Identification of Novel Colistin Resistance Genes in an Extremely Colistin Resistant *Pseudomonas aeruginosa* Clinical Isolate

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Abstract

Background

Pseudomonas aeruginosa (PA) readily acquires genomic mutations and exogenous genetic elements that confer antimicrobial resistance (AMR). With the rise in AMR, there are limited antibiotics available to treat multidrug-resistant (MDR) PA. As such, clinicians have returned to previously used antibiotics. Colistin, sidelined for neurotoxicity and nephrotoxicity, has returned to clinical practice as a viable but suboptimal option for MDR-PA treatment. The most common mechanism of resistance to colistin involves modifications of the lipid A moiety within the bacterial lipopolysaccharide (LPS) [1, 2]. Following the identification of a MDR PA isolate, BWH047, we experimentally determined its colistin MIC to be > 1,280 µg/mL and used genomic approaches to identify novel genetic mechanisms of extreme colistin resistance [3].

Methods

We created a random, saturated transposon (Tn) insertion library in PA BWH047 using the *Himar1* mariner system. After exposure of the library to 640 µg/mL colistin for 10 hours, genomic DNA was harvested, and the Tn insertion sites were sequenced. Insertion sequencing (INSeq) analysis was performed. We identified 27 genes conditionally important for BWH047 growth in the presence of colistin. We initially selected five targets *arnC, dedA, wapH, speE2,* and *bchE* and tested their impact on colistin resistance using standard broth microdilution methods.

Results

Of our deletion mutants, three showed loss of resistance to colistin. ArnC was chosen as a positive control as its role in colistin resistance in PA is well described [1]. Colistin MICs of BWH047 Δ*arnC*, Δ*dedA*, Δ*wapH*, Δ*speE2,* and Δ*bchE* were determined to be 0.5, 0.5, 1, > 1,280 and > 1280 µg/mL, respectively.

Conclusions

Here, we used INSeq to identify novel genes involved in extreme colistin resistance. Thus far, we have identified two new candidate genes *dedA* and *wapH*, critical for colistin resistance in PA BWH047. Neither gene has been associated with colistin resistance in PA. However, *dedA* orthologs in *Burkholderia thailandenesis* and *Klebsiella pneumoniae* have been shown to be important for colistin resistance [4,5]. The gene *wapH* is part of the LPS core oligosaccharide biosynthetic pathway and its discovery hints that additional alterations in the bacterial outer membrane may impact colistin resistance [6].

Background and Library Generation

Clinical and Isolate Background

- BWH047 was isolated from a BAL from a patient on protracted inhaled colistin therapy
- Kirby-Bauer and broth microdilution (BMD) testing revealed that BWH047 was a multidrug-resistant PA isolate
- To determine the actual minimal inhibitory concentration (MIC) for colistin, 10x BMD MICs were performed. The MIC of BWH047 was determined to be >1280 µg/mL [3]
- Genomic sequencing did not reveal the presence of any canonical colistin resistance mutations or the presence of *mcr* genes which confer colistin resistance. [3]

Mechanisms of Colistin Resistance

- Colistin, a cationic polypeptide disrupts negatively charged bacterial membranes leading to membrane instability and cell death.
- Common mechanisms of resistance include modification to LPS, specifically lipid A, with phosphoethanolamine (pEtN) and 4-amino-4-deoxy-L-arabinose (L-Ara4N) [1,2]
- Additional resistance mechanisms include:
  - Upregulation of efflux pumps
  - Upregulation of two-component systems
  - Increased biofilm formation

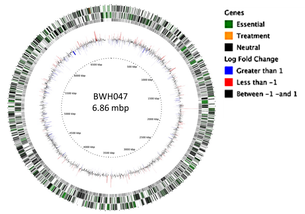


Figure 1: Creation of a Transposon Library in BWH047. A mariner-based transposon system was used to generate a saturated transposon-insertion library in BWH047 (~2,000,000 unique insertions)

INSeq Experimental Design

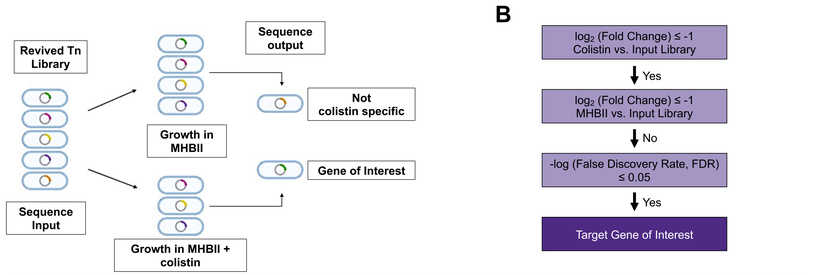


Figure 2: Execution of BWH047 INSeq Experiment. (A) The diverse BWH047 transposon library was subjected to treatment with ¼ MIC (320 µg/mL) and ½ MIC (640 µg/mL) colistin for 10 hours at 37˚C and the remaining bacteria sequenced. **(B)** Genes that had a $\log_2$ fold-change of ≤ -1 only in the presence of colistin (and not MHBII alone) and a -log false discovery rate (FDR) ≤ 0.05 were selected for additional analysis.

Identification of Target Genes of Interest

A

Target #	Gene	Putative Function
1	*hypothetical 00163*	hypothetical protein, CPBP family metalloprotease
2	*topA*	DNA topoisomerase
3	*arnB*	4-amino-4-deoxy-L-arabinose (L-Ara4N) aminotransferase
4	*arnC*	L-Ara4N arabinose transferase
5	*arnT*	L-Ara4N-arabinose-arabinosyl transferase
6	*rkpK*	UDP-glucose 6-dehydrogenase
7	*dedA*	conserved membrane protein
8	*phzA*	phenazine biosynthesis protein
9	*mexA*	RND multidrug efflux membrane protein
10	*algC*	phosphomannomutase/phosphorglucomutase
11	*bchE*	B12-binding methyltransferase, radical S-adenosyl-L-methionine protein
12	*hypothetical 02806*	hypothetical protein, GNAT family N-acetyltransferase
13	*wapH*	D-inositol-3-phosphate glycosyltransferase
14	*speE2*	polyamine aminopropyltransferase
15	*speH*	S-adenosylmethionine decarboxylase
16	*pgi*	glucose-6-phosphate isomerase
17	*hypothetical 04230*	soluble epoxide hydrolase
18	*hypothetical 04523*	hypothetical protein
19	*cysH*	phosphoadenosine phosphosulfate reductase
20	*oprF*	outer membrane porin F
21	*mexY*	RND multidrug efflux transporter
22	*zapE*	cell division protein
23	*ctpC*	cation transporting (cadmium, zinc, manganese) P-type ATPase
24	*lecA*	PA-1 galactophilic lectin
25	*ndhC*	NAD(P)H-quinone oxidoreductase, subunit
26	*nuoE*	NADH-quinone oxidoreductase, subunit
27	*hypothetical 06381*	hypothetical protein, tyrosine-type recombinase/integrase

B

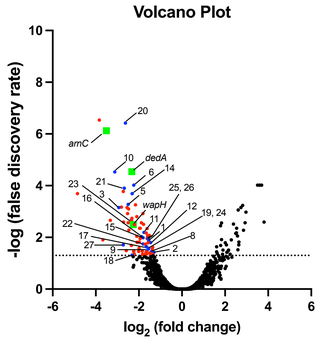


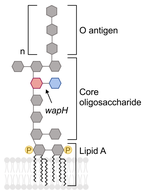
Figure 3: Identification of candidate gene targets. (A) Table listing all 27 gene targets conditionally essential for growth of BWH047 in the presence of colistin. **(B)** For all genes with insertion data (n~4600), $\log_2$ fold-change (x-axis) and –log false discovery rate (FDR, y-axis) were plotted to generate a volcano plot. All genes with $\log_2$ fold-change ≤ -1 and a –log FDR ≥ 1.3 (dashed line) are highlighted in red. The filtered list of target genes are highlighted in blue (n=27) and annotated with target numbers that match column 1 of the table. Notable genes whose deletion dramatically alters colistin MIC (n = 3) are presented as green squares.

Phenotypic Analysis

Strain	Colistin MIC (µg/mL)
BWH047	1280
Δ*hypo00163*	320
Δ*arnC*	0.5
Δ*dedA*	0.5
Δ*phzA*	640
Δ*mexA*	1280
Δ*bchE*	1280
Δ*wapH*	1
Δ*speE2*	1280
Δ*oprF*	320

Table 2: Colistin minimum inhibitory concentrations (MIC). Broth microdilution (BMD) for wildtype BWH047 and select mutants. We identified two genes, *hypothetical 00163* and *oprF* with modest reductions in colistin MIC (blue), and three genes (*arnC, dedA* and *wapH*) with dramatically reduced colistin MICs (red).

wapH



WapH

- Part of a LPS core oligosaccharide biosynthetic operon [6-8]
- Putative glycosyltransferase: catalyzes (black arrow) the addition of D-glucose (blue) to 2-amino-2-deoxy-galatose (red) on the core oligosaccharide [6-8]
- Not previously described as having a role in colistin resistance

Figure 4: Structure of LPS. PA LPS is composed of three domains: lipid A, core oligosaccharide and the O-antigen.

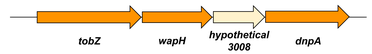
dedA

Strain	IPTG (mM)	Colistin MIC (µg/mL)
BWH047	-	1280
Δ*dedA*	-	0.5
Δ*dedA* + pPSV37	1	0.5
Δ*dedA* + pPSV37-*dedA*	1	128

DedA

- In *Burkholderia thailandensis* and *Klebsiella pneumoniae*, orthologs play a critical role in colistin resistance [4,5]
- *K. pneumoniae* Δ*dedA* had reduced L-Ara4N modification of lipid A [5]
- Ectopic expression of *dedA* off plasmid pPSV37 partially complements colistin MIC of Δ*dedA* mutant.

wapH operon



Strain	Colistin MIC (µg/mL)
BWH047	1280
Δ*tobZ*	160
Δ*dnpA*	1280

TobZ

- Putative carbamolytransferase that may be involved in LPS core oligosaccharide biosynthesis [7]
- Mutations in *tobZ* linked to colistin resistance [9]

DnpA

- Located downstream of *wapH*
- Possible role in PA persister cell phenotype [10]

Conclusions and Future Directions

- INSeq analysis validated known mechanisms of resistance and revealed two novel genes which abrogate the extreme colistin MIC of BWH047.
- LPS modification and biosynthesis plays a crucial role in colistin resistance.
- Additional genes targets of unknown function have been identified that modestly impact colistin MIC.
- LPS and lipid A analysis of BWH047 and Δ*dedA* and Δ*wapH* mutations
- Complementation of all mutations that impact colistin MIC
- Ongoing gene deletion and colistin MIC phenotypic analysis of remaining genes identified by INSeq analysis

References

1. Olaitan, A.O., Morand, S., and Rolain, J.M., Mechanisms of polymxin resistance: acquired and intrinsic resistance in bacteria. *Front Microbiol*. 2014. 5:643.
2. Fernandez L., et al., Characterization of the Polymyxin B Resistome of *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother*. 2013. 57(1):110-19.
3. Bachta, K.E.R., et al., Draft Genome Sequence of Pseudomonas aeruginosa Strain BWH047, a Sequence Type 235 Multidrug-Resistant Clinical Isolate. *Microbiol Resour Announc*. 2019. 8(29).
4. Panta, P.R., et al., A DedA Family Membrane Protein is Required for *Burkholderia thailandensis* Colistin Resistance. *Front Microbiol*. 2019. 10:2532.
5. Tiwari, V., et al., A *Klebsiella pneumoniae* DedA family membrane protein is required for colistin resistance and for virulence in a wax moth larvae. *Sci. Rep.* 2021. 11(1):24365.
6. Matewish, J.M., The Functional Role of Lipopolysaccharide, the Cell Envelope and Surface Proteins of *Pseudomonas aeruginosa*. Thesis. University of Guelph. Ontario, Canada. 2004.
7. King, J.D., et al., Review: Lipopolysaccharide biosynthesis in *Pseudomonas aeruginosa*. *Innate Immun*. 2009. 15(5): 261-312.
8. Lam, J.S., et al., Genetic and Functional Diversity of Pseudomonas aeruginosa Lipopolysaccharide. *Front Microbiol*. 2011. 2:118.
9. Jochumsen, T., et al., The evolution of antimicrobial peptide resistance in *Pseudomonas aeruginosa* is shaped by strong epistatic interactions. *Nat Commun*. 2016. 7:13002.
10. Khandekar, S., et al., The Putative De-N-acetylase DnpA Contributes to Intracellular and Biofilm-Associated Persistence of *Pseudomonas aeruginosa* Exposed to Fluoroquinolones. *Front Microbiol*. 2018. 9:1455.

Contact Information

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