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Loss of Hypermucoviscosity and Increased Fitness Cost in Colistin-Resistant *Klebsiella pneumoniae* Sequence Type 23 Strains

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In this study, we investigated the effects of colistin resistance on virulence and fitness in hypermucoviscous (HV) *Klebsiella pneumoniae* sequence type 23 (ST23) strains. Colistin-resistant mutants were developed from three colistin-susceptible HV *K. pneumoniae* ST23 strains. The lipid A structures of strains were analyzed by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry. Changes in HV were investigated using the string test, and extracellular polysaccharide production was quantified. The expression levels of the *phoQ*, *pmrD*, *pmrB*, *pbgP*, *magA*, and p-*rmpA2* genes, serum resistance, and biofilm-forming activity were determined. The fitness of colistin-resistant mutants compared to that of the parental strains was examined by determining the competitive index (CI). The colistin-resistant mutants exhibited reduced HV, which was accompanied by decreased formation of capsular polysaccharides (CPS) and reduced expression of genes (*magA* and p-*rmpA2*). While there was enhanced expression of *pmrD* and *pbgP* in all colistin-resistant derivatives, there were differences in the expression levels of *phoQ* and *pmrB* between strains. MALDI-TOF analysis detected the addition of aminoarabinose or palmitate to the lipid A moiety of lipopolysaccharide in the colistin-resistant derivatives. In addition, survival rates in the presence of normal human serum were decreased in the mutant strains, and CI values (0.01 to 0.19) indicated significant fitness defects in the colistin-resistant derivatives compared to the respective parental strains. In hypervirulent HV *K. pneumoniae* strains, the acquisition of colistin resistance was accompanied by reduced CPS production, impaired virulence, and a significant fitness cost.

Highly invasive strains of *Klebsiella pneumoniae* that exhibit the hypermucoviscosity/hypermucoviscous (HV) phenotype have been reported as human pathogens, causing liver abscesses in patients in America, Europe, South Korea, and Taiwan (1, 2). The HV phenotype of *K. pneumoniae* is due to capsular polysaccharide (CPS) production and the presence of virulence genes, such as *magA*, g-*rmpA*, p-*rmpA*, and p-*rmpA2* (3, 4). Several studies demonstrated that the sequence type 23 (ST23) clone was associated with the K1 capsular serotype in HV *K. pneumoniae* strains that were isolated from liver abscesses (3). Meanwhile, the K2 serotype was reported for ST86, ST375, and ST380 HV isolates (2).

Colistin (also known as polymyxin E) is an antimicrobial polypeptide that mediates bactericidal activity by interacting with the lipid A component of lipopolysaccharide (LPS) present on Gram-negative pathogens, including *K. pneumoniae*, leading to outer membrane disruption (5). It is known that the modification of LPS following the addition of 4-amino-4-deoxy-L-arabinose to lipid A is related to colistin resistance in *K. pneumoniae*. The modification of LPS is associated with the *pbgPE* operon, which is regulated by two-component regulatory systems, such as PmrAB and PhoPQ. Insertional inactivation of MgrB, a negative regulator of PhoPQ, was also detected in colistin-resistant *K. pneumoniae* strains (6).

While the use of colistin was abandoned in the early 1980s due to the serious nephrotoxic and neurotoxic effects associated with this compound (7), the subsequent emergence of multidrug-resistant *K. pneumoniae* strains has led to the revival of colistin therapy (8). With the increased use of this antibiotic, however, colistin-resistant *K. pneumoniae* strains have begun to be isolated worldwide (5). For example, 6.8% of *K. pneumoniae* strains in South Korea were reported to be colistin resistant (7). As a result, it is necessary to gain a better understanding of the virulence and fitness of colistin-resistant *K. pneumoniae* strains.

Although colistin resistance is associated with the CPS of *K. pneumoniae*, the mechanism by which CPS confers this resistance has yet to be fully elucidated (9). The effects of colistin resistance on virulence and on *in vitro* and *in vivo* fitness costs have been observed in other Gram-negative, rod-shaped pathogens, such as *Acinetobacter baumannii* and *Salmonella enterica* (10, 11). Furthermore, the biological fitness cost of colistin resistance in *Klebsiella pneumoniae* carbapenemase (KPC)-producing *K. pneumoniae* was found to be negligible (12). However, there is a lack of data regarding the effects of colistin resistance on virulence and fitness in HV *K. pneumoniae* strains.

In this report, we describe decreased HV in *in vitro*-induced colistin-resistant mutants of serotype K1 *K. pneumoniae* ST23 strains. The expression levels of several virulence-associated genes as well as CPS production, serum resistance, and biofilm formation were assessed in colistin-resistant mutants *in vitro* developed from three colistin-susceptible strains. In addition, growth curves and *in vitro* competition assays were performed to investigate whether there is a fitness cost associated with colistin resistance in HV *K. pneumoniae*.

MATERIALS AND METHODS

Bacterial strains and *in vitro* selection of induced colistin-resistant mutants. Three colistin-susceptible *K. pneumoniae* ST23 strains, 08-B063, 07-B-060, and 13703-3487, which were isolated from patients in three

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different hospitals of Korea, were used in this study. Colistin-resistant mutants (08-B063R, 07-B-060R, and 13703-3487R) were developed *in vitro* from the three colistin-susceptible strains. Development of colistin resistance was obtained through serial passage of susceptible strains in Luria-Bertani (LB) broth supplemented with increasing concentrations of colistin. Briefly, 10^6 CFU/ml from overnight cultures of colistin-susceptible strains was 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 subinhibitory concentration of colistin (0.25 mg/liter) and incubated overnight. Thereafter, *in vitro*-selected mutants from the previous stage were serially passaged daily in LB broth containing increasing concentrations of colistin (from 0.5 to 32 mg/liter). After the 9th serial passage, the colistin-resistant mutants (08-B063R, 07-B-060R, and 13703-3487R) were selected in LB broth containing 64 mg/liter of colistin, and the MICs of colistin for four colonies picked at random were confirmed. To compare the phenotypes of colistin-resistant mutants with those of a clinically arising colistin-resistant strain, an ST23 colistin-resistant clinical strain, K07-08-056, which was collected from South Korea in 2008, was included in this study. Its colistin MIC was 8 mg/liter.

Antimicrobial susceptibility testing. MICs of antimicrobial agents were determined using a broth microdilution method, according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (13). Eleven antimicrobial agents or combinations were tested: ampicillin, aztreonam, cefotaxime, ceftazidime, ciprofloxacin, colistin, gentamicin, imipenem, piperacillin-tazobactam, polymyxin B, and trimethoprim-sulfamethoxazole. Susceptibility was defined according to CLSI breakpoints, and *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were employed as reference strains. All tests were performed in duplicate, and each test included three biological replicates per strain.

Analysis of lipid A structure. Lipid A extraction was performed using an ammonium hydroxide-isobutyric acid method, and extracts were subjected to negative-ion matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry analysis (8). Briefly, lyophilized cells (10 mg) were resuspended in 400 μl of isobutyric acid–1 M ammonium hydroxide (5:3 [vol/vol]) and incubated at 100°C for 4 h. Samples were centrifuged (13,000 rpm for 15 min), and supernatants were transferred to new tubes, diluted with an equal volume of water, and lyophilized. Samples were then washed twice with methanol and centrifuged (4,000 rpm for 15 min). The insoluble lipid A pellet was solubilized in a chloroform–methanol–water mixture (3:1.5:0.25 [vol/vol/vol]). Analyses were performed using a Bruker Autoflex II MALDI-TOF mass spectrometer (Bruker Daltonics, Billerica, MA) in negative reflective mode with delayed extraction. The ion-accelerating voltage was set at 20 kV. To analyze the samples, a few microliters of the lipid A suspensions (1 mg/ml) was desalted with a few grains of ion-exchange resin (Dowex 50W-X8; H^+). Then, a 1-μl aliquot of the suspension was deposited on the target and covered with an identical amount of 2,5-dihydroxybenzoic acid matrix (Sigma-Aldrich, St. Louis, MO) dissolved in acetonitrile. Data were analyzed in negative reflectron mode with a 100-Hz laser repetition rate. Three hundred shots were accumulated for each spectrum.

Serotyping and string test analyses. K serotyping of *K. pneumoniae* using multiplex PCR was performed as described previously (4). The HV phenotypes of the *K. pneumoniae* strains were determined using the string test (2). For this test, bacterial colonies are stretched on an agar plate using an inoculation loop. Strains that produce colonies that can be stretched in a viscous string >5 mm are considered HV positive (2).

Extraction and quantification of extracellular polysaccharides. To evaluate CPS production by *K. pneumoniae* strains, CPS was extracted and quantified as previously described (14). Briefly, samples (500 μl) cultured in medium without colistin were mixed with 100 μl of 100 mM citric acid (pH 2.0) containing 1% Zwittergent 3-14 detergent (Sigma-Aldrich) and then incubated overnight at 50°C. After centrifugation (13,000 rpm for 15 min), 250-μl quantities of the resulting supernatants were transferred to new tubes, and absolute ethanol was added to a final proportion of 80% (vol/vol). The CPS was allowed to precipitate at 4°C for 30 min, and the samples were centrifuged for 5 min. Pellets were dried and dissolved in 100 μl of distilled water, and then 600 μl of 12.5 mM borax in H_2SO_4 was added to each sample. Samples were boiled for 5 min, and 3-hydroxydiphenol (Sigma-Aldrich) was added to a final proportion of 0.15% (vol/vol). The absorbance at 520 nm was then measured, and the amounts of CPS were determined from a standard curve of glucuronic acid (Sigma-Aldrich). CPS concentrations are expressed as micrograms per 10^9 CFU.

Sequence analysis. The nucleotide sequences of *magA*, g-*rmpA*, p-*rmpA*, and p-*rmpA2*, which are virulence genes associated with CPS production, were determined using the primers listed in Table 1. For sequencing of the chromosomal genes *magA* and g-*rmpA*, genomic DNA (gDNA) was extracted from the *K. pneumoniae* strains using a gDNA extraction kit (iNtRON, Seongnam-si, South Korea). To sequence the plasmid-borne p-*rmpA* and p-*rmpA2* genes, plasmid DNA was extracted from the *K. pneumoniae* strains using a plasmid minikit (Qiagen, Venlo, Netherlands). The *pmrAB*, *phoPQ*, and *mgrB* genes were also amplified and sequenced for all colistin-resistant mutants, as described previously (15).

mRNA expression analysis. The expression levels of the *phoQ*, *pmrD*, *pmrB*, *pbgP*, *magA*, and p-*rmpA2* genes were determined by quantitative real-time PCR (qRT-PCR), as described previously (15). For RNA preparation, bacterial cells were grown aerobically in Mueller-Hinton (MH) broth until mid-log phase. Total RNA was harvested using an RNeasy kit (Qiagen), and concentrations were quantified spectrophotometrically. Reverse transcription was performed using Omniscript reverse transcriptase (Qiagen). qRT-PCR was performed using SYBR green PCR master mix (Applied Biosystems, Foster City, CA) in a Thermal Cycler Dice Real Time System TP800 (TaKaRa, Otsu, Japan). The transcript levels of each gene were calculated from a standard curve obtained by PCR amplification of serially diluted genomic DNA using the respective qRT-PCR primers (16). Expression of the *rpoB* housekeeping gene was used to normalize the transcript levels.

Serum resistance assay. The serum resistance of each *K. pneumoniae* strain was determined according to the method of Siu et al. (3). Bacterial suspensions containing 1.5×10^6 CFU/ml were collected from mid-log-phase cultures, mixed at a 1:3 (vol/vol) ratios with pooled human serum (Innovative Research Inc., Novi, MI), and incubated at 37°C. Colony counts were obtained for the initial mixture and after 2 h of incubation, using the serial dilution method. Serum susceptibilities were characterized by calculating the survival rate of each strain by plotting the average survival percentage against the incubation time. *K. pneumoniae* strains were considered sensitive to serum if CFU counts dropped to 1% of the original mixture and were considered resistant if at least 90% of the organisms survived after 2 h of incubation (17).

Biofilm assays. Biofilm assays were performed as described previously, with slight modifications (18). Briefly, 150 μl of bacterial culture (1.5×10^7 CFU/ml) was added to wells in 96-well flat-bottomed polystyrene plates and incubated for 16 to 24 h at 37°C. At the end of the incubation period, planktonic bacteria were removed by washing twice with 200 μl of distilled water. Wells were dried, treated with 200 μl of 0.5% crystal violet stain for 20 min, and washed twice with distilled water. The bound dye was solubilized with 200 μl of 95% ethanol and quantified by measuring the optical density at 600 nm (OD_{600}). Each curve was performed in triplicate.

Growth curve assays. To examine *in vitro* fitness costs under noncompetitive conditions, growth rates were determined for *K. pneumoniae* strains. Growth curves were generated by diluting equal numbers of CFU of each isolate (approximately 5×10^6 CFU/ml) in LB broth and incubating at 37°C with constant shaking (180 rpm). The OD_{600} of each culture was then measured at 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 12, and 24 h postdilution (10). Each curve was performed in duplicate.

***In vitro* competition assays.** To compare the relative fitness of each colistin-resistant mutant with that of the parental susceptible strain, the *in vitro* competitive index (CI) of each mutant was measured (19). Samples

TABLE 1 Primers used in this study

Primer	Sequence (5′→3′)[a]	Amplicon size (bp)	Reference
Primers for sequencing			
magA-F	CAAGTTTCGAAGCTCTTGGTG	1,884	This study
magA-R	CAAAACAAGCCAAACTGAAAGG		
g-rmpA-F	TTTTGCGAAAGTATTTATTTGATAAGG	980	This study
g-rmpA-R	TAAATAAAAGAGATCCTTCACATCCCC		
p-rmpA-F	TTTACCGTGATTGATTGAATTTTT	929	This study
p-rmpA-R	TTGCGTCTATTCATCGCTTTT		
p-rmpA2-F	CCATGCAAACACAAACACAA	978	This study
p-rmpA2-R	TTCTCAACCTCCTTCTTTGAGC		
Primers for qRT-PCR			
RT-pbgP-F	AACTACTGACCATGGCGGCG	116	This study
RT-pbgP-R	GCCAGCCAGTTCACCACGAA		
RT-magA-F	GTCAGGCAGCTGTTGTGAACG	135	This study
RT-magA-R	CACTTCTCGTATTTGCGGCGA		
RT-p-rmpA2-F	ATTACGTATGAAGGCTCGATGG	129	This study
RT-p-rmpA2-R	AATGTTTTCCTTAACATTTTATAACCAT		
RT-rpoB-F	CGCGTATGTCCGATCGAAA	100	This study
RT-rpoB-R	GCGTCTCAAGGAAGCCATATTC		
Primers for allelic replacement			
Kan-F	AACAGTGAATTGGAGTTCGTCTTGTTATA	907	22
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] The sequences identical to the 3′ and 5′ ends of the cassette gene sequence are underlined.

of the wild-type and mutant strains were harvested from cultures during exponential growth and mixed at a 1:1 proportion (1.5×10^3 CFU of each strain). Each mixture was then used to inoculate 10 ml of LB broth and incubated at 37°C with shaking (180 rpm) for 16 to 18 h (10). The total CFU in each mixture and the proportion of the colistin-resistant isolates were determined by plating serial 10-fold dilutions on LB agar and LB agar supplemented with 10 mg/liter of colistin and incubation overnight at 37°C. The CI was defined as the ratio of the CFU of the resistant mutant to the CFU of the parental strain. Two independent competition experiments were performed to calculate the average values for each CI. By definition, a fitness of 1 indicates that the mutation has no fitness effect. Meanwhile, a ratio greater than or less than 1 indicates increased or decreased fitness, respectively. The relative fitness cost of clinically isolated colistin-resistant strain K07-08-056 was compared with that of a colistin-susceptible strain, 08-B063.

Construction of specific gene deletion mutants. To investigate the relationships between two-component regulatory systems and CPS production, we deleted the *phoQ* and *pmrB* genes in a colistin-resistant mutant, 08-B063R. Plasmid pKD46 was used to induce λ red recombinase production in the host cell to enhance homologous recombination (20). The tetracycline and kanamycin cassettes were obtained from pDMS 197 vector and *Enterococcus faecalis* ATCC 51299, respectively (21, 22). A PCR product containing a tetracycline and kanamycin resistance cassette flanked by ~500 bp of the regions surrounding the *phoQ* and *pmrB* genes was introduced into the 08-B063R harboring pKD46 by electroporation (22). 08-B063R Δ*phoQ* or 08-B063R Δ*pmrB* mutants were selected on LB agar plates supplemented with 10 mg/liter of tetracycline or 50 mg/liter of kanamycin, respectively. The replacement of target genes was confirmed by colony PCR and sequencing.

Statistics. Data are presented as means ± standard deviations. Statistical analysis was performed as appropriate using Student's *t* test or analysis of variance (ANOVA), followed by a Dunnett multiple-comparison test using Prism version 3.00 for Windows (GraphPad Software, San Diego, CA). *P* values of <0.05 were considered to be statistically significant.

RESULTS

Antimicrobial susceptibility profiles. The MICs of a panel of antimicrobials for *K. pneumoniae* strains are presented in Table 2. The parental strains were susceptible to the majority of the antimicrobial agents tested, including aztreonam, cefotaxime, ceftazidime, ciprofloxacin, colistin, gentamicin, imipenem, piperacillin-tazobactam, and trimethoprim-sulfamethoxazole. Conversely, each of these strains was resistant to ampicillin. Meanwhile, with the exception of colistin and polymyxin B, only subtle changes were observed in the MICs of antimicrobial agents for the *in vitro*-derived colistin-resistant mutants.

Expression of two-component regulatory systems and modification of lipid A. qRT-PCR analysis indicated increased expression of the *phoQ*, *pmrD*, *pmrB*, and *pbgP* genes in the three colistin-resistant mutants 08-B063R, 07-B-060R, and 13703-3487R compared to the expression levels observed in the respective parental susceptible strains (Fig. 1). Specifically, the transcript levels

TABLE 2 Antimicrobial susceptibility profiles of the three pairs of colistin-susceptible and colistin-resistant *Klebsiella pneumoniae* strains

Strain[a]	MIC (mg/liter)[b]										
	COL	PB	AMP	AZ	CTX	CAZ	CIP	GEN	IMI	P/T	SXT
08-B063	0.5	2	64	≤0.06	≤0.06	0.5	≤0.06	0.5	0.125	8/4	0.12/2.37
08-B063R	512	>64	>64	≤0.06	≤0.06	0.5	0.125	0.5	0.125	16/4	0.5/9.5
07-B-060	0.5	0.5	>64	≤0.06	≤0.06	0.5	≤0.06	0.5	0.25	16/4	0.5/9.5
07-B-060R	256	>64	>64	≤0.06	≤0.06	0.5	≤0.06	0.5	0.125	8/4	0.5/9.5
13703-3487	0.5	0.25	>64	≤0.06	≤0.06	0.25	≤0.06	1	0.25	8/4	2/38
13703-3487R	256	64	>64	≤0.06	≤0.06	0.25	≤0.06	0.5	0.125	8/4	1/19

[a] All strains are of ST23 in the Multi Locus Sequence Typing database.
[b] COL, colistin; PB, polymyxin B; AMP, ampicillin; AZ, aztreonam; CTX, cefotaxime; CAZ, ceftazidime; CIP, ciprofloxacin; GEN, gentamicin; IMI, imipenem; P/T, piperacillin-tazobactam; SXT, trimethoprim-sulfamethoxazole.

of the *phoQ*, *pmrD*, *pmrB*, and *pbgP* genes increased from 1.8- to 7.5-fold, 6.3- to 9.5-fold, 1.6- to 17.8-fold, and 26.4- to 58.8-fold in the colistin-resistant mutants compared to the parental strains, respectively.

pmrD and *pbgP* expression increased consistently in all colistin-resistant mutants compared with their respective colistin-susceptible strains. Strain 08-B063R exhibited markedly higher expression of *phoQ*, but not of *pmrB*, than did the parental strain. In contrast, the expression levels of *pmrB*, but not of the *phoQ* gene, were higher in the 07-B-060R and 13703-3487R strains than in their respective parental strains.

There were two predominant peaks (*m/z* 1,824 and *m/z* 1,840) of hexa-acylated species in the MALDI-TOF mass spectra of the lipid A structures of all three pairs of *K. pneumoniae* strains (Fig. 2). The hexa-acylated species at *m/z* 1,824 likely represents a hexa-acylated lipid A containing two glucosamines, two phosphates, four 3-OH-C_{14}s, and two C_{14}s. Meanwhile, the peak at *m/z* 1,840 likely represents a hexa-acylated lipid A containing two glucosamines, two phosphates, four 3-OH-C_{14}s, one C_{14}s, and one C_{14}:OH (hydroxymyristate) (8). Furthermore, the peaks at *m/z* 1,955 and *m/z* 1,971 likely corresponded to the addition of aminoarabinose (theoretical *m/z* of 131) to the two major hexa-acylated species, and the peaks at *m/z* 2,063 and *m/z* 2,079 likely corresponded to palmitoylated lipid A molecules (palmitate has a theoretical *m/z* of 238) (8). Although the addition of aminoarabinose to the *m/z* 1,824 species of lipid A was detected in two colistin-susceptible strains, 08-B063 and 07-B-060, this modification was not detected in the *m/z* 1,840 species of any of the susceptible strains. In contrast, the addition of aminoarabinose to the *m/z* 1,840 species was identified in two colistin-resistant mutants, 07-B-060R and 13703-3487R. Palmitoylation of both the *m/z* 1,824 and *m/z* 1,840 lipid A species was detected in each of the mutant and parental strains; however, the relative amount of palmitoylated lipid A increased significantly in colistin-resistant mutants

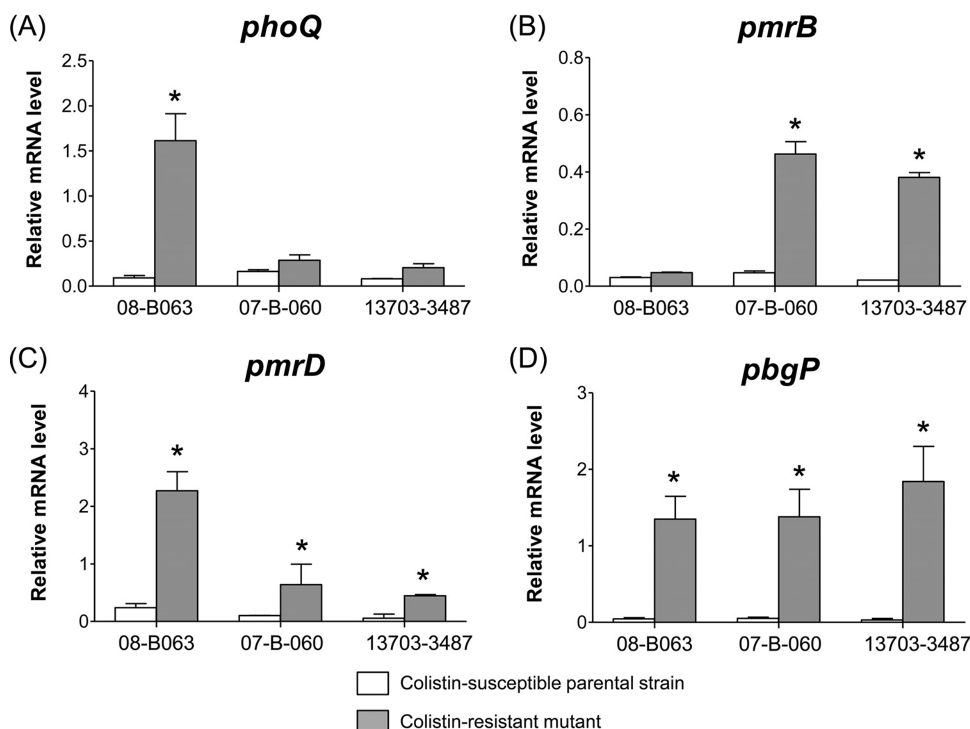

FIG 1 Comparison of the expression levels of *phoQ* (A), *pmrB* (B), *pmrD* (C), and *pbgP* (D) in the three pairs of *Klebsiella pneumoniae* ST23 strains. Error bars indicate the standard deviations for three triplicate samples. *, $P < 0.05$ versus the result for a corresponding colistin-susceptible parental strain.

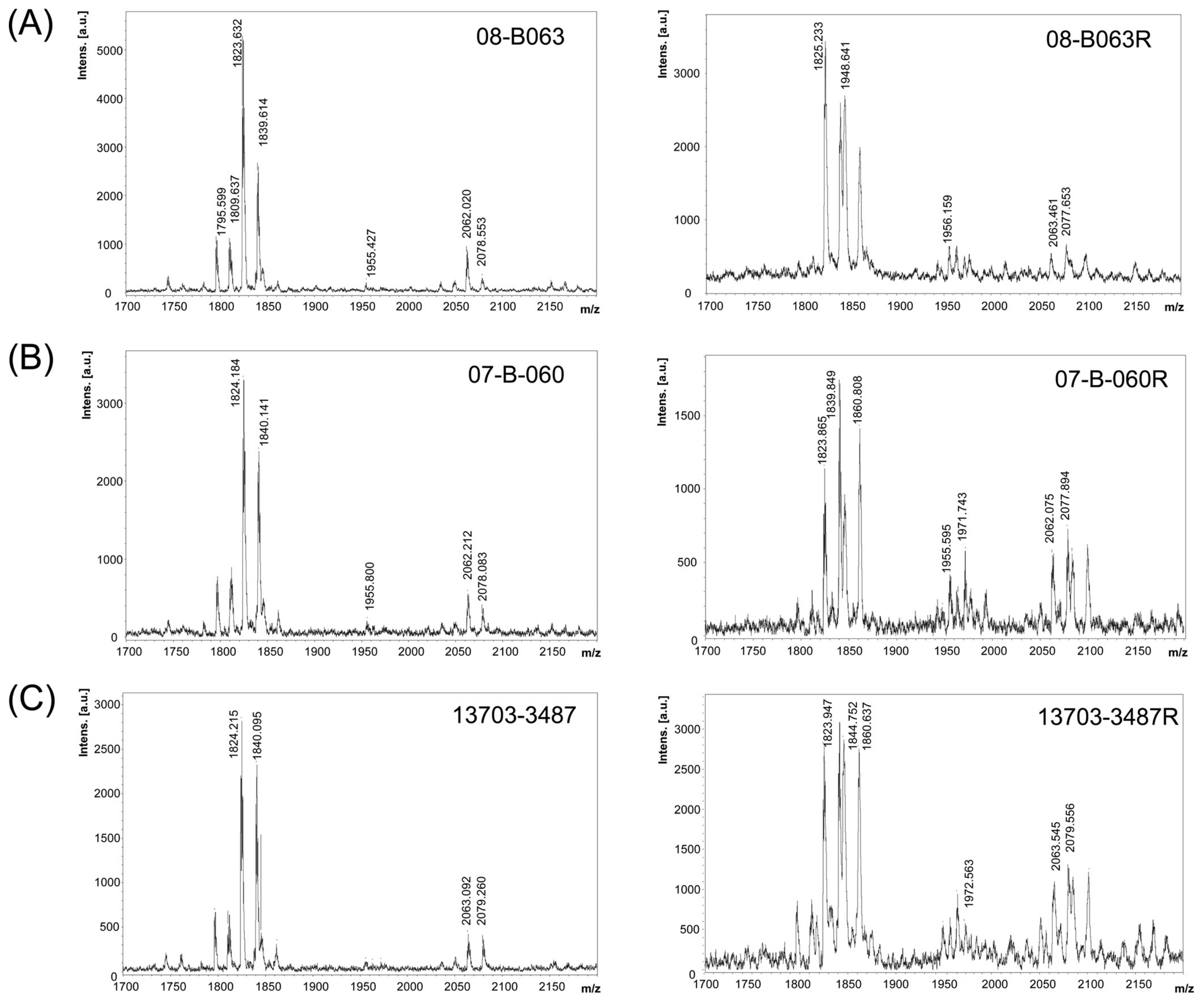

FIG 2 Lipid A structures of *Klebsiella pneumoniae* ST23 strains. Shown are the negative-ion matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry spectra of the lipid A moieties of lipopolysaccharide isolated from strains 08-B063 and 08-B063R (A), 07-B-060 and 07-B-060R (B), and 13703-3487 and 13703-3487R (C).

(Table 3). Likewise, the addition of aminoarabinose to the *m/z* 1,824 lipid A species was significantly greater in the 08-B063R and 07-B-060R strains than in the parental strains (Table 3).

Decreased HV phenotype and CPS formation in colistin-resistant mutants. All strains were identified as serotype K1 by PCR analysis, and the HV phenotype was observed in the three colistin-susceptible *K. pneumoniae* strains by string test (Fig. 3A). In contrast, while the 07-B-060R and 13703-3487R mutants exhibited positive string tests, the lengths of the bacterial strings from colonies of these strains were reduced in comparison to those of the parental strains. The clinically isolated colistin-resistant strain K07-08-056 also showed negativity in the string test (Fig. 3A). In addition, the colistin-resistant mutant 08-B063R and clinical colistin-resistant strain K07-08-056 appeared to have lost the HV phenotype entirely (Fig. 3B). Consistent with these results, CPS production by the colistin-resistant mutants decreased compared with that of the colistin-susceptible parental strains (Fig. 3C).

TABLE 3 Relative intensities of minor lipid A species in three pairs of *Klebsiella pneumoniae* ST23 strains

	Relative intensity of modified acylated species[a]			
	Addition of aminoarabinose (m/z 131) to:		Addition of palmitate (m/z 238) to:	
Strain	C_{14} (m/z 1,824)	C_{14}:OH (m/z 1,840)	C_{14} (m/z 1,824)	C_{14}:OH (m/z 1,840)
08-B063	2.06	ND[b]	17.35	13.76
08-B063R	14.67	ND	11.76	19.23
07-B-060	4.87	ND	16.86	17.90
07-B-060R	36.67	32.29	47.54	42.41
13703-3487	ND	ND	15.06	15.71
13703-3487R	ND	31.29	39.39	39.99

[a] Relative intensities of the modified acylated structures (addition of aminoarabinose or palmitate) compared with those of the major acyl group structures (backbone) are displayed. The intensity of the major acyl group structure is regarded as 100.
[b] ND, not detected.

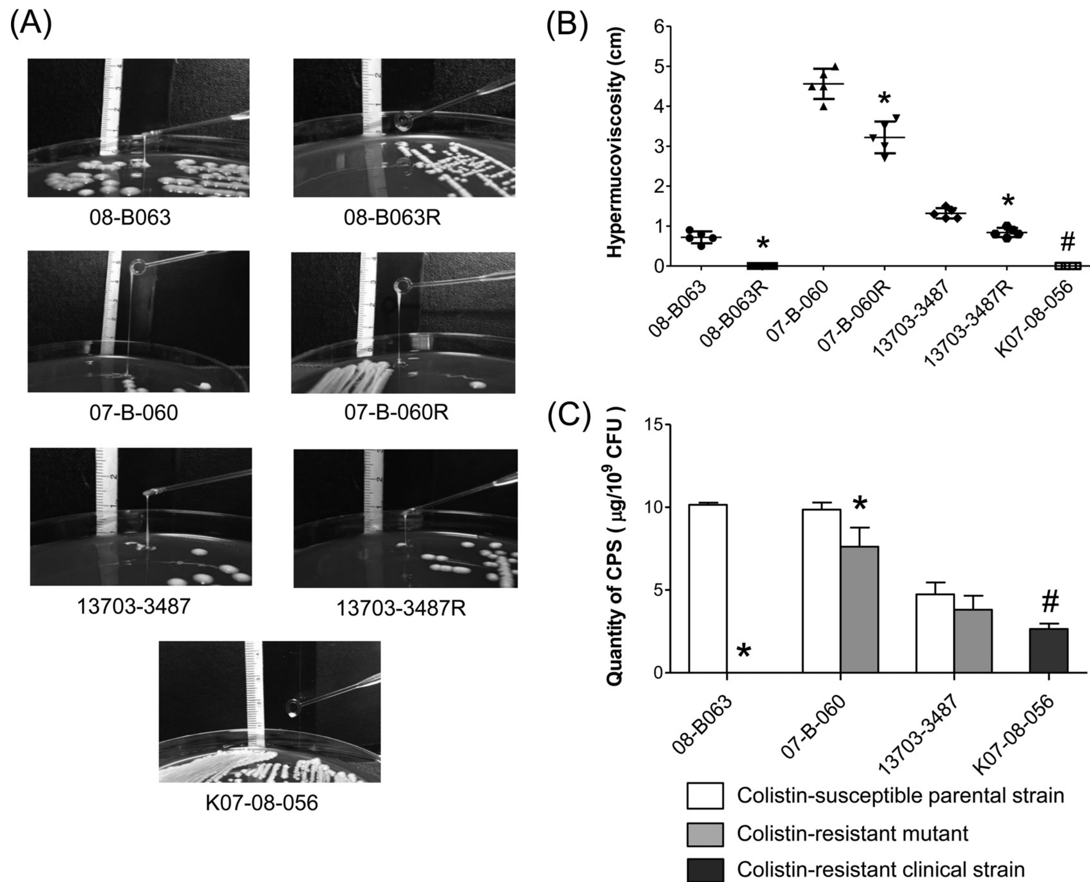

FIG 3 Mucoviscosity and capsular polysaccharide (CPS) production. (A and B) Mucoviscosity. The string test was used to assess the hypermucoviscosity of *K. pneumoniae* strains. A string of 5 mm or longer is defined as positive. (C) CPS production. CPS biosynthesis in the *K. pneumoniae* strains was determined by phenol-sulfuric acid assays. *, $P < 0.05$ versus the value for a corresponding colistin-susceptible parental strain; #, $P < 0.05$ versus the value for colistin-susceptible strain 08-B063. Error bars indicate the standard deviations for three triplicate samples.

Clinical colistin-resistant strain K07-08-056 also showed decreased CPS production compared with that of colistin-susceptible strain 08-B063. Especially noteworthy, no CPS production was detectable in strain 08-B063R.

Expression of genes related to the HV phenotype. Sequencing analyses detected no amino acid variations corresponding to the *magA*, g-*rmpA*, p-*rmpA*, and p-*rmpA2* genes among the six *K. pneumoniae* strains utilized in this study. However, qRT-PCR analysis detected a 34.1% to 40.3% decrease in *magA* expression in the three colistin-resistant *K. pneumoniae* mutants compared to the levels measured in the colistin-susceptible parental strains (Fig. 4A). Likewise, the expression levels of the plasmid-borne gene p-*rmpA2*, which also plays a role in the HV phenotype of *K. pneumoniae* (23), were decreased in the mutant strains in comparison to the progenitor strains; however, this decrease was less than that observed for *magA* (Fig. 4B).

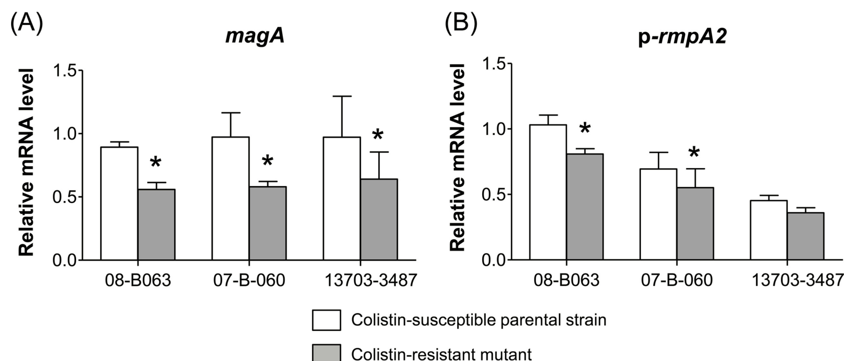

FIG 4 Relative transcriptional levels of *magA* (A) and p-*rmpA2* (B) in the three pairs of *Klebsiella pneumoniae* strains. Error bars indicate the standard deviations for three triplicate samples.

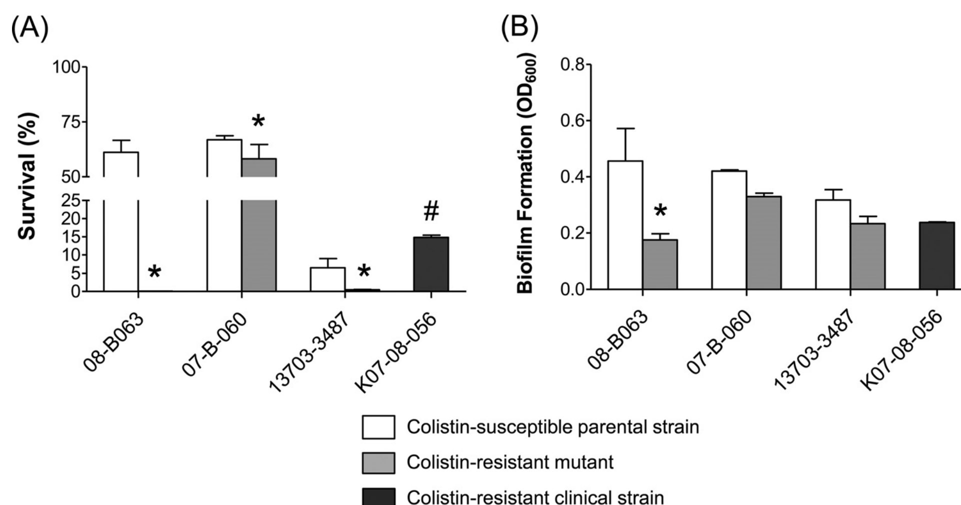

FIG 5 (A) Serum resistance assays of wild-type and colistin-resistant *Klebsiella pneumoniae* strains. Serum resistance is represented as percent viability (no. of colonies after 2 h of incubation/no. of colonies for the initial mixture). Error bars indicate standard deviations. Survival rates of each colistin-susceptible parental strain (white) and colistin-resistant mutant (gray) were evaluated and compared after incubation for 2 h with human serum. (B) Analysis of biofilm formation by colistin-susceptible and -resistant *Klebsiella pneumoniae* strains. Overnight cultures of *K. pneumoniae* strains were grown in fresh Luria-Bertani (LB) broth at a ratio of 1:100 in polystyrene plates at 37°C for 5 h. The bacteria were stained with crystal violet, washed to remove unbound cells, and eluted with 95% ethanol, and biofilm masses were detected by measuring the absorbance at 600 nm. *, $P < 0.05$ versus the value for a corresponding colistin-susceptible parental strain; #, $P < 0.05$ versus the value for colistin-susceptible strain 08-B063. Error bars indicate the standard deviations for three triplicate samples.

Reduced serum resistance and biofilm formation. Survival rates of the colistin-susceptible parental strains and the *in vitro*-selected colistin-resistant mutants were evaluated in the presence of normal human serum over a 2-h period (Fig. 5A). Two colistin-resistant derivatives, 08-B063R and 13703-3487R, exhibited markedly reduced survival rates in the presence of normal human serum compared their respective colistin-susceptible parental strains. Meanwhile, there was no significant decrease in the serum survival rate of strain 07-B-060R compared to that of the parental strain. Clinical colistin-resistant strain K07-08-056 showed a reduced survival rate against normal human serum compared with those of colistin-susceptible strains 08-B063 and 07-B-060.

Figure 5B depicts the results of biofilm formation assays for the three pairs of *K. pneumoniae* strains and a clinically isolated colistin-resistant strain. Compared with the control strain *P. aeruginosa* ATCC 27853, which produces relatively robust biofilms (OD_{600}, 1.52), the wild-type colistin-susceptible strains were not strong biofilm producers. Nevertheless, colistin-resistant mutants exhibited even smaller amounts of biofilm formation than their respective parental strains; however, the decreases in biofilm formation of strains 07-B-060R and 13703-3487R were not statistically significant. Biofilm-forming activity of clinical colistin-resistant strain K07-08-056 was lower than those of colistin-susceptible strains, but the difference in biofilm formation was not statistically significant.

Bacterial growth rate and fitness. To investigate whether colistin-resistant mutants exhibit a fitness defect compared to their respective parental strains, growth curves and *in vitro* competition experiments were performed (Fig. 6). Although there was some decrease in the growth rates of the 08-B063R and 07-B-060R strains compared to their progenitor strains during the stationary phase (Fig. 6A and B), the growth rates between colistin-susceptible strains and colistin-resistant mutants were not different significantly. The growth rate of clinical colistin-resistant strain K07-08-056 was also not different from those of colistin-susceptible strains.

While only modest decreases were observed in the growth rates of the mutant strains during single culture, the CI values indicated that the colistin-resistant mutants exhibited significant fitness defects when cocultured with the parental strains (CI values, 0.01 to 0.19; Fig. 6D). The clinical colistin-resistant strain K07-08-056 also showed a significant fitness cost compared with colistin-susceptible strain 08-B063 (CI value, 0.001).

Variations of *pmrAB*, *phoPQ*, and *mgrB* genes among colistin-resistant mutants. Colistin-resistant mutants developed from the same colistin-susceptible strain showed consistent phenotypes in string test, CPS production, serum resistance, biofilm formation, and fitness cost (data not shown). Amino acid variations of *pmrAB* and *phoPQ* in colistin-resistant mutants and a clinical colistin-resistant strain are shown in Table 4. Amino acid substitutions were different among colistin-resistant mutant lineages, but they were consistent among the mutants from the same colistin-susceptible strain, except 07-B-060. No amino acid substitutions were observed in *pmrA* and *mgrB* genes in this study.

Mucoviscosity and CPS production of *phoQ* or *pmrB* deletion mutants. Both the 08-B063R Δ*phoQ* and 08-B063R Δ*pmrB* mutants exhibited negative string test results (Fig. 7A). While CPS production in the 08-B063R Δ*phoQ* mutant was identified, no CPS was detected in the 08-B063R Δ*pmrB* mutant (Fig. 7B). Although CPS production in the 08-B063R Δ*phoQ* mutant was significantly higher than that in colistin-resistant mutant 08-B063R, it did not restore the level of that of the susceptible parental strain, 08-B063.

DISCUSSION

HV *K. pneumoniae* isolates are highly invasive and have emerged as a major cause of pyogenic liver abscesses as well as infections

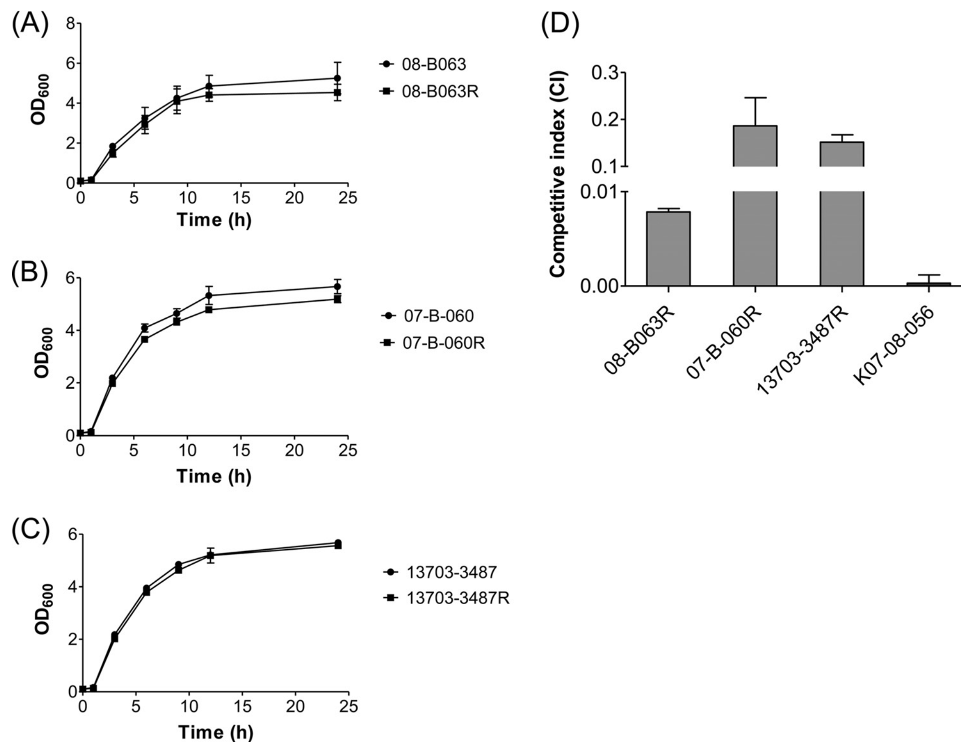

FIG 6 Growth rates and fitness costs. (A to C) Growth curves of colistin-susceptible (S) and -resistant (R) *Klebsiella pneumoniae* strains. *y* axis, optical densities (OD) of broth cultures at 600 nm; *x* axis, time of growth (hours). (D) Fitness costs of colistin-resistant mutants relative to those of their colistin-susceptible parental strains. The relative fitness of colistin-resistant clinical strains with 08-B063 was also measured. A CI value less than 1 indicates a fitness defect, and a value greater than 1 indicates a fitness benefit. Each of the three colistin-resistant mutants (CI values, 0.001 to 0.19) exhibited a marked fitness defect. Error bars indicate the standard deviations for three triplicate samples.

resulting in metastatic complications, including endophthalmitis, meningitis, and cerebral and pulmonary abscesses (24). Serotype K1 is common in HV *K. pneumoniae* isolates (2), and ST23 strains were found to comprise the majority of serotype K1 *K. pneumoniae* isolates exhibiting the HV phenotype (25). In South Korea, ST23 strains accounted for 97.3% of K1 serotype *K. pneumoniae* isolates collected from patients with community-acquired liver abscesses (26). Consistent with these observations, the three colistin-susceptible HV *K. pneumoniae* isolates used in this study were ST23 strains of the K1 serotype. Here we report that the acquisition of colistin resis-

TABLE 4 Amino acid alterations of PhoPQ, PmrAB, and MgrB of colistin-resistant mutants compared to their parental strains

		Amino acid alteration at:								
		PhoP residue			PhoQ residue(s)				PmrB residue(s)	
Parental strain	Resistant mutant	13	15	76	341–352	757	803	1252	256–302	1031
08-B063	R1				Deletion		Y268S			F344L
	R2				Deletion		Y268S			F344L
	R3				Deletion		Y268S			F344L
	R4				Deletion		Y268S			F344L
07-B-060	R1					N253D	Y268C		46-bp insert	
	R2						Y268C		46-bp insert	
	R3						Y268C		46-bp insert	
	R4						Y268C		46-bp insert	
13703-3487	R1			V26L				D418N	46-bp insert	
	R2			V26L				D418N	46-bp insert	
	R3			V26L				D418N	46-bp insert	
	R4			V26L				D418N	46-bp insert	
K07-08-056[a]		V5L	L7S	V26L						P344L

[a] Amino acid alterations of a colistin-resistant clinical strain compared to *K. pneumoniae* NTUH-K2044.

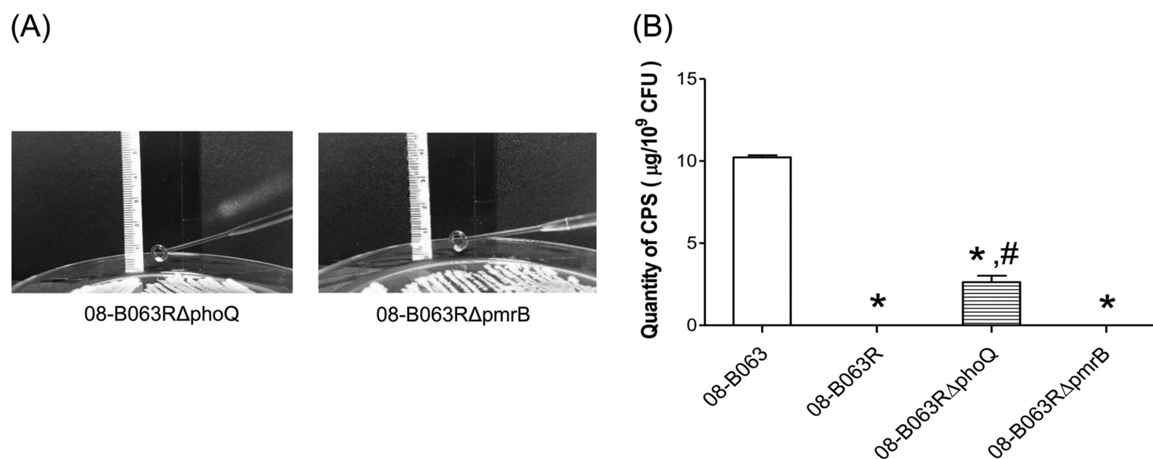

FIG 7 Mucoviscosity and CPS production of *phoQ* and *pmrB* deletion mutants. (A) Mucoviscosity of mutants. The string test was used to assess the hypermucoviscosity of mutants. A string length less than 5 mm was defined as negative. (B) CPS production of mutants. Error bars indicate the standard deviations for three triplicate samples. *, $P < 0.05$ versus value for colistin-susceptible strain 08-B063; #, $P < 0.05$ versus value for colistin-resistant strain 08-B063R.

tance may lead to the loss of HV, resulting in decreased virulence and increased biological cost in *K. pneumoniae* ST23 strains.

We confirmed that the acquisition of colistin resistance in *K. pneumoniae* is associated with upregulation of the genes encoding the PhoPQ or PmrAB two-component regulatory systems and subsequent increases in *pmrD* and *pbgP* expression. Our findings that only either the *phoQ* or the *pmrB* gene was overexpressed in a colistin-resistant mutant led us to propose that each of PhoPQ and PmrAB may be associated differentially with colistin resistance in *K. pneumoniae*, instead of the connection between two two-component regulatory systems. MALDI-TOF mass spectrometry (MS) analysis demonstrated that the addition of aminoarabinose and palmitate to the lipid A moiety of LPS was increased in the colistin-resistant mutants (27). These results indicate that the mutants derived from susceptible parents acquired colistin resistance by typical mechanisms, as described previously (28).

Our results showed that the HV phenotype was decreased or eliminated in the *in vitro*-selected colistin-resistant mutants and a clinically isolated colistin-resistant strain and that this decrease was accompanied by reduced CPS production. Consistent with these results, the expression levels of the *magA* and p-*rmpA2* genes, which regulate CPS production in *K. pneumoniae*, were reduced significantly in the mutant strains in comparison to their respective parental strains. Hypervirulent *K. pneumoniae* isolates are associated with high levels of CPS production and the presence of virulence genes such as *magA*, g-*rmpA*, p-*rmpA*, and p-*rmpA2* (3, 4). The serotype K1 polymerase gene *magA* is a crucial virulence gene that regulates CPS biosynthesis (29). Likewise, the *rmpA* genes, which are chromosomally (g-*rmpA* gene) or plasmid (p-*rmpA* and p-*rmpA2*) encoded, are involved in CPS production, which is responsible for enhanced colony mucoidy and virulence phenotypes (30). The correlation between CPS production and resistance to antimicrobial peptides, such as polymyxin B, has been reported. Campos et al. (9) demonstrated that CPS could inhibit the interaction of polymyxin B with the bacterial cell surface, and that antimicrobial peptides stimulate CPS production in the K2, K3, K35, and K47 serotypes of *K. pneumoniae*, which contrasts with our results. Recently, however, Srinivasan et al. (31) reported that *K. pneumoniae* NTUH-K2044, which, like the strains used in this study, is a K1 serotype ST23 strain, exhibited high levels of CPS production and was extremely susceptible to colistin. This group also revealed that an NTUH-K2044 Δ*kpnEF* mutant lost the HV phenotype and exhibited decreased CPS synthesis and decreased expression of CPS cluster genes; however, no significant decrease in colistin resistance was observed (31). Thus, CPS production may not directly influence the MIC of colistin for *K. pneumoniae*. *phoQ* and *pmrB* deletion mutants from a colistin-resistant mutant did not restore the CPS production of their susceptible strain, suggesting that two-component regulatory systems may have no direct relationships with CPS production in *K. pneumoniae*. Instead, colistin may act as an environmental stimulus that affects two-component regulatory systems, such as PmrAB or PhoPQ, which influence CPS production and HV indirectly.

The HV phenotype associated with CPS production is a well-known virulence determinant in *K. pneumoniae* (3, 4). In addition to reduced HV and CPS production, our colistin-resistant derivatives exhibited increased serum sensitivity and reduced biofilm formation. The bactericidal effect of serum is regarded as a host defense system and is mediated by complement proteins. Thus, the stimulation with colistin resulted in both colistin resistance and impaired virulence-related phenotypes in the mutant strains. Colonization of nosocomial bacteria is associated with the resistance of these organisms to complement-mediated killing (32), and CPS provides an advantage for mouse bladder colonization by a uropathogenic *E. coli* strain (33). Furthermore, Leying et al. reported that serum resistance correlated with the amount of K1 type CPS production. In contrast, alterations in LPS structure did not correlate with serum resistance (34). In our study, the dramatic decrease in serum resistance associated with the 08-B063R strain may have been the result of the loss of HV due to reduced CPS production.

Biofilm-producing bacteria exhibit enhanced infectivity and increased persistence, which result in higher colonization. Furthermore, biofilms are often associated with chronic infections due to their low susceptibility to host immune responses and to antimicrobial agents (35). Boddicker et al. demonstrated a correlation between biofilm formation and K2 type CPS production in

K. pneumoniae (36). In addition, Wu et al. reported that *treC* and *sugE* affect biofilm formation by modulating CPS production in the *K. pneumoniae* NTUH-K2044 strain (18). Accordingly, the reduction of biofilm-forming activity in colistin-resistant mutants may be associated with the observed decrease in CPS production after exposure to colistin. Thus, colistin-resistant derivatives exhibited several impaired virulence phenotypes *in vitro* that may be interrelated, including reductions in HV, CPS production, serum resistance, and biofilm formation. The colistin-resistant mutants developed from the same parental susceptible strain showed similar phenotypes, suggesting that the differences of phenotypic and genotypic alterations between colistin-resistant mutants might be dependent upon the parental strain. However, the *in vivo* relevance of these virulence defects, and the mechanism by which two-component systems, such as PhoPQ and PmrAB, regulate CPS production requires further investigation. Likewise, a study of the clinical outcomes of infections due to colistin-resistant *K. pneumoniae* isolates is necessary.

In this study, we also revealed that colistin resistance affects the fitness of *K. pneumoniae*. Reduced fitness in colistin-resistant organisms was reported in several previous studies, particularly for *A. baumannii* (10, 11). Beceiro et al. demonstrated that a colistin-resistant mutant of *A. baumannii* with no production of lipid A moiety exhibited a significant fitness reduction in both *in vitro* and *in vivo* competitive-growth experiments (10). However, they also showed that lipid A modification through phosphoethalolamine addition yielded only a subtle fitness cost (10), and another study found that there was no difference in the fitness of a *K. pneumoniae* strain that had acquired colistin resistance due to a mutation in *pmrB* compared to that of its colistin-susceptible progenitor strain (12). These findings suggest that different resistance mechanisms involve different fitness burdens. This inconsistency in the fitness burdens associated with colistin resistance may be due to strain-specific differences. While the parental strains used in this study are highly virulent and exhibit the HV phenotype, multiple alterations to physiological functions, including those associated with virulence and fitness, may have occurred during the development of colistin resistance. Such alterations would likely result in significant differences in our strains compared to those acquiring colistin resistance via a single mutation. Regardless, high fitness burdens in colistin-resistant strains may explain the low prevalence and restricted spread of colistin resistance in *K. pneumoniae*, particularly in HV ST23 strains, in clinical settings (7). However, since the fitness burden and virulence of antimicrobial-resistant strains is a crucial issue related to the dissemination of these organisms, further investigation is required (37).

In summary, this study elucidated the effects of colistin resistance on several virulence phenotypes in hypervirulent *K. pneumoniae* strains. We found that development of colistin resistance can occur via the upregulation of certain two-component regulatory systems and through modifications of the lipid A moiety of LPS, and that this resistance may result in defects in virulence-related phenotypes, including CPS production, HV, and serum resistance. In addition, we demonstrated an *in vitro* fitness defect in colistin-resistant mutants compared to the hypervirulent *K. pneumoniae* parental strains.

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