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Elevated Expression of GlpT and UhpT via FNR Activation Contributes to Increased Fosfomycin Susceptibility in *Escherichia coli* under Anaerobic Conditions

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Because a shortage of new antimicrobial agents is a critical issue at present, and with the spread of multidrug-resistant (MDR) pathogens, the use of fosfomycin to treat infections is being revisited as a "last-resort option." This drug offers a particular benefit in that it is more effective against bacteria growing under oxygen-limited conditions, unlike other commonly used antimicrobials, such as fluoroquinolones and aminoglycosides. In this study, we showed that *Escherichia coli* strains, including enterohemorrhagic *E. coli* (EHEC), were more susceptible to fosfomycin when grown anaerobically than when grown aerobically, and we investigated how the activity of this drug was enhanced during anaerobic growth of *E. coli*. Our quantitative PCR analysis and a transport assay showed that *E. coli* cells grown under anaerobic conditions had higher levels of expression of *glpT* and *uhpT*, encoding proteins that transport fosfomycin into cells with their native substrates, i.e., glycerol-3-phosphate and glucose-6-phosphate, and led to increased intracellular accumulation of the drug. Elevation of expression of these genes during anaerobic growth requires FNR, a global transcriptional regulator that is activated under anaerobic conditions. Purified FNR bound to DNA fragments from regions upstream of *glpT* and *uhpT*, suggesting that it is an activator of expression of *glpT* and *uhpT* during anaerobic growth. We concluded that the increased antibacterial activity of fosfomycin toward *E. coli* under anaerobic conditions can be attributed to elevated expression of GlpT and UhpT following activation of FNR, leading to increased uptake of the drug.

In addition to pathogens acquiring resistance to antimicrobial agents by genetic modifications, a reduction of the antimicrobial activity of drugs associated with environmental changes is a potential risk factor that impairs antimicrobial chemotherapy. Bacteria often encounter oxygen-limited situations during infection of the host. In many cases, the antibacterial activities of drugs, including fluoroquinolones and aminoglycosides, decrease when oxygen is depleted (1–4).

Fosfomycin recently regained attention as an antibiotic that could be effective against multidrug-resistant (MDR) pathogens, such as extended-spectrum β-lactamase (ESBL) producers, because there is no structural relationship between the drug and other commonly used antimicrobials, including fluoroquinolones, β-lactams, and aminoglycosides (5, 6). Fosfomycin is also used to decrease the risk of development of hemolytic-uremic syndrome (HUS), a fatal infectious disease caused by enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 (7, 8). As another great benefit, this drug is more effective under anaerobic conditions (9, 10). However, the reason for this increased effectiveness is poorly understood.

Fosfomycin is transported via GlpT and UhpT, which are glycerol-3-phosphate and glucose-6-phosphate symporters, respectively, and then inhibits MurA activity, which transfers phosphoenolpyruvate (PEP) to the 3′-hydroxyl group of UDP-*N*-acetylglucosamine in the initial step of bacterial cell wall biosynthesis (11–13). Susceptibility to fosfomycin can be affected by expression of the genes encoding GlpT, UhpT, and MurA. In some *E. coli* studies, mutations in genes encoding the positive regulators of *uhpT* expression, UhpA and CyaA, conferred resistance because the mutants had reduced uptake of fosfomycin (14, 15). Other studies showed that a clinical isolate resistant to fosfomycin produces a MurA variant that results in overexpression of MurA (16). In addition to these genetic mutations, the expression of *glpT* and *uhpT* can be altered reversibly in response to environmental changes. We recently found that CpxAR and TorSRT, which are two-component systems in EHEC, regulate the *glpT* and *uhpT* genes (17, 18). Fosfomycin as well as indole and trimethylamine-*N*-oxide (TMAO), which are secondary metabolites derived from the gut microbiota, activate these respective systems and repress the *glpT* and *uhpT* genes, resulting in increased tolerance to fosfomycin in EHEC. This implies that the CpxAR and TorSRT systems contribute to innate tolerance to fosfomycin in EHEC. We aimed to gain further insight into the molecular mechanism of *glpT* and *uhpT* gene expression associated with susceptibility to fosfomycin, as such an insight will help us to establish a strategy that enhances the utility of this drug.

For *E. coli* strains, including EHEC strains, available oxygen is depleted at enteric sites where they reside or cause infection. When oxygen is depleted, they can alter the expression of subsets

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Relevant genotype/phenotype[a]	Reference or source
Strains		
HH-H7-008	Parent strain (*tnaA*/*lacZI* deletion from EHEC O157:H7 [RIMD 0509952])	23
HH-H7-095	*glpT* mutant of HH-H7-008	18
HH-H7-097	*uhpT* mutant of HH-H7-008	This work
HH-H7-103	*glpT uhpT* double mutant of HH-H7-008	This work
HH-H7-150	*fnr* mutant of HH-H7-008	This work
HH-H7-170	*arcA* mutant of HH-H7-008	This work
MG1655	Wild-type *E. coli* K-12 reporter strain	24
Rosetta(DE3)	T7 expression strain; Cm^r	Novagen/EMD Bioscience
CFT073	Uropathogenic *E. coli* strain	25
GU1193	ESBL-producing *E. coli* clinical isolate	This work
Plasmids		
pKO3	Temperature-sensitive vector for gene targeting; *sacB* Cm^r	26
pTrc99K	Vector for IPTG-inducible expression; Km^r	27
pTrc99-6Hisfnr	N-terminally His_6-tagged Fnr overexpression plasmid; Km^r	This work
pTrc99-6HisfnrD154A	N-terminally His_6-tagged D154AFnr overexpression plasmid; Km^r	This work
pNN387	Single-copy plasmid with promoterless *lacZ*; Cm^r	28
pNNglpT-P	*glpT* promoter reporter; Cm^r	18
pNNuhpT-P	*uhpT* promoter reporter; Cm^r	This work

[a] Cm^r, chloramphenicol resistance; Km^r, kanamycin resistance.

of genes, including genes responsible for metabolism, and then switch their respiration from aerobiosis to anaerobiosis to efficiently obtain bioenergy (19). FNR (fumarate nitrate reduction) and Arc (aerobic respiration control) are the central regulatory systems for altering the gene expression profile to maximize cell growth under anaerobic conditions. The FNR protein is a transcriptional regulator containing an Fe-S cluster, and it serves as a redox sensor and is active during anaerobic growth (20). The protein contains cysteine clusters in the N-terminal region which are responsible for a conformational change in the protein to its active, DNA-binding form in response to anaerobiosis. The Arc system is a two-component system that is composed of ArcA, the response regulator, and ArcB, the sensor kinase. ArcB is autophosphorylated during the transition from aerobic to microaerobic growth and then transfers the phosphate to ArcA (21). The phosphorylated ArcA protein represses genes encoding enzymes to operate the tricarboxylic acid (TCA) cycle during aerobic respiration in cells while activating a subset of genes encoding enzymes involved in fermentative metabolism (22).

In this study, we initially showed that fosfomycin is more active against EHEC grown anaerobically than against that grown aerobically. We provided evidence, using genetic and biochemical approaches, that EHEC cells in anaerobic growth have higher expression levels of both *glpT* and *uhpT* and more uptake of fosfomycin, leading to increased susceptibility to this drug. We also found that elevated expression of *glpT* and *uhpT* during anaerobic growth required FNR. In addition to EHEC, we showed that other *E. coli* members are also more susceptible to fosfomycin under anaerobic conditions, for the same reason. These data provide insights into the mechanism of action of fosfomycin toward *E. coli* under anaerobic conditions.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Unless otherwise indicated, all bacteria were grown in Luria-Bertani (LB) medium. EHEC strains were grown aerobically in glass tubes with shaking at 160 rpm. For anaerobic culture, we grew EHEC in a sealed container with gas generators (Anaero-Pack-Anaero; Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan). We also used the nonpathogenic K-12 strain (24), a uropathogenic *E. coli* (UPEC) strain (25), and an ESBL-producing *E. coli* clinical isolate designated GU1193. The ESBL producer was originally isolated from a urinary catheter placed in a hospitalized patient and is highly resistant to cefotaxime, piperacillin, and levofloxacin (MICs of 32, 128, and 16 μg/ml, respectively). The cell growth was monitored by measuring the absorbance at 600 nm. For marker selection and maintenance of plasmids, antibiotics were added to the growth medium at the following concentrations: 150 μg/ml ampicillin, 15 μg/ml chloramphenicol, and 25 μg/ml kanamycin.

Cloning and mutant construction. In-frame deletions of *uhpT*, *fnr*, and *arcA* were constructed by sequence overlap extension PCR according to a previously described strategy (26), using the delta1/delta2 and delta3/delta4 primer pairs for each gene (Table 2). The upstream flanking DNA included 450 bp of sequence, the first four amino acid codons for *uhpT*, and the first three amino acid codons for *fnr* and *arcA*. The downstream flanking DNA included the last four amino acid codons for *uhpT*, the last three amino acid codons for *fnr*, the last two amino acid codons for *arcA*, the stop codon, and 450 bp of additional DNA. These deletion constructs were ligated into the BamHI- and SalI-digested temperature-sensitive vector pKO3 (26) and introduced into HH-H7-008, the parent strain (23). We selected sucrose-resistant, chloramphenicol-sensitive colonies at 30°C and confirmed the resulting mutant strains by PCR analysis and DNA sequencing.

To construct the His_6-D154AFnr expression plasmid pTrc99-6HisD154Afnr, we initially constructed pTrc99-6Hisfnr. The *fnr* gene was amplified with the primer pair shown in Table 2. The product was digested with NcoI and BamHI and ligated into the similarly digested pTrc99K plasmid (27) to generate pTrc99-6Hisfnr. The pTrc99-6HisfnrD154A plasmid was constructed by site-directed mutagenesis of pTrc99-6Hisfnr. The mutations were generated by using the primer pairs shown in Table 2 and a QuikChange site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA), with the following modification: we used KOD FX Neo polymerase (Toyobo Co., Ltd., Osaka, Japan) instead of *Pfu* Turbo DNA polymerase for PCR amplification.

To construct pNNuhpT-P, a *lacZ* reporter plasmid to measure *uhpT* promoter activity, we PCR amplified the 300-bp region upstream of the

TABLE 2 Primers used in this study

Primer	DNA sequence (5′-3′)	Use
uhpT-delta1	GCGGGATCCTGTGGCTGATGCCATTTGC	*uhpT* mutant construction
uhpT-delta2	CAGTTACGTTTATGCCACTGTCAAGAAAGCCAGCATGGGTTAC	*uhpT* mutant construction
uhpT-delta3	AGGAGTAACCCATGCTGGCTTTCTTGACAGTGGCATAAACGTAAC	*uhpT* mutant construction
uhpT-delta4	GCGGTCGACTTGCTCGGCGGCTTTGGTC	*uhpT* mutant construction
fnr-delta1	GCGAGATCTCATTTCAGCGCGAAGTCTG	*fnr* mutant construction
fnr-delta2	ATGCGGAAAAATCAGGCAACGTTCGGGATCATAGGTCTGCTC	*fnr* mutant construction
fnr-delta3	GCTTGAGCAGACCTATGATCCCGAACGTTGCCTGATTTTTCCGC	*fnr* mutant construction
fnr-delta4	GCGGTCGACCTTCGAAAGGACGGTTATGC	*fnr* mutant construction
arcA-delta1	GCGGGATCCTATCCTTCTGTTTACTTAGG	*arcA* mutant construction
arcA-delta2	GGTGGTAAAGCCGATTAATCTTCGGTCTGCATGTTTGCTACC	*arcA* mutant construction
arcA-delta3	TTTAGGTAGCAAACATGCAGACCGAAGATTAATCGGCTTTACC	*arcA* mutant construction
arcA-delta4	GCGGTCGACCAACGTTTACACCCAATGC	*arcA* mutant construction
pTrc-fnr-6His-F	GCGCCATGGTCCATCACCATCACCATCACCCGGAAAAGCGAATTATACG	pTrc99-6Hisfnr construction
pTrc-fnr-R	GCGGGATCCTCAGGCAACGTTACGCGTATG	pTrc99-6Hisfnr construction
fnrD154A-F	GGTGAAATCAAAGGCGCTCAGGACATGATCCTG	pTrc99-6HisfnrD154A construction
fnrD154A-R	CAGGATCATGTCCTGAGCGCCTTTGATTTCACC	pTrc99-6HisfnrD154A construction
uhpT-PF	GCGGCGGCCGCGCTTGTTTGCTTATCTGGGG	pNNuhpT-P construction and gel shift assay
uhpT-PR	GCGAAGCTTGGGTTACTCCTGAAATGAATAC	pNNuhpT-P construction and gel shift assay
glpT-PF	GCGGCGGCCGCTCACTTGATTGCGAGTCGCG	Gel shift assay
glpT-PR	GCGAAGCTTTGAAAGCCTCCGTGGCCCGTG	Gel shift assay
rhlR-PF	GCGGGATCCGACCAAGTCCCCGTGTCGTG	Gel shift assay
rhlR-PR	GCGGGATCCTCGCCATCATCCTGAGCATC	Gel shift assay
rrsA-qPCR-F	CGGTGGAGCATGTGGTTTAA	Quantitative real-time PCR
rrsA-qPCR-R	GAAAACTTCCGTGGATGTCAAGA	Quantitative real-time PCR
rpoD-qPCR-F	CAAGCCGTGGTCGGAAAA	Quantitative real-time PCR
rpoD-qPCR-R	GGGCGCGATGCACTTCT	Quantitative real-time PCR
glpT-qPCR-F	TGCCCGCAGGTTTGATTC	Quantitative real-time PCR
glpT-qPCR-R	CCATGGCACAAAGCCCATA	Quantitative real-time PCR
uhpT-qPCR-F	AAGCCGACCCTGGACCTT	Quantitative real-time PCR
uhpT-qPCR-R	ACGGTTTGAACCACATTTTGC	Quantitative real-time PCR
murA-qPCR-F	CACAATTTCCGGCGCTAAA	Quantitative real-time PCR
murA-qPCR-R	GCCAGTAGAGCGGCAAAAAG	Quantitative real-time PCR

uhpT gene by using the primers uhpT-PF and uhpT-PR and ligated the product into the NotI- and HindIII-digested pNN387 plasmid with promoterless *lacZ* (28). All constructs were confirmed by DNA sequencing.

Drug susceptibility assays. MIC assays were performed by the standard serial agar dilution method of the Clinical and Laboratory Standards Institute (CLSI) (29). The MIC was determined as the lowest concentration at which growth was inhibited. To examine bacterial survival rates in fosfomycin-containing broth, a 50-fold dilution of overnight standing culture was inoculated into fresh LB broth, and bacteria were grown to mid-logarithmic phase. One-milliliter portions of cultures were transferred to microcentrifuge tubes containing fosfomycin and incubated at 37°C without shaking for 1 h. For fosfomycin-free controls, separate 1-ml portions of the cultures were incubated in the absence of fosfomycin. Survival rates were determined as the percent CFU of fosfomycin-treated cells compared to fosfomycin-free control cells.

Fosfomycin active transport assays. Assays to test for the accumulation of fosfomycin in bacterial cells were conducted as previously described (17). Bacteria were grown in 20 ml of LB medium to late logarithmic phase and resuspended in 1 ml of LB medium. This suspension was incubated for 60 min at 37°C in the presence of 2 mg of fosfomycin per ml and then washed three times with hypertonic buffer (10 mM Tris [pH 7.3], 0.5 mM $MgCl_2$, and 150 mM NaCl) to remove the antibiotic. Cells were resuspended in 0.5 ml of distilled water and plated on LB agar to determine the number of CFU/ml. The resuspended bacteria were boiled at 100°C for 3 min to release the fosfomycin. After centrifugation, the antibiotic concentration in the supernatant was determined by a disc diffusion assay. In this assay, sterilized assay discs (13 mm; Whatman, Florham Park, NJ) were saturated with 0.1 ml of the supernatant and deposited onto LB agar plates overlaid with a 1:10 dilution of an overnight culture of *E. coli* MG1655 as a reporter strain (24). Commercial fosfomycin was used as a standard (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Fosfomycin concentrations in supernatants were quantified by measuring the diameter (in millimeters) of the inhibitory zones on the LB agar culture and are presented in nanograms per 10^7 cells.

RNA extraction and quantitative real-time PCR analyses. Bacteria were grown to the mid-logarithmic growth phase (optical density at 600 nm [OD_{600}], ~0.4) in LB medium. Total RNA extraction and cDNA synthesis were performed using an SV total RNA isolation system and a GoScript reverse transcription system as described by the manufacturer (Promega Corp., Madison, WI). Real-time PCR mixtures included 2.5 ng cDNA and 200 nM (each) primers in SYBR Select master mix (Applied Biosystems, Foster City, CA) and were run on an ABI Prism 7900HT Fast real-time PCR system. The constitutively expressed *rrsA* and *rpoD* genes were used as internal controls. Primers are listed in Table 2. Amplification plot and melting curve data are available upon request.

Overexpression and purification of D154AFNR. N-terminally histidine-tagged D154AFNR (His_6-D154AFnr) was expressed in and purified from *Escherichia coli* Rosetta(DE3) (Novagen/EMD Bioscience, Philadelphia, PA). Bacteria containing the recombinant plasmid were grown at 37°C to an OD_{600} of 0.4 in LB medium, 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) was then added, and culture growth was continued for 3 h. Cells were harvested and stored at −80°C overnight. The cell pellet was suspended in lysis buffer (20 mM Tris [pH 7.9], 500 mM NaCl, and 10% glycerol) and lysed by sonication. The lysate was centrifuged, and the resulting supernatant was mixed with Ni-nitrilotriacetic acid (Ni-NTA) agarose (Qiagen, Valencia, CA) for 1 h. The agarose was washed

with 50 mM imidazole twice, and then D154AFNR was eluted with 200 mM imidazole. The protein was >95% pure as estimated by SDS-PAGE and Coomassie brilliant blue staining. The protein concentration was determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA). The protein was diluted in gel shift assay buffer (20 mM Tris [pH 7.5], 50 mM KCl, 1 mM dithiothreitol, and 10% glycerol) to a concentration of 4 pmol/μl and then stored at 4°C.

Gel shift assays. To assess FNR binding to the *glpT* and *uhpT* promoter sequences in gel shift assays, we used 321-bp DNA probes containing the 300-bp regions upstream of the *glpT* and *uhpT* start codons, respectively. We also used a 323-bp DNA fragment of the *Pseudomonas aeruginosa rhlR* gene as a nonspecific control probe. The probe DNA fragments (0.30 pmol) were mixed with purified D154AFNR in a 10-μl reaction mixture. After incubation for 20 min at room temperature, samples were separated by electrophoresis on a 5% nondenaturing Tris-glycine-EDTA (10 mM Tris [pH 8.0], 380 mM glycine, and 1 mM EDTA) acrylamide gel in Tris-glycine-EDTA buffer at 4°C. The gel was incubated in 10,000-fold-diluted SYBR green I nucleic acid stain (Lonza, Walkersville, MD), and DNA was visualized under UV light at 300 nm.

Promoter assays. EHEC strains carrying pNN-glpT-P or pNN-uhpT-P, the LacZ reporter plasmid, were grown aerobically or anaerobically at 37°C in LB medium. To measure LacZ expression from pNN-uhpT-P, we added 25 μg/ml of glucose-6-phosphate to the medium, because the promoter activity of the *uhpT* gene was too low to be detected in the absence of glucose-6-phosphate. β-Galactosidase activities from LacZ expression in cell lysates were determined as described by Miller (30).

RESULTS

Anaerobically grown EHEC cells are more susceptible to fosfomycin. To test the susceptibility of EHEC to fosfomycin under anaerobic conditions, we determined the MICs of fosfomycin by an agar dilution method for the wild-type parent EHEC strain grown aerobically or anaerobically. We found that the MIC for anaerobically grown cells was 8-fold lower than that for aerobically grown cells (MIC of 0.5 μg/ml for anaerobically grown cells versus 4 μg/ml for aerobically grown cells) (Table 3). We also measured survival rates with fosfomycin for anaerobically and aerobically grown EHEC cells after treatment with 1.56 μg/ml of fosfomycin as described in Materials and Methods. Under aerobic culture conditions, the number of CFU after incubation in the presence of fosfomycin was 2.14% (±1.66%) of the number of CFU in the absence of fosfomycin (Fig. 1). Under anaerobic conditions, the survival rate of EHEC was only 0.016% (±0.009%) after fosfomycin treatment (Fig. 1). This is consistent with the results of MIC experiments and indicates that EHEC is more susceptible to fosfomycin when it is grown anaerobically.

TABLE 3 Fosfomycin MICs of EHEC O157:H7 and its derivatives

Strain	Fosfomycin MIC (μg/ml)	
	Aerobic	Anaerobic
Parent (HH-H7-008)	4	0.5
Δ*glpT* (HH-H7-095)	32	16
Δ*uhpT* (HH-H7-097)	8	2
Δ*glpT* Δ*uhpT* (HH-H7-103)	128	64
Δ*fnr* (HH-H7-150)	4	2
Δ*arcA* (HH-H7-170)	4	0.5

Anaerobically grown EHEC has higher transcription levels of *glpT* and *uhpT*, which result in increased intracellular accumulation of fosfomycin. In anaerobically grown cells, intracellular accumulation of fosfomycin might be increased, which would lead to a higher level of susceptibility to the drug. To examine if this hypothesis is correct, we compared intracellular levels of fosfomycin between anaerobically and aerobically grown cells by a transport assay as described in Materials and Methods. The level of fosfomycin in anaerobic culture was 12-fold higher than that in aerobic culture (82.3 ± 1.9 ng/10^7 cells for aerobic culture versus 974.2 ± 476.7 ng/10^7 cells for anaerobic culture) (Fig. 2). We also measured the transcript levels of *glpT* and *uhpT*, encoding transporters for fosfomycin uptake, in aerobically and anaerobically grown cells by quantitative PCR (qPCR) analysis. The *glpT* and *uhpT* expression levels in anaerobically grown cells were 70- and 13-fold higher, respectively, than those in aerobically grown cells (Fig. 3). As a control, no significant difference in transcript levels of *murA*, encoding a target protein for the drug, was observed between these strains.

To confirm that elevated expression of *glpT* and *uhpT* in anaerobically grown cells leads to increased susceptibility, we compared the levels of susceptibility to fosfomycin between aerobic and anaerobic cultures for *glpT* and *uhpT* gene deletion mutants. These mutants showed less susceptibility than the wild-type par-

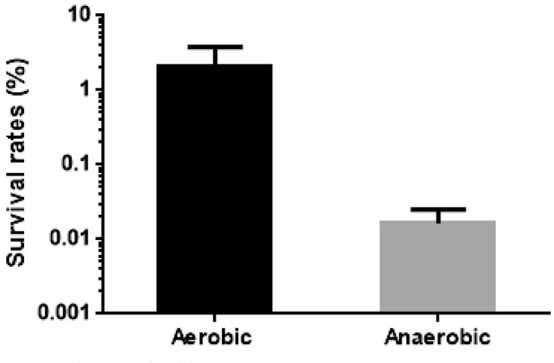

FIG 1 Survival rates of wild-type parent strains grown under aerobic or anaerobic conditions after incubation with or without 1.56 μg/ml fosfomycin. The survival rates are given as percentages of CFU/ml for strains after incubation with fosfomycin compared to those without fosfomycin. Data plotted are the means for three independent experiments; error bars indicate the standard deviations.

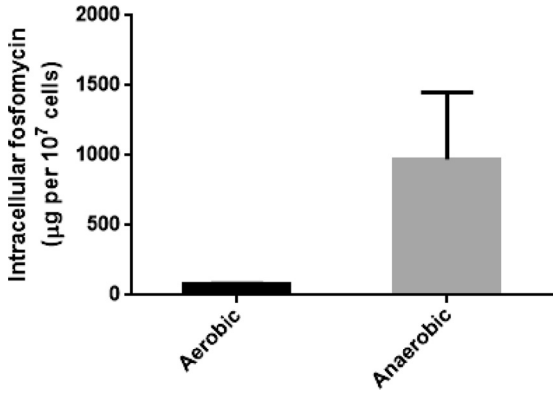

FIG 2 Intracellular accumulation of fosfomycin in wild-type parent strains grown under aerobic or anaerobic conditions. Accumulation among these strains is given as the amount of fosfomycin (nanograms) per 10^7 cells. Data plotted are the means for three independent experiments; error bars indicate the standard deviations.

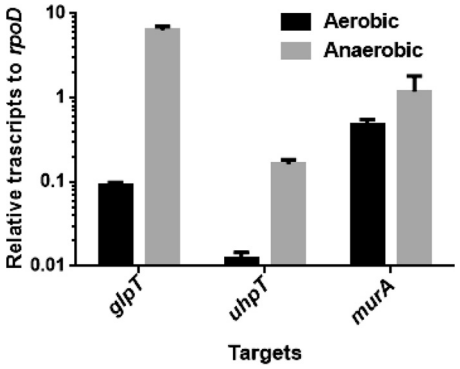

FIG 3 Transcript levels of the *glpT*, *uhpT*, and *murA* genes in wild-type parent strains grown under aerobic or anaerobic conditions. Transcript levels of *glpT*, *uhpT*, and *murA* are given as values relative to the *rpoD* (housekeeping gene) transcript level. Data plotted are the means for two biological replicates; error bars indicate the ranges.

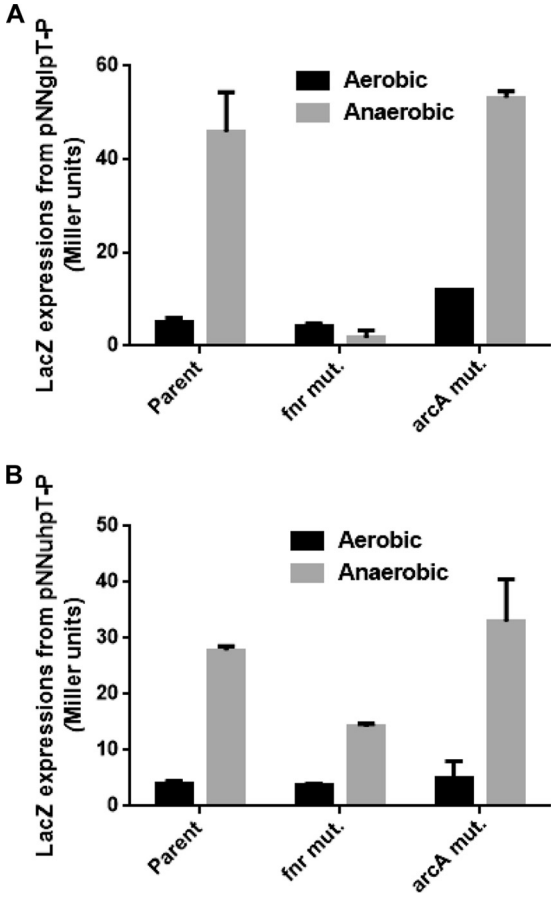

FIG 4 β-Galactosidase activities of EHEC wild-type parent, the *fnr* mutant, and the *arcA* mutant, containing *lacZ* reporter plasmids and grown under aerobic or anaerobic conditions. β-Galactosidase activities from LacZ expression in these strains correspond to *glpT* (A) or *uhpT* (B) promoter activities and are given in Miller units. Data plotted are the means for three independent experiments; error bars indicate the standard deviations.

ent under both aerobic and anaerobic conditions, suggesting that GlpT- and UhpT-dependent fosfomycin uptake indeed contributes to the growth inhibition. The MICs were as follows: 0.5 μg/ml for the parent anaerobic culture, 4 μg/ml for the parent aerobic culture, 16 μg/ml for the Δ*glpT* anaerobic culture, 32 μg/ml for the Δ*glpT* aerobic culture, 2 μg/ml for the Δ*uhpT* anaerobic culture, 8 μg/ml for the Δ*uhpT* aerobic culture, 64 μg/ml for the Δ*glpT* Δ*uhpT* anaerobic culture, and 128 μg/ml for the Δ*glpT* Δ*uhpT* aerobic culture (Table 3). MICs for these mutants under anaerobic conditions were only 2-fold (for the Δ*glpT* and Δ*glpT* Δ*uhpT* strains) or 4-fold (for the Δ*uhpT* strain) lower than those under aerobic conditions, while the decremental degree of the MIC in the wild-type background was 8-fold. These combined results indicate that increased susceptibility to fosfomycin during anaerobic growth in EHEC is partly due to an elevated expression of GlpT and UhpT that leads to increased intracellular accumulation of the drug.

The anaerobic transcriptional activator FNR is responsible for the elevation of *glpT* and *uhpT* expression levels under anaerobic conditions. FNR and ArcAB are major proteins that are responsible for induction of genes during anaerobic growth (20, 21). These proteins can be activated when oxygen is depleted. To examine the involvement of FNR and ArcAB in elevated expression of *glpT* and *uhpT* during anaerobic growth, we constructed *fnr* and *arcAB* deletion mutants and then determined the MICs of fosfomycin for these mutants when they were grown aerobically and anaerobically. We found that the *fnr* mutant showed a 4-fold lower susceptibility than that of the wild-type parent during anaerobic growth (MIC of 0.5 μg/ml for the wild-type parent versus 2 μg/ml for the *fnr* mutant), but there was no difference in the MIC between these strains during aerobic growth (MIC of 4 μg/ml for the wild-type parent and the *fnr* mutant) (Table 3). On the other hand, the deletion of *arcA* did not affect the MIC of fosfomycin even under anaerobic conditions.

We measured the promoter activities of *glpT* and *uhpT* via LacZ expression from the reporter plasmids pNN-glpT-P and pNN-uhpT-P, containing 300-bp regions upstream of the *glpT* and *uhpT* transcriptional start sites, respectively, fused to a promoterless *lacZ* gene. The wild-type parent carrying each reporter plasmid was grown aerobically or anaerobically in LB broth. We added glucose-6-phosphate as an inducer to measure LacZ expression from pNN-uhpT-P so that the basal level of promoter activity would be increased to a detectable level. Consistent with the qPCR results, LacZ expression levels from both pNN-glpT-P and pNN-uhpT-P in the wild-type parent grown under anaerobic conditions were 7.0- to 9.0-fold higher than those under aerobic conditions (Fig. 4A and B). However, LacZ expression from pNN-glpT-P in the *fnr* mutant grown under anaerobic conditions was at the same level as that in the wild-type parent and the *fnr* mutant grown under aerobic conditions (Fig. 4A). LacZ expression from pNN-uhpT-P in the *fnr* mutant grown anaerobically was significantly lower than that in the wild-type parent; however, unlike the level from the *glpT* promoter, the level was still 3-fold higher than that in the wild-type parent and the *fnr* mutant when these strains were grown aerobically (Fig. 4B). We also measured LacZ expression from pNN-glpT-P and pNN-uhpT-P in the *arcA* mutant background. Consistent with the results of MIC determination, LacZ expression in the mutant was elevated when the strain was grown anaerobically as well as in the wild-type parent; thus, the ArcAB system does not contribute to elevations of *glpT* and *uhpT* expression during anaerobic growth (Fig. 4A and B). These observations suggest that elevations of *glpT* and *uhpT* expression during

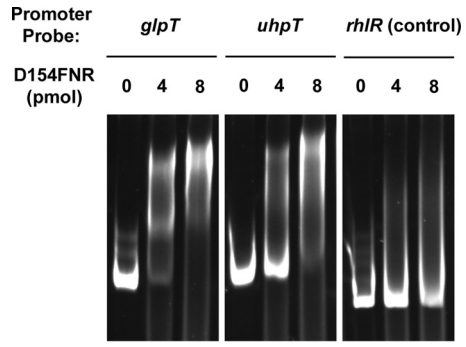

FIG 5 Gel shift assay showing binding of FNR to the *glpT* and *uhpT* promoters. The D154AFNR protein (0, 4, or 8 pmol) was added to reaction mixtures containing 0.3 pmol of DNA probe. DNA upstream of *rhlR* was used as a nonbinding (negative) control. Reaction mixtures were separated in polyacrylamide gels. Free and FNR-bound DNAs were visualized by SYBR green I staining under UV light (300 nm).

anaerobic growth require the *fnr* gene, but there might be yet another regulatory element for the *uhpT* gene.

FNR binds to *glpT* and *uhpT* upstream regions. To determine if FNR directly activates the expression of *glpT* and *uhpT*, we tested the ability of FNR to bind to the regions upstream of their promoters by performing a gel shift assay. The FNR protein remains in an inactive state in the presence of oxygen; therefore, we used the D154A mutant protein for this assay. The D154A mutant is an FNR protein with alanine substituted for aspartate at amino acid residue 154, and it is able to form a dimer which is in an active conformation even in the presence of oxygen and then to bind to the target DNA in the same fashion as that of wild-type FNR (31). We observed delayed mobilities of *glpT* and *uhpT* upstream region DNAs on electrophoresis, but not of *rhlR* from *Pseudomonas aeruginosa* (nonspecific control), suggesting that control of *glpT* and *uhpT* expression by FNR is direct (Fig. 5). We also noted that D154AFNR bound to the *glpT* promoter with a higher affinity than that for the *uhpT* promoter, because the mobility of the DNA fragment from the *glpT* upstream region was mostly shifted in the presence of 8 pmol of protein, whereas a significant amount of unshifted *uhpT* fragment was still observed at the same concentration of protein. These observations agree with the results of promoter assays showing that FNR contributes to the activation of *glpT* expression to a higher degree than that for *uhpT* expression.

Non-EHEC members are also more susceptible to fosfomycin under anaerobic conditions. To test whether other *E. coli* members are also more susceptible to fosfomycin under anaerobic conditions, we compared the MICs of fosfomycin in MG1655 (a nonpathogenic K-12 strain), the non-gastrointestinally infectious UPEC strain CFT073, and the ESBL-producing clinical isolate GU1193 cultured under aerobic and anaerobic conditions. All of them exhibited 8-fold lower MICs during anaerobic culture (Table 4). We also measured the transcript levels of *glpT* and *uhpT* by qPCR analysis for these *E. coli* strains grown aerobically or anaerobically. Similar to the results of the experiment with EHEC, both *glpT* and *uhpT* expression levels in anaerobically grown cells were higher than those in aerobically grown cells (Fig. 6A to C). We note that the degree of elevation of the *glpT* transcript in MG1655 was relatively lower than those in the other *E. coli* strains, although the reason for this is unclear (3-fold elevation for MG1655 versus 70-, 80-, and 50-fold elevations for EHEC O157, CFT073, and GU1193, respectively). To compare the sequences of the *fnr* open reading frame and the regions upstream of the *glpT* and *uhpT* genes among these *E. coli* strains, including the EHEC strain, we determined these sequences for GU1193 because the complete genome sequence for this strain has not been available. Among EHEC O157, MG1655, CFT073, and GU1193, the sequences were >95% identical. These observations suggest that MG1655, CFT073, and GU1193 are more susceptible to fosfomycin under anaerobic conditions due to elevated expression of GlpT and UhpT via FNR activation in the same fashion as that observed for EHEC.

DISCUSSION

The antibacterial activity of fosfomycin is relatively low compared with those of other commonly used bactericidal drugs under the typical standard laboratory conditions, which include aerobic culture in rich media (29). However, the activity of fosfomycin increases under anaerobic conditions, whereas those of other bactericidal drugs decrease (1–4, 9, 10). Oxygen-limited situations are very common for bacteria during the infection process, for instance, when they are in an enteric site and growing with other members in a microbial complex as a biofilm. Recently, combination treatment with fosfomycin and other drugs has been proposed to improve antibacterial therapy against pathogens growing in a biofilm (10, 32). Insights into the mechanism of action of fosfomycin toward bacteria growing under anaerobic conditions, such as understanding how fosfomycin is more effective against anaerobically grown cells, will aid us in establishing a method to enhance the efficacy of fosfomycin treatment. We focused mainly on EHEC here, because available oxygen is strictly limited at the enteric site where it infects the host and fosfomycin is traditionally used for treatment (7, 8). Our study initially showed that EHEC grown anaerobically is indeed more susceptible to this drug (Table 3; Fig. 1). We provided direct evidence that cells during anaerobic growth had elevated production of GlpT and UhpT and resulted in increased uptake of the drug (Table 3; Fig. 2 to 4). In addition, although we originally studied EHEC, fosfomycin was also more active against other *E. coli* members, including the laboratory K-12 strain, UPEC, and an ESBL-producing clinical isolate, under anaerobic conditions, with higher expression levels of GlpT and UhpT, as observed with EHEC (Table 4; Fig. 6).

The native function of GlpT and UhpT is to transport glycerol-3-phosphate and glucose-6-phosphate, respectively, into cells (12, 13). These compounds can be utilized as carbon sources for growth. In addition, EHEC can also use glycerol-3-phosphate as an electron donor for anaerobic respiration, where it is oxidized to dihydroxyacetone phosphate (DHAP) by glycerol-3-phosphate dehydrogenase and transfers electrons to the terminal reductases (33, 34). Therefore, GlpT and UhpT are closely related to biological fitness for EHEC, and repression of their genes indeed imposes a metabolic burden (17, 18). Thus, activation of expression of

TABLE 4 Fosfomycin MICs of other *E. coli* strains

Strain	Fosfomycin MIC (μg/ml)	
	Aerobic	Anaerobic
MG1655 (nonpathogenic K-12 strain)	4	0.5
CFT073 (UPEC)	8	1
GU1193 (ESBL producer)	8	1

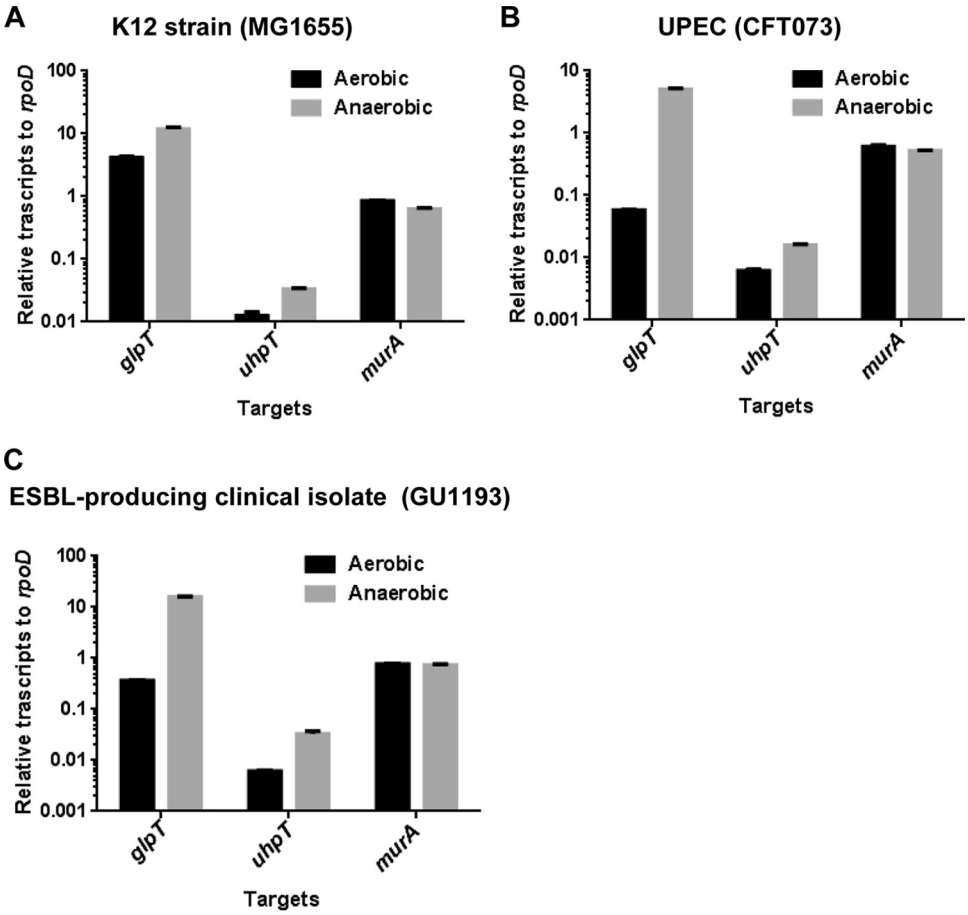

FIG 6 Transcript levels of the *glpT*, *uhpT*, and *murA* genes in the nonpathogenic K-12 strain (A), a UPEC strain (B), and an ESBL-producing clinical strain (C) grown under aerobic or anaerobic conditions. Transcript levels of *glpT*, *uhpT*, and *murA* are given as values relative to those for *rpoD* (housekeeping gene). Data plotted are the means for two biological replicates; error bars indicate the ranges.

these genes during anaerobic growth may be reasonable from a physiological standpoint for EHEC to reduce the metabolic cost. This concept is also supported by epidemiologic data showing that susceptibility rates have remained relatively stable despite the prevalent use of fosfomycin, though mutants that are resistant to fosfomycin can frequently be isolated from *in vitro* laboratory cultures (35–39). A loss of biological fitness, together with conferred resistance, offers the benefit that the development of resistance to the drug could occur with a low possible incidence even if its use increases in the future.

In this study, we also identified the regulatory element that is responsible for activation of *glpT* and *uhpT* expression associated with elevated susceptibility to fosfomycin during anaerobic growth. Induction of expression of these genes requires FNR (Fig. 4). The purified FNR protein bound to the regions upstream of *glpT* and *uhpT*; thus, FNR is an activator of these genes (Fig. 5). We note that the *fnr* mutant still had some level of *uhpT* expression; in addition, the affinity of FNR binding to *uhpT* was relatively lower than that for *glpT*. This implies that additional regulatory elements may participate in expression of the *uhpT* gene under anaerobic conditions. Previous studies of the nonpathogenic laboratory *E. coli* K-12 strain demonstrated that the *uhpT* gene is activated by UhpA together with cyclic AMP receptor protein (CRP) (40). UhpA is a response regulator that pairs with UhpB, the sensor kinase, to compose a two-component system, and it is activated in the presence of glucose-6-phosphate (41). On the other hand, CRP binds cyclic AMP (cAMP), and then the CRP-cAMP complex controls global genes related to bacterial metabolism (42). Unlike FNR, these transcriptional regulators are able to function under both aerobic and anaerobic conditions. UhpA is essential for expression of *uhpT* as a specific regulator (43). CRP-cAMP binds to a region that is relatively remote from the RNA polymerase binding site on the *uhpT* gene promoter, and then it stabilizes the complex of RNA polymerase, the promoter DNA, and UhpA (40, 44). In our experiment, UhpA also likely acted as an essential element for expression of *uhpT* during anaerobic growth in EHEC, because LacZ expression from pNN-uhpT-P was below the detectable limit even when cells were grown anaerobically. FNR might act as a cofactor for UhpA as well as CRP-cAMP. Our data will be useful in future studies to complete the picture by adding FNR to the regulatory model for *uhpT* gene control during anaerobic growth.

We conclude here that the increased antibacterial activity of fosfomycin toward *E. coli* strains, including EHEC, under anaerobic conditions can be attributed to an elevated expression of GlpT and UhpT that is caused by activation of FNR, which then leads to increased uptake of the drug. We believe that this study will aid us to more precisely understand the utility of this drug in

in vivo situations and may provide extensive information to make fosfomycin treatment more effective.

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