Identification of a Second Two-Component Signal Transduction System That Controls Fosfomycin Tolerance and Glycerol-3-Phosphate Uptake

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Particular interest in fosfomycin has resurfaced because it is a highly beneficial antibiotic for the treatment of refractory infectious diseases caused by pathogens that are resistant to other commonly used antibiotics. The biological cost to cells of resistance to fosfomycin because of chromosomal mutation is high. We previously found that a bacterial two-component system, CpxAR, induces fosfomycin tolerance in enterohemorrhagic *Escherichia coli* (EHEC) O157:H7. This mechanism does not rely on irreversible genetic modification and allows EHEC to relieve the fitness burden that results from fosfomycin resistance in the absence of fosfomycin. Here we show that another two-component system, TorSRT, which was originally characterized as a regulatory system for anaerobic respiration utilizing trimethylamine-*N*-oxide (TMAO), also induces fosfomycin tolerance. Activation of the Tor regulatory pathway by overexpression of *torR*, which encodes the response regulator, or addition of TMAO increased fosfomycin tolerance in EHEC. We also show that phosphorylated TorR directly represses the expression of *glpT*, a gene that encodes a symporter of fosfomycin and glycerol-3-phosphate, and activation of the TorR protein results in the reduced uptake of fosfomycin by cells. However, cells in which the Tor pathway was activated had an impaired growth phenotype when cultured with glycerol-3-phosphate as a carbon substrate. These observations suggest that the TorSRT pathway is the second two-component system to reversibly control fosfomycin tolerance and glycerol-3-phosphate uptake in EHEC, and this may be beneficial for bacteria by alleviating the biological cost. We expect that this mechanism could be a potential target to enhance the utility of fosfomycin as chemotherapy against multidrug-resistant pathogens.

Although fosfomycin is classified as an old antibiotic, it was recently revived as an antibiotic that could be effective against multidrug-resistant (MDR) pathogens, such as extended-spectrum- β -lactamase (ESBL) producers (1). This antibiotic has no structural relationship to other commonly used antibiotics; therefore, it is not affected by the development of cross-resistance in MDR pathogens (2,3). Fosfomycin is also used to decrease the risk of development of hemolytic-uremic syndrome (HUS), which is a fatal infectious disease caused by enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 (4–6).

Fosfomycin is an antagonist of phosphoenolpyruvate (PEP) and inhibits MurA activity, which transfers PEP to the 3'-hydroxyl group of UDP-*N*-acetylglucosamine in the initial step of bacterial cell wall biosynthesis (7). GlpT, which is a glycerol-3-phosphate transporter, and UhpT, which is a glucose-6-phosphate transporter, are involved in the uptake of fosfomycin (8–11). Mutations in the genes encoding these proteins that result in impaired fosfomycin binding or uptake confer resistance to fosfomycin. In some *E. coli* studies, mutations in genes encoding the positive regulators of *uhpT* expression, UhpA and CyaA, confer resistance because these mutants have a reduced uptake of fosfomycin (12,13). Other studies showed that a clinical isolate that is resistant to fosfomycin produces a MurA variant that results in the overexpression of MurA (14).

However, these mutations cause irreversible defects in GlpT, UhpT, and/or MurA production and thus affect GlpT- and UhpT-dependent carbon substrate uptake and MurA-catalyzed cell wall synthesis, which impose a fitness burden on the cells. Mutants that are resistant to fosfomycin can frequently be isolated *in vitro* in

rich media (15,16), while epidemiologic data indicate that susceptibility rates have remained relatively stable, despite the prevalent use of fosfomycin (17–19). Thus, the fitness cost of fosfomycin resistance may account for the relatively low level of fosfomycin resistance seen in the clinical setting. However, the Clinical and Laboratory Standards Institute (CLSI) reported that the *in vivo* antibacterial activity of fosfomycin against *E. coli* species is relatively lower than that of other commonly used antibiotics, such as β -lactams and fluoroquinolones (20). We asked if there is an innate mechanism of tolerance to this drug that does not rely on genetic modifications, as this could serve as a potential target to enhance the utility of fosfomycin.

Previously, we found that the CpxAR pathway, a two-component system (TCS), in EHEC induces fosfomycin tolerance (21).

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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 (EHEC O157:H7 strain RIMD 0509952 containing <i>tnaA</i> and <i>lacZI</i> deletions)	27
HH-H7-040	<i>cpxA</i> mutant from HH-H7-008	21
HH-H7-093	<i>cpxA cpxR</i> double mutant from HH-H7-008	21
HH-H7-095	<i>glpT</i> mutant from HH-H7-008	This work
HH-H7-121	<i>torR</i> mutant from HH-H7-008	This work
HH-H7-122	<i>torS</i> mutant from HH-H7-008	This work
HH-H7-123	<i>torT</i> mutant from HH-H7-008	This work
HH-H7-157	<i>cpxA torR</i> double mutant from HH-H7-008	This work
MG1655	<i>E. coli</i> K-12 wild-type reporter strain	28
Rosetta(DE3)	T7 expression strain, Cm ^r	Novagen/EMD Bioscience
CFT073	Uropathogenic <i>E. coli</i> strain	29
Plasmids		
pKO3	Temperature-sensitive vector for gene targeting, <i>sacB</i> Cm ^r	30
pTrc99A	Vector for IPTG-inducible expression, Ap ^r	31
pTrc99torR	TorR overexpression plasmid, Ap ^r	This work
pQE80	Vector for expression of His-tagged protein, Ap ^r	Qiagen
pQE80torR	N-terminal His ₆ -TorR overexpression plasmid, Ap ^r	This work
pNN387	Single-copy plasmid with promoterless <i>lacZ</i> , Cm ^r	32
pNNGlpT-P	<i>glpT</i> promoter reporter, Cm ^r	This work
pNNGlpT-P1	pNNGlpT-P with deletion of the <i>glpT</i> promoter in the TorR-binding site, Cm ^r	This work

^a Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance.

The CpxAR pathway is activated in the presence of sub-MICs of fosfomycin, and then a transient decrease in GlpT- and UhpT-dependent transport confers fosfomycin tolerance and reduces the fitness cost associated with it. The reversible mechanism, which does not rely on genetic mutations to confer fosfomycin tolerance, may be a beneficial strategy for bacteria to alleviate the fitness burden conferred by fosfomycin resistance in fosfomycin-free circumstances.

EHEC has ~30 pairs of proteins composing the TCS, including CpxAR (22). We are interested in the relationship between these TCS proteins and the reversible control of fosfomycin tolerance. In addition to CpxAR, we found that another TCS, the Tor system, controls fosfomycin tolerance. The Tor system regulates the utilization of trimethylamine-*N*-oxide (TMAO) as an electron acceptor for enterobacteria, including EHEC, during anaerobic respiration (23). TorS is the sensor kinase, and it is autophosphorylated by sensing the complex of TMAO and a periplasm-binding protein, TorT. TorS then transfers the phosphate to its cognate response regulator, TorR (24, 25). The phosphorylated TorR activates a subset of genes that encode proteins involving the TMAO reductase system (26).

In this study, we found that TorSRT coordinates the reversible control of fosfomycin tolerance. The TorR protein activated by the TMAO signal repressed the *glpT* gene encoding a glycerol-3-phosphate/fosfomycin cotransporter. The TorR-activated cells had reduced fosfomycin uptake, resulting in fosfomycin tolerance. However, the fosfomycin-tolerant cells had a decreased capability for glycerol-3-phosphate uptake, suggesting a trade-off between fosfomycin tolerance and glycerol-3-phosphate utilization.

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. To anaerobically grow EHEC strains, we used Hungate tubes (Bello Glass Inc., Vineland, NJ) equipped with a rubber stopper and a screw cap. For marker selection and plasmid maintenance, antibiotics were added to the growth medium at the following concentrations: 150 µg/ml ampicillin and 15 µg/ml chloramphenicol. For growth experiments using carbon source-limited medium, EHEC strains were grown in LB medium at 37°C with shaking for 12 to 16 h. The pellets were washed twice with glucose-free Dulbecco's modified Eagle medium (DMEM) and resuspended in half of the original culture volume with the same medium. The cell suspensions were diluted into fresh medium supplemented with 0.5% glycerol-3-phosphate or glucose at a 1:100 ratio. The bacteria were grown at 37°C with shaking, and cell growth was monitored by determination of the absorbance at 660 nm.

Cloning and mutant constructions. In-frame deletions of *torR*, *torS*, and *torT* were constructed by sequence overlap extension PCR according to a strategy described previously (30) with primer pairs torR-delta1/torR-delta2 and torR-delta3/torR-delta4 for *torR*, primer pairs torS-delta1/torS-delta2 and torS-delta3/torS-delta4 for *torS*, and primer pairs torT-delta1/torT-delta2 and torT-delta3/torT-delta4 for *torT* (Table 2). The upstream flanking DNA included 450 bp and the first 4 amino acid codons for *torR*, the first 5 amino acid codons for *torS*, and the first 4 amino acid codons for *torT*. The downstream flanking DNA included the last 10 amino acid codons for *torR*, the last 3 amino acid codons for *torS*, the last 10 amino acid codons for *torT*, the stop codon, and 450 bp of DNA. These deletion constructs were ligated into BamHI- and SalI-digested temperature-sensitive vector pKO3 and introduced into HH-H7-008, the parent strain (27). We selected sucrose-resistant, chloramphenicol-sensitive colonies at 30°C and confirmed the resulting mutant strains using PCR analysis and DNA sequencing. We also constructed a mutant with a deletion of the *glpT* gene by the same method using primer pairs glpT-delta1/glpT-delta2 and glpT-delta3/glpT-delta4.

To construct *torR* expression plasmid pTrc99torR, the *torR* gene was amplified with the primer pair pTrc-torR-F/pTrc-torR-R (Table 2). The product was digested with NcoI and BamHI and ligated into similarly digested plasmid pTrc99A. The His₆-TorR expression plasmid pQE80-torR was constructed by ligating the *torR* gene that had been PCR amplified with primers pQE-torR-F and pQE-torR-R into BamHI- and Hin-

TABLE 2 Primers used in this study

Primer	DNA sequence (5'–3')	Use
glpT-delta1	GCGGGATCCAGCGCGCACCGCTGTGCAG	<i>glpT</i> mutant construction
glpT-delta2	TCATGCCATTAGCCTCCGTTGCGAATACTCAACATTGAAAGCC	<i>glpT</i> mutant construction
glpT-delta3	CGGAGGCTTTCAATGTTGAGTATTCGCAACGGAGGCTAATGGC	<i>glpT</i> mutant construction
glpT-delta4	GCGGTCGACCTTCAAAGGTGTGCACCCGG	<i>glpT</i> mutant construction
torR-delta1	GCGGGATCCTCACCATGCCTGGAATATCC	<i>torR</i> mutant construction
torR-delta2	ATCAGCGGCTAAGAAATAACCTTCGTGATGTGGCATCAGAGGG	<i>torR</i> mutant construction
torR-delta3	TAAACCCCTCTGATGCCACATCACGAAGTTATTCTTAGCCGC	<i>torR</i> mutant construction
torR-delta4	GCGGTCGACAGCGTAACCTGTTGCAGGAG	<i>torR</i> mutant construction
torS-delta1	GCGGGATCCGCTACTACCGAGCAAAATGG	<i>torS</i> mutant construction
torS-delta2	GCTGATATTGATTCAAATCGCGTTGGTTAAATTCACGGTCGGTG	<i>torS</i> mutant construction
torS-delta3	AGTGACCCGACCGTGAATTTAAACACGCGATTGAATCAATATC	<i>torS</i> mutant construction
torS-delta4	GCGGTCGACTGATGACGATAAAAGCGACC	<i>torS</i> mutant construction
torT-delta1	GCGGGATCCTGGGCTGGAAATGGGCGCAG	<i>torT</i> mutant construction
torT-delta2	GATGAGGCTATCATGCGCGTGTCTTTTATCAGCACACATCAGC	<i>torT</i> mutant construction
torT-delta3	AGCCGCTGATGTGTGCTGATAAAACAGCACGCGCATGATAGCC	<i>torT</i> mutant construction
torT-delta4	GCGGTCGACATAGCGGTGGTATCAAAACC	<i>torT</i> mutant construction
pTrc-torR-F	GCGCCATGGCACATCACATTGTTATTGTTG	pTrc99torR construction
pTrc-torR-R	GCGGGATCCTCAGCACACATCAGCGGCTAAG	pTrc99torR construction
pQE-torR-F	GCGGGATCCCCACATCACATTGTTATTG	pQE80torR construction
pQE-torR-R	GCGAAGCTTTCAGCACACATCAGCGGC	pQE80torR construction
glpT-PF	GCGGCGGCGGCTCACTTGATTGCGAGTCGCG	Probe preparation for gel shift assay
glpT-PR	GCGAAGCTTTGAAAGCCTCCGTGGCCCGTG	Probe preparation for gel shift assay
torC-PF	GCGATGCATGGGCAAGTTGATATCCAGC	Probe preparation for gel shift assay
torC-PR	GCGGGTACCAATAGCCCCTGTAAAATTATG	Probe preparation for gel shift assay
rhIR-PF	GCGGGATCCGACCAAGTCCCCGTGTCGTG	Probe preparation for gel shift assay
rhIR-PR	GCGGGATCCTCGCCATCATCCTGAGCATC	Probe preparation for gel shift assay
glpT-PR2	TGAAAGCCTCCGTGGCCCGTGGTCTGATTCAAGTGAAGAGAACC	Deletion of TorR binding site in <i>glpT</i>
glpT-footprint1-6FAM	CGGCAGGTAAGCGCGCTTTG	Footprinting, 6-FAM-labeled primer
glpT-footprint2	CGCATATTCGCTCATAATTG	Footprinting, unlabeled primer
glpT-RACE1	GCCAGTCATTGAACCAGGCC	RACE unique primer
glpT-RACE2	GTGCACCATAGTACGACCGC	RACE unique primer
glpT-RACE3	CGCCAGAATCAAACCTGCGG	RACE unique primer
Oligo(dT)-Anchor	GACCACGCGTATCGATGTGCTGACTTTTTTTTTTTTTTTT	RACE oligo(dT) attached to an anchor
PCR Anchor	GACCACGCGTATCGATGTGCTGAC	RACE anchor primer
rrsA-qPCR-F	CGGTGGAGCATGTGGTTTAA	Quantitative real-time PCR
rrsA-qPCR-R	GAAAACCTCCGTGGATGTCAAGA	Quantitative real-time PCR
rpoD-qPCR-F	CAAGCCGTGGTCGGAATA	Quantitative real-time PCR
rpoD-qPCR-R	GGGCGCGATGCACTTCT	Quantitative real-time PCR
glpT-qPCR-F	TGCCCCGAGGTTTGATTC	Quantitative real-time PCR
glpT-qPCR-R	CCATGGCACAAGCCCAT	Quantitative real-time PCR
uhpT-qPCR-F	AAGCCGACCCTGGACCTT	Quantitative real-time PCR
uhpT-qPCR-R	ACGGTTTGAACCATTTTTCG	Quantitative real-time PCR
murA-qPCR-F	CACAATTTCCGGCGCTAAA	Quantitative real-time PCR
murA-qPCR-R	GCCAGTAGAGCGGCAAAAAG	Quantitative real-time PCR
torC-qPCR-F	TTGCCGAGCGTGAATGG	Quantitative real-time PCR
torC-qPCR-R	GCGACAGGTTGCCGAGTT	Quantitative real-time PCR

dIII-digested plasmid pQE80 (Table 2). The resulting *E. coli* construct produces TorR as an N-terminal hexahistidine-tagged protein in the presence of IPTG (isopropyl- β -D-thiogalactopyranoside).

To construct pNNGlpT-P, a *lacZ* reporter plasmid used to measure *glpT* promoter activity, we PCR amplified the 300-bp region upstream of the *glpT* gene with primers glpT-PF and glpT-PR and ligated the product into NotI- and HindIII-digested plasmid pNN387 with promoterless *lacZ*. We also constructed pNNGlpT-P1, which consisted of pNNGlpT-P from which the TorR-binding site was deleted. A DNA fragment from which the 18-bp region containing the TorR-binding site was removed was PCR amplified with primers glpT-PF and glpT-PR2, and the following PCR was performed with primers glpT-PF and glpT-PR. The PCR fragment was ligated into pNN387 as described above. All constructs were confirmed by DNA sequencing.

Drug susceptibility assays. MIC assays were performed by a serial agar dilution method that consisted of the standard method of the Japanese Society of Chemotherapy. Bacteria were grown for 20 h at 37°C in LB medium without shaking. Five microliters of 100-fold-diluted cultures (~5,000 cells) was inoculated onto an agar plate containing antibiotics, and the plate was incubated for 16 h at 37°C. The MICs were determined to be the lowest concentration at which growth was inhibited. To examine bacterial survival rates in fosfomycin-containing broth, a 50-fold dilution of culture that had been standing overnight was inoculated into fresh LB broth, and the bacteria were grown to mid-logarithmic phase. A 1-ml portion of the cultures was transferred into a microcentrifuge tube containing fosfomycin, and the tube was incubated at 37°C without shaking for 1 h. As a fosfomycin-free control, a separate 1-ml portion of the cultures was incubated in the absence of fosfomycin. Survival

rates are presented as the percentage of the number of CFU for fosfomycin-treated cells relative to the number of CFU for fosfomycin-free control cells.

RNA extraction and quantitative real-time PCR analyses. Bacteria were grown to mid-logarithmic growth phase (optical density at 600 nm [OD₆₀₀], ~0.5) in LB medium. TorR was overexpressed in the wild-type parent harboring pTrc99torR grown with 0.1 mM IPTG. TMAO was added to the LB medium at a concentration of 20 mM. 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). The real-time PCR mixture included 2.5 ng cDNA and 200 nM primers in SYBR Select master mix (Applied Biosystems, Foster City, CA), and PCRs were run on an ABI Prism 7900HT Fast real-time PCR system. Constitutively expressed *rrsA* and *rpoD* genes were used as an internal control. Primers are listed in Table 2. Amplification plot and melting curve data are available upon request.

Overexpression and purification of His₆-TorR. His₆-TorR was expressed in and purified from *Escherichia coli* Rosetta(DE3) (Novagen/EMD Bioscience, Philadelphia, PA). Bacteria containing recombinant plasmid were grown to an OD₆₀₀ of 0.4 at 37°C in LB medium, 0.5 mM IPTG 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, 10% glycerol) and lysed by sonication. The lysate was centrifuged, and the resulting supernatant was mixed with Ni-nitrilotriacetic acid–agarose (Qiagen, Valencia, CA) for 1 h. The agarose was washed twice with 50 mM imidazole, and then His₆-TorR was eluted with 500 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). Proteins were stored at –80°C.

Gel shift assays. To assess TorR binding to the *glpT* promoter sequence in gel shift assays, we used a 321-bp DNA probe containing the 300-bp region upstream of the *glpT* start codon. We also used a 318-bp DNA fragment from the 300-bp region upstream of the *torC* start codon as a positive-control probe and a 323-bp DNA fragment from the *Pseudomonas aeruginosa* *rhlR* gene as a nonspecific control probe. The purified His₆-TorR was phosphorylated in gel shift assay buffer (20 mM Tris [pH 7.5], 50 mM KCl, 1 mM dithiothreitol, 10% glycerol) containing 50 mM carbamoyl phosphate (Sigma-Aldrich, St. Louis, MO) and 10 mM magnesium chloride for 30 min at 30°C. Then, we expected that at least some portion of the TorR protein pool would be phosphorylated in the *in vitro* reaction. The probe DNA fragments (0.30 pmol) were mixed with phosphorylated His₆-TorR in a 10-μl reaction mixture. After incubation for 20 min at room temperature, samples were separated by electrophoresis on a 5% nondenaturing acrylamide Tris-glycine-EDTA (10 mM Tris [pH 8.0], 380 mM glycine, 1 mM EDTA) 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 the DNA was visualized under UV light at 300 nm.

DNase I footprinting. DNase I footprinting was performed using a previously described nonradiochemical capillary electrophoresis method on an ABI Prism genetic analyzer equipped with ABI Prism GeneScan analysis software (33). The 6-carboxyfluorescein (6-FAM)-labeled (5′) 255-bp DNA fragment (starting at 49 bp inside the *glpT*-coding region and ending 206 bp upstream of the *glpT* start codon) was generated by PCR amplification using a 6-FAM-labeled forward primer (primer *glpT*-footprint1-6FAM) and an unlabeled reverse primer (primer *glpT*-footprint2). The DNA fragment (0.45 pmol) was mixed with phosphorylated TorR (40 pmol) in a 50-μl reaction mixture containing the same buffer described above. After incubation for 20 min at room temperature, DNase I (0.3 U; Promega Corp., Madison, WI) was added. After incubation for 60 s at room temperature, samples were purified for GeneScan sequencing analysis.

Analysis by 5′ RACE. We used 1.5 μg of total RNA isolated from the mid-logarithmic-phase wild-type parent grown in LB medium and synthesized cDNA using a GoScript reverse transcription system with the unique primer *glpT*-RACE1 (starting at 559 bp inside the *glpT*-coding region). A poly(A) linker was attached to the 3′ terminus of cDNA using the terminal transferase (TdT; New England BioLabs, Ipswich, MA). Two rounds of PCR were performed by using an oligo(dT) primer attached to a 22-base anchor sequence at the 5′ end [primer Oligo(dT)-Anchor] and primer *glpT*-RACE2 (starting at 441 bp inside the *glpT*-coding region) for the first round and an anchor primer without the oligo(dT) sequence (PCR anchor) and primer *glpT*-RACE3 (starting at 309 bp inside the *glpT*-coding region) for the second round. Eventually, we got a single ~380-bp product by rapid amplification of cDNA ends (RACE). The product was TA cloned into pCR2.1 TOPO (Life technologies, Waltham, MA), and six clones were sequenced.

Promoter assays. To measure the promoter activity of the *glpT* genes, EHEC strains carrying the reporter plasmid were grown in LB medium. The wild-type parent and a Δ *torR* strain harboring the reporter plasmid were anaerobically grown to the early stationary phase. Each cell culture of 0.5 ml was transferred into a 1.5-ml microtube, and 50 μl of chloroform was added. After incubation for 5 min, the β-galactosidase activity in the supernatant was monitored using a Tropix Galacto-Light Plus kit according to the manufacturer's protocol (Applied Biosystems, Foster City, CA).

Fosfomycin active transport assays. Assays to test for fosfomycin accumulation in bacterial cells were conducted as previously described (21). Bacteria were grown to late logarithmic phase in 20 ml of LB medium 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 MgCl₂, 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 bacterial resuspension was boiled at 100°C for 3 min to release the fosfomycin. After centrifugation, the antibiotic concentration in the supernatant was determined by a diffusion disc assay. In this assay, sterilized assay discs (diameter, 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 (28). Commercial fosfomycin was used as a standard (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The fosfomycin concentration in the supernatants was quantified by the diameter (mm) of the inhibitory rings on the LB agar culture and is presented as the number of ng per 10⁷ cells.

RESULTS

Overexpression of the *torR* gene confers fosfomycin tolerance in EHEC. In our previous study, we found that the activation of CpxAR resulted in increased tolerance to fosfomycin (21). We tested other TCSs of EHEC to determine if they control fosfomycin tolerance. Overexpression of the response regulator can artificially lead to constitutive activation of the TCS even in the absence of a signal from the sensor protein (31, 33, 34). We overexpressed each response regulator from an IPTG-inducible promoter on pTrc99A, a multicopy plasmid, and then the MICs of fosfomycin for these response regulator-overexpressing strains were determined. We found that the *torR*-overexpressing strain showed a lower susceptibility to fosfomycin than the control strain carrying the empty plasmid (MICs, 4 mg/liter for the control strain versus 16 mg/liter for the *torR*-overexpressing strain) (Table 3). We also compared the survival rate of the *torR*-overexpressing strain with that of the control EHEC strain after treatment with 1.56 mg/liter of fosfomycin, as described in Materials and Methods. After incubation in the presence of fosfomycin, the number of CFU of the control strain was only 1.4% ± 0.3% of the number of CFU of the control strain in the absence of fosfomycin

TABLE 3 Fosfomycin MICs for the EHEC O157:H7 strain and its derivatives

Strain	Fosfomycin MIC (mg/liter)
Parent (HH-H7-008)	4
HH-H7-008/pTrc99A	4
HH-H7-008/pTrc99torR	16
HH-H7-008 $\Delta glpT$ (HH-H7-095)	16
HH-H7-008 $\Delta glpT$ /pTrc99A	16
HH-H7-008 $\Delta glpT$ /pTrc99torR	16
HH-H7-008 $\Delta cpxAR$ /pTrc99A	4
HH-H7-008 $\Delta cpxAR$ /pTrc99torR	16
HH-H7-008 $\Delta cpxA$ (HH-H7-040)	16
HH-H7-008 $\Delta cpxA \Delta torR$ (HH-H7-157)	16

(Fig. 1). On the other hand, the survival rate of the *torR*-overexpressing EHEC strain was $88.8\% \pm 11.9\%$ after fosfomycin treatment (Fig. 1). This finding is consistent with the findings of the MIC experiments and indicates that overexpression of *torR* increases the tolerance of EHEC O157:H7 to fosfomycin.

Overexpression of the *torR* gene suppresses *glpT* expression and results in reduced fosfomycin uptake. GlpT and UhpT are transporters for fosfomycin uptake, and MurA is a target for the drug; thus, they are determinants for susceptibility to fosfomycin. To determine whether TorR affects the level of *glpT*, *uhpT*, and/or *murA* gene expression or not, we compared the levels of these transcripts between the *torR*-overexpressing and control strains by quantitative PCR (qPCR) analysis. We found that the level of *glpT* expression in the *torR*-overexpressing cells was 15-fold lower than that in the control, whereas no significant difference in the levels of *uhpT* and *murA* expression was observed between these strains (Fig. 2).

To confirm that the depletion of GlpT reduces the susceptibility to fosfomycin, we constructed a mutant with an in-frame deletion of the *glpT* gene and tested the susceptibility to fosfomycin. The *glpT* deletion mutant had increased tolerance to fosfomycin compared to that of the parent strain. However, overexpression of

FIG 1 Survival rates of the wild-type parent strain harboring pTrc99A (vector control) or pTrc99torR (a TorR-overexpressing plasmid) after incubation with or without 1.56 $\mu\text{g/ml}$ fosfomycin. The survival rates are presented as the percentage of the number of CFU/ml for the strain after incubation with fosfomycin relative to that after incubation without fosfomycin. Data are plotted as the means from three independent experiments; error bars indicate standard deviations. ****, $P < 0.0005$.

FIG 2 Levels of *glpT*, *uhpT*, and *murA* gene transcripts in the wild-type parent strain harboring pTrc99A (vector control) or pTrc99torR (a TorR-overexpressing plasmid). The levels of the *glpT*, *uhpT*, and *murA* transcripts are presented relative to the *rpoD* gene (a housekeeping gene) transcript level. Data are plotted as the means from two biological replicates; error bars indicate ranges. ****, $P < 0.0005$.

TorR in the *glpT* mutant had no effect on fosfomycin tolerance relative to that of the strain carrying the vector control (Table 3). These observations suggest that fosfomycin tolerance in the *torR*-overexpressing strain is attributed to the suppression of GlpT production. We previously found that the Cpx system represses *glpT* expression. The response regulator CpxR was constitutively activated when CpxA phosphatase activity was abolished, and then fosfomycin tolerance was increased (21). Our data suggest that there is no cross talk between the Cpx and Tor pathways because the overexpression of TorR in the *cpxAR* mutant and the deletion of *cpxA* in the *torR* mutant still increased fosfomycin tolerance to the same degree as that in the wild-type parent background (Table 3).

We also measured the intracellular fosfomycin levels of the *torR*-overexpressing and control strains. The level of fosfomycin accumulation in the *torR*-overexpressing cells was 7.5-fold lower than that in cells of the control strain (7.0 ± 0.8 ng per 10^7 cells for the *torR*-overexpressing strain versus 52.6 ± 19.4 ng per 10^7 cells for the control strain) (Fig. 3). From these combined results, the induction of fosfomycin tolerance by *torR* overexpression can be

FIG 3 Intracellular accumulation of fosfomycin in the wild-type parent strain harboring pTrc99A (vector control) or pTrc99torR (a TorR-overexpressing plasmid). Accumulation is described as the amount of fosfomycin (ng) in 10^7 cells. Data are plotted as the means from three independent experiments; error bars indicate standard deviations. **, $P < 0.01$.

FIG 4 Cell growth of the wild-type parent strain harboring pTrc99A (vector control) or pTrc99torR (a TorR-overexpressing plasmid) in DMEM with 0.5% (wt/vol) glycerol-3-phosphate (glycerol-3P) (A) or glucose (B) as a carbon source to support growth. Growth was monitored by determination of the absorbance at 660 nm. The experiment was repeated twice, and similar results were obtained each time.

explained by the reduction of GlpT-dependent fosfomycin uptake.

TorR-overexpressing cells have limited glycerol-3-phosphate uptake. GlpT transports glycerol-3-phosphate into the cells as its innate function, and then EHEC can utilize it as a carbon source. We predicted that the *torR*-overexpressing strain has a growth defect in a medium containing glycerol-3-phosphate as the carbon source to support growth. We separately cultured EHEC carrying a *torR*-overexpressing plasmid or an empty vector in glucose-free DMEM supplemented with glycerol-3-phosphate as the carbon source to support growth. As shown in Fig. 4A, the growth rate of the *torR*-overexpressing strain was lower than that of the control strain in this medium. However, there was no significant difference in growth between these strains when they were grown in DMEM supplemented with glucose, in which growth does not depend on GlpT, instead of glycerol-3-phosphate (Fig. 4B).

TMAO represses *glpT* expression in a TorR-, TorS-, and TorT-dependent manner. Trimethylamine-*N*-oxide (TMAO) is a signal that activates the Tor pathway in EHEC anaerobiosis. TorT, a periplasmic receptor protein, forms a complex with TMAO. TorS is autophosphorylated by interaction with the TorT-TMAO complex and then transfers its phosphate to TorR (23–25). To test whether TMAO represses *glpT* expression through activation of the Tor pathway, we measured the levels of the *glpT* transcript in the wild-type EHEC and *torR* mutant strains grown anaerobically in the presence or absence of TMAO by qPCR analysis. The qPCR data showed that the level of expression of *glpT* in the wild-type parent was 2.5-fold lower when it was grown with TMAO than when it was grown without TMAO (Fig. 5A). On the other hand, the level of the *glpT* transcript in the *torR* mutant was not decreased by TMAO (Fig. 5A). We also confirmed that the level of expression of *torC*, which is activated by the Tor system, was increased in the wild-type parent grown in the presence of TMAO but not in the *torR* mutant (Fig. 5A). Similar to the results for the *torR* mutant, neither repression of *glpT* nor activation of *torC* by TMAO was observed in the *torS* and *torT* mutant strains because TorS and TorT are also required for Tor activation in the presence of TMAO (see Fig. S1 in the supplemental material). Consistent with the results of qPCR analysis, fosfomycin accumulation in EHEC grown with TMAO was 2.2-fold lower than that in EHEC grown without TMAO (472 ± 117 ng per 10^7 cells when the

strain was grown without TMAO versus 213 ± 83 ng per 10^7 cells when the strain was grown with TMAO) (Fig. 5B). These results indicate that TMAO activates the Tor pathway and then suppresses GlpT production.

FIG 5 Effect of TMAO-induced Tor system activation. (A) The levels of the *glpT* and *torC* transcripts in the wild-type parent and the *torR* mutant grown anaerobically with or without 20 mM TMAO were measured by qPCR. These transcript levels are presented relative to the *rpoD* gene (a housekeeping gene) transcript level. Data are plotted as the means from two biological replicates; error bars indicate ranges. (B) Intracellular accumulation of fosfomycin, described as the amount of fosfomycin (ng) in 10^7 cells, in the wild-type parent anaerobically grown with or without 20 mM TMAO. Data are plotted as the means from three independent experiments; error bars indicate standard deviations. *, $P < 0.05$; ****, $P < 0.0005$.

FIG 6 TorR binds the *glpT* promoter region containing predicted decameric sequence. (A) Results of a gel shift assay showing the binding of TorR to the *glpT* promoter. The TorR protein (0, 10, 20, or 40 pmol) was added to reaction mixtures containing 0.3 pmol of a DNA probe. DNA upstream of *torC* and *rhIR* was used as a positive control and a nonbinding (negative) control, respectively. Reaction mixtures were separated on polyacrylamide gels. Free and TorR-bound DNAs were visualized by SYBR green I staining under UV light at 300 nm. (B) DNase I footprinting of the *glpT* promoter region. A 255-bp, 6-FAM-labeled DNA fragment was incubated in the presence or absence of phosphorylated TorR (40 pmol) and subjected to DNase I digestion. The fluorescence intensities of the DNA fragments (y axis) are plotted relative to their size in bases (x axis). One region (outlined by the dashed line) was protected from DNase I digestion in the presence of 40 pmol TorR. (C) Sequence of the upstream *glpT* region. The double-headed arrows indicate the predicted TorR-binding sites, and the region protected from DNase I digestion is indicated by the box. The transcriptional start site is indicated by the arrow labeled +1. The *glpT* translational start codon and predicted -10 and -35 boxes are indicated.

TorR is a repressor for the *glpT* gene. To investigate if TorR directly regulates the expression of *glpT*, we purified TorR as an N-terminal hexahistidine-tagged protein and examined its ability to bind the promoter of the *glpT* gene by gel shift assays. We used a 321-bp DNA probe including a 300-bp region upstream of the *glpT* gene. We also used a similarly sized DNA probe from the region of the *torC* promoter including a known TorR-binding sequence as a positive control and a similarly sized DNA probe

from the region upstream of the *rhIR* gene from *P. aeruginosa* as a nonspecific control. TorR bound to both *glpT* and *torC* promoter DNA fragments but not the *rhIR* gene used as a nonspecific probe (Fig. 6A). We also confirmed that the binding to the *glpT* promoter depends on the phosphorylation of the TorR protein because no shift of the *glpT* DNA band was observed when nonphosphorylated protein (in the absence of carbamoyl phosphate) was used.

FIG 7 β -Galactosidase activities of the EHEC wild-type parent and the *torR* mutant containing *glpT-lacZ* reporter plasmids anaerobically grown with or without 20 mM TMAO. The β -galactosidase activities from LacZ expression in these strains correspond to *glpT* promoter activities and are described as the number of relative light units (RLU) in 10^6 cells. Data are plotted as the means from three independent experiments; error bars indicate standard deviations. ****, $P < 0.0005$.

TorR is proposed to bind to a decameric consensus sequence (5'-CTGTTTCATAT-3') (35). The promoter region of the *torC* gene has three consensus sequences that match completely and another one with a 3-base replacement to which the TorR protein binds with a relatively lower affinity (35). We found these consensus sequences with the 3-base replacement 41 bp upstream (CTGTTAATCA) (designated site 1) and 119 bp upstream (CAATTCACAT) (designated site 2) of the *glpT* translational start site, respectively (the consensus sequences are underlined). We then identified the TorR-binding region by DNase I footprinting. A 33-bp region including the site 1 element was partly protected from DNase I digestion by TorR, suggesting that the TorR protein binds to site 1 but not to site 2 (Fig. 6B). The *glpT* transcriptional start site has been proposed to be an A residue 76 bases downstream of the translational start site; the site 1 element is located 36 bases upstream of the transcriptional start site (36). We confirmed that the proposed transcriptional start site is correct by 5' RACE analysis, as described in Materials and Methods (Fig. 6C).

To verify the TorR-binding site in an *in vivo* experiment, we constructed two *glpT*-promoter-*lacZ* plasmids; one was designated pNNgIpT-P, which contains a 300-bp region upstream of the *glpT* translational start site fused to a promoterless *lacZ* gene, and the other was designated pNNgIpT-P1, which contains the proposed TorR-binding site on pNNgIpT-P from which an 18-bp region was removed. The LacZ activity in the wild-type parent containing plasmid pNNgIpT-P was decreased when the strain was anaerobically grown with TMAO, but this decrease did not occur in the *torR* mutant (Fig. 7). In addition, TMAO did not repress LacZ expression in the wild-type parent carrying pNNgIpT-P1 (Fig. 7). These observations indicate that TorR binds the decameric consensus sequence (CTGTTAATCA, where the underlined sequence is the consensus sequence) between the transcriptional and translational start sites of *glpT* and then represses *glpT* gene transcription probably by blocking transcription elongation.

Fosfomycin does not activate the Tor pathway. In our previous study, we showed that fosfomycin activates the Cpx pathway and induces tolerance to fosfomycin by repressing the expression of *glpT* (21). To investigate whether or not the Tor pathway is also activated by fosfomycin, we grew the wild-type parent in medium with and without 0.2 μ g/ml of fosfomycin and compared the levels of the *torC* transcript between these strains by qPCR analysis. Cell growth was not affected with this concentration of fosfomycin. No significant difference in *torC* transcript levels between the strains was seen when they were grown with and without fosfomycin (see Fig. S2 in the supplemental material).

Overexpression of *torR* orthologs in the nonpathogenic K-12 strain and a UPEC strain also induces tolerance to fosfomycin. To determine if the Tor system induces fosfomycin tolerance in other *E. coli* species, we used strain MG1655 from the nonpathogenic K-12 subgroup and nongastrointestinal infectious uropathogenic *E. coli* (UPEC) strain CFT073. The sequences of the open reading frames for the *torR* and *glpT* orthologs among the MG1655, EHEC O157, and UPEC CFT073 strains were >95% identical (28, 29). We cloned *torR* ortholog genes of MG1655 and CFT073 into plasmid pTrc99A and then overexpressed the plasmid in parallel hosts. Similar to the results of the experiment with EHEC O157, survival rates after fosfomycin treatment were higher for the *torR*-overexpressing strains than for the vector control ($9.1\% \pm 2.3\%$ for the MG1655 control strain versus $100.7\% \pm 4.3\%$ for *torR*-overexpressing strain MG1655 and $7.4\% \pm 2.1\%$ for the CFT073 control strain versus $45.3\% \pm 5.7\%$ for *torR*-overexpressing strain CFT073) (Fig. 8).

DISCUSSION

The decline in the use of fosfomycin, as well as the biological cost conferred by the acquisition of resistance to fosfomycin, has contributed to a lower incidence of fosfomycin resistance. In an era of a shortage of new antimicrobial agents, particular interest in fosfomycin has recently resurfaced. We aimed to get insight into the molecular mechanism by which innate tolerance is induced; such insight will aid us with the establishment of a method that enhances the efficacy of this drug.

Reversible mechanisms to control fosfomycin tolerance that do not depend on genetic modifications, including chromosomal mutations in *glpT*, *uhpT*, and/or *murA*, would allow EHEC to relieve the biological cost conferred by fosfomycin resistance in fosfomycin-free circumstances and could thus be beneficial for EHEC in respect to fitness.

We previously identified a reversible mechanism operated by the CpxAR two-component system (21). Activation of the Cpx pathway leads to increased tolerance to fosfomycin and decreased uptake of the carbon substrates glycerol-3-phosphate and glucose-6-phosphate due to repression of GlpT and UhpT production when a sub-MIC of fosfomycin is present.

In this study, we found that the Tor pathway induces fosfomycin tolerance (Table 3; Fig. 1). Activation of the Tor pathway reduced the level of *glpT* expression (Fig. 2, 4, and 5A) and decreased the amount of intracellular fosfomycin, which led to increased tolerance to fosfomycin (Fig. 3 and 5B). However, unlike the Cpx pathway, the Tor pathway was not activated by fosfomycin (see Fig. S2 in the supplemental material) and TorR directly impaired *glpT* transcription elongation, resulting in a decrement of GlpT protein production without cross talk with the Cpx pathway (Table 3; Fig. 6). We also found that the Tor orthologs in a nonpatho-

FIG 8 Survival rates of nonpathogenic K-12 (MG1655) (A) and uropathogenic (CFT073) (B) strains harboring pTrc99A (vector control) or pTrc99torR (a TorR-overexpressing plasmid) after incubation with or without 1.56 μ g/ml fosfomycin. The survival rates are described as the percentage of the number of CFU/ml for the strain after incubation with fosfomycin relative to that after incubation without fosfomycin. Data plotted are the means from three independent experiments; error bars indicate standard deviations. ****, $P < 0.0005$.

genic K-12 strain and a nondiarrheic uropathogenic strain (UPEC) also participate in the induction of fosfomycin tolerance (Fig. 8). The Tor-mediated fosfomycin tolerance mechanism may be conserved among strains of *E. coli* and related species.

The Tor pathway was originally characterized to be a regulatory system for the anaerobic reduction of TMAO (35). *E. coli* species, including EHEC, use TMAO as an electron acceptor for their anaerobic respiration, where TMAO is reduced to trimethylamine (TMA) to allow the cell to generate the proton motive force required for ATP synthesis (37). The Tor pathway is activated when TMAO is present in the medium. TorS is autophosphorylated when the complex of TMAO and TorT interacts with the sensing domain of this protein and then transfers the phosphate to its cognate response regulator, TorR (24). Phosphorylated TorR stimulates the promoter of the *torCAD* operon, encoding proteins that are responsible for TMAO respiratory system (38). The Tor pathway also activates the *tnaA* gene, encoding tryptophanase, which is proposed to counteract alkaline stress, and represses the *gadAB* and the *hdeABD* genes, which contribute to bacterial survival under acidic pH conditions (26). Regulation by Tor also contributes to protection against alkalization of the me-

dium after TMA production. In addition to these genes, we have found another gene regulated by the Tor system.

The TMA precursor is produced by gut microorganisms from carnitine, lecithin, or phosphatidylcholine contained in food (for example, red meat) and oxidized into TMAO in liver, and eventually, the oxidized molecule is transported via blood to the enteric site (39). Therefore, the activation of the Tor pathway by TMAO could occur when EHEC is in the animal gut. Animal studies have indicated that TMAO levels can be affected by the gut microflora, which produces TMA from dietary carnitine and phosphatidylcholine, resulting in an increased risk for cardiovascular disease (CVD) (40, 41). These results imply that the activity of the Tor system may be associated with the levels of TMAO derived from the TMA producers in the gut, which could affect the level of susceptibility to fosfomycin in *E. coli* species.

E. coli species can 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, including the TMAO reductase (37, 42). Thus, repression of GlpT production by Tor activation may lead to defects in not only carbon source availability but also anaerobic electron transport. However, if any alternative carbon sources and electron donors are present at sufficient levels in the gut, cells with fosfomycin tolerance conferred by transient Tor activation may be able to grow without being outcompeted by the susceptible members.

Genetic modifications, such as chromosomal mutations and acquisition of external genes, can provide bacteria with long-term drug resistance that leaves genetic scars and then often confers an irreversible fitness burden. We suggest that the mechanism of control of tolerance to antibiotics that depends on environmental changes that do not leave any genetic scars, which is often difficult to detect in clinical situations by current molecular techniques, may be an important clinical issue to keep in mind. However, this theory encourages us to create a concept that we can make fosfomycin treatment more effective if we can close reversible mechanisms, such as the Cpx and Tor pathways.

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