



Aminoarabinylation of Lipid A Is Critical for the Development of Colistin Resistance in *Pseudomonas aeruginosa*

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ABSTRACT Lipid A aminoarabinylation is invariably associated with colistin resistance in *Pseudomonas aeruginosa*; however, the existence of alternative aminoarabinylation-independent colistin resistance mechanisms in this bacterium has remained elusive. By combining reverse genetics with experimental evolution assays, we demonstrate that a functional lipid A aminoarabinylation pathway is critical for the acquisition of colistin resistance in reference and clinical *P. aeruginosa* isolates. This highlights lipid A aminoarabinylation as a promising target for the design of colistin adjuvants against *P. aeruginosa*.

KEYWORDS colistin, *Pseudomonas aeruginosa*, acquired resistance

The reintroduction of colistin in clinical practice as a last-resort treatment option for life-threatening multidrug-resistant Gram-negative infections has inevitably led to the spread of colistin-resistant isolates (1). Gram-negative bacteria acquire colistin resistance primarily through remodeling the lipid A moiety of lipopolysaccharide (LPS) by the covalent addition of 4-amino-4-deoxy-L-arabinose (L-Ara4N) and/or phosphoethanolamine (PEtN), which reduces LPS affinity for colistin. However, other polymyxin resistance mechanisms have been described, such as lipid A glycosylation or acylation, capsule formation, and overexpression of efflux pumps and/or basic outer membrane proteins, which can bind and mask the divalent cation-binding sites of LPS (2, 3).

Adaptive resistance to colistin in *Pseudomonas aeruginosa* clinical isolates is always associated with overexpression of the *arn* operon, encoding the enzymes for L-Ara4N addition to lipid A (4–6). In this bacterium, *arn* expression is controlled by a complex regulatory network involving at least five two-component systems (TCSs) (7–11). Accordingly, mutations within these TCSs resulting in constitutive activation of the *arn* operon are typically identified in colistin-resistant *P. aeruginosa* strains (6, 12, 13). A recent experimental evolution and comparative genomics study proposed that the evolution of high levels of colistin resistance in *P. aeruginosa* invariably involves mutations in crucial nodes, including two TCSs controlling *arn* expression and the outer membrane protein Opr86 (BamA), followed by mutations in genes unrelated to L-Ara4N accounting for high resistance levels (14). However, a previous study provided evidence that individual TCSs are not essential for the acquisition of colistin resistance in *P. aeruginosa*, leading to the hypothesis that alternative or compensatory mechanisms may exist (12). This is in line with the results of independent random transposon mutagenesis analyses or genome sequencing of colistin-resistant clinical isolates which identified some genes unrelated to L-Ara4N likely involved in polymyxin resistance in *P. aeruginosa* (11, 15, 16).

To definitely clarify the relevance of lipid A aminoarabinylation to the acquisition of colistin resistance in *P. aeruginosa*, as well as the existence of possible alternative colistin resistance mechanisms, we generated a mutant impaired in the first committed

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TABLE 1 MICs of colistin for *P. aeruginosa* parental and Δ *arnBCA* mutant strains

Strain	MIC (μ g/ml)
PAO1	0.5
PAO1 Δ <i>arnBCA</i>	0.5
PA14	0.5
PA14 Δ <i>arnBCA</i>	0.5
KK1	1
KK1 Δ <i>arnBCA</i>	0.5
KK27	1
KK27 Δ <i>arnBCA</i>	0.5
TR1	0.5
TR1 Δ <i>arnBCA</i>	0.5

steps of L-Ara4N biosynthesis in the reference strain PAO1 (PAO1 Δ *arnBCA*), as described in the supplemental material. This mutant showed the same colistin MIC of its parental strain (Table 1), implying that the *arn* operon does not affect the basal level of colistin resistance, at least in our experimental setting.

To verify the effect of *arn* deletion on acquired colistin resistance, we first determined the frequency of colistin-resistant spontaneous mutants by plating on Mueller-Hinton agar plates containing 5 or 10 μ g/ml colistin. The frequency of resistance was 3.3×10^{-7} and 1.4×10^{-7} for the parental strain PAO1 in the presence of 5 and 10 μ g/ml colistin, respectively, while it was 55- and 190-fold lower for PAO1 Δ *arnBCA* (6.1×10^{-9} and 7.4×10^{-10} with 5 and 10 μ g/ml colistin, respectively). As a control, no differences were observed in the frequency of spontaneous mutants resistant to an antibiotic with a different mechanism of action (gentamicin; data not shown). In 13 independent experiments, only 16 and 4 colonies were obtained for the PAO1 Δ *arnBCA* mutant on plates with 5 and 10 μ g/ml colistin, respectively. To confirm the colistin-resistant phenotype, the colistin MICs for these mutants were determined and compared to those of spontaneous mutants from the wild-type PAO1. Surprisingly, we found that 85% of spontaneous mutants of PAO1 Δ *arnBCA* had the same MIC as the parental strain (0.5 μ g/ml), and only 2 isolates showed a slight (2-fold) increase in colistin MIC (1 μ g/ml; see Fig. S1 in the supplemental material), indicating that all the spontaneous mutants obtained on plates for PAO1 Δ *arnBCA* were false positives. In contrast, colistin resistance in spontaneous mutants of the wild-type PAO1 was much more varied, with >65% of isolates showing an MIC 4- to 16-fold higher than that of the parental strain (Fig. S1), although none of the spontaneous mutants had a colistin MIC higher than the maximum colistin concentration present on agar plates (10 μ g/ml).

While the above-described results suggest that the L-Ara4N-deficient mutant is much less prone to develop colistin resistance by spontaneous mutation(s), the selection of spontaneous mutants on colistin-containing agar plates did not allow us to obtain highly resistant isolates. We therefore sequentially cultured PAO1 and PAO1 Δ *arnBCA* in the presence of increasing concentrations of colistin (from 0.25 to 16 μ g/ml, corresponding to 0.5 \times to 32 \times the MIC) in the attempt to select for mutants which acquire successive mutations leading to high-level colistin resistance (see the supplemental material for experimental details). In 15 independent assays, the PAO1 Δ *arnBCA* mutant never grew with colistin concentrations higher than 2 μ g/ml, while the wild-type PAO1 always acquired the ability to grow in the presence of 16 μ g/ml colistin (Fig. 1A). The reintroduction of *arnBCA* *in trans* restored the ability of PAO1 Δ *arnBCA* to develop colistin resistance (Fig. S2). MIC assays on a representative number of isolated colonies from cultures with the highest colistin concentration that allowed growth confirmed that the PAO1 Δ *arnBCA* mutant did not acquire high levels of resistance (colistin MIC, ≤ 2 μ g/ml), while colistin MICs for PAO1 derivatives ranged between 32 and 128 μ g/ml.

To rule out that the relevance of the *arn* operon for acquired colistin resistance is strain dependent, Δ *arnBCA* mutants were generated in different backgrounds, i.e., the reference clinical strain PA14, which was isolated from a burn patient and that is

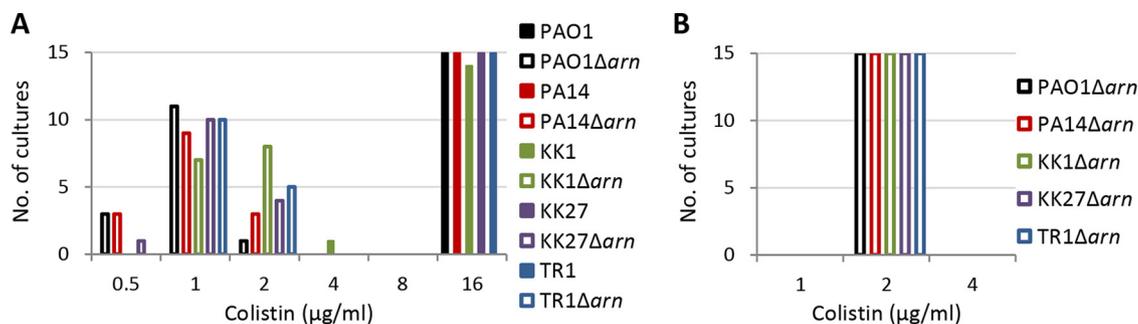


FIG 1 Maximum colistin resistance acquired by reference (PAO1 and PA14) or cystic fibrosis *P. aeruginosa* isolates (KK1, KK27, and TR1) and/or their corresponding Δ arnBCA mutants after sequential passages in Mueller-Hinton broth in the presence of increasing colistin concentrations (0.25 to 16 μ g/ml) in a short-term experiment (single passage at each colistin concentration) (A) or a long-term experiment (5 serial passages at each colistin concentration) (B) (see supplemental material for experimental details). Fifteen independent cultures were analyzed for each strain in each experiment. Cultures showing no visible growth after 5 days were considered extinct.

distantly related to PAO1 (17), and 3 cystic fibrosis isolates (KK1, KK27, and TR1; Table S1). As for PAO1, the colistin MIC for these Δ arnBCA mutants was comparable to or only 2-fold lower than that of the parental strains (Table 1), confirming that the *arn* operon marginally contributes to basal levels of colistin resistance. Conversely, the *arn* operon was found to be critical for acquired resistance to colistin also in these isolates, as all Δ arnBCA mutants failed to develop resistance to >2 μ g/ml colistin in 15 independent experimentally induced resistance assays. Their parental isolates readily acquired high-level resistance to colistin, with the exception of a single culture of the KK1 strain (Fig. 1A). The acquired colistin-resistant phenotype was stable, as the colistin MIC was not significantly reduced after several passages in colistin-free medium (Table S2). Notably, colistin MIC was strongly reduced upon deletion of the *arn*BCA genes in *in vitro*-evolved colistin-resistant PAO1 or PA14 isolates (Table S3), in line with the results from previous reports (5).

Taking into consideration the number of replicates and the level of colistin resistance reached by each replicate, the above-described assay involved relatively few generations for the Δ arnBCA mutants, ranging from 385 to 450 generations per mutant. To assess whether colistin resistance could be selected for in a higher number of generations, a long-term experiment was performed for the Δ arnBCA mutants by subculturing 15 independent cultures for each mutant for 5 serial passages in the presence of each increasing concentration of colistin (see supplemental material for experimental details). While prolonged exposure to increasing colistin concentrations stabilized the acquisition of a low-level resistance phenotype by Δ arnBCA mutants (up to 2 μ g/ml colistin), none of the cultures were able to grow in the presence of >2 μ g/ml colistin (Fig. 1B). This indicates that in our experimental setting, high levels of colistin resistance were not developed in the absence of a functional *arn* operon even after $>2,000$ generations per mutant (ca. 140 generations per independent culture). Recently, lipid A aminoarabinylation and the development of colistin resistance were observed in *arn*B-deleted *P. aeruginosa* (18), suggesting that the L-Ara4N biosynthesis pathway can somehow remedy the lack of ArnB in *P. aeruginosa*. Our study shows that this does not occur in ArnBCA-deficient cells.

In conclusion, this work demonstrates that a functional *arn* operon and, thus, aminoarabinylation of lipid A are required for acquired colistin resistance in both reference and clinical isolates of *P. aeruginosa*. Indeed, none of the Δ arnBCA mutants became resistant to ≥ 4 μ g/ml colistin, which corresponds to the epidemiological cutoff (ECOFF) of colistin for *P. aeruginosa* (http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Rationale_documents/Colistin_rationale_1.0.pdf), i.e., the highest MIC for isolates devoid of any detectable acquired resistance mechanisms (19). Thus, our data directly confirm that lipid A aminoarabinylation is a critical prerequisite for the acquisition of colistin resistance in this pathogen (5, 6, 9, 14). This evidence, together

with recent observations that the addition of PEtN to lipid A, by either endogenous (EptA) or plasmid-harbored PEtN transferases (MCR-1), has marginal effects on colistin resistance in *P. aeruginosa* (20, 21), implies that pharmacological inhibition of L-Ara4N biosynthetic enzymes could represent a suitable approach to extend the anti-*Pseudomonas* clinical lifetime of colistin.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.01820-17>.

SUPPLEMENTAL FILE 1, PDF file, 0.6 MB.

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REFERENCES

- 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>.
- Olaïtan AO, Morand S, Rolain JM. 2014. Mechanisms of polymyxin resistance: acquired and intrinsic resistance in bacteria. *Front Microbiol* 5:643. <https://doi.org/10.3389/fmicb.2014.00643>.
- Baron S, Hadjadj L, Rolain JM, Olaïtan AO. 2016. Molecular mechanisms of polymyxin resistance: knowns and unknowns. *Int J Antimicrob Agents* 48:583–591. <https://doi.org/10.1016/j.ijantimicag.2016.06.023>.
- 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>.
- 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>.
- Moskowitz SM, Brannon MK, Dasgupta N, Pier M, Sgambati N, Miller AK, Selgrade SE, Miller SJ, Denton M, Conway SP, Johansen HK, Høiby 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>.
- Macfarlane EL, Kwasnicka A, Ochs MM, Hancock RE. 1999. PhoP-PhoQ homologues in *Pseudomonas aeruginosa* regulate expression of the outer-membrane protein OprH and polymyxin B resistance. *Mol Microbiol* 34:305–316. <https://doi.org/10.1046/j.1365-2958.1999.01600.x>.
- 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>.
- Fernández L, Gooderham WJ, Bains M, McPhee JB, Wiegand I, Hancock RE. 2010. Adaptive resistance to the “last hope” antibiotics polymyxin B and colistin in *Pseudomonas aeruginosa* is mediated by the novel two-component regulatory system ParR-ParS. *Antimicrob Agents Chemother* 54:3372–3382. <https://doi.org/10.1128/AAC.00242-10>.
- Fernández L, Jenssen H, Bains M, Wiegand I, Gooderham WJ, Hancock RE. 2012. The two-component system CprRS senses cationic peptides and triggers adaptive resistance in *Pseudomonas aeruginosa* independently of ParRS. *Antimicrob Agents Chemother* 56:6212–6222. <https://doi.org/10.1128/AAC.01530-12>.
- Gutu AD, Sgambati N, Strasbourger P, Brannon MK, Jacobs MA, Haugen E, Kaul RK, Johansen HK, Høiby N, Moskowitz SM. 2013. Polymyxin resistance of *Pseudomonas aeruginosa phoQ* mutants is dependent on additional two-component regulatory systems. *Antimicrob Agents Chemother* 57:2204–2215. <https://doi.org/10.1128/AAC.02353-12>.
- Lee JY, Chung ES, Na IY, Kim H, Shin D, Ko KS. 2014. Development of colistin resistance in *pmrA*-, *phoP*-, *parR*- and *cprR*-inactivated mutants of *Pseudomonas aeruginosa*. *J Antimicrob Chemother* 69:2966–2971. <https://doi.org/10.1093/jac/dku238>.
- Wi YM, Choi JY, Lee JY, Kang CI, Chung DR, Peck KR, Song JH, Ko KS. 2017. Emergence of colistin resistance in *Pseudomonas aeruginosa* ST235 clone in South Korea. *Int J Antimicrob Agents* 49:767–769. <https://doi.org/10.1016/j.ijantimicag.2017.01.023>.
- Jochumsen N, Marvig RL, Damkjaer S, Jensen RL, Paulander W, Molin S, Jelsbak L, Folkesson A. 2016. The evolution of antimicrobial peptide resistance in *Pseudomonas aeruginosa* is shaped by strong epistatic interactions. *Nat Commun* 7:13002. <https://doi.org/10.1038/ncomms13002>.
- Fernández L, Alvarez-Ortega C, Wiegand I, Olivares J, Kocincová D, Lam JS, Martínez JL, Hancock RE. 2013. Characterization of the polymyxin B resistance of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 57:110–119. <https://doi.org/10.1128/AAC.01583-12>.
- Lee JY, Na IY, Park YK, Ko KS. 2014. Genomic variations between colistin-susceptible and -resistant *Pseudomonas aeruginosa* clinical isolates and their effects on colistin resistance. *J Antimicrob Chemother* 69:1248–1256. <https://doi.org/10.1093/jac/dkt531>.
- Pirnay JP, Bilocq F, Pot B, Cornelis P, Zizi M, Van Eldere J, Deschaght P, Vaneechoutte M, Jennes S, Pitt T, De Vos D. 2009. *Pseudomonas aeruginosa* population structure revisited. *PLoS One* 4:e7740. <https://doi.org/10.1371/journal.pone.0007740>.
- Chung ES, Lee JY, Rhee JY, Ko KS. 2017. Colistin resistance in *Pseudomonas aeruginosa* that is not linked to *arnB*. *J Med Microbiol* 66:833–841. <https://doi.org/10.1099/jmm.0.000456>.
- Ellington MJ, Ekelund O, Aarestrup FM, Canton R, Doumith M, Giske C, Grundman H, Hasman H, Holden MT, Hopkins KL, Iredell J, Kahlmeter G, Köser CU, MacGowan A, Mevius D, Mulvey M, Naas T, Peto T, Rolain JM, Samuelsen Ø, Woodford N. 2017. The role of whole genome sequencing in antimicrobial susceptibility testing of bacteria: report from the EUCAST subcommittee. *Clin Microbiol Infect* 23:2–22. <https://doi.org/10.1016/j.cmi.2016.11.012>.
- Nowicki EM, O'Brien JP, Brodbelt JS, Trent MS. 2015. Extracellular zinc induces phosphoethanolamine addition to *Pseudomonas aeruginosa* lipid A via the ColRS two-component system. *Mol Microbiol* 97:166–178. <https://doi.org/10.1111/mmi.13018>.
- Liu YY, Chandler CE, Leung LM, McElheny CL, Mettus RT, Shanks RMQ, Liu JH, Goodlett DR, Ernst RK, Doi Y. Structural modification of lipopolysaccharide conferred by *mcr-1* in Gram-negative ESKAPE pathogens. *Antimicrob Agents Chemother* 61: <https://doi.org/10.1128/AAC.00580-17>.