Investigation of Novel *pmrB* and *eptA* Mutations in Isogenic *Acinetobacter baumannii* Isolates Associated with Colistin Resistance and Increased Virulence *In Vivo*

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ABSTRACT Colistin resistance in *Acinetobacter baumannii* is of great concern and is a threat to human health. In this study, we investigate the mechanisms of colistin resistance in four isogenic pairs of *A. baumannii* isolates displaying an increase in colistin MICs. A mutation in *pmrB* was detected in each colistin-resistant isolate, three of which were novel (A28V, I232T, and ΔL9-G12). Increased expression of *pmrC* was shown by semi-quantitative reverse transcription-PCR (qRT-PCR) for three colistin-resistant isolates, and the addition of phosphoethanolamine (PEtN) to lipid A by PmrC was revealed by mass spectrometry. Interestingly, PEtN addition was also observed in some colistin-susceptible isolates, indicating that this resistance mechanism might be strain specific and that other factors could contribute to colistin resistance. Furthermore, the introduction of *pmrAB* carrying the short amino acid deletion ΔL9-G12 into a *pmrAB* knockout strain resulted in increased *pmrC* expression and lipid A modification, but colistin MICs remained unchanged, further supporting the strain specificity of this colistin resistance mechanism. Of note, a mutation in the *pmrC* homologue *eptA* and a point mutation in *ISAbal* upstream of *eptA* were associated with colistin resistance and increased *eptA* expression, which is a hitherto undescribed resistance mechanism. Moreover, no cost of fitness was observed for colistin-resistant isolates, while the virulence of these isolates was increased in a *Galleria mellonella* infection model. Although the mutations in *pmrB* were associated with colistin resistance, PEtN addition appears not to be the sole factor leading to colistin resistance, indicating that the mechanism of colistin resistance is far more complex than previously suspected and is potentially strain specific.

KEYWORDS *Acinetobacter*, antibiotic resistance, genome analysis, lipid A, virulence

Acinetobacter baumannii is of major concern in the hospital setting and causes a wide range of nosocomial infections, e.g., wound and urinary tract infections, pneumonia, and bloodstream infections (1). The ability of the bacterium to acquire or

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upregulate antimicrobial resistance determinants and its propensity for epidemic spread have led to a high frequency of multidrug-resistant (MDR) *A. baumannii* strains worldwide (2, 3). The World Health Organization has classified carbapenem-resistant *A. baumannii* as “priority one” on the global priority list of antibiotic-resistant bacteria for research and development of new antibiotics (4).

Colistin (polymyxin E) was first isolated from *Paenibacillus polymyxa* (formerly *Bacillus polymyxa* var. *colistinus*) in 1949 (5). Due to its nephrotoxicity, the clinical use of colistin was discontinued in the 1980s. Since effective antimicrobial agents are often lacking in the treatment of MDR pathogens like *A. baumannii*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*, colistin was reintroduced in the 1990s (6). However, colistin resistance is increasingly reported in *A. baumannii* isolates, and pandrug-resistant *A. baumannii* isolates have emerged (7–9).

Colistin is a cationic polypeptide antibiotic, and its primary target is the outer bacterial cell membrane. The molecule interacts with the negatively charged lipopolysaccharide (LPS) molecules of the cell membrane by displacement of positively charged ions (Mg^{2+} and Ca^{2+}), which destroys the integrity of the cell membrane, subsequently leading to cell death (6). The modification of the lipid A fraction of LPS in the bacterial cell membrane is the main colistin resistance mechanism. The modifications include the addition of 4-amino-4-deoxy-L-arabinose (LARA4N), phosphoethanolamine (PEtN), or galactosamine to lipid A (10–12). These modifications lower the net negative charge of the cell membrane, thereby affecting the binding of colistin and preventing the disruption of the cell membrane (6). *A. baumannii* is lacking the biosynthesis genes for LARA4N, and therefore it uses PEtN addition as the main colistin resistance mechanism, which is mediated by the chromosomally encoded *pmrCAB* operon. The PEtN transferase PmrC facilitates the addition of the compound to lipid A and is strictly regulated by the two-component system PmrAB (11, 13). Increased expression of *pmrC* is associated with a decrease in colistin susceptibility and is often due to mutations in the *pmrAB* regulators (13–14). Two other colistin resistance mechanisms have been described in *A. baumannii*. The first mechanism involves the complete loss of LPS due to mutations in or disruption of the LPS biosynthesis genes. Amino acid substitutions, frameshifts, or truncation of *lpxA*, *lpxC*, and *lpxD* have been described to lead to a complete loss of LPS and subsequently to colistin resistance in laboratory-derived and clinical *A. baumannii* isolates (15). The second, more recently reported mechanism is due to the disruption of the global regulator H-NS by insertion sequence *ISAbi125* (16). H-NS is predicted to negatively regulate the expression of the *pmrC* homologue *eptA*, which in turn modifies lipid A by PEtN addition (16). Moreover, a plasmid-mediated colistin resistance determinant (*mcr-1*) has been reported worldwide in many Gram-negative pathogens (e.g., *Escherichia coli*) (17), but so far it has not been detected in *A. baumannii*.

In this study, we investigate the mechanisms of colistin resistance in four isogenic pairs of *A. baumannii* isolates that were obtained from patients hospitalized in Germany, Greece, and Spain. We analyzed whole-genome sequencing (WGS) data to identify mutations in *pmrB* and *eptA* in isolates with a high colistin MIC. Moreover, we investigated the virulence of colistin-resistant isolates in a *Galleria mellonella* infection model.

RESULTS

Description of isolates. To investigate the mechanisms of colistin resistance, we selected four pairs of *A. baumannii* isolates obtained during the patients' hospital course (Table 1). The isolates were collected from patients hospitalized in Germany, Greece, and Spain. With the exception of isolate pair 2, all isolates were recovered while the patients were receiving colistin therapy. The isogenic background of all isolates representing an isolate pair was confirmed by WGS (allele differences, <10). The isolates exhibited an increase in colistin MIC by at least four log₂ dilutions as determined by broth microdilution. The isolates from Spain and Greece all belonged to the international clonal lineage IC2, while isolate pair 4 belonged to IC4. Isolates with

TABLE 1 Overview of isogenic *A. baumannii* isolate pairs and amino acid substitutions in PmrB and EptA

Isolate pair no.	Isolate	Patient identifier	Date of isolation (day of (day-mo-yr) isolation ^a)	Antibiotic treatment ^e	Sample type ^f	Origin	Clonal lineage ^c	Colistin MIC (mg/liter) ^b	Amino acid substitution in ^d :	
									PmrB	EptA
1	MB_2 MB_6C	01-001	13-05-2012 (3) 24-05-2012 (F)	Col	R S	Seville, Spain	IC2	2 256	ΔL9-G12	
2	MB_23C MB_177	01-016 01-041	25-10-2012 (3) 04-02-2013 (3)	Mem	S S	Seville, Spain	IC2	2 256	I232T	R127L
3	MB_90 MB_119	21-107 21-140	18-11-2013 (F) 06-02-2014 (3)	Col	R R	Athens, Greece	IC2	32 >256	A28V	
4	SG3161 SG3166	KBT6804357	03-2016 (nd) 03-2016 (nd)	Col	W R	Frankfurt, Germany	IC4	1 256	S17R	

^aHospital day after enrollment into the clinical trial. F, sample recovered at the end of the patient's antibiotic treatment; nd, not determined.

^bThe colistin MIC was determined by broth microdilution.

^cClonal lineages were determined by cgMLST.

^dAmino acid substitutions were detected by comparison of the amino acid sequences within isolates of an isolate pair.

^eCol, colistin; Mem, meropenem.

^fR, respiratory tract; S, stool; W, wound.

low colistin MICs preceded the isolates with high colistin MICs within an isolate pair (Table 1). Isolate pair 1 and 2 were recovered from patients hospitalized in Seville, Spain, and displayed an increase in colistin MICs from 2 to 256 mg/liter. In isolate pair 1, isolates were obtained from one patient, and in isolate pair 2, both isolates were recovered from different patients at day 3 of therapy. Both isolates from isolate pair 3, recovered from different patients hospitalized in Athens, Greece, were considered colistin resistant. Nevertheless, the colistin MICs differed by at least four log₂ dilutions (32 versus ≥256 mg/liter). Isolate pair 4 was recovered from a patient hospitalized in Frankfurt, Germany, and it exhibited a colistin MIC increase from 1 to 256 mg/liter. The patient was infected with both isolates at the same time when admitted to the hospital. All MIC differences were seen consistently in three independent experiments.

Resistome analysis. In addition, the MICs of 12 different antimicrobials were determined, and the resistome of each isolate was analyzed by ResFinder (Table S1, Fig. S1). In general, all isolates have an MDR phenotype, as they were resistant to at least nine different antimicrobials from six different antimicrobial classes (Table S1). Moreover, the MICs were very similar, and phenotype shifts from susceptible to resistant were only observed in isolate pair 1 for tigecycline. The acquired resistomes were identical in isolate pair 1 and 3, while isolate pair 2 differed in aminoglycoside and sulfonamide resistance determinants and isolate pair 4 differed in aminoglycoside and tetracycline resistance determinants (Fig. S1).

Analysis of *pmrCAB*, *eptA*, and other allele differences. Comparison of the draft genomes revealed differences in the *pmrCAB* operon between isolates representing an isolate pair. In the four isolates with high colistin MICs, the amino acid substitutions S17R, A28V, and I232T, as well as the 4-amino acid deletion ΔL9-G12, were detected in PmrB (Table 1). Both PmrA and PmrC were identical in isolates of an isolate pair. Moreover, a *pmrC* homologue (termed *eptA*) was present in isolate pairs 1, 2, and 3. However, *eptA* was not detected in isolate pair 4, *A. baumannii* ATCC 19606, or the *pmrAB* knockout, as determined by WGS or by PCR. The amino acid substitution R127L was detected in EptA in the colistin-resistant isolate MB_177 of isolate pair 2. Moreover, *ISAbal* was found in reverse orientation upstream of *eptA* in both isolates MB_23C and MB_177. While the insertion site was identical, a single point mutation (A→T) was observed in position 1091, close to the putative promoter region in *ISAbal* in isolate MB_177.

Besides mutations in the *pmrCAB* operon, disruption of H-NS or mutations in/disruptions of genes involved in LPS/lipid A biosynthesis have been associated with

FIG 1 Relative *pmrC* and *eptA* expression of all isolate pairs, determined by qRT-PCR. The number of *pmrC* transcripts (A) and *eptA* transcripts (B) of isolates with low colistin MICs (white) was related to that of isolates with higher colistin MIC (gray) after being normalized to the expression of the reference gene *rpoB* and the susceptible isolate, set as 1. Depicted are isolate pair 1 (MB_2 and MB_6C), isolate pair 2 (MB_23C and MB_177), isolate pair 3 (MB_90, MB_119), and isolate pair 4 (SG3161 and SG3166). Results are represented as mean $\pm$ standard error of the mean (SEM). Statistical analysis was done by performing an unpaired *t* test using the recorded absolute values. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

colistin resistance. Since no differences were detected, these gene products were considered not to be associated with colistin resistance in our isolates. Furthermore, the plasmid-mediated resistance determinant *mcr-1* or its homologues were not present. Other allele differences were detected in all isolate pairs except for isolate pair 4 (Table S2). Isolate pair 1 differed in three alleles, isolate pair 2 in eight alleles, and isolate pair 3 in four alleles. Most of these alleles included hypothetical and uncharacterized proteins, or components of bacterial metabolism, but also two putative transcription regulators differed in isolate pair 2.

Increased expression of *pmrC* and *eptA*. Since amino acid deletions or substitutions were detected in PmrB in all colistin-resistant isolates, we investigated their effect on the expression of *pmrC* by semi-quantitative reverse transcription-PCR (qRT-PCR). The relative expression was compared between isolates of an isolate pair (Fig. 1A). The expression of *pmrC* was significantly increased in the colistin-resistant isolates, MB_6C and SG3166. Compared to that in the colistin-susceptible isolates, *pmrC* was expressed

approximately 13× more than that in isolate MB_6C, while in isolate SG3166, a 10-fold increase in expression was observed. Although both isolates in isolate pair 3 were considered colistin resistant, the expression of *pmrC* was approximately 4× higher in isolate MB_119 compared to that in MB_90. Interestingly, the expression of *pmrC* was significantly decreased in MB_177 (the colistin-resistant isolate of isolate pair 2), so a different resistance mechanism is suggested in this isolate. Furthermore, the analysis of *eptA* expression by qRT-PCR revealed slightly increased expression in isolate MB_6C and decreased expression in isolate MB_119 compared to that in the isolates with low colistin MICs (Fig. 1B). In contrast, the expression of *eptA* was significantly higher in isolate MB_177 compared to that in MB_23C, although in both isolates, *ISAbal* was found in reverse orientation upstream of the gene. These findings suggest an association of point mutations in *ISAbal* or *eptA* with increased *eptA* expression and colistin resistance.

Lipid A modifications. An increased expression of *pmrC* or *eptA* is associated with the addition of PEtN to lipid A. To measure potential modifications of lipid A, LPS and lipid A was extracted from both mid-log-phase and overnight cultures of the isolate pairs and the reference strain *A. baumannii* ATCC 19606. Analysis by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF) revealed no significant differences between mid-log-phase and overnight cultures, as very similar mass spectra were obtained. The following major lipid A species were detected in the reference strain ATCC 19606 and all isolate pairs (Fig. 2A to E): hepta-acylated lipid A (m/z 1,910), hexa-acylated lipid A (m/z 1,728), penta-acylated lipid A lacking one fatty acid moiety (m/z 1,530), and tetra-acylated lipid A (m/z 1,404). However, peaks at m/z 1,530 and m/z 1,404 were small and unclear for some isolates (data not shown). The addition of PEtN (theoretical m/z 124) to lipid A is shown by the mass at m/z 2,034 (+1 PEtN) and m/z 2,157 (+2 PEtN). A single PEtN addition was found in isolate pair 1, exclusively in the colistin-resistant isolate MB_6C (Fig. 2B). In contrast, the mass at m/z 2,034 was detected in both isolates of isolate pair 2 (Fig. 2C). This was also observed in isolate pair 4 (Fig. 2E). These unexpected findings suggest a different or additional resistance mechanism in isolates MB_177 and SG3166. Furthermore, the relatively high colistin MICs of both isolates of isolate pair 3 were probably not due to PEtN addition, since a single PEtN addition was detected in isolates MB_90 and MB_119 (Fig. 2D). Interestingly, the addition of two PEtN molecules to lipid A (m/z 2,157) was observed only in isolate MB_119. Moreover, the addition of galactosamine to lipid A, which is indicated by a mass at m/z 2,071, was not observed in any isolate. Although PEtN addition was detected in colistin-resistant isolates, it seemed to be a strain-specific mechanism.

Similar growth kinetics for all isolates. Mutations in *pmrB* have been reported to carry a fitness cost (32). We therefore sought to investigate if our colistin-resistant isolates exhibited reduced growth and found that growth rates within an isolate pair did not differ (Fig. S2). In addition, growth rates and doubling times were calculated for all isolates, and this also revealed no differences within an isolate pair (data not shown). Therefore, the acquisition of colistin resistance did not appear to have a fitness cost as measured by growth kinetics.

Increased virulence in isolates with high colistin MICs. Colistin resistance is reportedly associated with decreased fitness and virulence (32–33). Therefore, we also investigated the virulence of these isolate pairs in the *G. mellonella* model of infection. The larvae were infected with the *A. baumannii* isolates, and survival, as well as melanization, was assessed (Fig. 3 and 4). Interestingly, the colistin-resistant isolates exhibited increased virulence (Fig. 3). In isolate pair 1, the survival rate of the larvae infected with MB_2 was 40% after 48 h, while none survived when infected with MB_6C ($P \leq 0.021$). After 96 h, the survival rate of larvae infected with MB_177 was also lower (30%) compared to that of those infected with MB_23C (60%) ($P \geq 0.04$). Although both isolates of isolate pair 3 are colistin resistant, a difference in virulence was also observed. While 50% of larvae infected with isolate MB_90 survived, the survival rate of

FIG 2 Mass spectra of lipid A extracted from overnight cultures of *A. baumannii* isolates. Represented are mass spectra in the range of m/z 1,650 to 2,200, and masses of important lipid A species are given in the spectra (24). (A) *A. baumannii* ATCC 19606, (B) isolate pair 1 (MB_2 and MB_6C), (C) isolate pair 2 (MB_23C and MB_177), (D) isolate pair 3 (MB_90 and MB_119), (E) isolate pair 4 (SG3161 and SG3166). An arrow indicates PetN addition indicated by a mass at m/z 2,034 or 2,157. Col^S, colistin-susceptible; Col^R = colistin-resistant.

FIG 3 Survival curves for *G. mellonella* larvae infected with different *A. baumannii* isolates. Illustrated are isolate pairs 1 to 4 (A to D). *G. mellonella* larvae were infected with 10^5 CFU/larva, and survival was observed over 96 h. Isolates with low colistin MICs are represented in blue, isolates with high colistin MICs in red, and the PBS control in black.

larvae infected with MB_119 was only 20% after 96 h ($P \leq 0.04$). No significant differences were detected in comparison of larvae infected with the isolates of isolate pair 4; both isolates resulted in low survival rates of approximately 30% after 96 h, although a higher percentage of larvae were killed in the first 24 h when infected with the colistin-resistant isolate SG3166 ($P \leq 0.17$).

Similar results were observed for melanization, which is used as an indicator for morbidity. Although all *A. baumannii* isolates induced high levels of melanization, higher melanization scores were observed in larvae infected with isolates with higher colistin MICs (Fig. 4). Larvae infected with the colistin-resistant isolate MB_6C displayed increased melanization over the entire time course (24 h to 96 h). Infection with isolates MB_177 and MB_119 led to unchanged or decreased rates of melanization after 24 h, but was elevated after 48 to 96 h. In contrast, larvae infected with isolate pair 4 revealed lower melanization scores for the colistin-susceptible isolate after 24 h and very similar and constant melanization scores between 48 and 96 h for both isolates, indicating no significant difference in morbidity. Both the decreased survival rates and the elevated melanization scores indicate a greater virulence in the colistin-resistant *A. baumannii* isolates MB_6C, MB_117, and MB_119.

Introduction of *pmrAB* variants into *A. baumannii* $\Delta pmrAB$. To further analyze the association of *pmrB* mutations with colistin resistance, a *pmrAB* knockout strain was created ($\Delta pmrAB$). The *pmrAB* variants from isolates MB_2 and MB_6C, as well as that from ATCC 19606 as a control, were introduced into $\Delta pmrAB$, and colistin MICs were determined (Table S3). Surprisingly, the colistin MICs were 0.5 to 1 mg/liter, regardless of the *pmrAB* variant introduced into the knockout strain. To confirm the expression of *pmrAB* on the plasmid and *pmrC* in $\Delta pmrAB$, gene expression was analyzed by qRT-PCR. Large numbers of *pmrA* and *pmrB* transcripts were detected in all cases, indicating expression of both genes on the plasmid (data not shown). Moreover, the expression of *pmrC* was significantly increased in $\Delta pmrAB$ complemented with *pmrAB* from isolate MB_6C ($\Delta L9$ -G12), whereas the expression did not differ in $\Delta pmrAB$ complemented with *pmrAB* from ATCC 19606 and isolate MB_2 (Fig. 5). Slightly increased *pmrC* expression

FIG 4 Infection of *G. mellonella* larvae with different *A. baumannii* isolates and its effect on melanization (morbidity). *G. mellonella* larvae were infected with 10^5 CFU/larva, and melanization was observed over 96 h. A higher melanization score indicates greater morbidity. The PBS control had a melanization score of 0. Depicted are isolate pairs 1 to 4 (A to D). Isolates with low colistin MICs are represented in white and isolates with high colistin MICs in black.

was detected in the wild-type ATCC 19606 compared to $\Delta pmrAB$, indicating a basal expression of *pmrC*.

Since *pmrC* was overexpressed in $\Delta pmrAB$ harboring pJN17/04::*pmrAB*-6, the modification of lipid A was analyzed by mass spectrometry (Fig. 6). The same major lipid A species already described for the isolate pairs (m/z 1,404, 1,530, 1,728, and 1,910) were also detected in the *pmrAB* knockout strains. The addition of a single PETN molecule indicated by the mass at m/z 2,034 was observed only in $\Delta pmrAB$ complemented with pJN17/04::*pmrAB*-6C (Fig. 6D), indicating that the *pmrB* mutation resulted both in increased *pmrC* expression and lipid A modification. Since the colistin MICs did not change, a different, hitherto unknown factor contributing to resistance or strain dependency are strongly suggested.

DISCUSSION

In the present study, we have investigated colistin resistance in four isogenic isolate pairs of *A. baumannii*, which gives us the ability to analyze the resistance mechanisms in very similar genetic backgrounds and exclude typical genetic variations seen in epidemiologically unrelated strains. Similar approaches have been conducted by, e.g., Dahdouh et al., Durante-Mangoni et al., and Beceiro et al., the latter reporting *pmrB* mutations, increased *pmrC* expression, and lipid A modification in colistin-resistant *A. baumannii* isolates (24, 34, 35). Many different amino acid substitutions or short amino acid deletions have been described in PmrB, both in clinical and in laboratory-derived *A. baumannii* isolates (14). We found three different single amino acid substitutions and one 4-amino acid deletion in PmrB. The substitutions S17R and A28V and the small deletion Δ L9-G12 are located in or in close proximity to the transmembrane domain of PmrB, which is involved in sensing extracellular stimuli and signal transduction. The substitution I232T is located near the histidine residue H228, which is important for autophosphorylation and transfer of the phosphoryl group (33, 36–38). Both locations

FIG 5 Relative *pmrC* expression of *A. baumannii* $\Delta pmrAB$ harboring various *pmrAB* variants. The number of *pmrC* transcripts of *A. baumannii* $\Delta pmrAB$ was related to that of *A. baumannii* ATCC 19606, as well as that of the knockout ($\Delta pmrAB$) harboring *pmrAB* variants from ATCC 19606 ($\Delta pmrAB + 19606$), MB_2 ($\Delta pmrAB + MB_2$), and MB_6C ($\Delta pmrAB + MB_6C$), respectively. The number of transcripts was normalized to the expression of the reference gene *rpoB*, and the knockout was set as 1. Results are represented as mean $\pm$ SEM. Statistical analysis was done by performing an unpaired *t* test using the recorded absolute values. **, $P < 0.01$.

are important for protein function, and substitutions/deletions in these regions are predicted to affect signal transduction and autophosphorylation (36).

To our knowledge, the substitutions A28V and I232T and the deletion $\Delta L9$ -G12 are derived from novel mutations in *pmrB* that have not been previously described. One would expect to see increased *pmrC* expression in these isolates, leading to the modification of lipid A through the addition of PEtN, which has been described several times before in the literature as a mechanism of colistin resistance (11, 13, 24). However, it seems that this rule cannot be applied to all isolates, and colistin resistance appears to be a much more complex mechanism than previously suspected. Only for the short amino acid deletion $\Delta L9$ -G12 in isolate pair 1 were the expected results of increased *pmrC* expression and PEtN addition to lipid A confirmed in this study. However, complementation of a *pmrAB* knockout with *pmrAB* harboring the deletion $\Delta L9$ -G12 did not result in colistin MIC changes, although *pmrC* expression was increased and lipid A modified. These data indicate the contribution of *pmrB* mutations to the upregulation of *pmrC* expression, but not necessarily to a phenotype change with regard to colistin susceptibility. Moreover, both isolates of isolate pair 3 were colistin resistant, but the amino acid substitution A28V in PmrB resulted in even higher colistin MICs and *pmrC* expression, which suggests an additive effect of mutations in *pmrB*, underlining the potential of mutation accumulation as a mechanism for increased antimicrobial resistance. In addition, a larger amount of PEtN added to lipid A is suggested in isolate MB_119, but it is likely that another factor contributes to the observed high-level resistance (39). Therefore, PEtN additions appear to be less crucial for a colistin-resistant phenotype than previously thought, and similar results were obtained for other isolate pairs. The amino acid substitution S17R in PmrB resulted in increased *pmrC* expression, which has been previously described in colistin-resistant isolates (38), but PEtN addition was detected in both isolates of isolate pair 4. The same has been observed for isolate pair 2, and although this can be partially explained by the involvement of *eptA*, these findings are rather surprising and have not been described in the literature so far. Furthermore, a *pmrAB* knockout complemented with *pmrAB* from isolate MB_6C displayed PEtN addition but no change in colistin MICs, which indicates that PEtN modification of lipid A is not the only factor contributing to colistin resistance. It is

FIG 6 Mass spectra of lipid A extracted from *A. baumannii* $\Delta pmrAB$ strains. Represented are mass spectra in the range of m/z 1,650 to 2,200, and masses of important lipid A species are given in the spectra (24). (A) *A. baumannii* $\Delta pmrAB$, (B) *A. baumannii* $\Delta pmrAB$ + pJN17/04::*pmrAB*-19606, (C) *A. baumannii* $\Delta pmrAB$ + pJN17/04::*pmrAB*-2, and (D) *A. baumannii* $\Delta pmrAB$ + pJN17/04::*pmrAB*-6C. An arrow indicates PETn addition indicated by a mass at m/z 2,034.

strongly suggested that another different and hitherto unknown factor(s) contributes to resistance (39), which so far cannot be explained by other allele differences detected in the isolate pairs. Another explanation might be a strain-specific effect of the mutation on *pmrC* expression (38), something that has also been observed with efflux and carbapenem resistance in *A. baumannii* (40–41).

In contrast to the other *pmrB* mutations, the substitution I232T in PmrB did not change *pmrC* expression. An amino acid change from threonine to isoleucine has been previously reported in the same position and was described to increase *pmrC* expression (38), which suggests the amino acid change I232T in PmrB does not affect the activity and autophosphorylation of the sensor kinase and is not associated with colistin resistance. Instead, the association of the *pmrC* homologue *eptA* with colistin resistance was suggested. The amino acid substitution R127L was detected in EptA, which might alter the efficiency and function of the enzyme. Moreover, *ISAbal* was detected upstream of *eptA* in isolate pair 2, which explains PETn addition to lipid A independently of *pmrCAB*. *ISAbal* is known to provide a strong promoter for gene expression and a point mutation close to the -35 region, and the upstream promoter element (UP-element) could alter the promoter strength and therefore explain the differential expression of *eptA* in isolate pair 2 (42). Moreover, the presence of an insertion sequence indicates the possible dissemination of *eptA* between other isolates or

bacterial species, as is frequently observed for β -lactamases (42). Besides *pmrB*, several allele differences were detected in isolate pair 2, two of which were located in putative transcription regulators. Both regulators could be involved in the regulation of *eptA* expression. In addition, an allele difference in the guanosine polyphosphate pyrophosphohydrolase/synthase (SpoT) was found. SpoT is involved in purine metabolism and synthesizes ppGpp, which has been described as a global transcriptional regulator in the stress response of bacteria and is associated with antimicrobial resistance in *E. coli* (43).

Several studies have described colistin-resistant *A. baumannii* isolates with reduced fitness and impaired virulence (32, 33, 44, 45), while other studies are in agreement with our observation of unchanged fitness or growth characteristics (34–35). Retention of fitness and virulence in colistin-resistant isolates has been attributed to the mechanism of resistance. A fitness loss was often associated with mutations in lipid A biosynthesis genes and complete loss of LPS, while alterations in *pmrCAB* did not affect the fitness or virulence of *A. baumannii* (46). In our study, unchanged or increased virulence and melanization in *G. mellonella* larvae was detected in colistin-resistant isolates. High melanization scores are an indicator for an immune response of the host (*G. mellonella*). Our results revealed higher scores in larvae infected with *A. baumannii* isolates displaying high colistin MICs. Therefore, we propose a correlation between the mechanism of colistin resistance and virulence. *pmrAB*-mediated LPS modification has been described to play a role in virulence in other organisms, such as *Salmonella* spp., by altering the innate immune recognition (e.g., by reduction of Toll-like receptor-LPS interaction) (47). Moreover, LPS and lipid A have been discussed as potential virulence factors in *A. baumannii*, and LPS derived from *A. baumannii* has been shown to stimulate innate immune responses in humans via Toll-like receptor 4 signaling (48–49). Therefore, it is not unexpected that lipid A modifications can be associated with a change in virulence in *A. baumannii*. Increased virulence of colistin-resistant strains could also be partially due to cross-resistance to antimicrobial peptides naturally produced by *G. mellonella* larvae as part of their innate immunity (50). Resistance to these peptides would reduce the effectiveness of the animal's immunity and thus result in the increased visible pathogenicity of the *A. baumannii* isolates.

In conclusion, we have identified four different *pmrB* mutations, three of which have not been described before (leading to A28V, I232T, and Δ L9-G12). All novel *pmrB* mutations were associated with increased virulence in *G. mellonella*. Furthermore, it was shown that *pmrAB* regulates *pmrC* expression and PEtN addition to lipid A, but that alone does not seem crucial for colistin resistance in *A. baumannii*. Instead, the association of the *pmrC* homologue *eptA* with colistin resistance was described, and a possible complex regulation of *eptA* expression is suggested. Nevertheless, the exact resistance mechanism for two isolates remains to be elucidated, as inconsistent results were obtained regarding PEtN addition, and the contribution of *eptA* to colistin resistance needs further investigation. These data open a window into the complexity of colistin resistance in *A. baumannii*, and more work is needed to fully understand the colistin resistance mechanisms in *A. baumannii*. Rather than a single rule for the mechanism of colistin resistance, it appears that strain-dependent factors must be taken into account. Moreover, a high cascade in the regulation of colistin resistance mechanisms, as found in other Gram-negative bacteria (14), could also be present in *A. baumannii*. Since this is still unknown, and global regulators are mostly unidentified, further analysis is required but beyond the scope of this work.

MATERIALS AND METHODS

Bacterial isolates. In total, 8 *A. baumannii* isolates were investigated. The isolates demonstrated an increase in colistin MIC by at least four dilution steps. In addition, isolates representing isolate pairs were required to demonstrate fewer than ten allele differences in core genome multilocus sequence typing (cgMLST). The isolates were obtained from four patients hospitalized in Frankfurt (Germany; *n* = 1), Athens (Greece; *n* = 1), and Seville (Spain; *n* = 2). The isolates from Greece and Spain were recovered from respiratory tract or stool samples obtained from patients with ventilator-

TABLE 2 List of oligonucleotides used in this study

Primer	Sequence (5' to 3')	Expt	Reference or source
C65	GAGTCTAATGGCGGTGGTTC	<i>rpoB</i> qRT-PCR	23
C66	ATTGCTTCATCTGCTGGTTG		
J15	GGTGGAAATGGGTCAATAACG	<i>pmrA</i> qRT-PCR	11
J16	GGTGTGCTGCTCTTTGACG		13
H52	TCGATGAAATTCTAGATACTCAAATG	<i>pmrB</i> qRT-PCR	13
H53	CCCAAATATCGATAAACAGATCTTC		
N3	TGTTGCTACCTATTATGCGG	<i>pmrC</i> qRT-PCR	This study
N4	ATTTTGAATTTGGTCGGGTG		
L39	GATCGCGTTGAACAATATCA		
L40	GCAATACAATAACCGTGGA		
N5	TCAGTTCTTTCTTAGGGGC	<i>eptA</i> qRT-PCR (together with N4)	
H48	GCGAGGAGCACATTTCTTAA	Sanger sequencing of <i>pmrCAB</i>	13
H49	TGTAGTCACTCAGATGCTGAA		
H51	GGCAAAGCAAGATGTAACCTTAA		
H54	TTGAAGCAGATCCGTCAAAG		
H55	TGCACCCAAATTTAAACCATC		
H57	ATGACAAAAATCTTGATGATTGAAGAT	Sanger sequencing of <i>pmrCAB</i>	24
H58	TTATGATTGCCCAAACGGTAG		
H59	GTGCATTATTCATTAACAAAAAC		
H62	CCTAAATCGATTTCTTTTGG		
H64	TTAGTTTACATGGGCACAA		
H65	GGTTGTTATTGAAGAAAGTAT		
M80	CAAGGAGCCATCGTCATATA	Sanger sequencing of <i>eptA</i>	This study
M81	ATGGTGTTTATCCGGGTAC		
N1	CATAAGAAGGCTCCGAAGAA		
N2	TGTCATCATAACATTCGCCA		
N6	GAGCATTTCAACTGGGGATA		
J76	AGAGCATCGAATCTCTACTTTAGCGTATGGTG	Markerless <i>pmrAB</i> knockout	
J77	AAACAAGAGCTTACGGTTCTAGGCTCGCTTTAGTT		
J78	AAGCGAGCCTAGAACCCTAAGCTCTGTTTCACTT		
J79	GTGGATCCCCGGGCGGATTTAATTGTCGGCTGG		
K58	GCCACTCATCGAGTAAACCGACTTGTGATACGAA	In-Fusion cloning of <i>pmrAB</i> into pJN17/04	This study
K59	ATGAATTACAACAGTTAGGTTTCATGATGGGTTGG		

associated pneumonia (VAP) between 2012 and 2015 as part of the MagicBullet clinical trial (9, 18). The isolates from Germany were recovered from wound and respiratory samples obtained in 2016 from a stroke patient.

Antimicrobial susceptibility testing. Susceptibility to colistin was determined by broth microdilution using premanufactured freeze-dried microtiter plates (Micronaut 5 Colistin plates; Merlin Diagnostika, Bornheim, Germany). The MIC was determined in cation-adjusted Mueller-Hinton broth (CAMHB) according to the international standard ISO 20776-1 (19). Colistin resistance was defined as an MIC of >2mg/liter, using the EUCAST resistance breakpoints for *A. baumannii*. Susceptibility to tetracycline, gentamicin (Sigma-Aldrich, Steinheim, Germany), amikacin, ciprofloxacin, meropenem, minocycline, rifampin, tigecycline (Molekula, Newcastle upon Tyne, UK), levofloxacin (Sanofi Aventis, Frankfurt, Germany), azithromycin (Pfizer Pharma GmbH, Berlin, Germany), and erythromycin (AppliChem, Darmstadt, Germany) was determined by agar dilution following the current CLSI guidelines (20). All susceptibility tests were repeated at least three times independently. *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 29213 were used as controls.

WGS and sequence data analysis. All *A. baumannii* isolates were subjected to WGS (21). Sequencing libraries were prepared using the Nextera XT library prep kit (Illumina GmbH, Munich, Germany) for a 250-bp paired-end sequencing run on an Illumina MiSeq sequencer. The resulting FASTQ files were quality trimmed and assembled *de novo* using Velvet with SeqSphere⁺ v.3.0 software (Ridom GmbH, Münster, Germany). The assembled genomes were aligned to the *A. baumannii* reference strain ACICU to determine differences in specific open reading frames in each draft genome sequence, as well as for cgMLST (21), and were further analyzed using ResFinder for resistome analysis (22). Besides the amino acid sequence of PmrCAB, the sequences of H-NS and of the LPS/lipid A biosynthesis components LpsB, LpxABCD, LpxHKLM, and KdtA/WaaA were compared between the isolates and to those of the reference strain ACICU.

PCR-based methods. All primers used in this study were designed using Primer3 software (<http://bioinfo.ut.ee/primer3/>) and are listed in Table 2. Crude cell lysates or genomic DNA were used as the templates for PCR. Differences determined by WGS in *pmrCAB* and *eptA* between isolates of an isolate pair were confirmed by Sanger sequencing (LGC Genomics GmbH, Berlin, Germany).

Semiquantitative reverse transcription-PCR. To measure the expression of *pmrCAB* and *eptA*, qRT-PCR was performed as previously described, using *rpoB* as reference gene (25). The specificity of the *pmrC* and *eptA* primers were confirmed by PCR. All qRT-PCRs were done in triplicates using freshly prepared RNA and cDNA and were repeated independently at least three times. The relative expressions

of *pmrCAB* and *eptA* were compared with either the isolates representing an isolate pair or with the *pmrAB* knockout strain, and statistical analysis was done by performing an unpaired *t* test using the recorded absolute values.

Markerless *pmrAB* knockout and cloning of *pmrAB*. A *pmrAB* knockout was created as previously described by Stahl et al. (26), using *A. baumannii* ATCC 19606 as the parent strain. The upstream and downstream regions of *pmrAB* were integrated into plasmid pBlISK_sacBkanR using the In-Fusion HD EcoDry cloning kit (TaKaRa Clontech, Saint-Germain-en-Laye, France) and primers J76 to J79 (Table 2). The successful deletion of *pmrAB* was confirmed by WGS. The *pmrAB* variants of *A. baumannii* ATCC 19606 as control and of isolates MB_2 and MB_6C were amplified using the primer pair K58 and K59 (Table 2), and the shuttle vector pJN17/04 was linearized as previously described (27). *pmrAB* was integrated into the plasmid using the In-Fusion HD EcoDry cloning kit. The plasmids pJN17/04::*pmrAB*-19606, pJN17/04::*pmrAB*-2, and pJN17/04::*pmrAB*-6C were introduced into *A. baumannii* Δ *pmrAB* by electroporation (27). Successful transformants were confirmed by PCR and maintained on Luria-Bertani (LB) agar supplemented with 10 mg/liter kanamycin (Molekula, Newcastle upon Tyne, UK).

Lipid A extraction and mass spectrometry. For LPS and lipid A extraction, all isolates and the reference strain *A. baumannii* ATCC 19606 were cultured in 250 ml of LB medium at 37°C and 220 rpm. To determine if the composition of lipid A varied between growth phases, the bacteria were cultured overnight or until the mid-log phase was reached (optical density at 600 nm [OD₆₀₀], 0.7 to 0.9). The cultures were centrifuged at 4,000 × *g* for 15 min, and the cell pellet was washed twice with phosphate-buffered saline (PBS). Approximately 200 to 400 mg of the cell pellets were subjected to LPS extraction following the Tri Reagent method with three additional chloroform extraction steps (28). Mass spectrometry analysis was done using a MALDI-TOF mass spectrometer (Bruker Biflex III; Bruker Daltonics, Bremen, Germany). Crude lipid A extract was resolved in chloroform:methanol:H₂O (4:4:1 [vol/vol]) and subjected to an ultrasound bath for 20 min, followed by a centrifugation step at 13,000 rpm for 2 min. Aliquots of 1 μ l sample were mixed 1:1 with the matrix (5-chloro-2-mercaptobenzothiazole and ammonium-EDTA), spotted directly onto the MALDI target plate, and dried in air. Mass spectra were measured using negative ion mode within a mass range of 500 to 3,000 Da (29).

Growth kinetics. Growth kinetics were performed in triplicates as previously described (27). Briefly, a McFarland suspension of 0.5 generated from a fresh overnight culture was used to inoculate 10 ml Mueller-Hinton (MH) broth. The cultures were incubated for six hours at 37°C and 200 rpm, and a sample was taken every hour. Samples were serially diluted in cold saline and plated onto MH agar, and CFU per milliliter values were determined after overnight incubation.

In vivo virulence in *G. mellonella*. The *in vivo* virulence of the *A. baumannii* isolates was investigated using the *G. mellonella* invertebrate model (TruLarv; Biosystems Technology, Exeter, UK) as previously described (30). For each *A. baumannii* isolate, 10 larvae were infected by injection with 10⁵ CFU/larva of the respective isolate into the left proleg. PBS was used in the control injections. The larvae were incubated aerobically at 37°C and scored for survival (live/dead) at 0, 24, 48, 72, and 96 h postinfection. Moreover, the melanization scores were recorded as an indicator of morbidity (31). All assays were performed in triplicates and results were pooled for analysis. Statistical analysis was carried out using an unpaired *t* test at 96 h, as described previously (30).

Accession number(s). All raw reads generated were submitted to the Sequence Read Archive of the National Center for Biotechnology Information (NCBI) under the accession numbers [SRP131448](#) and [SRP155178](#), as well as under the BioProject accession number [PRJNA482774](#).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.01586-18>.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

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We have no conflicts of interest to declare.

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