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Differential Role of Two-Component Regulatory Systems (*phoPQ* and *pmrAB*) in Polymyxin B Susceptibility of *Pseudomonas aeruginosa*

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Abstract

Polymyxins are often considered as a last resort to treat multidrug resistant *P. aeruginosa* but polymyxin resistance has been increasingly reported worldwide in clinical isolates. Polymyxin resistance in *P. aeruginosa* is known to be associated with alterations in either PhoQ or PmrB. In this study, mutant strains of *P. aeruginosa* carrying amino acid substitution, a single and/or dual inactivation of PhoQ and PmrB were constructed to further understand the roles of PhoQ and PmrB in polymyxin susceptibility. Polymyxin B resistance was caused by both inactivation and/or amino acid substitutions in PhoQ but by only amino acid substitutions of PmrB. Alterations of both PhoQ and PmrB resulted in higher levels of polymyxin B resistance than alteration of either PhoQ or PmrB alone. These results were confirmed by time-killing assays suggesting that high-level polymyxin resistance in *P. aeruginosa* is caused by alterations of both PhoQ and PmrB.

Keywords

Pseudomonas aeruginosa; Polymyxin B Resistance; *phoPQ*; *pmrAB*

1. Introduction

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen that is a frequent cause of hospital-acquired infections [1–5]. *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 [2]. Treatment usually involves the use of one or more antibiotics such as β-lactams, aminoglycosides, or quinolones. Combination therapy is usually recommended as it reduces the risk of antibiotic resistance and enhances the eradication rate. Despite the use of combination therapy, there are numerous reports of emergence of multidrug resistant *P. aeruginosa*. When multidrug *P. aeruginosa* occurs in critically ill patients, polymyxins are often the last resort for treatment of the infections [6,7]. However, polymyxin B associated multidrug resistant *P. aeruginosa* have been increasingly reported worldwide [8–11].

Polymyxins are a group of cationic polypeptide antibiotics consisting of five different compounds (A to E); only polymyxin B and E (colistin) have been used in clinical practice. Polymyxin B differs by only one amino acid from polymyxin E and both have essentially the same antibacterial spectrum, susceptibilities, and resistant mechanisms [12,13].

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Polymyxins interacts with lipopolysaccharide (LPS) of the outer membrane of Gram-negative bacteria and displace the calcium and magnesium bridges that stabilize the LPS disrupting the outer membrane producing cell death [14]. Based on *in vitro* studies using *P. aeruginosa* PAO1, it is known that polymyxin B resistance can be induced by *arnBCADTEF* that is modulated through a two-component regulatory systems of *phoPQ* and *pmrAB* under culture conditions limiting magnesium (e.g., 20 μM) [15–18]. In clinical isolates using normal culture conditions (e.g., Luria-Bertani or Mueller Hinton) for *P. aeruginosa*, polymyxin B resistance has been associated with alterations in either PhoQ or PmrB [1,19]. Polymyxin B resistant *P. aeruginosa* PAK selected *in vitro* by increasing concentrations of polymyxin B was found to have amino acid substitutions in PmrB [20]. Other studies using *P. aeruginosa* PAO1 suggested cross-talk between the *phoPQ* and *pmrAB* systems [17,18]. Although involvement of PhoQ and PmrB in polymyxin B resistance is known in *P. aeruginosa*, molecular details of polymyxin susceptibility remain unclear including the degree of polymyxin susceptibility and the correlation with alterations in PhoQ and/or PmrB. In this study, polymyxin susceptibility was examined in *P. aeruginosa* harboring inactivation and/or amino acid substitutions in PhoQ and/or PmrB.

2. Materials and Methods

2.1. Bacterial Strains and Culture Conditions

P. aeruginosa PAO1 and clinical isolates of *P. aeruginosa* (M38100B4, NY214, NY215, NY220) reported previously [1,19] were used for this study. *E. coli* strains (DH5α and SM10) were also used for general cloning experiments. All bacterial strains were routinely cultured in Luria-Bertani (LB) medium. When needed, antibiotics were supplemented in the LB medium as ampicillin (100 μg/mL), gentamicin (15 μg/mL), and tetracycline (10 μg/mL) for *E. coli*. Antibiotics were also added to LB medium to select *P. aeruginosa* gene-knockout strains using carbenicillin (100 μg/mL), gentamicin (80 μg/mL), and tetracycline (80 μg/mL). Divalent cation-adjusted Mueller-Hinton (MH) (Oxoid, Ogdensburg, New York) broth was used for antibiotic susceptibility testing. All antibiotics and other chemicals used in this study were purchased from Sigma (Sigma, St. Louis, MO). Solutions of these compounds were prepared by dissolving in sterile doubledistilled water or the solvent suggested by the manufacturer and sterilized by filtering through the 0.4 μm disposable membranes (Millipore, Billerica, Massa-chusetts). The final pH of prepared medium was ~7.0 after appropriate adjustments.

2.2. Antibiotic Susceptibility Testing

Antibiotic susceptibility was determined as minimal inhibitory concentrations (MICs) using the broth dilution method as described [25]. Briefly, polymyxin B was added to MH broth to achieve serial two-fold dilutions between 0.25 and 32 μg/mL using sterile 17 × 100 mm snapped-cap Falcon culture tubes (1 mL/tube; Fisher Scientific). Fresh overnight cultures of each bacterial strain were diluted in saline to an optical density at 600 nm of 0.08 to 0.12 (approximately 1×10^8 viable cells per mL). A portion of the adjusted cell suspension (5 μL for ~$10^{5–6}$ cells) was inoculated to each MH broth containing polymyxin B as indicated. The cell cultures were then incubated overnight (16 to 18 hrs) at 37°C without shaking. The MIC was defined as the lowest concentration that completely inhibited growth of the inoculums. MIC values were confirmed by three independent experiments.

2.3. Genomic DNA Extraction, PCR and DNA Sequence Analysis

Genomic DNA extraction, PCR amplification, and cloning experiment were performed as described [19]. PCR fragments were used to determine DNA sequences by a commercial DNA sequencing service (GENEWIZ, South Plainfield, NJ).

2.4. Construction of Gene-Knockout Strains

The gene *phoQ* was knocked-out by inserting a gentamicin resistance cassette (Gm) from pGMΩ1 [21] into *Nru*I of the 3060-bp DNA fragment containing *oprH- phoPQ*. The gene *pmrB* was knocked-out by inserting a tetracycline resistance cassette (Tc) derived from Tn5 (Epicentre Biotechnologies, Madison, WI) into *Pst*I of the 1437-bp DNA fragment containing *pmrB*. To delete the genes of *oprH-phoPQ* a 1906-bp *Pst*I fragment from the 3060-bp fragment containing *oprH-phoPQ* was replaced by the Gm cassette. To delete the genes of *pmrAB*, a 1468-bp *Pst*I fragment from the 3157-bp *Sal*I/*Sac*I fragment containing *pmrAB* was replaced by the Gm cassette. The DNA fragments carrying the gene-knockout or -deletion were inserted into the conjugative plasmid of pRTP1 [22] and the resulting plasmids were used to knockout the genes for *P. aeruginosa* by biparental conjugation methods as described [23]. The gene replacement by Gm or Tc was confirmed for authenticity by PCR methods as described [24].

2.5. Time-Killing Assay

P. aeruginosa strains were grown overnight in LB broth. The cells were diluted in saline to an optical density at 600 nm of 0.08 to 0.12 and 5 μL of each diluted cells (~$10^{5–6}$ cells) were inoculated into 1 mL of MH broth supplemented with appropriate amount of polymyxin B, following incubation at 37°C. Aliquots (100 μL) were withdrawn at specific time intervals (4, 8, and 12 hours) and spread on LB agar plates with further dilution before spreading on plain LB agar plates. After overnight incubation (16 to 18 hours) at 37°C, cells that survived were counted as colony forming units (CFU).

3. Results and Discussions

3.1. A Single Inactivation of PhoQ or PmrB in Polymyxin B Susceptibility

P. aeruginosa PAO1 and a clinical isolate of *P. aeruginosa* M38100B4 were used to study the effects of PhoQ- or PmrB-inactivation on polymyxin B susceptibility. *P. aeruginosa* PAO1 carrying PhoQ-inactivation showed an MIC of 4 μg/mL of polymyxin B whereas the parental PAO1 MIC was 0.5 μg/mL. Polymyxin B susceptibility of the PAO1 carrying the PhoQ-inactivation was fully restored by introducing an intact clone of *oprH-PhoPQ* from the parental PAO1. These results are consistent with the previous observation that truncated-PhoQ in the clinical isolate of *P. aeruginosa* yielded an MIC of 8 μg/mL of polymyxin B. The PAO1 carrying PmrB-inactivation showed no significant change of MIC (0.5 μg/mL of polymyxin B) and inactivation of both PhoQ and PmrB retained the effect of only inactivation of PhoQ (MIC 4 μg/mL), indicating that inactivation of PmrB did not affect polymyxin susceptibility. Previous reports however showed that a single amino acid substitution (M292T) in PmrB increased the MIC of polymyxin B to 8 μg/mL [1] and inactivation of PmrB in this isolate decreased the MIC to 0.5 μg/mL. The isolate carrying a single amino acid substitution in PmrB was used to inactivate PhoQ resulting in an MIC of 16 μg/mL with full complementation of susceptibility by an intact clone of *oprH- phoPQ* (Table 1). These results suggest that amino acid substitutions of PmrB rather than inactivation whereas either inactivation or amino acid substitutions of PhoQ results in polymyxin resistance. These results also suggest that although both PhoQ and PmrB play roles in polymyxin B resistance, their specific roles in polymyxin B susceptibility may be different.

3.2. Dual Alterations of PhoQ and PmrB in Polymyxin B Susceptibility

Amino acid substitutions in either PhoQ or PmrB showed MIC's of 4 to 8 μg/mL among clinical isolates of *P. aeruginosa* [1,19]. To examine the effects of alterations in both PhoQ and PmrB on polymyxin B susceptibility, *P. aeruginosa* PAO1 and the clinical *P.*

aeruginosa isolate M38100B4 were used following alterations in both PhoQ and PmrB. *P. aeruginosa* PAO1 carrying an PhoQ-inactivation and deletion of a full length of *pmrAB* (*phoQ*:: Tc/Δ*pmrAB*::Gm) was constructed and this dual mutant strain was transformed by plasmids carrying amino acid substitutions in PmrB, M292T for pAU129 and A247T/ Y345H for pAU154 as reported previously [1,19]. The dual mutant strain retained an MIC of 4 μg/mL which was the same MIC as found with PhoQ-inactivation alone. However, the mutant strain carrying the plasmids (pAU129 or pAU154) showed an MIC of 8 μg/mL whereas the MIC of the same mutant strain carrying the cloning vector (pUCP18) was unchanged (4 μg/mL) (Table 2). Similarly, the polymyxin B resistant clinical isolate M38100B4 carrying M292T in PmrB was used to construct a dual mutant strain carrying a deletion of the full length of *oprH-phoPQ*. This mutant strain was transformed by plasmids carrying amino acid substitutions or inactivated-PhoQ, V260G for pAU146, truncated-PhoQ for pAU147, H223R for pAU148 reported previously [19]. The clinical isolate M38100B4 in which the full length of *oprH-phoPQ* was deleted retained its MIC of 8 μg/mL. However, the same mutant strain carrying the amino acid substitutions in PhoQ or truncated-PhoQ showed an MIC of 16 μg/mL; the cloning vector alone had no changed in MIC (Table 2). These results suggest that the alterations in both PhoQ and PmrB cause higher level resistance to polymyxin B than that of alterations in either PhoQ or PmrB alone.

The effect of alterations of both PhoQ and PmrB was confirmed by time-killing assays using the PAO1 mutant strain (*phoQ*::Tc/Δ*pmrAB*::Gm) carrying a plasmid pAU129 (M292T in PmrB), the same mutant strain carrying a cloning vector (pUCP18), and the parental strain PAO1. As shown in Figure 1(a), the PAO1 mutant strain carrying alterations in both PhoQ and PmrB survived with significantly higher numbers of cells than the same strain carrying a single alteration (PhoQ inactivation). In contrast, the parental PAO1 was killed within 12 hours in the presence of 4 μg/mL of polymyxin B. Similarly, the clinical isolate M38100B4 carrying an inactivated-PhoQ (*i.e*., alterations in both PhoQ and PmrB) also survived at significantly higher numbers of cells than that of the parental M38100B4 (Figure 1(b)). These results confirmed the above observations that *P. aeruginosa* carrying alterations in both PhoQ and PmrB resulted in higher levels of polymyxin B resistance than that of alterations in either PhoQ or PmrB alone.

To further substantiate the effects of alterations in both PhoQ and PmrB 3 clinical isolates (NY214, NY215, and NY220) of *P. aeruginosa* reported previously (19) were used to induce high-level resistance to polymyxin B under polymyxin B selection pressure. The 3 clinical isolates were incubated in LB broth containing 4 μg/mL of polymyxin B for 24 hours and plated on LB agar plates containing 8 μg/mL of polymyxin B. A single colony from each culture was used for 3 additional passages on plain LB agar plates. Three colonies from the third passage of each culture showed high-level resistance to polymyxin B (MICs of 16 to 32 μg/mL). DNA sequence analysis revealed that the high-level resistant colonies harbored inactivation and/or amino acid substitutions of both PhoQ and PmrB (Table 3). These results clearly demonstrated that alterations in both PhoQ and PmrB resulted in high-level polymyxin B resistance among clinical *P. aeruginosa* isolates.

In this study, three different approaches were used to understand the roles of PhoQ and PmrB alterations in relation to polymyxin B susceptibility. The clinical isolate M38100B4 had polymyxin B resistance (MIC 8 μg/mL) due to a single amino acid substitution in PmrB which increased MIC from 8 to 16 μg/mL after inactivation of PhoQ. The second approach was alterations of both PhoQ and PmrB, which showed higher levels of polymyxin B resistance than those with alterations of either PhoQ or PmrB alone. The third was that the time-killing rate of strains carrying alterations of both PhoQ and PmrB was much slower than that of the strains carrying alterations in either PhoQ or PmrB alone. Lastly, 3 clinical isolates were used to substantiate the roles of alterations in both PhoQ and PmrB in

polymyxin B resistance. All of the observations demonstrate that the alterations in both PhoQ and PmrB result in higher levels of polymyxin B resistance than do alterations in either PhoQ or PmrB alone.

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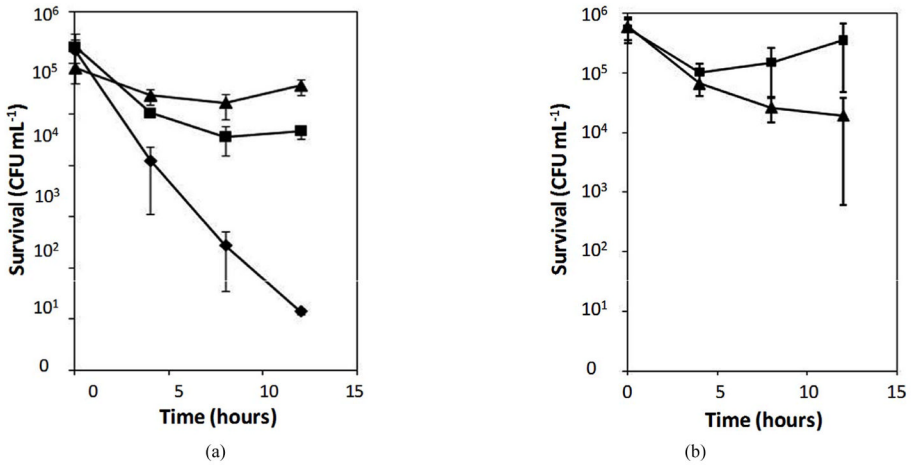

Figure 1.

Time-killing assays of *Pseudomonas aeruginosa*. Time-killing assays were performed using 4 and 8 μg/mL of polymyxin B for PAO1 (a) and clinical isolate M38100B4 (b), respectively, as described in Materials and Methods. The clinical isolate M38100B4 carries an amino acid substitution (M292T) in PmrB causing polymyxin B resistance (MIC 8 μg/mL) as reported (1). Error bars were obtained from three independent assays. (a) Diamond, a strain PAO1; triangle, a strain PAO1 (*phoQ*::Gm/Δ*pmrAB*::Tc)/pAU129 (*pmrAB* carrying M292T in PmrB); square, a strain PAO1 (*phoQ*::Gm/Δ*pmrAB*::Tc) /pAU126 (intact *pmrAB* from PAO1); (b) Triangle, a strain M38100B4; squares, a strain M38100B4 carrying inactivated-PhoQ. The clones of pAU126 and pAU129 were used from the previous reports (1).

Table 1

MICs of *P. aeruginosa* strains to polymyxin B.

Strains	MICs to polymyxins (μg/mL)[a]
PAO1	0.5
PAO1 (*phoQ*::Gm)	4
PAO1 (*phoQ*::Gm)/pAU124[b]	0.25
PAO1 (*pmrB*::Tc)	0.5
PAO1 (*phoQ*::Gm/*pmrB*::Tc)	4
M38100B4[c]	8
M38100B4 (*phoQ*::Tc)	16
M38100B4 (*phoQ*::Tc)/pAU124	8
M38100B4 (*pmrB*::Tc)	0.5

[a] MIC measurement repeated three times with identical results.

[b] pAU124 carries a full length of *oprH-phoPQ* from *P. aeruginosa* PAO1.

[c] M38100B4 carried a single amino acid substitution (M292T) in PmrB (1).

Table 2

MICs of mutant *P. aeruginosa* to polymyxin B.

Strains	MICs to polymyxin B (μg/mL)[a]
PAO1 (*phoQ*::Tc/Δ*pmrAB*::Gm)	4
PAO1 (*phoQ*::Tc/Δ*pmrAB*::Gm)/pAU129	8
PAO1 (*phoQ*::Tc/Δ*pmrAB*::Gm)/pAU154	8
PAO1 (*phoQ*::Tc/Δ*pmrAB*::Gm)/pUCP18 (vector)	4
M38100B4 (Δ*oprH-phoPQ*::Gm)	8
M38100B4 (Δ*oprH-phoPQ*::Gm)/pAU146	16
M38100B4 (Δ*oprH-phoPQ*::Gm)/pAU147	16
M38100B4 (Δ*oprH-phoPQ*::Gm)/pAU148	16
M38100B4 (Δ*oprH-phoPQ*::Gm)/pUCP18 (vector)	8

[a]MIC measurement repeated three times with identical results.

Note: pAU129, amino acid substitution (M292T) in PmrB; pAU154, amino acid substitutions (A247T; Y345H) in PmrB; pAU146, amino acid substitution (V260G) in PhoQ; pAU147, truncated-PhoQ; pAU148, amino acid substitution (H223R) in PhoQ (see details in the text).

Table 3

DNA sequence analysis of PhoQ and PmrB from polymyxin B resistant clinical isolates of *P. aeruginosa* and their MICs.

P. aeruginosa isolates[a]	Amino acid alteration[b]		MICs to polymyxin B (μg/mL)[c]
	PhoQ	PmrB	
NY214R1	Amino acid substitution at V260G	Amino acid substitution at M292T	16
NY214R2	Amino acid substitution at V260G	Amino acid substitution at M292T	16
NY214R3	Amino acid substitution at V260G	Amino acid substitution at M292T	16
NY215R1	Frame-shift mutation by 17 nucleotides deletion from 1278818 with truncated-protein at position 152nd amino acid	Amino acid substitution at A247T	16
NY215R2	Frame-shift mutation by 17 nucleotides deletion from 1278818 with truncated-protein at position 152nd amino acid	Amino acid substitution at A247T	16
NY215R3	Frame-shift mutation by 17 nucleotides deletion from 1278818 with truncated-protein at position 152nd amino acid	Amino acid substitution at Y345H	16
NY220R1	Amino acid substitution at V260G	Amino acid substitution at A247T and Y345H	32
NY220R2	Amino acid substitution at V260G	Amino acid substitution at A247T and Y345H	32
NY220R3	Amino acid substitution at H223R	Amino acid substitution at Y345H	16

[a] Polymyxin B resistant strains of NY214R, NY215R, and NY220R were induced from NY214, NY215, and NY220 reported previously (19); see details in the text.

[b] A full length of PhoQ (1347-bp; 448 amino acids) and of PmrB (1434-bp; 477 amino acids) was compared to that of *P. aeruginosa* PAO1 (www.pseudomonas.com).

[c] MIC measurement repeated three times with identical results.