Japan Antibiotics
Research Association

The Society for
Actinomycetes Japan

ARTICLE

In vitro resistance development gives insights into molecular resistance mechanisms against cefiderocol

Richard Kriz[1,2] · Kathrin Spettel [2,3] · Alina Pichler[1,2,5] · Katharina Schefberger[1] · Maria Sanz-Codina[4] ·
Felix Lötsch[3] · Nicole Harrison[1] · Birgit Willinger[3] · Markus Zeitlinger[4] · Heinz Burgmann[1] · Heimo Lagler[1]

Abstract

Cefiderocol, a novel siderophore cephalosporin, demonstrates promising in vitro activity against multidrug-resistant Gram-negative bacteria, including carbapenemase-producing strains. Nonetheless, only a few reports are available regarding the acquisition of resistance in clinical settings, primarily due to its recent usage. This study aimed to investigate cefiderocol resistance using an in vitro resistance development model to gain insights into the underlying molecular resistance mechanisms. Cefiderocol susceptible reference strains (*Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*) and a clinical *Acinetobacter baumannii* complex isolate were exposed to increasing cefiderocol concentrations using a high-throughput resistance development model. Cefiderocol susceptibility testing was performed using broth microdilution. Whole-genome sequencing was employed to identify newly acquired resistance mutations. Our in vitro resistance development model led to several clones of strains exhibiting cefiderocol resistance, with MIC values 8-fold to 512-fold higher than initial levels. In total, we found 42 different mutations in 26 genes, of which 35 could be described for the first time. Putative loss-of-function mutations were detected in the *envZ*, *tonB*, and *cirA* genes in 13 out of 17 isolates, leading to a decrease in cefiderocol influx. Other potential resistance mechanisms included multidrug efflux pumps (*baeS*, *czcS*, *nalC*), antibiotic-inactivating enzymes (*ampR*, *dacB*), and target mutations in penicillin-binding-protein genes (*mrcB*). This study reveals new insights into underlying molecular resistance mechanisms against cefiderocol. While mutations leading to reduced influx via iron transporters was the most frequent resistance mechanism, we also detected several other novel resistance mutations causing cefiderocol resistance.

These authors contributed equally: Richard Kriz, Kathrin Spettel

✉ Heimo Lagler
  heimo.lagler@meduniwien.ac.at

1 Division of Infectious Diseases and Tropical Medicine,
   Department of Medicine I, Medical University of Vienna,
   Vienna, Austria

2 Section Biomedical Science, Health Sciences, FH Campus Wien
   University of Applied Sciences, Vienna, Austria

3 Division of Clinical Microbiology, Department of Laboratory
   Medicine, Medical University of Vienna, Vienna, Austria

4 Department of Clinical Pharmacology, Medical University of
   Vienna, Vienna, Austria

5 Present address: Pediatric Laboratory, Department of Laboratory
   Medicine, Medical University of Vienna, Vienna, Austria

Introduction

The rise of multidrug-resistant (MDR) Gram-negative bacteria (GNB) presents an escalating public health threat resulting in a major global health burden including higher case fatality rates, morbidity, and increasing health expenses [1]. With the emergence of novel resistance patterns and new underlying resistance mechanisms, clinicians and researchers face increasing challenges in the treatment and management of these infections, necessitating the development of new antibiotics and a comprehensive understanding of the resistance mechanisms [2, 3]. According to international organizations, one of the major public health challenges of our time is the emergence of antimicrobial resistance [4, 5].

Cefiderocol is a newly developed catechol-substituted siderophore cephalosporin with a new mode of bacterial cell wall entry utilizing the bacterial iron transport system. Cefiderocol binds to extracellular iron, forming a chelate

SPRINGER NATURE

complex that is transported into the cell. In the periplasmic space, cefiderocol inhibits peptidoglycan synthase by binding to penicillin-binding protein 3 (PBP3), leading to subsequent cell lysis [6]. In addition to the beta-lactam core of cefiderocol, the C-3 side chain is similar to the side chain found in cefepime, which increases water solubility and resists degradation by beta-lactamases. Additionally, a catechol moiety attached to the C-3 side chain allows cefiderocol to form a chelating complex with ferric iron. On the other hand, the C-7 side chain of cefiderocol is the same as in ceftazidime and confers stability against beta-lactamases as well as increases activity against *Pseudomonas aeruginosa* [7]. Cefiderocol has been approved by the FDA in 2019 and by the EMA in 2020 for treatment of GNB infections in adults [8, 9].

As the first siderophore-cephalosporin, cefiderocol has shown potent activity against MDR Enterobacterales species, non-fermenting and carbapenemase-producing bacteria in previous studies [10, 11]. Especially for complicated infections with MDR-GNB including New Delhi metallo-beta-lactamase (NDM) producing Enterobacterales, cefiderocol could offer an effective treatment alternative.

Despite demonstrating promising in vitro efficacy, various resistance mechanisms have already been suggested for the development of cefiderocol resistance. These include reduced influx caused by mutations in siderophore receptors of the iron transport system and mutations in porins, target mutations in PBP genes and the presence of antibiotic-inactivating enzymes. Mutations in siderophore receptors (*piuA, cirA, fiuA*) and other components (*tonB, envZ*) involved in iron uptake have been observed in various species such as *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter cloacae*, and *Klebsiella pneumoniae* [12–14]. Loss-of-function mutations affecting porins have been identified in various species, such as Enterobacterales (OmpF, OmpD) and *Klebsiella spp*. (OmpK35, OmpK36, and OmpK37). Although porin mutations are associated with a small increase in cefiderocol minimal inhibitory concentration (MIC) (2-4 fold), this increase seems to be not sufficient for cefiderocol resistance [15–17]. Furthermore, a few studies have reported target mutations in the *pbp3* gene associated with cefiderocol resistance [18, 19].

A significant challenge in using clinical isolates for resistance research is that the original susceptible isolate is frequently unavailable following the in vivo development of resistance. When performing whole genome sequencing (WGS) in search of the causal underlying molecular resistance mechanism, a genome of an arbitrary reference isolate is typically used for sequence comparison. Consequently, this comparison often reveals a considerable number of mutations and polymorphisms, frequently reaching into the thousands. This abundance of variants presents a major

challenge in unequivocally identifying the mutation or resistance gene responsible for the development of resistance.

To solve this problem, one can use a resistance development model where initially susceptible isolates are exposed to an antimicrobial substance in vitro until resistance development occurs. Subsequently, the sequence of the initial susceptible isolate is compared to that of the newly acquired resistant isolate, and the newly acquired mutations are likely to be associated with the resistance development. One study focusing on a spatiotemporal in vitro resistance development approach of bacterial resistance to antibiotics was the study of Baym et al., which visualized impressively this process on an agar plate, allowing for real-time observation of evolutionary dynamics [20].

We chose a temporal approach for in vitro resistance development, utilizing a high-throughput model in microwell plates. The aim of the study was to induce cefiderocol resistance in vitro in susceptible *A. baumannii* complex, *Escherichia coli*, *K. pneumoniae* and *P. aeruginosa* strains and analyze the newly acquired resistance mutations to assess the putative underlying resistance mechanism using WGS.

Methods

Sampling

In this study we used the cefiderocol susceptible reference strains *E. coli* ATCC 25922, *K. pneumoniae* ATCC 10031, and *P. aeruginosa* ATCC 27853 as well as the clinical *A. baumannii* complex isolate 19-628. To confirm cefiderocol susceptibility antimicrobial susceptibility testing using iron-depleted (Chelex, Sigma-Aldrich, Darmstadt, Germany) and cation-adjusted Müller-Hinton-broth microdilution according to the ISO 20776-1 (2019).

In vitro antimicrobial resistance development model

A high throughput resistance development model was newly designed to induce antimicrobial resistance against cefiderocol in the four susceptible strains listed above. In total 96 replicates of each strain (5×10^7 CFU ml^{-1}) were exposed to increasing cefiderocol concentrations in cation-adjusted Mueller-Hinton broth (Becton-Dickinson, Franklin Lakes, USA) in 96-well plates for three days, see Fig. 1. The first batch of resistance development plates had an initial cefiderocol concentration of 0.125 mg l^{-1}, and every three days, 100 µl of the suspensions were transferred into a new 96-well plate with the cefiderocol concentration doubled. This cycle was repeated up to 12 times until a maximum cefiderocol concentration of 128–512 mg l^{-1}

Fig. 1 Overview of the methodological workflow of the resistance development model
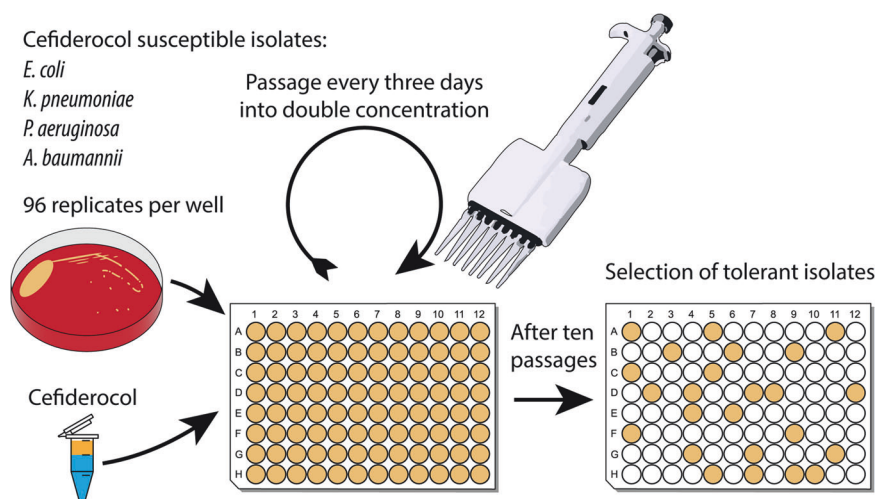
depending on the species was reached. After resistance development, 5 μl of the suspensions of all wells were streaked on Columbia agar with 5% sheep blood (Becton-Dickinson, Franklin Lakes, USA) to check for growth. The MIC of cefiderocol for all vital isolates was determined using broth microdilution. Resistance to cefiderocol was determined when the MIC exceeded 2 mg l^{-1} according to EUCAST breakpoint. Stable resistance was defined as a persistently elevated MIC observed after discontinuing the selection pressure and five subcultures.

Whole genome sequencing

We selected the four original cefiderocol susceptible isolates as well as four randomly selected cefiderocol resistance induced clones of *A. baumannii* complex, *E. coli*, and *K. pneumoniae* each, and five cefiderocol resistant clones of *P. aeruginosa* for further investigation with WGS. The DNA of these 21 isolates was extracted based on a phenol-chloroform method. Briefly, several colonies from overnight cultures on Columbia sheep blood 5% agar (Becton-Dickinson, Franklin Lakes, USA) were picked, and the cells were resuspended in 300 μl Tris buffer and 100 μl 5 M NaCl. Then, 400 μl phenol-chloroform-isoamyl alcohol (25:24:1) was added to the mixture. The suspensions were homogenized twice for 15 s at $5000 \times g$ in lysing tubes with beads. The supernatant was transferred to a new tube, and 300 μl chloroform was added. The sample was centrifuged at $18,000 \times g$ for 5 min, and the supernatant was transferred to a new tube. The last step was repeated, and 225 μl 10 M ammonium acetate was added and vortexed. The tube was then filled to 2 ml with 100% ice-cold ethanol. After centrifugation at 4 °C and $18,000 \times g$ for 30 min, the precipitated DNA pellet was washed twice with ice-cold 70% ethanol and resuspended in 50 μl Tris buffer. Following DNA extraction, the Illumina DNA Library Prep kit was

used to prepare the samples for WGS according to the manufacturer's instructions and were sequenced on Illumina MiSeq® Platform on V3 Flow Cell with a final library concentration of 8 pM.

For bioinformatical analysis, the raw reads were quality-trimmed using Trim-Galore 0.4.4_dev [21] and assembled using SPAdes v3.15.4 [22]. The isolates were examined for large deletions and recombination events using Mauve 2.4.0 [23]. The assembled genome of the clinical *A. baumannii* complex strain was annotated using pgap 2023-05-17 [24]. The annotated genomes of the ATCC strains were downloaded from https://genomes.atcc.org/. These genomes were used as a backbone for mapping the resistant isolates using Bowtie2 2.3.4.1 [25]. Variant calling was then performed using samtools 1.16.1 [26] and VarScan v2.4.4 [27], and the variants were annotated using SnpEff 5.0e [28].

Results and discussion

Phenotypical changes and resistance profiles

In the in vitro resistance development model, almost all replicates of certain bacterial species continued to grow even when exposed to escalating concentrations of cefiderocol. However, most of these replicates seem to be transiently tolerant cells, as not all of them exhibited a stable resistance. Other temporary resistance mechanisms may enable their survival, including the upregulation of drug efflux pumps or the downregulation of iron transporters, potentially triggered by the gradual increase in cefiderocol concentration.

Following the development of resistance in *P. aeruginosa* using a final cefiderocol concentration of 512 mg l^{-1}, 84 out of 96 replicates were viable when cultivated on Columbia agar. Of these 84 isolates, 23 *P. aeruginosa*

Table 1 Overview of number of replicates during each step of the study

	P. aeruginosa	*A. baumannii*	*E. coli*	*K. pneumoniae*
max cefiderocol concentration [mg l^{-1}]	512	512	256	128
Replicates exposed to cefiderocol	96	96	96	96
Replicates viable after exposure	84	31	27	5
Replicates resistant against cefiderocol	23	17	25	5
Replicates sequenced	5	4	4	4

isolates developed resistance to cefiderocol (Table 1). Among the five sequenced *P. aeruginosa* isolates, the MIC of cefiderocol increased from 0.5 mg l^{-1} to 4–16 mg l^{-1}.

Similarly, after resistance development in *A. baumannii* complex using a final cefiderocol concentration of 512 mg l^{-1}, 31 out of 96 wells showed growth when cultivated on Columbia agar. Of these 31 isolates, 17 *A. baumannii* complex isolates exhibited resistance to cefiderocol. Among the four sequenced *A. baumannii* complex isolates, the MIC of cefiderocol increased from ≤0.064 mg l^{-1} to 8–>32 mg l^{-1}.

In the case of *E. coli*, resistance was induced using a final cefiderocol concentration of 256 mg l^{-1}, resulting in 27 out of 96 replicates being viable. Among these 27 isolates, 25 *E. coli* isolates demonstrated resistance to cefiderocol. Of the four sequenced *E. coli* isolates, the MIC of cefiderocol increased from 0.125 mg l^{-1} to 16–32 mg l^{-1}.

Furthermore, *K. pneumoniae* isolates were subjected to resistance development using a final cefiderocol concentration of 128 mg l^{-1}, and five out of 96 replicates were viable. All five of these isolates demonstrated resistance against cefiderocol. Among the four sequenced *K. pneumoniae* isolates, the MIC of cefiderocol increased from ≤0.064 mg l^{-1} to 8–16 mg l^{-1}. We selected 17 cefiderocol resistant isolates (four *E. coli*, *K. pneumoniae* and *A. baumannii* complex and five *P. aeruginosa*) as well as the original corresponding cefiderocol susceptible wild-type isolates for further investigation with WGS.

Many isolates, especially *P. aeruginosa* and *A. baumannii*, showed high tolerability and growth even in high cefiderocol concentrations, although after removing selection pressure the MIC remained at susceptible levels. Therefore, it is important to distinguish between tolerance, persistence, and resistance, which are fundamental survival strategies in bacteria. Tolerance allows bacteria to temporarily endure adverse conditions. Persistence leads to the formation of cells that can resist even high concentrations of antibiotics, although they are practically inert, until the environment changes to more favorable conditions. Resistance involves genetic changes that provide long-term protection against specific stressors. Therefore, it cannot be assumed that all replicates that have grown at high cefiderocol concentrations have automatically acquired stable resistance. Instead, they have developed a transient tolerance and persistence since, in our model, they had the time

to gradually adapt to increasing concentrations. An explanation could be changes in gene expression of relevant resistance mechanisms, e.g. overexpression of drug efflux pumps or downregulation of iron transporters. Still, after removing the selection pressure, these isolates quickly reverted to their original susceptible phenotype. On the other hand, nearly all *K. pneumoniae* and *E. coli* isolates that were viable in high cefiderocol concentrations remained cefiderocol resistant even after multiple passaging without cefiderocol exposure, indicating stable mutations in resistance genes.

Cefiderocol pharmacokinetics and resistance development

Cefiderocol is administered as an intravenous infusion of 2 g every 8 h for 7–14 days, depending on the severity of the infection [29]. Cefiderocol protein binding (PB) has previously been reported as 40–60% [30, 31]. It has also been shown to penetrate various body tissues and fluids, including urine and bronchoalveolar lavage fluid [32, 33]. Understanding these factors is essential for ensuring its effectiveness while minimizing resistance development. This is crucial because suboptimal concentrations provide a fertile ground for resistance development.

Cefiderocol has shown no accumulation and the PK did not change with multiple dosing [34]. The maximum total serum concentration (C_{max}) after a cefiderocol 2 g single dose infusion over 3 h was 89.7 mg l^{-1} [35] and over 1 h was 156 mg l^{-1} [34]. Assuming a PB of 40%, this corresponds to a free concentration of 53.82 mg l^{-1} and 93.6 mg l^{-1}. Therefore, our in vitro model maximum exposure concentrations (128–512 mg l^{-1}) exceeded the C_{max} observed in humans. Additionally, the mutants generated with MIC values ranging from 4 to 32 mg l^{-1} remained below the average C_{max} [36].

It must be noted that our model does not replicate the real in vivo conditions during cefiderocol treatment. In our resistance development model, we employed optimal conditions for resistance development to generate as many resistant isolates as possible to investigate the underlying molecular resistance mechanisms. Therefore, no conclusions can be drawn that cefiderocol would exhibit rapid resistance development in vivo, since our model is not

tailored for estimating the in vivo resistance development rate.

Newly acquired resistance mutations

In this study, all our isolates that acquired cefiderocol resistance consistently showed an increase in MIC against cefiderocol after exposure, with the MIC increasing by 8-fold to 512-fold, see Table 2.

Table 2 and Fig. 2 provide comprehensive data on the MIC of cefiderocol before and after resistance development in the four control strains, as well as the detected putative resistance mechanisms and genetic changes that may be the cause for resistance acquisition to cefiderocol.

Using WGS we identified 42 different mutations within 26 distinct genes. Notably, 35 of these genetic alterations have not been previously described, to the best of our knowledge, representing an expansion of the understanding of resistance to cefiderocol in four different bacterial species.

Of the 17 sequenced resistant isolates, each harbored between one to four newly acquired variants compared to their corresponding wild-type sequences. Notably, the most common variants observed were V295G in *baeS* and R181* in *yhaJ*, present in all four *K. pneumoniae* isolates. The gene with the highest number of variants was *envZ*, exhibiting six different variants across all four *E. coli* and three *K. pneumoniae* isolates.

Reduced influx of cefiderocol due to impaired iron transport

In our study, we found several newly acquired mutations in genes related to reduced influx. We detected this mechanism 20 times in 13 out of 17 sequenced isolates, making it clear that reduced influx appears to be the predominant resistance mechanism. These mutations were mostly loss-of-function mutations, such as premature stop codons and frameshift mutations, which result in a truncated or highly altered protein and are likely to be associated with a loss of transporter function.

We detected six different mutations (L27P, R82C, I86S, V145G, T247I, T247P) in *envZ*, the sensor protein of the two-component regulatory system OmpR/EnvZ. *EnvZ* may affect cefiderocol uptake as it regulates the expression of genes involved in iron uptake, such as the porins OmpC and OmpF [37]. Mutations in *envZ* have been associated with cefiderocol resistance in in vitro derived isolates in a study [38] and especially mutations at amino acid position T247 seem to have a high impact on the function of the gene [39]. Overall, our findings suggest that mutations in *envZ* play a major role in cefiderocol resistance.

Furthermore, we detected three mutations including two loss-of-function variants, one premature stop codon as well as an inframe deletion (L241*, G244R, T434_T435del) in the gene *fptA* as well as a large deletion of the gene region *irtA-irtB-fptA-menF*. *FptA* is a ton B-dependent receptor for the uptake of the siderophore pyochelin and has already been associated with increased cefiderocol susceptibility when overexpressed [40]. Conversely, in our study, two isolates showed loss-of-function mutations in *fptA*, which likely decreased cefiderocol susceptibility.

We detected a frameshift mutation and a premature stop codon (G496fs, Q304*) in *cirA*, which is a TonB-dependent receptor responsible for the uptake of catecholate siderophores. Recent studies have shown that loss-of-function mutations in *cirA* can lead to decreased uptake of cefiderocol thus leading to resistance, in line with our study [12, 13, 15, 41].

We discovered three frameshift mutations (S46fs, S31fs, D180fs) in the gene *tonB* as well as a large deletion of a genomic region including *tonB* (*yciC-yciB*-hydrolase-*tonB*-*yciI*), resulting in a loss of protein function. TonB is an inner membrane protein that transduces energy needed for active transport to TonB-dependent receptors in the outer membrane. It is part of the regulating TonB-ExbB-ExbD complex, which plays a crucial role in the uptake of nutrients such as iron [42, 43]. In a previous study, frameshift mutations in *tonB* have already been reported with increased MIC levels to other siderophore-conjugated antibiotics [14].

We identified a premature stop codon (E116*) in the gene *ydiV*. Overexpression of *ydiV* can cause a change in the folding of the protein Fur, impairing its function as an inhibitor of iron receptors, which in turn leads to increased iron uptake [44].

Additionally, we determined a mutation (N302K) in the gene *iscS*, an iron-sulfur protein assembly protein, which is part of a sensing mechanism involved in a wide range of biological processes but the impact on cefiderocol resistance remains unclear [45].

We found two coding mutations as well as an upstream gene variant (T134P, S108fs) in the *piuC* gene, an outer membrane siderophore receptor. Mutations in this gene have previously been linked to resistance against other siderophore-conjugated beta-lactam antibiotics and cefiderocol [46, 47].

Additionally, we detected an upstream gene variant of the gene encoding for enterochelin esterase, which is responsible for the dissociation of the enterochelin iron complex, after entry into the cell [48].

All these mutations in various genes related to iron uptake are likely linked to a reduced influx of cefiderocol, leading to increased resistance to the drug. Therefore, the unique mechanism of cefiderocol to enter the cell seems

Table 2

762   R. Kriz et al.

Table 2 Overview of sequenced isolates, as well as their resistance profile and newly acquired genetic changes

Species	FDC MIC [mg l⁻¹] before RD	FDC MIC [mg l⁻¹] after RD	Putative resistance mechanism	Gene	Variant type	Amino acid change	Ref-erence
A. baumannii complex clinical isolate 19-628	<=0.064	32	protein biosynthesis	tuf	missense variant	H23Y	
			protease	sohB	frameshift variant	I28fs	
		>32	iron transport	piuC	upstream gene variant		
		8	cell division	ftsZ	missense variant	G110S	
			PBP	mrcB	missense variant	R363C	
		>32	secondary messenger	cyaA	frameshift variant	I178fs	
			RNA polymerase	rpoZ	frameshift variant	E18fs	
			iron transport	tonB	frameshift variant	D180fs	
E. coli ATCC 25922	0.125	16	iron transport	envZ	missense variant	V145G	(61)
			iron transport	tonB	frameshift variant	S46fs	
			iron transport	ydiV	stop gained	E116*	
		16	drug efflux	baeS	missense variant	I170S	(62)
			iron transport	envZ	missense variant	R82C	
		16	iron transport	envZ	missense variant	V145G	(61)
			iron transport	yciC-yciB-hydrolase-tonB-yciI	deletion		
		32	iron transport	envZ	missense variant	I86S	(63)
			mannose transport	manX	synonymous variant	A13A	
			iron transport	tonB	frameshift variant	S31fs	
K. pneumoniae ATCC 10031	<=0.064	16	drug efflux	baeS	missense variant	V295G	(38,62)
			iron transport	cirA	frameshift variant	G496fs	
			iron transport	envZ	missense variant	T247I	(39)
				yhaJ	stop gained	R181*	
		8	drug efflux	baeS	missense variant	V295G	(38,62)
			iron transport	cirA	stop gained	Q304*	
				yhaJ	stop gained	R181*	
		8	drug efflux	baeS	missense variant	V295G	(38,62)
			iron transport	envZ	missense variant	T247P	(39)
				yhaJ	stop gained	R181*	
		16	drug efflux	baeS	missense variant	V295G	(38,62)
			secondary messenger	cyaA	stop gained	Q148*	
			iron transport	envZ	missense variant	L27P	
				yhaJ	stop gained	R181*	
P. aeruginosa ATCC 27853	0.5	16	iron transport	fptA	deletion & stop gained	L241*	
			iron transport	piuC	missense variant	T134P	
			threonine metabolism	tdh	missense variant	T119I	
		16	drug efflux	czcS	missense variant	R246S	
			iron transport	fptA	missense variant	G244R	
			iron transport	piuC	frameshift variant	S108fs	
			threonine metabolism	tdh	missense variant	Y127C	
		16	iron transport	irtA-irtB-fptA-menF	deletion		
			beta lactamase	ampR	missense variant	D135G	
			iron transport	Enterochelin Esterase	upstream gene variant		
			drug efflux	nalC	frameshift variant	R17fs	
		4	drug efflux	nalC	missense variant	F55C	
				spoT	frameshift variant	I295fs	
		4	beta lactamase	dacB	missense variant	A451P	
			iron transport	fptA	inframe deletion	T434_T435del	
			metabolism	iscS	missense variant	N302K	
				pilN	stop gained	Q89*	

Colors: purple - alteration in iron transport; blue - alteration of penicillin binding protein; green - alteration of drug efflux pumps; orange - alteration in antibiotic inactivating enzymes; FDC - cefiderocol; MIC - minimal inhibitory concentration; RD - resistance development

SPRINGER NATURE
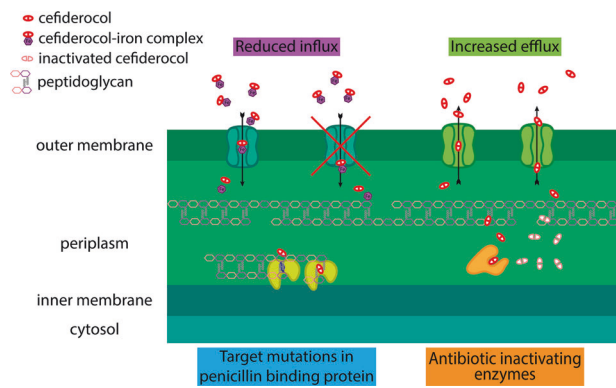
Fig. 2 Graphical representation of putative resistance mechanisms associated with cefiderocol resistance

also to be its vulnerable point, making it the primary target for potential resistance against this antibiotic.

Antibiotic-inactivating enzymes

Antibiotic-inactivating enzymes are a class of bacterial enzymes that can neutralize the effects of antibiotics by modifying or degrading them. These enzymes are the most common mechanism of antibiotic resistance and can be found in many different types of bacteria.

In our study, we found one mutation (D135G) in *P. aeruginosa* in *ampR*, the transcription factor of the antibiotic-inactivating enzyme *ampC*. This missense mutation is already described in two studies focusing on ceftolozane-tazobactam resistance as well as aztreonam resistance [49, 50] and may lead to a constitutive active gene expression of AmpC.

In our study, a mutation (A451P) in *dacB* gene (also known as PBP4) was found in one *P. aeruginosa* isolate. In a study by Moya et al. it was shown that knockout of *dacB* in *P. aeruginosa* leads to constitutive overexpression of AmpC beta-lactamase as well as activating the CreBC (BlrAB) two-component regulator [51]. This effect was also reported by Ito et al. regarding ceftazidime and cefepime, but not in cefiderocol [52].

Mutations in the beta-lactamase AmpC itself have also been verified as causing cefiderocol resistance by modifying the beta-lactam recognition site which in turn leads to a wider spectrum of degradable beta-lactams. Gomis-Font et al. characterized the mutations L320P, G183D, E247K and T96I, all of which led to an increase in cefiderocol MICs, while having differing effects on other beta-lactams [46]. Another study found deletions in the R2 loop of AmpC, which conferred resistance [53]. However, no mutation in AmpC could be detected in our isolates.

Still, the significance of antibiotic-inactivating enzymes cannot be assessed exhaustively within the scope of this study. The in vitro resistance development model used in this study did not allow for the transmission of genetic elements, such as plasmids, which are often responsible for the transportation of antibiotic-inactivating enzymes. While we found variants, which might be involved in the over-expression of beta-lactamases or lead to increased affinity of beta-lactamases to the antibiotic, it was not possible for the exposed strains to acquire new genes encoding antibiotic-inactivating enzymes via genetic exchange with other bacteria. Thus, the relevance of antibiotic-inactivating enzymes cannot be determined adequately in this study.

Increased drug efflux

Multidrug efflux pumps are a class of proteins that are present in the cell membrane of bacteria and are involved in the export of a wide range of antibiotics and other toxic compounds from the bacterial cell. These pumps play a significant role in the development of antibiotic resistance by removing the antibiotics from the cell before they can exert their bactericidal effects. In this study, mutations associated with multidrug efflux were found in seven isolates.

The mutation V295G was identified in *baeS*, one part of the two-component regulator *baeSR*, which controls expression of major facilitator superfamily efflux pumps, in all *K. pneumoniae* isolates as well as the amino acid substitution I170S in one *E. coli* isolate. Other studies have previously reported mutations in *baeS* including the V295G mutation in association with increased drug efflux as well as cefiderocol and colistin resistance [38, 54, 55].

Additionally, the mutation (R246S) was located in *czcS* in a *P. aeruginosa* isolate, a part of the two-component regulation system CzcR-CzcS, which regulates the heavy metal drug efflux pump CzcCBA. This drug efflux pump has also been associated with a cross-resistance between zinc and imipenem [56].

Furthermore, we identified mutations (R17fs, F55C) in the gene *nalC* in *P. aeruginosa* isolates, which negatively regulates the multidrug efflux pump MexAB-OprM [57]. As *nalC* downregulates the expression of drug efflux pumps, it is reasonable to assume that loss-of-function mutations can lead to reduced susceptibility. However, the relevance of these mutations in association with cefiderocol resistance remains unclear, as they only occurred in association with other putative resistance mechanisms, which could contribute to the resistance development against cefiderocol.

Target mutations in penicillin-binding protein

PBPs are transpeptidases responsible for the final stages of peptidoglycan synthesis in GNB and are the target proteins of cefiderocol. Target mutations in the *pbp* genes, leading to structural changes and following reduced binding affinity

between antibiotics and the target enzymes, have been implicated in resistance to several beta-lactam antibiotics, including cephalosporins. However, the role of *pbp* target mutations in cefiderocol resistance is not well established. Some studies have suggested that mutations in *pbp3* may contribute to cefiderocol resistance by altering the structure and function of the PBP3 enzyme as it shows the highest affinity for cefiderocol of all PBPs [15, 18, 19]. In our study, a missense mutation (R363C) was found in *mrcB*, which encodes PBP1b in *A. baumannii*. Although PBP1b shows lower affinity to cefiderocol than PBP3 in *A. baumannii* [15], this mutation seems to be most likely causing cefiderocol resistance in the isolate.

Other unknown mechanisms

We detected a premature stop codon (Q89*) in the gene *pilN*, which is a type 4 fimbrial biogenesis protein [58]. Another frameshift mutation (I295fs) was found in the metabolization gene *spoT* [59]. In the adenylate/guanylate cyclase gene *cyaA* we identified the mutations I178fs and Q148*. Further mutations were found in additional genes like in the elongation factor Tu (H23Y), *ftsZ* (G110S), *manX* (A13A), *rpoZ* (E18fs), *sohB* (I28fs) and *tdh* (T119I, Y127C). In four different *K. pneumoniae* clones, we detected the same premature stop codon (R181*) in the gene *yhaJ*, without any major evidence of a putative resistance mechanism [60]. These mutations, however, always occurred in combination with other mutations potentially explaining cefiderocol resistance.

Limitations

This is an in vitro study investigating the development of resistance to cefiderocol. Cefiderocol susceptible isolates were exposed to escalating concentrations of cefiderocol, starting with an initial concentration below both the MIC and the therapeutic concentration used in vivo. These in vitro conditions are considered ideal for the development of resistance but are not comparable to an in vivo setting during treatment. Therefore, it cannot be estimated how fast resistance development can occur in vivo during therapy with cefiderocol.

Additionally, the resistance development was conducted in CAMHB rather than in iron-depleted CAMHB. Our aim was to provide optimal conditions for the development of cefiderocol resistance in vitro. Given that our primary objective was to generate as many mutants as possible, this approach proved effective. However, it is possible that some of the resistance mechanisms would be more or less frequent under iron-depleted conditions. Investigating this in a further study would be valuable, as using an iron-depleted medium would better simulate in vivo conditions.

While we have repeatedly subcultured the strains they maintained stable cefiderocol resistance. Still, mutations in iron influx pathways may contribute to a loss of fitness, which may cause them to decline within the microbial ecosystem in vivo or under adverse conditions. Furthermore, the assessment of in vivo pathogenicity becomes a viable consideration, as potential loss of fitness could lead to reduced virulence in the event of infection. In this respect, employing an in vivo model to compare isolates before and after the acquisition of resistance may offer a valuable information regarding pathogenicity.

However, it has been observed that resistance development is possible with improper use of this antibiotic, and many different molecular resistance mechanisms have been identified. Therefore, in vitro resistance development models are very useful tools in identifying underlying molecular resistance mechanisms. Although this model reduces the noise of genetic variants, that must be considered for causing resistance, it is not an exhaustive analysis of the molecular resistance mechanisms. In vitro mutagenesis would be ideal to further analyze the involvement of each variant found in resistance development.

Conclusion

Our study provides a comprehensive look into the molecular basis of cefiderocol resistance using an in vitro resistance development model. We detected 35 novel resistance mutations putatively conferring resistance to cefiderocol in 26 different genes. These findings, alongside the identification of previously reported mutations associated with cefiderocol resistance, offer a better understanding of the molecular resistance mechanisms and relevant genes involved in cefiderocol resistant isolates.

The most frequent resistance mechanism was reduced influx via iron transporters, but we also detected a wide range of other putative resistance mutations previously not associated with cefiderocol resistance. Even though cefiderocol has demonstrated low resistance rates in clinical environments and exhibits potent activity against MDR-GNB, the potential emergence of resistance is still a looming concern, especially with escalated and inadequately monitored usage of this antimicrobial agent. Therefore, it is crucial to promote considerate use of antibiotics, implement effective surveillance and stewardship programs, and pursue resistance testing to cefiderocol to assess efficacy. As with any antimicrobial agent, adequate and indicated clinical usage is essential to preserve its effectiveness in future clinical settings.

Data availability

Compliance with ethical standards

References

1. Cassini A, Högberg LD, Plachouras D, Quattrocchi A, Hoxha A, Simonsen GS, et al. Attributable deaths and disability-adjusted life-years caused by infections with antibiotic-resistant bacteria in the EU and the European Economic Area in 2015: a population-level modelling analysis. Lancet Infect Dis. 2019;19:56–66.
2. Murray CJL, Ikuta KS, Sharara F, Swetschinski L, Robles Aguilar G, Gray A, et al. Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. Lancet. 2022;399:629–55.
3. Woolhouse M, Waugh C, Perry MR, Nair H. Global disease burden due to antibiotic resistance – state of the evidence. J Glob Health. 2016;6:010306.
4. European Centre for Disease Prevention and Control., World Health Organization. Antimicrobial resistance surveillance in Europe 2023: 2021 data. LU: Publications Office; 2023. Available from: https://data.europa.eu/doi/10.2900/63495.
5. WHO. Antibacterial agents in clinical and preclinical development: an overview and analysis. 2021. Available from: https://www.who.int/publications/i/item/9789240047655.
6. Ito A, Nishikawa T, Matsumoto S, Yoshizawa H, Sato T, Nakamura R, et al. Siderophore Cephalosporin Cefiderocol Utilizes Ferric Iron Transporter Systems for Antibacterial Activity against Pseudomonas aeruginosa. Antimicrob Agents Chemother. 2016;60:7396–401.
7. Sato T, Yamawaki K. Cefiderocol: Discovery, Chemistry, and In Vivo Profiles of a Novel Siderophore Cephalosporin. Clin Infect Dis. 2019;69:S538–43.
8. European Medicines Agency. European Medicines Agency. 2020. Fetcroja. Available from: https://www.ema.europa.eu/en/medicines/human/EPAR/fetcroja.
9. Food and Drug Administration. FDA approves new antibacterial drug to treat complicated urinary tract infections as part of ongoing efforts to address antimicrobial resistance. 2020. Available from: https://www.fda.gov/news-events/press-announcements/fda-approves-new-antibacterial-drug-treat-complicated-urinary-tract-infections-part-ongoing-efforts.
10. Delgado-Valverde M, Conejo MDC, Serrano L, Fernández-Cuenca F, Pascual Á. Activity of cefiderocol against high-risk clones of multidrug-resistant Enterobacterales, Acinetobacter baumannii, Pseudomonas aeruginosa and Stenotrophomonas maltophilia. J Antimicrob Chemother. 2020;75:1840–9.
11. Kohira N, West J, Ito A, Ito-Horiyama T, Nakamura R, Sato T, et al. In Vitro Antimicrobial Activity of a Siderophore Cephalosporin, S-649266, against Enterobacteriaceae Clinical Isolates, Including Carbapenem-Resistant Strains. Antimicrob Agents Chemother. 2016;60:729–34.
12. Klein S, Boutin S, Kocer K, Fiedler MO, Störzinger D, Weigand MA, et al. Rapid Development of Cefiderocol Resistance in Carbapenem-resistant Enterobacter cloacae During Therapy Is Associated With Heterogeneous Mutations in the Catecholate Siderophore Receptor cirA. Clin Infect Dis. 2022;74:905–8.
13. McElheny CL, Fowler EL, Iovleva A, Shields RK, Doi Y. In Vitro Evolution of Cefiderocol Resistance in an NDM-Producing Klebsiella pneumoniae Due to Functional Loss of CirA. Microbiol Spectr. 2021;9:e01779–21.
14. Moynié L, Luscher A, Rolo D, Pletzer D, Tortajada A, Weingart H, et al. Structure and Function of the PiuA and PirA Siderophore-Drug Receptors from Pseudomonas aeruginosa and Acinetobacter baumannii. Antimicrob Agents Chemother. 2017;61:e02531-16.
15. Ito A, Sato T, Ota M, Takemura M, Nishikawa T, Toba S, et al. In Vitro Antibacterial Properties of Cefiderocol, a Novel Siderophore Cephalosporin, against Gram-Negative Bacteria. Antimicrob Agents Chemother. 2018;62:e01454-17.
16. Poirel L, Sadek M, Kusaksizoglu A, Nordmann P. Co-resistance to ceftazidime-avibactam and cefiderocol in clinical isolates producing KPC variants. Eur J Clin Microbiol Infect Dis. 2022;41:677–80.
17. Rolston KVI, Gerges B, Shelburne S, Aitken SL, Raad I, Prince RA. Activity of Cefiderocol and Comparators against Isolates from Cancer Patients. Antimicrob Agents Chemother. 2020;64:e01955-19.
18. Malik S, Kaminski M, Landman D, Quale J. Cefiderocol Resistance in Acinetobacter baumannii: Roles of β-Lactamases, Siderophore Receptors, and Penicillin Binding Protein 3. Antimicrob Agents Chemother. 2020;64:e01221-20.
19. Wang Q, Jin L, Sun S, Yin Y, Wang R, Chen F, et al. Occurrence of High Levels of Cefiderocol Resistance in Carbapenem-Resistant Escherichia coli before Its Approval in China: a Report from China CRE-Network. Tyne DV, editor. Microbiol Spectr. 2022;10:e02670–21.
20. Baym M, Lieberman TD, Kelsic ED, Chait R, Gross R, Yelin I, et al. Spatiotemporal microbial evolution on antibiotic landscapes. Science. 2016;353:1147–51.
21. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet J. 2011;17:10.

22. Prjibelski A, Antipov D, Meleshko D, Lapidus A, Korobeynikov A. Using SPAdes De Novo Assembler. Curr Protoc Bioinforma. 2020;70:e102.

23. Darling AE, Mau B, Perna NT. progressiveMauve: multiple genome alignment with gene gain, loss and rearrangement. PLoS One. 2010;5:e11147.

24. Tatusova T, DiCuccio M, Badretdin A, Chetvernin V, Nawrocki EP, Zaslavsky L, et al. NCBI prokaryotic genome annotation pipeline. Nucleic Acids Res. 2016;44:6614–24.

25. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. Nat Methods. 2012;9:357–9.

26. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, et al. The Sequence Alignment/Map format and SAMtools. Bioinformatics. 2009;25:2078–9.

27. Koboldt DC, Chen K, Wylie T, Larson DE, McLellan MD, Mardis ER, et al. VarScan: variant detection in massively parallel sequencing of individual and pooled samples. Bioinformatics. 2009;25:2283–5.

28. Cingolani P, Platts A, Wang LL, Coon M, Nguyen T, Wang L, et al. A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of Drosophila melanogaster strain w [1118]; iso-2; iso-3. Fly. 2012;6:80–92.

29. Dagher M, Ruffin F, Marshall S, Taracila M, Bonomo RA, Reilly R, et al. Case Report: Successful Rescue Therapy of Extensively Drug-Resistant Acinetobacter baumannii Osteomyelitis With Cefiderocol. Open Forum Infect Dis. 2020;7:ofaa150.

30. Matsumoto S, Singley CM, Hoover J, Nakamura R, Echols R, Rittenhouse S, et al. Efficacy of Cefiderocol against Carbapenem-Resistant Gram-Negative Bacilli in Immunocompetent-Rat Respiratory Tract Infection Models Recreating Human Plasma Pharmacokinetics. Antimicrob Agents Chemother. 2017;61:e00700–17.

31. Fetcroja 1 g powder - Summary of Product Characteristics (SmPC). 2024. Available from: https://www.medicines.org.uk/emc/product/11771/smpc.

32. Miyazaki S, Katsube T, Shen H, Tomek C, Narukawa Y. Metabolism, Excretion, and Pharmacokinetics of [14C]-Cefiderocol (S-649266), a Siderophore Cephalosporin, in Healthy Subjects Following Intravenous Administration. J Clin Pharmacol. 2019;59:958–67.

33. Katsube T, Saisho Y, Shimada J, Furuie H. Intrapulmonary pharmacokinetics of cefiderocol, a novel siderophore cephalosporin, in healthy adult subjects. J Antimicrob Chemother. 2019;74:1971–4.

34. Saisho Y, Katsube T, White S, Fukase H, Shimada J. Pharmacokinetics, Safety, and Tolerability of Cefiderocol, a Novel Siderophore Cephalosporin for Gram-Negative Bacteria, in Healthy Subjects. Antimicrob Agents Chemother. 2018;62:https://doi.org/10.1128/aac.02163-17.

35. Sanabria C, Migoya E, Mason JW, Stanworth SH, Katsube T, Machida M, et al. Effect of Cefiderocol, a Siderophore Cephalosporin, on QT/QTc Interval in Healthy Adult Subjects. Clin Therapeutics. 2019;41:1724–1736.e4.

36. Katsube T, Echols R, Arjona Ferreira JC, Krenz HK, Berg JK, Galloway C. Cefiderocol, a Siderophore Cephalosporin for Gram-Negative Bacterial Infections: Pharmacokinetics and Safety in Subjects With Renal Impairment. J Clin Pharmacol. 2017;57:584–91.

37. Gerken H, Vuong P, Soparkar K, Misra R. Roles of the EnvZ/OmpR Two-Component System and Porins in Iron Acquisition in Escherichia coli. mBio. 2020;11:e01192-20.

38. Yamano Y, Takemura M, Nakamura R, Echols R. Frequency of resistance acquisition and resistance mechanisms to cefiderocol. In: Poster presented at: ID Week Annual Meeting. Poster 1455; 2020.

39. Dutta R, Yoshida T, Inouye M. The Critical Role of the Conserved Thr247 Residue in the Functioning of the Osmosensor EnvZ, a Histidine Kinase/Phosphatase, in Escherichia coli. J Biol Chem. 2000;275:38645–53.

40. Luscher A, Moynié L, Auguste PS, Bumann D, Mazza L, Pletzer D, et al. TonB-Dependent Receptor Repertoire of Pseudomonas aeruginosa for Uptake of Siderophore-Drug Conjugates. Antimicrob Agents Chemother. 2018;62:e00097-18.

41. Werth BJ, Ashford NK, Penewit K, Waalkes A, Holmes EA, Bryan A, et al. Evolution of cefiderocol resistance in Stenotrophomonas maltophilia using in vitro serial passage techniques. JAC Antimicrob Resistance. 2022;4:dlac011.

42. Gupta A, Landman D, Quale J. Relationship of TonB-dependent receptors with susceptibility to cefiderocol in clinical isolates of Pseudomonas aeruginosa. J Antimicrob Chemother. 2022;77:1282–5.

43. Schalk IJ, Mislin GLA, Brillet K. Chapter Two - Structure, Function and Binding Selectivity and Stereoselectivity of Siderophore–Iron Outer Membrane Transporters. In: Argüello JM, Lutsenko S, editors. Current Topics in Membranes. Academic Press; 2012. p. 37–66. Available from: https://www.sciencedirect.com/science/article/pii/B9780123943903000021.

44. Zhang F, Li B, Dong H, Chen M, Yao S, Li J, et al. YdiV regulates Escherichia coli ferric uptake by manipulating the DNA-binding ability of Fur in a SlyD-dependent manner. Nucleic Acids Res. 2020;48:9571–88.

45. Wu G, Li P, Wu X. Regulation of Escherichia coli IscS desulfurase activity by ferrous iron and cysteine. Biochem Biophys Res Commun. 2008;374:399–404.

46. Gomis-Font MA, Sastre-Femenia MÀ, Taltavull B, Cabot G, Oliver A. In vitro dynamics and mechanisms of cefiderocol resistance development in wild-type, mutator and XDR Pseudomonas aeruginosa. J Antimicrob Chemother. 2023;78:1785–94.

47. Kim A, Kutschke A, Ehmann DE, Patey SA, Crandon JL, Gorseth E, et al. Pharmacodynamic Profiling of a Siderophore-Conjugated Monocarbam in Pseudomonas aeruginosa: Assessing the Risk for Resistance and Attenuated Efficacy. Antimicrob Agents Chemother. 2015;59:7743–52.

48. Langman L, Young IG, Frost GE, Rosenberg H, Gibson F. Enterochelin System of Iron Transport in Escherichia coli: Mutations Affecting Ferric-Enterochelin Esterase. J Bacteriol. 1972;112:1142–9.

49. Ding L, Sun Y, Zhang Y, Shen S, Hu F. In Vivo Development of Aztreonam Resistance in Meropenem-Resistant Pseudomonas aeruginosa Owing to Overexpression of the blaPDC-16. Microbiol Spectr. 2023;11:e03080-22.

50. Rubio AM, Kline EG, Jones CE, Chen L, Kreiswirth BN, Nguyen MH, et al. In Vitro Susceptibility of Multidrug-Resistant Pseudomonas aeruginosa following Treatment-Emergent Resistance to Ceftolozane-Tazobactam. Antimicrob Agents Chemother. 2021;65:https://doi.org/10.1128/aac.00084-21.

51. Moya B, Dötsch A, Juan C, Blázquez J, Zamorano L, Haussler S, et al. β-Lactam Resistance Response Triggered by Inactivation of a Nonessential Penicillin-Binding Protein. PLOS Pathog. 2009;5:e1000353.

52. Ito A, Nishikawa T, Ota M, Ito-Horiyama T, Ishibashi N, Sato T, et al. Stability and low induction propensity of cefiderocol against chromosomal AmpC β-lactamases of Pseudomonas aeruginosa and Enterobacter cloacae. J Antimicrob Chemother. 2018;73:3049–52.

53. Kawai A, McElheny CL, Iovleva A, Kline EG, Sluis-Cremer N, Shields RK, et al. Structural Basis of Reduced Susceptibility to Ceftazidime-Avibactam and Cefiderocol in Enterobacter cloacae Due to AmpC R2 Loop Deletion. Antimicrob Agents Chemother. 2020;64:e00198-20.

54. Liu X, Chang Y, Xu Q, Zhang W, Huang Z, Zhang L, et al. Mutation in the two-component regulator BaeSR mediates cefiderocol resistance and enhances virulence in Acinetobacter baumannii. mSystems. 2023;0:e01291-22.

55. Simner PJ, Beisken S, Bergman Y, Ante M, Posch AE, Tamma PD. Defining Baseline Mechanisms of Cefiderocol Resistance in the Enterobacterales. Microb Drug Resistance. 2022;28:161–70.

56. Perron K, Caille O, Rossier C, Delden C, van, Dumas JL, Köhler T. CzcR-CzcS, a Two-component System Involved in Heavy Metal and Carbapenem Resistance in Pseudomonas aeruginosa. J Biol Chem. 2004;279:8761–8.

57. Cao L, Srikumar R, Poole K. MexAB-OprM hyperexpression in NalC-type multidrug-resistant Pseudomonas aeruginosa: identification and characterization of the nalC gene encoding a repressor of PA3720-PA3719. Mol Microbiol. 2004;53:1423–36.

58. Sampaleanu LM, Bonanno JB, Ayers M, Koo J, Tammam S, Burley SK, et al. Periplasmic Domains of Pseudomonas aeruginosa PilN and PilO Form a Stable Heterodimeric Complex. J Mol Biol. 2009;394:143–59.

59. Sarubbi E, Rudd KE, Xiao H, Ikehara K, Kalman M, Cashel M. Characterization of the spoT gene of Escherichia coli. J Biol Chem. 1989;264:15074–82.

60. Palevsky N, Shemer B, Connolly JPR, Belkin S. The Highly Conserved Escherichia coli Transcription Factor YhaJ Regulates Aromatic Compound Degradation. Front Microbiol. 2016;7:1490.

61. Castanheira M, Arends SJR, Davis AP, Woosley LN, Bhalodi AA, MacVane SH. Analyses of a Ceftazidime-Avibactam-Resistant Citrobacter freundii Isolate Carrying blaKPC-2 Reveals a Heterogenous Population and Reversible Genotype. mSphere. 2018;3:e00408-18.

62. Dean CR, Barkan DT, Bermingham A, Blais J, Casey F, Casarez A, et al. Mode of Action of the Monobactam LYS228 and Mechanisms Decreasing In Vitro Susceptibility in Escherichia coli and Klebsiella pneumoniae. Antimicrob Agents Chemother. 2018;62:https://doi.org/10.1128/aac.01200-18.

63. Adler M. Evolution of Carbapenem-Resistance in Escherichia coli. [Uppsala]: Uppsala Universitet; 2009. Available from: https://www.ibg.uu.se/digitalAssets/167/c_167900-l_3-k_adler-marlen-report.pdf.