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Octapeptin C4 and polymyxin resistance occur via distinct pathways in an epidemic XDR *Klebsiella pneumoniae* ST258 isolate

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Background: Polymyxin B and E (colistin) have been pivotal in the treatment of XDR Gram-negative bacterial infections; however, resistance has emerged. A structurally related lipopeptide, octapeptin C4, has shown significant potency against XDR bacteria, including polymyxin-resistant strains, but its mode of action remains undefined.

Objectives: We sought to compare and contrast the acquisition of resistance in an XDR *Klebsiella pneumoniae* (ST258) clinical isolate *in vitro* with all three lipopeptides to potentially unveil variations in their mode of action.

Methods: The isolate was exposed to increasing concentrations of polymyxins and octapeptin C4 over 20 days. Day 20 strains underwent WGS, complementation assays, antimicrobial susceptibility testing and lipid A analysis.

Results: Twenty days of exposure to the polymyxins resulted in a 1000-fold increase in the MIC, whereas for octapeptin C4 a 4-fold increase was observed. There was no cross-resistance observed between the polymyxin- and octapeptin-resistant strains. Sequencing of polymyxin-resistant isolates revealed mutations in previously known resistance-associated genes, including *crrB*, *mgrB*, *pmrB*, *phoPQ* and *yciM*, along with novel mutations in *qseC*. Octapeptin C4-resistant isolates had mutations in *mlaDF* and *pqiB*, genes related to phospholipid transport. These genetic variations were reflected in distinct phenotypic changes to lipid A. Polymyxin-resistant isolates increased 4-amino-4-deoxyarabinose fortification of lipid A phosphate groups, whereas the lipid A of octapeptin C4-resistant strains harboured a higher abundance of hydroxymyristate and palmitoylate.

Conclusions: Octapeptin C4 has a distinct mode of action compared with the polymyxins, highlighting its potential as a future therapeutic agent to combat the increasing threat of XDR bacteria.

Introduction

Infections by XDR bacteria are an increasing concern due to the lack of effective antibiotics, thereby resulting in high mortality.[1,2] Common therapeutic interventions include fosfomycin, tigecycline and polymyxins; however, resistance has emerged.[2–9] Few new antibiotics or combinations are clinically available to combat XDR infections, hence it is desirable to discover novel classes.[10]

Octapeptins are structurally similar to the polymyxins, with both lipopeptide classes consisting of a cyclic heptapeptide ring and linear tail capped with a fatty acid, containing multiple positively charged diaminobutyric acid (Dab) residues (Figure 1).[11–13] These Dab residues are critical for interactions of the polymyxins with the basal component of LPS, lipid A. Their mode of action involves the initial binding to lipid A, displacement of magnesium (Mg^{2+}) and calcium (Ca^{2+}), outer membrane permeabilization, leakage of cytoplasmic contents and subsequent cell death; however, the exact mechanism is yet to be discerned.[14,15] Polymyxin resistance leads to modification of the phosphate groups on lipid A with 4-amino-4-deoxyarabinose (Ara4N) and/or phosphoethanolamine (pEtN). This reduces polymyxin binding by removing the negative phosphate that attracts the cationic Dab residues, stabilizing the outer membrane and negating the infiltration of this antibiotic class.[16,17] Constitutive up-regulation of this pathway is achieved through chromosomal variations in the two-component regulatory systems (TCSs) *crrAB*, *pmrAB* and *phoPQ* and the negative regulator *mgrB* in *Klebsiella pneumoniae*.[8,9,18] The structurally similar octapeptins retain most of the key binding motifs and might be expected to employ a similar mode of action. The most significant structural difference between the polymyxins and octapeptins is a truncated linear exocyclic peptide (one residue instead of three) linked to a β-hydroxy-fatty acid (instead of an alkyl fatty

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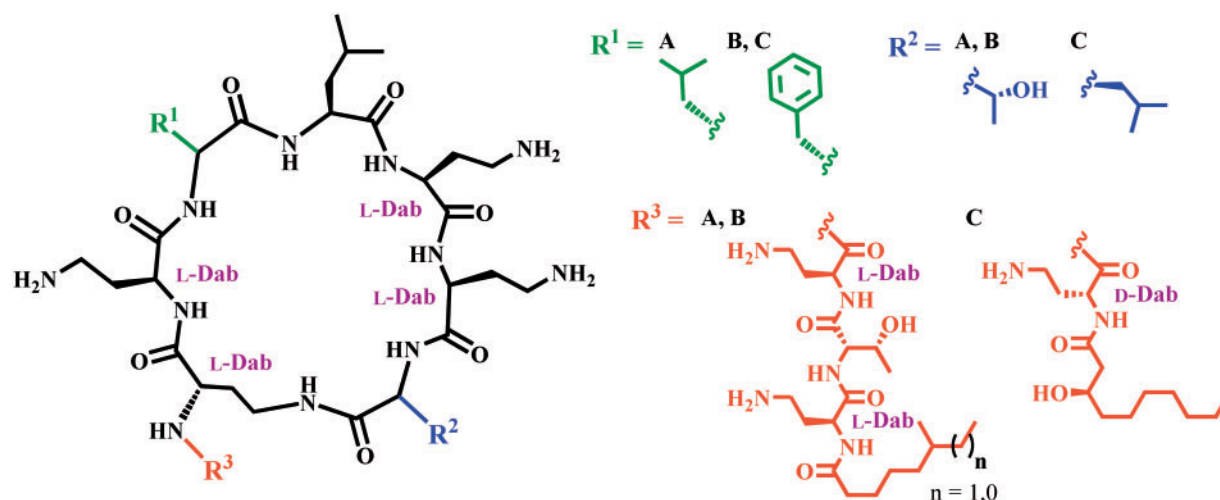


Colistin (A) and polymyxin B (B) differ by one amino acid [polymyxin B: phenylalanine and colistin: leucine (R[1])]. One defining feature of octapeptin C4 (C) is that it contains 8 amino acids rather than 10 in polymyxins. In addition, a leucine residue replaces threonine within the ring (R[2]), the exocyclic Dab residue is the D-enantiomer and the fatty acid tail contains a 3-hydroxy group (R[3]).

Figure 1. Structural comparison between the three lipopeptide antibiotics used in this study. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

acid, a critical component in polymyxin activity[19]) in the octapeptins.[11–13] More minor variations include L-Dab to D-Dab and L-Thr to L-Leu substitutions. Despite their similarity, prior research has revealed that octapeptins retain the ability to kill polymyxin-resistant (Pmx-R) bacteria and several exhibit broad-spectrum activity (against Gram-positive bacteria, fungi and protozoa).[11,20,21] Interestingly, several octapeptin *in vivo* mouse studies have shown activity against Pmx-R infections and less toxicity compared with polymyxins.[13,22–24]

We have recently reported the first syntheses of octapeptin C4[11] and A3,[22] followed by detailed biological characterization of octapeptin C4 that demonstrates its potential as a new 'last-resort' antibiotic.[23] In view of the limited understanding of the mechanism by which octapeptins target bacteria, and to help advance their preclinical development, we sought to investigate the differences driving development of octapeptin C4 and polymyxin resistance at a genetic level. Two studies have previously investigated the acquisition of resistance to octapeptins. One was performed using EM49 (a mixture of octapeptin classes A and B), which exhibited no increase in resistance after 10 passages for *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans*.[25] The other investigated lipid A modifications in octapeptin C4-resistant *P. aeruginosa* isolates obtained from a subculture surviving a single overnight treatment at 2 or 32 mg/L.[23]

The ST258 lineage of *K. pneumoniae* is commonly involved in outbreaks as it frequently harbours carbapenem resistance.[26–30] We have previously used WGS to investigate the acquisition of polymyxin resistance in an epidemic lineage of *K. pneumoniae* ST258 isolated from a Greek hospital.[26] An XDR polymyxin-susceptible isolate, 20_GR_12, was selected from this cohort and represents a strain in which a 'last-resort' antibiotic is employed. This isolate was passaged with an increasing concentration of polymyxins or octapeptin C4 for 20 days followed by antimicrobial susceptibility testing, WGS, complementation assays and analysis of lipid A composition to elucidate the potential mode of action.

Materials and methods

Bacterial strains and growth conditions

The clinical polymyxin-susceptible XDR *K. pneumoniae* ST258 (closely related to the NJST258_2 clade) isolate, 20_GR_12, was sourced through Hygeia General Hospital, Athens, Greece as previously described.[26] Cultures were grown in LB and, for single colony isolation, cultures were grown on either LB or nutrient agar (NA) plates.

Antimicrobial susceptibility testing

MIC was determined by the broth microdilution method according to CLSI guidelines.[31] Cultures were grown in CAMHB and, to assess cross-resistance of day 20 isolates, broth was supplemented with the concentration of antibiotic tolerated at that timepoint (Table S1, available as Supplementary data at *JAC* Online). Clinical breakpoints were determined in accordance with CLSI guidelines[32] and, for tigecycline, as per EUCAST (Version 8.0, 2018) (see http://www.eucast.org). As no clinical breakpoint has been reported for octapeptin C4, an MIC $\geq$32 mg/L was defined as resistant.

Selection of resistance

A single colony of 20_GR_12 was selected and grown overnight at 37°C, shaking at 220 rpm. This culture was grown to log phase (OD_{600} = 0.4–0.6) and plated into three separate 96-well polystyrene, non-treated plates (Sigma–Aldrich) with colistin, polymyxin B or octapeptin C4 (n = 6). Following overnight incubation, the well that harboured the densest growth ($OD_{600} \geq 1$) underwent a 1:1000 dilution and was transferred to a new plate with the concentration range adjusted accordingly. The highest concentration used for the polymyxins was 128 mg/L, and 32 mg/L for octapeptin C4. This process was performed for 20 days with the following 5 days of no antibiotic exposure. At day 20, the culture was further diluted (1:1000) and placed in non-supplemented broth to be incubated overnight, followed by

Table 1. MICs of several antibiotic classes for day 20 replicates compared with the initial isolate

	MIC (mg/L)[b]														
Strain[a]	CST	PMB	OctC4	AMX	ATM	FEP	CRO	CHL	CIP	GEN	MEM	PIP	TET	TGC	TMP
Initial	$\leq 0.25^{S}$	$\leq 0.125^{S}$	8	$>64^{R}$	$>64^{R}$	$\geq 16^{R}$	$>64^{R}$	$\geq 32^{R}$	$>64^{R}$	$\leq 4^{S}$	$\geq 32^{R}$	$>64^{I}$	$>64^{R}$	$\leq 2^{S,I}$	$>64^{R}$
CST_1	$>128^{R}$	$>128^{R}$	≤ 8	$>64^{R}$	$>64^{R}$	$\geq 16^{R}$	$>64^{R}$	$\leq 8^{S}$	$\geq 32^{R}$	4^{S}	$\geq 32^{R}$	$>64^{I}$	$>64^{R}$	$\leq 4^{S,R}$	$>64^{R}$
CST_2	$>128^{R}$	$>128^{R}$	≤ 4	$>64^{R}$	$>64^{R}$	$>64^{R}$	$>64^{R}$	4^{S}	$\geq 32^{R}$	$\leq 4^{S}$	$\geq 32^{R}$	$>64^{I}$	$>64^{R}$	2^{I}	$>64^{R}$
CST_3	$\geq 128^{R}$	$\geq 128^{R}$	≤ 4	$>64^{R}$	$>64^{R}$	$>64^{R}$	$>64^{R}$	8^{S}	$>64^{R}$	$\leq 4^{S}$	$>64^{R}$	$>64^{I}$	$>64^{R}$	$\leq 4^{I,R}$	$>64^{R}$
CST_4	$>128^{R}$	$>128^{R}$	≤ 8	$>64^{R}$	$>64^{R}$	$>64^{R}$	$>64^{R}$	$\leq 8^{S}$	$>64^{R}$	$\leq 4^{S}$	$>64^{R}$	$>64^{I}$	$>64^{R}$	$\leq 4^{I,R}$	$>64^{R}$
CST_5	$>128^{R}$	$>128^{R}$	2	$>64^{R}$	$>64^{R}$	$>64^{R}$	$>64^{R}$	$\leq 8^{S}$	$>64^{R}$	$\leq 4^{S}$	$>64^{R}$	$>64^{I}$	$>64^{R}$	$\leq 4^{I,R}$	$>64^{R}$
CST_6	$>128^{R}$	$>128^{R}$	≤ 8	$>64^{R}$	$>64^{R}$	$>64^{R}$	$>64^{R}$	8^{S}	$>64^{R}$	$\leq 4^{S}$	$>64^{R}$	$>64^{I}$	$>64^{R}$	$\geq 4^{R}$	$>64^{R}$
PMB_1	128^{R}	128^{R}	≤ 4	$>64^{R}$	$>64^{R}$	$>64^{R}$	32^{R}	4^{S}	32^{R}	$\leq 4^{S}$	$>64^{R}$	$>64^{I}$	$>64^{R}$	$\leq 4^{I,R}$	$>64^{R}$
PMB_2	$>128^{R}$	$>128^{R}$	8	$>64^{R}$	$>64^{R}$	$\leq 8^{I}$	$>64^{R}$	4^{S}	$>64^{R}$	$\leq 4^{S}$	$\leq 0.25^{S}$	$>64^{I}$	$>64^{R}$	$\leq 2^{S,I}$	$>64^{R}$
PMB_3	$>128^{R}$	$>128^{R}$	4	$>64^{R}$	$>64^{R}$	$\geq 32^{R}$	$>64^{R}$	8^{S}	$>64^{R}$	2^{S}	$\geq 32^{R}$	$>64^{I}$	$>64^{R}$	2^{I}	$>64^{R}$
PMB_4	$>128^{R}$	$>128^{R}$	4	$>64^{R}$	$>64^{R}$	$\geq 8^{I,R}$	$\geq 32^{R}$	$\leq 2^{S}$	48^{R}	2^{S}	$\geq 8^{R}$	$>64^{I}$	$>64^{R}$	2^{I}	$>64^{R}$
PMB_5	$>128^{R}$	$>128^{R}$	≤ 8	$>64^{R}$	$>64^{R}$	$\geq 8^{I,R}$	$\geq 32^{R}$	$\leq 8^{S}$	$>64^{R}$	$\leq 4^{S}$	$\geq 2^{I,R}$	$>64^{I}$	$>64^{R}$	$\leq 2^{S,I}$	$>64^{R}$
PMB_6	$>128^{R}$	$>128^{R}$	≤ 8	$>64^{R}$	$>64^{R}$	$\geq 16^{R}$	$>64^{R}$	8^{S}	$>64^{R}$	$\leq 4^{S}$	$\geq 32^{R}$	$>64^{I}$	$>64^{R}$	$\leq 4^{I,R}$	$>64^{R}$
OctC4_1	0.25^{S}	$\leq 0.25^{S}$	32	$>64^{R}$	$>64^{R}$	$\geq 16^{R}$	$>64^{R}$	8^{S}	$>64^{R}$	$\leq 2^{S}$	$\geq 32^{R}$	$>64^{I}$	$>64^{R}$	$\leq 2^{S,I}$	$>64^{R}$
OctC4_2	0.25^{S}	0.25^{S}	32	$>64^{R}$	$>64^{R}$	$\geq 8^{I,R}$	$>64^{R}$	8^{S}	$>64^{R}$	$\leq 2^{S}$	$\geq 32^{R}$	$>64^{I}$	$\leq 2^{S}$	$\leq 1^{S}$	$\geq 8^{R}$
OctC4_3	$\leq 0.5^{S}$	0.25^{S}	32	$>64^{R}$	$>64^{R}$	$\leq 4^{S,I}$	32^{R}	8^{S}	$>64^{R}$	$\leq 4^{S}$	$\leq 0.25^{S}$	$>64^{I}$	$>64^{R}$	2^{I}	$>64^{R}$
OctC4_4	$\leq 0.5^{S}$	0.25^{S}	>32	$>64^{R}$	$>64^{R}$	$\leq 4^{S,I}$	32^{R}	8^{S}	$>64^{R}$	1^{S}	$\leq 0.25^{S}$	$>64^{I}$	$\geq 2^{S,R}$	$\leq 2^{S,I}$	$\geq 4^{R}$
OctC4_5	0.5^{S}	0.5^{S}	32	$>64^{R}$	$>64^{R}$	$>64^{R}$	$>64^{R}$	$\leq 8^{S}$	$>64^{R}$	2^{S}	$>64^{R}$	$>64^{I}$	$>64^{R}$	2^{I}	$>64^{R}$
OctC4_6	$\leq 0.5^{S}$	$\leq 0.5^{S}$	32	$>64^{R}$	$>64^{R}$	$\leq 4^{S,I}$	$\geq 32^{R}$	$\leq 16^{S, I}$	$>64^{R}$	2^{S}	$\leq 0.25^{S}$	$>64^{I}$	$>64^{R}$	2^{I}	$>64^{R}$

Resistance determined as per CLSI guidelines, except EUCAST guideline used for tigecycline (^{S}, susceptible; ^{I}, intermediate; ^{R}, resistant). Fluctuations in MIC values ($n = 4$) are displayed by two letters defining the resistance level. Grey shading indicates resistant MIC values. Resistance to octapeptin C4 defined at an MIC ≥ 32 mg/L, as no clinical breakpoint has been reported for this compound.

[a]The initial polymyxin-susceptible isolate (20_GR_12) and this strain subjected to 20 days of treatment with colistin (CST), polymyxin B (PMB) or octapeptin C4 (OctC4) (_1, _2, _3, _4, _5 and _6 indicate replicate numbers).

[b]MIC determined for: CST, colistin; PMB, polymyxin B; OctC4, octapeptin C4; AMX, amoxicillin; ATM, aztreonam; FEP, cefepime; CRO, ceftriaxone; CHL, chloramphenicol; CIP, ciprofloxacin; GEN, gentamicin; MEM, meropenem; PIP, piperacillin; TET, tetracycline; TGC, tigecycline; and TMP, trimethoprim.

an MIC test to evaluate resistance stability. Fold-change significance was determined via GraphPad Prism 7 with a one-way ANOVA with a Tukey's multiple comparisons test where significance was $P < 0.05$.

Lipid A modifications

Lipid A was extracted using the ammonium hydroxide–isobutyric acid protocol as previously described.[33] Day 20 cultures were grown overnight in LB supplemented with antibiotic (Table S1). Overnight inocula were subcultured (1:100) into 100 mL of LB broth and grown to an $OD_{600} = 0.8–1$. Cultures were pelleted (15008 **g**, 20 min, 4°C), washed with 1× PBS (15008 **g**, 15 min, 4°C) and freeze-dried.[33] Ten milligrams of lyophilized cells was suspended in isobutyric acid:ammonium hydroxide (5:3 v/v) at 100°C for 4 h, supernatants isolated by centrifugation (12470 **g**, 15 min), diluted with water (1:1 v/v) and lyophilized. Extracts then underwent two methanol washes (1180 **g**, 15 min) and extracted lipid A (1 mg/L) was solubilized in methanol (5 mM ammonium acetate). Samples were infused at a low rate of 5 μL/min into a QSTAR Elite (Applied Biosystems) hybrid quadrupole Time-of-Flight (TOF) mass spectrometer. Data were exported from Analyst (SCIEX), normalized to the highest mass intensity and graphed in GraphPad Prism 7.

DNA extractions and library preparation

Glycerol stocks from day 20 isolates were grown on NA plates. Single colonies were grown in antibiotic supplemented broth (Table S1), incubated overnight and DNA was extracted using the DNeasy Blood and Tissue Kit (QIAGEN) according to the manufacturer's guidelines. Two colonies were selected from day 0 and four colonies from four replicates per treatment group. Quantification of DNA was acquired using Qubit®3.0 (Thermo Fisher Scientific) and 1 ng of DNA underwent library preparation with the Nextera XT kit (Illumina) as per the manufacturer's instructions. Quality control was checked with a 2100 Bioanalyzer (Agilent Technologies) and LabChip GX (PerkinElmer).

Sequencing and analysis

Libraries were sequenced on an Illumina NextSeq with 150 bp paired-end sequencing reads with ≥95× coverage with the exception of CST_2 (colony 1) (48×). Trimmomatic[34] was used to trim paired-end reads and SPAdes v3.10.1 implemented for assembly.[35] Annotation of assembled genomes was accomplished using the Rapid Annotation using Subsystem Technology (RAST).[36] The Centre for Genomic Epidemiology (CGE) tools were implemented to delineate laterally acquired resistance genes (ResFinder 3.0)[37] and plasmids (PlasmidFinder 1.3).[38] Reads were aligned using BWA-MEM[39], analysed through FreeBayes[40] and impact of change determined through SnpEff.[41] Nucleotide sequences have been deposited under NCBI BioProject PRJNA415530 (www.ncbi.nlm.nih.gov/bioproject/415530).

Complementation assays

Genes speculated to cause resistance underwent complementation as previously described.[26,42] Briefly, genes harbouring a potential variation contributing to resistance were amplified using the 2X Phusion HF master mix (Thermo Fisher) with the primers listed in Table S2. The gene was cloned

Table 2. Detection of acquired resistance genes and plasmid replicons compared with the initial isolate

	Antibiotic class impacted[b]																		Plasmid[c]				
	A				B							F	Q			S	T	Tr					
Strain[a]	*aac(6')-Ib*	*aph(3')-Ia*	*aph(3'')-Ib*	*aph(6)-Id*	bla_{KPC-2}	bla_{KPC-9}	bla_{LEN-12}	bla_{OXA-9}	bla_{SHV-12}	bla_{TEM-1A}	bla_{TEM-1B}	*fosA*	*aac(6')-Ib-cr*	*oqxA*	*oqxB*	*sul2*	*tet*(A)	*dfrA14*	I	II	III	IV	V
Initial	2	2	2	2	2		2	2			2	2	2	2	2	2	2	2	2	2	2	2	2
CST_1	4	4	4	4	4		4	4		1	3	4	4	4	4	4	4	4	4	4	4	4	4
CST_2	4	4	4	4	4		4	4			4	4	4	4	4	4	4	4	4	4	4	4	4
CST_3	4	4	4	4	4		4	4			4	4	4	4	4	4	4	4	4	4	4	4	4
CST_4	4	4	4	4	4		4	4			4	4	4	4	4	4	4	4	4	4	4	4	4
PMB_1	4	4	4	4	4		4	4		1	3	4	4	4	4	4	4	4	4	4	4	4	4
PMB_2	4		4	4			4				4	4	4	4	4	4	4	4	4	4	4	4	
PMB_3	4	3	4	4	3		3	3	1		4	4	4	4	4	4	4	4	4	4	4	4	3
PMB_4	4	4	4	4	4		4	4		1	3	4	4	4	4	4	4	4	4	4	4	4	4
OctC4_1	4	4	4	4	1		3	4	1		4	4	4	4	4	4	4	4	4	4	4	4	4
OctC4_2	4	4			3		4	4		4		4	4	4	4				4	4	4		4
OctC4_3	4	4	2	2		4	3	4	1	2	2	4	4	4	4	2	2	2	4	4	4	2	4
OctC4_4	4		1	1			4				1	4	4	4	4	1	1	1	4			1	4

Number represents the presence of the gene or plasmid, of four colonies, harbouring this attribute.
[a]The initial polymyxin-susceptible isolate (20_GR_12) and this strain subjected to 20 days of treatment with colistin (CST), polymyxin B (PMB) or octapeptin C4 (OctC4) (_1, _2, _3 and _4 indicate replicate numbers).
[b]Acquired resistance genes as determined by ResFinder 3.0. (≥90% identity and ≥60% length) for: A, aminoglycoside; B, β-lactam; F, fosfomycin; Q, quinolone; S, sulphonamide; T, tetracycline; and Tr, trimethoprim.
[c]Plasmid replicons detected by PlasmidFinder 1.3 (≥95% identity): I, ColRNAI; II, IncFIB(K)-Kpn3; III, IncFII(K); IV, IncN; and V, IncX3.

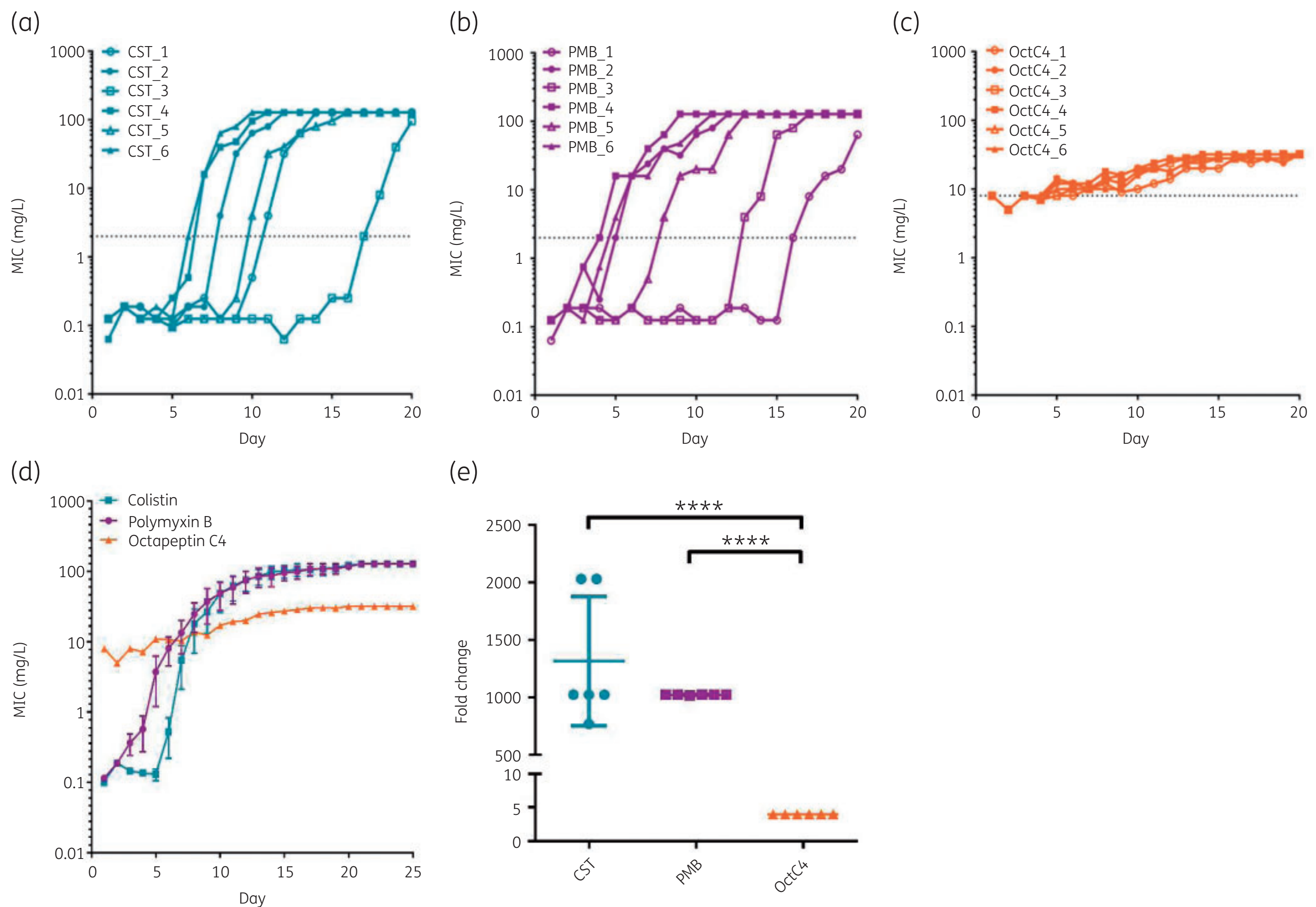


Figure 2. Acquired resistance in XDR *K. pneumoniae* over time for polymyxins and octapeptin C4. (a) Colistin. (b) Polymyxin B. (c) Octapeptin C4. (d) Overall comparison of acquired resistance for 20 day antibiotic exposure and the following 5 days without exposure (mean ± SEM, $n = 6$). (e) Fold change of colistin, polymyxin B and octapeptin C4 day 0 and day 20 MICs (mean ± SD) (****$P < 0.001$). The broken lines represent breakpoints (2 mg/L for polymyxins, 8 mg/L set for octapeptin C4 to highlight divergence from day 0). Highest concentration used was 128 mg/L for polymyxins and 32 mg/L for octapeptin C4. CST, colistin; PMB, polymyxin B; OctC4, octapeptin C4. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

into the pCR-BluntII-TOPO using the Zero Blunt TOPO PCR cloning kit (Invitrogen). The plasmid was transformed in electrocompetent *E. coli* TOP10 via electroporation, grown on Mueller-Hinton agar (MHA) (kanamycin: 50 mg/L) at 37°C and plasmids extracted using the QIAprep Spin Miniprep Column kit (QIAGEN). Plasmids were transformed into the initial susceptible strain (20_GR_12) and incubated on MHA (zeocin: 1500 mg/L). Furthermore, the WT gene was amplified from the initial strain and placed into the resistant day 20 isolates followed by MIC determination.

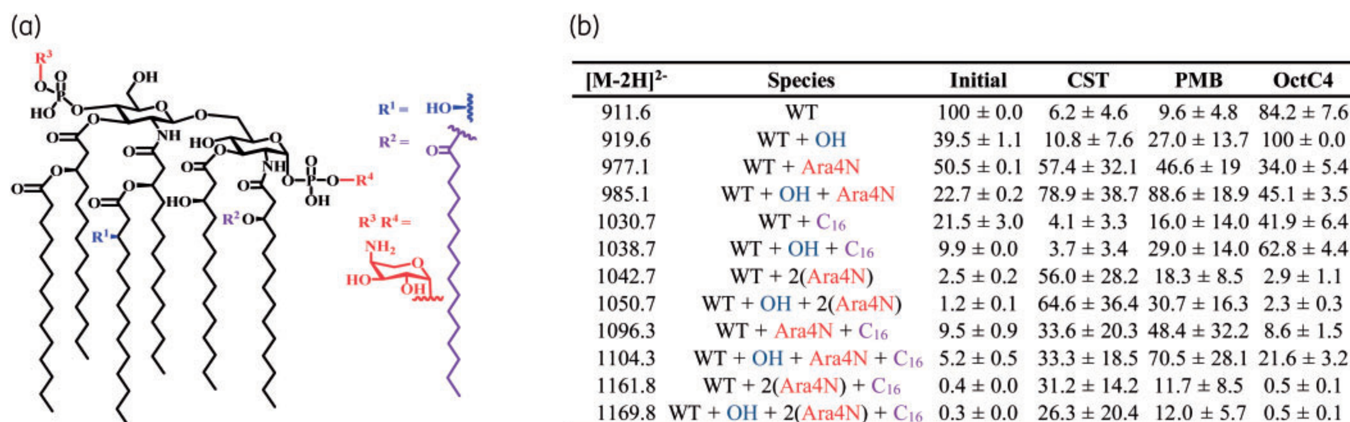
Results

Rapid resistance acquisition for polymyxins dissimilar to octapeptin C4

Selected resistance over the 20 day time course revealed significant variability between polymyxins and octapeptin C4 (Figure 2d). Initially, an MIC of 0.125 mg/L was measured for both colistin and polymyxin B. The majority of replicates treated with the polymyxins had a clinical resistance phenotype of >2 mg/L by day 10 (Figure 2a and b). Every replicate had a rapid escalation in MIC to >64 mg/L, generally within 5 days from the point when the MIC reached 0.5 mg/L. In contrast, octapeptin C4 resistance progressed steadily amongst replicates (Figure 2c) with only a 4-fold increase (from an initial MIC of 8 mg/L) compared with a ≥1000-fold increase for the polymyxins (Figure 2e). Although octapeptin C4 resistance remained stable, the extent of growth started to diminish during the last passages in the presence of 32 or 16 mg/L octapeptin C4.

Lack of cross-reactivity and reduction of resistance in octapeptin C4-selected isolates

Day 20 isolate MICs of a broad array of antibiotic classes were determined to evaluate whether acquired resistance conferred cross-resistance or resulted in regained susceptibility (Table 1). No cross-reactivity was apparent between polymyxins and octapeptin C4. Non-susceptibility to amoxicillin, aztreonam, ceftriaxone, ciprofloxacin, piperacillin and trimethoprim was ubiquitous amongst treatment groups. Chloramphenicol resistance was observed in



[M-2H]²⁻	Species	Initial	CST	PMB	OctC4
911.6	WT	100 ± 0.0	6.2 ± 4.6	9.6 ± 4.8	84.2 ± 7.6
919.6	WT + OH	39.5 ± 1.1	10.8 ± 7.6	27.0 ± 13.7	100 ± 0.0
977.1	WT + Ara4N	50.5 ± 0.1	57.4 ± 32.1	46.6 ± 19	34.0 ± 5.4
985.1	WT + OH + Ara4N	22.7 ± 0.2	78.9 ± 38.7	88.6 ± 18.9	45.1 ± 3.5
1030.7	WT + C_{16}	21.5 ± 3.0	4.1 ± 3.3	16.0 ± 14.0	41.9 ± 6.4
1038.7	WT + OH + C_{16}	9.9 ± 0.0	3.7 ± 3.4	29.0 ± 14.0	62.8 ± 4.4
1042.7	WT + 2(Ara4N)	2.5 ± 0.2	56.0 ± 28.2	18.3 ± 8.5	2.9 ± 1.1
1050.7	WT + OH + 2(Ara4N)	1.2 ± 0.1	64.6 ± 36.4	30.7 ± 16.3	2.3 ± 0.3
1096.3	WT + Ara4N + C_{16}	9.5 ± 0.9	33.6 ± 20.3	48.4 ± 32.2	8.6 ± 1.5
1104.3	WT + OH + Ara4N + C_{16}	5.2 ± 0.5	33.3 ± 18.5	70.5 ± 28.1	21.6 ± 3.2
1161.8	WT + 2(Ara4N) + C_{16}	0.4 ± 0.0	31.2 ± 14.2	11.7 ± 8.5	0.5 ± 0.1
1169.8	WT + OH + 2(Ara4N) + C_{16}	0.3 ± 0.0	26.3 ± 20.4	12.0 ± 5.7	0.5 ± 0.1

(a) Modifications that were detected in WT hexa-acylated lipid A. This included hydroxylation of a myristate (R^1), palmitoylation (R^2) and the addition of 4-amino-4-deoxy-arabinose (Ara4N) to either of the phosphate groups (R^3 and R^4). (b) Doubly charged lipid A species detected for the initial isolate ($n=2$) and treatment groups ($n=6$). Values represent mean ± SD of the relative peak intensities.

Figure 3. Lipid A modifications identified after 20 days of exposure to colistin, polymyxin B or octapeptin C4. CST, colistin; PMB, polymyxin B; OctC4, octapeptin C4. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

the initial isolate but normally diminished over the time course across treatment groups. In some instances, cefepime susceptibility was restored (replicates OctC4_3, OctC4_4 and OctC4_6). These replicates also regained susceptibility to meropenem, as did PMB_2. Replicate OctC4_2 also exhibited susceptibility to tetracycline and tigecycline but replicate OctC4_4 showed variability towards these antibiotics, with both resistant and susceptible MICs depending on the colonies selected.

Octapeptin C4-resistant isolates harbour an increase in hydroxymyristate and palmitoylate dissimilar to Ara4N lipid A modifications in Pmx-R strains

In the initial isolate, MS/MS analysis of extracted lipid A fractions showed that the major singly charged peak was at *m/z* 1824.2, which corresponded to a hexa-acylated lipid A species composed of two phosphate groups, two glucosamines and four 3-hydroxymyristoyl groups (3-OH-C_{14}), with two of these further acylated with myristate (C_{14}) (Figure 3a and Figure S1). This mass correlated with a doubly charged species of *m/z* 911.6 with greater intensity, herein designated as the WT lipid A. Lesser quantities of various modifications accompanied the WT lipid A, including a hydroxyl modification of a myristate (*m/z* 919.6, WT + $C_{14:OH}$), palmitoylation (*m/z* 1030.7, WT + C_{16}) and Ara4N (*m/z* 977.1, WT + Ara4N) (Figure 3b). The detection of Ara4N species may indicate the initial strain is heteroresistant, with a resistant subpopulation existing within a phenotypically susceptible isolate. Pmx-R isolates showed near complete loss of WT lipid A and fortification of Ara4N on phosphate groups, mainly in hydroxymyristate species [*m/z* 985.1, WT + $C_{14:OH}$ + Ara4N; *m/z* 1042.7, WT + 2(Ara4N); *m/z* 1050.7, WT + $C_{14:OH}$ + 2(Ara4N)] (Figure 3b, Figure S2 and Figure S3). The other commonly reported resistance modification to lipid A, pEtN (*m/z* 973.2), was never observed. Lipid A from the octapeptin C4-selected isolates differed from the Pmx-R isolates and was similar to the WT profile (major peak of the hydroxymyristate derivative), but with a significant 5-fold increase in representation of palmitoylation (Figure 3b and Figure S4). The Ara4N modification was enhanced compared with WT, but not to the extent seen with Pmx-R isolates.

Plasmid loss associated with octapeptin C4 resistance

To ascertain the genetic basis for resistance and subsequent phenotypic traits, four day 20 replicates were selected from each treatment group. Clonal expansion of genomic variations was monitored by selecting four colonies per replicate along with two colonies from the initial isolate. The initial isolate harboured resistance genes targeting aminoglycosides, β-lactams, fosfomycin, quinolones, sulphonamides, tetracycline and trimethoprim (Table 2). Five plasmid replicons were identified including ColRNAI, IncFIB(K)-Kpn3, IncFII(K), IncN and IncX3. The only replicates with other resistance gene alterations in polymyxin-treated groups were PMB_2 [loss of *aph(3′)-Ia*, bla_{KPC-2}, bla_{OXA-9} and IncX3 replicon, $n = 4$] and PMB_3 (loss of IncX3, $n = 1$) (Table 2). High variability was observed for octapeptin C4-exposed replicates including the absence of *aph(3′)-Ia*, *aph(3′′)-Ib*, *aph(6)-Id*, bla_{KPC-2}, bla_{OXA-9}, bla_{TEM-1B}, *sul2*, *tet*(A) and *dfrA14*. Furthermore, plasmid replicon loss was apparent in three of the four replicates including IncFIB(K)-Kpn3, IncFII(K) and IncN.

Chromosomal variations in LPS pathways associated with polymyxin resistance whilst phospholipid (PL) transport associated with octapeptin C4 resistance

Genomic alterations identified in replicates treated with polymyxin or octapeptin C4 differed significantly. In Pmx-R replicates, affected genes were predominantly associated with LPS processing and lipid A modifications, including *crrB*, *hepIII*, *lptC*, *mgrB*, *pmrB*, *phoPQ* and *yciM* (Table 3). An additional TCS gene, *qseC*, was also disrupted in PMB_3 (S8R, I283L) and PMB_4 (L40F). Although similar genes were impacted across replicates, the mutation positions differed. Additionally, an accumulation of variations in LPS pathways was apparent within a single replicate. Variants were observed in all four colonies indicating clonal expansion.

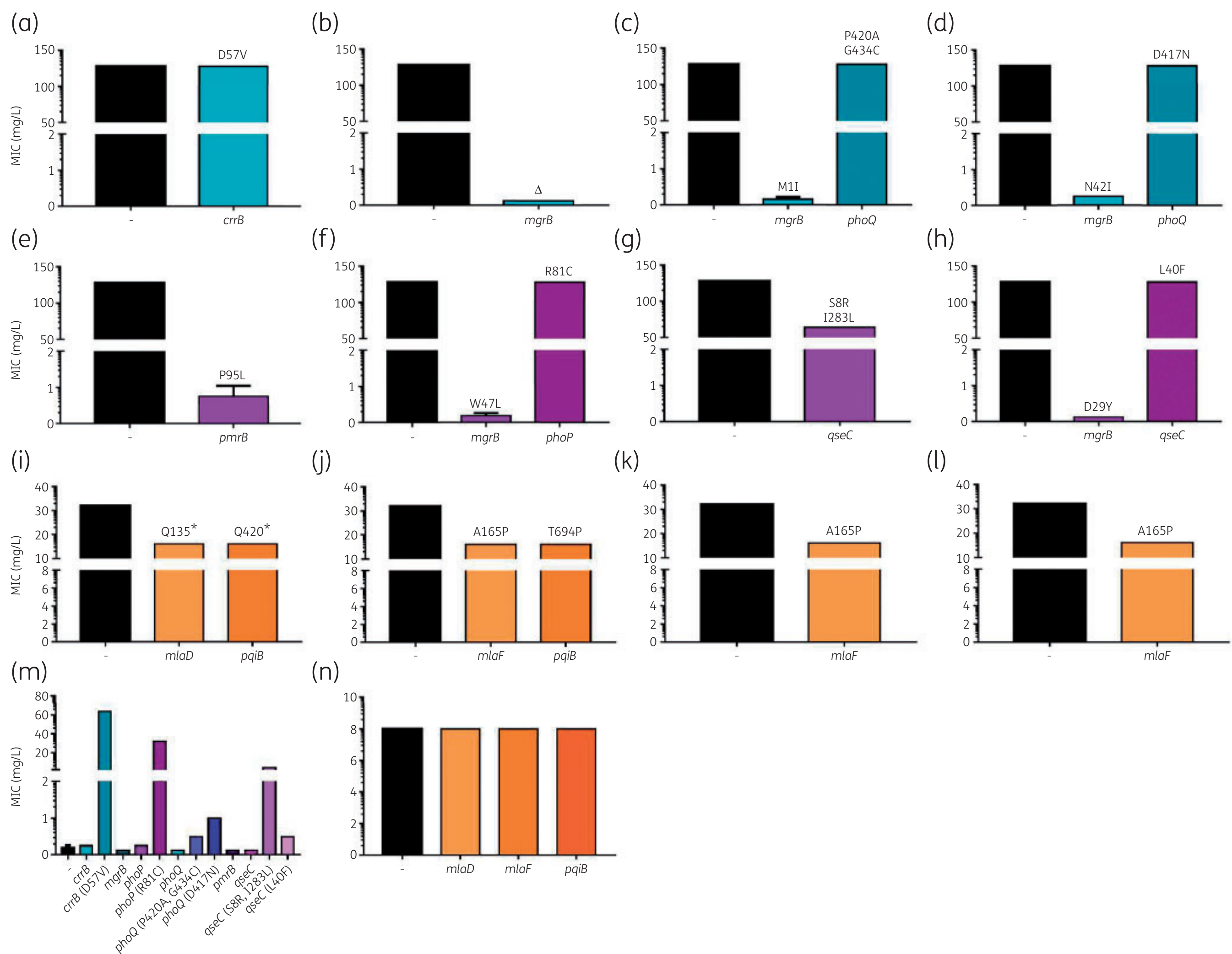


Figure 4. Complementation assays to delineate contribution to resistance of variations detected in day 20 treated strains. (a–d) Colistin treatment groups complemented with WT gene. (a) CST_1 with pTOPO-*crrB*. (b) CST_2 with pTOPO-*mgrB*. (c) CST_3 with pTOPO-*mgrB* or pTOPO-*phoQ*. (d) CST_4 with pTOPO-*mgrB* or pTOPO-*phoQ*. (e–h) Polymyxin B treatment groups complemented with WT gene. (e) PMB_1 with pTOPO-*pmrB*. (f) PMB_2 with pTOPO-*mgrB* or pTOPO-*phoP*. (g) PMB_3 with pTOPO-*qseC*. (h) PMB_4 with pTOPO-*mgrB* or pTOPO-*qseC*. (i–l) Octapeptin C4 treatment groups complemented with WT gene. (i) OctC4_1 with pTOPO-*mlaD* or pTOPO-*pqiB*. (j) OctC4_2 with pTOPO-*mlaF* or pTOPO-*pqiB*. (k) OctC4_3 with pTOPO-*mlaF*. (l) OctC4_4 with pTOPO-*mlaF*. (m) 20_GR_12, the initial strain, complemented with WT genes and genes harbouring mutations potentially causing polymyxin resistance. (n) Complementation of octapeptin C4 resistance-associated WT genes in 20_GR_12. A - symbol indicates that no complementation was conducted and represents the initial MIC. The *y*-axis split signifies the breakpoint for polymyxins (2 mg/L) and initial MIC for octapeptin C4 (8 mg/L). Values represent mean ± SD ($n = 4$). Text above bars (a–l) indicates the amino acid change(s) in the selected resistant isolate. Δ represents a complete gene deletion and * is a stop codon. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

Complementation assays were conducted to unveil these genes' contributions to resistance (Figure 4). Polymyxin susceptibility was restored in CST_2 (complete deletion of *mgrB*), CST_3 (M1I), CST_4 (N42I), PMB_2 (W47L) and PMB_4 (D29Y) once complemented with pTOPO-*mgrB* (Figure 4b–d, f and h). The PmrB (P95L) variant in PMB_1 was validated to contribute to resistance (Figure 4e). Alterations in CrrB (D57V), PhoP (R81C) and QseC (S8R, I283L) were confirmed to cause resistance once these mutated genes were introduced into the initial strain (Figure 4m). Subtle increases in polymyxin MIC were detected for PhoQ (P420A, G434C), PhoQ (D417N) and QseC (L40F) but did not surpass the breakpoint MIC (Figure 4m). This confirms the presence of multiple resistance-conferring mutations in a single isolate with several contributing to polymyxin tolerance.

Several octapeptin C4 replicates harboured changes in *mlaDF*, *pqiB* and *traH* in all four colonies. Additional genes that were altered in two colonies per replicate included *azoR*, *hinT* and *rpsA*. Strikingly, *mlaF* (A165P) was impacted in three different octapeptin C4 replicates at the same position (Table 3). Complementation assays that introduced pTOPO-*mlaD*, -*mlaF* or -*pqiB* into octapeptin C4-resistant replicates reduced the MIC by 2-fold; however, consistently only partial growth was observed at 8 mg/L (Figure 4i–l). Introduction of WT genes into the initial isolate revealed that the vector and gene did not influence the MIC and

Table 3. Genomic alterations detected in day 20 resistant isolates

Strain[a]	Gene	Gene description	Nt change[b]	Amino acid change[c]
CST_1 (4)	*crrB*	two-component hybrid sensor and regulator	A170T	D57V
CST_2 (4)	*hepIII*	LPS heptosyltransferase III	A238Δ^{fs}	R80G^{tr}
CST_2 (4)	*mgrB*	putative inner membrane protein	1-144Δ	1-47Δ
CST_3 (4)	*mgrB*	putative inner membrane protein	G3A	M1I
CST_3 (4)	*phoQ*	sensor protein	C1258G, G1300T	P420A, G434C
CST_4 (3)	*epsJ*	glycosyltransferase	T932G	L310STOP
CST_4 (3)	*lptC*	LPS export system protein	Δ498A^{fs}	N166K^{tr}
CST_4 (4)	*mgrB*	putative inner membrane protein	G-41T, A125T	N42I
CST_4 (4)	*phoQ*	sensor protein	G1249A	D417N
PMB_1 (4)	*pmrB*	sensor protein	C284T	P95L
PMB_2 (4)	*dnaJ*	chaperone protein	A892C	T298P
PMB_2 (4)	*mgrB*	putative inner membrane protein	G140T	W47L
PMB_2 (4)	*phoP*	transcriptional regulatory protein	C241T	R81C
PMB_2 (4)	*hepIII*	LPS heptosyltransferase III	TGAAGAGACCCG153Δ	Y51STOP
PMB_3 (4)	*qseC*	sensory histidine kinase	GCCTGAGCCTGC17Δ^{fs}, A847C	S8R, I283L
PMB_4 (4)	*mgrB*	putative inner membrane protein	G85T	D29Y
PMB_4 (4)	*qseC*	sensory histidine kinase	CTGGATAAGCTG118Δ^{fs}	L40F
PMB_4 (4)	*yciM*	LPS regulatory protein	T128G	V43G
OctC4_1 (4)	*mlaD*	uncharacterized ATP-binding cassette (ABC) transporter, periplasmic component	C403T	Q135STOP
OctC4_1 (4)	*pqiB*	paraquat-inducible protein B	C1258T	Q420STOP
OctC4_1 (4)	*traH*	conjugal transfer protein	G417T	M139I
OctC4_2 (4)	*pqiB*	paraquat-inducible protein B	A2080C	T694P
OctC4_2 (2)	*rpsA*	small subunit (SSU) ribosomal protein S1p	T1031A	L344Q
OctC4_3 (2)	*hinT*	YcfF/hinT protein: purine nucleoside phosphoramidase	Δ240C^{fs}	D81R^{tr}
OctC4_4 (2)	*azoR*	flavin mononucleotide (FMN)-dependent NADH-azoreductase	T152A	L51Q
OctC4_2 (4), OctC4_3 (4), OctC4_4 (4)	*mlaF*	uncharacterized ABC transporter, ATP-binding protein	GCCGC493Δ^{fs}	A165P^{tr}

[a]Strain represented as: treatment group (colistin, CST; polymyxin B, PMB; octapeptin C4, OctC4)_replicate number (number of colonies impacted from the four selected).
[b]Nt variations present in ≥90% of reads and ≥50× coverage compared with the initial strain, 20_GR_12. Δ symbolizes a deletion, – in front of the nt position indicates an alteration upstream and ^{fs} represents a frameshift mutation.
[c]The introduction of a truncation in the protein downstream of the alteration is noted as ^{tr}.

confirmed that these alterations were responsible for the resistance observed (Figure 4m and n).

Discussion

Polymyxins can lead to high levels of resistance during therapeutic use, likely driven by suboptimal exposure in the clinic due to the risk of nephrotoxicity.[43] Resistance in *K. pneumoniae* appears to be stable and incurs a minimal fitness cost.[44,45] These clinical characteristics were reflected in our study whereby once the isolate could tolerate 0.5 mg/L of either colistin or polymyxin B, the clinical breakpoint was vastly exceeded within 48 hours, well within the duration of clinical antibiotic therapy. This rapid progression of resistance was not observed for octapeptin C4, in which only comparatively minor increases in MIC were observed. The slow progression in resistance profile could be an advantageous characteristic of octapeptin C4 as a potential clinical intervention.

Following 20 days of increasing sublethal antibiotic exposure, no cross-resistance was apparent between polymyxins and octapeptin C4. Colistin and polymyxin B resulted in similar resistance profiles with the only deviation seen in sample PMB_2, in which susceptibility to meropenem was regained. This was due to the absence of bla_{KPC-2} and bla_{OXA-9}, along with the homogeneous loss of IncX. Clinically, meropenem is being used in combination with polymyxins, and these results suggest that, in some cases, meropenem may overcome polymyxin resistance.[46,47] Furthermore, previous research has identified the loss of bla_{KPC} plasmids in Pmx-R clinical isolates and suggests that this loss is due to a potential fitness cost.[48] Our results also show plasmid loss in octapeptin C4-exposed replicates, and this corresponded to a reduction in resistance to cefepime, meropenem and tetracycline. However, these findings are preliminary and whether this resembles a fitness cost associated with octapeptin C4 exposure or results from repeated passaging under no selective pressure warrants further investigation. Similarly, chloramphenicol susceptibility was restored in day 20 isolates exposed to polymyxins or octapeptin C4. Whether this is the result

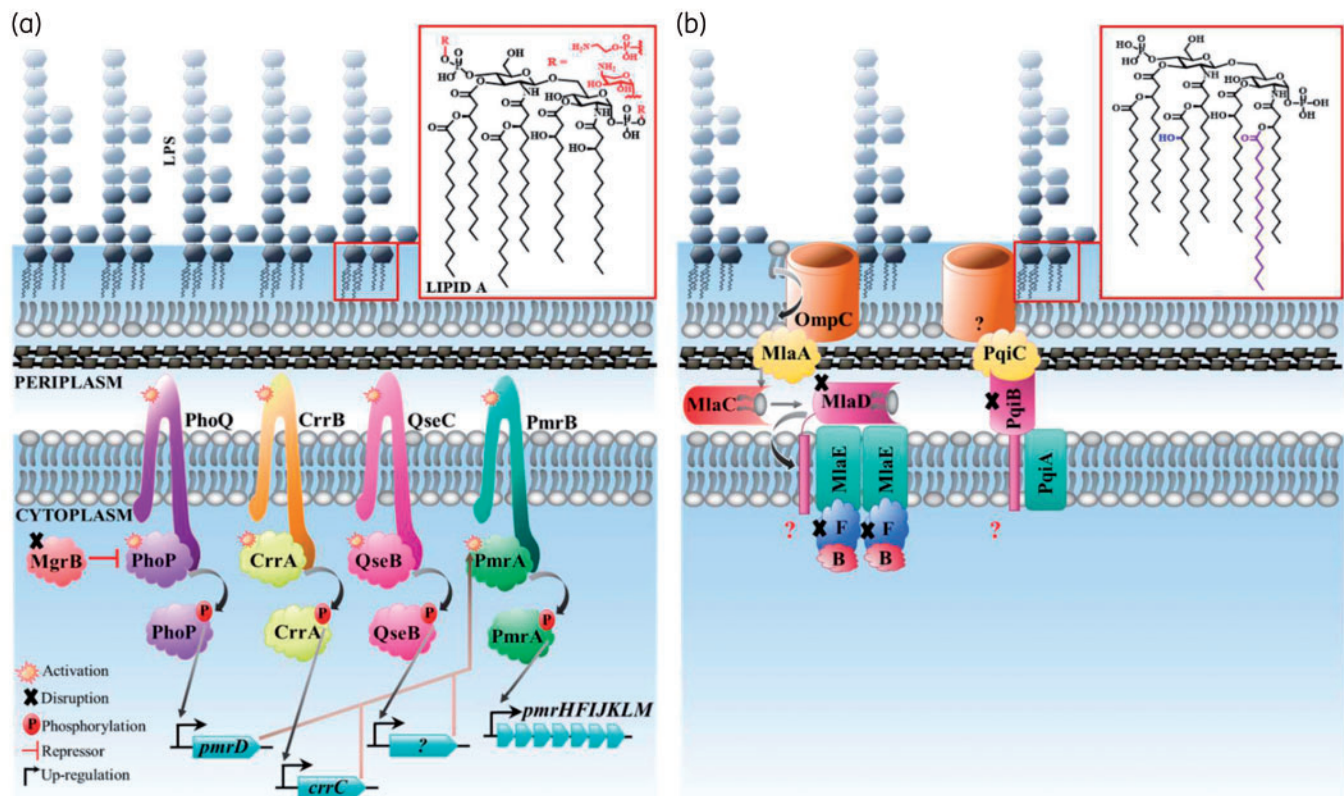


Figure 5. Proposed pathway associated with *K. pneumoniae* polymyxin and octapeptin C4 resistance observed in this study. (a) To facilitate resistance to polymyxins, genomic variations are acquired in TCSs. These encompass CrrAB, QseBC, PmrAB and PhoPQ with MgrB acting as a negative repressor. Once this pathway is activated during resistance, sensor histidine kinases (CrrB, QseC, PmrB and PhoQ) will phosphorylate response regulators (CrrA, QseB, PmrA and PhoP) and allow for the expression of target genes [*crrC*, unknown (?), *pmrD* and *pmrHFIJKLM*]. Disruptions in MgrB allow for the up-regulation of this pathway resulting in the expression of *pmrHFIJKLM*, which allows for Ara4N to be attached to phosphate groups on lipid A. The pEtN lipid A modification, facilitated via the *pmrCAB* operon, was not observed in this study. (b) The major disruptions identified during octapeptin C4 resistance were in the Mla and Pqi pathway. OmpC removes PLs from the outer membrane and transfers these to MlaA. PLs are transported across the periplasm via MlaC and transported to the MlaBDEF complex where the subsequent fate of PLs is unknown. An unknown porin complexes with PqiC to transport metabolites and potentially PLs across the periplasm via the PqiAB complex. Mutations in these pathways elevate the octapeptin C4 MIC and subsequently hydroxymyristate and palmitoylate are added to lipid A to potentially stabilize the outer membrane. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

of repeated passaging or a novel loss of chloramphenicol resistance via gaining resistance to the three lipopeptides is yet to be discerned.

The mutations observed in selected Pmx-R ST258 strains can be compared to those we have previously identified in closely related Pmx-R clinical ST258 isolates (2_GR_12, 4_GR_12, 10_GR_13, 13_GR_14 and 14_GR_14).[26] Similarly, the vast majority of resistance was attributed to *mgrB* (60%), albeit not via an IS element disruption. Additional mutations were identified in *phoPQ* accompanying the *mgrB* disruption, which was also apparent in this study [CST_3 (*mgrB*: M1I, *phoQ*: P420A, G434C), CST_4 (*mgrB*: N42I, *phoQ*: D417N), PMB_2 (*mgrB*: W47L, *phoP*: R81C)]. Other mutations in *crrB*, *mgrB*, *pmrB*, *phoPQ* and *yciM* have previously been described in Pmx-R strains.[17,18,49] Overall, this indicates that *in vitro* experiments can give rise to genomic changes similar to those observed in the clinic. The notion that one alteration in a TCS drives resistance, the circumstance for the majority of clinical isolates, is well accepted.[50] However, our findings contradict this concept although the high concentration of polymyxin used for our *in vitro* resistance selection could be influencing this finding.

We also identified alterations in another TCS, QseBC, which is known to facilitate cross-talk with PmrAB in *E. coli*.[51] In *E. coli*, PmrB acts as a non-cognate partner to the QseBC TCS and has the ability to not only phosphorylate PmrA, but also QseB. The absence of QseC was shown to impact virulence due to the accumulation of phosphorylated QseB and, in particular, alterations in the histidine kinase domain attenuates its ability to dephosphorylate QseB.[51,52] Furthermore, the deletion of *qseC* and *pmrA*, promoting phosphorylation of QseB by PmrB, stimulated tolerance to polymyxin B.[53] This signalling pathway is not well-characterized in *K. pneumoniae*. We observed partial tolerance to PMB when a frameshift mutation was apparent at nt 118; however, full resistance in PMB_4 was promoted by alterations in *mgrB* (D29Y) and *yciM* (V43G), which has recently been identified to cause resistance.[49] Similarly, PMB_3 harboured a frameshift in *qseC* (GCCTGAGCCTGC17Δ^{fs}), although an additional I283L change in the histidine kinase region resulted in an MIC of 4 mg/L. This did not explain the full resistance profile exhibited by PMB_3 and due to the presence of both alleles during complementation the true extent of resistance cannot be

deduced. Considering PMB_3 still resulted in the addition of Ara4N to lipid A, we speculate that due to the perturbation in the QseC kinase this is increasing the accumulation of phosphorylated QseB and allows for the up-regulation of transcriptional targets. Subsequent transcription could be activating PmrA, similar to other TCSs in *K. pneumoniae*, allowing for the expression of the *pmrHFIJKLM* operon (Figure 5a).

The Mla pathway was impacted in octapeptin C4-exposed replicates. These genes are responsible for PL importation from the outer membrane.[54] Removal of *mlaC* in *E. coli* was previously identified to increase the abundance of palmitoylated lipid A to stabilize the outer membrane, which correlated with the phenotype in our study. Similarly, prior research exposing *P. aeruginosa* to octapeptin C4 (32 mg/L) revealed an increase in palmitoylated lipid A.[23] Literature reports have demonstrated that octapeptins have the capacity to bind to PLs[55] and it is likely that octapeptin C4 utilizes this pathway in order to traverse to the outer membrane (Figure 5b). The involvement of PqiB in membrane integrity has only recently been characterized in *E. coli*.[56] PqiB was identified to connect to PqiC and potentially deliver substrate(s) from the outer to inner membrane. The contribution of the Pqi and Mla pathway appeared to be additive when evaluating the MIC reduction in OctC4_1 and OctC4_2. Further genes impacted, though not homogeneously amongst the colonies, included *rpsA* (40S ribosomal protein), *azoR* (quinone reductase), *traH* (plasmid conjugal transfer protein) and *hinT* (purine nucleoside phosphoramidase), which may indicate several intracellular targets.[57–60] The lack of mutations associated with Ara4N modifications to lipid A is consistent with the lipid A profile of the octapeptin C4-resistant isolates. This observation supports the hypothesis that the octapeptins work by a different mode of action compared with the polymyxins, one that does not require an initial binding to lipid A and explains the lack of cross-resistance between the two classes of lipopeptides. However, further studies are required to determine if this occurs ubiquitously for *K. pneumoniae* and if the same phenomenon is observed for other Gram-negative pathogens. The development of resistance in *in vitro* experiments entails several caveats compared with the clinical *in vivo* condition, including a limited regulation of cell growth and antibiotic concentration and exposure to a concentration of antibiotic that may not reflect an *in vivo* scenario.[61,62] Hence, it would be of interest to discern the development of octapeptin resistance *in vivo*. Nonetheless, the slow progression of resistance, potential fitness cost if resistance develops and the alternative mechanism of infiltration of octapeptin C4 highlight the potential for octapeptins as future antibiotics.

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Transparency declarations

M. A. C. currently holds a fractional Professorial Research Fellow appointment at the University of Queensland with his remaining time as CEO of Inflazome Ltd, a company with headquarters in Dublin, Ireland that is developing drugs to address clinical unmet needs in inflammatory disease by targeting the inflammasome. All other authors: none to declare.

Author contributions

M. E. P., M. A. C., L. J. M. C. and M. A. T. B. conceived this study. M. E. P., M. D. C. and D. G. performed the sequencing analysis and M. E. P., M. S. B. and S. R. performed the experiments. M. E. P. wrote the paper with input from the other authors.

Supplementary data

Tables S1 and S2 and Figures S1 to S4 are available as Supplementary data at *JAC* Online.

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