Acquisition of *Acinetobacter baumannii* colistin resistance due to loss of lipopolysaccharide results in increased susceptibility to clinically relevant antibiotics without altering susceptibility to antimicrobial peptides

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INTRODUCTION

Acinetobacter baumannii **is a Gram-negative coccobacillus that can cause different types of infections as a nosocomial pathogen including pneumonia, bacteremia, meningitis and skin and soft tissue infection, among others. The number of multidrug and pandrug resistant strains has increased alarmingly in recent years. In many cases, the cationic peptide antibiotic colistin is one of the few clinically available antibiotics that retains activity against these isolates. Unfortunately, an increasing number of colistin-resistant strains have been reported, severely complicating the clinical management of these infections.**

Colistin is a cationic peptide that interacts with the negatively charged lipid A moiety of lipopolysaccharide (LPS) and disorganizes the outer membrane of the bacterium. Two mechanisms have been shown to confer colistin resistance in *A. baumannii*, ethanolamine modification of lipid A due to mutation in the *pmrAB* locus resulting in an increased positive charge of LPS, and complete loss of LPS expression due to mutation of the genes involved in LPS biosynthesis, such as *lpxA*, *lpxC* or *lpxD*. LPS is located on the outer leaflet of Gram-negative outer membranes and serves as a major structural component and an important barrier to the entry of extracellular molecules. For this reason, the complete loss of LPS has the potential to alter antibiotic susceptibility to additional antibiotics, which may elucidate additional therapeutic options for the treatment of infections caused by *A. baumannii* acquiring colistin resistance via LPS loss.

In the present work we analyze the resistant profile of MDR clinical *A. baumannii* strains which have acquired colistin resistance via LPS loss, through mutation of LPS biosynthesis genes, or via LPS modification due to mutation in the PmrAB system.

CONCLUSION AND DISCUSSION

The findings presented in this study indicate that the acquisition of resistance to colistin via LPS loss results in dramatic increases in susceptibility to azithromycin, rifampicin and vancomycin. The increase in susceptibility observed with vancomycin upon LPS loss is of interest as this antibiotic is traditionally used with Gram positive bacterial infections due to the intrinsic resistance seen with most Gram negative species.

The data presented here demonstrating that the loss of LPS results in increased permeability of the *A. baumannii* bacterial membrane make it tempting to suggest that the observed increase in antibiotic susceptibility is, at least in part, explained by this increased permeability, in consonance with data of previous work. This idea is supported by prior studies demonstrating that LPS is one of the main barriers contributing to the membrane permeability of hydrophobic compounds. That study, performed with *Escherichia coli* containing thermosensitive mutations in the *lpxA* and *lpxD* genes, demonstrated that LPS plays a role in the passive entry of macrolides and rifamycins, which may explain the results seen here with azithromycin and rifampicin.

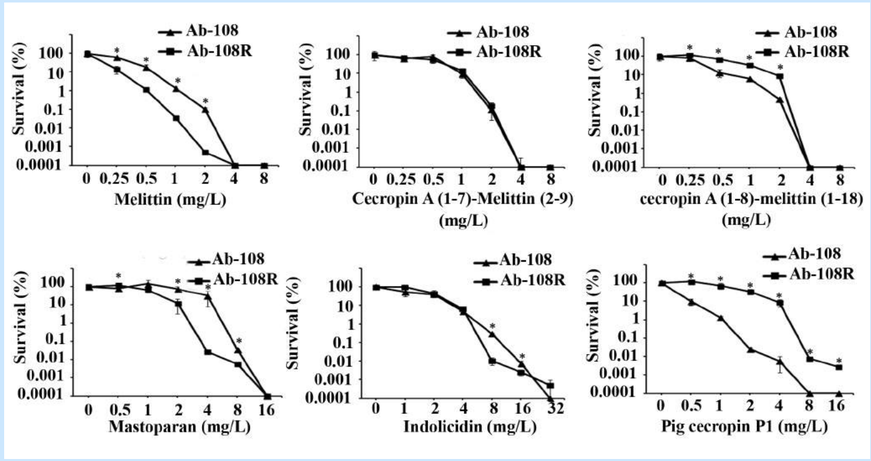
Our results also suggest that cationic peptides can be an alternative to be considered against colistin resistant strains regardless of the via by which bacteria have acquired resistance to colistin.

Table 1. **Minimum Inhibitory Concentrations of *A. baumannii* strains for 15 antibiotics**

Strain	Description	EU/10⁶ cells[a]	Minimum inhibitory concentration mg/L (Susceptible or Resistant)														
			CST	MEM	IPM	FEP	CAZ	AZM	AMK	GEN	CIP	RIF	TIC	AMP	TGC	S	VAN
Ab-84	MDR clinical isolate	17.78 ±3.54	≤0.25 (S)	256 (R)	256 (R)	64 (R)	1024 (R)	16	8 (S)	>128 (R)	64 (R)	2	>256 (R)	>256 (R)	2 (I)	16 (R)	256
Ab-108	MDR clinical isolate	18.53 ±4.80	≤0.25 (S)	256 (R)	256 (R)	128 (R)	1024 (R)	16	512 (R)	>128 (R)	64 (R)	4	>256 (R)	>256 (R)	1 (S)	32 (R)	512
Ab-167	MDR clinical isolate	20.02 ±2.36	≤0.25 (S)	256 (R)	256 (R)	128 (R)	2048 (R)	8	256 (R)	>128 (R)	128 (R)	4	>256 (R)	>256 (R)	4 (R)	64 (R)	256
Ab-176	MDR clinical isolate	42.97 ±21.52	≤0.25 (S)	256 (R)	256 (R)	128 (R)	2048 (R)	8	256 (R)	>128 (R)	64 (R)	4	>256 (R)	>256 (R)	2 (I)	64 (R)	256
Ab-84R	Ab-84 with 40 nucleotide insertion at 321 nucleotide of *lpxC*	< 1	>128 (R)	4 (I)	16 (R)	64 (R)	32 (R)	≤0.25	≤0.5 (S)	128 (R)	64 (R)	≤0.125	>256 (R)	>256 (R)	1 (S)	32 (R)	1
Ab-108R	Ab-108 with T614A mutation in *lpxA* producing I205N	< 1	>128 (R)	64 (R)	64 (R)	>256 (R)	256 (R)	≤0.25	64 (R)	>128 (R)	64 (R)	≤0.125	>256 (R)	>256 (R)	4 (R)	128 (R)	8
Ab-167R	Ab-167 with *ISAba1* insertion at nucleotide 321 of *lpxC*	< 1	>128 (R)	8 (R)	32 (R)	16 (I)	32 (R)	≤0.25	32 (I)	128 (R)	32 (R)	≤0.125	>256 (R)	>256 (R)	2 (I)	64 (R)	4
Ab-176R	Ab-176 with G739T mutation in *lpxD* producing premature stop codon	< 1	>128 (R)	4 (I)	8 (R)	16 (I)	32 (R)	≤0.25	64 (R)	>128 (R)	32 (R)	≤0.125	>256 (R)	>256 (R)	1 (S)	16 (R)	0.5
ATCC 19606	*A. baumannii* type strain	1064.82 ±1410.18	≤0.25 (S)	1 (S)	0.5 (S)	8 (S)	8 (S)	1	4 (S)	1 (S)	≤0.2 5 (S)	2	16 (S)	>256 (R)	1 (S)	2 (S)	>64
CS01	MDR clinical isolate		≤0.25 (S)	64 (R)	32 (R)	32 (R)	128 (R)	8	2 (S)	1 (S)	32 (R)	>256	>256 (R)	>256 (R)	1 (S)	4 (S)	32
CR17	Clinical derivative CS01 containing a M12K mutations in *pmrA*		64 (R)	32 (R)	32 (R)	16 (I)	64 (R)	8	≤0.5 (S)	0.5 (S)	32 (R)	>256	>256 (R)	>256 (R)	0.5 (S)	4 (S)	32

All strains are *A. baumannii*. MDR: multidrug resistant. EU: Endotoxic Units. CST: colistin; MEM: meropenem; IPM: imipenem; FEP: cefepime; CAZ: ceftazidime; AZM: azithromycin; AMK: amikacin; GEN: gentamicin; CIP: ciprofloxacin; RIF: rifampicin; TIC: ticarcillin; AMP: ampicillin; TGC: tigecycline; S: sulbactam; VAN: vancomycin. S: susceptible; R: resistant; I: Intermediate. [a]Data represent the mean ± the standard error of the mean of three independent assays.

Figure 2. **Killing assay with six antimicrobial peptides**



RESULTS

1. Effect of LPS loss on antibiotic susceptibility.

MICs for 15 clinically relevant antibiotics were determined for the multidrug resistant parent strains and their colistin-resistant, LPS-deficient derivatives (Table 1). For gentamicin, ciprofloxacin, ticarcillin, ampicillin, tigecycline and sulbactam, susceptibility was unchanged or varied minimally between the parental strain and the LPS-deficient derivatives over the ranges used for MIC determination. Susceptibility increased moderately for amikacin (4-16 fold), ceftazidime (4-64 fold), imipenem (4-32 fold), cefepime (0-8 fold), and meropenem (4-64 fold) upon LPS loss.
The most dramatic changes in susceptibility occurred with azithromycin (>32 fold change for all strains), rifampicin (>32 fold change for all strains) and vancomycin (64-512 fold change). In the cases of azithromycin and rifampicin, the LPS-deficient strains were extremely susceptible to these antibiotics with MIC values below the lower limit of the range of antibiotic concentrations used for testing (0.25 mg/L for azithromycin and 0.125 mg/L for rifampicin). Vancomycin, an antibiotic to which *A. baumannii* is intrinsically resistant (ATCC 19606, Table 1), had dramatically increased activity against the LPS-deficient strains, with three strains (Ab-84R, Ab-167R and Ab-176R).

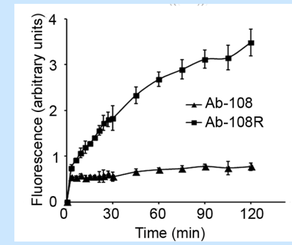


Figure 1. Cell permeability

2. Colistin resistance due to LPS loss increases membrane permeability.

Ethidium bromide accumulation assays were performed in order to measure changes in membrane permeability of the four colistin-resistant derivatives compared to their parental strains. As shown in Figure 1, LPS-deficient derivatives demonstrated more rapid accumulation of intracellular ethidium bromide compared to the parental strains, and in all cases fluorescence was higher at 120 minutes for all LPS-deficient derivatives compared to the parent strains. These results indicate that LPS loss results in higher membrane permeability, similar to results described previously.

3. Antimicrobial peptides retain activity against colistin-resistant strains.

In order to analyze the effect of cationic peptides as candidates against colistin-resistant strains, we measured the bactericidal activity of six antimicrobial peptides. Bacterial killing assays were performed using increasing concentrations of melittin, cecropin A (1-7)-melittin (2-9), cecropin A (1-8)-melittin (1-18), mastoparan, indolicidin and pig cecropin P1. Our results summarized in Figure 2 show that concentrations ranging from 8 to 32 mg/L of the six antimicrobial peptides were able to reduce bacterial viability more than 3 $\log_{10}$.