance mechanisms in *K. pneumoniae* is critically important.

Colistin, which is also called polymyxin E, is a cationic peptide antibiotic.[3] Because this compound is positively charged, colistin can bind the lipid A moiety of bacterial LPS.[4,5] Colistin causes leakage of the cell membrane, resulting in a bactericidal effect. To counter the activity of colistin, *K. pneumoniae* modifies LPS by incorporation of 4-amino-4-deoxy-L-arabinose (Ara4N) and phosphoethanolamine (PEtN), alterations that are mediated by products of the *pmrHFIJKLM* operon and *pmrC*, respectively.[6–8] These modifications neutralize the negative charge of the bacterial LPS, resulting in reduced affinity for colistin. Previous studies indicated that the *pmrHFIJKLM* operon and *pmrC* are directly regulated by PmrAB and PhoPQ, respectively.[7,9,10] MgrB also has been shown to negatively regulate PhoPQ by inhibiting the phosphorylation of PhoQ.[11,12] Alterations of *mgrB*, *pmrB* and *phoQ* have been reported to enhance LPS modification, resulting in colistin resistance in *K. pneumoniae*.[11–14]

In recent studies, amino acid substitutions in CrrB were reported to be responsible for colistin resistance in *K. pneumoniae*.[15–17] These missense mutations of CrrB induce the expression of *crrC*,

such that increased accumulation of CrrC, acting through *pmrAB*, causes increased expression of the *pmrHFIJKLM* operon and *pmrC*.[16] Thus, amino acid substitutions in CrrB yield enhanced LPS modification, resulting in increased resistance to colistin.

The pathway leading from amino acid substitutions in CrrB to colistin resistance is similar to that induced by alterations of MgrB, PmrB and PhoQ, since mutations of these regulators result in colistin resistance by LPS modification with Ara4N or PEtN.[11,14,16,18] However, clinical isolates with CrrB missense mutations typically exhibit higher colistin resistance (MICs $\geq$512 mg/L) than clinical strains rendered colistin resistant by other mechanisms (Table S1, available as Supplementary data at *JAC* Online).[16,18] These observations imply that CrrAB might also induce mechanism(s) of colistin resistance other than those mediated by increased expression of the *pmrHFIJKLM* operon and *pmrC*.

To identify these hypothetical other mechanism(s) of colistin resistance induced by CrrB missense mutations, a transposon mutant library of the A4528 *crrB*(N141I) strain was constructed. N141I was an amino acid substitution that was identified in colistin-resistant isolates and located in the putative histidine kinase of CrrB.[16] Screening of this library identified 13 loci (other than *crrAB*, *crrC* and the *pmrHFIJKLM* operon) whose mutation yields colistin resistance; we describe here the further characterization of one such locus.

Materials and methods

Bacterial isolates and culture conditions

The A4528 *crrB*(N141I) strain was constructed from *K. pneumoniae* A4528 by using site-directed mutagenesis to introduce a single nucleotide mutation into the *crrB* locus of the parent.[16] The resulting strain was then used to study colistin resistance in *K. pneumoniae*, since this strain was used to construct various mutants that were related to colistin resistance. To clarify the mechanism(s) of colistin resistance induced by the amino acid substitution in CrrB, the A4528 *crrB*(N141I) strain was subjected to transposon mutagenesis to establish a transposon mutant library (see below).

In previous work, transcript levels of various colistin-resistance-related genes were characterized using the A4528 *crrB*(N141I) strain and this transcriptional analysis was confirmed by repeating these assays in eight colistin-resistant clinical isolates (Col4, Col7, Col20, Col21, Col22, Col28, Col36 and Col44) harbouring known missense mutations in relevant genes (Table S1).[16,18] To compare these transcriptional analyses, mRNA expressions of four colistin-susceptible isolates (A4528, ref. 64, N4252 and N5906) that are known to harbour the *crrAB* genes were measured.[16] These strains were used in the present study.

Cloning and genetic manipulation were performed by standard methodologies using *Escherichia coli* DH10B as the host. Except as noted below, both *K. pneumoniae* and *E. coli* were grown in LB medium, supplemented when necessary with 50 mg/L kanamycin or 100 mg/L ampicillin.

Construction of transposon mutant library

A mini-Tn*5* transposon was used for transposon mutagenesis. The transposon donor was conjugated with the A4528 *crrB*(N141I) strain using the technique described in previous studies.[19,20] Following transposon mutagenesis, a total of 2976 transposon mutants were collected. To evaluate the diversity of this library, 48 mutants were randomly selected and the transposon insertion sites were determined by semi-random PCR and DNA sequencing.[21] These 48 isolates corresponded to 46 independent transposon mutants; the remaining two mutants were duplicates of other mutants (data not shown). This result suggested that 95.8% (46/48) of the total 2976 transposon mutants should represent independent insertion events. However, the real diversity of the transposon mutant library was difficult to calculate, since inserted sequences may result in polar effects.[22]

The transposon mutant library was screened for isolates with increased susceptibility to colistin. Specifically, the library was replica inoculated to medium with and without colistin (1024 mg/L) to identify colonies that were unable to grow in the presence of high-concentration colistin. The resulting isolates were recovered from medium lacking colistin, repurified and further characterized by MICs, insertion site and transcriptional analysis as detailed below.

Detections of transcriptional junctions and 5′ ends of cDNAs

To determine whether *crrC*, *H239_3063*, *H239_3064* and *H239_3065* were transcribed as an operon, PCR was used to detect the presence in cDNA of intra- and intergenic fragments proximal to these loci. Total RNA was isolated from the A4528 *crrB*(N141I) strain using the RNeasy Mini Kit (Qiagen) and 700 ng of total RNA was subjected to cDNA synthesis using SuperScript IV Reverse Transcriptase (Invitrogen). Specific primer pairs (3062-3063-F and 3062-3063-R for *crrC* to *H239_3063*; 3063-3064-F and 3063-3064-R for *H239_3063* to *H239_3064*; 3064-3065-F and 3064-3065-R for *H239_3064* to *H239_3065*; 3065-3066-F and 3065-3066-R for *H239_3065* to *H239_3066*; and H239_3065-flank-F and CrrCAB-seqR4 as a positive control) were employed to perform PCR; primers are listed in Table S2.

To identify the upstream end of the *crrC-H239_3063-H239_3064-H239_3065* transcript, total RNA was reverse transcribed to cDNA using the SMARTer™ RACE cDNA Amplification Kit (Takara). These amplicons were cloned into the pJET1.2 plasmid (Thermo Scientific) and the resulting plasmid inserts were sequenced. The obtained DNA sequences were mapped to the A4528 genome to determine the operon's transcription start site.

Electrophoretic mobility shift assay (EMSA)

To express CrrA protein, the coding region of CrrA was amplified by PCR using primer pairs CrrA-pET-28C-F and CrrA-pET-28C-R (Table S2). The resulting clone was transformed to *E. coli* BL21 (DE3). The recombinant CrrA protein was induced by IPTG and was purified with His Mag Sepharose Ni beads (GE Healthcare). DNA fragments F1 and F2 were amplified by PCR using primer pairs CrrA-inverse-F and EMSA-Frag-1-R, and EMSA-Frag-2-F and EMSA-Frag-2-R, respectively. Proteins and DNA were reacted in reaction buffer (10 mM Tris–HCl pH 7.5, 50 mM KCl, 1 mM DTT, 10 mM $MgCl_2$ and 5% glycerol) for 1 h. The resulting reaction mixtures were subjected to electrophoresis and then stained with ethidium bromide.

Determination of susceptibility to antibiotics

The MICs of different antibiotics were determined by broth microdilution according to CLSI protocols. Aliquots of 5×10^4 cfu *K. pneumoniae* were inoculated onto CAMHB (BBL) plates supplemented with different concentrations of colistin, chloramphenicol, ciprofloxacin, tetracycline, cefotaxime or tigecycline, and the plates were incubated at 37°C. PABN (25 mg/L) was used to determine MICs when bacterial susceptibilities to colistin were examined without activities of efflux pumps. MICs were determined after overnight growth. The MICs for the *E. coli* ATCC 25922 strain were determined in parallel, serving as quality control.

Determination of mRNA expression levels by qRT–PCR

An aliquot (400 ng) of total RNA from each strain was subjected to cDNA synthesis using SuperScript IV Reverse Transcriptase. The cDNAs of *wbbM*, *wzt*, *dedA*, *tolA*, *rbsK*, *ompR*, *kdsA*, *envC*, *AEJ99441.1*, *H239_3063*, *H239_3064*, *H239_3065* and 23S rRNA (used as an internal control) were quantified using Power SYBR® Green Master Mix (Thermo Scientific) and an ABI 7900 Real-Time PCR system according to the manufacturer's

instructions. Sequences of the transcript-specific primers used for qRT–PCR are listed in Table S2. The relative RNA expression levels were calculated according to the ΔΔCt method, with normalization to 23S rRNA levels.[23]

Genetic manipulations for gene deletion and complementation

Coding regions and flanking fragments for the *crrA*, *H239_3063*, *H239_3064* and *H239_3065* loci from the A4528 *crrB*(N141I) strain were amplified by PCR using primer pairs crrA-flank-F and crrA-flank-R for *crrA*, H239_3063-flank-F and H239_3063-flank-R for *H239_3063*, H239_3064-flank-F and H239_3064-flank-R for *H239_3064* and H239_3065-flank-F and H239_3065-flank-R for *H239_3065*. The resulting products were cloned (separately) into the pJET1.2 plasmid. The coding regions of the respective ORFs were then removed by inverse PCR with primer pairs crrA-inverse-F and crrA-inverse-R for *crrA*, H239_3063-inverse-F and H239_3063-inverse-R for *H239_3063*, H239_3064-inverse-F and H239_3064-inverse-R for *H239_3064* and H239_3065-inverse-F and H239_3065-inverse-R for *H239_3065*. The ORF-deleted fragments were amplified by PCR (with the flanking primer pairs indicated above) and subcloned (separately) into the blunted NotI-digested pKO3-km plasmid.[24] The primer sequences for genetic manipulations are listed in Table S2. The resulting pKO3-km-derived plasmids were transformed (separately) into the A4528 *crrB*(N141I) strain by electroporation to generate the deletion mutants, using the previously described method.[25] Final mutants were confirmed by PCR and sequencing.

To construct the *H239_3064* complementation strain, the *H239_3064* locus was cloned into a pGEM-T plasmid (Promega) that carries a *lac* promoter along with a gene providing kanamycin resistance (plac). The fragment spanning the *H239_3064* locus was amplified from the A4528 strain by PCR with primer pair H239_3063-inverse-F and H239_3065-inverse-R (Table S2) and the amplicon was cloned into EcoRI-digested plac. The resulting plasmid (plac-H239_3064) was transformed into the A4528 *crrB*(N141I) Δ*H239_3062* strain by electroporation and plasmid-bearing (complemented) strains were then selected using kanamycin.

Detection of fluorescence accumulation in bacteria

The analysis was modified from the previous studies.[26,27] Each strain was cultured to mid-log phase. The bacterial pellet was washed with PBS and adjusted to an OD_{600} of 0.5. Ethidium bromide (final concentration 10 mg/L) and CCCP (final concentration 25 mg/L) were added and incubation was performed for 1 h. To activate efflux pumps, the bacterial pellet was resuspended with PBS supplemented with 5% glucose. The bacteria were collected at different timepoints and resuspended with 1 mM glycine/HCl (pH 2.3). The fluorescence of supernatants was detected by a Beckman Coulter PARADIGM with 535 nm excitation and 595 nm emission.

Results

Transposon mutant library of the A4528 crrB(N141I) strain

Clinical strains with *crrB* missense mutations exhibited higher colistin MICs than those for strains harbouring mutations in other genes (Table S1). However, *pmrH* transcript levels were elevated in colistin-resistant strains with alterations of *crrB*, *mgrB*, *phoQ* and *pmrB*. No significant difference in *pmrH* transcript levels between these strains was observed.[18] To identify the unknown colistin resistance mechanism(s) induced in *crrB* missense mutants, a transposon mutant library of A4528 *crrB*(N141I) was constructed. A total of 2967 transposon mutants of A4528 *crrB*(N141I) were collected and this library was screened for isolates with increased susceptibility to colistin. Subsequent characterization identified 47 mutants with colistin MICs that were significantly decreased compared with that for the A4528 *crrB*(N141I) parent strain (Table 1). The insertion location of the transposon in each of these 47 mutants was defined by semi-random PCR and sequencing to identify flanking sequences. The largest number of hits (20 of 47 total) corresponded to insertions in *crrAB* ($n = 12$) or *crrC* ($n = 8$) (Table 1). Among the remaining mutants, the largest classes were insertions in LPS synthesis-associated loci (*glf*, *wbbM*, *wzt* and *uge*; $n = 13$ total) and LPS modification-associated loci (*pmrHFIJKLM* operon; $n = 3$) (Table 1). These data indicated that the screening method was reliable, since *crrAB*, *crrC* and the *pmrHFIJKLM* operon were all associated with colistin resistance in the previous study.[18] Nine additional loci (*dedA*, *usg*, *tolA*, *kdsA*, *rbsK*, *ompR*, *envC*, *H239_3064* and *AEJ99441.1*) were recovered as insertion sites in this transposon screen (Table 1). We hypothesized that some or all of these nine loci might be involved in the additional colistin resistance observed in *K. pneumoniae crrB* missense mutants.

Table 1. Genetic locations of transposon, putative functions and the number of mutants identified in this study

Locations of transposon	Functions	Number of mutants
crrAB	regulators of *pmrHFIJKLM* operon	12
crrC	regulator of *pmrHFIJKLM* operon	8
pmrHFIJKLM	Ara4N modification	3
glf	UDP-galactopyranose mutase	1
wbbM	glycosyl transferase	5
wzt	sugar ABC transporter ATP-binding protein	6
uge	uridine diphosphate galacturonate 4-epimerase	1
tolA	membrane-anchored protein	1
kdsA	2-dehydro-3-deoxyphosphooctonate aldolase	1
rbsK	carbohydrate kinase	2
ompR	osmolarity response regulator	1
envC	septal ring factor	1
dedA	putative integral membrane protein	1
usg	putative semialdehyde dehydrogenase	1
H239_3064[a]	putative RND-type efflux pump	2
AEJ99441.1[b]	hypothetical protein	1

[a]Locus tag of the UHKPC45 strain in the NCBI database.
[b]Locus tag of the KCTC 2242 strain in the NCBI database.

Transcription of H239_3064 is induced by missense mutations in crrB

Our previous study demonstrated that *crrAB* regulates the *pmrHFIJKLM* operon via *crrC*.[16] To determine whether the remaining loci identified in the present study were induced in the presence of *crrB* missense mutations, transcription of the genes identified by transposon insertions was compared between A4528 *crrB*(N141I) and its A4528 parent. The mRNA levels of *usg* were not separately quantified, since the *usg* and *dedA* loci are believed to be

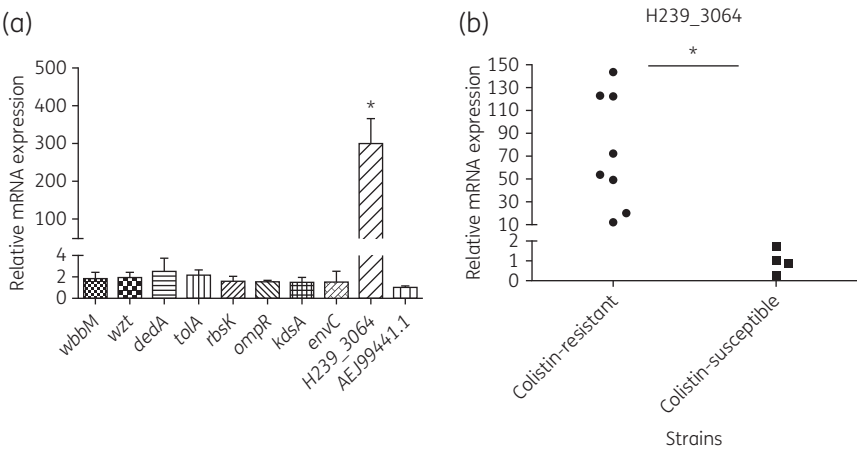

Figure 1. (a) mRNA levels of the respective loci were calculated by normalizing expression in A4528 *crrB*(N141I) to that in A4528 WT. (b) Relative mRNA levels of the *H239_3064* locus were quantified in colistin-resistant strains harbouring a *crrB* missense mutation; values were normalized to those in the respective colistin-susceptible strains. The mRNA expression in each strain was measured by qRT–PCR. Data are presented as mean ± SEM from three independent experiments. Statistical analysis was performed using a two-tailed Student's *t*-test (*$P < 0.05$).

transcribed together. Transcription levels of the LPS synthesis-associated loci were also determined as part of this experiment. Given that *glf*, *wbbM*, *wzt* and *uge* are located within the same region of the genome, analysis focused on *wbbM* and *wzt* as representative loci. This transcriptional analysis showed that mRNA expression of one of the targeted loci, *H239_3064*, was significantly enhanced in the A4528 *crrB*(N141I) strain compared with expression of this locus in the A4528 WT strain (Figure 1a). To confirm this observation, mRNA levels of *H239_3064* were quantified in clinical isolates known to harbour *crrB* missense mutations and these levels were compared with those in colistin-susceptible strains. The results revealed that *H239_3064* transcripts accumulated to significantly higher levels in colistin-resistant isolates carrying *crrB* missense mutations (Figure 1b). These data suggested that the *H239_3064* locus is normally down-regulated by *crrAB* and may be involved in colistin resistance in *K. pneumoniae*.

crrC and H239_3064 are co-transcribed

To characterize the mechanistic role(s) of *H239_3064*, loci adjacent to the *H239_3064* locus in the A4528 strain were sequenced and subjected to further analysis. Moreover, *H239_3063* and *H239_3065* transcripts accumulated to significantly higher levels in colistin-resistant isolates carrying *crrB* missense mutations (Figure S1). Given their proximity and shared orientation, the *crrC*, *H239_3063*, *H239_3064* and *H239_3065* loci were postulated to be co-transcribed as an operon (Figure 2). To examine this hypothesis, cDNA from the A4528 *crrB*(N141I) strain was analysed. We found that fragments corresponding to intergenic regions spanning *crrC* to *H239_3063*, *H239_3063* to *H239_3064* and *H239_3064* to *H239_3065* were PCR amplified from A4528 *crrB*(N141I) cDNA; an intergenic fragment spanning *H239_3065* to *H239_3066* was not recovered in the same PCR assay (Figure S2). Rapid amplification of cDNA ends also indicated that the transcription start site of this transcript is located upstream of *crrC* (Figure 2). Furthermore, analysis using the BPROM software (http://www.softberry.com/berry.phtml) identified consensus −10 and −35 promoter motifs adjacent to this transcript start site (Figure 2).

To demonstrate that *crrB* could regulate the *crrC* operon through *crrA*, deletion of *crrA* in A4528 *crrB*(N141I) and EMSA of CrrA were performed. The results indicated that deletion of *crrA* in the A4528 *crrB*(N141I) strain reduced the MIC of colistin (Table 2). The mRNA expressions of *crrC*, *H239_3063*, *H239_3064* and *H239_3065* in the A4528 *crrB*(N141I) Δ*crrA* strain were decreased, compared with those of the A4528 *crrB*(N141I) strain (Figure S3). Furthermore, EMSA indicated recombinant CrrA could react with the F1 fragment (promoter region of the *crrC* operon), resulting in a shift of the DNA fragment (Figure 3). However, the F2 fragment (*crrC* transcriptional region) was not bound by CrrA (Figure 3). Together, these results indicated that amino acid substitutions in CrrB yield enhanced transcription of *crrC* through *crrA* as well as that of the *H239_3063*, *H239_3064* and *H239_3065* loci. Given that co-transcribed bacterial loci often participate in shared biological functions, we postulated that *H239_3063*, *H239_3064* and *H239_3065* may all be involved in colistin resistance.

According to similarity of amino acid sequences, putative functions of H239_3063, H239_3064 and H239_3065 were identified. The ABC transporter transmembrane region was identified in H239_3063. H239_3064 was predicted to be an RND-type efflux pump, as indicated by the presence of an HAE1 domain. H239_3065 was a putative *N*-acetyltransferase. The *crrC* operon was also identified in *Citrobacter amalonaticus* and *Enterobacter ludwigii* by sequence homologies.

H239_3064 locus contributes to colistin resistance

To test whether *H239_3063*, *H239_3064* and *H239_3065* influence colistin resistance in *K. pneumoniae*, individual mutants harbouring deletions in each of these loci were created in the A4528 *crrB*(N141I) background. Colistin susceptibilities of the resulting mutants were determined. The results revealed that the colistin MIC for A4528 *crrB*(N141I) Δ*H239_3064* was 8-fold lower than that for A4528 *crrB*(N141I) (Table 2). Deletion of the *H239_3063* locus in A4528 *crrB*(N141I) yielded a nominal but non-significant increase in susceptibility to colistin (Table 2). Double deletion of the

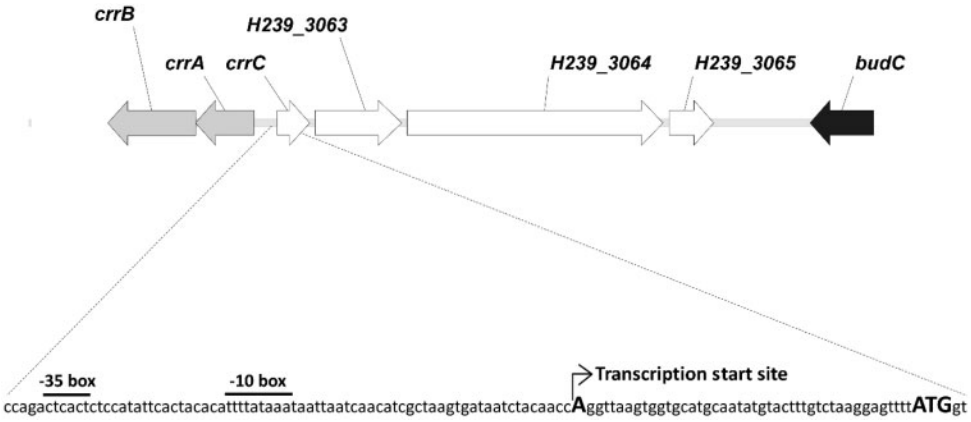

Figure 2. Schematic diagram of genome organization in the vicinity of the *H239_3064* locus. Directions of arrows indicate transcriptional orientation. The transcription start site was identified by rapid amplification of cDNA ends and putative −10 and −35 promoter motifs were identified using online tools. The capitalized ATG corresponds to the start codon of the *crrC* ORF.

Table 2. MIC of colistin for the A4528 *crrB*(N141I) strain with deletion and complementation of the *H239_3063*, *H236_ 3064* and *H236_ 3065* loci

Strain	MIC of colistin[a] (mg/L)
A4528 WT	1
A4528 *crrB*(N141I)	2048
A4528 *crrB*(N141I) Δ*H239_3063*	1024
A4528 *crrB*(N141I) Δ*H239_3064*	256
A4528 *crrB*(N141I) Δ*H239_3065*	2048
A4528 *crrB*(N141I) Δ*H239_3063-H239_3064*	128
A4528 *crrB*(N141I) Δ*H239_3064*/plac[b]	256
A4528 *crrB*(N141I) Δ*H239_3064*/plac-H239_3064[c]	2048
A4528 WT/plac[b]	1
A4528 WT/plac-H239_3064[c]	4
ATCC 25922[d]	1

[a]Susceptibilities to antibiotics were determined from independent triplicate experiments.
[b]The plasmid plac is described in the Materials and methods section.
[c]The coding region of *H239_3064* was cloned into the plac plasmid and resulted in the plac-H239_3064 plasmid.
[d]The MIC for the *E. coli* ATCC 25922 strain was determined in parallel, serving as quality control.

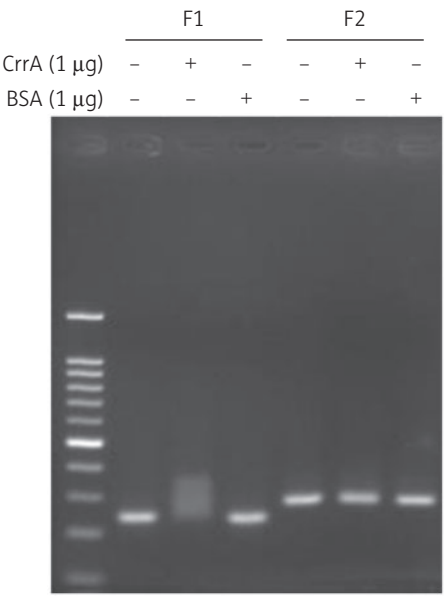

Figure 3. EMSA experiment with CrrA. DNA fragments F1 (promoter region of *crrC* operon) and F2 (*crrC* transcriptional region) were reacted with water, recombined CrrA protein or non-related protein (BSA). The reaction mixtures were subjected to electrophoresis and then stained with ethidium bromide.

H239_3063 and *H239_3064* loci in A4528 *crrB*(N141I) resulted in a 16-fold change in colistin MIC (Table 2). Deletion of the *H239_3065* locus in A4528 *crrB*(N141I) did not result in a significant change in the MIC of colistin (Table 2). Although mRNA expression of the *crrC* operon was slightly influenced by genetic manipulation, no significant polar effect was observed (Figure S4).

Complementation of A4528 *crrB*(N141I) Δ*H239_3064* with a plasmid-borne *H239_3064* locus restored resistance to colistin and mRNA expression of *H239_3064* (Table 2) (Figure S3). Furthermore, complementation of the A4528 WT strain with plac-H239_3064 reduced susceptibility to colistin (Table 2). These results demonstrated that increased expression of *H239_3064* contributes to colistin resistance in *K. pneumoniae*.

Increased expression of H239_3064 provides increased resistance to tetracycline and tigecycline

Based on homology, H239_3064 is predicted to be an RND-type efflux pump. PABN is a well-known efflux pump inhibitor (EPI) and previous studies indicated that EPI enhances bacterial susceptibility to antibiotics.[28–31] To test whether H239_3064 was inhibited by PABN, colistin MICs were determined in the presence of PABN. However, addition of PABN at this concentration did not enhance the colistin susceptibility of the A4528 *crrB*(N141I) strain (data not shown). Moreover, it is possible that the increased expression of *H239_3064* may result in increased efflux (and hence increased susceptibility) to compounds other than colistin. To examine

whether *H239_3064* influences susceptibilities to other antibiotics, A4528-derived strains were tested for MICs of chloramphenicol, ciprofloxacin, tetracycline, cefotaxime and tigecycline. Compared with the A4528 parent strain, A4528 *crrB*(N141I) exhibited decreased susceptibility to tetracycline and tigecycline; deletion of *H239_3064* in the A4528 *crrB* missense mutant strain attenuated this phenomenon (Table 3). However, the A4528 *crrB*(N141I) strain, with or without the *H239_3064* locus, did not show altered susceptibility to chloramphenicol, ciprofloxacin or cefotaxime (Table 3).

H239_3064 locus contributes to ethidium bromide accumulation

To demonstrate that *H239_3064* was a putative RND-type efflux pump, fluorescence accumulation experiments were performed. The results indicated that ethidium bromide accumulation was reduced in both A4528 *crrB*(N141I) and A4528 *crrB*(N141I) Δ*H239_3064* after re-energization of bacteria (Figure 4). Significantly, ethidium bromide accumulation of A4528 *crrB*(N141I) Δ*H239_3064* was more than that of A4528 *crrB*(N141I) within 60 min (Figure 4). These results indicated that *H239_3064* plays a role in ethidium bromide accumulation and *H239_3064* might be a transporter of the RND-type efflux pump type.

Discussion

Our previous study indicated that *crrAB*, *crrC* and the *pmrHFIJKLM* operon are major mediators of colistin resistance in the A4528 *crrB*(N141I) strain and, as expected, these loci were re-isolated in the present study.[16] The additional loci identified in the present study included *dedA*, which encodes a putative integral membrane protein; the previous study had demonstrated that *dedA* is essential for growth during exposure to colistin.[32] *usg* was also identified by a transposon insertion in the present study; notably, *usg* is located upstream of *dedA* in the *K. pneumoniae* genome, so insertion at *usg* may have polar effects on *dedA* expression. Multiple additional loci associated with colistin resistance were also identified for the first time in the present study. Several of the loci that were identified in the current study's screen of transposon mutants were LPS synthesis-associated genes, including *glf*, *wbbM*, *wzt* and *uge*.[33–36] This observation suggested that defects in LPS synthesis may interfere with LPS modification, thereby resulting in decreased colistin resistance. Other loci encoding membrane-associated proteins (*tolA* and *ompR*) were identified in the present study; loss of these proteins may impair the permeability and/or structure of the bacterial membrane, which would influence susceptibility to colistin.[37,38] However, the remaining loci could not be systematically classified, and further studies will be needed to define how these loci influence colistin resistance.

The *crrC*, *H239_3063*, *H239_3064* and *H239_3065* loci were shown here to be co-transcribed, thus forming an operon (Figure 2). These loci therefore may contribute to a shared biological function. However, mutations of *H239_3063* and *H239_3065* did not yield significant changes in colistin resistance (Table 2); definition of the actual function of *H239_3063* and *H239_3065* will require further investigation. Our previous study showed that approximately half of *K. pneumoniae* clinical isolates lack *crrAB*.[16] Notably, the *crrC* operon (*crrC*, *H239_3063*, *H239_3064* and *H239_3065*) is also absent from the genome of the standard NTUH-K2044 strain (NCBI reference sequence NC_012731.1).

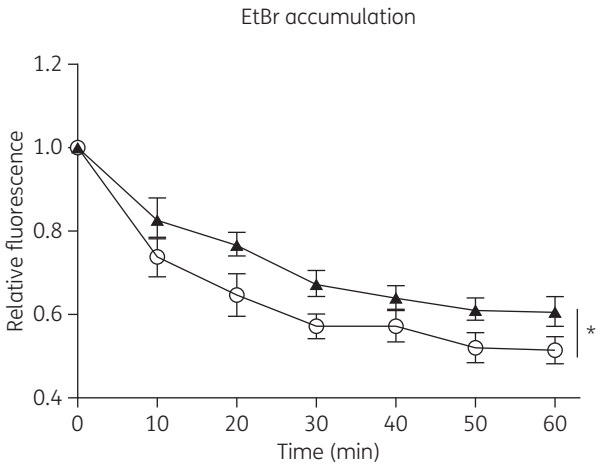

Figure 4. A4528 *crrB*(N141I) (circles) and A4528 *crrB*(N141I) Δ*H239_3064* (triangles) treated with ethidium bromide (EtBr) were collected at different timepoints after bacteria were re-energized by glucose. The fluorescence was measured at 535 nm excitation and 595 nm emission. Data are presented as mean ± SEM from three independent experiments. Statistical analysis was performed using a two-tailed Student's *t*-test (*$P < 0.05$).

Table 3. Susceptibilities of A4528-devrived strains to antibiotics

	MIC[a] (mg/L)				
Strain	chloramphenicol	ciprofloxacin	tetracycline	cefotaxime	tigecycline
A4528 WT	4	0.03125	1	0.0625	1
A4528 *crrB*(N141I)	4	0.03125	2	0.0625	2
A4528 *crrB*(N141I) Δ*H239_3064*	4	0.03125	1	0.0625	1
A4528 *crrB*(N141I) Δ*H239_3064*/plac[b]	NA	NA	1	NA	1
A4528 *crrB*(N141I) Δ*H239_3064*/plac-H239_3064[c]	NA	NA	2	NA	2

NA, not available.
[a]Susceptibilities to antibiotics were determined from independent triplicate experiments.
[b]The plasmid plac is described in the Materials and methods section.
[c]The coding region of *H239_3064* was cloned into the plac plasmid and resulted in the plac-H239_3064 plasmid.

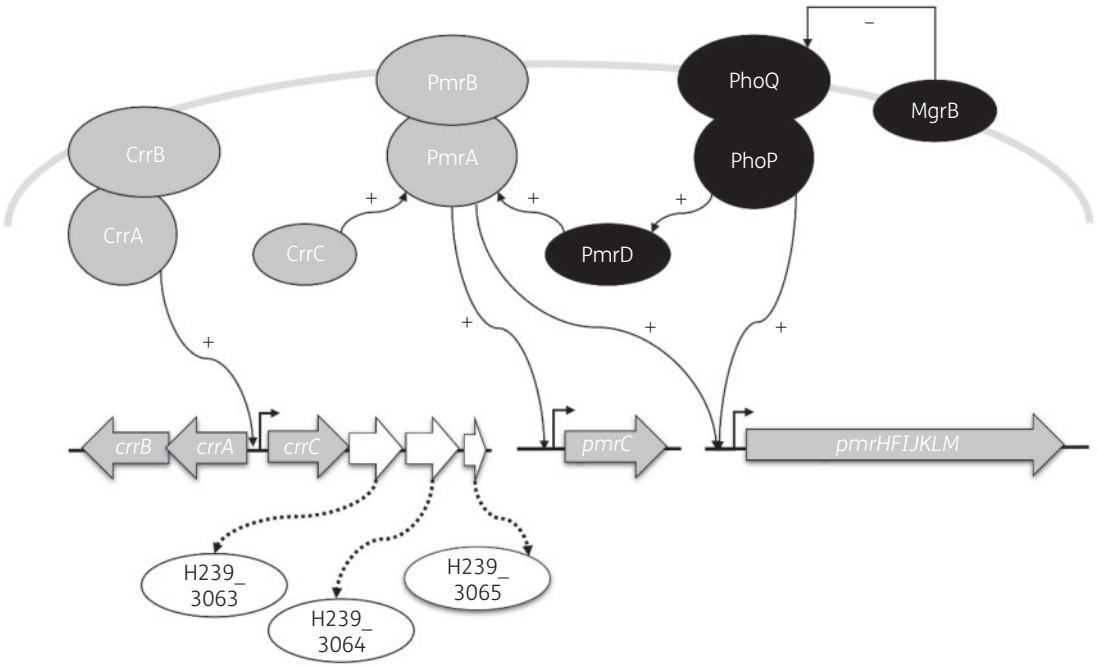

Figure 5. Schematic diagram of colistin resistance mechanisms in *K. pneumoniae*. Mutations of MgrB, PhoPQ and PmrAB induce LPS modifications with Ara4N and PEtN through effects on expression of the *pmrHFIJKLM* operon and *pmrC*. Amino acid substitutions in CrrB alter regulation of *pmrAB* through effects on CrrC expression, resulting in overexpression of the *pmrHFIJKLM* operon and *pmrC*. Expression of H239_3064, a putative efflux pump, is also induced by CrrB missense mutations and the pump contributes to decreased susceptibility to colistin.

These observations indicate that this region is not essential for bacterial growth and so is variably present in the *K. pneumoniae* population. Most colistin-resistant strains with amino acid substitutions of CrrB were ST11 and ST258 isolates.[15,16,18] Therefore, prevalence of the *crrAB* and *crrC* operon might be related to genetic evolution, since the genomic sequences of these two types are close.[39]

The encoded protein of H239_3064 shares 49% amino acid identity with *K. pneumoniae* AcrB, a known efflux pump.[40] Although H239_3064 appears to be an RND-type efflux pump, its associated fusion protein and outer membrane protein are unknown. In the present study, the *H239_3064* locus was shown to contribute to colistin resistance, as demonstrated by deletion and complementation experiments. Moreover, *H239_3064* might be an efflux pump-type transporter, since deletion of *H239_3064* in the A4528 *crrB*(N141I) strain increased fluorescence accumulation. H239_3064 might directly pump out colistin, or substrate(s) that are pumped out by H239_3064 could influence the bacterial surface charge, resulting in altered susceptibility to colistin.[41]

Tigecycline, like colistin, is among the last-resort antibiotics reserved for the treatment of CRKP infection. The decreased susceptibility to tigecycline observed here (Table 3) is therefore an unfortunate secondary effect of increased expression of *H239_3064*. Although increased expression of *H239_3064* did not result in a dramatic change in susceptibility to tigecycline, the observed decrease in tigecycline susceptibility may facilitate selection for increased resistance to tigecycline during clinical treatment with the combination of colistin and tigecycline.

In summary, the present study demonstrated that *crrB* missense mutants exhibit increased expression of a putative RND-type efflux pump, H239_3064, and showed that this locus contributes to colistin resistance. These results explain why colistin-resistant strains harbouring *crrB* missense mutants display higher colistin MICs than clinical strains harbouring mutations in *mgrB*, *phoPQ* and *pmrAB* (Figure 5). Furthermore, the current work further showed that increased transcription of the *H239_3064* locus results in decreased susceptibility to tetracycline and tigecycline, an effect that may have clinical relevance.

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Transparency declarations

None to declare.

Supplementary data

Tables S1 and S2 and Figures S1 to S4 are available as Supplementary data at *JAC* Online.

References

1 Munoz-Price LS, Poirel L, Bonomo RA *et al*. Clinical epidemiology of the global expansion of *Klebsiella pneumoniae* carbapenemases. *Lancet Infect Dis* 2013; **13**: 785–96.

2 Chiu SK, Wu TL, Chuang YC *et al*. National surveillance study on carbapenem non-susceptible *Klebsiella pneumoniae* in Taiwan: the emergence and rapid dissemination of KPC-2 carbapenemase. *PLoS One* 2013; **8**: e69428.

3 Biswas S, Brunel JM, Dubus JC *et al*. Colistin: an update on the antibiotic of the 21st century. *Expert Rev Anti Infect Ther* 2012; **10**: 917–34.

4 Li J, Nation RL, Milne RW *et al*. Evaluation of colistin as an agent against multi-resistant Gram-negative bacteria. *Int J Antimicrob Agents* 2005; **25**: 11–25.

5 Bialvaei AZ, Samadi Kafil H. Colistin, mechanisms and prevalence of resistance. *Curr Med Res Opin* 2015; **31**: 707–21.

6 Yan A, Guan Z, Raetz CR. An undecaprenyl phosphate-aminoarabinose flippase required for polymyxin resistance in *Escherichia coli*. *J Biol Chem* 2007; **282**: 36077–89.

7 Lee H, Hsu FF, Turk J *et al*. The PmrA-regulated pmrC gene mediates phosphoethanolamine modification of lipid A and polymyxin resistance in *Salmonella enterica*. *J Bacteriol* 2004; **186**: 4124–33.

8 Olaitan AO, Morand S, Rolain JM. Mechanisms of polymyxin resistance: acquired and intrinsic resistance in bacteria. *Front Microbiol* 2014; **5**: 643.

9 Cheng HY, Chen YF, Peng HL. Molecular characterization of the PhoPQ-PmrD-PmrAB mediated pathway regulating polymyxin B resistance in *Klebsiella pneumoniae* CG43. *J Biomed Sci* 2010; **17**: 60.

10 Chen HD, Groisman EA. The biology of the PmrA/PmrB two-component system: the major regulator of lipopolysaccharide modifications. *Annu Rev Microbiol* 2013; **67**: 83–112.

11 Cannatelli A, D'Andrea MM, Giani T *et al*. In vivo emergence of colistin resistance in *Klebsiella pneumoniae* producing KPC-type carbapenemases mediated by insertional inactivation of the PhoQ/PhoP mgrB regulator. *Antimicrob Agents Chemother* 2013; **57**: 5521–6.

12 Cannatelli A, Giani T, D'Andrea MM *et al*. MgrB inactivation is a common mechanism of colistin resistance in KPC-producing *Klebsiella pneumoniae* of clinical origin. *Antimicrob Agents Chemother* 2014; **58**: 5696–703.

13 Olaitan AO, Diene SM, Kempf M *et al*. Worldwide emergence of colistin resistance in *Klebsiella pneumoniae* from healthy humans and patients in Lao PDR, Thailand, Israel, Nigeria and France owing to inactivation of the PhoP/PhoQ regulator mgrB: an epidemiological and molecular study. *Int J Antimicrob Agents* 2014; **44**: 500–7.

14 Miller AK, Brannon MK, Stevens L *et al*. PhoQ mutations promote lipid A modification and polymyxin resistance of *Pseudomonas aeruginosa* found in colistin-treated cystic fibrosis patients. *Antimicrob Agents Chemother* 2011; **55**: 5761–9.

15 Wright MS, Suzuki Y, Jones MB *et al*. Genomic and transcriptomic analyses of colistin-resistant clinical isolates of *Klebsiella pneumoniae* reveal multiple pathways of resistance. *Antimicrob Agents Chemother* 2015; **59**: 536–43.

16 Cheng YH, Lin TL, Lin YT *et al*. Amino acid substitutions of CrrB responsible for resistance to colistin through CrrC in *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* 2016; **60**: 3709–16.

17 Jayol A, Nordmann P, Brink A *et al*. High-level resistance to colistin mediated by various mutations in the *crrB* gene among carbapenemase-producing *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* 2017; **61**: e01423-17.

18 Cheng YH, Lin TL, Pan YJ *et al*. Colistin resistance mechanisms in *Klebsiella pneumoniae* strains from Taiwan. *Antimicrob Agents Chemother* 2015; **59**: 2909–13.

19 Fang CT, Chuang YP, Shun CT *et al*. A. novel virulence gene in *Klebsiella pneumoniae* strains causing primary liver abscess and septic metastatic complications. *J Exp Med* 2004; **199**: 697–705.

20 Herrero M, de Lorenzo V, Timmis KN. Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. *J Bacteriol* 1990; **172**: 6557–67.

21 Salama NR, Shepherd B, Falkow S. Global transposon mutagenesis and essential gene analysis of *Helicobacter pylori*. *J Bacteriol* 2004; **186**: 7926–35.

22 Wetmore KM, Price MN, Waters RJ *et al*. Rapid quantification of mutant fitness in diverse bacteria by sequencing randomly bar-coded transposons. *MBio* 2015; **6**: e00306-15.

23 Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C^T}$ method. *Methods* 2001; **25**: 402–8.

24 Link AJ, Phillips D, Church GM. Methods for generating precise deletions and insertions in the genome of wild-type *Escherichia coli*: application to open reading frame characterization. *J Bacteriol* 1997; **179**: 6228–37.

25 Lin TL, Yang FL, Yang AS *et al*. Amino acid substitutions of MagA in *Klebsiella pneumoniae* affect the biosynthesis of the capsular polysaccharide. *PLoS One* 2012; **7**: e46783.

26 Smith HE, Blair JM. Redundancy in the periplasmic adaptor proteins AcrA and AcrE provides resilience and an ability to export substrates of multidrug efflux. *J Antimicrob Chemother* 2014; **69**: 982–7.

27 Srinivasan VB, Singh BB, Priyadarshi N *et al*. Role of novel multidrug efflux pump involved in drug resistance in *Klebsiella pneumoniae*. *PLoS One* 2014; **9**: e96288.

28 Barrero MA, Pietralonga PA, Schwarz DG *et al*. Effect of the inhibitors phenylalanine arginyl β-naphthylamide (PAβN) and 1-(1-naphthylmethyl)-piperazine (NMP) on expression of genes in multidrug efflux systems of *Escherichia coli* isolates from bovine mastitis. *Res Vet Sci* 2014; **97**: 176–81.

29 Yu EW, Aires JR, McDermott G *et al*. A periplasmic drug-binding site of the AcrB multidrug efflux pump: a crystallographic and site-directed mutagenesis study. *J Bacteriol* 2005; **187**: 6804–15.

30 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.

31 Ni W, Li Y, Guan J *et al*. Effects of efflux pump inhibitors on colistin resistance in multidrug-resistant Gram-negative bacteria. *Antimicrob Agents Chemother* 2016; **60**: 3215–8.

32 Jana B, Cain AK, Doerrler WT *et al*. The secondary resistome of multidrug-resistant *Klebsiella pneumoniae*. *Sci Rep* 2017; **7**: 42483.

33 Nassau PM, Martin SL, Brown RE *et al*. Galactofuranose biosynthesis in *Escherichia coli* K-12: identification and cloning of UDP-galactopyranose mutase. *J Bacteriol* 1996; **178**: 1047–52.

34 Kos V, Whitfield C. A membrane-located glycosyltransferase complex required for biosynthesis of the D-galactan I lipopolysaccharide O antigen in *Klebsiella pneumoniae*. *J Biol Chem* 2010; **285**: 19668–87.

35 Izquierdo L, Merino S, Regue M *et al*. Synthesis of a *Klebsiella pneumoniae* O-antigen heteropolysaccharide (O12) requires an ABC 2 transporter. *J Bacteriol* 2003; **185**: 1634–41.

36 Gierczynski R, Kaluzewski S, Zasada AA *et al*. Occurrence of selected genes of the *Klebsiella pneumoniae* clusters waa and wb for lipolysaccharide synthesis in reference and epidemic strains. *Med Dosw Mikrobiol* 2005; **57**: 383–93.

37 Llamas MA, Ramos JL, Rodriguez-Herva JJ. Mutations in each of the tol genes of *Pseudomonas putida* reveal that they are critical for maintenance of outer membrane stability. *J Bacteriol* 2000; **182**: 4764–72.

38 Slauch JM, Silhavy TJ. Genetic analysis of the switch that controls porin gene expression in *Escherichia coli* K-12. *J Mol Biol* 1989; **210**: 281–92.

39 Qi Y, Wei Z, Ji S *et al*. ST11, the dominant clone of KPC-producing *Klebsiella pneumoniae* in China. *J Antimicrob Chemother* 2011; **66**: 307–12.

40 Murakami S, Nakashima R, Yamashita E *et al*. Crystal structures of a multidrug transporter reveal a functionally rotating mechanism. *Nature* 2006; **443**: 173–9.

41 Dreier J, Ruggerone P. Interaction of antibacterial compounds with RND efflux pumps in *Pseudomonas aeruginosa*. *Front Microbiol* 2015; **6**: 660.