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The emergence of cefiderocol resistance in *Pseudomonas aeruginosa* from a heteroresistant isolate during prolonged therapy

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ABSTRACT Cefiderocol is a siderophore cephalosporin designed to target multi-drug-resistant Gram-negative bacteria. Previously, the emergence of cefiderocol non-susceptibility has been associated with mutations in the chromosomal cephalosporinase (PDC) along with mutations in the PirA and PiuA/D TonB-dependent receptor pathways. Here, we report a clinical case of cefiderocol-resistant *P. aeruginosa* that emerged in a patient during treatment. This resistance was associated with mutations not previously reported, suggesting potential novel pathways to cefiderocol resistance.

KEYWORDS gram-negative bacteria, siderophores, *Pseudomonas aeruginosa*, cefiderocol

Cefiderocol is a novel siderophore antibiotic designed to target multi-drug-resistant (MDR) Gram-negative bacteria. Cefiderocol overcomes traditional efflux and porin-mediated resistance, by binding to extracellular iron and utilizing TonB-dependent iron-siderophore transporters (TBDT) to gain access to the periplasmic space (1). *In vitro* studies have established PirA and PiuA/PiuD as the major TBDT pathways responsible for cefiderocol uptake in *Pseudomonas aeruginosa*, and we have previously reported mutations in these pathways in cefiderocol non-susceptible clinical isolates (2, 3). In addition, the size of the large siderophore moiety decreases cefiderocol's vulnerability to degradation by beta-lactamases, such as the AmpC cephalosporinase intrinsic to *P. aeruginosa* (4). These factors have made it a promising last-line agent in the treatment of MDR *P. aeruginosa* infections. However, there are concerns of poor clinical outcomes and treatment failures with cefiderocol monotherapy despite *in vitro* susceptibility (5). Heteroresistance, or the survival of a small subpopulation of bacteria at or above the minimum inhibitory concentration (MIC) of an antibiotic, has been postulated as a mechanism for this observation (6). In this report, we identify treatment-emergent cefiderocol resistance in a patient associated with gene mutations not previously reported arising from a heteroresistant isolate.

A man in his 30s with a medical history of type II diabetes mellitus was admitted to an outside facility for acute respiratory distress syndrome (ARDS) due to SARS-CoV-2. He required venovenous extracorporeal membrane oxygenation over a 100-day period with minimal improvement. During this stay, he developed a bronchopleural fistula with associated right-sided empyema, and cultures grew MDR *P. aeruginosa*. Initial susceptibility testing demonstrated resistance to amikacin, meropenem, ceftazidime-avibactam, and ceftolozane-tazobactam (C/T). The patient underwent treatment with chest tube placement and an 8-week course of cefiderocol therapy; however, there was minimal clinical improvement, and cultures from the sputum and pleural fluid remained positive. He was transferred for lung transplantation evaluation. Repeat sputum culture 2 days after transfer showed persistent MDR *P. aeruginosa* (isolate BSL5) and at this time a new

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course of therapy was initiated with cefiderocol (2 g intravenously every 6 hours for augmented renal clearance) plus inhaled colistin (75 mg q12h by nebulization). Cultures of the pleural fluid were obtained after 31 days (BSL9), and 48 days (BSL15) of cefiderocol therapy due to lack of clinical improvement on directed antibiotics. The patient was ultimately deemed ineligible for transplant and was transitioned to comfort care after 81 days of hospitalization.

Cefiderocol susceptibilities of the isolates were initially screened by gradient strips, and subsequently confirmed by broth microdilution in iron-depleted Mueller-Hinton (ID-MH) media and Kirby-Bauer disk diffusion testing on standard Mueller-Hinton agar per Clinical Laboratory Standards Institute (CLSI) guidelines. In addition, population analysis profile (PAP) testing was performed *via* agar dilution using serial cefiderocol concentrations (0, 4, 8, and 16 ug/mL) on Mueller-Hinton agar. The number of surviving colonies at each cefiderocol concentration (read at 24 and 48 hours) was subtracted from the number of colonies on the growth control plate to calculate the change in CFU (ΔCFU). The ΔCFU was plotted across each cefiderocol concentration, and the area under the curve (AUC) was calculated using the trapezoidal method with a baseline of −10 in Prism (GraphPad, Boston, MA, USA). We defined heteroresistance as less than a 6 $\log_{10}$ decrease in bacterial growth at the broth microdilution cefiderocol breakpoint (4 ug/mL) or above, according to the criteria of Band and Weiss (7). The strains PAO1 (susceptible) and C1814 (heteroresistant) were used as controls (8). Genomic DNA was isolated from each strain and sequencing was performed on the MiSeq platform (Illumina, Inc.) with 2 × 300 bp paired-end reads. Variant calling was performed using snippy (https://github.com/tseemann/snippy) using PAO1 as a reference. Genomes were assembled using Spades v.3.15.5 and resistome was determined using AMRFinderPlus v.3.11.14 (9, 10). Sequence data for the isolates are available on the National Center for Biotechnology Information database under the Bioproject accession number PRJNA1034644.

Cefiderocol susceptibility testing by broth microdilution in ID-MH media showed categorical agreement with initial screening (Table 1). Population analysis profiles showed a progressive loss of cefiderocol susceptibility with BSL5 displaying a susceptible phenotype and BSL15 displaying a frankly resistant phenotype (Fig. 1). BSL9 met the criteria for heteroresistance, with a small subpopulation surviving at a cefiderocol concentration of 16 µg/mL. Statistical analyses revealed a significant difference between BSL9 survival versus both PAO1 and BSL5 (*P*-value = 0.005 and 0.02, respectively).

Whole-genome sequencing data revealed all three isolates were related, with 54, 49, and 65 variants present exclusively in BSL5, BSL9, and BSL15, respectively (all non-synonymous changes are listed in Table S1). Each isolate shared the same mutations in the *oprD, ampC* [encoding the Pseudomonas-derived cephalosporinase (PDC)], *ampR*, *pirA, pirR, pirS, piuA,* and *piuC* genes (Table S2). No mutations were found in *ftsI* encoding penicillin-binding protein 3. All three isolates carried the $bla_{\text{VIM-4}}$ gene, encoding the Verona integron-encoded metallo-β-lactamase. A version of the modified carbapenem

TABLE 1 Susceptibilities of the isolates investigated[g]

Strain	MEM MIC (µg/mL)[a]	C/T MIC (µg/mL)[b]	CZA MIC (µg/mL)[a]	CST MIC (µg/mL)[c]	FDC MIC (µg/mL)[d]	DD (mm)[e]	PAP-AUC
PAO1	ND	ND	ND	ND	0.25	31	40.63
hR control	ND	ND	ND	ND	1	21	124.9
BSL5	>8	>256	48	4	2	26	45.35
BSL9	>8	>256	ND[f]	4	4	24	90.58
BSL15	>8	>256	ND[f]	4	>32	13	158.9

[a]Clinical microbiology laboratory.
[b]Research laboratory, gradient diffusion strip.
[c]Research laboratory, broth microdilution in cation adjusted Mueller-Hinton.
[d]Research laboratory, broth microdilution in iron-depleted Mueller-Hinton media.
[e]Kirby-Bauer disk diffusion zone diameter, in millimeters.
[f]Presumed resistant.
[g]C/T, ceftolozane-tazobactam; CZA, ceftazidime-avibactam; CST, colistin; FDC, cefiderocol; hR, heteroresistant; MEM, meropenem; PAP-AUC, population analysis profile-area under the curve; ND, not determined.

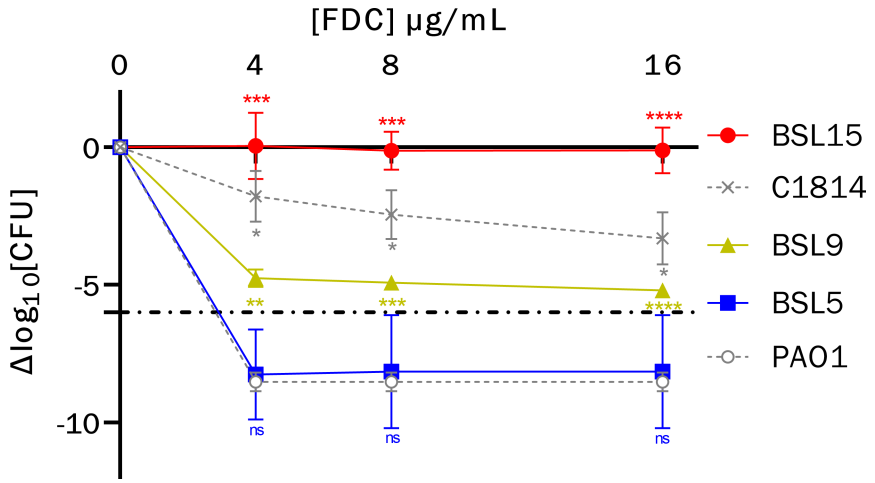

FIG 1 Population analysis profiles of sequentially collected multi-drug-resistant *P. aeruginosa*. Isolate profiles were compared to representative susceptible control (PAO1, open circle) and heteroresistant control (C1814, X) phenotypes. Dash-dot line represents the cutoff for heteroresistance. Error bars represent 95% confidence intervals across triplicate runs with a controlled inoculum of approximately 10^7–10^8. *P*-values for comparison of individual isolate growth relative to PAO1: ns, not significant, * ≤0.05, ** ≤0.01, *** ≤0.001, **** ≤0.0001.

inactivation method using cefiderocol disks did not suggest the VIM-4 enzyme significantly contributed to the cefiderocol resistance phenotype (Table S3) (11). Compared to isolates BSL5 and BSL9, the resistant isolate BSL15 harbored 15 unique non-synonymous mutations in a coding region, three of which led to insertion, deletion, or frameshift mutations (Table 2). The first was in PA5279, predicted to encode a protein of the DUF484 family with a GAF domain, part of a six open reading frame operon and upstream of a site-specific recombinase, implicated in zinc-associated pyoverdine production and pyocin production (12, 13). However, a pyoverdine fluorescence assay showed a higher pyoverdine production in BSL5 as compared to BSL15, suggesting that an increase in pyoverdine synthesis was not related to the decreased cefiderocol susceptibility of

TABLE 2 Nucleotide changes in a coding region unique to BSL15

POS[a]	Effect	Nucleotide change[b]	Amino acid change	Gene loci[c]
473432	Missense variant	c.242A > C	p.Glu81Ala	PA0426
750530	Missense variant	c.574C > G	p.Leu192Val	PA0690
750652	Missense variant	c.696_697delGGinsCA	p.Asp233Asn	PA0690
994768	Inframe insertion	c.70_71insAGGCTGCCGGGAGT GTCGTCGAGACGGGGCAAG	p.Gln24_Ala25insAlaAlaGlySer ValValGluThrGlyGlnGlu	PA0911
1566317	Missense variant	c.2351_2352delCGinsTT	p.Ser784Phe	PA1436
2262929	Missense variant	c.154G > A	p.Ala52Thr	PA2064
2263733	Missense variant	c.1244_1245delGCinsAT	p.Ser415Asn	PA2065
2647470	Missense variant	c.939_941delGTTinsCTG	p.Phe314Cys	PA2392
2685507	Missense variant	c.1672G > A	p.Val558Ile	PA2402
3596196	Inframe deletion	c.256_294delCGCCTGGTCGACG GCACCTACCTGCCGCGCCCCCACCAC	p.Arg86_His98del	PA3206
3686843	Missense variant	c.1808C > A	p.Thr603Lys	PA3294
4476328	Missense variant	c.374A > G	p.His125Arg	PA3995
4732282	Missense variant	c.4517C > A	p.Pro1506Gln	PA4225
5726546	Missense variant	c.690_693delGACTinsAGCC	p.Thr231Ala	PA5088
5944026	Frameshift variant	c.422delA	p.His141fs	PA5279

[a]Position in reference to *P. aeruginosa* PAO1 chromosome.
[b]Numbering indicates nucleotide from start codon.
[c]Gene loci references homologous gene in PAO1 chromosome.

FDC15. The second was in the *alpE* gene (PA0911), part of the AlpBCDE programmed cell death pathway implicated in *P. aeruginosa* virulence (14). The third gene encodes the CpxS sensor histidine kinase (PA3206), which regulates the cell envelope stress response in *E. coli* and has been shown to activate the MexAB-OprM efflux pump in *P. aeruginosa* (15). Interestingly, *in silico* analysis shows a consensus CpxR binding motif in the intergenic region between *piuA* and *piuC*, suggesting the potential for the CpxRS system to regulate *piuA* expression, although this has not been confirmed experimentally. There was also a reversion to wild type in BSL15 of a disruptive insertion mutation present in BSL5 and BSL9 that led to the insertion of asparagine at AA position 44 of the ClpS protein (PA2621).

The medical community has cited increasing concerns about rapidly emerging cefiderocol non-susceptibility and unanticipated treatment failure (3, 5, 16). Prior reports suggest that the emergence of cefiderocol non-susceptibility in *P. aeruginosa* occurs in the setting of mutations affecting PBP3, AmpC, or TonB-dependent receptors PirA and PiuA/D. In the present case, we describe the emergence of cefiderocol resistance *via* a heteroresistant intermediate after prolonged antibiotic exposure. This was associated with new potential pathways for the emergence of cefiderocol non-susceptibility. Further studies will be needed to characterize the specific contributions of these genes to the cefiderocol resistance phenotype.

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ADDITIONAL FILES

The following material is available online.

Supplemental Material

Supplemental Table S1 (AAC01009-23-S0001.pdf). Non-synonymous polymorphisms in each isolate.
Supplemental Table S2 (AAC01009-23-S0002.pdf). Non-synonymous mutations in resistance associated genes.
Supplemental Table S3 (AAC01009-23-S0003.pdf). Modified carbapenem inactivation method using cefiderocol disks.

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