


Evolution of cefiderocol resistance in *Stenotrophomonas maltophilia* using *in vitro* serial passage techniques

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Objectives: Cefiderocol is a siderophore cephalosporin active against MDR Gram-negatives including *Stenotrophomonas maltophilia*. Cefiderocol resistance remains uncommon and incompletely understood. We selected for cefiderocol-resistant *S. maltophilia in vitro* and characterized the genetic mechanisms and potential for cross-resistance to other antimicrobials.

Methods: We selected cefiderocol resistance in three clinical strains of *S. maltophilia* by serial passage in escalating concentrations of cefiderocol. Emergent cefiderocol-resistant isolates were subjected to repeat susceptibility testing against a panel of relevant antimicrobials. Isolates with confirmed MIC changes were whole genome sequenced.

Results: Each parent strain was initially susceptible to cefiderocol (MICs of 0.03125, 0.03125 and 0.125 mg/L), and one initially tested susceptible to ceftazidime/avibactam (MIC 4 mg/L). We recovered evolved isolates achieving cefiderocol resistance at MICs of 8–32 mg/L from each parental strain. Some cefiderocol resistant isolates reverted following one to four drug-free passages. Ceftazidime/avibactam MICs of passaged isolates repeatedly increased to ≥ 256 mg/L, and while other MICs were largely unchanged, trimethoprim/sulfamethoxazole MICs declined 4-fold in two strains. WGS revealed one evolved isolate carrying six coding mutations, while four were isogenic mutants of *tonB*, *tolQ*, *smf-1* and the *smeT* promoter. Mutation of the *smeT* promoter downregulated the *smeDEF* efflux pump and reduced susceptibility to penicillins but increased susceptibility to several other classes including sulphonamides. Other mutations occurred in genes putatively involved in iron metabolism including *smlt1148* and *cirA*.

Conclusions: *S. maltophilia* strains evolved cefiderocol resistance through different genetic pathways, but often involved iron transport. Future work is required to fully understand the role(s) of other genes in cefiderocol resistance.

Introduction

Cefiderocol is a siderophore cephalosporin with activity against Gram-negatives including many carbapenem-resistant strains, although most isolates included in studies have been Enterobacteriales or *P. aeruginosa*.^{1,2} There is limited information about cefiderocol resistance among *Stenotrophomonas maltophilia*,^{1–5} a difficult-to-treat pathogen with many innate resistance mechanisms including chromosomally encoded class A serine and class B metallo- β -lactamases that render most β -lactams ineffective.⁶ *S. maltophilia* is an important nosocomial pathogen among oncology patients that may also cause pneumonia in patients with obstructive lung disease or cystic fibrosis.

The treatment of choice for *S. maltophilia* is trimethoprim/sulfamethoxazole, however, its utility is sometimes reduced by resistance or tolerability, especially among haematology and oncology patients.⁷ Due to its unique mechanism of action, cefiderocol is promising for the treatment of *S. maltophilia*. Since cefiderocol has recently been introduced and has a novel mechanism of action, clinical isolates that are resistant to cefiderocol are uncommon. Consequently, the full spectrum of mutations and mechanisms that could mediate cefiderocol resistance are incompletely understood, especially for *S. maltophilia*. Our study objective was to identify and describe possible mutational mechanisms of cefiderocol resistance in *S. maltophilia* using *in vitro* serial passage techniques and WGS.

Methods

Serial passage, susceptibility testing and phenotype stability

Using three independent, cefiderocol-susceptible *S. maltophilia* blood isolates obtained from the University of Washington Medical Center (W358, W359, and W364), we selected for cefiderocol resistance by serial passage in escalating concentrations of cefiderocol in cation-adjusted iron-depleted Mueller–Hinton broth beginning at 0.5× the MIC (CA-IDMHB; provided by Shionogi Inc. through International Health Management Associates). Once growth was visible (within 8 h to 2 days) a sample of the broth was diluted 1:1000 into fresh CA-IDMHB with twice the previous concentration of cefiderocol. Fifty microlitres was plated onto Mueller–Hinton agar and a Kirby–Bauer disc containing 30 mg of cefiderocol was added to screen for the emergence of isolates with reduced susceptibility. Emergent isolates with reduced zone sizes (<16 mm) on screening plates were subjected to repeat MIC testing in duplicate by broth microdilution (ATCC 27853 used as QC strain) to cefiderocol, ceftazidime, trimethoprim/sulfamethoxazole and minocycline, as well as ceftazidime/avibactam by MIC test strip (Liofilchem®). MICs were read after incubation at 37°C for 24 h. Passages continued for 21 days and cefiderocol concentrations ranged from 0.03 mg/L on Day 1 to as high as 32 mg/L by Day 21. Recovered isolates were passaged on drug-free medium for four passages with repeated MIC testing to assess stability of the resistance phenotype.

WGS

WGS was performed on a selection of mutant isolates displaying elevated MICs of cefiderocol relative to their parental strains using the MiSeq platform with 300 cycle chemistries (Illumina, San Diego, CA, USA) to an average read depth of 86× (minimum depth of 22× and down-sampled to a maximum depth of 100×), as previously described.⁸ Genomes were analysed essentially as described by Roach et al.,⁸ except using ABySS 2.0.2⁹ for *de novo* assembly, bwa-mem (v0.7.12)¹⁰ for alignment, and SAMtools (v1.1)¹¹ for variant calling. Variant calling was performed using reference genomes identified as most closely matching the sequenced isolates. Mutations were manually verified using the Integrative Genomics Viewer,¹² then annotated with sequence features using SnpEFF.¹³ Copy number variants were investigated using CNOGpro.¹⁴ Sequence data from this study are available from the NCBI Sequence Read Archive under accession PRJNA707063 (<https://www.ncbi.nlm.nih.gov/bioproject/PRJNA707063/>). Mutations in coding regions and susceptibility data are summarized in Table 1.

RT-PCR

To assess the impact of the mutation in the *smeT* promoter observed in W357-2-10 we used RT-PCR to measure differences in the expression of the *smeDEF* operon regulator *smeT*, and separately, the operon itself using the first and last structural genes in that unit (*smeD* and *smeF*). RNA was extracted from mid-log phase cultures using RNeasy Bacterial Kit (Qiagen), and iScript Reverse Transcriptase was used for cDNA generation. Results were analysed using the double-delta Ct method, normalizing gene expression of *sme* gene transcripts against *gyrA* expression. Primer sequences for *smeT*,¹⁵ *smeD*,¹⁶ *smeE*¹⁷ and *gyrA*¹⁷ are published elsewhere.

Phenotype microarray

We investigated potential differences in candidate phenotypes between W357-2-10 and W357 using a phenotype microarray system (Biolog) due to the stability of the cefiderocol resistance phenotype and then unclear contribution of the *smeT* promoter mutation observed. Both strains were inoculated into panels PM9-PM13 and cellular respiration was monitored

for 48 h as described previously.¹⁸ The parent and mutant respiration curves were integrated and compared then summarized in Table S1 and Figure S1 (available as [Supplementary data](#) at JAC-AMR Online).

Results

Isolates recovered from serial passage experiments and their MICs are listed in Table 1. We recovered isolates with reduced susceptibility to cefiderocol in all three genetic backgrounds. Ceftazidime/avibactam MICs increased from 4 mg/L, 48 mg/L and 64 mg/L to ≥256 mg/L in the evolved strains. Cross-resistance to other agents was limited, but trimethoprim/sulfamethoxazole MICs declined by 4-fold in two of the three genetic backgrounds (W357 and W364).

Whole genome analysis revealed isogenic mutations of *tonB*, *smf-1*, *tolQ* and the *smeT* promoter in 4/5 isolates, without other identifiable changes (Table 1). The remaining isolate carried six separate coding mutations.

The *smeT*-promoter mutant displayed the greatest increase in cefiderocol MIC from baseline (1024-fold), with MIC elevations of 128–256-fold above baseline persisting for ≥4 passages on drug-free medium. Mutants of *smf-1*, *tolQ* and *tonB* displayed smaller and less stable MIC elevations.

We also identified several other isolates from resistance screening plates that, despite elevated MICs, did not acquire identifiable mutations or changes in gene copy number, which has been suggested as a potential mechanism of heteroresistance. Such isolates' resistance phenotypes were unstable and rapidly lost when subcultured on drug-free medium.

RT-PCR revealed that the *smeT* promoter mutation in W357-2-10 was associated with a >1.3-fold increase in *smeT* expression and a ~10-fold reduction in *smeDEF* expression (Figure 1). Phenotype microarrays of W357 and W357-2-10 (Figure S1 and Table S1) indicate that, overall, W357-2-10 was more resistant to penicillins and vancomycin, while W357 was more resistant to a variety of intracellularly active agents (chloramphenicol, nalidixic acid, sulphonamides, tetracyclines) as well as polymyxin B and benzethonium chloride.

Discussion

In this study we determined that cefiderocol resistance evolved in *S. maltophilia in vitro* can emerge via a variety of genetic pathways and with variable degrees of phenotypic stability. Our results suggest that reduced susceptibility could be inducible in some passaged isolates, without any detectible genetic changes, but the basis and significance of these elusive and unstable phenotypes is unclear. While we did not observe mutations in genes associated with canonical β-lactam resistance, such as those that encode penicillin-binding proteins or β-lactamases, we did observe some concurrent increases in MICs of ceftazidime and ceftazidime/avibactam among our resistant isolates. The passaged isolate with the highest resistance carried six separate coding mutations, but most harboured isogenic mutations of single genes (Table 1).

The *smeT* promoter mutant (W357-2-10) exhibited the greatest and most stable resistance phenotype of all the single-gene mutants identified in the study. This mutant exhibited cross-resistance to ceftazidime/avibactam. Phenotype microarray data also showed that W357-2-10 had reduced susceptibility

Table 1. Changes in susceptibility (MICs, mg/L) to various antimicrobials among isolates recovered from cefiderocol serial passage experiments

Strain	Time to isolate emergence (days)	FDC	CAZ/AVI	CAZ	MIN	TMP/SMX	FDC stability: no. of drug-free passes				Variants		
							2	3	4	gene	nucleic acid	amino acid	
W357-Parent	—	0.03125	4	128	4	0.5/9.5	0.03125	0.0625	0.03125	—	—	—	—
W357-2-10	15	32	≥256	256	2	0.125/2.375	8	4.0-8.0	4.0-8.0	<i>smeT</i>	c.-49_-48insTTGT	(promoter)	—
W358-Parent	—	0.125	64	≥256	0.5	0.125/2.375	0.0625	0.0625	0.03125	—	—	—	—
W358-1-25	21	32	≥256	≥256	0.5	0.25/4.75	16	16	8.0-16.0	<i>smlt1148</i>	c.2413A>C	p.Thr805Pro (promoter)	—
W358-2-2	12	8	≥256	≥256	0.5	0.125/2.375	1.0-2.0	1	1	<i>tesB</i>	c.-33T>C	(promoter)	—
W358-2-15	18	16	≥256	≥256	0.5	0.25/4.75	0.0625	0.0625	0.0625	<i>panD</i>	c.258_260delCAT	p.Ile87del	—
W364-Parent	—	0.03125	48	64-128	1	1/19	0.03125	0.03125	0.03125	<i>smlt3318</i>	c.383T>A	p.Leu128Gln (promoter)	—
W364-8	16	8	≥256	256	1	0.25/4.75	8	2.0-4.0	2	<i>cysK1</i>	c.-59G>A	(promoter)	—
										<i>cirA</i>	c.112_122dupTCCCCTCCGC	p.Arg42fs	—
										<i>smf-1</i>	c.394A>G	p.Thr132Ala	—
										<i>tolQ</i>	c.647C>A	p.Pro216Gln	—
										—	—	—	—
										<i>tonB</i>	c.376_384dupGCTCCGCCG	p.Ala126_Pro128dup	—

FDC, cefiderocol; CAZ, ceftazidime; CAZ/AVI, ceftazidime/avibactam; MIN, minocycline; TMP/SMX, trimethoprim/sulfamethoxazole. Cefiderocol MICs were repeated after passage from the freezer onto drug-free medium for up to four passes. Variants list the gene name, followed by the DNA variant and when relevant the amino acid changes.

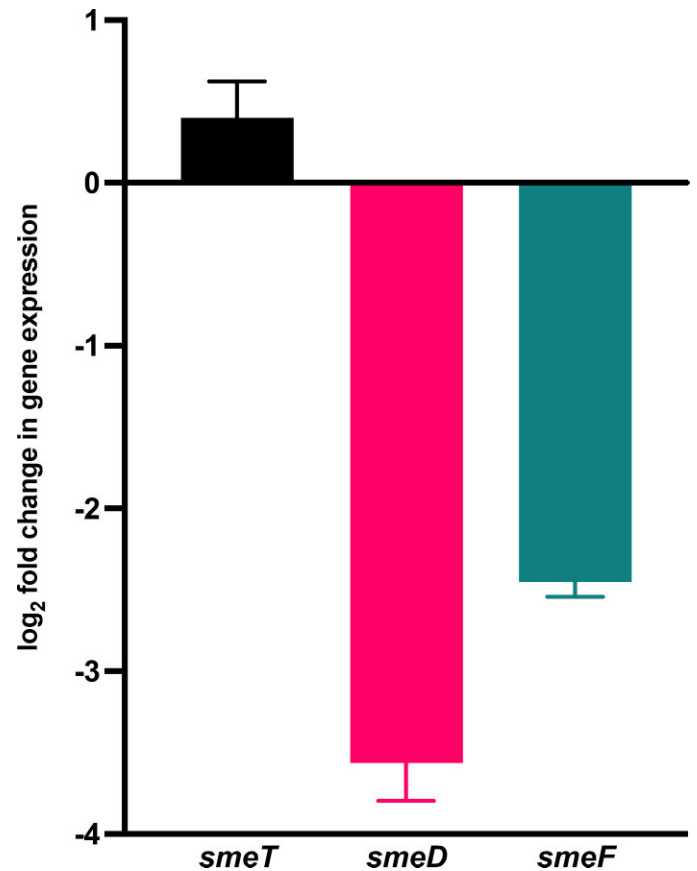


Figure 1. Log₂ fold change in expression of *smeTDEF* operon elements in mutant W357-2-10 relative to parent strain W357. Expression of *sme* elements was determined by RT-PCR and normalized to *gyrA* expression and is shown relative to the gene expression in the parental strain. Error bars indicate standard deviation of measurements.

to oxacillin, nafcillin and cloxacillin, but other β-lactams on the panels (amoxicillin, ampicillin, azlocillin, carbenicillin, cefazolin, ceftriaxone, cefuroxime, cephalothin and penicillin) had similar activity at the concentrations tested, which are proprietary (Biolog). Although none of these agents is generally presumed to be meaningfully active against *S. maltophilia*, such differences suggest some degree of β-lactam cross-resistance. *SmeT* is a repressor of the *S. maltophilia* TetR family MDR efflux pump *smeDEF*.^{19,20} *smeDEF* overexpression, typically related to *smeT* mutation, has been associated with resistance to a variety of antimicrobials including tetracyclines, trimethoprim/sulfamethoxazole and fluoroquinolones.²¹ In contrast, we observed a mutation in the *smeT* promoter region accompanying reductions in trimethoprim/sulfamethoxazole MICs. Furthermore, the candidate phenotypes from the microarray data showed that the strain with the *smeT* promoter mutation was potentially more susceptible to demeclocycline, doxycycline, nalidixic acid and four sulphonamides. Collectively, these susceptibility profiles suggest that a reduction in *SmeDEF* activity, which was confirmed by our RT-PCR showing upregulated *smeT* and decreased *smeDEF* expression, contributes to cefiderocol resistance. It is presently unclear how reduced *smeDEF* expression contributes

to this phenotype, but the effect may be indirect since previous work suggests β -lactams are poor substrates for SmeDEF.

It was unsurprising to recover a mutation of iron transporter *tonB* given the known role of TonB-dependent receptors in active transport of siderophore drug conjugates.²² Ceftazidime resistance has also been linked to TonB function, so while the relevant strain was initially resistant to ceftazidime, *tonB* mutations could theoretically mediate cross-resistance between siderophore conjugates and traditional β -lactams.²³

It is unclear how *smf-1*, which affects fimbriae and surface adhesion, or transmembrane transporter *tolQ*, affect cefiderocol susceptibility, and these mechanisms warrant further investigation. Of the six mutated genes observed in W358-1-25 two have a plausible link to cefiderocol resistance: *cirA*, which is a TonB-dependent receptor family protein previously implicated in cefiderocol resistance,²⁴ and *smlt1148*, a putative iron transport protein. Other mutations involve amino acid and fatty acid metabolism in ways that may contribute to metabolic resistance phenotypes.²⁵

Conclusions

Our study suggests that disparate mutations, many in genes relating to iron transport, occur with the emergence of cefiderocol resistance in *S. maltophilia* *in vitro*. We also report a counter-intuitive relationship between the *smeDEF* transporter downregulation and cefiderocol resistance, which warrants further study. Future work should also focus on whether cefiderocol exposure, especially clinically meaningful kinetic exposures, can select for changes in MDR efflux pumps that could affect other classes of antimicrobials.

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Transparency declarations

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Author contributions

B.J.W., A.B. and S.J.S. conceived of and designed the study. N.K.A. and B.J.W. performed the serial passage, resistance screening and susceptibility testing. K.P., A.W., E.A.H. and S.J.S. performed the WGS and analysis, and the RT-PCR. B.J.W. wrote the first draft, and all authors reviewed the data, prepared the manuscript and approved the final version.

Supplementary data

Table S1 and Figure S1 are available as [Supplementary data](#) at *JAC-AMR* Online.

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