

1 **OmpF Downregulation Mediated by Sigma E or OmpR Activation Confers**  
2 **Cefalexin Resistance in *Escherichia coli* in the Absence of Acquired  $\beta$ -**  
3 **Lactamases.**

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5 **Maryam ALZAYN<sup>1,2</sup>, Punyawee DULYAYANGKUL<sup>1</sup>, Naphat SATAPOOMIN<sup>1</sup>,**  
6 **Kate J. HEESOM<sup>3</sup>, Matthew B. AVISON<sup>1\*</sup>**

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8 **<sup>1</sup>School of Cellular & Molecular Medicine, University of Bristol, Bristol, UK**

9 **<sup>2</sup>Biology Department, Faculty of Science, Princess Nourah Bint Abdulrahman**  
10 **University, Riyadh, Saudi Arabia**

11 **<sup>3</sup>University of Bristol Proteomics Facility, Bristol, UK**

12

13 **\* Correspondence to: School of Cellular & Molecular Medicine, University of**  
14 **Bristol, Bristol, United Kingdom. [matthewb.avison@bris.ac.uk](mailto:matthewb.avison@bris.ac.uk)**

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17 **Running Title: OmpR and Sigma E mediated Cefalexin Resistance in *E. coli***

18

19 **Abstract**

20 Cefalexin is a widely used 1<sup>st</sup> generation cephalosporin, and resistance in  
21 *Escherichia coli* is caused by Extended-Spectrum (e.g. CTX-M) and AmpC  $\beta$ -  
22 lactamase production and therefore frequently coincides with 3<sup>rd</sup> generation  
23 cephalosporin resistance. However, we have recently identified large numbers of *E.*  
24 *coli* isolates from human infections, and from cattle, where cefalexin resistance is not  
25  $\beta$ -lactamase mediated. Here we show, by studying laboratory selected mutants,  
26 clinical isolates, and isolates from cattle, that OmpF porin disruption or  
27 downregulation is a major cause of cefalexin resistance in *E. coli*. Importantly, we  
28 identify multiple regulatory mutations that cause OmpF downregulation. In addition to  
29 mutation of *ompR*, already known to downregulate OmpF and OmpC porin  
30 production, we find that *rseA* mutation, which strongly activates the Sigma E regulon,  
31 greatly increasing DegP production, which degrades OmpF, OmpC and OmpA  
32 porins. Furthermore, we reveal that mutations affecting lipopolysaccharide structure,  
33 exemplified by the loss of GmhB, essential for lipopolysaccharide heptosylation, also  
34 modestly activate DegP production, resulting in OmpF degradation. Remarkably,  
35 given the critical importance attached to such systems for normal *E. coli* physiology,  
36 we find evidence for DegP-mediated OmpF downregulation, *gmhB* and *rseA* loss of  
37 function mutation in *E. coli* isolates derived from human infections. Finally, we show  
38 that these regulatory mutations enhance the ability of group 1 CTX-M  $\beta$ -lactamase to  
39 confer reduced carbapenem susceptibility, particularly those mutations that cause  
40 OmpC in addition to OmpF downregulation.

## 41 **Introduction**

42 Cefalexin is a 1<sup>st</sup> generation cephalosporin widely used in human, companion, and  
43 farmed animal medicine. In 2016 in Bristol, United Kingdom, and surrounding  
44 regions, (a population of 1.5 million people) 27.6 cefalexin courses were dispensed  
45 per 1000 patient population (2.8% of all dispensed items). Whilst dispensing rates  
46 had dropped by 19.5% since 2013, the proportion of *Escherichia coli* from  
47 community-origin urine samples resistant to cefalexin in this region rose from 7.06%  
48 to 8.82% (1).

49 Cefalexin resistance in *E. coli* is caused by hyper-production of the chromosomally-  
50 encoded class 1 cephalosporinase gene *ampC*, or acquisition of plasmid AmpC  
51 (pAmpC), or Extended Spectrum  $\beta$ -lactamases (ESBLs). These are also  
52 mechanisms of 3<sup>rd</sup> generation cephalosporin resistance (3GC-R). We recently  
53 reported that among community-origin urinary *E. coli* from Bristol and surrounding  
54 regions collected in 2017/18, 69% of cefalexin resistant isolates were 3GC-R,  
55 suggesting that cefalexin resistance in the absence of ESBL/AmpC production is  
56 common (2). A similar observation was made when analysing faecal samples from  
57 dairy cattle in the same region, where only 30% of samples containing cefalexin  
58 resistant *E. coli* yielded 3GC-R isolates (3). Hyper-production of common acquired  
59 penicillinases such as TEM-1 and OXA-1 does not confer cefalexin resistance in *E.*  
60 *coli* (4). Furthermore, the involvement of efflux pump over-production, e.g. AcrAB-  
61 TolC in *E. coli* has not been reported, but OmpF porin loss is known to reduce  
62 cefalexin susceptibility (5). Indeed, early work showed cefalexin more efficiently uses  
63 OmpF than OmpC porin to enter *E. coli* (6).

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

71

## 72 **Results and Discussion**

73 Cefalexin resistance in *E. coli* is associated with OmpF/OmpC porin downregulation  
74 due to *ompR* mutation.

75 One spontaneous cefalexin resistant mutant was selected from each of three *E. coli*  
76 parent strains: EC17, ATCC25922 and PSA. Cefalexin MICs against these isolates  
77 and their mutant derivatives are reported in **table 1**. In each case, to identify the  
78 possible cause of cefalexin resistance, LC-MS/MS whole-cell proteomics was  
79 performed comparing each mutant with its parent. No mutant over-produced the  
80 chromosomally-encoded AmpC  $\beta$ -lactamase (**Tables S1-S3**), and no  
81 promoter/attenuator sequence mutations upstream of *ampC* were identified in any of  
82 the mutants, based on WGS (**Figure S1**). The only significant ( $p < 0.05$ ;  $> 2$ -fold)  
83 protein abundance change common to all three wild-type/mutant pairs was  
84 downregulation of OmpF porin production (**Table 2, Tables S1-S3**). There was no  
85 evidence of AcrAB-TolC efflux pump over-production in the proteomics data for  
86 mutant (**Tables S1-S3**). Despite OmpF porin downregulation, comparison of *ompF*-  
87 containing WGS contigs from wild-type/mutant pairs revealed no mutations in *ompF*

88 or within 10 kb up- or downstream. We therefore concluded that there is a *trans*-  
89 regulatory mutation affecting OmpF abundance in each mutant.

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

102

103 DegP over-production due to RseA anti-Sigma E mutation is associated with OmpF  
104 porin downregulation and cefalexin resistance in *E. coli*.

105 Nine proteins, including OmpF, were significantly differentially regulated in the same  
106 direction in the cefalexin resistant mutants derived from isolates EC17 and  
107 ATCC25922, each relative to their parent strain. Three proteins (BamD, DegP and  
108 YgiM) were upregulated and six (NmpC, DctA, ArcA, OmpF and YhiL) were  
109 downregulated (**Tables S1,S2**). We were interested to note that one upregulated  
110 protein in both mutants was DegP (**Table 2**), which is a protease known to degrade  
111 porin proteins (9,10). Interestingly, in the PSA-derived *ompR* mutant with

112 downregulated OmpF and OmpC, described above, DegP production was 2-fold  
113 lower than in the wild-type parent, suggesting a feedback response to porin  
114 downregulation (**Table 2**). DegP production was increased 7-fold in the ATCC25922-  
115 derived mutant and OmpF was downregulated 5.9-fold, as was OmpC (6.7-fold) and  
116 OmpA (5.6-fold) (**Table 2**); which is a typical Sigma E response (11). In the EC17-  
117 derived mutant, DegP was upregulated a more modest 4.4-fold, and here, OmpF  
118 was downregulated 2.9-fold, but OmpC and OmpA were not significantly ( $p < 0.05$ )  
119 downregulated, suggesting a weaker Sigma E response (**Table 2**). This led to the  
120 suggestion that OmpC downregulation, seen in the PSA-derived and ATCC25922-  
121 derived cefalexin resistant mutants alongside OmpF downregulation (**Table 2**) is not  
122 necessary for cefalexin resistance. To confirm this, we disrupted *ompF* in  
123 ATCC25922 and found this to be sufficient for cefalexin resistance (**Table 1**).  
124 Additional downregulation of OmpC is not necessary.

125 Analysis of WGS data identified that the ATCC25922-derived mutant expressing a  
126 phenotype typical of a strong Sigma E response had a mutation predicted to cause a  
127 Trp33Arg mutation in RseA, which is a known Sigma E anti-sigma factor (12,13).  
128 Loss of RseA is expected to release Sigma E so that it can bind, among others, to  
129 the *degP* promoter, increasing transcription, leading to porin degradation and  
130 cefalexin resistance (11). We disrupted *rseA* in ATCC25922 and confirmed that this  
131 mutation does cause cefalexin resistance (**Table 1**).

132

133 Perturbation of Lipopolysaccharide heptosylation due to *gmhB* mutation causes  
134 cefalexin resistance in *E. coli*.

135 The EC17-derived cefalexin resistant mutant which also appears to have a Sigma E  
136 response, though weaker than the *rseA* mutant, was shown through WGS analysis  
137 to have a deoxythymidine nucleotide insertion after nucleotide 348 of *gmhB*,  
138 predicted to cause a frameshift affecting the encoded protein beyond amino acid  
139 117. This gene encodes the enzyme D-alpha,beta-D-heptose-1,7-bisphosphate  
140 phosphatase, which is part of a pathway responsible for producing heptose for  
141 lipopolysaccharide biosynthesis (14). Loss of enzymes involved in this system are  
142 associated with increased outer membrane permeability, but interestingly, deletion of  
143 *gmhB* does not disrupt full length LPS production or damagingly compromise the  
144 outer membrane permeability barrier (14,15). The obvious conclusion is that this  
145 perturbation in envelope structure activates the Sigma E regulon, resulting in OmpF  
146 degradation by DegP. We disrupted *gmhB* in ATCC25922 and found that this  
147 mutation causes cefalexin resistance (**Table 1**). In the ATCC25922 background, the  
148 *rseA* and *gmhB* mutants were similar, in MIC terms, to the *ompF* mutant (**Table 1**).  
149 This further supports the conclusion that despite other porin production changes  
150 caused by *rseA* mutation and *ompR* mutation, as identified above, it is OmpF  
151 downregulation that is driving the cefalexin resistance phenotype observed in these  
152 three in vitro selected mutants.

153

154 Loss and downregulation of OmpF in cefalexin resistant *E. coli* from cattle and  
155 humans and evidence for *rseA* and *gmhB* mutations in human clinical isolates.

156 We chose two cefalexin resistant but 3GC-S isolates at random from our previous  
157 survey of dairy farms (3), and two from our previous survey of human urinary *E. coli*  
158 (2). Cefalexin resistance was confirmed by MIC (**Table 1**). WGS revealed disruption

159 of *ompF* in both farm isolates: in Farm-1, a Tn5 insertion disrupted *ompF*, truncating  
160 OmpF after amino acid 316. In Farm-2 a frameshift mutation disrupted OmpF after  
161 amino acid 96.

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

180 According to WGS, ST131 isolate UTI-2 did not have a mutation in *gmhB*, *rseA*,  
181 *ompR*, or *ompF* relative to the ST131 control isolate UTI-80710. Therefore, the  
182 regulatory mutation leading to elevated DegP levels, reduced OmpF levels, and  
183 cefalexin resistance in UTI-2 has not been identified. However, given the complexity



184 of Sigma E activation signals and the impact that many different changes in  
185 envelope structure can have on it (16), it is possible that clinical isolates do carry  
186 mutations that activate this regulon. Indeed, searches of the NCBI database  
187 identified carbapenem resistant human *E. coli* isolate E300, identified in Japan (17),  
188 which has an 8 nt insertion, leading to a frameshift in *rseA* at nucleotide 34  
189 (Accession Number AP022360). Furthermore, two human clinical isolates were  
190 found to have a single nucleotide insertion leading to a frameshift in *gmhB* after  
191 nucleotide 126; one from China (Accession Number CP008697) and one from the  
192 USA (Accession Number CP072911); and three commensal *E. coli* from the USA  
193 (18) were found to have frameshift mutations at various positions in *gmhB*  
194 (Accession Numbers CP051692, CP054319, and CP054319). Accordingly, we  
195 conclude that mutations likely to cause the same phenotypes found in our laboratory-  
196 selected cefalexin-resistant mutants are also found in clinical and commensal *E. coli*  
197 from across the world.

198

199 Influence of *ompF* porin loss and downregulation on late generation cephalosporin  
200 and carbapenem susceptibility in the presence of various CTX-M  $\beta$ -lactamases.

201 Our final aim was to test the impact of *ompF* loss and downregulation, due to OmpR  
202 mutation or activation of Sigma E, on late generation cephalosporin or carbapenem  
203 MIC in *E. coli* producing CTX-M  $\beta$ -lactamases. To do this we introduced, using  
204 conjugation, natural plasmids carrying various *bla*<sub>CTX-M</sub> variants commonly identified  
205 in human and cattle 3GC-R *E. coli* in South West England: encoding CTX-M-1, CTX-  
206 M-14 and CTX-M-15 (2,19). We measured MICs of 3GCs and 4GCs used in humans  
207 (ceftazidime, cefepime) or cattle (ceftiofur, cefquinome), and the carbapenem

208 ertapenem against CTX-M plasmid transconjugants of *E. coli* parent strains and their  
209 *ompF*, *rseA* or *ompR*, mutant derivatives (**Table 3**).

210 In wild-type ATCC25922, as expected, CTX-M-1 and CTX-M-15 conferred resistance  
211 to all four cephalosporins tested, CTX-M-14 did not confer ceftazidime resistance,  
212 and none of the enzymes conferred ertapenem resistance. Disruption of *ompF* or  
213 *rseA* did not change the susceptibility profile but there were some MIC changes.  
214 Disruption of *ompF* caused a two-doubling increase in ertapenem MIC against  
215 transconjugants producing CTX-M-1 and CTX-M-15, but there was no change in MIC  
216 against the CTX-M-14 transconjugant. Disruption of *rseA* caused a similar impact on  
217 ertapenem MIC against CTX-M-1 or CTX-M-15 producers, but additionally caused a  
218 two-doubling increase in MIC against the CTX-M-14 producer. It is likely that this  
219 additional effect is due to the downregulation in OmpC additionally seen in the *rseA*  
220 mutant (**Table 2**), OmpC being a key carbapenem porin (20).

221 Resistance profiles seen in wild-type *E. coli* isolate PSA CTX-M plasmid  
222 transconjugants were almost identical to transconjugants of isolate ATCC25922  
223 (**Table 3**). However, carriage of plasmids encoding CTX-M-1 or CTX-M-15 conferred  
224 ertapenem non-susceptibility in the PSA *ompR* mutant, the ertapenem MIC being  
225 one doubling higher than against CTX-M-1 or CTX-M-15 transconjugants of the  
226 ATCC25922 *rseA* mutant derivative (**Table 3**). The greater impact of *ompR* mutation  
227 than *rseA* mutation on reducing OmpC levels (**Table 2**) likely explains this  
228 difference. Indeed, *ompR* mutation has previously been associated with ertapenem  
229 non-susceptibility in ESBL producing *E. coli* (8,20).

230

231 Conclusions

232 Cefalexin is a widely used antibacterial in human and veterinary medicine, and so  
233 cefalexin resistance is of considerable clinical importance. Despite this, mechanisms  
234 of resistance have not been given very much attention, particularly in the post-  
235 genomic age. We were surprised to find that, in our recent surveys of human and  
236 cattle cefalexin resistant isolates, acquired cephalosporinase (pAmpC or ESBL) or  
237 chromosomal AmpC hyper-production were not the cause of cephalalexin resistance  
238 in a large proportion of isolates (2,3). We show here strong evidence that OmpF loss  
239 or downregulation is a key mechanism of cefalexin resistance in *E. coli* in the  
240 absence of  $\beta$ -lactamase production. Whilst OmpF loss contributes to resistance to a  
241 wide range of antibacterials (5), our findings show that cefalexin resistance is  
242 unusual in being caused solely by OmpF loss. Furthermore, we show that OmpF  
243 downregulation can also confer this phenotype. This may explain why *ompF* loss-of-  
244 function mutations are found among *E. coli* from clinical samples, but our work  
245 suggests there may also be numerous different regulatory mutations found among  
246 clinical isolates, each downregulating OmpF.

247 Such is the wide range of regulatory systems controlling OmpF production, both at  
248 transcriptional, translational, and post-translational levels (7, 16, 21), it is not  
249 surprising that cefalexin resistance mutations arise in many different genes in the  
250 laboratory, as seen here. Importantly, these mutations may contribute to resistance  
251 to other antibacterials, when partnered with other mechanisms. Indeed, some of  
252 these mutations also affect OmpC levels, and if this is a sufficiently large effect (e.g.  
253 in the *ompR* mutant identified here) that can give rise to carbapenem non-  
254 susceptibility if the mutant acquires a common ESBL such as CTX-M-15. Similar  
255 *ompR* mutants have been seen in the clinic (8). The other regulatory mutations  
256 affecting OmpF levels found in the laboratory-selected mutants reported here work

257 through Sigma E mediated DegP over-production. It is well known that DegP  
258 degrades porins (10, 16), but it has not previously been reported that DegP-  
259 mediated degradation of OmpF is sufficient to cause resistance to any antibacterial  
260 drug.

261 Our findings are also potentially important because they suggest that OmpF is more  
262 susceptible to DegP mediated proteolysis *in vivo* in *E. coli* than the other two main  
263 porins, OmpC and OmpA. In the *rseA* mutant with “maximal” Sigma E activation and  
264 DegP upregulation, OmpF, OmpC and OmpA levels all fell, but in the *gmhB* mutant  
265 overproducing DegP to a lesser extent, only OmpF levels significantly fell (**Table 2**).  
266 This was still sufficient to cause cefalexin resistance but had a smaller effect on  
267 ertapenem MIC in the presence of CTX-M-15 production (**Table 3**), likely due to the  
268 OmpF-specific effect on porin downregulation.

269 It is known that mutations affecting outer membrane and lipopolysaccharide structure  
270 activate Sigma E, because they affect envelope integrity (16). It has not previously  
271 been shown, however, that mutations disrupting *gmhB* can do this, and further, that  
272 this can cause cefalexin resistance. We considered that despite such mutations  
273 arising in the laboratory, this perhaps overstates their clinical relevance, because  
274 disruption of the Gmh system causes significant attenuation and increased  
275 susceptibility to envelope stresses, though significantly, loss of GmhB has the  
276 mildest effect in this regard (15). Accordingly, we were very interested to find  
277 cefalexin resistant human urinary ST131 isolates having OmpF downregulation, and  
278 in one case, DegP upregulation, suggestive of a GmhB negative phenotype, though  
279 *gmhB* was intact and the nature of the mutation responsible will be the focus of  
280 future work. However, importantly, we did find clear evidence of *rseA* and *gmhB*  
281 loss-of-function mutations among clinical and commensal *E. coli* from secondary

282 analysis of WGS data. Therefore, we provide here strong evidence that mutations  
283 constitutively activating Sigma E, including those which do this by altering  
284 lipopolysaccharide structure, can be tolerated by *E. coli* in a clinical setting. These  
285 mutations, and possibly others yet to be identified, cause clinically-relevant cefalexin  
286 resistance in the absence of  $\beta$ -lactamase production through DegP-mediated OmpF  
287 proteolysis.

288

## 289 **Experimental**

### 290 Bacterial isolates, selection of resistant mutants and susceptibility testing

291 Three  $\beta$ -lactam susceptible *E. coli* isolates were used: the type-strain ATCC25922;  
292 the human urinary isolate EC17, provided by Dr Mandy Wootton, Public Health  
293 Wales; and a ciprofloxacin resistant isolate, PSA, from faecal samples collected on a  
294 dairy farm (3). To select cefalexin-resistant derivatives of these isolates, 100  $\mu$ l of  
295 overnight culture grown in Nutrient Broth were spread onto Mueller Hinton agar  
296 containing 16  $\mu$ g.ml<sup>-1</sup> cefalexin and each plate incubated for 24 h. In addition, four  
297 cefalexin resistant but 3GC-S isolates were used: one each from faecal samples  
298 from two dairy farms (Farm-1, Farm-2) as collected previously (3); two human  
299 urinary isolates (UTI-1, UTI-2) also as collected previously (2). The control isolate  
300 UTI-80710 is 3GC-R due to CTX-M-15 production (2) and was selected based on its  
301 production of wild-type OmpF porin levels (see text). Microtiter MIC assays were  
302 performed and interpreted according to CLSI guidelines (22, 23).

### 303 Proteomics

304 One millilitre of an overnight Cation Adjusted Mueller Hinton Broth (CA-MHB) culture  
305 was transferred to 50 ml CA-MHB and cells were grown at 37°C to 0.6-0.8 OD<sub>600</sub>.

306 Cells were pelleted by centrifugation (10 min, 4,000×g, 4°C) and resuspended in 35  
307 ml of 30 mM Tris-HCl, pH 8 and broken by sonication using a cycle of 1 s on, 0.5 s  
308 off for 3 min at amplitude of 63% using a Sonics Vibracell VC-505TM (Sonics and  
309 Materials Inc., Newton, Connecticut, USA). The sonicated samples were centrifuged  
310 at 8,000×g for 15 min at 4°C to pellet intact cells and large cell debris. Protein  
311 concentrations in all supernatants were quantified using the Biorad Protein Assay  
312 Dye Reagent Concentrate according to the manufacturer's instructions. Proteins (1  
313 µg/lane) were separated by SDS-PAGE using 11% acrylamide, 0.5% bis-acrylamide  
314 (Biorad) gels and a Biorad Min-Protein Tetracell chamber model 3000X1. Gels were  
315 resolved at 200 V until the dye front had moved approximately 1 cm into the  
316 separating gel. Proteins in all gels were stained with Instant Blue (Expedeon) for 5  
317 min and de-stained in water. LC-MS/MS data was collected as previously described  
318 (24). The raw data files were processed and quantified using Proteome Discoverer  
319 software v1.4 (Thermo Scientific) and searched against bacterial genome and  
320 horizontally acquired resistance genes as described previously (25).

### 321 Whole genome sequencing and analyses

322 WGS was performed by MicrobesNG (<https://microbesng.uk/>) on a HiSeq 2500  
323 instrument (Illumina, San Diego, CA, USA) using 2x250 bp paired end reads. Reads  
324 were trimmed using Trimmomatic (26) and assembled into contigs using SPAdes  
325 3.13.0 (27). Contigs were annotated using Prokka 1.2 (28). Resistance genes and  
326 sequence types, according to the Achtman scheme (29) were assigned using the  
327 ResFinder (30) and MLST 2.0 on the Center for Genomic Epidemiology  
328 (<http://www.genomicepidemiology.org/>) platform. Pairwise contig alignments to  
329 identify mutations versus parent isolate, which were sequenced in parallel, was with  
330 EMBOSS Stretcher ([https://www.ebi.ac.uk/Tools/psa/emboss\\_stretcher/](https://www.ebi.ac.uk/Tools/psa/emboss_stretcher/))

331 Insertional inactivation of genes and conjugation of CTX-M-encoding plasmids.

332 Insertional inactivation of *ompF*, *rseA*, or *gmhB* was performed using the pKNOCK  
333 suicide plasmid (31). DNA fragments were amplified with Phusion High-Fidelity DNA  
334 Polymerase (NEB, UK) from *E. coli* ATCC25922 genomic DNA by using primers  
335 *ompF*-KO-FW (5'-CAAGGATCCTGATGGCCTGAACTTC-3') with a BamHI  
336 restriction site, underlined, and *ompF*-KO-RV (5'-  
337 CAAGTCGACTTCAGACCAGTAGCC-3') with a Sall site; *rseA*-KO-FW (5'-  
338 CGCGGATCCTGCAGAAAACCAGGGAAAGC-3') with a BamHI site and *rseA*-KO-  
339 RV (5'-TGCACTGCAGCCATTTGGGTAAGCTGTGCC-3') with a PstI site; *gmhB*-KO-  
340 FW (5'-TATACTAGTCACGGCTATGTCCATGAGA-3') with a SpeI site, and *gmhB*-  
341 KO-RV (5'-TATGTCGACTCGGTCAGCGTTTCAAAC-3') with a Sall site. Each PCR  
342 products were ligated into pKNOCK-GM (31) at the BamHI and Sall (for *ompF*),  
343 BamHI and PsaI (for *rseA*), or SpeI and Sall (for *gmhB*) sites. Each recombinant  
344 plasmid was then transferred by conjugation into *E. coli* ATCC25922 previously  
345 transformed to kanamycin resistance by introducing pK18 (32) by electroporation.  
346 Mutants were selected for gentamicin non-susceptibility (10 µg.ml<sup>-1</sup>), with kanamycin  
347 (30 µg.ml<sup>-1</sup>) being used to counter-select against the donor. Mutations were  
348 confirmed by PCR using primers *ompF*-F (5'-ATGATGAAGCGCAATAAT-3') and  
349 BT543 (5'-TGACGCGTCCTCGGTAC-3'); *rseA*-F (5'-AGCCGCTATCATGGATTGTC-  
350 3') and BT87 (5'-TGACGCGTCCTCGGTAC-3'); *gmhB*-F (5'-  
351 TAAATCAATCAGGTTTATGC-3') and BT543.

352 Conjugation of natural CTX-M-encoding plasmids from cattle *E. coli* isolates (19)  
353 YYZ70-1 (CTX-M-15), YYZ16-1 (CTX-M-1) and PSA37-1 (CTX-M-14) into *E. coli*  
354 derivatives was performed by mixing on agar. Donor and recipient strains were  
355 grown overnight on LB agar plates with selection. A loopful of colonies for each was

356 resuspended separately into 1 ml of PBS and centrifuged at 12,000xg for 1 min. The  
357 pellet was then resuspended in 1 ml 100 mM CaCl<sub>2</sub> and incubated on ice for 30 min.  
358 A 3:1 v/v ratio of recipient to donor cell suspension was made and 4 µl of the mixture  
359 were spotted onto non-selective LB agar, which was incubated for 4-5 h at 37°C.  
360 Spots of mixed growth were scraped into a micro centrifuge tube containing 500 µl  
361 PBS and 30 µl of this mixture were spread onto a selective plate and incubated  
362 overnight at 37°C. The donor *E. coli* used were isolates PSA or ATCC25922, and  
363 their derivatives. PSA is resistant to ciprofloxacin, so 4 µg.ml<sup>-1</sup> ciprofloxacin was  
364 used as counter-selection against the donor. For ATCC25922 and derivatives, pK18  
365 (32) was introduced by electroporation prior to conjugation to allow counter selection  
366 using kanamycin (50 µg.ml<sup>-1</sup>). Selection for the transconjugant was with 10 µg.ml<sup>-1</sup>  
367 cefotaxime.

368

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371

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378



379 **Transparency declaration**

380 The authors declare no conflict of interests.

381

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

PSA	PSA (M)	ATCC25922	ATCC25922 (M)	EC17	EC17 (M)	ATCC25922 <i>ompF</i>	ATCC25922 <i>rseA</i>	ATCC25922 <i>gmhB</i>	Farm-1	Farm-2	UTI-1	UTI-2
16	32	16	32	8	32	32	64	64	64	64	32	32

“M” designates mutants selected for growth on cefalexin.

Shaded values represent resistant based on CLSI breakpoints (23), otherwise susceptible.

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

	OmpF			OmpC			OmpA			DegP		
	Mean	SEM	P (WT/M)	Mean	SEM	P (WT/M)	Mean	SEM	P (WT/M)	Mean	SEM	P (WT/M)
<b>PSA</b>	0.23	0.05		5.93	1.00		3.24	0.32		0.06	0.01	
<b>PSA (M)</b>	0.00	0.00	0.005	0.17	0.04	0.002	3.69	0.74	>0.25	0.03	0.01	0.03
<b>ATCC25922</b>	1.69	0.10		1.66	0.26		5.55	0.41		0.08	0.01	
<b>ATCC25922 (M)</b>	0.29	0.14	0.0006	0.24	0.05	0.003	1.00	0.17	0.0003	0.54	0.10	0.005
<b>EC17</b>	1.15	0.15		2.49	0.50		3.79	0.29		0.17	0.03	
<b>EC17 (M)</b>	0.39	0.16	0.01	2.25	0.66	>0.25	4.47	0.78	>0.25	0.74	0.10	0.003

Abundance relative to total ribosomal proteins is presented for each protein, n=3 biological replicates for each isolate/mutant. Raw data are presented in **tables S1-S3**.

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

Strain Name	MIC $\mu\text{g.ml}^{-1}$				
	Cefepime	Cefquinome	Ceftazidime	Ceftiofur	Ertapenem
ATCC25922(pK18)	0.25	0.125	0.25	0.25	0.016
ATCC25922(pK18) CTX-M-1	>128	>128	16	>128	0.0625
ATCC25922(pK18) CTX-M-15	>128	>128	32	>128	0.125
ATCC25922(pK18) CTX-M-14	64	>128	2	>128	0.0313
ATCC25922(pK18) <i>ompF</i>	0.25	0.125	0.5	0.5	0.016
ATCC25922(pK18) <i>ompF</i> CTX-M-1	>128	>128	32	>128	0.25
ATCC25922(pK18) <i>ompF</i> CTX-M-15	>128	>128	64	>128	0.5
ATCC25922(pK18) <i>ompF</i> CTX-M-14	>128	>128	4	>128	0.0313
ATCC25922(pK18) <i>rseA</i>	0.25	0.25	0.5	0.5	0.0313
ATCC25922(pK18) <i>rseA</i> CTX-M-1	>128	>128	16	>128	0.5
ATCC25922(pK18) <i>rseA</i> CTX-M-15	>128	>128	32	>128	0.5
ATCC25922(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

CTM-M variants were delivered on natural plasmids by conjugation. Plasmid pK18 (32) 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 “non-susceptible” (resistant or intermediate) according to CLSI breakpoints (23).



