

PmrAB, a Two-Component Regulatory System of *Pseudomonas aeruginosa* That Modulates Resistance to Cationic Antimicrobial Peptides and Addition of Aminoarabinose to Lipid A

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Spontaneous polymyxin-resistant mutants of *Pseudomonas aeruginosa* were isolated. The mutations responsible for this phenotype were mapped to a two-component signal transduction system similar to PmrAB of *Salmonella enterica* serovar Typhimurium. Lipid A of these mutants contained aminoarabinose, an inducible modification that is associated with polymyxin resistance. Thus, *P. aeruginosa* possesses a mechanism that induces resistance to cationic antimicrobial peptides in response to environmental conditions.

Cationic antimicrobial peptides (CAPs) are a widely conserved host defense mechanism of plants and animals. Their antimicrobial effects can be attributed to their amphipathic, detergent-like nature, which enables individual CAP molecules to interact with both anionic and hydrophobic components of the bacterial envelope. CAPs bind to lipopolysaccharide (LPS), a major component of the gram-negative cell surface, through interactions with phosphates and fatty acids of LPS core and lipid A moieties (31). These molecules cross the outer membrane and periplasm, disrupt the membrane potential of the inner membrane, and thereby cause cell death (25). In vertebrates, the CAPs that pathogens encounter at epithelial surfaces are a major component of innate immunity, an ancient system of host defense that is stimulated via receptors that recognize pathogen-associated molecular patterns (29).

Pseudomonas aeruginosa is an opportunistic pathogen of humans that causes infections in those with host defense defects such as epidermal injury, immunodeficiency, and impaired epithelial clearance mechanisms. In the human host, *P. aeruginosa* is exposed to endogenous CAPs such as β -defensins (37) and cathelicidins (5) at epithelial surfaces. It may also encounter exogenous CAPs in this setting, when agents such as the polymyxins, acylated cyclic CAPs synthesized by the gram-positive soil bacterium *Bacillus polymyxa*, are used as antibiotics. Since the discovery and initial clinical use of the polymyxins more than 50 years ago, both clinical (12, 23, 26) and experimental (7, 16, 32) *P. aeruginosa* polymyxin resistance has been reported. *P. aeruginosa* possesses proteases that can degrade some CAPs (35); in addition, physiological (or “adaptive”) polymyxin resistance may occur in response to membrane stresses such as divalent cation limitation (7, 13, 27, 30) and polymyxin exposure (9, 16, 36), the latter being associated with the modulation of lipid A fatty acid composition (9). The *P. aeruginosa* PhoPQ two-component system contributes to the

induction of these resistance phenotypes; however, its role appears to be complex (13, 27), and the potential roles of other regulatory systems related to PmrAB, a response regulator-sensor kinase pair that regulates polymyxin resistance in *Salmonella enterica* serovar Typhimurium (18, 34), have not been defined.

Isolation of polymyxin-resistant mutants of *P. aeruginosa*. Conditions that physiologically induce polymyxin resistance have not been fully defined for *P. aeruginosa* and could involve multiple regulatory systems. In order to identify regulators important for this resistance, spontaneous mutants of wild-type *P. aeruginosa* (strain PAK; obtained from S. Lory) were isolated from late-exponential-phase cultures by selection on Luria-Bertani (LB) plates containing 20 to 50 μ g of polymyxin B (USB/Amersham) per ml. After incubation for 72 to 96 h at ambient temperature (approximately 25°C), 12 initial isolates were colony purified, of which 6 displayed a stable resistance pattern, as indicated by growth on LB plates containing polymyxin B (20 μ g/ml) after two passages through LB broth lacking CAPs. Among these six isolates, two distinct phenotypes were observed, differing primarily in terms of growth rate and degree of polymyxin resistance. For each phenotype, representative mutant strains, designated PAK pmrB6 and PAK pmrB12 , were selected for characterization. The polymyxin-resistant strains grew more slowly on solid media than the wild-type strain but had growth rates in liquid media that were similar to that of the wild-type strain (generation time in LB broth at 37°C at mid-log phase, \approx 50 min). The polymyxin resistance of PAK pmrB6 and PAK pmrB12 strains was confirmed by a quantitative bactericidal assay performed as described previously (39), with incubation in the presence of polymyxin B for 30 min, dilution, and plating for the enumeration of surviving CFU. For all bactericidal assays, each strain was tested in triplicate at each peptide concentration. Relative to that for the wild-type strain, the polymyxin concentrations resulting in a 50% reduction in the number of CFU (50% lethal dose [LD₅₀]) for PAK pmrB6 and for PAK pmrB12 were about 6 and 16 times as high, respectively (Table 1).

Cross-resistance of polymyxin-resistant strains to addi-

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TABLE 1. Resistance of *P. aeruginosa* PAK*pmrB* strains to CAPs

CAP	PAK <i>pmrB</i> ⁺ LD ₅₀ (μg/ ml)	PAK <i>pmrB6</i>		PAK <i>pmrB12</i>	
		LD ₅₀ (μg/ml)	Relative resistance	LD ₅₀ (μg/ml)	Relative resistance
Polymyxin B	0.5	3	6	8	16
β-Defensin-2	0.5	1.5	3	6	12
Protegrin-1	1	6	6	60	60
C18G	1	16	16	>200	>200

tional CAP classes. In other gram-negative bacteria, resistance to polymyxin may confer cross-resistance to other structural classes of CAPs due to structural modification of a common drug target (e.g., LPS), regulatory mutation, or both. Therefore, polymyxin-resistant strains of *P. aeruginosa* were tested for cross-resistance to additional CAPs. Quantitative bactericidal assays (39) were performed with the following modifications: cells were diluted to 2×10^4 CFU per ml prior to assay, and assays of human β-defensin activity were performed in 1.4% tryptic soy broth with 10 mM Na phosphate (pH 7.4) rather than Mueller-Hinton broth. The PAK*pmrB6* and PAK*pmrB12* strains displayed cross-resistance to defensins, protegrin, and α-helical peptides, as reflected by relative LD₅₀s (compared to those of the PAK parental strain) for human β-defensin-2 (4), protegrin-1 (39), and C18G, an α-helical peptide derived from the carboxy terminus of platelet factor IV (11), that ranged from 3 to >200 (Table 1). In addition, these strains were also resistant to human β-defensin-1, rabbit α-defensin NP1, and the α-helical cathelicidins CAP18, SMAP29, and LL37 (data not shown). These results indicate that the *P. aeruginosa* PmrAB system regulates resistance to a variety of structural classes of CAP.

Identification of the *P. aeruginosa* PmrAB homologue. Potential homologues of the *S. enterica* serovar Typhimurium *pmrAB* locus were identified by BLAST homology comparisons (1) of the *P. aeruginosa* Genome Project database (www.pseudomonas.com). This analysis revealed strong matches for *pmrA* in the *P. aeruginosa* open reading frames designated PA2479 (probability score of $6e^{-46}$) and PA4776 ($4e^{-48}$) and corresponding matches for *pmrB* in PA2480 ($4e^{-26}$) and PA4777 ($2e^{-28}$). Therefore, insertion mutations targeting these loci were constructed in the polymyxin-resistant strains. A loss of resistance was associated only with the disruption of the locus corresponding to PA4776 (*pmrA*) and PA4777 (*pmrB*) in *P. aeruginosa*, which encode a response regulator and a sensor histidine kinase displaying, respectively, 44 and 32% identity and 59 and 48% similarity to their *S. enterica* serovar Typhimurium homologues. A PAK*pmrB6*-derived strain with a gentamicin resistance cassette inserted within *pmrA* was as susceptible to killing by polymyxin as the parental PAK strain (Fig. 1A). The disruption of *pmrA* in PAK*pmrB12* gave similar results (data not shown). The polymyxin resistance phenotypes of these strains were completely dependent on an intact *pmrAB* locus. In contrast, the disruption of *phoP* in strain PAK*pmrB6* did not diminish the resistance phenotype (Fig. 1A).

Constitutive expression of the polymyxin resistance phenotype due to mutations in *pmrB*. To test their ability to confer constitutive polymyxin resistance on recipient *P. aeruginosa*

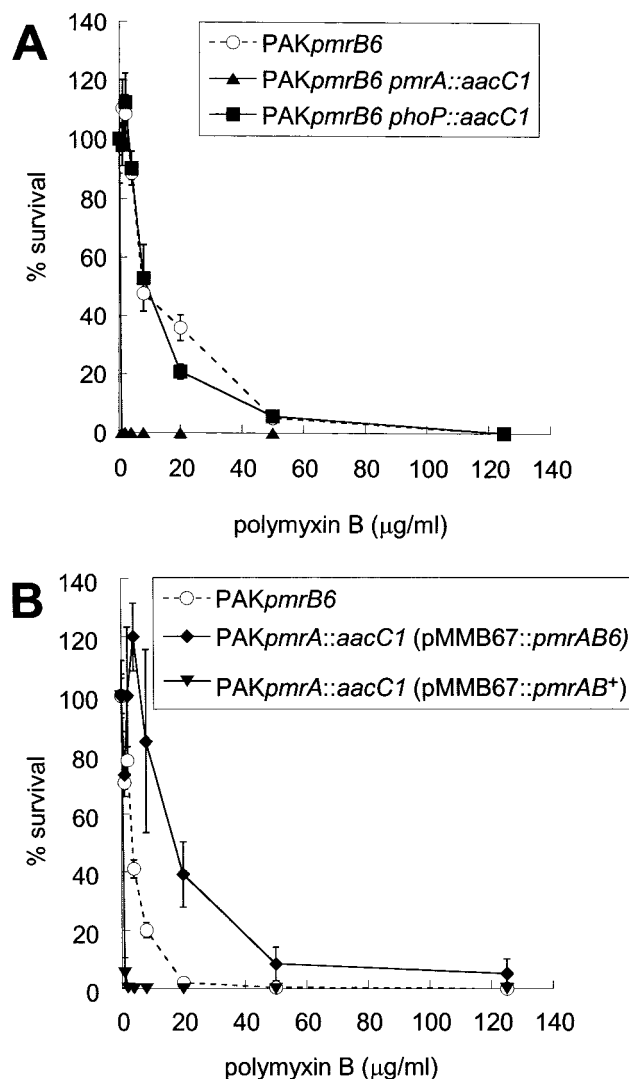


FIG. 1. Role of the *pmrAB* locus in *P. aeruginosa* polymyxin resistance. (A) Resistance of strains with an *aacC1* (Gm^r) cassette insertion. Triangles, PAK*pmrB6 pmrA::aacC1*; circles, PAK*pmrB6*; squares, PAK*pmrB6 phoP::aacC1*. (B) Resistance of strains with an episomal copy of the indicated *pmrAB* allele. Diamonds, PAK*pmrA::aacC1* (pMMB67HE::*pmrAB6*); circles, PAK*pmrB6*; inverted triangles, PAK*pmrA::aacC1* (pMMB67HE::*pmrAB*⁺).

strains, *pmrAB* alleles from strains PAK*pmrB6* and PAK*pmrB12* were amplified by PCR from chromosomal DNA and cloned into the IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible broad-host-range expression plasmid pMMB67HE (15). Strains carrying a plasmid with these alleles were then tested for resistance to polymyxin. Bacteria were grown in the presence of IPTG under Mg²⁺-replete conditions that do not induce physiological polymyxin resistance. The *pmrAB6* allele (but not the wild-type *pmrAB* allele or *pmrA6* alone) conferred constitutive resistance on the *pmrAB*-null strain PAK*pmrA::aacC1* (Fig. 1B). These results suggested the presence of a mutation in the *pmrB6* allele. Similar results were obtained for the *pmrB12* allele, indicating the presence of a mutation in *pmrB12*. Sequencing of these *pmrB* alleles revealed distinct missense mutations, L243Q in PAK*pmrB6* (single nucleotide

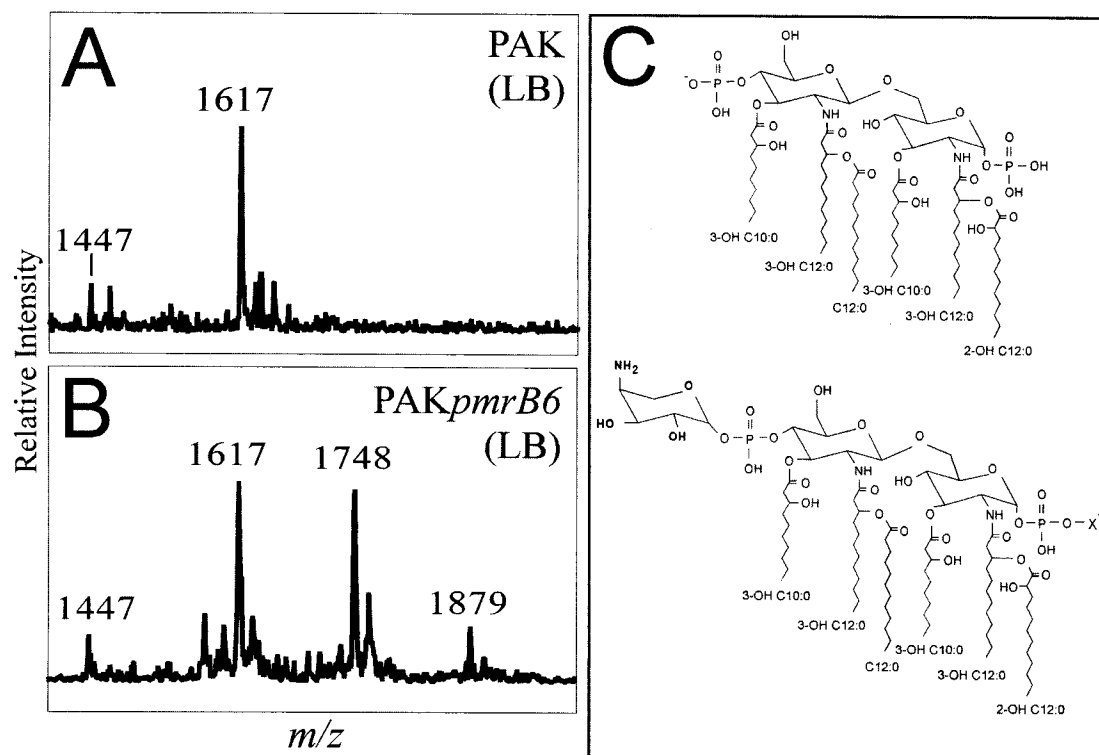


FIG. 2. Association of *pmrB* mutations with constitutive addition of aminoarabinose to *P. aeruginosa* lipid A. (A) MALDI-TOF negative-ion mode analysis of lipid A purified from *P. aeruginosa* strain PAK. In the negative-ion mode, observed molecular species lack at least one proton: $[M-H]^-$. The difference between $[M-H]^-$ at m/z 1,447 and m/z 1,617 ($\Delta m/z = 170$) in the mass spectrum indicates the loss of 3-hydroxydecanoate from position 3 of the reducing diglucosamine (right-hand ring in panel C). (B) Mass spectrum for lipid A from strain PAK $pmrB6$. The difference between $[M-H]^-$ at m/z 1,617, 1,748, and 1,879 ($\Delta m/z = 131$) indicates the addition of aminoarabinose to the 1 and 4' phosphates of lipid A. (C) Structure of *P. aeruginosa* lipid A without and with aminoarabinose. The X^- symbol at the right side of the lower structure represents either H (corresponding to m/z 1,748) or aminoarabinose (corresponding to m/z 1,879). The fatty acids depicted are 3-hydroxydecanoate (3-OH $C_{10:0}$), laurate ($C_{12:0}$), 2-hydroxylaurate (2-OH $C_{12:0}$), and 3-hydroxylaurate (3-OH $C_{12:0}$).

substitutions T5365486A) and A248V in PAK $pmrB12$ (C5365501T), in the histidine box motif of the sensor kinase, adjacent to the putative active-site histidine, H249. The H-box motif mediates phosphotransfer to the response regulator (PmrA) following sensor kinase activation and dimerization. Although the effect of any given H-box mutation cannot be readily predicted, the *P. aeruginosa pmrB* mutations are quite similar to activating mutations previously identified in *ntxB* (*glnL*), the nitrogen regulator II sensor kinase of *Escherichia coli* (2). Specifically, both the *pmrB6* allele and the *glnL1012* allele carry mutations at a position corresponding to a conserved leucine 6 residues towards the amino terminus from the active-site histidine, and the *pmrB12* allele and *glnL1004* allele carry identical mutations at a position corresponding to a conserved alanine immediately amino terminal to this histidine. Because the PmrAB system is known to stimulate aminoarabinose synthesis in *S. enterica* serovar Typhimurium, it is plausible that the *pmrB* H-box mutations selectively impair PmrB phosphatase activity, leading to constitutive activation of the PmrA regulon.

The *pmrAB* locus modulates the addition of aminoarabinose to lipid A in *P. aeruginosa*. Loci within the genome of *P. aeruginosa* strain PAO1, designated PA3552 to PA3559 (*pmrHFJKLME*), PA3540 (*algD*), and PA2022 (*ugd*), are ho-

mologues of PmrA-regulated *S. enterica* serovar Typhimurium genes that encode aminoarabinose synthetic enzymes essential for polymyxin resistance (17, 19). Because the polymyxin-resistant strains were thus expected to have aminoarabinose-modified LPS, lipid A was purified from them and analyzed. *P. aeruginosa* strains PAK, PAK $pmrB6$, PAK $pmrB12$, PAK $pmrB6\Delta pmrPAB::aacC1$, and PAK $pmrB12\Delta pmrPAB::aacC1$ were grown to stationary phase under conditions that do not induce physiologic aminoarabinose addition (LB broth with 1 mM $MgCl_2$ but without polymyxin B). Lipid A was isolated and analyzed as described previously (13) by negative-ion-matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (MS) by using a BIFLEX-III mass spectrometer (Bruker Daltonics Inc., Billerica, Mass.). PAK lipid A had mass peaks corresponding to the previously determined structures of *P. aeruginosa* lipid A (6, 22) containing five (m/z 1,447) or six (m/z 1,617) fatty acid substitutions (Fig. 2A). In contrast, PAK $pmrB6$ lipid A had additional mass peaks at m/z 1,748 and m/z 1,879 (Fig. 2B) corresponding to the addition of one or two 4-aminoarabinose moieties (change in m/z [$\Delta m/z$], 131) to the wild-type lipid A structure (Fig. 2C). The mass spectrum for PAK $pmrB12$ lipid A was indistinguishable from that of PAK $pmrB6$ (data not shown). Analyses of mass spectra for lipid A isolated from the PAK $pmrB6\Delta pmrPAB::aacC1$

and PAK $\Delta pmrB12\Delta pmrPAB::aacC1$ strains, in which the *pmrPAB* genes have been replaced by a gentamicin cassette, gave results similar to those for the wild type, indicating the PmrAB dependence of this modification in these strains. Because MALDI-TOF analysis is not quantitative, differences in polymyxin resistance observed among mutant strains may be due to differences in aminoarabinose content. Alternatively, differences may be due to changes in labile modifications of lipid A (e.g., phosphoethanolamine) lost during sample preparation (41) or to changes in nonlipid A surface structures, such as proximal LPS core sugar phosphates (21) or LPS-associated lipoproteins (20). Despite these possibilities, these results indicate that the *P. aeruginosa* PmrAB system mediates the addition of aminoarabinose to lipid A and provides additional support for the importance of this outer membrane modification in the polymyxin resistance of gram-negative bacteria.

The isolation of spontaneous polymyxin-resistant mutants of *P. aeruginosa*, described here for the first time, was a prerequisite to defining the PmrAB two-component system as an important regulator of *P. aeruginosa* resistance to polymyxin and other CAPs. The polymyxin resistance phenotypes of *P. aeruginosa* strains were associated with mutations in the H-box motif of the PmrB sensor kinase. Both the polymyxin resistance of these mutants and the addition of aminoarabinose to their lipid A under Mg^{2+} -replete conditions were dependent on the mutated *pmrAB* locus. These regulatory mutants were highly resistant to a variety of CAPs, indicating that the *P. aeruginosa* PmrAB system can induce CAP resistance.

The physiologic conditions that induce *P. aeruginosa* CAP resistance through PmrAB two-component signaling are not known. In *S. enterica* serovar Typhimurium, PmrB may function to sense the ionization state of iron (40); however, its periplasmic domain, the presumed site of this sensing capability, lacks homology to that of *P. aeruginosa*. Unlike *S. enterica* serovar Typhimurium, the viability of *P. aeruginosa* at high ferrous iron concentrations is not diminished by a disruption of *pmrAB*, and extracellular iron does not induce polymyxin resistance in a *pmrAB*-dependent fashion (S. M. Moskowitz and S. I. Miller, unpublished results). Moreover, the *P. aeruginosa* PAO1 genome lacks a homologue of the *pmrD* gene (33), an important regulator of PmrAB activation in *S. enterica* serovar Typhimurium (24). Thus, the activation of PmrAB in *P. aeruginosa* may differ significantly from that in *S. enterica* serovar Typhimurium.

The levels of transcriptional regulation of the *pmrAB* locus in *P. aeruginosa* strains also appear to differ. Polymyxin B and other CAPs induce transcription of the *P. aeruginosa pmrAB* locus in a PhoPQ-independent fashion (28). In contrast, in *S. enterica* serovar Typhimurium, subinhibitory concentrations of CAPs induce CAP resistance in a PhoPQ-dependent fashion (3). Nonetheless, both divalent cation deficiency (7, 13, 27) and acidity (Moskowitz and Miller, unpublished results) induce mild polymyxin resistance in wild-type *P. aeruginosa*, similar to effects in *S. enterica* serovar Typhimurium that are mediated by the PhoPQ and PmrAB systems, respectively (38). Thus, in both organisms, the induction of CAP resistance, triggered by various physiologic conditions acting through these two-component systems, may represent an important step in adaptation to host environments.

P. aeruginosa strains isolated from infants with cystic fibrosis

(8) have aminoarabinose addition to lipid A (13) as an early adaptation, consistent with the notion that CAPs impose selective pressure on *P. aeruginosa* in the airways of individuals with cystic fibrosis (37). Moreover, inhaled polymyxin E (colistin) is commonly used to treat cystic fibrosis airway infection in Europe and Australia (12, 14, 26), and its intravenous use has also been advocated for the treatment of multidrug-resistant *P. aeruginosa* (10). Colistin-resistant strains of *P. aeruginosa* isolated from patients receiving inhaled colistin as routine maintenance therapy (12, 14) have alterations in the lipid A structure, including the addition of aminoarabinose (S. M. Moskowitz, R. K. Ernst, and S. I. Miller, unpublished results). Such lipid A modifications indicate potential targets for the development of novel antipseudomonal agents that could act synergistically with polymyxin.

Nucleotide sequence accession numbers. Sequences of PAK *pmrB* wild-type, *pmrB6*, and *pmrB12* alleles have been registered with the GenBank database under accession numbers AY493419 to AY493421.

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