

# Emergence of Colistin-Resistance in Extremely Drug-Resistant *Acinetobacter baumannii* Containing a Novel *pmrCAB* Operon During Colistin Therapy of Wound Infections

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**Background.** Colistin resistance is of concern since it is increasingly needed to treat infections caused by bacteria resistant to all other antibiotics and has been associated with poorer outcomes. Longitudinal data from in vivo series are sparse.

**Methods.** Under a quality-improvement directive to intensify infection-control measures, extremely drug-resistant (XDR) bacteria undergo phenotypic and molecular analysis.

**Results.** Twenty-eight XDR *Acinetobacter baumannii* isolates were longitudinally recovered during colistin therapy. Fourteen were susceptible to colistin, and 14 were resistant to colistin. Acquisition of colistin resistance did not alter resistance to other antibiotics. Isolates had low minimum inhibitory concentrations of an investigational aminoglycoside, belonged to multi-locus sequence type 94, were indistinguishable by pulsed-field gel electrophoresis and optical mapping, and harbored a novel *pmrCIA1B* allele. Colistin resistance was associated with point mutations in the *pmrA1* and/or *pmrB* genes. Additional *pmrC* homologs, designated *eptA-1* and *eptA-2*, were at distant locations from the operon. Compared with colistin-susceptible isolates, colistin-resistant isolates displayed significantly enhanced expression of *pmrCIA1B*, *eptA-1*, and *eptA-2*; lower growth rates; and lowered fitness. Phylogenetic analysis suggested that colistin resistance emerged from a single progenitor colistin-susceptible isolate.

**Conclusions.** We provide insights into the in vivo evolution of colistin resistance in a series of XDR *A. baumannii* isolates recovered during therapy of infections and emphasize the importance of antibiotic stewardship and surveillance.

**Keywords.** Colistin-resistant *Acinetobacter baumannii*; infection control; translational research.

Polymyxin antibiotics such as colistin are increasingly relied upon as agents of last resort to treat infections caused by bacteria resistant to all other antibiotics [1, 2].

Colistin's bactericidal activity is thought to occur through a detergent-like effect, in which the compound interacts with the hydrophilic lipid A component of lipopolysaccharide, disrupting the outer membrane and causing cell death [3]. A proposed alternative mechanism involves hydroxyl radical production [4]. Colistin resistance is concerning because there are no new antibiotics active against gram-negative bacteria are anticipated in the near future [5], and colistin resistance has been associated with poorer clinical outcomes [6].

*Acinetobacter baumannii* is an important nosocomial pathogen with a propensity to acquire resistance to multiple antibiotics [7]. Two mechanisms for colistin resistance in *A. baumannii* have been proposed. The

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first involves either mutations in the genes encoding lipid A biosynthesis—*lpxA*, *lpxC*, and *lpxD* [8]—or disruption of *lpxA* and *lpxC* expression by insertion sequence *ISAb11* [9]. These alterations appear to cause a complete loss of lipopolysaccharide, resulting in decreased outer-membrane integrity and increased susceptibility to other antibiotics. The second mechanism is more common and involves mutations in *pmrA* and *pmrB*, leading to modification of bacterial lipopolysaccharide [10–12]. Unlike changes in *lpxACD*, mutations in *pmrCAB* do not result in increased susceptibility to other antimicrobials. It has been suggested that acquisition of colistin resistance is associated with reduced virulence and fitness, although this was based on the study of a single resistant clone obtained in vitro [13].

Our objective was to perform an in-depth characterization of a longitudinal series of colistin-resistant *A. baumannii* isolates recovered during concurrent colistin therapy of severely war-wounded patients. Our aims were to determine the genetic relatedness of the strains, to gain insight into the molecular mechanisms underlying the development of colistin resistance in vivo, and to screen for a putative association between acquired colistin resistance and change in baseline resistance to other antibiotics or fitness of the pathogen.

## METHODS

This report is the result of a quality-improvement initiative authorized by the US Army Medical Command and respective institutional review boards to intensify infection-control measures.

Antibiotic susceptibility was determined according to Clinical Laboratory Standards Institute S100-M22 guidelines (Table 1) [15]. Primers (Supplementary Table 1) were designed using Primer Express, version 2.0 (Applied Biosystems), and validated using MIQE (Minimum Information for Publication of Quantitative Real-Time Polymerase Chain Reaction [PCR] Experiments) guidelines [17]. Pulsed-field gel electrophoresis (PFGE), optical genome mapping (OGM), in vitro growth rates and competition assays, sequencing of the *pmrCAB* operon, whole-genome sequencing, phylogenetic analysis, conventional and real-time PCR, and gene expression assays were performed as described in the Supplementary Methods.

## RESULTS

Twenty-eight *A. baumannii* isolates (14 colistin-susceptible isolates and 14 colistin-resistant isolates) were recovered from 7 patients over 11 months (Table 1). They were collected during infections that occurred in 2 clusters: 6 patients were in cluster 1, and 1 was in cluster 2. The second cluster occurred 3 months after the last isolate from the first cluster was recovered. All isolates were resistant to at least 1 drug from  $\geq 4$  antimicrobial classes and thus were classified as XDR isolates [18]. All isolates had low minimum inhibitory concentrations (MICs) of the

investigational aminoglycoside arbekacin (Table 1). Isolates were susceptible to minocycline but displayed varying susceptibility to tigecycline. Colistin resistance was not associated with altered susceptibility to other agents.

All patients in the study had sustained severe trauma in combat operations outside the United States, were  $<35$  years old, were previously healthy without any comorbidities, and had been hospitalized in an intensive care unit or surgical ward. All patients had recently received antibiotics, but not colistin, for complicated infections during initial hospitalization in Afghanistan, transient hospitalization in Germany, and definitive hospitalization in the United States.

Clinical isolates were indistinguishable by PFGE (Figure 1A) and formed a separate cluster based on a 90% similarity cutoff when compared against 1500 *A. baumannii* collected from 24 geographically separated hospitals in the global Military Health System. OGM showed that the strains were  $>99\%$  homologous and contained a number of chromosomal insertions, deletions, and rearrangements relative to *A. baumannii* reference strains AB0057, ACICU, and AYE (Figure 1B). The outbreak strain belonged to a rare multilocus sequence type (ST), ST94 [19, 20].

All isolates had a *pmrCAB* operon whose sequence diverged from those of published *A. baumannii* *pmrCAB* operons (Supplementary Table 1). The absence of insertion sequences or transposons in the vicinity of the operon indicates that sequence divergence was due to evolutionary mechanisms rather than to acquisition of foreign DNA. The *pmrB* gene was identical to that of *A. baumannii* AB0057. However, PmrA and PmrC were more closely related to their *Acinetobacter nosocomialis* homologs than to *A. baumannii* proteins: 99% versus 98% identity and 97% versus 88% identity, respectively (Table 2). The difference between 99% and 98%, although small, is significant: *A. nosocomialis* PmrA (99% identity) differs from PmrA1 by a single conservative amino acid difference, and *A. baumannii* PmrA (98% identity) has 2 conservative and 2 nonconservative amino acid changes relative to *A. baumannii* PmrA1. Consequently, we designated these genes *pmrA1* and *pmrC1*. No complete or draft *A. baumannii* genome in GenBank had the *pmrC1* allele.

Isolates had 2 additional *pmrC*-related genes, designated ethanolamine phosphotransferase A-1 (*eptA-1*) and A-2 (*eptA-2*), elsewhere in the chromosome. When calculated from the *oriC*, the *pmrC1A1B* operon was located from 795 449 to 799 147 bp; *eptA-1* is located from 1 652 432 to 1 654 079 bp, and *eptA-2* from 2 762 837 to 2 764 484 bp. These genes were more similar to the canonical *pmrC* gene than to *pmrC1* (Table 2). Four of 11 *A. baumannii* strains with complete genome sequences had only *pmrC*; among these were AB0057 and AB307-0294, the isolates most closely related to the outbreak strain. Five strains had both *pmrC* and *eptA-2*, and 1, *A. baumannii* 1656-2, had *pmrC*, *eptA-1*, and *eptA-2* (Supplementary Table 2). Of 116 draft *A. baumannii* genomes in the NCBI database (available at: <http://www.ncbi.com>), 20% possessed 2 *eptA* homologs

**Table 1. Clinical and Phenotypic Characteristics of *Acinetobacter baumannii* Clinical Isolates**

Strain <sup>a</sup>	Patient	Date <sup>b</sup>	Source <sup>c</sup>	Exposure <sup>e</sup>	Phenotype <sup>f</sup>	Colistin				MIC, by Drug, µg/mL <sup>d</sup>				
						Microbroth MIC, µg/mL <sup>g</sup>		Etest MIC, µg/mL	TREK MIC, µg/mL					
						With Tween 80	Without Tween 80			TGC	ARB	PMB	MIN	SAM
						3405	1	1	Wound	No	S	0.25	1	0.125
3387	1	3	Wound	No	S	0.125	1	0.125	≤0.25	4	1	0.19	2	>16/8
3543	1	3	Wound	No	S	0.125	1	0.25	≤0.25	2	0.5	0.25	1.5	>16/8
3544	1	3	Wound	No	S	0.125	1	0.19	≤0.25	4	2	0.25	1.5	>16/8
3546	1	4	Tissue	No	S	0.25	1	0.19	≤0.25	4	1	0.25	1.5	>16/8
3527	1	5	Wound	Yes	S	0.25	2	0.125	≤0.25	4	2	0.5	2	8/4
3941	1	11	Wound	Yes	R	64	128	192	>4	8	1	64	2	8/4
3942	1	15	Wound	No	R	64	128	128	>4	8	1	64	1.5	16/8
4778	2	15	Urine	No	R	64	128	>128	>4	12	1	8	1.5	16/8
4106	3	17	Wound	No	R	64	64	8	>4	12	2	16	2	4/2
4119	3	17	Wound	No	R	>64	256	128	>4	16	2	96	2	4/2
4112	4	17	Wound	No	R	64	64	128	>4	16	1	48	2	<4/2
4118	5	22	Wound	No	R	64	256	128	>4	2	1	64	2	4/2
4611	6	22	Wound	No	R	>64	256	>256	>4	3	1	32	1	8/4
5540	7	35	Blood	No	S	0.125	1	0.75	≤0.25	2	2	0.19	1.5	>16/8
5544	7	35	Respiratory	Yes	S	0.125	1	0.5	≤0.25	4	2	0.25	1.5	16/8
5545	7	35	Tissue	Yes	S	0.125	1	0.38	≤0.25	4	2	0.25	1.5	16/8
6263	7	36	Respiratory	Yes	S	0.125	1	0.38	≤0.25	4	2	0.25	1.5	>16/8
6264	7	36	Tissue	Yes	S	0.125	1	0.19	≤0.25	4	2	0.19	1.5	>16/8
6265	7	36	Urine	Yes	S	0.125	2	0.19	≤0.25	3	2	0.19	1	>16/8
6266	7	37	Respiratory	Yes	S	0.25	1	0.38	≤0.25	4	2	0.5	1.5	>16/8
6267	7	38	Respiratory	Yes	R	16	16	3	2	2	2	2	1	4/2
6268	7	38	Urine	Yes	R	16	16	32	>4	3	2	4	2	>16/8
6269	7	43	Respiratory	Yes	R	64	128	>256	>4	2	2	4	1	8/4
6270	7	43	Respiratory	Yes	R	8	16	4	>4	4	1	4	2	8/4
6271	7	43	Respiratory	Yes	R	64	128	>256	>4	2	2	4	2	8/4
6272	7	43	Respiratory	Yes	R	8	16	6	>4	3	2	4	1	8/4
6273	7	43	Respiratory	Yes	S	0.0625	1	0.25	≤0.25	4	1	2	1.5	8/4

Abbreviations: ARB, arbekacin; MIC, minimum inhibitory concentration; MIN, minocycline; PMB, polymyxin B; SAM, ampicillin-sulbactam; TGC, tigecycline.

<sup>a</sup> Deidentified designation used to track isolates throughout the MRSN.

<sup>b</sup> Expressed in weeks after the first isolate was recovered from patient 1.

<sup>c</sup> Clinical site where the isolate was recovered.

<sup>d</sup> All strains were resistant to amikacin, gentamicin, imipenem, meropenem, tobramycin, and vancomycin. MICs of TGC, PMB, and MIN were determined in triplicate, using the Etest. SAM MICs were determined using 3 identification systems (see Methods). MICs of arbekacin were determined in triplicate by microbroth dilution as described elsewhere [14].

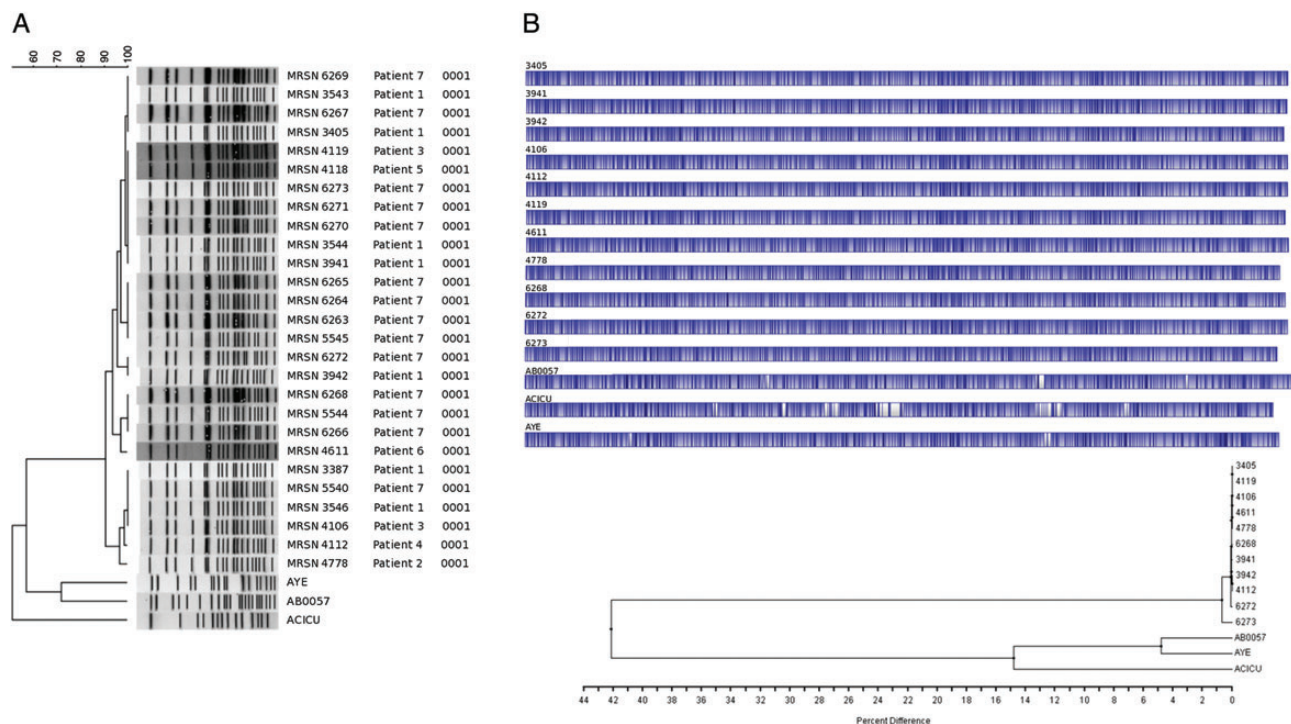
<sup>e</sup> "Yes" indicates treatment with colistin when the isolate was recovered.

<sup>f</sup> Based on MICs of colistin, as recommended by the Clinical and Laboratory Standards Institute [15].

<sup>g</sup> Determined in quintuplicate, using Clinical and Laboratory Standards Institute guidelines [15], with and without the addition of Tween 80 at a final concentration of 0.002% [16].

in addition to *pmrC*, and another 4% contained  $\geq 3$  *eptA* homologs along with *pmrC*. It is noteworthy that in all strains from this series, *eptA-1* and *eptA-2* are associated with prophages. Phages with partial homology to *Acinetobacter* phage YMC/09/02/B1251 ABA BP [21] lie 3.1 kb upstream from *eptA-1* and 1.8 kb upstream from *eptA-2* but are only

approximately 85% identical to each other. In addition, *eptA-1* is located approximately 0.9 kb upstream from an integrase with homology to the recombinases of IS-123 and IS-143. The corresponding nucleotide sequences have been uploaded to Genbank (KC700024, *eptA-1* context; KC700023, *eptA-2* context).



**Figure 1.** Pulsed-field gel electrophoresis (PFGE) and optical genome mapping profiles of selected strains. *A*, PFGE profiles showing the relatedness of the 27 clinical isolates and of *Acinetobacter baumannii*–reference strains. DNA was digested with *Apal* and separated by gel electrophoresis. All strains were considered indistinguishable by use of a 90% cutoff. *B*, Optical maps and associated dendrogram of 12 strains compared with in silico optical maps of *A. baumannii* AB0057, AYE, and ACICU generated from GenBank sequences. Strain relatedness was calculated using the unweighted pair group method with arithmetic mean. Blue areas represent areas of alignment, compared with MRSN 3405, and white areas represent unaligned regions. Vertical black bars represent *NcoI* restriction cut sites.

We developed a duplex PCR assay that allows specific detection of *pmrC1*. The reaction generated 2 products: a 511-bp amplicon from *pmrC1* and a 318-bp fragment derived from *pmrC*, *eptA-1*, or *eptA-2* (Supplementary Figure 1). Unexpectedly, among 110 clinical isolates of *A. baumannii* from our collection that differed with respect to pulse type, 12 tested

positive for *pmrC1* (data not shown). This indicates that *pmrC1* is not a unique feature of ST94.

All 14 colistin-resistant isolates had nonsynonymous point mutations in the *pmrA1* and/or *pmrB* genes when compared to the colistin-susceptible strains, amounting to 5 distinct mutations (Table 3). Phylogenetic analysis was performed using

**Table 2. Identity and Similarity Among Ethanolamine Phosphotransferases Produced by *Acinetobacter baumannii* and *Acinetobacter nosocomialis***

Protein	Percentage Identity; Percentage Similarity					
	PmrC	PmrC1	PmrC-R	EptA	EptA-1	EptA-2
PmrC	100	87.6; 94.0	87.8; 93.4	93.9; 97.0	93.2; 96.8	93.9; 96.8
PmrC1	...	100	97.3; 98.5	83.6; 91.4	83.1; 91.4	83.2; 91.2
PmrC-R	...	...	100	83.9; 91.2	83.5; 91.2	83.6; 91.0
EptA	...	...	...	100	97.4; 98.7	99.6; 99.8
EptA-1	...	...	...	...	100	97.1; 98.5
EptA-2	...	...	...	...	...	100

Abbreviations: EptA, *A. baumannii* MDR-TJ EptA; EptA-1, *A. baumannii* MRSN3405 EptA homolog (same chromosomal context as *eptA* in *A. baumannii* MDR-TJ); EptA-2, *A. baumannii* MRSN3405 EptA homolog (different chromosomal context as *eptA* in *A. baumannii* MDR-TJ); PmrC, *A. baumannii* AB0057 PmrC; PmrC1, *A. baumannii* MRSN3405 PmrC1; PmrC-R, *A. nosocomialis* RUH2624 PmrC.



high-quality single-nucleotide polymorphisms (SNPs) only (ie, SNPs that were unambiguously present in all reads from the sequencing data. On the basis of this criterion, 18 high-quality SNPs were identified by comparing whole-genome sequences from 21 strains. Phylogenetic analysis was used to infer relatedness between isolates on the basis of high-quality SNP sets that include (Figure 2A) or exclude (Figure 2B) the 5 mutations identified in *pmrA1* and *pmrB*. The analysis revealed that strains from clusters 1 and 2 were closely related, with MRSN (Multidrug-Resistant Organism Repository and Surveillance Network) 4611 representing an intermediary strain between both groups (Figure 2). Therefore, both clusters appear to be derived from a common colistin-susceptible progenitor strain despite having been isolated 3 months apart. Colistin resistance evolved in cluster 1 via multiple independent mutations in the *pmrB* gene of a susceptible ancestor, such as MRSN 3405. MRSN 3942 was isolated from the index patient and is closely related to MRSN 4778 (patient 2), MRSN 4106 (patient 3), MRSN 4119 (patient 3), MRSN 4112 (patient 4), and MRSN 4118 (patient 5), with the major exception that MRSN 3942 carries a T51A mutation in *pmrB*, while the others have a C695T substitution (Table 3). The final sequenced strain from cluster 1, MRSN 4611, was collected from patient 6 twenty-two weeks after recovery of the first isolate. MRSN 4611 lies on a distinct branch of the phylogenetic tree (Figure 2) and is the only strain to have the G788T mutation in *pmrB*. MRSN 4611 has 4 private SNPs as well as 2 variants shared by all strains from cluster 2. These data suggest that MRSN 4611 and the cluster 2 isolates had a common ancestor that arose from MRSN 3405 (Figure 2).

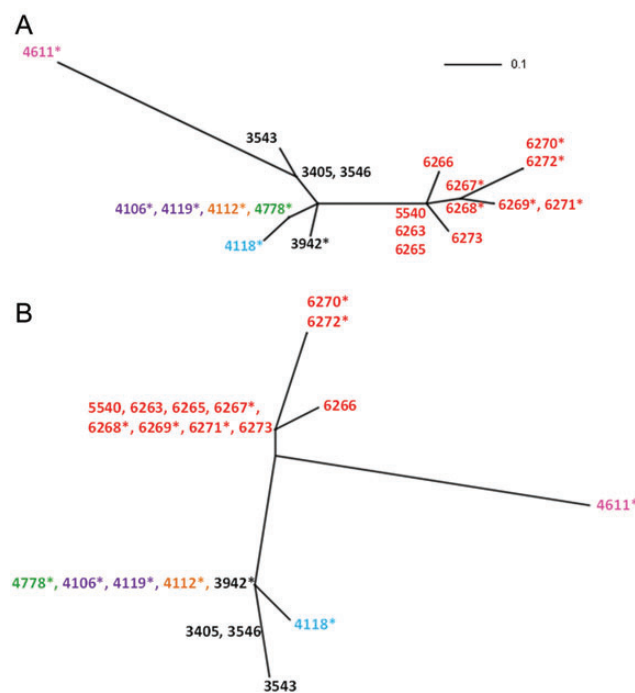
Cluster 2 composes a distinct phylogenetic branch because of 4 high-quality SNPs present in all members (Figure 2). As

mentioned, 2 of these SNPs are also found in MRSN 4611. Other SNPs provide further insights into the evolution of cluster 2. The putative colistin-susceptible founder strain in this patient, MRSN 5540, obtained from a blood culture, shares the same high-quality SNPs as MRSN 6263 and MRSN 6265, isolated a week later from respiratory and urine samples, respectively (Table 1). The 2 other colistin-susceptible strains, MRSN 6266 and MRSN 6273, each differ from MRSN 5540 by 1 high-quality SNP (Figure 2B). Consistent with being direct descendants of MRSN 5540, MRSN 6267 and MRSN 6268 can be distinguished from this isolate by just a single high-quality SNP that maps to *pmrA1*. MRSN 6269 and MRSN 6271 have the *pmrA1* mutation and a mutation in *pmrB*, while MRSN 6270 and MRSN 6272 have the *pmrA1* mutation, as well as 2 additional shared high-quality SNPs, demonstrating the continual evolution of this strain in vivo.

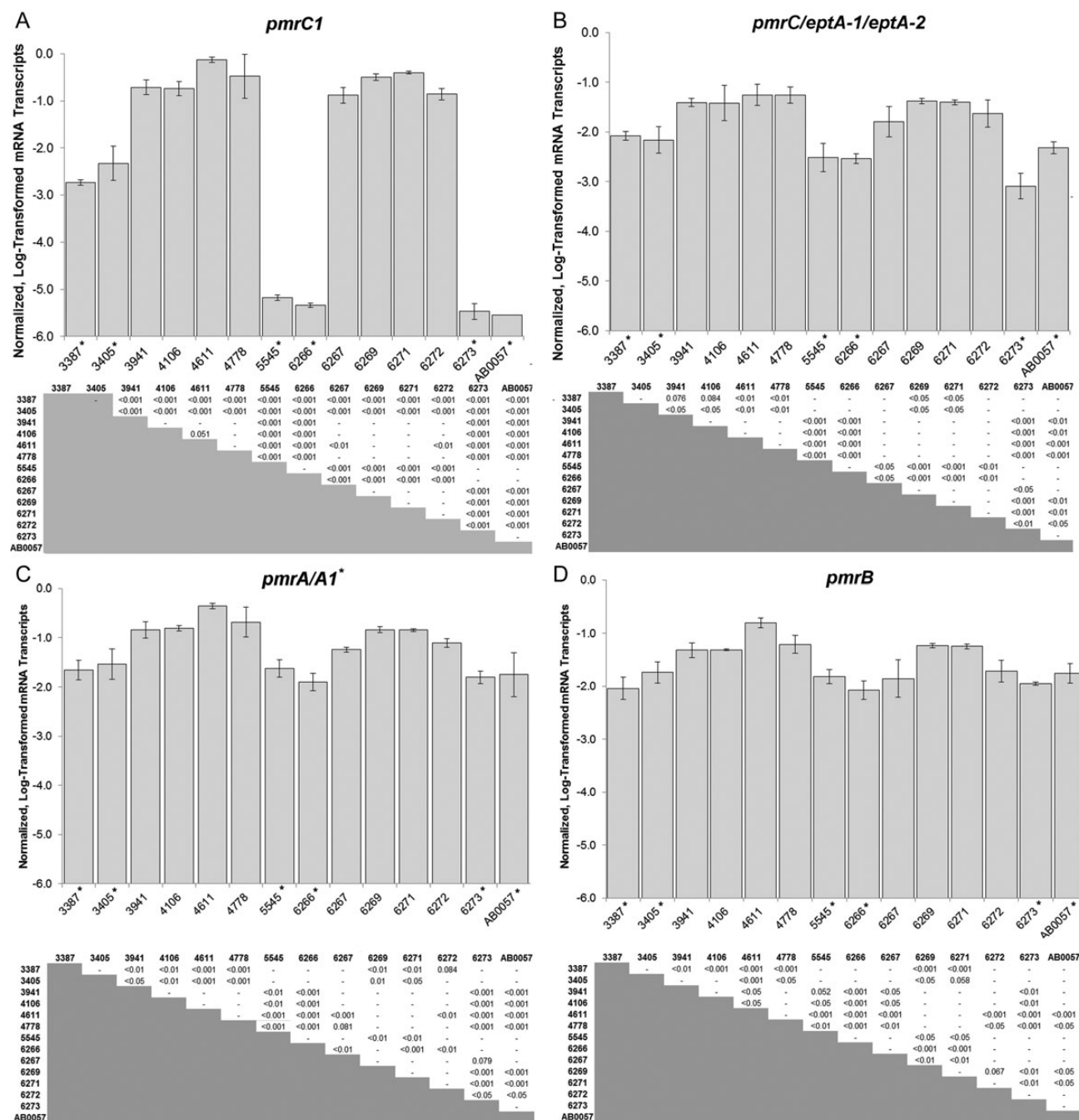
Absolute transcript levels of *pmrC*, *pmrC1*, *pmrA*, *pmrA1*, *pmrB*, *eptA-1*, and *eptA-2* were measured in 13 clinical isolates

**Table 3. Nucleotide and Amino Acid Substitutions in Colistin-Resistant Isolates**

MRSN	Patient	<i>pmrA1</i>	PmrA1	<i>pmrB</i>	PmrB
3941	1	...	...	T51A	S17R
3942	1	...	...	T51A	S17R
4106	3	...	...	C695T	T232I
4112	4	...	...	C695T	T232I
4118	5	...	...	C695T	T232I
4119	3	...	...	C695T	T232I
4611	6	...	...	G788T	R263L
4778	2	...	...	C695T	T232I
6267	7	A24T	E8D	...	...
6268	7	A24T	E8D	...	...
6269	7	A24T	E8D	T346C	Y116H
6270	7	A24T	E8D	...	...
6271	7	A24T	E8D	T346C	Y116H
6272	7	A24T	E8D	...	...



**Figure 2.** Phylogenetic analysis of outbreak strains. A, Unrooted phylogenetic tree of 21 outbreak strains based on 18 high-quality and unambiguous single-nucleotide polymorphisms (SNPs), including *pmrC1A1B* mutations, identified by whole-genome sequencing. B, An unrooted tree based on the 13 high-quality and unambiguous SNPs that lie outside the *pmrC1A1B* locus. The branching pattern of each tree indicates inferred evolutionary relationships among strains. Branch length is proportional to the number of SNPs between strains. The scale bar represents the mean number of nucleotide changes per base. Label color indicates the patient (black, patient 1; green, patient 2; purple, patient 3; orange, patient 4; blue, patient 5; magenta, patient 6; and red, patient 7). Colistin-resistant isolates are indicated by an asterisk.



**Figure 3.** Transcription analysis of *pmrA*, *pmrA1*, *pmrB*, *pmrC1*, *pmrC*, *eptA-1*, and *eptA-2*. Normalized, log-transformed messenger RNA (mRNA) levels from *pmrC1* (A), *pmrC/eptA-1/eptA-2* (B), *pmrA/A1* (C), and *pmrB* (D) from 5 colistin-susceptible clinical isolates, 8 colistin-resistant clinical isolates, and *Acinetobacter baumannii* AB0057. Data represent the average from 3 independent experiments; error bars represent 1 SD. One-way analysis of variance with the Tukey multiple-comparison correction was used to determine whether transcript levels from a gene differed among the strains. The results of statistical analysis in all multiple comparisons are tabulated below each graph. The hyphens indicate that the transcript levels did not differ significantly ( $P \geq .05$ ). \*Primers for *pmrC* were complementary to *eptA-1* and *eptA-2*. Data represent the combined transcript levels for *eptA-1* and *eptA-2* in ST94 strains and *pmrC* transcript levels in *A. baumannii* AB0057 only. Primers for *pmrA* cannot amplify from *pmrA1* and vice versa. *pmrA* transcript levels in *A. baumannii* AB0057 have been added to the *pmrA1* transcript data to facilitate direct comparisons.

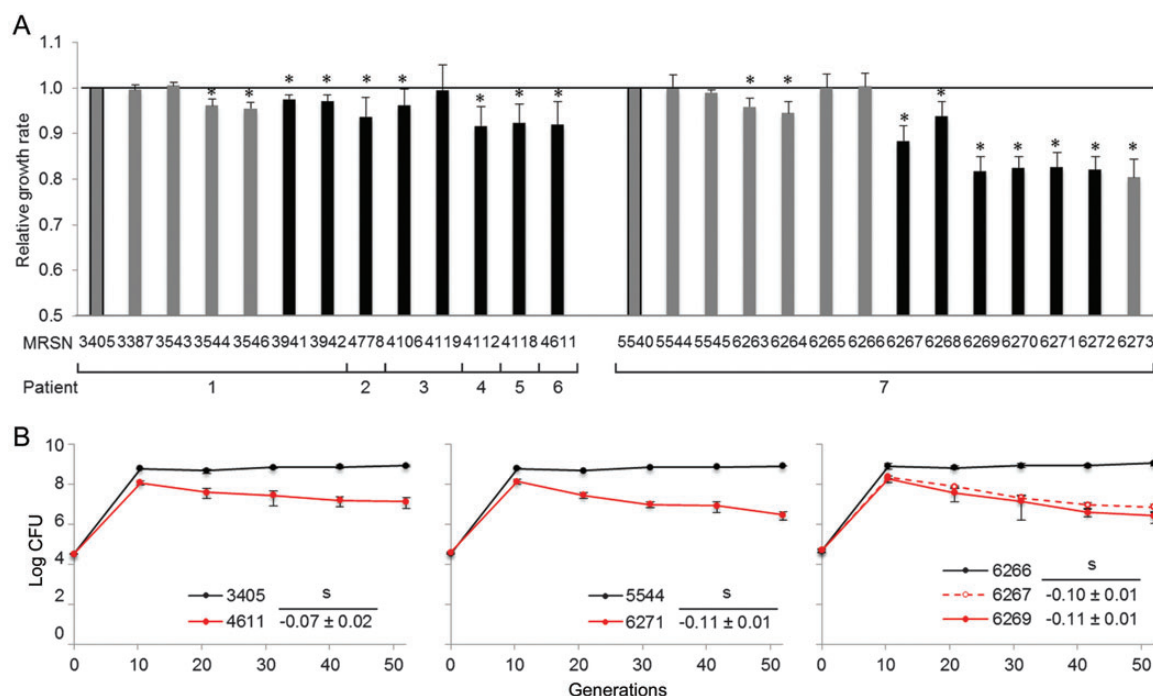
and *A. baumannii* AB0057 by quantitative real-time PCR (Figure 3). Transcripts from *pmrC1A1B* varied considerably within the same strain. Of interest, transcript levels of *pmrA1*

and *pmrB* averaged 10-fold higher than those from *pmrC1* in colistin-susceptible strains (Figure 3), suggesting that, although these genes form an operon, there are additional regulatory

mechanisms that exert a more subtle transcription control of each of the genes. This additional regulation is also observable in AB0057, where *pmrA* and *pmrB* transcripts were 4–8-fold more abundant than those of *pmrC* (Figure 3). It is important to note that in the colistin-susceptible isolates, transcript levels of *eptA-1* and *eptA-2*, unlike those of *pmrC1*, did not differ significantly from those of *pmrA1* and *pmrB*, providing further evidence for additional regulatory elements controlling *pmrC1A1B* expression. Transcription of all genes was significantly higher in colistin-resistant strains, compared with their susceptible counterparts (Figure 3), and was unaffected by the presence of colistin in the growth medium (data not shown). Transcription from *pmrC1* was up to 800-fold higher in colistin-resistant strains, compared with their colistin-susceptible counterparts. Expression of *eptA-1* and *eptA-2* was also significantly enhanced in resistant strains (Figure 3). These results support the hypothesis that both *eptA-1* and *eptA-2* are at least partially controlled by the PmrAB 2-component regulatory system and may play a role in colistin resistance.

Whether the acquisition of antibiotic resistance affects fitness can be determined by measuring growth rates [22, 23].

Growth rates of isolates from cluster 1, relative to that of susceptible MRSN 3405, began to decrease progressively at week 3, whereas the strains remained colistin susceptible. However, the greatest decrease (8% fitness loss) was observed for colistin-resistant strains isolated from patients 2, 4, 5, and 6 (Figure 4). In cluster 2, colistin-susceptible isolates, except for MRSN 6263 and MRSN 6264, which had a 4% fitness loss, had growth rates similar to that of the first colistin-susceptible isolate from patient 7. Strains with a single mutation, E8D in PmrA showed approximately 10% reduced fitness. Strain MRSN 6269, with mutations E8D in PmrA and Y116H in PmrB, had a greater fitness decrease of 18%. All colistin-resistant strains subsequently isolated from patient 7 displayed similar reductions in fitness. For a more sensitive evaluation, in vitro competition experiments were performed between closely related colistin-susceptible and colistin-resistant isolates: MRSN 3405 versus MRSN 4611, MRSN 5544 versus MRSN 6271, and MRSN 6266 versus MRSN 6267 (harboring 1 mutation, E8D in PmrA1) or MRSN 6269 (with mutations E8D in PmrA and Y116H in PmrB; Figure 4B). In all cases, the resistant isolate displayed a marked competitive disadvantage.



**Figure 4.** Evaluation of fitness. *A*, Growth rates of 13 colistin-susceptible strains (gray bars) and 14 colistin-resistant strains (black bars) were determined at the beginning of the exponential phase. The relative growth rate represents the ratio of growth of every strain from patient 1 to 6 to that of MRSN (Multidrug-Resistant Organism Repository and Surveillance Network) 3405 or from patient 7 to MRSN 5540 (taken as 1). The values are the means for 3 cultures from 5 independent experiments with error deviations. \*Significant difference  $P < .01$  of the mean value. *B*, For competition, colistin-susceptible strains (black dots) were cocultured at a 1:1 ratio with resistant isolates (red dots) over 5–6 cycles.  $\log_{10}$  colony-forming units (CFU) of the resistant and susceptible populations at the end of each transfer (approximately 10 generations) were plotted against the number of generations. The selection coefficient(s) was calculated as the slope of the linear regression model  $\ln(CI)/\ln(d)$ , where  $CI$  is the CFU ratio of the resistant and susceptible population at  $t_1$  divided by the same ratio at  $t_0$ , and  $d$  is the dilution factor. The data represent the mean number of CFU ( $\pm$ SD) in 2 independent cultures from 2–3 independent experiments.

## DISCUSSION

Active surveillance of multidrug-resistant organisms provided the opportunity to study the evolution of colistin resistance in a longitudinal series of *A. baumannii* isolates collected from patients undergoing antibiotic therapy. The *A. baumannii* outbreak strain was unrelated to known pulse types of *A. baumannii* and belonged to the rare multilocus sequence type ST94, which has been reported only twice to date [19, 20]. Whole-genome phylogenetic analysis suggests that all isolates were closely related and represent the expansion of a single colistin-susceptible strain.

Colistin had been used since 2003 at the facility where this outbreak occurred. Based on a query of the database of microbiology results, there were no instances of colistin-resistant *A. baumannii* isolated from clinical infections from 2003 to 2012. Furthermore, there are no published reports of colistin-resistant *Acinetobacter* within the Military Health System during this period. While it is tempting to suggest that the *A. baumannii* strain described herein has a propensity to develop colistin resistance more rapidly than strains carrying the canonical *pmrCAB* operon, we were unable to demonstrate this in vitro (data not shown). However, it is noteworthy that the compound used for in vitro testing (colistin sulfate) differs from the prodrugs used in vivo (colistin methanesulfonate or colistimethate compounds) [24]. Various factors affect the rate and amount of prodrug conversion in the body. Therefore, the results of in vitro exposure assays may not be generalizable to an organism that encounters evolving colistin concentrations in vivo.

Isolates carried a novel allele of the *pmrCAB* gene cluster, designated *pmrC1A1B*. *PmrB* was identical to *pmrB* in *A. baumannii* AB0057, but both *pmrC1* and *pmrA1* contained nonsynonymous mutations, compared with the *pmrC* and *pmrA* genes from reference strains.

An analysis of complete and draft genome sequences of *A. baumannii* demonstrated that *pmrC1* is unique to our isolates. The absence of transposable elements in the surrounding region argues against horizontal transfer and suggests that this allele likely diverged from the majority allele through evolutionary pressure. In a random sample of 110 *A. baumannii* clinical isolates collected by our surveillance network, >10% carried *pmrC1*. Although this result indicates that *pmrC1* is relatively common in *A. baumannii*, it is important to note that current biochemical testing cannot distinguish between *A. baumannii*, *A. nosocomialis*, and *Acinetobacter pittii*. Studies suggest that the latter 2 strains can account for 10%–40% of all clinical isolates identified as *A. baumannii* [25–27], and these *pmrC1*-carrying isolates may belong to these species.

All colistin-resistant isolates carried mutations in the *pmrC1A1B* operon. Four mutations were identified in *PmrB* (S17R, T232I, R263L, and Y116H), and 1 was identified in *PmrA1* (E8D). The *PmrB* changes have not yet been described (although R263P and R263C were reported by Arroyo et al [11]).

The S17R and Y116H *PmrB* substitutions occurred outside the histidine-kinase domain and may influence the interactions between *PmrA* and *PmrB*. Mutations in this region of the protein, including S119T, S14L, and T13N, have been identified in colistin-resistant strains [10–12]. The E8D substitution in *PmrA1* is identical to a *PmrA* mutation in a colistin-resistant *A. baumannii* clinical isolate [28].

The data suggest that multiple independent colistin resistant clones arose from the initial susceptible strain, represented by MRSN 3405, during therapy. Isolates from patients 1, 2, and 3 were closely related, based on high-quality SNPs, but acquisition of colistin resistance occurred through different mutations. In contrast, MRSN 4611 from patient 6 showed evolutionary divergence from MRSN 3405. MRSN 4611 shares 2 high-quality SNPs that are found in all cluster 2 isolates, suggesting that this strain and the isolates from the second outbreak had a common progenitor descended from the founder *A. baumannii* clone. Because the earliest isolates from cluster 2 are colistin susceptible, the parental strain from cluster 1 may have reverted to colistin susceptibility in the absence of selective pressure before it reemerged 3 months later in patient 7. When this patient underwent colistin therapy, *A. baumannii* became drug resistant via new *pmrA1* and *pmrB* mutations. It should be noted that the 18 SNPs we used for phylogenetic analysis may not represent the complete set of genomic differences among the outbreak strains. We are currently producing a finished genome of the initial isolate, which will serve as reference sequence for identifying all SNPs, indels, and rearrangements that arose during the in vivo evolution of the outbreak strain.

Importantly, because bacteria from clinical samples are cultured from single, pure colonies, subpopulations of *Acinetobacter* with various mutations in *pmrA* and *pmrB* could coexist in the same patient and go undetected. We were able to identify multiple mutations in the outbreak isolates by collecting serial samples from patients over an extended period.

In addition to the variant *pmrC1* gene, the outbreak strain had 2 *pmrC* homologs, *eptA-1* and *eptA-2*. These genes have >95% identity with canonical *pmrC* from *A. baumannii* AB0057 but <90% identity with *pmrC1*. Prophages are located in the vicinity of both genes in all strains, and *eptA-1* lies 900 bp upstream from a putative integrase gene. This provides tantalizing evidence that both genes may have been horizontally acquired or duplicated within the ancestral strain. *eptA-1* and *eptA-2* showed significantly enhanced expression in colistin-resistant strains, but because the real-time primers cannot discriminate between the genes, expression data presumably reflect transcription of both genes. These data support the hypothesis by Arroyo et al that additional copies of *pmrC* might be found in 2 of the clinical isolates from their study, based on the observation that, compared with the reference strain, both strains showed approximately 6-fold higher transcription from *pmrC* when *pmrB* was knocked out [11].



A comprehensive search for these genes in full and complete draft genomes of *A. baumannii* demonstrated that 6 of the 11 complete genome sequences contain *eptA-2* and that 1 also contains *eptA-1*. Furthermore, examination of 116 draft genomes shows that approximately 20% of *A. baumannii* may possess, in addition to *pmrC*, 2 *eptA* homologues and that 4% might harbor  $\geq 3$ . The existence of the third or fourth *eptA*-related gene is based on an incomplete gene sequence, so uncertainties remain. The potential contribution of *EptA-1* and *EptA-2* to colistin resistance needs further investigation because the evidence we provide here is circumstantial. Unraveling the role of these proteins may provide a greater understanding of the role of phosphoethanolamine modification of lipid A in polymyxin resistance.

Colistin-resistant clinical isolates displayed variable levels of fitness loss, with a moderate reduction in growth rates associated with resistance in the initially isolated strains. This might have allowed the resistant phenotype to emerge. However, in later strains isolated from the same patient, the cost of resistance was high. Competition was performed over 5 cycles of growth and with stably resistant clones, demonstrating a clear competitive disadvantage associated with this type of colistin resistance. There was no evidence to support the notion that colistin-resistant isolates suffered compensatory mutations that increased virulence or fitness [28, 29]. On the contrary, there was increasing loss in fitness in successive colistin-resistant strains. Furthermore, colistin-resistant strains were recovered only once from patients 2, 3, 4, 5, and 6, suggesting that such strains were unable to colonize or invade these patients.

Unlike previous reports [29–31], colistin resistance in these isolates was not associated with increased susceptibility to other antibiotics, including vancomycin (sometime referred to as the seesaw effect) [32]. All colistin-resistant *A. baumannii* infections were successfully treated with extended infusion of various combinations of tigecycline, minocycline, and/or colistin, and/or high-dose carbapenems. The isolates were also resistant to all aminoglycosides currently available in the United States but had low MICs of arbekacin, an aminoglycoside used in Japan to treat methicillin-resistant *Staphylococcus aureus* that shows promising activity against multidrug resistant gram-negative bacteria [33].

The relationship between our surveillance network and physicians, microbiologists, and infection preventionists enables collection of all organisms isolated from a patient. Typically, subsequent isolates are referred to the first or an earlier specimen from the same anatomic location. In one patient, detection of the emergence of colistin resistance would have been delayed using this customary referral approach. Following identification of colistin resistance, infection-control measures were further intensified. However, for combating antibiotic resistance due to mutational events, reducing or eliminating selection pressure is just as important as intensification of transmission-based precautions.

In summary, our findings underscore the genomic fluidity of *A. baumannii* and highlight the value of combining active surveillance with translational research. This capability permits longitudinal surveillance of clinical isolates and lends insight into their adaptive evolution under antibiotic pressure. Finally, our findings support calls for better antibiotic stewardship because colistin, an antibiotic that until recently had fallen into disuse owing to nephrotoxicity, is now a therapeutic mainstay in patients for whom there are no alternatives.

## NUCLEOTIDE SEQUENCES

Sequences of wild-type and mutant alleles of the *pmrC1A1B* operon have been submitted to GenBank under accession numbers KC009850–KC009855. The *eptA-1* and *eptA-2* sequences were submitted as accession numbers KC161249 and KC161248, respectively. The corresponding genetic context of *eptA-1* and *eptA-2* have been submitted separately to GenBank as accession numbers (KC700024, *eptA-1* context; KC700023, *eptA-2* context).

## Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (<http://jid.oxfordjournals.org/>). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

## Notes

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