Dissecting Colistin Resistance Mechanisms in Extensively Drug-Resistant Acinetobacter baumannii Clinical Isolates

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ABSTRACT Nosocomial infections with Acinetobacter baumannii are a global problem in intensive care units with high mortality rates. Increasing resistance to first- and second-line antibiotics has forced the use of colistin as last-resort treatment, and increasing development of colistin resistance in A. baumannii has been reported. We evaluated the transcriptional regulator PmrA as potential drug target to restore colistin efficacy in A. baumannii. Deletion of pmrA restored colistin susceptibility in 10 of the 12 extensively drug-resistant A. baumannii clinical isolates studied, indicating the importance of PmrA in the drug resistance phenotype. However, two strains remained highly resistant, indicating that PmrA-mediated overexpression of the phosphoethanolamine (PetN) transferase PmrC is not the exclusive colistin resistance mechanism in A. baumannii. A detailed genetic characterization revealed a new colistin resistance mechanism mediated by genetic integration of the insertion element ISAbal upstream of the PmrC homolog EptA (93% identity), leading to its overexpression. We found that eptA was ubiquitously present in clinical strains belonging to the international clone 2, and ISAbal integration upstream of eptA was required to mediate the colistin-resistant phenotype. In addition, we found a duplicated ISAbal-eptA cassette in one isolate, indicating that this colistin resistance determinant may be embedded in a mobile genetic element. Our data disprove PmrA as a drug target for adjuvant therapy but highlight the importance of PetN transferase-mediated colistin resistance in clinical strains. We suggest that direct targeting of the homologous PetN transferases PmrC/EptA may have the potential to overcome colistin resistance in A. baumannii.

IMPORTANCE The discovery of antibiotics revolutionized modern medicine and enabled us to cure previously deadly bacterial infections. However, a progressive increase in antibiotic resistance rates is a major and global threat for our health care system. Colistin represents one of our last-resort antibiotics that is still active against most Gram-negative bacterial pathogens, but increasing resistance is reported worldwide, in particular due to the plasmid-encoded protein MCR-1 present in pathogens such as Escherichia coli and Klebsiella pneumoniae. Here, we showed that colistin resistance in A. baumannii, a top-priority pathogen causing deadly nosocomial infections, is mediated through different avenues that result in increased activity of homologous phosphoethanolamine (PetN) transferases. Considering that MCR-1 is also a PetN transferase, our findings indicate that PetN transferases might be the Achilles heel of superbugs and that direct targeting of them may have the potential to preserve the activity of polymyxin antibiotics.

KEYWORDS Acinetobacter baumannii, antibiotic resistance, colistin, eptA, ethanolamine transferase, mcr-1, pmrA

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Antimicrobial resistance is a serious threat to global health systems, resulting in the loss of treatment options to fight a growing number of bacterial infections (1). Considering the paucity of newly developed antibiotics in the last decades, old antibiotics such as polymyxins have been increasingly used to treat infections caused by multidrug-resistant (MDR) Gram-negative pathogens (2–4). Nowadays, the polymyxin antibiotics polymyxin E (colistin) and polymyxin B represent the last resort for the treatment of serious Gram-negative infections, such as infections caused by carbapenem-resistant Enterobacteriaceae, MDR Pseudomonas aeruginosa, and MDR Acinetobacter baumannii (5, 6). Unfortunately, the increasing use of polymyxins to treat serious infections caused by these pathogens leads to a spread of resistance to these last-line drugs (7). There is a high unmet medical need for new drugs effective against Gram-negative bacteria to treat infections caused by these pathogens (8). Besides this, an alternative strategy resides in the recovery of colistin efficacy by blocking bacterial colistin resistance mechanisms. Antibiotic adjuvant therapies consist in the combination of a potent antibiotic with a nonantibiotic agent interfering with specific antibiotic resistance or virulence mechanisms. This strategy may provide a new tool to fight infections caused by drug-resistant pathogens by restoring or boosting the efficacy of an approved antibiotic (9).

Colistin resistance is conferred by lipopolysaccharide (LPS) modifications at the outer cell envelope. Reduction of the negative charge on LPS results in a reduced affinity of colistin to LPS (10). The two main LPS modifications conferring colistin resistance are the addition of 4-amino-4-deoxy-L-arabinose (AraN) and phosphoethanolamine (PetN) to the lipid A (11). The expression of LPS-modifying enzymes is regulated by the concerted action of several two-component systems (TCSs). In Enterobacteriaceae, PhoPQ and PmrAB TCSs regulate the expression of colistin resistance mechanisms, whereas in P. aeruginosa the PhoPQ, PmrAB, ParRS, ColRS, and CprRS TCSs seem to be involved (11). Plasmid-mediated colistin resistance has been recently reported in Enterobacteriaceae due to the PetN transferase MCR-1. The presence of MCR-1 on a plasmid leads to its rapid geographical and interspecies spread (12, 13). Nevertheless, mcr-1 seems to be restricted to Enterobacteriaceae species and has never been detected in A. baumannii. In A. baumannii, colistin resistance is mediated by PetN addition to the lipid A, and this resistance mechanism is regulated by the PmrAB TCS. In contrast to other pathogens, the AraN lipid A modification pathway is not present in A. baumannii (11), rendering A. baumannii a suitable pathogen to develop an adjuvant therapy approach to rejuvenate colistin efficacy by blocking the PmrAB TCS.

Colistin resistance in A. baumannii clinical isolates is associated with alterations in the pmrCAB operon. The pmrC gene codes for a PetN transferase, and pmrA and pmrB code for the TCS (14). It has been shown that mutations in the PmrAB TCS induce the overexpression of pmrC, leading to the modification of lipid A with PetN and colistin resistance (14–18). Because PmrA is the transcriptional regulator that triggers PmrC overexpression, inhibition of PmrA with a small molecule may potentially block PmrC overexpression and therefore switch off colistin resistance in A. baumannii (19). This study was designed to evaluate the clinical relevance of PmrA as a drug target to restore colistin efficacy in A. baumannii. We demonstrate that in the absence of PmrA-mediated expression of PmcC, transposition of an insertion sequence (IS) element leads to overexpression of the alternative highly similar PetN transferase EptA, which also confers colistin resistance in A. baumannii clinical isolates. Our results show that in all studied clinical isolates, overexpression of at least one PetN transferase (PmcC or various EptA variants) was responsible for colistin resistance, indicating that PetN transferases may be a suitable drug target to overcome colistin resistance in A. baumannii.

RESULTS

PmrA is not essential for colistin resistance in A. baumannii clinical isolates. We deleted pmrA from the genome of a panel of 12 colistin-resistant A. baumannii strains to evaluate the transcriptional regulator PmrA as a potential drug target to rejuvenate
Colistin efficacy in *A. baumannii*. The strains in the panel consisted of recently isolated colistin-resistant clinical strains collected from diverse geographical origins. They belong to three distinct and highly successful clonal lineages, the international clone 1 (ST1), international clone 2 (ST2), and ST25 clonal lineages (Table 1)(20–22). All strains were classified as extensively drug resistant according to the criteria of Magiorakos et al. (23). These data underscore the clinical relevance and the diversity of the strain panel. The colistin-susceptible *A. baumannii* ATCC 17978 strain was included as a reference strain. In all strains, *pmrA* was deleted by applying a previously described method that allows efficient scarless genome engineering even in extensively resistant *A. baumannii* clinical isolates (24).

The colistin sensitivity of the parental clinical isolates and their corresponding Δ*pmrA* knockout mutants (Δ*pmrA*) was determined by broth microdilution method. *pmrA* deletion reduced MICs 64- to 1,024-fold in 10 out of 12 initially colistin-resistant clinical isolates (83%), thus restoring susceptibility to colistin (MIC, ≤2 μg/ml) (Table 2). To our surprise, however, two strains (BV94 and BV189) retained colistin resistance even in the absence of *pmrA*.

We investigated the differences between strains that became susceptible after *pmrA* deletion and those that remained resistant by analyzing the sequence variations of the PmrAB TCS in the strain panel. The PmrA and PmrB sequences of the deletion and those that remained resistant by analyzing the sequence variations of the

| Strain designation | MIC (μg/ml) of drug: | PmrB mutations (amino acid substitutions) |
|--------------------|----------------------|-------------------------------------------|
|                    | Wild type            | Δ*pmrA*                                   |                                      |
| ATCC 17978         | 0.25                 | 0.25                                      | Reference                             |
| BV94               | 64                   | 32                                        | Wild type                             |
| BV95               | 32                   | 0.5                                       | L274W                                 |
| BV172              | 256                  | 1                                         | Q43L and L267F                        |
| BV173              | 128                  | 1                                         | A138T and A226V                       |
| BV174              | 64                   | 1                                         | Q277R                                 |
| BV175              | 256                  | 0.5                                       | L267W                                 |
| BV185              | 256                  | 0.25                                      | P233S                                 |
| BV186              | 16                   | 0.25                                      | Q277R                                 |
| BV187              | 16                   | 0.25                                      | Q277R                                 |
| BV189              | 64                   | 64                                        | Wild type                             |
| BV190              | 256                  | 0.5                                       | A138T and A226V                       |
| BV191              | 256                  | 0.25                                      | A138T and P233S                       |

*Susceptibility breakpoint, ≤2 μg/ml. Susceptible, italics; resistant, bold.*
lineages, respectively. Nonsynonymous mutations were found only in the PmrB sensor kinase (Table 2). Interestingly, the two strains with an unaltered PmrB sequence were those that remained colistin resistant after pmrA deletion (BV94 and BV189). Our data suggest that colistin resistance in these two strains is not conferred by PmrA-mediated PmrC overexpression. We confirmed this hypothesis by quantifying the expression of pmrC using quantitative reverse transcription-PCR (qRT-PCR) (Fig. 1). The control strain ATCC 17978 and the two refractory strains BV94 and BV189 showed only marginal levels of pmrC expression. In contrast, the 10 other strains showed pmrC overexpression, and this overexpression was abolished in the ΔpmrA mutant.

Taken together, colistin resistance in A. baumannii is predominantly conferred by mutations in the PmrB TCS sensor kinase that lead to overexpression of PmrC, as shown in 10 out of 12 clinical strains. However, some isolates (2 out of 12 strains in our panel) may use an alternative colistin resistance mechanism independent of PmrA-mediated PmrC overexpression to resist the antibacterial activity of colistin.

EptA, a PmrC homolog, is present in the A. baumannii strains of international clone 2. Lesho et al. described the presence of the alternative PetN transferase EptA in A. baumannii (17). EptA and PmrC are homologous proteins with 93% amino acid identity, suggesting similar enzymatic activities. However, the role of EptA in A. baumannii colistin resistance is still unclear (17). To investigate the prevalence of eptA in A. baumannii, we took advantage of sequence differences between pmrC and eptA at the N- and C-terminal ends of the open reading frames and designed oligonucleotides (oVT152/oVT153) that can discriminate eptA from pmrC (Fig. 2A; see also Table S1 in the supplemental material). Using these eptA-specific primers, we detected eptA in all our international clone 2 strains but not in international clone 1 strains (Table 3 and Fig. S1). This finding was further confirmed by screening 12 additional isolates from the BioVersys strain collection (data not shown).

The integrated insertion element ISAbal causes eptA overexpression in BV94 and BV189. Two isoforms of eptA, eptA-1 and eptA-2 (GenBank accession numbers KC700024 and KC700023, respectively) have been described at different locations in the genome of various A. baumannii strains (17). Taking advantage of the different flanking regions, we designed primers able to discriminate eptA-1 from eptA-2 (oVT198/oVT199 and oVT201/oVT202, respectively) (Fig. 2A and Table S1). By genotyping the strain panel, we demonstrated that all strains that belong to the international clone 2 contained eptA-1 and four of them contained an additional copy of eptA-2 (Table 3 and Fig. S1). Interestingly, the PCR products obtained for eptA-2 in BV94 and eptA-1 in BV189 were approximately 1 kb larger than the expected fragment size. Sequencing of the PCR products identified the insertion element ISAbal upstream of eptA-2 and eptA-1 in BV94 and BV189, respectively. The ISAbal orientation enabled its strong promoter (Pout)
to drive eptA overexpression as previously described for other antibiotic resistance determinants (Fig. 3) (25, 26).

Using probes specific for eptA or pmrC, we quantified their respective expression levels in our strains. eptA was 550- and 25-fold higher expressed in BV94 and BV189, respectively, than the homologous isoform pmrC in the control strain ATCC 17978 (which does not contain eptA) (Fig. 2B). This eptA overexpression was not altered in the ΔpmrA mutant strains, suggesting that eptA expression in both strains is independent of the PmrAB TCS. These data suggest that an ISAbal-driven eptA overexpression may represent an alternative and PmrAB-independent colistin resistance mechanism in A. baumannii clinical strains.

**ISAbal-driven eptA overexpression confers colistin resistance in A. baumannii clinical isolates.** To validate the hypothesis that ISAbal-driven eptA overexpression confers colistin resistance in A. baumannii clinical isolates, we deleted eptA-1 in the clinical isolates BV189 and BV94 and determined MIC values. Indeed, BV189 (which carries ISAbal upstream of eptA-1) became colistin susceptible upon eptA-1 deletion,

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**TABLE 3** Distribution of the eptA variants in the strain panel

| Strain designation | MLST | eptA variant(s)                |
|--------------------|------|--------------------------------|
| ATCC 17978         | 77   | eptA-1, ISAbal-eptA-2, ISAbal-eptA-3 |
| BV94               | 2    | eptA-1                         |
| BV95               | 25   |                                |
| BV172              | 2    | eptA-1                         |
| BV173              | 2    | eptA-1                         |
| BV174              | 2    | eptA-1, eptA-2                 |
| BV175              | 2    | eptA-1                         |
| BV185              | 2    | eptA-1                         |
| BV186              | 2    | eptA-1, eptA-2                 |
| BV187              | 2    | eptA-1, eptA-2                 |
| BV189              | 2    | ISAbal-eptA-1                 |
| BV190              | 1    |                                |
| BV191              | 2    | eptA-1                         |
indicating an essential role of IS\textit{AbaI}\textsubscript{-}\textit{eptA-1} in conferring colistin resistance in this strain (Table 4). In contrast, BV94, carrying both \textit{eptA-1} and \textit{eptA-2} isoforms but carrying an IS\textit{AbaI} insertion only upstream of \textit{eptA-2}, remained colistin resistant after deletion of \textit{eptA-1}, suggesting the key role of IS\textit{AbaI} insertion for colistin resistance in \textit{A. baumannii}. To further confirm the importance of IS\textit{AbaI}, we constructed the double mutant BV94\textsubscript{Δ}eptA-1/ΔeptA-2 and evaluated its colistin susceptibility. A 4-fold MIC reduction was observed in the BV94\textsubscript{Δ}eptA-1/ΔeptA-2 mutant compared to BV94 and BV94\textsubscript{Δ}eptA-1, indicating that \textit{eptA-2} with an upstream IS\textit{AbaI} is involved in the colistin resistance mechanism of BV94. However, we were surprised to see that BV94\textsubscript{Δ}eptA-1/ΔeptA-2 remained resistant to colistin with a MIC of 16 µg/ml, indicating that there must be yet another colistin resistance mechanism present in this isolate.

**Three different \textit{eptA} variants can confer colistin resistance in \textit{A. baumannii}.** We genotyped the BV94\textsubscript{Δ}eptA-1/ΔeptA-2 double mutant and confirmed the successful deletion of \textit{eptA-1} and \textit{eptA-2}. However, we detected the presence of at least one additional \textit{eptA} variant.

**TABLE 4 Recovery of colistin susceptibility after deletion of different \textit{eptA} isoforms**

| Strain | Colistin MIC (µg/ml)* |
|--------|-----------------------|
|        | Wild type | Δ\textit{eptA-1} | Δ\textit{eptA-1}/Δ\textit{eptA-2} | Δ\textit{eptA-1}/Δ\textit{eptA-2}/Δ\textit{eptA-3} |
| BV189  | 128        | 0.5              |                           |                                                |
| BV94   | 64         | 64               | 16                        |                                                |

*Susceptibility breakpoint, 2 µg/ml. Susceptible, italics; resistant, bold.
copy (eptA-3) in the double mutant (Fig. S2). We performed a fusion primer and nested integrated PCR experiment (FPNI-PCR) to amplify the genomic flanking regions of the additional eptA-3 variant (27). Sequencing revealed the ISAbal insertion element and the gene ABK1_2603 present upstream and downstream of eptA-3, respectively (Fig. 3). This eptA-3 gene context in BV94 was identical to the eptA-2 gene context present in the A. baumannii strain 1656-2 (GenBank accession number NC_017162). However, further upstream there were marked differences. ISAbal-eptA-3 in BV94 was adjacent to the gene ABK1_3144, while ISAbal-eptA-2 in 1656-2 was adjacent to a different gene (Fig. 3). We could not determine the downstream flanking region of ISAbal-eptA3 ABK1_2603 in multiple attempts. Nevertheless, the 9-bp target site duplications (TSD) created by ISAbal transposition could not be identified directly outside the ISAbal upstream eptA-3 (25). In contrast, TSD were present next to the right and left inverted repeats of the ISAbal upstream eptA-1 and eptA-2, which is consistent with a single transposition event. These observations indicate that ISAbal did not insert upstream eptA-3 in a single transposition event, and therefore, ISAbal-eptA-3 in BV94 might be a result of an ISAbal-eptA-2 cassette gene duplication, implying that the ISAbal-eptA colistin resistance determinant is contained in a mobile genetic element.

We finally deleted the DNA fragment between ABK1_3144 and ABK1_3143 containing eptA-3 to confirm that ISAbal-eptA-3 was responsible for the high residual colistin resistance in BV94ΔeptA-1/ΔeptA-2. In addition, we performed PCR-based eptA genotyping on the resulting triple mutant BV94ΔeptA-1/ΔeptA-2/ΔnaxD to exclude the presence of yet another eptA copy (Fig. S2). The loss of all 3 eptA isoforms in BV94ΔeptA-1/ΔeptA-2/ΔnaxD rendered this triple mutant susceptible to colistin, indicating that colistin resistance in BV94 was entirely conferred by the overexpression of EptA-isoforms (Table 4).

**Targeting PetN transferases may overcome colistin resistance in A. baumannii.** We have shown that colistin resistance was mediated in 10 out of 12 analyzed clinical strains by PmrA-mediated overexpression of PmrC. In the remaining two strains, ISAbal-driven EptA expression conferred colistin resistance. Taken together, in all tested clinical isolates colistin resistance was mediated by the overexpression of PetN transferases, suggesting that inhibition of these homologous enzymes with small molecules may have the potential to overcome colistin resistance in A. baumannii. Chin and colleagues recently suggested that the acetyl-galactosamine (GalNAc) deacetylase NaxD plays a role in colistin resistance in A. baumannii (28). In this report, the expression of NaxD, which was regulated by the PmrAB TCS, mediated galactosamine (GalN) addition to lipida, conferring colistin resistance in A. baumannii. We performed additional experiments to exclude the possibility that the colistin resensitization observed in our clinical isolates after deletion of pmrA was based on a modulation of naxD expression and not pmrC expression. We first confirmed the PmrAB-controlled naxD expression based on qRT-PCR data for BV191 and BV191ΔpmrA. BV191 has a mutated PmrB that likely triggers PmrA-mediated pmrC overexpression (Table 2). Similarly, naxD expression was 15-fold higher in the colistin-resistant strain BV191 than the susceptible strain ATCC 17978 (Fig. 4). In BV191ΔpmrA, lacking the response regulator PmrA, pmrC and naxD overexpression was abolished, confirming that both genes were regulated by the PmrAB TCS. Notably, pmrC overexpression was 20-fold higher than naxD overexpression, suggesting a minor contribution of NaxD compared to PmrC in colistin resistance. To confirm the major role of PmrC in PmrA-mediated colistin resistance and to exclude that another PmrA-regulated gene, such as naxD, is involved in colistin resistance, we directly deleted the effector pmrC from the genome of BV191. The loss of PmrC rendered BV191 susceptible to colistin (MIC of 0.5 μg/ml) and resulted in a similar phenotype as in BV191ΔpmrA (Table 2). In contrast, naxD was still 15-fold overexpressed in BV191ΔpmrC (Fig. 4). This result suggests that overexpression of naxD is not sufficient to confer colistin resistance in BV191 and indicates that PmrC is the main effector of PmrA-mediated colistin-resistant A. baumannii strains, such as BV191.
DISCUSSION

Bacteria have evolved multiple ways to escape the hazardous action of antibiotics. In nosocomial infections, the individual strain history of antibiotic exposures during patient treatment may result in the development and accumulation of different resistance mechanisms in different strains of the same species. Therefore, it is important to study resistance mechanisms on multiple strains. Moreover, it is crucial to study these mechanisms on strains that developed resistance during patient treatment due to the discrepancy that may be observed between in vitro- and in vivo-developed mechanisms. For instance, A. baumannii polymyxin resistance is commonly mediated by LPS loss when A. baumannii is exposed to the drug in vitro, but this mechanism is not viable in vivo due to the strong fitness cost that it engenders (16, 29).

In this study, we dissected the mechanisms conferring colistin resistance in 12 clinically relevant A. baumannii strains. To our knowledge, this is the first time that colistin resistance is genetically characterized in a panel of A. baumannii clinical strains that developed resistance during patient treatment and not strains that artificially acquired resistance by in vitro selection/passaging. This gap in knowledge originates from the difficulties in manipulating the genome of A. baumannii colistin-resistant clinical strains. Indeed, as exemplified in our strain panel, such strains are generally resistant to all other antibiotics because colistin is used as a last option in the treatment of A. baumannii infections, only when other antibiotics fail. To break the barrier of antibiotic resistance in these strains, we applied a genome editing method based on a nonantibiotic resistance marker, which is efficient regardless of the resistance profile of the strain (24).

We demonstrated two different ways to overexpress PetN transferases that cause colistin resistance in A. baumannii clinical isolates (Fig. 5). The predominant colistin resistance mechanism found in 83% of the studied clinical isolates was mediated by pmrC overexpression. The overexpression of pmrC in these strains was entirely caused by mutations in the sensor kinase PmrB, although previous studies also found mutations in the response regulator PmrA (14, 15, 17). We found 7 different PmrB variants among the 10 PmrC-mediated colistin-resistant strains, indicating the diversity of mutations that lead to PmrC overexpression. Except for the A226V and P233S mutations, the identified PmrB mutations were not yet reported in A. baumannii (11, 15, 16).

Interestingly, we found two clinical isolates in which colistin resistance was conferred by a genomic insertion of ISAba1, resulting in a strong overexpression of the pmrC homolog eptA. eptA-1 and eptA-2 genes have been previously identified in A. baumannii; however, their distribution, expression regulation, and role in colistin resistance were not assessed (17). Our study revealed that A. baumannii strains of the international clone 2, which represent the most problematic strains in hospitals, carry at least one
eptA variant. In contrast, international clone 1 strains did not carry eptA. Our data further show that eptA expression is not regulated by the PmrAB TCS, but instead, integration of IS\textit{AbaI} upstream of any eptA isoform is required to confer the resistance phenotype, presumably by IS\textit{AbaI}-driven eptA overexpression. Consequently, detection of an eptA gene alone is not sufficient to classify \textit{A. baumannii} strains as colistin resistant.

The analysis of PmrA as a potential drug target confirmed the importance of this protein in mediating colistin resistance in \textit{A. baumannii}. However, the high prevalence of eptA and the ability of IS\textit{AbaI} to integrate upstream of eptA and drive its expression independently of the PmrAB TCS disproved PmrA as a direct drug target for resensitization of \textit{A. baumannii} to colistin (Fig. 5). An adjuvant therapy consisting of a PmrA inhibitor in combination with colistin would most likely select for IS\textit{AbaI}-driven EptA-overexpressing colistin-resistant strains. As demonstrated by the two clinical isolates BV94 and BV189, such strains are already present in hospitals. One of the strains also contained a duplicated IS\textit{AbaI}-eptA cassette, suggesting that this functional cassette mediating colistin resistance was present on a mobile element. The presence of a mobile colistin-resistance-mediating cassette increases the probability of intra- and interspecies transfer of the resistance pathways by the integration into plasmids. This phenomenon was recently illustrated with plasmid-carried PetN transferase \textit{mcr-1}, which was initially found in China but rapidly has spread globally and in different species (12, 13). Nevertheless, \textit{mcr-1} seems to be limited to \textit{Enterobacteriaceae} species and has never yet been detected in \textit{A. baumannii}.

One of the major colistin resistance pathways in \textit{Enterobacteriaceae} and \textit{P. aeruginosa} is the addition of AraN to lipid A (11). Although we describe here two different ways to overexpress PetN transferases, our results suggest that colistin resistance in clinical \textit{A. baumannii} isolates is exclusively conferred by PetN addition to lipid A. A recent study suggested that a GalN-based modification of lipid A may be involved in colistin resistance in \textit{A. baumannii} (28). In contrast, our results suggest that alteration of the lipid A structure by addition of PetN plays the major role in colistin resistance in \textit{A. baumannii}. It has also been described that loss of LPS may confer colistin resistance in
A. baumannii (30). However, most of the LPS-deficient colistin-resistant mutants were obtained in vitro after colistin evolution, and it has been shown that these mutants are hypersusceptible to other antibiotic classes and are avirulent (16, 29). Emergence of LPS-deficient colistin-resistant mutants in patients is therefore unlikely.

In conclusion, the overexpression of homologous PetN transferases caused colistin resistance in all studied clinical isolates, but in some cases this occurred independently of PmrAB. The crystal structure of Neisseria meningitidis PetN transferase has been recently reported, and this enzyme has been proposed as a drug target for antivirulence and antiresistance drug development to treat Neisseria gonorrhoeae and N. meningitidis infections (31, 32). Our data suggest that a direct inhibitor of homologous PetN transferases PmrC and EptA may have the potential to overcome colistin resistance in A. baumannii clinical strains (Fig. 5).

MATERIALS AND METHODS

**Bacterial strains, MIC, MLST, and oligonucleotides.** The A. baumannii reference strain ATCC 17978 and 12 extensively drug-resistant A. baumannii clinical isolates from the BioVersys proprietary strain collection were used in this study. The microdilution method was used to determine MICs according to the CLSI guidelines (33). Multiple locus sequence type (MLST) was determined according to the Pasteur scheme using specific primers (source: http://pubmlst.org/abaumannii/) (20). Oligonucleotides used in this study are listed in Table S1 in the supplemental material.

**Genomic deletions of pmrA, eptA-1, eptA-2, and pmrC in A. baumannii clinical isolates.** Scarless deletions of pmrA, pmrC, and the eptA isoforms were performed using a two-step recombination method previously described (24).

DNA fragments corresponding to 700-bp up- and downstream genomic regions of the genes to be deleted were amplified by PCR and cloned in the multiple cloning site of the knockout platform pVT77. Oligonucleotides oVT49/oVT50 and oVT51/oVT52 were used to amplify the up- and downstream regions, respectively, of pmrA. The resulting DNA fragments were ligated and introduced into pVT77 previously digested by EcoRI and BamHI. Similarly, oligonucleotides oVT235/oVT236 and oVT237/oVT238 were used to amplify the flanking regions of eptA-1, and oligonucleotides oVT305/oVT306 and oVT307/oVT242 were used to amplify the flanking regions of eptA-2. The resulting DNA fragments for eptA-1 and eptA-2 were introduced into pVT77 previously digested by Xhol and XbaI using NEBuilder HiFi DNA assembly (New England Biolabs). For eptA-3 deletion, the genomic regions flanking the duplicated cassette were amplified using oVT390/oVT391 and oVT392/oVT393. The resulting DNA fragments were cloned into pVT77 previously digested with EcoRI and XbaI using NEBuilder HiFi DNA assembly. Last, the flanking regions of pmrC were amplified using oVT324/oVT325 and oVT326/oVT327, and the resulting DNA fragments were cloned into pVT77 previously digested with KpnI and PstI using NEBuilder HiFi DNA assembly.

The cloned knockout plasmids were transformed in E. coli conjugative strain MFDPir to proceed with the construction of markerless deletion in A. baumannii, as previously described (24). Briefly, after conjugation, genomic plasmid integration was selected on LB agar plates containing 100 μg/ml sodium tellurite. Clones were screened for up- or downstream integration by PCR using primer oVT8, which anneals on the plasmid, and oVT91, oVT243, oVT311, or oVT326, which anneals upstream of pmrA, eptA-1, eptA-2, or pmrC, respectively. For eptA-3, clones were screened using primers oVT8/oVT396 and oVT174/oVT397 for upstream and downstream integration, respectively. Clones containing up- and downstream plasmid integrations were transferred on LB agar plates containing 100 μg/ml 3’-deoxythymidine to select for plasmid removal from the genome. Clones were screened for gene deletion and plasmid removal by PCR using primers oVT91/oVT92, oVT243/oVT244, oVT246/oVT311, oVT396/oVT397, and oVT328/oVT14 for pmrA, eptA-1, eptA-2, eptA-3, and pmrC, respectively. The genomic gene deletions were finally confirmed by DNA sequencing (Microsynth AG, Balgach, Switzerland).

**Genotyping of pmrA, pmrB, and eptA.** A genomic DNA sequence including pmrA and pmrB was PCR amplified from all the strains of the panel using oVT91 and oCX292, and the PCR products were sent for sequencing (Microsynth AG, Balgach, Switzerland). The genotyping of eptA isoforms was performed by PCR using eptA-specific primers oVT152 and oVT153, which anneal on all eptA isoforms but not on pmrC (Fig. 2A). PCR using primers oVT198/oVT199 and oVT201/oVT202, which anneal on the flanking sides of eptA-1 and eptA-2, respectively, were used to discriminate between eptA isoforms (Fig. 2A).

**qRT-PCR.** Quantitative reverse transcription-PCR was performed as previously described (24). The specific expression of the PetN transferases encoded by pmrC and eptA was evaluated using oVT162/oVT163 and oVT164/oVT165 primers, respectively (Fig. 2A). The expression of naxD was evaluated using oVT314/oVT315 primers. Expression levels were normalized to that of the housekeeping gene rpoD using the comparative threshold cycle (ΔΔCT) method. The expression of rpoD was evaluated using rpoD-qRT-PCR using primers specific for rpoD.

**FPNII-PCR.** Fusion primer and nested integrated PCR was performed as previously described (27). This method relies on a three-step PCR using arbitrary degenerated oligonucleotides fused to known adaptors and three sequence-specific oligonucleotides, which consist in our case of eptA-specific oligonucleotides. FPNI-PCR experiments were performed on the BV94ΔeptA-1/ΔeptA-2 mutant with two sets of three eptA-specific oligonucleotides, oligonucleotides oVT343, oVT344, and oVT345 and oligonucleotide oVT346.
otides oVT340, oVT341, and oVT342, to identify the sequence up- and downstream of the new eptA copy, respectively. The degenerated primers and the known adaptor primers were directly taken from the previously described method (27). Briefly, the first round of PCRs was performed using the degenerated primers and oVT343 for upstream identification and oVT340 for downstream identification. The second round of PCRs was performed with the first adaptor primer FSP1 and oVT344 for upstream identification and oVT341 for downstream identification. The last round of PCRs was performed with the second adaptor primer FSP2 and oVT345 for upstream identification and oVT342 for downstream identification. The brightest and most distinct PCR products obtained were sent for sequencing (Microsynth AG, Balgach, Switzerland).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/mBio.01083-19.

FIG S1, PDF file, 0.3 MB.
FIG S2, PDF file, 0.3 MB.
TABLE S1, PDF file, 0.2 MB.

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