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Multiple Genetic Mutations Associated with Polymyxin Resistance in *Acinetobacter baumannii*

Tze Peng Lim,[a,b] Rick Twee-Hee Ong,[c] Pei-Yun Hon,[a] Jane Hawkey,[d] Kathryn E. Holt,[d] Tse Hsien Koh,[b] Micky Lo-Ngah Leong,[a] Jocelyn Qi-Min Teo,[b] Thean Yen Tan,[e] Mary Mah-Lee Ng,[a] Li Yang Hsu[a,c]

Yong Loo Lin School of Medicine, National University Health System, Singapore[a]; Singapore General Hospital, Singapore[b]; Saw Swee Hock School of Public Health, National University Health System, Singapore[c]; The University of Melbourne, Melbourne, Australia[d]; Changi General Hospital, Singapore[e]

We studied polymyxin B resistance in 10 pairs of clinical *Acinetobacter baumannii* isolates, two of which had developed polymyxin B resistance *in vivo*. All polymyxin B-resistant isolates had lower growth rates than and substitution mutations in the *lpx* or *pmrB* gene compared to their parent isolates. There were significant differences in terms of antibiotic susceptibility and genetic determinants of resistance in *A. baumannii* isolates that had developed polymyxin B resistance *in vivo* compared to isolates that had developed polymyxin B resistance *in vitro*.

Acinetobacter baumannii is a Gram-negative bacillus (GNB) whose strains are increasingly extensively drug resistant (XDR) due to their wide repertoire of antimicrobial resistance mechanisms and ability to acquire new resistance determinants (1–3). Polymyxins are currently the last-line therapeutic option for the treatment of XDR *A. baumannii* infections. Although surveillance data suggest that <1.0% of current *A. baumannii* strains are resistant to polymyxins (4, 5), clinical treatment failures have been reported. *A. baumannii* primarily acquires resistance to polymyxins by alterations to or complete loss of lipopolysaccharide (LPS) (8, 9) mediated via activation of two-component regulatory systems that cause the constitutive activation of LPS-modifying genes (10, 11). Polymyxin-resistant mutants developed *in vitro* appear to exhibit increased susceptibility to various other classes of antimicrobial compounds but do not necessarily exhibit a corresponding decrease in virulence (12).

We undertook this study to investigate the genetic causes of polymyxin resistance and cell wall physical characteristics in *in vivo*- and *in vitro*-derived polymyxin-resistant XDR *A. baumannii* strains via whole-genome sequencing and electron microscopy and also to assess the biological costs of acquisition of polymyxin resistance.

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Detailed methodology descriptions are provided in the supplemental material. In brief, 10 epidemiologically unrelated clinical XDR *A. baumannii* isolates obtained between 2006 and 2009 were selected. Two XDR *A. baumannii* isolates (isolates 1 and 2) had acquired polymyxin resistance *in vivo*. The other eight clinical isolates (isolates 3 to 10) were polymyxin B sensitive, with polymyxin B-resistant mutants generated *in vitro* via passaging on polymyxin B-impregnated Mueller-Hinton agar (MHA) plates at 3 times the MIC for 20 cycles.

MICs of a broad panel of antibiotics (Table 1) were obtained by broth microdilution for all pairs of isolates, with rifampin MICs obtained by a modified broth macrodilution method according to CLSI guidelines (13). The polymyxin B-resistant mutants were subjected to a further 20 days of passaging on drug-free and polymyxin B-impregnated MHA, with susceptibility testing repeated at 10- and 20-day time points to determine the stability of resistant phenotypes.

In vitro growth rates were determined for all isolates, and the exponential growth of the bacterial population over 24 h was analyzed using an adapted mathematical model (14).

The paired polymyxin-susceptible and -resistant isolates were sequenced on an Illumina HiSeq2000 platform, assembled *de novo* using VelvetOptimiser software (https://github.com/tseemann/VelvetOptimiser), and oriented with respect to a finished reference *A. baumannii* genome (NC_017162.1) with Mauve software (15). Gene annotation was performed using PROKKA (16). *In silico* multilocus sequence typing (MLST) was used for predictions for each isolate with the MLST 1.7 online software tool (https://cge.cbs.dtu.dk/services/MLST/), selecting the Institut Pasteur MLST scheme (17).

For each XDR *A. baumannii* lineage, the sequenced reads for both polymyxin-susceptible and polymyxin-resistant isolates were mapped to the draft genome assembly of the susceptible isolate by the use of BWA-MEM (18). Single-nucleotide polymorphisms (SNPs) and small insertions and deletions (indels) were subsequently identified with SAMtools (19) and annotated using SnpEff software (20).

To identify insertion sequences (IS) that differed between the isolates in each pair, we screened all draft genome assemblies to identify every IS present using the ISFinder database (21), determining the presence or absence of these sequences in each isolate read set using ISMapper (22).

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Address correspondence to Li Yang Hsu, liyang_hsu@yahoo.com.

T.P.L. and R.T.-H.O. contributed equally to this article.

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TABLE 1 Antibiotic susceptibility testing results for pre- and post-polymyxin B-exposure *Acinetobacter baumannii calcoaceticus* complex clinical isolates

Antibiotic(s)	MIC(s) (mg/liter)[a]									
	Isolate 1	Isolate 2	Isolate 3	Isolate 4	Isolate 5	Isolate 6	Isolate 7	Isolate 8	Isolate 9	Isolate 10
Ampicillin-sulbactam	≥128/64 (64/32)	≥128/64 (32/16)[b]	64/32 (1/0.5)[b]	64/32 (8/4)[b]	64/32 (8/4)[b]	16/8 (4/2)[b]	16/8 (4/2)[b]	32/16 (8/4)[b]	≥128/64 (8/4)[b]	64/32 (16/8)
Piperacillin-tazobactam	≥256/4	≥256/4	≥256/4 (≤1/4)[b]	≥256/4 (≤1/4)[b]	≥256/4 (≤1/4)[b]	≥256/4 (≤1/4)[b]	≥256/4 (≤1/4)[b]	≥256/4 (≤1/4)[b]	≥256/4 (≤1/4)[b]	≥256/4 (≤1/4)[b]
Ceftazidime	≥128	≥128	≥128 (≤0.5)[b]	≥128 (8)[b]	≥128 (4)[b]	≥128 (0.5)[b]	≥128 (2)[b]	≥128 (2)[b]	≥128 (≤0.5)[b]	≥128 (16)[b]
Cefepime	≥64	≥64	≥64 (1)[b]	≥128 (1)[b]	≥128 (2)[b]	≥128 (≤0.5)[b]	≥128 (≤0.5)[b]	≥128 (1)[b]	≥128 (≤0.5)[b]	≥128 (4)[b]
Doripenem	≥16	≥16	≥16	≥16 (≤0.06)[b]	≥16 (0.25)[b]	≥16 (0.12)[b]	≥16 (0.25)[b]	≥16 (0.12)[b]	≥16 (0.12)[b]	≥16 (0.25)[b]
Imipenem	≥64 (32)	≥64	32 (1)[b]	16 (0.25)[b]	≥64 (1)[b]	32 (0.25)[b]	32 (0.5)[b]	32 (0.5)[b]	32 (0.5)[b]	32 (0.5)[b]
Meropenem	≥64 (32)	≥64	≥64 (0.5)[b]	16 (≤0.12)[b]	≥64 (0.5)[b]	32 (≤0.12)[b]	32 (≤0.12)[b]	32 (≤0.12)[b]	≥64 (≤0.12)[b]	≥64 (≤0.12)[b]
Aztreonam	32	≥128	≥128 (1)[b]	≥128 (1)[b]	≥128 (4)[b]	≥128 (≤0.5)[b]	≥128 (≤0.5)[b]	64 (4)[b]	≥128 (≤0.5)[b]	≥128 (4)[b]
Gentamicin	≥64	≥64	≥64	≥64 (8)[b]	≥64 (16)[b]	≥64 (≤0.25)[b]	≥64 (0.5)[b]	≥64	≥64 (8)[b]	≥64 (8)[b]
Amikacin	≥128	≥128	≥128 (64)	≥128 (64)	≥128	64 (≤0.1)[b]	64 (≤0.1)[b]	≥128 (≤0.1)[b]	≥128 (32)[b]	≥128 (64)
Ciprofloxacin	≥16	≥16	≥16	≥16 (8)	≥16	≥16	≥16	≥16	≥16 (8)	≥16
Tigecycline	8 (16)	4 (4)	1 (0.25)[b]	8 (1)[b]	4 (0.5)[b]	≥32 (0.5)[b]	≥32 (0.5)[b]	8 (0.5)[b]	8 (0.128)[b]	4 (0.064)[b]
Polymyxin B	2 (≥32)[b]	1 (≥32)[b]	4 (≥32)[b]	0.5 (≥32)[b]	0.5 (≥32)[b]	1 (≥32)[b]	1 (≥32)[b]	0.5 (≥32)[b]	0.5 (16)[b]	0.5 (≥32)[b]

[a] Numbers in parentheses indicate the change in MIC in the post-polymyxin B-exposure isolate.
[b] A 3-fold or greater change in the drug MIC was seen with the post-polymyxin B-exposure isolate.

Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) techniques were employed to visualize and analyze the polymyxin B resistance mechanisms that developed during XDR *A. baumannii* post-polymyxin B exposure.

All preexposure isolates were resistant to all antibiotics tested except polymyxin B (Table 1). Increased susceptibility to other antibiotics was generally observed for the *in vitro* post-polymyxin B-exposure isolates. These phenotypes were stable even after 20 days of passaging. However, the *in vivo*-derived polymyxin B-resistant isolates (isolates 1B and 2B) showed no significant change in drug MIC results.

We identified between 1 and 35 nonsynonymous mutations and addition of insertion sequence (IS) elements within various genes in each post-polymyxin B-exposure isolate (Table 2). None of the mutations were common between the isolates in any pair, but nonsynonymous genetic mutations were identified in the lipid biosynthesis *lpx* genes of four postexposure isolates (6B, 7B, 9B, and 10B), including a premature stop codon in *lpxA* of isolate 9B and a 7-bp sequence insertion resulting in a codon frameshift in *lpxC* of isolate 10B. Four postexposure isolates (3B, 4B, 5B, and 8B) had an IS (ISAba1) within the *lpxA* or *lpxC* gene. Both isolates that developed polymyxin B resistance *in vivo* (isolates 1B and 2B) harbored mutations at the *pmrB* locus. Isolate 2B harbored the greatest number of SNPs, most of which were not in genes associated with cell membrane synthesis or regulation, compared to its sensitive parent isolate.

Representative TEM and SEM images are shown in the figures in the supplemental material. Generally, there were no objective differences between the preexposure and postexposure isolates in TEM. SEM showed that the preexposure isolates had classical smooth and intact surfaces in a diploid structure whereas the polymyxin-B-resistant mutants were more compact in appearance and had multiple dents or craters on their surfaces.

The best-fit growth rate constants for the post-polymyxin B-exposure isolates were lower than those measured for the respective parent isolates (Fig. S3 in the supplemental material), suggesting lower growth rates and lower fitness.

We found significant differences between the isolates based on how the polymyxin resistance was derived, although the number tested was too small for definite conclusions to be reached. The laboratory-induced polymyxin B-resistant isolates became more susceptible to other classes of antibiotics than the preexposure isolates (9, 12), but the isolates that developed resistance *in vivo* did not exhibit this phenomenon, consistent with other case reports (23–25).

We identified two insertions of IS*15* in the *mutS* gene in clinical polymyxin-resistant isolate 2B, which harbored an excess of SNPs. Truncation of the *mutS* gene in this isolate may thus explain the large number of SNPs identified in isolate 2B, in similarity to previous reports in *A. baumannii* (26).

Our EM results had demonstrated morphological differentiation suggestive of LPS loss and altered expression of outer membrane proteins between polymyxin B-susceptible and polymyxin B-resistant *A. baumannii* isolates (9, 27). This observation was consistent with published reports of *Escherichia coli* studies where the bacterial cell envelope had been damaged by novel antimicrobial peptides (28).

In conclusion, the more complex selection pressures that occur *in vivo* likely result in polymyxin-resistant mutants that are different from and perhaps more fit than the *in vitro*-derived polymyx-

TABLE 2 Genome-wide mutations found in post-polymyxin B-exposure *Acinetobacter baumannii calcoaceticus* complex clinical isolates with reference to their pre-polymyxin B-exposure counterparts

Isolate (inferred ST)	Gene or gene location[a]	Annotation or product	Mutation	Reference sequence inference/genomic coordinates of IS "hits"
1 (ST-1)	*pmrB*	Two-component sensor kinase signal peptide	P233S	YP_003730963.1
	NA	Signal peptide	L105F	YP_001712355.1
	NA	Family finger-like domain protein	C8W	YP_003731514.1
	pntA-1	Pyridine nucleotide transhydrogenase alpha subunit	T157I	YP_047601.1
	NA	TEM-type beta-lactamase	Codon deletion IPFFAAFCL11I	YP_002317692.1
	ABA1_00178	Laminin-binding surface protein	IS*15* insertion	192134–192126
2 (ST-2)	NA	RND transporter/multidrug resistance efflux pump	R172H	YP_001707896.1
	NA	Glycosyl transferase	S33F	YP_045879.1
	NA	Putative porin protein associated with imipenem resistance	Stop codon Q36*	YP_003731166.1
	pmrB	Two-component sensor kinase	R263H	YP_003730963.1
	NA	Glycosyltransferase	Frame shift E147E	YP_001083155.1
	mutS	DNA mismatch repair protein	IS*15* insertion	1792809–1792801, 1793624–1793633
	Between *papD* and *fimA*	P pilus assembly protein, pilin FimA	ISAba1 insertion	2395586–2395591
3 (ST-2)	NA	Two-component sensor kinase homologous to *pmrB*	R64C	YP_003730836.1
	NA	Phosphatidylserine synthase	P234L	YP_005527355.1
	filE	NA	Codon deletion TAPTAP129−	YP_001845298.1
	lpxA	UDP-N-acetylglucosamine acyltransferase	ISAba1 insertion	2690056–2690049
4 (ST-1)	NA	Outer membrane phospholipase A	Frame shift/base deletion at codon 31 (GT/G)	YP_005526168.1
	NA	RND transporter/multidrug resistance efflux pump	Codon insertion T21TPAPA	YP_001707896.1
	lpxC	N-Acetylglucosamine deacetylase	ISAba1 insertion	3758548–3758539
5 (ST-1)	*lpxC*	N-Acetylglucosamine deacetylase	ISAba1 insertion	3758550–3758559
6 (ST-1)	*lpxD*	UDP-3-O-(3-hydroxymyristoyl) glucosamine N-acyltransferase	S167F	YP_005515126.1
	baeR	OmpR family transcriptional regulator	G60A	YP_045370.1
	NA	DNA polymerase III subunit tau	EP435	YP_002319726.1
7 (ST-1)	*rpsE*	30S ribosomal protein S6	Y80D	YP_047025.1
	lpxA	UDP-N-acetylglucosamine acyltransferase	G56V	YP_003731736.1
8 (ST-1)	NA	Hypothetical protein	Codon insertion D282DDK	WP_000179573
	lpxC	N-Acetylglucosamine deacetylase	ISAba1 insertion	Amino acid positions 155–159
9 (ST-1)	*lpxA*	UDP-N-acetylglucosamine acyltransferase	Stop codon E84*	YP_003731736.1
	NA	Pilus assembly protein FilE	Codon deletion TAPTAPTAP126	YP_001714906.1
	Upstream of *rpoC*	RpoC DNA-directed RNA polymerase, beta′ subunit	ISC1041 insertion	375323–375326
10 (ST-1)	NA	Putative metal transporter	Codon deletion HH171	YP_005523968.1
	lpxC	N-Acetylglucosamine deacetylase	Frame shift with a 7-bp insertion resulting in incomplete codon insertion (Q252QSS)	YP_047978.1
	NA	Peptidase M16C-associated family protein	ISAba125/ISC1041 insertion	2202796–2202801
	NA	Hypothetical protein	ISAba125 insertion	3016296–3016300
	NA	Penicillin-binding protein 1A	ISAba1 insertion	3618404–3618395

[a] NA, not available; RND, resistance-nodulation-division.

in-resistant mutants (12, 23–25), suggesting that current *in vitro*-derived polymyxin-resistant *A. baumannii* mutants are relatively poor surrogates for the study of polymyxin resistance and its impact on virulence or biofitness.

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