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Arnaud Bolard, Monika Schniederjans, Susanne Haüssler, Pauline Triponney, Benoit Valot, et al.. Production of Norspermidine Contributes to Aminoglycoside Resistance in pmrAB Mutants of *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy*, 2019, 63 (10), pp.e01044-19. 10.1128/AAC.01044-19 . hal-02438158

**HAL Id: hal-02438158**

**<https://hal.science/hal-02438158v1>**


Submitted on 30 Oct 2024

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# Production of Norspermidine Contributes to Aminoglycoside Resistance in *pmrAB* Mutants of *Pseudomonas aeruginosa*

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**ABSTRACT** Emergence of resistance to polymyxins in *Pseudomonas aeruginosa* is mainly due to mutations in two-component systems that promote the addition of 4-amino-4-deoxy-L-arabinose to the lipopolysaccharide (LPS) through upregulation of operon *arnBCADTEF-ugd* (*arn*) expression. Here, we demonstrate that mutations occurring in different domains of histidine kinase PmrB or in response regulator PmrA result in coresistance to aminoglycosides and colistin. All seventeen clinical strains tested exhibiting such a cross-resistance phenotype were found to be *pmrAB* mutants. As shown by gene deletion experiments, the decreased susceptibility of the mutants to aminoglycosides was independent from operon *arn* but required the efflux system MexXY-OprM and the products of three genes, PA4773-PA4774-PA4775, that are cotranscribed and activated with genes *pmrAB*. Gene PA4773 (annotated as *speD2* in the PAO1 genome) and PA4774 (*speE2*) are predicted to encode enzymes involved in biosynthesis of polyamines. Comparative analysis of cell surface extracts of an *in vitro* selected *pmrAB* mutant, called AB16.2, and derivatives lacking PA4773, PA4774, and PA4775 revealed that these genes were needed for norspermidine production via a pathway that likely uses 1,3-diaminopropane, a precursor of polyamines. Altogether, our results suggest that norspermidine decreases the self-promoted uptake pathway of aminoglycosides across the outer membrane and, thereby, potentiates the activity of efflux pump MexXY-OprM.

**KEYWORDS** PmrAB, *Pseudomonas aeruginosa*, aminoglycosides, colistin, norspermidine, polyamines

*Pseudomonas aeruginosa* is a common cause of health care-associated infections (1). Because of an increasing number of extensively drug-resistant (XDR) strains, polymyxins (colistin and polymyxin B) and aminoglycosides are used more and more as last-line antibiotics to treat infected patients (2, 3). Therefore, emergence of resistance to one or both of these antibiotic families under treatment may leave clinicians with very few or no more therapeutic options and result in clinical failures.

Though still infrequent (3), polymyxin resistance in *P. aeruginosa* is due to enzymatic addition of 4-amino-4-deoxy-L-arabinose (Ara4N) to lipid A of the lipopolysaccharide (LPS), with a subsequent decrease in the electrostatic interaction between the polycationic polymyxins and the phosphate residues of the LPS (4, 5). Ara4N modification of the LPS is determined by an operon, *arnBCADTEF-ugd* (hereafter called *arn*), whose expression is controlled by several two-component regulatory systems (TCS) including PmrAB, PhoPQ, ParRS, ColRS, and CprRS. Various mutations in these phosphorelays have been demonstrated or more simply suspected to activate *arn* transcription and,

**Citation** Bolard A, Schniederjans M, Haüssler S, Triponney P, Valot B, Plésiat P, Jeannot K. 2019. Production of norspermidine contributes to aminoglycoside resistance in *pmrAB* mutants of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 63:e01044-19. <https://doi.org/10.1128/AAC.01044-19>.

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**Received** 24 May 2019

**Returned for modification** 28 June 2019

**Accepted** 25 July 2019

**Accepted manuscript posted online** 5 August 2019

**Published** 23 September 2019

**TABLE 1** Effects of *pmrB* mutations on antibiotic susceptibility

Strain	Plasmid	Protein PmrB <sup>b</sup>	MIC ( $\mu\text{g ml}^{-1}$ ) for: <sup>a</sup>							
			CST	GEN	AMK	TOB	TCC	CAZ	IPM	CIP
PAO1		Wild-type	0.5	1	2	0.25	16	2	1	0.12
AB8.2		V28G	<b>128</b>	<b>8</b>	<b>8</b>	<b>2</b>	4	1	0.5	0.25
AB16.1		F408L	<b>128</b>	<b>8</b>	<b>8</b>	<b>2</b>	16	2	1	0.12
AB16.2		$\Delta$ L172	<b>128</b>	<b>8</b>	<b>8</b>	<b>2</b>	8	1	0.5	0.12
PAO1 $\Delta$ <i>pmrAB</i>		$\Delta$	0.5	1	2	0.25	16	2	1	0.12
PAO1 $\Delta$ <i>pmrAB</i>	pME6012		0.5	1	2	0.25	16	2	1	0.12
PAO1 $\Delta$ <i>pmrAB</i>	pABWT	Wild-type	0.5	1	2	0.25	16	2	1	0.12
PAO1 $\Delta$ <i>pmrAB</i>	pAB8.2	V28G	<b>128</b>	<b>8</b>	<b>8</b>	<b>2</b>	8	1	0.5	0.25
PAO1 $\Delta$ <i>pmrAB</i>	pAB16.1	F408L	<b>128</b>	<b>8</b>	<b>8</b>	<b>2</b>	8	1	0.5	0.25
PAO1 $\Delta$ <i>pmrAB</i>	pAB16.2	$\Delta$ L172	<b>128</b>	<b>8</b>	<b>8</b>	<b>2</b>	8	1	0.5	0.25

<sup>a</sup>The MIC data are representative of three independent experiments. Values in bold are at least 4-fold higher than those of strain PAO1. CST, colistin; GEN, gentamicin; AMK, amikacin; TOB, tobramycin; TCC, ticarcillin (plus clavulanic acid at a fixed concentration of  $2 \mu\text{g ml}^{-1}$ ); CAZ, ceftazidime; IPM, imipenem; CIP, ciprofloxacin.

<sup>b</sup>Amino acid sequence refers to PmrB protein of strain PAO1.

thereby, to enhance colistin resistance in clinical strains of *P. aeruginosa* (6). Sensor PmrB is a hot spot for such mutations (7–13).

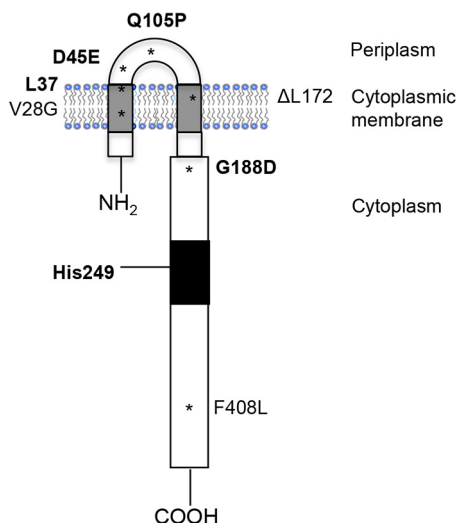
Three genes of the PmrAB regulon (PA4773, PA4774, and PA4775) are activated when planktonic bacteria are grown upon  $\text{Mg}^{2+}$  starvation at acidic pH in the presence of extracellular DNA (eDNA) or with antimicrobial peptides, such as colistin, indolicidin, and CP11 (14–17). Inactivation of gene PA4774 (annotated as spermidine synthase gene *speE2* in the *Pseudomonas* Genome database) (18) was found to alter the outer membrane permeability barrier to polymyxin B, CP10A, and gentamicin (14). Another study revealed that the resistance developed by *P. aeruginosa* toward polymyxin B, gentamicin, and tobramycin when cultured with eDNA or at acidic pH required functional PA4773 and *arn* genes (16). It was concluded that genes PA4773 (*S*-adenosylmethionine transferase gene *speD2* in the *Pseudomonas* Genome database) and PA4774 are part of an alternative spermidine biosynthesis pathway, which once activated via TCS PmrAB protects *P. aeruginosa* against antimicrobial peptides and aminoglycosides by increasing the amounts of the positively charged spermidine at the cell surface (14).

Nonenzymatic resistance of clinical strains of *P. aeruginosa* to aminoglycosides is often associated with constitutive upregulation of efflux system MexXY-OprM (19). Loss-of-function mutations in gene *mexZ*, which encodes a TetR-like repressor of operon *mexXY*; alteration of components of the protein synthesis machinery; or amino acid substitutions in TCS ParRS can positively impact *mexXY* activity and thus promote resistance to pump MexXY-OprM substrates, such as aminoglycosides, cefepime, and fluoroquinolones, from 2- to 4-fold. In addition, ParRS mutants exhibit an increased resistance to colistin as a result of operon *arn* induction and to carbapenems due to repressed transcription of the porin OprD gene (20).

The present study investigates a novel type of aminoglycoside-colistin cross-resistant *pmrAB* mutant that overexpresses both the *mexXY* operon and the PA4773-PA4774-PA4775 gene cluster.

## RESULTS

**Mutations of sensor PmrB provides cross-resistance to aminoglycosides and colistin.** Characterization of resistant mutants of reference strain PAO1 selected on agar plates containing  $8 \mu\text{g ml}^{-1}$  ( $16 \times \text{MIC}$ ; mutant rates =  $9.10^{-7}$ ) and  $16 \mu\text{g ml}^{-1}$  ( $32 \times \text{MIC}$ ;  $6.10^{-8}$ ) of colistin, showed that all the analyzed clones were coresistant to colistin and aminoglycosides (256-fold and up to 8-fold more than PAO1, respectively), while some of them were slightly more susceptible (2-fold) to  $\beta$ -lactams (Table 1). Sequencing of genes *pmrA*, *pmrB*, *parS*, *parR*, *cprS*, *cprR*, *phoP*, *phoQ*, *colS*, and *colR* known to be involved in regulation of operon *arn* expression revealed the presence of single amino acid substitutions in histidine kinase PmrB of randomly chosen mutants AB8.2 (V28G)



**FIG 1** Schematic representation of histidine kinase (HK) PmrB. The mutations responsible for cross-resistance to aminoglycosides and colistin in *in vitro*-selected mutants and clinical strains are marked with asterisks. The first and second transmembrane domains of PmrB are colored in gray (from amino acid position 15 to 37 and from 161 to 183, respectively). Also represented are the periplasmic domain (from 38 to 160), the HAMP linker domain (from 186 to 238), the dimerization/phosphoacceptor (HisKA) domain (from 239 to 304, colored in black) that contains the conserved active site histidine 249, and the histidine kinase-like ATPase (HATPase) domain (from 348 to 459). The domains are available from the SMART protein database (<http://smart.embl-heidelberg.de>).

and AB16.1 (F408L) and an amino acid deletion in the protein of mutant AB16.2 ( $\Delta$ L172) (Fig. 1). To confirm the impact of these mutations on aminoglycoside resistance, we complemented the *pmrAB*-negative strain PAO1 $\Delta$ *pmrAB* with plasmid vector pME6012 carrying the *pmrAB* alleles from PAO1, AB8.2, AB16.1, and AB16.2 (yielding constructs pABWT, pAB8.2, pAB16.1, and pAB16.2, respectively). Compared with those of the wild-type control, all of the mutated *pmrB* genes conferred significant resistance to gentamicin ( $8 \mu\text{g ml}^{-1}$ ;  $8\times$  MIC), amikacin ( $8 \mu\text{g ml}^{-1}$ ;  $4\times$  MIC), and tobramycin ( $2 \mu\text{g ml}^{-1}$ ;  $8\times$  MIC) in addition to colistin ( $128 \mu\text{g ml}^{-1}$ ;  $256\times$  MIC) (Table 1). Conversely, deletion of gene *pmrA* or *pmrB* restored a wild-type susceptibility phenotype in mutant AB16.2, thus providing further evidence that the cross-resistance of *pmrB* mutants required both components of the signal transducing system PmrAB (Table 2). The observation that mutants PAO1 $\Delta$ *pmrA* and PAO1 $\Delta$ *pmrB* exhibited the same resistance levels as that of parent strain PAO1 also indicated that PmrAB does not play a role in the intrinsic resistance of *P. aeruginosa* to antibiotics, including polymyxins (Table 2). To assess a possible contribution of LPS modification operon *arn* to aminoglycoside

**TABLE 2** Drug susceptibility of PAO1 and derived mutants

Strain	Gene deletion	MIC ( $\mu\text{g ml}^{-1}$ ) for: <sup>a</sup>			
		CST	GEN	AMK	TOB
PAO1		0.5	1	2	0.25
	<i>pmrA</i>	0.5	1	2	0.25
	<i>pmrB</i>	0.5	1	2	0.25
	<i>arn</i>	0.5	1	2	0.25
	<i>parS</i>	0.25	1	2	0.25
	<i>mexXY</i>	0.5	<u>0.06</u>	<u>0.5</u>	0.125
AB16.2		<b>128</b>	<b>8</b>	<b>8</b>	<b>2</b>
	<i>pmrA</i>	0.5	1	2	0.25
	<i>pmrB</i>	0.5	1	2	0.25
	<i>arn</i>	1	<b>8</b>	<b>8</b>	<b>2</b>
	<i>parS</i>	<b>4</b>	<b>8</b>	<b>8</b>	<b>2</b>
	<i>mexXY</i>	<b>4</b>	0.06	<u>0.25</u>	<u>0.06</u>

<sup>a</sup>Values in bold (underlined) are at least 4-fold higher (lower) than those of strain PAO1.

**TABLE 3** Effects of *pmrB* mutations on susceptibility of *P. aeruginosa* clinical strains to colistin and aminoglycosides

Strain (plasmid)	Sequence variation(s) in protein PmrB <sup>a</sup>	MIC ( $\mu\text{g ml}^{-1}$ ) for: <sup>b</sup>			
		CST	GEN	AMK	TOB
Clinical strain					
543	S2P, A4T, G68S, <b>G86V</b> , Y345H, <b>G362S</b>	<b>8</b>	>256	>256	>256
2243	<b>Q105P</b> , Y345H	<b>128</b>	>256	>256	>256
2739	S2P, A4T, <b>V6A</b> , V15I, G68S, <b>V264A</b> , Y345H	<b>16</b>	>256	>256	>256
3038	<b>D47N</b> , Y345H	<b>64</b>	<b>4</b>	<b>8</b>	<b>16</b>
3091	S2P, A4T, <b>V6A</b> , V15I, G68S, Y345H	<b>128</b>	>256	<b>32</b>	>256
3092	S2P, A4T, <b>V6A</b> , V15I, <b>L37P</b> , G68S, Y345H	<b>128</b>	>256	<b>128</b>	>256
3795	<b>G188D</b> , Y345H	<b>128</b>	<b>8</b>	<b>32</b>	<b>2</b>
3890	S2P, A4T, <b>D45E</b> , Y345H	>128	<b>4</b>	<b>32</b>	<b>2</b>
3921	Y345H	>128	>256	>256	>256
4536	<b>V136E</b> , Y345H	<b>4</b>	>256	<b>8</b>	<b>16</b>
4586	Y345H	<b>64</b>	<b>32</b>	>256	<b>64</b>
4660	<b>G121P</b> , <b>V313A</b> , Y345H	<b>64</b>	<b>16</b>	<b>64</b>	>256
4782	S2P, A4T, V15I, <b>H33Y</b> , G68S, Y345H	<b>4</b>	<b>4</b>	<b>64</b>	<b>64</b>
5058	S2P, A4T, <b>D45N</b> , G68S, Y345H, <b>G362S</b>	<b>4</b>	>256	<b>16</b>	<b>32</b>
5071	<b>F168L</b> , Y345H	<b>16</b>	<b>4</b>	<b>16</b>	>256
5101	<b>R92H</b> , <b>G123S</b> , Y345H	<b>32</b>	>256	>256	>256
5115	<b>R92H</b> , <b>G123S</b> , Y345H	<b>16</b>	<b>4</b>	<b>16</b>	<b>64</b>
3095	S2P, A4T, <b>V6A</b> , V15I, G68S, Y345H	1	1	2	0.25
PAO1 construct					
PAO1		0.5	1	2	0.25
PAO1 $\Delta$ <i>pmrAB</i>		0.5	1	2	0.25
PAO1 $\Delta$ <i>pmrAB</i> (pAB2243)	<b>Q105P</b> , Y345H	<b>128</b>	<b>16</b>	<b>32</b>	<b>4</b>
PAO1 $\Delta$ <i>pmrAB</i> (pAB3092)	S2P, A4T, <b>V6A</b> , V15I, <b>L37P</b> , G68S, Y345H	<b>128</b>	<b>8</b>	<b>8</b>	<b>2</b>
PAO1 $\Delta$ <i>pmrAB</i> (pAB3795)	<b>G188D</b> , Y345H	<b>128</b>	<b>8</b>	<b>8</b>	<b>2</b>
PAO1 $\Delta$ <i>pmrAB</i> (pAB3890)	S2P, A4T, <b>D45E</b> , Y345H	<b>16</b>	<b>4</b>	<b>8</b>	<b>2</b>
PAO1 $\Delta$ <i>pmrAB</i> (pAB3095)	S2P, A4T, <b>V6A</b> , V15I, G68S, Y345H	0.5	1	2	0.25

<sup>a</sup>The PmrB sequence of reference strain PAO1 was used as a reference. The amino acid changes highlighted in boldface are absent from strains PAO1, PA14, and LESB58.

<sup>b</sup>MIC values are representative of three independent experiments. Values in bold are at least fourfold higher than those of strain PAO1 or its mutant PAO1 $\Delta$ *pmrAB*.

resistance of *pmrB* mutants, we measured gene *arnA* expression by reverse transcriptase quantitative PCR (RT-qPCR) in AB8.2, AB16.1 and AB16.2 and removed the whole *arn* locus from the PAO1 and AB16.2 genomes (yielding derivatives PAO1 $\Delta$ *arn* and AB16.2 $\Delta$ *arn*, respectively). All of the three studied mutants were found to overexpress gene *arnA* strongly (from 424- to 654-fold versus PAO1). Whereas the parental susceptibility to colistin was almost completely restored in mutant AB16.2 $\Delta$ *arn* (MIC = 1  $\mu\text{g ml}^{-1}$ ; Table 2), MICs of aminoglycosides remained unchanged, confirming our hypothesis of an Arn-independent mechanism of aminoglycoside resistance in *pmrB* mutants. In a previous work, we showed that mutational activation of TCS ParRS results in constitutive upregulation of operon *mexXY* and increased resistance to various pump MexXY-OprM substrates, such as aminoglycosides (20). To assess the role played by this TCS in aminoglycoside-colistin cross-resistance, we deleted operon *parRS* in AB16.2 (yielding strain AB16.2 $\Delta$ *parRS*) and PAO1 (PAO1 $\Delta$ *parRS*). As indicated in Table 2, these deletions only impacted colistin MICs with a 16-fold reduction in AB16.2 $\Delta$ *parRS* compared with that of AB16.2 (4 versus 64  $\mu\text{g ml}^{-1}$ ) and a 2-fold decrease in that of PAO1 (as already reported in reference 20). Altogether, these results supported the notion that other PmrAB-dependent mechanisms were responsible for the resistance of *pmrB* mutants to aminoglycosides.

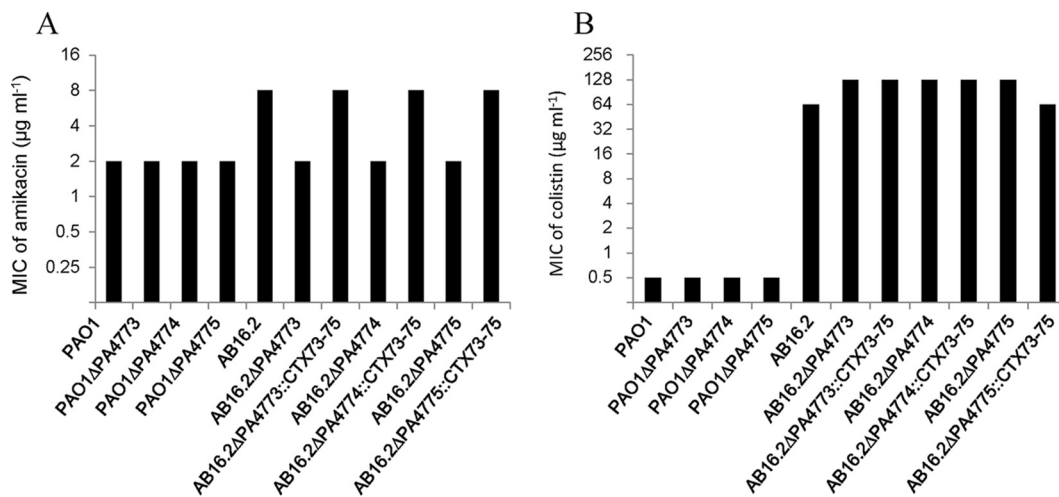
**Aminoglycoside-colistin cross-resistant *pmrAB* mutants occur in the clinical setting.** To get an insight into the relevance of such mutants in patients, we selected 28 colistin-nonsusceptible clinical strains (MICs from 4 to 256  $\mu\text{g ml}^{-1}$ ) from our lab collection. Seventeen of them (60.7%) appeared to be at least 4-fold more resistant to gentamicin, tobramycin, and amikacin than reference strain PAO1 (Table 3). Sequencing of gene *pmrB* in these 17 strains revealed the presence of several nonsynonymous mutations relative to the PAO1 gene sequence (Table 3). A number of predicted amino acid variations in protein PmrB were identical to those of aminoglycoside-colistin-

susceptible strains PA14 (S2P, A4T, G68S, V15I, Y345H) and LESB58 (Y345H) and were considered as common polymorphism. Four *pmrAB* alleles encoding five so far uncharacterized amino acid variations in PmrB were amplified from isolates 2243 (Q105P), 3092 (V6A/L37P), 3795 (G188D), and 3890 (D45E) and cloned in vector pME6012. In addition, we cloned the *pmrAB* locus of a drug-susceptible clinical strain (3095) that codes for a PmrB variant exhibiting a V6A substitution along with several nonsignificant amino acid changes. The resulting recombinant plasmids were used to complement strain PAO1 $\Delta$ *pmrAB* (Table 3). Reminiscent of our previous findings, the nonpolymorphic mutations in PmrB (L37P, D45E, Q105P, G188D) except V6A led to cross-resistance to aminoglycosides and colistin in transcomplemented mutant PAO1 $\Delta$ *pmrAB* (Table 3).

Since response regulator PmrA can also be constitutively activated by mutations (4), we sequenced its gene in the two strains showing nonsignificant amino acid variations in sensor PmrB, namely 3091 and 4586. Beside the L71R change also present in strain PA14, isolate 3091 harbored a L21I substitution in PmrA. Cloning and expression of the *pmrAB* genes from 3091 in PAO1 $\Delta$ *pmrAB* caused an increase in the MICs of gentamicin (8  $\mu$ g ml<sup>-1</sup>; 8-fold higher than the wild-type level), amikacin (8  $\mu$ g ml<sup>-1</sup>; 4-fold), tobramycin (2  $\mu$ g ml<sup>-1</sup>; 8-fold), and colistin (128  $\mu$ g ml<sup>-1</sup>; 256-fold), demonstrating that coresistance to aminoglycosides and polymyxins can arise when either PmrB or PmrA is mutationally activated. Since the PmrA protein from strain 4586 displayed the same sequence as PAO1, we considered its resistance phenotype to aminoglycosides and polymyxins as being independent of PmrAB.

**MexXY-OprM is required for aminoglycoside resistance and contributes to colistin resistance in *pmrB* mutants.** The transcript levels of gene *mexY* that code for resistance-nodulation-cell division (RND) transporter MexY were measured in strain PAO1 $\Delta$ *pmrAB* complemented with different *pmrAB* alleles described above (i.e., carrying PmrAB activating mutations). Compared with PAO1 $\Delta$ *pmrAB* overexpressing the wild-type *pmrAB* genes from plasmid pABWT, *mexY* transcription was found to be only marginally increased (from 1.4- to 2.8-fold) upon complementation with 6 of the 7 mutated alleles (see Fig. S1 in the supplemental material), while the resistance to aminoglycosides (from 4- to 8-fold) (Tables 1 and 3) and other MexXY-OprM substrates, ciprofloxacin and cefepime (2-fold, data not shown), was augmented significantly. The allele from the remaining strain 2243 (that encodes a Q105P PmrB variant) was associated with a 16.6-fold upregulation of *mexY* relative to PAO1 $\Delta$ *pmrAB*(pABWT) and a stronger increase in MICs of aminoglycosides (from 16- to 32-fold), ciprofloxacin and cefepime (4-fold; data not shown). To further investigate the role of MexXY-OprM in the phenotype of *pmrB* mutants, we deleted operon *mexXY* in mutant AB16.2 and in PAO1. Because this efflux system contributes to the natural resistance of *P. aeruginosa* to aminoglycosides (21), the *mexXY* deletion rendered strain PAO1 hypersusceptible to these antimicrobials (compare mutant PAO1 $\Delta$ *mexXY* with PAO1 in Table 2). Very similar results were obtained when genes *mexXY* were removed from AB16.2 (AB16.2 $\Delta$ *mexXY* versus AB16.2 in Table 2). MICs of aminoglycosides decreased from 32-fold (amikacin, tobramycin) to 128-fold (gentamicin), clearly indicating that the elevated resistance of *pmrB* mutants to these antibiotics depends on the activity of MexXY-OprM, though operon *mexXY* expression remains unaffected by the *pmrB* mutations (Fig. S1). We, therefore, hypothesized that another mechanism could potentiate the extrusion of aminoglycosides out of bacteria by MexXY-OprM. Indeed, synergistic interplays between efflux systems and factors modulating the outer membrane permeability to antibiotics, such as porins, are common in multidrug-resistant Gram-negative bacteria (19).

Unexpectedly, inactivation of operon *mexXY* strongly reduced colistin MIC (16-fold) leaving a residual resistance of 4  $\mu$ g ml<sup>-1</sup> in AB16.2 $\Delta$ *mexXY* while it had virtually no effect on the susceptibility level of parent PAO1 (Table 2). Although polymyxins are not in the list of known substrates of MexXY-OprM (22), our data supported the notion that aminoglycosides and colistin might be pumped more efficiently outside bacteria if their intracellular penetration was impaired by a decreased permeability of the cell envelope.

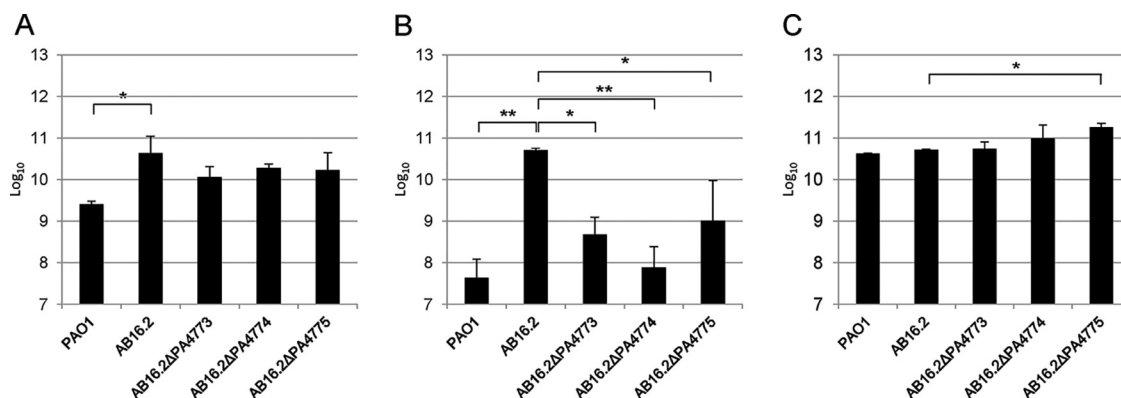


**FIG 2** Contribution of genes PA4773, PA4774, and PA4775 to amikacin (A) and colistin (B) resistance. Deletion mutants AB16.2ΔPA4773, AB16.2ΔPA4774, and AB16.2ΔPA4775 were transcomplemented with a DNA fragment carrying the wild-type genes PA4773-PA4774-PA4775 inserted in chromosomal site *attB* (yielding constructs AB16.2ΔPA4773::CTX73-75, AB16.2ΔPA4774::CTX73-75, and AB16.2ΔPA4775::CTX73-75, respectively). The data presented are representative of 3 independent MIC determinations.

### Aminoglycoside resistance is linked to genes PA4773-PA4774-PA4775 in *pmrB* mutants.

To identify the mechanisms that could synergize MexXY-OprM activity in our mutants, we compared the transcriptomes of AB16.2 and PAO1. A total of 233 genes turned out to be dysregulated, of which 201 exhibited an increased expression and 32 a reduced expression of  $\geq 3$ -fold linked to PmrAB activation (see Table S1 in the supplemental material). As expected, operon *arn* and all of the genes of the *pmrAB* cluster that includes PA4773 (*speD2*), PA4774 (*speE2*), PA4775, PA4776 (*pmrA*), and PA4777 (*pmrB*) were strongly activated in AB16.2. In *P. aeruginosa* genomes (18), genes PA4773 to PA4777 form three transcriptional units. Protein PA4773 shares 24.2% sequence identity with *S*-adenosylmethionine decarboxylase SpeD (encoded by gene PA0654 in strain PAO1), and PA4774 has a sequence 38.3% identical to that of spermidine synthase SpeE (gene PA1687). Protein PA4775 has no known homologs. Involvement of these three proteins in the resistance phenotype of *pmrB* mutants was examined by deleting each encoding gene in AB16.2 and PAO1. We obtained no evidence of the contribution of these proteins to the natural resistance of *P. aeruginosa* to aminoglycosides or polymyxins (compare PAO1 and its PAO1Δ derivatives in Fig. 2A and B; see also Fig. S2 in the supplemental material). However, while it did not affect colistin resistance significantly, inactivation of any of these proteins restored a wild-type susceptibility to aminoglycosides in AB16.2. Conversely, mutants AB16.2ΔPA4773, AB16.2ΔPA4774, and AB16.2ΔPA4775 recovered the initial phenotype of AB16.2 when a DNA fragment carrying genes PA4773-PA4774-PA4775 was inserted in chromosomal site *attB* (Fig. 2A; see also Fig. S2). At this point, our results showed that the elevated resistance of *pmrB* mutants to aminoglycosides was dependent upon PA4773-PA4774-PA4775 and efflux system MexXY-OprM.

**Norspermidine contributes to aminoglycoside resistance.** Since genes PA4773 and PA4774 are predicted to encode enzymes involved in synthesis of polyamines, we measured the amounts of 1,3-diaminopropane (a precursor of polyamines), putrescine, cadaverine, norspermidine, spermidine, and spermine bound to the cell surface of strains PAO1, AB16.2, AB16.2ΔPA4773, AB16.2ΔPA4774, and AB16.2ΔPA4775 by using liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS). As shown in Fig. S3 in the supplemental material, we found no significant differences in the areas under the peaks of cadaverine, putrescine, and spermine between the strains. In contrast, 15.8-fold more spermidine was detected in the extracts from mutant AB16.2 ( $10.6 \log_{10}$ ) relative to that from strain PAO1 ( $9.4 \log_{10}$ ) (Fig. 3A). These amounts were



**FIG 3** Amounts of spermidine (A), norspermidine (B), and 1,3-diaminopropane (C) in cell surface extracts of strain PAO1 and derived mutants. The histograms represent the area under the peak values of each compound as determined by LC-ESI-MS. The data correspond to means of normalized values (log scale) ± the standard deviation (SD) of three independent experiments. Tukey's test results are indicated as follows: \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

only partially reduced in the mutants lacking PA4773, PA4774, or PA4775, suggesting that the major part of the spermidine present in the extracts had been elaborated via another pathway. More importantly, the LC-ESI-MS analysis revealed the presence of much higher quantities (>1,000-fold) of norspermidine at the surface of AB16.2 ( $10.7 \log_{10}$ ) than of PAO1 ( $7.6 \log_{10}$ , a value close to the limit of detection) (Fig. 3B). The observation that the deletion of gene PA4773 ( $8.6 \log_{10}$ ), PA4774 ( $7.8 \log_{10}$ ), or PA4775 ( $9.0 \log_{10}$ ) was associated with lower norspermidine levels in AB16.2 (126-, 794-, and 50-fold, respectively) (Fig. 3B) supports the notion that when overexpressed in *pmrB* mutants, the three genes enable the formation of this polyamine and contribute to aminoglycoside resistance. Finally, 1,3-diaminopropane turned out to be 3.2-fold more abundant in extracts from mutant AB16.2ΔPA4775 ( $11.2 \log_{10}$ ) than from AB16.2 ( $10.7 \log_{10}$ ) (Fig. 3C), which might suggest that protein PA4775 converts this precursor to fuel the norspermidine biosynthesis pathway.

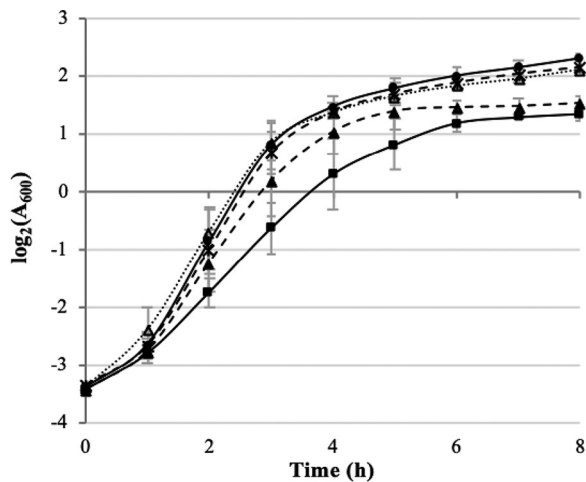
**Impact of norspermidine synthesis on bacterial growth.** Addition of exogenous norspermidine is known to inhibit bacterial growth in various species, including *P. aeruginosa* (23). Since mutant AB16.2 formed smaller colonies than parental strain PAO1 on Mueller-Hinton (MH) agar medium, we compared the growth curves of the two strains with those of mutants AB16.2ΔPA4773, AB16.2ΔPA4774, and AB16.2ΔPA4775. As shown in Fig. 4, these experiments confirmed the impaired development of mutant AB16.2. Suppression of genes PA4773 and PA4774 in this mutant completely restored the parental fitness, while the deletion of PA4775 only partially improved its growth, which is consistent with the hypothesis that norspermidine production in mutant AB16.2 is deleterious.

**DISCUSSION**

Mutations in TCS PmrAB are a well-known cause of colistin resistance in *P. aeruginosa* (6). However, their impact on bacterial susceptibility to aminoglycosides has not been addressed specifically until the present study. Lopez-Causapé et al. observed the emergence of *pmrB* mutations in strain PAO1 submitted to increasing concentrations of tobramycin in addition to other gene alterations (e.g., *fusA1*, *nuoD*), but the role of mutated PmrB in coresistance to aminoglycosides and polymyxins was not investigated further (24). In another study, a concomitant increase in tobramycin (2-fold) and colistin (64-fold) MICs was noted in strain PA14 upon complementation with an A4T/L323H PmrB variant provided by allelic replacement (25).

Our work demonstrates that mutations affecting the histidine kinase PmrB or response regulator PmrA elicit a cross-resistance to these antibiotics in *in vitro*-selected mutants and clinical strains. Alteration of this TCS has been reported to result in a wide range of colistin MICs in *P. aeruginosa* (from 2 to 128 mg/liter) due to different levels of operon *arn* activation (4, 10). All of the PmrAB variants described here conferred a quite



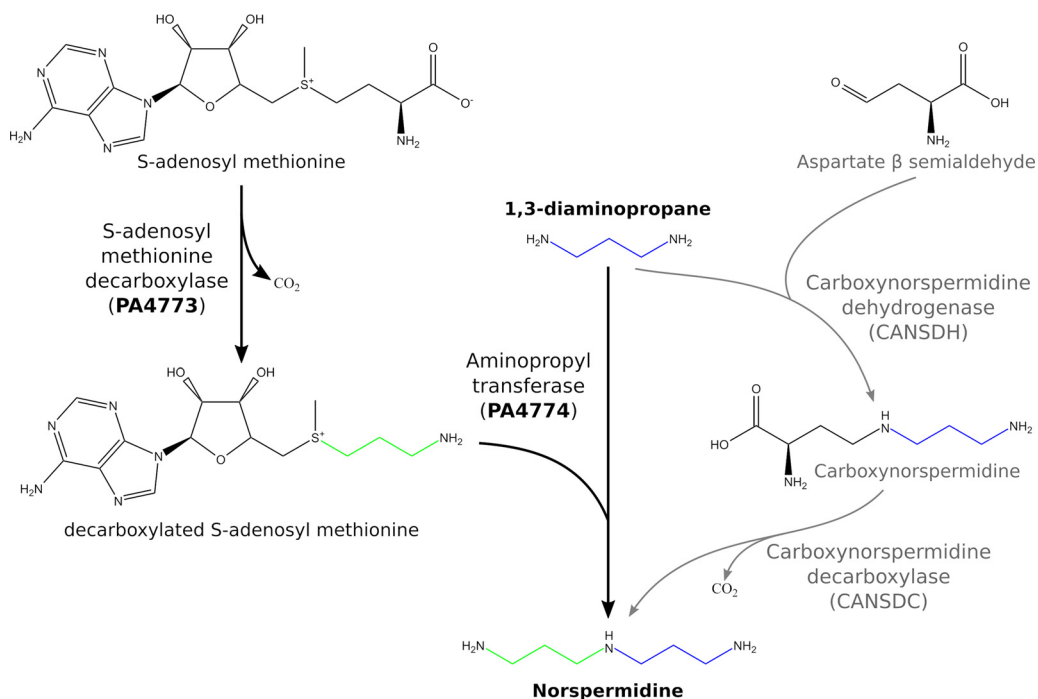


**FIG 4** Effects of gene PA4773, PA4774, and PA4775 overexpression on bacterial growth. Growth of strains PAO1 (circles, solid line), AB16.2 (squares, solid line), AB16.2 $\Delta$ PA4773 (crosses, dashed line), AB16.2 $\Delta$ PA4774 (open triangles, dotted line), and AB16.2 $\Delta$ PA4775 (triangles, dashed line) in Mueller-Hinton broth at 37°C was measured spectrophotometrically at  $A_{600}$ . Error bars indicate SD of three biological replicates.

high resistance to colistin ( $\text{MIC} \geq 16 \mu\text{g ml}^{-1}$ ) when expressed in strain PAO1 $\Delta$ *pmrAB*, which might suggest that aminoglycoside resistance arises only when the TCS is activated by specific mutations. However, our observation that various amino acid substitutions occurring in different domains of PmrB (Fig. 1) and in PmrA provide the same cross-resistance profile does not support this assumption. Furthermore, this phenotype was also observed in mutants selected on low concentrations of colistin ( $4 \mu\text{g ml}^{-1}$  instead of 8 or  $16 \mu\text{g ml}^{-1}$ ) (data not shown). Thus, aminoglycoside resistance seems to be a common feature of *pmrAB* mutants.

As highlighted by gene deletion experiments, aminoglycoside resistance of *pmrB* mutants was independent from the LPS modification pathway determined by operon *arn* (i.e., independent from the mechanism of polymyxin resistance) and did not result from constitutive overexpression of genes *mexXY*. Intriguingly, a Q105P change in PmrB increased *mexXY* expression and aminoglycoside resistance (2-fold) in PAO1 $\Delta$ *pmrAB* compared with other PmrB variations (see Fig. S1 in the supplemental material; Table 3). This PmrAB-dependent activation of pump MexXY-OprM remains to be investigated. It could potentially involve ParRS, as cross talks between TCS are common in bacteria to allow adapted stress responses (26). Operon *mexXY* transcription is indeed upregulated when ParRS is activated by mutations or bacterial exposure to colistin (20). Though gene deletion experiments established that this TCS does not mediate aminoglycoside resistance in mutant AB16.2, they have to be done on PAO1 $\Delta$ *pmrAB*(pAB2243) to rule out any implication of ParRS in *mexXY* dysregulation linked to the PmrB Q105P variant.

As stated above, the presence of efflux pump MexXY-OprM was required for aminoglycoside resistance in *pmrAB* mutants, but genes *mexXY* do not need to be constitutively activated (Fig. S1). Since protein synthesis inhibitors induce *mexXY* transcription through the ArmZ/MexZ regulatory pathway when ribosomes stall (27), one could assume that aminoglycosides are stronger inducers of *mexXY* expression in *pmrAB* mutants than in wild-type bacteria. However, deletion of genes PA4773, PA4774, and PA4775 in mutant AB16.2 provided a simpler explanation in line with the amounts of several polyamines extracted from the cell surface (Fig. 3A). Several lines of evidence suggest that this three-gene cluster, which is cotranscribed with and under the control of *pmrAB* genes, is involved in norspermidine biosynthesis. To our knowledge, this linear and flexible aliphatic molecule that carries three amine groups positively charged at physiological pH has never been detected before in *P. aeruginosa*. In contrast to spermidine, a triamine involved in multiple physiological functions, norspermidine is



**FIG 5** Norspermidine biosynthesis. The putative pathway of norspermidine synthesis in *P. aeruginosa* and the previously identified pathway of norspermidine biosynthesis in *Vibrionales* are represented with black and gray lines respectively.

considered as rather uncommon in bacteria except in *Vibrionales* (28). In *Vibrio cholerae*, norspermidine is synthesized from 1,3-diaminopropane through the aspartate  $\beta$ -semialdehyde-dependent pathway involving enzymes carboxyspermidine dehydrogenase (CASDH) and carboxyspermidine decarboxylase (CANSDC) (29) (Fig. 5). BLAST searches failed to find evident homologs encoded by the *P. aeruginosa* genome (30). Some bacteria such as the hyperthermophiles *Thermus thermophilus* and *Thermotoga maritima* are able to produce norspermidine through an *S*-adenosylmethionine-dependent route (31, 32). This pathway relies on the activity of an aminopropyltransferase enzyme able to add an aminopropyl residue from decarboxylated *S*-adenosylmethionine to various polyamines including 1,3-diaminopropane. This metabolic route is widely used in bacteria to form spermidine from putrescine (31). However, its role in the synthesis of other polyamines has been reported only in *T. thermophilus*, and *T. maritima*, which produce an aminopropyltransferase enzyme (named triamine/agmatine aminopropyl transferase [TAAPT]) able to cope with substrates other than putrescine (33, 34). One explanation for the presence of norspermidine in *P. aeruginosa* might be that the putative enzyme SpeE2 encoded by gene PA4774 promotes the transfer of an aminopropyl residue to 1,3-diaminopropane to form norspermidine (Fig. 5). Supporting this hypothesis, we found that, with a sequence identity of 60%, protein PA4774 is the closest homolog of aminopropyltransferase from *T. thermophilus* HB8 (NCBI Protein accession number [1UIR\\_A](#)) (34). Additional experiments are now necessary to confirm that the substrate specificity of PA4774 includes 1,3-diaminopropane. If correct, this scenario could explain why higher amounts of this metabolite were detected in cell extracts of mutant AB16.2 $\Delta$ PA4775 than in AB.16.2.

While norspermidine was strongly overproduced in *pmrAB* mutants through the PA4773-PA4774-PA4775 pathway, spermidine synthesis was also increased to similar levels via another route. The presence of high spermidine amounts at the cell surface was reported to have a protective role against aminoglycosides when bacteria are exposed to an acid environment or to eDNA, through the activation of TCS PmrAB (16). That the decreased resistance of mutants AB16.2 $\Delta$ PA4773, AB16.2 $\Delta$ 4774, and AB16.2 $\Delta$ 4775 to aminoglycosides correlated with reduced norspermidine levels (while

that of spermidine remained almost unchanged) strongly suggest that norspermidine plays the main protective role against these antibiotics in *pmrAB* mutants. An attractive hypothesis supported by some experimental data (16) would be that norspermidine and spermidine modulate the self-promoted uptake pathway of aminoglycosides through the outer membrane, by reducing the net negative charge of the cell surface. Whether these two structurally close polyamines individually confer a resistance to specific inhibitors sharing a polycationic structure is an interesting issue that warrants further studies.

Mutation-driven activation of PmrAB was associated with therapeutically significant levels of aminoglycoside and polymyxin resistance in *in vitro* mutants, according to EUCAST breakpoints (Tables 1 and 3). Consistent with these results, 16 of 17 clinical strains exhibiting a cross-resistance to both antibiotic families turned out to be *pmrAB* mutants. The impaired growth of these bacteria *in vitro*, due to polyamine production, might reduce their ability to cause acute infections. However, their presence in clinical samples evidently shows that their survival in patients is not compromised by mutations in PmrAB.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** Bacterial strains and plasmids used in this study are listed in Table 4. Bacteria were routinely grown in Mueller-Hinton broth (MHB) with adjusted concentrations of divalent cations  $\text{Ca}^{2+}$  (from 20 to 25  $\mu\text{g ml}^{-1}$ ) and  $\text{Mg}^{2+}$  (from 10 to 12.5  $\mu\text{g ml}^{-1}$ ) (Becton, Dickinson and Company, Sparks, MD), or on Mueller-Hinton agar (MHA; Becton, Dickinson and Company, Sparks, MD). Eighteen clinical strains of *P. aeruginosa* isolated between 2015 and 2018 in 14 French hospitals were studied, of which 17 were colistin resistant (MIC,  $>2 \mu\text{g ml}^{-1}$ ). Wild-type reference strain PAO1 (from K. Stover's laboratory) was used to select colistin-resistant mutants and to perform gene complementation experiments. Unless otherwise stated, bacterial cultures were performed at 37°C.

PmrAB mutants were selected by spreading  $10^8$  CFU of log-phase PAO1 cells on Mueller-Hinton agar (MHA) plates supplemented with 8 and 16  $\mu\text{g ml}^{-1}$  colistin, respectively. Plasmid vectors were maintained in subcultures of *E. coli* with 50  $\mu\text{g ml}^{-1}$  kanamycin, 15  $\mu\text{g ml}^{-1}$  tetracycline, 100  $\mu\text{g ml}^{-1}$  ampicillin, or 50  $\mu\text{g ml}^{-1}$  streptomycin as selection markers. Transconjugants and transformants of *P. aeruginosa* were selected on MHA or *Pseudomonas* isolation agar (PIA; Becton, Dickinson and Company, Sparks, MD) by using 200  $\mu\text{g ml}^{-1}$  tetracycline, 2,000  $\mu\text{g ml}^{-1}$  streptomycin, or 150  $\mu\text{g ml}^{-1}$  ticarcillin. Bacterial growth curves were established in triplicate from freshly diluted cultures in 30 ml of MHB (initial absorbance  $A_{600} = 0.1$ ) and incubated at 37°C with vigorous shaking. The absorbance was monitored hourly up to 8 h. Standard deviations were calculated at each time point.

**Antimicrobial susceptibility testing.** The MICs of selected antibiotics were determined by microdilution in MHB and interpreted according to the guidelines of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (<http://www.eucast.org>).

**Transcomplementation of mutant PAO1 $\Delta$ *pmrAB*.** The *pmrAB* loci from strain PAO1, *in vitro*-selected mutants, and clinical strains were amplified by PCR from genomic DNA extracts (Wizard genomic DNA purification kit; Promega Corporation, Charbonnières-les-Bains, France) with specific primers PCR*pmrAB*1 and PCR*pmrAB*4 (see Table S2 in the supplemental material). The amplicons were first cloned into vector pCR-Blunt. Then, 2,286-bp fragments carrying the *pmrAB* alleles were subcloned into EcoRI-linearized plasmid pME6012 (35). The sequences of cloned alleles were checked on both strands by using specific primers (3130 Genetic Analyzer; Applied Biosystems) (Table S2). Recombinant plasmids were introduced by electrotransformation into mutant PAO1 $\Delta$ *pmrAB* (36), and the resulting transformants were selected on MHA supplemented with 200  $\mu\text{g ml}^{-1}$  tetracycline.

**Chromosomal complementation with PA4773-PA4774-PA4775 gene cluster.** The locus with its promoter region was amplified by PCR from a whole DNA extract of strain PAO1, with primers PCRiPA4773A1 and PCRiPA4775A4 (Table S2). The 3,231-bp amplicon was cloned into vector pCR-Blunt and then subcloned into plasmid mini-CTX1 previously cleaved with endonucleases BamHI/NotI (37). The recombinant plasmid was transferred from *E. coli* CC118 to *P. aeruginosa* strains by conjugation as previously reported (37), and the transconjugants were selected on PIA medium containing 200  $\mu\text{g ml}^{-1}$  tetracycline. Excision of the tetracycline resistance cassette was achieved by expressing recombinase Fip from plasmid pFLP2. This plasmid, which carries levansucrase gene *sacB*, was finally cured by growing bacteria on minimal agar medium M9 containing 5% sucrose. Insertion of the PA4773-PA4774-PA4775 fragment in chromosomal site *attB* was confirmed by PCR sequencing experiments (Table S2).

**Construction of deletion mutants in strain PAO1.** Gene deletion mutants were obtained by using overlapping PCRs and recombination events, as previously described by Kaniga et al. (38). Briefly, using the primers indicated in Table S2, the DNA regions flanking the target genes were amplified as single DNA fragments. These fragments were subsequently cloned into plasmid pCR-Blunt and subcloned into suicide vector pKNG101 in *E. coli* CC118 $\lambda$ *pir* (38). The resulting plasmids were introduced into *P. aeruginosa* strains by triparental mating using helper strain *E. coli* HB101(pRK2013) (39). Transconjugants were selected on PIA supplemented with 2,000  $\mu\text{g ml}^{-1}$  streptomycin. Excision of pKNG101 was obtained by selection on M9 minimal agar medium supplemented with 5% sucrose. The deletions were checked by PCR and sequencing using specific primers (Table S2).

**TABLE 4** Strains and plasmids used in the study

Strain or plasmid	Relevant characteristic(s) <sup>a</sup>	Source or reference
<b>Strain</b>		
<i>E. coli</i>		
DH5α	F <sup>-</sup> φ80 <i>lacZ</i> ΔM15 Δ( <i>lacZYA-argF</i> )U169 <i>recA1 endA1 hsdR17</i> (r <sub>K</sub> <sup>-</sup> , m <sub>K</sub> <sup>+</sup> ) <i>phoA supE44 thi-1 gyrA96 relA1 λ<sup>-</sup></i>	Invitrogen
CC118	Δ( <i>ara-leu</i> ) <i>araD ΔlacX74 galE galk phoA20 thi-1 rpsE rpoB argE</i> (Am) <i>recAI</i>	44
CC118 λ <i>pir</i>	CC118 lysogenized with λ <i>pir</i> phage	45
HB101	<i>supE44 hsdS20</i> (r <sub>B</sub> <sup>-</sup> , m <sub>B</sub> <sup>-</sup> ) <i>recA13 ara-14 proA2 lacY1 galk2 rpsL20 xyl-5 mtl-1 leuB6 thi-1</i>	46
<i>P. aeruginosa</i>		
PAO1	Wild-type reference strain	30
AB8.2	PAO1 spontaneous <i>pmrB</i> mutant (V28G)	This study
AB16.1	PAO1 spontaneous <i>pmrB</i> mutant (F408L)	This study
AB16.2	PAO1 spontaneous <i>pmrB</i> mutant (ΔL172)	This study
PAO1Δ <i>pmrB</i>	PAO1 with in-frame deletion of gene <i>pmrB</i>	This study
PAO1Δ <i>pmrA</i>	PAO1 with in-frame deletion of gene <i>pmrA</i>	This study
PAO1Δ <i>pmrAB</i>	PAO1 with in-frame deletion of operon <i>pmrAB</i>	20
PAO1ΔPA4773	PAO1 with in-frame deletion of gene PA4773	This study
PAO1ΔPA4774	PAO1 with in-frame deletion of gene PA4774	This study
PAO1ΔPA4775	PAO1 with in-frame deletion of gene PA4775	This study
PAO1Δ <i>arn</i>	PAO1 with in-frame deletion of operon <i>arnBCADTEF-ugd</i> ( <i>arn</i> )	This study
PAO1Δ <i>mexXY</i>	PAO1 with in-frame deletion of operon <i>mexXY</i>	47
PAO1Δ <i>parRS</i>	PAO1 with in-frame deletion of operon <i>parRS</i>	20
AB16.2Δ <i>pmrB</i>	AB16.2 with in-frame deletion of gene <i>pmrB</i>	This study
AB16.2Δ <i>pmrA</i>	AB16.2 with in-frame deletion of gene <i>pmrA</i>	This study
AB16.2ΔPA4773	AB16.2 with in-frame deletion of gene PA4773	This study
AB16.2ΔPA4774	AB16.2 with in-frame deletion of gene PA4774	This study
AB16.2ΔPA4775	AB16.2 with in-frame deletion of gene PA4775	This study
AB16.2Δ <i>arn</i>	AB16.2 with in-frame deletion of operon <i>arnBCADTEF-ugd</i> ( <i>arn</i> )	This study
AB16.2Δ <i>mexXY</i>	AB16.2 with in-frame deletion of operon <i>mexXY</i>	This study
AB16.2Δ <i>parRS</i>	AB16.2 with in-frame deletion of operon <i>parRS</i>	This study
AB16.2ΔPA4773::73-75	AB16.2ΔPA4773 complemented with PA4773 to PA4775 locus	This study
AB16.2ΔPA4774::73-75	AB16.2ΔPA4774 complemented with PA4773 to PA4775 locus	This study
AB16.2ΔPA4775::73-75	AB16.2ΔPA4775 complemented with PA4773 to PA4775 locus	This study
<b>Plasmid</b>		
pKNG101	Marker exchange suicide vector in <i>P. aeruginosa</i> ; <i>sacBR mobRK2 oriR6K</i> ; Str <sup>r</sup>	38
pRK2013	Helper plasmid for mobilization of non-self-transmissible plasmids; ColE1 Tra <sup>+</sup> Mob <sup>+</sup> ; Kan <sup>r</sup>	39
pCR-Blunt	Blunt-end cloning vector; <i>ccdB lacZα</i> ; Zeo <sup>r</sup> Kan <sup>r</sup>	Life Technologies
pME6012	Broad-host-range expression plasmid; Tet <sup>r</sup>	35
mini-CTX1	Self-proficient integration vector, Ω-FRT-attP-MCS, <i>ori</i> , <i>int</i> , <i>oriT</i> ; Tet <sup>r</sup>	37
pFLP2	Source of FLP recombinase; Tic <sup>r</sup>	48
pABWT	pME6012 carrying genes <i>pmrAB</i> from strain PAO1	This study
pAB8.2	pME6012 carrying genes <i>pmrAB</i> from mutant 8.2	This study
pAB16.1	pME6012 carrying genes <i>pmrAB</i> from mutant 16.1	This study
pAB16.2	pME6012 carrying genes <i>pmrAB</i> from mutant 16.2	This study
pAB2243	pME6012 carrying genes <i>pmrAB</i> from strain 2243	This study
pAB3092	pME6012 carrying genes <i>pmrAB</i> from strain 3092	This study
pAB3795	pME6012 carrying genes <i>pmrAB</i> from strain 3795	This study
pAB3890	pME6012 carrying genes <i>pmrAB</i> from strain 3890	This study
pAB3095	pME6012 carrying genes <i>pmrAB</i> from strain 3095	This study
pAB3091	pME6012 carrying genes <i>pmrAB</i> from strain 3091	This study
pKNGΔ <i>pmrAB</i>	BamHI/ApaI 1,028-kb fragment composed of sequences flanking the 5' and 3' ends of <i>pmrAB</i> , cloned in pKNG101	20
pKNGΔ <i>pmrB</i>	BamHI/XbaI 1,074-kb fragment composed of sequences flanking the 5' and 3' ends of <i>pmrB</i> , cloned in pKNG101	This study
pKNGΔ <i>pmrA</i>	ApaI 851-bp fragment composed of sequences flanking the 5' and 3' ends of <i>pmrA</i> , cloned in pKNG101	This study
pKNGΔPA4773	BamHI/ApaI 1,045-kb fragment composed of sequences flanking the 5' and 3' ends of PA4773, cloned in pKNG101	This study
pKNGΔPA4774	BamHI/ApaI 1,070-kb fragment composed of sequences flanking the 5' and 3' ends of PA4774, cloned in pKNG101	This study
pKNGΔPA4775	BamHI/ApaI 1,027-kb fragment composed of sequences flanking the 5' and 3' ends of PA4775, cloned in pKNG101	This study
pKNGΔ <i>arn</i>	BamHI/ApaI 1,135-kb fragment composed of sequences flanking the 5' and 3' ends of <i>arnBCADTEF-ugd</i> ( <i>arn</i> ), cloned in pKNG101	This study

(Continued on next page)

TABLE 4 (Continued)

Strain or plasmid	Relevant characteristic(s) <sup>a</sup>	Source or reference
pKNGΔ <i>mexXY</i>	BamHI/ApaI 1,756-kb fragment composed of sequences flanking the 5' and 3' ends of <i>mexXY</i> , cloned in pKNG101	47
pKNGΔ <i>parRS</i>	ApaI 1,045-kb fragment composed of sequences flanking the 5' and 3' ends of <i>parRS</i> , cloned in pKNG101	20
mini-CTX::PA4773-75	PA4773 to PA4775 locus cloned in mini-CTX1 at sites BamHI/NotI	This study

<sup>a</sup>Str<sup>r</sup>, marker of streptomycin resistance; Kan<sup>r</sup>, kanamycin resistance; Zeo<sup>r</sup>, zeocin resistance; Tet<sup>r</sup>, tetracycline resistance; Tic<sup>r</sup>, ticarcillin resistance.

**RT-qPCR.** Total RNA was extracted and purified (RNeasy plus kit, Qiagen) from cultures of strain PAO1 or its mutants grown to mid-log phase ( $A_{600} = 0.8$ ) in MHB, as previously reported (40). Two micrograms of RNA extracts treated with DNase (Qiagen) were reverse transcribed into cDNA with ImProm-II reverse transcriptase (RT) according to the manufacturer's recommendations (Promega, Madison, WI). The mRNA amounts of target genes were estimated by real-time quantitative PCR (RT-qPCR) in a Rotor Gene RG6000 instrument (Qiagen, Courtaboeuf, France) by using the QuantiFast SYBR green PCR kit (Qiagen), specific RT-qPCR primers (listed in Table S2), and a 1:10 dilution of cDNA as the template. Absolute values of gene expression were normalized for each strain with those of housekeeping gene *uvrD* and expressed as a ratio (fold change) to that of wild-type strain PAO1, used as a reference (41). Mean gene expression values were calculated from two independent cultures, each assayed in duplicate.

**RNA-Seq transcriptome.** Strain PAO1 and mutant AB16.2 were incubated at 37°C with shaking (250 rpm) in drug-free MHB until an  $A_{600}$  of  $0.8 \pm 0.05$ . RNA was extracted from cell pellets (duplicates for each sample) using the RNeasy minikit (Qiagen) in combination with QIAshredder columns (Qiagen). The preparation and sequencing of the cDNA libraries were done as described previously (42). Libraries were sequenced with 50 cycles in single end mode on an Illumina HiSeq 2500 device. Computational analysis was done according to the method reported by Dotsch et al. (42), with some modifications. The reads were aligned to the PAO1 reference genome using Stampy (43).

**LC-ESI-MS analysis of cell surface extracts.** Overnight cultures in MHB were diluted in fresh medium and grown at 35°C with shaking to mid-log phase ( $A_{600} = 0.8$ ). Surface-associated polyamines were extracted as previously described, except that the pellet was resuspended in a final 500- $\mu$ l volume of buffer (14). Analysis of polyamines was performed on the platform of BioPark (Archamps Technopole, France) from 100  $\mu$ l of bacterial extract added to 90  $\mu$ l of *N*-(succinimidyl)oxycarbonylmethyl)tris(2,4,6-trimethoxyphenyl)phosphonium bromide 20 mM (TMPP; Sigma-Aldrich, Saint Louis, MO). The primary amines were labeled with TMPP, which is known to make amines more amenable to detection by mass spectrometry by improving the molecule ionization in the electrospray. Addition of TMPP increased the mass of 572.18 Da to the nominal mass of any molecule with a primary or secondary amine. The mixture was incubated for 1 h at room temperature, and the reaction was stopped by addition of 150  $\mu$ l of NH<sub>4</sub>OH (1 M). After 30 min of incubation at room temperature, 160  $\mu$ l of trifluoroacetic acid (TFA) (10%) was added, and the samples were desalted by solid phase extraction (SPE) (Omics bond elut C<sub>18</sub> 100- $\mu$ l tip; Waters) according to the manufacturer's protocol. Amines were eluted using 50  $\mu$ l of mix containing 60% acetonitrile (ACN) and 0.1% TFA and dried down by speed vacuum. The pellet was resuspended in 20  $\mu$ l of 2% ACN and 0.1% TFA, and 2  $\mu$ l of the mixture was loaded on a standard reverse-phase chromatography column using an Ultimate 300 nanoflow high-performance liquid chromatography system coupled to a Q-Exactive Orbitrap with a 75  $\mu$ m by 150 mm Acclaim PepMap 100, C<sub>18</sub>, 3- $\mu$ m nanoViper column (Thermo Scientific, Bremen, Germany). The amines were eluted by a gradient from 2% to 35% ACN in 0.1% (vol/vol) formic acid, and subsequently from 35% to 85% over a period of 35 min. Then, the amines were detected by a mass spectrometer connected at the column exit to an electrospray ionization interface (ES-MS). The Q-Exactive Orbitrap acquired a full-range scan from 310 to 2,000 Th (70,000 resolution; AGC, target 3.10<sup>6</sup>; maximum IT, 200 ms). An equimolar mix of 1,3-diaminopropane, putrescine dihydrochloride, cadaverine, norspermidine, spermidine, and spermine was analyzed in similar conditions and used as standard. Extracted ion chromatograms were used to identify the *m/z* ions expected for the TMPP amines. Areas under the peaks were collected to compare the amounts of amines in different strains.

**Statistical analysis.** The reported gene read counts were used to estimate differential gene expression between the mutant AB16.2 and PAO1, making use of package DESeq in R (project for statistical computing). Comparisons of polyamine levels were performed using R software (v 3.3.3). The three independent replicates were normalized to remove block effects and then log<sub>10</sub> transformed. For each polyamine and strain tested, an analysis of variance (ANOVA) test was applied. Statistically significant ( $P < 0.05$ ) differences between the strains were checked with Tukey's honestly significant difference (HSD) test.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.01044-19>.

**SUPPLEMENTAL FILE 1**, PDF file, 0.3 MB.

## ACKNOWLEDGMENTS

We are grateful to Loïc Andrey for his excellent technical assistance.

This work was supported by the French ministry of Health through the Santé publique France agency.

## REFERENCES

- Luyt CE, Hekimian G, Koulenti D, Chastre J. 2018. Microbial cause of ICU-acquired pneumonia: hospital-acquired pneumonia versus ventilator-associated pneumonia. *Curr Opin Crit Care* 24:332–338. <https://doi.org/10.1097/MCC.0000000000000526>.
- Del Barrio-Tofino E, Lopez-Causapé C, Cabot G, Rivera A, Benito N, Segura C, Montero MM, Sorli L, Tubau F, Gomez-Zorrilla S, Tormo N, Dura-Navarro R, Viedma E, Resino-Foz E, Fernandez-Martinez M, Gonzalez-Rico C, Alejo-Cancho I, Martinez JA, Labayru-Echverria C, Duenas C, Ayestaran I, Zamorano L, Martinez-Martinez L, Horcajada JP, Oliver A. 2017. Genomics and susceptibility profiles of extensively drug-resistant *Pseudomonas aeruginosa* isolates from Spain. *Antimicrob Agents Chemother* 61:e01589–17. <https://doi.org/10.1128/AAC.01589-17>.
- Sader HS, Huband MD, Castanheira M, Flamm RK. 2017. *Pseudomonas aeruginosa* antimicrobial susceptibility results from four years (2012 to 2015) of the international network for optimal resistance monitoring program in the United States. *Antimicrob Agents Chemother* 61:e02252–16. <https://doi.org/10.1128/AAC.02252-16>.
- Moskowitz SM, Ernst RK, Miller SI. 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. <https://doi.org/10.1128/jb.186.2.575-579.2004>.
- Nummila K, Kilpelainen I, Zahringer U, Vaara M, Helander IM. 1995. Lipopolysaccharides of polymyxin B-resistant mutants of *Escherichia coli* are extensively substituted by 2-aminoethyl pyrophosphate and contain aminoarabinose in lipid A. *Mol Microbiol* 16:271–278. <https://doi.org/10.1111/j.1365-2958.1995.tb02299.x>.
- Jeannot K, Bolard A, Plésiat P. 2017. Resistance to polymyxins in Gram-negative organisms. *Int J Antimicrob Agents* 49:526–535. <https://doi.org/10.1016/j.ijantimicag.2016.11.029>.
- Abraham N, Kwon DH. 2009. A single amino acid substitution in PmrB is associated with polymyxin B resistance in clinical isolate of *Pseudomonas aeruginosa*. *FEMS Microbiol Lett* 298:249–254. <https://doi.org/10.1111/j.1574-6968.2009.01720.x>.
- Barrow K, Kwon DH. 2009. Alterations in two-component regulatory systems of phoPQ and pmrAB are associated with polymyxin B resistance in clinical isolates of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 53:5150–5154. <https://doi.org/10.1128/AAC.00893-09>.
- Schurek KN, Sampaio JL, Kiffer CR, Sinto S, Mendes CM, Hancock RE. 2009. Involvement of pmrAB and phoPQ in polymyxin B adaptation and inducible resistance in non-cystic fibrosis clinical isolates of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 53:4345–4351. <https://doi.org/10.1128/AAC.01267-08>.
- Moskowitz SM, Brannon MK, Dasgupta N, Pier M, Sgambati N, Miller AK, Selgrade SE, Miller SI, Denton M, Conway SP, Johansen HK, Hoiby N. 2012. PmrB mutations promote polymyxin resistance of *Pseudomonas aeruginosa* isolated from colistin-treated cystic fibrosis patients. *Antimicrob Agents Chemother* 56:1019–1030. <https://doi.org/10.1128/AAC.05829-11>.
- Miller AK, Brannon MK, Stevens L, Johansen HK, Selgrade SE, Miller SI, Hoiby N, Moskowitz SM. 2011. PhoQ mutations promote lipid A modification and polymyxin resistance of *Pseudomonas aeruginosa* found in colistin-treated cystic fibrosis patients. *Antimicrob Agents Chemother* 55:5761–5769. <https://doi.org/10.1128/AAC.05391-11>.
- Sautrey G, Zimmermann L, Deleu M, Delbar A, Souza Machado L, Jeannot K, Van Bambeke F, Buyck JM, Decout JL, Mingeot-Leclercq MP. 2014. New amphiphilic neamine derivatives active against resistant *Pseudomonas aeruginosa* and their interactions with lipopolysaccharides. *Antimicrob Agents Chemother* 58:4420–4430. <https://doi.org/10.1128/AAC.02536-13>.
- Lee JY, Ko KS. 2014. Mutations and expression of PmrAB and PhoPQ related with colistin resistance in *Pseudomonas aeruginosa* clinical isolates. *Diagn Microbiol Infect Dis* 78:271–276. <https://doi.org/10.1016/j.diagmicrobio.2013.11.027>.
- Johnson L, Mulcahy H, Kanevets U, Shi Y, Lewenza S. 2012. Surface-localized spermidine protects the *Pseudomonas aeruginosa* outer membrane from antibiotic treatment and oxidative stress. *J Bacteriol* 194:813–826. <https://doi.org/10.1128/JB.05230-11>.
- McPhee JB, Bains M, Winsor G, Lewenza S, Kwasnicka A, Brazas MD, Brinkman FS, Hancock RE. 2006. Contribution of the PhoP-PhoQ and PmrA-PmrB two-component regulatory systems to Mg<sup>2+</sup>-induced gene regulation in *Pseudomonas aeruginosa*. *J Bacteriol* 188:3995–4006. <https://doi.org/10.1128/JB.00053-06>.
- Wilton M, Charron-Mazenod L, Moore R, Lewenza S. 2016. Extracellular DNA acidifies biofilms and induces aminoglycoside resistance in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 60:544–553. <https://doi.org/10.1128/AAC.01650-15>.
- McPhee JB, Lewenza S, Hancock RE. 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. <https://doi.org/10.1046/j.1365-2958.2003.03673.x>.
- Winsor GL, Griffiths EJ, Lo R, Dhillon BK, Shay JA, Brinkman FS. 2016. Enhanced annotations and features for comparing thousands of *Pseudomonas* genomes in the *Pseudomonas* genome database. *Nucleic Acids Res* 44:D646–D653. <https://doi.org/10.1093/nar/gkv1227>.
- Li XZ, Plésiat P, Nikaido H. 2015. The challenge of efflux-mediated antibiotic resistance in Gram-negative bacteria. *Clin Microbiol Rev* 28:337–418. <https://doi.org/10.1128/CMR.00117-14>.
- Muller C, Plésiat P, Jeannot K. 2011. A two-component regulatory system interconnects resistance to polymyxins, aminoglycosides, fluoroquinolones, and beta-lactams in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 55:1211–1221. <https://doi.org/10.1128/AAC.01252-10>.
- Aires JR, Köhler T, Nikaido H, Plésiat P. 1999. Involvement of an active efflux system in the natural resistance of *Pseudomonas aeruginosa* to aminoglycosides. *Antimicrob Agents Chemother* 43:2624–2628. <https://doi.org/10.1128/AAC.43.11.2624>.
- Masuda N, Sakagawa E, Ohya S, Gotoh N, Tsujimoto H, Nishino T. 2000. Contribution of the MexX-MexY-OprM efflux system to intrinsic resistance in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 44:2242–2246. <https://doi.org/10.1128/aac.44.9.2242-2246.2000>.
- Qu L, She P, Wang Y, Liu F, Zhang D, Chen L, Luo Z, Xu H, Qi Y, Wu Y. 2016. Effects of norspermidine on *Pseudomonas aeruginosa* biofilm formation and eradication. *Microbiologyopen* 5:402–412. <https://doi.org/10.1002/mbo3.338>.
- Lopez-Causapé C, Rubio R, Cabot G, Oliver A. 2018. Evolution of the *Pseudomonas aeruginosa* aminoglycoside mutational resistance *in vitro* and in the cystic fibrosis setting. *Antimicrob Agents Chemother* 62:e02583–17. <https://doi.org/10.1128/AAC.02583-17>.
- Schniederjans M, Koska M, Haussler S. 2017. Transcriptional and mutational profiling of an aminoglycoside-resistant *Pseudomonas aeruginosa* small-colony variant. *Antimicrob Agents Chemother* 61:e01178–17. <https://doi.org/10.1128/AAC.01178-17>.
- Agrawal R, Sahoo BK, Saini DK. 2016. Cross-talk and specificity in two-component signal transduction pathways. *Future Microbiol* 11:685–697. <https://doi.org/10.2217/fmb-2016-0001>.
- Jeannot K, Sobel ML, El Garch F, Poole K, Plésiat P. 2005. Induction of the MexXY efflux pump in *Pseudomonas aeruginosa* is dependent on drug-ribosome interaction. *J Bacteriol* 187:5341–5346. <https://doi.org/10.1128/JB.187.15.5341-5346.2005>.
- Hamana K. 1997. Polyamine distribution patterns within the families *Aeromonadaceae*, *Vibrionaceae*, *Pasteurellaceae*, and *Halomonadaceae*, and related genera of the gamma subclass of the *Proteobacteria*. *J Gen Appl Microbiol* 43:49–59. <https://doi.org/10.2323/jgam.43.49>.
- Lee J, Sperandio V, Frantz DE, Longgood J, Camilli A, Phillips MA, Michael AJ. 2009. An alternative polyamine biosynthetic pathway is widespread in bacteria and essential for biofilm formation in *Vibrio cholerae*. *J Biol Chem* 284:9899–9907. <https://doi.org/10.1074/jbc.M900110200>.

30. Stover CK, Pham XQ, Erwin AL, Mizoguchi SD, Warren P, Hickey MJ, Brinkman FSL, Hufnagle WO, Kowalik DJ, Lagrou M, Garber RL, Goltry L, Tolentino E, Westbrook-Wadman S, Yuan Y, Brody LL, Coulter SN, Folger KR, Kas A, Larbig K, Lim R, Smith K, Spencer D, Wong G-S, Wu Z, Paulsen IT, Reizer J, Saier MH, Hancock REW, Lory S, Olson MV. 2000. Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature* 406:959–964. <https://doi.org/10.1038/35023079>.
31. Michael AJ. 2016. Biosynthesis of polyamines and polyamine-containing molecules. *Biochem J* 473:2315–2329. <https://doi.org/10.1042/BCJ20160185>.
32. Michael AJ. 2016. Polyamines in eukaryotes, bacteria, and archaea. *J Biol Chem* 291:14896–14903. <https://doi.org/10.1074/jbc.R116.734780>.
33. Korolev S, Ikeguchi Y, Skarina T, Beasley S, Arrowsmith C, Edwards A, Joachimiak A, Pegg AE, Savchenko A. 2002. The crystal structure of spermidine synthase with a multisubstrate adduct inhibitor. *Nat Struct Biol* 9:27–31. <https://doi.org/10.1038/nsb737>.
34. Ohnuma M, Ganbe T, Terui Y, Niitsu M, Sato T, Tanaka N, Tamakoshi M, Samejima K, Kumasaka T, Oshima T. 2011. Crystal structures and enzymatic properties of a triamine/agmatine aminopropyltransferase from *Thermus thermophilus*. *J Mol Biol* 408:971–986. <https://doi.org/10.1016/j.jmb.2011.03.025>.
35. Heeb S, Itoh Y, Nishijyo T, Schnider U, Keel C, Wade J, Walsh U, O’Gara F, Haas D. 2000. Small, stable shuttle vectors based on the minimal pVS1 replicon for use in Gram-negative, plant-associated bacteria. *MPMI* 13: 232–237. <https://doi.org/10.1094/MPMI.2000.13.2.232>.
36. Choi KH, Kumar A, Schweizer HP. 2006. A 10-min method for preparation of highly electrocompetent *Pseudomonas aeruginosa* cells: application for DNA fragment transfer between chromosomes and plasmid transformation. *J Microbiol Methods* 64:391–397. <https://doi.org/10.1016/j.mimet.2005.06.001>.
37. Hoang TT, Kutchma AJ, Becher A, Schweizer HP. 2000. Integration-proficient plasmids for *Pseudomonas aeruginosa*: site-specific integration and use for engineering of reporter and expression strains. *Plasmid* 43:59–72. <https://doi.org/10.1006/plas.1999.1441>.
38. Kaniga K, Delor I, Cornelis GR. 1991. A wide-host-range suicide vector for improving reverse genetics in Gram-negative bacteria: inactivation of the *blaA* gene of *Yersinia enterocolitica*. *Gene* 109:137–141. [https://doi.org/10.1016/0378-1119\(91\)90599-7](https://doi.org/10.1016/0378-1119(91)90599-7).
39. Ditta G, Stanfield S, Corbin D, Helinski DR. 1980. Broad host range DNA cloning system for Gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. *Proc Natl Acad Sci U S A* 77:7347–7351. <https://doi.org/10.1073/pnas.77.12.7347>.
40. Dumas JL, van Delden C, Perron K, Köhler T. 2006. Analysis of antibiotic resistance gene expression in *Pseudomonas aeruginosa* by quantitative real-time-PCR. *FEMS Microbiol Lett* 254:217–225. <https://doi.org/10.1111/j.1574-6968.2005.00008.x>.
41. Jo JT, Brinkman FS, Hancock RE. 2003. Aminoglycoside efflux in *Pseudomonas aeruginosa*: involvement of novel outer membrane proteins. *Antimicrob Agents Chemother* 47:1101–1111. <https://doi.org/10.1128/aac.47.3.1101-1111.2003>.
42. Dotsch A, Eckweiler D, Schniederjans M, Zimmermann A, Jensen V, Scharfe M, Geffers R, Haussler S. 2012. The *Pseudomonas aeruginosa* transcriptome in planktonic cultures and static biofilms using RNA sequencing. *PLoS One* 7:e31092. <https://doi.org/10.1371/journal.pone.0031092>.
43. Lunter G, Goodson M. 2011. Stampy: a statistical algorithm for sensitive and fast mapping of Illumina sequence reads. *Genome Res* 21:936–939. <https://doi.org/10.1101/gr.111120.110>.
44. Manoel C, Beckwith J. 1985. TnpA: a transposon probe for protein export signals. *Proc Natl Acad Sci U S A* 82:8129–8133. <https://doi.org/10.1073/pnas.82.23.8129>.
45. Herrero M, de Lorenzo V, Timmis KN. 1990. Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in Gram-negative bacteria. *J Bacteriol* 172:6557–6567. <https://doi.org/10.1128/jb.172.11.6557-6567.1990>.
46. Lacks S, Greenberg B. 1977. Complementary specificity of restriction endonucleases of *Diplococcus pneumoniae* with respect to DNA methylation. *J Mol Biol* 114:153–168. [https://doi.org/10.1016/0022-2836\(77\)90289-3](https://doi.org/10.1016/0022-2836(77)90289-3).
47. Guénard S, Muller C, Monlezun L, Benas P, Broutin I, Jeannot K, Plésiat P. 2014. Multiple mutations lead to MexXY-OprM-dependent aminoglycoside resistance in clinical strains of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 58:221–228. <https://doi.org/10.1128/AAC.01252-13>.
48. Hoang TT, Karkhoff-Schweizer RR, Kutchma AJ, Schweizer HP. 1998. A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. *Gene* 212:77–86. [https://doi.org/10.1016/S0378-1119\(98\)00130-9](https://doi.org/10.1016/S0378-1119(98)00130-9).