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Pathways Regulating the *pbgP* Operon and Colistin Resistance in *Klebsiella pneumoniae* Strains

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In this study, we investigated colistin resistance mechanisms associated with the regulation of the *pbgP* operon in *Klebsiella pneumoniae*, using four isogenic pairs of colistin-susceptible strains and their colistin-resistant derivatives and two colistin-resistant clinical isolates. Amino acid sequence alterations of PhoPQ, PmrAB, and MgrB were investigated, and mRNA expression levels of *phoQ*, *pmrB*, *pmrD*, and *pbgP* were measured using quantitative real-time PCR. The *phoQ* and *pmrB* genes were deleted from two colistin-resistant derivatives, 134R and 063R. We found that *phoQ*, *pmrD*, and *pbgP* were significantly upregulated in all colistin-resistant derivatives. However, *pmrB* was significantly upregulated in only two colistin-resistant derivatives and one clinical strain. *pmrB* was not overexpressed in the other strains. The minimum inhibitory concentration of colistin was drastically lower in both *phoQ*- and *pmrB*-deleted mutants from a colistin-resistant derivative (134R) that was overexpressing *phoQ* and *pmrB*. However, colistin susceptibility was restored only in a *phoQ*-deleted mutant from a colistin-resistant derivative (063R) without overexpression of *pmrB*. In conclusion, two different regulations of the *pbgP* operon may associate with the development of colistin-resisant *K. pneumoniae*.

Keywords: Colistin resistance, *Klebsiella pneumoniae*, two-component regulatory systems

Introduction

Klebsiella pneumoniae is an opportunistic nosocomial pathogen that causes urinary tract infections, pneumonia, and septicemia in intensive care units [24]. Multidrug-resistant (MDR) *K. pneumoniae* infections that have increased morbidity and mortality are a growing global concern [26]. Carbapenem-resistant *K. pneumoniae* isolates, including KPC- or NDM-producing isolates, have recently increased worldwide [22]. The increasing occurrence of MDR or carbapenem-resistant *K. pneumoniae* has led to a reliance on colistin as a last-resort treatment option [12]. Colistin is a cationic amphipathic lipopeptide with a heptapeptide ring and a fatty acid tail [17]. The polycationic peptide ring on polymyxin interacts with the lipid A moiety of bacterial lipopolysaccharide (LPS), causing disorganization of the bacterial outer membrane [11]. Although colistin is one of few antimicrobial agents with low resistance rates, colistin-resistant *K. pneumoniae* isolates have been reported worldwide, including in Korea [13, 20, 23, 25, 28].

Colistin resistance is associated with LPS modifications that result in changes to the charge of the bacterial outer membrane [2]. This LPS modification relies on the activation of the PhoPQ and PmrAB two-component regulatory systems in gram-negative bacteria such as *K. pneumoniae* [5, 23]. Upregulation of PhoPQ and PmrAB two-component regulatory systems increases the expression of the gene encoding proteins for biosynthesis and transfer of 4-amino-4-deoxy-L-arabinose (Ara4N) to lipid A [18, 30]. In *K. pneumoniae*, PhoP activates the *pbgP* (also called *pmrHFIJKLM* and *arn*) operon directly, or indirectly through the activation of PmrD, which in turn activates the PmrA responsible for activation of the *pbgP* operon [5, 19]. MgrB was recently demonstrated to be a negative regulator associated with the upregulation of the PhoPQ system that eventually leads to colistin resistance [2, 25].

Although genetic alterations in PhoPQ, PmrAB, and MgrB have been associated with colistin resistance in

K. pneumoniae [2, 3, 4, 14, 15, 23], the complete mechanism underlying colistin resistance in *K. pneumoniae* is currently unclear owing to the species-specific nature of the PhoPQ and PmrAB two-component regulatory system pathway. In this study, we describe differential pathways of PhoPQ and PmrAB two-component regulatory systems to affect the *pbgP* operon and colistin resistance in different *K. pneumoniae* strains, using isogenic strains and clinical isolates.

Materials and Methods

Bacterial Strains, Plasmids, and Growth Conditions

The bacterial strains and plasmids used in the present study are listed in Table 1. Four colistin-susceptible *K. pneumoniae* strains (134, 039, 063, and 068) and two colistin-resistant *K. pneumoniae* clinical isolates (073 and 4405) obtained from patients in Korea were used in this study. The four strains were demonstrated to belong to different sequence types (STs) (ST730, ST11, ST23, and ST152) in a multilocus sequence typing analysis [9]. Colistin-susceptible strains were repeatedly cultured with increasing concentrations of colistin in Luria-Bertani (LB) broth. Briefly, 10^6 CFU/ml from overnight cultures of colistin-susceptible strains were used to inoculate LB medium lacking colistin, and incubated overnight with vigorous shaking at 37°C. Cultures were then diluted 1:100 in fresh medium containing a sub-inhibitory concentration of colistin (0.25 mg/l) and incubated overnight. Thereafter, in-vitro-selected mutants from the previous stage were serial-passaged daily in LB broth containing increasing concentrations of colistin (from 0.5 to 32 mg/l). At the end of the induction period, isogenic colistin-resistant derivatives (134R, 039R, 063R, and 068R) were selected in LB broth containing 64 mg/l of colistin, and the minimum inhibitory concentrations (MICs) of colistin for three colonies picked at random were confirmed [6]. Colistin MICs for the colistin-resistant derivatives ranged from 256 μg/ml to >8,192 μg/ml (Table 2).

Antimicrobial Susceptibility Testing

Colistin MICs were determined by the broth microdilution protocol according to the Clinical and Laboratory Standards

Table 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Description	Reference
Strain		
K. pneumoniae		
134	Clinical isolate; colistin-susceptible	This study
039	Clinical isolate; colistin-susceptible	This study
063	Clinical isolate; colistin-susceptible	This study
068	Clinical isolate; colistin-susceptible	This study
073	Clinical isolate; colistin-resistant	This study
4405	Clinical isolate; colistin-resistant	This study
134R	Selected colistin-resistant derivative from 134	This study
039R	Selected colistin-resistant derivative from 039	This study
063R	Selected colistin-resistant derivative from 063	This study
068R	Selected colistin-resistant derivative from 068	This study
134RΔ*phoQ*	Δ*phoQ*::Tc, 134R background; Tet^r; The *phoQ* gene was inactivated.	This study
134RΔ*pmrB*	Δ*pmrB*::Km, 134R background; Km^r; The *pmrB* gene was inactivated.	This study
063RΔ*phoQ*	Δ*phoQ*::Tc 063R background; Tet^r; The *phoQ* gene was inactivated.	This study
063RΔ*pmrB*	Δ*pmrB*::Km, 063R background; Km^r; The *pmrB* gene was inactivated.	This study
Escherichia coli		
DH5α	Host strain harboring pKD46 vector F- *endAl hsdR17* (r_K- M_K^+) *supE44 thi-1 recAl gyrA relAl* ϕ80*dlacZAM15* Δ(*lacZYA-argF*)*U169*	[22]
K-12	Host strain harboring pDMS197 vector; F- lambda- *ilvG- rfb-50 rph-1*	[23]
Enterococcus faecalis (ATCC 51299)	Km cassette source for one-step mutagenesis protocol, Km^r	[24]
Plasmids		
pKD46	*araBp-gam-bet-exo*, *bla*(*ApR*), *repA101*(*ts*), *oriR101*, λ Red recombinase expression, Cm^r	[22]
pDMS197	oriT oriV sacB TcR, Tet cassette source for one-step mutagenesis protocol, Tet^r	[23]

Table 2. Genotype, antimicrobial susceptibility profiles, and amino acid alterations of PhoPQ, PmrAB, and MgrB in four pairs of isogenic *K. pneumoniae* strains and colistin-resistant clinical isolates.

Strains	ST	Colistin MIC (μg/ml)	Amino acid alteration[a]
134	730	1	
134R		256	None
039	11	0.5	
039R		>8,192	None
063	23	0.5	
063R		512	Y268S, Deletion of 5 amino acids at position 14 in PhoQ
068	152	0.5	
068R		1,024	Insertion of IS5-like element in MgrB
073	37	>64	V24A and D150G in PhoQ, R256G in PmrB
4405	11	>64	D150G in PhoQ, R256G in PmrB

[a]Amino acid sequences in colistin-resistant derivatives were compared with the amino acid sequences of their parental colistin-susceptible strains. Amino acid sequences of two colistin-resistant clinical isolates (073 and 4405) were compared with the sequences of a *K. pneumoniae* reference strain, NTUH-K2044.

Institute methods [8].

Construction of Specific Gene-Deleted Mutants

The *phoQ* and *pmrB* genes were deleted from colistin-resistant derivatives 134R and 063R. A pKD46 plasmid induced lambda Red recombinase production in the host cell to enhance homologous recombination [7]. The pKD46 plasmid was electroporated into colistin-resistant derivatives (1.8 kV, 25 μF, 200 Ω) and grown at 30°C with L-arabinose. The tetracycline and kanamycin cassettes were obtained from the pDMS 197 vector and *Enterococcus faecalis* ATCC 51299, respectively [10, 27]. Following the PCR-mediated antimicrobial resistance cassette insertion mutagenesis method [16], a PCR product containing a tetracycline and kanamycin resistance cassette flanked by ~500 bp of the regions surrounding the *phoQ* or *pmrB* gene was introduced into the electroporated 134R and 063R isolates, respectively [16, 27]. 134RΔ*phoQ*, 134Δ*pmrB*, 063RΔ*phoQ*, and 063RΔ*pmrB* mutants were obtained on plates containing 50 μg/ml tetracycline and 10 μg/ml kanamycin. Transformants were confirmed by PCR and sequencing to detect modified sizes of the genes.

Sequence Analysis and mRNA Expression Analysis

PCR and sequencing were conducted using the primers listed in Table 3 to determine amino acid alterations of PhoPQ, PmrAB, and MgrB in colistin-resistant derivatives and clinical isolates. The gene expression levels of *phoQ*, *pmrB*, *pmrD*, and *pbgP* were investigated using quantitative real-time PCR (qRT-PCR) (Table 3). Bacterial cells were grown aerobically in Mueller-Hinton broth until mid-log phase for RNA preparation. Total RNA was harvested using an RNeasy kit (Qiagen, Germany) and reverse transcription was performed using Omniscript reverse transcriptase (Qiagen). qRT-PCR was performed using SYBR green PCR master mix (Applied Biosystems, USA) in a Thermal Cycler Dice Real Time System TP800 (Takara, Japan). Expression of the *rpoB* housekeeping gene was used to normalize transcript levels.

Genome Sequencing and Confirmation of Genetic Variants

To identify the genetic alterations in colistin-resistant derivatives, whole-genome sequencing was performed using next-generation sequencing technology. Genomic DNA isolated from overnight cultures of four pairs of colistin-susceptible strains and their colistin-resistant derivatives was sequenced using Illumina Hiseq2000 Preliminary Performance Parameters, with 100X coverage (Macrogen, South Korea). After sequencing, raw sequence data were filtered based on quality score and then assembled onto the *K. pneumoniae* NTUH-K2044 (NC_012731.1) and *K. pneumoniae* MGH 78578 (NC_009648.1) reference genomic sequences using BWA ver. 0.5.9-r16. Sequence features, including single nucleotide polymorphisms and short insertions and deletions, in colistin-resistant strains were identified through next-generation sequencing and confirmed through Sanger sequencing.

Results

Amino Acid Alterations in Colistin-Resistant Derivatives and Clinical isolates

Four colistin-resistant mutants were obtained from their parental colistin-susceptible *K. pneumoniae* strains. Colistin MICs increased from 0.5–1 μg/ml to 256 μg/ml and >8,192 μg/ml (Table 2). Amino acids of PhoPQ, PmrAB, and MgrB were compared between colistin-susceptible and colistin-resistant isogenic strains. Whereas two colistin-resistant derivatives (134R and 039R) showed no amino acid substitutions, mutations were found in the other two derivatives (063R and 068R). We found an Y268S substitution and a deletion of five amino acids in PhoQ in the 063R strain and the insertion of an IS5-like element in MgrB in

Table 3. Primers used in this study.

Primer	Sequence (5′ → 3′)[a]	Amplicon size (bp)	Reference
Primers for sequencing			
PhoP-F	ATGCCGTAGGCCAGGGAGAG	964	This study
PhoP-R	CCGCTGGGCGTTTACTGA		
PhoQ-F	CGTTCGATAAAGTCGGGCCA	1,716	This study
PhoQ-R	GCGAGAAAGCCACACCATCG		
PmrA-F	GCAACCGCTTCCCGTACCTC	1,030	This study
PmrA-R	CGGTATTTCCGCGCACTGTC		
PmrB-F	GAGGATAGCGCCCATGCAAA	1,310	This study
PmrB-R	AACGAGCCGGCGACCAATAC		
MgrB-F	CAG CCA GCG ATG CCA GAT TT	380	Cannatelli *et al*. [2]
MgrB-R	CCTGGCGTGATTTTGACACGA		
Primers for qRT-PCR			
phoQ-F	GATGGGAAAACGGCGCTATT	100	This study
phoQ-R	TGCCCTGCTCATCGTAGATAAG		
pmrD-F	TGGAGTGGTGGGTAAAAAAAGTACA	100	This study
pmrD-R	GCTTCAATCTCTGCGATCATCTC		
pmrB-F	TTGGCCTGGCGCTGTATATC	100	This study
pmrB-R	GCGTGCGGTTCTCCAGTT		
pbgP-F	AACTACTGACCATGGCGGCG	116	This study
pbgP-R	GCCAGCCAGTTCACCACGAA		
rpoB-F	CGCGTATGTCCGATCGAAA	100	This study
rpoB-R	GCGTCTCAAGGAAGCCATATTC		
Primers for allelic replacement			
Kan-F	AACAGTGAATTGGAGTTCGTCTTGTTATA	907	Song and Ko [27]
Kan-R	GCTTTTTAGACATCTAAATCTAGGTA		
pmrB-KF-LF	CCCTGCTGTCACGGCTGATG	528	This study
pmrB-KF-LR	GACGAACTCCAATTCACTGTTTGCGGTTCTCCAGTTCGCTT		
pmrB-KF-RF	AGATTTAGATGTCTAAAAAGCGTCTCTGGCGATGCGACGTT	501	This study
pmrB-KF-RR	GCGTTGCGGCCTTCTTAATG		
Tet-F	GCACATCAAGGCCAAGCCC	1,541	This study
Tet-R	GCGCATTCACAGTTCTCCGC		
phoQ-KF-LF	ATCCGTAACCGCGGCAAAGT	544	This study
phoQ -KF-LR	GGGCTTGGCCTTGATGTGCAAGCCATTGCGTTTCAGCCA		
phoQ-KF-RF	GCGGAGAACTGTGAATGCGCTGTCGGCGAGCAGAACGATT	537	This study
phoQ-KF-RR	GGGGGTCGATAAAATTGGCG		

[a]Sequences identical to the 3′ and 5′ ends of the cassette gene sequence are underlined.

the 068R strain (Table 2). Two clinically obtained wild-type colistin-resistant isolates (073 and 4405) showed amino acid alterations of PhoQ and PmrB (Table 2).

Expression of Two-Component Regulatory Systems and *pbgP*

phoQ, *pmrD*, and *pbgP* were significantly upregulated in all colistin-resistant derivatives (Figs. 1A, 1C, and 1D). However, *pmrB* was significantly upregulated in only two colistin-resistant derivatives, 134R and 039R (Fig. 1B).

Expression levels of *phoQ*, *pmrB*, *pmrD*, and *pbgP* were also measured in two colistin-resistant clinical isolates, 073 and 4405, that belonged to different clones, ST37 and ST11, respectively (Fig. 2). All four genes were overexpressed in

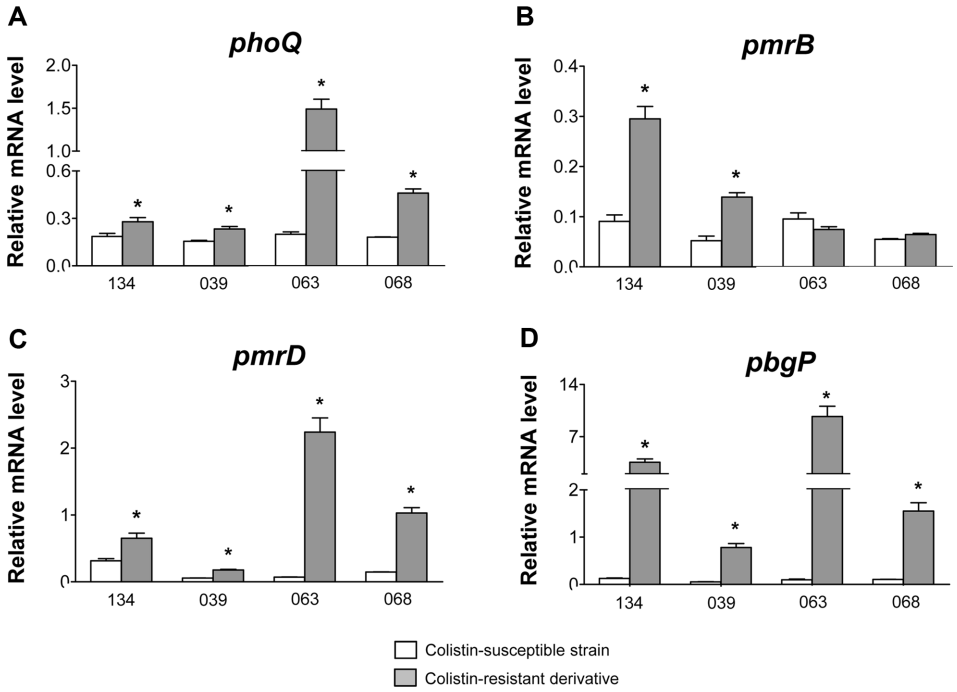

Fig. 1. Gene expression analysis of *phoQ* (**A**), *pmrB* (**B**), *pmrD* (**C**), and *pbgP* (**D**) in four pairs of isogenic strains.
Vertical bars represent standard deviations. Asterisks (*) indicate significant differences in expression ($p < 0.05$).

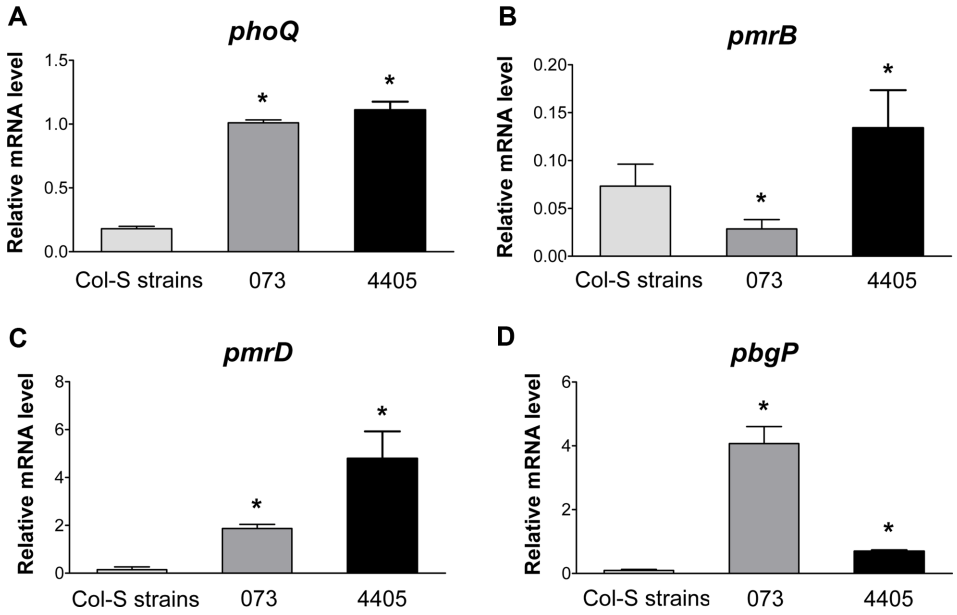

Fig. 2. Gene expression analysis of *phoQ* (**A**), *pmrB* (**B**), *pmrD* (**C**), and *pbgP* (**D**) in colistin-resistant clinical isolates.
Vertical bars represent standard deviations. Asterisks (*) indicate significant differences in expression ($p < 0.05$) relative to average gene expression levels in four colistin-susceptible *K. pneumoniae* strains used in this study.

4405, as they were in 134R and 039R. However, *pmrB* was not overexpressed in 073, similar to its expression in 063R and 068R.

Effects of *phoQ* or *pmrB* Deletion

The *phoQ* or *pmrB* gene was deleted in two colistin-resistant derivatives, 134R and 063R. Although both PhoQ

and PmrB were overexpressed in 134R, only PhoQ was overexpressed in 063R. In both *phoQ*- and *pmrB*-deleted mutants of 134R (134RΔ*phoQ* and 134RΔ*pmrB*), colistin MICs decreased to 1 μg/ml from 256 μg/ml (Table 4). The *phoQ*-deleted mutant of 063R (063RΔ*phoQ*) showed decreased colistin MIC (0.25 mg/l), but the colistin MIC of the *pmrB*-deleted mutant (063RΔ*pmrB*) was unchanged (Table 4).

The expression levels of *phoQ, pmrB, pmrD*, and *pbgP* were also evaluated in *phoQ*- and *pmrB*-deleted mutants of colistin-resistant derivatives. The deleted mutants from two colistin-resistant derivatives showed similar expression patterns except in their levels of *pbgP* expression (Fig. 3). Although *pbgP* expression decreased significantly in 134RΔ*pmrB* relative to 134R (Fig. 3A), it did not decrease in 063RΔ*pmrB* relative to 063R (Fig. 3B). This finding is comparable with the results that the colistin MIC decreased in 134RΔ*pmrB* but not in 063RΔ*pmrB*.

Table 4. Colistin MICs of colistin-resistant derivatives and their *phoQ* or *pmrB* deletion mutants.

Strains	Colistin MIC (μg/ml)
134	1
134R	256
134RΔ*phoQ*	1
134RΔ*pmrB*	1
063	0.5
063R	512
063RΔ*phoQ*	0.25
063RΔ*pmrB*	512

Whole-Genome Sequencing

Next-generation sequencing produced 13–17 million nucleotides for each of the eight strains. They covered 90.37–98.37-fold. We were able to map reads from three isolates to 68.61–88.82% and 72.44–81.42% of the *K. pneumoniae* NTUH-K2044 and *K. pneumoniae* MGH 78578 (NC_009648.1) reference genomes, respectively.

In 134R and 039R, only alterations in intergenic regions were found. In B0608-134R, an IS*5*-like element was inserted between KPN_RS11130 and KPN_RS11135 (or *crrA*). In 039R, an IS*5*-like element was inserted between KPN_RS16170 (or *mgrB*) and KPN_RS16175.

In 063R, four alterations were found in three genes. In *phoQ*, two alterations were found: A803C leading to an Y268S amino acid substitution, and a 12 bp deletion at the 341th nucleotide position. In the intergenic region between KP1_RS16895 and KP1_RS16900, a 1 bp deletion at the 51st nucleotide position was also found. In KP1_RS00290, which encodes a repressor of the *galETK* operon, a single nucleotide substitution, T104G, caused the amino acid change V35G. Strain 068R showed disruptions in MgrB caused by IS*5*-like element insertions. In addition, one nucleotide alteration (G593T), leading to the amino acid substitution G198V, was found in KPN_RS1140, which encodes an RstA regulator (sensor histidine protein kinase). Only restricted alterations between colistin-susceptible WT strains and their resistant counterparts indicated that they are isogenic to each other.

Discussion

The activation of the PhoPQ and/or PmrAB two-component regulatory system is known to operate in

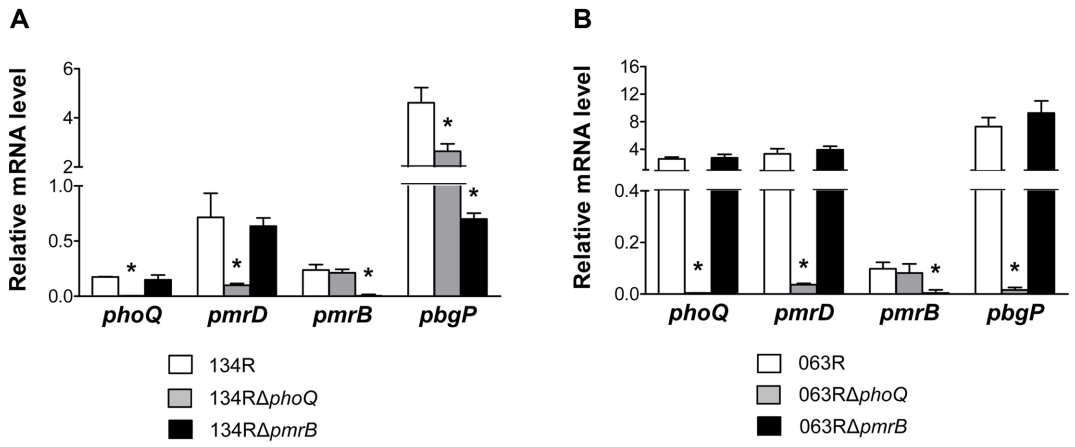

Fig. 3. Gene expression analysis of *phoQ*, *pmrD*, *pmrB*, and *pbgP* in *phoQ*- and *pmrB*-deleted mutants of 134R (**A**) and 063R (**B**).
Vertical bars represent standard deviations. Asterisks (*) indicate significant differences ($p < 0.05$) versus colistin-resistant 134R and 063R strains.

regulators and sensors associated with colistin resistance in gram-negative bacteria [21]. It has been suggested that there are diverse controlling mechanisms associated with the PhoPQ and PmrAB two-component regulatory systems in gram-negative bacteria [19]. In *Salmonella enterica*, the PhoPQ and PmrAB two-component regulatory systems are mediated by PmrD (the connector-mediated pathway), but PmrD regulated by the PhoPQ system does not activate the PmrAB system in *E. coli* [29]. In *Yersinia pestis*, no PmrD has been found, and PhoPQ and PmrAB act independently [19]. In *Acinetobacter baumannii*, only the PmrAB system was identified, unlike in *Pseudomonas aeruginosa*, which contains both systems [1].

It is well known that the PhoPQ-PmrD-PmrAB-mediated pathway regulates colistin resistance in *K. pneumoniae* [18]. Both PhoPQ and PmrAB two-component regulatory systems regulate the *pbgP* operon, and thus increased expression of them implies acquisition of colistin resistance in *K. pneumoniae*. However, Wright *et al*. [30] recently reported colistin-resistant *K. pneumoniae* strains with unaltered PhoPQ or PmrAB expression. Our results in the present study suggest that different mechanisms mediate colistin resistance in *K. pneumoniae*.

In two colistin-resistant derivatives, 134R and 039R, PhoPQ and PmrAB activation may mediate with PmrD, as shown in a previous study [5]. Significant overexpression of *phoQ*, *pmrB*, and *pmrD* was identified in 134R, and drastic decreases in colistin MICs were found in both *phoQ*-deleted and *pmrB*-deleted mutants. These results support the feedforward connector loop pathway suggested by Mitrophanov *et al*. [19] and Cheng *et al*. [5, 30]. That is, *pbgP* activation associated with the development of colistin resistance may be regulated through three pathways in one strain: direct regulation by PhoPQ, direct regulation by PmrAB, and PmrD-mediated regulation by PhoPQ and PmrAB systems. Direct activation of *pbgP* by PhoPQ may yield a relatively low effect on colistin resistance in 134R, considering the decreased *pbgP* expression level in single deletion mutants. PmrD mediation may potentiate the two two-component regulatory systems. However, no mutations of two two-component regulatory systems were found in these mutants, which may indicate that other mechanisms affect *pbgP* expression and finally colistin resistance. A colistin-resistant clinical isolate (4405) seems to have the same resistance mechanisms as these colistin-resistant derivatives.

However, PmrAB was not activated in the other two colistin-resistant derivatives, 063R and 068R, and one colistin-resistant clinical isolate, 073. The lack of a decrease in the colistin MIC in 063Δ*pmrB* indicates that PmrAB has no association with colistin resistance in these strains, despite the drastic decrease in the colistin MIC in 063Δ*phoQ*. In addition, *pbgP* activation was blocked only in 063Δ*phoQ*. This result was shown in several strains reported by Wright *et al*. [30]. In these mutants, mutations in the *phoQ* and *mgrB* genes were found. These mutations may affect their colistin resistance. In the strains belonging to this category, there may be only one pathway regulating *pbgP* activation associated with the development of colistin resistance.

Thus, *K. pneumoniae* strains may develop colistin resistance by two or more different regulatory methods of the *pbgP* operon. In some strains in our study, both the PhoPQ and PmrAB two-component regulatory systems may regulate the *pbgP* operon directly, or PhoPQ activates the *pbgP* operon indirectly through the activation of PmrD. However, only the PhoPQ system may regulate the *pbgP* operon in other strains. Although direct *pbgP* activation by PhoPQ has been shown in *Y. pestis*, it was not found in the lineage of *S. enterica*, *S. flexneri*, and *E. coli*. Thus, direct *pbgP* activation by PhoPQ may be the ancestral form that was lost after divergence from the *K. pneumoniae* lineage. Therefore, the presence of three pathways in one *K. pneumoniae* strain represents the co-existence of ancestral and derived forms. The lack of PmrD mediation in some *K. pneumoniae* strains may also support the hypothesis that the PhoPQ and PmrAB two-component regulatory systems are a transition or modified state. The *pmrD* gene in these strains might be acquired and thus may be activated by PhoPQ, but might not obtain the ability to activate PmrA. Later, the connection between the PmrAB system and the *pbgP* operon might have been interrupted. In addition, it is also possible that other pathways to colistin resistance in *K. pneumoniae* may exist.

In summary, we confirmed previous findings that each *K. pneumoniae* strain has unique mechanisms to regulate the *pbgP* operon and to develop colistin resistance [15]. The PhoPQ and PmrAB two-component regulatory systems in *K. pneumoniae* may be evolutionarily transient forms that diversify into different pathways. Further research is required to investigate the potential abilities of the different regulatory pathways to invoke different physiological effects, such as responses to environmental stress, virulence, and fitness.

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