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EXPERIMENTAL THERAPEUTICS

Cephem Potentiation by Inactivation of Nonessential Genes Involved in Cell Wall Biogenesis of β-Lactamase-Producing *Escherichia coli*

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ABSTRACT Reversal of antimicrobial resistance is an appealing and largely unexplored strategy in drug discovery. The objective of this study was to identify potential targets for "helper" drugs reversing cephem resistance in *Escherichia coli* strains producing β-lactamases. A CMY-2-encoding plasmid was transferred by conjugation to seven isogenic deletion mutants exhibiting cephem hypersusceptibility. The effect of each mutation was evaluated by comparing the MICs in the wild type and the mutant harboring the same plasmid. Mutation of two genes encoding proteins involved in cell wall biosynthesis, *dapF* and *mrcB*, restored susceptibility to cefoxitin (FOX) and reduced the MICs of cefotaxime and ceftazidime, respectively, from the resistant to the intermediate category according to clinical breakpoints. The same mutants harboring a CTX-M-1-encoding plasmid fell into the intermediate or susceptible category for all three drugs. Individual deletion of *dapF* and *mrcB* in a clinical isolate of CTX-M-15-producing *E. coli* sequence type 131 (ST131) resulted in partial reversal of ceftazidime and cefepime resistance but did not reduce MICs below susceptibility breakpoints. Growth curve analysis indicated no fitness cost in a Δ*mrcB* mutant, whereas a Δ*dapF* mutant had a 3-fold longer lag phase than the wild type, suggesting that drugs targeting DapF may display antimicrobial activity, in addition to synergizing with selected cephems. DapF appeared to be a potential FOX helper drug target candidate, since *dapF* inactivation resulted in synergistic potentiation of FOX in the genetic backgrounds tested. The study showed that individual inactivation of two nonessential genes involved in cell wall biogenesis potentiates cephem activity according to drug- and strain-specific patterns.

KEYWORDS extended-spectrum β-lactamase (ESBL), cephem resistance, helper drug

Resistance to cephems, a group of β-lactam antibiotics including cephalosporins and cephamycins, is clinically important, as these antimicrobial agents, especially oxyimino-cephalosporins, are commonly used in clinical practice, are easy to administer, and have low toxicity (1). With treatment options limited to "last-resort" antimicrobial agents, mainly carbapenems (2), no adequate therapeutic options exist to face the worldwide increase of infections caused by cephem-resistant *Enterobacteriaceae* producing extended-spectrum β-lactamase (ESBL) or AmpC β-lactamase, which have a considerable clinical and economic impact (3). There is an urgent need for new therapeutic strategies to manage both community- and hospital-acquired infections caused by these bacteria. One of the possible strategies is to suppress resistant subpopulations by combination therapy (4). This approach includes combinations with "helper" drugs designed to enhance antimicrobial activity (5). β-Lactamase inhibitors are a prominent example of helper drugs used in clinical practice. Numerous inhibitors with novel mechanisms of inhibition are currently being developed against β-lactamase-producing strains that have

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TABLE 1 MICs of FOX and CTX in *E. coli* wild-type BW25113 and in 16 derivative isogenic Keio mutants

	MIC[a] (μg/ml)																
Drug	Wild type	Δ*mrcB*	Δ*dacA*	Δ*ycfM*	Δ*ybgF*	Δ*dapF*	Δ*ycjU*	Δ*fis*	Δ*rimK*	Δ*rplA*	Δ*dksA*	Δ*bamB*	Δ*surA*	Δ*tolC*	Δ*acrA*	Δ*recA*	Δ*yciM*
FOX	4	0.25	1	0.5	1	0.5	2	4	4	4	2	4	16[b]	1	2	2	8
CTX	0.031	0.016	0.016	0.031	0.031	0.008	0.016	0.031	0.031	0.031	0.008	0.016	0.031	0.016	0.016	0.031	0.031

[a]The MICs of drugs for which individual gene deletion resulted in a synergistic effect (interaction index ≤ 0.25) are underlined.
[b]Deletion of *surA* had an antagonistic effect on FOX (interaction index ≥ 4).

resistance to traditional inhibitors, such as clavulanic acid, tazobactam, and sulbactam (6). A new β-lactam–β-lactamase inhibitor combination, ceftazidime-avibactam, has recently been approved for clinical use (7). Efflux pump inhibitors (EPIs) are another example of helper drugs suppressing resistance mechanisms, but despite the numerous EPIs described in the scientific literature (8), none of them has reached clinical development, mainly due to toxicity. In addition to suppression of resistance, helper drugs can also reduce the likelihood of resistance development during antimicrobial therapy (9).

The antimicrobial susceptibility phenotype of a given bacterial species depends on the concerted activities of several elements, which as a whole are known as the intrinsic resistome (10, 11). The various components of the *Escherichia coli* intrinsic resistome have been inferred by previous studies measuring the susceptibility and fitness of in-frame gene deletion mutants in the presence of antimicrobial agents (12, 13). Despite the potential of the intrinsic resistome to serve as a source for helper drug targets, no studies have investigated whether inactivation of these genes restores susceptibility in resistant strains by reducing the MIC below the clinical breakpoint. In this study, we evaluated the helper drug target potential of 16 selected genes important for intrinsic resistance to cephems in *E. coli* (12, 13). The effect of each gene deletion on cephem activity was studied by comparing the MICs of the deletion mutant and the wild type in different *E. coli* genetic backgrounds harboring plasmids encoding clinically important ESBLs (CTX-M-1 and CTX-M-15) or AmpC β-lactamase (CMY-2). We identified two gene deletions, Δ*dapF* and Δ*mrcB*, that potentiate cephem activity against β-lactamase-producing strains in a drug-specific fashion.

RESULTS

Effects of gene deletions on acquired cephem resistance. The MICs of cefoxitin (FOX) and cefotaxime (CTX) in the *E. coli* wild-type strain BW25113 and in the 16 derivative isogenic Keio mutants selected for this study are shown in Table 1. Based upon the magnitude of the MIC change determined by each gene deletion relative to the wild type, six and two gene deletions exhibited synergy with FOX and CTX, respectively. One gene deletion (Δ*surA*) had an antagonistic effect with FOX. Seven gene deletions resulted in MIC reductions greater than 2-fold (Table 1), and their effects were studied in the same genetic background (BW25113) containing a CMY-2-encoding plasmid. As expected, the plasmid conferred resistance to ceftazidime (CAZ), CTX, and FOX in BW25113 (Table 2). However, in the presence of the same plasmid, two individual gene deletions (Δ*mrcB* and Δ*dapF*) reduced the MICs of FOX and CTX by

TABLE 2 MICs of FOX, CTX, and CAZ in wild-type BW25113 *E. coli* and derivative isogenic Keio mutants carrying a CMY-2-encoding plasmid

	MIC[a] (μg/ml)									
Drug	Wild type	Δ*mrcB*	Δ*dacA*	Δ*ycfM*	Δ*ybgF*	Δ*dapF*	Δ*dksA*	Δ*tolC*	Susceptibility breakpoint	Resistance breakpoint
FOX	32	**8**	32	16	16	**2**	16	32	≤8	≥32
CTX	8	4	8	16	8	2	4	8	≤1	≥4
CAZ	32	8	32	32	32	32	16	32	≤4	≥16

[a]MICs equal to or below the CLSI susceptibility breakpoints for *Enterobacteriaceae* (37) are indicated in boldface. The MICs of drugs for which individual gene deletion resulted in a synergistic effect (interaction index ≤ 0.25) are underlined.

TABLE 3 MICs of FOX, CTX, and CAZ in *E. coli* wild-type BW25113 and in two derivative isogenic Keio mutants carrying a CTX-M-1-encoding plasmid (Δ*dapF* and Δ*mrcB*)

Cephem	MIC[a] (μg/ml)				
	Wild type	Δ*mrcB*	Δ*dapF*	Susceptibility breakpoint	Resistance breakpoint
FOX	**4**	**0.5**	**0.5**	≤8	≥ 32
CTX	16	2	**1**	≤1	≥ 4
CAZ	**2**	**0.25**	**0.25**	≤4	≥ 16

[a]MICs below the CLSI susceptibility breakpoints for *Enterobacteriaceae* (37) are indicated in boldface. The MICs of drugs for which individual gene deletion resulted in a synergistic effect (interaction index ≤ 0.25) are underlined.

more than 2-fold compared to wild-type strain BW25113. Both deletions exhibited synergistic interactions with FOX, while CAZ and CTX synergistic interactions were limited to Δ*mrcB* and Δ*dapF*, respectively (Table 2). According to the CLSI breakpoints, these two mutants were susceptible to FOX and fell into the intermediate category for CAZ and CTX, respectively (Table 2). The effects of these two gene deletions were further evaluated in the presence of a CTX-M-1-encoding plasmid to check whether the effect of each gene deletion is restricted to the specific β-lactamase type. The MICs in both Δ*dapF* and Δ*mrcB* mutants harboring the CTX-M-1-encoding plasmid were 8- to 16-fold lower than in the wild-type strain containing the same plasmid (Table 3). CTX resistance was completely reversed by deletion of *dapF*, while the *mrcB* deletion resulted in only partial reversal of resistance to the cephalosporin. Both deletions exhibited synergistic interactions with CAZ and FOX, although both the wild type and the mutants producing CTX-M-1 fell into the susceptible category, according to the CLSI breakpoints for the drugs (Table 3).

Based on the susceptibility results, *dapF* and *mrcB* were deleted in a clinical isolate of CTX-M-15-producing *E. coli* sequence type 131 (ST131) (UR40) to determine if the two individual gene deletions could potentiate cephems in this epidemic clone. Both the wild type and the mutants producing CTX-M-15 were susceptible to FOX, according to the CLSI breakpoints (Table 4). Complete and partial reversal of CAZ resistance was observed in Δ*dapF* and Δ*mrcB* mutants, respectively. The two mutants fell into the intermediate category for cefepime (FEP) but retained resistance to ceftriaxone (CRO) and CTX, most likely because the wild-type strain displays very high MICs of these two oxyimino-cephalosporins (MICs, >64 and >128 μg/ml, respectively). Deletion of *dapF* had a synergistic effect with all five tested cephems, whereas only synergy with FOX was detected following *mrcB* deletion (Table 4).

Real-time growth analysis of *E. coli* ST131 wild type and mutants. Real-time growth curve analysis was performed to generate information regarding the fitness of Δ*dapF* and Δ*mrcB* mutants compared to the wild type and to assess how these gene deletions affected growth in the presence of FOX. This cephamycin was selected because it was the only cephem that exhibited synergy with both deletions (Table 4).

TABLE 4 MICs of CRO, CTX, CAZ, FOX, and FEP in a clinical isolate of *E. coli* ST131 (UR40) harboring a CTX-M-15-encoding plasmid and in two derivative isogenic mutants (Δ*dapF* and Δ*mrcB*)

Drug	MIC[a] (μg/ml)				
	Wild-type UR40	Δ*mrcB*	Δ*dapF*	Susceptibility breakpoint	Resistance breakpoint
CRO	>128	128	64	≤1	≥ 4
CTX	>64	64	32	≤1	≥ 4
CAZ	16	8	**4**	≤4	≥ 16
FOX	**8**	**<2**	**<2**	≤8	≥ 32
FEP	16	8	4	≤2	≥ 16

[a]MICs equal to or below the CLSI susceptibility breakpoints for *Enterobacteriaceae* (37) are indicated in boldface. The MICs of drugs for which individual gene deletion resulted in a synergistic effect (interaction index ≤ 0.25) are underlined.

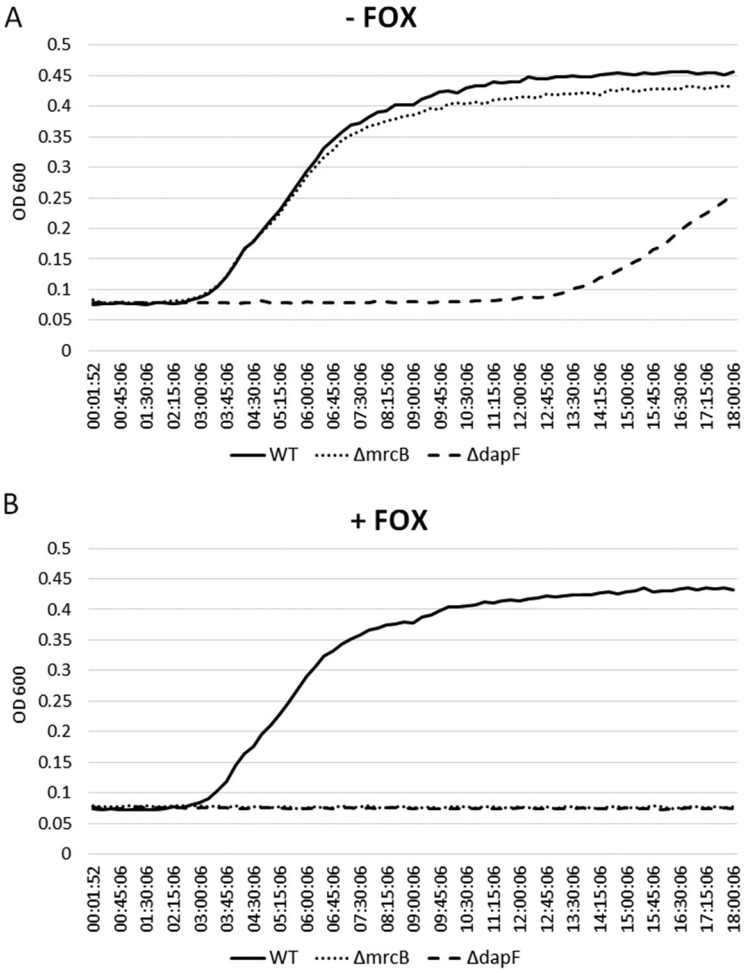

FIG 1 Growth curves of wild-type (WT) *E. coli* ST131 producing CTX-M-15 and derivative deletion mutants (Δ*dapF* and Δ*mrcB*) in the absence (A) and presence (B) of 2 μg/ml FOX.

Analysis of the lag phase of the two mutants and the wild type in FOX-free medium showed no effect of *mrcB* deletion on bacterial growth. However, Δ*dapF* displayed a 3-fold-longer lag phase than the wild type and the Δ*mrcB* mutant (Fig. 1A). As expected, supplementation of the medium with 2 μg/ml of FOX had no effect on the growth of the wild type but inhibited growth of both mutants (Fig. 1B).

MrcB and DapF homology between *Enterobacteriaceae* and humans. Considerable homology in amino acid sequence (100% coverage and 87 to 99% identity relative to the sequence in *E. coli*) was detected for both proteins between *Enterobacteriaceae* species: *Klebsiella pneumoniae* (87% for MrcB and 95% for DapF), *Salmonella enterica* (91% for MrcB and 96% for DapF), *Shigella flexneri* (99% for MrcB and 99% for DapF), and *Citrobacter freundii* (93% for MrcB and 98% for DapF). In contrast, no human homologues were identified for MrcB and DapF.

DISCUSSION

We employed a novel, straightforward approach to identify potential antimicrobial helper drug targets in ESBL/AmpC-producing *E. coli*. This approach applied knowledge generated from previous high-throughput drug-gene interaction profiling studies showing that individual gene deletions can cause hypersusceptibility in wild-type *E. coli* (12, 13). Here, the effects of such gene deletions on cephem susceptibility were studied in resistant *E. coli* producing different types of clinically important β-lactamases. Interestingly, deletion of *mrcB* or *dapF* enhanced the activity of oxyimino-cephalosporins (CAZ, CTX, and FEP) and cephamycins (FOX) according to drug- and strain-specific patterns. Complete reversal of

resistance, defined as a MIC reduction from the resistant to the susceptible category according to clinical breakpoints, was observed only for FOX, by inactivation of either gene in the CMY-2-producing strain (Table 2), and for CAZ, by *dapF* inactivation in the CTX-M-15-producing strain (Table 4). Both gene products are involved in cell wall biogenesis, i.e., *mrcB* encodes a penicillin-binding protein (PBP1b) and *dapF* encodes an epimerase involved in the synthesis of peptidoglycan precursors.

DapF appears to be a promising FOX helper drug target, since deletion mutants in *dapF* displayed FOX potentiation and were consistently susceptible to the cephamycin regardless of the strain genetic background and β-lactamase type (Tables 2, 3, and 4). Although the CTX-M-1 and CTX-M-15 β-lactamases did not confer resistance in the wild-type strain, *dapF* deletion mutants consistently showed MICs $\geq$6-fold lower than that of the wild type, which is a desirable feature for a helper drug target. Deletion of the gene also restored full susceptibility to CAZ in the CTX-M-15-producing strain (Table 4) but did not reduce the MIC of CAZ in the strain that produced CMY-2 (Table 2). We do not know why the MIC of CAZ was not lowered in the presence of CMY-2, but this observation suggests that combining CAZ with DapF inhibitors would not enhance CAZ efficacy against strains expressing this AmpC type β-lactamase. DapF, a cytoplasmic diaminopimelate (DAP) epimerase, is part of the DAP pathway that converts diaminopimelic acid (LL-DAP) to a better peptidoglycan precursor (meso-DAP), which incorporates into the cell wall 1,000 times more efficiently than LL-DAP (14, 15). Deletion of *dapF* increases the LL-DAP pool compared to meso-DAP, likely reducing the peptidoglycan synthesis rate, which in turn may explain the reduced fitness of the Δ*dapF* ST131 mutant in the growth assay (Fig. 1). It should be noted that the increased cephem susceptibility observed in this mutant may well be influenced by its reduced fitness. The poor fitness of Δ*dapF* ST131 suggests that compounds interfering with DapF function may exhibit a growth-inhibitory effect *in vivo*. The absence of DapF homologues in mammals (15, 16) is another characteristic favorable for developing a FOX helper drug targeting this protein.

Similar to Δ*dapF*, deletion of *mrcB* reversed FOX resistance in the CMY-2-producing strain and increased susceptibility to both FOX and oxyimino-cephalosporins in the CTX-M-producing strains (Tables 3 and 4). However, unlike Δ*dapF*, *mrcB* deletion decreased the MIC of CAZ in the CMY-2-producing strain (Table 2) and did not affect the strain's fitness (Fig. 1). Previous studies have shown reduced fitness of Δ*mrcB* in stationary-phase competition assays using model *E. coli* strains and decreased tolerance for bile in *Salmonella enterica* serovar Typhi (17, 18). The *mrcB* gene product, PBP1b, is one of the two major bifunctional penicillin-binding proteins in *E. coli*, with both transglycosylase and transpeptidase enzymatic activities. PBP1b is uniquely important for biofilm formation and motility and is implicated in outer membrane constriction during cell division (19–21). While the enhanced cephem activity observed in Δ*mrcB* could be due to reduced integrity of the peptidoglycan layer (22), the known synthetic lethality between *mrcB* and *mrcA* (PBP1a) (20, 23–25) suggests a more specific mechanism. In particular, the potentiation of FOX in the Δ*mrcB* mutant implies that PBP1a may be a primary target of the cephamycin. Interestingly, *mrcA* deletion did not cause increased cephem sensitivity, suggesting a more critical role of PBP1b in cephem resistance (12, 13, 23). PBP1b function requires interaction with the outer membrane lipoprotein YcfM (20, 24, 26). In the absence of its interaction partner, aberrant YcfM interactions with other proteins may introduce additional envelope stress in the presence of cephems (20). These data suggest that lack of PBP1b-YcfM interaction not only reduces peptidoglycan synthesis, but may also induce collateral damage in the presence of cephems. It can be hypothesized that compounds interfering with PBP1b-YcfM interaction may inhibit PBP1b functions and produce secondary deleterious effects.

This study identified an antagonistic effect of *surA* deletion on FOX antimicrobial activity but no interaction between the gene deletion and CTX in the CMY-2-producing strain (Table 1). This observation suggests that the antimicrobial activity of FOX partially depends upon SurA. This protein is a periplasmic chaperone that

assists outer membrane porin maturation and outer membrane biogenesis (27). Inactivation of *surA* results in fewer porins in the outer membrane and defects in this membrane barrier (28, 29). The observed antagonism between *surA* deletion and FOX may not be solely due to the reduced outer membrane porin-dependent diffusion of FOX into the periplasmic space, since the activities of other cephems were not affected in Δ*surA*. Other, unknown factors, possibly related to SurA chaperone functions, could be responsible for the increased MIC of FOX in this gene deletion mutant.

In conclusion, this study identified two potential cephem helper drug target candidates, DapF and PBP1b. Cephem activity was potentiated by inactivation of the genes encoding these proteins, although full reversal of resistance was obtained only for selected drugs and was generally influenced by the strain's genetic background and the β-lactamase type, which may be regarded as undesirable features for a novel helper drug. The only exception was *dapF* inactivation, which synergistically potentiated FOX antimicrobial activity regardless of the strain's genetic background and β-lactamase type. The reduced fitness of the Δ*dapF* mutant can be regarded as a desirable feature, since drugs targeting DapF are likely to display their own antimicrobial activity, in addition to synergizing with cephems. Moreover, the high homology among *Enterobacteriaceae* and low similarity with human orthologues are also favorable features for the helper drug target potential of DapF. On the basis of these *in vitro* findings, more research is needed to assess the possible use of the protein as a target for the discovery of helper drugs that enhance FOX activity when used in combination.

MATERIALS AND METHODS

Antimicrobial agents and media. All the antimicrobial agents and growth media used in this study were purchased from Sigma-Aldrich (Denmark) and Becton Dickinson (USA), respectively. Bacteria were routinely grown on Luria-Bertani agar (LA) and in Luria-Bertani broth (LB). Susceptibility testing and growth curve analysis were performed in cation-adjusted Mueller-Hinton Broth II (MHB II). SOC tryptone-based medium (30) was used to enhance the recovery of transformants after electroporation. PCR primers were synthesized by TAG (Copenhagen, Denmark).

Bacterial isolates and plasmids. Sixteen Keio mutants (31) and the wild-type *E. coli* K-12 (BW25113) were purchased from the Coli Genetic Stock Center, Yale University (http://cgsc.biology.yale.edu/). These mutants were selected based on the findings of two previous studies using the Keio collection (12, 13). Our selection included (i) mutants that had reduced fitness (3-fold or greater) in the presence of different cephems, such as cefaclor, cefoxitin, cefsulodin, and ceftazidime (Δ*mrcB*, Δ*dacA*, Δ*ycfM*, Δ*ybgF*, Δ*dapF*, Δ*fis*, Δ*rplA*, and Δ*bamB*) (13), and (ii) mutants that had increased susceptibility to 8 or more antimicrobial agents, including at least one cephem (Δ*ybgF*, Δ*dapF*, Δ*ycjU*, Δ*rimK*, Δ*rplA*, Δ*dksA*, Δ*surA*, Δ*tolC*, Δ*acrA*, Δ*recA*, and Δ*yciM*) (12). Donor strains carrying IncI1 plasmids encoding different β-lactamases (CMY-2 and CTX-M-1) were used for the conjugation experiments (32, 33) (Table 5). A representative of the epidemic *E. coli* ST131 clone harboring $bla_{CTX\text{-}M\text{-}15}$ on an IncF plasmid (strain UR40) (34) was used for genetic engineering.

Transfer of β-lactamase-encoding plasmids into Keio mutants. The well-characterized IncI1 CMY-2-encoding plasmid from *E. coli* R7AC (32, 35) was transferred individually into each selected Keio mutant and the wild-type strain by conjugation and transformation, respectively. A second IncI1 plasmid encoding CTX-M-1 (Table 5) was transferred by conjugation into two selected mutants, Δ*mrcB* and Δ*dapF*. Briefly, 500 μl of overnight donor and recipient cultures was centrifuged, resuspended in 50 μl LB, and incubated at 37°C. After 90 min of mating, the suspension was streaked on a selective LA plate containing 25 μg/ml kanamycin (Kan) and 1 μg/ml CTX. Due to the lack of a marker for counterselection, the same plasmids could not be introduced into the Keio wild-type strain BW25113 by conjugation and thus were transformed by electroporation according to a standard protocol (30). The wild-type transformants obtained were used as a reference to assess the effects of each gene deletion in the Keio mutant transconjugants containing the same plasmid.

Targeted gene deletions in CTX-M-15-producing ST131. Two genes, *mrcB* and *dapF*, were deleted individually in UR40 (ST131) using the λ-red recombination system (36). Briefly, a FLP recombination target (FRT)-flanked chloramphenicol resistance cassette was amplified from a template plasmid, pKD3, using primer pairs tagged with sequences containing 39 bases up- or downstream of the target gene. As the UR40 strain was resistant to ampicillin (Amp) and Kan, a tetracycline resistance (Tet^r) marker was cloned into the original Amp resistance (Amp^r) marker of the temperature-sensitive helper plasmid, pKD46, carrying λ-red recombinase genes γ, β, and *exo* under control of an arabinose inducible promoter, *araC*-P_{araB}. The Tet^r marker was PCR amplified from pBR322 using primers containing 5′ XmnI digestion sites and digested with XmnI (Thermo, USA). In parallel, pKD46 was digested with XmnI and subsequently dephosphorylated with alkaline phosphatase (Thermo, USA). Finally, the two fractions were ligated using T4 DNA ligase (Thermo, USA), resulting in the

TABLE 5 Bacterial strains and plasmids used in this study

Strain	Plasmid	β-Lactamase enzyme	Origin	Relevant genotype or features[a]	Reference
BW25113			Laboratory strain, K-12 derivative	F^- Δ(*araD-araB*)567 Δ*lacZ*4787(::*rrnB*-3) λ^- *rph*-1 Δ(*rhaDrhaB*)568 *hsdR*514	31
R7AC	IncI1	CMY-2	Dog feces	ST297	32
UR40	IncF	CTX-M-15	Human urine	ST131	34
2007-10-2348-1	IncI1	CTX-M-1	Cattle feces	ST49	33
JW0145-1				BW25113 Δ*mrcB*::Kan	31
JW0627-1				BW25113 Δ*dacA*::Kan	31
JW5157-1				BW25113 Δ*ycfM*::Kan	31
JW0732-1				BW25113 Δ*ybgF*::Kan	31
JW5592-1				BW25113 Δ*dapF*::Kan	31
JW1310-1				BW25113 Δ*ycjU*::Kan	31
JW0141-1				BW25113 Δ*dksA*::Kan	31
JW2496-3				BW25113 Δ*bamB*::Kan	31
JW0052-1				BW25113 Δ*surA*::Kan	31
JW5503-1				BW25113 Δ*tolC*::Kan	31
JW0452-3				BW25113 Δ*acrA*::Kan	31
JW2669-1				BW25113 Δ*recA*::Kan	31
JW0836-1				BW25113 Δ*rimK*::Kan	31
JW3947-1				BW25113 Δ*rplA*::Kan	31
JW1272-3				BW25113 Δ*yciM*::Kan	31
JW3229-1				BW25113 Δ*fis*::Kan	31
	pKD46-Amp^r		Synthetic plasmid	λ-red genes (γ, β, and *exo*) and *araC*-P_{araB}; Amp^r	36
	pKD46-Tet^r		Synthetic plasmid	pKD46-Amp^r derivative; Tet^r	This study
UR40 Δ*dapF*	IncF	CTX-M-15		UR40 (ST131) *dapF*::Cam	This study
UR40 Δ*mrcB*	IncF	CTX-M-15		UR40 (ST131) *mrcB*::Cam	This study

[a]Amp, ampicillin; Kan, kanamycin; Cam, chloramphenicol.

construction of pKD46 with the Tet^r resistance marker. The newly constructed pKD46-Tet^r plasmid was transformed into the UR40 strain, and recombinase proteins were induced by adding arabinose to the culture. Subsequently, the induced culture was used to prepare electrocompetent cells following a standard protocol (30), and the amplified FRT-flanked resistance cassette was electroporated into the competent cells, followed by recovery in SOC medium and selection on LA plates supplemented with 24 μg/ml chloramphenicol. Finally, gene deletions were confirmed by PCR amplification and sequencing (Macrogen Inc.). Primer sequences are available upon request.

Antimicrobial susceptibility testing. MICs of FOX, CTX, CAZ, CRO, and FEP were determined by broth microdilution using two different types of commercial 96-well plates (ESB1F and COMPAN1F; Trek Diagnostic Systems Inc.) according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (37). Each MIC value was reported as the average of at least two biological replicates. Complete reversal of antimicrobial resistance was defined as a reduction of the MIC to equal to or below the CLSI susceptibility breakpoint. Reduction of the MIC to the intermediate category was categorized as partial reversal of resistance. To enable quantitative assessment of antibiotic–gene-deletion interaction, the fractional inhibitory concentration index calculation (38) was modified to calculate the interaction index, defined as follows: antibiotic MIC_{mutant}/antibiotic $MIC_{wild\ type}$. A stringent interpretation of interaction index values of ≤0.25 and ≥4 was used to indicate synergistic and antagonistic interaction, respectively. Values from >0.5 to <4 were considered "no interaction," similar to the standard designations of fractional inhibitory concentration index values (39). In addition, the synergistic and antagonistic designations also correspond to a 4-fold minimum MIC decrease or increase, respectively, consistent with interpretations in standard checkerboard assays (38).

Growth curve analyses. Growth curve analyses were performed using BioScreen (Oy Growth Curves Ab Ltd., Finland). Broth microcultures of the mutants and the wild type in MHB II were distributed into a 100-well plate with or without 2 μg/ml FOX and incubated in a BioScreen chamber for 18 to 24 h at 37°C under continuous shaking. The FOX concentration, 4-fold below the MIC of the wild-type UR40 strain, was chosen based on the interaction index for synergy, 0.25. The optical density at 600 nm (OD_{600}) of each microculture was measured and recorded every 15 min, after a standstill of 5 s in automatic mode. The recorded ODs were transferred to an Excel file and subsequently used to plot growth curves.

***In silico* study of protein homology.** The amino acid sequences of *E. coli* MrcB and DapF were downloaded in FASTA format from Uniprot Web resources (http://www.uniprot.org) (uniprot entries P02919 [MrcB] and P0A6K1 [DapF]) and were aligned with those of other *Enterobacteriaceae* (taxonomy identification number [taxid], 91347) and human (taxid, 9606) homologues using protein BLAST on the website of the National Center for Biotechnology Information (NCBI). Except for expansion of the maximum target sequences, default BLAST parameters were used, with an expected cutoff value of 10. The accession numbers used for the protein sequences were WP_016529988.1 (MrcB) and ACI11814.1 (DapF) for *K. pneumoniae*, WP_000918132.1 (MrcB) and WP_001160672.1 (DapF) for *S. enterica*, EIQ27511.1 (MrcB) and EIQ17403.1 (DapF) for *S. flexneri*, and CAA90232.1 (MrcB) and WP_038634512.1 (DapF) for *C. freundii*.

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