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Retention of virulence following adaptation to colistin in *Acinetobacter baumannii* reflects the mechanism of resistance

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Objectives: Colistin resistance in *Acinetobacter baumannii* has been associated with loss of virulence and a negative impact on isolate selection. In this study, exposure of clinical isolates to suboptimal concentrations of colistin was used to explore the capacity to develop resistance by diverse mechanisms, and whether acquired resistance always reduces fitness and virulence.

Methods: Twelve colistin-susceptible clinical *A. baumannii* isolates were exposed to a sub-MIC concentration of colistin over 6 weeks with weekly increases in concentration. Stable resistance was then phenotypically investigated with respect to antibiotic/biocide resistance, virulence in *Galleria mellonella* and growth rate. Putative mechanisms of resistance were identified by targeted sequencing of known resistance loci.

Results: Eight *A. baumannii* isolates acquired resistance to colistin within 1 week with MICs ranging from 2 to >512 mg/L. By 6 weeks 11 isolates were resistant to colistin; this was linked to the development of mutations in *pmr* or *lpx* genes. Strains that developed mutations in *lpxACD* showed a loss of virulence and increased susceptibility to several antibiotics/disinfectants tested. Two of the colistin-resistant strains with mutations in *pmrB* retained similar virulence levels to their respective parental strains in *G. mellonella*.

Conclusions: Acquisition of colistin resistance does not always lead to a loss of virulence, especially when this is linked to mutations in *pmrB*. This underlines the importance of understanding the mechanism of colistin resistance as well as the phenotype.

Keywords: *Acinetobacter baumannii*, colistin adaptation, *Galleria mellonella*, antibiotic resistance

Introduction

Acinetobacter baumannii is a Gram-negative pathogen of increasing importance associated with nosocomial infections. The burden of *A. baumannii* disease is complicated by isolates being resistant to many different classes of antibiotics (MDR). In 2011, over 25% of all reported clinical *A. baumannii* infections in the UK were resistant to more than three frontline antibiotics, with resistance to carbapenems increasing rapidly.[1–3] Many new drug resistance mechanisms such as bla_{NDM-1} have already been found in *Acinetobacter*, which has left an over-reliance on older drugs, such as the polymyxins.[4,5] In several cases, polymyxins are the only effective antibiotics available for treatment of *A. baumannii* infections. However, resistance is on the increase with high levels of colistin-resistant strains now frequently isolated in South America, Asia and Eastern Europe.[6–8] There have also been incidents of isolates that are now characterized as pan-drug resistant.[9]

Colistin (polymyxin E) is a bactericidal antibiotic and functions by the interaction of a cationic peptide (colistin) with the lipid A moiety of the anionic LPS and displacement of bacterial counter ions [magnesium (Mg^{2+}) and calcium (Ca^{2+})] that normally stabilize the negatively charged LPS molecules. This leads to a rapid increase in the permeability of the outer membrane and subsequent leakage of cellular contents and, therefore, cell death.[10,11]

Resistance to colistin across Gram-negative bacteria is commonly associated with alterations in the bacterial outer membrane. These may include reduction in LPS, reduced amounts of Mg^{2+} and Ca^{2+} in the cell envelope and alteration of lipid levels. In *A. baumannii*, mutations in the two-component regulatory system PmrAB have provided the focus for acquired colistin resistance. Genetic changes in *pmrA* and *pmrB* have been shown to lead to an increase in colistin resistance in *A. baumannii* and other Gram-negative MDR organisms, e.g. *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*.[12–14] Changes in this operon result in lipopolysaccharide remodelling with the replacement of lipid A with 4-amino-4-deoxy-L-arabinose and/or phosphoethanolamine, leading to a loss of negative charge on the bacterial outer membrane. This, in turn, leads to a decreased affinity for the binding of colistin.[15,16]

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Other genetic changes known to affect colistin susceptibility are mutations in the lipid biosynthesis genes, *lpxA*, *lpxC* or *lpxD*,[17] which may result in the production of a colistin-resistant strain that is LPS deficient.[18]

It has been well documented that acquisition of resistance is associated with a fitness cost, especially with modifications that alter the integrity of the cellular membrane.[19] Colonization with colistin-resistant *A. baumannii* has been reported without any obvious signs of clinical infection,[20] and there are examples where colistin resistance has led to impaired virulence of *A. baumannii*.[21–23] The same group also reported isolating a clinical strain of *A. baumannii* that developed resistance after antibiotic treatment with colistin, but retained its infecting capacity.[24] Therefore, it is unclear what the biological fitness cost, if any, is after acquisition of colistin resistance.

In the present study we have generated colistin-resistant *A. baumannii* strains and used the invertebrate infection model *Galleria mellonella* to show that acquired resistance to colistin does not necessarily lead to loss of virulence and fitness as measured by growth rate.

Methods

Bacterial strains and culture conditions

The WT *A. baumannii* strains used in this study have been described previously and include representatives of international clones I–III.[25] The exceptions are the clinical isolates TDG1 and TDG2 and the environmental isolate NTU1264. All strains were grown in tryptic soy broth (TSB) with aeration or on tryptic soy agar (TSA) plates at 37°C unless otherwise stated.

Determination of MICs

The MICs of various antibiotics and disinfectants for *A. baumannii* strains were determined using a broth microdilution method with a starting inoculum of 1×10^5 cfu/mL. The OD_{600} was measured after 20 h of static incubation at 37°C and the MIC was defined as the lowest concentration of antibiotic or disinfectant at which no bacterial growth was observed.

Continuous exposure to colistin

A. baumannii strains were grown at an initial concentration of 0.25 mg/L colistin in 3 mL of TSB, 250 rpm, 37°C. Every other day cultures were passaged into fresh TSB with the same concentration of colistin. Each week, cultures were frozen for later analysis and the MIC of colistin was determined for all strains and, regardless of the MIC value, the concentration of colistin was doubled, reaching a final concentration of 8 mg/L after 6 weeks. Colistin-resistant strains were passaged on TSA plates 10 times and reassessed for stability of the mutation.

Sequencing of lpxACD and pmrAB

lpxA, *lpxC*, *lpxD*, *pmrA* and *pmrB* from *A. baumannii* strains were amplified using the following primers: ABlpxAregAS (5′-CAATATCCAAAGTCTGAAGAAGC-3′), ABlpxAregS (5′-TGATGGCATTGTAGCAGCAAC-3′), ABlpxC seq1 (5′-GTTACAAAGCATGGTGAAAC-3′), ABlpxC seq1R (5′-TGACTTATGTCACACTCACG-3′), ABlpxDseq1 (5′-ATGAAAGTGCAACAATATCG-3′), ABlpxDseq1R (5′-CTATTTACGCAAATTAAAG-3′), ABqseBregS (5′-GTTTGCTGGATGTAAAAACTCAGG-3′), ABqseBregAS (5′-GATGACACTGAAAATTGAGGTGC-3′) and qseCregAS(5′-CATAACTTATGGACAGGCTGG-3′), qseCregS (5′-ATGTTCACCATTTAAGAGCG-3′) or 2750-F, 2750-R.[26] They were sequenced at Beckmann Genomics (Takeley, UK) using the same primers and additional primers qseC528s (5′-TGAGTTAAAAGAACGCGATTC-3′) and qseC728as (5′-GGTTTGTAAGTTCAATGCAGT C-3′) for *pmrB*. Sequences were analysed using DNASTAR Lasergene 10. To predict whether amino acid substitutions in PmrAB and LpxACD affect protein function, Sorting Intolerant From Tolerant (SIFT) scores were calculated (http://sift.jcvi.org).

G. mellonella killing assays

Wax moth larvae (*G. mellonella*) were purchased from Livefood UK Ltd (Rooks Bridge, Somerset, UK) and were maintained on wood chips in the dark at 14°C. *Galleria* were stored for not longer than 2 weeks. Bacterial infection of *G. mellonella* was as described previously.[27]

Statistical analyses

Unpaired two-tailed Student's *t*-tests were performed for the statistical analysis. All analysis was performed on three or more independent experiments using Graphpad Prism Software.

Results

Generation and stability of colistin resistance

It was possible to generate increased resistance to colistin for *A. baumannii* by the continuous culturing of cells in increasing concentrations of colistin in all isolates except strain NTU1264, which died after <1 week of exposure (Table S1, available as Supplementary data at *JAC* Online). The MICs for the colistin-resistant isolates after 6 weeks ranged from 32 to >512 mg/L with most strains showing resistance to ≥256 mg/L. The rate of acquisition of high levels of colistin resistance differed between strains with most showing a gradual increase in resistance. However, some strains (UKA2, NCTC 13424) acquired high levels of colistin resistance (>128 mg/L) after just 1 week of exposure. The exposure experiment was repeated to ascertain whether similar patterns of resistance for each strain emerged. Although all strains again became resistant to colistin, the rate at which resistance was generated in individual strains differed between the two experiments (data not shown). All MICs of colistin for week 6 isolates were confirmed by performing broth microdilution MIC analysis in Mueller–Hinton broth. All values were found to be identical or within a 2-fold difference from those obtained in TSB.

To understand whether the mechanisms that led to a decrease in susceptibility to colistin were stable, each strain was passaged at least 10 times in the absence of colistin and then the MIC of colistin was rechecked. The MIC for all strains was found to be the same as before passaging, which showed that the changes leading to colistin resistance were stable (data not shown). To determine whether a clonal population had been generated, the 6 week broth cultures were streaked out and six colonies from several colistin-resistant strains were isolated and tested for colistin susceptibility. Colistin resistances as measured by MIC for all colonies from each strain were found to be identical (data not shown).

Antibiotic/disinfectant MICs for colistin-resistant mutants

Several strains (ATCC 17978, NCTC 13424, W1, AYE, UKA2, UKA8 and UKA18) from the initial exposure experiments were subjected to further analysis. Resistance to several antibiotics was

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investigated and, since resistance to colistin results from changes to the cell membrane that might influence disinfectant and antiseptic resistance, the MICs of many clinically relevant disinfectant compounds and formulations were also determined (Table 1). With the obvious exception of colistin, mutants showed increased susceptibility to several classes of antibiotic. Increased susceptibility was observed in some strains for topical antiseptics, particularly benzalkonium chloride. There was also increased susceptibility to several surface disinfectants with reduced levels of tolerance to phenol-based and cationic disinfectants. The exception was strain UKA8, with colistin adaptation leading to little or no change in susceptibility to these disinfectants. Susceptibility to H_2O_2, peracetic acid, H_2O_2/peracetic acid-based disinfectant, H_2O_2-based disinfectant and chlorine-based disinfectant showed little or no change as assessed by MIC determination for most strains.

Virulence of colistin-resistant mutants

To test for potential loss of virulence following colistin adaptation, the invertebrate model *G. mellonella* was infected with several colistin-adapted strains. *Galleria* has already been shown by us, and others, to be a good model to study *A. baumannii* virulence.[25,28,29] *Galleria* larvae were infected with 1×10^6 cfu for each of the colistin-adapted mutants and compared with WT infection (Figure 1 and Figure S1). UKA2 and UKA18, which are avirulent strains, showed no change in virulence and strains ATCC 17978, NCTC 13424 and AYE appeared to have lost their virulence after adaptation to colistin. However, strain W1, which was amongst the most virulent strains of *A. baumannii* tested, and UKA8 showed no loss of virulence despite an increase in colistin resistance. After infection at a lower dose (1×10^5 cfu) there was no statistically significant difference between the W1 colistin-adapted strain and its WT, although the UKA8 colistin-adapted strain showed slightly reduced virulence at this infectious dose, which was not significant ($P>0.5$; data not shown).

To investigate whether this retention of virulence was due to maintenance of fitness/growth rate, all strains were grown in Graces insect medium at 37°C for 24 h. Colistin-adapted strains UKA8 and W1 had comparable growth rates to their WTs, but all other strains tested showed reduced growth in this medium after exposure to colistin (data not shown).

Genetic changes in pmrAB/lpxA following colistin adaptation

Previous research on colistin adaptation has identified genetic changes in *pmrB* and, to a lesser extent, *pmrA*, *lpxA*, *lpxC* and *lpxD* as important in the generation of colistin resistance.[12,17,18,30] These genes from colistin-adapted strains were sequenced and compared with their respective WTs. A variety of mutations were found in these genes (Table 2). In the virulent W1 strain, there was a stable duplication of amino acids Ser17 to Phe26 in PmrB present in the colistin-adapted strain. UKA8 had undergone a single amino acid substitution (T235I) also in PmrB. W1 (W1 Col^2) and UKA8 (UKA8 Col^2) strains from the second colistin exposure dataset also had their respective genes sequenced to see if the same mutations had occurred. UKA8 had a frameshift mutation in *lpxD* and W1 showed an amino acid substitution (S17R) in PmrB. This amino acid was within the region that had been duplicated in the previous exposure. Subsequent virulence testing revealed that UKA8 Col^2 was avirulent and W1 Col^2 showed ~50% *Galleria* mortality at the high challenge dose (Figure 1).

To further quantify when these genetic mutations occurred for the strains that retained their virulence in *Galleria*, the *pmrB* was sequenced from weeks 1 to 6 after exposure to colistin. The UKA8 col strain developed the T235I mutation during the first week of exposure, but the amino acid duplication in W1 Col strain was only observed after 6 weeks of exposure to colistin. To understand how these mutations contribute to virulence in *Galleria*, strains from W1 and UKA8 from weeks 1 to 6 after exposure to colistin were injected into *Galleria* (Figure 2). For UKA8, although the mean value increased, this was not statistically significant ($P>0.5$) from weeks 1 to 6, but for the W1 strain, virulence was attenuated by week 3, but was restored at week 6. The growth rate for all weeks appeared to be unaffected relative to the WT. Mechanisms responsible for initial adaptation to colistin exposure remain to be elucidated.

Discussion

A number of colistin-resistant *A. baumannii* strains were generated and used to investigate the relationship between colistin resistance, biocide cross-resistance and virulence in *Galleria* as a measure of biological fitness. Here, we have shown that the generation of resistance to colistin does not always lead to growth retardation or loss of virulence. Although *Galleria* are not vertebrate models, for other microorganisms it is increasingly recognized that there is correlation between virulence in *Galleria* and in mammalian models,[27,31,32] and strains of *A. baumannii* that have been shown to be virulent in mice are virulent in *Galleria*.[25]

Laboratory-generated colistin-resistant isolates generally showed increased susceptibility to various classes of antibiotics with aminoglycoside susceptibility particularly affected. Resistance to certain disinfectants (e.g. phenolic-based disinfectants) decreased, but susceptibility to others (e.g. H_2O_2-based disinfectants) was unaffected. Interestingly, those strains (W1 and UKA8) that were less affected by challenge with azithromycin, cefepime and teicoplanin, which is often an indication of a change in LPS structure, were those that retained biological fitness and virulence in *Galleria*, indicating that membrane integrity is perhaps maintained better in these strains.

Colistin resistance in *A. baumannii*, as already mentioned, is associated with changes in the LPS structure. LPS is a critical factor in infection since it is a component that is often recognized by the host's immune system. Colistin resistance mechanisms in *A. baumannii* have focused on the *lpx* and/or *pmr* genes. Mutations in these genes have previously been shown to have a likely fitness cost with decreased virulence shown in the mouse model of infection and have a lower invasiveness compared with colistin-susceptible strains.[24,33] Research has also shown that colistin-resistant strains were also outcompeted by susceptible strains in patients following withdrawal of colistin therapy.[34]

Here, we have shown that mutations in *pmrB* do not lead to loss of virulence or fitness as assessed by growth kinetics. With many different genes affecting LPS production and stability there are potentially several targets where genetic mutation would affect LPS structure and colistin susceptibility. Several of the mutations described here are similar to those described previously, e.g. ISAba1 transposon insertion into *lpxC* and the frameshift in *lpxD* resulting in a truncated protein, indicating that, whilst

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Table 1. MICs of various antibiotics and disinfectants for colistin-adapted strains

	ATCC 17978		NCTC 13424		W1			AYE		UKA2		UKA8			UKA18	
	WT	mut	WT	mut	WT	mut	mut^2	WT	mut	WT	mut	WT	mut	mut^2	WT	mut
Colistin	0.5–1	>64	0.5–1	>64	1–2	32–64	64 to >64	1	32	0.5–1	>64	1	>64	>64	1–2	64 to >64
Amikacin	4	1	>512	>512	32	8	32	128	8	8	0.5–1	16	1	≤0.5	32	2
Gentamicin	2	1	>512	>512	128	8	16–32	>512	256	>512	256	512	64	64	>512	64
Piperacillin/ tazobactam	4–8	2	256	4	8	2	4–8	32	1–2	32	8	4–8	2	1	64	1
Meropenem	≤0.5	≤0.5	64	1	1–2	≤0.5	≤0.5	≤0.5	≤0.5	4	≤0.5	≤0.5	≤0.5	≤0.5	64	≤0.5
Ciprofloxacin	≤0.5	≤0.5	32	8	64	16	32–64	64	8	64	8	64	32	16	>512	4
Azithromycin	2	0.5	>64	64	4	4	4	8	0.06–0.125	8	0.125	4	1–2	0.5	32	≤0.06
Cefepime	4	4	>64	4	16	8	8–16	>64	64 to >64	16–32	2	32	8	0.5	>64	1
Teicoplanin	>64	64	>64	1	>64	64 to >64	>64	>64	0.5–1	>64	4–8	>64	64	1	>64	2
HDPCM	2	1	4	≤0.5	2–4	1	4	2	≤0.5–1	1–2	≤0.5	4	4	≤0.5	4	≤0.5
BCl	8	4	8	1	8	2	8	8	2	4–8	1–2	8	4–8	1	8	1
CHD	8	8	16	4	16	8–16	16	8–16	8	16	4–8	8–16	4–8	2	32	2
Phe (%wc)	6.25–3.125	3.125	3.125	≤0.1	3.125	0.2–0.39	3.125	3.125	0.39	3.125	0.1–0.2	3.125	1.56	0.39	6.25–3.125	≤0.1
Quat (%wc)	0.39	≤0.1	0.39	≤0.1	0.78	0.78	0.78	0.78	≤0.1	0.78	≤0.1	0.39	0.39	≤0.1	0.39	≤0.1
HPAA (%wc)	0.78	0.19	1.56	0.78	3.125	3.125	3.125	3.125	1.56–3.125	3.125	3.125	3.125	1.56	0.19	1.56	0.19
HBD (%wc)	1.56	0.09	1.56	0.39	1.56	1.56	1.56	1.56	1.56	1.56–3.125	1.56	1.56	1.56	0.09	1.56–0.78	0.19
Chl (ppm)	500	500	500	250	250	250	500	250	125	250	250	500	500	250	500	250
H_2O_2 (%)	0.06	0.03	0.06	0.03	0.06	0.06	0.03	0.06	0.03	0.06	0.06	0.03	0.03	0.03	0.06	0.03
PAA (%)	0.04	0.04	0.08	0.04	0.08	0.08	0.08	0.08	0.08	0.08	0.04	0.08	0.08	0.04	0.04	0.02
EtOH (%)	6.25	6.25	6.25	6.25	3.125	0.78	3.125–6.25	3.125	0.78–1.56	3.125	0.78–1.56	3.125	3.125	6.25	6.25	6.25

Disinfectants used were hexadecylpyridinium chloride monohydrate (HDPCM), benzalkonium chloride (BCl), chlorhexidine digluconate (CHD), phenol-based disinfectant (Phe), quaternary ammonium disinfectant (Quat), H_2O_2/peracetic acid-based disinfectant (HPAA), H_2O_2-based disinfectant (HBD), chlorine-based disinfectant (Chl), hydrogen peroxide (H_2O_2), peracetic acid (PAA) and ethanol (EtOH). All values are given in mg/L unless otherwise stated. %wc = % working concentration. mut indicates strains isolated after 6 weeks of colistin adaptation and mut^2 indicates those strains investigated from the second round of adaptation to colistin. All MICs are shown as the range from at least three independent experiments. Values highlighted by light grey or dark grey shading indicate at least a 4-fold increase or decrease in MIC, respectively, relative to WT.

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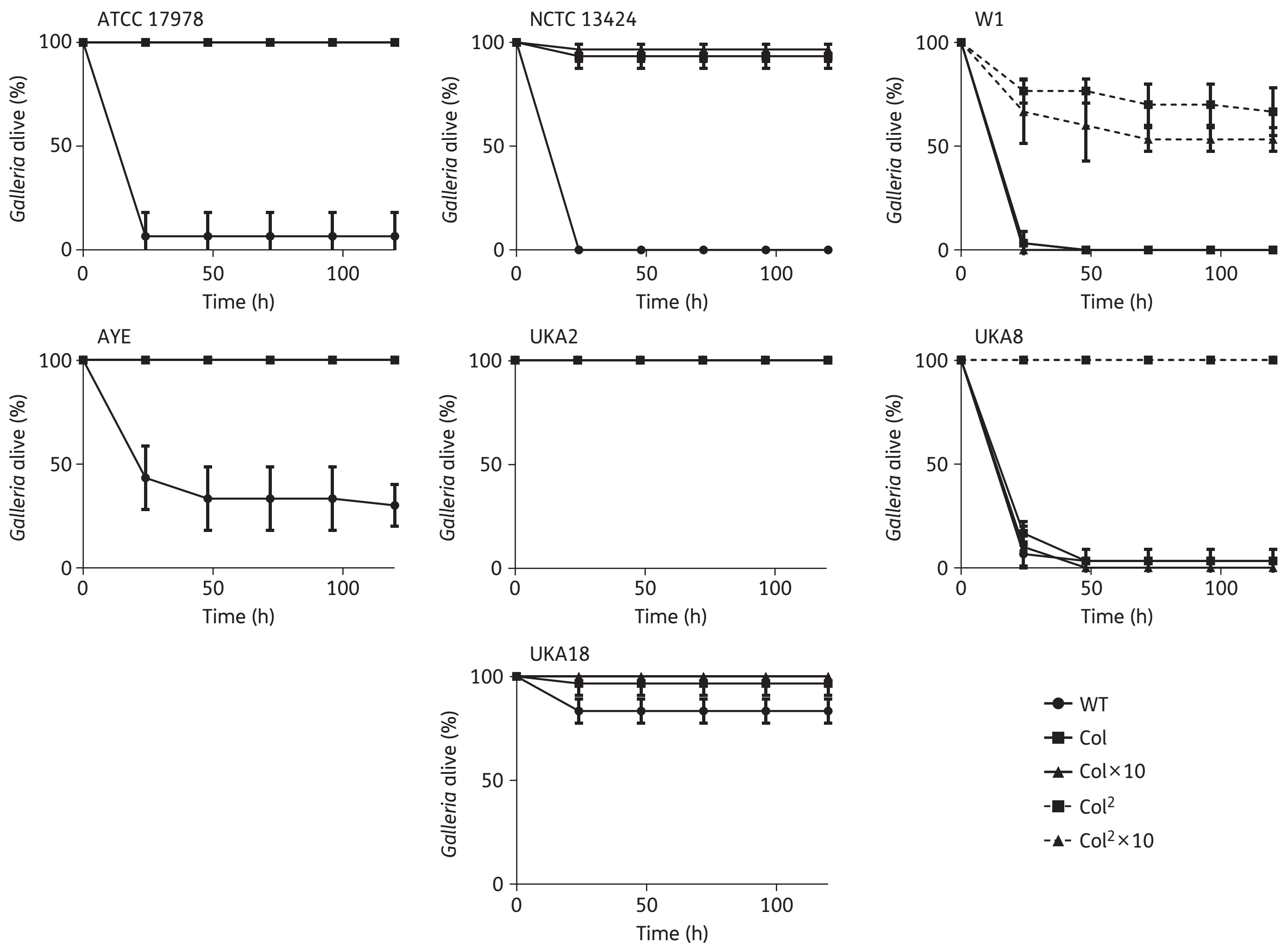

Figure 1. Virulence of *A. baumannii* colistin-adapted strains in *G. mellonella*. Groups of 10 larvae were challenged with 1×10^6 cfu, the number of live versus dead larvae was determined every 24 h post-infection up to 120 h and the number of live larvae was expressed as a percentage. WT, colistin-adapted after 6 weeks (Col) and colistin-adapted then passaged 10 times (Col×10) strains are shown on the graphs for seven separate strains as indicated. Colistin-adapted (Col^2) and colistin-adapted then passaged 10 times (Col^2×10) strains analysed from the second round of adaptation are shown where applicable. Individual values at 24 and 120 h post-infection are shown in Figure S1.

resistance to colistin can occur through a variety of mechanisms, some are perhaps more common.[17,18] Although the function of the C-terminal domain in LpxD is unknown, its removal abolishes the enzymatic function.[35] Mutations observed, in this study, in LpxC are all in conserved amino acid residues and include a mutation of the critically important active site residue F191. Allelic differences in the different *lpxC* mutants are apparent in the susceptibility to different biocides, including H_2O_2/peracetic acid-based disinfectant and H_2O_2-based disinfectant, although the impact of these alleles on LPS structure is unclear. Every isolate that, during acquisition of colistin resistance, gained a mutation in *lpxA*, *lpxC* or *lpxD* was avirulent in *Galleria* and had increased susceptibility to many frontline antibiotics for *A. baumannii* treatment. Therefore, it is increasingly obvious why these genes are attractive targets for antibiotic design[36,37] and our study validates the potential of studying such inhibitors in the *Galleria* infection model. Previous research has shown that *lpx* mutants were highly attenuated in mouse and *Caenorhabditis elegans* infection models, but mutations in *pmrB* resulted in no attenuation in mice.[38]

It is plausible that UKA8 and W1 colistin-resistant strains, which have a point mutation or a 30 bp duplication in *pmrB*, respectively, have undergone the development of secondary-site or compensatory mutations and it is interesting that the 30 bp *pmrB* duplication occurred only at week 6, which was after development of colistin resistance, but associated with re-acquisition of WT virulence in *Galleria*. Compensatory mutations enable the resistant strain to recover its fitness and virulence through mutation in one or more genes unrelated to the initial development of antibiotic resistance. WGS of the colistin-adapted strains at weeks 1–5 will enable identification of potential mutations linked to initial acquisition of resistance as well as specific mutations relating to loss and regain of virulence in *Galleria*. Many colistin-resistant *A. baumannii* strains show single-amino-acid changes in *pmrB*, including several in the HisKA domain (amino acids 216–276).[12,39–41] Analysis of all

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Table 2. Mutations in *A. baumannii* strains involved in colistin resistance

Strain	Gene	Nucleotide change	Amino acid change	Effect	Growth retardation	Virulence in *Galleria*
AYE Col	*lpxC*	SNP nt 758 T to A	**I253N**		yes	no
17978 Col	*lpxA*	SNP nt 646 G to T	E216Stop	truncated 215 amino acid protein	yes	no
UKA2 Col	*lpxC*	SNP nt 573 T to G	**F191L**		yes	no
W1 Col	*pmrB*	insertion of 30 nt after nt 78	duplication of S17 to F26	elongated transmembrane domain	no	WT
W1 Col[2]	*pmrB*	SNP nt 51 T to A	S17R		slight	reduction
UKA8 Col	*pmrB*	SNP nt 704 C to T	**T235I**		no	WT
UKA8 Col[2]	*lpxD*	1 nt deletion after nt 954	frameshift after amino acid 318	truncated 325 amino acid protein	yes	no
UKA18 Col	*lpxC*	ISAba1 transposon insertion after nt 395		inactivation of *lpxC*	yes	no
13424 Col	*lpxC*	SNP nt 245 C to A	**A82E**		yes	no

Growth retardation and virulence are shown for comparison to show that those that retained virulence in *Galleria* and had an unaffected growth rate had mutations in *pmrB*. All mutations in the *lpx* genes caused a loss of virulence. WT means WT virulence. Amino acid substitutions predicted to affect protein function by SIFT analysis (<0.05) are shown in bold. No mutations in *pmrA* were detected.

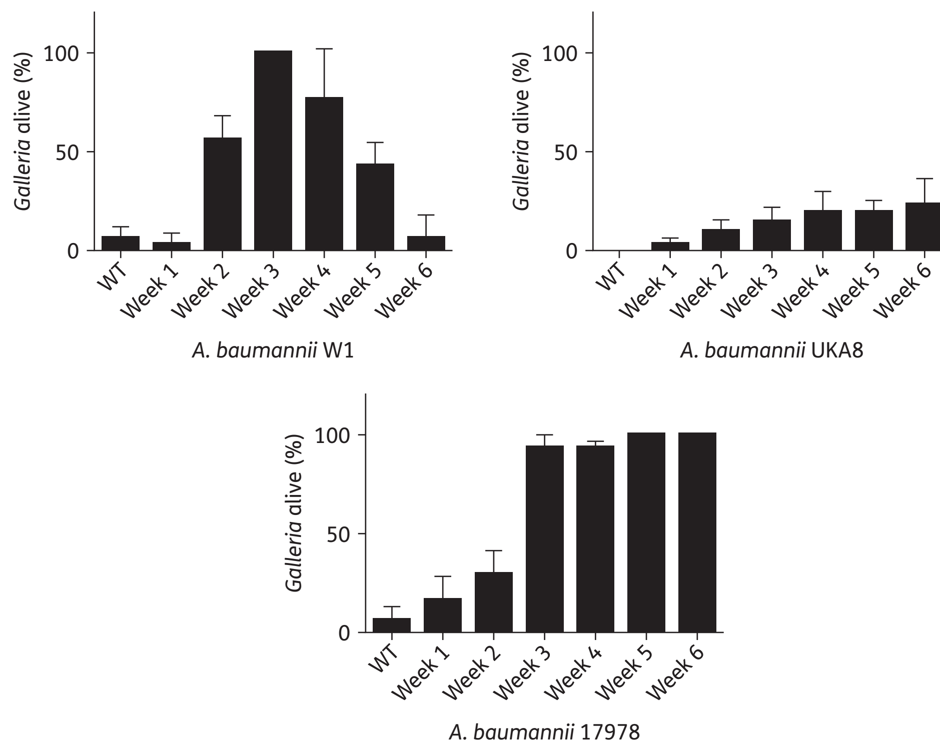

Figure 2. Virulence of *A. baumannii* strains after adaption to colistin over 6 weeks. *G. mellonella* larvae were challenged with *A. baumannii* strains indicated at an inoculum of 1×10^6 cfu. At 24 h post-infection, the number of live versus dead larvae was determined and the number of larvae alive was expressed as a percentage.

known amino acid changes in PmrB revealed that amino acid changes occur throughout the protein, but several amino acids around the His phosphorylation site of the HisK domain are prime candidates for mutational changes. The two colistin-adapted strains in this study that retained WT virulence and fitness had mutations in *pmrB*. Of particular interest is the amino acid duplication in W1; this occurs in a predicted transmembrane region (amino acids 10–29). Mutations within this region have previously been implicated in colistin resistance,[12,40,42] and in *Salmonella* mutations in the transmembrane region of PmrB led to a 4-fold increase in colistin resistance.[43] To our knowledge this is the first description of a duplication within a transmembrane domain in

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this class of global regulators and future studies will seek to understand how this duplication mediates colistin resistance and retention of virulence.

Overall the study has highlighted the potential for a wide range of clonally distinct *A. baumannii* isolates to adapt to colistin exposure with the development of stable resistance. The differences in virulence between mutants with different resistance mechanisms highlight the importance of understanding mechanisms of antibiotic resistance as well as the phenotype, when determining potential clinical significance. The interplay between primary and compensatory mutations in achieving this evolutionary balance will be the subject of future studies.

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Transparency declarations

None to declare.

Supplementary data

Table S1 and Figure S1 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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