Genomic variations between colistin-susceptible and -resistant *Pseudomonas aeruginosa* clinical isolates and their effects on colistin resistance

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**Objectives:** The emergence of colistin-resistant *Pseudomonas aeruginosa* is becoming a serious concern worldwide. We investigated genetic variations involved in the acquisition and loss of colistin resistance in three clinical isogenic *P. aeruginosa* isolates (GKK-1, GKK-2 and GKK-3) recovered from a single patient and assessed their impacts on colistin resistance.

**Methods:** We applied whole genome sequencing technology to identify single nucleotide polymorphisms and insertions or deletions in two colistin-resistant isolates compared with a susceptible isolate.

**Results:** Thirty-seven non-synonymous mutations in 33 coding sequences were detected in the colistin-resistant isolates GKK-1 and GKK-3. Only one gene (PA1375) was significantly down-regulated in both colistin-resistant isolates; this gene encodes erythronate-4-phosphate dehydrogenase. Among the eight genes that were up-regulated in the colistin-resistant isolates, three encoded hypothetical proteins (PA1938, PA2928 and PA4541) and five were predicted to be involved in core biological functions, encoding a cell wall-associated hydrolase (PA1199), a response regulator EraR (PA1980), a sensor/response regulator hybrid (PA2583), a glycosyltransferase (PA5447) and an arabinose efflux permease (PA5548). All mutants with allelic replacement of these candidate genes, apart from one (PA1375), exhibited increases in colistin susceptibility, ranging from 2- to 16-fold. Colistin susceptibility decreased in complemented strains compared with the mutants; however, in three cases, resistance did not reach wild-type level.

**Conclusions:** This study demonstrates genetic differences between *P. aeruginosa* isogenic isolates and identifies novel determinants that may be associated with the acquisition of colistin resistance. These findings will lay the foundation for a complete understanding of the molecular mechanisms of colistin resistance in *P. aeruginosa*.

**Keywords:** whole genome sequencing, two-component regulatory systems, glycosyltransferases

**Introduction**

*Pseudomonas aeruginosa* is one of the major organisms responsible for nosocomial infections such as pneumonia, urinary tract infections, surgical site infections and bloodstream infections. Infections caused by *P. aeruginosa* are very difficult to treat, due to its common intrinsic resistance to many antimicrobial agents. In addition to intrinsic resistance, *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 *P. aeruginosa* has led to a resurgence in the use of polymyxin antibiotics such as polymyxin B and colistin (polymyxin E) as therapeutic agents. Polymyxins are a family of antimicrobial cyclic oligopeptides synthesized by the Gram-positive organism *Bacillus polymyxa*. The two clinically available forms, polymyxin B sulphate and colistimethate (a prodrug of colistin), are administered to cystic fibrosis patients either intravenously or by inhalation. Polymyxins bind to lipopolysaccharide (LPS), the major constituent of the Gram-negative outer membrane, thus promoting membrane permeabilization and the diffusion of peptides across the periplasm. The insertion of polymyxins into the inner membrane disrupts cellular respiration and results in cell lysis and death. With the increased use of polymyxins, the emergence of polymyxin-resistant *P. aeruginosa* isolates has been reported around the world. Resistance to polymyxins is associated with specific modification of the lipid A component of LPS, resulting in a reduction of the net negative charge of the outer membrane.
Colistin resistance in \textit{P. aeruginosa}

In \textit{P. aeruginosa}, LPS modification is triggered by Mg\textsuperscript{2+}-limiting conditions and upon exposure to subinhibitory concentrations of antimicrobial peptides.\textsuperscript{9,10} In addition, it has been reported that two two-component regulation systems, PhoPQ and PrmAB, respond to Mg\textsuperscript{2+}-limiting conditions, resulting in polymyxin resistance in \textit{P. aeruginosa}.	extsuperscript{11} Recently, the ParRS and CprRS two-component regulatory systems have also been found to play a role in polymyxin resistance in \textit{P. aeruginosa}.	extsuperscript{12,13} Although the two-component systems PrmAB, PhoPQ, ParRS and CprRS are known to be involved in polymyxin resistance in \textit{P. aeruginosa}, the precise molecular details of these resistance mechanisms have remained unclear, including a correlation of polymyxin susceptibility with amino acid alterations in these two-component systems.

Recent advances in whole genome sequencing technology and comparative genetic analysis have permitted detailed investigations of genetic differences between pairs of bacterial isolates. In particular, the mechanisms and evolution of antimicrobial resistance in pathogens have been explored in many studies by using whole genome sequencing.\textsuperscript{14–20} Comparisons of whole genome sequences between antibiotic-resistant and -susceptible isolates enable the identification of mutations associated with antimicrobial resistance.

In this study, we investigated the genetic differences between colistin-resistant and -susceptible \textit{P. aeruginosa} isogenic isolates recovered from a single patient,\textsuperscript{21} using comparative genome sequencing. In addition, we explored whether the identified genetic differences are associated with the acquisition of colistin resistance in \textit{P. aeruginosa} using molecular techniques. Our results show that several additional mechanisms, including novel two-component regulatory systems, are involved in the complicated regulatory network mediating adaptive resistance to colistin in \textit{P. aeruginosa}.

Materials and methods

\textbf{Bacterial strains, plasmids and growth conditions}

Whole genome sequence drafts were obtained from two colistin-resistant \textit{P. aeruginosa} isolates (GKK-1 and GKK-3) and a colistin-susceptible isolate (GKK-2) (Table S1, available as Supplementary data at \textit{JAC} Online). These isolates were obtained sequentially from a single patient with bilateral ureteral stones and hydronephrosis in both kidneys, who developed resistance to colistin during treatment. These isolates were identified as isogenic, only differing in their polymyxin resistances. While the MICs of colistin and polymyxin B for the colistin-resistant isolates were 8 and 4 mg/L, respectively, those of the colistin-susceptible isolate (GKK-2) were 1 mg/L for both colistin and polymyxin B. A detailed clinical history of the patient and preliminary characterization of the isolates have been described previously.\textsuperscript{21} The genetically manipulated strains and plasmids present in colistin-resistant isolates were confirmed by Sanger sequencing. To predict whether the identified amino acid substitutions were likely to affect protein function, sorting intolerant from tolerant (SIFT) scores were calculated (http://sift.jcvi.org). Based on the amino acids appearing at each position, SIFT calculates the probability that an amino acid substitution at a position is tolerated, depending on the most frequent amino acid being tolerated. If the normalized value is \(\leq 0.05\), the substitution is predicted to be deleterious.\textsuperscript{22,23}

\textbf{Quantitative real-time PCR (qRT–PCR)}

The expression levels of selected mRNA transcripts were quantified in the three \textit{P. aeruginosa} isolates by qRT–PCR essentially as previously described,\textsuperscript{24} with a few modifications. In brief, total RNA from the three \textit{P. aeruginosa} isolates was extracted from mid-log-phase bacterial cultures (optical densities at 600 nm of \(\text{OD}_{600} \approx 0.5\)) using the Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Contaminating genomic DNA was removed from total RNA samples with the Ambion DNA-free Kit (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer’s protocols. Purified RNA was quantified spectrophotometrically. Reverse transcription reactions were performed in accordance with the recommended protocol for Omniscript reverse transcriptase (Qiagen). Quantification of the transcripts was performed by using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) with an ABI7300 Sequence Detection System (Applied Biosystems). Primers were designed for this study with Primer3 and are shown in Table S2 (available as Supplementary data at \textit{JAC} Online). Expression of the 30S ribosomal housekeeping gene \textit{rpsl} (rpsl) was assessed in parallel for the calculation of fold changes according to the threshold cycle (\(C_{T}\)) method. Experiments were repeated with three independent cultures, each tested in duplicate. Differences in the gene expression levels of target genes between colistin-resistant and -susceptible isolates were analysed by one-way ANOVA using SPSS 15.0 software. Differences were considered to be significant at \(P \leq 0.05\).

For allelic replacement and overexpression mutants, expression levels of \textit{pmrA}, \textit{phoP} and \textit{annB} were also determined as described above. Expression levels were compared with those of the appropriate wild-type isolates: P80 for the allelic replacement mutants and P155 for the overexpression mutants.

\textbf{Construction of mutants with allelic replacement}

Since no antibiotic markers to select mutants were found in strains GKK-1 and GKK-3,\textsuperscript{21} a colistin-resistant \textit{P. aeruginosa} clinical isolate P80 (Table S1)
was used to construct mutants with allelic replacement. Allelic replacement mutagenesis was performed with minor modifications of a previously described method.\textsuperscript{25} Briefly, \textasciitilde450 bp chromosomal DNA segments, containing left and right regions of the target genes, were PCR amplified from chromosomal DNA using specific oligonucleotide primers (Table S2). Reverse primers for left fragments and forward primers for right fragments were designed to contain 20 nucleotides identical to the 5’- and 3’-regions of the cassette. Ultimately, a constructed fusion PCR fragment replaced a portion of the target gene with a cassette gene. For replacement of the target genes and the selection of mutants, we used the kanamycin resistance gene cassette (904 bp) containing the aphIII gene and its promoter region. This cassette was amplified from Enterococcus faecalis ATCC 51299 chromosomal DNA. After two-step PCR, the resulting fusion PCR products (\textasciitilde500 ng/μL) were directly transformed into competent cells harbouring the helper plasmid pHK1014, which contains the aacC1 gene for selection of transformants, the origin of replication from pUCP18\textsuperscript{26} and the λRed recombinase for stimulating homologous recombination at the target site from pKD46,\textsuperscript{27,28} Homologous recombination between the construct and the target gene in the chromosome was forced using the chemical transformation method.\textsuperscript{29} Kanamycin-resistant transformants were selected on LB agar plates supplemented with 200 mg/L kanamycin. Targeted gene replacement events were confirmed by colony PCR. Cell lysates from mutant strains were used as PCR templates, along with primers L-F and R-R, to verify the correct incorporation of a gene replacement construct into the genome. The correct incorporation of a fused construct (left fragment–kanamycin resistant cassette–right fragment) resulted in a larger or smaller PCR product (\textasciitilde1.8 kb) obtained from a mutant strain compared with that of the wild-type strain.

**Complementation of target genes in their mutants and overexpression of target genes in a colistin-susceptible P. aeruginosa clinical isolate**

Each gene was amplified by PCR using genomic DNA from the colistin-resistant isolate GKK-1 as a template with specific oligonucleotide primers (Table S2). Primers were engineered to carry specific restriction sites for cloning and were designed with Primer3. The fragments resulting from the PCR were cut with the appropriate restriction enzymes and cloned into pUC195,\textsuperscript{30} digested with the same restriction enzymes. Each ligation mixture was then used to transform E. coli DH5α competent cells for cloning. In order to obtain complemented strains, plasmids were transformed into the appropriate mutants by electroporation with a Gene Pulser apparatus (Bio-Rad). Transformation mixtures were plated onto LB plates containing 50 mg/L gentamicin and 50 mM L-arabinose. Plasmids were also introduced separately into electrocompetent cells of the colistin-susceptible strain (P155) to generate overexpression strains.

**Results**

**Non-synonymous mutations in the colistin-resistant P. aeruginosa isolates GKK-1 and GKK-3, compared with GKK-2**

Next-generation sequencing produced \textasciitilde2.7 billion, 2.4 billion and 2.6 billion nucleotides for GKK-1, GKK-2 and GKK-3, respectively. They covered 279-, 250- and 279-fold, respectively. We were able to map reads from three isolates to 78.9%, 78.5% and 79.6% of the P. aeruginosa PAO1 reference genome, respectively. Comparative genome sequence analysis identified 166 nucleotide alterations (160 SNPs and 6 short indels) between the colistin-resistant isolates and a colistin-susceptible isolate (Table S3, available as Supplementary data at JAC Online). Specifically, these nucleotides were the same in both colistin-resistant isolates (GKK-1 and GKK-3), but differed in the colistin-susceptible isolate (GKK-2). Of these 166 nucleotide alterations, 114 (113 SNPs and 1 deletion in colistin-resistant isolates) were found in coding sequences (CDSs) and 52 alterations (47 SNPs and 5 indels) were identified in intergenic regions. Only 39 (38 SNPs and 1 deletion) of 114 nucleotide alterations located in CDSs were expected to be non-synonymous mutations resulting in substitutions of amino acids. Of these, two successive mutations were also found in two CDSs (PA2928 and PA4554) and two kinds of amino acid substitutions were identified in four CDSs (PA1086, PA1874, PA4088 and PA4554) (Table 1). Thus, 37 amino acid substitutions were distributed among 33 CDSs. Compared with the colistin-susceptible isolate, GKK-2, 84 and 81 nucleotide alterations (in 74 and 71 CDSs, respectively) were found solely in GKK-1 and GKK-3, respectively.

In this study, amino acid alterations were also found in PmrAB and PhoPQ two-component regulatory systems, which are known to be related to colistin resistance; however, these alterations were not simultaneously identified in both colistin-resistant isolates (GKK-1 and GKK-3). In PmrB, G20C and V136G were identified in GKK-1 and GKK-3, respectively. In PhoP, V99M was identified only in GKK-1. Colistin resistance may occur in an independent manner\textsuperscript{26} and, thus, mutations in just one of the resistant isolates may be important in colistin resistance. In this study, however, we focused on genes showing identical non-synonymous mutations in both colistin-resistant P. aeruginosa isolates (GKK-1 and GKK-3) to investigate the common mutations associated with colistin resistance. We reasoned that these genes may provide us with the best insight into the link between genetic mutations and the development of colistin-resistant phenotypes in P. aeruginosa. Although several two-component regulatory systems such as PmrAB, PhoPQ, ParRS, CprRS and ColRS have been reported to be associated with colistin resistance, no mutations were found in the pmrA, phoQ, parR, parS, cprR, cprS, colR and colS genes.

**SIFT scores**

Subsequently, SIFT scores were calculated for 37 amino acid substitutions to predict whether these substitutions, identified in both colistin-resistant isolates, affect protein function. As shown in Table 1, only 9 of the 37 non-synonymous mutations were predicted to be deleterious (SIFT score \textasciitilde0.05), with the other 28 mutations predicted to be tolerant mutations, probably having less impact on protein function. This result suggests that not all non-synonymous mutations in colistin-resistant isolates might be directly involved in colistin resistance per se.

**Comparison of gene expression**

Expression levels of 33 genes, harbouring non-synonymous substitutions in both colistin-resistant isolates, were compared between colistin-resistant and -susceptible isolates by qRT–PCR (data not shown). Comparisons of transcript levels revealed that nine genes showed significantly different expression levels between colistin-resistant and -susceptible isolates: eight genes (PA1199, PA1938, PA1980, PA2583, PA2928, PA4541, PA5447 and PA5548) were expressed at greater levels in both colistin-resistant isolates (3- to 9-fold increases) and one gene (PA1375)
was expressed at a lower level in both colistin-resistant isolates, with an ≈3-fold decrease (Figure 1).

In contrast, the other 24 genes showed no significant differences in gene expression between the colistin-resistant and -susceptible isolates or exhibited inconsistent gene expression patterns. We hypothesized that the nine genes exhibiting differences in gene expression in both colistin-resistant isolates may play a role in adaptive colistin resistance and further investigated their effects on colistin susceptibility in *P. aeruginosa* through allelic replacement of the gene, complementation and overexpression studies.

### Effects on colistin susceptibility of gene inactivation and complementation

We attempted to construct allelic replacement mutants of all nine candidate genes in a colistin-resistant clinical isolate (P80). We successfully replaced eight of the nine candidate genes in the P80 background; however, we were unable to generate a PA1938-deleted mutant, even after repeated attempts.

All mutants, apart from one (P80ΔPA1375), showed a decrease in the MIC of colistin, ranging from 2- to 16-fold, compared with the wild-type strain (Table 2). Allelic replacement of PA2928, a

### Table 1. Non-synonymous mutations between colistin-susceptible and -resistant *P. aeruginosa* isolates

| Locus ID<sup>a</sup> | Gene name | Product | Genome position | PAO1 and GKK-2 | GKK-1 and GKK-3 | Amino acid substitution(s) | SIFT score |
|---------------------|-----------|---------|-----------------|---------------|-----------------|---------------------------|-----------|
| PA0207              | transcriptional regulator | 236883  | T               | G             | H222Q                                 | 0.05<sup>b</sup> |
| PA0246              | major facilitator superfamily transporter | 279066  | T               | C             | I112V                                 | 0.23      |
| PA0549              | hypothetical protein | 608475  | T               | C             | Y107H                                 | 0.16      |
| PA0979              | hypothetical protein | 1062225 | G               | C             | Q49E                                  | 0.06      |
| PA1086              | flagK flagellar hook-associated protein FlgK | 1172411 | C               | G             | S83R                                  | 0.03<sup>b</sup> |
| PA1199              | lipoprotein | 1300783 | G               | A             | V176M                                 | 0.01<sup>b</sup> |
| PA1223              | transcriptional regulator | 1326908 | GAGTT           | —             | N10 and L11 deletion                   | —         |
| PA1375              | pdxB erythronate-4-phosphate dehydrogenase | 1493012 | A               | G             | E367G                                  | 0.37      |
| PA1537              | short-chain dehydrogenase | 1675230 | C               | G             | D213H                                  | 0.03<sup>b</sup> |
| PA1600              | cytochrome c | 1741955 | C               | T             | R429Q                                  | 0.15      |
| PA1874              | hypothetical protein | 2041238 | A               | G             | N1600D                                 | 0.71      |
| PA1938              | hypothetical protein | 2119550 | G               | C             | A209P                                  | 1.00      |
| PA1980              | eraR response regulator EraR | 2165882 | G               | A             | V6I                                     | 0.03<sup>b</sup> |
| PA2200              | hypothetical protein | 2419044 | G               | A             | D108N                                  | 0.57      |
| PA2214              | major facilitator superfamily transporter | 2436282 | T               | C             | V294A                                  | 0.01<sup>b</sup> |
| PA2360              | hypothetical protein | 2607389 | T               | C             | T82A                                   | 1.00      |
| PA2385              | PvdQ 3-oxo-C12-homoserine lactone acylase PvdQ | 2638238 | C               | T             | A190T                                  | 0.32      |
| PA2474              | hypothetical protein | 2792602 | C               | G             | L33V                                   | 0.78      |
| PA2583              | sensor/response regulator hybrid | 2921447 | T               | C             | T375A                                  | 0.30      |
| PA2928              | hypothetical protein | 3284242–3 | GT             | AG            | D119A                                  | 0.04<sup>b</sup> |
| PA3063              | pelB PelB | 3472842 | G               | C             | V1076L                                 | 0.45      |
| PA3297              | ATP-dependent helicase | 3692354 | G               | A             | S884L                                  | 0.01<sup>b</sup> |
| PA3595              | major facilitator superfamily transporter | 4037077 | T               | C             | V340A                                  | 0.66      |
| PA4088              | aminotransferase | 4571518 | C               | A             | A197D                                  | 0.59      |
| PA4394              | hypothetical protein | 4924552 | C               | G             | V178L                                  | 0.06      |
| PA4541              | hypothetical protein | 5086443 | A               | G             | V1334A                                  | 0.81      |
| PA4554              | pilY1 type 4 fimbrial biogenesis protein PilY1 | 5101692 | A               | G             | N373S                                  | 0.35      |
| PA4554<sup>a</sup>  | pilY1 type 4 fimbrial biogenesis protein PilY1 | 5103521–2 | GC             | AG            | A947S                                  | 0.96      |
| PA4627              | hypothetical protein | 5194470 | C               | T             | T65I                                   | 0.01<sup>b</sup> |
| PA5175              | cysQ 3,5-bisphosphate nucleotidase CysQ | 5828822 | A               | G             | V29A                                  | 1.00      |
| PA5430              | hypothetical protein | 6112014 | C               | G             | L84V                                   | 1.00      |
| PA5447              | wbpZ glycosyltransferase | 6168557 | G               | A             | A259T                                  | 0.53      |
| PA5534              | hypothetical protein | 6227989 | T               | C             | T86A                                   | 0.48      |
| PA5548              | major facilitator superfamily transporter | 6242938 | A               | G             | K363R                                  | 0.34      |

<sup>a</sup>Bold font indicates genes showing differences in expression levels between colistin-resistant and -susceptible isolates.

<sup>b</sup>A significant difference in gene function, as predicted by the SIFT score.
Figure 1. Transcript levels of nine genes showing significant differences in expression levels in the colistin-resistant isolates GKK-1 and GKK-3, compared with the colistin-susceptible isolate GKK-2. (a) PA1199, (b) PA1375, (c) PA1938, (d) PA1980, (e) PA2583, (f) PA2928, (g) PA4541, (h) PA5447 and (i) PA5548. Error bars represent the standard deviations of three biological repeats, each performed in duplicate.

Table 2. MICs of colistin for the eight mutants in the P80 background and their respective complemented strains

| Locus ID | Gene name | in colistin-resistant isolate P80 background | in colistin-susceptible isolate P155 background |
|----------|-----------|--------------------------------------------|-----------------------------------------------|
|          |           | wild-type P80 | mutant | complemented strain | wild-type P155 | overexpression strain |
| PA1199   |           | 8            | 2      | 8                    | 0.5            | 2                        |
| PA1375   | pdxB      | 8            | 8      | 8                    | 0.5            | 0.5                      |
| PA1980   | eraR       | 8            | 4      | 8                    | 0.5            | 0.5                      |
| PA2583   |           | 8            | 4      | 8                    | 0.5            | 4                        |
| PA2928   |           | 8            | 0.5    | 4                    | 0.5            | 8                        |
| PA4541   |           | 8            | 2      | 4                    | 0.5            | 4                        |
| PA5447   | wbpZ       | 8            | 4      | 8                    | 0.5            | 2                        |
| PA5548   |           | 8            | 2      | 4                    | 0.5            | 8                        |

aGene inactivation and complementation were unsuccessful for PA1938, even after repeated attempts.

bIncreased or decreased MICs by ≥2-fold compared with those for the wild-type strain or mutant are indicated in bold font.
gene encoding a protein of unknown function, caused the largest decrease in the MIC of colistin, from 8 to 0.5 mg/L. Allelic replacement of PA1199, PA4541 and PA5548, genes encoding a lipoprotein, a hypothetical protein and a major facilitator superfamily transporter, respectively, also greatly reduced colistin resistance (MICs, 8 mg/L to 2 mg/L). No decrease in the MIC of colistin for P80ΔPA1375 was expected, since PA1375 was down-regulated in colistin-resistant isolates; therefore, its allelic replacement might not increase colistin susceptibility.

Eight candidate genes were complemented, in order to verify that the increased colistin susceptibilities observed in the mutants resulted from allelic replacement of the gene. In all complemented strains, the MICs of colistin increased compared with the mutants (Table 2). In the complemented strains of P80ΔPA1199, P80ΔPA1980, P80ΔPA2583 and P80ΔPA5447 (P80ΔPA1199-C, P80ΔPA1980-C, P80ΔPA2583-C and P80ΔPA5447-C, respectively), the MIC of colistin was restored to that for the wild-type isolate (P80). In P80ΔPA2928-C, P80ΔPA4541-C and P80ΔPA5548-C, the MICs of colistin increased to 4 mg/L; however, these strains did not acquire wild-type levels of colistin resistance.

**Effect of gene overexpression in a colistin-susceptible isolate**

To investigate the contributions of specific mutations found in colistin-resistant isolates and the effects of overexpressing candidate genes, we cloned eight genes from GKK-1 (except PA1938) into the L-arabinose-inducible expression vector pJN105; subsequently, we transformed these constructs into a colistin-susceptible clinical isolate (P155). In six transformed strains, P155-pGKK1199, P155-pGKK2583, P155-pGKK2928, P155-pGKK4541, P155-pGKK5447 and P155-pGKK5548, colistin susceptibility decreased by 4- to 16-fold compared with wild-type P155 (Table 2). However, two transformed strains of PA1375 and PA1980 (P155-pGKK1375 and P155-pGKK1980, respectively) showed no change in the MICs of colistin.

**Impact of candidate genes on expression levels of pmrA, phoP and arnB**

The addition of 4-amino-4-deoxy-l-arabinose (L-Ara4N) to the phosphate group of lipid A is known to be associated with colistin resistance in *P. aeruginosa* and the *arnB* operon encodes enzymes responsible for the biosynthesis and attachment of L-Ara4N to lipid A.10,31 To investigate the effects of the eight candidate genes on the PmrAB and PhoPQ two-component regulatory systems and lipid A modification, expression levels of *pmrA*, *phoP* and *arnB* were determined in the mutants and overexpression strains. No significant dysregulation of the *phoP* gene was observed in any of the mutants (Figure 2b) and no significant differences or even decreases in expression of the *phoP* gene were identified in any of the overexpression strains (Figure 2e). However, expression levels of the *pmrA* and *arnB* genes simultaneously decreased and increased in some mutants and overexpression strains (Figure 2a, c, d and f).

The PA1199-, PA2928- and PA5548-deleted mutants showed considerably lower expression levels of both *pmrA* and *arnB*, exhibiting 4- to 7-fold decreases compared with their

![Figure 2](https://academic.oup.com/jac/article-abstract/69/5/1248/683364)

Figure 2. Gene expression analysis of *pmrA*, *phoP* and *arnB* in mutants (a–c) for eight colistin resistance determinants and their complemented strains (d–f). Error bars represent the standard deviations of two biological repeats, each performed in duplicate. WT, wild-type.
wild-type colistin-resistant counterpart (P80) (Figure 2a and c). P80\(\Delta PA2583\) showed 3- and 1.7-fold reductions in the expression levels of \(pmrA\) and \(arnB\), respectively, but these reductions were not significant (\(P=0.014\) and \(P=0.033\), respectively). P80\(\Delta PA1375\), P80\(\Delta PA1980\), P80\(\Delta PA4541\) and P80\(\Delta PA5447\) showed no differences in either \(pmrA\) or \(arnB\) expression levels.

As in the deletion mutants, PA1199-, PA2928- and PA5548-overexpression strains of P155 showed significant increases in the expression levels of \(pmrA\) and \(arnB\), ranging from 4- to 15-fold, compared with their colistin-susceptible wild-type counterpart (P155) (Figure 2d and f). In addition to these three overexpression strains, P155-pGKK2583 also showed increases in the expression levels of both \(pmrA\) and \(arnB\) (7- and 2-fold, respectively). However, the other four overexpression strains (P155-pGKK1375, P155-pGKK1980, P155-pGKK4541 and P155-pGKK5447) exhibited no significant expression differences from their wild-type counterpart.

Discussion

In this study, we compared the whole genome sequences of multiple \(P.\ aeruginosa\) clinical isolates obtained from one patient undergoing colistin treatment; these isolates varied with respect to colistin susceptibility. These isolates are believed to be isogenic, since they show the same genotype as judged by PFGE, multilocus sequence typing and antibiograms, with the exception of polymyxins.

A notable finding of the present study is that the three \(P.\ aeruginosa\) isolates showed remarkable nucleotide variations. In total, 467 nucleotide variations (279 in CDSs and 188 in intergenic regions) were identified in at least one colistin-resistant isolate. Of these, 166 SNPs were common to both colistin-resistant isolates (GKK-1 and GKK-3). Such a high number of mutations may be driven by the presence of mutator phenotypes, although we did not investigate the mutator phenotype in our strains. In addition, 37 non-synonymous substitutions were identified in 33 genes; these substitutions were common to both colistin-resistant \(P.\ aeruginosa\) isolates (GKK-1 and GKK-3). The high sequence variability of isolates circulating during colistin treatment was not observed with \(Acinetobacter\ baumannii\).

It has been postulated that repeated isolation of colistin-resistant \(P.\ aeruginosa\) isolates may be due to recombination by a different isolate; alternatively, colistin may provoke genomic variation in \(P.\ aeruginosa\). Since our primary aim was to identify the genetic determinants of colistin resistance in \(P.\ aeruginosa\), we focused our analysis on the 33 genes with common amino acid substitutions in both colistin-resistant \(P.\ aeruginosa\) isolates.

The expression levels of 9 of these 33 genes were dysregulated in both colistin-resistant isolates compared with a colistin-susceptible isolate. We attempted to construct mutants for these nine genes with allelic replacement and successfully obtained mutants for eight genes; we were unsuccessful in generating a PA1938-deletion mutant. Of the genes examined, only \(pdxB\) (PA1375), which encodes erythronate-4-phosphate dehydrogenase and is involved in the biosynthesis of pyridoxine (vitamin B6), showed decreased expression in colistin-resistant isolates. However, neither allelic replacement nor overexpression of \(pdxB\) resulted in a detectable effect on colistin resistance. Recent work suggests that \(pdxB\) plays a role in regulating susceptibility to several cationic antimicrobial peptides, including colistin; however, in our study, \(pdxB\) did not appear to affect colistin resistance. The other seven genes exhibited increased colistin susceptibility in their mutants and restored colistin resistance in their complemented strains.

In addition, decreased colistin susceptibility was observed in overexpression strains of six genes, with the exception of PA1980 (\(eraR\)), a gene encoding the response regulator \(EraR\). Thus, we have identified six genes associated with adaptive colistin resistance in \(P.\ aeruginosa\): PA1199, PA2583, PA2928, PA4541, PA5447 and PA5548. Of these six genes, four (PA1199, PA2583, PA2928 and PA5548) affected the expression levels of \(pmrA\) and \(arnB\) both in their mutants (Figure 2a and c) and in the overexpression strains (Figure 2d and f). Thus, we hypothesize that these four genes are associated with expression of the PmrAB two-component system and affect the \(arnB\) operon, which adds L-Ara4N to the phosphate group of lipid A. Attachment of L-Ara4N to lipid A alters the negative charge of the outer membrane, invoking colistin resistance. It is also of note that \(phoP\) expression did not vary between any of the gene mutants or overexpression strains. Therefore, we conclude that the PhoPQ two-component regulatory system is probably not involved in the process of lipid A modification in these gene replacement or overexpression mutants.

PA1199 encodes a lipoprotein that is known to act as a cell wall-associated hydrolase. This lipoprotein possesses 69% similarity to the ybdO endopeptidase of \(E.\ coli\), which is known to be involved in peptidoglycan synthesis for bacterial growth. PA2583 encodes a sensor/response regulator hybrid acting in the two-component regulatory system and is 61% similar to the BvgS protein of \(Bordetella pertussis\), which regulates the virulence properties of \(B.\ pertussis\). PA5447 encodes a major facilitator superfamily transporter, which acts as an arabinoside efflux permease and shows 45% similarity to the putative multidrug efflux transporter yceJ, located in a region of lincomycin resistance genes in \(Bacillus subtilis\). The function of PA2928 is unknown. The relationships between these genes, the PmrAB two-component system and the \(arnB\) operon would be interesting to investigate.

The other three genes (PA1980, PA4541 and PA5447) may influence colistin susceptibility in a manner that is independent of LPS modification mediated by the PmrAB and PhoPQ two-component systems and the \(arnB\) operon. PA1980 (\(eraR\)) encodes a response regulator of the \(EraRS\) two-component regulatory system. In addition to PmrAB and PhoPQ, two additional two-component systems (ParRS and CprRS) have recently been reported to be associated with colistin resistance in \(P.\ aeruginosa\). Although further studies are required, our results suggest that additional two-component regulatory systems, including EraRS, may be involved in colistin resistance in \(P.\ aeruginosa\). PA5447, otherwise known as \(wpb\), encodes a glycosyltransferase participating in LPS biosynthesis. Although it is unknown how PA5447 expression is regulated, its up- or down-regulation would probably affect the structure of LPS, thereby resulting in differences in colistin susceptibilities in bacterial pathogens. Although the precise function of the PA5447 product is not yet known, it is predicted to be either a membrane protein or a secreted factor according to the Pseudomonas Genome Database (www.pseudomonas.com). It may be worthwhile to investigate its association with colistin resistance.
In this study, we identified nine genes with amino acid alterations and altered expression levels common in colistin-resistant P. aeruginosa isolates compared with a colistin-susceptible isolate. Four genes, encoding a cell-wall-associated hydrolase (PA1199), a sensor/response regulator hybrid (PA2583), an arabinoxyloketon urease (PA5548) and a protein with unknown function (PA2928), may affect colistin susceptibility by regulating LPS modification through a mechanism mediated by the PmrAB two-component system. Three other genes, which encode the response regulator EraR (PA1980), a glycosyltransferase participating in LPS biosynthesis (PA5447) and a protein with unknown function (PA4541), may influence colistin susceptibility in a manner that is not associated with LPS modification. Importantly, these genes have not been reported to be involved with colistin resistance in any previous studies. Thus, adaptive colistin resistance in P. aeruginosa is mediated by a complicated regulatory network that probably emerges through diverse genetic alterations. Our findings will provide further understanding of the molecular mechanisms by which colistin resistance emerges in P. aeruginosa.

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Transparency declarations

None to declare.

Supplementary data

Tables S1, S2 and S3 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

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