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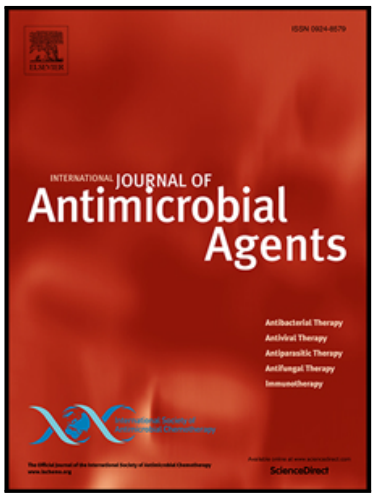


# Identification and Characterisation of Fosfomycin Resistance in Escherichia coli Urinary Tract Infection Isolates from Australia

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**Highlights**

- This study reports on two fosfomycin-resistant *Escherichia coli* urinary-tract infection isolates from Australia.
- This study identified the *fosA4* gene in two clinical *E. coli* isolates - the *fosA4* gene has previously been reported in the literature in only one clinical *E. coli* isolate, in 2014, in Japan.
- This study identified other antimicrobial resistance genes harboured by the fosfomycin-resistant *E. coli* isolates.

# Identification and Characterisation of Fosfomycin Resistance in *Escherichia coli* Urinary Tract Infection Isolates from Australia

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**Keywords:**

fosfomycin resistance, *E. coli*, urinary-tract infections

**ABSTRACT**

Of 1,033 *E. coli* urinary tract infection isolates collected from females >12 years of age in Australia, in 2019, only two isolates were resistant to fosfomycin with an MIC >256 mg/L. Despite having different multilocus sequence types, the two isolates harboured an identical plasmid-encoded *fosA4* gene. The *fosA4* gene has previously been identified in a single clinical *E. coli* isolate, cultured in Japan in 2014. Each fosfomycin-resistant isolate harboured two conjugative plasmids which possessed an array of genes conferring resistance to aminoglycosides, beta-lactams, macrolides, quinolones, sulphonamides, and/or trimethoprim.

## 1. INTRODUCTION

Fosfomycin is a broad-spectrum antibiotic which binds irreversibly to UDP-*N*-acetylglucosamine enolpyruvyl transferase (MurA), an enzyme involved in the early stages of peptidoglycan synthesis [1]. Following the increasing prevalence of extended-spectrum β-lactamases (ESBLs) and carbapenem-resistant Enterobacterales (CRE), fosfomycin has been approved for the treatment of uncomplicated urinary tract infections (UTIs) caused by *Escherichia coli* in many countries, including Australia [2].

Resistance to fosfomycin can occur via three mechanisms, (1) expression of an altered form of MurA with reduced affinity to fosfomycin, (2) defects in the expression of membrane transporter proteins, and (3) expression of fosfomycin-inactivating enzymes encoded by *fos* genes [3]. The *fos* genes can be chromosomal but can also be located on transposable elements and conjugative plasmids, potentiating spread through horizontal gene transfer events. In *E. coli*, the *fos* genes encode FosA or FosC2, both of which modify fosfomycin by the addition of glutathione to the epoxide ring. FosA can be encoded by *fosA, fosA2, fosA3, fosA4, fosA5* or *fosA6* and FosC2 is encoded by *fosC2* [4]. Other *fos* genes, not typically found in *E. coli*, include *fosB*, *fosB1*, *fosB2*, *fosB3*, *fosD*, *fosE*, *fosG*, *fosH*, *fosI*, *fosK* and *fosX* [4].

Several studies have assessed the activity of fosfomycin on *E. coli* isolates causing UTIs but such data is lacking for UTI isolates from Australia. In 2017, fosfomycin tromethamine was approved by the Therapeutic Goods Administration in Australia for the treatment of acute uncomplicated UTIs caused by fosfomycin-sensitive pathogens in women >12 years of age. In this study, we perform a cross-sectional study to identify and characterize the fosfomycin-resistant UTI *E. coli* isolates.

## 2. MATERIALS AND METHODS

### 2.1. Bacterial Isolates

In 2019, a total of 1,033 non-duplicate *E. coli* urinary isolates from females >12 years of age were collected from six institutions around Australia – PathWest Laboratory Medicine WA (Western Australia), Concord Hospital (New South Wales), Pathology Queensland Central Laboratory (Queensland), Sullivan Nicolaides Pathology (Queensland), Monash Pathology, Monash Health (Victoria), and The Canberra Hospital (Australian Capital Territory). Isolates were transported on Amies transport media, subcultured onto blood agar and stored frozen in 15% glycerol at -80°C. Isolates were subcultured onto blood agar for a maximum of 16 hours before susceptibility testing and extraction of genomic DNA.

### 2.2. Antibiotic Susceptibility Testing

All isolates were tested by EUCAST disk diffusion [5] for fosfomycin susceptibility using a 200 µg disk containing 50 µg glucose-6-phosphate on Muller-Hinton solid media. Isolates with an inhibition zone of diameter ≥24 mm were considered susceptible and isolated colonies within the inhibition zone were ignored. For isolates with an inhibition zone of diameter <24 mm, the fosfomycin minimum inhibitory concentration (MIC) was determined by EUCAST agar dilution method in the presence of 25 mg/L glucose-6-phosphate. Results were interpreted according to the EUCAST Breakpoints Tables v10.0, 2020 (S: ≤32 mg/L, R: >32 mg/L) [6]. Isolates identified as fosfomycin-resistant by agar dilution were further tested for resistance to an array of antibiotics using the VITEK® 2 AST-N246 susceptibility panel.

### 2.3. Whole Genome Sequencing

Short read sequencing was performed on isolates with a fosfomycin MIC >32 mg/L using the NextSeq® platform (Illumina, San Diego, USA) and 150 bp paired-end chemistry. Genomic DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, 69506) and 1.0 ng of DNA was used as input to the Illumina Nextera XT library preparation protocol. Raw reads

were assembled using SPAdes [7] and antimicrobial resistance genes were detected using ResFinder 3.2 [8]. Long-read sequencing for strains EC1 and EC2 was carried out using a Nanopore R.9.4.1 flowcell on a MinION Mk1B. Basecalling was carried out using guppy (3.4.5) using the dna_r9.4.1_450bps_hac model. Reads for strain EC1 (547-fold mapped coverage) were filtered by quality and size (>q10; >5kb) using NanoFilt (v2.6.0). All EC2 nanopore reads (128-fold mapped coverage) were used in assembly. *De novo* assemblies were carried out using Flye (v2.6). Nanopore reads were used to iteratively polish each genomes five times using Racon (v1.4.15). Illumina reads for EC1 (79-fold mapped coverage) and EC2 (67-fold mapped coverage) were used to iteratively polish each genome five times using Pilon (v1.23). Genome assemblies and sequence reads for strains EC1 and EC2 have been deposited to the NCBI Sequence Read Archive under BioProject PRJNA643009. Gene prediction and sequence alignment were performed using Prodigal [9] and BLAST [10], respectively. Sequence types were designated according to the Achtman multilocus sequence typing (MLST) system using the *Escherichia* MLST website [11].

## 3. RESULTS

### 3.1. Antimicrobial susceptibility

Of the 1,033 *E. coli* isolates included in the study, seven isolates had a zone diameter <24 mm by disk diffusion (supplementary data). Of these, five isolates were fosfomycin-susceptible with an MIC of 16 mg/L for one isolate and 32 mg/L for four isolates. The remaining two isolates, EC1 and EC2, were fosfomycin-resistant with an MIC >256 mg/L.

### 3.2. Fosfomycin-resistant Isolates

Whole genome sequencing identified EC1 as ST69 and EC2 as ST10 according to the *E. coli* (Achtman) MLST scheme. The EC1 isolate harboured three plasmids, pEC1-1, pEC1-2 and pEC1-3 of sizes 122 kb (Accession No. CP058575), 77 kb (Accession No. CP058576) and 14 kb (Accession No. CP058577), respectively. The pEC1-1 and pEC1-2 plasmids were

conjugative plasmids and harboured the *tra* genes necessary for conjugal transfer. The EC2 isolate harboured two plasmids, pEC2-1 and pEC2-2 of sizes 109 kb (Accession No. CP058572) and 89 kb (Accession No. CP058573), respectively. The two EC2 plasmids possessed genes required for conjugal transfer.

### 3.3. Fosfomycin resistance mediated by the *fosA4* gene

The *fosA4* gene was identified in the two fosfomycin-resistant isolates and was located on the pEC1-2 and pEC2-1 conjugative plasmids. The *fosA*4 nucleotide sequences (417 bp) were identical in both isolates and the genes were located on a 3.5 kb genetic island. The two genetic islands were also identical (100% nucleotide identity) and each contained a direct repeat of the movable IS26 element on either end (Figure 1). No other genes associated with fosfomycin resistance were identified.

### 3.4. Antimicrobial genes other than *fosA4*

In addition to *fosA4*, other antimicrobial resistance genes were detected in the two fosfomycin-resistant isolates. The genes were located on the conjugative plasmids or on the chromosome and are listed in Table 1. The VITEK® 2 results for EC1 showed resistance to ampicillin, amoxicillin/clavulanic acid, ticarcillin/clavulanic acid, cefazolin, cefoxitin, ceftazidime, gentamicin, tobramycin, trimethoprim and trimethoprim/sulfamethoxazole. All resistance phenotypes, with the exception of resistance to cephalosporins, could be explained by the presence of at least one of the antimicrobial resistance genes identified in EC1. The VITEK® 2 results for EC2 showed resistance to ampicillin and norfloxacin. Ampicillin resistance in EC2 was due to the presence of the $bla_{\mathrm{TEM}}$ genes, while norfloxacin resistance was due to the presence of a S83L mutation in *gyrA*.

## 4. DISCUSSION

Fosfomycin, discovered in 1969, is an example of an older ‘forgotten’ antimicrobial agent, that has been reintroduced for use in therapy as a result of the rise in antimicrobial resistance

and the introduction of fewer new drugs to the market. The antimicrobial is used for the treatment of uncomplicated UTIs which are most commonly caused by *E. coli*. In this study, the first national study to understand the local resistance profile, the *E. coli* fosfomycin resistance rate was 0.2% (2/1,033).

Although fosfomycin resistance is relatively rare (<1%) in *E. coli* [12], an increase in fosfomycin-resistant *E. coli* has been detected worldwide due to the rapid dissemination of *fos* genes. The most frequently identified *fos* gene in fosfomycin-resistant *E. coli* is *fosA3* which has been identified on plasmids in East Asian countries from clinical specimens, healthy individuals and domestic animals [4]. Spread of the *fosA3* gene has been attributed to mobile IS26 elements. In this study, we identified the *fosA4* gene in the fosfomycin resistant *E. coli* isolates which is most closely related to and shares 94% amino acid identity with *fosA3*. In the published literature, *fosA4* has been identified in only one clinical *E. coli* isolate, from Japan in 2014 [13]. However, a BLAST search identified the *fosA4* sequence in plasmids from *E. coli* and other species such as *Salmonella enterica, Klebsiella pneumoniae, Acinetobacter baumannii* and *Kluyvera georgiana*. The origin of *fosA4* has been traced back to *K. georgiana*, a species phenotypically similar to *E. coli* and which is found in water, soil, sewage and healthcare environments [14].

The *fosA4* gene identified in this study was located on a 3.5 kb genetic island which is likely to be involved in the spread of the *fosA4* gene. This genetic island was identical (100% nucleotide identity) to a region identified on the pSGB23 (GenBank accession number NZ_CP023167) plasmid from the *Salmonella enterica* subsp. *enterica* serovar Saintpaul strain [15]. A BLAST search detected the 3.5 kb genetic island in the absence of one IS26 element on plasmids identified in other Enterobacterales including *Citrobacter freundii*, *Enterobacter cloacae*, *Klebsiella pneumoniae* and *Shigella flexneri*. The genetic island in the absence of one IS26 element was also identified on the chromosome of *Acinetobacter*

*baumannii* and *Proteus mirabilis* which confirms chromosomal integration of the genetic island is possible.

Antimicrobial resistance genes other than *fosA4* were identified in this study which explained the resistance phenotype of EC1 and EC2. However, some resistance genes were not phenotypically expressed.

In conclusion, this study identified two fosfomycin-resistant UTI *E. coli* isolates which harboured the *fosA4* gene on a genetic island flanked by two direct repeats of the movable IS26 element. Since both isolates possessed different MLST profiles, the genetic island has likely been acquired via two independent horizontal gene transfer events from other *E. coli* strains or from other species harbouring the island. To minimize the emergence and spread of resistance via horizontal gene transfer, it is recommended fosfomycin be reserved for the treatment of acute uncomplicated UTI patients when the standard first-line drugs are not an option.

**Declarations**

**Funding:** The fosfomycin antimicrobial resistance surveillance study was funded by Mayne Pharma.

**Competing Interests:** B.A. Rogers has consulted for Mayne Pharma with regards to the registration of fosfomycin in Australia with the Therapeutic Goods Administration.

**Ethical Approval:** Not required

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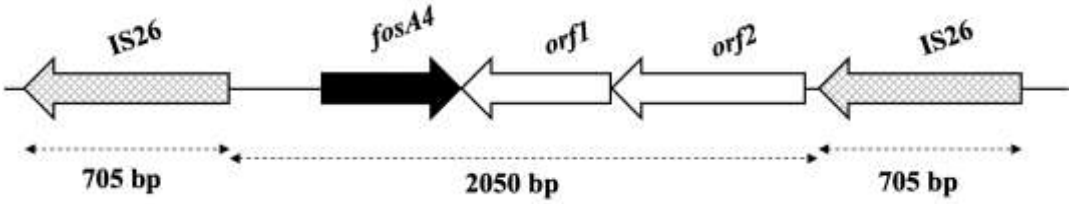


**Figure 1. Genetic environment of *fosA4*.**

The black arrow represents the fosfomycin resistance *fosA4* gene (417 bp), the white arrows represent open-reading frames (*orf1* encodes a protein of uncharacterised function and *orf2* encodes a transcriptional regulator) and the arrows with grey gridlines represent movable IS26 elements. This island was identified on pEC1-2 and pEC2-1.

Table 1. Antibiotic resistance genes identified in the fosfomycin-resistant isolates.

| Isolate | DNA | Antibiotic resistance genes |
|---|---|---|
| EC1 | chromosome | *mdfA* |
| | pEC1-1 | *aac(3)-IId, aph(3")-Ib, aph(6)-Id,* $bla_{TEM1-B}$*, mphA, sul2, tetA* |
| | pEC1-2 | $bla_{DHA-6}$*, dfrA17, fosA4, mphA, qnrB4, sul1* |
| EC2 | chromosome | *mdfA* |
| | pEC2-1 | *aadA1,* $bla_{TEM1-B}$*, fosA4, sul3* |
| | pEC2-2 | $bla_{TEM1-C}$ |

Table 2. Resistance phenotype and genotype of EC1 and EC2

| **Isolate** | **Phenotypic Resistance** | **Genetic marker/mutation** | **Location of genetic marker/mutation** |
|---|---|---|---|
| EC1 | Fosfomycin | *fosA4* | pEC1-2 |
| | **Beta lactams**:<br>Ampicillin<br>Amoxicillin/Clavulanic acid<br>Ticarcillin/Clavulanic acid<br>Cefazolin<br>Cefoxitin<br>Ceftazidime | $bla_{\text{TEM1-B}}$ | pEC1-1 |
| | **Aminoglycosides**:<br>Gentamicin<br>Tobramycin | *aac(3)-IId, aph(3")-Ib, aph(6)-Id* | pEC1-1 |
| | Trimethoprim | *dfrA17* | pEC1-2 |
| | Trimethoprim/Sulfamethoxazole | *dfrA17, sul1*<br>*sul2* | pEC1-2<br>pEC1-1 |
| EC2 | Fosfomycin | *fosA4* | pEC2-1 |
| | Ampicillin | $bla_{\text{TEM1-C}}$ | pEC2-2 |
| | Norfloxacin | *gyrA* S83L mutation | chromosome |