SUPPLEMENTARY MATERIAL

METHODS

***Bacterial strains, chemicals, and media used*.** The *E. coli* clinical isolates included here were obtained from the CANWARD collection (1). Between 2007 and 2016, ten fosfomycin-resistant *E. coli* were identified. The three *E. coli* isolates (two from urine and one from blood; EC623771-EC623773) with a fosfomycin MIC of >512 µg/ml were selected for further evaluation, as described below.

The strains and plasmids used in this study are listed in **Table 1**. *E. coli* K12 BW25113 was obtained from the Coli Genetic Stock Centre (Yale University, CT, USA) (2). Strains were grown at 37^{o}C in Luria–Bertani (LB) broth with aeration at 170 rpm, LB agar plates, or Mueller–Hinton (MH) agar plates. Ampicillin was included in all LB and MH media at a final concentration of 100 µg/ml to maintain transformed plasmids. Cloned *fosA* gene expression from transformed plasmids was induced with IPTG at a final concentration of 0.1 mM in LB and 1 mM in MH broth. Ampicillin was purchased from VWR (Radnor, PA, USA). IPTG was purchased from Cedarlane (Burlington, ON, Canada). Fosfomycin analytical grade powder was supplied by Paladin Labs (Montreal, QC, Canada).

Clinical isolate whole genome sequencing. Whole genome sequencing of the three fosfomycin–resistant *E. coli* isolates was performed on an Illumina MiSeq system. DNA libraries were prepared with Nextera XT reagents and then sequenced using V2 chemistry. Sequencing data has been deposited in GenBank as BioProject PRJNA511988. The accession numbers for *E. coli* strains EC623771 (*fosA3*), EC623772 (*fosA7.5*^{Q86E}), and EC623773 (*fosA8*) are SAMN13659120, SAMN13659121, and SAMN13659122, respectively. Genome assembly and annotation were performed with the Integrated Rapid Infectious

Disease Analysis (IRIDA) platform (version 19.09) (3). Briefly, this pipeline combines Shovill assembly, with Prokka annotation and QUAST assembly assessment. Resequencing of *E. coli* EC623772 was performed on a MinION Mk1b system (Oxford Nanopore Technologies; ONT). The sequencing library was prepared with the ONT Ligation Sequencing Kit using genomic DNA that has been sheared to a mean fragment size of 8 kb with a gTube (Covaris, Inc). The sequencing run used a FLO-MIN106D flow cell (pore version R9.4.1) and MinKNOW software (release 19.12.5). Base calling was performed with Guppy (version 3.2.10). Flye (v.2.8.1) was used for read assembly and yielded 11 contigs, including the ≈103 kb closed circular plasmid sequence containing the *fosA7.5*^{Q86E} allele (4).

FosA sequence sources. The *fosA* reference sequences used in this study were obtained from published literature and public databases, including NCBI's Bacterial Antimicrobial Resistance Reference Gene Database (BioProject PRJNA313047) and the Comprehensive Antibiotic Resistance Database (CARD; https://card.mcmaster.ca/). The list of sequences and sources is summarized in **Table S2**. Multiple approaches were used to identify *fosA* alleles in the EC623771, EC623772, and EC623773 datasets. Sequences were analyzed by ResFinder on the Center for Genomic Epidemiology website (https://cge.cbs.dtu.dk/services/), annotated contigs were reviewed for genes labelled '*fosA*' or 'glutathione transferase', and contig sequences were compared to a reference set of *fosA* sequences using Geneious (version 7, Biomatters Ltd., Auckland New Zealand).

Construction of strains and plasmids. Synthesis and cloning of *fos* genes into the expression plasmid pMS119EH was performed using Bio Basic Inc. Gene Synthesis services (Markham, ON, Canada). The ampicillin–resistant P*tac* expression plasmid pMS119EH (5) was used as the parental vector for all constructs. Each *fos* gene was synthesized with an in–

frame C–terminal hexahistidine affinity tag (His_6-tag) according to the sequences listed in **Figure S4.** All *fos* genes were cloned into the pMS119EH multiple cloning site at 5' EcoRI and 3' XbaI cut sites and all gene sequences were verified by Sanger sequencing. Plasmids were transformed into *E. coli* BW25113 competent cells using standard protocols (6) and cryopreserved in LB with 20% dimethylsulfoxide.

***SDS–Tricine–PAGE and Western Blotting*.** Overnight (16 h) cultures of *E. coli* transformants grown in LB broth with ampicillin were standardized to 1.0 absorbance units (OD_{600}), then 50 µL of cells was used to inoculate 5 ml LB broth with ampicillin, and cultures were incubated at 37^{o}C until reaching 0.5 absorbance units. IPTG was added at a final concentration of 0.1 mM, and cultures were incubated at 25^{o}C for another 3 hours. 2 ml of cells were harvested by centrifugation (15,000 x g, 1 min), re–suspended in 0.4 ml of 8 M urea buffer (100 mM NaH_2PO_4, 10 mM Tris, 8M urea, pH 8.0) and vortexed briefly. The supernatants were recovered after centrifugation at 15,000 x g, for 10 min. Protein concentration from supernatants was determined using a modified Lowry protein assay (7). 10 µL of protein extracts (10 µg) were separated using 12% sodium dodecylsulfate (SDS)–Tricine polyacrylamide gel electrophoresis (PAGE), and visualized with ultraviolet (UV) light using 0.5% 2,2,2–trichloroethanol (TCE) (8), then transferred onto a nitrocellulose membrane using Western Blotting (9). The membrane was blocked for 1 h in Tris–buffered saline (20 mM Tris, 500 mM NaCl, pH 7.5) containing 5% skim milk powder and washed in Tris–buffered saline containing 0.05% tween–20. Western blotted FosA-His_6 protein accumulation from total cell protein extracts was colorimetrically detected using an anti–Hexahistidine horseradish peroxidase (HRP)–conjugated antibody (Thermo Fisher Scientific) with the HRP conjugate substrate kit (Bio–Rad).

Antimicrobial susceptibility testing. *In vitro* susceptibility of *E. coli* transformants to fosfomycin was determined by CLSI agar dilution (10, 11), CLSI disk diffusion (11, 12), and Etest (bioMérieux, Marcy l'Etoile, France). Agar dilution plates contained doubling–dilutions of fosfomycin from 0.5 to 512 µg/ml (Mueller–Hinton agar supplemented with 25 µg/ml of glucose–6–phosphate) (10, 11); 200 µg fosfomycin disks (containing 50 µg of glucose–6–phosphate) (11, 12) and Etest were tested on Mueller–Hinton agar.

The Mueller–Hinton agar used to test transformants was supplemented with 100 µg/ml ampicillin (selection) and 1 mM IPTG to induce protein expression of genes under control of the P*tac* promotor. Inoculated agar plates were incubated in ambient air at 35°C ± 2°C for 16–18 h. Fosfomycin MICs were determined in triplicate for each transformant using each of the three susceptibility testing methods.

Agar dilution MICs and disk diffusion zone sizes were read following CLSI directives (11). Etest endpoints were read following manufacturer instructions. CLSI fosfomycin agar dilution MIC breakpoints (≤64 µg/ml = susceptible, 128 µg/ml = intermediate, ≥256 µg/ml = resistant) and disk diffusion zone size breakpoints (≥16 mm = susceptible, 13–15 mm = intermediate, ≤12 mm = resistant) were used to interpret the results of antimicrobial susceptibility testing. Quality control testing included ATCC strains *E. coli* 25922 and *Pseudomonas aeruginosa* 27853.

Multiple sequence alignment and phylogenetic analysis. Multiple sequence alignment and phylogenetic analysis of FosA protein sequences were performed with MEGA7 (13). Dendrograms **(Figures 1A and 1B)** were constructed using the Neighbor-Joining method and confidence intervals were assigned using the interior branch test (500 replicates) (14, 15).

The sequence alignment of FosA proteins shown in **Figure 1C** was conducted used Clustal Omega (16) and visualized by Jalview version 2.10.5 software (17).

Homology modelling of FosA protein sequences. The crystal structure of FosA1 ($FosA^{Tn2921}$) from *Serratia marcescens* (PDB: 1nbp) was identified as the closest template based on the RMSD values (2.3 ± 1.8 Å to 2.6 ± 2.0 Å) and C–score values (ranging from 1.03 to 1.19) for each FosA protein sequence using the I–TASSER homology modelling online webserver (18, 19). The overlapped superposition of all FosA homology model sequences generated by I–TASSER shown in **Figure S3**, was performed using PyMOL software version 2.2.3 (20).

REFERENCES

1. Zhanel GG, Adam HJ, Baxter MR, Fuller J, Nichol KA, Denisuik AJ, Golden AR, Hink R, Lagacé-Wiens PRS, Walkty A, Mulvey MR, Schweizer F, Bay D, Hoban DJ, Karlowsky JA. 2019. 42936 pathogens from Canadian hospitals: 10 years of results (2007-16) from the CANWARD surveillance study. J Antimicrob Chemother 74:iv5–iv21.
2. Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, Datsenko KA, Tomita M, Wanner BL, Mori H. 2006. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol Syst Biol 2:1–11.
3. Matthews TC, Bristow FR, Griffiths EJ, Petkau A, Adam J, Dooley D, Kruczkiewicz P, Curatcha J, Cabral J, Fornika D, Winsor GL, Courtot M, Bertelli C, Roudgar A, Feijao P, Mabon P, Enns E, Thiessen J, Keddy A, Isaac-Renton J, Gardy JL, Tang P, The IRIDA Consortium, Carriço JA, Chindelevitch L, Chauve C, Graham MR, McArthur AG, Taboada EN, Beiko RG, Brinkman FS, Hsiao WW, Van Domselaar G. 2018. The Integrated Rapid Infectious Disease Analysis (IRIDA) platform. BioRxiv 1–34.
4. Kolmogorov M, Yuan J, Lin Y, Pevzner PA. 2019. Assembly of long, error-prone reads using repeat graphs. Nat Biotechnol 37:540–546.
5. Fürste JP, Pansegrau W, Frank R, Blöcker H, Scholz P, Bagdasarian M, Lanka E. 1986. Molecular cloning of the plasmid RP4 primase region in a multi-host-range *tacP* expression vector. Gene 48:119–131.
6. Green R, Rogers EJ. 2013. Chemical transformation of *E. coli*. Methods Enzymol 529:329–336.
7. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with the folin phenol reagent. J Biol Chem 193:265–275.
8. Bay DC, Turner RJ. 2012. Spectroscopic analysis of small multidrug resistance protein EmrE in the presence of various quaternary cation compounds. Biochim Biophys Acta -

Biomembr 1818:1318–1331.
9. Towbin H, Staehelin T, Gordon J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci U S A 76:4350–4354.
10. Clinical and Laboratory Standards Institute. 2018. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. M07, 11th edition. CLSI. Wayne, PA.
11. Clinical and Laboratory Standards Institute. 2019. Performance standards for antimicrobial susceptibility testing. M100, 29th edition. CLSI. Wayne, PA.
12. Clinical and Laboratory Standards Institute. 2018. Performance standards for antimicrobial disk susceptibility tests. M02, 13th edition. CLSI. Wayne, PA.
13. Kumar S, Stecher G, Tamura K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Mol Biol Evol 33:1870–1874.
14. Saitou N, Nei M. 1987. The nighbor-joining method: A new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425.
15. Dopazo J. 1994. Estimating errors and confidence intervals for branch lengths in phylogenetics trees by a bootstrap approach. J Mol Evol 38:300–304.
16. Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, Lopez R, McWilliam H, Remmert M, Söding J, Thompson JD, Higgins DG. 2011. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Mol Syst Biol 7:1–6.
17. Waterhouse AM, Procter JB, Martin DMA, Clamp M, Barton GJ. 2009. Jalview Version 2-A multiple sequence alignment editor and analysis workbench. Bioinformatics 25:1189–1191.
18. Yang J, Yan R, Roy A, Xu D, Poisson J, Zhang Y. 2014. The I-TASSER suite: protein structure and function prediction. Nat Methods 12:7–8.
19. Pakhomova S, Rife CL, Armstrong RN, Newcomer ME. 2004. Structure of fosfomycin resistance protein FosA from transposon *Tn2921*. Protein Sci 13:1260–1265.
20. Schrödinger LLC. The PyMOL molecular graphics system, version 2.2.3.
21. Mathur P, Veeraraghavan B, Devanga Ragupathi NK, Inbanathan FY, Khurana S, Bhardwaj N, Kumar S, Sagar S, Gupta A. 2018. Multiple mutations in lipid-A modification pathway & novel *fosA* variants in colistin-resistant *Klebsiella pneumoniae*. Futur Sci OA 4:FSO319.

Table S1. Resistance genes and plasmids in *E. coli* urinary isolates.

Strain	MLST	Resistance genes	Plasmids
E. coli EC623771	ST-131	*aac(3)-IId, blaCTX-M-65, blaTEM-1B, fosA3*	IncFIA, IncFIB, IncFII, IncN
E. coli EC623772	ST-354[a]	*aac(3)-IId, aph(3'')-Ib, aph(6)-Id, blaCMY-2, qnrB19, sul2, tet(A), fosA7.5*	Col(pHAD28), IncC, IncFIA, IncFIB
E. coli EC623773	ST-457	*aac(3)-IId, aadA1, aadA2, aph(3'')-Ib, aph(6)-Id, blaTEM-1B, cmlA1, dfrA12, floR, sul2, tet(A), fosA8*	Col(MG828), Col8282, ColpVC, IncFIB, IncFII, IncN, IncX1, p0111

[a]Not in PubMLST Achtman Database, novel ST within the ST-354 clonal complex

Abbreviations: MLST; Multi-locus sequence typing

Table S2. Accession numbers for FosA proteins analyzed in this study.

FosA Allele	Source	Protein Sequence ID
FosA1	*Serratia marcescens*	WP_038415208.1
FosA2	*Enterobacter cloacae*	WP_025205684.1
FosA3	*Escherichia coli*	WP_014839980.1
FosA3	*Escherichia coli* (EC623771)	[This Study]
FosA4	*Escherichia coli*	WP_034169466.1
FosA5	*Klebsiella pneumoniae*	WP_012579083.1
FosA6	*Escherichia coli*	WP_069174570.1
FosA7	*Salmonella enterica*	WP_000941934.1
FosA7.2	*Salmonella enterica*	WP_000941935.1
FosA7.3	*Salmonella enterica*	WP_023231494.1
FosA7.4	*Salmonella enterica*	WP_023216493.1
FosA7.6	*Salmonella enterica*	WP_061377147.1
FosA7.7	*Salmonella enterica*	WP_058653118.1
FosA7.8	*Salmonella enterica*	WP_079820715.1
FosA7	*Salmonella enterica*	WP_079825509.1
FosA7	*Klebsiella aerogenes*	WP_072383501.1
FosA7	*Klebsiella oxytoca*	WP_049094497.1
FosA7	*Klebsiella pneumoniae*	WP_110225974.1
FosA7	*Escherichia coli*	WP_097497719.1
FosA7	*Citrobacter koseri*	WP_058668522.1
FosA7	*Citrobacter freundii*	WP_071684814.1
FosA7	*Citrobacter freundii*	WP_087879153.1
FosA8	*Leclercia adecarboxylata*	WP_063277905.1
FosA8	*Escherichia coli* (EC623773)	[This Study]
FosA9	*Klebsiella variicola*	WP_114473955.1
$FosA7^{M}$	*Klebsiella pneumoniae*	[Mathur 2018] (21)
$FosA8^{M}$	*Klebsiella pneumoniae*	WP_105321914.1
$FosA9^{M}$	*Klebsiella pneumoniae*	WP_134874959.1
$FosA10^{M}$	*Klebsiella pneumoniae*	WP_004177548.1
$FosA11^{M}$	*Klebsiella pneumoniae*	WP_002887377.1
$FosA12^{M}$	*Klebsiella pneumoniae*	WP_004146118.1
$FosA7.5^{WT}$	*Escherichia coli*	WP_000941933.1
$FosA7.5^{Q86E}$	*Escherichia coli* (EC623772)	[This Study]
$FosA7.5^{W92G}$	*Escherichia coli*	WP_094163054.1

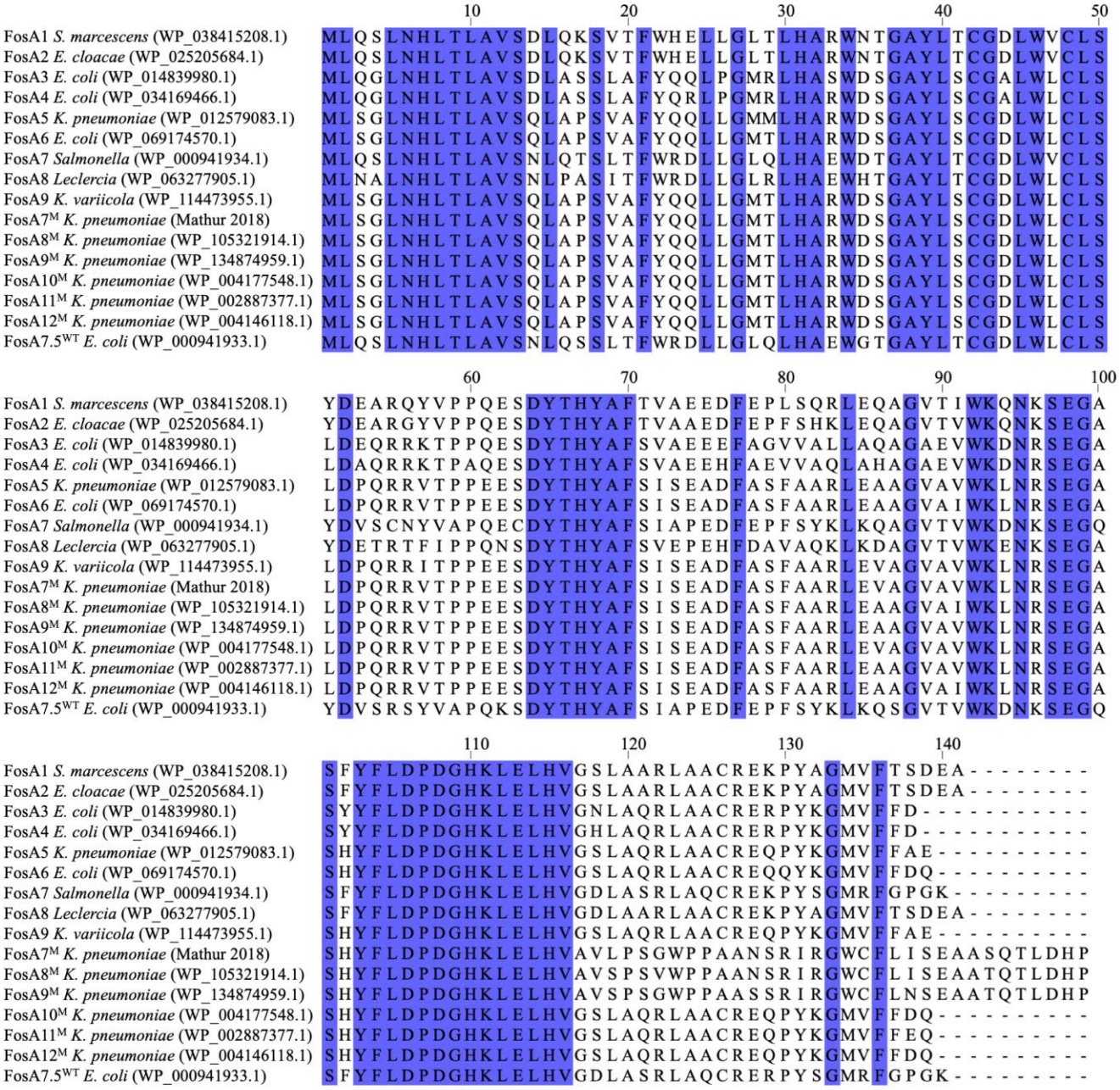

Figure S1. Multiple sequence alignment of FosA1-A12 protein sequence variants. Blue colouring in the alignment indicates conserved residues identified amongst FosA1–12 family members. The alignment was generated using Jalview version 2.10.5 (17).

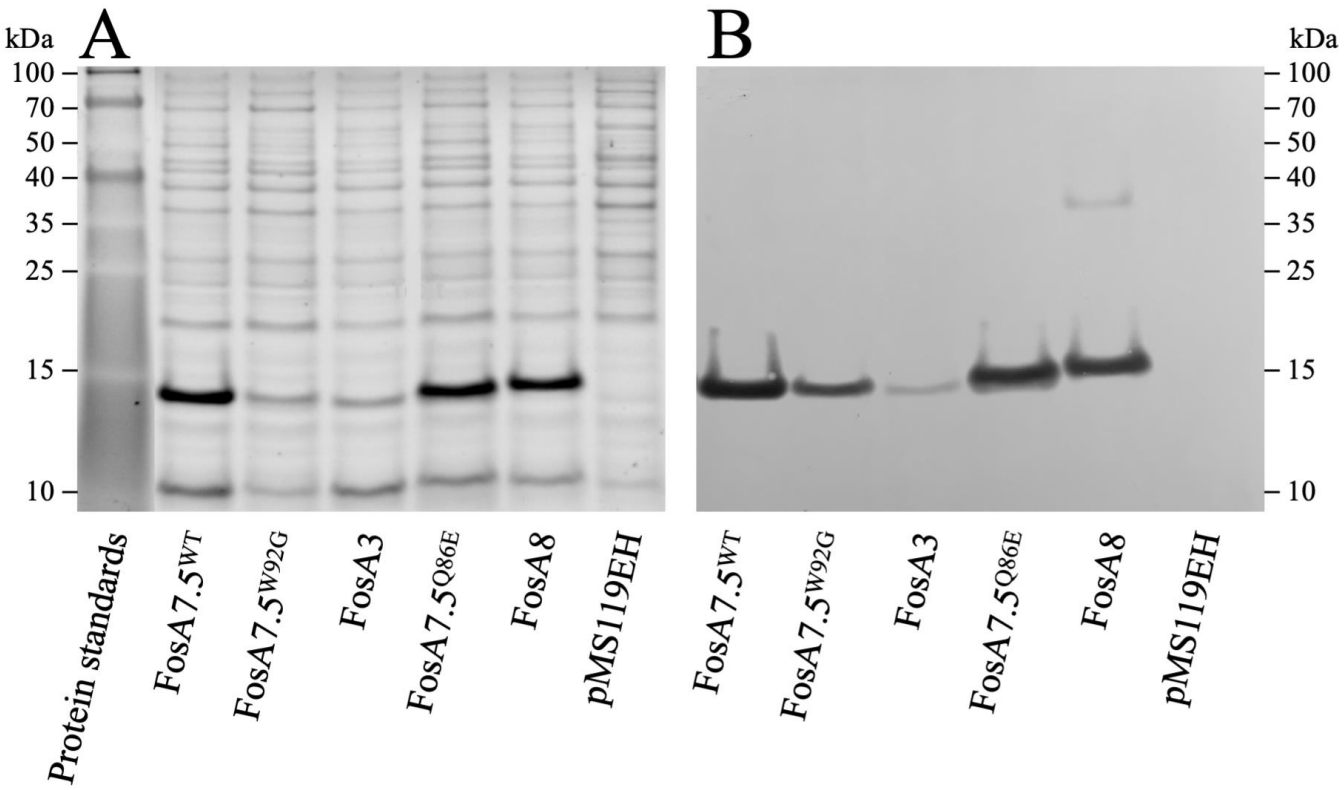

Figure S2. Induction of FosA proteins in *E. coli* transformants. A) TCE–visualized protein extracts. Proteins were fractionated on a 12% SDS–PAGE gel containing 0.5% TCE and visualized under ultraviolet light (8). B) Western blot of protein extracts (9). Proteins were detected using a His probe–HRP–conjugated antibody and the HRP conjugate substrate kit (Bio–Rad).

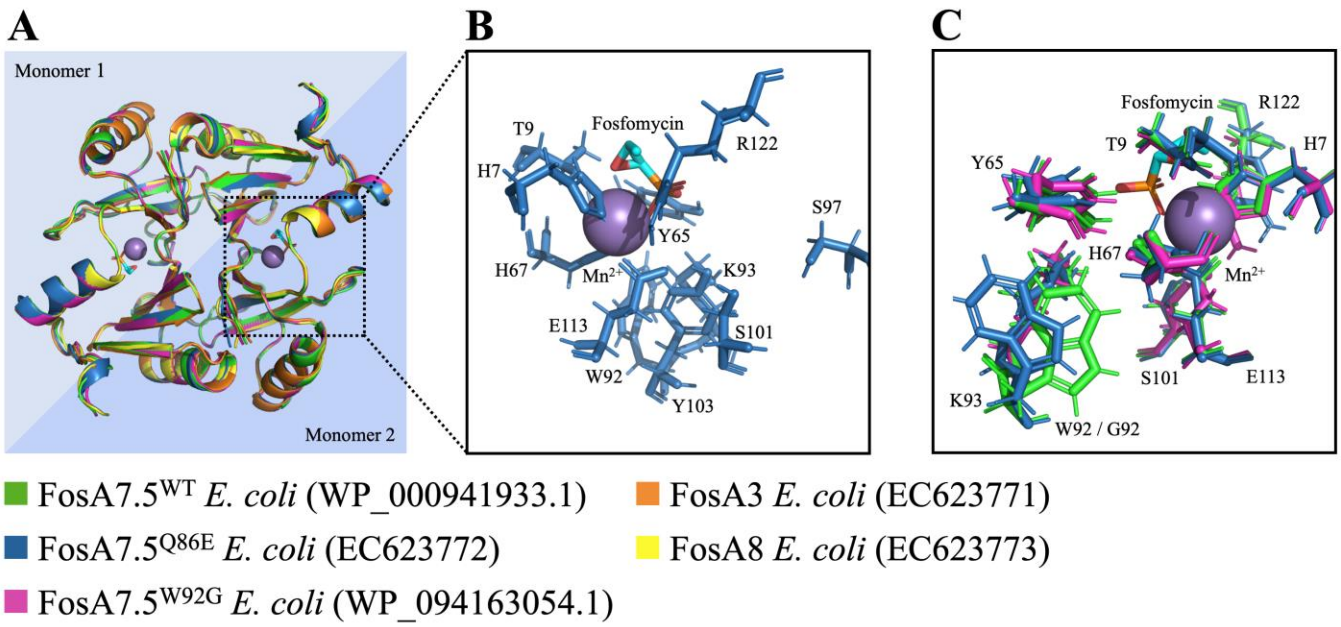

Figure S3. Structural visualization of FosA protein homology models. **A**) An overview of aligned superimposed FosA protein dimers are shown where a bound fosfomycin and Mn^{2+} ion are shown as a colored stick diagram and sphere respectively. **B**) A zoomed in stick diagram view of the active site of FosA7.5^{Q86E} from *E. coli* EC623772. **C)** A stick diagram of superimposed active sites from FosA7.5^{WT}, FosA7.5^{Q86E}, and FosA7.5^{W92G} rotated 120 degrees from panel B. These images were created using the program PyMOL version 2.2.3 (20). Colors listed below each panel correspond to FosA sequences shown in all panels.

>FosA7.5WT_E.coli_WP_000941933.1
gaattcaggagaaataatATGCTTCAATCTCTGAACCACTTAACGCTTGCTGTCAGTAATTTGCAAAGT
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TCTCGACCCGGACGGGCACAAACTGGAACTGCATGTGGGCGATCTGGCCGCGCGTCTGGCGGCGT
GTCGGGAGAAGCCTTACGCGGGAATGGTTTTTACGTCAGATGAAGCGggcggctctcatcatcat
catcatcattctTAAtctaga

Figure S4. Fasta files for BioBasic gene synthesis**.** Lowercase letters indicate restriction sites

and hexahistidine tags.