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Alterations in Two-Component Regulatory Systems of *phoPQ* and *pmrAB* Are Associated with Polymyxin B Resistance in Clinical Isolates of *Pseudomonas aeruginosa*▽

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Polymyxins are often the only option to treat acquired multidrug-resistant *Pseudomonas aeruginosa*. Polymyxin susceptibility in *P. aeruginosa* PAO1 is associated with the lipopolysaccharide structure that is determined by *arnBCADTEF* and modulated by *phoPQ* and *pmrAB*. We examined five clonally unrelated clinical isolates of polymyxin B-resistant *P. aeruginosa* to investigate the molecular basis of polymyxin resistance. All isolates grew with 4 μg/ml polymyxin B (MIC, 8 μg/ml), whereas *P. aeruginosa* PAO1 grew with 0.25 μg/ml polymyxin B (MIC, 0.5 μg/ml). The resistant isolates were converted to susceptible ones (the MICs fell from 8 to 0.5 μg/ml) following the introduction of *phoPQ* (four isolates) and *pmrAB* (one isolate), which had been cloned from strain PAO1. DNA sequence analysis revealed that a single-nucleotide substitution in three isolates replaced a single amino acid of PhoQ, the deletion of 17 nucleotides in one isolate truncated the protein of PhoQ, and two nucleotide substitutions in one isolate replaced two amino acids of PmrB. The involvement of these amino acid substitutions or the truncated protein of PhoQ and PmrB in polymyxin B resistance was confirmed using strain PAO1 lacking *phoPQ* or *pmrAB* that was transformed by *phoPQ* or *pmrAB* containing the amino acid substitutions or the truncated protein. The resistant clinical isolates were sensitized by the inactivation of *arnBCADTEF* (the MICs fell from 8 to 0.5 μg/ml). These results suggest that polymyxin B resistance among clinical isolates of *P. aeruginosa* is associated with alterations in two-component regulatory systems of *phoPQ* or *pmrAB*.

Pseudomonas aeruginosa is a nosocomial gram-negative opportunistic pathogen that causes a variety of infections (e.g., urinary tract, respiratory, skin, soft tissue, etc.) (3, 12, 18, 19). *P. aeruginosa* accounts for 11 to 14% of all nosocomial infections and is a major problem for people hospitalized with cancer, cystic fibrosis, or burns (3). Treatment usually involves the use of one or more antibiotics, such as β-lactams, aminoglycosides, or quinolones. Combination therapy usually is recommended for *P. aeruginosa* infections, as it decreases the risk of antibiotic resistance and enhances the eradication rate. Despite the use of combination therapy, there are numerous reports of the emergence of multidrug-resistant *P. aeruginosa*. Polymyxins (polymyxin B and colistin) often have been the last resort to treat such isolates (1, 3, 27). However, polymyxin B resistance in multidrug-resistant clinical isolates has been reported (4, 6, 11, 25).

The mode of action and the resistance mechanism to polymyxin B has been studied extensively using the reference *P. aeruginosa* strain PAO1. Polymyxin B is a polycationic lipopeptide antibiotic that interacts with a negatively charged lipid A moiety of the lipopolysaccharide (LPS) of gram-negative bacteria and leads to cell lysis and death (26). Resistance to polymyxin B is caused by the inhibition of the interactions between the antibiotic and the lipid A moiety of the LPS, and the inhibition is based on modifications of lipid A so that it is less negatively charged. LPS modification occurs by the addition of 4-amino-4-deoxy-L-arabinose to lipid A under limiting nutrient conditions, such as 20 μM magnesium or calcium, and is directed by an *arnBCADTEF* operon modulated by two-component regulatory systems of *phoPQ* and *pmrAB* (9, 13, 14, 20). In normal growth conditions, the two-component regulatory systems strictly repress *arnBCADTEF*, resulting in a phenotype of intrinsic susceptibility to polymyxins (8, 16, 17). Therefore, it is postulated that any interruption of the regulatory systems derepresses *arnBCADTEF* with resulting resistance to polymyxins. Indeed, in vitro-acquired polymyxin B-resistant *P. aeruginosa* PAK carried a single-amino-acid substitution in PmrB (20).

Although polymyxin-resistant clinical isolates of *P. aeruginosa* have been reported increasingly worldwide (5, 6, 10, 11, 25), the genetic basis for the resistance in these clinical isolates is unclear. This study aimed to examine the molecular details of polymyxin B resistance among clinical isolates of *P. aeruginosa*. We characterized five polymyxin B-resistant clinical isolates of *P. aeruginosa* and found that four isolates carried a single-amino-acid substitution or protein truncation in PhoQ, and one isolate carried dual amino acid substitutions in PmrB. The involvement of the amino acid substitutions or protein truncation in polymyxin B resistance was confirmed by introducing *phoPQ* or *pmrAB* containing the amino acid substitutions or protein truncation into *P. aeruginosa* PAO1 lacking *phoPQ* or *pmrAB*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Previously identified polymyxin B-resistant clinical isolates of *P. aeruginosa* were obtained from State University of New York (SUNY) Downstate Medical Center (Brooklyn, NY) (11), and the

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TABLE 1. Antibiotic susceptibility of *P. aeruginosa* clinical isolates

Antibiotic	MIC (μg/ml) for *P. aeruginosa* strain[a]:					
	PAO1	NY214	NY215	NY217	NY219	NY220
Azlocillin	2	8	8	16	8	16
Carbenicillin	64	256	256	>256	256	>256
Ceftriaxone	4	16	32	16	32	32
Ticarcillin	8	32	32	32	32	32
Ciprofloxacin	0.5	8	4	1	0.5	8
Nalidixic acid	64	>256	>256	64	64	>256
Gentamicin	0.25	4	32	4	4	4
Tobramycin	0.25	2	2	1	2	8
Polymyxin B	0.5	8	8	8	8	8

[a] MIC measurements were repeated three times with identical results.

reference *P. aeruginosa* strain PAO1 also was used. *Escherichia coli* DH5α was used for general cloning experiments. *P. aeruginosa* and *E. coli* DH5α were grown routinely in Luria-Bertani (LB) medium. When needed, ampicillin (100 μg/ml), chloramphenicol (10 μg/ml), gentamicin (15 μg/ml), or tetracycline (10 μg/ml) was added to the LB medium for *E. coli*; carbenicillin (100 μg/ml), gentamicin (100 μg/ml), or tetracycline (80 μg/ml) also was added to the LB medium for *P. aeruginosa*. Divalent cation-adjusted Mueller-Hinton (MH) (Oxoid, Ogdensburg, New York) broth (pH 7.0) was used for antibiotic susceptibility testing. All antibiotics and other chemicals used in this study were purchased from Sigma (St. Louis, MO).

Antibiotic susceptibility testing. Antibiotic susceptibility was determined as MICs using the broth dilution method as guided by the CLSI (2). The following polymyxin B susceptibility breakpoints were used: resistance, MIC ≥ 8 μg/ml; susceptible, MIC ≤ 2 μg/ml.

Genotype analysis. Genotype analysis was performed by PCR-based random amplified polymorphic DNA (RAPD) fingerprint using arbitrary primers of 5′-ACGGCCGACC-3′ (primer 1) and 5′-GCTGGGCCGA-3′ (primer 2) as described previously (15). RAPD products were separated by electrophoresis in 1.5% agarose gels with Tris-acetate-EDTA running buffer at 9 V/cm for 3 h. Molecular size standards were included on the agarose gels (1-kb ladder; Invitrogen).

Cloning of full-length *pmrAB* and *phoPQ*. The full length of *pmrAB* was cloned from a cosmid clone that was generated from *P. aeruginosa* PAO1 and obtained from the Pseudomonas Genetic Stock Center (www.pseudomonas.med.ecu.edu/). The cosmid clone pMO0010107 was digested by HindIII and SacI to isolate a 9.2-kb DNA fragment containing *pmrAB*, which was inserted into the same restriction enzyme sites of a *P. aeruginosa*/*E. coli* shuttle vector, pUCP18 (22), and was named pAU126. Since a cosmid clone containing full-length *phoPQ* was not available from the Pseudomonas Genetic Stock Center, full-length *oprH-phoPQ* was amplified from *P. aeruginosa* PAO1 using the PCR primer pair 5′-CAGGCAGATCACGAGAAACA-3′ and 5′-CAGCCGAACAGACTTCAGCG-3′ (3,060 bp from positions 1276710 to 1279770; http://www.pseudomonas.com/). The PCR fragment was inserted into the shuttle vector pUCP18 and was named pAU124.

Full-length *oprH-phoPQ* (3,060 bp) from clinical isolates (NY214, NY215, NY217, and NY219) was cloned by PCR methods using the same PCR primer pair as that used above and were named pAU146, pAU147, pAU148, and pAU149, respectively. A portion of *pmrB* from the clinical isolate NY220 also was cloned by PCR methods. A PCR primer pair (5′-GCGGTGGAGGCGGTACCGCTGG-3′ and 5′-GGGAATTCTCAGATATGTGACCGCCCGC-3′) was used to amplify the PCR fragment (803 bp from positions 5365390 to 5366193; http://www.pseudomonas.com/) that comprised the putative histidine box motif (20) and the C terminus with KpnI/SacI restriction enzyme sites at both ends of the PCR fragment. The PCR fragment digested by KpnI/SacI then was replaced by the same restriction fragment from the *pmrAB* clone (pAU126). The new *pmrAB* clone containing the histidine box motif and C terminus from NY220 was named pAU154. All cloned PCR amplicons were subjected to confirmatory sequencing (SeqWright DNA Sequencing Services, Houston, TX) to rule out the presence of unwanted mutations introduced by PCR.

Gene replacement. A gentamicin (Gm) resistance cassette from pGMΩ1 (21) was inserted into BsmI/NruI to delete 1,870 bp from *oprH-phoPQ* (pAU124). The Gm cassette also was inserted into PstI to delete 1,466 bp from *pmrAB* (pAU126). The DNA fragments carrying a deletion of *oprH-phoPQ* and *pmrAB* were inserted into the conjugative plasmid pRTP1 (23), and the resulting plasmids were used to delete the genes of *P. aeruginosa* PAO1 by biparental conjugation as described previously (7). A portion of the gene *arnB* also was amplified using a PCR primer pair of 5′-CGGCCCCCTGGCGGAGCGAT-3′ and 5′-AGGTCGGCCAGGTTGTATTT-3′ (1,077 bp from positions 3979487 to 3980564; http://www.pseudomonas.com/). The PCR fragment was cloned into pBluescript SK (Stratagene, La Jolla, CA), and a tetracycline (Tc) resistance cassette from Tn*5* (21) was inserted into the HincII site of the PCR fragment. The PCR fragment inactivated by the Tc cassette was used to knock out the *arnB* gene as mentioned above. The gene knockouts were confirmed for authenticity by PCR methods as described previously (24).

Nucleotide sequence accession numbers. The nucleotide sequences of *phoQ* from polymyxin B-resistant clinical isolates of *P. aeruginosa* (NY214, NY215, NY217, and NY219) and *pmrB* from a polymyxin B-resistant clinical isolate of *P. aeruginosa* (NY220) have been submitted to the GenBank nucleotide sequence databases under the accession numbers GQ266138, GQ266139, GQ266140, GQ266141, and GQ266142, respectively.

RESULTS AND DISCUSSION

Antibiotic susceptibility of clinical isolates. The antibiotic susceptibility of the five clinical isolates of *P. aeruginosa* was determined as MICs of polymyxin B, β-lactams (azlocillin, carbenicillin, ceftriaxone, and ticarcillin), fluoroquinolones (ciprofloxacin and nalidixic acid), and aminoglycosides (gentamicin and tobramycin). The polymyxin B MICs for all of the isolates were 8 μg/ml. Also, the MICs of all other antibiotics except fluoroquinolones were higher (at least fourfold) than those for the reference strain *P. aeruginosa* PAO1. The fluoroquinolone MICs for the two isolates NY217 and NY219 were similar to those for the reference strain PAO1 (Table 1). Genotypic relatedness among the five clinical isolates also was examined by RAPD genotype fingerprinting patterns. Two arbitrary PCR primers showed quite a distinct genotype pattern for each isolate, including strain PAO1 (Fig. 1). This result suggests that the isolates are of different lineages.

Genetic complementation of polymyxin B resistance by *phoPQ* and *pmrAB*. It has been reported that polymyxin B susceptibility is associated with the LPS structure that is directed by *arnBCADTEF* and modulated by two-component regulatory systems of *pmrAB* and *phoPQ* (13, 14, 16). To examine the role of the two-component regulatory systems in polymyxin B resistance, full-length *phoPQ* (pAU124) and *pmrAB* (pAU126) cloned from the reference strain PAO1 were

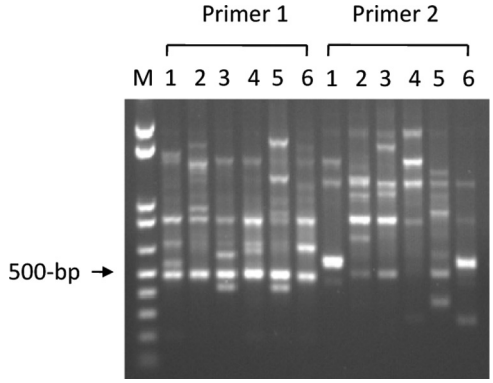

FIG. 1. Genotype fingerprinting analysis of clinical isolates of *P. aeruginosa*. RAPD fingerprinting genotype analysis was performed using arbitrary PCR primers 1 and 2 as described in Materials and Methods. Lane 1 shows reference strain PAO1, and lanes 2 to 6 are clinical isolates of NY214, NY215, NY217, NY219, and NY220, respectively. Lane M is a 0.1- to 1-kb ladder (Invitrogen).

TABLE 2. Complementation analysis of polymyxin B-resistant clinical isolates of *P. aeruginosa*

Clinical isolate	Polymyxin B MIC (μg/ml) for strain harboring:		
	pAU124[a] (*phoPQ*)	pAU126 (*pmrAB*)	pUCP18 (cloning vector)
NY214	1	4 (8)[b]	8
NY215	0.5	8	8
NY217	1	8	8
NY219	1	8	8
NY220	8	1 (2)	8

[a] Full-length *phoPQ* and *pmrAB* were cloned from *P. aeruginosa* PAO1 as described in Materials and Methods.
[b] Numbers in parentheses denote repeated MIC measurements performed more than three times.

introduced into each of the five clinical isolates, and the polymyxin B MICs for the clones were compared to those for the parental isolates. Polymyxin B MICs for four isolates (NY214, NY215, NY217, and NY219) harboring pAU124 were reduced to 0.5 or 1 μg/ml. However, the MICs for the same isolates harboring pAU126 were unchanged. The polymyxin B MIC for isolate NY220 harboring pAU126 was reduced to 1 or 2 μg/ml, whereas the MIC for the same isolate harboring pAU124 was unchanged. The MICs for the five clinical isolates were unchanged by the cloning vector (pUCP18) (Table 2). These results suggest that polymyxin B resistance from the four clinical isolates (NY214, NY215, NY217, and NY219), which is sensitized by an intact *phoPQ*, is associated with a *phoPQ* mutation, and that polymyxin B resistance from the isolate NY220, which is sensitized by an intact *pmrAB*, is associated with a *pmrAB* mutation.

DNA sequence analysis of *phoQ* and *pmrB* from the polymyxin B-resistant clinical isolates. In vitro-acquired polymyxin B-resistant *P. aeruginosa* PAK showed nucleotide sequence substitutions that caused amino acid substitutions in a histidine sensor kinase of PmrB (20). To examine whether any amino acid substitution in the histidine sensor kinases of PhoQ and PmrB was associated with polymyxin B resistance, the genes of the resistant isolates were compared to those of the strain PAO1. Results revealed that a single-nucleotide substitution in *phoQ* substituted a single amino acid for isolates NY214 (V to G), NY217 (H to R), and NY219 (V to G) or a nucleotide deletion (17-bp deletion) in *phoQ* that shifted a reading frame at the 152nd amino acid and the truncated protein for isolate NY215. Nucleotide substitutions in *pmrB* substituted two amino acids (A to T and Y to H) for the sequence of isolate NY220 (Table 3). Nucleotide sequences of full-length *phoQ* from isolate NY220 and a putative histidine box motif of *pmrB* (542 bp from positions 5365240 to 5365782) from the isolates NY214, NY2215, NY217, and NY219 also were determined. Results showed that none of the DNA sequences resulted in amino acid substitutions for PhoQ and PmrB compared to the sequence of the strain PAO1 (Table 3).

We found that four of the polymyxin B-resistant clinical isolates carried alterations in PhoQ, and the other one carried alterations in PmrB. Although the majority of the alterations were amino acid substitutions, one isolate showed a truncated protein for PhoQ. The insertion inactivation of PhoQ in *P. aeruginosa* PAO1 showed the increased expression of *arnBCADTEF* (8), suggesting that the clinical isolate (NY215) carrying truncated PhoQ has the increased expression of *arnBCADTEF*. In addition to the amino acid substitutions in PhoQ or PmrB, the complete inactivation of PhoQ also is one of genetic alterations responsible for polymyxin B resistance.

Expression of *phoQ* and *pmrB* from the polymyxin B-resistant clinical isolates. Since the genes *phoQ* and *pmrB* are controlled by their upstream genes (*oprH* and *pmrA*) (13, 16), full-length *oprH-phoPQ* from NY214, NY215, NY217, and NY219 and full-length *pmrAB* from NY220 were cloned. Mutant strains of PAO1(Δ*oprH-phoPQ*) or PAO1(Δ*pmrAB*) were constructed as described in Materials and Methods. The mutant PAO1 strains were transformed by each clone of *oprH-phoPQ* or *pmrAB* from the resistant clinical isolates including PAO1, and the transformants were used to determine polymyxin B susceptibility. MICs for the two mutant strains (Δ*oprH-phoPQ* and Δ*pmrAB*) and the same mutant strains harboring each clone of *oprH-phoPQ* or *pmrAB* from PAO1 were unchanged compared to those for the parental strain PAO1, suggesting that lacking the two-component regulatory

TABLE 3. DNA sequence analysis of *phoQ* and *pmrB* from polymyxin B-resistant clinical isolates of *P. aeruginosa*

Isolate	Amino acid alteration for:	
	PhoQ[a]	PmrB[b]
NY214	Nucleotide substitution (T at position 1779141 to G) resulting in amino acid substitution V260G	No change
NY215	17 nucleotides deletion from position 1278818 generated a frameshift with a truncated protein at the 152nd amino acid	No change
NY217	Nucleotide substitution (A at position 1279029 to G) resulting in amino acid substitution H223R	No change
NY219	Nucleotide substitution (T at position 1779141 to G) resulting in amino acid substitution	No change
NY220	No change	Nucleotide substitution (G at position 5365488 to A) resulting in amino acid substitution A247T; nucleotide substitution (T at position 5365792 to C) resulting in amino acid substitution Y345H

[a] The sequence of full-length *phoQ* (1,347 bp; 448 amino acids) was compared to that of *P. aeruginosa* PAO1 (www.pseudomonas.com).
[b] A putative histidine box (H-box; 550 bp) as designated by Moskowitz et al. (20) was analyzed for NY214, NY215, NY217, and NY219; the full-length sequence of *pmrB* (1,434 bp; 477 amino acids) was analyzed for NY220.

TABLE 4. Polymyxin B susceptibility in mutant *P. aeruginosa* strains

P. aeruginosa strain	Polymyxin B MIC[a] (μg/ml)
PAO1	0.5
PAO1(Δ*opr*H-*phoPQ*::Gm)	0.5
PAO1(Δ*opr*H-*phoPQ*::Gm)/pAU124 (*oprH-phoPQ* from PAO1)	0.25
PAO1(Δ*opr*H-*phoPQ*::Gm)/pAU146 (*oprH-phoPQ* from NY214)	4
PAO1(Δ*opr*H-*phoPQ*::Gm)/pAU147 (*oprH-phoPQ* from NY215)	4
PAO1(Δ*opr*H-*phoPQ*::Gm)/pAU148 (*oprH-phoPQ* from NY217)	4
PAO1(Δ*opr*H-*phoPQ*::Gm)/pAU149 (*oprH-phoPQ* from NY219)	4
PAO1(Δ*opr*H-*phoPQ*::Gm)/pUCP18 (vector)	0.25
PAO1(Δ*pmrAB*::Tc)	0.25
PAO1(Δ*pmrAB*::Tc)/pAU126 (*pmrAB* from PAO1)	≤0.25
PAO1(Δ*pmrAB*::Tc)/pAU154 (*pmrAB* from NY220)	4
PAO1(Δ*pmrAB*::Tc)/pUCP18	0.25
PAO1(*arnB*::Tc)	0.5
NY214(*arnB*::Tc)	0.5
NY215(*arnB*::Tc)	0.5
NY217(*arnB*::Tc)	0.5
NY219(*arnB*::Tc)	ND[b]
NY220(*arnB*::Tc)	0.5

[a] MIC measurements were repeated three times with identical results.
[b] ND, not determined.

systems has no effect on polymyxin B susceptibility. However, the mutant strain (Δ*oprH-phoPQ*) harboring *oprH-phoPQ* from the four resistant clinical isolates (NY214, NY215, NY217, and NY219) restored their polymyxin B resistance (MICs from 0.5 to 4 μg/ml), and the mutant strain (Δ*pmrAB*) harboring *pmrAB* from the resistant clinical isolate NY220 also restored its polymyxin B resistance (MICs from 0.25 to 4 μg/ml). The MICs for the mutant strains harboring the cloning vector (pUCP18) were unchanged (Table 4).

Involvement of *arnBCADTEF* in polymyxin B resistance of clinical isolates. To clarify the involvement of LPS modifications in the polymyxin B resistance of the clinical isolates, *arnB* was inactivated by inserting a Tc resistance cassette. All clinical isolates with the inactivation of *arnB* became susceptible to polymyxin B at the same level as that of susceptible *P. aeruginosa* PAO1 (MICs fell from 8 to 0.5 μg/ml) (Table 4). These results suggest that the polymyxin B resistance of the clinical isolates is fully explained by the LPS modifications directed by *arnBCADTEF*.

Overall, we found alterations in signal sensor kinases of PhoQ or PmrB from polymyxin B-resistant clinical isolates and confirmed that these alterations were associated with resistance to polymyxin B. These findings suggest that polymyxin B resistance in clinical isolates of *P. aeruginosa* usually is caused by alterations in either PhoQ or PmrB, which may have become evident in response to the increased use of polymyxins in recent years.

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