AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Clinical Microbiology®

BACTERIOLOGY

Frequency and Mechanisms of Spontaneous Fosfomycin Nonsusceptibility Observed upon Disk Diffusion Testing of *Escherichia coli*

Aaron E. Lucas,[a] Ryota Ito,[a,b] Mustapha M. Mustapha,[a] Christi L. McElheny,[a] Roberta T. Mettus,[a] Sarah L. Bowler,[a] Serena F. Kantz,[a] Marissa P. Pacey,[a] A. William Pasculle,[c] Vaughn S. Cooper,[d,e] Yohei Doi[a,b,e]

[a]Division of Infectious Diseases, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA

[b]Department of Microbiology, Fujita Health University, Toyoake, Aichi, Japan

[c]Clinical Microbiology Laboratory, University of Pittsburgh Medical Center, Pittsburgh, Pennsylvania, USA

[d]Department of Molecular Microbiology and Genetics, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA

[e]Center for Innovative Antimicrobial Therapy, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA

ABSTRACT Fosfomycin maintains activity against most *Escherichia coli* clinical isolates, but the growth of *E. coli* colonies within the zone of inhibition around the fosfomycin disk is occasionally observed upon susceptibility testing. We aimed to estimate the frequency of such nonsusceptible inner colony mutants and identify the underlying resistance mechanisms. Disk diffusion testing of fosfomycin was performed on 649 multidrug-resistant *E. coli* clinical isolates collected between 2011 and 2015. For those producing inner colonies inside the susceptible range, the parental strains and their representative inner colony mutants were subjected to MIC testing, whole-genome sequencing, reverse transcription-quantitative PCR (qRT-PCR), and carbohydrate utilization studies. Of the 649 *E. coli* clinical isolates, 5 (0.8%) consistently produced nonsusceptible inner colonies. Whole-genome sequencing revealed the deletion of *uhpT* encoding hexose-6-phosphate antiporter in 4 of the *E. coli* inner colony mutants, while the remaining mutant contained a nonsense mutation in *uhpA*. The expression of *uhpT* was absent in the mutant strains with *uhpT* deletion and was not inducible in the strain with the *uhpA* mutation, unlike in its parental strain. All 5 inner colony mutants had reduced growth on minimal medium supplemented with glucose-6-phosphate. In conclusion, fosfomycin-nonsusceptible inner colony mutants can occur due to the loss of function or induction of UhpT but are rare among multidrug-resistant *E. coli* clinical strains. Considering that these mutants carry high biological costs, we suggest that fosfomycin susceptibility of strains that generate inner colony mutants can be interpreted on the basis of the zone of inhibition without accounting for the inner colonies.

KEYWORDS fosfomycin, susceptibility testing, disk diffusion, mechanism of resistance

Escherichia coli organisms have increasingly become resistant to commonly used antimicrobial agents. Of the *E. coli* strains causing catheter-associated urinary tract infections in the United States, over 30% are now resistant to fluoroquinolones, and up to 16% are resistant to cephalosporins, largely due to the production of extended-spectrum β-lactamases (ESBLs) and plasmid-mediated cephalosporinases (1). Fosfomycin is a broad-spectrum cell wall synthesis inhibitor produced by *Streptomyces* spp. and *Pseudomonas syringae*. It exerts antibacterial activity by inactivating the cytosolic *N*-acetylglucosamine enolpyruvyl transferase (MurA),

Received 25 August 2017 **Returned for modification** 19 September 2017 **Accepted** 25 October 2017

Accepted manuscript posted online 1 November 2017

Citation Lucas AE, Ito R, Mustapha MM, McElheny CL, Mettus RT, Bowler SL, Kantz SF, Pacey MP, Pasculle AW, Cooper VS, Doi Y. 2018. Frequency and mechanisms of spontaneous fosfomycin nonsusceptibility observed upon disk diffusion testing of *Escherichia coli*. J Clin Microbiol 56:e01368-17. https://doi.org/10.1128/JCM.01368-17.

Editor Nathan A. Ledeboer, Medical College of Wisconsin

Address correspondence to Yohei Doi, yod4@pitt.edu.

A.E.L. and R.I. contributed equally to this article.

which prevents the formation of *N*-acetylmuramic acid, an essential component of peptidoglycan (2). It maintains activity against the majority of *E. coli* clinical strains and is now one of the first-line agents endorsed for the empirical treatment of uncomplicated urinary tract infection (3).

The emergence of fosfomycin-resistant mutants is frequently observed in *E. coli* under *in vitro* conditions (4). This often results from defects in either the GlpT (glycerol-3-phosphate) or UhpT (hexose-6-phosphate) transporter systems utilized by fosfomycin for cell entry, which may occur thorough loss-of-function mutations in *glpT* or *uhpT* and their regulatory genes themselves or sometimes in genes that maintain cAMP levels that positively regulate *glpT*, such as *cyaA* and *ptsI* (5). While data regarding resistance mechanisms underlying fosfomycin-resistant clinical strains remain relatively scarce, a defective GlpT or UhpT system and, in some instances, specific substitutions in MurA have been implicated (5–7). The overall resistance rates of fosfomycin remain very low among *E. coli* clinical strains, likely due to the fitness costs incurred by these GlpT or UhpT transporter mutations, which have been further described to result in decreased growth rates in both laboratory medium and urine (5, 8). More recently, the plasmid-mediated fosfomycin resistance gene *fosA3* encoding glutathione *S*-transferase, which inactivates fosfomycin by catalyzing the covalent addition of glutathione to C-1 of fosfomycin and opening its epoxide ring, has been reported from *E. coli* clinical and animal strains mostly in East Asia, but its impact appears relatively confined to the region so far (2, 9, 10).

Susceptibility testing of fosfomycin requires the addition of 25 μg/ml of glucose-6-phosphate (G6P) in Mueller-Hinton (MH) medium, which induces the hexose phosphate transport system and increases as well as stabilizes the activity of fosfomycin (11). For disk diffusion testing, the currently approved disk formulation is 200 μg of fosfomycin and 50 μg of G6P, with zone diameters of $\leq$12 mm defining resistance, 13 to 15 mm defining an intermediate, and $\geq$16 mm defining susceptibility per the Clinical and Laboratory Standards Institute guidelines (12). However, the interpretation of the disk diffusion susceptibility testing results can be complicated by scattered colonies that emerge within the zone of inhibition in up to 41% of *E. coli* clinical strains (13). The resistance mechanism underlying these mutants has not been reported, and the clinical significance of these colonies within the zone of inhibition and how these findings should be interpreted remain uncertain (13, 14).

The present study was undertaken with the aims of estimating the frequency of the emergence of fosfomycin-nonsusceptible mutants that may confound the interpretation of the susceptibility testing results and also identifying the specific resistance mechanisms of these mutants that emerge upon disk diffusion testing of fosfomycin among multidrug-resistant *E. coli* clinical strains.

MATERIALS AND METHODS

Susceptibility testing. A total of 649 unique cephalosporin-resistant *E. coli* clinical isolates collected at the University of Pittsburgh Medical Center between 2011 and 2015 were subjected to susceptibility testing with fosfomycin using the standard disk diffusion method (15). The fosfomycin disks (containing 200 μg of fosfomycin and 50 μg of G6P) and BBL MH agar II were purchased from BD (Franklin Lakes, NJ). When colonies were observed inside the inhibition zone, the test was repeated for reproducibility, and strains that consistently produced inner colonies in the nonsusceptible range (i.e., $\leq$15 mm) were included in the further studies. Fosfomycin MICs were determined by the agar dilution method with the addition of 25 μg/ml of G6P (12).

Whole-genome sequencing. The parental clinical strains and representative inner colonies, one per parental strain, were subjected to NextSeq whole-genome sequencing (Illumina, San Diego, CA). The reads were assembled *de novo* using the de Bruijn assembler SPAdes (16) and annotated using the prokaryotic annotation pipeline Prokka (17). Pairwise gene content differences were assessed initially using blastn queries of annotated genes against contigs, and then sequence reads were aligned to raw sequencing reads using SRST2, a tool for assessing the presence of genes from raw sequencing reads (18). Large ($>$0.5 kb) chromosomal deletions were detected by extracting regions of low coverage ($<$5 reads) after aligning reads from the derivative strain to assembled contigs of the parent strain using BWA-MEM (http://bio-bwa.sourceforge.net/) and SAMtools (19). Single nucleotide polymorphism (SNP) differences and small indels were assessed using nullarbor (https://github.com/tseemann/nullarbor) and breseq, respectively (20), with the parent strain of each pair as the reference. *In silico* multilocus sequence

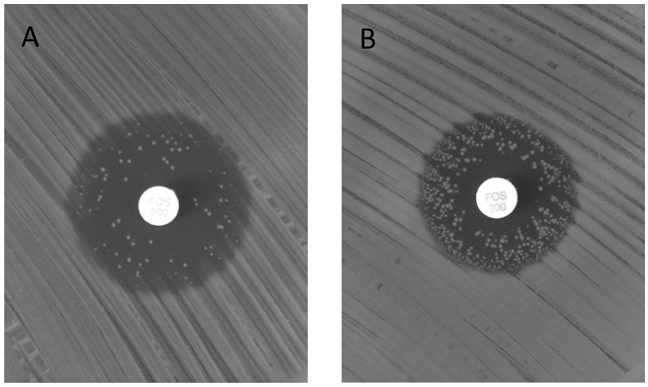

FIG 1 Examples of *E. coli* clinical strains that consistently generate fosfomycin-nonsusceptible mutants within the zone of inhibition upon disk diffusion testing. (A) *E. coli* S61. (B) *E. coli* S65.

typing (MLST) was performed using the Center for Genomic Epidemiology website (https://cge.cbs.dtu.dk/services/MLST/).

Carbohydrate utilization assay. The strains were grown in MH broth at 37°C with shaking overnight. The pellets were then collected, washed twice, and suspended in saline. M9 minimum medium agar containing 0.2% G6P as the sole carbon source was inoculated with this suspension. The growth was observed after incubating at 35°C for 48 h. Strains lacking growth or showing poor growth without the formation of colonies were considered deficient in UhpT function.

Total RNA extraction. Total RNA was extracted from cultures grown in lysogeny broth (LB) at 37°C until they reached an optical density at 600 nm (OD_{600}) of 0.5. RNA was extracted using TRIzol reagent (Thermo Fisher, Waltham, MA) according to the manufacturer's instructions. Contaminating DNA was removed using the Turbo DNA-free kit (Thermo Fisher), and RNA was quantitated by absorbance at 260 nm.

Quantitative RT-PCR analysis. Reverse transcription-quantitative PCR (qRT-PCR) was performed using the Power SYBR green RNA-to-CT 1-step kit (Thermo Fisher). Twenty-microliter reactions were performed in a 96-well format, and reaction mixtures contained either *rplB* primers (forward, GCTGCG CGTTCTGGGTAA; reverse, TGAACGCCCCACGGAGTT) or *uhpT* primers (forward, GTTTTATCGGCCTGCGT TAC; reverse, AGAGGAAAATGTTGGCGAAG) at 200 nM and the reaction mix diluted to 1×. RNA was then added to a final amount of 50 ng per reaction. The cycle conditions were as follows: 1 cycle at 48°C for 30 min, 1 cycle at 95°C for 10 min, and 40 cycles of 95°C for 15 s and 56°C for 1 min. Melting curves were generated by 80 cycles of 60°C for 15 s with 0.5°C increments. The results were calculated using the comparative threshold cycle (C_T) method, where relative amounts of RNA were normalized to that of the *rplB* gene (21). Changes in fluorescence were monitored using an Applied Biosystems 7500 real-time system (Thermo Fisher). Nontemplate and nonreverse transcriptase controls were included for each run. qRT-PCRs were performed in triplicates with two separate RNA preps with comparable results.

qRT-PCR with the induction of *uhpT* was performed as previously reported (7). Briefly, the cells of overnight cultures were collected and washed twice with saline. The pellets were then incubated in M9 minimal medium with or without 0.2% G6P for 30 min at 37°C. RNA extraction and analysis of qRT-PCR were performed as described above.

Mutation frequency. We performed a Luria-Delbruck fluctuation test to determine the mutation frequencies of each of the parent clinical strains (22). LB (3 ml) was inoculated with approximately 50 cells and grown overnight at 37°C. The following day, 100 μl of each culture was spread on LB agar containing 25 μg/ml of G6P with either 50 μg/ml or 200 μg/ml of fosfomycin. As a control, 100 μl of each culture was also spread on LB agar plates containing 100 μg/ml of rifampin. The resistant colonies were counted after incubating overnight at 37°C. A cell viability count was also performed to calculate the total number of cells. The mutation frequency was calculated by dividing the number of resistant cells by the total number of cells.

Accession number(s). The raw sequence reads underlying the analysis presented in the work have been deposited under accession numbers SRX2676277 to SRX2676282 and SRX2676285 to SRX2676288.

RESULTS

Frequency of mutants with reduced fosfomycin susceptibility. Of the 649 cephalosporin-resistant clinical isolates screened (495 ESBL producing and 154 AmpC or KPC producing), only 20 isolates (3.1%) generated zones of inhibition in the susceptible range but also grew nonsusceptible inner colonies surrounding the fosfomycin disks. Upon repeated disk testing, 5 of these isolates were found to consistently produce isolated colonies within the 15-mm diameter around the fosfomycin disk which defines nonsusceptibility (Fig. 1). Of the 5 patients from whom these isolates were identified, a history of treatment with fosfomycin was recorded for one patient

TABLE 1 Summary of the clinical isolates and mutants and status of the genes associated with fosfomycin resistance

Strain	Fosfomycin MIC (μg/ml)	ST	*murA*	*ptsI*	*cyaA*	*glpT*	*uhpA*	*uhpC*	*uhpT*	Growth without glucose-6-phosphate	Mutation frequency
S59	16	131								+	$\sim10^{-10}$
S60	64		Intact	Intact	Intact	Intact	Absent	Absent	Absent	−	
S61	2	405								+	$\sim10^{-8}$
S62	512		Intact	Intact	Intact	Intact	Absent	Absent	Absent	−	
S65	256	131								+	$\sim10^{-7}$
S66	>1,024		Intact	Intact	Intact	Intact	Truncated	Intact	Intact	−	
S67	2	4981								+	$\sim10^{-11}$
S68	64		Intact	Intact	Intact	Intact	Absent	Absent	Absent	−	
S69	8	624								+	$\sim10^{-11}$
S70	64		Intact	Intact	Intact	Intact	Absent	Absent	Absent	−	

who had received an oral dose 25 days prior to the isolation of strain S65. For each of these 5 strains, an inner colony closest to the fosfomycin disk was subcultured and used for subsequent investigation. These inner colony subcultures showed stable and homogenous nonsusceptibility without reverting to the susceptible phenotype. Therefore, a total of 10 strains (5 otherwise fosfomycin-susceptible clinical strains, as defined by disk diffusion testing, and the corresponding mutants with nonsusceptibility identified as inner colonies) were subjected to further investigation. The MICs of these 5 parental clinical strains ranged between 2 and 256 μg/ml, whereas the MICs of the corresponding mutant strains ranged between 64 and >1,024 μg/ml (Table 1). The wide range of agar dilution MICs observed for the parental clinical strains likely reflected the outgrowth of spontaneous nonsusceptible mutants.

MLST. Two of the 5 strains belonged to sequence type (ST) 131, which is the global epidemic multidrug-resistant (MDR) clone (23). The remaining 3 strains belonged to ST405 (founder of clonal group [CG] 405), ST4981, and ST624 (part of CG648). CG405 and CG648 are also recognized as international MDR clonal lineages (24).

Sequencing of the paired clinical strains and mutants with reduced fosfomycin susceptibility. The 10 strains were sequenced by NextSeq (Illumina), and the genomic differences between the clinical strains and their corresponding strains with reduced susceptibility were identified. There were no SNPs or insertion/deletions in *glpT*, *murA*, *ptsI*, or *cyaA*, which are genes that have been associated with fosfomycin resistance. On the other hand, 4 of the 5 mutant strains lacked the *uhpT* gene encoding hexose-6-phosphate transporter, which was present and intact in the corresponding clinical strains (Fig. 2). The first mutant (S60) had an ~13-kb deletion of *uhpA-uhpB-uhpC-uhpT-adeD-adeQ-yicN-nepI-yicS* genes. The second mutant (S62) had an ~22-kb deletion of *uhpA-uhpB-uhpC-uhpT-adeD-adeQ-yicN-nepI* genes. The fourth mutant (S68) had an ~16-kb deletion of *yidK-yidJ-yidI-yidG-yidF-emrD-tisB-istR-ivbL-ilvB-ilvN-uhpA-uhpB-uhpC-uhpT-adeD* genes. The fifth mutant (S70) had an ~10-kb deletion of *uhpA-uhpB-uhpC-uhpT-adeD* genes. *uhpT* was present and intact in the third (S66) mutant. However, S66 contained a nonsense mutation leading to a premature stop codon in *uhpA*, truncating the gene from 591 bp to 283 bp. *UhpA* is required for the expression of *uhpT in vivo* (25).

Expression of *uhpT*. The expression of *uhpT* was 12.4- to 74-fold lower in the four mutants (S60, S62, S68, and S70) missing *uhpT* in the genome than in their corresponding parental strains, likely indicating nonspecific amplification at higher cycles. S66 showed 3.7-fold lower expression of *uhpT* than the corresponding clinical strain. However, in the qRT-PCR analysis with G6P induction, *uhpT* expression from S65 was strongly induced (4,779-fold ± 2,994-fold) by G6P, whereas significantly lower induction of *uhpT* (2.2-fold ± 0.4-fold) was observed in the corresponding mutant S66 ($P = 0.002$ by an unpaired *t* test).

Mutation frequency. The mutation frequencies of the 5 clinical strains ranged from 10^{-7} to 10^{-11} when selected on 50 μg/ml fosfomycin plates containing 25 μg/ml G6P. Three *uhpT* deletion mutants had no SNP differences compared with their parental genomes, while the remaining one (S70) had a single SNP compared to the parent

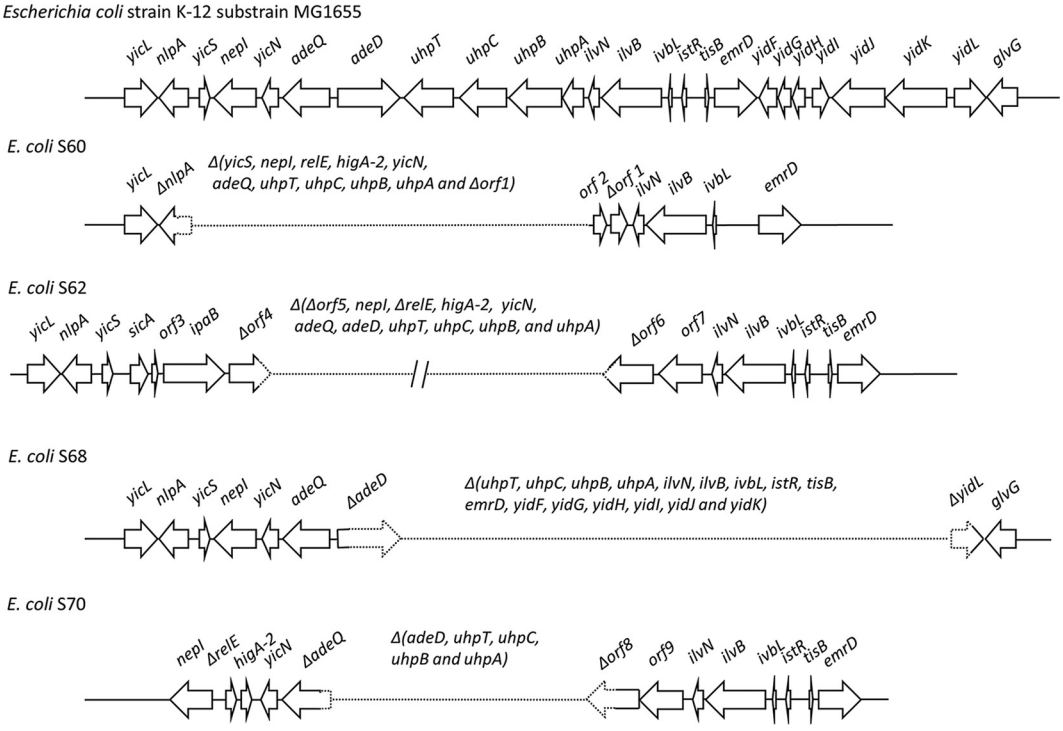

FIG 2 Genetic environment of the *uhpT* region in the 4 nonsusceptible mutants missing *uhpT*. orf 2, IS*1*-family transposase.

genome. On the other hand, the genome of the *uhpA* mutant (S66) with a mutation frequency of approximately 10^{-7} was found to have 51 SNPs compared with the parental genome (S65). Strain S65 produced abundant inner colonies approaching the fosfomycin disk (~100 colonies) upon multiple disk diffusion tests relative to the strains with *uhpT* deletions (~3 to 20 colonies in the nonsusceptible range) (Table 1, Fig. 1).

Carbohydrate utilization. All 10 strains grew on M9 minimal agar medium supplemented with 0.1% glucose (positive control), whereas none grew on M9 minimal agar medium without supplementation (negative control). All 5 clinical strains grew on M9 minimum agar medium supplemented with 0.2% G6P. However, none of the 5 fosfomycin-nonsusceptible mutants grew on this medium, confirming the lack of functional UhpT activity in these mutants.

DISCUSSION

Fosfomycin is increasingly used for the treatment of uncomplicated urinary tract infection because of the increasing resistance of *E. coli* to other oral agents (e.g., ciprofloxacin and trimethoprim-sulfamethoxazole) (3). Fosfomycin maintains *in vitro* activity against the vast majority of *E. coli* clinical strains, with reported susceptibility rates ranging between 94 and 99% (26–28). It is well acknowledged that resistance to fosfomycin can readily emerge spontaneously *in vitro* when conducting susceptibility testing (13, 29, 30). As fosfomycin use increases, the demand for susceptibility testing of this agent is expected to rise. In clinical microbiology laboratories where fosfomycin susceptibility testing is not performed routinely, the most practical methods of testing include the disk diffusion method or Etest. However, the frequency at which the spontaneous nonsusceptible mutants emerge within the zone of inhibition and the underlying mechanisms of fosfomycin nonsusceptibility in this context have not been reported.

The findings from our study put this observation into context. While inner colonies within the inhibition zone were observed in as many as 5.7% of the cephalosporin-resistant *E. coli* clinical isolates at our hospitals, those that could affect interpretation

(i.e., for susceptible-to-intermediate [minor error] or susceptible-to-resistant [major error] isolates) occurred only in 3.1% of isolates initially and in 0.8% upon repeat testing. Therefore, the occurrence of diagnostic uncertainty caused by spontaneous fosfomycin-nonsusceptible mutants that emerge in the process of disk diffusion testing appears to be relatively rare.

Fosfomycin resistance is rare in *E. coli* but can occur from decreased transport across the outer membrane, from a reduced affinity of the target enzyme MurA, or from the production of FosA-family enzymes that inactivate fosfomycin (31). Of these mechanisms, the loss of function of hexose-6-phosphate transporter UhpT or glycerol-3-phosphate transporter GlpT, through changes either in the transporter genes themselves or in their regulatory genes, is the most common pathway to fosfomycin resistance (7, 14, 21, 32–34). Changes in the phosphoenolpyruvate protein phosphotransferase I gene (*ptsI*) and adenylyl cyclase gene (*cyaA*) are also reported to impact the expression of UhpT and GlpT through the modulation of cAMP levels (31). More recently, the exact contribution of each of these genes in resistance was demonstrated by using specific single or double gene knockout strains of *E. coli* (35). In our investigation, the 5 mutants with reduced fosfomycin susceptibility either lacked the expression of hexose-6-phopshate transporter gene *uhpT* or had poor induction of *uhpT* due to a nonsense mutation in *uhpA*, which is essential for the induction of the transporter. A nonsense mutation in *uhpA* occurred in a strain that demonstrated a hypermutator phenotype and produced isolated yet abundant fosfomycin-nonsusceptible inner colonies. For all 4 strains without *uhpT* expression, large independent deletions spanning 10 to 22 kb including *uhpT* were identified by whole-genome sequencing. Therefore, for nonhypermutator *E. coli* strains, *en bloc* deletion of the genomic region containing *uhpT* and its regulatory genes appears to be the predominant pathway leading to spontaneous fosfomycin nonsusceptibility.

Fosfomycin resistance due to defective UhpT or GlpT is reported to confer high fitness cost, and strains with such resistance are known to be outcompeted by strains that are susceptible to fosfomycin (5). The European Committee on Antimicrobial Susceptibility Testing (EUCAST) now recommends ignoring isolated inner colonies within the zone of inhibition when interpreting fosfomycin susceptibility by the use of disk diffusion testing (http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_7.1_Breakpoint_Tables.pdf). We could not correlate our observations clinically, as only 1 of the 5 patients with strains producing fosfomycin-nonsusceptible inner colonies had a history of treatment with this agent. However, our data, combined with the known biological costs of this resistance mechanism, appear to support this EUCAST approach until further clinical correlations are made.

In summary, fosfomycin-nonsusceptible inner colonies that emerge upon disk diffusion susceptibility testing of *E. coli* are due to the loss of functional UhpT, but the incidence of such mutants that actually affect susceptibility interpretation is low. Coupled with the knowledge that fosfomycin-nonsusceptible *E. coli* strains due to transporter defects carry high biological costs, we suggest that *E. coli* strains that generate isolated nonsusceptible colonies within the zone of inhibition in the susceptible range can be interpreted as susceptible to fosfomycin.

ACKNOWLEDGMENTS

The work presented here was supported by research grants from the National Institutes of Health (R21AI123747 and R01AI104895 to Y.D. and U01AI124302 to V.S.C.).

REFERENCES

1. Weiner LM, Webb AK, Limbago B, Dudeck MA, Patel J, Kallen AJ, Edwards JR, Sievert DM. 2016. Antimicrobial-resistant pathogens associated with healthcare-associated infections: summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2011–2014. Infect Control Hosp Epidemiol 37:1288–1301. https://doi.org/10.1017/ice.2016.174.
2. Sastry S, Doi Y. 2016. Fosfomycin: resurgence of an old companion. J Infect Chemother 22:273–280. https://doi.org/10.1016/j.jiac.2016.01.010.
3. Gupta K, Hooton TM, Naber KG, Wullt B, Colgan R, Miller LG, Moran GJ, Nicolle LE, Raz R, Schaeffer AJ, Soper DE, Infectious Diseases Society of America, European Society for Microbiology and Infectious Diseases. 2011. International clinical practice guidelines for the treatment of acute

uncomplicated cystitis and pyelonephritis in women: a 2010 update by the Infectious Diseases Society of America and the European Society for Microbiology and Infectious Diseases. Clin Infect Dis 52:e103-20. https://doi.org/10.1093/cid/ciq257.
4. Venkateswaran PS, Wu HC. 1972. Isolation and characterization of a phosphonomycin-resistant mutant of *Escherichia coli* K-12. J Bacteriol 110:935–944.
5. Nilsson AI, Berg OG, Aspevall O, Kahlmeter G, Andersson DI. 2003. Biological costs and mechanisms of fosfomycin resistance in *Escherichia coli*. Antimicrob Agents Chemother 47:2850–2858. https://doi.org/10.1128/AAC.47.9.2850-2858.2003.
6. Takahata S, Ida T, Hiraishi T, Sakakibara S, Maebashi K, Terada S, Muratani T, Matsumoto T, Nakahama C, Tomono K. 2010. Molecular mechanisms of fosfomycin resistance in clinical isolates of *Escherichia coli*. Int J Antimicrob Agents 35:333–337. https://doi.org/10.1016/j.ijantimicag.2009.11.011.
7. Ohkoshi Y, Sato T, Suzuki Y, Yamamoto S, Shiraishi T, Ogasawara N, Yokota SI. 2017. Mechanism of reduced susceptibility to fosfomycin in *Escherichia coli* clinical isolates. Biomed Res Int 2017:5470241. https://doi.org/10.1155/2017/5470241.
8. Karageorgopoulos DE, Wang R, Yu XH, Falagas ME. 2012. Fosfomycin: evaluation of the published evidence on the emergence of antimicrobial resistance in Gram-negative pathogens. J Antimicrob Chemother 67:255–268. https://doi.org/10.1093/jac/dkr466.
9. Wachino J, Yamane K, Suzuki S, Kimura K, Arakawa Y. 2010. Prevalence of fosfomycin resistance among CTX-M-producing *Escherichia coli* clinical isolates in Japan and identification of novel plasmid-mediated fosfomycin-modifying enzymes. Antimicrob Agents Chemother 54:3061–3064. https://doi.org/10.1128/AAC.01834-09.
10. Guo Q, Tomich AD, McElheny CL, Cooper VS, Stoesser N, Wang M, Sluis-Cremer N, Doi Y. 2016. Glutathione-*S*-transferase FosA6 of *Klebsiella pneumoniae* origin conferring fosfomycin resistance in ESBL-producing *Escherichia coli*. J Antimicrob Chemother 71:2460–2465. https://doi.org/10.1093/jac/dkw177.
11. Barry AL, Fuchs PC. 1991. *In vitro* susceptibility testing procedures for fosfomycin tromethamine. Antimicrob Agents Chemother 35:1235–1238. https://doi.org/10.1128/AAC.35.6.1235.
12. Clinical and Laboratory Standards Institute. 2016. Performance standards for antimicrobial susceptibility testing, 26th ed. M100-S26. Clinical and Laboratory Standards Institute, Wayne, PA.
13. Kaase M, Szabados F, Anders A, Gatermann SG. 2014. Fosfomycin susceptibility in carbapenem-resistant *Enterobacteriaceae* from Germany. J Clin Microbiol 52:1893–1897. https://doi.org/10.1128/JCM.03484-13.
14. Pasteran F, Lucero C, Rapoport M, Guerriero L, Barreiro I, Albornoz E, Veliz O, Corso A. 2012. Tigecycline and intravenous fosfomycin zone breakpoints equivalent to the EUCAST MIC criteria for *Enterobacteriaceae*. J Infect Dev Ctries 6:452–456. https://doi.org/10.3855/jidc.2238.
15. Clinical and Laboratory Standards Institute. 2015. Performance standards for antimicrobial disk susceptibility tests; approved standard, 12th ed. M02-A12. Clinical and Laboratory Standards Institute, Wayne, PA.
16. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, Lesin VM, Nikolenko SI, Pham S, Prjibelski AD, Pyshkin AV, Sirotkin AV, Vyahhi N, Tesler G, Alekseyev MA, Pevzner PA. 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol 19:455–477. https://doi.org/10.1089/cmb.2012.0021.
17. Seemann T. 2014. Prokka: rapid prokaryotic genome annotation. Bioinformatics 30:2068–2069. https://doi.org/10.1093/bioinformatics/btu153.
18. Inouye M, Dashnow H, Raven LA, Schultz MB, Pope BJ, Tomita T, Zobel J, Holt KE. 2014. SRST2: rapid genomic surveillance for public health and hospital microbiology labs. Genome Med 6:90. https://doi.org/10.1186/s13073-014-0090-6.
19. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R, 1000 Genome Project Data Processing Subgroup. 2009. The sequence alignment/map format and SAMtools. Bioinformatics 25:2078–2079. https://doi.org/10.1093/bioinformatics/btp352.
20. Barrick JE, Colburn G, Deatherage DE, Traverse CC, Strand MD, Borges JJ, Knoester DB, Reba A, Meyer AG. 2014. Identifying structural variation in haploid microbial genomes from short-read resequencing data using breseq. BMC Genomics 15:1039. https://doi.org/10.1186/1471-2164-15-1039.
21. Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25:402–408. https://doi.org/10.1006/meth.2001.1262.
22. Luria SE, Delbruck M. 1943. Mutations of bacteria from virus sensitivity to virus resistance. Genetics 28:491–511.
23. Mathers AJ, Peirano G, Pitout JD. 2015. *Escherichia coli* ST131: the quintessential example of an international multiresistant high-risk clone. Adv Appl Microbiol 90:109–154. https://doi.org/10.1016/bs.aambs.2014.09.002.
24. Pitout JD. 2012. Extraintestinal pathogenic *Escherichia coli*: a combination of virulence with antibiotic resistance. Front Microbiol 3:9. https://doi.org/10.3389/fmicb.2012.00009.
25. Wright JS, Olekhnovich IN, Touchie G, Kadner RJ. 2000. The histidine kinase domain of UhpB inhibits UhpA action at the *Escherichia coli uhpT* promoter. J Bacteriol 182:6279–6286. https://doi.org/10.1128/JB.182.22.6279-6286.2000.
26. de Cueto M, Lopez L, Hernandez JR, Morillo C, Pascual A. 2006. *In vitro* activity of fosfomycin against extended-spectrum-β-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae*: comparison of susceptibility testing procedures. Antimicrob Agents Chemother 50:368–370. https://doi.org/10.1128/AAC.50.1.368-370.2006.
27. Lai B, Zheng B, Li Y, Zhu S, Tong Z. 2014. *In vitro* susceptibility of *Escherichia coli* strains isolated from urine samples obtained in mainland China to fosfomycin trometamol and other antibiotics: a 9-year surveillance study (2004–2012). BMC Infect Dis 14:66. https://doi.org/10.1186/1471-2334-14-66.
28. Johnson JR, Drawz SM, Porter S, Kuskowski MA. 2013. Susceptibility to alternative oral antimicrobial agents in relation to sequence type ST131 status and coresistance phenotype among recent *Escherichia coli* isolates from U.S. veterans. Antimicrob Agents Chemother 57:4856–4860. https://doi.org/10.1128/AAC.00650-13.
29. Tsuruoka T, Yamada Y. 1975. Charactertization of spontaneous fosfomycin (phosphonomycin)-resistant cells of *Escherichia coli* B *in vitro*. J Antibiot (Tokyo) 28:906–911. https://doi.org/10.7164/antibiotics.28.906.
30. Ballestero-Tellez M, Docobo-Perez F, Rodriguez-Martinez JM, Conejo MC, Ramos-Guelfo MS, Blazquez J, Rodriguez-Bano J, Pascual A. 2017. Role of inoculum and mutant frequency on fosfomycin MIC discrepancies by agar dilution and broth microdilution methods in *Enterobacteriaceae*. Clin Microbiol Infect 23:325–331. https://doi.org/10.1016/j.cmi.2016.12.022.
31. Castañeda-García A, Blázquez J, Rodríguez-Rojas R. 2013. Molecular mechanisms and clinical impact of acquired and intrinsic fosfomycin resistance. Antibiotics 2:217–236. https://doi.org/10.3390/antibiotics2020217.
32. Oteo J, Orden B, Bautista V, Cuevas O, Arroyo M, Martinez-Ruiz R, Perez-Vazquez M, Alcaraz M, Garcia-Cobos S, Campos J. 2009. CTX-M-15-producing urinary *Escherichia coli* O25b-ST131-phylogroup B2 has acquired resistance to fosfomycin. J Antimicrob Chemother 64:712–717. https://doi.org/10.1093/jac/dkp288.
33. Li Y, Zheng B, Li Y, Zhu S, Xue F, Liu J. 2015. Antimicrobial susceptibility and molecular mechanisms of fosfomycin resistance in clinical *Escherichia coli* isolates in mainland China. PLoS One 10:e0135269. https://doi.org/10.1371/journal.pone.0135269.
34. Tseng SP, Wang SF, Kuo CY, Huang JW, Hung WC, Ke GM, Lu PL. 2015. Characterization of fosfomycin resistant extended-spectrum β-lactamase-producing *Escherichia coli* isolates from human and pig in Taiwan. PLoS One 10:e0135864. https://doi.org/10.1371/journal.pone.0135864.
35. Ballestero-Tellez M, Docobo-Perez F, Portillo-Calderon I, Rodriguez-Martinez JM, Racero L, Ramos-Guelfo MS, Blazquez J, Rodriguez-Bano J, Pascual A. 2017. Molecular insights into fosfomycin resistance in *Escherichia coli*. J Antimicrob Chemother 72:1303–1309. https://doi.org/10.1093/jac/dkw573.