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# Temocillin Resistance in the *Enterobacter cloacae* Complex Is Conferred by a Single Point Mutation in BaeS, Leading to Overexpression of the AcrD Efflux Pump

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1 **Temocillin resistance in *Enterobacter cloacae* complex is conferred by a single point**  
2 **mutation in BaeS leading to an overexpression of AcrD efflux pump**

3

4 Running title: Temocillin resistance in ECC

5

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18

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20

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23

24 **ABSTRACT**

25 *Enterobacter cloacae* complex (ECC) has become a major opportunistic pathogen with antimicrobial  
26 resistance issues. Temocillin, an 'old' carboxypenicillin remarkably stable towards  $\beta$ -lactamases, has  
27 been used as an alternative for the treatment of multidrug-resistant ECC infections. Here, we aimed  
28 at deciphering the never-investigated mechanisms of temocillin resistance acquisition in  
29 *Enterobacterales*. By comparative genomic analysis of two clonally-related ECC clinical isolates, one  
30 susceptible (Temo\_S; MIC of 4 mg/L), and the other resistant (Temo\_R; MIC of 32mg/L), we found  
31 that they only differed by 14 SNPs including one non-synonymous mutation (Thr175Pro) in the two-  
32 component system (TCS) sensor histidine kinase BaeS. By site-directed mutagenesis in *E. coli* CFT073,  
33 we demonstrated that this unique change in BaeS was responsible for a significant (16-fold) increase  
34 in temocillin MIC. Since the BaeSR TCS regulates the expression of two RND-type efflux pumps  
35 (namely AcrD and MdtABCD) in *E. coli* and *Salmonella*, we evidenced by qRT-PCR that *mdtB*, *baeS*,  
36 and *acrD* genes were significantly overexpressed (15-, 11-, and 3-fold, respectively) in Temo\_R. To  
37 confirm the role of each efflux pump in this mechanism, multi-copy plasmids harboring *mdtABCD* or  
38 *acrD* were introduced into either Temo\_S or the reference strain *E. cloacae* subsp. *cloacae* ATCC  
39 13047. Interestingly, only the overexpression of *acrD* conferred a significant increase (from 8- to 16-  
40 fold) of the temocillin MIC. Altogether, we have shown that temocillin resistance in ECC can result  
41 from a single BaeS alteration, likely resulting in the permanent phosphorylation of BaeR leading to  
42 AcrD overexpression and temocillin resistance by enhanced active efflux.

43

## 44 INTRODUCTION

45 Widely encountered in natural environments, members of the *Enterobacter cloacae* complex  
46 (ECC) are also commensals of the gut microbiota of both animals and humans (1). The ECC  
47 presents a high genetic diversity and comprises 12 different genetic clusters: C-I (*Enterobacter*  
48 *asburiae*), C-II (*Enterobacter kobei*), C-III (*E. cloacae* subsp. *hoffmannii*), C-IV (*E. roggenkampii*),  
49 C-V (*Enterobacter ludwigii*), C-VI (*Enterobacter hormaechei* subsp. *oharae* or *E. hormaechei*  
50 subsp. *xiangfangensis*), C-VII (*E. hormaechei* subsp. *hormaechei*), C-VIII (*E. hormaechei* subsp.  
51 *steigerwaltii*), C-IX (*E. bugandensis*), C-X (*Lelliota nimipressuralis*), C-XI (*E. cloacae* subsp.  
52 *cloacae*), and C-XII (*E. cloacae* subsp. *dissolvens*) (2-4). From a clinical point of view, ECC has  
53 recently become a major opportunistic pathogen associated with many hospital-acquired  
54 infections and outbreaks. Often multidrug-resistant, ECC belongs to the ESPAKE group  
55 (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter*  
56 *baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.), which comprises nosocomial  
57 pathogens with limited antimicrobial treatment options (5). All ECC members harbor a  
58 chromosomal *bla*<sub>AmpC</sub> gene coding for an AmpC-type cephalosporinase conferring an intrinsic  
59 resistance to aminopenicillins and first- and second-generation cephalosporins (6). Due to its  
60 high genomic plasticity, ECC is also able to easily develop acquired resistances. For instance,  
61 there is a large proportion of ECC clinical isolates resistant to third-generation cephalosporins  
62 (3GC) due to AmpC overproduction and/or acquisition of plasmid-mediated extended-  
63 spectrum  $\beta$ -lactamases (ESBLs) (7). Therefore, only a limited number of carbapenem-sparing  
64 therapeutic options remain for the treatment of 3GC-resistant ECC infections, including  
65 neglected and disused antibiotics such as colistin, fosfomycin, and temocillin (8).

66 Temocillin, a 6- $\alpha$ -methoxy derivative of ticarcillin, is an antibiotic commercialized in the early  
67 1980s and only used in a few European countries (Belgium, France, Luxembourg, and the UK)

68 mainly due to the concomitant marketing of 3GCs (8,9). The use of this antibiotic in clinics has  
69 been based on its multiple advantageous characteristics such as (i) a narrow spectrum almost  
70 restricted to *Enterobacterales*, (ii) a remarkable stability towards hydrolysis by numerous  $\beta$ -  
71 lactamases including ESBLs and AmpC-type cephalosporinases, and (iii) a minimal risk of  
72 *Clostridioides difficile* infection by the preservation of intestinal microbiota (10). In addition,  
73 the frequency of resistant mutants *in vitro* appears low (ranging from  $1 \times 10^{-8}$  to  $1 \times 10^{-10}$ ) (11-  
74 13) whereas no *in vivo* development of temocillin resistance has been detected after  
75 treatment so far (13,14). Therefore, it has recently become a useful alternative to  
76 carbapenems for the treatment of infections caused by ESBL-producing *Enterobacterales*  
77 (9,10). Surprisingly, mechanisms of temocillin resistance have been very poorly studied with  
78 the exception of intrinsic resistance in *Pseudomonas aeruginosa*, mainly driven by active efflux  
79 (15,16). Whereas hydrolysis of temocillin by class B- and D-type  $\beta$ -lactamases is well known,  
80 mechanisms of acquired chromosomal-encoded resistance have never been reported in  
81 *Enterobacterales*.

82 Two-component systems (TCS) are universal signal transduction systems in bacteria and  
83 participate in various important cellular processes such as pathogenicity, antibiotic resistance,  
84 environmental stresses, quorum sensing, and biofilm production (17,18). These  
85 environmental response systems are typically composed of a sensor histidine kinase (HK) and  
86 a cognate response regulator (RR) (19). HKs are usually transmembrane proteins, which are  
87 able to perceive environmental signals. When stimulated, HK triggers its autophosphorylation.  
88 The phosphate group is then transferred to an aspartate residue on the cognate RR located in  
89 the cytoplasm (20). The phosphorylated RR can therefore bind to promoter regions of target  
90 genes leading to expression regulation (20). Among TCS identified in *Enterobacterales*, several  
91 of them are known to be involved in antibiotic resistance such as BaeSR, CpxAR, EnvZ/OmpR,

92 EvgAS, PhoPQ, PmrAB, and RcsBCD (21). Nonetheless, only two TCSs have been characterized  
93 so far in ECC, namely PhoPQ and CrrAB, which are both involved in colistin heteroresistance  
94 (22-24).

95 Here, by comparing a temocillin-susceptible and -resistant ECC clinical isolates, we identified  
96 a mutation in the HK BaeS of the TCS BaeSR. By using knockout mutants and overexpressing  
97 strains of genes known to be regulated by BaeSR, we identified the genetic basis of acquired  
98 temocillin resistance in ECC, which will pave the way for a better understanding of  
99 mechanisms of antimicrobial resistance deployed by this worrisome pathogen.

100 **RESULTS**

101 ***E. asburiae* isolates have different antibiotic susceptibility profiles**

102 Two clinical isolates, first identified as belonging to the *E. cloacae* complex by MALDI-TOF mass  
103 spectrometry, were recovered from the same urinary specimen (Table 1). By *hsp60*  
104 sequencing, both isolates were shown to belong to the cluster C-I (namely *E. asburiae*) and  
105 were presumably clonally related. A comparison of antibiotic susceptibility profiles showed  
106 that these isolates only differed by their susceptibility to three  $\beta$ -lactams: ticarcillin, temocillin,  
107 and aztreonam (Table 2). Indeed, *E. asburiae* Temo\_R exhibited 32-, 8-, and 4-fold higher MICs  
108 of ticarcillin, temocillin, and aztreonam, respectively, compared to those determined for the  
109 *E. asburiae* Temo\_S strain (Table 2). Note that no change in MICs was observed in the presence  
110 of Phe-Arg- $\beta$ -naphthylamide (PA $\beta$ N) for these three antibiotics (data not shown).

111 ***E. asburiae* isolates are clonally related**

112 By genome sequencing, we determined that the two strains belonged to the sequence type  
113 ST24 and confirmed that they were isogenic with a difference of only 14 single-nucleotide  
114 polymorphisms (SNPs). Detailed analysis revealed that around one third of the SNPs (5/14)  
115 occurred within noncoding regions. On the seven SNPs appearing in coding regions, six led to  
116 synonymous mutations, and three were responsible for an amino acid change (Table 3). The  
117 three non-synonymous mutations were found in three different genes coding for a  
118 hypothetical protein (Leu118Phe), the TCS sensor HK BaeS (Thr175Pro), and a beta-1,3-  
119 glucosyltransferase (Ile38Val) (Table 3).

120 **Unique Thr175Pro change in BaeS confers temocillin resistance**

121 TCS systems, including BaERS, have been associated with antibiotic resistance. Therefore, the  
122 consequence of the amino-acid change found in the BaeS sequence was of high interest to  
123 investigate in the context of temocillin resistance. In order to confirm the role of the

124 Thr175Pro mutation in BaeS, we introduced it in the *baeS* gene of *E. coli* CFT073 by site-  
125 directed mutagenesis. As expected, we demonstrated experimentally that this unique point  
126 mutation was alone responsible for a significant (16-fold) increase in MICs of temocillin,  
127 ticarcillin, and aztreonam (Table 2). Note that the mutation was located in a transmembrane  
128 domain of BaeS, which is not in the vicinity of the histidine residue (His<sub>250</sub>) within the  
129 cytoplasmic catalytic domain (Figure S1).

### 130 **Temocillin resistance is due to overexpression of AcrD efflux pump system**

131 The BaeSR TCS regulates the expression of two efflux pump systems (namely AcrAD-TolC and  
132 MdtABCD) belonging to the RND (Resistance-Nodulation-cell Division) superfamily in *E. coli*  
133 and *Salmonella* (25,26). In addition, *baeS* is part of the *mdtABCD-baeSR* operon (27). Then, we  
134 compared by qRT-PCR the expression of *mdtB* and *acrD* genes as well as *baeS* in both *E.*  
135 *asburiae* Temo\_R and Temo\_S clinical isolates. We evidenced that these three genes were  
136 significantly overexpressed in *E. asburiae* Temo\_R as compared to *E. asburiae* Temo\_S with a  
137 fold change of 15, 11, and 3 for *mdtB*, *baeS*, and *acrD*, respectively. These results strongly  
138 suggested that decreased susceptibility to ticarcillin, temocillin, and aztreonam in *E. asburiae*  
139 Temo\_R was likely related to an increase of the efflux mediated by MdtABCD and/or AcrD  
140 RND-type efflux pump systems.

141 To confirm the role of these two RND-type efflux pump systems, both *mdtABCD* and *acrD*  
142 genes (together with their own promoters) were cloned into the pBAD202/D-TOPO vector and  
143 expressed in *E. asburiae* Temo\_S strain. We observed that the overexpression of *acrD*, but not  
144 *mdtABCD*, was able to confer temocillin resistance (64 mg/L) with a 16-fold increase in MIC  
145 (Table 2). The overexpression of *acrD* also led to an increase in MICs of ticarcillin (32-fold) and  
146 aztreonam (4-fold) while overexpression of *mdtABCD* only conferred a four-fold increase in  
147 ticarcillin MIC (Table 2). The same mutants and *trans*-complemented strains were also



148 obtained in the reference strain *E. cloacae* subsp. *cloacae* ATCC 13047 (C-XI). Although we  
149 showed that the deletion of *baeS* (ECL\_03405), *mdtB* (ECL\_03403) or *acrD* (ECL\_03767) did  
150 not modify susceptibility profiles to  $\beta$ -lactams, we confirmed that the sole overexpression of  
151 *acrD* conferred significant increase (8 fold) in MICs of temocillin (Table 2).

152

153 **DISCUSSION**

154 This study deciphers for the first time the genetic basis of temocillin resistance in  
155 *Enterobacterales*, which is a cross-resistance to carboxypenicillins (ticarcillin and temocillin)  
156 and aztreonam. Indeed, mechanisms of temocillin intrinsic resistance/acquired susceptibility  
157 have only been studied in *P. aeruginosa* (15,16). In this species, it was demonstrated that  
158 temocillin was a substrate of the RND-type efflux system MexAB-OprM while its uptake  
159 marginally requires anion-specific porins OpdK and OpdF (15,16).

160 Here, we showed that temocillin resistance was due to a unique point non-synonymous  
161 mutation in the *baeS* gene (leading to the Thr175Pro change) encoding the HK of the TCS  
162 BaeSR. In *E. coli*, BaeSR is a well-known TCS involved in the regulation of the envelope stress  
163 response (28,29). It is worth notifying that this TCS can be induced by a pleiotropic number of  
164 compounds including indole, copper, zinc, ethanol, sodium tungstate tannin, and flavonoid  
165 (26,30-37). BaeSR is also one of the most studied TCS associated with multidrug resistance in  
166 *E. coli*, *Salmonella*, and *A. baumannii*. However, it has never been linked to temocillin  
167 resistance and intriguingly enough, has never been studied in ECC, species in which only 2 TCS  
168 have been described so far to be linked with antibiotic resistance. Indeed, PhoPQ and CrrAB  
169 have been both associated with colistin hetero-resistance (22-24).

170 BaeSR system has been linked to antibiotic resistance in different bacterial species throughout  
171 the regulation of genes encoding for efflux pump systems. In *E. coli* and *Salmonella*, the  
172 overexpression of BaeR has been shown to regulate expression of *acrD* and *mdtA*, both part  
173 of the AcrAD-TolC and MdtABC efflux pumps, conferring resistance to different classes of  
174 antibiotics such as novobiocin, some  $\beta$ -lactams or deoxycholate (25-27,38,39). In addition,  
175 BaeR overexpression slightly reduced susceptibility to cephalosporins by decreasing the  
176 expression of outer membrane proteins in *E. coli* (40), whereas in *Salmonella*, overexpression

177 of BaeR also increased polymyxin susceptibility by altering the expression of polymyxin  
178 resistance-associated genes (41). In *A. baumannii*, BaeSR affects polymyxin susceptibility by  
179 regulating the expression of efflux pump systems such as MacAB and AdeIJK while it is also  
180 involved in tigecycline resistance by activating the expression of AdeABC, AdeIJK, and MacAB-  
181 TolC (42-44).

182 As opposed to other antibiotics, very few studies have linked temocillin with RND-type efflux  
183 pumps with only the AcrAD-TolC system being involved in its export. The AcrD RND-type efflux  
184 pump functions in cooperation with AcrA and TolC, and the AcrAD-TolC system and effluxes  
185 structurally-diverse compounds including SDS, novobiocin, and deoxycholate as well as  
186 aminoglycosides and dianionic  $\beta$ -lactams such as carbenicillin, oxacillin, nafcillin, and  
187 aztreonam (25,45-47). Very recently, it was also demonstrated in *S. Typhimurium* that AcrD  
188 expelled unreported substrates such as temocillin, dicloxacillin, cefazolin, and fusidic acid (48).  
189 Interestingly, the overexpression of *acrD* in an  $\DeltaacrB\DeltaacrD$  *E. coli* strain increased MICs of  
190 both temocillin and aztreonam by 8-fold (48).

191 In ECC, temocillin resistant mutant carried a Thr175Pro point mutation in the BaeS-encoding  
192 gene, known to regulate together with BaeR, efflux pump systems in other bacterial species.  
193 Recently in *E. coli*, five different gain-of-function mutations in *baeS* were identified as  
194 constitutively activating the BaeSR TCS with increased expression of MdtABC efflux pump (49).  
195 These modifications likely alter the BaeS structure with constitutive elevation of kinase activity  
196 and/or reduction of phosphatase activity, which in turn cause permanent phosphorylation of  
197 BaeR. Phosphorylated BaeR will then be able to bind *mdtABCD-baeSR* and *acrD* promoter  
198 regions and increase corresponded genes expression (49). In this study, DGE analysis through  
199 RNAseq revealed that the mutation C310T in the *baeS51* mutant (leading to Arg104Cys amino  
200 acid change) was responsible for a 37- and 6-fold change in expression of *mdtABCD-baeSR* and

201 *acrD*, respectively, as compared to the parental strain (49). This difference of change in  
202 expression is similar to what we observed here between *E. asburiae* Temo\_R and Temo\_S  
203 strains, possibly due to the expression of the *mdtABCD-baeSR* operon, which is directly  
204 controlled by BaeR whereas *acrD* is indirectly regulated (50).

205 In conclusion, we describe for the first the genetic basis of temocillin resistance in  
206 *Enterobacterales* through a single mutation (Thr175Pro) in BaeS and as a consequence, an  
207 increase of AcrD-mediated efflux (Figure 1).

208 **MATERIALS AND METHODS**

209 **Bacterial strains and growth conditions.** Bacterial strains used in this study are listed in Table  
210 1. The two clinical isolates (Temo\_S and Temo\_R) were recovered from a single urine sample  
211 from the same patient. The *E. cloacae* subsp. *cloacae* ATCC 13047 reference strain (51) was  
212 used to construct deletion and *trans*-complemented strains while *E. coli* CFT073 (52) was used  
213 to construction the *baeS*<sup>Thr175Pro</sup> point-mutation strain by site-directed mutagenesis. All strains  
214 were cultured with constant agitation (200 rpm) at 35°C in Luria-Bertani (LB) medium.

215 **Antimicrobial susceptibility testing.** MICs were determined using the broth microdilution  
216 reference method according to the guidelines recommended by the European Committee on  
217 Antimicrobial Susceptibility (EUCAST; <http://www.eucast.org/>). The following antibiotics were  
218 tested: amoxicillin, ticarcillin, temocillin, piperacillin, ceftazidime, ceftazidime/avopiban, cefepime,  
219 aztreonam, ertapenem, gentamicin, ciprofloxacin, tigecycline, colistin, and chloramphenicol.  
220 All experiments were performed in biological triplicate. MICs of ticarcillin, temocillin, and  
221 aztreonam were also determined by broth microdilution in the absence or presence of the  
222 efflux pump inhibitor PAβN at the concentration of 40 mg/L.

223 **Whole-genome sequencing and bioinformatic analysis.** The genome sequences of both *E.*  
224 *asburiae* Temo\_S and Temo\_R clinical isolates were determined by WGS at the “Plateforme  
225 de microbiologie mutualisée” (P2M) of the Pasteur International Bioresources network  
226 (Institut Pasteur, Paris, France). DNA was extracted using the MagNAPure 96 system (Roche  
227 Diagnostics GmbH, Mannheim, Germany). The libraries were prepared using the Nextera XT  
228 kit (Illumina, San Diego, CA), and the sequencing was performed using an Illumina NextSeq  
229 500 system generating 150-bp paired-end reads.

230 FastQ files were trimmed using the fqCleanER tool  
231 (<https://gitlab.pasteur.fr/GIPhy/fqCleanER>), and the qualities of the filtered FastQ files were

232 evaluated using the FastQC software  
233 (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). Genomes were de novo  
234 assembled using the SPAdes v 3.12 (53) while the quality of assemblies was assessed using the  
235 Quast software (54).

236 The identification to the species level was performed using the rMLST algorithm (55). The  
237 sequence type (ST) was resolved using the appropriate MLST schema (56). The cluster  
238 attribution based on the partial sequence of the *hsp60* gene was determined using blastn  
239 algorithm (2). The identification to the species level was also confirmed by average nucleotide  
240 identity (ANI) analysis using all reference ECC genomes (3) using the mash tool. Acquired  
241 resistance genes and point mutations were detected using Resfinder and PointFinder  
242 databases, respectively (<https://cge.food.dtu.dk/services/ResFinder/>; 57). The genome of the  
243 Temo\_S strain was annotated using the RAST server and then used as the reference genome  
244 to perform variant calling between the two Temo\_R and Temo\_S strains using Freebayes after  
245 a mapping of the reads using Burrows-Wheeler transform (58).

246 The genomic sequences of both *E. asburiae* Temo\_S and Temo\_R clinical isolates were  
247 deposited in GenBank as BioProject no. PRJNA902787.

248 **Construction of the knockout mutants and *trans*-complemented strains in *E. cloacae*.** The  
249 deletion of the genes encoding the histidine kinase BaeS (ECL\_03405), the response effector  
250 BaeR (ECL\_03406), and the two RND pumps MdtB (ECL\_03403) and AcrD (ECL\_03767) were  
251 performed in ATCC 13047 using the Red helper plasmid pKOBEG as previously described (59-  
252 63) (Tables 1 and S1). The RND pump-encoding genes (MdtABCD or ECL\_03403-ECL\_03407;  
253 and AcrD or ECL\_03767) including their own promoters were amplified by PCR (Table S1) and  
254 obtained amplicons were TA-cloned into the overexpression plasmid, pBAD202 (low-copy  
255 number plasmid: ~20 copies/cell) Directional TOPO (Invitrogen, Villebon-sur-Yvette, France).

256 *E. coli* TOP10 cells (Invitrogen) carrying pBAD202 with correctly oriented inserts were then  
257 selected on LB plates with 40 mg/L of kanamycin. After extraction, plasmids carrying the RND  
258 efflux pump-encoding genes were transformed in *E. cloacae* ATCC 13047 strain or *E. asburiae*  
259 Temo\_S clinical isolate (Table 1).

### 260 **Site-Directed Mutagenesis**

261 To confirm the role of the Thr175Pro mutation in *baeS*, single-nucleotide allelic replacement  
262 was carried out using the suicide vector pDS132 in *E. coli* CFT073 (52,64). The cloning steps  
263 were performed in *E. coli* DH5 $\alpha$   $\lambda$ pir strain to allow replication of the plasmid (Table S1). The  
264 recombinant plasmids were then purified and introduced in *E. coli* CFT073 by electro-  
265 transformation. Clones that integrated the plasmid were selected by plating cells on LB plates  
266 containing 30 mg/L of chloramphenicol. After overnight growth at 35°C, one colony was  
267 picked, diluted in 10 mM MgSO<sub>4</sub> solution, and serial dilutions were plated on LB agar plates  
268 with 5% sucrose and without NaCl to force plasmid excision. After overnight incubation at  
269 35°C, 100 clones were streaked on chloramphenicol-containing LB agar plates and on LB agar  
270 with 5% sucrose and without NaCl. Several clones were tested by PCR-sequencing to identify  
271 those carrying the mutation (Table S1).

272 **Quantification of gene expression by RT-qPCR.** Total RNAs were extracted from *E. asburiae*  
273 Temo\_S and Temo\_R strains grown to the late-exponential phase by using the Direct-Zol RNA  
274 mini-prep kit (Zymo Research, Irvine, CA). Residual chromosomal DNA was removed by  
275 treating samples with the TURBO DNA-free kit (Life Technologies, Saint-Aubin, France).  
276 Samples were quantified using the NanoDrop One spectrophotometer (Thermo Fisher  
277 Scientific, Courtabœuf, France). cDNA was synthesized from total RNAs (~1  $\mu$ g) using the  
278 QuantiTect reverse transcription kit (Qiagen) according to the manufacturer's instructions.  
279 Transcript levels were determined by the  $\Delta\Delta$ Ct method using *rpoB* as a housekeeping control

280 gene. The expression, and the fold change (FCs) of *mdtB*, *baeS*, and *acrD* genes were calculated  
281 by Ct comparison between Temo\_S and Tem\_R clinical isolates. Experiments were performed  
282 at least in three biological replicates. Student t-test was employed and *p*-values less than 0.05  
283 were considered as statistically significant.



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288

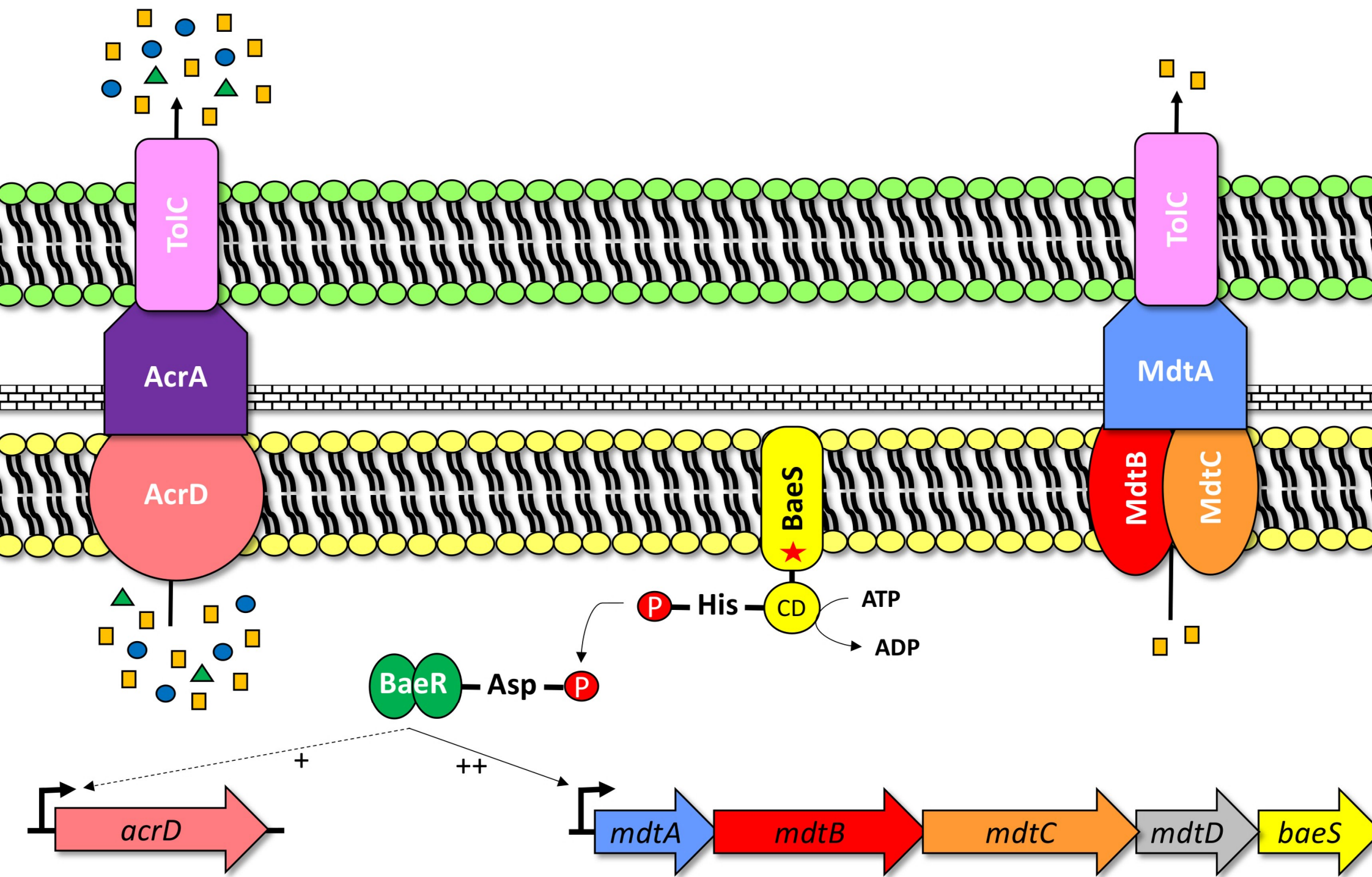
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453 **LEGEND OF THE FIGURE**

454 **FIG 1** Mechanism of temocillin resistance in ECC through the two-component system BaeSR.  
455 The alteration of BaeS by a single mutation (Thr175Pro; red star), responsible for a constitutive  
456 elevation of kinase activity and/or reduction of phosphatase activity, is responsible for  
457 permanent phosphorylation of BaeR. Phosphorylated BaeR can then activate directly (solid  
458 line arrow) *mdtABCD-baeSR* and indirectly (dotted line arrow) *acrD* expression. As a  
459 consequence, the expression of those genes contribute to increase the active efflux of  
460 ticarcillin (yellow rectangle) and temocillin (blue round)/ticarcillin/aztreonam (green triangle)  
461 through MdtABC-TolC and AcrAD-TolC RND-type efflux pump systems, respectively.



**TABLE 1** Strains and plasmid used in the study

Strain or plasmid	Genotype/relevant characteristic(s)	Reference
<b>Strains</b>		
<i>E. asburiae</i> Temo_S	Human clinical isolate Ecc20200302 (urine, 2020/02/28), Tem <sup>S</sup>	This study
<i>E. asburiae</i> Temo_R	Human clinical isolate Ecc20200303 (urine, 2020/02/28), Tem <sup>R</sup>	This study
Tem_S/pBAD	<i>E. asburiae</i> Temo_S <i>trans</i> -complemented strain carrying pBAD202/D-TOPO	This study
Tem_S/pBAD $\Omega$ mdtABCD	<i>E. asburiae</i> Temo_S <i>trans</i> -complemented strain carrying pBAD202/D-TOPO $\Omega$ mdtABCD (ECL_03401-ECL_03404)	This study
Tem_S/pBAD $\Omega$ acrD	<i>E. asburiae</i> Temo_S <i>trans</i> -complemented strain carrying pBAD202/D-TOPO $\Omega$ acrD (ECL_03767)	This study
<i>E. cloacae</i> subsp. <i>cloacae</i> ATCC 13047	Reference strain ATCC 13047 (cluster XI)	51
ATCC13047 $\Delta$ baeS	ATCC 13047 deleted for <i>baeS</i> (ECL_03405)	This study
ATCC13047 $\Delta$ mdtB	ATCC13047 deleted for <i>mdtB</i> (ECL_03403)	63
ATCC13047 $\Delta$ acrD	ATCC13047 deleted for <i>acrD</i> (ECL_03767)	63
ATCC13047/pBAD	ATCC 13047 <i>trans</i> -complemented strain carrying pBAD202/D-TOPO	63
ATCC13047/pBAD $\Omega$ mdtABCD	ATCC 13047 <i>trans</i> -complemented strain carrying pBAD202/D-TOPO $\Omega$ mdtABCD (ECL_03401-ECL_03404)	63
ATCC13047/pBAD $\Omega$ acrD	ATCC 13047 <i>trans</i> -complemented strain carrying pBAD202/D-TOPO $\Omega$ acrD (ECL_03767)	63
<i>E. coli</i> CFT073	Wild-type susceptible clinical isolate (phylogenetic group B2)	52
CFT073_ <i>baeS</i> <sup>Thr175Pro</sup>	CFT073 derivative with allelic replacement of <i>baeS</i> by <i>baeS</i> <sup>Thr175Pro</sup>	This study
<i>E. coli</i> DH5 $\alpha$ $\lambda$ pir	Reference strain ( <i>supE4</i> $\Delta$ <i>lacU169</i> ( $\Phi$ 80 <i>lacZ</i> $\Delta$ M15) <i>hsdR17</i> <i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>relA1</i> $\lambda$ pir)	Laboratory collection
<b>Plasmids</b>		
pBAD202/D-TOPO	General expression vector with arabinose-inducible promoter, Kan <sup>R</sup>	Life Technologies
pKOBEG	Recombination vector, phage $\lambda$ <i>recY</i> $\beta$ $\alpha$ operon under the control of the pBAD promoter, Cm <sup>R</sup>	60
pKD4	Plasmid containing an FRT-flanked kanamycin, Kan <sup>R</sup>	59
pCP20_Gm	FLP-mediated recombination vector, Gm <sup>R</sup>	61
pDS132	Conditional replication vector; <i>R6K</i> origin, <i>mobRP4</i> transfer origin, sucrose-inducible <i>sacB</i> , Cm <sup>R</sup>	64

Cm<sup>R</sup>, chloramphenicol resistant; Gm<sup>R</sup>, gentamicin resistant; Kan<sup>R</sup>, kanamycin resistant; Tem<sup>R</sup>, temocillin resistant; Tem<sup>S</sup>, temocillin susceptible.

**TABLE 2** MIC values of antibiotics for *E. cloacae* clinical isolates, *E. cloacae* ATCC 13047, and *E. coli* CFT073 (and their derivative strains)

Strain	MIC (mg/L) <sup>a,b</sup>													
	AMX	TEM	TIC	PIP	FOX	CTX	FEP	ATM	ERT	GEN	CIP	TGC	COL	CHL
<b><i>E. asburiae</i></b>														
Temo_S	>256	4	2	4	>256	0.5	0.03	0.03	0.25	0.12	<0.01	0.5	32	4
Temo_R	>256	<b>32 (8)</b>	<b>64 (32)</b>	4	>256	0.5	0.03	<b>0.12 (4)</b>	0.25	0.12	<0.01	0.5	64	4
Temo_S/pBAD	>256	4	2	2	>256	0.5	0.03	0.03	0.25	0.12	<0.01	0.5	32	4
Temo_S/pBAD $\Omega$ mdtABCD	>256	4	<b>8 (4)</b>	2	>256	0.5	0.03	0.03	0.25	0.12	<0.01	0.5	16	4
Temo_S/pBAD $\Omega$ acrD	>256	<b>64 (16)</b>	<b>64 (32)</b>	2	>256	0.5	0.03	<b>0.12 (4)</b>	0.25	0.12	<0.01	0.5	32	4
<b><i>E. cloacae</i> subsp. <i>cloacae</i></b>														
ATCC 13047	>256	8	4	8	>256	2	0.03	0.5	0.25	0.25	0.03	0.5	>256	4
ATCC13047 $\Delta$ baeS	>256	4	4	8	>256	2	0.03	0.5	0.25	0.25	0.03	0.5	256	4
ATCC13047 $\Delta$ mdtB	>256	8	4	8	>256	2	0.03	0.5	0.25	0.25	0.03	0.5	256	4
ATCC13047 $\Delta$ acrD	>256	8	4	8	>256	2	0.03	0.25	0.25	0.25	0.03	0.5	>256	4
ATCC13047/pBAD	>256	8	4	8	>256	2	0.03	0.5	0.25	0.25	0.03	0.5	>256	4
ATCC13047/pBAD $\Omega$ mdtABCD	>256	8	<b>16 (4)</b>	8	>256	2	0.03	0.5	0.5	0.25	0.03	0.5	>256	4
ATCC13047/pBAD $\Omega$ acrD	>256	<b>64 (8)</b>	<b>128 (16)</b>	8	>256	2	0.03	<b>2 (4)</b>	0.25	0.25	0.03	0.5	>256	4
<b><i>E. coli</i></b>														
CFT073	4	4	4	2	2	0.12	0.06	0.06	0.06	0.12	<0.01	0.12	0.12	4
CFT073_baeS <sup>Thr175Pro</sup>	4	<b>64 (16)</b>	<b>64 (16)</b>	2	2	0.12	0.06	<b>1 (16)</b>	0.06	0.12	<0.01	0.12	0.12	4

<sup>a</sup> AMX, amoxicillin; ATM, aztreonam; CHL, chloramphenicol; CIP, ciprofloxacin; COL, colistin; CTX, cefotaxime; ERT, ertapenem; FEP, cefepime; FOX, ceftoxitin; GEN, gentamicin; PIP, piperacillin; TEM, temocillin; TIC, ticarcillin; TGC, tigecycline.

<sup>b</sup> Numbers in boldface are MICs that are significantly different (fold changes of  $\geq 4$  are indicated in parentheses).



**TABLE 3** Nucleotide changes identified between *E. asburiae* Temo\_S and Temo\_R isogenic isolates

Gene <sup>a</sup>	Predicted product	Nucleotide change <sup>a</sup>	Amino acid change	Homolog in <i>E. cloacae</i> subsp. <i>cloacae</i> ATCC 13047 <sup>b</sup>
OSB85_02280	General stress protein YmdF	24A>C 36A>T	None None	ECL_02630
OSB85_09375	Hypothetical protein	354G>T 390T>C	Leu118Phe None	Not found
OSB85_12740	IS3 family transposase	120C>T 132C>T	None None	Not found
OSB85_20490	Two-component system sensor histidine kinase BaeS	523A>C	Thr175Pro	ECL_03405
OSB85_21180	Beta-1,3-glucosyltransferase	112A>G 117C>T	Ile38Val None	Not found

<sup>a</sup> Gene names and positions from the annotated sequence of the *E. asburiae* Temo\_S clinical isolate (GenBank accession no. SAMN31775694).

<sup>b</sup> Gene names are from the annotated sequence of the *E. cloacae* subsp. *cloacae* ATCC 13047 (GenBank accession no. NC\_014121).

**TABLE 3** Nucleotide changes identified between *E. asburiae* Temo\_S and Temo\_R isogenic isolates

Gene <sup>a</sup>	Predicted product	Nucleotide change <sup>a</sup>	Amino acid change	Homolog in <i>E. cloacae</i> subsp. <i>cloacae</i> ATCC 13047 <sup>b</sup>
OSB85_02280	General stress protein YmdF	24A>C 36A>T	None None	ECL_02630
OSB85_09375	Hypothetical protein	354G>T 390T>C	Leu118Phe None	Not found
OSB85_20490	Two-component system sensor histidine kinase BaeS	523A>C	Thr175Pro	ECL_03405
OSB85_21180	Beta-1,3-glucosyltransferase	112A>G 117C>T	Ile38Val None	Not found

<sup>a</sup> Gene names and positions from the annotated sequence of the *E. asburiae* Temo\_S clinical isolate (GenBank accession no. SAMN31775694).

<sup>b</sup> Gene names are from the annotated sequence of the *E. cloacae* subsp. *cloacae* ATCC 13047 (GenBank accession no. NC\_014121).