



OmpF Downregulation Mediated by Sigma E or OmpR Activation Confers Cefalexin Resistance in *Escherichia coli* in the Absence of Acquired β -Lactamases

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ABSTRACT Cefalexin is a widely used first-generation cephalosporin, and resistance in *Escherichia coli* is caused by extended-spectrum (e.g., CTX-M) and AmpC β -lactamase production and therefore frequently coincides with third-generation cephalosporin resistance. However, we have recently identified large numbers of *E. coli* isolates from human infections, and from cattle, where cefalexin resistance is not β -lactamase mediated. Here, we show, by studying laboratory-selected mutants, clinical isolates, and isolates from cattle, that OmpF porin disruption or downregulation is a major cause of cefalexin resistance in *E. coli*. Importantly, we identify multiple regulatory mutations that cause OmpF downregulation. In addition to mutation of *ompR*, already known to downregulate OmpF and OmpC porin production, we find that *rseA* mutation, which strongly activates the sigma E regulon, greatly increases DegP production, which degrades OmpF, OmpC, and OmpA. Furthermore, we reveal that mutations affecting lipopolysaccharide structure, exemplified by the loss of GmhB, essential for lipopolysaccharide heptosylation, also modestly activate DegP production, resulting in OmpF degradation. Remarkably, given the critical importance attached to such systems for normal *E. coli* physiology, we find evidence for DegP-mediated OmpF downregulation and *gmhB* and *rseA* loss-of-function mutation in *E. coli* isolates derived from human infections. Finally, we show that these regulatory mutations enhance the ability of group 1 CTX-M β -lactamase to confer reduced carbapenem susceptibility, particularly those mutations that cause OmpC in addition to OmpF downregulation.

KEYWORDS lipopolysaccharide, porins, sigma factors

Cefalexin is a first-generation cephalosporin widely used in human, companion, and farmed animal medicine. In 2016, in Bristol, United Kingdom, and surrounding regions (a population of 1.5 million people), 27.6 cefalexin courses were dispensed per 1,000 patient population (2.8% of all dispensed items). While dispensing rates had dropped by 19.5% since 2013, the proportion of *Escherichia coli* from community-origin urine samples resistant to cefalexin in this region rose from 7.06% to 8.82% (1).

Cefalexin resistance in *E. coli* is caused by hyperproduction of the chromosomally encoded class 1 cephalosporinase gene *ampC*, acquisition of plasmid AmpC (pAmpC), or extended-spectrum β -lactamases (ESBLs). These are also mechanisms of third-generation cephalosporin resistance (3GC-R). We recently reported that among community-origin urinary *E. coli* from Bristol and surrounding regions collected in 2017 and 2018, 69% of cefalexin-resistant isolates were 3GC-R, suggesting that cefalexin resistance in the absence of ESBL/AmpC production is common (2). A similar observation was made when analyzing fecal samples from dairy cattle in the same region, where only 30% of samples containing cefalexin-resistant *E. coli* yielded 3GC-R isolates (3).

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TABLE 1 MICs ($\mu\text{g}\cdot\text{ml}^{-1}$) of cefalexin against *E. coli* isolates and mutant derivatives^b

Isolate or mutant	Cefalexin MIC ($\mu\text{g}\cdot\text{ml}^{-1}$)
PSA	16
PSA (M) ^a	32
ATCC 25922	16
ATCC 25922 (M)	32
EC17	8
EC17 (M)	32
ATCC 25922 <i>ompF</i>	32
ATCC 25922 <i>rseA</i>	64
ATCC 25922 <i>gmhB</i>	64
Farm 1	64
Farm 2	64
UTI-1	32
UTI-2	32

^aM, mutants selected for growth on cefalexin.

^bShaded values represent resistant based on CLSI breakpoints (24); otherwise, nonshaded values represent susceptible.

Hyperproduction of commonly acquired penicillinases such as TEM-1 and OXA-1 does not confer cefalexin resistance in *E. coli* (4). Furthermore, the involvement of efflux pump overproduction, e.g., AcrAB-TolC in *E. coli*, has not been reported, but OmpF porin loss is known to reduce cefalexin susceptibility (5). Indeed, early work showed cefalexin more efficiently uses OmpF than OmpC porin to enter *E. coli* (6).

One aim of the work reported here was to characterize cefalexin resistance mechanisms in *E. coli* lacking acquired β -lactamases by studying resistant mutants selected *in vitro*. A second aim was to characterize mechanisms of cefalexin resistance seen in 3GC-susceptible (3GC-S) human urinary and cattle isolates from our earlier surveillance studies (2, 3). A third aim was to determine if the cefalexin resistance mechanisms identified here enhance CTX-M-mediated β -lactam resistance.

RESULTS AND DISCUSSION

Cefalexin resistance in *E. coli* is associated with OmpF/OmpC porin downregulation due to *ompR* mutation. One spontaneous cefalexin-resistant mutant was selected from each of three *E. coli* parent strains, EC17, ATCC 25922, and PSA. Cefalexin MICs against these isolates and their mutant derivatives are reported in Table 1. In each case, to identify the possible cause of cefalexin resistance, liquid chromatography-tandem mass spectrometry (LC-MS/MS) whole-cell proteomics was performed comparing each mutant with its parent. No mutant overproduced the chromosomally encoded AmpC β -lactamase (Tables S1 to S3 in the supplemental material), and no promoter/attenuator sequence mutations upstream of *ampC* were identified in any of the mutants, based on whole-genome sequencing (WGS) (Fig. S1). The only significant ($P < 0.05$; >2-fold) protein abundance change common to all three wild-type/mutant pairs was downregulation of OmpF porin production (Table 2; Tables S1 to S3). There was no evidence of AcrAB-TolC efflux pump overproduction in the proteomics data for mutants

TABLE 2 LC-MS/MS proteomic comparisons of porin proteins, OmpA, and DegP abundance in *E. coli* isolates versus cefalexin-resistant mutant derivatives^a

Isolate or mutant	OmpF			OmpC			OmpA			DegP		
	Mean	SEM	<i>P</i> (WT/M)	Mean	SEM	<i>P</i> (WT/M)	Mean	SEM	<i>P</i> (WT/M)	Mean	SEM	<i>P</i> (WT/M)
PSA	0.23	0.05		5.93	1.00		3.24	0.32		0.06	0.01	
PSA (M)	0.00	0.00	0.005	0.17	0.04	0.002	3.69	0.74	>0.25	0.03	0.01	0.03
ATCC 25922	1.69	0.10		1.66	0.26		5.55	0.41		0.08	0.01	
ATCC 25922 (M)	0.29	0.14	0.0006	0.24	0.05	0.003	1.00	0.17	0.0003	0.54	0.10	0.005
EC17	1.15	0.15		2.49	0.50		3.79	0.29		0.17	0.03	
EC17 (M)	0.39	0.16	0.01	2.25	0.66	>0.25	4.47	0.78	>0.25	0.74	0.10	0.003

^aAbundance relative to total ribosomal proteins is presented for each protein; $n = 3$ biological replicates for each wild-type/mutant pair (WT/M). Raw data are presented in Tables S1 to S3 in the supplemental material.

(Tables S1 to S3). Despite OmpF porin downregulation, comparison of *ompF*-containing WGS contigs from wild-type/mutant pairs revealed no mutations in *ompF* or within 10 kb up- or downstream. We therefore concluded that there is a *trans*-regulatory mutation affecting OmpF abundance in each mutant.

Since the two-component system OmpR/EnvZ is known to control porin gene transcription in *E. coli* (7), we searched among WGS data for mutations in the genes encoding this regulator, and a mutation was found in *ompR* in the cefalexin-resistant derivative of isolate PSA, predicted to cause a Gly63Ser change in OmpR. A Gly63Val substitution in OmpR has previously been shown to cause OmpF and OmpC porin downregulation in *E. coli* (8), and proteomics confirmed that OmpC was also downregulated in the PSA-derived cefalexin-resistant mutant relative to PSA, but another major outer membrane protein OmpA was not (Table 2). Accordingly, we conclude that OmpR mutation explains cefalexin resistance due to OmpF (and possibly OmpC) downregulation in the mutant derivative of isolate PSA. However, *ompR* and *envZ* were found to be wild type in the other two cefalexin-resistant mutants, suggesting alternative regulatory mutations.

DegP overproduction due to RseA anti-sigma E mutation is associated with OmpF porin downregulation and cefalexin resistance in *E. coli*. Eight proteins, including OmpF, were significantly differentially regulated in the same direction in the cefalexin-resistant mutants derived from isolates EC17 and ATCC 25922, each relative to their parent strain. Three proteins (BamD, DegP, and YgiM) were upregulated, and five (NmpC, DctA, ArcA, OmpF, and YhiL) were downregulated (Tables S1 and S2). We were interested to note that one upregulated protein in both mutants was DegP (Table 2), which is a protease known to degrade porin proteins (9, 10). Interestingly, in the PSA-derived *ompR* mutant with downregulated OmpF and OmpC described above, DegP production was 2-fold lower than in the wild-type parent, suggesting a feedback response to porin downregulation (Table 2). DegP production was increased 7-fold in the ATCC 25922-derived mutant, and OmpF was downregulated 5.9-fold, as were OmpC (6.7-fold) and OmpA (5.6-fold) (Table 2), which is a typical sigma E response (11). In the EC17-derived mutant, DegP was upregulated a more modest 4.4-fold, and here, OmpF was downregulated 2.9-fold, but OmpC and OmpA were not significantly downregulated ($P > 0.25$), suggesting a weaker sigma E response (Table 2). This led to the suggestion that OmpC downregulation, seen in the PSA-derived and ATCC 25922-derived cefalexin-resistant mutants alongside OmpF downregulation (Table 2), is not necessary for cefalexin resistance. To confirm this, we disrupted *ompF* in ATCC 25922 and found this to be sufficient for cefalexin resistance (Table 1). Additional downregulation of OmpC is not necessary.

Analysis of WGS data identified that the ATCC 25922-derived mutant expressing a phenotype typical of a strong sigma E response had a mutation predicted to cause a Trp33Arg mutation in RseA, which is a known sigma E anti-sigma factor (12, 13). Loss of RseA is expected to release sigma E so that it can bind, among others, to the *degP* promoter. This increases *degP* transcription, leading to porin degradation and cefalexin resistance (11). We disrupted *rseA* in ATCC 25922 and confirmed that this mutation does cause cefalexin resistance (Table 1).

Perturbation of lipopolysaccharide heptosylation due to *gmhB* mutation causes cefalexin resistance in *E. coli*. The EC17-derived cefalexin-resistant mutant, which also appears to have a sigma E response, though weaker than the *rseA* mutant, was shown through WGS analysis to have a deoxythymidine nucleotide insertion after nucleotide 348 of *gmhB*, predicted to cause a frameshift affecting the encoded protein beyond amino acid 117. This gene encodes the enzyme D-alpha,beta-D-heptose-1,7-bisphosphate phosphatase, which is part of a pathway responsible for producing heptose for lipopolysaccharide (LPS) biosynthesis (14). Loss of enzymes involved in this system is associated with increased outer membrane permeability, but interestingly, deletion of *gmhB* does not disrupt full-length LPS production or damagingly compromise the outer membrane permeability barrier (14, 15). The obvious conclusion is that this perturbation in envelope structure activates the sigma E regulon, resulting in OmpF degradation by DegP. We disrupted *gmhB* in ATCC 25922 and found that this mutation

causes cefalexin resistance (Table 1). In the ATCC 25922 background, the *rseA* and *gmhB* mutants were similar, in MIC terms, to the *ompF* mutant (Table 1). This further supports the conclusion that despite other porin production changes caused by *rseA* mutation and *ompR* mutation, as identified above, it is OmpF downregulation that is causing the cefalexin resistance phenotype observed in these three *in vitro*-selected mutants.

Loss and downregulation of OmpF in cefalexin-resistant *E. coli* from cattle and humans and evidence for *rseA* and *gmhB* mutations in human clinical isolates. We chose two cefalexin-resistant but 3GC-S isolates at random from our previous survey of dairy farms (3) and two from our previous survey of human urinary *E. coli* (2). Cefalexin resistance was confirmed by MIC (Table 1). WGS revealed disruption of *ompF* in both farm isolates: in farm 1, a Tn5 insertion disrupted *ompF*, truncating OmpF after amino acid 316. In farm 2, a frameshift mutation disrupted OmpF after amino acid 96.

The *ompF* gene was intact in both human urinary isolates, which were identified by WGS as being sequence type 131 (ST131). Proteomics did, however, show significant ($P < 0.05$) downregulation of OmpF abundance relative to ribosomal proteins compared with the control human isolate EC17 (1.43 ± 0.16 [mean \pm SEM]; $n = 3$) and a very closely phylogenetically related control ST131 urinary isolate, collected in parallel (3), UTI-80710 (1.15 ± 0.09 ; $n = 3$), in both cefalexin-resistant urinary isolates. In UTI-1, OmpF downregulation was ~ 2 -fold relative to both controls (OmpF abundance, 0.70 ± 0.12 ; $n = 3$), but in UTI-2, OmpF was ~ 10 -fold downregulated relative to both controls (OmpF abundance, 0.13 ± 0.03 ; $n = 3$). Notably, UTI-1 also had a nonsense mutation at codon 82 in *ompC*. As expected, therefore, OmpC was undetectable by proteomics in UTI-1, but OmpC abundance relative to ribosomal proteins in UTI-2 (2.64 ± 0.84 ; $n = 3$) was not significantly lower ($P > 0.25$) than in control isolates UTI-80710 (3.14 ± 0.31 ; $n = 3$) and EC17 (2.55 ± 0.61 ; $n = 3$). Most interestingly, UTI-2 produced > 2 -fold elevated ($P < 0.05$) levels of DegP (abundance relative to ribosomal proteins, 0.42 ± 0.04 ; $n = 3$) compared with control isolates EC17 (0.20 ± 0.04 ; $n = 3$) and UTI-80710 (0.13 ± 0.02 ; $n = 3$), suggestive of a phenotype like that of the *gmhB* mutant described above. UTI-1 did not produce DegP at levels significantly different from control ($P > 0.25$).

According to WGS, ST131 isolate UTI-2 did not have a mutation in *gmhB*, *rseA*, *ompR*, or *ompF* relative to the ST131 control isolate UTI-80710. Therefore, the regulatory mutation leading to elevated DegP levels, reduced OmpF levels, and cefalexin resistance in UTI-2 has not been identified. However, given the complexity of sigma E activation signals and the impact that many different changes in envelope structure can have on it (16), it is possible that clinical isolates do carry mutations that activate this regulon. Indeed, searches of the NCBI database identified carbapenem-resistant human *E. coli* isolate E300, identified in Japan (17), which has an 8-nucleotide (nt) insertion, leading to a frameshift in *rseA* at nucleotide 34 (GenBank accession number [AP022360](#)). Furthermore, two human clinical isolates were found to have a single nucleotide insertion leading to a frameshift in *gmhB* after nucleotide 126, one from China (GenBank accession number [CP008697](#)) and one from the United States (GenBank accession number [CP072911](#)), and three commensal *E. coli* from the United States (18) were found to have frameshift mutations at various positions in *gmhB* (GenBank accession numbers [CP051692](#), [CP054319](#), and [CP054319](#)). Accordingly, we conclude that mutations likely to cause the same phenotypes found in our laboratory-selected cefalexin-resistant mutants are also found in clinical and commensal *E. coli* from across the world.

Influence of *ompF* porin loss and downregulation on late-generation cephalosporin and carbapenem susceptibility in the presence of various CTX-M β -lactamases. Our final aim was to test the impact of *ompF* loss and downregulation, due to OmpR mutation or activation of sigma E, on late-generation cephalosporin or carbapenem MIC in *E. coli* producing CTX-M β -lactamases. To do this, we introduced, using conjugation, natural plasmids carrying various *bla*_{CTX-M} variants commonly identified in human and cattle 3GC-R *E. coli* in southwest England, which encoded CTX-M-1, CTX-M-14, and

TABLE 3 Influence of *ompF*, *rseA*, and *ompR* mutations on late-generation cephalosporin and carbapenem MICs against *E. coli* producing CTX-M variants^a

Strain name (CTX-M variant)	MIC ($\mu\text{g}\cdot\text{ml}^{-1}$) of:				
	Cefepime	Cefquinome	Ceftazidime	Ceftiofur	Ertapenem
ATCC 25922(pK18)	0.25	0.125	0.25	0.25	0.016
ATCC 25922(pK18) (CTX-M-1)	>128	>128	16	>128	0.0625
ATCC 25922(pK18) (CTX-M-15)	>128	>128	32	>128	0.125
ATCC 25922(pK18) (CTX-M-14)	64	>128	2	>128	0.0313
ATCC 25922(pK18) <i>ompF</i>	0.25	0.125	0.5	0.5	0.016
ATCC 25922(pK18) <i>ompF</i> (CTX-M-1)	>128	>128	32	>128	0.25
ATCC 25922(pK18) <i>ompF</i> (CTX-M-15)	>128	>128	64	>128	0.5
ATCC 25922(pK18) <i>ompF</i> (CTX-M-14)	>128	>128	4	>128	0.0313
ATCC 25922(pK18) <i>rseA</i>	0.25	0.25	0.5	0.5	0.0313
ATCC 25922(pK18) <i>rseA</i> (CTX-M-1)	>128	>128	16	>128	0.5
ATCC 25922(pK18) <i>rseA</i> (CTX-M-15)	>128	>128	32	>128	0.5
ATCC 25922(pK18) <i>rseA</i> (CTX-M-14)	>128	>128	4	>128	0.125
PSA	0.125	0.125	0.5	0.25	0.016
PSA CTX-M-1	>128	>128	32	>128	0.125
PSA CTX-M-15	>128	>128	32	>128	0.25
PSA CTX-M-14	128	>128	8	>128	0.0313
PSA (M) (<i>ompR</i>)	0.125	0.25	0.5	0.5	0.0625
PSA (M) (<i>ompR</i>) (CTX-M-1)	>128	>128	16	>128	1
PSA (M) (<i>ompR</i>) (CTX-M-15)	>128	>128	16	>128	1
PSA (M) (<i>ompR</i>) (CTX-M-14)	>128	>128	4	>128	0.5

^aCTM-M variants were delivered on natural plasmids by conjugation. Plasmid pK18 (33) was added to provide a marker (kanamycin resistance) to allow selection for recipients in conjugation. Isolate PSA is fluoroquinolone resistant, so this was not necessary. Shading is "nonsusceptible" (resistant or intermediate) according to CLSI breakpoints (24).

CTX-M-15 (2, 19). We measured MICs of 3GCs and 4GCs used in humans (ceftazidime, cefepime) or cattle (ceftiofur, cefquinome), and the carbapenem ertapenem against CTX-M plasmid transconjugants of *E. coli* parent strains and their *ompF*, *rseA*, or *ompR* mutant derivatives (Table 3).

In wild-type ATCC 25922, as expected, CTX-M-1 and CTX-M-15 conferred resistance to all four cephalosporins tested, CTX-M-14 did not confer ceftazidime resistance, and none of the enzymes conferred ertapenem resistance. Disruption of *ompF* or *rseA* did not change the susceptibility profile, but there were some MIC changes. Disruption of *ompF* caused a fourfold increase in ertapenem MIC against transconjugants producing CTX-M-1 and CTX-M-15, but there was no change in MIC against the CTX-M-14 transconjugant. Disruption of *rseA* caused a similar impact on ertapenem MIC against CTX-M-1 or CTX-M-15 producers but additionally caused a fourfold increase in MIC against the CTX-M-14 producer. It is likely that this effect is due to the additional downregulation in OmpC seen in the *rseA* mutant (Table 2), with OmpC being a key carbapenem porin (20).

Resistance profiles seen in wild-type *E. coli* isolate PSA CTX-M plasmid transconjugants were almost identical to transconjugants of isolate ATCC 25922 (Table 3). However, carriage of plasmids encoding CTX-M-1 or CTX-M-15 conferred ertapenem nonsusceptibility in the PSA *ompR* mutant, the ertapenem MIC being twofold higher than against CTX-M-1 or CTX-M-15 transconjugants of the ATCC 25922 *rseA* mutant derivative (Table 3). The greater impact of *ompR* mutation than *rseA* mutation on reducing OmpC levels (Table 2) likely explains this difference. Indeed, *ompR* mutation has previously been associated with ertapenem nonsusceptibility in ESBL-producing *E. coli* (8, 20).

Conclusions. Cefalexin is a widely used antibacterial in human and veterinary medicine, and so, cefalexin resistance is of considerable clinical importance. Despite this, mechanisms of resistance have not been given very much attention, particularly in the postgenomic age. We were surprised to find that, in our recent surveys of human and cattle cefalexin-resistant isolates, acquired cephalosporinase (pAmpC or ESBL) or chromosomal AmpC hyperproduction was not the cause of cefalexin resistance in a large proportion

of isolates (2, 3). We show here strong evidence that OmpF loss or downregulation is a key mechanism of cefalexin resistance in *E. coli* in the absence of β -lactamase production. While OmpF loss contributes to resistance to a wide range of antibacterials (5), our findings show that cefalexin resistance is unusual in being caused solely by OmpF loss. Furthermore, we show that OmpF downregulation can also confer this phenotype. This may explain why *ompF* loss-of-function mutations are found among *E. coli* from clinical samples, but our work suggests there may also be numerous different regulatory mutations found among clinical isolates, each downregulating OmpF. It has been previously reported that cefalexin diffuses better through OmpF than OmpC (6), and recent general rules of porin permeation show that β -lactams with a relatively high negative charge, as is true of cefalexin, permeate slowly through porins, meaning that entry favors OmpF, with its larger and less cation-selective pore (21).

Such is the wide range of regulatory systems controlling OmpF production, both at transcriptional, translational, and posttranslational levels (7, 16, 22), that it is not surprising that cefalexin resistance mutations arise in many different genes in the laboratory, as seen here. Importantly, these mutations may contribute to resistance to other antibacterials when partnered with other mechanisms. Indeed, some of these mutations also affect OmpC levels, and if this is a sufficiently large effect (e.g., in the *ompR* mutant identified here) it can cause carbapenem nonsusceptibility, provided the mutant acquires a common ESBL such as CTX-M-15. Similar *ompR* mutants have been seen in the clinic (8). This is because, as shown previously (21), ertapenem has a fast permeation rate through porins, meaning that, unlike cefalexin, it permeates well through both OmpF and OmpC (21). The other regulatory mutations affecting OmpF levels found in the laboratory-selected mutants reported here work through sigma E-mediated DegP overproduction. It is well-known that DegP degrades porins (10, 16), but it has not previously been reported that DegP-mediated degradation of OmpF is sufficient to cause resistance to any antibacterial drug.

Our findings are also potentially important because they suggest that OmpF is more susceptible to DegP-mediated proteolysis *in vivo* in *E. coli* than the other main porin, OmpC, and the abundant outer membrane protein OmpA. In the *rseA* mutant with “maximal” sigma E activation and DegP upregulation, OmpF, OmpC, and OmpA levels all fell. In contrast, in the *gmhB* mutant overproducing DegP, to a lesser extent than the *rseA* mutant, only OmpF levels significantly fell (Table 2). This was still sufficient to cause cefalexin resistance but had a smaller effect on ertapenem MIC in the presence of CTX-M-15 production (Table 3), likely due to the OmpF-specific effect on porin downregulation since, as mentioned above, ertapenem can permeate well through OmpC (21).

It is known that mutations affecting outer membrane and lipopolysaccharide structure activate sigma E because they affect envelope integrity. They can also affect the insertion and stability of porins in the outer membrane (16). It has not previously been shown, however, that mutations disrupting *gmhB* can activate sigma E and, further, that this can cause cefalexin resistance. We considered that, despite such mutations arising in the laboratory, this perhaps overstates their clinical relevance because disruption of the Gmh system causes significant attenuation and increased susceptibility to envelope stresses, though, significantly, loss of GmhB has the mildest effect in this regard (15). Accordingly, we were very interested to find cefalexin-resistant human urinary ST131 isolates having OmpF downregulation, and in one case, DegP upregulation, suggestive of a GmhB-negative phenotype, though *gmhB* was intact, and the nature of the mutation responsible will be the focus of future work. However, importantly, we did find clear evidence of *rseA* and *gmhB* loss-of-function mutations among clinical and commensal *E. coli* from secondary analysis of WGS data. Therefore, we provide here strong evidence that mutations constitutively activating sigma E, including those which do this by altering lipopolysaccharide structure, can be tolerated by *E. coli* in a clinical setting. These mutations, and possibly others yet to be identified, cause

clinically relevant cefalexin resistance in the absence of β -lactamase production through DegP-mediated OmpF proteolysis.

MATERIALS AND METHODS

Bacterial isolates, selection of resistant mutants, and susceptibility testing. Three β -lactam-susceptible *E. coli* isolates were used, the type strain ATCC 25922; the human urinary isolate EC17, provided by Mandy Wootton, Public Health Wales; and a ciprofloxacin-resistant isolate, PSA, from fecal samples collected on a dairy farm (3). To select cefalexin-resistant derivatives of these isolates, 100 μ l of overnight culture grown in nutrient broth was spread onto Mueller-Hinton agar containing 16 μ g·ml⁻¹ cefalexin and each plate incubated for 24 h. In addition, four cefalexin-resistant but 3GC-S isolates were used, one each from fecal samples from two dairy farms (farm 1 and farm 2) as collected previously (3) and two human urinary isolates (UTI-1, UTI-2), also as collected previously (2). The control isolate UTI-80710 is 3GC-R due to CTX-M-15 production (2) and was selected based on its production of wild-type OmpF porin levels (see Results and Discussion above). Microtiter MIC assays were performed and interpreted according to CLSI guidelines (23, 24).

Proteomics. One milliliter of an overnight cation-adjusted Mueller-Hinton Broth (CA-MHB) culture was transferred to 50 ml CA-MHB, and cells were grown at 37°C to an optical density at 600 nm (OD₆₀₀) of 0.6 to 0.8. Cells were pelleted by centrifugation (10 min, 4,000 \times g, 4°C) and resuspended in 35 ml of 30 mM Tris-HCl, pH 8, and broken by sonication using a cycle of 1 s on, 0.5 s off for 3 min at amplitude of 63% using a Sonics Vibracell VC-505 (Sonics and Materials Inc., Newton, CT, USA). The sonicated samples were centrifuged at 8,000 \times g for 15 min at 4°C to pellet intact cells and large cell debris. Protein concentrations in all supernatants were quantified using the Bio-Rad protein assay dye reagent concentrate according to the manufacturer's instructions. Proteins (1 μ g/lane) were separated by SDS-PAGE using 11% acrylamide, 0.5% bis-acrylamide (Bio-Rad) gels, and a Bio-Rad Mini-Protean Tetra Cell chamber. Gels were resolved at 200 V until the dye front had moved approximately 1 cm into the separating gel. Proteins in all gels were stained with Instant Blue (Expedeon) for 5 min and destained in water. LC-MS/MS data were collected as previously described (25). The raw data files were processed and quantified using Proteome Discoverer software v1.4 (Thermo Scientific) and searched against bacterial genome and horizontally acquired resistance genes as described previously (26).

Whole-genome sequencing and analyses. WGS was performed by MicrobesNG on a HiSeq 2500 instrument (Illumina, San Diego, CA, USA) using 2 \times 250-bp paired-end reads. Reads were trimmed using Trimmomatic (27) and assembled into contigs using SPAdes 3.13.0 (28). Contigs were annotated using Prokka 1.2 (29). Resistance genes and sequence types, according to the Achtman scheme (30), were assigned using the ResFinder (31) and MLST 2.0 on the Center for Genomic Epidemiology (<http://www.genomicepidemiology.org/>) platform. Pairwise contig alignments to identify mutations versus parent isolate, which were sequenced in parallel, were with EMBOSS Stretcher (https://www.ebi.ac.uk/Tools/psa/emboss_stretcher/).

Insertional inactivation of genes and conjugation of CTX-M-encoding plasmids. Insertional inactivation of *ompF*, *rseA*, or *gmbB* was performed using the pKNOCK suicide plasmid (32). DNA fragments were amplified with Phusion High-Fidelity DNA polymerase (NEB, UK) from *E. coli* ATCC 25922 genomic DNA by using primers *ompF*-KO-FW (5'-CAAGGATCCTGATGGCCTGAACCTC-3') with a BamHI restriction site, underlined, and *ompF*-KO-RV (5'-CAAGTTCGACTTCAGACCAGTAGCC-3') with a Sall site; *rseA*-KO-FW (5'-CGCGGATCC TGCAGAAAACAGGGAAAGC-3') with a BamHI site and *rseA*-KO-RV (5'-TGCACTGCAGCCATTGGGTAAGCTG TGCC-3') with a PstI site; *gmbB*-KO-FW (5'-TATACTAGTCACGGCTATGTCATGAGA-3') with a SpeI site; and *gmbB*-KO-RV (5'-TATGTCGACTCGGTACGGCTTCAAAC-3') with a Sall site. All PCR products were ligated into pKNOCK-GM (32) at the BamHI and Sall (for *ompF*), BamHI and PstI (for *rseA*), or SpeI and Sall (for *gmbB*) sites. Each recombinant plasmid was then transferred by conjugation into *E. coli* ATCC 25922 previously transformed to kanamycin resistance by introducing pK18 (33) by electroporation. Mutants were selected for gentamicin nonsusceptibility (10 μ g·ml⁻¹), with kanamycin (30 μ g·ml⁻¹) being used to counterselect against the donor. Mutations were confirmed by PCR using primers *ompF*-F (5'-ATGATGAAGCGAATAAT-3') and BT543 (5'-TGACGCGTCTCGGTAC-3'), *rseA*-F (5'-AGCCGCTATCATGGATTGC-3') and BT87 (5'-TGACGCGTCTCGGTAC-3'), and *gmbB*-F (5'-TAAATCAATCAGGTTTATGC-3') and BT543.

Conjugation of natural CTX-M-encoding plasmids from cattle *E. coli* isolates (19) YYZ70-1 (CTX-M-15), YYZ16-1 (CTX-M-1), and PSA37-1 (CTX-M-14) into *E. coli* derivatives was performed by mixing on agar. Donor and recipient strains were grown overnight on LB agar plates with selection. A loopful of colonies for each was resuspended separately into 1 ml of phosphate-buffered saline (PBS) and centrifuged at 12,000 \times g for 1 min. The pellet was then resuspended in 1 ml 100 mM CaCl₂ and incubated on ice for 30 min. A 3:1 (vol/vol) ratio of recipient to donor cell suspension was made, and 4 μ l of the mixture was spotted onto nonselective LB agar, which was incubated for 4 to 5 h at 37°C. Spots of mixed growth were scraped into a microcentrifuge tube containing 500 μ l PBS, and 30 μ l of this mixture was spread onto a selective plate and incubated overnight at 37°C. The donor *E. coli* used were isolates PSA or ATCC 25922, and their derivatives. PSA is resistant to ciprofloxacin, so 4 μ g·ml⁻¹ ciprofloxacin was used as counterselection against the donor. For ATCC 25922 and derivatives, pK18 (33) was introduced by electroporation prior to conjugation to allow counterselection using kanamycin (50 μ g·ml⁻¹). Selection for the transconjugant was with 10 μ g·ml⁻¹ cefotaxime.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.

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