The RND efflux pump EefABC is highly conserved within lineages of *E. coli* commonly associated with infection.

Running Title: EefABC; the seventh RND pump in *E. coli*

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Abstract

Tripartite resistance-nodulation-division (RND) efflux pumps confer multidrug resistance (MDR) in Gram-negative bacteria and are critical for many physiological functions including virulence and biofilm formation. The common laboratory strain of *E. coli,* K-12 MG1655 has six recognised RND transporters participating in tripartite pump formation (AcrB, AcrD, AcrF, CusA, MdtBC, and MdtF). However, by studying >20,000 *E. coli* genomes we show that *E. coli* belonging to phylogroups B2, D, E, F and G, which are commonly associated with infection, possess an additional, seventh RND transporter, EefB. It is found in a five gene operon, *eefRABCD,* which also encodes a TetR family transcription factor, a periplasmic adapter protein, an outer membrane factor and major facilitator superfamily pump. In contrast, *E. coli* from phylogroups A, B1 and C, generally containing environmental and commensal strains, do not encode the operon and instead encode an uncharacterised ORF, *ycjD*. In phylogroups where the *eefRABCD* operon is present it was very highly conserved. In fact, conservation levels were comparable to that of the major *E. coli* RND efflux

system AcrAB-TolC, suggesting a critical biological function. Protein modelling shows that this pump is highly divergent from endogenous *E. coli* RND systems with unique structural features, while showing similarities to efflux systems found in *Pseudomonas aeruginosa*. However, unlike other major RND efflux systems, EefABC does not appear to transport antimicrobials and instead may be important for infection or survival in the host environment.

Importance

Efflux pumps are molecular machines that export molecules out of bacterial cells. The efflux pumps belonging to the RND family are particularly important as they export antibiotics out of Gram-negative bacterial cells, contributing to antibiotic resistance. The important human pathogen, *E. coli*, has been previously reported to have six RND pumps. However, we show that phylogroups of *E. coli* commonly associated with infection encode a seventh RND pump, EefABC which is highly conserved, suggesting an important biological function. While the function of EefABC in *E. coli* remains to be resolved, it does not seem to transport antimicrobial compounds. These findings are important because they reveal a new RND pump, potentially involved in virulence and survival in the host, that could represent a new therapeutic target. Additionally, it again shows that laboratory type strains of common bacterial pathogens are not representative of those that are infection causing.

Introduction

Escherichia coli is a leading cause of invasive bacterial infections in humans, causing a range of diseases from urinary tract infections to haemorrhagic shock (Holmes et al., 2021). However, *E. coli* is also found in the wider environment and is a common commensal, colonising the gastrointestinal tract of both humans and animals. *E. coli* is a genetically diverse species that is divided into phylogroups (A, B1, B2, C, D, E, F and G) which are determined by genetic similarity (Denamur et al., 2021). Though virulence is not limited to specific phylogroups (Clermont et al., 2000, Gordon and Cowling, 2003), extra-intestinal infection is most commonly associated with phylogroups B2 and D (Denamur et al., 2021), while commensal or environmental lifestyles are mainly associated with phylogroups A and B1 (Tenaillon et al., 2010). In addition to phylogroups, *E. coli* isolates are also classified by sequence types (ST),

determined by multi-locus sequence typing (MLST) (Maiden et al., 1998). Pandemic clonal STs such as ST131 are responsible for high incidence rates of extra-intestinal *E. coli* infections and the carriage of genes conferring multidrug resistance (Shaik et al., 2017, Li et al., 2023).

Gram-negative bacteria, such as *E. coli*, possess efflux pumps of the resistance-nodulation-division (RND) family which are best known for the export of antimicrobials and biocides (Chen et al., 2003, Sanchez et al., 1997, Nishino and Yamaguchi, 2001, Alav et al., 2021) with overexpression of these pumps conferring multidrug resistance (MDR) (Swick et al., 2011, Chetri et al., 2019). RND pumps form tripartite complexes with periplasmic adaptor proteins (PAP) and outer membrane factors (OMF) to span the inner membrane, periplasmic space, and the outer membrane (Alav et al., 2021). In addition to MDR, RND pumps have been implicated in a wide range of additional functions, including virulence (Nishino et al., 2006, Blair et al., 2015, Padilla et al., 2010), biofilm production (Baugh et al., 2014), decreased susceptibility to dyes, bile salts and fatty acids (Nishino and Yamaguchi, 2001, Mateus et al., 2021), export of polyamines and quorum sensing molecules (Chan et al., 2007), export of bacterial metabolites (Wang-Kan et al., 2021), copper and ion homeostasis (Grass and Rensing, 2001, Franke et al., 2001), and motility (Bazzini et al., 2011, Yang et al., 2011).

Many Gram-negative bacteria encode multiple RND efflux pumps, with differing substrate specificities which are often expressed only under specific environmental conditions. However, the number of RND pumps is highly variable between bacterial lineages. The human-restricted pathogen *Neisseria gonorrhoeae* possesses only a single RND pump (MtrCDE), while bacterial species that can colonise multiple habitats generally possess more. *Salmonella enterica* serovar Typhimurium has five RND pumps, whereas *E. coli* is generally reported to have six: AcrB, AcrD, AcrF, MdtBC, MdtF and CusA (Nishino and Yamaguchi, 2001). However, the number of RND pumps can also vary within a genus. For example, we have recently shown that species across the *Acinetobacter* genus possess between two and nine RND pumps, with species most-commonly associated with human infection tending to encode more RND efflux pumps (Darby et al., 2023). A study by Ma *et al.* found loss of MtrC function correlated with increased drug susceptibility in *N. gonorrhoeae* isolated from the cervical environment (Ma et al., 2020). Moreover, we recently demonstrated that AcrF in *E. coli* O157:H7 is non-functional due to a conserved insertion containing two stop

103 codons (Pugh et al., 2023), showing that within a species not all efflux pumps present
104 are always functional.
105 The expression of RND efflux pumps is controlled by a complex network of
106 positive and negative regulators including repressors of the TetR family. We previously
107 reported the presence of the TetR family regulator EefR in four of ten *E. coli* strains
108 studied (Colclough et al., 2019). The *eefR* gene was encoded alongside genes
109 annotated as *eefA* and *eefB* that are predicted to encode a PAP and RND pump,
110 respectively. This RND pump was first reported in *Enterobacter aerogenes* (now
111 reclassified as *Klebsiella aerogenes*), where *eefA, eefB* and *eefC* were found in a three
112 gene operon without the regulator, *eefR* (Masi et al., 2005). In that study, the *eefABC*
113 operon was not expressed under laboratory conditions due to transcriptional silencing
114 by H-NS (Masi et al., 2005), however overexpression was found to confer resistance
115 to erythromycin (Masi et al., 2006). The EefABC RND pump has also been reported
116 in *Klebsiella pneumoniae,* where it has been linked to virulence, as deletion of *eefA*
117 was found to reduce both colonisation of the gastrointestinal tract and tolerance to low
118 pH (Coudeyras et al., 2008).
119 In *E. coli* the *eefABC* operon is absent from the widely studied strain K-12 but
120 has been reported in a single highly drug resistant environmental isolate*, E. coli* SMS-
121 3-5. In this isolate the efflux pump was part of a larger gene cluster which included the
122 regulator *eefR* and a major facilitator superfamily (MFS) efflux pump *eefD* (Fricke et
123 al., 2008). To date, the wider prevalence of the *eefRABCD* operon across the diversity
124 of the *E. coli* population and its function in *E. coli* are both still unknown. Here we
125 demonstrate that: 1) EefABC is exclusively found in clinically relevant phylogroups of
126 *E. coli* and is highly conserved, 2) homology modelling reveals that the pump has
127 several distinctive structural features compared to other RND efflux pumps, and 3)
128 neither EefABC, nor EefD transport clinically relevant antimicrobials but can transport
129 dyes.
130
131 **Materials and methods**
132 **Prevalence and conservation of *eefRABCD* across *E. coli* and related species**
133 The *eefB* gene from *E. coli* SMS-3-5 (CP000970.1) was aligned to *E. coli* genomes
134 (taxid 562) within the NCBI RefSeq Genome Database.
135 A total of 20,013 *E. coli* genome assemblies from 38 STs (Achtman scheme)
136 (Wirth et al., 2006) were downloaded from Enterobase (Zhou et al., 2020) for the

137 determination of *eef* conservation across *E. coli*. Duplicates were removed based
138 upon MASH (v 2.2.2) distances (Ondov et al., 2016). A custom blast database (BLAST
139 v 2.10.0) (Camacho et al., 2009) was generated from the sequences of *eefR, eefA,*
140 *eefB, eefC* and *eefD* of *E. coli* ATCC 25922 (CP009072.1) and used with ABRicate (v
141 0.9.8) (Seemann, 2017) to identify the presence and conservation of *eef* genes across
142 the *E. coli* assemblies. Where an *eef* gene was found to be split across multiple contigs
143 within an assembly, the assembly was removed from analysis as it was not possible
144 to confirm from the assemblies alone whether this was due to a sequencing or
145 assembly error, or the interruption of the gene.
146 The *eefRABCD* gene sequences from *E. coli* ATCC 25922 were also used to
147 determine whether *Shigella* species encode the operon. Assemblies of *Shigella boydii*
148 (*n* = 495)*, Shigella dysenteriae* (*n* = 497)*, Shigella flexneri* (*n* = 499)*,* and *Shigella*
149 *sonnei* (*n* = 500) were downloaded from Enterobase. As with *E. coli,* duplicates were
150 identified using MASH and removed prior to running ABRicate. Assemblies containing
151 *eef* genes split over multiple contigs were removed from the analysis as with *E. coli.*
152 The *eefRABCD* genes from *E. coli* ATCC 25922 were also aligned to *Salmonella* (taxid
153 590)*, Photorhabdus* (taxid 29487)*, Yersinia* (taxid 629)*, Serratia* (taxid 613)*,*
154 *Pseudomonas* (taxid 286), *Enterobacter* (taxid 547) and *Acinetobacter* (taxid 469)
155 genomes to identify any homologs in related Gammaproteobacteria, however this was
156 achieved using the NCBI RefSeq Genome Database.
157
158 **The phylogenetic context of *eefRABCD***
159 Five assemblies of each *E. coli* ST (excluding ST84 where *n* = 2) and *E. fergusonii*
160 were chosen at random to generate a phylogenetic tree. Assemblies were annotated
161 using Prokka (v 1.14.6) (Seemann, 2014) with subsequent GFF files used as input for
162 Roary (v 3.13.0) (Page et al., 2015). The core gene alignment produced by Roary was
163 used to construct a GTR-gamma tree with 100 bootstraps using RaXmL (v 8.2.12)
164 (Stamatakis, 2014). Trees were visualised and annotated using iTOL (Letunic and
165 Bork, 2019).
166
167 **Genomic context of *eefRABCD***
168 Due to limited availability of RefSeq genomes for some *E. coli* STs used in this work,
169 seven were chosen at random. Reference sequences were downloaded from NCBI
170 NC_004431.1 (ST73), NC_007946.1 (ST95), NZ_HG941718.1 (ST131),

171 NC_002695.2 (ST11), NC_000913.3 (ST10), NC_011751.1 (ST69), and
172 NZ_CP035350.1 (ST617). The location of the *eef* operon was identified in the ST73,
173 ST95, ST131 and ST11 and downloaded along with the flanking 10,000 bp. The
174 homologous regions in ST10, ST69 and ST617 were also identified and downloaded.
175 Alignments of the genomic regions in all seven reference sequences was performed
176 using EasyFig (v 2.2.2) (Sullivan et al., 2011).
177 Genomes of additional bacterial species; *E. albertii* (CP070290.2), *E.*
178 *marmotae* (CP056165.1), *K. aerogenes* (NZ_CP041925.1), *K. pneumoniae*
179 (NC_016845.1), *Enterobacter vonholyi* (VTUC01000001.1), *Enterobacter dykesii*
180 (VTTY01000003.1), *Enterobacter wuhouensis* (SJOO01000006.1), *Enterobacter*
181 *kobei* (KI973153.1), *Enterobacter chengduensis* (CP043318.1), *S. boydii*
182 (CP026836.1), *S. dysenteriae* (CP026774.1), *S. flexneri* (AE005674.2) and *S. sonnei*
183 (CP055292.1) were downloaded from NCBI. The visualisation of the genomic context
184 of the *eef* operon and homologs was achieved using EasyFig. Mapping of insertion
185 sequences in *S. dysenteriae* was done using ISEScan (v 1.7.2.3) (Xie and Tang,
186 2017).
187
188 **Homology modelling and structural analysis**
189 Multiple sequence alignments (MSA) were prepared using MAFFT and NJ/UPGMA
190 phylogeny algorithms as implemented in MAFFT v.7 server (https://mafft.cbrc.jp/)
191 (Katoh et al., 2019). Phylo.io was used for phylogenetic guide tree visualisation
192 (Robinson et al., 2016). Structural annotations of the MSA sequences were done with
193 Espript 3 (http://espript.ibcp.fr) (Robert and Gouet, 2014).
194 For homology modelling, I-TASSER (Yang et al., 2015) was used in manual
195 mode with assignment of templates and structural alignment, supplemented by
196 SWISS-MODEL (Waterhouse et al., 2018). The following structural templates have
197 been used for the specific protein modelling. EefA modelling: MexA (Uniprot P52477)
198 2V4D.pdb (Symmons et al., 2009); AcrA (Uniprot P0AE06) 5V5S.pdb (Wang et al.,
199 2017). AcrA was used as a template due to the smaller gaps in the alignment and
200 better quality of available full-length template. EefB modelling: MexB (Uniprot P52002)
201 3W9I.pdb (Nakashima et al., 2013); AcrB (Uniprot P31224) 2GIF.pdb (Seeger et al.,
202 2006). EefC modelling: TolC (Uniprot P02930) 1EK9.pdb (Koronakis et al., 2000);
203 OprM (Uniprot Q51487) 4Y1K.pdb (Monlezun et al., 2015); OprJ (Uniprot Q51397)
204 5AZS.pdb (Yonehara et al., 2016); OprN (Uniprot Q9I0Y7) 5AZO.pdb, 5AZP.pdb

205 (Yonehara et al., 2016); 5IUY.pdb (Ntsogo Enguéné et al., 2017). EefD modelling:
206 EmrD (Uniprot P31442) 2GFP.pdb (Yin et al., 2006); MdfA (Uniprot P0AEY8)
207 4ZP0.pdb (Heng et al., 2015).
208 For the models of the protein oligomers and the complete EefABC tripartite
209 pump, rigid-body structural docking of the homology models was used guided by the
210 available cryo-EM structure (5O66.pdb; (Wang et al., 2017)), the results of which were
211 cross-validated manually and obvious steric clashes removed using Coot (Emsley et
212 al., 2010). Additional structural analysis and visualisation was performed with Pymol
213 (PyMOL Molecular Graphics System, Version 1.71 Schrödinger, LLC).
214
215 **Strains, plasmids and culture conditions**
216 All strains used in this work are listed in Table S1. Strains were grown in lysogeny
217 broth (LB, Merck) at 37˚C with aeration unless stated otherwise.
218
219 **Cloning of *eefABCD***
220 The *eefABC* operon was amplified from the chromosome of *E. coli* ATCC 25922
221 (NCTC 12241) using Q5 polymerase (New England Biolabs) and primers which
222 incorporated the *Nde*I and *Xho*I restriction sites (Table S2). The amplicon was cloned
223 into both pET21a (ampicillin resistant) and pET24a (kanamycin resistant) plasmids
224 (Invitrogen) which are identical aside from their resistance cassette. No IPTG induction
225 was used in this work.
226 The *eefD* gene was amplified from the *E. coli* ATCC 25922 chromosome using
227 Q5 (New England Biolabs) and primers that incorporated the *ApaLI* and *PstI* restriction
228 sites (Table S2). The amplicon was then cloned into pACYC177 (ATCC).
229
230 **Deletion of *eefABC* and *eefD* in *E. coli* ATCC 25922**
231 Deletion of the genes encoding the RND system of the *eefRABCD* operon was
232 achieved by homologous recombination (Kim et al., 2014). However, due to the size
233 of the *eefABC* operon, first *eefB* was interrupted, followed by the remaining *eefA* and
234 *eefC* genes. An *eefD* knockout was generated independently using the same method.
235 All primers are listed in Table S2.
236
237 **Determination of minimum inhibition concentration (MIC) of antimicrobials,**
238 **metals, and dyes**

239 Bacterial susceptibility to a range of antimicrobials and dyes was determined using the
240 agar doubling dilution method described by the Clinical and Laboratory Standards
241 Institute (CLSI, 2020). *E. coli* ATCC 25922 was used as a control to confirm
242 antimicrobial efficacy in line with EUCAST guidelines (EUCAST, 2021). For the
243 susceptibility to metals, bile salts and polyamines, a broth microdilution method was
244 used (EUCAST, 2021).
245
246 **Accumulation and efflux of ethidium bromide**
247 Ethidium bromide (EtBr) accumulation was measured as previously described (Smith
248 and Blair, 2014). Briefly, EtBr was added to cells and the increase in fluorescence
249 was measured over time.
250 Efflux activity was also assessed as previously described (Smith and Blair,
251 2014). Here, cells were incubated in the presence of EtBr and carbonyl cyanine m-
252 chlorophenyl hydrazone (CCCP) until fluorescence saturation was reached. Re-
253 energisation was achieved with glucose and the rate of reduction in fluorescence
254 was measured.
255
256 **Results**
257 **The *eefRABCD* operon is present in *E. coli* phylogroups associated with**
258 **infection**
259 To first understand how widespread the *eefRABCD* operon is across *E. coli,* the NCBI
260 RefSeq Genome Database was utilised. The *eefB* gene from *E. coli* SMS-3-5 was
261 aligned to genomes belonging to *E. coli* (taxid 562) using NCBI nucleotide blast. The
262 top 100 matches in the *eefB* alignment had 100% sequence coverage and ≥97.7%
263 sequence identity (data not shown), suggesting that the *eefB* gene was present more
264 widely across *E. coli*. One of the strains found to possess *eefB* was the well
265 characterised strain ATCC 25922. The *eef* operon in both *E. coli* SMS-3-5 and ATCC
266 25922 were found to be very similar and as a result, the operon from ATCC 25922
267 was used as the reference in future work.
268 Next we looked at the distribution of *eefRABCD* across *E. coli*. 20,013
269 assemblies were downloaded from the Enterobase database representing 38 STs.
270 Using MASH distances, 766 assemblies were identified as duplicates and removed
271 from the data set. The presence of *eefR, eefA, eefB, eefC* and *eefD* in the remaining

272 19,247 assemblies was determined using ABRicate (Table S3). Interestingly there
273 was a clear divide between phylogroups where the *eefRABCD* operon was identified
274 and those where it was completely absent (Fig. 1). The operon was not found in ST
275 groups belonging to phylogroups A, B1 and C, which are more traditionally classified
276 as environmental isolates. However, the *eefRABCD* operon was present in all ST
277 groups of phylogroups B2, F and G, which are strongly associated with human
278 infection and MDR. Notably, a single ST within phylogroups D and E lacked the
279 operon, ST69 and ST182 respectively. Despite these two exceptions, a distinct divide
280 between the A-B1-C and B2-D-E-F-G clades was identified, implying a clear
281 evolutionary relationship.
282
283 **The *eefRABCD* operon is highly conserved across phylogroups B2, D, E, F and**
284 **G**
285 In *E. coli* assemblies positive for an *eefRABCD* gene, the entire operon was always
286 present, with gene sequences highly conserved. Using *E. coli* ATCC 25922 as a
287 reference, gene coverage for each component of the *eef* operon was always greater
288 that 98% (Table S6). The nucleotide percentage identity varied between genes and
289 across phylogroups though despite subtle variations, *eefR, eefA, eefB* and *eefD*
290 averaged >99% overall. Interestingly, *eefC,* the gene coding for the OMF, was
291 marginally more variable than the four other genes within the operon (Table 1).
292 To contextualise the extent of *eefRABCD* conservation, the conservation of
293 *acrA, acrB* and *tolC*, which encode the critically important RND efflux pump AcrAB-
294 TolC in *E. coli*, was determined. In general, the nucleotide identity of the *eef* operon
295 was conserved to a similar level as *acrAB-tolC*, all genes (excluding *eefC* in ST354)
296 were >97% identical to those in *E. coli* ATCC 25922 (Table 1). However, conservation
297 of both the *acrAB-tolC* and *eefABC* RND systems varied slightly at both the ST and
298 phylogroup level. This suggests a strong selection pressure on the operon, indicating
299 an important biological function.
300 Phylogroup B2 contains many clinically significant *E. coli* STs including the
301 MDR ST131. Across phylogroup B2, *eefB was* generally more conserved than *acrB*
302 with the *eef* genes having higher homology to the reference than the *acrAB-tolC*
303 genes. However, in phylogroups D, E and F the opposite was seen as the *acrAB-tolC*
304 genes were more conserved than the *eefRABCD* genes. Though only one phylogroup

305 G ST was used in the analysis, in ST738 *eefR, eefC* and *eefD* had the highest
306 homology to the reference, with *acrA, acrB, eefA* and *eefB* all equally conserved.
307
308 **Phylogroups A, B1 and C have highly conserved *sapF-fabI* intergenic region in**
309 **place of *eefRABCD***
310 While *eefRABCD* was found to be highly conserved across phylogroups B2, D, E, F
311 and G, it was completely absent from phylogroups A, B1 and C. To explore this further
312 RefSeq genomes of strains with and without the operon were downloaded from NCBI
313 and aligned using EasyFig (Fig. 2). In assemblies encoding the *eef* operon, it was
314 always found at the same genomic location; that is between the essential gene *fabI*
315 and the non-essential gene *sapF*. FabI is an enoyl- [acyl-carrier-protein] reductase
316 that is involved in fatty acid production (Bergler et al., 1994), while SapF is a putrescine
317 export protein belonging to the *sapBCDF* system (Sugiyama et al., 2016).
318 Interestingly, in assemblies where the *eefRABCD* operon was absent, a hypothetical
319 gene, *ycjD,* was annotated as present in the same genomic location. The *ycjD* open
320 reading frame (ORF) was 354 nucleotides long and ran in the opposite orientation to
321 *eefRABCD*.
322 A larger alignment of the 97 assemblies from phylogroups A, B1 and C (and
323 ST69 and ST182) used to construct the phylogenetic tree demonstrated that the *ycjD*
324 hypothetical gene is highly conserved across phylogroups A, B1 and C
325 (Supplementary Data file). The alignment of the *sapF-ycjD-fabI* region also
326 demonstrated that ST69 and ST182 possess an intergenic region highly homologous
327 to the STs belonging to phylogroups A, B1 and C, though subtle differences were
328 present (Fig. 3). The open reading frame annotated as *ycjD* in K-12, is 12 nucleotides
329 longer in both ST69 and ST182 assemblies (Fig. 3). Taken together, these findings
330 suggest that *eef* has been lost from *E. coli* on at least two independent instances.
331
332 **Distribution of *eefRABCD* in Gram-negative bacteria**
333 To see if the *eefRABCD* operon was present more widely across *Escherichia* species,
334 the ATCC 25922 *eef* operon was aligned to sequences from the *Escherichia* genus
335 (taxid 561), with *E. coli* (taxid 562) sequences excluded, using the NCBI RefSeq
336 database. Only *Escherichia marmotae* and *Escherichia albertii* were found to possess
337 the *eefRABCD* operon (Fig. S1) while *E. fergusonii, Escherichia ruysiae*, *Escherichia*
338 *vulneris, and E. hermannii* did not encode it.

339 Due to the genetic similarity of *Shigella* and *E. coli* (van den Beld and Reubsaet,
340 2012, Brenner et al., 1972), the presence of the *eef* operon across *Shigella spp*. was
341 investigated. Gene fragments were detected in a small number of *S. boydii* and *S.*
342 *flexneri* assemblies but only *S. dysenteriae* was found to consistently possess the
343 operon (Fig. S2, Table S5). However, whilst genes belonging to the *eefRABCD* operon
344 were detected in 363 of the 486 *S. dysenteriae* assemblies included in this study, only
345 a single assembly was positive for *eefR.* Moreover, whilst *eefA* was present in all
346 assemblies possessing the operon, sequence coverage averaged at 24.6%. Assembly
347 annotation identified the presence of an insertion sequence in the place of *eefR* and
348 *eefA* (Fig. S3), explaining the absence and truncation of *eefR* and *eefA* respectively,
349 across the *S. dysenteriae* assemblies. In comparison, no insertion sequences were
350 detected within the *eef* operon of *E. coli* ATCC 25922, nor in the 10,000 base pairs
351 up- or down-stream of the operon.
352 As the EefABC efflux pump has been reported in both *K. aerogenes* and *K.*
353 *pneumoniae* (Coudeyras et al., 2008, Masi et al., 2005) with differing operon
354 architecture (Fig. S4), the presence of the operon across related
355 Gammaproteobacterial genera was determined. Only low homology orthologs of EefA
356 and EefB were identified in *Yersinia* and *Serratia* while no conserved homologues
357 were identified in *Salmonella*, *Acinetobacter*, *Pseudomonas* or *Photorhabdus* (Table
358 S6). In *Enterobacter,* conservation of the operon differed between species (Fig. S5).
359 Some species, such as *E. chengduensis*, encoded *eefRABC,* but not *eefD*, however
360 sequence identity was only 73% (blastn) indicating that the *E. coli* and *Enterobacter*
361 *eef* operons are not homologous, and instead the association is likely due to historical
362 literature and gene nomenclature.
363
364 **Sequence and structural prediction analysis reveals unique features of the**
365 **EefABC pump**
366 The *eefABC* operon appears to encode a tripartite RND-efflux pump similar to the
367 AcrAB-TolC assembly in *E. coli*. As there is no experimental structural information
368 currently available on any of the components of EefABC, we conducted sequence
369 analysis to identify potential structural templates and subsequently performed
370 homology modelling using the highest scoring templates.
371 Comparison with other OMFs of known structure, revealed the closest relatives
372 within the wider OMF-family to be the *Pseudomonas* proteins OprM and OprJ (Fig.

373 S6), followed by CusC, and hence OprM/J were used as structural templates for
374 homology modelling (Fig. S7 and S8 and supplementary text). Structural alignment of
375 EefC with OprM/J produced an alignment with very few gaps allowing for direct
376 mapping of the aligned sequences of EefC onto OprM/J. EefC has significantly shorter
377 extracellular loops in comparison to TolC, in particular the L2 loop which occludes
378 TolC opening (Vaccaro et al., 2008), suggesting a more-open state of the EefC
379 channel (Fig. 4a). Such OMF loops are prominent targets of protective antibodies
380 (Domínguez-Medina et al., 2020) and the non-protruding loops may serve to avoid
381 antibody restriction and LPS occlusion. Additionally, the N-terminal tail of EefC is 30
382 residues longer than that seen in TolC, making it more similar to the structure of
383 OprN/J (Fig. S8 and Fig. S9).

384 The comparison of the gating-loop regions in EefC and TolC reveals that they
385 are highly divergent, indicating a locking mechanism markedly different from that
386 observed in TolC, and hence EefC is unlikely to function with any PAPs that normally
387 pair with TolC, and likely only interacts with its cognate PAP EefA. Specifically, several
388 key residues in the helix7/helix8 hairpin of TolC, responsible for gating the TolC
389 channel are different in EefC, e.g. the R367 (TolC), forming part of the so-called
390 "primary gate" that seals the TolC channel by binding to the conserved D153 (TolC;
391 D206 in EefC) and thus anchoring to the helix 4 (Andersen et al., 2002a, Bavro et al.,
392 2008), in EefC is substituted by a small non-polar residue G412, making such
393 interaction impossible (Fig. S8).

394 In addition, the electrostatic properties of the EefC channel are predicted to be
395 dramatically different to TolC and other OMFs, which is likely to impact heavily the ion
396 and drug selectivity of the channel (Marshall and Bavro, 2020). Firstly, the so-called
397 "secondary gate" of the channel, formed of the prominently conserved double
398 aspartate ring (D371; D373) which forms the basis of cation-selectivity in TolC
399 (Andersen et al., 2002b, Schulz and Kleinekathöfer, 2009), is fully absent in EefC and
400 instead is substituted by small-hydroxylated residues (T416 and T419), a feature
401 which appears to be unique to EefC. Secondly, EefC possesses additional bulky
402 aliphatic residues L415 and L418, which effectively hydrophobically seal the
403 periplasmic end of the channel (Fig. S8).

404 The PAP EefA was analysed in a similar way, revealing a sequence identity of
405 53.85% to the *Pseudomonas* MexA; but only 50.13% against the *E. coli* AcrA. Due to
406 the availability of more complete full-length experimental templates, EefA homology

407 models were created using both MexA (2v4d.pdb; (Symmons et al., 2009)) and AcrA
408 (5v5s.pdb; (Wang et al., 2017)) as templates, (utilising both I-TASSER and Swiss-
409 model tools) (Fig. 4b; Fig. S10). While neither of the models delivered the same level
410 of confidence as those for EefC, the alignments with the known PAP structures are
411 readily interpretable, allowing identification of the protein features. A detailed
412 discussion of the EefA structure is given in the Supplementary text with the major
413 findings summarised here.

414 Alignment of EefA and AcrA results in a direct amino-acid match with only 2
415 gaps in the alignment, one in the unstructured N-terminal tail, and another at position
416 Q221 (EefA), which has a 4-residue-long deletion relative to AcrA (Fig. 4b; Fig. S10**)**.
417 This region corresponds to the C-terminal end of α-helix 3, which is flanking the β-
418 barrel domain in PAPs, however it is not predicted to affect RND-binding (McNeil et
419 al., 2019). Despite overall similarity with MexA/AcrA there are distinctive differences
420 in the organisation of the EefA, notably in its α-hairpin domain (Fig. 4b; Fig. S11).
421 While the RLS(D) motif, which is thought to be critical for PAP-OMF interaction (Kim
422 et al., 2010, Song et al., 2014, Alav et al., 2021) appears to be preserved in EefA,
423 (R120/L124/S131/D136), there are a number of significant changes in the adjacent
424 residues, notably R123 (K131 in AcrA); V125 (L133); D134 (E142), which would likely
425 result in steric clashes that would preclude direct compatibility with TolC, and such
426 residues are likely to be playing a discriminatory role engaging with EefC (Fig. S11).

427 The RND-transporter component, EefB, is a large integral membrane protein,
428 predicted to form a functional trimer similar to other transporters in the family (Fig. 4c).
429 Alignment of the EefB with other RND transporters of known function including AcrB
430 (*E. coli*); MtrD (*N. gonorrhoeae*); MexB (*P. aeruginosa*); CusA (*Campylobacter jejuni*)
431 and AdeB (*A. baumanii*) revealed that they are highly similar, with EefB being the most
432 similar to AcrB and MexB (57.0% and 56.8% identity respectively), while CusA and
433 MtrD being most divergent (Fig. S12). The MexB structure was used as a template to
434 generate a high-fidelity homology model of EefB, as there were fewer gaps in the
435 alignment (0.5% vs 0.7% for AcrB) and slightly higher residue overlap with EefB (1038
436 *vs* 1033 residue overlap respectively).

437 Overall, the alignment of EefB with MexB (and AcrB) produces very few gaps
438 (the longest is 2 residues long), allowing for unequivocal attribution of secondary
439 structure elements (Fig. S12). As can be seen from the side-by-side comparison of
440 the EefB and AcrB, both present a virtually identical architecture (Fig. S13), with the

441 only notable differences being the shortened loop connecting the TM helices α16 and
442 α17 in EefB (residues 498-507); and the shorter C-terminal tail. Consistent with this,
443 the critical proton-relay residues found in MexB D407, D408, K939 and T976 (Guan
444 and Nakae, 2001) are conserved in EefB (D408, D409, K935 and T972 respectively)
445 and the predicted structures of the access and distal binding pockets of EefB suggest
446 a closer relation to the MexB/AcrB than to MexY type transporters.

447 The last member of the operon is EefD, a member of the MFS family of efflux
448 pumps that according to the Transporter Classification Database (TCDB) belongs to
449 the 2.A.1.2 group of MFS transporters, related to MDR-function, which also includes
450 Bcr/CflA, EmrD and MdfA (Reddy et al., 2012, Quistgaard et al., 2016) (Fig. S14). This
451 resulted in high-confidence homology models (I-TASSER C-score = 1.82), revealing
452 a classic 6+6 transmembrane helical arrangement closely related to the general
453 topology of MdfA, although with significant differences in the substrate binging cavity,
454 suggesting the substrate range will be notably different between EefD and MdfA (Fig.
455 4d; Fig. S15) (Adler and Bibi, 2002, Heng et al., 2015, Nagarathinam et al., 2018).
456 These discrepancies make predictions of the possible substrates of EefD problematic,
457 although some overlap with MdfA can be expected (Lewinson et al., 2003), e.g.
458 lipophilic cations such as ethidium.

459 We used the homology models described above to dock the components into
460 a complete tripartite pump using the cryo-EM structures of AcrAB-TolC (Wang et al.,
461 2017) as a guide. EefABC can indeed be assembled using the same architecture with
462 minimal steric clashes, as can be seen in Fig. 5.

463

464 ***E. coli* EefABC is not a drug transporter**

465 The high levels of conservation of the *eefRABCD* operon within clinically relevant
466 lineages of *E. coli* suggests it has an important biological function and its substrate
467 profile may differ from other *E. coli* RND pumps due to its unique structure.
468 Antimicrobials are known substrates of RND efflux pumps such as AcrB, and
469 overexpression of RND pumps can confer MDR in both the laboratory and clinic.
470 Therefore, the effect of *eefABC* and *eefD* expression on *E. coli* susceptibility to a range
471 of antimicrobials and dyes was determined. Due to the previously mentioned
472 homology between *E. coli* ATCC 25922 and *E. coli* SMS-3-5, and the well
473 characterised antimicrobial susceptibility profile of ATCC 25922 this strain was used
474 for EefABC characterisation experiments.

475 Deletion of *eefB* in *E. coli* ATCC 25922 did not increase susceptibility to any
476 antimicrobial or dye tested. Subsequent inactivation of *eefA* and *eefC* to give an
477 *eefABC* knockout also had no effect on the susceptibility of *E. coli* ATCC 25922 to
478 antibiotics (Table 2).
479 As loss of EefABC function did not alter the drug susceptibility profile of ATCC
480 25922, *eefABC* was cloned into the pET21a and pET24a plasmids and expressed in
481 *E. coli* MG1655, which does not naturally encode the system. Expression of *eefABC*
482 in *E. coli* MG1655 did not alter susceptibility to any of the antimicrobials tested. AcrB
483 is the dominant RND pump in *E. coli* and this can mask phenotypic changes
484 associated with other efflux systems so it was deleted. However, expression of
485 *eefABC* in the absence of *acrB* still did not reveal any significant changes in
486 susceptibility but did result in a decrease in susceptibility to ethidium bromide and
487 rhodamine 6G suggesting these dyes can be transported by the pump (Table S7).
488 The MFS pump EefD was also characterised, however both inactivation in *E.*
489 *coli* ATCC 25922 (loss of function) and expression in *E. coli* MG1655 (gain of function)
490 had no effect on antimicrobial and dye susceptibilities. Expression of *eefD* alone in
491 MG1655 Δ*acrB* was also not found to affect MICs.
492 A subset of the RND pumps (including CusABC) are known to pump metal ions
493 and form the subfamily of heavy metal efflux (HME)-pumps (Gupta et al., 1999, Long
494 et al., 2012, Lecointre et al., 1998, Klenotic et al., 2020). Due to the structural similarity
495 of EefC to CusC, the susceptibility to heavy metals was also measured. Yet when
496 either *eefB* or *eefABC* were deleted from ATCC 25922, the susceptibility to cobalt,
497 nickel, zinc and iron were not significantly different to the wild-type ATCC 25922.
498
499 **EefABC can export ethidium bromide**
500 To confirm the cloned EefABC pump is functional, and that ethidium bromide is a
501 substrate, the intracellular accumulation and efflux rate of ethidium bromide was
502 measured. Expression of EefABC with EefD in the absence of AcrB significantly
503 decreased intracellular accumulation of EtBr and increased the rate at which EtBr was
504 pumped out of cells (Fig. S16 and S7). In addition, deletion of *eefD* significantly slowed
505 efflux of ethidium bromide, which together, shows this inner membrane pump is
506 needed for transport of the substrate across the inner membrane.
507

508 **Discussion**

509 The number of RND pumps present in different Gram-negative bacterial species
510 varies and there is increasing evidence that prevalence of RND efflux pumps can also
511 vary between isolates of the same species (Darby et al., 2023, Leus et al., 2020,
512 Nowak et al., 2015, Nemec et al., 2007, Wieczorek et al., 2013). Yet it is still broadly
513 assumed that all *E. coli* isolates possess six RND efflux pumps despite recent work
514 showing that not all six pumps are always functional (Anes et al., 2015, Pugh et al.,
515 2023). Here we further demonstrate that the assumption of six RND pumps is
516 inaccurate; STs belonging to the phylogroups of *E. coli* that are most commonly
517 associated with invasive infection (B2, D, E, F and G) encode a seventh, highly
518 conserved RND pump operon (*eefRABCD*), while the operon was completely absent
519 from phylogroups A, B1 and C, which are generally associated with environmental or
520 commensal lifestyles. The level of conservation suggests that EefRABCD has a
521 biologically important function resulting in a high degree of selection pressure while
522 the distribution in phylogroups commonly associated with infection suggests the
523 system could have a role in infection or survival in the host environment.

524 Across phylogroups A, B1 and C a 354-nucleotide ORF is found in place of
525 *eefRABCD.* This putative gene *ycjD* runs in the opposite orientation to *eefRABCD* and
526 is highly conserved between STs of these phylogroups. This putative gene is also
527 present in ST69 and ST182 which belong to phylogroup D and E respectively though
528 in these two STs a 12-nucleotide insertion is present at the 3' end of the gene. Studies
529 from another group support the hypothesis that transcriptional activity is happening at
530 this ORF as public data from their transcriptional start site, term-seq and ribosomal
531 profiling studies show antisense transcriptional and translational activity at the 5' end
532 of *ycjD* gene (Adams et al., 2021, Thomason et al., 2015, Weaver et al., 2019). It is
533 worth noting, that while *ycjD* is indeed coding a domain of unknown function (DUF),
534 the UniproKB AlphaFold structural prediction for it strongly suggests that it is linked to
535 DNA modification/restriction, as it belongs to the endonuclease/DNA methylase fold.

536 The EefABC efflux pump was first described in the opportunistic human
537 pathogen *K. aerogenes* and subsequently in *K. pneumoniae*, though operon structure
538 differs between the two *Klebsiella* species (Coudeyras et al., 2008, Masi et al., 2005).
539 In *K. aerogenes* overexpression of *eefABC* decreased susceptibility to erythromycin
540 and ticarcillin (Masi et al., 2006) whilst overexpression of *K. pneumoniae eefA* and
541 *eefB* in an *E. coli* (*ΔacrAB, ΔydhE*) background decreased susceptibility to oxacillin,

542 erythromycin, novobiocin, acriflavine, ethidium bromide and cholate (Ni et al., 2020).
543 However, in this study, neither gain nor loss of *eefABC* function from *E. coli* altered
544 susceptibility to any antibiotics tested, though over-expression did decrease
545 susceptibility to ethidium bromide. Moreover, expression of *eefABC* (in the absence of
546 AcrB) decreased intracellular accumulation of ethidium bromide demonstrating that
547 the pump is functional. No effect was seen upon inactivation of the pump but this is
548 likely because AcrB was still present which would mask any effect. However, deletion
549 of the inner membrane component *eefD* alone, caused significantly slower EtBr efflux
550 even in the presence of AcrB suggesting it has a significant role in transport of
551 substrates across the inner membrane. The apparent difference in substrate profile
552 between *Klebsiella* and *E. coli* could be due to differences in nucleotide sequence or
553 operon structure subsequently altering the regulation of the operon or function of the
554 orthologous protein. In *K. aerogenes* the operon has been demonstrated to be H-NS
555 silenced in laboratory conditions (Masi et al., 2006, Masi et al., 2005), though due to
556 the genomic location of the *eef* operon in *E. coli* and the resulting proximity of the *eefR*
557 promoter to that of *fabI* which encodes an essential enoyl- [acyl-carrier-protein]
558 reductase it is unlikely that the operon is silenced *in vivo* in *E. coli.*
559 When looking across the *Escherichia* genus, *eefRABCD* was only present in *E.*
560 *marmotae* and *E. albertii,* though the operon was identified in *S. dysenteriae,* which
561 has high genetic similarity to *E. coli.* Here, *eefR* was generally absent due to the
562 presence of an insertion sequence in place of *eefR* and *eefA.* A further insertion
563 sequence was identified downstream of *eefD.* It has been shown previously that
564 *Shigella* species have higher numbers of insertion sequences when compared to other
565 Gammaproteobacteria such as *E. coli* (Touchon and Rocha, 2007). High numbers of
566 insertion sequences is linked to recent host-specification as the integration of IS
567 elements into the genomes is associated with early stage genome degradation (Moran
568 and Plague, 2004, Hawkey et al., 2020, Yang et al., 2005). The presence of an
569 insertion sequence in place of *eefR* and *eefA* and downstream of *eefD* may therefore
570 suggest the operon is in the process of being lost or degraded in *S. dysenteriae*. Many
571 Gram-negative bacteria including *Salmonella* sp. (taxid 590) did not encode an
572 ortholog of the Eef system but orthologs of EefA and EefB were identified in *Serratia*
573 and *Yersinia* species, suggesting related systems could be dispersed through the
574 Gammaproteobacteria.

575 Overall, our analysis of EefABC revealed unexpected similarities to the tripartite
576 pumps OprM-MexAB and OprJ-MexCD in *Pseudomonas* (Masuda et al., 2000), rather
577 than to any endogenous *E. coli* paralogues, which may suggest that these pumps have
578 been acquired *via* a lateral gene transfer event, similar to some other plasmid encoded
579 efflux/secretion systems e.g. the EAEC virulence plasmid pAA2 (Imuta et al., 2008).
580 Given the close similarities of the EefABC system to both MexAB-OprM and
581 MexCD-OprJ and based on previous profiling of these *Pseudomonas* pumps (Masuda
582 et al., 2000), we initially hypothesised that these pumps could have overlapping
583 substrate ranges. However, despite the overall similarity, our tests did not reveal any
584 direct effect of EefABC on transport of antimicrobials, suggesting alternative function.
585 As *E. coli* also possesses HME-RND systems such as CusABC we also
586 investigated the possibility that EefABC may be involved in metal ion tolerance, but
587 we were unable to identify a metal ion substrate for the efflux pump (Table 8). As
588 mentioned above, the EefC lacks the double aspartate "gates" which have been
589 implicated in the coordination of metal ions in the case of TolC (Higgins et al., 2004,
590 Bavro et al., 2008) (Fig. S8). This alone however could not be used to rule out ion-
591 efflux function, as it has previously been noted (Kulathila et al., 2011) that CusC does
592 not (Alcalde-Rico et al., 2016)by itself show any specific features associated with
593 monovalent ion selectivity, with that function rather being associated with the RND
594 transporter, in that case CusA. Indeed, while CusA presents a clear relay of
595 methionine clusters to bind and export Cu(I) and Ag(I) ions (Delmar et al., 2013), there
596 is no identifiable pattern of such residues in the case of EefB. On the other hand,
597 divalent-ion specific pumps such as ZneA, have more restricted binding sites formed
598 from negatively charged residues (Pak et al., 2013), but again, the residues
599 participating in ion coordination are not conserved in EefB.
600 Aside from conferring resistance to antimicrobials, RND efflux pumps have
601 myriad other physiological roles involving export of both exogenous and endogenous
602 substrates (Henderson et al., 2021). For example, RND efflux pumps in various Gram-
603 negative bacteria have been linked to virulence (Wang-Kan et al., 2017, Nishino et al.,
604 2006). While we have so far been unable to assign clear function to EefRABCD, given
605 the striking distribution and conservation of *eefRABCD* only in phylogroups of *E. coli*
606 associated with disease it is possible that this pump has roles associated with
607 virulence or survival in the host environment and work is continuing to assign biological
608 function.

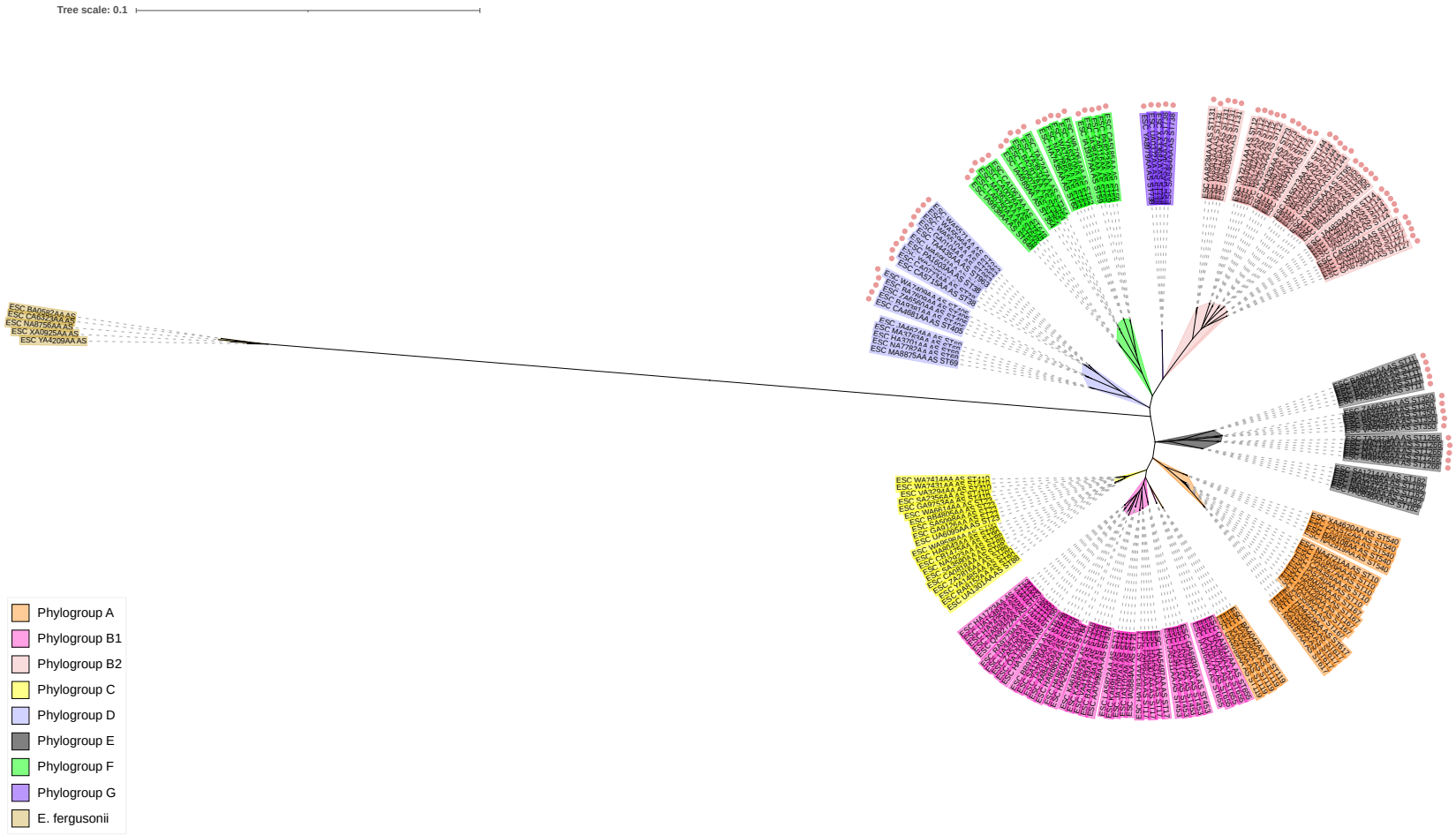

Figure 1. Phylogenetic structure of the assemblies used in this analysis and the distribution of *eefRABCD*

The tree was created using five assemblies per ST (exception ST84 *n=2*). Assemblies were chosen randomly. The tree was rooted using five *E. fergusonii* assemblies. The leaves are annotated with the ST group of the assembly and coloured coded by phylogroup, colours used to highlight phylogroups are shown in the legend on the bottom left corner. STs positive for the *eefRABCD* operon are marked with ●. Tree was generated using RaXmL and visualised on iTol.

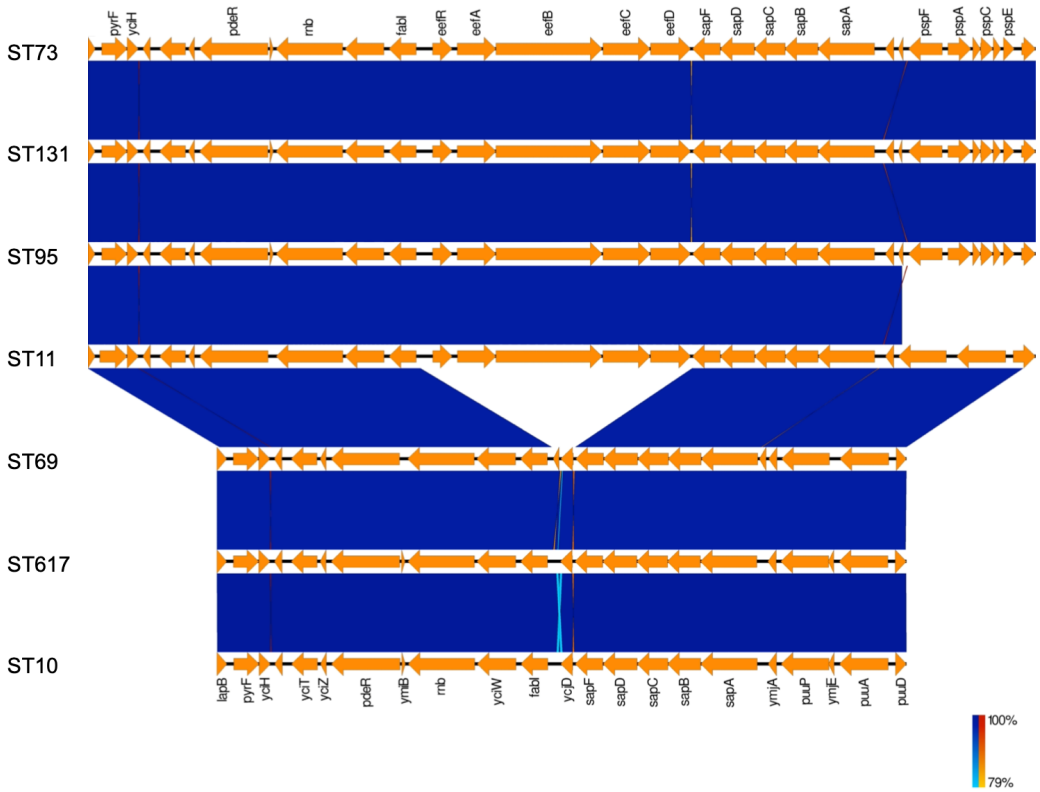

Figure 2. The genomic region of strains with and without *eefRABCD*. EasyFig alignment of the *eef* region in ST73 (B2), ST131 (B2), ST95 (B2), ST11 (E), ST69 (D), ST617 (A), ST10 (A). The *eefRABCD* operon was consistently identified between *fabI* and *sapF.*

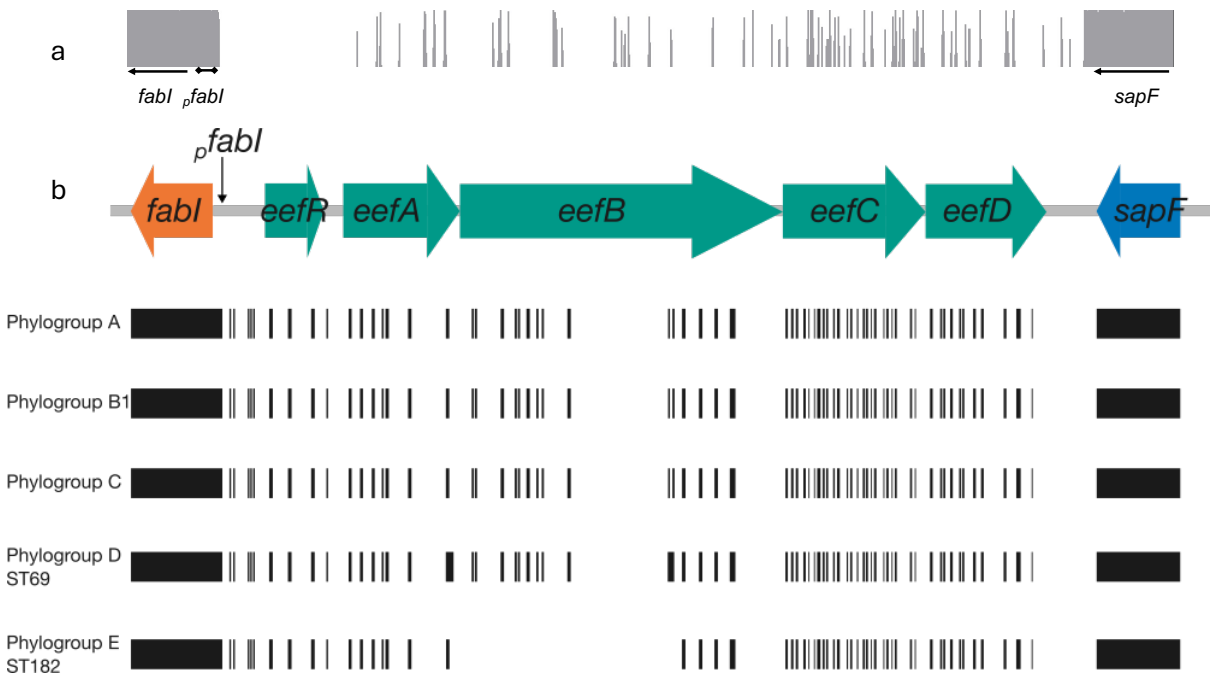

Figure 3. Diagrammatic representation of *eefRABCD* homology in strains that lack the operon.

In *E. coli* ST positive for *eefRABCD*, the operon was consistently located between *fabI* and *sapF*. To determine whether *E. coli* assemblies that lacked the operon had conserved regions of the *eef* operon, the equivalent *fabI-sapF* intergenic region of assemblies negative for *eefRABCD* were aligned with *fabI-eefRABCD-sapF* from *E. coli* ATCC 25922. **A) Consensus identity of the *fabI-sapF* intergenic region in *E. coli* that lack the *eef* operon.** Between all STs, and despite phylogroup, the *fabI-sapF* intergenic regions aligned to the *eefRABCD* operon in a highly conserved manner. **B) Cartoon representation of operon fragment conservation in *E. coli* ST that lack the *eef* operon.** Closer inspection of the alignment (Supplementary file 1) found that phylogroups A, B1 and C had almost identical *fabI-sapF* intergenic regions, while ST69 and ST189 had marginally different homology patterns. Taken together, these data suggest the operon may have been lost in up to three independent events.

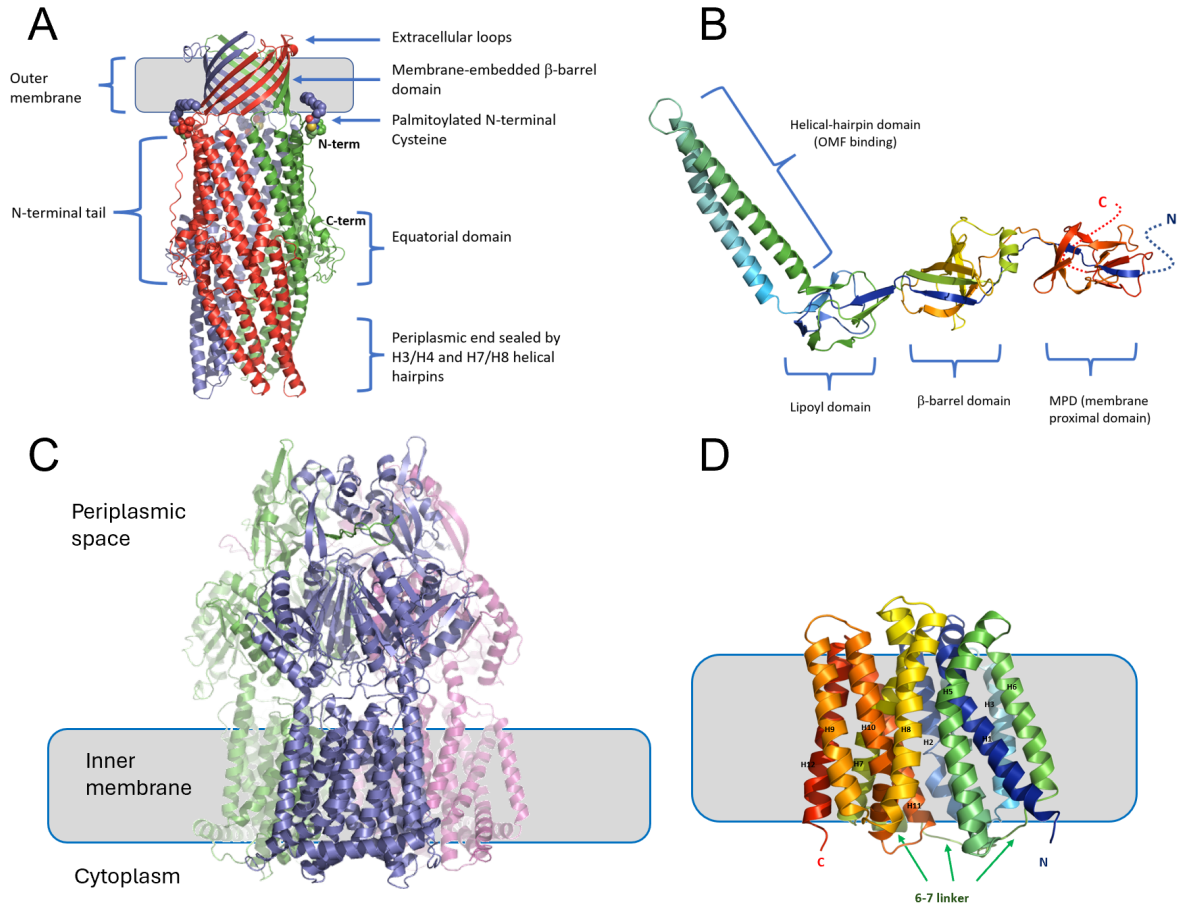

Figure 4. Multi-panel representation of the Eef components. Not to scale.

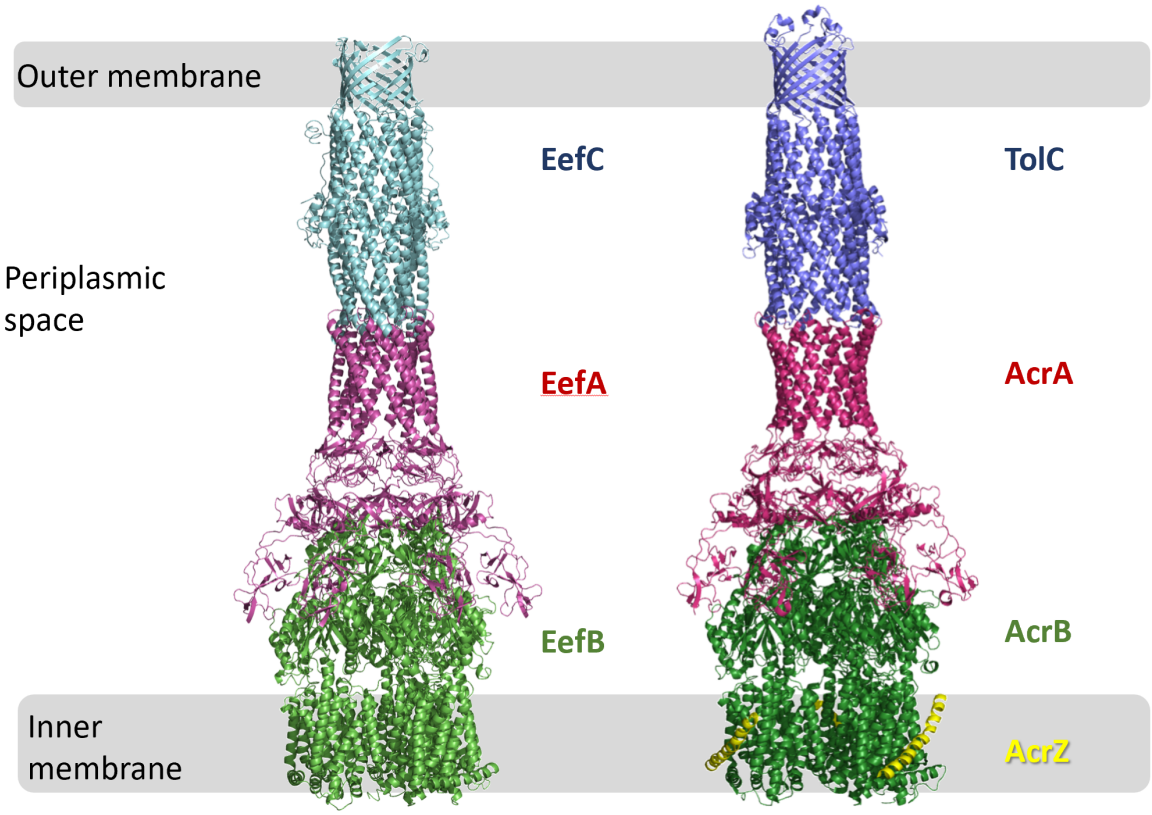

Figure 5. Comparison of the predicted structures of the assembled EefABC and the experimental cryo-EM structure of AcrABZ-TolC (based on 5066.pdb; (Wang et al., 2017))

Table 1. Conservation of *acrAB-tolC* and *eefRABCD* across *E. coli* phylogroups

Phylogroup	ST group	Mean nucleotide identity to *E. coli* ATCC 25922 (%)							
		eefR	*eefA*	*eefB*	*eefC*	*eefD*	*acrA*	*acrB*	*tolC*
B2	ST12	99.82	99.98	99.68	99.48	99.40	99.83	98.70	98.95
	ST127	99.12	99.64	99.61	99.42	99.48	99.75	99.46	98.57
	ST131	99.65	99.54	99.58	99.13	99.48	99.75	99.49	98.83
	ST14	98.91	98.49	99.41	99.12	99.57	99.83	99.70	98.83
	ST144	99.65	99.91	99.74	99.70	99.74	99.83	99.71	98.79
	ST73	100.00	100.00	100.00	100.00	99.97	100.00	100.00	99.93
	ST95	99.82	99.91	99.77	99.45	99.48	99.83	98.73	99.05
D	ST38	99.12	98.65	98.42	97.39	98.96	99.25	98.69	98.43
	ST405	99.45	98.32	99.00	99.20	99.14	99.33	98.67	98.05
	ST69	-	-	-	-	-	99.16	98.76	98.29
	ST963	99.12	98.66	98.42	97.38	98.97	99.25	98.70	98.65
E	ST11	99.12	97.95	98.06	97.53	98.37	99.33	98.53	98.52
	ST182	-	-	-	-	-	99.17	98.64	98.38
	ST350	99.47	98.03	98.38	97.62	98.25	99.08	98.70	98.44
	ST1266	99.47	98.22	98.46	97.57	98.19	99.08	98.76	98.65
F	ST354	98.93	98.84	99.52	97.07	99.66	99.08	98.97	98.37
	ST59	98.93	99.11	99.10	99.01	99.48	99.05	98.78	98.43
	ST62	99.12	98.75	99.39	98.76	99.23	99.15	98.79	98.10
	ST648	99.65	99.47	99.03	98.91	99.47	99.00	98.73	97.53
G	ST738	99.65	98.84	98.91	99.34	99.83	98.86	99.14	98.52
Average gene conservation		**99.39**	**99.02**	**99.14**	**98.67**	**99.26**	**99.38**	**98.98**	**98.56**

The extent of gene conservation for *acrA, acrB, tolC, eefR, eefA, eefB, eefC,* and *eefD* across phylogroups B2, D, E, F, and G are highlighted as follows ≥96% , ≥97% ≥98% ≥99%

Table 2. **Susceptibility of *E. coli* to antimicrobials following loss and gain of *eefABC* and *eefD***

																				MIC (μ
Strain	**AZT**	**BAC**	**CAR**	**CEF**	**CHL**	**CIP**	**CLI**	**ERY**	**EB**	**FA**	**GEN**	**MER**	**MOX**	**NA**	**NOV**	**RIF**	**SPE**	**TET**	**TIC**	
ATCC 25922	0.12	32	16	0.06	4	0.03	128	64	512	512	0.5	0.016	0.03	2	32	>32	8	1	8	
ATCC 25922 ΔeefB	0.12	32	16	0.06	4	0.016	128	64	512	512	0.5	0.016	0.03	2	32	>32	8	1	8	
ATCC 25922 ΔeefABC	0.25	32	16	0.06	2	0.03	128	64	512	512	0.5	0.016	0.03	2	32	>32	8	1	8	
ATCC 25922 ΔeefD	0.12	32	16	0.06	8	0.06	256	64	512	1024	0.5	0.016	0.06	8	64	>32	8	1	8	
MG1655 + pET21a	0.12	64	>1024	0.06	8	0.016	128	32	1024	512	0.12	0.03	0.06	8	128	>32	4	1	>1024	
MG1655 + pET21a *eefABC*	0.12	64	>1024	0.06	8	0.016	128	32	1024	1024	0.25	0.03	0.06	8	128	>32	4	1	>1024	
MG1655 + pET24a	0.06	64	8	0.06	8	0.016	128	32	1024	512	0.25	0.03	0.03	8	128	>32	8	1	4	
MG1655 + pET24a *eefABC*	0.06	64	8	0.06	8	0.016	128	32	1024	512	0.25	0.016	0.06	8	128	>32	4	1	4	
MG1655 + pACYC177	0.06	64	>1024	0.06	8	0.016	128	32	1024	1024	0.5	0.03	0.06	8	128	>32	4	1	>1024	
MG1655 + pACYC177 *eefD*	0.06	64	8	0.06	8	0.016	128	32	1024	1024	0.5	0.03	0.06	8	128	>32	4	1	4	
MG1655 ΔacrB + pET21a	0.12	4	>1024	<0.016	1	0.008	2	2	8	8	0.12	0.03	<0.008	2	2	32	4	0.25	>1024	
MG1655 ΔacrB + pET21a *eefABC*	0.06	8	>1024	0.03	1	0.008	2	2	**32**	16	0.12	0.03	<0.008	2	4	32	4	0.25	>1024	
MG1655 ΔacrB + pET24a	0.12	4	8	<0.016	1	0.008	2	2	8	8	0.25	0.03	<0.008	2	2	32	4	0.25	4	
MG1655 ΔacrB + pET24a *eefABC*	0.06	8	8	<0.016	2	0.008	2	4	**32**	16	0.25	0.03	<0.008	2	4	32	4	0.25	4	
MG1655 ΔacrB + pACYC177	0.12	4	>1024	<0.016	1	0.008	2	2	8	8	0.5	0.03	<0.008	2	2	32	4	0.25	>1024	
MG1655 ΔacrB + pACYC177 *eefD*	0.12	4	8	<0.016	1	0.008	2	2	8	8	0.5	0.03	<0.008	2	2	32	4	0.25	4	
MG1655 ΔacrB + pET21a eefABC pACYC177 *eefD*					2	0.008														

AZT – aztreonam, BAC – benzalkonium chloride, CAR – carbenicillin, CEF – cefotaxime, CHL – chloramphenicol, CIP – ciprofloxacin, CLI – clindamycin, ERY – erythromycin, EB – ethidium bromide, FA -fusidic acid, GEN – gentamicin, MER – meropenem, MOX – moxifloxacin, NA – nalidixic acid, NOV – novobiocin, RIF – rifampicin, SPE – spectinomycin, TET – tetracycline, TIC – ticarcillin

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Competing interest statement

None to declare