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Mutations and expression of PmrAB and PhoPQ related with colistin resistance in *Pseudomonas aeruginosa* clinical isolates

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ABSTRACT

To comprehend the resistance of colistin resistance, we investigated the relationships between amino acid alterations and expression of PmrAB and PhoPQ and colistin resistance in 16 colistin-nonsusceptible clinical *Pseudomonas aeruginosa* isolates. In addition, we obtained induced colistin-resistant mutants and their colistin-susceptible revertants. Expression levels of the *pmrA*, *phoP*, *parR*, *cprR*, and *pmrH* genes were determined for them. Nine amino acid substitutions unique to 10 colistin-nonsusceptible *P. aeruginosa* (CNPA) isolates were identified: 7 in PmrB and 1 each in PmrA and PhoQ. However, 6 CNPA isolates did not show amino acid substitutions compared with colistin-susceptible *P. aeruginosa* isolates. Among 16 CNPA isolates, 7 and 8 isolates displayed activated expression of *pmrA* and *phoP*, respectively. Activated expression of *pmrA* and/or *phoP* was identified in 13 isolates of CNPA isolates, but some had no noticeable PmrAB and PhoPQ amino acid substitutions. In addition, in vitro selected colistin-resistant mutants (P5R and P155R) showed higher expression level in *pmrA*, *phoP*, and *pmrH* than their parent strains (P5 and P155) and colistin-susceptible, revertant strains (P5R-rev and P155R-rev). However, expression of the *parR* and *cprR* genes was not consistent. Our data may indicate that amino acid substitutions of PmrAB or PhoPQ do not have an immediate connection with decreased susceptibility of colistin in *P. aeruginosa* isolates, although activated expression of *pmrAB* and/or *phoPQ* resulting in overexpression of *pmrH* may be required for colistin resistance. Expression of *pmrAB* or *phoPQ* related with colistin nonsusceptibility may not explained by a single mechanism, which may suggest that colistin resistance appears easily by diverse pathways in clinical settings as well as in laboratory.

1. Introduction

Pseudomonas aeruginosa causes serious hospital-acquired infections in immunocompromised patients and fatal lung disease in patients with cystic fibrosis. *P. aeruginosa* is 1 of the major organisms responsible for nosocomial infections such as pneumonia, urinary tract infections (UTIs), surgical site infections, and bloodstream infections (Lister et al., 2009). *P. aeruginosa* has an ability to develop resistance to multiple classes of antimicrobial agents, provoking the emergence of multidrug-resistant (MDR) isolates. The emergence and spread of MDR Enterobacteriaceae isolates including *P. aeruginosa* has led to the resurgence of use of polymyxin antibiotics such as polymyxin B and colistin as therapeutic agents. With increasing use of polymyxins, polymyxin-resistant *P. aeruginosa* isolates have been reported from around the world (Falagas et al., 2008; Landman et al., 2005; Wang et al., 2006).

Polymyxin resistance is associated with specific modification of the lipid A component of the outer membrane lipopolysaccharide (LPS), resulting in a reduction of the net negative charge of the outer membrane. *P. aeruginosa* exhibits lipid A modifications under Mg^{2+}-limiting conditions (Moskowitz et al., 2004). It has been reported that two 2-component regulation systems, PhoP-PhoQ and PmrA-PmrB, respond to Mg^{2+}-limiting conditions, resulting in polymyxin resistance in *P. aeruginosa* (McPhee et al., 2006). In addition, it has recently been reported that amino acid alterations in PhoPQ and PmrAB are associated with polymyxin B resistance in clinical *P. aeruginosa* isolates (Abraham and Kwon, 2009; Barrow and Kwon, 2009; Schurek et al., 2009). Recently, ParRS and CprRS 2-component regulatory systems have also been found to play a role in polymyxin resistance in *P. aeruginosa* (Fernández et al., 2010; Fernández et al., 2012). However, those studies analyzed polymyxin B resistance-induced strains or only a small number of polymyxin B–resistant isolates.

In this study, we investigated the amino acid substitution of PmrA-PmrB and PhoP-PhoQ in colistin-nonsusceptible *P. aeruginosa* (CNPA) and colistin-susceptible *P. aeruginosa* (CSPA) clinical isolates from South Korea. Additionally, we compared and analyzed the difference of *pmrA* and *phoP* expression by colistin susceptibility among clinical isolates, in vitro selected mutants, and their susceptible revertants.

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2. Materials and methods

2.1. P. aeruginosa isolates

Sixteen colistin-nonsusceptible (8 colistin-resistant and 8 colistin-intermediate) and 6 CSPA isolates were included in this study (Lee et al., 2011). These isolates were among those collected during a nationwide surveillance study on bacteremia and UTIs between November 2006 and August 2007. In vitro antimicrobial susceptibility testing was performed by a broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2011). Colistin-nonsusceptible *P. aeruginosa* isolates were selected on the basis of intermediate susceptibility (MIC = 4 mg/L) or resistance (MIC ≥8 mg/L) to colistin (CLSI, 2011). *Escherichia coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were employed as reference strains.

2.2. In vitro selection of colistin-resistant mutants and their colistin-susceptible, revertant strains

To investigate the genetic alteration of *pmrAB* or *phoPQ* caused by the change of colistin susceptibility, we cultivated induced colistin-resistant mutants. These were developed from 2 colistin-susceptible isolates, P5 and P155, through serial passage in increasing colistin concentrations (Park et al., 2011). As a result, 2 in vitro selected colistin-resistant mutants, P5R and P155R, were obtained. Their colistin MICs exceeded 64 mg/L. We also obtained their colistin-susceptible, revertant strains, P5R-rev and P155R-rev, by serial passage of in vitro selected colistin-resistant mutants (P5R and P155R) in drug-free medium. After 14 serial passages of P155R and 22 passages of P5R, complete colistin-susceptible revertants (MICs, 0.5 mg/L) could be obtained.

2.3. pmrAB and phoPQ sequencing

Full-length *pmrAB* (2123 bp) and *phoPQ* (2021 bp) were amplified and sequenced using the primers listed in Table 1. Amino acid sequences of 16 CNPA isolates were compared with those of other CSPA isolates and CSPA reference strain PAO1 (GenBank accession number, AE004091.2). To predict whether amino acid substitutions in PmrAB and PhoPQ affect protein function, Sorting Intolerant From Tolerant (SIFT) scores were calculated (http://sift.jcvi.org). Additionally, we performed the SMART analysis (http://smart.embl.de/) for the determination of domain architectures in PmrA, PmrB, PhoP, and PhoQ.

2.4. Quantitative reverse transcription PCR (qRT-PCR)

Expression levels of *pmrA*, *phoP*, *parR*, *cprR*, and *pmrH* were determined by qRT-PCR as described previously with some modification (Dumas et al., 2006). In brief, total RNA of 16 CNPA and 5 CSPA isolates were extracted from the mid-log phase bacterial culture (optical density at 600 nm of approximately 0.5) using the Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. Reverse transcription reactions were performed in accordance with the protocol for the use of Omniscript reverse transcriptase (Qiagen). Quantification of *pmrA*, *phoP*, *parR*, *cprR*, and *pmrH* transcripts was performed by using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) on ABI7300 Sequence Detection System (Applied Biosystems). Expression of the 30S ribosomal gene *rpsL* was assessed in parallel to normalize the transcriptional levels of target genes. Experiments were repeated with 2 independent cultures, each tested in duplicate.

2.5. Statistical analysis

Differential gene expression of *pmrA* and *phoP* between CNPA and CSPA was analyzed with Student's *t* test, using a normal significance level $P \leq 0.05$. The SPSS version 11.5 for Windows (SPSS Inc, Chicago, IL, USA) was used for statistical analysis.

Table 1
Primers used in this study.

Gene	Primer	Sequence (5′ → 3′/5′ → 3′)	Amplicon size (bp)	Reference
Primers for sequencing				
pmrAB	Pmr-F1/R1	TGCAGGACCTCTACGAACTG/ AGCTCGGCAACCAGTTGATC	772	This study
	Pmr-F2a/R2	TTCGATCTCGACGAACTGCA/ ACCATGAACCTGCTGCTGTT	874	This study
	Pmr-F3/R3	TGCTGTCGAGCCTCAACCTG/ CATCGACAAGGACATCGAGC	658	This study
	Pmr-F4/R4	TTGATGGAGCAGATCCTGCT/ AAGATGATCCGTTACTACGA	689	This study
phoPQ	Pho-F1/R1	GACAACAACACCAAGCTGTT/ TGAGGATCAGGATCGGGAAG	593	This study
	Pho-F2/R2	AGCGAATACCACCACGACC/ GAGCATCAGACGGATGCGCA	567	This study
	Pho-F3/R3	TGATGGAACAGCTCTATCCC/ TTGAGCAGGCTCTCGAACT	634	This study
	Pho-F4/R4	GAGGAGTTCTTCGTGTTCGA/ GTCGCGATAGACCTTGTCCA	606	This study
	Pho-F5/R5	AGCAGATAGGCTATCAGTTG/ CCACTGAACATCCGCTCGAA	603	This study
Primers for qRT-PCR				
rpsL	RpsL-QF/QR	GTGGTGAAGGTCACAACCTG/ CCTGCTTACGGTCTTTGACA	135	Lee et al., 2012
pmrA	Pmr-QF/QR	CACCAGGTGACCCTGTCC/ CGTAGAGGCTCTGCTCCAGT	124	Lee et al., 2012
phoP	Pho-QF/QR	TCTACCGGGTCAGCGAATAC/ GATCAGGATCGGGAAGGACT	122	Lee et al., 2012
parR	Par-QF/QR	AGAATGGTCTGCAGGTGTGC/ CGGCTTGATCACGTAGTCGT	134	This study
cprR	Cpr-QF/QR	CGCCTGGAAGATCCTTGAGT/ CACGTTGAGGGTGTTGCTTT	115	This study
pmrH	PmrH-QF/QR	GTTCGTCAGCGACGACAGT/ AAACCGGGCTCGATAACTTC	129	This study

3. Results

3.1. Amino acid variations of PmrAB and PhoPQ

Specific mutations in the PmrAB and PhoPQ 2-component systems are known to be associated to the colistin resistance in *P. aeruginosa*. In this study, we determined whether these mutations confer colistin resistance in several clinical isolates by analyzing 666-bp sequences of *pmrA* gene, 1434 bp of *pmrB* gene, 678 bp of *phoP* gene, and 1347 bp of *phoQ* gene against 16 CNPA and 6 CSPA isolates. Nucleotide variations were found in 17 sites of *pmrA* gene, 43 sites of *pmrB* gene, 7 sites of *phoP* gene, and 25 sites of *phoQ* gene. Most nucleotide substitutions were synonymous, and amino acid alterations were found in 3 sites of PmrA, 12 sites of PmrB, and 2 sites of PhoQ (Table 2). While 8 amino acid substitutions were found both in CNPA and CSPA isolates, 9 amino acid substitutions were identified in 10 CNPA isolates only (Table 2). Most of the amino acid substitutions unique to CNPA isolates were found in PmrB, Val15Ile, Met48Leu, Ala67Thr, Gly68Asp, Asp70Asn, His340Arg, and Thr343Ala. In PmrA, Leu157Gln was found only in 1 colistin-intermediate *P. aeruginosa* isolates. Ala143Val of PhoQ was also found only in 1 colistin-resistant *P. aeruginosa* isolate. However, no amino acid substitutions were found in PhoP, and neither frameshift mutations nor deletions were identified in this study. Among 16 CNPA isolates, 7 isolates (P30, P135, P147, P129, P165, P205, and P212) did not have amino acid variations that differed from the CSPA isolates.

We also determined the sequences of the *pmrAB* and *phoPQ* genes against in vitro selected colistin-resistant mutants using 2 CSPA isolates and their respective revertant strains to determine if