Tet38 Efflux Pump Contributes to Fosfomycin Resistance in *Staphylococcus aureus*

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ABSTRACT Fosfomycin inhibits MurA following uptake by the GlpT transporter of glycerol-3-phosphate in *Escherichia coli*. In *Staphylococcus aureus*, plasmid overexpression of the Tet38 efflux pump and a *glpT* mutant resulted in increased MICs and decreased accumulation of fosfomycin, with MICs affected by glycerol-3-phosphate. In contrast, a *tet38* mutant had a lower MIC and increased accumulation of fosfomycin, suggesting that Tet38 acts as an efflux transporter of fosfomycin.

KEYWORDS *S. aureus*, efflux pump, Tet38, fosfomycin, G3P

The expanded use of fosfomycin is being evaluated because of increasing resistance to other antimicrobial agents. Fosfomycin is distinct among the currently available antimicrobials used in clinical medicine in targeting the MurA protein involved in cell wall synthesis (1–3). Resistance to fosfomycin in *Staphylococcus aureus* has been shown to be due to *fosB*, a plasmid-located gene that encodes a thiol-S-transferase that inactivates fosfomycin (4, 5), or to mutations in fosfomycin uptake transporters GlpT and UhpT (1, 6).

Uptake of fosfomycin and glycerol-3-phosphate (G3P) by GlpT has been demonstrated directly in *Escherichia coli* but not studied directly in *S. aureus* (7, 8). The transport of fosfomycin by GlpT is weakly competitively inhibited by G3P (8).

Tet38 is a chromosomally encoded major facilitator superfamily efflux transporter of *S. aureus* that has a substrate profile that includes structurally diverse compounds and that has a role in *S. aureus* binding to and uptake by epithelial cells (9–11). Fosfomycin resistance in *S. aureus* has not been shown to be due to efflux mechanisms, but because of its broad substrate profile, we tested the possibility that Tet38 affects susceptibility to fosfomycin. We show here that susceptibility to and accumulation of fosfomycin are affected by *tet38* in a manner suggesting that fosfomycin is an efflux substrate of Tet38 and that this effect is modulated by G3P.

All strains and plasmids used in this study are listed in Table 1. *S. aureus* was cultivated in Luria-Bertani broth (LB; Difco, Sparks, MD) at 37°C, unless otherwise stated. All antibiotics and compounds were purchased from the Sigma-Aldrich Chemical Co. (St. Louis, MO). All primers in this study were synthesized from Eton Bioscience, Inc., Boston, MA.

The MIC was determined as the lowest concentration of antibiotic in a series of 2-fold dilutions that yielded no visible growth after incubation at 37°C for 24 h. The addition of G3P was at a 5 mM final concentration.

Construction of mutant strain QT03 (*glpT::Tn*). QT03 was constructed by phage ϕ 85 transduction using the *S. aureus* donor USA300 (*glpT::Tn*) (Nebraska Transposon Mutant Library) and RN6390 as a recipient (10, 12). The transductants were selected on Trypticase soy agar plates supplemented with erythromycin (10 μ g/ml), and the modified sequence was confirmed by DNA sequencing.

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TABLE 1 Bacterial strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Genotype, description, or DNA sequence ^a	Reference or source
Strains		
<i>S. aureus</i>		
RN6390	8325-4 wild type	9
QT03	8325-4 <i>glpT::Tn</i>	This study
QT7	8325-4 <i>tet38</i> mutant	9
USA300 (<i>glpT::Tn</i>)	<i>glpT::Tn</i> , Em ^r	12
RN6390(pLI50)	Cm ^r	This study
RN6390(pLI50- <i>glpT</i>)	<i>glpT</i> overexpressor, Cm ^r	This study
RN6390(pLI50- <i>tet38</i>)	<i>tet38</i> overexpressor, Cm ^r	This study
QT03(pLI50- <i>glpT</i>)	<i>glpT</i> complemented, Cm ^r	This study
QT7(pLI50- <i>tet38</i>)	<i>tet38</i> complemented, Cm ^r	This study
<i>E. coli</i>		
TOP10	F [−] <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) φ80 <i>lacZ</i> Δ <i>M15</i> Δ <i>lacX74</i> <i>nupG</i> <i>recA1</i> <i>araD139</i> Δ(<i>ara-leu</i>)7697 <i>galE15</i> <i>galK16</i> <i>rpsL</i> (Str ^r) <i>endA1</i> λ [−]	9
Plasmids		
pLI50	Shuttle plasmid <i>E. coli</i> - <i>S. aureus</i> , Cm ^r	10
Primers by target (restriction site)		
<i>tet38</i>		
Forward (HindIII)	TCATTGGTGTAGAAGCTTATGATTATGAAT	
Reverse (BamHI)	CGCGAATTTATATCTATGGATCCTTATTTT	
<i>glpT</i>		
Forward (HindIII)	TTTATCATAAGCTTTTAATGATGAAC	
Reverse (BamHI)	TAAGGAGGATTTTGGATCCATGAATTT	

^aEm^r, erythromycin resistance; Cm^r, chloramphenicol resistance. Restriction sites for the primer sequences are underlined.

Construction of *glpT* and *tet38* overexpressor strains. *glpT* (SA0325) and *tet38* (SA0132) were amplified with primers designed from *S. aureus* N315 and were cloned into plasmid pLI50 (Table 1) (13). Plasmids pLI50-*glpT* and pLI50-*tet38* were first constructed in *E. coli* TOP10, and then the transformants were selected on LB agar supplemented with 100 μg/ml ampicillin. The recombinant plasmids were subsequently reextracted and reintroduced into *S. aureus* RN4220 and then into *S. aureus* RN6390. Transformants were selected on LB agar supplemented with 10 μg/ml chloramphenicol and grown at 37°C. The *glpT* and *tet38* sequences were confirmed by DNA sequencing.

Accumulation assay. The accumulation assay was based on the technique described by Kurabayashi et al., with some modifications (14). *S. aureus* cells were grown in LB medium until an optical density at 600 nm (OD₆₀₀) of 0.6, were harvested and resuspended in 2 ml of LB supplemented with 10-fold the MIC of fosfomycin, and then were incubated at 37°C for 1 h. The bacteria were washed thrice with phosphate-buffered saline, resuspended in 500 μl of water, boiled for 3 min, and centrifuged to collect the pellets. The supernatants (40 μl) were deposited on 6-mm discs and placed on LB plates spread with *S. aureus* RN6390, according to the agar diffusion technique (10). Commercial fosfomycin was used to create a standard curve (diameter of zone of inhibition/μg fosfomycin) used to determine the amount of fosfomycin extracted from equivalent amounts of bacterial cells.

Effect of *tet38* and *glpT* on activity of fosfomycin. Compared to the parental strain RN6390, plasmid-mediated overexpression of *tet38* resulted in a 4-fold increase in the MIC of fosfomycin, and QT7 (*tet38* mutant) had a 2-fold decrease in MIC (Table 2), suggesting that Tet38 acts as an efflux transporter of fosfomycin. In contrast, plasmid overexpression of *glpT* did not affect the MIC of fosfomycin, but QT03 (*glpT* mutant) had an 8-fold increase in MIC, suggesting that GlpT is involved in the uptake of fosfomycin in *S. aureus* as it is in *E. coli*. Plasmid-expressed *tet38* complemented the *tet38* mutant,

TABLE 2 Susceptibility of *S. aureus* to fosfomycin, tetracycline, and palmitoleic acid

<i>S. aureus</i> strain	MIC ($\mu\text{g/ml}$) ^a					
	FOS		TET		PAL	
	–G3P	+G3P	–G3P	+G3P	–G3P	+G3P
RN6390	1	1	0.125	0.06	128	128
QT7 ^b	0.5	0.5	0.06	0.06	128	128
QT03 ^b	8	4	0.125	0.125	128	128
RN6390(pLI50) ^c	1	1	0.125	0.06	128	128
RN6390(pLI50- <i>tet38</i>)	4	1	0.5	0.25	1024	256
RN6390(pLI50- <i>glpT</i>)	1	1	0.125	0.125	128	128
QT7(pLI50) ^c	0.5	0.5	0.06	0.06	128	128
QT7(pLI50- <i>tet38</i>)	4	1	0.25	0.125	512	256
QT03(pLI50) ^c	8	4	0.125	0.125	128	128
QT03(pLI50- <i>glpT</i>)	1	1	0.125	0.125	128	128

^aFOS, fosfomycin; TET, tetracycline; PAL, palmitoleic acid; G3P, glycerol-3-phosphate at 5 mM.^bQT7, *tet38* mutant; QT03, *glpT* mutant.^cAll strains harboring plasmid pLI50 were grown in the presence of 10 $\mu\text{g/ml}$ chloramphenicol and at 37°C.

and plasmid-expressed *glpT* complemented the *glpT* mutant (Table 2). The *tet38* and *glpT* transcripts were increased 10-fold by quantitative reverse transcription-PCR (qRT-PCR) in strains carrying the plasmids with the respective cloned genes compared to the strains without these plasmids (data not shown).

Since fosfomycin transport by GlpT is competitively affected by its primary substrate G3P in *E. coli*, we tested the effect of G3P on the activity of fosfomycin. At a concentration of 5 mM, the addition of G3P resulted in a 4-fold reduction in the MIC of fosfomycin for the *tet38*-overexpressing strain and a 2-fold reduction for QT03 (*glpT* mutant), but it had no effect on the parental strain (Table 2). To determine if the effect of G3P was specific for fosfomycin, we tested its effect on two other substrates of Tet38, tetracycline and palmitoleic acid. The addition of G3P resulted in a 2-fold reduction in the tetracycline MIC for both wild-type and *tet38* overexpressor strains and a 4-fold reduction in the palmitoleic acid MIC for the *tet38* overexpressor strain. Thus, the addition of G3P also affects *tet38*-mediated resistance to two other substrates in addition to fosfomycin. Reserpine is a known inhibitor of a number of *S. aureus* major facilitator superfamily (MFS) pumps. At a final concentration of 10 $\mu\text{g/ml}$, reserpine reduced the MICs of tetracycline and palmitoleic acid by 2-fold and the MIC of fosfomycin by 4-fold in RN6390 and USA300 (data not shown).

Accumulation of fosfomycin in *S. aureus* mutants and overexpressors. Using a previously described bioassay for bacterial cell accumulation of fosfomycin (14), we determined if the effects of *tet38* and *glpT* on fosfomycin MICs could be correlated with drug accumulation. Fosfomycin accumulation was decreased 2- to 3-fold in the *tet38* overexpressor strain and QT03 (*glpT* mutant) (Table 3), findings consistent with changes in fosfomycin MICs and supporting a role for Tet38 as an efflux transporter and GlpT as an uptake transporter of fosfomycin in *S. aureus*.

TABLE 3 Accumulation of fosfomycin in *S. aureus* cells

<i>S. aureus</i> strain	Fosfomycin amt (μg) ^a	Fosfomycin concn ($\mu\text{g}/\mu\text{l}$) ^b
RN6390	24.5 $\pm$ 3	0.61 $\pm$ 0.5
QT7 (<i>tet38</i> mutant)	25.0 $\pm$ 2	0.63 $\pm$ 0.3
QT03 (<i>glpT</i> mutant)	14.5 $\pm$ 2	0.36 $\pm$ 0.5
RN6390(pLI50)	24.5 $\pm$ 1	0.61 $\pm$ 0.2
RN6390(pLI50- <i>tet38</i>)	8.0 $\pm$ 1	0.23 $\pm$ 0.2
RN6390(pLI50- <i>glpT</i>)	26.5 $\pm$ 2	0.65 $\pm$ 0.4

^aThe amount of intracellular fosfomycin of *S. aureus* strains was quantified by first measuring the diameter (in millimeters) of the inhibition zones of *S. aureus* RN6390 agar culture (40 μl of supernatant per disc, 10⁶ bacterial cells), and then the amount of drug in micrograms was calculated based on a standard fosfomycin curve (micrograms per millimeter). The assays were repeated three times using three separate biological samples.^bThe concentrations of fosfomycin were calculated using 40 μl as the deposited volume of supernatant per *S. aureus* strain.

Summary. We previously demonstrated that Tet38 extrudes tetracycline and palmitoleic acid (10, 11) and that its expression is increased in subcutaneous abscesses (15). Fosfomycin also appears to be an efflux substrate of Tet38. Tet38 transport of fosfomycin and Tet38 substrates is affected by G3P, another substrate of GlpT, a known importer of fosfomycin (6, 8, 14), suggesting that G3P can act as a competitive inhibitor of Tet38 substrates, including fosfomycin. Our data indicate that fosfomycin accumulation and activity are reduced when *tet38* is overexpressed and suggest that fosfomycin activity is likely to be reduced under conditions that induce the expression of *tet38*, such as in abscess infections (15).

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