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MECHANISMS OF RESISTANCE

Identification and Characterization of a Novel FosA7 Member from Fosfomycin-Resistant *Escherichia coli* Clinical Isolates from Canadian Hospitals

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ABSTRACT Here, we characterize the *fosA* genes from three *Escherichia coli* clinical isolates recovered from Canadian patients. Each *fosA* sequence was individually overexpressed in *E. coli* BW25113, and antimicrobial susceptibility testing was performed to assess their role in fosfomycin resistance. The findings from this study identify and functionally characterize FosA3, FosA8, and novel FosA7 members and highlight the importance of phenotypic characterization of *fosA* genes.

KEYWORDS fosfomycin, *fosA7*, beta-lactamase, *Escherichia coli*, novel *fosA*

Escherichia coli is a common urinary tract pathogen. Treatment of infections caused by extended-spectrum beta-lactamase (ESBL)-producing or multidrug-resistant (MDR) *E. coli* can be problematic, as therapeutic options may be limited. Due to the increasing prevalence of ESBL and MDR *E. coli*, there has been renewed interest in the use of fosfomycin (1–4). Fosfomycin is a phosphoenolpyruvate analogue that disrupts bacterial cell wall synthesis by inhibiting UDP-*N*-acetylglucosamine-3-enolpyruvyl transferase (MurA), an enzyme involved in synthesis of *N*-acetylmuramic acid (5). Resistance to fosfomycin occurs by three main mechanisms: (i) alteration of fosfomycin drug uptake transporter genes (*glpT* and *uhpT*), (ii) modification or overexpression of *murA*, or (iii) acquisition of a fosfomycin-inactivating (*fos*) enzyme (5). Fos enzymes are of the greatest concern, since the *fos* genes that encode them can be found on plasmids, allowing for their dissemination by horizontal gene transfer (4, 5). In Canada, fosfomycin resistance and *fos* gene detection among *E. coli* clinical isolates are rare and have not been well described to date (6).

In this study, we characterize the *fosA* genes from three fosfomycin-resistant *E. coli* isolates (two from urine and one from blood) from a Canadian collection of clinical strains and describe a novel FosA7.5 variant within the FosA7 group. We also discuss the phylogenetic relationships among previously identified FosA1-A12 members.

Whole-genome sequencing of the three Canadian isolates (EC623771 [GenBank BioSample no. SAMN13659120], EC623772 [GenBank BioSample no. SAMN13659121], and EC623773 [GenBank BioSample no. SAMN13659122]) was performed on an Illumina MiSeq system using Nextera XT DNA libraries. Contigs were assembled and annotated using the IRIDA version 19.09 assembly and annotation pipeline, which combines Shovill-based assembly and QUAST quality assessment with Prokka annotation (7). Sequence analysis revealed *fosA* genes in all three genomes (see Table S1 in the supplemental material). The *E. coli* EC623771 *fosA* gene was 100% identical to *fosA3* (8), and the *E. coli* EC623773 *fosA* gene was 100% identical to *fosA8* (9) (Fig. 1). The *E. coli*

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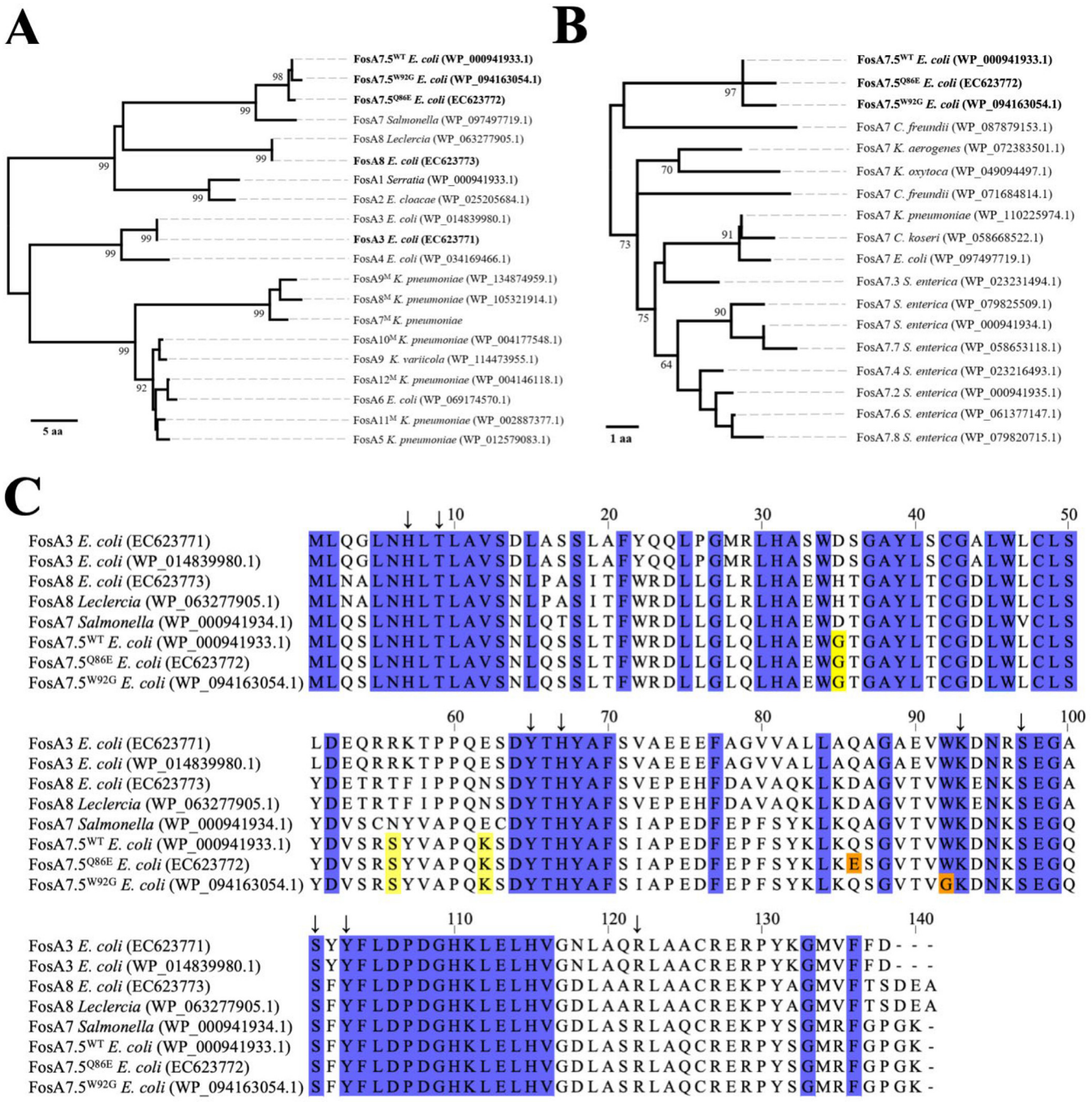

FIG 1 Phylogenetic and sequence analysis of clinically isolated *E. coli* FosA sequences and their comparison to previously identified FosA variants. (A) Phylogenetic analysis of FosA1 to FosA12 protein sequences using the neighbor-joining distance-based method. Branch lengths represent amino acid differences as distance (scale bar). (B) Phylogenetic comparison of FosA7 family protein sequences using the same method as described in panel A. (C) Multiple sequence alignment of FosA3, FosA8, and FosA7 protein sequence variants. Blue coloring in the alignment indicates conserved residues identified among FosA1 to 12 family members. Amino acid differences that distinguish the FosA7.5 group from FosA7 are shown in yellow. Differences among FosA7.5 sequences are highlighted in orange. Arrows indicate active site residues. The alignment was generated using Jalview v2.10.5 (21).

EC623772 *fosA* gene was not identical to any previously characterized *fosA* sequence but demonstrated >99% sequence identity to two publicly deposited sequences, a *fosA* gene from a canine isolate of *E. coli* (WP_094163054.1) and a reference sequence annotated as *fosA7.5* (WP_000941933.1) (Table S2). We decided to name the novel *E. coli* EC623772 *fosA* variant and the *E. coli* WP_094163054.1 variant *fosA7.5*, following the numbering convention that has been previously used for annotating *fosA7* genes in the

TABLE 1 Sequences, strains, and plasmids examined in this study

Strains and plasmids	Characteristics[a]	Reference or source
Strains		
E. coli EC623771	Fosfomycin-resistant isolate	CANWARD
E. coli EC623772	Fosfomycin-resistant isolate	CANWARD
E. coli EC623773	Fosfomycin-resistant isolate	CANWARD
E. coli BW25113	*F*$^-$, Δ*(araD-araB)567*, Δ*lacZ4787(::rrnB-3)*, λ$^-$, *rph-1*, Δ*(rhaD-rhaB)568*, *hsdR514*	NBRP[b]
E. coli pMS119EH	*E. coli* BW25113 transformed with pMS119EH	This study
E. coli FosA3	*E. coli* BW25113 transformed with pMS-FosA3	This study
E. coli FosA8	*E. coli* BW25113 transformed with pMS-FosA8	This study
E. coli FosA7.5^{WT}	*E. coli* BW25113 transformed with pMS-FosA7.5^{WT}	This study
E. coli FosA7.5^{Q86E}	*E. coli* BW25113 transformed with pMS-FosA7.5^{Q86E}	This study
E. coli FosA7.5^{W92G}	*E. coli* BW25113 transformed with pMS-FosA7.5^{W92G}	This study
Plasmids		
pMS119EH	NruI-NdeI deletion of pJF119EH vector	22
pMS-FosA3	C-terminus His6-tagged *fosA3* EC623771 gene cloned in pMS119EH	This study
pMS-FosA8	C-terminus His6-tagged *fosA8* EC623773 gene cloned in pMS119EH	This study
pMS-FosA7.5^{WT}	C-terminus His6-tagged *fosA7.5* WP_000941933.1 gene cloned in pMS119EH	This study
pMS-FosA7.5^{Q86E}	C-terminus His6-tagged *fosA7.5* EC623772 gene cloned in pMS119EH	This study
pMS-FosA7.5^{W92G}	C-terminus His6-tagged *fosA7.5* WP_094163054.1 gene cloned in pMS119EH	This study

[a]His_6, hexahistidine; C-terminus, carboxy-terminus.
[b]National BioResource Project, Keio Collection (23).

NCBI Bacterial Antimicrobial Resistance Reference Gene Database. Neighbor-joining analysis was performed to further investigate the relationship of the FosA variants from our clinical isolates with previously described FosA1 to FosA12 sequences. (Fig. 1A and B) (8–17). This confirmed the similarity of the three FosA7.5 sequences.

Relative to FosA7.5 from *E. coli* WP_000941933.1, FosA7.5 from *E. coli* EC623772 has a Q86E change, whereas the FosA7.5 from *E. coli* WP_094163054.1 shows a W92G change at a highly conserved amino acid position. Hence, we refer to the "wild-type" *fosA7.5* sequence as *fosA7.5^{WT}*, the novel *E. coli* EC623772 *fosA* variant as *fosA7.5^{Q86E}*, and the canine WP_094163054.1 variant as *fosA7.5^{W92G}*. Despite its name, FosA7.5 is distinct from the canonical FosA7 sequence and differs from other FosA7 members at amino acid sites G35, S56, and K62. FosA7 was originally identified in *Salmonella enterica* serovar Heidelberg (15), and most closely related variants are associated with *S. enterica*, whereas FosA7.5, along with the EC623772 and canine variants, is restricted to *E. coli*. These sequences are also distinct from FosA7-like sequences from *Klebsiella* spp. and *Citrobacter* spp. (Fig. S1, Table S2).

Twelve FosA variants (FosA1 to FosA12) were previously described in peer-reviewed publications (8–17). It should be noted that there is currently some inconsistency in the FosA literature regarding the naming of FosA enzyme variants. Notably, FosA7, FosA8, FosA9, and FosA10 have each been used twice to describe different variants (9, 15–18). After the published description of FosA7 in *Salmonella* Heidelberg, Mathur et al. described six FosA variants in *Klebsiella pneumoniae* and named them FosA7 through FosA12 (referred to in Fig. 1, Table 1, and the following text as FosA7^M to FosA12^M) (17). Phylogenetic analysis suggests that FosA7^M, FosA8^M, and FosA9^M may make a distinct branch of FosA enzymes, but it is unclear if three separate designations are warranted (Fig. 1A). More recently, *E. coli* FosA8, FosA9, and FosA10 genes were described in three papers (9, 16, 18). Based on our phylogenetic and sequence analyses in Fig. 1, FosA10^M, FosA11^M, and FosA12^M are very similar to one another as well as to FosA5, FosA6, and the newer FosA9 allele (16). Notably, the original descriptions of FosA5 (13), FosA6 (14), and FosA9 (16) indicate that they were mobilized to *E. coli* from *Klebsiella*. Thus, all of these alleles may represent a family of genes derived from *Klebsiella*.

Consistent with previous reports, the *fosA3* (EC623771) and *fosA8* (EC623773) genes identified in our isolates are associated with plasmid sequences. The *fosA7.5^{Q86E}* allele in EC623772 is flanked on both sides by insertion sequences that confounded our initial attempts to determine the location of this gene. Available genome assemblies (e.g., *E. coli* Ec40743 [CP041919.1] and *E. coli* 210205630 [CP015912]) suggest that the *fosA7.5^{WT}*

TABLE 2 Fosfomycin susceptibility testing results for *E. coli* transformants

E. coli plasmid transformant[c]	Agar dilution MIC (μg/ml)	Disk diffusion zone diam (mm)	Etest MIC (μg/ml)	Result
FosA3	>512	6[a]	>1,024	Resistant
FosA8	>512	6	>1,024	Resistant
FosA7.5^{WT}	>512	6	>1,024	Resistant
FosA7.5^{Q86E}	>512	6	>1,024	Resistant
FosA7.5^{W92G}	32	30[b]	2	Susceptible
pMS119EH	2–4	30	0.5	Susceptible

[a]6 mm is equivalent to no zone diameter.
[b]CLSI, 30 mm; EUCAST, 36 mm.
[c]All transformants were induced with 1 mM IPTG.

allele is located on the *E. coli* chromosome. However, resequencing of *E. coli* EC623772 with a MinION system (Oxford Nanopore Technologies) and assembly with Flye version 2.8.1 (19) revealed that *fosA7.5^{Q86E}* is located on a 103-kb plasmid. Sequence comparison using publicly available databases shows that the backbone of this 103-kb plasmid, excluding the *fosA7.5^{Q86E}* region, is conserved with other plasmids from *E. coli*, *Salmonella*, and *Klebsiella* (e.g., GenBank plasmids CP044142.1, JN983043.1, and MF582638.1).

To verify that the genes from the three Canadian clinical isolates conferred resistance to fosfomycin, *fosA3* (EC623771), *fosA8* (EC623773), and the three *E. coli fosA7.5* sequences were gene synthesized by Bio Basic, Inc. (Canada), and individually cloned into the low copy expression vector pMS119EH. All *fosA* genes were cloned with a C-terminal hexahistidine affinity tag (His_6-tag) and then overexpressed in the *E. coli* K-12 strain BW25113 with isopropyl β-D-1-thiogalactopyranoside (IPTG) induction (Table 1). Western blotting demonstrated successful FosA protein expression and accumulation of each *E. coli* transformant (Fig. S2). Each transformant underwent fosfomycin antimicrobial susceptibility testing using agar dilution and disk diffusion according to CLSI standards and an Etest, and the results are shown in Table 2. The *fosA3*, *fosA8*, and *fosA7.5^{Q86E}* genes cloned from the Canadian clinical isolates, as well as the wild-type *fosA7.5^{WT}* allele, all conferred resistance to fosfomycin (MIC values of >512 μg/ml and >1,024 μg/ml for agar dilution and Etest, respectively). The only exception was *E. coli* transformed with the *fosA7.5^{W92G}* variant, which remained susceptible to fosfomycin at MIC values of 32 μg/ml and 2 μg/ml using the agar dilution and Etest methods, respectively.

As we noted key amino acid differences between FosA7.5 members, we generated homology models of FosA3, FosA8, and the three FosA7.5 variants using the I-TASSER Web server (20) to determine if any protein structural alterations impacting the FosA active site may explain why the *fosA7.5^{W92G}* transformant was susceptible to fosfomycin (Fig. S3). Dimeric FosA protein homology models were generated from the FosA1 *Serratia marcescens* (PDB: 1nbp) crystal structure to model the complete active site spanning the dimer interface. All FosA7.5 variant models demonstrated tight overall alignment to previously characterized FosA3 and FosA8 based on lowest root mean square deviation (RMSD) values (1.722 to 2.011 Å). The W92G amino acid change in FosA7.5^{W92G} (GenBank accession number WP_094163054.1) appeared to generate a larger pocket near the fosfomycin binding site when aligned to other FosA7.5 models, suggesting that the replacement of tryptophan by a smaller glycine residue may reduce the enzymatic activity of this variant. FosA7.5^{W92G} may allow greater substrate movement or amino acid flexibility within the enzyme's active site by replacing this conserved tryptophan that we observed in FosA alignments at this residue position (Fig. S1).

In conclusion, we identified three *fosA* genes in three *E. coli* clinical isolates (EC623771 to EC623773) recovered from Canadian patients. In addition to confirming the role of *fosA3* and *fosA8* as determinants of fosfomycin resistance (8, 9), we identified and characterized multiple variants of *fosA7.5*. Unlike other *fosA7* alleles, which are associated with *Salmonella*, distribution of *fosA7.5* is primarily restricted to *E. coli*. Ongoing surveillance for fosfomycin resistance is crucial to ensure that this antimicrobial remains effective as a first-line therapy for urinary tract infections.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1 MB.

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