







Implications of two-component systems *EnvZ/OmpR* and *BaeS/BaeR* in *in vitro* temocillin resistance in *Escherichia coli*

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Background: *BaeS/BaeR* is a two-component system of *Escherichia coli* that controls the expression of porins and efflux pumps. Its role in beta-lactam resistance is limited.

Objectives: To study the role of *baeS/baeR* two-component system in temocillin resistance in *E. coli*.

Methods: *E. coli* strain BW25113 and single-gene deletion mutants related to two-component systems were collected from the KEIO collection. Double-gen deletion mutants were generated. Temocillin-resistant mutant frequencies were determined at 32 mg/L. *E. coli* BW25113 mutants were selected by selective pressure from serial passages. Biological costs were analysed by growth curves. Genomes of the generated mutants were sequenced. The expression level of the *mdtA*, *mdtB*, *mdtC*, *acrD* and *tolC* in the $\Delta baeS$ mutant was determined by RT-PCR (with/without temocillin exposure).

Results: The frequency of temocillin mutants ranged from 2.12×10^{-8} to 4.51×10^{-8} in single-porin mutants. No mutants were recovered from *E. coli* BW25113 ($>10^{-9}$). Selection of temocillin-resistant variants by serial passage yielded mutants up to 128 mg/L. Mutations were found in the *baeS* gene. Temocillin MICs ranged from 4 to 32 mg/L (highest MICs for $\Delta baeS$ and $\Delta ompR$). The efflux pumps *mdtA*, *mdtB*, *mdtC* and *acrD* pumps were over-expressed 3–10-fold in the presence of temocillin in $\Delta baeS$ compared to control.

Conclusions: Mutations in the sensor histidine kinase, *baeS*, may be involved in temocillin resistance through the expression of the efflux pumps *mdtABC* and *acrD*. In addition, the low mutation rate may be a good predictor of temocillin activity.

Introduction

Antimicrobial resistance is one of the biggest threats facing public health in infectious diseases. In recent years, the increase in multidrug-resistant *Enterobacteriales* has been a challenge in patient management due to the reduction in therapeutic options. Despite the importance in promoting research and development of new antibiotics, investment from the pharmaceutical industry and biotech companies is declining and, in the last decade, the FDA has approved only 17 new systemic antibiotics.^{1,2} Owing to the lack of new

antibiotic molecules, ‘old antibiotics’ such as colistin and some aminoglycosides have been rescued to treat infections caused by multidrug-resistant bacteria.³ In this context, the use of temocillin, a semi-synthetic 6- α -methoxy derivative of ticarcillin marketed in the 1980s with a spectrum of action against *Enterobacteriales* and currently available for use in countries such as Belgium, France, Germany, Luxembourg and the UK has been recovered.⁴

Resistance to β -lactams in *Enterobacteriales* is mainly mediated by the production of ESBL, AmpC and carbapenemases.⁵ The temocillin stability against ESBL- and AmpC-type β -lactamases

and some carbapenemases such as KPC-type, has made it an alternative to antibiotics of last resort such as carbapenems, avoiding its overuse.⁶ However, although the main β -lactam resistance mechanisms are mediated by enzyme production, changes in membrane permeability also play an important role through alterations in the expression of outer membrane porins (Omps) and/or efflux pumps, causing resistance in *Enterobacteriales*.^{7,8} However, these types of study are scarce in temocillin, whose resistance mechanisms remain largely unknown.

Porins and efflux pumps are channels located in the plasma membrane of Gram-negative bacteria required for the exchange of small molecules and nutrients, whose expression is regulated by changes in external stress conditions.⁹ Some of these are tightly regulated by two-component systems, consisting of a membrane-bound histidine kinase that senses changes in the environment and sends a response to a regulator that modulates the differential expression of the genes involved.¹⁰ The *envZ/ompR* system is a two-component system in which *envZ* has the role of a histidine kinase sensor and *ompR* acts as a regulator of two porins, *ompC* and *ompF*. There are other porins, including *ompA*, *ompX* and *ompW*, but although their regulation is also modulated by environmental conditions, they appear to be independent of the control of the *envZ/ompR* system.¹¹ While *ompA* plays an important role in maintaining the membrane integrity,¹² *ompW* and *ompX* have been implicated in different responses to external stresses, including iron homeostasis.¹³ In addition to the *envZ/ompR*, another two-component system, *baeS/baeR*, has also been implicated in drug resistance in *E. coli*.¹⁴ The sensor kinase BaeS and the response regulator BaeR modulate the expression of inner efflux pumps *mdtABC* and *acrD*,¹⁵ which, in conjunction with the outer membrane efflux pump *tolC*, allow the efflux of a variety of compounds from the outer membrane.¹⁶ Moreover, recent studies suggest that single point mutations in the BaeS increase temocillin resistance in *Enterobacter cloacae* complex, although more studies are needed to confirm this in other species of *Enterobacteriales*.¹⁷

Therefore, the objective of this study was to determine the role of porins and efflux pumps in resistance mechanisms to temocillin in *E. coli* under the control of the two-component systems *envZ/ompR* and *baeS/baeR*.

Materials

Bacterial strains

The wild-type *E. coli* BW25113 strain and single-gene deletion mutants related to two-component systems that express porins *envZ/ompR* ($\Delta envZ$, $\Delta ompR$, $\Delta ompA$, $\Delta ompC$, $\Delta ompF$, $\Delta ompW$, $\Delta ompR\Delta ompX$) and efflux pumps *baeS/baeR* ($\Delta baeS$, $\Delta baeR$, $\Delta tolC$, $\Delta acrD$, $\Delta mdtA$, $\Delta mdtB$ and $\Delta mdtC$), were collected from the KEIO collection.¹⁸ The double deletion mutants, $\Delta ompR\Delta ompF$, $\Delta ompR\Delta ompW$, $\Delta ompR\Delta ompX$, $\Delta ompR\Delta ompA$, $\Delta ompC\Delta ompR$, $\Delta ompC\Delta ompF$, $\Delta ompW\Delta ompC$, $\Delta ompX\Delta ompC$, $\Delta ompA\Delta ompC$, $\Delta ompF\Delta ompX$ and $\Delta ompX\Delta ompW$, were generated by phage P1vir transduction (Coli Genetic Stock Center, Yale University) from the previously selected strains, as described.¹⁹

Temocillin mutant frequency determination

Temocillin-resistant mutant frequencies were evaluated for the wild-type *E. coli* BW25113 and the single-gene deletion

porin mutants ($\Delta ompR$, $\Delta ompF$, $\Delta ompC$, $\Delta ompA$, $\Delta ompX$ and $\Delta ompW$). Mutant frequencies were determined in quadruplicate as previously described.²⁰ Briefly, bacterial cultures were plated on drug-free MHA and MHA plates supplemented with 32 mg/L of temocillin (Eumedica, Belgium), colonies were enumerated after 24 h incubation at 37°C and mutant frequencies were calculated as the ratio of temocillin-resistant mutant cfu to total cfu.

In vitro selection of temocillin resistance via serial passages and bacterial fitness

Selection of resistance to temocillin in *E. coli* BW25113 was performed by serial passage in 2-fold temocillin concentration in each subculture, in a range of 2–128 mg/L. Serial passage experiments were performed on MHA plates supplemented with each temocillin concentration. The fitness cost was measured by the growth time (doubling time in h) in *E. coli* BW25113, the mutants recovered at each temocillin concentration above 16 mg/L and the mutants obtained in temocillin frequency assay. Growth curves were generated in 96-well plates at 37°C and read every hour at OD₆₀₀ (Infinite Nano 200 Pro; TECAN, Switzerland). Analyses were performed by fitting a non-parametric form (smoothing factor 0.55) the experimental growth curves using *QuvE* v.1.1 and the means values of each mutant were compared to control *E. coli* BW25113 by Student's *t*-test ($P \pm 0.05$).²¹

Whole-genome sequencing

The bacterial genomes of the mutants recovered from the serial passage study at concentrations >16 mg/L temocillin and the mutants obtained from the mutant frequency assay at 32 mg/L temocillin were sequenced using the MiSeq platform system (Illumina, San Diego, CA, USA). DNA extractions were performed with automatic system MagCore HF16 Plus (RCBBioscience, New Taipei City, Taiwan). Sample library was prepared with the Nextera XT DNA library preparation kit (Illumina, CA, USA) and DNA sequencing was carried out with the MiSeq Reagent Kit V3 (600 cycles) and the Illumina MiSeq sequencer (2×300 paired-end reads). The reads were quality filtered and *de novo* assembling of raw reads into contigs using were performed using CLC Genomic Workbench v.9.1 (Qiagen, Hilden, Germany). Mutations were called using the Breseq variant report v.0.35 via Galaxy/Pasteur (<https://galaxy.pasteur.fr>) using annotated genome of *E. coli* BW25113 as reference (NZ_CP009273.1).²²

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing to temocillin (Eumedica, Belgium), ertapenem (Sigma Aldrich, USA), tobramycin (Sigma Aldrich), fosfomicin (Santa Cruz Biotechnology, USA) and ciprofloxacin (Sigma Aldrich, USA) was determined by microdilution assay according to ISO 20776-1:2019 (European Committee for Standardization 2006). Microdilution was performed in 96-well plates containing concentration between 128 and 0.06 mg/L in MHB medium for temocillin, tobramycin and fosfomicin and between 16 and 0.002 mg/L for ertapenem and ciprofloxacin. The MIC of each antibiotic was selected by the mean of three determinations considering as the lowest concentration showing no visible growth. To study possible cross-resistance for *E. coli*

BW25113, $\Delta baeS$ mutant, mutants obtained after sequential passages and single-gene deletion porin mutants, disc diffusion was performed for ampicillin, cefuroxime, cefoxitin, cefotaxime, ceftazidime, cefepime, aztreonam, ertapenem, imipenem and meropenem in accordance with EUCAST guidelines. Also, hetero-resistance phenotype to temocillin, done by gradient strip assays following the manufacturer's instructions, to *E. coli* ATCC 25922 was used as a control strain.

Real-time quantitative RT-PCR (RT-qPCR)

The genes encoding pumps regulated by the two-component *baeS/baeR* system (*mdtA*, *mdtB*, *mdtC* and *acrD*) and the not-directly related genes *tolC* and *acrA*, were selected to verify the expression level of these. Expression levels were measured by RT-qPCR (LifeCycler, Roche, Switzerland) in the $\Delta baeS$ mutant using *E. coli* BW25113 as control, with or without exposure to temocillin concentrations of 16 and 2 mg/L, respectively. Total RNA was extracted using QIAcubesQiagen (Venlo, the Netherlands), following the manufacturer's instructions. cDNA was synthesized from total RNA samples using transcriptor first strand cDNA synthesis kit (Roche, Switzerland). Real-time PCR was performed in the LightCycler 480 Instrument II (Roche, Switzerland) with specific primers (Supplementary Table S1, available as Supplementary data at JAC Online). *gyrB* was chosen as a housekeeping gene. The relative expression levels of each gene were calculated using the $2^{-\Delta\Delta Ct}$ method. Experiments were performed in triplicate.

Results

Temocillin-resistant mutant characterization

The temocillin mutant frequencies for the isogenic collection are shown in Table 1. Inactivation of porin genes resulted in the selection of mutants at 32 mg/L in $\Delta ompR$, $\Delta ompF$, $\Delta ompC$, $\Delta ompX$, $\Delta ompW$ ($\Delta ompR$ -32, $\Delta ompF$ -32, $\Delta ompC$ -32, $\Delta ompX$ -32, $\Delta ompW$ -32). No $\Delta ompA$ -derived mutants were obtained. Temocillin mutant frequencies in these isolates ranged from 2.12×10^{-8} to 4.51×10^{-8} (Table 1). Mutants recovered from mutant frequencies assay showed a MIC to temocillin between 64–128 mg/L (Table 1). However, when one-step mutant frequency assay at a concentration of 32 mg/L of temocillin was performed in *E. coli* BW25113, no mutants were recovered. After serial passages, *E. coli* BW25113 was able to grow in duplicate at each temocillin concentrations of 4, 8, 16, 32, 64 and 128 mg/L. All mutants recovered from the serial passage assay showed a 2–32-fold increase MIC to temocillin over control *E. coli* BW25113 (Table 1). Regarding fitness cost analyses, mutants recovered at 16, 32, 64 and 128 mg/L significantly increased their doubling time between 1.5- and 2.9-fold longer than *E. coli* BW25113, with mutant BW25113-64 and the BW25113-128 showing the longest doubling time. The mutants obtained in the temocillin frequency assay showed no significant difference in their doubling time compared to the control *E. coli* BW25113 (Table 1).

Mutations relative to *E. coli* BW25113 were compared using Breseq mutation prediction pipeline. Mutants generated at 32, 64 and 128 mg/L of temocillin, in the serial passages assay, contained a significant number of reads carrying the mutation Q163E (CAG→GAG) ($P < 0.001$) in the histidine kinase sensor

baeS at position 2 156 843 in the chromosome, whereas this mutation was not found in the mutant generated at 16 mg/L (Table 1). Analysis of the mutations in the isolates obtained from the mutant frequency assay, also identified point mutations in *baeS*, specifically S167I (AGC→ATC) in the *ompR* and *ompW* mutants, V270G (GTG→GGG) in the *ompC* mutant, D156N (GAT→AAT) in the *ompF* mutant. In the *ompX* mutant, a 6 bp deletion was found between positions 2156469 and 2156476 (Table 1).

Antimicrobial susceptibility

The analysis of a *E. coli* BW25113 mutant isogenic collection allowed to us determine the impact of multiple porins and efflux pumps in temocillin resistance. MICs to temocillin ranged from 1 to 32 mg/L, with the highest MICs $\Delta baeS$ (MIC 32 mg/L) and $\Delta ompR$ (MIC 16 mg/L) mutants with an increase of 8- and 4-fold increase, respectively, compared to *E. coli* BW25113 (MIC of 4 mg/L) (Table 2). The lowest MIC was recorded in $\Delta tolC$ (MIC of 1 mg/L) with a 4-fold decrease in temocillin concentration compared to *E. coli* BW25113. Other antibiotics whose resistance is associated with changes in membrane permeability with loss of porins (ertapenem), efflux pumps (ciprofloxacin and tobramycin) or defects in the synthesis of transporters (fosfomycin), were also tested in the isogenic collection. For ertapenem, the MICs ranged from 0.003 to 0.5 mg/L. Of the single-gene deletion mutants, the loss of the *ompF* and *ompR* genes resulted in a 4- and 64-fold increase, in MIC, respectively. MICs to ciprofloxacin, fosfomycin and tobramycin in these mutants ranged from 0.001–0.125 mg/L, 1–4 and 0.25–2 mg/L, respectively, in these mutants. The *tolC* mutant showed a 2-fold decreased in MICs to these antimicrobials compared to *E. coli* BW25113. The temocillin MICs of the double-porin mutants ranged from 4 to 16 mg/L. Mutants with deletions in *ompR* reached an MIC at temocillin of 16 mg/L, with no differences with respect to the single *ompR* mutant. The double loss of the *ompC* and *ompF* porins did not increase the MIC at temocillin with respect to the *E. coli* BW25113 control. With respect to other antibiotics, double-porin mutants (except double-gene mutants of *ompA* or *ompW*) increased their MIC to ertapenem 4- to 64-fold compared to the control. There were no significant MIC changes to ciprofloxacin or tobramycin in these double-gene mutants. With respect to cross-resistance, a reduction in the size of ampicillin inhibition halo (3–4 mm, changing clinical category from susceptible to resistance) was observed in the mutants obtained with serial passages and a reduction in aztreonam inhibition halo (4 mm with no changes in clinical category) was observed for the BW-64 and BW-128 mutants. No cross-resistance was found for the rest of the β -lactams studied (Supplementary Table S2). Regarding heteroresistance phenotype, colonies within the ampicillin inhibition halo were observed in the strains studied, including for the control *E. coli* BW-25113 (Supplementary Table S2). Colonies or double inhibition halos were also observed near the ellipse of the temocillin gradient strips for all the strains studied (Supplementary Figure S1).

Efflux pumps expression

The genes encoding efflux pumps, regulated by the two-component *baeS/baeR* system in addition to *tolC* and *acrA*,

Table 1 . Temocillin MIC (mg/L), position and mutations found in *baeS* gene in *E. coli* BW25113 and isogenic collection generated by serial passage in increasing temocillin concentration and mutant obtained from porin deficiency isolates in the mutant frequency assay (32 mg/L), temocillin mutant frequency (percentage of reads) and fitness cost measured by duplication time (h). Bold indicates amino acid change and position

Strain name	MIC (mg/L)	Information of mutations in <i>baeS</i> gene					
		Position	Codon change	Frequency	P value	Temocillin mutant frequency (mean + SD)	Duplication time (h)
<i>E. coli</i> BW25113	4	—	—	—	—	>10 ⁻⁹	0.67 ± 0.15
BW25113-16	16	ND	ND	ND	ND	—	1.03 ± 0.22 ^a
BW25113-32	32	2 156 843	Q163E (CAG→GAG)	96%	0.053	—	1.48 ± 0.93 ^a
BW25113-64	64	2 156 843	Q163E (CAG→GAG)	100%	0.001	—	1.94 ± 1.29 ^a
BW25113-128	128	2 156 843	Q163E (CAG→GAG)	98%	0.034	—	1.54 ± 0.92 ^a
ΔompR-32	64	2 156 856	S167I (AGC→ATC)	100%	0.001	4.51 × 10 ⁻⁸ ± 2.06 × 10 ⁻⁸	0.76 ± 0.18
ΔompC-32	64	2 157 165	V270G (GTG→GGG)	100%	0.001	2.74 × 10 ⁻⁸ ± 1.07 × 10 ⁻⁸	0.72 ± 0.17
ΔompF-32	128	2 156 822	D156N (GAT→AAT)	100%	0.001	3.77 × 10 ⁻⁸ ± 2.76 × 10 ⁻⁸	0.70 ± 0.19
ΔompX-32	64	2,156,469-2,156,476	Δ6 bp	100%	0.001	2.94 × 10 ⁻⁸ ± 1.66 × 10 ⁻⁸	0.68 ± 0.17
ΔompW-32	64	2 156 856	S167I (AGC→ATC)	100%	0.001	2.12 × 10 ⁻⁸ ± 1.44 × 10 ⁻⁸	0.65 ± 0.14

ND, not detected.

^aP ≤ 0.05.

Table 2. Antimicrobial susceptibility (MIC, mg/L) of *E. coli* BW25113 mutant isogenic collection

Strain name	MIC (mg/L)					
	TEM	ERT	CIP	FOT	TOB	
ATCC25922	16	0.008	0.008	1	0.5	
BW25113	4	0.008	0.008	1	0.5	
ΔenvZ	8	0.004	0.008	1	1	
ΔompR	16	0.5	0.016	4	1	
ΔompA	8	0.004	0.008	2	1	
ΔompC	4	0.004	0.008	2	0.5	
ΔompF	16	0.06	0.016	2	2	
ΔompX	8	0.016	0.008	1	1	
ΔompW	8	0.004	0.002	1	2	
ΔbaeS	32	0.006	0.008	1	1	
ΔbaeR	8	0.004	0.008	4	2	
ΔacrD	8	0.004	0.125	1	1	
ΔmdtA	8	0.016	0.125	2	0.5	
ΔmdtB	8	0.016	0.004	2	0.5	
ΔmdtC	8	0.016	0.004	1	0.5	
ΔtolC	1	0.004	0.002	1	0.25	
ΔompR-ΔompC	16	0.25	0.006	8	0.5	
ΔompR-ΔompF	16	0.5	0.016	8	0.5	
ΔompR-ΔompA	8	0.125	0.016	2	0.25	
ΔompR-ΔompW	16	0.125	0.016	4	0.5	
ΔompC-ΔompF	8	0.5	0.016	8	0.5	
ΔompC-ΔompA	4	0.016	0.006	2	0.25	
ΔompC-ΔompW	4	0.06	0.006	2	0.5	
ΔompC-ΔompX	4	0.006	0.006	1	0.5	
ΔompF-ΔompX	16	0.03	0.03	2	0.5	
ΔompF-ΔompW	8	0.03	0.03	32	1	
ΔompA-ΔompX	8	0.016	0.016	4	0.5	
ΔompA-ΔompW	4	0.016	0.016	1	1	
ΔompW-ΔompX	4	0.016	0.016	2	1	

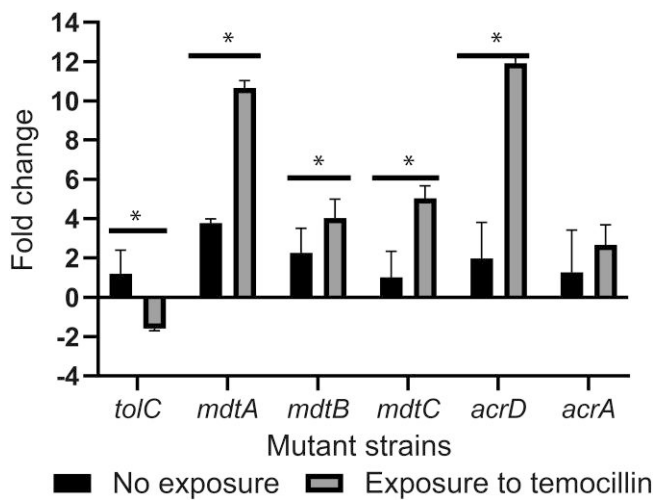


Figure 1. Fold change expression of the efflux pump components *tolC*, *mdtA*, *mdtB*, *mdtC*, *acrD* and *acrA* in the $\Delta baeS$ compared to *E. coli* BW25113 with and without temocillin exposure.

were measured in response to temocillin in the $\Delta baeS$ mutant compared to the control *E. coli* BW25113. Without temocillin exposure, the expression of the studied efflux pumps did not change in the $\Delta baeS$ mutant compared to the control *E. coli* BW25113. When single mutant $\Delta baeS$ and *E. coli* BW25113 were exposed to 1/2xMIC of temocillin, *mdtA*, *mdtC* and *acrD* genes were overexpressed by 7-, 4- and 10-fold, respectively, in $\Delta baeS$ compared to the expression of these efflux pumps in the control *E. coli* BW25113. The expression levels of *tolC* and *acrA*, did not change on exposure to temocillin (Figure 1).

Discussion

This work provides insight into the resistance mechanisms to temocillin in *E. coli* through the involvement of the two-component systems *envZ/ompR* and *baeS/baeR*, finding that mutations or deletions in the sensor histidine kinase *BaeS* lead to an increase in MIC to temocillin, mediated by overexpression in the efflux pumps *acrD*, *mdtABC*.

Temocillin is a recently rescued forgotten antibiotic due to its resistance to hydrolysis by Ambler class A and C β -lactamases.⁶ However, the molecular basis of resistance to temocillin remains largely unknown and although alterations in membrane permeability and the enzymatic barrier are known to contribute to antibiotic resistance in *E. coli*,^{12,23} there are few studies elucidating it with temocillin.

It is proposed that the frequency of selection of mutants *in vitro* to temocillin is low, as is the selection of resistant mutants detected *in vivo*.²⁴ In terms of the frequency of spontaneous mutant appearance, the frequency of appearance of single-porin mutants was 10^{-8} , in contrast to the results obtained with *E. coli* BW25113 where no temocillin-resistant mutant was recovered. These data agree with other studies where the frequency of occurrence of temocillin mutants reached 10^{-8} .²⁵ However, mutants were recovered from *E. coli* BW25113 during serial passage. In all these recovered mutants, the MIC to temocillin was

increased between 2- and 32-fold, compared to control *E. coli* BW25113. Analysis of the relative mutations in mutants obtained at 32, 64 and 128 mg/L of temocillin, showed various changes in the *BaeS* histidine kinase sensor as well as in the porin mutants recovered from the mutant frequency assay. In this work, the doubling time of the mutants recovered in the serial passaging assay with temocillin was slightly longer than that of the control *E. coli* BW25113. It is known that strains with mutations conferring antibiotic resistance often have a lower growth rate, but these mutations may confer a survival advantage that selects for the resistant population.²⁶ Although temocillin resistance was not reversed and the MIC to temocillin remained stable in the mutants, it is unclear how this fitness cost could impact clinical isolates or if this biological cost could be compensated for by other mutations.

The regulation of the membrane permeability through reducing uptake by downregulating the expression of porins, or by increasing the efflux of compounds by overexpressing efflux pumps, is mediated by two-component systems.²⁷ The two-component systems, *EnvZ/OmpR* and *BaeS/BaeR*, regulate membrane permeability via porins and efflux pumps, respectively. In this study, the MIC to temocillin was studied in single mutants of several porins in an isogenic collection. Although it was originally thought that resistance to temocillin could be due to a reduced entry of the molecule into the cell through the porins of the outer membranes,²⁸ single mutants of $\Delta ompC$, $\Delta ompF$, $\Delta ompA$, $\Delta ompX$ and $\Delta ompW$ did not show a significant increase in their MIC to temocillin compared to the control *E. coli* BW25113. An increase in 4xMIC was only observed for the single mutant $\Delta ompR$, the main regulator of *OmpC* and *OmpF*, porins for which changes in their expression have been described to confer a decreased susceptibility to some beta-lactams such as ertapenem.²⁹ Indeed, loss of the *ompF* and *ompR* genes resulted in 4- and 64-fold increases in MIC to ertapenem, respectively. Whereas there are studies linking resistance to other beta-lactams, such as ceftriaxone, in *S. enterica*, mediated by *OmpW* and *OmpX*-like porins,³⁰ no increase in MIC to temocillin was observed for the loss of these in our study. Similarly, no changes in MICs to temocillin were observed in the double-porin mutants, which obtained the same MIC values as their single mutants.

The *BaeS/BaeR* two-component system has also been linked to antimicrobial resistance in *E. coli*.³¹ Moreover, it has been described that overproduction of *BaeR* can also cause novobiocin resistance through overexpression of the drug efflux system *mdtABC*.³² In this work, mutation and deletions cause overexpression of the efflux pump *mdtABC*, and especially of *acrD*. Moreover, $\Delta baeS$ mutant increased its MIC to temocillin up to 8-fold in compared to the control. In addition to novobiocin resistance, increased erythromycin resistance has also been described by gain-of-function mutations in *baeS* that constitutively activate the *baeSR* two-component regulatory system to increase expression of the efflux pump *mdtABC*.³³ Although in Enterobacteriales both *baeS* and *baeR* have been implicated in resistance to ciprofloxacin through activation of the histidine kinase sensor³⁴ and to ceftriaxone through loss of *baeR*,³⁰ the involvement of the *baeS/baeR* two-component system in antibiotic resistance remains unclear. Recently, Guérin *et al.* showed that temocillin resistance in the *Enterobacter asburiae* can result from a single *BaeS* alteration, probably resulting in the

permanent phosphorylation of BaeR and leading to AcrD overexpression and temocillin resistance through enhanced active efflux.¹⁷ However, because of the presence of polymorphisms present in *baeS* from different Enterobacteriales species and even within the same species, it is not possible to compare these point-single mutations.

This work has certain limitations. This study used the KEIO collection, which is derived from a non-uropathogenic intestinal strain of *E. coli* K12. It is unclear how the selected mutations may affect other *E. coli* strains from different sources with varying responses to factors such as nutrient intake and cellular homeostasis, which are involved in the regulation of resistance mechanisms such as porins and efflux pumps. Also, this study was performed entirely under *in vitro* conditions and it is unknown what effects of two-component systems may affect temocillin resistance *in vivo*. Further studies are needed to clarify the *in vivo* implications of this temocillin resistance.

In conclusion, alterations in *E. coli* membrane porins may not be involved in temocillin resistance. However, this work has shown that mutations in the histidine kinase sensor *BaeS* can alter the expression of plasma membrane efflux pumps in *E. coli* by overexpressing the efflux pumps *mdtABC* and *acrD* contributing to temocillin resistance. Although this resistance has some biological cost in terms of growth rate, it does not appear to contribute significantly to the development of the mutants. In addition, the low rate of mutant emergence may be a good predictor of temocillin activity.

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Transparency declarations

None to declare.

Supplementary data

Figure S1 and Tables S1 and S2 are available as [Supplementary data](#) at JAC Online.

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