



HAL
open science

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

François Guérin, François Gravey, Sophie Reissier, Malo Penven, Charlotte Michaux, Simon Le Hello, Vincent Cattoir

► To cite this version:

François Guérin, François Gravey, Sophie Reissier, Malo Penven, Charlotte Michaux, et al.. Temocillin Resistance in the *Enterobacter cloacae* Complex Is Conferred by a Single Point Mutation in BaeS, Leading to Overexpression of the AcrD Efflux Pump. *Antimicrobial Agents and Chemotherapy*, 2023, 67 (6), pp.e00358-23. 10.1128/aac.00358-23 . hal-04117033

HAL Id: hal-04117033

<https://hal.science/hal-04117033>

Submitted on 29 Jun 2023

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution 4.0 International License

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

6 François Guérin^{a,b*}, François Gravey^{c,d}, Sophie Reissier^{a,b}, Malo Penven^{a,b}, Charlotte Michaux^b,
7 Simon Le Hello^{c,d}, Vincent Cattoir^{a,b*}

8

9 ^aDepartment of Clinical Microbiology, Rennes University Hospital, 35033 Rennes, France

10 ^bUniversity of Rennes, Inserm UMR 1230 BRM, 35043 Rennes, France

11 ^cNormandie Univ, UNICAEN, UNIROUEN, Inserm UMR 1311 DYNAMICURE, 14000 Caen, France

12 ^dMicrobiology Department, CHU Caen, 14000 Caen, France

13

14 ***Correspondence:** Dr François Guérin or Prof. Vincent Cattoir, CHU de Rennes, Service de
15 Bactériologie-Hygiène Hospitalière, 2 rue Henri Le Guilloux, 35033 Rennes Cedex 9, France.

16 +33-2-99-28-98-28, Fax: +33-2-99-28-41-59, Email: francois.guerin@chu-rennes.fr or

17 vincent.cattoir@chu-rennes.fr.

18

19 Keywords: ECC; *E. asburiae*; BaeSR, AcrD, MdtABC.

20

21 Word count: abstract = 249 words; text = 2,872 words; 3 Tables; 1 Figure; 64 References; 2

22 Supplemental materials.

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 β -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*_{AmpC} 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 β -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- α -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 β -
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 1×10^{-8} to 1×10^{-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 β -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 β -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- β -naphthylamide (PA β 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 (His₂₅₀) 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 β -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 β -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 β -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*^{Thr175Pro} 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, ceftaxime, 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 PA β N 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 α λ 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 MgSO₄ 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 μ 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.

284 **ACKNOWLEDGMENTS**

285 A part of this work was performed using bioinformatical resources provided by CRIANN
286 (Normandy, France). The three-year doctoral contract of François Gravey was funded by a grant
287 from the Normandy Region.

288

- 290 1. Sanders WE, Jr., Sanders CC. 1997. *Enterobacter* spp.: pathogens poised to flourish at the turn of the
291 century. Clin Microbiol Rev 10:220-241.
- 292 2. Hoffmann H, Roggenkamp A. 2003. Population genetics of the nomenclature species *Enterobacter cloacae*. Appl
293 Environ Microbiol 69:5306-5318.
- 294 3. Sutton GG, Brinkac LM, Clarke TH, Fouts DE. 2018. *Enterobacter hormaechei* subsp. *hoffmannii* subsp.
295 nov., *Enterobacter hormaechei* subsp. *xiangfangensis* comb. nov., *Enterobacter roggenkampii* sp. nov.,
296 and *Enterobacter muelleri* is a later heterotypic synonym of *Enterobacter asburiae* based on
297 computational analysis of sequenced *Enterobacter* genomes. F1000Res 7:521.
- 298 4. Wu W, Feng Y, Zong Z. 2021. Precise species identification for *Enterobacter*: a genome sequence-based
299 study with reporting of two novel species, *Enterobacter quasiroggenkampii* sp. nov. and *Enterobacter*
300 *quasimori* sp. nov. mSystems 5:e00527-20.
- 301 5. De Oliveira DMP, Forde BM, Kidd TJ, Harris PNA, Schembri MA, Beatson SA, Paterson DL, Walker MJ.
302 2020. Antimicrobial resistance in ESKAPE pathogens. Clin Microbiol Rev 33.
- 303 6. Mezzatesta ML, Gona F, Stefani S. 2012. *Enterobacter cloacae* complex: clinical impact and emerging
304 antibiotic resistance. Future Microbiol 7:887-902.
- 305 7. Davin-Regli A, Lavigne JP, Pages JM. 2019. *Enterobacter* spp.: update on taxonomy, clinical aspects, and
306 emerging antimicrobial resistance. Clin Microbiol Rev 32.
- 307 8. Giske CG. 2015. Contemporary resistance trends and mechanisms for the old antibiotics colistin,
308 temocillin, fosfomicin, mecillinam and nitrofurantoin. Clin Microbiol Infect 21:899-905.
- 309 9. Livermore DM, Tulkens PM. 2009. Temocillin revived. J Antimicrob Chemother 63:243-245.
- 310 10. Alexandre K, Fantin B. 2018. Pharmacokinetics and pharmacodynamics of temocillin. Clin
311 Pharmacokinet 57:287-296.
- 312 11. Slocombe B, Basker MJ, Bentley PH, Clayton JP, Cole M, Comber KR, Dixon RA, Edmondson RA, Jackson
313 D, Merrikin DJ, Sutherland R. 1981. BRL 17421, a novel beta-lactam antibiotic, highly resistant to beta-
314 lactamases, giving high and prolonged serum levels in humans. Antimicrob Agents Chemother 20:38-
315 46.
- 316 12. Adams-Haduch JM, Potoski BA, Sidjabat HE, Paterson DL, Doi Y. 2009. Activity of temocillin against KPC-
317 producing *Klebsiella pneumoniae* and *Escherichia coli*. Antimicrob Agents Chemother 53:2700-2701.
- 318 13. Soubirou JF, Rossi B, Couffignal C, Ruppe E, Chau F, Massias L, Lepeule R, Mentre F, Fantin B. 2015.
319 Activity of temocillin in a murine model of urinary tract infection due to *Escherichia coli* producing or
320 not producing the ESBL CTX-M-15. J Antimicrob Chemother 70:1466-1472.
- 321 14. Alexandre K, Chau F, Guerin F, Massias L, Lefort A, Cattoir V, Fantin B. 2016. Activity of temocillin in a
322 lethal murine model of infection of intra-abdominal origin due to KPC-producing *Escherichia coli*. J
323 Antimicrob Chemother 71:1899-1904.
- 324 15. Buyck JM, Guenard S, Plesiat P, Tulkens PM, Van Bambeke F. 2012. Role of MexAB-OprM in intrinsic
325 resistance of *Pseudomonas aeruginosa* to temocillin and impact on the susceptibility of strains isolated
326 from patients suffering from cystic fibrosis. J Antimicrob Chemother 67:771-775.
- 327 16. Chalhoub H, Pletzer D, Weingart H, Braun Y, Tunney MM, Elborn JS, Rodriguez-Villalobos H, Plesiat P,
328 Kahl BC, Denis O, Winterhalter M, Tulkens PM, Van Bambeke F. 2017. Mechanisms of intrinsic resistance
329 and acquired susceptibility of *Pseudomonas aeruginosa* isolated from cystic fibrosis patients to
330 temocillin, a revived antibiotic. Sci Rep 7:40208.
- 331 17. Mitrophanov AY, Groisman EA. 2008. Signal integration in bacterial two-component regulatory systems.
332 Genes Dev 22:2601-2611.
- 333 18. Rajput A, Seif Y, Choudhary KS, Dalldorf C, Poudel S, Monk JM, Palsson BO. 2021. Pangenome analytics
334 reveal two-component systems as conserved targets in ESKAPEE pathogens. mSystems 6.
- 335 19. West AH, Stock AM. 2001. Histidine kinases and response regulator proteins in two-component signaling
336 systems. Trends Biochem Sci 26:369-376.
- 337 20. Zschiedrich CP, Keidel V, Szurmant H. 2016. Molecular mechanisms of two-component signal
338 transduction. J Mol Biol 428:3752-3775.
- 339 21. Tierney AR, Rather PN. 2019. Roles of two-component regulatory systems in antibiotic resistance. Future
340 Microbiol 14:533-552.
- 341 22. Band VI, Crispell EK, Napier BA, Herrera CM, Tharp GK, Vavikolanu K, Pohl J, Read TD, Bosinger SE, Trent
342 MS, Burd EM, Weiss DS. 2016. Antibiotic failure mediated by a resistant subpopulation in *Enterobacter*
343 *cloacae*. Nat Microbiol 1:16053.

- 344 23. Guerin F, Isnard C, Sinel C, Morand P, Dhalluin A, Cattoir V, Giard JC. 2016. Cluster-dependent colistin
345 hetero-resistance in *Enterobacter cloacae* complex. J Antimicrob Chemother 71:3058-3061.
- 346 24. Pantel L, Guerin F, Serri M, Gravey F, Houard J, Maurent K, Attwood M, Noel A, MacGowan A, Racine E,
347 Cattoir V, Gualtieri M. 2022. Exploring cluster-dependent antibacterial activities and resistance
348 pathways of NOSO-502 and colistin against *Enterobacter cloacae* complex species. Antimicrob Agents
349 Chemother 66:e0077622.
- 350 25. Hirakawa H, Nishino K, Hirata T, Yamaguchi A. 2003. Comprehensive studies of drug resistance mediated
351 by overexpression of response regulators of two-component signal transduction systems in *Escherichia*
352 *coli*. J Bacteriol 185:1851-1856.
- 353 26. Nishino K, Nikaido E, Yamaguchi A. 2007. Regulation of multidrug efflux systems involved in multidrug
354 and metal resistance of *Salmonella enterica* serovar Typhimurium. J Bacteriol 189:9066-9075.
- 355 27. Nagakubo S, Nishino K, Hirata T, Yamaguchi A. 2002. The putative response regulator BaeR stimulates
356 multidrug resistance of *Escherichia coli* via a novel multidrug exporter system, MdtABC. J Bacteriol
357 184:4161-4167.
- 358 28. Nagasawa S, Ishige K, Mizuno T. 1993. Novel members of the two-component signal transduction genes
359 in *Escherichia coli*. J Biochem 114:350-357.
- 360 29. Raffa RG, Raivio TL. 2002. A third envelope stress signal transduction pathway in *Escherichia coli*. Mol
361 Microbiol 45:1599-1611.
- 362 30. Hirakawa H, Inazumi Y, Masaki T, Hirata T, Yamaguchi A. 2005. Indole induces the expression of
363 multidrug exporter genes in *Escherichia coli*. Mol Microbiol 55:1113-1126.
- 364 31. Wang D, Fierke CA. 2013. The BaeSR regulon is involved in defense against zinc toxicity in *E. coli*.
365 Metallomics 5:372-383.
- 366 32. Zoetendal EG, Smith AH, Sundset MA, Mackie RI. 2008. The BaeSR two-component regulatory system
367 mediates resistance to condensed tannins in *Escherichia coli*. Appl Environ Microbiol 74:535-539.
- 368 33. Touchon M, Hoede C, Tenaillon O, Barbe V, Baeriswyl S, Bidet P, Bingen E, Bonacorsi S, Bouchier C,
369 Bouvet O, Calteau A, Chiapello H, Clermont O, Cruveiller S, Danchin A, Diard M, Dossat C, Karoui ME,
370 Frapy E, Garry L, Ghigo JM, Gilles AM, Johnson J, Le Bouguenec C, Lescat M, Mangenot S, Martinez-
371 Jehanne V, Matic I, Nassif X, Oztas S, Petit MA, Pichon C, Rouy Z, Ruf CS, Schneider D, Tourret J, Vacherie
372 B, Vallenet D, Medigue C, Rocha EP, Denamur E. 2009. Organised genome dynamics in the *Escherichia*
373 *coli* species results in highly diverse adaptive paths. PLoS Genet 5:e1000344.
- 374 34. Nishino K, Honda T, Yamaguchi A. 2005. Genome-wide analyses of *Escherichia coli* gene expression
375 responsive to the BaeSR two-component regulatory system. J Bacteriol 187:1763-1772.
- 376 35. Yamamoto K, Ogasawara H, Ishihama A. 2008. Involvement of multiple transcription factors for metal-
377 induced *spy* gene expression in *Escherichia coli*. J Biotechnol 133:196-200.
- 378 36. Zhou L, Lei XH, Bochner BR, Wanner BL. 2003. Phenotype microarray analysis of *Escherichia coli* K-12
379 mutants with deletions of all two-component systems. J Bacteriol 185:4956-4972.
- 380 37. Leblanc SK, Oates CW, Raivio TL. 2011. Characterization of the induction and cellular role of the BaeSR
381 two-component envelope stress response of *Escherichia coli*. J Bacteriol 193:3367-3375.
- 382 38. Baranova N, Nikaido H. 2002. The BaeSR two-component regulatory system activates transcription of
383 the *yegMNOB* (*mdtABCD*) transporter gene cluster in *Escherichia coli* and increases its resistance to
384 novobiocin and deoxycholate. J Bacteriol 184:4168-4176.
- 385 39. Hirakawa H, Nishino K, Yamada J, Hirata T, Yamaguchi A. 2003. Beta-lactam resistance modulated by the
386 overexpression of response regulators of two-component signal transduction systems in *Escherichia*
387 *coli*. J Antimicrob Chemother 52:576-582.
- 388 40. Wang S, You C, Memon FQ, Zhang G, Sun Y, Si H. 2021. BaeR participates in cephalosporins susceptibility
389 by regulating the expression level of outer membrane proteins in *Escherichia coli*. J Biochem 169:101-
390 108.
- 391 41. Sun F, Qi C, Wei Q, Zhang L, Fu H, Jiang X, Lu F, Li L. 2022. BaeR overexpression enhances the
392 susceptibility of *acrB* deleted *Salmonella enterica* serovar Typhimurium to polymyxin. Vet Microbiol
393 274:109552.
- 394 42. Boinett CJ, Cain AK, Hawkey J, Do Hoang NT, Khanh NNT, Thanh DP, Dordel J, Campbell JI, Lan NPH,
395 Mayho M, Langridge GC, Hadfield J, Chau NVV, Thwaites GE, Parkhill J, Thomson NR, Holt KE, Baker S.
396 2019. Clinical and laboratory-induced colistin-resistance mechanisms in *Acinetobacter baumannii*.
397 Microb Genom 5.
- 398 43. Lin MF, Lin YY, Lan CY. 2015. The role of the two-component system BaeSR in disposing chemicals
399 through regulating transporter systems in *Acinetobacter baumannii*. PLoS One 10:e0132843.

- 400 44. Lin MF, Lin YY, Yeh HW, Lan CY. 2014. Role of the BaeSR two-component system in the regulation of
401 *Acinetobacter baumannii* *adeAB* genes and its correlation with tigecycline susceptibility. BMC Microbiol
402 14:119.
- 403 45. Rosenberg EY, Ma D, Nikaïdo H. 2000. AcrD of *Escherichia coli* is an aminoglycoside efflux pump. J
404 Bacteriol 182:1754-1756.
- 405 46. Yamasaki S, Nagasawa S, Hayashi-Nishino M, Yamaguchi A, Nishino K. 2011. AcrA dependency of the
406 AcrD efflux pump in *Salmonella enterica* serovar Typhimurium. J Antibiot (Tokyo) 64:433-437.
- 407 47. Kobayashi N, Tamura N, van Veen HW, Yamaguchi A, Murakami S. 2014. Beta-lactam selectivity of
408 multidrug transporters AcrB and AcrD resides in the proximal binding pocket. J Biol Chem 289:10680-
409 10690.
- 410 48. Cuesta Bernal J, El-Delik J, Gottig S, Pos KM. 2021. Characterization and molecular determinants for
411 beta-lactam specificity of the multidrug efflux pump AcrD from *Salmonella typhimurium*. Antibiotics
412 (Basel) 10.
- 413 49. Cho H, Misra R. 2021. Mutational activation of antibiotic-resistant mechanisms in the absence of major
414 drug efflux systems of *Escherichia coli*. J Bacteriol 203:e0010921.
- 415 50. Choudhary KS, Kleinmanns JA, Decker K, Sastry AV, Gao Y, Szubin R, Seif Y, Palsson BO. 2020. Elucidation
416 of regulatory modes for five two-component systems in *Escherichia coli* reveals novel relationships.
417 mSystems 5.
- 418 51. Ren Y, Ren Y, Zhou Z, Guo X, Li Y, Feng L, Wang L. 2010. Complete genome sequence of *Enterobacter*
419 *cloacae* subsp. *cloacae* type strain ATCC 13047. J Bacteriol 192:2463-2464.
- 420 52. Mobley HL, Green DM, Trifillis AL, Johnson DE, Chippendale GR, Lockatell CV, Jones BD, Warren JW.
421 1990. Pyelonephritogenic *Escherichia coli* and killing of cultured human renal proximal tubular epithelial
422 cells: role of hemolysin in some strains. Infect Immun 58:1281-1289.
- 423 53. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, Lesin VM, Nikolenko SI, Pham S,
424 Prjibelski AD, Pyshkin AV, Sirotkin AV, Vyahhi N, Tesler G, Alekseyev MA, Pevzner PA. 2012. SPAdes: a
425 new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol 19:455-
426 477.
- 427 54. Gurevich A, Saveliev V, Vyahhi N, Tesler G. 2013. QUAST: quality assessment tool for genome
428 assemblies. Bioinformatics 29:1072-1075.
- 429 55. Jolley KA, Bliss CM, Bennett JS, Bratcher HB, Brehony C, Colles FM, Wimalaratna H, Harrison OB,
430 Sheppard SK, Cody AJ, Maiden MCJ. 2012. Ribosomal multilocus sequence typing: universal
431 characterization of bacteria from domain to strain. Microbiology (Reading) 158:1005-1015.
- 432 56. Miyoshi-Akiyama T, Hayakawa K, Ohmagari N, Shimojima M, Kirikae T. 2013. Multilocus sequence typing
433 (MLST) for characterization of *Enterobacter cloacae*. PLoS One 8:e66358.
- 434 57. Zankari E, Hasman H, Cosentino S, Vestergaard M, Rasmussen S, Lund O, Aarestrup FM, Larsen MV.
435 2012. Identification of acquired antimicrobial resistance genes. J Antimicrob Chemother 67:2640-2644.
- 436 58. Li H, Durbin R. 2009. Fast and accurate short read alignment with Burrows-Wheeler transform.
437 Bioinformatics 25:1754-1760.
- 438 59. Datsenko KA, Wanner BL. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12
439 using PCR products. Proc Natl Acad Sci U S A 97:6640-6645.
- 440 60. Derbise A, Lesic B, Dacheux D, Ghigo JM, Carniel E. 2003. A rapid and simple method for inactivating
441 chromosomal genes in *Yersinia*. FEMS Immunol Med Microbiol 38:113-116.
- 442 61. Doublet B, Douard G, Targant H, Meunier D, Madec JY, Cloeckert A. 2008. Antibiotic marker
443 modifications of lambda Red and FLP helper plasmids, pKD46 and pCP20, for inactivation of
444 chromosomal genes using PCR products in multidrug-resistant strains. J Microbiol Methods 75:359-361.
- 445 62. Guerin F, Isnard C, Cattoir V, Giard JC. 2015. Complex regulation pathways of AmpC-mediated beta-
446 lactam resistance in *Enterobacter cloacae* complex. Antimicrob Agents Chemother 59:7753-7761.
- 447 63. Guerin F, Lallement C, Isnard C, Dhalluin A, Cattoir V, Giard JC. 2016. Landscape of resistance-nodulation-
448 cell division (RND)-type efflux pumps in *Enterobacter cloacae* complex. Antimicrob Agents Chemother
449 60:2373-2382.
- 450 64. Philippe N, Alcaraz JP, Coursange E, Geiselmann J, Schneider D. 2004. Improvement of pCVD442, a
451 suicide plasmid for gene allele exchange in bacteria. Plasmid 51:246-255.
- 452

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.

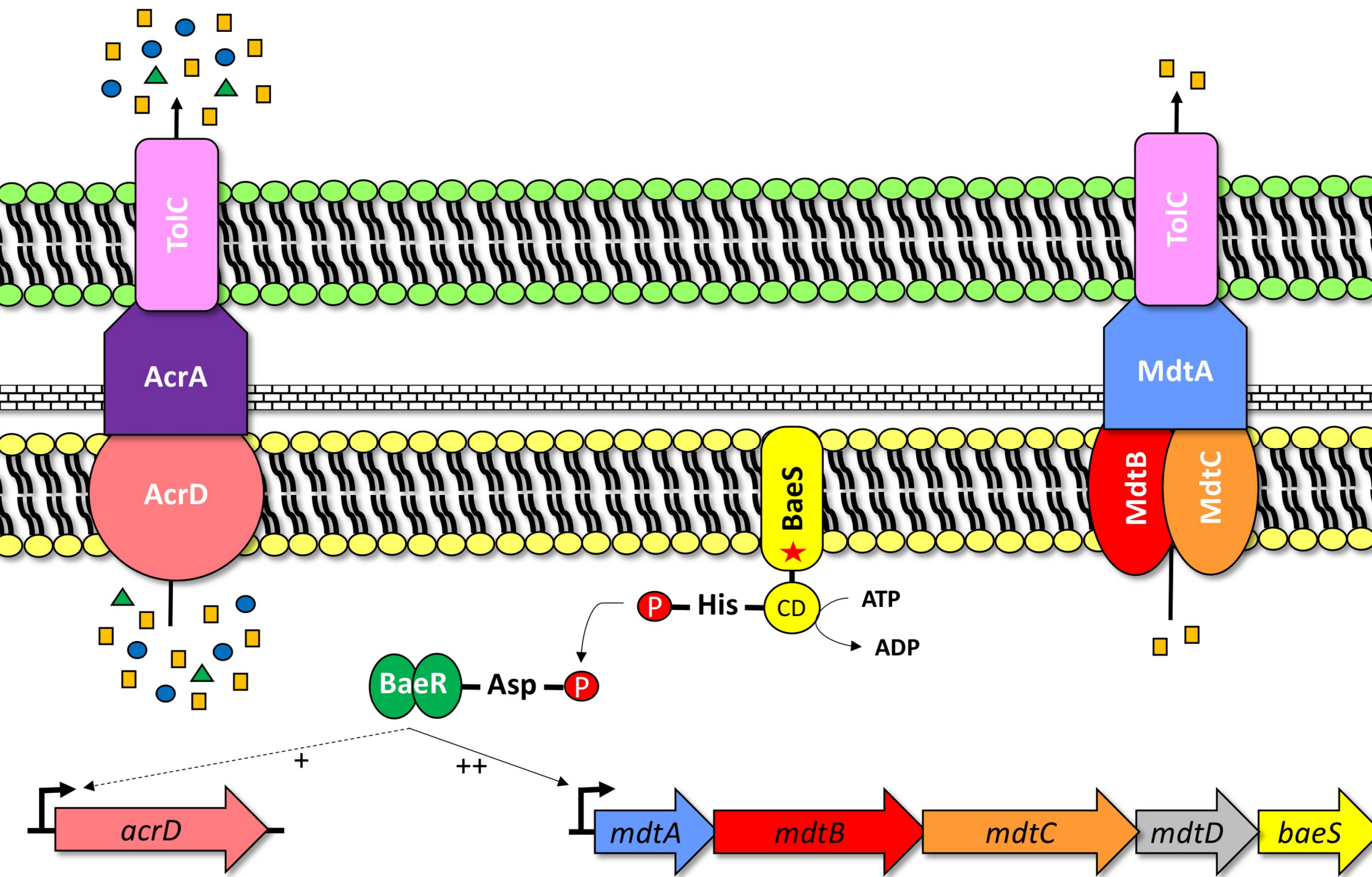


TABLE 1 Strains and plasmid used in the study

Strain or plasmid	Genotype/relevant characteristic(s)	Reference
Strains		
<i>E. asburiae</i> Temo_S	Human clinical isolate Ecc20200302 (urine, 2020/02/28), Tem ^S	This study
<i>E. asburiae</i> Temo_R	Human clinical isolate Ecc20200303 (urine, 2020/02/28), Tem ^R	This study
Tem_S/pBAD	<i>E. asburiae</i> Temo_S <i>trans</i> -complemented strain carrying pBAD202/D-TOPO	This study
Tem_S/pBADΩ <i>mdtABCD</i>	<i>E. asburiae</i> Temo_S <i>trans</i> -complemented strain carrying pBAD202/D-TOPOΩ <i>mdtABCD</i> (ECL_03401-ECL_03404)	This study
Tem_S/pBADΩ <i>acrD</i>	<i>E. asburiae</i> Temo_S <i>trans</i> -complemented strain carrying pBAD202/D-TOPOΩ <i>acrD</i> (ECL_03767)	This study
<i>E. cloacae</i> subsp. <i>cloacae</i> ATCC 13047	Reference strain ATCC 13047 (cluster XI)	51
ATCC13047Δ <i>baeS</i>	ATCC 13047 deleted for <i>baeS</i> (ECL_03405)	This study
ATCC13047Δ <i>mdtB</i>	ATCC13047 deleted for <i>mdtB</i> (ECL_03403)	63
ATCC13047Δ <i>acrD</i>	ATCC13047 deleted for <i>acrD</i> (ECL_03767)	63
ATCC13047/pBAD	ATCC 13047 <i>trans</i> -complemented strain carrying pBAD202/D-TOPO	63
ATCC13047/pBADΩ <i>mdtABCD</i>	ATCC 13047 <i>trans</i> -complemented strain carrying pBAD202/D-TOPOΩ <i>mdtABCD</i> (ECL_03401-ECL_03404)	63
ATCC13047/pBADΩ <i>acrD</i>	ATCC 13047 <i>trans</i> -complemented strain carrying pBAD202/D-TOPOΩ <i>acrD</i> (ECL_03767)	63
<i>E. coli</i> CFT073	Wild-type susceptible clinical isolate (phylogenetic group B2)	52
CFT073_ <i>baeS</i> ^{Thr175Pro}	CFT073 derivative with allelic replacement of <i>baeS</i> by <i>baeS</i> ^{Thr175Pro}	This study
<i>E. coli</i> DH5α λ <i>pir</i>	Reference strain (<i>supE4 ΔlacU169</i> (Φ80 <i>lacZΔM15</i>) <i>hsdR17 recA1 endA1 gyrA96 thi-1 relA1 λpir</i>)	Laboratory collection
Plasmids		
pBAD202/D-TOPO	General expression vector with arabinose-inducible promoter, Kan ^R	Life Technologies
pKOBEG	Recombination vector, phage λ <i>recYβα</i> operon under the control of the pBAD promoter, Cm ^R	60
pKD4	Plasmid containing an FRT-flanked kanamycin, Kan ^R	59
pCP20_Gm	FLP-mediated recombination vector, Gm ^R	61
pDS132	Conditional replication vector; <i>R6K</i> origin, <i>mobRP4</i> transfer origin, sucrose-inducible <i>sacB</i> , Cm ^R	64

Cm^R, chloramphenicol resistant; Gm^R, gentamicin resistant; Kan^R, kanamycin resistant; Tem^R, temocillin resistant; Tem^S, 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) ^{a,b}													
	AMX	TEM	TIC	PIP	FOX	CTX	FEP	ATM	ERT	GEN	CIP	TGC	COL	CHL
<i>E. asburiae</i>														
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	32 (8)	64 (32)	4	>256	0.5	0.03	0.12 (4)	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 Ω mdtABCD	>256	4	8 (4)	2	>256	0.5	0.03	0.03	0.25	0.12	<0.01	0.5	16	4
Temo_S/pBAD Ω acrD	>256	64 (16)	64 (32)	2	>256	0.5	0.03	0.12 (4)	0.25	0.12	<0.01	0.5	32	4
<i>E. cloacae</i> subsp. <i>cloacae</i>														
ATCC 13047	>256	8	4	8	>256	2	0.03	0.5	0.25	0.25	0.03	0.5	>256	4
ATCC13047 Δ baeS	>256	4	4	8	>256	2	0.03	0.5	0.25	0.25	0.03	0.5	256	4
ATCC13047 Δ mdtB	>256	8	4	8	>256	2	0.03	0.5	0.25	0.25	0.03	0.5	256	4
ATCC13047 Δ 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 Ω mdtABCD	>256	8	16 (4)	8	>256	2	0.03	0.5	0.5	0.25	0.03	0.5	>256	4
ATCC13047/pBAD Ω acrD	>256	64 (8)	128 (16)	8	>256	2	0.03	2 (4)	0.25	0.25	0.03	0.5	>256	4
<i>E. coli</i>														
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 ^{Thr175Pro}	4	64 (16)	64 (16)	2	2	0.12	0.06	1 (16)	0.06	0.12	<0.01	0.12	0.12	4

^a 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.

^b Numbers in boldface are MICs that are significantly different (fold changes of ≥ 4 are indicated in parentheses).

TABLE 3 Nucleotide changes identified between *E. asburiae* Temo_S and Temo_R isogenic isolates

Gene ^a	Predicted product	Nucleotide change ^a	Amino acid change	Homolog in <i>E. cloacae</i> subsp. <i>cloacae</i> ATCC 13047 ^b
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

^a Gene names and positions from the annotated sequence of the *E. asburiae* Temo_S clinical isolate (GenBank accession no. SAMN31775694).

^b 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 ^a	Predicted product	Nucleotide change ^a	Amino acid change	Homolog in <i>E. cloacae</i> subsp. <i>cloacae</i> ATCC 13047 ^b
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

^a Gene names and positions from the annotated sequence of the *E. asburiae* Temo_S clinical isolate (GenBank accession no. SAMN31775694).

^b Gene names are from the annotated sequence of the *E. cloacae* subsp. *cloacae* ATCC 13047 (GenBank accession no. NC_014121).