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Multifaceted mechanisms of colistin resistance revealed by genomic analysis of multidrug-resistant *Klebsiella pneumoniae* isolates from individual patients before and after colistin treatment ☆

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SUMMARY

Objectives: Polymyxins (i.e., polymyxin B and colistin) are used as a last-line therapy to combat multidrug-resistant (MDR) *Klebsiella pneumoniae*. Worryingly, polymyxin resistance in *K. pneumoniae* is increasingly reported worldwide. This study identified the genetic variations responsible for high-level colistin resistance in MDR *K. pneumoniae* clinical isolates.

Methods: Sixteen MDR *K. pneumoniae* isolates were obtained from stool samples of 8 patients before and after colistin treatment. Their genomes were sequenced on Illumina MiSeq to determine genetic variations.

Results: Fifteen of 16 isolates harboured IS*Kpn26*-like element insertion at nucleotide position 75 of *mgrB*, abolishing its negative regulation on *phoPQ*; while colistin-susceptible ATH7 contained intact *mgrB* and *phoQ*. Interestingly, each of the 7 *mgrB*-disrupted, colistin-susceptible isolates contained a nonsynonymous substitution in PhoQ (G39S, L239P, N253T or V446G), potentially impairing its function and intergenically suppressing the effect caused by *mgrB* inactivation. Additionally, three of the 7 corresponding *mgrB*-disrupted, colistin-resistant isolates harboured a secondary nonsynonymous substitution in PhoQ (N253P, D438H or T439P).

Conclusions: This is the first report of *phoQ* mutations in *mgrB*-disrupted, colistin-susceptible *K. pneumoniae* clinical isolates. We also discovered multiple *phoQ* mutations in *mgrB*-disrupted, colistin-resistant strains. Our findings highlight the multifaceted molecular mechanisms of colistin resistance in *K. pneumoniae*.

Introduction

Multidrug-resistant (MDR) *Klebsiella pneumoniae* is a severe threat to human health worldwide and urgently requires novel treatment options.[1] *K. pneumoniae* is a major cause of hospital-acquired infections including pneumonia, bloodstream infections, and infections in newborns and critically-ill patients.[2–4] The increasing reports of MDR *K. pneumoniae* isolates that are resistant to all classes of antibiotics, including the last-line polymyxins, are most disconcerting.[3,5–7]

Polymyxins were originally discovered in the 1940s and abandoned in the 1970s due to potential nephrotoxicity and neurotoxicity; however, over the last decade they have been revived as a last resort to treat severe infections caused by Gram-negative 'superbugs'.[8–10] Of the major polymyxin families (e.g., polymyxins A to E), only polymyxin B and colistin (i.e., polymyxin E) are used in the clinic.[10] Polymyxins are a class of polycationic lipopeptide antibiotics with a narrow spectrum of activity against Gram-negative bacteria.[11] The exact mode of action of polymyxins is not clear, but involves initial electrostatic and hydrophobic interactions between the cationic polymyxin molecule and the anionic lipid A

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of lipopolysaccharides (LPS) in bacterial outer membrane (OM), followed by the replacement of divalent cationic ions (e.g., Mg^{2+} and Ca^{2+}), disorganisation of cell envelope, cellular component leakage, and eventually cell death.[12] Alternative mechanisms were also reported, including inhibition of membrane-bound type II NADH-quinone oxidoreductase and generation of cytotoxic hydroxyl radicals.[13,14]

In *K. pneumoniae*, polymyxin resistance is mostly mediated by modifications of lipid A with 4-amino-4-deoxy-L-arabinose (L-Ara4N, *arnBCADTEF-ugd* loci) and/or phosphoethanolamine (pEtN, *pmrC* or plasmid-borne *mcr-1*).[15–17] The chromosomally encoded lipid A modifications can be induced by several two-component systems (TCSs), including PhoPQ and PmrAB.[18,19] MgrB is a small transmembrane protein and exerts negative feedback regulation on *phoPQ*.[20] Loss-of-function mutations of *mgrB* invoked constitutive induction of *phoPQ*, thereby upregulating the lipid A modification and conferring polymyxin resistance.[20–26] Moreover, *mgrB* mutations were not necessarily associated with a significant fitness cost and could be stably maintained in the absence of selective pressure.[27] Interestingly, a recent study showed the presence of partial suppressor mutations of *phoQ* (N253T and V446G) in *mgrB*-disrupted, colistin-resistant *K. pneumoniae* clinical isolates, indicating that these additional *phoQ* mutations could constitute a fitness advantage.[23] However, it is unclear whether these varations are prevalent in *mgrB*-inactivated MDR *K. pneumoniae* clinical isolates.

In this study, we report the comparative genomic analysis of the 16 colistin-susceptible and -resistant carbapenemase-producing *K. pneumoniae* isolates obtained from 8 patients before and after colistin treatment. The results provide key insights into the acquisition of polymyxin resistance in this problematic 'superbug'.

Materials and methods

Bacterial strains, media and antimicrobial susceptibility test

Sixteen isolates were obtained from stool samples of eight patients with an infection of carbapenemase-producing *K. pneumoniae* before and after colistin treatment in Athens, Greece. Antimicrobial compounds tested in this study are listed in Table S1. Colistin MICs were determined using broth microdilution according to the Clinical and Laboratory Standards Institute guideline.[28]

Genome sequencing and assembly

Bacterial genomic DNA was extracted from log-phase cultures grown in cation-adjusted Muller–Hinton broth (CAMHB, Oxoid) using a DNeasy Blood and Tissue Kit (QIAGEN, Dusseldorf, Germany). Electrophoresis and Qubit (Life Technologies, USA) were used to assess the quality and quantity of DNA samples, respectively. DNA libraries were constructed using a Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, USA), and indexed with TruSeq Dual Indexing Reagent (Illumina). Single-end 75 bp DNA sequencing was performed on an Illumina MiSeq (Micromon, Clayton, Australia). Quality-trimmed reads were employed for *de novo* genome assembly with SPAdes,[29,30] and the results were evaluated using QUAST 4.5.[31] With Mauve Contigs Mover,[32] the assembled contigs were ordered with complete reference genomes of TGH13 and AUSMDU00008079, the two closest isolates based on phylogenetic analysis (below). Genome annotation was conducted with the ordered contigs using Prokka,[33] and insertion sequences (ISs) were predicted using ISEScan.[34] The assembly of each *K. pneumoniae* genome was uploaded to the Resistance Gene Identifier (RGI) 3.2.1 web portal from the Comprehensive Antimicrobial Resistance Database (CARD) to predict antimicrobial resistance genes using Strict algorithm.[35] Specifically, the Strict algorithm detected the homologs of the known antimicrobial resistance genes in CARD database.[35] For each genome, the predicted antimicrobial resistance genes were grouped according to the Drug Class categorisation. Heatmap was generated to show the presence and absence of classes of antimicrobial resistance genes. IslandViewer 4 was employed to predict genomic islands.[36]

Sequence typing and phylogenetic analysis

The 16 assemblies and 143 *K. pneumoniae* complete genomes from GenBank (Table S2) were submitted to Multilocus Sequencing Typing (MLST) database to determine sequence types (STs),[37] and were compared to determine the core genome using Harvest tool.[38] The derived core genome alignment was employed to estimate Maximum-Likelihood (ML) tree using RAxML 8.2.9 with GTRGAMMA model (150 bootstrap replicates, MRE-based bootstrapping criterion).[39] The tree was visualised using iTOL v4.4.2.[40]

Genetic variation detection

For each of the 16 isolates, quality-trimmed reads were aligned to the reference (TGH13 or AUSMDU00008079) using SubRead.[41] Nesoni was used to identify, filter and annotate single nucleotide polymorphism (SNP).[42] Specifically, SNPs were identified using a diploid analysis with the predominant variants showing in $\geq$ 66.7% of the mapped reads and having a quality score $>$ 20. Structure variations were initially identified using GRIDSS (quality score $\geq$ 1,000 and having assemblies from both sides of the breakpoint) with the reference genomes and further checked by Artemis bamview.[43,44] Gene presence/absence variation was analysed using Roary with a sequence identity of $\geq$ 99%.[45] Mutations in *mgrB* and *phoQ* were validated by PCR amplification and Sanger sequencing using the primers in Table S3. PROVEAN (PRotein Variation Effect ANalyzer) was employed to predict the function impact of an amino acid substitution in PhoQ.[46]

Results

Antimicrobial susceptibility

For 36 antibiotics of 14 major classes, 15 isolates showed resistance to 35 antibiotics except ATH21, and were thus classified as extensively drug-resistant (XDR) according to the breakpoints from the European Committee on Antimicrobial Susceptibility Testing (Table S1).[47] Strain ATH21 was susceptible to imipenem (MIC = 0.25 mg/L) and gentamicin (MIC = 1 mg/L). Our broth microdilution results showed that all the 8 colistin-susceptible strains had colistin MICs $\leq$ 0.5 mg/L; whereas the other 8 colistin-resistant isolates had colistin MICs $\geq$ 64 mg/L (Table 1), representing 128 to 1024-fold increase.

Genome sequencing and assembly

Genome sequencing of the 16 *K. pneumoniae* isolates yielded 2.85–3.10 million reads per sample, equivalent to 181–241 MB data per isolate (Table S4). After filtering, 2.08–2.78 million quality-trimmed reads were obtained for each sample with the length of 67–72 bp, representing approximately 30-fold coverage of the genomes (Table S4). *De novo* assembly yielded 238–399 contigs ($\geq$500 bp) for each isolate with the total length ranging from 5,364,335 to 5,748,458 bp, GC content in 56.9–57.3% and N50 of 33,800–66,326 bp (Table 2). Overall, 5,079–5,524 genes and 5,017–5,466 protein-encoding sequences were annotated from the 16 draft genomes (Table 2). The genomes were deposited in DDBJ/ENA/GenBank under the accession numbers shown in Table 2.

Table 1
MICs of polymyxin B and colistin against MDR *K. pneumoniae* isolates.

Patient	Strain	MIC (mg/L)	
		Colistin	Polymyxin B
A	ATH7	0.25	<0.125
	ATH8	>128	128
B	ATH9	0.25	0.5
	ATH10	64	32
C	ATH15	<0.125	<0.125
	ATH16	128	128
D	ATH17	<0.125	0.25
	ATH18	128	128
E	ATH21	0.25	0.5
	ATH22	64	32
F	ATH23	0.5	0.5
	ATH24	64	64
G	ATH25	0.5	0.5
	ATH26	128	64
H	ATH30	0.5	0.5
	ATH29	64	32

Phylogenetic analysis

MLST analysis with the 7 housekeeping genes *gapA, infB, mdh, pgi, phoE, rpoB* and *tonB* classified ATH9/ATH10 to ST147 and the other 14 isolates to ST258. A phylogenetic tree (Fig. 1) was constructed based on the core genome alignment using the 16 draft assemblies and 143 complete genomes from GenBank (Table S2). Strains TGH13 (Genbank Accession No. CP012745, ST147) and AUSMDU00008079 (GenBank Accession No. CP022691-CP022694, ST258) were phylogenetically closest to ATH9/ATH10 and the other 14 isolates, respectively (Fig. 1). Therefore, TGH13 and AUSMDU0008079 genomes were employed as references to identify genetic variations.

Mobile elements

Across the 16 isolates, 61 putative plasmids were assembled, with 1 (ATH10) to 11 (ATH18) plasmids per isolate and the plasmid size ranging from 1.1 to 200 Kb (Table S5). Seventeen plasmids were assigned to known incompatibility (Inc) groups including ColRNAI, IncFIB, IncFIC, IncFII, IncHI1B and IncX3; the most common Inc group was ColRNAI (detected using ColRNAI_DQ298019 probe) which was present in 6 colistin-susceptible and 5 colistin-resistant isolates.[48] Additionally, 392 insertion sequences (ISs) were predicted with the length ranging from 294 to 3,030 bp, representing 55–58 unique ISs from 20 families (Fig. 2 and Table S6). IS*NCY* accounted for 33.5% of the total ISs and was the most abundant (Fig. 2). Strains ATH10, ATH16 and ATH17 contained much less ISs (<10) than the others (Fig. 2). Strains ATH7/ATH8, ATH23/ATH24 and ATH30/ATH29 contained similar IS elements, indicating their close phylogenetic relationships.

Genomic content and resistance genes

Among the 16 isolates, 449 genes were predicted to confer resistance to different antimicrobial classes including fluoroquinolones (*gyrAB, parC* and *qnrA1*), fosfomycin (*uhpT* and *fosA6*), aminoglycosides [*aac(6′)-Ib10, aac(6′)-Ib7, aadA, aadA2, ant(2″)-Ia, aph(3′)-Ia, aph(3″)-Ib* and *aph(6)-Id*], chloramphenicol (*catI*), diaminopyrimidines (*dfrA12* and *dfrA14*), macrolides (*mphA* and *mrx*) and sulfonamides (*sul1* and *sul2*) (Fig. 3 and Table S7). Totally, 146 efflux pump genes (e.g., *emrB, marA, msbA, msrB, ompK37, oqxAB, patA* and *vgaC*) were identified in the 16 isolates, and likely rendered the isolates resistant to a broad range of antibiotics; 80 regulators (e.g., *emrR, hns, baeR*) were discovered and assumed to regulate the expression of efflux pump genes. Notably, all the 16 genomes harboured β-lactamase genes, including $bla_{\text{KPC-2}}$, $bla_{\text{SHV-11}}$, $bla_{\text{SHV-5}}$, $bla_{\text{TEM-1}}$ and $bla_{\text{VIM-1}}$. In addition, 36–60 genomic island fragments per isolate were predicted, with the length ranging from 4.0 to 386.9 Kb (Table S8). Among them, 104 island fragments (12.4% of 840 island fragments in the 16 isolates) harboured a total of 203 resistance genes (3–17 resistance genes per isolate), conferring resistance to β-lactams, aminoglycosides, chloramphenicol, and diaminopyrimidines.

Genetic variations in colistin-susceptible and -resistant isolates from the same patients

By mapping the reads to their corresponding reference genomes, 2,762 SNPs were identified among the 16 isolates with 120–195 SNPs per isolate. Totally, 303 (10.9%) SNPs were exclusively discovered in either colistin-susceptible or -resistant isolates; among them, 266 SNPs were present in 100% of the sequencing reads (Tables 3 and S9; Fig. 4). Interestingly, 186 unique SNPs were identified between ATH25 and ATH26, indicating their significant genetic discrepancies (Table S9). Strains ATH7 and ATH21 contained a stop-gain (E132Stop) and a frameshift variation (I115fs) in *ramR*, respectively; both might abolish its transcriptional repression on the downstream regulator *ramA* and potentially induced

Table 2
Draft assemblies of the 16 *K. pneumoniae* isolates.

Isolate	Accession No.	Assembly size (bp)	No. of contigs (≥500 bp)	GC content (%)	No. of plasmids	No. of genes	MLST type
ATH7	VJXP00000000	5,708,072	287	57.04	6	5,485	ST258
ATH8	VJXO00000000	5,710,068	320	57.03	4	5,484	ST258
ATH9	VJXN00000000	5,446,905	334	57.30	1	5,155	ST147
ATH10	VJXM00000000	5,364,335	258	57.30	1	5,079	ST147
ATH15	VJXL00000000	5,681,214	334	57.08	3	5,439	ST258
ATH16	VJXK00000000	5,682,327	335	57.08	2	5,443	ST258
ATH17	VJXJ00000000	5,716,616	376	56.94	5	5,492	ST258
ATH18	VJXI00000000	5,713,722	399	56.95	11	5,486	ST258
ATH21	VJXH00000000	5,521,083	327	57.20	2	5,272	ST258
ATH22	VJXG00000000	5,633,725	285	57.12	4	5,368	ST258
ATH23	VJXF00000000	5,605,330	238	57.11	4	5,351	ST258
ATH24	VJXE00000000	5,604,784	256	57.11	3	5,359	ST258
ATH25	VJXD00000000	5,748,458	329	57.02	6	5,524	ST258
ATH26	VJXC00000000	5,631,750	305	57.10	3	5,411	ST258
ATH29	VJXA00000000	5,600,153	307	57.11	3	5,349	ST258
ATH30	VJXB00000000	5,600,123	264	57.11	3	5,348	ST258

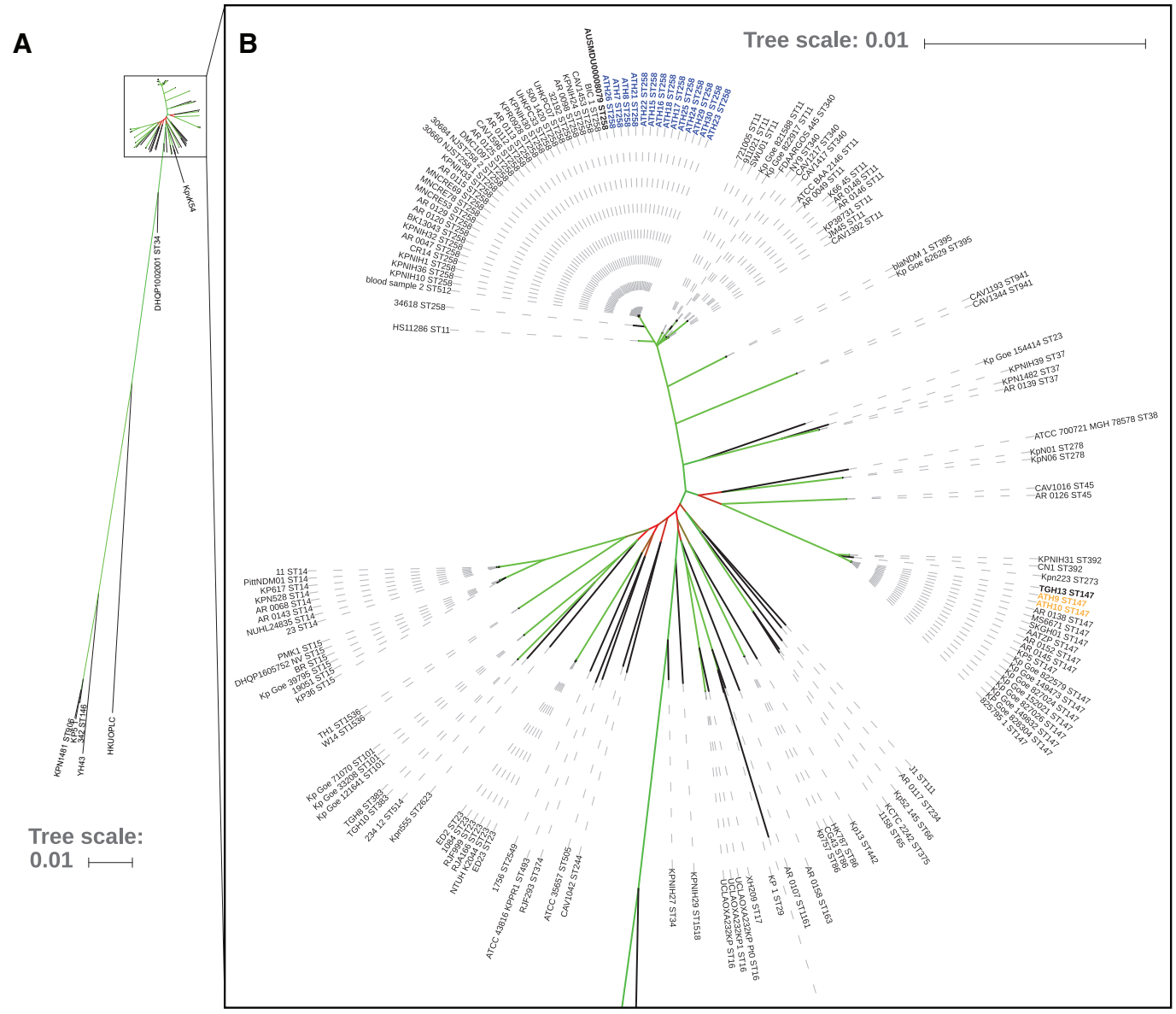

Fig. 1. ML-based phylogeny of *K. pneumoniae*, containing the 16 isolates and 143 isolates with complete genomes (A). Bootstrap values (percentage) are indicated by a colour scale from red (1%) to green (100%). Specifically, the closely related *K. pneumoniae* genomes are shown in (B). The ST147 isolates ATH9 and ATH10 are highlighted in orange; and the 14 ST258 isolates are in blue. Reference strains for genetic variation analysis are in bold. The sequence type is shown after the isolate name for each genome. Scale bars in (A) and (B) indicate the number of substitutions per nucleotide site.

the expression of AcrAB efflux pump, thereby rendering strains resistant to tigecycline.[49]

Overall, 102 DNA breakpoints were predicted in 16 isolates, with 4–10 per isolate (Table S10). Comparative genomic analysis determined a core genome consisting 4,224 genes (≥99% identity in amino acid sequence), and a pan genome of totally 6,708 genes (Table S11). ATH9 and ATH10 showed significant differences from the other isolates in genome content, consistent with their distinct sequence type classification (ST147, Fig S1).

Genetic variations associated with colistin susceptibility and resistance

Single nucleotide variations were identified in the genes that are associated with colistin resistance in *K. pneumoniae*, including TCSs and lipid A modification genes (Table 4). The ST147 isolates ATH9 and ATH10 contained a unique missense variation (T140P) in *pmrB* compared to their reference TGH13. All 14 ST258 isolates contained a V53G variation in *phoP* compared to the reference AUSMDU00007089; whereas the two ST147 isolates ATH9 and ATH10 possessed an intact *phoP* compared to the reference TGH13. Missense variations of *phoQ* were identified in 10 of 16 isolates (Table 4 and Fig. 5). In 4 colistin-susceptible isolates ATH9, ATH23, ATH25 and ATH30, single missense variations (G39S, V446G or L239P) were identified in *phoQ*; while the corresponding colistin-resistant isolates (ATH10, ATH24, ATH26 and ATH29) obtained from the same patients after colistin treatment possessed an intact *phoQ* (Table 4). The other three colistin-susceptible ST258 isolates (ATH15, ATH17 and ATH21) shared a common missense variation $phoQ^{N253T}$ that was caused by a nucleotide substitution (758A>C). Their corresponding colistin-resistant isolates had either an additional missense variation (D438H in ATH16 and T439P in ATH18), or a variation (N253P in ATH22) caused by dinucleotide substitution (757_758delAAinsCC) in *phoQ*. This dinucleotide

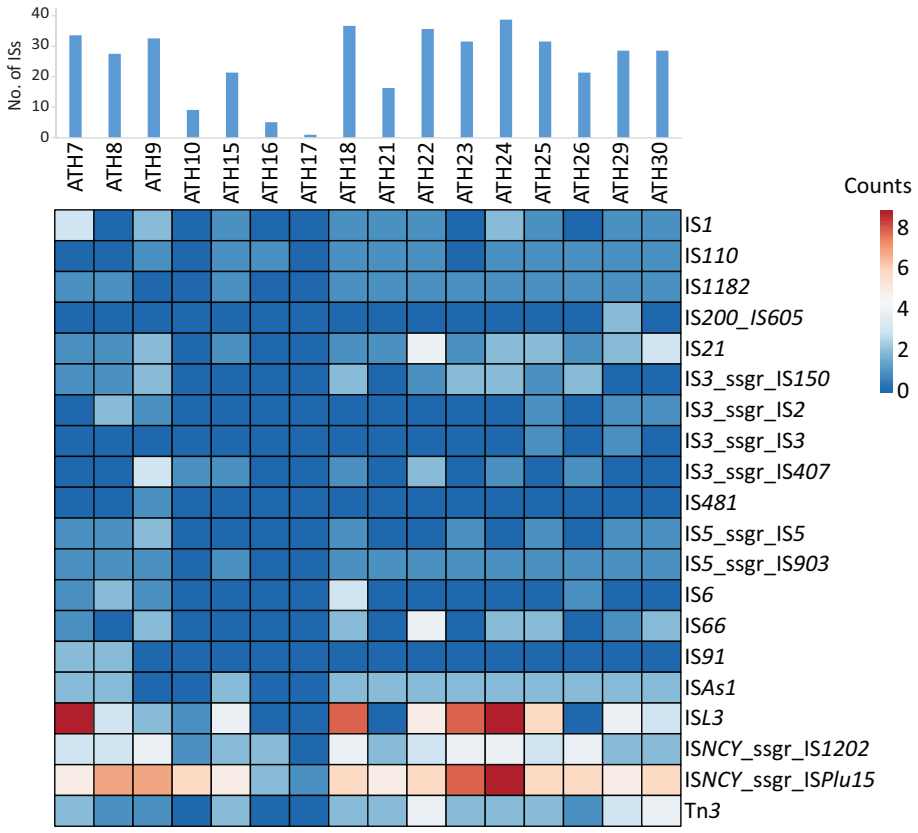

Fig. 2. Predicted insertion sequences in the 16 *K. pneumoniae* isolates.

Table 3
SNPs identified by comparative genomic analysis.

Isolate	Total No. of SNPs compared to the reference genome	No. of unique SNPs	No. of SNPs between colistin-susceptible and -resistant strains
ATH7	121	8	15
ATH8	120	7	
ATH9	157	5	14
ATH10	161	9	
ATH15	193	6	13
ATH16	194	7	
ATH17	188	4	15
ATH18	195	11	
ATH21	159	19	32
ATH22	153	13	
ATH23	188	5	10
ATH24	188	5	
ATH25	179	89	186
ATH26	187	97	
ATH30	188	6	18
ATH29	191	12	

substitution occurred very likely via acquiring a second nucleotide substitution (757A>C) in the same codon (AAC for Asn253) of *phoQ*. In addition, a premature mutation (E307Stop) was discovered in *pmrC* of ATH9 and ATH10, indicating that both were unable to modify lipid A with pEtN. Strain ATH21 contained a specific missense variation (R306S) in *arnB* (UDP-4-amino-4-deoxy-L-arabinose:oxoglutarate aminotransferase) and an in-frame deletion (E176del) in *ugd* (UDP-glucose 6-dehydrogenase). Both genes belonged to the *arnBCADTEF-ugd* loci that are responsible for lipid A modification with L-Ara4N.[20] Both ST147 isolates ATH9 and ATH10 shared a common variation in the *arn* promoter (−58A>G). Isolate ATH7 contained an S204Y variation in the LPS heptosyltransferase gene *rfaQ* (Table S9).

Very surprisingly, 15 *K. pneumoniae* isolates exhibited integration of an IS element in *mgrB* with the only exception of ATH7 (Table 4 and Fig. 5). Further PCR amplification and Sanger sequencing confirmed that the insertion occurred at the 75 nt position of *mgrB* in the forward direction among 13 isolates, the same position but in the reverse direction in ATH9 and ATH10. The 1,196-bp IS element discovered in this study belonged to IS*5* family and showed 99% nucleotide identity with IS*Kpn26*, therefor designated IS*Kpn26*-like element.[50]

Discussion

In this study, we undertook comparative genomic analysis with 16 colistin-susceptible and -resistant clinical isolates and identified the genetic variations potentially responsible for high-level colistin resistance in *K. pneumoniae*. The *K. pneumoniae* ST258 group is an important cause of MDR hospital outbreaks,[51] while ST147 is a newly emerged human-related *K. pneumoniae* sequence type and also associated with MDR outbreaks.[52,53] Among the 16 isolates, 14 were classified as ST258, whereas ATH9 and ATH10 were grouped to ST147 (Table 2). Consistent with their XDR profiles, the assembled genomes harboured a variety of antibiotic resistance genes (Table S7 and Fig. 3). Plasmids, ISs and genomic islands were predicted and highly likely they contributed to the acquisition of MDR (Tables S5, S6 and S8; Fig. 2). With TGH13 and AUSMDU00008079 genomes as references, SNPs, gene presence/absence variations, and structural variations were identified in 16 isolates (Tables 3 and S9–11; Figs. 4 and S1). Key genetic variations associated with polymyxin resistance were identified, including an IS*Kpn26*-like element insertion in *mgrB* at 75-nt and several nonsynonymous variations in *phoQ, pmrC, arnB* and *ugd* (Table 4 and Fig. 5).

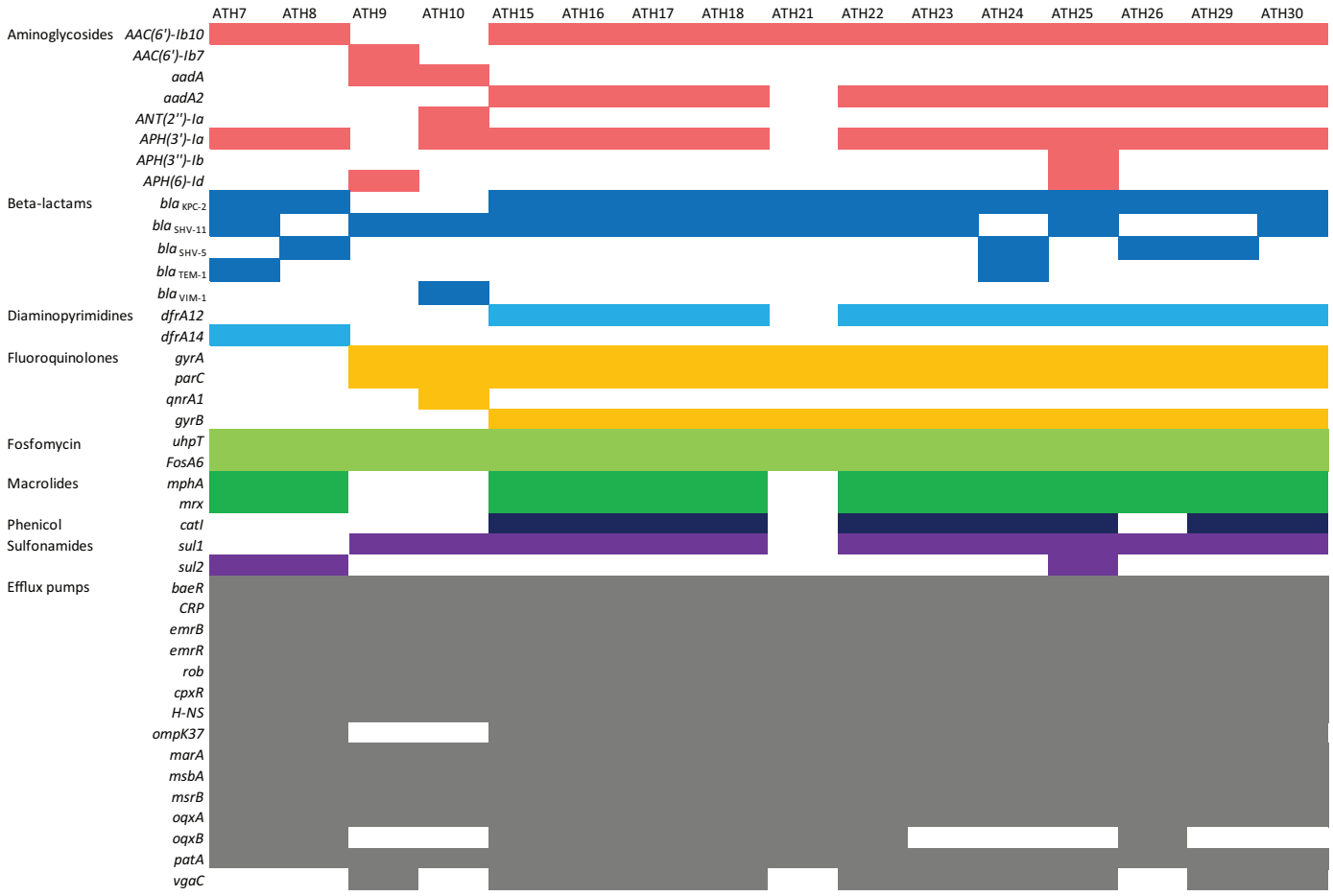

Fig. 3. The absence (blank) and presence (colour) of the predicted antibiotic resistance genes in the 16 *K. pneumoniae* isolates.

K. pneumoniae commonly evolves colistin resistance via pEtN and L-Ara4N modifications on the lipid A component of LPS.[15–17] The expression of relevant genes (*pmrC* and *arn* operon) are controlled by TCSs PmrAB and PhoPQ.[18,20] Environmental stimuli (e.g. high concentrations of ferric iron for PmrB, low concentrations of divalent cationic ions, and presence of cationic antimicrobial peptides for PhoQ) can trigger the auto-phosphorylation of membrane-bound kinases PmrB and PhoQ, which in turn activate the cognate regulators PmrA and PhoP by phosphorylation, respectively.[54] In *K. pneumoniae*, the activated PhoP promotes the transcription of a large number of genes, including host adaptation genes, *arn* operon and connecter *pmrD*.[18,55,56] PmrD in turn binds to phosphorylated PmrA, and thereafter leading to persistent expression of PmrA-activated genes (e.g., *pmrCAB, ugd*).[18,55,56] Mutations in *pmrAB* and *phoPQ* can render *K. pneumoniae* resistant to polymyxins.[18,22] In our study, all the colistin-susceptible and their corresponding colistin-resistant isolates had identical *pmrAB* and *phoP*, indicating that they might not contribute to polymyxin resistance. Whereas the genetic variations in *phoQ* were identified in 7 colistin-susceptible and 3 colistin-resistant isolates (Table 4), suggesting that the *phoQ* mutations may not always play a role in acquisition of polymyxin resistance. Specifically, as shown in Fig. 5, the 7 colistin-susceptible isolates (ATH9, ATH15, ATH17, ATH21, ATH23, ATH25 and ATH30) had a mutated *phoQ* compared to their reference genomes, while the corresponding colistin-resistant isolates obtained from the same patients after colistin treatment contained either an intact (ATH10, ATH24, ATH26 and ATH29) or a secondary mutation in *phoQ* (ATH16, ATH18 and ATH22). Our results indicates that certain genetic variations in *phoQ* may not contribute to colistin resistance, while other genetic variations may lead to high-level colistin resistance.

Interestingly, apart from *phoQ* variations, in 15 isolates we identified the insertion of the IS*Kpn26*-like element at 75-nt of *mgrB* either in the forward (13 isolates) or reverse direction (2 isolates) (Table 4). MgrB is a small membrane protein with only 47 amino acids and is a negative regulator of PhoPQ (Fig. 5).[22] Previous studies showed that inactivation of *mgrB* resulted in the upregulation of PhoPQ, which in turn induced lipid A modifications and high-level polymyxin resistance in *K. pneumoniae*.[21,22] Epidemiologically, *mgrB* inactivation (e.g., non-synonymous mutations, insertion, deletion and integration with IS element) is widely reported in carbapenemase-producing *K. pneumoniae*.[7,15,21,23,26,57] These 8 'pairs' of isolates are not isogenic strains that are commonly used in molecular microbiological studies, but were collected before and after colistin treatment in individual patients. Together, our results showed that the mechanisms of colistin resistance in the 8 isolates (ATH8, ATH10, ATH16, ATH18, ATH22, ATH24, ATH26 and ATH29) were mainly mediated by (i) inactivation of *mgrB* only (ATH8, ATH10, ATH24, ATH26 and ATH29), and (ii) inactivation of *mgrB* plus second-site mutations in *phoQ* (ATH16, ATH18 and ATH22) as shown in Table 4. Previous studies showed *mgrB* disruption (*mgrB*::N25IS*Kpn26*-like) caused polymyxin resistance in ST258 Greek isolates.[23] Missense variations of *phoQ* (N253T, V446G, L239P and G39S) were identified in *mgrB*-disrupted isolates (ATH9, ATH15, ATH17, ATH21, ATH23, ATH25 and ATH30) (Table 4), which potentially rendered these strains susceptible to polymyxins. Specifically, as shown in Fig 5, these *phoQ* SNPs occurred in the transmembrane domain (G39S), HAMP domain (present

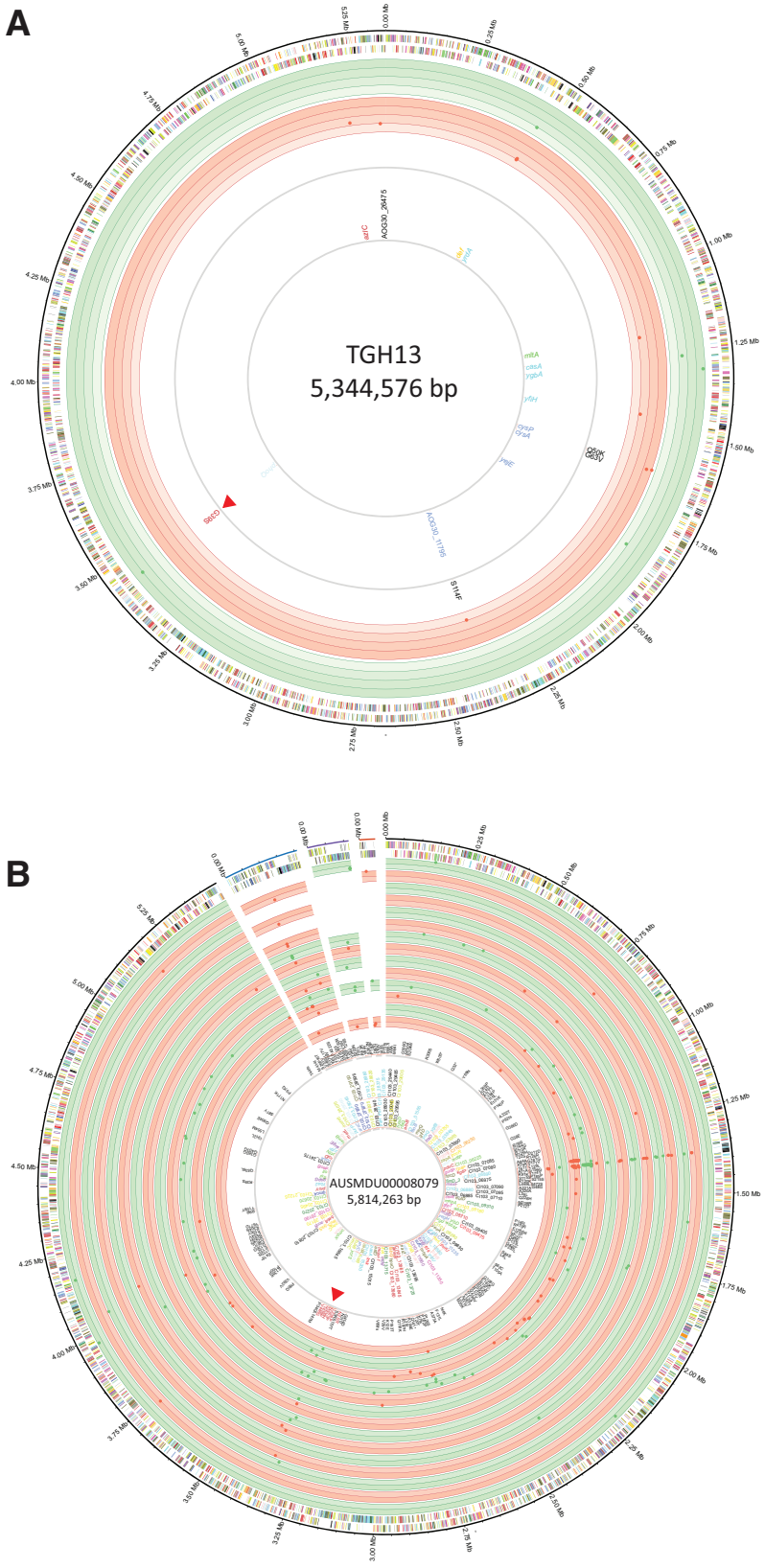

Fig. 4. Unique nonsynonymous SNPs of the 16 *K. pneumoniae* isolates. The circular tracks, from outermost to innermost, represent the genes on positive and negative strands of the reference genomes, SNPs in colistin-susceptible (green) and -resistant isolates (red), amino acid substitutions, and the affected genes. The genes are colour-coded according to the predicted Clusters of Orthologous Groups (COG) functional categories of reference genomes using eggNOG tool.[64] For SNP tracks, from outermost to innermost they are ATH9 and ATH10 in (A), and ATH7, ATH8, ATH15, ATH16, ATH17, ATH18, ATH21, ATH22, ATH23, ATH24, ATH25, ATH26, ATH 30 and ATH29 in (B); in each track, the SNPs are denoted by solid circles according to their quality score, with a range of 20–100 (from the lightest to darkest shading). The *phoQ* SNPs are highlighted by red arrows. Within 1.35–1.40 Mb (B), 73 SNPs were identified mostly in CI103_06880 (integrase) and CI103_07080–7095 (hypothetical proteins). No SNP track is depicted in (B) if there is no SNPs on reference plasmids.

Table 4
Genetic variations contributing to colistin resistance.

Isolate	Colistin susceptibility	Category of resistance mechanism	*mgrB*	*pmrB*	*phoP*	*phoQ*	*pmrC*	*arnB*	*ugd*
			CI103_10160/ AOG30_10755[a]	CI103_03610/ AOG30_18810	CI103_16945/ AOG30_17125	CI103_16950/ AOG30_17130	CI103_18860/ AOG30_18800	CI103_01375/ AOG30_01390	CI103_08920/ AOG30_09695
ATH7	S				V53G (158T>G[b])				
ATH8	R	I	*mgrB*::N25IS*Kpn26*-like		V53G (158T>G)				
ATH15	S		*mgrB*::N25IS*Kpn26*-like		V53G (158T>G)	N253T (758A>C)			
ATH16	R	II	*mgrB*::N25IS*Kpn26*-like		V53G (158T>G)	N253T (758A>C), D438H (1312G>C)			
ATH17	S		*mgrB*::N25IS*Kpn26*-like		V53G (158T>G)	N253T (758A>C)			
ATH18	R	II	*mgrB*::N25IS*Kpn26*-like		V53G (158T>G)	N253T (758A>C), T439P (1315A>C)			
ATH21	S		*mgrB*::N25IS*Kpn26*-like		V53G (158T>G)	N253T (758A>C)		R306S (916C>A)	E176Del (527_529delAAG)
ATH22	R	II	*mgrB*::N25IS*Kpn26*-like		V53G (158T>G)	N253P (757_758delAAinsCC)			
ATH23	S		*mgrB*::N25IS*Kpn26*-like		V53G (158T>G)	V446G (1337T>G)			
ATH24	R	I	*mgrB*::N25IS*Kpn26*-like		V53G (158T>G)				
ATH25	S		*mgrB*::N25IS*Kpn26*-like		V53G (158T>G)	L239P (716T>C)			
ATH26	R	I	*mgrB*::N25IS*Kpn26*-like		V53G (158T>G)				
ATH30	S		*mgrB*::N25IS*Kpn26*-like		V53G (158T>G)	V446G (1337T>G)			
ATH29	R	I	*mgrB*::N25IS*Kpn26*-like		V53G (158T>G)				
ATH9	S		*mgrB*::N25IS*Kpn26*-like	T140P (418A>C)	−38G>T	G39S (115G>A)	E307Stop (919G>T)	−58A>G	
ATH10	R	I	*mgrB*::N25IS*Kpn26*-like	T140P (418A>C)	−38G>T		E307Stop (919G>T)	−58A>G	

[a] Loci in AUSMDU0008079/TGH13 genome.
[b] Nucleotide variations.

in histidine kinases, adenylate cyclases, methyl accepting proteins and phosphatases, N253T and L239P) and histidine kinase domain (V446G). Among them, N253T and V446G were previously discovered along with *mgrB* disruption in colistin-resistant *K. pneumoniae* clinical isolates.[23] Individually complementation of mutant *phoQ*^{N253T} or *phoQ*^{V446G} in *mgrB*-disrupted isolate reduced the polymyxin MICs, suggesting that N253T and V446G might exert partial suppressor effect on *mgrB* inactivation.[23] Previous structural analysis also showed that substitution of N253 by a residue with a shorter side chain (e.g., Thr in the present study) would decrease PhoQ activity.[58] Therefore, the *phoQ* mutations (N253T, V446G, L239P and G39S) identified in our study might cause dysfunction of PhoQ and stopped the downstream events on lipid A modifications, and therefore the *mgrB*-disruption did not cause colistin resistance in these susceptible isolates (i.e., ATH9, ATH15, ATH17, ATH21, ATH23, ATH25 and ATH30). This is the first report of intergenic suppressions in *mgrB*-disrupted, colistin-susceptible, carbapenemase-producing MDR *K. pneumoniae* clinical isolates.

Previous studies showed that *mgrB* disruption is not necessarily associated with significant fitness cost.[27,59,60] The induction of *phoPQ* expression via *mgrB* disruption not only confers *K. pneumoniae* resistance to polymyxins and cationic antimicrobial peptides, but also enhances virulence by attenuating the host defence response activation.[59,60] Interestingly, a few other studies reported reduced virulence,[61] loss of hypermucoviscosity,[62] and declined growth in *mgrB*-disrupted *K. pneumoniae* strains,[63] indicating that the fitness cost and benefits of *mgrB* inactivation are strain-dependent. In our colistin-susceptible strains, *mgrB* disruption was accompanied with *phoQ* mutations, suggesting that *mgrB* disruption alone might result in certain fitness cost during host adaptation and *K. pneumoniae* may retain its fitness by acquiring function-impairing *phoQ* mutations. Whereas under colistin selection pressure, the PhoQ function in 3 isolates (ATH16, ATH18 and ATH22) could be 'restored' by the second-site mutations (D438H, T439P and N253P [caused by second nucleotide substitution 757A>C following 758A>C]), thereby leading to lipid A modifications and colistin resistance. Other genetic variations may also contribute to the suppression effect on *mgrB*-disruption in colistin-susceptible isolates. In colistin-susceptible ATH21, the *arnB*^{R306S} and *ugd*^{E176del} might abolish lipid A modification with L-Ara4N (Table 4). In colistin-susceptible isolate ATH7, the *rfaQ*^{S204Y} variation might affect the heptosyltransferase activity and then impair lipid A - core oligosaccharide biosynthesis (Table S9). In contrast, these variations were absent in the colistin-resistant isolates ATH22 and ATH8. Genetic variations of *pmrB*^{T140P}, *pmrC*^{E307Stop}, *phoP*^{V53G} and −58A>G in *arnB* promoter were also identified in both colistin-susceptible and -resistant isolates (Table 4), indicating that they are irrelevant to colistin resistance.

In summary, key genetic variations were identified in 16 colistin-susceptible and -resistant MDR *K. pneumoniae* clinical isolates by genome sequencing and comparative genomic analyses. These variations involved an IS*Kpn26*-like element insertion in *mgrB* (at nucleotide position 75) and 7 missense mutations in *phoQ* (G39S, L239P, N253T, N253P, D438H, T439P and V446G). Importantly, this is the first report of *phoQ* mutations accompanied with disrupted-*mgrB* in colistin-susceptible *K. pneumoniae*

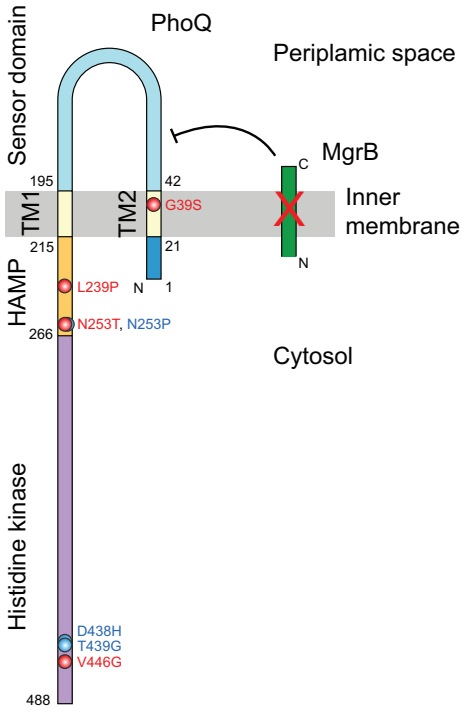

Fig. 5. Mutations in PhoQ and MgrB. The *mgrB* inactivation (via I*SKpn26*-like integration) is denoted by red '×'. The nonsynonymous SNPs in *phoQ* are indicated by blue (identified exclusively in colistin-resistant strains) and red (the rest) dots. The function domains of PhoQ were assigned based on the literature.[65]

clinical isolates, and we also discovered secondary *phoQ* mutations in *mgrB*-disrupted, colistin-resistant strains. Our findings highlight the multifaceted molecular mechanisms of colistin resistance in *K. pneumoniae*.

Declaration of competing interest

The authors declare no conflict of interest.

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Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jinf.2019.07.009.

References

1. World Health Organization. *Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics* 2017. Available from: https://www.who.int/medicines/publications/WHO-PPL-Short_Summary_25Feb-ET_NM_WHO.pdf?ua=1.
2. Munoz-Price LS, Poirel L, Bonomo RA, Schwaber MJ, Daikos GL, Cormican M, et al. Clinical epidemiology of the global expansion of *Klebsiella pneumoniae* carbapenemases. *Lancet Infect Dis* 2013;**13**(9):785–96.
3. Pragasam AK, Shankar C, Veeraraghavan B, Biswas I, Nabarro LE, Inbanathan FY, et al. Molecular mechanisms of colistin resistance in *Klebsiella pneumoniae* causing bacteremia from India—a first report. *Front Microbiol* 2016;**7**:2135.
4. Brooks B, Olm MR, Firek BA, Baker R, Thomas BC, Morowitz MJ, et al. Strain-resolved analysis of hospital rooms and infants reveals overlap between the human and room microbiome. *Nat Commun* 2017;**8**(1):1814.
5. Navon-Venezia S, Kondratyeva K, Carattoli A. *Klebsiella pneumoniae*: a major worldwide source and shuttle for antibiotic resistance. *FEMS Microbiol Rev* 2017;**41**(3):252–75.
6. Rojas LJ, Salim M, Cober E, Richter SS, Perez F, Salata RA, et al. Colistin resistance in carbapenem-resistant *Klebsiella pneumoniae*: laboratory detection and impact on mortality. *Clin Infect Dis* 2017;**64**(6):711–18.
7. Cheng YH, Lin TL, Pan YJ, Wang YP, Lin YT, Wang JT. Colistin resistance mechanisms in *Klebsiella pneumoniae* strains from Taiwan. *Antimicrob Agents Chemother* 2015;**59**(5):2909–13.
8. Velkov T, Roberts KD, Nation RL, Thompson PE, Li J. Pharmacology of polymyxins: new insights into an 'old' class of antibiotics. *Future Microbiol* 2013;**8**(6):711–24.
9. Landman D, Georgescu C, Martin DA, Quale J. Polymyxins revisited. *Clin Microbiol Rev* 2008;**21**(3):449–65.
10. Li J, Nation RL, Turnidge JD, Milne RW, Coulthard K, Rayner CR, et al. Colistin: the re-emerging antibiotic for multidrug-resistant Gram-negative bacterial infections. *Lancet Infect Dis* 2006;**6**(9):589–601.
11. Velkov T, Roberts KD, Thompson PE, Li J. Polymyxins: a new hope in combating gram-negative superbugs. *Future Med Chem* 2016;**8**(10):1017–25.
12. Urakawa H, Yamada K, Komagoe K, Ando S, Oku H, Katsu T, et al. Structure–activity relationships of bacterial outer-membrane permeabilizers based on polymyxin B heptapeptides. *Bioorg Med Chem Lett* 2010;**20**(5):1771–5.
13. Deris ZZ, Akter J, Sivanesan S, Roberts KD, Thompson PE, Nation RL, et al. A secondary mode of action of polymyxins against Gram-negative bacteria involves the inhibition of NADH-quinone oxidoreductase activity. *J Antibiot (Tokyo)* 2014;**67**(2):147–51.
14. Sampson TR, Liu X, Schroeder MR, Kraft CS, Burd EM, Weiss DS. Rapid killing of *Acinetobacter baumannii* by polymyxins is mediated by a hydroxyl radical death pathway. *Antimicrob Agents Chemother* 2012;**56**(11):5642–9.
15. Wright MS, Suzuki Y, Jones MB, Marshall SH, Rudin SD, van Duin D, et al. Genomic and transcriptomic analyses of colistin-resistant clinical isolates of *Klebsiella pneumoniae* reveal multiple pathways of resistance. *Antimicrob Agents Chemother* 2015;**59**(1):536–43.
16. Liu YY, Wang Y, Walsh TR, Yi LX, Zhang R, Spencer J, et al. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. *Lancet Infect Dis* 2016;**16**(2):161–8.
17. Leung LM, Cooper VS, Rasko DA, Guo Q, Pacey MP, McElheny CL, et al. Structural modification of LPS in colistin-resistant, KPC-producing *Klebsiella pneumoniae*. *J Antimicrob Chemother.* 2017;**72**(11):3035–42.
18. 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.
19. Kim SY, Choi HJ, Ko KS. Differential expression of two-component systems, *pmrAB* and *phoPQ*, with different growth phases of *Klebsiella pneumoniae* in the presence or absence of colistin. *Curr Microbiol* 2014;**69**(1):37–41.
20. Olaitan AO, Morand S, Rolain JM. Mechanisms of polymyxin resistance: acquired and intrinsic resistance in bacteria. *Front Microbiol* 2014;**5**:643.
21. Cannatelli A, Giani T, D'Andrea MM, Di Pilato V, Arena F, Conte V, et al. MgrB inactivation is a common mechanism of colistin resistance in KPC-producing *Klebsiella pneumoniae* of clinical origin. *Antimicrob Agents Chemother* 2014;**58**(10):5696–703.
22. Cannatelli A, D'Andrea MM, Giani T, Di Pilato V, Arena F, Ambretti S, 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**(11):5521–6.
23. Pitt ME, Elliott AG, Cao MD, Ganesamoorthy D, Karaiskos I, Giamarellou H, et al. Multifactorial chromosomal variants regulate polymyxin resistance in extensively drug-resistant *Klebsiella pneumoniae*. *Microb Genom* 2018;**4**. doi:10.1099/mgen.0.000158.
24. Otter JA, Doumith M, Davies F, Mookerjee S, Dyakova E, Gilchrist M, et al. Emergence and clonal spread of colistin resistance due to multiple mutational mechanisms in carbapenemase-producing *Klebsiella pneumoniae* in London. *Sci Rep* 2017;**7**(1):12711.
25. Choi MJ, Ko KS. Mutant prevention concentrations of colistin for acinetobacter baumannii, pseudomonas aeruginosa and *Klebsiella pneumoniae* clinical isolates. *J Antimicrob Chemother* 2014;**69**(1):275–7.
26. Olaitan AO, Diene SM, Kempf M, Berrazeg M, Bakour S, Gupta SK, 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**(6):500–7.
27. Cannatelli A, Santos-Lopez A, Giani T, Gonzalez-Zorn B, Rossolini GM. Polymyxin resistance caused by *mgrB* inactivation is not associated with significant biological cost in *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* 2015;**59**(5):2898–900.
28. Clinical and Laboratory Standards Institute. *Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, M07-Ed11*. Wayne, PA, USA: CLSI; 2018.

29. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, et al. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J Comput Biol* 2012;**19**(5):455–77.
30. Antipov D, Hartwick N, Shen M, Raiko M, Lapidus A, Pevzner PA. plasmidSPAdes: assembling plasmids from whole genome sequencing data. *Bioinformatics* 2016;**32**(22):3380–7.
31. Gurevich A, Saveliev V, Vyahhi N, Tesler G. QUAST: quality assessment tool for genome assemblies. *Bioinformatics* 2013;**29**(8):1072–5.
32. Rissman AI, Mau B, Biehl BS, Darling AE, Glasner JD, Perna NT. Reordering contigs of draft genomes using the mauve aligner. *Bioinformatics* 2009;**25**(16):2071–3.
33. Seemann T. Prokka: rapid prokaryotic genome annotation. *Bioinformatics* 2014;**30**(14):2068–9.
34. Xie Z, Tang H. ISEScan: automated identification of insertion sequence elements in prokaryotic genomes. *Bioinformatics* 2017;**33**(21):3340–7.
35. Jia B, Raphenya AR, Alcock B, Waglechner N, Guo P, Tsang KK, et al. CARD 2017: expansion and model-centric curation of the comprehensive antibiotic resistance database. *Nucleic Acids Res* 2017;**45**(D1):D566–DD73.
36. Bertelli C, Laird MR, Williams KP, Lau BY, Hoad G, et al., Simon Fraser University Research Computing G IslandViewer 4: expanded prediction of genomic islands for larger-scale datasets. *Nucleic Acids Res* 2017;**45**(W1):W30–WW5.
37. MLST database for *Klebsiella pneumoniae* [Internet]. 2018. Available from: http://bigsdb.web.pasteur.fr/klebsiella/.
38. Treangen TJ, Ondov BD, Koren S, Phillippy AM. The harvest suite for rapid core-genome alignment and visualization of thousands of intraspecific microbial genomes. *Genome Biol* 2014;**15**(11):524.
39. Stamatakis A. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* 2014;**30**(9):1312–13.
40. Letunic I, Bork P. Interactive tree of life (iTOL) v4: recent updates and new developments. *Nucleic Acids Res* 2019;**47**(W1):W256–9.
41. Liao Y, Smyth GK, Shi W. The subread aligner: fast, accurate and scalable read mapping by seed-and-vote. *Nucleic Acids Res* 2013;**41**(10):e108.
42. Harrison P., Seemann T. *From high-throughput sequencing read alignments to confident, biologically relevant conclusions with nesoni*2009. Available from: http://www.vicbioinformatics.com/nesoni_ba2009_poster.pdf.
43. Cameron DL, Schroder J, Penington JS, Do H, Molania R, Dobrovic A, et al. GRIDSS: sensitive and specific genomic rearrangement detection using positional de bruijn graph assembly. *Genome Res* 2017;**27**(12):2050–60.
44. Carver T, Harris SR, Berriman M, Parkhill J, McQuillan JA. Artemis: an integrated platform for visualization and analysis of high-throughput sequence-based experimental data. *Bioinformatics* 2012;**28**(4):464–9.
45. Page AJ, Cummins CA, Hunt M, Wong VK, Reuter S, Holden MT, et al. Roary: rapid large-scale prokaryote pan genome analysis. *Bioinformatics* 2015;**31**(22):3691–3.
46. Choi Y, Chan AP. PROVEAN web server: a tool to predict the functional effect of amino acid substitutions and indels. *Bioinformatics* 2015;**31**(16):2745–7.
47. The European Committee on Antimicrobial Susceptibility Testing. *Breakpoint tables for interpretation of MICs and zone diameters, version 8.0*2018. Available from: http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_8.0_Breakpoint_Tables.pdf.
48. Carattoli A, Zankari E, Garcia-Fernandez A, Voldby Larsen M, Lund O, Villa L, et al. *In silico* detection and typing of plasmids using plasmidfinder and plasmid multilocus sequence typing. *Antimicrob Agents Chemother* 2014;**58**(7):3895–903.
49. Chiu SK, Huang LY, Chen H, Tsai YK, Liou CH, Lin JC, et al. Roles of *ramR* and *tet*(A) mutations in conferring tigecycline resistance in carbapenem-resistant *Klebsiella pneumoniae* clinical isolates. *Antimicrob Agents Chemother* 2017;**61**(8) e00391–e00317.
50. Novovic K, Trudic A, Brkic S, Vasiljevic Z, Kojic M, Medic D, et al. Molecular epidemiology of colistin-resistant, carbapenemase-producing *Klebsiella pneumoniae* in Serbia from 2013 to 2016. *Antimicrob Agents Chemother* 2017;**61**(5) e02550–e02516.
51. Deleo FR, Chen L, Porcella SF, Martens CA, Kobayashi SD, Porter AR, et al. Molecular dissection of the evolution of carbapenem-resistant multilocus sequence type 258 *Klebsiella pneumoniae*. *Proc Natl Acad Sci USA* 2014;**111**(13):4988–93.
52. Lascols C, Peirano G, Hackel M, Laupland KB, Pitout JD. Surveillance and molecular epidemiology of *Klebsiella pneumoniae* isolates that produce carbapenemases: first report of OXA-48-like enzymes in North America. *Antimicrob Agents Chemother* 2013;**57**(1):130–6.
53. Guo L, An J, Ma Y, Ye L, Luo Y, Tao C, et al. Nosocomial outbreak of OXA-48-producing *Klebsiella pneumoniae* in a Chinese hospital: clonal transmission of ST147 and ST383. *PLoS ONE* 2016;**11**(8):e0160754.
54. Bader MW, Sanowar S, Daley ME, Schneider AR, Cho U, Xu W, et al. Recognition of antimicrobial peptides by a bacterial sensor kinase. *Cell* 2005;**122**(3):461–72.
55. Mitrophanov AY, Jewett MW, Hadley TJ, Groisman EA. Evolution and dynamics of regulatory architectures controlling polymyxin B resistance in enteric bacteria. *PLoS Genet* 2008;**4**(10):e1000233.
56. Luo SC, Lou YC, Rajasekaran M, Chang YW, Hsiao CD, Chen C. Structural basis of a physical blockage mechanism for the interaction of response regulator PmrA with connector protein PmrD from *Klebsiella pneumoniae*. *J Biol Chem* 2013;**288**(35):25551–61.
57. Giordano C, Barnini S, Tsioutis C, Chlebowicz MA, Scoulica EV, Gikas A, et al. Expansion of KPC-producing *Klebsiella pneumoniae* with various *mgrB* mutations giving rise to colistin resistance: the role of IS*L3* on plasmids. *Int J Antimicrob Agents* 2018;**51**(2):260–5.
58. Matamouros S, Hager KR, Miller SI. HAMP domain rotation and tilting movements associated with signal transduction in the PhoQ sensor kinase. *mBio* 2015;**6**(3) e00616–e00615.
59. Kidd TJ, Mills G, Sa-Pessoa J, Dumigan A, Frank CG, Insua JL, et al. A *Klebsiella pneumoniae* antibiotic resistance mechanism that subdues host defences and promotes virulence. *EMBO Mol Med* 2017;**9**(4):430–47.
60. Crawford MA, Fisher DJ, Leung LM, Lomonaco S, Lascols C, Cannatelli A, et al. CXC chemokines exhibit bactericidal activity against multidrug-resistant Gram-negative pathogens. *mBio* 2017;**8**(6) e01549–e01517.
61. Wand ME, Bock LJ, Sutton JM. Retention of virulence following colistin adaptation in *Klebsiella pneumoniae* is strain-dependent rather than associated with specific mutations. *J Med Microbiol* 2017;**66**(7):959–64.
62. Bardet L, Baron S, Leangapichart T, Okdah L, Diene SM, Rolain JM. Deciphering heteroresistance to colistin in a *Klebsiella pneumoniae* isolate from Marseille, France. *Antimicrob Agents Chemother* 2017;**61**(6) e00356–e00317.
63. Zhang H, Zhao D, Shi Q, Quan J, Li X, Yu Y. The *mcr-1* gene has no effect on colistin resistance when it coexists with inactivated *mgrB* gene in *Klebsiella pneumoniae*. *Microb Drug Resist* 2018;**24**(8):1117–20.
64. Huerta-Cepas J, Szklarczyk D, Forslund K, Cook H, Heller D, Walter MC, et al. eggNOG 4.5: a hierarchical orthology framework with improved functional annotations for eukaryotic, prokaryotic and viral sequences. *Nucleic Acids Res* 2016;**44**(D1):D286–93.
65. Lemmin T, Soto CS, Clinthorne G, DeGrado WF, Dal Peraro M. Assembly of the transmembrane domain of *E. coli* PhoQ histidine kinase: implications for signal transduction from molecular simulations. *PLoS Comput Biol* 2013;**9**(1):e1002878.