ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Oct. 2009, p. 4345–4351 Vol. 53, No. 10
0066-4804/09/$08.00+0 doi:10.1128/AAC.01267-08


# Involvement of *pmrAB* and *phoPQ* in Polymyxin B Adaptation and Inducible Resistance in Non-Cystic Fibrosis Clinical Isolates of *Pseudomonas aeruginosa*▽

Kristen N. Schurek,[1] Jorge L. M. Sampaio,[2] Carlos R. V. Kiffer,[2] Sumiko Sinto,[2] Caio M. F. Mendes,[2] and Robert E. W. Hancock[1]*

*Centre for Microbial Diseases & Immunity Research, University of British Columbia, 2259 Lower Mall, Vancouver, British Columbia, Canada,[1] and Fleury Medicina e Saúde, Setor de Microbiologia, Av. General Valdomiro de Lima 508, São Paulo, Brazil[2]*

Received 22 September 2008/Returned for modification 21 November 2008/Accepted 18 July 2009

**During investigation of susceptibility testing methods for polymyxins, 24 multidrug-resistant clinical isolates of *Pseudomonas aeruginosa* were observed to have a distinct, reproducible phenotype in which skipped wells were observed during broth microdilution testing for polymyxin B. Possible mechanisms underlying this phenotype were investigated. The effects of various concentrations of polymyxin B on growth, the expression of resistance genes, and outer-membrane permeability were observed. Real-time PCR was performed to compare the expression, in response to selected concentrations of polymyxin B, of genes related to the PhoP-PhoQ and PmrA-PmrB two-component regulatory systems in polymyxin B-susceptible isolate PAO1, polymyxin B-resistant isolate 9BR, and two isolates (19BR and 213BR) exhibiting the skipped-well phenotype. 19BR and 213BR appeared to have similar basal levels of expression compared to that of PAO1 for *phoQ*, *arnB*, and PA4773 (from the *pmrAB* operon), and in contrast, 9BR had 52- and 280-fold higher expression of *arnB* and PA4773, respectively. The expression of *arnB* and PA4773 increased in response to polymyxin B in a concentration-dependent manner for 9BR but not for 19BR and 213BR. For these isolates, expression was significantly increased for *arnB* and PA4773, as well as *phoQ*, only upon exposure to 2 μg/ml polymyxin B but not at a lower concentration of 0.125 μg/ml. The sequencing of the *pmrAB* and *phoPQ* operons for all three isolates revealed a number of unique mutations compared to that for PAO1. 1-*N*-phenylnaphthylamine (NPN) was used to study the effect of preincubation with polymyxin B on the self-promoted uptake of polymyxin B across the outer membrane. The preincubation of cells with 2 μg/ml polymyxin B affected baseline membrane permeability in 19BR and 213BR and also resulted in a reduced rate of NPN uptake in these isolates and in PAO1 but not in 9BR. The results presented here suggest that the skipped-well isolates have the ability to adapt to specific concentrations of polymyxin B, inducing known polymyxin B resistance genes involved in generating alterations in the outer membrane.**

The emergence of multidrug-resistant gram-negative organisms and the simultaneous lack of new clinically available antimicrobial agents have led to a resurgence of older compounds such as the polymyxins (6). These agents, including polymyxin B and colistin, have highly potent activity against gram-negative organisms, including *Pseudomonas aeruginosa*, but were previously abandoned due to a reported high incidence of nephrotoxicity and neurotoxicity (12).

Resistance to polymyxin B is predominantly associated with decreased uptake into the bacterial cell resulting from a reduced capacity for initial binding (23). Polymyxin B and other polycationic compounds enter the cell via a process known as self-promoted uptake (10, 11). Polymyxin B binds to outer-membrane lipopolysaccharide (LPS) displacing $Mg^{2+}$ and disrupting the $Mg^{2+}$ cross bridges between anionic LPS molecules in the outer membrane, thus leading to membrane destabilization. This leads to an increased permeability of the outer membrane, allowing further uptake of the antibiotic. In *P. aeruginosa*, the ability of polymyxin to permeabilize outer membranes reflects its ability to bind to LPS with higher affinity than the native cross-bridging cation $Mg^{2+}$ (20, 22).

A polymyxin B adaptive resistance phenotype was first reported by Gilleland and Murray in 1976 (9) when the wild-type PAO1 strain was passaged in minimal medium containing low $Mg^{2+}$ and exposed to increasing concentrations of polymyxin B. Since this time, many studies have focused on the structural basis of adaptive resistance (7, 8). Under various environmental conditions, *P. aeruginosa* has been found to synthesize different forms of the lipid A component of LPS (5). In particular, under $Mg^{2+}$-limiting conditions, *P. aeruginosa* exhibits lipid A modifications, including the addition of aminoarabinose and palmitate. These modifications have been associated with polymyxin B resistance (17, 18).

It is well established that two distinct two-component regulators, PhoP-PhoQ and PmrA-PmrB, respond to limiting $Mg^{2+}$ conditions, resulting in polymyxin B resistance in *P. aeruginosa* (14–16). Under $Mg^{2+}$-limiting conditions, PhoP-PhoQ autoregulates the *oprH-phoP-phoQ* operon (15), and similarly, PmrA-PmrB autoregulates the PA4773-5–*pmrAB*–PA4778 operon (17). Furthermore, the *arnBCADTEF*-PA3559 operon (PA3552-PA3559), which is responsible for the addition of aminoarabinose to lipid A (18), is separately regulated by each of these

* Corresponding author. Mailing address: Department of Microbiology and Immunology, University of British Columbia, No. 251, 2259 Lower Mall, Lower Mall Research Station, Vancouver, British Columbia V6T 1Z4, Canada. Phone: (604) 822-2682. Fax: (604) 827-5566. E-mail: bob@cmdr.ubc.ca.

▽ Published ahead of print on 27 July 2009.

two-component regulatory systems and is upregulated under $Mg^{2+}$-limiting conditions (17). Both the PA4773-PA4778 and the PA3552-PA3559 operons have shown independent upregulation in response to various cationic antimicrobial peptides in laboratory mutants (18). Mutations in PmrB and the presence of aminoarabinose have been directly associated with polymyxin B resistance (18).

During a comparative study of MIC methods for testing polymyxin B and colistin susceptibility, it was observed that 24 of 243 multidrug-resistant clinical isolates of *P. aeruginosa* demonstrated skipped wells in the broth microdilution method for either polymyxin B, colistin, or both (a "skipped well" is an isolated well showing no growth of bacteria despite the fact that a well with a higher concentration demonstrates growth). According to Clinical and Laboratory Standards Institute (CLSI) guidelines, one skipped well is acceptable and MIC readings should be taken based on the well with the highest antibiotic concentration exhibiting growth (3). The assumption is that a technical error has occurred. The multidrug-resistant isolates described here skipped one to five wells, and bacteria recovered from wells at the higher polymyxin concentrations (i.e., after the skipped wells) also exhibited a polymyxin MIC profile containing skipped wells. The phenomenon of skipped wells has previously been described and was termed cocarde growth (1, 2, 23). In this study, we set out to determine the potential causes for this type of resistance profile in the identified *P. aeruginosa* clinical isolates.

## MATERIALS AND METHODS

**Bacterial strain selection and identification.** All isolates were collected as part of a Brazilian surveillance study between 2002 and 2004 which defined multidrug resistance as resistance to meropenem, ciprofloxacin, polymyxin B, and at least one β-lactam from the set of cefepime, ceftazidime, or piperacillin-tazobactam. Samples were collected from intensive-care and oncology units from 21 hospitals throughout seven states. Each isolate represents a single sample from one patient. The isolates were identified as *P. aeruginosa* based on established protocols for each hospital and reconfirmed as *P. aeruginosa* according to identification procedures established by the *Manual of Clinical Microbiology* (19). The samples were maintained on glass beads and frozen at −80°C. MICs were determined for meropenem, ciprofloxacin, cefepime, ceftazidime, and piperacillin-tazobactam. A total of 243 isolates were identified as being multidrug resistant. Polymyxin B and colistin MICs were determined for these multidrug-resistant isolates. Twenty-four isolates skipped wells in the first round of MICs, and three isolates (9BR, 19BR, and 213BR), taken from different hospitals over the course of a 3-year period, were selected for further analysis. *P. aeruginosa* strain PAO1 was used as a wild-type strain.

**Antimicrobial susceptibilities.** MICs were determined as described by CLSI guidelines (M7-A6 and M100-S15), using broth microdilution (3). Polymyxin B MICs were determined by triplicate experiments for each isolate with concentrations ranging from 0.03 to 32 μg/ml in cation-adjusted Mueller-Hinton broth (CAMHB) and were incubated at 37°C for 48 h.

**Growth curves.** Growth curves were determined in triplicate for strains PAO1, 9BR, 19BR, and 213BR in the absence of polymyxin B as well as in the presence of both 0.125 μg/ml and 2 μg/ml of polymyxin B. These concentrations of polymyxin B were chosen here and for all subsequent experiments since growth appeared in the MIC wells at each of these concentrations, with 0.125 μg/ml being selected to represent a concentration below the liberal MIC and 2 μg/ml representing a concentration of polymyxin B occurring after the skipped wells but below the conservative MIC. Overnight cultures were diluted 1:20 into CAMHB containing appropriate polymyxin B concentrations, and the optical density at 600 nm ($OD_{600}$) was measured every 20 min for 24 h using the TECAN Spectrafluor Plus microplate reader.

**RNA extraction and cDNA synthesis.** A total of 100 μl of each isolate was harvested directly from the MIC plate from (i) the well containing no polymyxin, (ii) the well containing 0.125 μg/ml polymyxin, and (iii) the well containing 2 μg/ml polymyxin and was inoculated into 10 ml CAMHB containing the respective concentrations of polymyxin (i.e., 0, 0.125, and 2 μg/ml). The isolates were incubated with shaking at 37°C and were grown to an $OD_{600}$ of 0.5. RNA was isolated using a Qiagen RNeasy mini kit according to the manufacturer's instructions (Qiagen, Inc., Canada) and stored at −80°C with 0.2 U/μl SUPERase-In RNase inhibitor (Ambion, Inc., Austin, TX). RNA quality was assessed by agarose gel electrophoresis and by spectrophotometry. Four micrograms of total RNA was added to a reaction mixture to make cDNA and used for quantitative real-time PCR (qPCR). Quantitative PCR was performed on the genes PA1180 (*phoQ*), PA3552 (*arnB*), and PA4773 (the first gene of the *pmrAB* operon), and *rpsL* was used as a control. Each isolate was isolated from individual MIC plates in triplicate on separate days, and qPCR was performed on each repeat at least in duplicate.

**Polymyxin B outer-membrane interaction studies.** The hydrophobic fluorescent probe 1-*N*-phenylnaphthylamine (NPN) was used as described by Loh et al. to study the interaction of polymyxin B with the outer membranes of clinical isolates compared to that of PAO1 (13). Overnight cultures grown at 37°C in CAMHB containing either 0, 0.125, or 2 μg/ml polymyxin B were diluted 1:100 in fresh CAMHB containing the respective polymyxin B concentrations. Fifty-milliliter samples of mid-log-phase cells were harvested by centrifugation at 3,000 × *g* for 10 min and washed once with and resuspended in 5 mM sodium HEPES buffer (pH 7.2) with 5 μM carbonyl cyanide *m*-chlorophenylhydrazone (an uncoupler) at an $OD_{600}$ of 0.5. Fluorescence was measured at an excitation wavelength of 350 nm and an emission wavelength of 420 nm using a Perkin Elmer LS 50B fluorescent spectrophotometer. NPN was added to a final concentration of 10 μM to obtain baseline fluorescence. Polymyxin B was added to final concentrations of 0.25, 0.5, 1, 2, 4, 8, 16, 32, and 64 μg/ml.

**Sequencing of *pmrAB* and the *pmr* promoter region, including PA4773.** Genomic DNA was isolated using a DNeasy blood and tissue kit (Qiagen, Inc., Canada) according to the manufacturer's instructions from overnight cultures grown at 37°C in CAMHB for isolates PAO1 and 9BR and CAMHB plus 2 μg/ml polymyxin B for isolates 19BR and 213BR. For the PA4773-PA4778 operon, a set of PCR primers was designed to encompass the entire noncoding upstream region of PA4773 as well as PA4773 itself (1,069 bp), and a second set of primers was designed to amplify the region flanking *pmrAB* (2,349 bp). The OprH-PhoPQ operon was amplified as three overlapping PCR fragments for coverage of all three genes as well as the upstream noncoding region. Sequencing was performed using Applied Biosystems BigDye Terminator v3.1 chemistry and run on an Applied Biosystems Prism 377 automated sequencer.

**Genotyping.** *P. aeruginosa* isolates PAO1, 9BR, 19BR, and 213BR were typed by pulsed-field gel electrophoresis (PFGE) of SpeI-digested chromosomal DNA. SpeI restriction fragments were separated using the CHEF-DR III system (Bio-Rad Laboratories, Hercules, CA) and PFGE profiles and then digitized on the Gel Doc 1000 (Bio-Rad Laboratories) with Quantity One software (Bio-Rad Laboratories), and the results were analyzed using Bionumerics version 3.5 software (Applied Maths, Austin, TX). A dendrogram was constructed by the unweighted-pair group method using arithmetic averages with the Dice coefficient.

## RESULTS AND DISCUSSION

**Antimicrobial susceptibilities and the skipped-well phenomenon.** The occurrence of skipped wells during polymyxin B MIC testing was observed for 24 isolates. This did not coincide with the occurrence of skipped wells during colistin MIC testing since only eight of these isolates presented this phenotype. Polymyxin B broth microdilution assays producing skipped wells were found to be reproducible in only 10 to 15% of trials from the original stock cultures; however, when samples were harvested from the microtiter plate wells with growth at concentrations higher than the skipped wells, a reproducible skipped-well phenotype was observed. This indicates that only a proportion of bacteria in the original stock cultures had the skipped-well phenomenon but that these bred true. The antimicrobial susceptibilities of the selected clinical isolates 9BR, 19BR, and 213BR are presented in Table 1. Isolate 19BR did not grow in concentrations of 0.25 and 0.5 μg/ml polymyxin B, and isolate 213BR did not grow in 0.5 and 1 μg/ml. To determine the effect of polymyxin B on the growth of these isolates,

TABLE 1. Antibiotic susceptibilities of multidrug-resistant clinical isolates of *P. aeruginosa* exhibiting a skipped-well phenotype to polymyxin B[a]

| Isolate | MIC (μg/ml) for indicated antibiotic | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Liberal | | Conservative | | Etest | | | | | |
| | Polymyxin B | Colistin | Polymyxin B | Colistin | Polymyxin B | Meropenem | Ceftazidime | Piperacillin-Tazobactam | Ciprofloxacin | Cefepime |
| 9BR | 64 | 32 | 64 | 32 | 4 | >32 | >256 | 96 | >32 | >256 |
| 19BR | 0.25 | 4 | 8 | 4 | 1.5 | >32 | >256 | 64 | >32 | >256 |
| 213BR | 0.5 | 2 | 8 | 32 | 1.5 | >32 | >256 | 96 | >32 | >256 |

[a] The liberal MIC indicates the well containing the lowest concentration of antibiotic without visible growth. The conservative MIC is the MIC that takes into account the growth after the skipped wells.

growth was assessed, in the presence of 0.125 and 2 μg/ml polymyxin B, for skipped-well isolates 19BR and 213BR compared to a polymyxin B-susceptible laboratory wild-type strain PAO1 and isolate 9BR that was reproducibly polymyxin B resistant without skipped wells (Fig. 1). The presence of 0.125 μg/ml polymyxin B, a concentration below the liberal MIC of each isolate, had no effect on the growth of any of the four isolates, while the presence of 2 μg/ml polymyxin B extended the lag phase of both skipped-well isolates 19BR and 213BR by approximately 6 and 4 h, respectively. The extended lag phases observed for skipped-well isolates 19BR and 213BR in the presence of 2 μg/ml polymyxin B were consistent with the need for a period of adaptation to permit growth at higher concentrations of polymyxin B.

**Determining the contributions of PhoP-PhoQ and PmrA-PmrB to the skipped-well phenomenon.** As both adaptive and

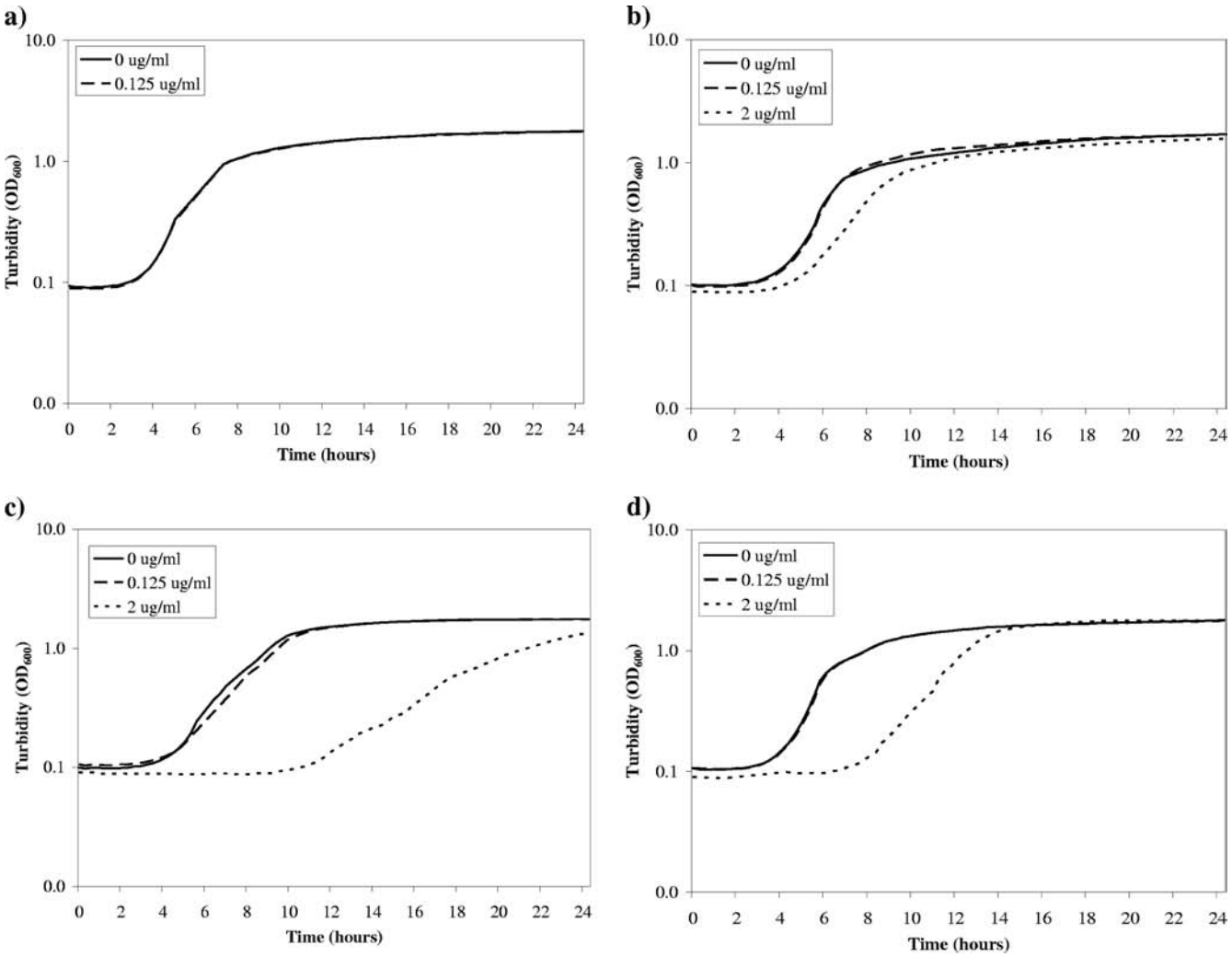


FIG. 1. Effect of 0.125 μg/ml and 2 μg/ml polymyxin B on the growth of *P. aeruginosa* isolates PAO1 (a), 9BR (b), 19BR (c), and 213BR (d).

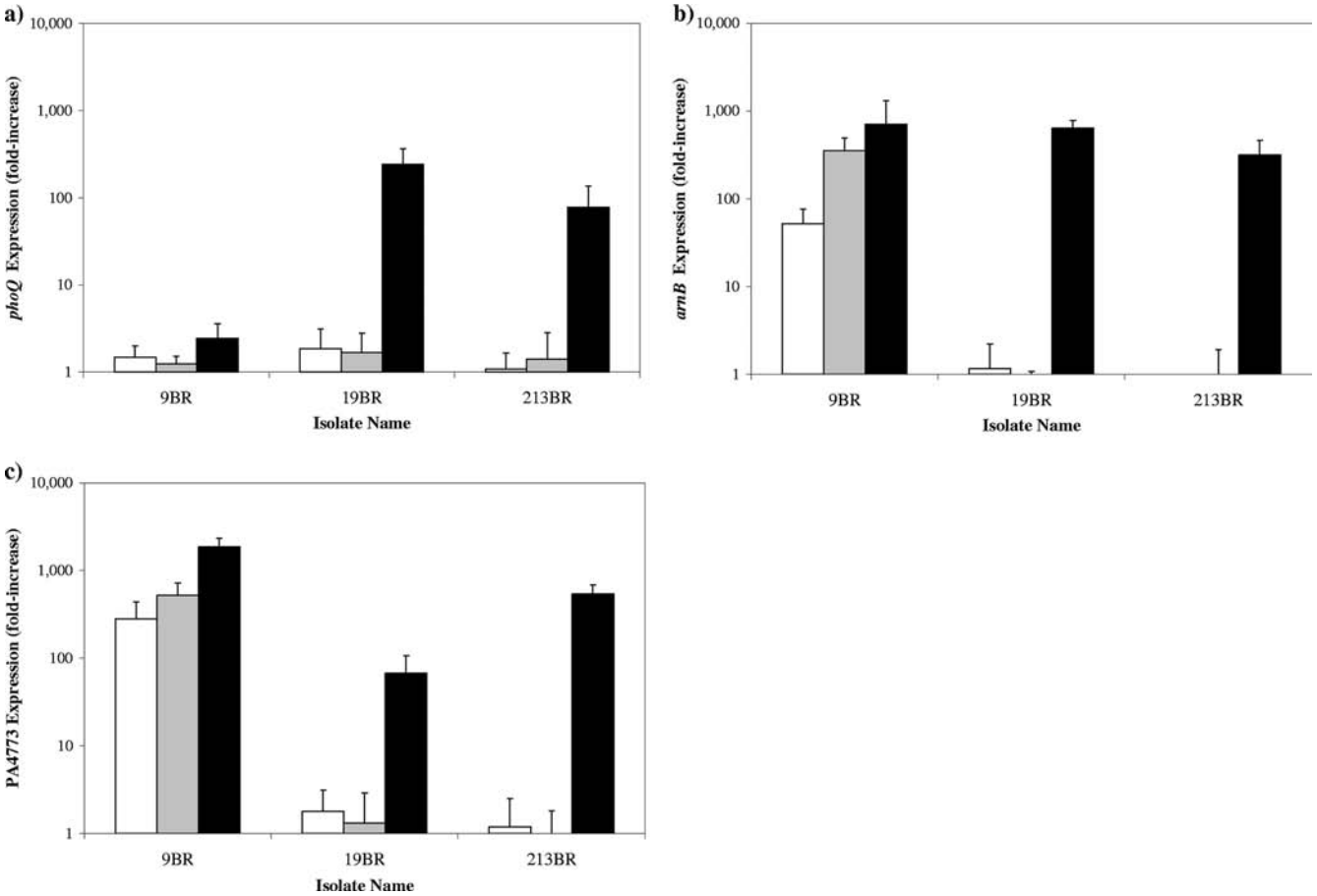


FIG. 2. Effect of polymyxin B on the expression of *phoQ* (a), *arnB* (b), and PA4773 (c) of isolates 9BR, 19BR, and 213BR measured as the *n*-fold change compared to the expression of the respective genes in PAO1 in the absence of polymyxin B. White bars represent 0 μg/ml, gray bars represent 0.125 μg/ml, and black bars represent 2 μg/ml polymyxin B. Error bars represent the standard deviations of three biological repeats, each performed in duplicate.

mutational resistance to polymyxin B have previously been linked to the PhoP-PhoQ and PmrA-PmrB two-component response regulatory systems and their downstream regulatory effects on the *arnBCADTEF*-PA3559 operon (17, 18), we investigated the potential contributions of these operons to the skipped-well resistance phenotype described here. The genes *phoQ*, *arnB*, and PA4773 (the first gene of the *pmrAB* operon) were selected for qPCR analysis as representative of the transcripts of each of the three operons. Figure 2 depicts the expression of these three genes in the skipped-well isolates 19BR and 213BR as well as the polymyxin B-resistant isolate 9BR relative to their expression in the susceptible PAO1 strain in the absence of polymyxin B. For the skipped-well isolates 19BR and 213BR in the absence of polymyxin B, the expression levels of all three genes of interest were comparable to the expression levels observed in PAO1. In contrast, the expression levels in the polymyxin B-resistant isolate 9BR of *arnB* and PA4773, but not *phoQ*, were significantly higher ($P < 0.05$) than in PAO1, with 52- to 280-fold increases, respectively. While expression was not affected by subinhibitory (0.125 μg/ml) polymyxin B treatment for any of the studied genes in the susceptible PAO1 strain (data not shown) or the two skipped-well isolates, the polymyxin B-resistant isolate exhibited a concentration-dependent increase in the expression of *arnB* and PA4773. At 2 μg/ml polymyxin B, although the wild type did not grow, the skipped-well isolates demonstrated a dramatic increase in the expression of *phoQ*, *arnB*, and PA4773 (*pmrAB* operon), thus suggesting a role for all three of these genes in the skipped-well phenomenon. In a study investigating lipid A modifications in cystic fibrosis (CF), environmental, and non-CF isolates of *P. aeruginosa*, Ernst et al. found that aminoarabinose was present only in isolates obtained from CF patients (4). The authors further indicated that at the modification levels found in early CF isolates of *P. aeruginosa*, this modification alone did not appear to cause significant levels of resistance; however, the modification was always found to be present in colistin-resistant CF isolates (4). The high levels of expression of the aminoarabinose modification operon seen in isolate 9BR suggests that this LPS modification is also likely to be present in polymyxin B-resistant non-CF isolates.

As the regulatory operon PA4773-PA4775-*pmrAB*-PA4778 as well as its downstream effector operon *arnBCADTEF*-PA3559 demonstrated increased expression in both the stably resistant isolate 9BR and the two skipped-well isolates 19BR and 213BR in response to polymyxin B, we believed that regulatory mutations in the upstream region of the PA4773-

TABLE 2. Missense mutations in PA4773, PmrA, PmrB, OprH, PhoP, and PhoQ and single-nucleotide base-pair changes in the promoter regions of operons PA4773-PA4778 and *oprH-phoPQ* observed in isolates 9BR, 19BR, and 213BR compared to wild-type PAO1[a]

| Isolate | Positions and nucleotide substitutions | | Amino acid substitution(s) | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Upstream region of PA4773 | Upstream region of OprH | PA4773 | PmrA | PmrB | OprH | PhoP | PhoQ |
| 9BR | Δ5361280–5361380; 5361407, G→A | 1276229, G→A; 1276365, G→C[b]; 1276493, C→T[bc]; 1276883, T→C[bc] | None | L71R[b] | ΔD45; Y345H[bc] | None | S179P[b] | *225E[bc]; T234A[bc]; S249P[b]; P367S[b] |
| 19BR | Δ5361279–5361379; 5361407, G→A | 1276229, G→A; 1276365, G→C[b]; 1276493, C→T[bc]; 1276883, T→C[bc] | None | L71R[b] | Y345H[bc] | None | S179P[b] | *225E[bc]; T234A[bc]; S249P[b]; P367S[b] |
| 213BR | Δ5361279–5361379; 5361407, G→A | 1276229, G→A; 1276365, G→C[b]; 1276493, C→T[bc]; 1276883, T→C[bc] | None | L71R[b] | Y345H[bc] | None | S179P[b] | *225E[bc]; T234A[bc]; S249P[b]; P367S[b] |

[a] Δ, deletion; *, insertion.
[b] This variant is also found in LESB58.
[c] This variant is also found in PA14.

PA4775-*pmrAB*-PA4778 operon may be involved in the polymyxin B resistance observed for all of these isolates. When the noncoding region upstream of the PA4773-PA4775-*pmrAB*-PA4778 operon was amplified and sequenced, a large 101-bp deletion and a single base-pair change were observed for all three clinical isolates compared to the wild-type PAO1 strain (Table 2). To determine if these mutations were unique to these isolates, a comparison was also made to other sequenced strains, including another wild-type strain, PA14, and the earliest-archived CF Liverpool epidemic strain, LESB58 (24). A number of single-base-pair changes were observed when comparing each of these strains (including the previously sequenced PA14 and LESB58 strains) to PAO1; however, neither the single-base-pair change nor the large deletion was observed in PAO1, PA14, or LESB58. Interestingly, LESB58 did have a 101-bp insertion compared to PAO1 and PA14 within the location where the deletion was observed in the Brazilian strains. A study of a second Liverpool epidemic strain, LES431, demonstrated that this isolate exhibited moderate upregulation of *pmrA* and *pmrB* (6.7 and 9.2-fold, respectively, as determined by microarray) compared to that of PAO1 in LB medium (21). The alterations in this promoter site, along with the increased expression of the PA4773-PA4775-*pmrAB*-PA4778 operon in both the Brazilian and the LES strains implicate this region in the regulation of this operon. The finding that all three clinical isolates from Brazil possessed the identical mutations in this region was surprising, as the isolates exhibited both distinct phenotypes and differing expression patterns; however, the PFGE genotyping seen in Fig. 3 does indicate that these three strains are related, with a Dice coefficient of >85%, but are not clonal.

As previous work found two phenotypically distinct polymyxin B-resistant mutants selected from the wild-type PAK strain on LB plates containing 20 to 50 μg/ml polymyxin B each possessed a single mutation (L243Q and A248V) in the histidine box of PmrB near the active site H249 and contained aminoarabinose under noninducing conditions (18), we also sought to characterize the PmrAB locus of these isolates. In sequencing PA4773, PmrA, and PmrB, the three strains under investigation were again identical to one another, with the exception of a single amino acid deletion in 9BR (Table 2). Compared to PAO1, a missense mutation in PmrA (L71R) was observed, and this mutation was also found in LESB58. The missense mutation Y345H was also observed in the wild-type strain PA14 as well as in LESB58 and so is not likely to be involved in polymyxin resistance. No mutations near the previously reported polymyxin B resistance mutations (18) in the histidine box of PmrB were observed.

As the major difference in the expression data between the skipped-well polymyxin B-resistant isolates and the constitutively polymyxin B-resistant isolates was found to be in the induced expression of *phoQ*, we anticipated major differences in the sequence data at this locus; however, no differences between these three isolates were observed (Table 2).

**Polymyxin B interaction studies.** As modifications to LPS influence the ability of polymyxin B to interact with the outer membrane of *P. aeruginosa*, NPN assays were performed to compare the ability of polymyxin B to interact with the membranes of the skipped-well isolates to that of the polymyxin B-susceptible PAO1 strain and the polymyxin B-resistant isolate 9BR. NPN is a fluorescent probe that fluoresces weakly in aqueous solution but strongly in nonpolar or hydrophobic environments. Under normal conditions, NPN is excluded from the outer membrane due to the tight cross-bridging of LPS by divalent cations and does not fluoresce. Upon the disruption of the outer membrane, NPN partitions into the outer-membrane interior, and an increase in fluorescence can be observed. The

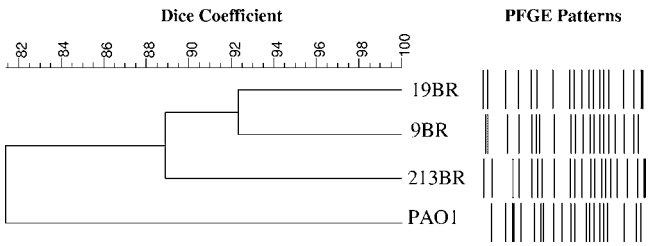


FIG. 3. Genetic relatedness of isolates 9BR, 19BR, 213BR, and PAO1 according to the Dice coefficient of similarity.

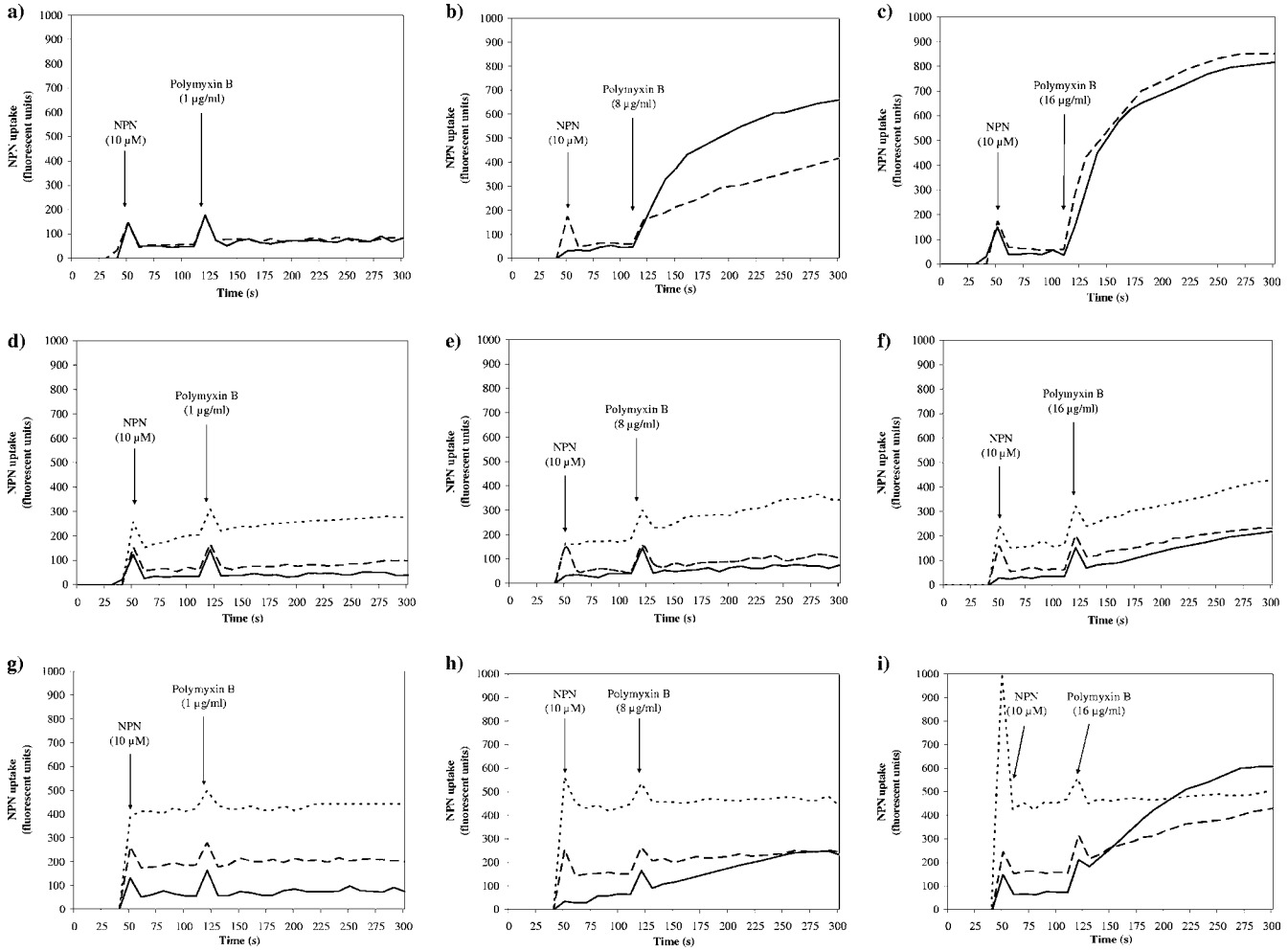


FIG. 4. Effect of preincubation with 0.125 μg/ml and 2 μg/ml polymyxin B on the membrane permeability of *P. aeruginosa* isolates PAO1 (a to c), 9BR (d to f), and 19BR (g to i). Solid lines indicate unexposed cells, dashed lines indicate cells preincubated with 0.125 μg/ml polymyxin B, and dotted lines indicate cells preincubated with 2 μg/ml polymyxin B.

initial rate of increase in fluorescence varies with the concentration of polymyxin B and defines the susceptibility of the outer membrane to permeabilization, which in turn relates to the efficiency of self-promoted uptake. As expected, polymyxin B was able to permeabilize the membranes of all isolates tested in a concentration-dependent manner (Fig. 4), with the outer membrane of PAO1 being permeabilized at a much lower concentration of 4 μg/ml polymyxin B (data not shown) and to a greater extent at all concentrations tested compared to the outer membrane of 9BR, which was affected at only concentrations of ≥32 μg/ml (data not shown). The permeabilization of the outer membrane of the skipped-well isolate 19BR fell between the susceptible and the resistant isolate and was observed at polymyxin B concentrations of ≥8 μg/ml.

Interestingly, preincubation with polymyxin B had differing effects on the efficiency of self-promoted uptake as well as the background levels of outer-membrane permeability of each isolate. Preincubation with 0.125 μg/ml of polymyxin B had little to no effect on the background permeability of the susceptible PAO1 strain (Fig. 4a to c) or the resistant 9BR strain (Fig. 4d to f), while background levels for 19BR increased significantly. Further increases in the background outer-membrane permeability were observed after preincubation with 2 μg/ml polymyxin B for both 9BR and 19BR. With regard to the effect on self-promoted uptake, at intermediate concentrations of polymyxin B, as represented by 8 μg/ml polymyxin B in Fig. 4, the preincubation of PAO1 with 0.125 μg/ml did retard the initial rate of uptake. Similar results were observed for 19BR as well as for 213BR at 16 μg/ml (data not shown). These data indicate that exposure to subinhibitory concentrations of polymyxin B caused significant structural alterations in the outer membranes of the skipped-well isolates 19BR and 213BR which affected their ability to interact with polymyxin B, consistent with the dramatic alterations in the expression levels of *arnB*, which is the first gene of the aminoarabinosylation operon that is known to influence polymyxin self-promoted uptake and susceptibility by reducing the negative charge on LPS. While slight to moderate increases in the baseline permeability of the outer membrane of 9BR to NPN were observed after preincubation with polymyxin B, no differences in the rate of NPN uptake were observed after the further addition of polymyxin

B, thus indicating that, in contrast to the skipped-well isolates, the preexposure of this resistant isolate to polymyxin B had little effect on the ability of the outer membrane to bind additional polymyxin B.

**Concluding remarks.** The data shown here support the notion that clinical isolates of *P. aeruginosa* have various abilities to adapt to the presence of polymyxins. While the polymyxin B-resistant isolate observed in this study appeared to constitutively overexpress the PmrA-PmrB two-component regulatory system and was capable of only moderate adaptation as indicated by a concentration-dependent upregulation of the PmrA-PmrB system and by minor changes in baseline NPN permeability upon exposure to polymyxin B, the skipped-well isolates surveyed appeared to have a heightened ability to respond to higher concentrations of polymyxin B than to lower concentrations, although of note, the levels of expression of these genes did not reach the same high levels as 9BR. This enhanced adaptability was evidenced by the extended lag phase, by dramatic changes in the expression of both the PhoP-PhoQ and the PmrA-PmrB systems, and by apparent structural changes in response to polymyxin B, affecting the permeabilization of the outer membranes of these isolates. Of note, the structural changes in the outer membrane of the skipped-well isolate, presumed on the basis of the NPN results, occurred in a concentration-dependent manner in response to preincubation with 0.125 μg/ml and 2 μg/ml polymyxin B, while the expression of *arnB*, *phoQ*, and PA4773 appeared to be increased only upon exposure to 2 μg/ml polymyxin B. This discordance between the two assays may indicate the involvement of other systems affecting membrane permeability in response to low concentrations of polymyxin B in the skipped-well isolates. A number of mutations were observed in the regulatory regions controlling *pmrAB* and *phoPQ* as well as in these genes themselves when the isolates were compared to wild-type isolates; however, only a single amino acid deletion in PmrB was observed in the constitutively resistant isolate compared to the skipped-well isolates. The contributions of these mutations to the observed phenotypes have yet to be determined. The organisms displaying the skipped-well phenomenon appear to have the heightened ability to sense specific polymyxin B concentrations and induce the PhoP-PhoQ and PmrA-PmrB systems, resulting in polymyxin resistance and the skipped-well phenotype. Thus, the ability of these organisms to grow at specific concentrations above those of the skipped wells relies on the differential effects of inhibition by particular polymyxin concentrations and the speed of induction of these operons. Most importantly, these data are the first to associate the PhoP-PhoQ and PmrA-PmrB systems and the LPS modification operon with polymyxin-inducible polymyxin B resistance in non-CF clinical isolates.

## ACKNOWLEDGMENTS

This work was supported by grants from the Canadian Cystic Fibrosis Foundation and the Canadian Institutes of Health Research. K.N.S. holds a Natural Sciences and Engineering Council of Canada postgraduate scholarship and a Michael Smith Foundation for Health Research senior graduate studentship. R.E.W.H. holds a Canada Research chair.

## REFERENCES

1. **Bliss, E. A., C. A. Chandler, and E. B. Schoenbach.** 1949. In vitro studies of polymyxin. Ann. N. Y. Acad. Sci. **51:**944–951.
2. **Chabbert, Y. A., and J. C. Patte.** 1961. Type and characteristics of the bacterial resistance to colistine. Ann. Inst. Pasteur (Paris) **100**(Suppl.)**:**44–52. (In French.)
3. **Clinical and Laboratory Standards Institute.** 2006. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 7th ed. Approved standard M7-A7. Clinical and Laboratory Standards Institute, Wayne, PA.
4. **Ernst, R. K., S. M. Moskowitz, J. C. Emerson, G. M. Kraig, K. N. Adams, M. D. Harvey, B. Ramsey, D. P. Speert, J. L. Burns, and S. I. Miller.** 2007. Unique lipid A modifications in *Pseudomonas aeruginosa* isolated from the airways of patients with cystic fibrosis. J. Infect. Dis. **196:**1088–1092.
5. **Ernst, R. K., E. C. Yi, L. Guo, K. B. Lim, J. L. Burns, M. Hackett, and S. I. Miller.** 1999. Specific lipopolysaccharide found in cystic fibrosis airway *Pseudomonas aeruginosa*. Science **286:**1561–1565.
6. **Falagas, M. E., and S. K. Kasiakou.** 2005. Colistin: the revival of polymyxins for the management of multidrug-resistant gram-negative bacterial infections. Clin. Infect. Dis. **40:**1333–1341.
7. **Gilleland, H. E., and R. S. Conrad.** 1982. Chemical alterations in cell envelopes of polymyxin-resistant mutants of *Pseudomonas aeruginosa* grown in the absence or presence of polymyxin. Antimicrob. Agents Chemother. **22:** 1012–1016.
8. **Gilleland, H. E., and L. B. Farley.** 1982. Adaptive resistance to polymyxin in *Pseudomonas aeruginosa* due to an outer membrane impermeability mechanism. Can. J. Microbiol. **28:**830–840.
9. **Gilleland, H. E., and R. G. Murray.** 1976. Ultrastructural study of polymyxin-resistant isolates of *Pseudomonas aeruginosa*. J. Bacteriol. **125:**267–281.
10. **Hancock, R. E. W., and D. S. Chapple.** 1999. Peptide antibiotics. Antimicrob. Agents Chemother. **43:**1317–1323.
11. **Hancock, R. E. W., T. Falla, and M. Brown.** 1995. Cationic bactericidal peptides. Adv. Microb. Physiol. **37:**135–175.
12. **Koch-Weser, J., V. W. Sidel, E. B. Federman, P. Kanarek, D. C. Finer, and A. E. Eaton.** 1970. Adverse effects of sodium colistimethate. Manifestations and specific reaction rates during 317 courses of therapy. Ann. Intern. Med. **72:**857–868.
13. **Loh, B., C. Grant, and R. E. W. Hancock.** 1984. Use of the fluorescent probe 1-*N*-phenylnaphthylamine to study the interactions of aminoglycoside antibiotics with the outer membrane of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **26:**546–551.
14. **Macfarlane, E. L., A. Kwasnicka, and R. E. W. Hancock.** 2000. Role of *Pseudomonas aeruginosa* PhoP-phoQ in resistance to antimicrobial cationic peptides and aminoglycosides. Microbiology **146:**2543–2554.
15. **Macfarlane, E. L., A. Kwasnicka, M. M. Ochs, and R. E. W. Hancock.** 1999. PhoP-PhoQ homologues in *Pseudomonas aeruginosa* regulate expression of the outer-membrane protein OprH and polymyxin B resistance. Mol. Microbiol. **34:**305–316.
16. **McPhee, J. B., M. Bains, G. Winsor, S. Lewenza, A. Kwasnicka, M. D. Brazas, F. S. L. Brinkman, and R. E. W. Hancock.** 2006. Contribution of the PhoP-PhoQ and PmrA-PmrB two-component regulatory systems to $Mg^{2+}$-induced gene regulation in *Pseudomonas aeruginosa*. J. Bacteriol. **188:**3995–4006.
17. **McPhee, J. B., S. Lewenza, and R. E. W. Hancock.** 2003. Cationic antimicrobial peptides activate a two-component regulatory system, PmrA-PmrB, that regulates resistance to polymyxin B and cationic antimicrobial peptides in *Pseudomonas aeruginosa*. Mol. Microbiol. **50:**205–217.
18. **Moskowitz, S. M., R. K. Ernst, and S. I. Miller.** 2004. PmrAB, a two-component regulatory system of *Pseudomonas aeruginosa* that modulates resistance to cationic antimicrobial peptides and addition of aminoarabinose to lipid A. J. Bacteriol. **186:**575–579.
19. **Murray, P. R.** 2003. Manual of clinical microbiology, 8th ed. ASM Press, Washington, DC.
20. **Peterson, A. A., R. E. W. Hancock, and E. J. McGroarty.** 1985. Binding of polycationic antibiotics and polyamines to lipopolysaccharides of *Pseudomonas aeruginosa*. J. Bacteriol. **164:**1256–1261.
21. **Salunkhe, P., C. H. Smart, J. A. Morgan, S. Panagea, M. J. Walshaw, C. A. Hart, R. Geffers, B. Tummler, and C. Winstanley.** 2005. A cystic fibrosis epidemic strain of *Pseudomonas aeruginosa* displays enhanced virulence and antimicrobial resistance. J. Bacteriol. **187:**4908–4920.
22. **Schindler, M., and M. J. Osborn.** 1979. Interaction of divalent cations and polymyxin B with lipopolysaccharide. Biochemistry **18:**4425–4430.
23. **Traub, W. H.** 1982. Polymyxin B-induced cocarde growth phenomenon of *Serratia marcescens* due to cationic detergent-like activity of polymyxin B. Chemotherapy **28:**363–368.
24. **Winstanley, C., M. G. Langille, J. L. Fothergill, I. Kukavica-Ibrulj, C. Paradis-Bleau, F. Sanschagrin, N. R. Thomson, G. L. Winsor, M. A. Quail, N. Lennard, A. Bignell, L. Clarke, K. Seeger, D. Saunders, D. Harris, J. Parkhill, R. E. Hancock, F. S. Brinkman, and R. C. Levesque.** 2009. Newly introduced genomic prophage islands are critical determinants of in vivo competitiveness in the Liverpool Epidemic Strain of *Pseudomonas aeruginosa*. Genome Res. **19:**12–23.