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Effect of *Staphylococcus aureus* Tet38 native efflux pump on *in vivo* response to tetracycline in a murine subcutaneous abscess model

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Objectives: *Staphylococcus aureus* native efflux pump Tet38 confers resistance to tetracycline when overexpressed. *tet38* expression is selectively upregulated in infection sites. This study evaluated the effect of Tet38 on tetracycline response in a murine subcutaneous abscess model.

Methods: *S. aureus* MW2 and its *tet38* mutant were injected subcutaneously on the opposite flanks of each mouse. The infected mice were treated with tetracycline (10 mg/kg) or PBS (control) intraperitoneally every 12 h. The efficacy of tetracycline against *S. aureus* was measured by the relative change in viable bacteria in the abscesses 24 h after infection compared with the initial inoculum. Plasmid-based *tet38*-complemented strains were created and used to infect the mice followed by tetracycline or PBS treatment.

Results: The increased bacterial loads of *S. aureus* MW2 and its *tet38* mutant in the abscess after 24 h were similar. Tetracycline produced significant decreases of both MW2 and the *tet38* mutant compared with control. Although the tetracycline MICs for MW2 and the *tet38* mutant did not differ *in vitro*, the antibacterial effect of tetracycline was significantly 2-fold greater in the *tet38* mutant compared with the MW2 parental strain *in vivo* with a decrease of 0.67 ± 0.21 and 0.35 ± 0.19 $\log_{10}$ cfu/abscess, respectively ($P < 0.05$). The increased tetracycline activity in the *tet38* mutant was complemented by plasmid-encoded *tet38*.

Conclusions: This study demonstrated that selective increased expression of *tet38* in an abscess can affect tetracycline efficacy against *S. aureus in vivo*, highlighting an effect of native efflux pumps on response to therapy not reflected by testing *in vitro*.

Introduction

Staphylococcus aureus is an important human pathogen that commonly causes skin and soft-tissue infections with subcutaneous abscess formation.[1] Tetracyclines have been used as empirical antibiotic therapies for *S. aureus* skin infections, including those due to strains resistant to methicillin,[2] but resistance to tetracyclines has emerged.[3] Resistance to tetracyclines is often associated with plasmid-mediated genes encoding active efflux pumps or proteins that protect ribosomes from drug action.[4] Tet38, in contrast, is a chromosomally encoded native efflux pump in *S. aureus* that when overexpressed from a plasmid confers a 32-fold increase and 4–8-fold increase in MICs of tetracycline and antibacterial fatty acids, respectively.[5,6] Expression of *tet38* is upregulated in sites of infection, such as murine abscesses and endocardial vegetations,[7,8] and thus may have greater effects *in vivo* than under laboratory conditions with limited expression. Although the physiological roles of Tet38 overexpression, independent of its protection from tetracycline action, may relate to its ability to confer resistance to antibacterial fatty acids, the trigger for overexpression in the abscess environment has not been fully evaluated. Various efflux pumps have been well characterized in *S. aureus*, few studies have examined the effect of these efflux pumps on response to therapy in infection models.[9,10] The association of upregulated expression of efflux pumps with clinically significant antibiotic resistance has been well established in Gram-negative organisms.[11,12] These transporters can act synergistically with the permeability barrier of the outer membrane, which enhances multiple drug resistances and can result in antibiotic treatment failure.[13] Examples of the well-studied efflux systems include *Escherichia coli* AcrAB–TolC[14] and *Pseudomonas aeruginosa* MexAB–OprM and related systems.[15,16] Because Gram-positive pathogens lack the outer membrane of Gram-negative bacteria that can enhance the effect of efflux pumps, we assessed whether the chromosomally encoded Tet38 efflux pump, which is selectively overexpressed *in vivo* could affect the response to antibiotic treatment of *S. aureus* infection. In the present study, we assessed the *in vivo* activity of tetracycline against MRSA strain MW2 and an otherwise isogenic *tet38* mutant in a murine

Table 1. Bacterial strains, plasmids and primers used in this study

	Genotype, characteristic(s) or sequences	Reference or source
S. aureus strains		
RN4220	restriction-deficient mutagenized RN450	7
MW2	CA-MRSA (USA 400 lineage)	7
MW2 *tet38*	*tet38* partial deletion mutant with *cat* gene insertion	7
MW2 (pMSP)	MW2 carrying empty plasmid vector pMSP3535	this study
MW2 *tet38* (pMSP)	MW2 *tet38* carrying empty plasmid vector pMSP3535	this study
MW2 *tet38* (pTet38)	*tet38*-complemented strain	this study
E. coli strains		
E. coli DH5a	general host for pMSP3535 vector	Invitrogen
Plasmids		
pMSP3535	Gram-positive bacterial shuttle vector, ERY^{Ra}	20
pMSP::*tet38* (pTet38)	pMSP3535 with *tet38* gene, ERY^{R}	this study
PCR primers (5′–3′)		
tet38 XhoI-For[b]	GCTACTCGAGTGGATGCGTATGGGTATTTTAG	this study
tet38 BamHI-Rev	GCTAGGATCCTTATTTTTCAGATTGTGTCCAACG	this study
pMSP-For	AATGCAGGTTAACCTGGCTTATC	this study
pMSP-Rev	TGCATCACCACGCATTACAA	this study
qRT–PCR primers (5′–3′)		
gmk-RT For	ACTAGGGATGCGTTTGAAGC	7
gmk-RT Rev	TCATGACCTTCGTCCATTGT	7
tet38-RT For	TGACAGGTGTGGCTATTGGT	this study
tet38-RT Rev	TTGCCTGGGAAATTTAATGC	this study

[a]ERY, erythromycin. Plasmid selection: 300 mg/L erythromycin for *E. coli* and 10 mg/L erythromycin for *S. aureus*.
[b]Restriction sites are indicated by underlining.

subcutaneous abscess model, which represents a common site of infection with *S. aureus*.

Materials and methods

Strains, plasmids and primers

The bacterial strains, plasmids and primers used in this study are listed in Table 1. The MW2 *tet38* mutant was generated in previous study.[7] To construct *tet38*-complemented strains, the *tet38* gene including its promoter region was amplified by PCR, then cloned into the pMSP3535 vector. Plasmids pMSP3535 and pMSP3535::*tet38* (pTet38) were transformed into *S. aureus* RN4220 and then into MW2 and the MW2 *tet38* mutant following selection with 10 mg/L erythromycin. The presence of *tet38* was confirmed by PCR and DNA sequencing.

Ethics

All animal studies were approved by Institutional Animal Care and Use Committees, Office of Laboratory Animal Welfare and followed the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care.

Mouse abscess model and tetracycline treatment in vivo

Swiss Webster male mice aged 6–8 weeks (weight 25–30 g, Charles River Laboratories Wilmington, MA, USA) were housed in biosafety level 2 facilities located at Massachusetts General Hospital (Boston, MA, USA). The mouse subcutaneous abscesses model was as previously described.[6] Briefly, *S. aureus* strains MW2 and its isogenic *tet38* mutant ($\sim 1\times10^6$ cfu in a volume of 0.2 mL) were injected subcutaneously on the opposite flanks of each mouse after anaesthesia. Infected mice were randomly allocated to the tetracycline treatment group or PBS control group (nine mice per group). Tetracycline (10 mg/kg) or PBS was injected intraperitoneally immediately and 12 h after infection. Twenty-four hours after infection (12 h after the last treatment), mice were euthanized and the abscesses were excised and homogenized. The viable bacterial burden was determined by counting cfu. The relative fold change ($\log_{10}$ cfu/abscess) was measured by comparing the viable bacteria recovered from the abscesses with the initial inoculum. All the experiments were performed at least three times. Statistical differences were analysed using the non-parametric Wilcoxon signed-rank test and $P < 0.05$ was considered significant.

RNA isolation and real-time quantitative RT–PCR

Total *S. aureus* RNA was isolated using the Qiagen RNeasy mini kit following the manufacturer's instructions. *tet38* expression was measured by quantitative RT–PCR (qRT–PCR) with the housekeeping gene *gmk* as internal control.[7] The primers are listed in Table 1. All the experiments were performed in triplicate, with three independent biological samples. The difference in relative expression was analysed using the Mann–Whitney *U*-test and $P < 0.05$ was considered significant.

Results and discussion

Tet38 selectively reduces tetracycline activity in vivo

The MICs of tetracycline *in vitro* did not differ between MW2 and its *tet38* mutant (0.25 mg/L). Because we had also shown previously that *tet38* was selectively overexpressed in an abscess environment,[7] we sought to evaluate whether *tet38* has an effect on response to tetracycline *in vivo* using an abscess model. The viable

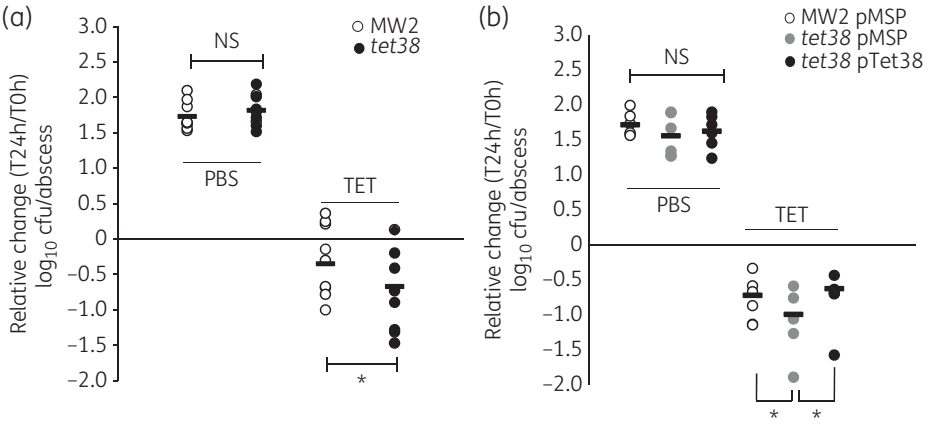

Figure 1. Tetracycline effect on MW2, the *tet38* mutant and *tet38*-complemented strains in a mouse subcutaneous abscess model. (a) Relative change in $\log_{10}$ cfu/abscess of MW2 and the *tet38* mutant in the control group (PBS) and the TET. (b) Relative change in $\log_{10}$ cfu/abscess of *tet38*-complemented strains in the control group (PBS) and the TET. Mice were infected with ∼6 $\log_{10}$ cfu of bacteria in each abscess; tetracycline at 10 mg/kg or PBS was administered intraperitoneally every 12 h. Each circle represents the relative change in bacterial load in each abscess ($\log_{10}$ cfu/abscess) at 24 h after infection compared with the initial inoculum ($T = 0$ h). Horizontal bars represent the means of each group. NS, not significant ($P > 0.05$). *$P < 0.05$. TET, tetracycline-treated group.

bacteria recovered from either MW2- or MW2 *tet38*-infected abscesses in the PBS group after 24 h were similar, with 1.73±0.07 and 1.82±0.07 $\log_{10}$ cfu/abscess increase compared with the inoculum, respectively ($P = 0.192$) (Figure 1a). Tetracycline at 10 mg/kg had *in vivo* activity in mice infected with MW2 and those infected with the *tet38* mutant compared with the PBS-treated mice. Importantly, the recovered bacterial load of the *tet38* mutant was ∼2-fold less than that of the MW2 parental strain (with decrease of 0.35±0.19 versus 0.67±0.21 $\log_{10}$ cfu/abscess, $P = 0.028$) (Figure 1a). The *tet38* mutant also demonstrated a bacterial load 2-fold lower than that of MW2 when treated with tetracycline at 5 mg/kg, but the difference did not reach statistical significance ($P = 0.066$, data not shown). Thus, the *tet38* mutant was more sensitive to tetracycline treatment *in vivo*, indicating that the presence of Tet38 improved the survival of *S. aureus* in the abscesses of mice treated with tetracycline, an effect that is not apparent with *in vitro* testing.

To validate further that the decreased activity of tetracycline against the *tet38* mutant was due to the disruption of *tet38*, we compared tetracycline *in vivo* activity against a *tet38*-complemented strain (*tet38* pTet38) with MW2 and the *tet38* mutant carrying the empty vector. The expression of *tet38* in *tet38* pTet38 was stable with a 6.3±1.5-fold increase compared with *tet38* pMSP3535 as evaluated by qRT–PCR ($P < 0.05$). The plasmids were stable in abscesses as determined by plating the strains recovered from the abscesses on tryptone soya agar plates containing erythromycin. Twenty-four hours after infection, the recovered viable bacteria of MW2 pMSP, *tet38* pMSP and *tet38* pTet38 from abscesses in PBS-treated mice were similar with an increase of 1.72±0.07, 1.56±0.12 and 1.62±0.1 $\log_{10}$ cfu/abscess, respectively ($P > 0.05$) (Figure 1b). In tetracycline-treated mice, *tet38* pMSP exhibited a higher reduction in bacterial burden in abscesses (1±0.16 $\log_{10}$ cfu/abscess) than WT MW2 pMSP (0.72±0.13 $\log_{10}$ cfu/abscess, $P < 0.05$) and *tet38*-complemented strain *tet38* pTet38 (0.63±0.16 $\log_{10}$ cfu/abscess, $P < 0.05$) (Figure 1b), further validating the effect of Tet38 on the response to tetracycline *in vivo*.

It has been shown that expression levels of efflux pumps influenced antibiotic efficacy against infections caused by Gram-negative pathogens.[14–16] The possible clinical relevance of pump-related drug resistance in *S. aureus* is supported by observations revealing augmented expression of various pumps such as NorA and NorB at sites of infection[17] and carriage of plasmid-borne efflux pumps QacA/B.[18] There have, however, been no data assessing the direct association of chromosomally encoded efflux pump overexpression with treatment response *in vivo* in a Gram-positive pathogen that lacks the outer membrane barrier that can enhance the effect of efflux pumps. This study demonstrated that the chromosomally encoded Tet38 efflux pump, which is selectively upregulated in the infection sites, reduces the tetracycline efficacy against *S. aureus* in subcutaneous abscesses, whereas no difference in MICs was observed *in vitro*. The findings support the concept that the selective expression of resistance genes in an abscess or other infection environment can affect response to therapy in a manner that may not be readily detected by routine susceptibility testing *in vitro*, reflecting a perhaps under-appreciated insidious effect of efflux pumps on response to therapy. As in all animal studies, the extrapolation of the results to responses to human infections is uncertain.

Triggers for tet38 upregulation in abscesses

Efflux pumps not only confer antibiotic resistance but also have physiological roles in response to the host environment.[19] Our previous study showed that *tet38* was selectively upregulated by 24-fold in the murine abscess model compared with *in vitro*.[7] To look for those conditions present in the abscess environment that may be signals for *tet38* overexpression, we evaluated *tet38* expression by exposure *in vitro* to a variety of signals mimicking those in an abscess environment, including starvation (growth in PBS), reduced oxygen tension, acid stress (pH 5.5) and free iron restriction. None of these conditions, however, induced the upregulation of *tet38* transcription (data not shown). We have previously showed that *tet38* expression can be induced by certain

fatty acids, which are also present in abscesses.[6] Therefore, the upregulation of *tet38* in abscesses may relate to its exposure to antibacterial fatty acids or yet undefined host factors that exist in the abscess environment. The additional effects of efflux pumps with natural substrates on bacterial fitness *in vivo* further highlight their importance as a challenge to antimicrobial therapy.

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Transparency declarations

None to declare.

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