

Mechanisms of fosfomycin resistance observed in clinical isolates of *Escherichia coli* from Canada: CANWARD 2007–2022

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Background: Oral fosfomycin is commonly prescribed as a single 3 g dose to empirically treat uncomplicated cystitis. This study investigated the mechanisms and frequency of fosfomycin resistance among clinical isolates of *Escherichia coli* from Canada.

Methods: In total, 5012 clinical isolates of *E. coli* collected by the CANWARD surveillance programme from 2007 to 2022 were tested for susceptibility to fosfomycin using the CLSI reference agar dilution method. MICs were interpreted by 2024 CLSI M100 breakpoints. The 19 fosfomycin-resistant (MIC ≥ 256 mg/L) and 45 fosfomycin-intermediate (MIC 128 mg/L) isolates identified, and 69 fosfomycin-susceptible (MIC ≤ 1 –64 mg/L) control isolates underwent WGS.

Results: In total, 98.7% (4948/5012) of all *E. coli* isolates tested were fosfomycin-susceptible; 0.4% were fosfomycin-resistant and 0.9% were fosfomycin-intermediate. ST131 accounted for 42.1%, 40.0% and 20.3%, respectively, of fosfomycin-resistant, fosfomycin-intermediate and fosfomycin-susceptible isolates. Six (31.6%) fosfomycin-resistant isolates and one (2.2%) fosfomycin-intermediate isolate carried a *fosA* gene. All *fosA* genes were plasmid mediated and all but one *fosA*-carrying plasmid harboured multiple other antimicrobial resistance genes. A nonsense mutation in, or complete deletion of, one or more genes in the fosfomycin transport systems GlpT and UhpT were observed in 63.2% (37/57) of *fosA*-negative fosfomycin-resistant and fosfomycin-intermediate isolates but were not detected in any of the 69 fosfomycin-susceptible isolates tested.

Conclusions: In Canada, the occurrence of mobile fosfomycin resistance determinants (*fosA* genes) in clinical isolates of *E. coli* is low ($\sim 0.14\%$ of all isolates). Our results highlight the importance of the functional transporter systems GlpT and UhpT for the *in vitro* activity of fosfomycin.

Introduction

Urinary tract infections (UTIs) are a common affliction, particularly of reproductive age women. Estimates indicate that approximately half of all women will experience at least one UTI during their lifetime.¹ *Escherichia coli* accounts for $\sim 80\%$ of community-acquired UTIs in otherwise healthy women, and $\sim 50\%$ of hospital-associated infections.² *In vitro* resistance to trimethoprim/sulfamethoxazole, β -lactams and fluoroquinolones, and MDR are frequently observed phenotypes when antimicrobial susceptibility testing of urinary isolates of *E. coli* is performed.³

Oral fosfomycin is a first-line empirical agent for the treatment of uncomplicated cystitis and is typically prescribed as a single 3 g dose. Its use may warrant particular consideration when MDR *E. coli* is suspected or proven to be the aetiological agent of uncomplicated cystitis.⁴ Fosfomycin imitates both glycerol-3-phosphate (G3P) and glucose-6-phosphate (G6P) to gain access into the cytoplasm of *E. coli* cells via GlpT, the G3P transporter, and UhpT, the G6P transporter.⁵ In the cytoplasm, fosfomycin acts as a phosphoenolpyruvate (PEP) analogue, where it binds to and inhibits MurA (UDP-N-acetylglucosamine enolpyruvyl transferase), the enzyme that catalyses the initial reaction in the peptidoglycan synthesis pathway.⁶ Fosfomycin activity is

not diminished by mechanisms commonly responsible for resistance to β -lactams (e.g. β -lactamases, PBP mutations), which target later steps in peptidoglycan synthesis.⁶

Three primary mechanisms of fosfomycin resistance are known: target (MurA) modification, enzymatic inactivation of fosfomycin, and impaired uptake of fosfomycin into bacterial cells.⁵ Modification of MurA, preventing fosfomycin binding to it, is a common mechanism of resistance identified by *in vitro* selection experiments; however, this mechanism is rarely present in clinical isolates resistant to fosfomycin.⁷ Recently, the enzymatic inactivation of fosfomycin through plasmid-mediated *fosA* genes has been the focus of many studies.^{8,9} More than 10 different *fosA* variants have been identified to date, with *fosA3* being the most common.^{8,9} New variants continue to be reported, such as *fosA11* in *Providencia rettgeri*.¹⁰ The third, and currently the most common mechanism of resistance in clinical isolates, is reduced fosfomycin uptake. Chromosomal mutations in the genes encoding GlpT and UhpT transport system proteins reduce fosfomycin permeability. Furthermore, chromosomal mutations of local regulators of transporter genes, such as *uhpA*, *uhpB* and *uhpC*, are also known to result in reduced fosfomycin uptake. Earlier studies have also shown that mutations in the chromosomal genes *cyaA* and *ptsI*, which are responsible for cAMP levels, can play a role in fosfomycin resistance because low cAMP levels down-regulate expression of the two fosfomycin transporters.^{11,12}

A previous study of 868 urinary isolates of *E. coli* from Canadian hospital laboratories collected between 2010 and 2013, reported that resistance to fosfomycin was rare, with only 0.1% of isolates being resistant.¹³ The current study reports the frequency of fosfomycin resistance among 5012 clinical isolates of *E. coli* collected by the CANWARD surveillance programme from 2007 to 2022, and investigates mechanisms of fosfomycin resistance in all fosfomycin-resistant and fosfomycin-intermediate isolates identified, as well as a subset of fosfomycin-susceptible isolates, using WGS. To enrich our cohort of fosfomycin-resistant and fosfomycin-intermediate resistant isolates we chose to test urinary isolates as well as isolates from non-urinary sites (i.e. blood, respiratory and wound isolates).

Materials and methods

Bacterial isolates

The CANWARD surveillance programme is an annual Canadian Antimicrobial Resistance Alliance (CARA)/Health Canada-partnered study that collects bacterial pathogens cultured in tertiary-care hospital laboratories in 8 of the 10 Canadian provinces.¹⁴ Isolates are collected consecutively, one per patient, from both inpatients and outpatients attending emergency rooms, hospital clinics, medical/surgical wards and ICUs. Each laboratory submits annual quotas of respiratory, wound, urine and bloodstream isolates. CANWARD receives annual approval by the University of Manitoba Research Ethics Board (H2009:059).

Antimicrobial susceptibility testing

From 2007 to 2022, the CANWARD surveillance programme tested 5012 clinical isolates of *E. coli* for susceptibility to fosfomycin using the CLSI reference agar dilution method on Mueller–Hinton agar supplemented with 25 mg/L glucose-6-phosphate.^{15–17} The isolates were cultured from respiratory, wound, urine and blood specimens. Fosfomycin was supplied by Paladin Labs (Montreal, Canada). Stock solutions and dilutions were

prepared in CAMHB as described by the CLSI.¹⁵ Quality control was performed following CLSI recommendations, and fosfomycin MICs were interpreted using 2024 CLSI M100 breakpoints (resistant ≥ 256 mg/L; intermediate 128 mg/L; susceptible ≤ 64 mg/L) for urinary isolates of *E. coli*.^{16,17} All isolates deemed fosfomycin resistant ($n=19$), all isolates deemed fosfomycin intermediate ($n=45$) and a similarly sized set of fosfomycin-susceptible isolates with MICs ranging from ≤ 1 to 64 mg/L ($n=69$), to function as controls, were chosen for WGS. To enrich our cohort of fosfomycin-resistant and fosfomycin-intermediate resistant isolates we chose to test urinary isolates as well as isolates from non-urinary sites (i.e. blood, respiratory and wound isolates). For simplicity we chose to apply urinary CLSI breakpoints (SIR) to urinary and non-urinary isolates. The 69 control *E. coli* isolates with fosfomycin MICs ranging from ≤ 1 to 64 mg/L were chosen to represent isolates with varying fosfomycin MICs and were randomly selected albeit representing all of the geographical areas of CANWARD.

Statistics were conducted using a 2×2 contingency table and Fisher's exact test.

WGS

Genomic DNA was extracted using Qiagen DNeasy 96 Blood and Tissue Kits (Hilden, Germany). Short-read libraries were created with TruSeq Nano DNA HT sample preparation kits (Illumina, San Diego, CA, USA). Isolates were sequenced using an Illumina NextSeq platform. Paired-end, 151 bp indexed reads were generated using the Illumina NextSeq platform. FastQC v0.11.9 (<https://github.com/s-andrews/FastQC>) was used to assess quality control metrics for Illumina reads. Contigs were assembled using Shovill v1.1.0 (<https://github.com/tseemann/shovill>). Oxford Nanopore Technologies sequencing libraries were prepped using the Rapid Barcoding kit (SQK-RBK004) and R9.4.1 flowcells. Reads were basecalled using the super accurate model (SUP) with Guppy v6.5.7. De novo assemblies were completed using Unicycler v0.5.0 (<https://github.com/rrwick/Unicycler>). Bacterial species were confirmed using the RefSeq Masher Matches tool (<https://github.com/phac-nml/refseq-masher>). StarAMR v0.11.0¹⁸ was used to detect antimicrobial resistance genes using the ResFinder database v2024-08-06¹⁹ and STs using the MLST database v2.23.0 (<https://github.com/tseemann/mlst>).

A custom script was used to locate 18 genes of interest [*murA*, *crp*, *crp*, *cyaA*, *pta*, *ptsH*, *ptsI*, *glpT*, *uhpT*, *uhpA*, *uhpB*, *uhpC*, *fruK*, *plsX*, *purB*, *hflD*, *mutL* and *mutS* from *E. coli* K12 MG1655 (NC_000913.3)] along with *fosA* genes (*fosL1*, *fosA2*, *fosA3*, *fosA4*, *fosA5*, *fosA6*, *fosA7*, *fosA7.5* and *fosA8*) in the assembled sequence data of each isolate. Briefly, reference sequences were blasted against isolate assemblies using blastn v2.15.0²⁰ with a minimum length of 100 bases and minimum 92% sequence identity. The nucleotide hits were then extracted, re-oriented to be on the same strand, and translated to protein sequences. Stop codons resulting in premature truncation of proteins were identified; if no stop codons were found, the protein sequences were aligned to the reference using MUSCLE v5.1²¹ and analysed for amino acid mutations relative to the reference (substitutions, deletions or insertions). Nucleotide hits near the edges of contigs were investigated manually. For gene targets that were not detected in the assembly based on the above search criteria we refer to them in the text as 'complete deletions'.

Plots were created in R v4.4.2²² using the tidyverse packages²³ and ComplexHeatmap v2.22.²⁴ Sequencing read data are available on NCBI SRA under BioProject number PRJNA1303168 (reviewer link: <https://dataview.ncbi.nlm.nih.gov/object/PRJNA1303168?reviewer=68qmmpp0jh6amm97n2hnc737>).

Results

Of the 5012 clinical isolates of *E. coli* collected by the CANWARD surveillance programme from 2007 to 2022 and tested for

susceptibility to fosfomycin, 0.4% ($n=19$) were fosfomycin resistant; nine isolates had a fosfomycin MIC of 256 mg/L, two isolates had a fosfomycin MIC of 512 mg/L and eight isolates had a fosfomycin MIC >512 mg/L. Forty-five isolates (0.9%) were fosfomycin intermediate (MIC 128 mg/L). The 69 fosfomycin-susceptible isolates chosen for WGS comprised 29, 11, 5, 3, 7, 4 and 10 isolates with, respectively, fosfomycin MICs of ≤ 1 , 2, 4, 8, 16, 32 and 64 mg/L. Of the 19 fosfomycin-resistant isolates, 1 was collected in each of 2007, 2008, 2009, 2010, 2014, 2015 and 2016; 2 isolates were collected in each of 2017, 2018 and 2022; and 6 isolates were collected in 2021. The 45 fosfomycin-intermediate isolates were obtained as follows: 2007, 3; 2008, 3; 2009, 4; 2010, 4; 2011, 2; 2012, 3; 2013, 3; 2014, 2; 2015, 4; 2016, 3; 2017, 3; 2018, 4; 2019, 0; 2020, 1; 2021, 4 and 2022, 2. Fosfomycin MICs and full descriptions of the WGS data for all 133 *E. coli* isolates included in this study are listed in Table S1 (available as [Supplementary data](#) at JAC-AMR Online).

Among fosfomycin-resistant isolates, ST131 ($n=8$; 42.1%) was the most common ST, followed by ST648 ($n=2$; 10.5%); other STs accounted for only single isolates of fosfomycin-resistant *E. coli* (Table S1). ST131 was also the most common ST among both fosfomycin-intermediate ($n=18$; 40.0%) and fosfomycin-susceptible isolates ($n=14$; 20.3%). We looked at the association of ST131 with ESBLs as a whole and then if there were any associations with either ST131 or ESBLs between fosfomycin-susceptible or non-susceptible (intermediate or resistant) isolates. We observed that ST131 was significantly more associated with an ESBL compared with non-ST131 isolates ($P<0.0001$). ST131 was significantly associated with fosfomycin non-susceptible isolates compared with susceptible isolates ($P=0.0138$). Finally, the presence of an ESBL was also significantly associated with fosfomycin non-susceptible isolates compared with susceptible isolates ($P=0.0075$).

Fosfomycin-resistant isolates tended to carry more antimicrobial resistance genes than fosfomycin-intermediate or fosfomycin-susceptible isolates (Figure 1). Among the 19 fosfomycin-resistant isolates, 89.5% also carried resistance genes to aminoglycosides, β -lactams and quinolones; 78.9% carried tetracycline resistance genes; 73.7% carried sulfonamide resistance genes; 63.2% carried trimethoprim resistance genes; and 47.4% carried macrolide resistance genes. Common resistance genes across both fosfomycin-resistant and fosfomycin-intermediate isolates included: *sul1* (47.4% of fosfomycin-resistant isolates; 46.7% of fosfomycin-intermediate isolates), *sul2* (52.6%; 20.0%), *bla*_{TEM-1B/C} (47.4%; 31.1%), *bla*_{CTX-M} (57.9%; 30.6%), *tetA/tetB/tetC/tetM* (84.2%; 48.9%), *aph(3'')-Ib* (52.6%; 22.2%), *aph(6)-Id* (52.6%; 22.2%), *mph(A)* (47.4%; 31.1%), *dfrA* (63.2%; 48.9%) and *aadA* (68.4%; 37.8%). *bla*_{CTX-M-14} and *bla*_{CTX-M-15} were the two most common *bla*_{CTX-M} types among both fosfomycin-resistant and fosfomycin-intermediate isolates (Table S1).

Six (31.6%) fosfomycin-resistant isolates and one (2.2%) fosfomycin-intermediate isolate carried a *fosA* gene (Table S2). The *fosA* genes present in fosfomycin-resistant isolates were *fosA3* ($n=2$), *fosA* ($n=1$), *fosA4* ($n=1$), *fosA7.5* ($n=1$) and *fosA8* ($n=1$); *fosA7.5* was identified in one fosfomycin-intermediate isolate. Using long-read sequencing, *FosA*-type plasmids carrying *fosA* genes were resolved (Table S2). The plasmids ranged in size from 47 to 232 kb. Plasmid replicon types were variable and

included IncN, IncC, IncI1, IncFII, IncFII/N and IncHI2/HI2A. Only replicon type IncFII was observed in multiple plasmids (carrying *fosA*, *fosA3* and *fosA8* genes), which were not closely related (data not shown). All but one *fos* gene-containing plasmid also carried other antimicrobial resistance genes; at least one of these additional genes was always a β -lactamase (i.e. *bla*_{CTX-M-65}, *bla*_{CTX-M-55}, *bla*_{TEM-1B}, *bla*_{TEM-232}, *bla*_{TEM-214} or *bla*_{CMY-2}).

Amino acid mutations in *murA*, *crp*, *rrr*, *pta*, *glpT*, *uhpT*, *cyaA*, *ptsH*, *ptsI*, *uhpA*, *uhpB*, *uhpC*, *fruK*, *plsX*, *purB*, *hflD*, *mutL* and *mutS* were determined by aligning protein sequences to the reference sequences from *E. coli* K12 MG1655 (Table S1); proportions of isolates (excluding isolates carrying a *fosA* gene) with amino acid mutations in six genes (*murA*, *glpT*, *uhpT*, *uhpA*, *uhpB*, *uhpC*) stratified by fosfomycin MIC are shown in Figure 2.

Among the 69 fosfomycin-susceptible isolates, there were only 6 isolates with notable gene mutations or alterations; each of the 6 isolates exhibited only a single gene mutation or alteration. Two isolates had a Pro99→Ser substitution within *murA*, which has been loosely associated with fosfomycin resistance.²⁵ However, Seok *et al.*²⁵ reported this mutation in combination with *fosA3*. In our report, isolates with a Pro99→Ser change had a fosfomycin MIC of ≤ 1 mg/L or 8 mg/L, suggesting that this mutation alone does not mediate fosfomycin resistance. The remaining four isolates had MICs of 16 to 32 mg/L and contained a premature stop codon in one of *ptsI*, *uhpA*, *uhpB* or *uhpC*. These alterations may elevate fosfomycin MICs by affecting the activation of *cyaA* or expression of *uhpT*. Of note, 16 isolates with fosfomycin MICs of 16 to 64 mg/L had no mutations in any of the genes we characterized (Table S2).

For the 57 isolates of *E. coli* that were fosfomycin resistant or fosfomycin intermediate and did not carry a *fosA* gene, we focused our analysis on alterations in the GlpT and UhpT transporters and the UhpABC system. Amino acid substitutions were detected in GlpT in 5 of the 57 isolates; the specific substitutions were: Gly389→Glu ($n=2$), Gly395→Arg ($n=1$), Pro218→Leu ($n=1$) and Arg45→His ($n=1$) (Table S1). None of these mutations have previously been reported, nor has their impact been characterized; these amino acid substitutions were only observed in isolates with fosfomycin MICs ≥ 128 mg/L. Previously described amino acid substitutions were commonly found (e.g. Glu448→Lys; Leu297→Phe; Glu443→Gln; Gln444→Glu; Ala16→Thr)¹² but these have been previously reported to have no impact on protein function.²⁶ Additionally, seven isolates contained nonsense mutations in *glpT* (all at different positions). Less variability was observed in amino acid substitutions within UhpT. The substitution Glu350→Gln ($n=23$; 40.4%), previously described as having no impact on protein function,²⁶ was also identified in 36.6% of fosfomycin-susceptible isolates in this study. The only other substitution detected among isolates not susceptible to fosfomycin was Asp320→Tyr ($n=1$), which has not been previously described. Four other isolates had nonsense mutations, each at a unique position. Two additional isolates had 38 and 33 amino acid deletions within UhpT. In 13 isolates, the *uhpT* gene was missing entirely; in all but 2 of these 13 isolates, the *uhpA*, *uhpB* and *uhpC* genes were also missing. Additional nonsense mutations in *uhpA*, *uhpB* and *uhpC* genes were commonly observed in isolates not susceptible to fosfomycin ($n=13$). Overall, 37 of 57 (63.2%) isolates not susceptible to fosfomycin had a nonsense mutation or complete deletion of one or

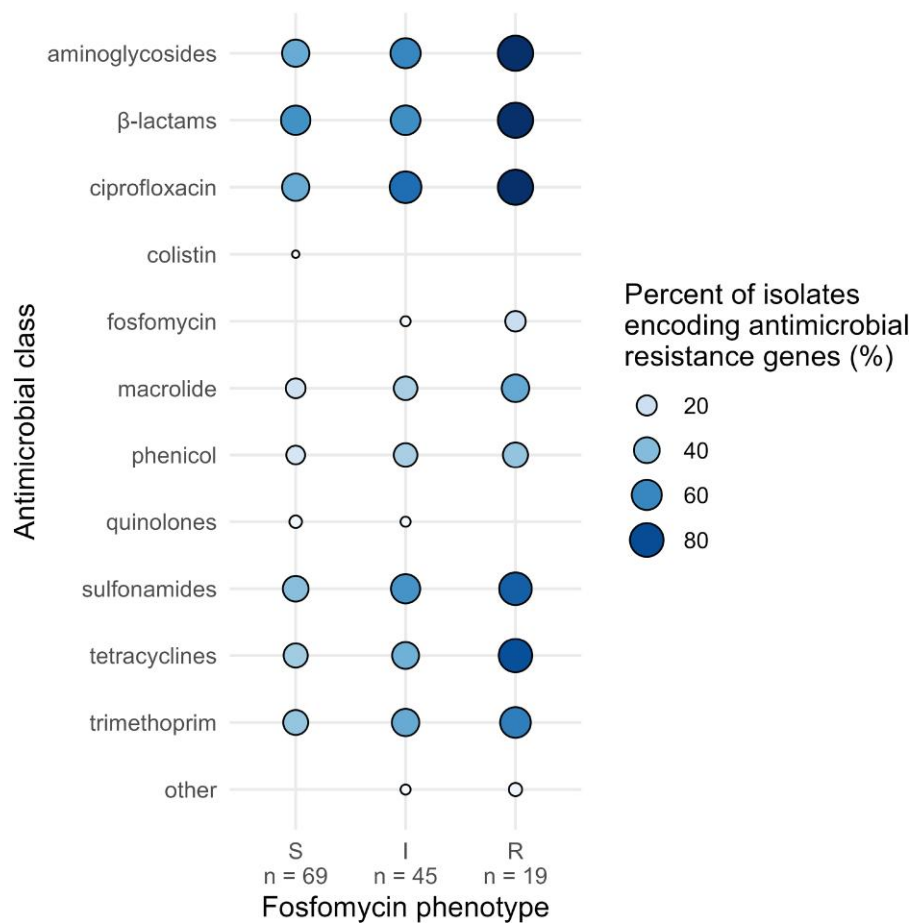


Figure 1. Proportions of *E. coli* isolates encoding antimicrobial resistance genes categorized by antimicrobial class and stratified by fosfomycin-susceptible (S), -intermediate (I) and -resistant (R) phenotypes. Fosfomycin MIC categories were defined by 2024 CLSI M100 breakpoints: susceptible, ≤64 mg/L; intermediate, 128 mg/L; and resistant, ≥256 mg/L. The ‘other’ antimicrobial class includes *rpoB* (n=1) and *ampC* promoter (n=1) mutations.

more of *glpT*, *uhpT* or *uhpABC*. These mutations or deletions were not observed in any fosfomycin-susceptible isolates and were found in every instance but three, in non-FosA-containing fosfomycin-resistant isolates. Of interest, these three *fosA*-negative fosfomycin-resistant isolates contained undescribed mutations in *GlpT* [Gly389→Glu (n=2) and Arg45→His (n=1)] but had no other detected mutations within the *GlpT*/*UhpT* transporters and *UhpABC* system. Future work on these specific mutations should be conducted to determine whether they contributed meaningfully to fosfomycin resistance in these isolates.

Genes required for cAMP regulation were investigated (Table S1). Transcription of *glpT* and *uhpT* is under control of *CyaA*.⁷ Mutations in *CyaA* and their effects on fosfomycin MICs remain unclear.¹² One study suggested substitution in Ser142→Asn contributed to increased MICs,²⁷ another indicated that Gly222→Ser, Ser356→Leu and Gly359→Glu changes resulted in loss of protein structure or function.²⁶ In our study, we found 119 of all 133 (89.5%) isolates studied had one or more of these substitutions and did not correlate with fosfomycin MICs. Activation of *CyaA* requires the presence of G6P and the

phosphotransferase system (PTS) system, which includes *ptsH* and *ptsI*. A study on amino acid substitutions within *ptsI* revealed a number of positions that were described as having no impact on protein function and subsequently did not contribute to the fosfomycin MIC (i.e. Arg367→Lys, Ala306→Thr and Val25→Ile).²⁶ In our study, we did not find any additional amino acid substitutions among the fosfomycin-resistant and fosfomycin-intermediate isolates studied. Additionally, we did not observe mutations or substitutions in *ptsH*. Data on *pta* have suggested that deletion of this gene decreases susceptibility to fosfomycin.^{27,28} We did not observe any deletions in this gene; however, we did note a limited number of amino acid substitutions in fosfomycin-resistant and fosfomycin-intermediate isolates, specifically Ala454→Thr (n=1), Arg401→Cys (n=1) and Val450→Thr (n=1), but the implications of these mutations are unknown. We did not observe deletions or mutations in *crr* or *crp*.

Previous work has shown that deletion of *purB*, *hflD*, *mutL*, *mutS*, *fruK* or *plsX* genes is associated with an increased fosfomycin MIC.²⁹ In our study, among all 133 isolates studied, we observed only a single instance where one of these genes (*mutS*)

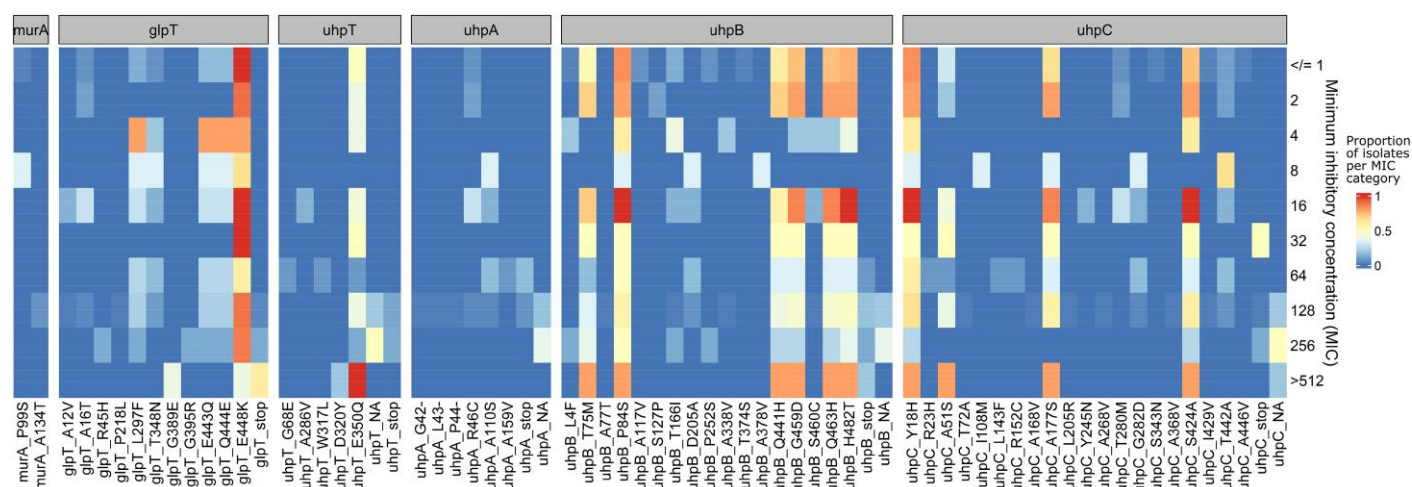


Figure 2. Prevalence of amino acid mutations identified for six major genes (*murA*, *glpT*, *uhpT*, *uhpA*, *uhpB* and *uhpC*) stratified by fosfomycin MIC for 126 isolates of *E. coli*. The figure excludes the seven isolates of *E. coli* identified with a *fosA*. Mutations are annotated with the amino acid change position relative to the reference *E. coli* K12 MG1655 gene. ‘Stop’ indicates a premature stop codon; ‘NA’ indicates the gene was missing from the assembly. Isolate proportion values of 0 (darkest shade of blue) indicate that the mutation was not present in any isolates, and values of 1 (darkest shade of red) indicate that the mutation was present in all isolates. Large mutations in the *uhpC* and *uhpA* genes were excluded for three isolates and one isolate, respectively, to improve figure readability.

was absent from an isolate (Table S1); that isolate had a fosfomycin MIC of 128 mg/L and no additional mutations associated with an increased fosfomycin MIC. We found that amino acid substitutions in *purB*, *hflD*, *mutL*, *mutS*, *fruK* and *plsX* genes were common; however, some changes were only observed in fosfomycin-resistant and fosfomycin-intermediate isolates. Those changes were *plsX*, Pro286→Ser ($n=1$); *hflD*, Asp7→Asn ($n=1$); *mutL*, Ala306→Gly ($n=1$), Ile195→Val ($n=1$) and Ser505→Pro ($n=1$); and *mutS*, Gly696→Glu ($n=1$) and Thr414→Ala ($n=1$). The implications of amino acid substitutions in these genes remain to be elucidated.

Discussion

The frequency of fosfomycin resistance among clinical isolates of *E. coli* in Canada remains low (<1%) when resistance is defined by interpreting reference method agar dilution MICs with current CLSI breakpoints for fosfomycin tested against urinary isolates of *E. coli*.^{15,16} In the current study of 5012 clinical isolates of *E. coli* from 2007 to 2022, only 0.4% were fosfomycin resistant (MIC ≥ 256 mg/L) and 0.9% were fosfomycin intermediate (MIC 128 mg/L). Only 6 of the 19 (31.6%) fosfomycin-resistant isolates carried a *fosA* gene. *FosA* plasmid size and replicon types were variable, with only IncFII observed in multiple plasmids; however, none of the replicon types were related, likely indicating multiple introductions of plasmid-mediated *fosA* genes into *E. coli* isolates over the study period. Several studies have examined *FosA3* and reported its association with multiple plasmid replicon types, including IncN and IncFII,^{30–32} as we observed in our fosfomycin-resistant isolates. Additionally, *FosA3* is known to be associated with CTX-M types, including CTX-M-55 and CTX-M-65, which we have confirmed with our data.^{30–32} *FosA8* was first described in 2019 on an IncN plasmid and is believed to have originated from *Leclercia adecarboxylata*.³³ The *FosA8* plasmid we identified was also on an IncN plasmid. With the exception of one isolate,

all *FosA*-containing plasmids were associated with additional antimicrobial resistance genes that included at least one β -lactamase gene. Therefore, the risk of selection for *fosA*-carrying isolates of *E. coli* by use of antimicrobial agents other than fosfomycin clearly exists and requires monitoring. Furthermore, the association of *E. coli* ST131 and ESBLs, specifically *bla*_{CTX-M} types, has been well described.³⁴ Here we report on a significant association between fosfomycin non-susceptible *E. coli* ST131 as well as ESBL production compared with fosfomycin-susceptible isolates. This combination of β -lactamase and clonal type may be contributing to increasing fosfomycin non-susceptible isolates in Canada.

Our results highlight the importance of the functional transporter systems, GlpT and UhpT, for fosfomycin activity. Several substitution mutations in GlpT were found solely in fosfomycin-resistant isolates and have not previously been reported, specifically Arg45→His, Gly395→Arg, Gly389→Glu and Pro218→Leu. Huang *et al.*³⁵ have previously shown that when Arg45, a conserved amino acid in bacterial GlpT proteins, is replaced by lysine, GlpT substrate transport activity is significantly decreased. This finding suggests that another amino acid substitution for Arg45, as we observed, has the potential to limit fosfomycin transport by GlpT and result in resistance. Similarly, a substitution mutation at amino acid Gly389, a backbone amino acid in GlpT known to recognize and bind the glycerol moiety of G3P, may also limit fosfomycin binding to and transport by GlpT.³⁶ Future work is needed to confirm these mutations and their direct association with elevated fosfomycin MICs. Nonsense mutations were also observed in some *glpT* genes resulting in a truncated, non-functional gene. There were nonsense mutations in five fosfomycin-resistant isolates; the locations of these mutations were dissimilar for all isolates. Previous studies have shown that premature stop codons resulting in the loss of function of GlpT are linked to fosfomycin-resistant and fosfomycin-intermediate phenotypes.¹² One study reported that the deletion

of nucleotide 401 resulted in a premature stop codon for *glpT* and was associated with fosfomycin resistance in *E. coli*.³⁷ Further work is needed to better describe the effects of nonsense mutations in *GlpT* on fosfomycin resistance.

In our investigation, the inducible transport system UhpT, coupled with regulatory genes *uhpA*, *uhpB* and *uhpC*, harboured crucial mutations impacting fosfomycin activity in both fosfomycin-resistant and -intermediate isolates of *E. coli*. The function of the UhpT system is intricately regulated by the phosphorelay system, UhpABC. Consequently, mutations affecting UhpABC function influence the overall expression levels of *uhpT*.⁸ *uhpA*, *uhpB* and *uhpC* exhibit a high mutation frequency,³⁸ a trend corroborated by our sequencing results. Mutations in the UhpT system leading to fosfomycin resistance predominantly manifest as nonsense mutations, complete operon deletions or partial operon deletions. Cattoir et al.⁸ previously reported that isolates of *E. coli* with such nonsense mutations and deleted operons displayed fosfomycin MICs >256 mg/L. In the isolates we studied, we did not observe discernible patterns among these mutations in the UhpABC system. However, 22.8% of fosfomycin-resistant and fosfomycin-intermediate isolates had a missing *uhpT* gene in combination with a partial or completely missing *uhpA*, *uhpB* or *uhpC* gene.

This study is not without limitations. To enrich our cohort of fosfomycin-resistant and fosfomycin intermediate-resistant isolates we chose to test urinary isolates as well as isolates from non-urinary sites (i.e. blood, respiratory and wound isolates). We chose to supplement our urinary isolates with non-urinary isolates as fosfomycin-resistant *E. coli* are so uncommon (only 0.4% in our study) in Canada. We also specifically chose to apply urinary CLSI breakpoints (SIR) to urinary and non-urinary isolates. This was performed for simplicity as we were most interested in investigating fosfomycin resistance mechanisms in *E. coli* isolates with very high MICs to fosfomycin (≥ 128 mg/L) to assess resistance mechanisms and particularly *fosA* genes. It should be mentioned that there is a contrast between EUCAST and CLSI fosfomycin breakpoints, with EUCAST's limited breakpoints and cautions around non-urinary sources. If we had used EUCAST fosfomycin breakpoints, the number of *E. coli* isolates deemed resistant and intermediate isolates would have been higher.

We conclude that fosfomycin-resistant (0.4%; MIC ≥ 256 mg/L) and fosfomycin-intermediate (0.9%; MIC 128 mg/L) isolates of *E. coli* remain rare in Canada and that the occurrence of *FosA* mobile resistance elements is low (0.14%; 7/5012). Our results highlight the importance of *GlpT* and *UhpT* transport systems for the activity of fosfomycin. Research elucidating the importance of the novel mutations associated with fosfomycin resistance identified in the current study may be important.

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Disclaimer

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Supplementary data

Tables S1 and S2 are available as Supplementary data at JAC-AMR Online.

References

- Kot B. Antibiotic resistance among uropathogenic *Escherichia coli*. *Pol J Microbiol* 2019; **68**: 403–15. <https://doi.org/10.33073/pjm-2019-048>
- Foxman B, Brown P. Epidemiology of urinary tract infections: transmission and risk factors, incidence, and costs. *Infect Dis Clin North Am* 2003; **17**: 227–41. [https://doi.org/10.1016/S0891-5520\(03\)00005-9](https://doi.org/10.1016/S0891-5520(03)00005-9)
- Adamus-Biatek W, Baraniak A, Wawszczak M et al. The genetic background of antibiotic resistance among clinical uropathogenic *Escherichia coli* strains. *Mol Biol Rep* 2018; **45**: 1055–65. <https://doi.org/10.1007/s11033-018-4254-0>
- Fajfr M, Balik M, Cermakova E et al. Effective treatment for uncomplicated urinary tract infections with oral fosfomycin, single center four year retrospective study. *Antibiotics* 2020; **9**: 511. <https://doi.org/10.3390/antibiotics9080511>
- Zurfluh K, Treier A, Schmitt K et al. Mobile fosfomycin resistance genes in Enterobacteriaceae—an increasing threat. *Microbiol Open* 2020; **9**: e1135. <https://doi.org/10.1002/mbo3.1135>
- Falagas ME, Athanasiaki F, Voulgaris GL et al. Resistance to fosfomycin: mechanisms, frequency and clinical consequences. *Inter J Antimicrob Agents* 2019; **53**: 22–8. <https://doi.org/10.1016/j.ijantimicag.2018.09.013>
- Castañeda-Barba S, Top EM, Stalder T. Plasmids, a molecular cornerstone of antimicrobial resistance in the One Health era. *Nat Rev Microbiol* 2024; **22**: 18–32. <https://doi.org/10.1038/s41579-023-00926-x>
- Cattoir V, Pourbaix A, Magnan M et al. Novel chromosomal mutations responsible for fosfomycin resistance in *Escherichia coli*. *Front Microbiol* 2020; **11**: 575031. <https://doi.org/10.3389/fmicb.2020.575031>
- Galindo-Méndez M, Navarrete-Salazar H, Baltazar-Jiménez F et al. Emergence of fosfomycin resistance by plasmid-mediated *fos* genes in uropathogenic ESBL-producing *E. coli* isolates in Mexico. *Antibiotics* 2022; **11**: 1383. <https://doi.org/10.3390/antibiotics11101383>
- Lu W, Zhou S, Ma X et al. *FosA11*, a novel chromosomal-encoded fosfomycin resistance gene identified in *Providencia rettgeri*. *Microbiol Spectr* 2024; **12**: e0254223. <https://doi.org/10.1128/spectrum.02542-23>
- Cattoir V, Guérin F. How is fosfomycin resistance developed in *Escherichia coli*? *Fut Microbiol* 2018; **13**: 1693–6. <https://doi.org/10.2217/fmb-2018-0294>
- Mattioni Marchetti V, Hrabak J, Bitar I. Fosfomycin resistance mechanisms in Enterobacterales: an increasing threat. *Front Cell Infect Microbiol* 2023; **13**: 1178547. <https://doi.org/10.3389/fcimb.2023.1178547>

- 13 Zhanell GG, Zhanell MA, Karlowsky JA. Oral and intravenous fosfomycin for the treatment of complicated urinary tract infections. *Can J Infect Dis Med Microbiol* 2020; **2020**: 8513405. <https://doi.org/10.1155/2020/8513405>
- 14 Zhanell GG, Adam HJ; Canadian Antimicrobial Resistance Alliance (CARA) and CANWARD. Ten years of the CANWARD study (2007–16). *J Antimicrob Chemother* 2019; **74**: iv2–4. <https://doi.org/10.1093/jac/dkz282>
- 15 CLSI. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically—Twelfth Edition: M07*. 2024.
- 16 CLSI. *Performance Standards for Antimicrobial Susceptibility Testing: Informational Supplement—Thirty-Fourth Edition: M100*. 2024.
- 17 Karlowsky JA, Baxter MR, Walkty AJ et al. In vitro activity of fosfomycin against bacterial pathogens isolated from urine specimens in Canada from 2007 to 2020: CANWARD surveillance study. *J Antimicrob Chemother* 2022; **77**: 3035–8. <https://doi.org/10.1093/jac/dkac275>
- 18 Bharat A, Petkau A, Avery BP et al. Correlation between phenotypic and in silico detection of antimicrobial resistance in *Salmonella enterica* in Canada using Staramr. *Microorg* 2022; **10**: 292. <https://doi.org/10.3390/microorganisms10020292>
- 19 Bortolaia V, Kaas RS, Ruppe E et al. ResFinder 4.0 for predictions of phenotypes from genotypes. *J Antimicrob Chemother* 2020; **75**: 3491–500. <https://doi.org/10.1093/jac/dkaa345>
- 20 Altschul SF, Gish W, Miller W et al. Basic local alignment search tool. *J Mol Biol* 1990; **215**: 403–10. [https://doi.org/10.1016/S0022-2836\(05\)80360-2](https://doi.org/10.1016/S0022-2836(05)80360-2)
- 21 Edgar RC. Muscle5: high-accuracy alignment ensembles enable unbiased assessments of sequence homology and phylogeny. *Nat Commun* 2022; **13**: 6968. <https://doi.org/10.1038/s41467-022-34630-w>
- 22 R Core Team. *R: a Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, 2024.
- 23 Wickham H, Averick M, Bryan J et al. Welcome to the tidyverse. *J Open Source Soft* 2019; **4**: 1686. <https://doi.org/10.21105/joss.01686>
- 24 Gu Z. Complex heatmap visualization. *iMeta* 2022; **1**: e43. <https://doi.org/10.1002/imt2.43>
- 25 Seok H, Choi JY, Wi YM et al. Fosfomycin resistance in *Escherichia coli* isolates from South Korea and in vitro activity of fosfomycin alone and in combination with other antibiotics. *Antibiotics* 2020; **9**: 112. <https://doi.org/10.3390/antibiotics9030112>
- 26 Sorlozano-Puerto A, Lopez-Machado I, Albertuz-Crespo M et al. Characterization of fosfomycin and nitrofurantoin resistance mechanisms in *Escherichia coli* isolated in clinical urine samples. *Antibiotics* 2020; **9**: 534. <https://doi.org/10.3390/antibiotics9090534>
- 27 Ohkoshi Y, Sato T, Suzuki Y et al. Mechanism of reduced susceptibility to fosfomycin in *Escherichia coli* clinical isolates. *BioMed Res Inter* 2017; **2017**: 5470241. <https://doi.org/10.1155/2017/5470241>
- 28 Hirakawa H, Takita A, Sato Y et al. Inactivation of *ackA* and *pta* genes reduces GlpT expression and susceptibility to fosfomycin in *Escherichia coli*. *Microbiol Spectr* 2023; **11**: e05069-22. <https://doi.org/10.1128/spectrum.05069-22>
- 29 Ma Y, Pirollo M, Giarratana L et al. Chromosomal genes modulating fosfomycin susceptibility in uropathogenic *Escherichia coli*: a genome-wide analysis. *Antimicrob Agents Chemother* 2025; **69**: e01417-24. <https://doi.org/10.1128/aac.01417-24>
- 30 Benzerara Y, Gallah S, Hommeril B et al. Emergence of plasmid-mediated fosfomycin-resistance genes among *Escherichia coli* isolates, France. *Emerg Infect Dis* 2017; **23**: 1564–7. <https://doi.org/10.3201/eid2309.170560>
- 31 Wang J, Zeng Z-L, Huang X-Y et al. Evolution and comparative genomics of F33A–B– plasmids carrying *bla*_{CTX-M-55} or *bla*_{CTX-M-65} in *Escherichia coli* and *Klebsiella pneumoniae* isolated from animals, food products, and humans in China. *mSphere* 2018; **3**: e00137-18. <https://doi.org/10.1128/mSphere.00137-18>
- 32 Biggel M, Zurfluh K, Treier A et al. Characteristics of *fosA*-carrying plasmids in *E. coli* and *Klebsiella* spp. isolates originating from food and environmental samples. *J Antimicrob Chemother* 2021; **76**: 2004–11. <https://doi.org/10.1093/jac/dkab119>
- 33 Poirel L, Vuillemin X, Kieffer N et al. Identification of FosA8, a plasmid-encoded fosfomycin resistance determinant from *Escherichia coli*, and its origin in *Leclercia adecarboxylata*. *Antimicrob Agents Chemother* 2019; **63**: e01403-19. <https://doi.org/10.1128/aac.01403-19>
- 34 Nicolas-Chanoine MH, Bertrand X, Madec JY. *Escherichia coli* ST131, an intriguing clonal group. *Clin Microbiol Rev* 2014; **27**: 543–74. <https://doi.org/10.1128/CMR.00125-13>
- 35 Huang Y, Lemieux MJ, Song J et al. Structure and mechanism of the glycerol-3-phosphate transporter from *Escherichia coli*. *Science* 2003; **301**: 616–20. <https://doi.org/10.1126/science.1087619>
- 36 Law CJ, Enkavi G, Wang D-N et al. Structural basis of substrate selectivity in the glycerol-3-phosphate: phosphate antiporter GlpT. *Biophys J* 2009; **97**: 1346–53. <https://doi.org/10.1016/j.bpj.2009.06.026>
- 37 Li Y, Zheng B, Li Y et al. Antimicrobial susceptibility and molecular mechanisms of fosfomycin resistance in clinical *Escherichia coli* isolates in mainland China. *PLoS One* 2015; **10**: e0135269. <https://doi.org/10.1371/journal.pone.0135269>
- 38 Nilsson AI, Berg OG, Aspevall O et al. Biological costs and mechanisms of fosfomycin resistance in *Escherichia coli*. *Antimicrob Agents Chemother* 2003; **47**: 2850–8. <https://doi.org/10.1128/AAC.47.9.2850-2858.2003>