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

Yi-Hsiang Cheng¹, Tzu-Lung Lin¹, Yi-Tsung Lin²,³ and Jin-Town Wang¹,⁴*

¹Department of Microbiology, National Taiwan University College of Medicine, Taipei, Taiwan; ²Division of Infectious Diseases, Department of Medicine, Taipei Veterans General Hospital, Taipei, Taiwan; ³Institute of Emergency and Critical Care Medicine, National Yang-Ming University, Taipei, Taiwan; ⁴Department of Internal Medicine, National Taiwan University Hospital, Taipei, Taiwan

*Corresponding author. Department of Microbiology, National Taiwan University College of Medicine, Taipei, Taiwan. Tel: +886-2-23123456, ext. 88292; Fax: +886-2-23948718; E-mail: wangjt@ntu.edu.tw

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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 CrdB 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 crrR 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 crrRAB, 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 CrmA would further support the characterization of pmrC.

The pathway leading from amino acid substitutions in CrmA to colistin resistance is similar to that induced by alterations of MgrB, PmrB and Phek, since mutations of these regulators result in colistin resistance by LPS modification with Ara4N or PetN.11,14,16,18 However, clinical isolates with CrmA missense mutations typically exhibit higher colistin resistance (MICs ≥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 CrmA 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 CrmA missense mutations, a transposon mutant library of the A4528 crmA(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 CrmA.16 Screening of this library identified 13 loci (other than crmA, crmA 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 crmA(N141I) strain was constructed from K. pneumoniae A4528 by using site-directed mutagenesis to introduce a single nucleotide mutation into the crmA 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 CrmA, the A4528 crmA(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 crmA(N141I) strain and this transcriptional analysis was confirmed by repeating these assays in eight colistin-resistant clinical isolates (Co4a, Co7, Co10, Co12, Co22, Co128, Co36 and Co44) harbouring known 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 crmA 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-Tn5 transposon was used for transposon mutagenesis. The transposon donor was conjugated with the A4528 crmA(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 of cDNA of intra- and intergenic fragments proximal to these loci. Total RNA was isolated from the A4528 crmA(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.23

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 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 MgCl2 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 5×104 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 AeJ99441.1, H239_3063, H239_3064 and H239_3065 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 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 blunt 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 strain by electroporation and plasmid-bearing (complemented) strains were then selected using kanamycin.

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 from the A4528 strain by PCR with primer pair H239_3063-inverse-F and H239_3063-inverse-R (Table S2) and the amplicon was cloned into EcoRI-digested plac. The resulting plasmid (plac-H239_3064) was transformed into the A4528 strain (Table S2). The resulting strain was 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 OD600 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/HC1 (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, mgbB, 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 (gft, 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.

**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

| Locations of transposon | Functions | Number of mutants |
|-------------------------|-----------|-------------------|
| crrAB                   | regulators of pmrHFIJKLM operon | 12               |
| crrC                    | regulator of pmrHFIJKLM operon | 8                |
| pmrHFIJKLM              | Ara4A modification | 3                |
| gft                     | 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-deoxyphosphochoanate 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_3064a              | putative RND-type efflux pump | 2               |
| AEJ99441.1b             | hypothetical protein | 1               |

aLocus tag of the UHKPC45 strain in the NCBI database.
bLocus tag of the KCTC 2242 strain in the NCBI database.
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 crB(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 crB 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 crB missense mutations (Figure 1b). These data suggested that the H239_3064 locus is normally down-regulated by crAB 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 crB 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 crB(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 crB(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/beryl.plhtml) identified consensus –10 and –35 promoter motifs adjacent to this transcript start site (Figure 2).

To demonstrate that crB could regulate the crrC operon through crA, deletion of crrA in A4528 crB(N141I) and EMSA of CrA were performed. The results indicated that deletion of crrA in the A4528 crB(N141I) strain reduced the MIC of colistin (Table 2). The mRNA expressions of crrC, H239_3063, H239_3064 and H239_3065 in the A4528 crB(N141I) ΔcrrA strain were decreased, compared with those of the A4528 crB(N141I) strain (Figure S3). Furthermore, EMSA indicated recombinant CrA 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 CrA (Figure 3). Together, these results indicated that amino acid substitutions in CrB yield enhanced transcription of crrC through crA 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 ludwigi 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 crB(N141I) background. Colistin susceptibilities of the resulting mutants were determined. The results revealed that the colistin MIC for A4528 crB(N141I) ΔH239_3064 was 8-fold lower than that for A4528 crB(N141I) (Table 2). Deletion of the H239_3063 locus in A4528 crB(N141I) yielded a nominal but non-significant increase in susceptibility to colistin (Table 2). Double deletion of the
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) D 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.

H239_3063 and H239_3065 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).

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) ΔH239_3063 | 1024                  |
| A4528 crrB(N141I) ΔH239_3064 | 256                  |
| A4528 crrB(N141I) ΔH239_3065 | 2048                  |
| A4528 crrB(N141I) ΔH239_3063-H239_3064 | 128                  |
| A4528 crrB(N141I) ΔH239_3064/plac b | 256                  |
| A4528 crrB(N141I) ΔH239_3064/plac-H239_3064 c | 2048                  |
| A4528 WT/plac b         | 1                        |
| A4528 WT/plac-H239_3064 c | 4                      |
| ATCC 25922 d            | 1                        |

aSusceptibilities to antibiotics were determined from independent tripli-cate experiments.

bThe plasmid plac is described in the Materials and methods section.

cThe coding region of H239_3064 was cloned into the plac plasmid and resulted in the plac-H239_3064 plasmid.

dThe MIC for the E. coli ATCC 25922 strain was determined in parallel, serving as quality control.

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, ceftaxime 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 ceftaxime (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) ΔH239_3064 after re-energization of bacteria (Figure 4). Significantly, ethidium bromide accumulation of A4528 crrB(N141I) ΔH239_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. 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. 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. 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. However, the remaining loci could not be systematically classified, and further studies will be needed to define how these loci influence colistin resistance.

Notably, the crr operon (crr, H239_3063, H239_3064 and H239_3065) was also absent from the genome of the standard NTUH-K2044 strain (NCBI reference sequence NC_012731.1).

**Table 3. Susceptibilities of A4528-derived strains to antibiotics**

| Strain                        | MIC (mg/L)         |
|-------------------------------|--------------------|
|                              | chloramphenicol    | ciprofloxacin | tetracycline | ceftaxime | tigecycline |
| A4528 WT                      | 4                  | 0.03125       | 1           | 0.0625    | 1          |
| A4528 crrB(N141I)             | 4                  | 0.03125       | 2           | 0.0625    | 2          |
| A4528 crrB(N141I) ΔH239_3064  | 4                  | 0.03125       | 1           | 0.0625    | 1          |
| A4528 crrB(N141I) ΔH239_3064/plac | NA                  | NA           | 1           | NA        | 1          |
| A4528 crrB(N141I) ΔH239_3064/H239_3064 | NA                  | NA           | 2           | NA        | 2          |

NA, not available.

*Susceptibilities to antibiotics were determined from independent triplicate experiments.

The plasmid plac is described in the Materials and methods section.

The coding region of H239_3064 was cloned into the plac plasmid and resulted in the plac-H239_3064 plasmid.

![Figure 4.](image-url)
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. Therefore, prevalence of the crrAB and crrC operon might be related to genetic evolution, since the genomic sequences of these two types are close.

The encoded protein of H239_3064 shares 49% amino acid identity with K. pneumoniae AcrB, a known efflux pump. 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.

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.

**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.

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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.

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