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MECHANISMS OF RESISTANCE

Urinary Tract Conditions Affect Fosfomycin Activity against *Escherichia coli* Strains Harboring Chromosomal Mutations Involved in Fosfomycin Uptake

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ABSTRACT The steps by which *Escherichia coli* strains harboring mutations related to fosfomycin (FOS) resistance arise and spread during urinary tract infections (UTIs) are far from being understood. The aim of this study was to evaluate the effects of urine, pH, and anaerobiosis on FOS activity against a set of isogenic strains carrying the most prevalent chromosomal mutations conferring FOS resistance (Δ*uhpT*, Δ*glpT*, Δ*cyaA*, and Δ*ptsI*), either singly or in combination. We also studied fosfomycin-resistant *E. coli* clinical isolates from patients with UTI. Our results demonstrate that urinary tract physiological conditions might have a profound impact on FOS activity against strains with chromosomal FOS resistance mutations. Specifically, acidic pH values and anaerobiosis convert most of the strains categorized as resistant to fosfomycin according to the international guidelines to a susceptible status. Therefore, urinary pH values may have practical interest in the management of UTIs. Finally, our results, together with the high fitness cost associated with FOS resistance mutations, might explain the low prevalence of fosfomycin-resistant *E. coli* variants in UTIs.

KEYWORDS fosfomycin activity, fosfomycin resistance, chromosomal mutations, UTI, *Escherichia coli*, urinary tract infection

Fosfomycin (FOS) is a phosphonic acid derivative produced by a broad variety of *Streptomyces* and *Pseudomonas* species (1). Since the discovery of FOS in 1969 (2), this natural antibiotic has attracted considerable clinical and scientific interest due to its broad-spectrum bactericidal activity against Gram-positive and Gram-negative bacteria (3–5). FOS distributes adequately to different tissues, such as the bladder, kidneys, lungs, bones, cerebrospinal fluid, and heart valves (6–10). Moreover, given the worrisome increase of multidrug-resistant pathogens (11), FOS has been reconsidered as a treatment option in numerous clinical guidelines and trials for the treatment of a wide range of infections (4, 12–15).

FOS has been used widely as a first-line agent for the empirical treatment of uncomplicated urinary tract infections (UTIs) (5). In *Escherichia coli*, the most prevalent causative organism of UTI (16, 17), FOS is actively transported into the bacterial cytoplasm via glycerol-3-phospate (GlpT) and glucose-6-phosphate (G6P) (UhpT) transporters (18). Because the presence of G6P acts as an inducer of the UhpT transporter, FOS susceptibility testing is performed in the presence of G6P to induce FOS suscep-

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tibility. Once FOS has reached the cytoplasm, it acts as a phosphoenolpyruvate (PEP) analogue, binding to MurA (UDP-*N*-acetylglucosamine enolpyruvyl transferase) covalently, preventing the formation of UDP-*N*-acetylglucosamine-3-enolpyruvate from PEP and *N*-acetylglucosamine (18, 19), and thus interfering with the first step of bacterial cell wall biosynthesis.

FOS resistance determinants are either chromosomal or plasmid mediated (1, 5). Chromosomally mediated FOS resistance can be achieved by reducing permeability to FOS through mutations in genes encoding the GlpT and UhpT transporters or their regulators. Permeability can also be reduced by mutations in the *cyaA* and/or *ptsI* genes, which control the intracellular cAMP levels necessary for activation of FOS transporters (20, 21). Regarding the FOS target, mutations in *murA* that decrease the affinity of MurA for FOS also reduce susceptibility (22). In addition, overexpression of *murA* has also been related to FOS resistance (23). However, few reports of clinical isolates have shown mutations in the *murA* gene, and none in the catalytic site of MurA, because most of them drastically reduce bacterial cell viability (24).

Ballestero-Téllez and colleagues (25) recently demonstrated that the presence of single chromosomal mutations producing loss of function, as well as some of their combinations, confer low-level FOS resistance (LLFR) but not clinical resistance according to international guidelines. Although the presence of LLFR mutations yields an FOS-susceptible phenotype, they may act as gateways for highly resistant subpopulations by the selection of additional LLFR mutations (25, 26).

Despite the increased use of FOS for treatment of UTIs, the prevalence of clinical isolates with low- and high-level FOS resistance is still very low (27, 28). In particular, the low prevalence of strains harboring chromosomal mutations conferring FOS resistance has been attributed to the high biological cost, entailing a reduced fitness that compromises competition with the normal microbiota in the human host (29). The effect of FOS resistance mutations on fitness is particularly interesting in the case of uropathogenic *E. coli* (UPEC), because if the cost is high, the resistant bacteria will not grow at the minimal rate needed to establish infection (30, 31). However, FOS-resistant clinical isolates containing mutations in the above-cited chromosomal genes have been described (30, 32, 33). Moreover, because fitness costs can easily be ameliorated by compensatory mutations, as shown for other antibiotic resistance genes (34), additional explanations for the low prevalence of FOS-resistant strains can be invoked.

Recently, we showed that low-level-quinolone-resistant (LLQR) *E. coli* strains are already resistant to high concentrations of ciprofloxacin (CIP) under urinary tract conditions, including the presence of urine, urinary pH, and anaerobiosis (35, 36). The bladder environment is mainly anaerobic, with a concentration of dissolved oxygen (DO) in urine of about 4.2 ppm; the concentration is also variable and mainly reflects the renal metabolic state (37). Moreover, in patients with urinary infections, the urine DO concentration is significantly reduced as a result of oxygen consumption by the microbes (37). However, although the molecular mechanisms of chromosomal FOS resistance and their effects on bacterial fitness are relatively well known, there is a paucity of information about the impact of the urinary tract environment on the antimicrobial activity of FOS against strains harboring chromosomal FOS resistance mutations.

Given the above information, the main objective of this study was to evaluate the impact of the urinary tract environment on the antimicrobial activity of FOS against a set of well-characterized isogenic strains harboring the most frequent chromosomal FOS resistance mutations. A series of FOS-resistant *E. coli* clinical strains isolated from patients with UTI was also studied.

RESULTS

Effects of urine and pH on fitness of *E. coli* isogenic strains. Figure 1 shows maximal growth rates per hour. Concerning growth in Mueller-Hinton (MH) broth, only the Δ*cyaA* Δ*glpT* and Δ*ptsI* Δ*cyaA* strains showed significant reductions in the maximal growth rate compared to that of BW25113. Notably, all of the strains with single and

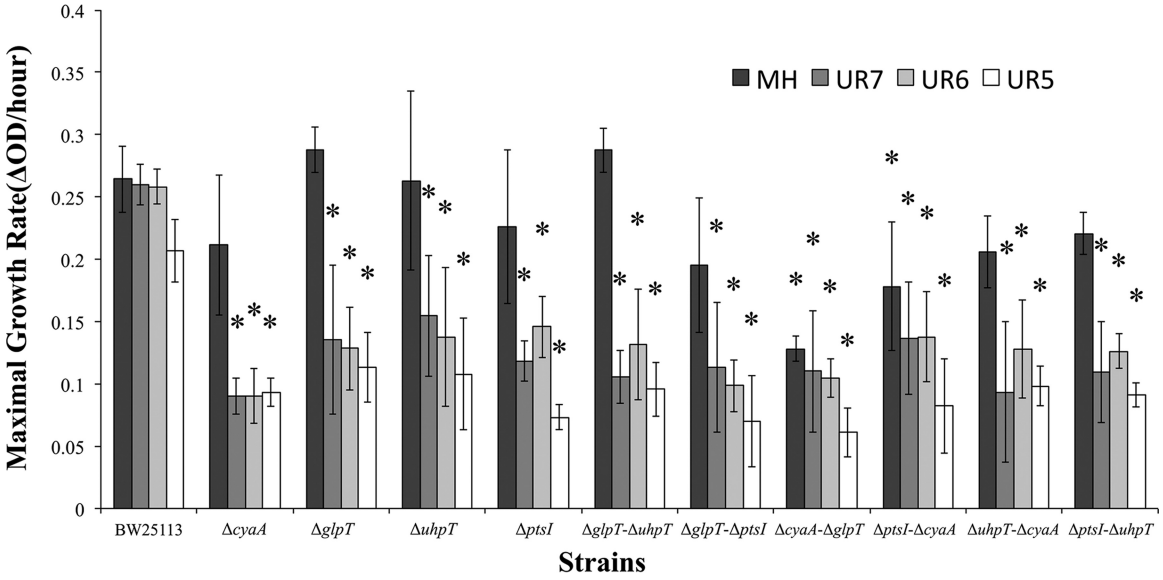

FIG 1 *In vitro* maximal growth rates (ΔOD per hour) for strain BW25113 and 10 isogenic LLFR and FOS-resistant strains in MH broth and urine at different pH values. Error bars represent interquartile ranges. Asterisks denote statistically significant differences ($P < 0.05$ [Mann-Whitney U test]) between the indicated strains and wild-type strain BW25113 under the same conditions.

double deletions showed statistically significant decreases in maximal growth rates in urine at different pH values compared to those for strain BW25113.

Effects of pH and anaerobiosis on FOS activity. Table 1 shows that under standard conditions (MH broth, pH 7.4, aerobic), all strains harboring double deletions were fully resistant to FOS according to EUCAST criteria (37). Among the single mutants, only the Δ*uhpT* strain presented a MIC over the cutoff value.

A remarkable impact of pH on FOS activity was observed. When susceptibility tests were performed at pH 8, all strains showed 2- to 16-fold MIC increases. However, at acidic pHs, most of the MIC values fell below the susceptibility cutoff. At pH 6, only the Δ*glpT* Δ*uhpT*, Δ*uhpT* Δ*cyaA*, and Δ*ptsI* Δ*uhpT* strains remained resistant. This effect was even more evident at pH 5, demonstrating that low pH values lead to higher FOS activity against *E. coli*.

Regarding the effect of anaerobiosis, Table 1 (nO_2 columns) shows that growth under anaerobiosis increased the effect of FOS at all pH values tested, with 2- to

TABLE 1 MICs of fosfomycin with G6P (25 μg/ml) against isogenic *E. coli* strains in MH broth at different pH values[a]

Strain	FOS MIC (μg/ml) in MH broth at pH:							
	8		7.4		6		5	
	O_2	nO_2	O_2	nO_2	O_2	nO_2	O_2	nO_2
ATCC 25922	8	1	2	1	1	0.5	1	0.5
BW25113	4	2	2	1	1	1	2	1
Δ*cyaA*	**64**	16	16	16	8	16	16	16
Δ*glpT*	32	8	2	1	1	1	0.5	1
Δ*uhpT*	**128**	16	**64**	16	16	8	16	16
Δ*ptsI*	8	2	4	2	4	1	4	2
Δ*glpT* Δ*uhpT*	**512**	**256**	**256**	**64**	**128**	**64**	32	32
Δ*glpT* Δ*ptsI*	**512**	**64**	**128**	**64**	32	16	16	16
Δ*cyaA* Δ*glpT*	**1,024**	**128**	**128**	32	32	16	16	16
Δ*ptsI* Δ*cyaA*	**1,024**	**128**	**128**	32	32	32	16	32
Δ*uhpT* Δ*cyaA*	**1,024**	**128**	**512**	**128**	**128**	**64**	**64**	32
Δ*ptsI* Δ*uhpT*	**1,024**	8	**128**	8	**128**	8	**128**	8

[a]MIC values indicating resistance (according to EUCAST guidelines) are shown in bold. O_2, aerobic conditions; nO_2, anaerobic conditions.

TABLE 2 MICs of fosfomycin with G6P (25 μg/ml) against isogenic *E. coli* strains in MH broth and urine at different pH values[a]

Strain	FOS MIC (μg/ml)							
	MH broth at pH 7.4		Urine at pH 7		Urine at pH 6		Urine at pH 5	
	O_2	nO_2	O_2	nO_2	O_2	nO_2	O_2	nO_2
ATCC 25922	2	1	4	2	1	1	2	1
BW25113	2	1	4	2	2	1	2	2
Δ*cyaA*	16	16	32	16	32	16	32	16
Δ*glpT*	2	1	16	2	8	4	1	1
Δ*uhpT*	**64**	16	**64**	16	32	8	16	2
Δ*ptsI*	4	2	**64**	32	32	32	32	32
Δ*glpT* Δ*uhpT*	**256**	**64**	**128**	**64**	**64**	32	8	8
Δ*glpT* Δ*ptsI*	**128**	**64**	32	8	16	8	16	4
Δ*cyaA* Δ*glpT*	**128**	32	**64**	**64**	**64**	**64**	**64**	**64**
Δ*ptsI* Δ*cyaA*	**128**	32	**128**	**64**	**64**	32	32	32
Δ*uhpT* Δ*cyaA*	**512**	**128**	**256**	**128**	**128**	**128**	**128**	**64**
Δ*ptsI* Δ*uhpT*	**128**	8	**256**	32	**256**	32	**64**	8

[a]MIC values indicating resistance (according to EUCAST guidelines) are shown in bold. O_2, aerobic conditions; nO_2, anaerobic conditions.

128-fold reductions of MIC values compared to those under aerobic conditions. At pH 7.4 and with anaerobiosis, only three mutants (Δ*glpT* Δ*uhpT*, Δ*glpT* Δ*ptsI*, and Δ*uhpT* Δ*cyaA*) remained resistant, with MICs just slightly over the susceptibility breakpoint. Further decreases of pH reduced the number of resistant strains, abolishing resistance completely at pH 5. Therefore, the combination of acidification and anaerobiosis increases susceptibility to FOS, even in strains with high resistance levels.

MIC determinations under urinary physiological conditions. Some MIC changes were observed in urine at pH 7 under aerobic conditions compared to the MICs in MH broth at pH 7.4 (Table 2). Most of the strains showed minor MIC variations, but two single variants (Δ*glpT* and Δ*ptsI*) displayed significant MIC increases (8-fold and 16-fold, respectively).

As previously observed in MH broth, acidification and anaerobic conditions increased the activity of FOS in the presence of urine. This activity was maximal at pH 5, with the notable exception of that of the Δ*cyaA* and Δ*ptsI* single variants. Both strains maintained elevated MICs, though they were below the cutoff value for resistance. Also, the Δ*cyaA* Δ*glpT* and Δ*uhpT* Δ*cyaA* double mutant strains remained resistant, but with a MIC value (64 μg/ml) very close to the cutoff according to EUCAST. UTI conditions did not affect the activity of FOS against strains containing particular mutations. For instance, the susceptibility of strains harboring the Δ*cyaA* mutation (alone or combined) was poorly affected by the tested conditions.

The combination of urine, acidic pH, and anaerobiosis had a large effect on FOS activity, making most strains susceptible at pH 5 according to the international guidelines.

Effects of urine, pH, and anaerobiosis on FOS activity against *E. coli* clinical isolates. To analyze the possibility that the effects observed were specific to strain BW25113, we evaluated the effects of urinary tract physiological conditions on fosfomycin-resistant *E. coli* clinical isolates from patients with UTI. Five FOS-resistant isolates were found among 404 UTI isolates (1.2%). The phenotypic and genotypic characteristics of these strains are shown in Table 3. As shown in Table 4, urine increased the MIC at neutral pH in all cases. Notably, acidification and anaerobiosis increased FOS activity in three of the five resistant strains (ECF33, ECF168, and ECF318). Interestingly, these are the only strains exhibiting mutations in the *uhpT* gene (ECF33 and ECF318) or the UhpT regulator genes *uhpA*, *uhpB*, and *uhpC* (ECF168 and ECF318).

DISCUSSION

FOS resistance has previously been related to a high biological cost, entailing reduced fitness, which compromises the competition with the normal microbiota in the

TABLE 3 Characteristics of fosfomycin-resistant *E. coli* clinical strains

Strain	MIC (μg/ml)		Amino acid substitution(s) encoded in[a]:								
	Microdilution	Etest	*murA*	*glpT*	*uhpA*	*uhpB*	*uhpC*	*uhpT*	*ptsI*	*cyaA*	*crp*
ECF33	512	>256	ND	Deletion	ND	ND	ND	Deletion	K367R	ND	ND
ECF145	256	64	ND	Deletion	ND	ND	ND	ND	K367R	ND	ND
ECF168	64	64	ND	F297L, N348T, Q443E, E444Q	P79S	A205D	Y18H, G282D, T435A	ND	K367R	S142N, deletion	ND
ECF318	256	>256	ND	C99G, F297L, Q443E, E444Q, K448E	P79S	ND	I14M, Q17Y	Q351E	K367R	S142N, E349A, T352S, K356S, E359G, D362E	ND
ECF330	256	>256	ND	K448E	ND	ND	ND	ND	K367R	S142N, E349A, T352S, K356S, E359G, D362E	ND

[a]ND, not detected.

human host (29). The effect of FOS resistance mutations on fitness is of particular interest in the case of UPEC strains, because if the cost is sufficiently high, resistant bacteria will not grow at the minimal rate needed to establish infection (30). We evaluated the impacts of urinary physiological conditions on the growth of isogenic strains displaying low and high levels of resistance to FOS and carrying the most prevalent FOS chromosomal mutations, singly and in combination, and we compared the results obtained to those for strains grown in MH broth. Interestingly, all the isogenic strains showed significant reductions in the maximal growth rate in urine compared to that of wild-type strain BW25113. Thus, the low prevalence of FOS-resistant strains can be explained in part by the high biological cost that these mutations promote under urinary tract conditions in the absence of FOS, confirming previous results (28). In this way, mutations related to the *cyaA* and *ptsI* genes lead to lower levels of cAMP, reducing UhpT and GlpT channel expression, pilus biosynthesis, or virulence factors (28). Concerning the presence of double deletions, the Δ*cyaA* Δ*glpT* and Δ*ptsI* Δ*cyaA* strains showed the highest reductions in the maximal growth rate in MH broth.

We previously showed that FOS MIC determination may not be an accurate predictor of FOS efficacy (25, 38). Susceptibility testing gives a measure of growth inhibition (MIC) under specific *in vitro* conditions. However, its clinical usefulness requires the extrapolation of these MIC values into a prediction of clinical outcome (39). MH broth is the medium of choice for susceptibility testing of commonly isolated aerobic or facultative organisms (40). This medium shows acceptable batch-to-batch reproducibility and a low concentration of inhibitors, with a stable pH value of 7.2 to 7.4, supporting satisfactory growth of most common pathogens. Nevertheless, the scenario in which antibiotics must act during UTI treatment is quite different. In the urinary tract, the presence of urine, low pH values, and an anaerobic atmosphere has been related to modulation of antibiotic effectiveness (35, 41, 42). Therefore, the activity of FOS under laboratory and UTI conditions is expected to be different.

Our results confirm that low pH values increase FOS activity (41, 43). In an acidic

TABLE 4 MICs of fosfomycin with G6P (25 μg/ml) against *E. coli* clinical strains in MH broth and urine at different pH values[a]

Strain	FOS MIC (μg/ml)							
	MH broth at pH 7.4		Urine at pH 7		Urine at pH 6		Urine at pH 5	
	O_2	nO_2	O_2	nO_2	O_2	nO_2	O_2	nO_2
ECF33	**512**	**64**	**512**	**256**	64	**128**	**64**	16
ECF145	**256**	**256**	**512**	**1,024**	**512**	**512**	**512**	**512**
ECF168	**64**	**64**	**128**	16	32	8	16	8
ECF318	**256**	**64**	**512**	**128**	**64**	32	32	16
ECF330	**256**	**128**	**1,024**	**1,024**	**512**	**512**	**512**	**128**

[a]MIC values indicating resistance (according to EUCAST guidelines) are shown in bold. O_2, aerobic conditions; nO_2, anaerobic conditions.

urine (pH values of 5 to 6), FOS is partially protonated and in a more lipophilic state, allowing FOS to enter bacteria and resulting in higher antimicrobial activity (43). As we previously demonstrated, the urine pH for patients with UTI caused by *E. coli* is mainly acidic (85.38% of patients have a urine pH of ≤6.5) (35). Therefore, urine physiological pH values may improve the effect of FOS in these patients. Additionally, growth under anaerobic conditions has also been related to increased antibacterial activity of FOS due to elevated expression of GlpT and UhpT after activation of FNR, leading to increased FOS uptake (44). The results obtained in our study support this finding, with 2- to 32-fold MIC reductions observed under anaerobic conditions. However, the effect of urine on FOS activity is weak. These data agree with those of Bergogne-Bérézin et al., who showed that urine slightly decreases the *in vitro* susceptibility to FOS (45).

The observed effect is not exclusive to strain BW25113 and its derivatives, as urine acidification and anaerobiosis increased FOS activity against three of the five FOS-resistant clinical strains tested. Interestingly, the three strains had mutations or deletions in the *uhpT* gene or in genes related to UhpT expression.

Despite the increased use of FOS for treatment of UTIs, there is a very low prevalence of FOS-resistant *E. coli* strains, particularly those harboring chromosomal mutations conferring FOS resistance (27, 28). Overall, our results demonstrate that urinary tract physiological conditions might have a profound impact on FOS activity, specifically against strains with common FOS resistance mutations. Here we have shown that urine acidification and anaerobiosis increase FOS activity on *E. coli* strains with low- and high-level FOS resistance due to mutations in chromosomal genes, adding an additional explanation for the low prevalence of FOS-resistant *E. coli* variants in UTIs.

The existence of strains harboring chromosomal FOS resistance mutations isolated from patients with UTI nevertheless suggests that mutations that compensate for the cost of FOS resistance are being selected in clinical settings. Also, clinical features may play an important role in the survival and selection of FOS-resistant mutant strains. For instance, pregnant women have an increased glomerular filtration rate and higher urinary calcium excretion throughout pregnancy, with higher urine pH values in the second and third trimesters (46). Thiazide diuretic intake is also associated with a higher urine pH by reducing urinary uric acid excretion (47). Furthermore, there are genetic disorders that are related to urine alkalization. Gitelman syndrome is an autosomal recessive disorder of the thiazide-sensitive sodium chloride cotransporter, expressed at the distal convoluted tubule, which is accompanied by an inappropriately high urine pH (48). Thus, there are cases in which an alkaline pH of urine can partially compromise the activity of FOS and select for mutations conferring low-level FOS resistance. Further work needs to be done to understand the mechanisms by which strains with FOS resistance mutations are selected during treatment of UTI with FOS. Experiments with an animal model of UTI are being performed in order to establish the *in vivo* correlation with the *in vitro* results.

The effect of urinary tract conditions on FOS activity is just the opposite of that on ciprofloxacin, i.e., ciprofloxacin activity decreases with acidity and anaerobiosis (35), making it possible to choose between two alternative treatments. Thus, urinary pH values may have practical interest in the management of UTIs.

MATERIALS AND METHODS

Strains and culture. *E. coli* BW25113 and 10 isogenic strains, constructed by Ballesteros-Téllez et al. (25) and carrying the most prevalent FOS chromosomal mutations (Δ*glpT*, Δ*uhpT*, Δ*cyaA*, and Δ*ptsI*), singly and in combination, were studied. *E. coli* ATCC 25922 was also used as a control strain for susceptibility assays. Additionally, five FOS-resistant *E. coli* strains isolated from patients with UTI were included.

From March to May 2016, 404 UPEC strains from patients attending the Virgen del Rocío University Hospital were selected using a systematic random sampling. Bacterial isolates were identified to the species level by use of matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS), and antibiotic susceptibility was determined using a MicroScan WalkAway Plus system (Siemens Healthcare Diagnostics, West Sacramento, CA). For all the strains that showed resistance to FOS according to EUCAST criteria (49), Etest (Liofilchem, Italy) was performed in order to verify this pheno-

type. Strains categorized as resistant by microdilution and Etest were included in this study. The study was approved by the ethical committee of the Hospital Universitario Virgen del Rocío.

Mueller-Hinton (MH) broth (Conda Pronadisa, Spain) at pH 7.4 was used as a control medium. Urine obtained from 5 healthy volunteers who had not received antibiotic treatment during the previous 6 months was used as culture medium. Urine samples were pooled and sterilized by filtration through 0.22-mm-pore-size filters (polyethersulfone [PES] membrane) (VWR, United Kingdom) and stored at −20°C. Before sample use, the urine pH was adjusted to values of 5.0, 6.0, and 7.0 with HCl or NaOH (Sigma-Aldrich, Spain), and samples were sterilized again by filtration. Sterility was verified by incubating aliquots of each sample at 37°C for 24 h.

Growth rate measurements. *In vitro* growth rates were determined for *E. coli* BW25113 and mutant derivatives, as follows. Overnight cultures were diluted 1:100 in MH broth and cultured at 37°C and 180 rpm for 2 h to reach the exponential growth phase. Approximately 10^5 cells were then inoculated into fresh medium (MH broth or urine at pH values of 5, 6, and 7.4). Plates were incubated on an automated microplate reader (Infinite M200; Tecan, Männedorf, Switzerland) at 37°C for 24 h, and the absorbance at 595 nm for each well was measured every 30 min after strong shaking. Growth assays were performed on three different days (five replicates per day), using clear, flat-bottomed 96-well plates (Nunc, Roskilde, Denmark) containing 100 μl of either pH-adjusted urine or MH broth. To determine the maximal growth rate (50), the difference between every two consecutive optical density (OD) values was calculated for the exponential growth phase, using a total of 15 replicates per strain. The median of the 15 highest ΔOD values was calculated for each strain, corresponding to the maximal growth rate. Statistical differences between mutants and wild-type BW25113 were calculated using the Mann-Whitney U test.

Susceptibility testing. MICs of FOS were determined in triplicate by using broth microdilution methods according to EUCAST guidelines (49). Also, gradient MIC strip experiments (MIC test strips [Liofilchem, Italy] supplemented with G6P) were performed on MH agar, and plates were incubated under aerobic and anaerobic conditions (Aanerogen; Oxoid) for 24 h at 37°C. To evaluate the effect of pH, MICs were determined in MH broth at pH 5, 6, 7.4, and 8. To reproduce UTI physiological conditions, MICs of FOS were also measured in urine at pH values of 5, 6, and 7 (pH 8 could not be tested in urine because massive precipitation of salts precluded bacterial growth detection). Plates were incubated for 24 h under aerobic and anaerobic conditions at 37°C.

Molecular detection of FOS resistance genes. Genes conferring resistant to fosfomycin (*murA*, *glpT*, *uhpA*, *uhpB*, *uhpC*, *uhpT*, *uhpA*, *ptsI*, *cyaA*, and *crp*) were amplified by PCR and then sequenced for the six FOS-resistant clinical isolates. FOS-susceptible *E. coli* strain BW25113 was used as a control for PCR and sequencing. Primers used in this study were those from our previous work (23).

Statistics. All statistical analyses were carried out using R software (Free Software Foundation's GNU General Public License), specifically the R commander package (R, version 3.3.3). Differences in maximal growth rates were determined using the Mann-Whitney nonparametric method. *P* values of ≤0.05 were considered statistically significant.

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