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MECHANISMS OF RESISTANCE

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

AQ: au

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ABSTRACT Lipid A aminoarabinosylation is invariably associated with colistin resistance in *Pseudomonas aeruginosa*; however, the existence of alternative aminoarabinosylation-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 aminoarabinosylation pathway is critical for the acquisition of colistin resistance in reference and clinical *P. aeruginosa* isolates. This highlights lipid A aminoarabinosylation 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 aminoarabinosylation 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 (μg/ml)
PAO1	0.5
PAO1 Δ*arnBCA*	0.5
PA14	0.5
PA14 Δ*arnBCA*	0.5
KK1	1
KK1 Δ*arnBCA*	0.5
KK27	1
KK27 Δ*arnBCA*	0.5
TR1	0.5
TR1 Δ*arnBCA*	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 μg/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 μg/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 μg/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 μg/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 μg/ml), and only 2 isolates showed a slight (2-fold) increase in colistin MIC (1 μg/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 μg/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 μg/ml, corresponding to 0.5× to 32× 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 μg/ml, while the wild-type PAO1 always acquired the ability to grow in the presence of 16 μg/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 μg/ml), while colistin MICs for PAO1 derivatives ranged between 32 and 128 μg/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

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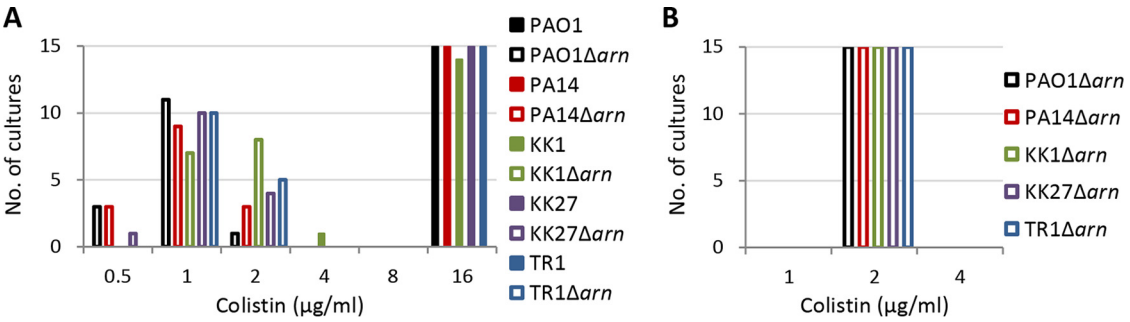

FIG 1 Maximum colistin resistance acquired by reference (PAO1 and PA14) or cystic fibrosis *P. aeruginosa* isolates (KK1, KK27, and TR1) and/or their corresponding Δ*arnBCA* mutants after sequential passages in Mueller-Hinton broth in the presence of increasing colistin concentrations (0.25 to 16 μg/ml) 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 μg/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 *arnBCA* 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 μg/ml colistin), none of the cultures were able to grow in the presence of >2 μg/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 aminoarabinosylation and the development of colistin resistance were observed in *arnB*-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, aminoarabinosylation 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 μg/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 aminoarabinosylation is a critical prerequisite for the acquisition of colistin resistance in this pathogen (5, 6, 9, 14). This evidence, together

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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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