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Evolution of β-lactamase-mediated cefiderocol resistance

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Background: Cefiderocol is a novel siderophore β-lactam with improved hydrolytic stability toward β-lactamases, including carbapenemases, achieved by combining structural moieties of two clinically efficient cephalosporins, ceftazidime and cefepime. Consequently, cefiderocol represents a treatment alternative for infections caused by MDR Gram-negatives.

Objectives: To study the role of cefiderocol on resistance development and on the evolution of β-lactamases from all Ambler classes, including KPC-2, CTX-M-15, NDM-1, CMY-2 and OXA-48.

Methods: Directed evolution, using error-prone PCR followed by selective plating, was utilized to investigate how the production and the evolution of different β-lactamases cause changes in cefiderocol susceptibility determined using microbroth dilution assays (MIC and IC_{50}).

Results: We found that the expression of bla_{OXA-48} did not affect cefiderocol susceptibility. On the contrary, the expression of bla_{KPC-2}, bla_{CMY-2}, $bla_{CTX-M-15}$ and bla_{NDM-1} substantially reduced cefiderocol susceptibility by 4-, 16-, 8- and 32-fold, respectively. Further, directed evolution on these enzymes showed that, with the acquisition of only 1–2 non-synonymous mutations, all β-lactamases were evolvable to further cefiderocol resistance by 2- (NDM-1, CTX-M-15), 4- (CMY-2), 8- (OXA-48) and 16-fold (KPC-2). Cefiderocol resistance development was often associated with collateral susceptibility changes including increased resistance to ceftazidime and ceftazidime/avibactam as well as functional trade-offs against different β-lactam drugs.

Conclusions: The expression of contemporary β-lactamase genes can potentially contribute to cefiderocol resistance development and the acquisition of mutations in these genes results in enzymes adapting to increasing cefiderocol concentrations. Resistance development caused clinically important cross-resistance, especially against ceftazidime and ceftazidime/avibactam.

Introduction

The novel and recently introduced cephalosporin cefiderocol is a promising treatment option for infections caused by MDR and carbapenemase-producing Gram-negatives based on two distinctive structural features.[1] Firstly, the cephalosporin molecule is linked to a catechol moiety (siderophore), promoting binding of iron and thus facilitating uptake through the bacterial iron transport systems. This 'Trojan horse strategy' leads to increased periplasmic concentrations and avoids porin-mediated resistance mechanisms.[1] Secondly, the ceftazidime- and cefepime-related side chains of cefiderocol provide improved hydrolytic stability against various β-lactamases, including carbapenemases (Figure S1, available as Supplementary data at *JAC* Online).[2,3] Indeed, cefiderocol hydrolysis was shown to be catalysed several orders of magnitudes less by various carbapenemases, such as KPC-3, NDM-1 and VIM-2, compared with similar β-lactam drugs, such as ceftazidime.[2] While clinical resistance to cefiderocol has mainly been associated with mutations in iron transporter systems,[4–6] the presence of different β-lactamases, such as PER, SHV, BEL and NDM-type, has shown to affect bacterial susceptibility against cefiderocol.[7] Further, a two amino acid deletion in the R2 loop of AmpC of a clinical *Enterobacter* spp. isolate led to reduced susceptibility towards cefiderocol as well as ceftazidime/avibactam.[8,9] Moreover, KPC variants conferring increased ceftazidime/avibactam resistance resulted in cross-resistance against

cefiderocol.[10,11] Further, increased copy number and expression of bla_{NDM-5} in *Escherichia coli* was shown to be associated with the development of cefiderocol resistance.[12] Additionally, the synergistic effects between cefiderocol with β-lactamase inhibitors indicate that the expression of various β-lactamase genes might play a crucial role in cefiderocol resistance development.[13] Thus, there is a clear potential for the selection of new or pre-existing β-lactamase variants exhibiting increased resistance towards cefiderocol.

However, a general understanding of the contribution and evolvability of these enzymes to changes in bacterial cefiderocol resistance is currently still lacking. Here, we provide a systematic study addressing this knowledge gap by asking to which extent the expression of clinical and contemporary β-lactamase genes from different Ambler classes plays a role in the evolution of cefiderocol resistance? Moreover, can the exposure to cefiderocol lead to cross-resistance and re-sensitization (collateral sensitivity) towards other β-lactams and β-lactam-inhibitor combinations? To this end, the genes of five β-lactamases, KPC-2 and CTX-M-15 (Ambler class A), NDM-1 (class B), CMY-2 (class C) and OXA-48 (class D), were expressed in *E. coli* using a low-copy number vector system (~15 copies/cell) since these β-lactamases are often plasmid associated.[14] First, changes in susceptibility due to the expression of β-lactamase genes were analysed by determining the IC_{50} and the standard MIC against a panel of different β-lactams. Next, we used directed evolution to probe the evolutionary potential of these β-lactamases by constructing mutational libraries and selecting clones with increased cefiderocol resistance. We show that the expression of β-lactamase genes from various Ambler classes affects cefiderocol susceptibility and these enzymes possess evolutionary potential to reduce cefiderocol susceptibility, which is often associated with collateral effects.

Materials and methods

Antibiotics and other agents

Cefiderocol was purchased from MedChemExpress (Monmouth Junction, NJ, USA). If not otherwise stated, other antibiotics and media were purchased from Sigma–Aldrich (St Louis, MO, USA). Strains used and constructed in this study are summarized in Table S1. Restriction enzymes, DNA polymerases and T4 ligase were purchased from ThermoFisher (Waltham, MA, USA). Primer sequences used in this study are summarized in Table S2.

Strain construction

Previously, we constructed a low-copy number vector (pUN) with a chloramphenicol resistance marker (pA15 origin with ~15 copies/cell).[15,16] The chloramphenicol marker carried a NcoI restriction site, which was removed by site-directed mutagenesis using GoldenGate cloning. In brief, whole vector amplification was performed with Phusion polymerase and primers P9/P10 (Table S2). The PCR product was digested using LguI and DpnI. Recirculation was performed using a T4 ligase and MP21-05 (*E. coli* E.cloni® 10G) was transformed with the ligated product. Clones were selected on LB plates containing 25 mg/L chloramphenicol and verified by Sanger sequencing (Genewiz, Leipzig, Germany).

This modified vector allowed us to sub-clone all β-lactamase genes, using a NcoI restriction site at the start codon and the XhoI restriction site directly after the stop codon. The gene sequences of bla_{CMY-2}, $bla_{CTX-M-15}$ and bla_{NDM-1} were synthesized by Genewiz according to the gene sequences NG_048935.1, NG_048814.1 and NG_049326.1, respectively. bla_{OXA-48} and bla_{KPC-2} originated from *E. coli* 50579417 and *Klebsiella pneumoniae* K47-25, respectively.[17,18]

Primers were designed, replacing the native NdeI restriction site at the start codon of the β-lactamase genes with a NcoI cutting site by inserting a glycine after the starting methionine amino acid (Table S2). For subcloning, the vector backbone was amplified using the primers P3/P4 and Phusion polymerase. Similarly, bla_{OXA-48} (P1/P2), bla_{KPC-2} (P41/42), bla_{CMY-2} (P52/53), $bla_{CTX-M-15}$ (P48/49) and bla_{NDM-1} (P50/51) were amplified, followed by a NcoI/XhoI digestion. The digested vector backbone and insert were T4 ligated and MP21-05 was transformed with the ligated product. The NcoI and NotI restriction sites within bla_{KPC-2} were removed using primers P43F/R and P44F/R, respectively, and GoldenGate cloning as described above.

After selective plating on cefiderocol agar plates, mutant alleles were amplified using primers P7/P8, sub-cloned into an isogenic pUN vector backbone, under the same promoter,[19] and transformed into MP21-05. This was done to control for chromosomal- and plasmid-mediated effects (e.g. changes in copy number and expression). Genes harbouring the corresponding single and double mutants, which could not be subcloned directly, were constructed by GoldenGate cloning, as described above, using the primers stated in Table S2. All changes were confirmed by Sanger sequencing.

Directed evolution and selective plating

Mutational libraries were constructed by error-prone PCR using 10 ng vector DNA, GoTag DNA polymerase (Promega, Madison, WI, USA), 25 mM $MgCl_2$ (Promega), 10 μM of primers P7/P8 and either 50 μM oxo-dGTP or 1 μM dPTP. PCR products were DpnI digested for 1 h at 37°C. Five nanograms of each product was used for a second PCR, which was performed as described above, but without mutagenic nucleotides. The second PCR product was then digested using NcoI and XhoI and ligated in a 1:3 ratio with the digested and purified vector backbone. MP21-05 was transformed with the ligation mixture, recovered in LB broth for 1 h at 37°C and plated on 25 mg/L chloramphenicol LB agar plates. Library sizes were determined by cell counts and mutation frequencies were determined using Sanger sequencing (Genewiz). The MP21-05 cultures harbouring the corresponding mutational libraries of β-lactamase genes were plated (~10^6 cfu) on LB agar plates containing increasing concentrations of cefiderocol and grown overnight at 37°C. Colonies grown were recovered on plates with the highest cefiderocol concentration and their genotype characterized by Sanger sequencing (Genewiz). Sequences were aligned using ESPript (v. 3).[20]

Dose–response curves and MIC determination

Dose–response curves were determined and their IC_{50} values calculated using GraphPad Prism (v. 9) as previously published.[16] MICs were determined by broth microdilution using in-house-designed premade Sensititre microtiter plates (TREK Diagnostic Systems/Thermo Fisher Scientific, East Grinstead, UK) according to the manufacturer's instruction and standard inoculum of 5×10^5 cfu/mL. The plates were incubated statically for 20 h at 37°C.

Results

Evolution of β-lactamase-mediated cefiderocol resistance

To comparatively study the effect of different β-lactamases on cefiderocol resistance development, we expressed five β-lactamase genes (bla_{CMY-2}, $bla_{CTX-M-15}$, bla_{NDM-1}, bla_{OXA-48} and bla_{KPC-2}) in a low-copy number vector in an isogenic *E. coli* E.cloni® 10G (MP21-05) background and determined changes in cefiderocol

MICs (Table 1). We found that the expression of bla_{OXA-48} and bla_{KPC-2} conferred no (≤2-fold) or marginal (4-fold) reduction in susceptibility towards cefiderocol, respectively. In contrast, expression of bla_{CMY-2}, $bla_{CTX-M-15}$ and bla_{NDM-1} substantially reduced cefiderocol susceptibility by 16-, 8- and 32-fold, respectively. Thus, our data show that the expression of contemporary and clinically relevant β-lactamase genes can be critical and contribute to cefiderocol resistance, which is in-line with previous observations.[7,10,13,21,22]

Further, the observed effect on cefiderocol susceptibility by the expression of β-lactamase genes suggests an evolutionary potential for the adaption towards increasing cefiderocol concentrations. To study this, we created mutational libraries, comprising at least 5000 mutants of each β-lactamase, using error-prone PCR with an average mutation rate of 1–2 non-synonymous mutations per gene. Mutational libraries were selected on agar plates with cefiderocol concentrations 2- to 16-fold above their WT MICs (KPC-2: 2–4 mg/L; CTX-M-15: 4 mg/L; NDM-1: 8 mg/L: CMY-2: 4 mg/L; OXA-48: 0.25 mg/L). Up to eight colonies were randomly selected per β-lactamase from plates containing the highest cefiderocol concentration, and changes in the target genes were characterized by Sanger sequencing. Among isolated variants, we selected a subset of single and double mutants, with amino acid changes either close to the active site, in structural elements important in substrate specificity (e.g. Ω loop), or described in naturally evolving variants for subsequent characterization (Figures S2–S6): two OXA-48 double mutants (F72L/S212A and F156S/T213A), three KPC-2 single mutants (D179A/G/Y), two single (S308R and L317P) and one CMY-2 double mutant (S308N/D309G), two NDM-1 double mutants (Q119R/D267G and Q94R/Q119H), as well as one single (E271K) and one double CTX-M-15 mutant (N192K/S220R). In addition, to identify the contribution of each individual amino acid change within the selected double mutants, the corresponding single mutants were constructed (Table S1).

Standard MIC assays have a limited resolution and may not capture marginal changes in susceptibility that, from an evolutionary perspective, have shown to be crucial for the selection of antibiotic resistance.[16,23] To provide an increased resolution to our susceptibility measurements, we determined the cefiderocol susceptibility changes using dose–response curves (Figure 1) and calculated the corresponding IC_{50} values (Table 2). We found that, with the acquisition of only one amino acid substitution, all β-lactamases evolved to confer significantly increased resistance (herein defined as reduced susceptibility compared with WT allele) against cefiderocol where IC_{50} values typically increased by 2- to 8-fold (Brown–Forsythe and Welch ANOVAs for samples with different SDs, see Table S3). Interestingly, our data also show synergy between strains producing different single mutants during the evolution of OXA-48 (F72L/S212A and F156S/T213A), CMY-2 (S308N/D309G) and NDM-1 (Q119R/D267G) where the IC_{50} values, conferred by the double mutants, were significantly higher than either single mutant alone (Table S3). On the contrary, *E. coli* producing CTX-M-15:E192K and NDM-1:Q94R did not contribute to cefiderocol resistance development in neither single nor double mutants and are thus likely to be hitch-hikers.

To assess the IC_{50} changes in a more clinical microbiological context, we further performed standard MIC susceptibility assays. Using this approach, significant cefiderocol MIC differences (>2-fold changes) were only observed for *E. coli* expressing bla_{OXA-48}, bla_{KPC-2} and bla_{CMY-2} mutants compared with their WT alleles (Table 1). On the contrary, all mutants of CTX-M-15 and NDM-1 conferred unchanged cefiderocol MIC values, despite their significant changes in IC_{50}. Taken together, tested β-lactamases of all Ambler classes can evolve to confer decreased susceptibility against cefiderocol, judged by their IC_{50} values, while exhibiting cryptic phenotypes from a clinical microbiological point of view (no changes in MIC). However, these marginal changes in resistance have been shown to be highly selectable, especially under low or sub-optimal β-lactam concentrations[16] and can provide a gateway for developing clinical resistance.[23–26]

Cefiderocol resistance display changes in collateral susceptibility

Cefiderocol is an oxyimino-cephalosporin combining chemical moieties of ceftazidime and cefepime (Figure S1). Evolution of β-lactamase-mediated resistance towards ceftazidime and ceftazidime combinations with β-lactamase inhibitors, such as avibactam, has been reported to cause collateral changes, e.g. cross-resistance and collateral sensitivity conferred by different enzymes, including KPC and OXA-48.[10,15,27] To understand whether collateral effects occur during the evolution towards cefiderocol resistance, we determined MICs against a panel of different β-lactams, covering all β-lactam classes (Table 1). Our MIC data show that a 4- to 8-fold increase in cefiderocol MIC in OXA-48 and KPC-2 mutants caused the development of strong cross-resistance against ceftazidime, with ceftazidime MICs elevated by >4- to >16-fold. In addition, all three selected KPC-2 mutants conferred cross-resistance against ceftazidime/avibactam with >8- to >16-fold increased MIC values. These observations are in-line with previous studies where the selection of KPC-2 on ceftazidime/avibactam caused the emergence of KPC-2 mutants conferring cross-resistance against cefiderocol.[10,27] Similarly, the selection for OXA-48 mutants displaying increased activity against ceftazidime resulted in mutants identical or similar (e.g. F72L and F156C/V) to the ones identified in this study,[16] suggesting that the exposure to either ceftazidime or cefiderocol causes functional cross-resistance in both KPC-2 and OXA-48. No effect of the OXA-48 mutants on ceftazidime/avibactam resistance development was found and cross-resistance to other cephalosporins, such as cefepime and cefotaxime, was not detected. In addition, no cross-resistance was observed towards ceftazidime/avibactam with the CMY-2 and CTX-M-15 variants.

We also observed that evolved cefiderocol resistance comes with a range of significant evolutionary trade-offs. For all three carbapenemases (OXA-48, KPC-2 and NDM-1), we found significant collateral sensitivities towards carbapenems. This was particularly true for the serine carbapenemases OXA-48 and KPC-2, where cefiderocol resistance development caused strong collateral sensitivity effects with reduced carbapenem MICs. The strongest effect was seen for meropenem with an MIC reduction of up to 32-fold in the KPC-mutant-producing strains. A smaller collateral sensitivity effect was observed within the NDM-1: Q119R/D267G-producing strain where the meropenem MIC was

Table 1. MIC determination

	Variants	MP strain no.	CFD	CAZ	CZA	C/T	CTX	FEP	ATM	MEM	IPM	ETP	MEV	IMR	TZP	TMC
E. coli	–	21-05	0.06	<0.25	<0.12	<0.25	<0.12	<0.12	<0.25	0.03	0.25	0.12	<0.06	0.5	<1	<8
CTX-M-15	WT	24-80	0.5	8	<0.12	<0.25	>16	2	8	0.03	0.25	<0.12	<0.06	0.25	<1	16
	N192K	29-15	1	4	<0.12	<0.25	>16	2	8	0.03	0.25	<0.12	<0.06	0.12	<1	<8
	S220R	29-16	0.25	0.5	<0.12	<0.25	1	<0.12	1	0.03	0.12	<0.12	0.25	0.12	<1	<8
	E271K	29-07	0.5	2	<0.12	<0.25	4	0.25	4	0.03	0.25	<0.12	<0.06	<0.12	<1	<8
	N192K/S220R	29-08	1	4	<0.12	<0.25	>16	1	16	0.03	0.25	<0.12	<0.06	0.12	<1	<8
KPC-2	WT	24-44	0.25	2	<0.12	1	2	0.5	8	1	2	1	<0.06	0.25	>32	<8
	D179A	24-69	1	16	1	0.5	0.25	<0.12	<0.25	0.03	0.25	0.12	<0.06	0.25	<1	<8
	D179G	24-71	2	16	1	0.5	0.5	0.25	<0.25	0.06	0.25	0.12	<0.06	0.25	<1	<8
	D179Y	24-70	2	32	2	1	2	0.5	<0.25	0.03	0.25	<0.12	<0.06	0.25	<1	<8
NDM-1	WT	24-81	2	>32	>32	>16	>16	4	<0.25	4	4	2	4	2	>32	16
	Q94R	29-18	2	>32	>32	>16	>16	4	<0.25	2	4	1	2	4	>32	16
	Q119R	29-10	2	>32	>32	>16	>16	4	<0.25	2	4	0.5	4	4	32	64
	D267G	29-17	2	>32	>32	>16	>16	4	<0.25	1	4	0.5	1	4	>32	16
	Q119R/D267G	29-09	2	>32	>32	>16	>16	4	<0.25	0.5	2	0.25	0.25	2	>32	64
	Q94R/Q119H	29-11	2	>32	>32	>16	>16	4	<0.25	2	8	0.5	0.5	2	>32	32
CMY-2	WT	12-69	0.5	4	<0.12	<0.25	2	<0.12	0.5	0.03	0.25	0.12	<0.06	0.25	<1	<8
	S308R	29-04	2	8	<0.12	<0.25	2	0.25	0.5	0.03	0.25	<0.12	<0.06	0.12	<1	<8
	S308N	29-13	1	4	<0.12	<0.25	2	0.25	0.5	0.03	0.25	<0.12	<0.06	0.12	<1	<8
	D309G	29-14	0.5	4	<0.12	<0.25	2	<0.12	0.5	0.03	0.25	0.12	<0.06	0.25	<1	<8
	L317P	29-06	1	8	<0.12	<0.25	2	0.25	<0.25	0.03	0.12	<0.12	<0.06	0.5	<1	<8
	S308N/D309G	29-05	2	8	<0.12	<0.25	2	0.5	0.5	0.03	0.12	0.12	<0.06	0.25	<1	<8
OXA-48	WT	21-01	0.06	<0.25	<0.12	<0.25	<0.12	<0.12	<0.25	0.25	1	<0.12	0.12	1	32	64
	F72L/S212A	22-37	0.5	1	<0.12	<0.25	<0.12	<0.12	<0.25	0.03	0.25	<0.12	<0.06	0.25	<1	<8
	F156S/T213A	24-41	0.5	2	<0.12	<0.25	<0.12	<0.12	<0.25	0.06	0.5	<0.12	<0.06	0.25	<1	32

CFD, cefiderocol; CAZ, ceftazidime; CZA, ceftazidime/avibactam; C/T, ceftolozane/tazobactam; CTX, cefotaxime; FEP, cefepime; ATM, aztreonam; MEM, meropenem; IPM, imipenem; ETP, ertapenem; MEV, meropenem/vaborbactam; IMR, imipenem/relebactam; TZP, piperacillin/tazobactam; TMC, temocillin.

The concentrations of avibactam, tazobactam and relebactam were fixed at 4 and 8 mg/L for vaborbactam.

β-lactamase genes are expressed in *E. coli* E.cloni® (MP21-05) and MIC values are reported in mg/L.

reduced by 8-fold. In addition, for both OXA-48 and KPC-2, the expression of mutant alleles resulted in a >32-fold reduction in piperacillin/tazobactam MIC. Other collateral sensitivity changes with MIC reductions >2-fold include ceftazidime (CTX-M-15: S220R and E271K), cefotaxime (KPC-2:D179A/G, CTX-M-15: S220R and CTX-M-15:E271K) and aztreonam (CMY-2:L317P, KPC-2 D179x and CTX-M-15:S220R).

Discussion

There have been observations that expression of β-lactamase genes can impact the bacterial susceptibility and the evolution of cefiderocol resistance.[7–10,12,13,21,22] Indeed, we showed that the production of WT β-lactamases from various Ambler classes can significantly alter cefiderocol susceptibility in *E. coli* (Table 1). Beyond that, we probed the evolutionary potential of all tested β-lactamases to adapt toward increasing cefiderocol resistance (Table 2). With the acquisition of only 1–2 amino acid changes, all β-lactamases evolved to confer increased resistance against cefiderocol. Interestingly, we observed that the extent by which cefiderocol resistance developed was highly dependent on the initial WT β-lactamase activity. Enzymes conferring an initial low-level resistance profile against cefiderocol, such as OXA-48 and KPC-2, showed the highest improvement. On the contrary, enzymes conferring initially higher cefiderocol resistance, such as CMY-2, NDM-1 and CTX-M-15, demonstrated substantial less improvements (Tables 1 and 2). Indeed, selected mutants for these enzymes did not significantly improve cefiderocol resistance

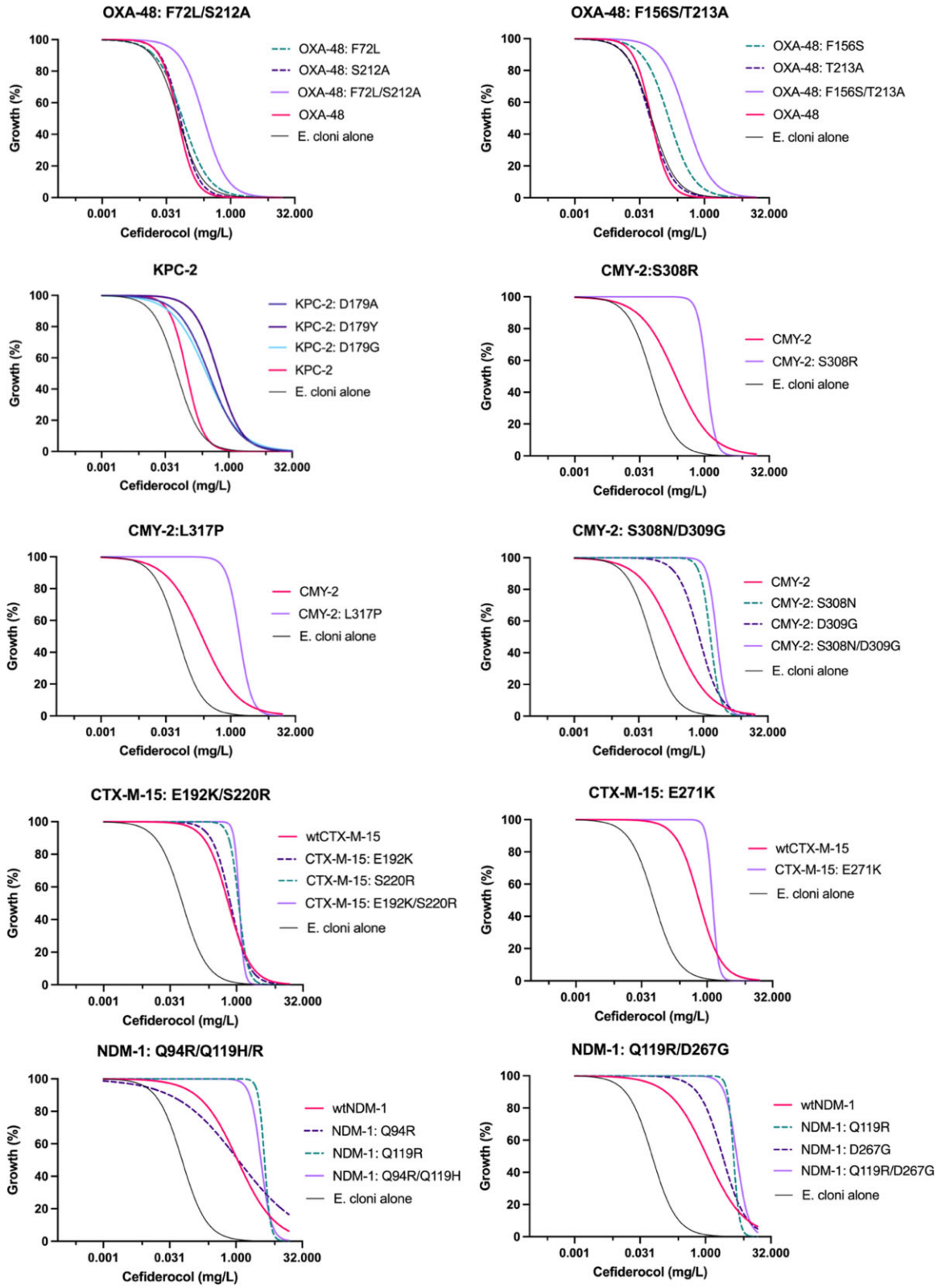

Figure 1. Cefiderocol dose–response curves. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

Table 2. IC_{50} determination

MP strain no.	Name	IC_{50} (mg/L)	SEM (mg/L)
MP21-05	*E. coli* E.cloni®	0.060	0.004
MP24-80	CTX-M-15	0.653	0.075
MP29-15	CTX-M-15:N192K	0.731	0.08
MP29-16	CTX-M-15:S220R	1.122	0.083
MP29-07	CTX-M-15:E271K	1.339	0.08
MP29-08	CTX-M-15:N192K/S220R	1.16	0.142
MP24-44	KPC-2	0.102	0.004
MP24-69	KPC-2:D179A	0.338	0.034
MP24-71	KPC-2:D179G	0.299	0.027
MP24-70	KPC-2:D179Y	0.558	0.037
MP24-81	NDM-1	1.087	0.172
MP29-10	NDM-1:Q94R	1.126	0.312
MP29-17	NDM-1:Q119R	4.448	0.253
MP29-18	NDM-1:D267G	2.489	0.323
MP29-09	NDM-1:Q94R/Q119H	3.683	0.238
MP29-11	NDM-1:Q119R/D267G	5.284	0.418
MP12-69	CMY-2	0.216	0.031
MP29-04	CMY-2:S308R	1.677	0.112
MP29-13	CMY-2:S308N	1.466	0.108
MP29-14	CMY-2:D309G	0.807	0.083
MP29-06	CMY-2:L317P	1.596	0.102
MP29-05	CMY-2:S308N/D309G	2.06	0.096
MP21-01	OXA-48	0.058	0.003
MP22-05	OXA-48:F72L	0.076	0.005
MP22-19	OXA-48:S212A	0.066	0.005
MP22-06	OXA-48:F156S	0.148	0.016
MP22-07	OXA-48:T213A	0.055	0.004
MP22-37	OXA-48:F72L/S212A	0.237	0.021
MP24-41	OXA-48:F156S/T213A	0.381	0.033

SEM represents the standard error based on at least three replicates.

within *E. coli* judged by a standard MIC assay. However, we found that the expression of most mutant alleles was able to significantly elevate resistance when measured in an IC_{50} set-up. Such cryptic changes in susceptibility have been previously described to play an important role in the evolution of β-lactamases and are highly selectable, especially under sub-optimal β-lactam concentrations.[16,23] We acknowledge the fact that only single and double mutants were studied, and further work needs to be done to explore the full evolutionary potential of these enzymes. In addition, the impact of these mutations with respect to structure and catalytic activity, as well as other possible aspects, such as stability and translational efficiency, needs to be further elucidated.

Evolution of β-lactamase-mediated resistance to ceftazidime/avibactam and cefepime has been shown to concurrently cause cross-resistance or reduced susceptibility to cefiderocol.[8–11,27] Here, we observed a similar phenomenon where cefiderocol and ceftazidime resistance increased parallelly to the same extent within OXA-48- and KPC-2-producing strains (Table 1) and ceftazidime/avibactam resistance in KPC-2. No cross-resistance against other oxyimino-cephalosporins or β-lactams was identified, indicating that the structural similarity between cefiderocol and ceftazidime plays an important role for the development of cross-resistance.

In contrast, widespread collateral sensitivity against other β-lactams, including carbapenems and penicillin-inhibitor combinations, was found in strains expressing mutant alleles with increased cefiderocol resistance (Table 1). We observed the strongest trade-offs during cefiderocol resistance development of OXA-48 and KPC-2 against carbapenems and aztreonam (KPC-2). Such collateral sensitivity/functional trade-offs can open the path for alternative treatment strategies, and they have been successfully exploited in the clinical setting with a carbapenem/β-lactamase inhibitor combination against ceftazidime/avibactam- and cefiderocol-resistant *K. pneumoniae* harbouring the natural KPC-31.[27,28] However, the molecular causes of these collateral effects remain poorly understood. A study on the ceftazidimase OXA-163, which possesses lower carbapenem activity compared with OXA-48, suggests that molecular evolution shapes drug incompatibility, resulting in multiple binding modes that give rise to these trade-offs.[29] For the latest carbapenem/β-lactamase inhibitor combinations, such as meropenem/vaborbactam and imipenem/relebactam, any collateral effects were seemingly related to the impact on the carbapenem susceptibility and not towards the inhibitor.

F72 and F156 in OXA-48 have been previously characterized as mutational hot-spot allowing for marginally increased catalytic ability to accelerate ceftazidime hydrolysis.[15] Here, we re-identified mutations at these positions (F72L and F156S) showing their involvement in cefiderocol resistance development. While OXA-48:F72L was reported in environmental samples,[30,31] most characterized OXA-48-like variants, which confer increased ceftazidime resistance, exhibit multiple amino acid deletions within the β5–β6 loop.[32] It remains to be determined whether these variants, such as OXA-163, also confer increased resistance against cefiderocol. In contrast, the D179x amino acid changes within the Ω-loop of KPC-type have been described in naturally evolving enzymes (KPC-78, KPC-86 and KPC-31; Figure S3). For CMY-2, amino acid changes clustered round the R2 loop, which has been shown to be host to the R2-side chain of β-lactam drugs.[33] Consequently, mutations and deletions within the R2 loop have been associated with increased resistance towards cephalosporins, such as cefepime and ceftazidime.[34,35] Also here, several of the amino acid changes or positions reported in this study have been associated with naturally evolving variants (e.g. CMY-133 and CMY-17). This underlines the fact that variants conferring improved cefiderocol resistance are already present in clinical isolates, and that these variants can be co-selected under, e.g. ceftazidime/avibactam treatment.[22] In addition, these enzymes are encoded on transferable plasmids allowing these genes to spread—a process that may be facilitated by the increasing usage of cefiderocol.

Taken together, this study provides a proof-of-principle showing that the expression of β-lactamase genes from various Ambler classes can substantially contribute to cefiderocol resistance and that many β-lactamases possess the evolutionary potential to adapt to increasing cefiderocol concentrations under laboratory conditions. Similar to other cephalosporins, this evolutionary process comes with collateral effects against β-lactam drugs, including both cross-resistance and re-sensitization.

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Transparency declarations

None to declare.

Supplementary data

Tables S1 to S3 and Figures S1 to S6 are available as Supplementary data at *JAC* Online.

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