Novel Chromosomal Mutations Responsible for Fosfomycin Resistance in Escherichia coli

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Fosfomycin resistance in Escherichia coli results from chromosomal mutations or acquisition of plasmid-mediated genes. Because these mechanisms may be absent in some resistant isolates, we aimed at decipher the genetic basis of fosfomycin resistance in E. coli. Different groups of isolates were studied: fosfomycin-resistant mutants selected in vitro from E. coli CFT073 (MIC = 1 mg/L) and two groups (wildtype and non-wildtype) of E. coli clinical isolates. Single-nucleotide allelic replacement was performed to confirm the implication of novel mutations into resistance. Induction of uhpT expression by glucose-6-phosphate (G6P) was assessed by RT-qPCR. The genome of all clinical isolates was sequenced by MiSeq (Illumina). Two first-step mutants were obtained in vitro from CFT073 (MICs, 128 mg/L) with single mutations: G469R in uhpB (M3); F384L in uhpC (M4). Second-step mutants (MICs, 256 mg/L) presented additional mutations: R282V in galU (M7 from M3); Q558* in lon (M8 from M4). Introduction of uhpB or uhpC mutations by site-directed mutagenesis conferred a 128-fold increase in fosfomycin MICs, whereas single mutations in galU or lon were only responsible for a 2-fold increase. Also, these mutations abolished the induction of uhpT expression by G6P. All 14 fosfomycin-susceptible clinical isolates (MICs, 0.5–8 mg/L) were devoid of any mutation. At least one genetic change was detected in all but one fosfomycin-resistant clinical isolates (MICs, 32 – 256 mg/L) including 8, 17, 18, 5, and 8 in uhpA, uhpB, uhpC, uhpT, and glpT genes, respectively. In conclusion, novel mutations in uhpB and uhpC are associated with fosfomycin resistance in E. coli clinical isolates.

Keywords: E. coli, fosfomycin-resistant, uhpB, uhpC, galU, lon

INTRODUCTION

Fosfomycin, a phosphonic acid derivative discovered in 1969, has become the first-choice antibiotic for the ‘single-dose’ oral treatment of uncomplicated urinary tract infections (UTIs) (Falagas et al., 2016). It is a bactericidal antibiotic with a broad spectrum of activity that interferes with the first step of peptidoglycan synthesis in both Gram-positive and Gram-negative bacteria (Castaneda-Garcia et al., 2013; Falagas et al., 2016). As a phosphoenolpyruvate analog, fosfomycin inhibits the cytosolic...
UDP-N-acetylglucosamine enolpyruvyltransferase (also named MurA) by covalent binding to key residue C115 of the enzyme active site, preventing the formation of N-acetylmuramic acid (Kahan et al., 1974). This low-molecular-weight antibiotic enters into the bacterial cell via two transport uptake systems: the glycerol-3-phosphate permease (encoded by glpT) constitutively expressed and, the hexose phosphate uptake transporter (encoded by uhpT) inducible by extracellular glucose-6-phosphate (G6P) (Castaneda-Garcia et al., 2013). While transcription of glpT and uhpT is regulated by glpR and uhpRABC, respectively, their expression also requires high levels of cyclic AMP (cAMP) combined with, as a complex, the cAMP receptor protein (CRP) (Castaneda-Garcia et al., 2013). cAMP levels depend on the activity of CyaA adenyl cyclase and are regulated by the phosphotransferase enzyme PtsI (Castaneda-Garcia et al., 2013).

Despite its widespread clinical use for many years in several countries, the prevalence of fosfomycin resistance is still low among *E. coli* clinical isolates, usually below 3% (4,5). Concerning multi-drug-resistant (MDR) isolates as ESBL-producing *E. coli*, levels of susceptibility to fosfomycin remain as high as 80% (Falagas et al., 2016, 2019; Aghamali et al., 2019). By contrast, the selection of fosfomycin-resistant mutants is much easier under *in vitro* conditions at high mutation frequencies (ca. 10⁻⁸–10⁻⁷) (Karageorgopoulos et al., 2012). This paradox is partially due to a significant resistance-associated fitness cost with decrease *in vitro* rate and attenuated virulence *in vivo* (Marchese et al., 2003; Nilsson et al., 2003; Pourbaix et al., 2017), and higher fosfomycin activity under urinary tract physiological conditions (i.e., urine acidification and anaerobiosis counterbalanced by negligible amounts of urinary G6P) that enhance expression of GlpT and UhpT (Martin-Gutierrez et al., 2018; Pourbaix et al., 2019).

Due to the unique mechanism of action of fosfomycin, there are no cross-resistances with other antibacterial agents (Falagas et al., 2016; Silver, 2017). However, three specific mechanisms of fosfomycin resistance were described in *E. coli*: impaired drug uptake, enzymatic drug inactivation and target modification (Cattoir and Guérin, 2018). Reduced drug uptake is the most frequent resistance mechanism for *in vitro* mutants and clinical isolates. It results from chromosomal mutations that alter the function or expression of GlpT and/or UhpT transporters. These mutations (mutations, insertions, deletions) can arise either in structural genes (i.e., glpT and uhpT) or in genes coding for regulators (i.e., uhpR, cyaA, and ptsI) (Castaneda-Garcia et al., 2013; Silver, 2017). More recently, there is the emergence of plasmid-mediated metallo-dependent enzymes (including FosA, FosB, and FosX) that inactivate the drug, of which FosA3 is, by far, the most frequently variant in *E. coli* (Yang et al., 2019). Much more uncommon, fosfomycin resistance can be mediated by qualitative and/or quantitative modifications of MurA (Silver, 2017).

The aim of this study was to (1) investigate the genetic basis of fosfomycin resistance in *E. coli* mutants selected *in vitro* that had no mutations in genes previously reported to be involved in resistance (i.e., glpT, uhpT, uhpR, murA, cyaA, and ptsI), (2) demonstrate experimentally the role of novel mutations identified in four different genes, and (3) determine their prevalence among a collection fosfomycin-resistant *E. coli* clinical isolates recently collected in France.

### MATERIALS AND METHODS

#### Bacterial Strains

Three different groups of *E. coli* strains were used in this study (Tables 1, 2). The first group consisted of fosfomycin-resistant mutants (CFT073_M3 to CFT073_M8) obtained from the parental strain *E. coli* CFT073 (uropathogenic strain belonging to phylgroup B2) (Welch et al., 2002) after serial passages on Mueller–Hinton (MH) medium (Difco, Becton Dickinson, Rungis, France) containing increased concentrations of fosfomycin (from 32 to 128 mg/L) in the presence of G6P (25 mg/L). The two other groups consisted of *E. coli* epidemiologically unrelated clinical isolates (wildtype and non-wildtype phenotype of resistance to fosfomycin, according to the epidemiological cut-off established at 8 mg/L) responsible for UTIs in patients hospitalized in two French university hospitals between 2012 and 2017.

Bacterial strains were routinely grown at 35°C in Luria–Bertani (LB) broth or agar supplemented with appropriate antibiotics, unless otherwise specified. When required, *E. coli* were grown on media supplemented with 100 mg/L ampicillin, 40 mg/L kanamycin or 25 mg/L chloramphenicol.

#### Antimicrobial Susceptibility Testing

MICs of fosfomycin were determined by using the agar dilution reference method described by the European Committee on Antimicrobial Susceptibility Testing¹. Briefly, bacterial suspension was prepared to match the turbidity of the 0.5 McFarland in sterile physiological water (ca. 10⁸ CFU/mL). Agar dilution was performed using MH agar plates containing 25 mg/L G6P. Cell suspensions were further diluted in MH broth and were delivered onto plates using a Steer replicator, which delivered ca. 10⁴ CFU for each isolate. Concentrations tested ranged from 256 to 0.125 mg/L. *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 25923 were used as control strains and were run in parallel with every experiment. Each MIC determination was performed at least three times. The current susceptibility breakpoint of fosfomycin for *Enterobacteriaceae* is a MIC ≤ 32 mg/L according to the EUCAST guidelines (see text footnote 1).

#### *In vitro* Bacterial Growth Rate

Growth rates at 35°C were measured in Luria–Bertani (LB) broth and Nutrient broth (NB) at pH 5 or 7 as well as in sterile-filtered pooled human male urine (pH = 6.5). The bacteria were grown aerobically overnight at 35°C and approximately 10⁵ colony-forming units (CFUs) were inoculated into 200 µL of growth medium on a bioscreen plate and the optical density at 600 nm was read each 5 min for 24 h with a multimode reader Infinite 200 Pro (Tecan, Männendorf, Switzerland). Maximal growth rate (MGR) of each strain was calculated as the inflexion point of first

¹www.eucast.org
| Strains | Characteristics | Fosfomycin MIC (mg/L) | Mutation(s) in: |
|---------|-----------------|----------------------|----------------|
|         |                 | uhpA | uhpB | uhpC | uhpT | glpT | murA | cyaA | ptsI | galU | lon |
| E. coli CFT073 | Wild-type susceptible strain (phylogenetic B2) | 1 | – | – | – | – | – | – | – | – | – |
| **In vitro mutants** | | | | | | | | | | | |
| E. coli CFT073_M3 | First-step resistant mutant derived from CFT073 | 128 | – | G469R | – | – | – | – | – | – | – |
| E. coli CFT073_M4 | First-step resistant mutant derived from CFT073 | 128 | – | – | F384L | – | – | – | – | – | – |
| E. coli CFT073_M7 | Second-step resistant mutant derived from CFT073_M3 | 256 | – | G469R | – | – | – | – | – | R282V | – |
| E. coli CFT073_M8 | Second-step resistant mutant derived from CFT073_M4 | 256 | – | – | F384L | – | – | – | – | – | Q558* |
| **Knockout mutants and trans-complemented strains** | | | | | | | | | | | |
| E. coli CFT073 ΔuhpT | CFT073 derivative with complete deletion of uhpT | 128 | – | – | del<sup>a</sup> | – | – | – | – | – | – |
| E. coli CFT073 ΔuhpB | CFT073 derivative with complete deletion of uhpB | 128 | – | del | – | – | – | – | – | – | – |
| E. coli CFT073 ΔuhpB_pBAD202 | CFT073 ΔuhpB carrying empty pBAD202 vector | 128 | – | del | – | – | – | – | – | – | – |
| E. coli CFT073 ΔuhpB_pBAD202-uhpB | CFT073 ΔuhpB carrying pBAD202<sub>ΔuhpB</sub> | 1 | – | _<sup>b</sup> | – | – | – | – | – | – | – |
| E. coli CFT073 ΔuhpC | CFT073 derivative with complete deletion of uhpC | 128 | – | – | del | – | – | – | – | – | – |
| E. coli CFT073 ΔuhpC_pBAD202 | CFT073 ΔuhpC carrying empty pBAD202 vector | 128 | – | – | del | – | – | – | – | – | – |
| E. coli CFT073 ΔuhpC_pBAD202-uhpC | CFT073 ΔuhpC carrying pBAD202<sub>ΔuhpC</sub> | 1 | – | _<sup>b</sup> | – | – | – | – | – | – | – |
| E. coli CFT073 ΔgalU | CFT073 derivative with complete deletion of galU | 2 | – | – | – | – | – | del | – | – | – |
| **Site-directed mutants** | | | | | | | | | | | |
| E. coli CFT073_uhpB<sub>G469R</sub> | CFT073 derivative with allelic replacement of uhpB by uhpB<sub>G469R</sub> | 128 | – | G469R | – | – | – | – | – | – | – |
| E. coli CFT073_uhpC<sub>F384L</sub> | CFT073 derivative with allelic replacement of uhpC by uhpC<sub>F384L</sub> | 128 | – | – | F384L | – | – | – | – | – | – |
| E. coli CFT073_galU<sub>R282V</sub> | CFT073 derivative with allelic replacement of galU by galU<sub>R282V</sub> | 2 | – | – | – | – | – | – | R282V | – | – |
| E. coli CFT073_lon<sub>Q558*</sub> | CFT073 derivative with allelic replacement of lon by lon<sub>Q558*</sub> | 2 | – | – | – | – | – | – | – | – | Q558* |

<sup>a</sup>del, deletion of the entire gene. <sup>b</sup>Wildtype gene in multicopy.
| Strain | Phylogenetic group | β-lactam resistance phenotype<sup>a</sup> | Fosfomycin MIC (mg/L) | Mutation(s) in: | Presence of fos gene(s) |
|--------|--------------------|------------------------------------------|-----------------------|-----------------|-------------------------|
|        |                    |                                          |                       | uhpA  | uhpB  | uhpC  | gtpT  | murA  | cyaA  | ptsI  | galU  | lon   |
| B60    | B2                 | ESBL                                     | 1                     | –     | –     | –     | –     | –     | –     | –     | –     | –     |
| B65    | B2                 | ESBL                                     | 1                     | –     | –     | –     | –     | –     | –     | –     | –     | –     |
| B69    | B2                 | ESBL                                     | 8                     | –     | –     | –     | –     | –     | –     | –     | –     | –     |
| B88    | B2                 | ESBL                                     | 1                     | –     | –     | –     | –     | –     | –     | –     | –     | –     |
| B108   | B2                 | WT                                       | 2                     | –     | –     | –     | –     | –     | –     | –     | –     | –     |
| B119   | B2                 | WT                                       | 0.5                   | –     | –     | –     | –     | –     | –     | –     | –     | –     |
| B120   | B2                 | WT                                       | 1                     | –     | –     | –     | –     | –     | –     | –     | –     | –     |
| B135   | B2                 | WT                                       | 0.5                   | –     | –     | –     | –     | –     | –     | –     | –     | –     |
| B140   | B2                 | WT                                       | 0.5                   | –     | –     | –     | –     | –     | –     | –     | –     | –     |
| B145   | B2                 | WT                                       | 1                     | –     | –     | –     | –     | –     | –     | –     | –     | –     |
| B151   | B2                 | WT                                       | 1                     | –     | –     | –     | –     | –     | –     | –     | –     | –     |
| C43    | B2                 | PASE                                     | 1                     | –     | –     | –     | –     | –     | –     | –     | –     | –     |
| C53    | B2                 | PASE                                     | 2                     | –     | –     | –     | –     | –     | –     | –     | –     | –     |
| C103   | B1                 | ESBL                                     | 1                     | –     | –     | –     | –     | –     | –     | –     | –     | –     |
|        |                    |                                          |                       |       |       |       |       |       |       |       |       |       |
|        |                    |                                          |                       |       |       |       |       |       |       |       |       |       |
| B56    | B2                 | ESBL                                     | 64                    | –     | –     | P169S | –     | –     | –     | –     | –     | –     |
| B97    | B2                 | ESBL                                     | 64                    | –     | –     | G397D | –     | –     | –     | –     | –     | –     |
| B175   | B2                 | WT                                       | 128                   | –     | –     | T72I  | –     | –     | L125F | –     | –     | –     |
| C05    | B2                 | WT                                       | 128                   | –     | –     | Q210* | –     | –     | –     | –     | –     | –     |
| C06    | E                  | ESBL                                     | > 256                 | Deleted operon | –     | –     | Q213* | –     | –     | –     | –     | –     |
| C09    | B1                 | PASE                                     | 256                   | –     | T374S | –     | –     | C141Y | –     | –     | –     |
| C10    | D                  | PASE                                     | 256                   | –     | –     | 1082_2557del | 736_737insT | –     | –     | –     | –     |
| C20    | B2                 | WT                                       | 128                   | 281delG | T166L, P252S | –     | –     | –     | –     | –     | –     |
| C21    | B2                 | WT                                       | 128                   | Deleted operon | –     | –     | –     | –     | –     | –     | –     |
| C33    | B2                 | WT                                       | 64                    | –     | 265_268del | –     | –     | –     | –     | –     | –     |
| C35    | D                  | WT                                       | 256                   | S104* | D205A | –     | –     | –     | –     | –     | –     |
| C38    | B2                 | HCASE                                     | > 256                 | –     | –     | –     | –     | P139Q | –     | –     | –     |
| C41    | D                  | PASE                                     | 256                   | –     | A223V | Y18H  | –     | –     | –     | –     | –     |
| C44    | D                  | PASE                                     | 64                    | A110S | D205A, A223V | G244D | –     | –     | –     | –     | –     |
| C49    | B1                 | PASE                                     | 32                    | –     | W198* | I108M | –     | –     | –     | –     | –     |
| C50    | B2                 | PASE                                     | 64                    | –     | H313Y | –     | –     | –     | –     | –     | –     |
| C51    | B2                 | PASE                                     | 64                    | –     | 1068delT | –     | Y223C | –     | –     | –     | –     |

(Continued)
| Strain | Phylagenetic group | β-lactam resistance phenotype | Fosfomycin MIC (mg/L) | Mutation(s) in: | Presence of fos gene(s) |
|--------|-------------------|-----------------------------|-----------------------|-----------------|---------------------|
|        |                   |                             |                       | uhpA | uhpB | uhpC | uhpT | glpT | murA | cyaA | ptsL | galU | lon |
| C55    | A                 | WT                          | 128                   | –    | –    | –    | 1068delT | –    | Y223C | –    | –    | –    | –    | –    |
| C62    | D                 | WT                          | 256                   | A110S| T166L| T374S| Q132*    | –    | –    | –    | –    | –    | –    | –    |
| C63    | D                 | PASE                        | 128                   | –    | T166L| P252S| 966_1239del| 101_1392del| –    | –    | –    | –    | –    | –    |
| C64    | B2                | HCASE                       | 64                    | –    | Q60* | –    | –        | –    | –    | –    | –    | –    | –    | –    |
| C68    | D                 | PASE                        | 128                   | –    | –    | Q76* | –        | –    | –    | –    | –    | –    | –    | –    |
| C73    | D                 | PASE                        | >256                  | Deleted operon| –    | –    | –        | –    | –    | –    | –    | –    | –    | –    |
| C75    | A                 | ESBL                        | 128                   | –    | R75C | –    | –        | –    | –    | –    | –    | –    | –    | –    |
| C80    | D                 | WT                          | 128                   | –    | P252S| –    | Q153S, Q353S| –    | –    | –    | –    | –    | –    | –    |
| C82    | B1                | WT                          | 64                    | –    | –    | –    | I108M   | Y60F | –    | –    | –    | –    | –    | –    |
| C84    | B1                | WT                          | 64                    | –    | –    | P36* | –        | Q7*  | –    | –    | –    | –    | –    | –    |
| C90    | B1                | WT                          | 256                   | –    | 559_1105del| –    | P97L   | –    | –    | –    | –    | –    | –    | –    |
| C91    | D                 | PASE                        | 128                   | Deleted operon| –    | –    | –        | –    | –    | –    | –    | –    | –    | –    |
| C93    | B2                | WT                          | 256                   | –    | 459_532del| –    | –       | –    | –    | –    | –    | –    | –    | –    |
| C98    | B2                | PASE                        | 64                    | –    | –    | –    | –        | –    | –    | –    | –    | –    | –    | –    |
| C100   | B2                | WT                          | >256                  | Deleted operon| –    | –    | –        | –    | –    | –    | –    | –    | –    | –    |
| C105   | B2                | WT                          | 64                    | –    | –    | 647_656del| –    | –    | –    | –    | –    | –    | –    |
| C106   | B1                | HCASE                       | 32                    | –    | –    | –    | Q66*    | Y223C| –    | –    | –    | –    | –    | –    |
| C110   | B2                | HCASE                       | 128                   | A110S| 411_423del| –    | A51S   | –    | –    | –    | –    | –    | –    | –    |
| C113   | B2                | PASE                        | 128                   | 129_129del| –    | A51S   | –    | –    | –    | –    | –    | –    | –    |
| C114   | B2                | WT                          | 256                   | –    | –    | Q132* | –        | –    | –    | –    | –    | –    | –    | –    |
| C115   | B2                | PASE                        | 256                   | Q28* | –    | –    | –        | –    | –    | –    | –    | –    | –    | –    |
| C116   | B2                | PASE                        | >256                  | –    | P218L| –    | –        | –    | –    | –    | –    | –    | –    | –    |
| C127   | B2                | ESBL                        | 256                   | –    | P218L| –    | –        | –    | –    | –    | –    | –    | –    | –    |

*ESBL, extended-spectrum β-lactamase; HCASE, hyperproduction of cephalosporinase; PASE, penicillinase; WT, wild-type. The epidemiological cut-off of fosfomycin in E. coli is 8 mg/L.
by-product of the curve of growth. For each strain and condition, MGR was measured in duplicate in three separate experiments.

**Construction of the Knockout Mutants**

The disruption of the genes coding for putative transporters (*glpT* and *uhpT*) and their regulators (*uhpA*, *uhpB*, and *uhpC*) were performed using the method previously described, with some modifications, using the Red helper plasmid pKOBEG (Datsenko and Wanner, 2000; Derbise et al., 2003). This vector is a low-copy-number plasmid that contains a gene for chloramphenicol resistance selection, a temperature-sensitive origin of replication, and a gene encoding a recombinase. Briefly, pKOBEG was first introduced into CFT073 competent cells by electroporation, and after incubation for 24 h at 30°C, a selectable kanamycin resistance cassette (flanked by flippase recognition target [FRT] sequences) was amplified by PCR using DNA of pKD4 plasmid as the template. The primers used included 5′ extensions with homology for the candidate genes (around 50 bases) (Table 3). The PCR product was introduced into the pKOBEG-harboring CFT073 by electroporation, and after homologous recombination, the disruption of the candidate gene was obtained. Selected clones were cured for the pKOBEG plasmid following a heat shock, creating the kanamycin-resistant variant. In order to have deletion mutants free of the antibiotic marker, strains then were transformed with the pCP20_Gm plasmid, which is able to express the FLP nuclease that recognizes the FRT sequences present on either side of the *kan* gene (Doublet et al., 2008). Lastly, the mutants were verified by Sanger sequencing.

**Construction of Trans-Complemented Strains**

The *uhpB* and *uhpC* wildtype genes were amplified by PCR using specific primers (Table 3) and each amplicon was TA cloned into the overexpression plasmid, pBAD202 directional TOPO (Invitrogen, Courtaboeuf, France). *E. coli* TOP10 cells (Invitrogen) carrying pBAD202 recombinants containing correctly oriented inserts were selected on LB plates with 40 mg/L kanamycin. After purification, recombinant plasmids pBAD202*ΔuhpB* and pBAD202*ΔuhpC* were used to transform by electroporation *ΔuhpB* and *ΔuhpC* mutants, respectively.

**Site-Directed Mutagenesis**

Single-nucleotide allelic replacement was carried out using the suicide vector pDS132 in order to confirm the role of novel mutations (Philippe et al., 2004). The cloning steps of the desired gene alleles into pDS132 were performed in *E. coli DH5α λpir* strain to allow replication of the plasmid. The recombinant plasmids were then purified and introduced in *E. coli CFT073* by electro-transformation. The first step of allelic exchange was selection of plasmid integration into the recipient chromosome by plating cells on chloramphenicol-containing LB plates. After overnight growth at 35°C, one colony was picked, diluted in 10 mM MgSO₄ solution, and serial dilutions were plated on LB agar plates with 5% sucrose and without NaCl. This plating step allowed selection of plasmid excision from the chromosome by a second cross-over. After overnight incubation at 35°C, 100 clones were streaked on chloramphenicol-containing LB agar plates and on LB agar with 5% sucrose and without NaCl. Several clones were screened by PCR-sequencing in order to identify those carrying the desired allele.

**RNA Extraction and RT-qPCR**

The levels of expression of *uhpT* were determined by RT-qPCR using specific primers (Table 3). *E. coli* cells were grown for 24 h in LB broth, and the cells were harvested and washed twice with M9 minimum salt solution as previously described (Ohkoshi et al., 2017). The suspended cells were used to inoculate to M9 minimum salt solution with or without 0.2% G6P supplementation and incubated for 30 min at 35°C. Total RNAs were extracted from all clinical isolates using the Direct-zol RNA miniprep kit (Zymo Research, Irvine, CA, United States). Residual chromosomal DNA was removed by treating samples with the Turbo DNA-free kit (Life Technologies, Saint-Aubin, France). Samples were quantified using the BioSpec-nano spectrophotometer (Shimadzu, Noisiel, France), and the integrity was assessed using the Agilent 2100 bioanalyzer according to the manufacturer’s instructions. cDNA was synthesized from total RNA (~25 ng) using the QuantiFast SYBR green RT-PCR kit (Qiagen), and transcript levels were determined by the ΔΔ threshold cycle (ΔΔCt) method using the rrsA (16S rRNA) gene as a housekeeping control gene (Table 3).

**WGS and Bioinformatic Analysis**

Genomic DNA was isolated using the Quick-DNA fungal/bacterial miniprep kit (Zymo Research, Irvine, CA, United States). DNA libraries were prepared using the NEBNext Ultra DNA library prep kit for Illumina (New England Biolabs, Ipswich, MA, United States) and sequenced as paired-end reads (2 × 300 bp) using an Illumina MiSeq platform and the MiSeq reagent kit version 3. The Illumina reads were assembled using the CLC Genomics Workbench software (Qiagen). The annotation of chromosome and plasmids was performed using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP)². The nucleotide sequences were also submitted to ResFinder server³ (version 3.1) to identify fosfomycin resistance mutations and acquired genes. Raw and processed data generated in this study were deposited in GenBank as bioproject no. PRJNA625505.

**RESULTS**

**In vitro Fosfomycin-Resistant Mutants of *E. coli CFT073***

Four different mutants were selected *in vitro* from the parental strain *E. coli CFT073*, including two single-step and two second-step mutants (Table 1). The two first-step mutants harbored only one mutation each: CFT073_M3 possessed a non-synonymous

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²www.ncbi.nlm.nih.gov/genome/annotation_prok/
³https://cge.cbs.dtu.dk/services/ResFinder/
mutation in *uhpB* (leading to the substitution G469R) and CFT073_M4 had a non-synonymous mutation in *uhpC* (leading to the substitution F384L). Both mutations were associated with a 128-fold increase in fosfomycin MICs (*Table 1*), and mutants were categorized as resistant according to the EUCAST breakpoints. Concerning the two-step mutants, CFT073_M7 and CFT073_M8 were obtained on agar plates supplemented with 128 mg/L of fosfomycin from CFT073_M3 and CFT073_M4, respectively. Both exhibited a two-fold increase in fosfomycin MICs (256 mg/L), and harbored one more mutation each: a non-synonymous mutation in *galU* (leading to the substitution R282V) in CFT073_M7, and a nonsense mutation in *lon* (leading to Q558*)* in CFT073_M8 (*Table 1*).

Overall, bacterial growth rates were reduced as the pH was lower and exhibited their lowest levels in urine (*Figure 1*). MGR of CFT073_M4 was significantly decreased as compared to that of CFT073 (*P* < 0.05, unpaired *t*-test), except in urine (*Figure 1*). Interestingly, CFT073_M7 had a significant decreased MGR as compared with CFT073 in LB and NB at pH 5 (*P* < 0.01, unpaired *t*-test) and also in urine at pH 6.5 (*P* < 0.001, unpaired *t*-test).

### Table 3: Deoxynucleotide primers used in the study.

| Primer | Nucleotide sequence (5′ to 3′) | Purpose |
| --- | --- | --- |
| pkD4_uhpB_F | CTCCGCGTTAATACGCCGTTGAATGCGCTGCAGCTGAGCTGCTTC | *uhpB* deletion |
| pkD4_uhpB_R | GAGATTGAAACACCACCGCGCAGCGTCAGGAAATGGCCTGACATATCCGCTCAG | |
| pkD4_uhpC_F | GAGACCAAGAACAGCTCGAGATGCGCTGCAGCTGAGCTGCTTC | *uhpC* deletion |
| pkD4_uhpC_R | TCTCCGCGTTAATACGCCGTTGAATGCGCTGCAGCTGAGCTGCTTC | |
| pkD4_uhpT_F | CATCGAGCTTACGCTTTCTCAACAGTTGCGCAAGCCACCCTGCTGCAGCTGAGCTGCTTC | *uhpT* deletion |
| pkD4_uhpT_R | AGTTCAGTATGCGACTGCTGAAATTTCTTTCGCGCGCAGCATATATTCGCTCAG | |
| pkD4_galU_F | CGTTCAGACGCTGATCTTCTTATGCGATCGGCTGCAACATGATTGCTGAGCTGCTTC | *galU* deletion |
| pkD4_galU_R | CCGGACGACGCGCGACGCGGATTGTTATGCGTACAAGTGCTGAGCTGCTTC | |
| pDS132_F | CTGTTGCATGGGCATAAAGA | Verification of cloning in pDS132 |
| pDS132_R | AGGAACACTTAACGGCTGAC | |
| Mut3/uhpB_G469R/xbaI-F1p | CCGCTCTAGAGGTTATTTCTCACCCTGCTGCAGCTGAGCTGCTTC | Site-directed mutagenesis for *uhpB* |
| Mut3/uhpB_G469R/xbaI-R1p | CCGCTCTAGAGGTTATTTCTCACCCTGCTGCAGCTGAGCTGCTTC | |
| uhpB_F | ACTGGGCGTCAGTAACGACG | Verification of *uhpB* sequence |
| uhpB_R | ATGGCGCATCGGCAGGCGCT | |
| Mut4/uhpC_F384L/xbaI-F1p | CCGCTCTAGAGGTTATTTCTCACCCTGCTGCAGCTGAGCTGCTTC | Site-directed mutagenesis for *uhpC* |
| Mut4/uhpC_F384L/xbaI-R1p | CCGCTCTAGAGGTTATTTCTCACCCTGCTGCAGCTGAGCTGCTTC | |
| Mut4/uhpC_F384L/F2p | CCGCTCTAGAGGTTATTTCTCACCCTGCTGCAGCTGAGCTGCTTC | |
| Mut4/uhpC_F384L/XbaI-R2p | CCGCTCTAGAGGTTATTTCTCACCCTGCTGCAGCTGAGCTGCTTC | |
| uhpC_F | TGTCTGCAACGACGCGCTGCTGCAGCTGAGCTGCTTC | |
| uhpC_R | GATGAACGTCCAGGCAAAACCT | Verification of *uhpC* sequence |
| galU_R282V/xbaI-F1p | CCGCTCTAGAGGTTATTTCTCACCCTGCTGCAGCTGAGCTGCTTC | Site-directed mutagenesis for *galU* |
| galU_R282V/R1p | TACGGTATTCAACGAAGGCTGAC | |
| galU_R282V/F2p | CACGGTATTCAACGAAGGCTGAC | |
| galU_R282V/XbaI-R2p | CACGGTATTCAACGAAGGCTGAC | |
| galU_F | TATACGGTATTCAACGAAGGCTGAC | Verification of *galU* sequence |
| galU_R | CACGGTATTCAACGAAGGCTGAC | |
| lon_Q558X/xbaI-F1p | CCGCTCTAGAGGTTATTTCTCACCCTGCTGCAGCTGAGCTGCTTC | Site-directed mutagenesis for *lon* |
| lon_Q558X/R1 | CATCAAGGTTGCGCGGAGCTGCTGCAGCTGAGCTGCTTC | |
| lon_Q558X/F2 | CATCAAGGTTGCGCGGAGCTGCTGCAGCTGAGCTGCTTC | |
| lon_Q558X/XbaI-R2p | CATCAAGGTTGCGCGGAGCTGCTGCAGCTGAGCTGCTTC | |
| lon1_F | GCCATTCACGCTGCTGCAGCTGAGCTGCTTC | Verification of *lon* sequence |
| lon1_R | GCCATTCACGCTGCTGCAGCTGAGCTGCTTC | |
| lon2_F | GCCATTCACGCTGCTGCAGCTGAGCTGCTTC | |
| lon2_R | GCCATTCACGCTGCTGCAGCTGAGCTGCTTC | |
| pBAB202_uhpB_F | CAGAAACCGCAACGAGGAAATGGG | Cloning of *uhpB* in pBAD202 |
| pBAB202_uhpB_R | CAGAAACCGCAACGAGGAAATGGG | |
| pBAB202_uhpC_F | CAGAAACCGCAACGAGGAAATGGG | Cloning of *uhpC* in pBAD202 |
| pBAB202_uhpC_R | CAGAAACCGCAACGAGGAAATGGG | |
| RT-qPCR_uhpT_F | ACCCTGAGGTTGCGCGGAGCTGCTGCAGCTGAGCTGCTTC | Quantification of *uhpT* expression |
| RT-qPCR_uhpT_R | ACCCTGAGGTTGCGCGGAGCTGCTGCAGCTGAGCTGCTTC | |
| RT-qPCR_rrsA_F | ACCCTGAGGTTGCGCGGAGCTGCTGCAGCTGAGCTGCTTC | Quantification of *rrsA* expression |
| RT-qPCR_rrsA_R | ACCCTGAGGTTGCGCGGAGCTGCTGCAGCTGAGCTGCTTC | |

*Primers described in Peng et al., 2014.*
(Figure 1). There was no difference in MGRs for CFT073_M3 and CFT073_M8 (Figure 1).

**Role of the Novel Mutations Into Fosfomycin Resistance**

To confirm the role of uhpB, uhpC, galU, and lon and their corresponding mutations in fosfomycin resistance, several approaches were used. First, knockout mutants were constructed, as well as their corresponding trans-complemented strains. Both ΔuhpB and ΔuhpC mutants were resistant to fosfomycin, with MICs at 128 mg/L (Table 1). As expected, the trans-complementation of ΔuhpB and ΔuhpC mutants with their respective isogenic copies restored the fosfomycin susceptibility, with MICs at 1 mg/L (Table 2). Whereas we failed to construct a Δlon mutant, a deleted mutant was obtained for galU that only exhibited a two-fold increase in MICs of fosfomycin (Table 2).

Second, we constructed site-directed mutants of CFT073 by single-nucleotide allelic replacement, to introduce the same mutations as those observed in mutants obtained in vitro by antibiotic selection. The introduction of a unique mutation in uhpB (G469R) or in uhpC (F384L) was responsible for a significant increase in MICs (from 1 to 128 mg/L) in both cases (Table 1), confirming experimentally their role into fosfomycin resistance. The unique mutation in galU (R282V) conferred a two-fold increase in MIC of fosfomycin as did the sole mutation in lon (Q558∗) (Table 1). The latter results are consistent with the increase of MICs of fosfomycin in second-step mutants as compared to single-step mutants (256 vs. 128 mg/L, respectively).

To understand the mechanism(s) by which these mutations confer higher fosfomycin MICs, we compared by RT-qPCR the differential expression of uhpT in the absence or presence of 0.2% G6P. After induction, uhpT expression was strongly enhanced (244-fold ± 47) in the CFT073 parental strain, as expected, whereas it was significantly lower in all mutants M3 (1.1-fold ± 0.4), M4 (1.4-fold ± 0.1), M7 (1.2-fold ± 0.2), and M8 (1.5-fold ± 0.1) (all P < 0.007 by an unpaired t) (Figure 2). This lack of induction by G6P was also observed with deleted and site-directed mutants for uhpB and uhpC (Figure 2). The deletion of glpT in CFT073 had no significant effect on G6P-mediated induction of uhpT expression, as expected, and it was also the case in CFT073_galU^{R282V} and CFT073_lon^{Q558∗} mutants (Figure 2). Surprisingly, the change in uhpT expression after G6P induction was significantly higher in galU-deleted mutant than in the parental strain (407 ± 33 vs. 244-fold ± 47; P = 0.0082) (Figure 2).

**Prevalence of Novel Mutations in Fosfomycin-Resistant E. coli Clinical Isolates**

To know if these mutations have been underestimated until now, we assessed their prevalence in a panel of 40 unrelated non-wildtype (MICs > 8 mg/L) E. coli clinical isolates (Table 2). We also studied a collection of 14 wildtype (MICs ≤ 8 mg/L) clinical isolates in which we verified the absence of mutations, as expected (Table 2).

Of the 40 non-wildtype isolates, no plasmid-mediated fosfomycin resistance genes (especially fosA3) were detected (Table 2). By contrast, at least one mutation/insertion/deletion was identified in almost all (n = 39) isolates, whereas only one isolate (C98, MIC at 64 mg/L) did not possess any change in uhpA, uhpB, uhpC, uhpT, glpT, murA, cyaA, ptsI, galU or lon genes (Table 2). Only two isolates (C49 and C106) were categorized as susceptible to fosfomycin (MICs at the susceptibility breakpoint, 32 mg/L) and harbored two mutations each (Table 2). Five isolates had a full deletion of the uhp operon, including one with one additional non-sense mutation in glpT

![Figure 1](https://example.com/image1.png)
FIGURE 2 | Changes in uhpT expression after induction with 0.2% of G6P in E. coli CFT073 parental strain and its derivative mutants. Transcript levels of uhpT are shown as relative values compared to those of rrsA (16S rRNA) gene. Data plotted correspond to the means and SDs of three biological replicates.

(Q213*). Besides these five cases, a genetic change was identified in uhpA, uhpB, uhpC, uhpT, glpT, cyaA, and ptsI in 8, 17, 18, 5, 8, 1, and 1 isolates, respectively (Table 2). Even though half of isolates presented several mutations in up to three genes, some unique mutations were sufficient to confer fosfomycin resistance (MICs ranging from 64 to >256 mg/L) such as in uhpB (Q60*, Q76*, 265_268del, P169S, P218L, and H313Y), uhpC (459_532del, Q132*, Q210* and G397D), uhpA (Q28* and R75C), and glpT (P139Q) (Table 2). Finally, no mutations were detected in galU and lon genes among the 40 clinical isolates tested.

DISCUSSION

The Uhp hexose phosphate transport pathway and its regulation are well described in E. coli (Kadner, 1973; Kadner and Winkler, 1973; Kadner and Shattuck-Eidens, 1983; Weston and Kadner, 1987, 1988; Island et al., 1992; Island and Kadner, 1993; Wright et al., 2000; Verhamme et al., 2001, 2002). UhpT is a member of the Major Facilitator Superfamily (MFS) containing 12 transmembrane protein segments, and it is responsible for the accumulation of G6P into the bacterial cells. The UhpT system is tightly controlled by the UhpABC phosphorelay system UhpABC, which is necessary for high-level expression of uhpT. UhpC is also an MFS member that shares approximately 30% amino acid sequence identity with UhpT. UhpC is a membrane-bound protein that senses external G6P in the periplasm and interacts with UhpB, stimulating its kinase activity. UhpB is a membrane-bound histidine kinase (HK) in a two-component system that possesses eight predicted transmembrane helices and a C-terminal cytoplasmic domain containing the conserved sequence elements common to HK proteins (i.e., the H-box around the phosphorylated histidine, the N-box, and the G-box comprising the ATP-binding and phosphate transfer region) (Parkinson and Kofoid, 1992). Upon interaction with UhpC, UhpB autophosphorylates the conserved histidine residue (His313), with subsequent phosphorylation at Asp54 of its cognate response regulator UhpA. Phosphorylated UhpA increases the affinity for its specific DNA binding sites, hence promoting transcription of uhpT.

Many mutants defective in the hexose phosphate transport were isolated between 1970s and 1990s, but shortcomings can be found in these old studies, such as the imprecise position of the mutation/deletion/insertion due to the poor annotation of the uhp region sequence, the absence of determination of fosfomycin MICs, and the ‘artificial nature’ of many in vitro mutants that were obtained by transposon insertion (Mu, Tn10), or a resistance cassette (Kadner, 1973; Kadner and Shattuck-Eidens, 1983; Weston and Kadner, 1987, 1988; Island et al., 1992). Also, mutations/insertions can have different impacts on fosfomycin susceptibility since some of them do not impair uhpT expression and others confer constitutive expression (Weston and Kadner, 1987; Island and Kadner, 1993). Deleted mutants with a kanamycin resistance cassette in uhpA, uhpB, or uhpC from the E. coli BW25113 parental strain only conferred a modest increase in fosfomycin MICs to 8, 8, and 4 mg/L (Castaneda-Garcia et al., 2009), respectively, which is different from
Figure 3 | Schematic representation of the structure of UhpB and UhpC proteins with position of mutations identified in our study (in black and red) and previously described by Ballestero-Téllez (34) (in blue) and by Martin-Gutiérrez (10) (in green). Mutations in red correspond to single mutations associated with fosfomycin resistance in clinical isolates. Mutations with arrows correspond to single mutations (G469R in UhpB and F384L in UhpC) demonstrated experimentally to be responsible for fosfomycin resistance. Amino acids of transmembrane segments are indicated for UhpB (500 amino acids) and UhpC (439 amino acids). For UhpB, the putative conserved H-, N-, and G-boxes are also represented. #, non-synonymous mutation; *, non-sense mutation; Δ, deletion.

Our findings. Altogether, it suggests that 'artificial' insertionional mutants do not represent systematically how bacteria develop fosfomycin resistance.

Unexpectedly, we found here novel mutations in *uhpB* and *uhpC* in mutants, which are not often detected in fosfomycin-resistant clinical isolates. Indeed, fosfomycin resistance in *E. coli* clinical isolates is usually due to chromosomal mutations in *uhpT*, *uhpA*, *glpT*, *murA*, *cytA*, and *ptsI* genes (Nilsson et al., 2003; Oteo et al., 2009; Takahata et al., 2010; Li et al., 2015b; Tseng et al., 2015; Ohkoshi et al., 2017; Lucas et al., 2018; Seok et al., 2020), and little is known about the involvement of mutations in other genes, especially those in *uhpB* and *uhpC* that have been exceptionally reported (Castaneda-Garcia et al., 2013).

Recently, mutations in *uhpB* or *uhpC* were described in *E. coli* BW25133-derived laboratory mutants Δ*cytA*, Δ*glpT-cytA*, Δ*glpT-ptsI*, and Δ*ptsI-cytA* recovered in vitro after time-kill experiments with fosfomycin (Ballestero-Tellez et al., 2017) and in two *E. coli* clinical isolates (Martin-Gutierrez et al., 2018). All the mutants were resistant to high levels to fosfomycin (MICs > 1,024 mg/L) and possessed the following mutations one or two mutations in *uhpB* (48del, W181*, L255*, and Q262*) and *uhpC* (T27*, T72P and 541_548del) (Ballestero-Tellez et al., 2017). In the two clinical isolates, one *uhpB* mutation (D205A) and three *uhpC* mutations (Y18H, G282D, T435A) were found in the first while two *uhpC* mutations (I14M, Q17Y) were found in the second (Martin-Gutierrez et al., 2018). We found here two mutations at the exact same position (D205A in UhpB and T72 in UhpC) of these previous studies (Figure 3), which is in favor of their role in fosfomycin resistance. In our study, *uhpB* mutations were distributed all along the 500-amino-acid-long protein in either periplasmic (*n* = 3), transmembrane (*n* = 5), or cytoplasmic (*n* = 6) regions, including one in the autophosphorylation H-box (H313Y) and another in the conserved G-box (G469R) that part of the ATP-binding domain (Figure 3). Concerning *uhpC* mutations, they were more frequently detected within the transmembrane segments (8/15) than into the cytoplasm (*n* = 6) or periplasm (*n* = 1) portions of the 439-amino-acid protein, suggesting that it could impair external G6P sensing through the membrane (Figure 3). Among fosfomycin-resistant clinical isolates, five had...
a full deletion of the uhp region (uhpA-uhpB-uhpC-uhpT), as reported (Lucas et al., 2018).

A majority of clinical isolates harbored uhpB and uhpC mutations that were widely distributed over the protein sequences. It is likely that all these mutations impact fosfomycin susceptibility differently, as described for insertion mutants exhibiting variable Uhp phenotypes (Weston and Kadner, 1988; Island et al., 1992; Island and Kadner, 1993). We also demonstrated that deletions and mutations in uhpB and uhpC were responsible for an absence of induction by G6P of uhpT expression, as described in several ΔuhpA, ΔuhpB, and ΔuhpC laboratory mutants and one clinical isolate with a truncated UhpA (Weston and Kadner, 1988; Island et al., 1992; Island and Kadner, 1993; Wright et al., 2000; Lucas et al., 2018). This confirms the role of UhpB and UhpC as G6P-response regulators required for the induction of uhpT expression. In addition, it appears that the mutation in uhpC (leading to the substitution F384L) also alters in vitro bacterial growth rate in LB and NB (regardless the pH) but not in urine, suggesting that it may occur in vivo.

Besides uhpB and uhpC mutations, two novel mutations were also identified in the two-step in vitro mutants. The first mutation occurred in galU that codes for a 302-amino-acid-long protein named UTP-glucose-1-phosphate uridylyltransferase, which catalyzes synthesis of UDP-D-glucose from UTP and α-D-glucose 1-phosphate (Weissborn et al., 1994). It is a central precursor for synthesis of cell surface carbohydrates, colanic acid, trehalose, cellulose, capsule- and membrane-derived oligosaccharides, and also has a major role in galactose metabolism (Ebrecht et al., 2015). Then, the deletion of galU has many consequences on different carbon metabolic pathways: for instance, they are unable to ferment galactose and fail to incorporate glucose and galactose into bacterial cell membranes, resulting in the incomplete synthesis of lipopolysaccharides (Fukasawa et al., 1962; Sundararajan et al., 1962). Also, the absence of galU leads to a reduced level of TolC into the outer membrane (Sharma et al., 2009), which might be related to antibiotic susceptibility. Here, we identified a non-synonymous mutation (R282V) in the C-terminal region of GalU that is outside the enzyme active site formed by the key residues T20, R21, and K202 (Ebrecht et al., 2015). Then, it is difficult to explain the implication of R282V mutation into the fosfomycin MIC two-fold increase. Note that it seems that this mutation also impacts on bacterial fitness when grown in acidic pH or in urine, suggesting that it may be difficult to develop in vivo.

The second mutation appeared in lon coding for an ATP-dependent serine protease that plays a major role in protein quality control, degrading incorrect proteins, and has an important role into many biological processes in bacteria (Tsilibaris et al., 2006). It degrades abnormal and misfolded proteins, but has also regulatory proteins as substrates, such as MarA and SoxS (Griffith et al., 2004). Here, we identified a lon mutation giving rise to a premature stop codon (Q558*), and then a truncated protein, probably not functional. Indeed, with a length of 784 amino acids in E. coli, a large part of the C-terminal domain is lacking (Amerik et al., 1991). Therefore, we can assume that this truncated protein is inactive since the Ser679-Lys722 catalytic dyad is absent (Botos et al., 2004). Interestingly, it was demonstrated that mutations in lon were implicated in the development of multiple antibiotic resistance phenotype related to the efflux pump system AcrAB-TolC, and to the OmpF porin (Nicoloff et al., 2006, 2007; Duval et al., 2009; Nicoloff and Andersson, 2013; Bhaskarla et al., 2016). MarA, SoxS, and Rob, positively control the expression of acrAB, tolC, and micF, and micF regulatory RNA post-transcriptionally represses the translation of ompF mRNA (Li et al., 2015a). In a lon mutant, the accumulation of MarA and SoxS could enhance the micF-mediated inhibition of the OmpF production, that could impact on fosfomycin activity since OmpF can facilitate the spontaneous diffusion of the antibiotic across the outer membrane (Golla et al., 2019).

In conclusion, we demonstrated here experimentally the role of novel mutations in four genes implicated in fosfomycin resistance, and the prevalence of uhpB and uhpC mutations among fosfomycin-resistant E. coli clinical isolates.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

AUTHOR CONTRIBUTIONS

VC and FG conceptualized the study. VC, AP, MM, FC, VL, BF, and FG contributed to methodology. VC and FG provided the formal analysis and visualization. VC, AP, MM, FC, VL, BF, and FG carried out the investigation. VC, BF, and FG were responsible for the resources. VC, BF, BF, and FG wrote the manuscript. All authors read and approved the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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