

# Role of the Tet38 Efflux Pump in *Staphylococcus aureus* Internalization and Survival in Epithelial Cells

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We previously identified the protein Tet38 as a chromosomally encoded efflux pump of *Staphylococcus aureus* that confers resistance to tetracycline and certain unsaturated fatty acids. Tet38 also contributes to mouse skin colonization. In this study, we discovered a novel regulator of *tet38*, named tetracycline regulator 21 (TetR21), that bound specifically to the *tet38* promoter and repressed pump expression. A  $\Delta tetR21$  mutant showed a 5-fold increase in *tet38* transcripts and an 8-fold increase in resistance to tetracycline and fatty acids. The global regulator MgrA bound to the *tetR21* promoter and indirectly repressed the expression of *tet38*. To further assess the full role of Tet38 in *S. aureus* adaptability, we tested its effect on host cell invasion using A549 (lung) and HMEC-1 (heart) cell lines. We used *S. aureus* RN6390, its  $\Delta tet38$ ,  $\Delta tetR21$ , and  $\Delta mgrA$  mutants, and a  $\Delta tet38 \Delta tetR21$  double mutant. After 2 h of contact, the  $\Delta tet38$  mutant was internalized in 6-fold-lower numbers than RN6390 in A549 and HMEC-1 cells, and the  $\Delta tetR21$  mutant was internalized in 2-fold-higher numbers than RN6390. A slight increase of 1.5-fold in internalization was found for the  $\Delta mgrA$  mutant. The growth patterns of RN6390 and the  $\Delta mgrA$  and  $\Delta tetR21$  mutants within A549 cells were similar, while no growth was observed for the  $\Delta tet38$  mutant. These data indicate that the Tet38 efflux pump is regulated by TetR21 and contributes to the ability of *S. aureus* to internalize and replicate within epithelial cells.

Efflux mechanisms are widely recognized as major contributors to resistance to many classes of antimicrobial agents via a diverse group of transporters also called efflux pumps (1, 2). These pumps are implicated in a variety of physiological roles, including the extrusion of drugs and other natural substrates (3, 4). Variations in the expression of efflux pumps can be influenced by several factors, such as antibiotics, natural compounds, and environmental conditions. Examples of such induction phenomena were reported in the expression of the efflux pump genes *norA*, *norB*, and *tet38* of *Staphylococcus aureus* in response to low free iron, acidity/low-oxygen conditions, and tetracycline and fatty acids, respectively (5–7). Recently, the resistance-nodulation-division (RND) family efflux pump FarE of the fatty acid resistance system *farR-farE* of *S. aureus* was reported to be induced by linoleic and arachidonic acids (8). The overexpressed FarE efflux pump extrudes and confers resistance to these two fatty acids. FarE expression is controlled by the regulator FarR, a member of the AcrR family of regulators (8). Several efflux pumps, such as CmeAB of *Campylobacter jejuni* and AcrAB of *Escherichia coli*, have also been shown to have dual functions, conferring resistance to antimicrobial compounds and contributing to the bacterium's ability to invade and survive in host cells or specific host environments (9, 10).

In our previous studies, we demonstrated that Tet38, a chromosomally encoded efflux pump of *S. aureus*, causes resistance to tetracycline and fatty acids and contributes to the ability of *S. aureus* to colonize mouse skin, as well as survive in the environment of an abscess (7, 11). We also demonstrated that the global regulator MgrA was an indirect negative regulator of *tet38* expression (12). The regulation of *tet38* expression and its role in *S. aureus* colonization are not well understood. *S. aureus* expresses *agr*-, *sar*-, and *sae*-dependent surface components, such as fibronectin-binding proteins, that enable it to adhere to and invade the host cells by binding to the host cell  $\alpha_5\beta_1$  integrins (13–15). The interaction of *S. aureus* with host cells provokes the rearrangement of the host cell actin cytoskeleton, which leads to the inter-

nalization of the pathogen into these cells (16). *S. aureus* is internalized in epithelial cells in a time- and dose-dependent manner. This invasion and the subsequent bacterial intracellular replication result in cell apoptosis (17–19). Although fibronectin-binding proteins have been shown to be important for the internalization of *S. aureus* into epithelial cells (13, 20), mutants lacking these proteins had residual binding and internalization capabilities, suggesting that other factors were also involved. We show here that Tet38 also contributes to both internalization and survival within epithelial cells and that its expression is regulated by, in addition to MgrA, a newly characterized transcriptional regulator, tetracycline regulator 21 (TetR21), which also mediates the induction of *tet38* by tetracycline and select fatty acids.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** Bacterial strains were cultivated in Trypticase soy broth (TSB) (Difco, Sparks, MD) at 37°C, unless otherwise stated. *E. coli* strains were grown at 37°C in Luria-Bertani (LB) broth containing ampicillin (100  $\mu$ g/ml). Bacterial strains and plasmids are listed in Table 1.

**Antibiotics, chemical compounds, and MICs.** Lysostaphin, tetracycline, chloramphenicol, erythromycin, ciprofloxacin, norfloxacin, anhydrotetracycline, lysostaphin, linoleic acid, palmitoleic acid, palmitic acid, and undecanoic acid were from Sigma Chemical Co., St. Louis, MO. Am-

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TABLE 1 Bacterial strains, plasmids, and cell lines used in this study

Strain, plasmid, or cell line	Genotype or relevant characteristic(s)	Reference or source
<i>S. aureus</i> strains		
RN6390	8325-4 wild type	22
QT1	8325-4 <i>mgrA::cat</i> or $\Delta$ <i>mgrA</i>	25
QT5	8325-4 <i>norB::cat</i>	12
QT7	8325-4 <i>tet38::cat</i> or $\Delta$ <i>tet38</i>	12
QT21	8325-4 $\Delta$ <i>tetR21</i>	This study
QT22	QT7(pLI50- <i>tet38</i> )	This study
QT23	8325-4 <i>tet38::cat</i> $\Delta$ <i>tetR21</i>	This study
RN6390(pLI50- <i>tet38</i> )	<i>tet38</i> overexpressor	This study
QT1(pLI50- <i>mgrA</i> )	Complementing QT1 strain	This study
QT21(pLI50- <i>tetR21</i> )	Complementing QT21 strain	This study
MW2	CA-MRSA (USA400 lineage)	11
MW2 $\Delta$ <i>tetR21</i>	CA-MRSA mutant, $\Delta$ <i>tetR21</i>	This study
MW2 $\Delta$ <i>tet38</i>	CA-MRSA mutant, $\Delta$ <i>tet38</i>	This study
<i>E. coli</i> strains		
BL21(DE3)	<i>E. coli</i> B F <sup>-</sup> <i>dcm ompT hsdS</i> (r <sub>B</sub> <sup>-</sup> m <sub>B</sub> <sup>-</sup> ) <i>gal</i> $\lambda$ (DE3)	Stratagene
Top10	F <sup>-</sup> <i>mcrA</i> $\Delta$ ( <i>mrr-hsdRMS-mcrBC</i> ) $\phi$ 80 <i>lacZ</i> $\Delta$ M15 $\Delta$ <i>lacX74 nupG recA1 araD139</i> $\Delta$ ( <i>ara-leu</i> )7697 <i>galE15 galK16 rpsL</i> (Str <sup>r</sup> ) <i>endA1</i> $\lambda$ <sup>-</sup>	Invitrogen
DH10B	<i>E. coli</i> for cloning	23
BL21(DE3)(pTrcHisA- <i>mgrA</i> )	<i>MgrA</i> overexpressor	25
BL21(DE3)(pTrcHisA- <i>tetR21</i> )	<i>TetR21</i> overexpressor	This study
Plasmids		
pTrcHisA	Cloning and His tag-expressing vector in <i>E. coli</i>	Invitrogen
pIMAY	<i>E. coli/S. aureus</i> temp-sensitive plasmid	23
Cell lines		
A549	ATCC CCL-185, human lung adenocarcinoma	ATCC
HMEC-1	Immortalized human dermal microvascular endothelial cell line	27

picillin and isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) were from Fisher Scientific, Pittsburgh, PA. Protease inhibitor tablets were from Life Technologies, Grand Island, NY. The MICs of fatty acids and antibiotics were determined by TSB microdilution as described previously (21).

**Induction of *tet38* expression by fatty acids.** Cultures of *S. aureus* RN6390 and its mutants were inoculated from an overnight culture and incubated until the optical density at 600 nm (OD<sub>600</sub>) reached 0.5 (~2.5 to 3 h). Linoleic acid, palmitoleic acid, palmitic acid, undecanoic acid, or tetracycline at concentrations of 0.5-fold, 0.25-fold, and 0.10-fold MIC for each strain were added to the culture, followed by incubation at 37°C for 1 h. At intervals of 10 min, 1 ml of the culture was collected and centrifuged at 15,000  $\times$  g in a microcentrifuge. An identical bacterial culture without fatty acid or tetracycline was incubated in parallel to serve as a control. Bacteria in cultures without antibacterial compounds grew slightly faster (OD<sub>600</sub> of 0.60 after 3 h of growth) than the induced cultures. Bacteria were lysed, and RNA prepared for real-time quantitative reverse transcription (qRT-PCR) as previously described (22).

**Real-time qRT-PCR.** Total *S. aureus* RNA was extracted from lyso-staphin-treated cells using the RNeasy midi kit (Qiagen, Valencia, CA). cDNAs were synthesized using the Verso cDNA synthesis kit (Thermo Scientific, ABgene, Epsom, Surrey, United Kingdom), followed by real-time qRT-PCR assays using EvaGreen dye and the CFX96 real-time system (Bio-Rad, Hercules, CA). Primers designed for the qRT-PCR assays were synthesized at Tufts University, Boston, MA, and are listed in Table 2. The housekeeping gene *gmk* was used as an internal control. All samples were analyzed in triplicate, and expression levels normalized against *gmk* gene expression, which remained unchanged following exposure to fatty acids and tetracycline. The assays were repeated with three independent biological samples. Statistical analyses were performed based on the Student *t* test to determine the significance of the gene expression values.

**Transcript levels of *fnbA* and *fnbB* of *S. aureus* in vitro cultures.** We designed primers for real-time qRT-PCRs for the *fnbA* and *fnbB* genes based on DNA sequences of the published genome of *S. aureus* NCTC8325. RNAs were extracted from *S. aureus* strain RN6390 and mutant strains QT7 and QT21. Primers were synthesized by Tufts Core Facility (Table 2).

**Construction of a *tetR21* mutant.** We constructed a *tetR21* in-frame deletion mutant based on the technique described by Monk et al. (23). The following primers were synthesized by the Tufts DNA Core Facility: forward primer 21AF/KpnI, 5'-ATCAGGTACCTTAACCCATTAAATTTATTAGA-3' (the KpnI restriction site is underlined); reverse primer 21BR, 5'-CTTCTTTCAAAAATAAATCAACCTCCTTGT-3'; forward primer 21CF, 5'-ACAAGGAGGTTGATTTATTTTTGAAAGAAGTAAGTAATACTCGCTTTTCC-3', with the complementary sequence of primer 21BR (underlined); and reverse primer 21DR/SacI, 5'-TTTTGAGCTCAAA TTAATATCAGCTTAGGTAAAT-3' (the SacI restriction site is underlined). Primers 21AF/KpnI and 21BR were used to amplify a 429-bp sequence upstream from the open reading frame (ORF) SA2165, and a 459-bp downstream sequence was amplified by the pair 21CF and 21DR/SacI. The two PCR products were diluted 20-fold, and 1  $\mu$ l of each was used as the template for a second PCR with primers 21AF/KpnI and 21DR/SacI. The 888-bp PCR product carried a deleted SA2165 gene with KpnI and SacI restriction sites in the flanking end regions. The PCR product was cloned into plasmid pIMAY and transformed into *E. coli* DH10B. The transformants were grown at 37°C on LB plates containing chloramphenicol at 10  $\mu$ g/ml. The construct pIMAY-SA2165 $\Delta$  was extracted and reintroduced into *S. aureus* strain RN4220 and then into *S. aureus* RN6390 for subsequent allelic exchange. *S. aureus* transformants were grown at 28°C in the presence of chloramphenicol at 10  $\mu$ g/ml. DNA sequencing was performed at each transformation step to verify the plasmid construction. We named the new con-

TABLE 2 Primers used in this study

Purpose and primer or amplified gene	Direction	Sequence	Fragment size (bp)
<b>Real-time qRT-PCR</b>			
<i>gmk</i>	Forward	TCAGGACCATCTGGAGTAGGTAAAG	108
	Reverse	TTCACGGATTTGACGTGTTG	
<i>mgrA</i>	Forward	AGCTGAAGCGACTTTGTCAGATGC	110
	Reverse	AGCGTGAACGTTCCGAAGTCGA	
<i>tetR21</i>	Forward	ATTTTACTTACATTACGAGGATAAA	100
	Reverse	GCGTAACAAATCAAATTGAGTATATG	
<i>tet38</i>	Forward	ATGAATGTTGAATATTCTAA	106
	Reverse	TGGCTACAGAAATCAAT	
<b>Internal region SA0131-<i>tet38</i> (SA0132) of <i>S. aureus</i> N315</b>			
SA0131	Forward	AGGTGTGTTTACAGTGAGCG	200
<i>tet38</i>	Reverse	CATCAGCAATGGCTACAGAA	
<b><i>tetR21</i> cloning</b>			
<i>tetR21</i> -F(BamHI)		TATTTGGATCCTTGAAAGAAGATAGGCCGA <sup>a</sup>	624
<i>tetR21</i> -R(EcoRI)		AGCGAGTAGAATTCTTAAGTTAGTGAAT <sup>a</sup>	
<b>Promoter binding assays</b>			
Biotinylated- <i>tet38</i> PF		TACAATAATTTTTACTCAA	240
<i>tet38</i> -R		ATTGGTTTAAATGTGTGGTGT	
Biotinylated- <i>norB</i> PF		ATAAGGTAAGATAACTAGCA	150
<i>norB</i> -R		ATCTCTATTTGCCTCCCTATA	
Biotinylated- <i>tetR21</i> PF		TTACCAATGTTAACATTTT	320
<i>tetR21</i> -R		ACCTCCTTGTGTTATTGAAC	
Biotinylated- <i>mgrA</i> PF		TACCGAATTCATTCATGATG	320
<i>mgrA</i> -R		CTGCAGACATACTATCCGTT	
<b><i>S. aureus</i> adhesins</b>			
<i>fnbA</i> -F		ATCCGCCGAACAACATACCT	110
<i>fnbA</i> -R		TGAAAAGGTTAAAGCAGTGG	
<i>fnbB</i> -F		GGAGCGGCCTCAGTATTCTT	114
<i>fnbB</i> -R		AGCTGAACCTCCCACTTCC	

<sup>a</sup> Restriction enzymes are underlined.

struct pIMAY-*tetR21*Δ. To integrate the construct pIMAY-*tetR21* into the chromosome, the *S. aureus* RN6390 transformants were diluted between 10-fold and 1,000-fold and then plated with chloramphenicol at 10 μg/ml and grown at 37°C. We verified the absence of extrachromosomal plasmid by PCR using primers designed from the plasmid pIMAY. Colonies were selected, plated on brain heart infusion (BHI) agar plates supplemented with anhydrotetracycline at 1 μg/ml, and grown at 28°C for 48 h. Chloramphenicol-sensitive colonies were selected and verified by PCR of the flanking regions of ORF SA2165 and by DNA sequencing. The deletion mutant was named QT21.

We constructed the *tet38*-, *mgrA*-, and *tetR21*-complemented strains by introducing the plasmids pLI50-*tet38*, pLI50-*mgrA*, and pLI50-*tetR21* into the mutants QT7, QT1, and QT21, respectively. The *tet38*-complemented strain was named QT22.

**Construction of a Δ*tet38* Δ*tetR21* double mutant by phage φ85 transduction.** *tet38::cat* from donor QT7 was transferred into the recipient *tetR21* mutant by phage transduction using phage φ85, as previously described (12). The phage titer was 10<sup>8</sup> PFU/ml. Colonies of interest were selected on Trypticase soy agar (TSA) plates containing sodium citrate (10 μg/ml) and chloramphenicol (5 μg/ml) and were characterized by restriction mapping and Southern hybridization analysis. The double mutant was named QT23.

**DNA mobility shift analyses.** Primers designed to amplify the putative promoter regions of *norB*, *tet38*, *tetR21*, and *mgrA* are listed in Table 2. The sense primers were biotinylated by the Tufts University Core Facility (Boston, MA). The promoter of *mgrA* was designed based on data published by Ingavale et al. (24).

To obtain the TetR21 protein for the gel shift binding assays, the *tetR21* gene was amplified from the genome of *S. aureus* RN6390 using primers *tetR21*-F and *tetR21*-R (Table 2). The PCR product was then cloned into plasmid pTrcHisA for protein expression as previously done for the MgrA protein (25). DNA sequencing was carried out to verify the DNA sequence of the cloned gene.

Purification of His-tagged TetR21 used Ni affinity chromatography. After column loading with cell lysate, the column was first washed with buffer A (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 5% glycerol) containing 10 mM imidazole, and TetR21-His was then eluted with buffer A containing 100 mM imidazole. SDS-polyacrylamide gel electrophoresis (PAGE) indicated 95% homogeneity of the purified TetR21 protein. Purified TetR21 (200 ng, 7.2 pmol) was mixed with biotin-labeled promoter DNAs (4 ng, 20 fmol), and the gel mobility shift assays were carried out using a LightShift chemiluminescence electrophoretic mobility shift assay (EMSA) kit (Pierce, Rockford, IL), as recommended by the manufacturer. Following incubation, the binding mixture was analyzed by 5% non-denaturing PAGE as previously described (25). The optimum quantities of proteins and DNA were determined and subsequently used in assays determining the effects of tetracycline and fatty acids on the ability of TetR21 to bind to the *tet38* promoter.

Tetracycline and fatty acids at concentrations 10-fold below their MICs were mixed with the TetR21 protein in buffer A supplemented with protease inhibitors (1 tablet per 50 ml of buffer A) (Life Technologies, Grand Island, NY) for 60 min prior to the gel shift binding assays with *tet38* promoter. The protease inhibitor cocktail contained inhibitors of major proteases, such as serine, cysteine, and aspartic acid proteases, and

aminopeptidase. The TetR21 protein used as a control without incubation with drugs was submitted to the same 60-min incubation with buffer A supplemented with protease inhibitors. To monitor any degradation of TetR21 following preincubation with drugs, the proteins were submitted to SDS-PAGE in parallel with the gel shift binding assays.

**Invasion assays.** Invasion assays were performed using the ATCC CCL-185 human lung adenocarcinoma cell line A549 and the immortalized human microvascular endothelial cell line HMEC-1 (26, 27). The *S. aureus* parental strain RN6390 was compared with mutants QT1, QT5, QT7, and QT21. The *tet38*-complemented strain QT22 and the *tet38* over-expressor RN6390(pLI50-*tet38*) were used as additional controls for the effect of *tet38* on bacterial internalization. Strain MW2 was compared with RN6390 and with mutants MW2 $\Delta$ *tet38* and MW2 $\Delta$ *tetR21* to verify the effects of *tet38* on the clinical isolate and its mutants (Table 1). The assays were based on the technique described by Cheung and Bayles (28).

**Bacterial preparation.** A 20-ml fresh culture of *S. aureus* was prepared from an overnight culture and grown to an OD<sub>600</sub> of 0.4. The bacteria were washed twice with Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Fisher Scientific, Waltham, MA), referred to hereinafter as assay medium. The bacterial pellet was resuspended in a 20-ml volume of fresh assay medium.

**Cell culture preparation.** A549 cells were cultured in assay medium in a 75-ml tissue culture flask until 90% confluence, as previously described by the manufacturer (ATCC), and were then seeded into 24-well plates (Costar) in assay medium and grown again to 90% confluence.

The HMEC-1 cells were cultured in the endothelial cell basal medium MCDB 131 supplemented with 10 mM L-glutamine and 10% fetal bovine serum (FBS) (Fisher Scientific, Waltham, MA).

**Internalization assays.** Infections were initiated at a multiplicity of infection (MOI) of 10:1 (10<sup>6</sup> washed bacteria/10<sup>5</sup> cells), using bacteria in logarithmic growth phase (OD<sub>600</sub> of 0.4). The 24-well plates were quickly centrifuged for 1 min at 500 × g to allow bacterial adhesion to the cell monolayer. The bacterium-cell mixtures were incubated at 37°C in 5% CO<sub>2</sub> for 120 min. At intervals of 30, 60, and 120 min, infected monolayer cells in corresponding wells were washed twice with assay medium to remove residual nonadherent bacteria and then incubated for 60 min at 37°C in assay medium with 200 μg/ml gentamicin. The plates were washed again with fresh medium, and the epithelial cells were lysed with 250 μl of Triton X-100 (1×). The bacteria were diluted in phosphate-buffered saline (PBS) and plated on LB agar plates, and colony counts were performed to determine the numbers of viable intracellular bacteria.

**Quantitation of internalized bacteria.** We carried out the experiments using the techniques described by Pfortner et al. with some modifications (29). Bacterial internalizations were performed as described above. After 45 min of bacterium-epithelial cell contact, gentamicin (200 μg/ml) was added to each well, followed by incubation at 37°C to eliminate the extracellular bacteria. For samples obtained at each time point from 0.5 h to 4 h, gentamicin was removed, and the cells were washed twice with assay medium and then lysed with 250 μl of Triton X-100 (1×), and colony counts were performed.

## RESULTS

**Identification of the *tetR21* gene of *S. aureus*.** We searched the genome of *S. aureus* strain N315 for possible regulator(s) of the *tet38* efflux pump and found two putative ORFs (SA2165 and SA2358) that were annotated as hypothetical proteins that showed similarity with the TetR transcriptional regulator family. We proceeded with the ORF SA2165, which encoded a putative protein with 207 amino acid residues and a predicted molecular mass of 24.5 kDa (GenBank accession number BAB43467). This hypothetical protein carries two regions that show amino acid motifs similar to the two conserved domains of the TetR transcriptional regulator family (pfam00440), which are the HTH-XRE super-

family and the TetR-C-8 superfamily. The first region (residues K20 to D60) was similar to the helix-turn-helix (HTH) portion of the N-terminal domain (E value of 4.27e−06), and the second region (residues N92 to W165) was similar to the transcriptional regulator C-terminal domain (E value of 1.44e−07) of the tetracycline regulator family. The SA2165 protein shows an overall similarity to the AcrR cluster of proteins (E value of 2.89e−12). All the E values reported from the BLAST search were minimal, which indicated a significant level of similarity between ORF SA2165 and the TetR regulator family. The AcrR protein is a repressor that regulates the expression of the multidrug efflux pump *acrAB* genes of *E. coli* under global stress conditions (30). Based on this similarity, we renamed ORF SA2165 as TetR21. Identical *tetR21* genes are found in the genomes of over 3,000 *S. aureus* genomes already sequenced, including strain NCTC8325. The *tetR21* gene from the genome of RN6390 (NCTC8325-4 background) was cloned, and the encoded protein expressed from plasmid pTrcHisA.

**TetR21 affects the susceptibility of *S. aureus* to antibiotics and fatty acids.** We previously showed that overexpression of *tet38* from plasmid pLI50-*tet38* caused 5- to 8-fold increases in the MICs of palmitoleic and undecanoic acids and a 16-fold increase in the MIC of tetracycline (7). Relative to the MIC for the parental strain, the MIC for the *tet38* mutant QT7 had a limited, 2-fold change for all compounds except norfloxacin and ciprofloxacin, suggesting limited expression of *tet38* under basal conditions *in vitro*. Deletion of the *tetR21* gene in the mutant QT21 resulted in 8-fold increases in the MICs of tetracycline and chloramphenicol, a 4-fold increase in the MIC of erythromycin, and 2-fold and 3-fold increases in the MICs of palmitoleic and undecanoic acid, respectively. Twofold increases in the MICs of norfloxacin and ciprofloxacin were also seen (Table 3).

The double mutant QT23 ( $\Delta$ *tet38*  $\Delta$ *tetR21*) showed MICs of tetracycline and fatty acids with values similar to those of the *tet38* mutant QT7. The MICs of chloramphenicol, erythromycin, ciprofloxacin, and norfloxacin remained the same as those of the *tetR21* mutant QT21. These data suggest that TetR21 may regulate *tet38* expression, the product of which confers resistance to tetracycline and fatty acids, but may also affect the expression of other determinants controlling the susceptibility to chloramphenicol and erythromycin.

**Effect of *tetR21* on the expression of *tet38*.** The effect of *tetR21* on the transcript levels of *tet38* was measured by real-time qRT-PCR using total RNAs from strain RN6390 and mutants QT7 ( $\Delta$ *tet38*), QT1 ( $\Delta$ *mgrA*), and QT21 ( $\Delta$ *tetR21*). Without induction by fatty acids or tetracycline, the transcript levels of *tet38* increased 5-fold in both mutant QT1 and mutant QT21 compared to its level in RN6390 (Table 4). These data suggested that *tet38* expression was under the control of MgrA and TetR21. No change was found in the transcript levels of *tetR21* or *mgrA* in mutant QT1 and mutant QT21, respectively (Table 4). We further evaluated the transcriptional variation of other efflux pump genes in the absence of an intact TetR21 and found that the transcript levels of *norA*, *norC*, *norD*, *mepA*, and *abcA* were not affected in mutant QT21. One exception was *norB*, which showed a 2-fold increase in its transcripts (data not shown).

To assess the effects of fatty acids on the transcript levels of *tet38*, *tetR21*, and *mgrA*, we exposed *S. aureus* RN6390, QT1, and QT21 to linoleic, palmitoleic, and undecanoic acids at 0.5-, 0.25-, and 0.10-fold MIC for each strain for up to 1 h. In RN6390, at 10-fold below the MIC, the *tet38* transcripts increased 4-fold with



TABLE 3 Susceptibility of *S. aureus* strains to tetracycline, fatty acids, quinolones, chloramphenicol, and erythromycin

Strain	MIC ( $\mu\text{g/ml}$ ) of <sup>a</sup> :							
	Undecanoic acid	Palmitoleic acid	Linoleic acid	NOR	CIP	TET	CHL	ERY
RN6390	500	500	1,000	0.5	0.25	0.06	4	0.06
QT1 ( $\Delta mgrA$ )	1,500	1,500	2,500	4	2	4	4	0.06
QT7 ( $\Delta tet38$ )	250	250	500	0.5	0.25	0.03	2	0.03
QT21 ( $\Delta tetR21$ )	1,500	1,000	2,000	1	0.5	0.5	32	0.25
QT23 ( $\Delta tet38 \Delta tetR21$ )	250	500	1,000	1	0.5	0.06	32	0.25
RN6390(pLI50)	500	500	1,000	0.5	0.25	0.06	4	0.06
QT1(pLI50)	1,500	1,500	2,500	4	2	4	4	0.06
QT1(pLI50- <i>mgrA</i> )	500	500	1,000	0.5	0.25	0.06	4	0.06
QT21(pLI50)	1,500	1,000	2,000	1	0.5	0.5	32	0.25
QT21(pLI50- <i>tetR21</i> )	500	500	1,000	0.5	0.25	0.06	4	0.06

<sup>a</sup> NOR, norfloxacin; CIP, ciprofloxacin; TET, tetracycline; CHL, chloramphenicol; ERY, erythromycin.

palmitoleic and undecanoic acids and 3-fold with linoleic acid and showed no change with palmitic acid (Table 4). The transcript levels of *tetR21* and *mgrA* of RN6390 remained unchanged after induction with all lipids. The mutants QT1 ( $\Delta mgrA$ ) and QT21 ( $\Delta tetR21$ ) showed approximately the same levels of *mgrA*, *tetR21*, and *tet38* transcripts with or without induction (Table 4). No induction of *tet38*, *tetR21*, or *mgrA* was found with exposure to palmitic acid at any of the three below-MIC concentrations.

Similarly, exposure of RN6390 to tetracycline at 10-fold below the MIC led to an increase of 4-fold in *tet38* transcripts. Exposure of QT1 and QT21 to tetracycline at 10-fold below the MIC did not substantially change the transcript levels of *tet38*, *mgrA*, or *tetR21* compared to the levels in the absence of tetracycline (Table 4). We chose the 10-fold-below-MIC concentrations of tetracycline and fatty acids for the gel shift assays to prevent any unintended damage to the TetR21 protein during the incubation period. These amounts of tetracycline and fatty acids would be sufficient to assess the effect of each compound on the TetR21-*tet38* promoter binding. Additional induction assays were carried out using complemented QT1 and QT21 strains, and the results indicated that the mutants exhibited gene expression patterns similar to that of RN6390 (Table 4). Statistical analyses were performed based on the Student *t* test to determine the significance of the gene expression values.

**Gel mobility shift assays.** DNA-protein gel mobility shift assays were carried out using the TetR21 purified protein and the promoters of efflux pump genes as previously described (12, 31–34). The putative *tet38* promoter is located within a 240-bp DNA intergenic region separating the *S. aureus* strain N315 ORF SA0130 (encoding a putative trehalose operon transcriptional regulator, on the complementing DNA strand) and the two N315 ORFs SA0131 and SA0132 (*tet38*). SA0131 encodes a putative purine nucleoside phosphorylase that is separated from *tet38* by 6 nucleotides. RT-PCR using primers encompassing the internal region of the two genes (Table 2) generated a 200-bp PCR product, indicating that the two adjacent ORFs share the same promoter region and are cotranscribed. The upstream 240-bp region of DNA carries a putative promoter, based on a search using the Neural Network Promoter Prediction server (35).

TetR21 protein bound to biotinylated *tet38* promoter, resulting in a band shift, and was displaced by excess unlabeled *tet38* promoter DNA. Nonspecific herring sperm DNA in 100-fold excess, in contrast, did not affect the TetR21-mediated *tet38* promoter band shift, showing the specificity of TetR21 binding to

*tet38* promoter (Fig. 1A). TetR21 also bound to the *norB* promoter but did not bind to the promoters of efflux pump genes *norA*, *norC*, *norD*, and *abcA* (Fig. 1B). Additional gel mobility shift assays were carried out with TetR21 and MgrA proteins and *tetR21* and *mgrA* promoters. The *tetR21* promoter was based on prediction by the Neural Network Promoter Prediction server, and the *mgrA* promoter was based on the primer extension data published by Ingavale et al. (24). The results showed that TetR21 and MgrA each bound to the *tetR21* promoter (Fig. 1C). In contrast, TetR21 did not bind to the *mgrA* promoter and MgrA did not bind to *tet38* promoter (data not shown). The absence of binding of MgrA to *tet38* promoter was also reported in our previous study (12).

**Effects of tetracycline and palmitoleic acid on TetR21 binding to *tet38* promoter.** Tetracycline has been shown to disrupt the binding between other TetR regulators and the gene promoters they regulate (36). We assessed the ability of tetracycline and palmitoleic acid, both substrates of Tet38, to prevent the binding of TetR21 to *tet38* promoter. Palmitoleic acid at 0.10-fold MIC induced the highest level of *tet38* transcript under the conditions used in this study (Table 4). We used the MIC for the wild-type RN6390 and preincubated TetR21 with tetracycline (0.006  $\mu\text{g/ml}$  or 1.8  $\mu\text{M}$ ) for a period of 60 min. At 60 min, 200 ng (7.2  $\mu\text{M}$ ) of protein was applied to gel mobility shift assays with *tet38* promoter. The control protein was treated in the same manner as the tested protein. We found that tetracycline at this level prevented TetR21 binding to *tet38* promoter after 60 min of preincubation (Fig. 2A).

We repeated the same experiments with palmitoleic acid at 50  $\mu\text{g/ml}$  (200  $\mu\text{M}$ ). TetR21 was preincubated with fatty acids for 60 min prior to being in contact with *tet38* promoter for a gel mobility shift assay. As was found with tetracycline, palmitoleic acid disrupted the TetR21-*tet38* promoter complex after 60 min of preincubation (Fig. 2A). Palmitic acid was used as a negative control since this saturated fatty acid did not affect *tet38* expression, and preincubation of TetR21 with palmitic acid (780  $\mu\text{M}$ ) for 60 min did not affect the formation of the TetR21-*tet38* promoter complex (Fig. 2A). In parallel, TetR21 protein (200 ng, 7.2  $\mu\text{M}$  each assay) was incubated with tetracycline, palmitoleic acid, and palmitic acid individually and then separately applied to SDS-PAGE followed by Coomassie blue staining to assess the integrity of the protein. No major protein degradation was found, indicating that tetracycline and these fatty acids did not result in degradation of the TetR21 protein under these conditions (Fig. 2B). A similar experiment with unde-

TABLE 4 Differences in the *tet38*, *tetR21*, and *mgrA* transcript levels in *S. aureus* strains following exposure to fatty acids and tetracycline

Strain, compound (MIC [ $\mu\text{g/ml}$ ])	Fold MIC used for induction	Relative expression (mean fold change $\pm$ SD) of <sup>a</sup> :		
		<i>tet38</i>	<i>tetR21</i>	<i>mgrA</i>
RN6390 (wild type)	No induction	1.0 $\pm$ 0.1	1.0 $\pm$ 0.1	1.0 $\pm$ 0.1
Tetracycline (0.06)	0.5	2.8 $\pm$ 0.2	0.9 $\pm$ 0.2	1.0 $\pm$ 0.1
	0.25	3.1 $\pm$ 0.2	1.0 $\pm$ 0.1	1.0 $\pm$ 0.2
	0.10	<b>4.0</b> $\pm$ 0.3	1.0 $\pm$ 0.1	1.0 $\pm$ 0.1
	0.05	2.0 $\pm$ 0.2	1.0 $\pm$ 0.2	1.0 $\pm$ 0.3
Palmitoleic acid (500)	0.5	2.5 $\pm$ 0.3	1.0 $\pm$ 0.2	1.0 $\pm$ 0.1
	0.25	<b>4.0</b> $\pm$ 0.3	1.0 $\pm$ 0.1	1.0 $\pm$ 0.2
	0.10	2.0 $\pm$ 0.2	1.0 $\pm$ 0.2	1.0 $\pm$ 0.1
Undecanoic acid (500)	0.5	1.5 $\pm$ 0.1	1.0 $\pm$ 0.1	1.0 $\pm$ 0.1
	0.25	<b>4.1</b> $\pm$ 0.4	1.0 $\pm$ 0.1	1.0 $\pm$ 0.1
	0.10	1.8 $\pm$ 0.4	1.0 $\pm$ 0.1	1.0 $\pm$ 0.1
Linoleic acid (1,000)	0.5	2.5 $\pm$ 0.5	1.0 $\pm$ 0.1	1.0 $\pm$ 0.1
	0.25	3.0 $\pm$ 0.4	1.0 $\pm$ 0.2	1.0 $\pm$ 0.1
	0.10	1.0 $\pm$ 0.1	1.0 $\pm$ 0.2	1.0 $\pm$ 0.1
Palmitic acid (2,000)	0.5	1.0 $\pm$ 0.1	1.0 $\pm$ 0.2	1.0 $\pm$ 0.1
	0.25	1.0 $\pm$ 0.1	1.0 $\pm$ 0.1	1.0 $\pm$ 0.1
	0.10	1.0 $\pm$ 0.1	1.0 $\pm$ 0.2	1.0 $\pm$ 0.1
QT1 ( $\Delta mgrA$ )	No induction	<b>5.0</b> $\pm$ 0.5	0.9 $\pm$ 0.1	
Tetracycline (4.0)	0.5	5.2 $\pm$ 0.4	0.8 $\pm$ 0.2	
	0.25	5.1 $\pm$ 0.4	0.7 $\pm$ 0.2	
	0.10	5.0 $\pm$ 0.3	0.9 $\pm$ 0.1	
	0.05	5.0 $\pm$ 0.2	0.9 $\pm$ 0.4	
Palmitoleic acid (1,500)	0.5	5.1 $\pm$ 0.4	1.0 $\pm$ 0.1	
	0.25	5.2 $\pm$ 0.5	1.2 $\pm$ 0.3	
	0.10	5.0 $\pm$ 0.5	1.1 $\pm$ 0.1	
Undecanoic acid (1,500)	0.5	4.5 $\pm$ 0.3	0.8 $\pm$ 0.3	
	0.25	5.1 $\pm$ 0.4	0.9 $\pm$ 0.1	
	0.10	5.1 $\pm$ 0.3	1.1 $\pm$ 0.1	
Linoleic acid (2,500)	0.5	5.1 $\pm$ 0.4	1.0 $\pm$ 0.2	
	0.25	5.0 $\pm$ 0.2	1.0 $\pm$ 0.1	
	0.10	5.0 $\pm$ 0.2	1.0 $\pm$ 0.2	
Palmitic acid (5,000)	0.5	5.0 $\pm$ 0.1	1.0 $\pm$ 0.2	
	0.25	5.0 $\pm$ 0.1	1.0 $\pm$ 0.2	
	0.10	5.0 $\pm$ 0.1	1.0 $\pm$ 0.2	
QT21 ( $\Delta tetR21$ )	No induction	<b>5.0</b> $\pm$ 0.5		1.0 $\pm$ 0.1
Tetracycline (0.5)	0.5	4.8 $\pm$ 0.5		1.0 $\pm$ 0.1
	0.25	5.1 $\pm$ 0.3		1.0 $\pm$ 0.2
	0.10	5.0 $\pm$ 0.4		1.0 $\pm$ 0.1
	0.05	4.5 $\pm$ 0.4		1.0 $\pm$ 0.1
Palmitoleic acid (1,000)	0.5	4.5 $\pm$ 0.3		1.0 $\pm$ 0.2
	0.25	5.0 $\pm$ 0.5		1.0 $\pm$ 0.2
	0.10	4.8 $\pm$ 0.2		1.0 $\pm$ 0.1
Undecanoic acid (1,500)	0.5	4.5 $\pm$ 0.3		1.0 $\pm$ 0.1
	0.25	4.8 $\pm$ 0.3		1.0 $\pm$ 0.1
	0.10	4.8 $\pm$ 0.3		1.0 $\pm$ 0.1
Linoleic acid (2,000)	0.5	5.0 $\pm$ 0.4		1.0 $\pm$ 0.1
	0.25	4.5 $\pm$ 0.5		1.0 $\pm$ 0.1
	0.10	4.8 $\pm$ 0.3		1.0 $\pm$ 0.1
Palmitic acid (5,000)	0.5	5.0 $\pm$ 0.2		1.0 $\pm$ 0.2
	0.25	4.8 $\pm$ 0.4		1.0 $\pm$ 0.2
	0.10	5.0 $\pm$ 0.4		1.0 $\pm$ 0.1
RN6390(pLI50)	No induction	1.0 $\pm$ 0.1	1.0 $\pm$ 0.1	1.0 $\pm$ 0.1
Tetracycline	0.10	3.9 $\pm$ 0.4	1.0 $\pm$ 0.1	1.0 $\pm$ 0.1
Palmitoleic acid	0.10	<b>4.0</b> $\pm$ 0.4	1.0 $\pm$ 0.2	1.0 $\pm$ 0.1
Undecanoic acid	0.10	3.8 $\pm$ 0.3	1.0 $\pm$ 0.1	1.0 $\pm$ 0.2
Linoleic acid	0.10	2.6 $\pm$ 0.3	1.0 $\pm$ 0.1	1.0 $\pm$ 0.2
Palmitic acid	0.10	1.0 $\pm$ 0.2	1.0 $\pm$ 0.2	1.0 $\pm$ 0.1

(Continued on following page)

TABLE 4 (Continued)

Strain, compound (MIC [ $\mu\text{g/ml}$ ])	Fold MIC used for induction	Relative expression (mean fold change $\pm$ SD) of <sup>a</sup> :		
		<i>tet38</i>	<i>tetR21</i>	<i>mgrA</i>
QT1 (pLI50- <i>mgrA</i> )	No induction	1.0 $\pm$ 0.2	1.0 $\pm$ 0.2	6.0 $\pm$ 0.5
Tetracycline	0.10	3.5 $\pm$ 0.4	1.0 $\pm$ 0.1	6.0 $\pm$ 0.4
Palmitoleic acid	0.10	<b>3.7</b> $\pm$ 0.4	1.0 $\pm$ 0.1	6.0 $\pm$ 0.4
Undecanoic acid	0.10	3.2 $\pm$ 0.4	1.0 $\pm$ 0.1	6.0 $\pm$ 0.5
Linoleic acid	0.10	2.2 $\pm$ 0.4	1.0 $\pm$ 0.1	6.0 $\pm$ 0.3
Palmitic acid	0.10	1.0 $\pm$ 0.1	1.0 $\pm$ 0.1	6.0 $\pm$ 0.2
QT21 (pLI50- <i>tetR21</i> )	No induction	1.0 $\pm$ 0.3	5.0 $\pm$ 0.2	1.0 $\pm$ 0.4
Tetracycline	0.10	3.5 $\pm$ 0.3	5.0 $\pm$ 0.5	1.0 $\pm$ 0.1
Palmitoleic acid	0.10	<b>3.7</b> $\pm$ 0.4	5.0 $\pm$ 0.3	1.0 $\pm$ 0.1
Undecanoic acid	0.10	3.0 $\pm$ 0.3	5.0 $\pm$ 0.3	1.0 $\pm$ 0.2
Linoleic acid	0.10	2.0 $\pm$ 0.2	5.0 $\pm$ 0.5	1.0 $\pm$ 0.1
Palmitic acid	0.10	1.0 $\pm$ 0.1	5.0 $\pm$ 0.5	1.0 $\pm$ 0.1

<sup>a</sup> The relative gene expression is determined as the ratio of gene transcripts of mutant versus wild-type strains and the ratio of transcripts with versus without induction by fatty acids or tetracycline. The *gmk* gene was used as an internal control. Each assay was done in triplicate, and RNAs were collected from three independent biological samples. All values represent the means of three independent experiments. Values in boldface represent differences that are statistically significant based on Student's *t* test ( $P < 0.05$ ). The differences between wild-type and mutant genes and the increases in transcript levels for gene expression after induction versus without induction were calculated.

canoic acid at a concentration of 50  $\mu\text{g/ml}$  (268  $\mu\text{M}$ ) could not be interpreted because TetR21 was degraded despite the presence of protease inhibitors.

**Effects of *tet38*, *tetR21*, and *mgrA* on *S. aureus* internalization, survival, and replication within host cells.** Our previous work demonstrated that the Tet38 efflux pump affected the ability of *S. aureus* to colonize mouse skin. To determine whether Tet38 affects the ability of *S. aureus* to invade epithelial and endothelial cells, we developed invasion assays using epithelial cell line A549 and endothelial cell line HMEC-1. *S. aureus* parental strain RN6390 was compared with mutants QT1 ( $\Delta\textit{mgrA}$ ), QT7 ( $\Delta\textit{tet38}$ ), and QT21 ( $\Delta\textit{tetR21}$ ) to assess their ability to invade and survive in these cell lines. All strains used in these assays grew similarly in TSB (data not shown). The complemented strain QT22 (QT7 with plasmid pLI50-*tet38*) was added to the assays to validate the effect of Tet38 on the internalization process. After 2 h of contact between cells and bacteria, we treated the cells with gentamicin at 200  $\mu\text{g/ml}$ , washed them with PBS, disrupted the cells with Triton X-100, and plated the internalized bacteria on LB agar. We found that QT7, which lacks a functional Tet38, had 6-fold less viable bacteria within A549 cells than the parental strain RN6390. In contrast, QT21, which lacks a functional TetR21 regulator, exhibited 2-fold more viable bacteria within A549 cells than RN6390. The QT1 mutant, which lacks functional MgrA, was similar (1.5-fold) to the wild-type RN6390 in the number of viable bacteria within A549 cells. We found a similar pattern using the HMEC-1 endothelial cell line, which showed a 5-fold-lower and a 2-fold-greater number of internal bacteria for QT7 and QT21, respectively. The complemented strain QT22 showed the same level of intracellular bacteria as RN6390 for both cell lines (Fig. 3A). Thus, *tet38* contributes to the ability of *S. aureus* to survive in epithelial and endothelial cells.

We followed the intracellular replication of *S. aureus* over time after treatment of A549 cells with gentamicin to eliminate the remaining extracellular bacteria. Gentamicin remained in the medium over the course of the extended incubation and was washed away prior to cell lysis for colony counts. Based on the results of the initial experiments, we determined that 45 min would allow sufficient bacteria inside the A549 cells for subsequent enumera-

tion. At 45 min of contact with an MOI of 10:1, the amounts of internalized bacteria of strains RN6390, QT1, and QT21 were similar (300 to 305 bacteria) and were 3-fold higher than the internalized amount of mutant QT7 (108 bacteria) (Fig. 3B). These data indicated that the MgrA and TetR21 regulators did not influence the initial internalization of *S. aureus* into A549 cells but that the absence of the Tet38 efflux pump resulted in an early lower level of survival. Bacterial intracellular replication was then monitored over 5 h. The numbers of intracellular bacteria remained stable for all strains for up to 120 min. Between 120 and 240 min, the numbers of bacteria of strains RN6390, QT1, and QT21 increased between 2-fold and 2.5-fold, followed by a plateau period, whereas the bacterial numbers of QT7 decreased slightly over the full time

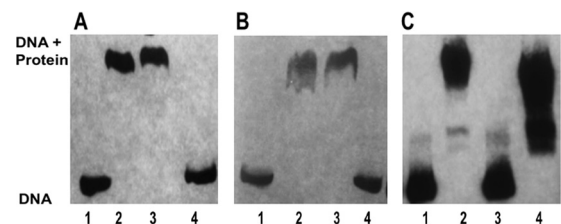
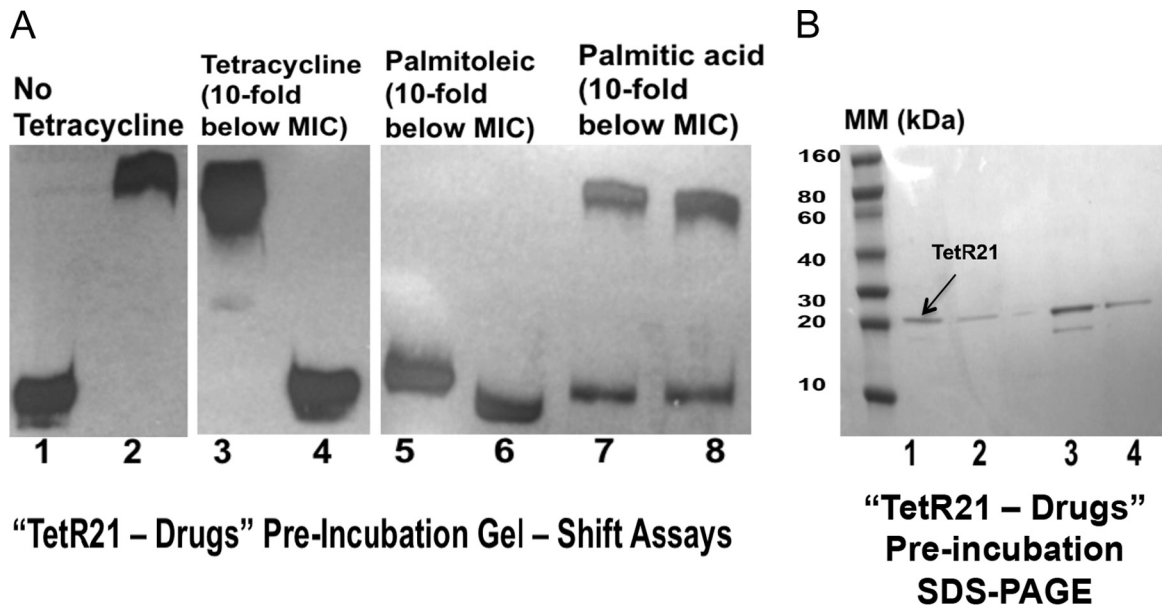


FIG 1 Binding of TetR21 to promoter DNA of *tet38*, *norB*, and *tetR21* and binding of MgrA to *tetR21* DNA. (A) Gel mobility shift analyses of the interactions of the purified TetR21 protein with the biotinylated *tet38* promoter. Protein (200 ng, 7.2 pmol) and DNA (4 ng, 20 fmol) were in contact for 30 min at room temperature, followed by electrophoresis through a 5% acrylamide gel. Competing unlabeled herring sperm DNA (nonspecific, 100-fold excess) and unlabeled *tet38* promoter (specific, 100-fold excess) were used to determine the specificity of the promoter binding assay. Lane 1, *tet38* promoter; lane 2, *tet38* + TetR21; lane 3, *tet38* + TetR21 + 100-fold herring DNA; lane 4, *tet38* + TetR21 + 100-fold unlabeled *tet38*. (B) Gel mobility shift analyses of the interactions of the purified TetR21 protein with the biotinylated *norB* promoter. Competing unlabeled herring sperm DNA (nonspecific, 100-fold excess) and unlabeled *norB* promoter (specific, 100-fold excess) were used to determine the specificity of the *norB* promoter binding. Lane 1, *norB* promoter; lane 2, *norB* + TetR21; lane 3, *norB* + TetR21 + 100-fold herring DNA; lane 4, *norB* + TetR21 + 100-fold unlabeled *norB*. (C) Gel mobility shift analyses of the interactions of the purified TetR21 and MgrA protein with the biotinylated *tetR21* promoter. The same procedure was carried out as described above. Lane 1, *tetR21* promoter; lane 2, *tetR21* + TetR21; lane 3, *tetR21* promoter; lane 4, *tetR21* + MgrA.



**FIG 2** Effects of tetracycline, palmitoleic acid, and palmitic acid on the *tet38*-TetR21 complex. (A) *tet38* promoter and TetR21 protein gel shift binding assays. TetR21 was preincubated with tetracycline, palmitoleic acid, or palmitic acid prior to being in contact with the *tet38* promoter. At 60 min, 200 ng of TetR21 protein was removed and used in a gel shift assay with *tet38* promoter. The drug concentrations (10-fold below the MICs) were as follows: tetracycline, 0.006  $\mu\text{g/ml}$  (1.8  $\mu\text{M}$ ); palmitoleic acid, 50  $\mu\text{g/ml}$  (200  $\mu\text{M}$ ); palmitic acid, 200  $\mu\text{g/ml}$  (780  $\mu\text{M}$ ). Lane 1, *tet38* DNA; lane 2, *tet38* + TetR21; lane 3, *tet38* + TetR21 with no tetracycline; lane 4, TetR21 + tetracycline for 60 min before addition of *tet38* DNA; lane 5, *tet38* DNA; lane 6, TetR21 + palmitoleic acid for 60 min before addition of *tet38* DNA; lane 7, TetR21 + palmitic acid for 60 min before addition of *tet38* DNA; lane 8, *tet38* + TetR21 with no fatty acid. (B) TetR21 protein before and after incubation with drugs and SDS-PAGE protein gels. The protein gel results indicated absence of major protein degradation following incubation with tetracycline or fatty acids for 60 min. Lane 1, purified TetR21 protein; lane 2, TetR21 incubated with tetracycline (1.8  $\mu\text{M}$ ); lane 3, TetR21 incubated with palmitoleic acid (200  $\mu\text{M}$ ); lane 4, TetR21 incubated with palmitic acid (780  $\mu\text{M}$ ). A smaller protein band appeared in the sample in lane 3, which suggests minor degradation after incubation of TetR21 with palmitoleic acid. The amount of intact protein was still sufficient to produce a mobility shift in the *tet38* DNA.

period. The complemented strain QT22, which carried the plasmid-cloned *tet38* in QT7, showed a growth pattern similar to that of RN6390 (Fig. 3B). We carried out the assay using the *tet38* overexpressor RN6390(pLI50-*tet38*) and found that this overexpressor generated a number of CFUs per monolayer similar to that of the QT21 mutant (720 versus 750 CFU/monolayer, respectively; data not shown in Fig. 3B). The  $\Delta\text{norB}$  efflux pump mutant QT5 was also tested in this assay, and it behaved similarly to RN6390, indicating that NorB did not play a role in bacterial intracellular replication (data not shown). *S. aureus* MW2 and its mutants were added to the intracellular replication assays to assess the effects of *tet38* in other *S. aureus* strains. We found that MW2 $\Delta\text{tet38}$  survived 6-fold less efficiently than MW2, and MW2 $\Delta\text{tetR21}$  replicated as efficiently as MW2 (Fig. 3C). The complemented strain MW2 $\Delta\text{tet38}$ (pLI50-*tet38*) recovered the ability to internalize inside host cells, and the mutant MW2 $\Delta\text{tetR21}$  showed a 2.5-fold increase in internalization (data not shown). These data, taken together, indicate that Tet38 contributes to the ability of *S. aureus* to survive within epithelial cells. The findings demonstrate an effect on intracellular replication over time. Furthermore, the stable numbers of viable bacteria of mutant and parental strains over several hours and the presence of differences in numbers of viable intracellular bacteria between mutant and parental strains as early as 45 min suggest that Tet38 may also have an effect on internalization itself.

***tet38* and *tetR21* do not affect the expression of *fnbA* and *fnbB* adhesins.** Since fibronectin-binding proteins mediate the adhesion and invasion of *S. aureus* in several cell types, including epithelial and endothelial cells (13), we carried out quantitative

real-time RT-PCRs using primers designed from genes *fnbA* and *fnbB* and total RNAs extracted from RN6390, QT7, and QT21 to assess potential differences in the expression of these adhesins. The transcript levels of *fnbA* and *fnbB* remained unchanged between the three *S. aureus* strains RN6390, QT7, and QT21. These data indicated that, under these conditions, *tet38* and *tetR21* did not affect the expression of *fnbA* and *fnbB*.

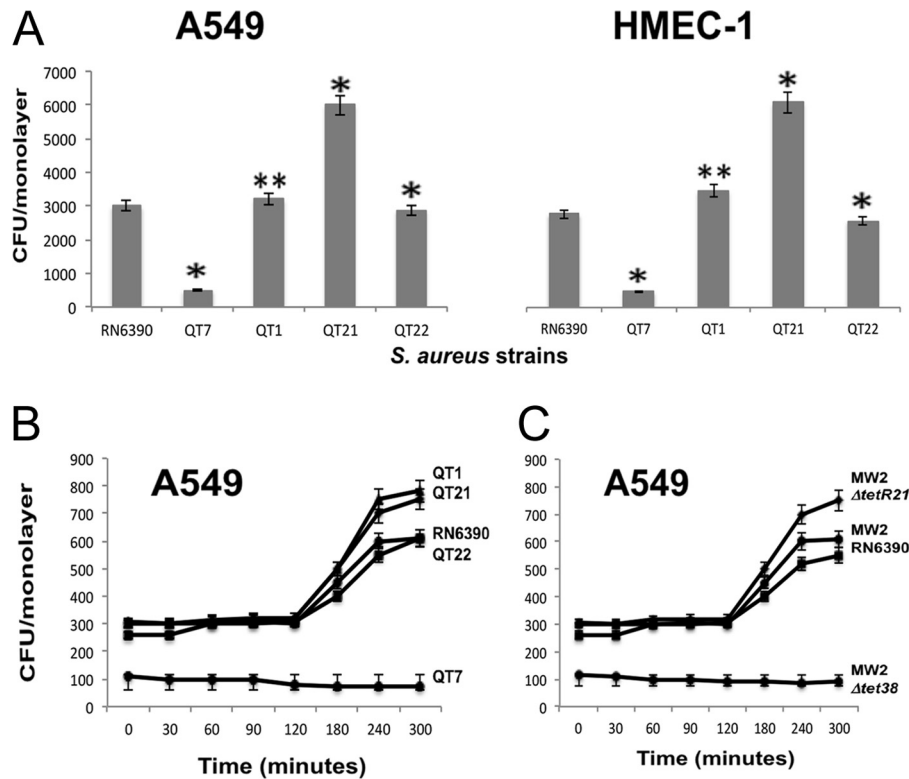
## DISCUSSION

*tet38* is highly conserved among *S. aureus* strains, based on published genomes of both methicillin-susceptible and -resistant strains. We previously found that Tet38 conferred resistance to tetracycline and certain unsaturated fatty acids, such as linoleic, palmitoleic, and undecanoic acids, but not to palmitic acid or the polyamine spermidine (7).

In this study, we identified a new TetR-like regulator of the *tet38* efflux pump gene that we named TetR21. The *tetR21* mutant exhibited a 5-fold increase in *tet38* transcripts, which correlated with an 8-fold increase in resistance to tetracycline, a 2-fold increase in resistance to palmitoleic acid, and a 3-fold increase in resistance to undecanoic acid. Unexpectedly the *tetR21* mutant also exhibited an 8-fold and a 4-fold increase in the MIC of chloramphenicol and erythromycin, respectively. These data suggested that TetR21 acted as a repressor of *tet38* expression and may also regulate the expression of other bacterial resistance determinants in addition to Tet38. The nature of these additional resistance determinants regulated by *tetR21* is the subject of ongoing investigation.

We then demonstrated that TetR21 directly and specifically





**FIG 3** Internalization and survival of *S. aureus* within epithelial and endothelial cells. (A) Internalization and survival of *S. aureus* within A549 and HMEC-1 cells. The internalization assays were carried out as described in Materials and Methods with a bacterial multiplicity of infection (MOI) of 10:1. After 2 h of internalization, cell monolayers were treated with gentamicin (200  $\mu\text{g/ml}$ ), detached, and lysed, and colony counts (CFU/monolayer) were performed. All values are the mean results  $\pm$  standard deviations (SD) from three independent experiments. \*, statistically significantly different from the wild type as determined by Student's *t* test ( $P < 0.05$ ); \*\*, not statistically significantly different. *S. aureus* strains were RN6390 (wild type), QT7 ( $\Delta tet38$ ), QT1 ( $\Delta mgrA$ ), QT21 ( $\Delta tetR21$ ), and QT22 ( $\Delta tet38$  complemented). A549, lung adenocarcinoma epithelial cells; HMEC-1, human microvascular endothelial cells. (B) Intracellular replication of *S. aureus* strains in A549 cells. Gentamicin was added after 45 min of contact between bacteria and A549 cells as described in Materials and Methods. Intracellular survival assays of *S. aureus* were carried out over a period of 300 min. Viable bacterial counts for strains RN6390, QT1, QT21, and QT22 increased after 120 min and stabilized at 5 h. Viable bacterial counts for strain QT7 at first remained unchanged and then decreased slightly over the course of 5 h. All values are the mean results  $\pm$  SD from three independent experiments. (C) Intracellular replication of clinical *S. aureus* strains in A549 cells. Clinical strain MW2 and mutants MW2 $\Delta tet38$  and MW2 $\Delta tetR21$  show the same pattern of intracellular replication as strains RN6390, QT7 and QT21, shown in panel B. All values are the mean results  $\pm$  SD from three independent experiments.

bound to the *tet38* promoter. Notably, the presence of tetracycline at a concentration 10-fold below its MIC provoked a reduction in the binding of TetR21 to *tet38* promoter in gel mobility shift assays, suggesting that the binding of tetracycline to TetR21 resulted in disruption of repressor binding and was responsible for the induction of *tet38* expression by tetracycline. The same phenomenon was observed when we used palmitoleic acid but not with palmitic acid (a saturated fatty acid) at 10-fold below the MIC. We have previously shown that the global regulator MgrA negatively regulated the expression of *tet38* (12). We demonstrated here that MgrA bound to the promoter of *tetR21* but not to that of *tet38*, suggesting that it functions as a direct regulator of *tetR21* expression, thereby contributing to TetR21 repression of *tet38* expression. The nature of the role played by MgrA toward *tetR21* expression was not clear, since a lack of MgrA did not lead to an obvious change in the level of *tetR21* expression in either direction.

Many multidrug efflux pumps in Gram-negative bacteria like *E. coli*, *K. pneumoniae*, and *E. cloacae* are controlled by special regulatory systems that mediate responses to hostile environments (37). In addition, studies of Gram-negative efflux pumps

revealed that the expression of several transporters of the RND (resistance-nodulation-division) family of multidrug (MDR) transporters were induced by and transported cellular metabolites as part of the bacterial cell physiology (38–40). Since Tet38 conferred resistance to fatty acids, a natural host defense component, we observed the effects of certain lipids on the gene expression levels of *tet38* and its regulators *tetR21* and *mgrA*. Exposure of *S. aureus* RN6390 to linoleic, palmitoleic, and undecanoic acids led to 4-fold increases in *tet38* transcript levels *in vitro*, while no change was detected for the *tetR21* and *mgrA* transcripts. Furthermore, we found that the *tet38* transcript levels remained unchanged after exposure of *mgrA* and *tetR21* mutants to fatty acids, suggesting that induction requires intact MgrA and TetR21 regulators.

To understand further the role played by the Tet38 efflux pump and the effects of TetR21 and MgrA on *S. aureus* survival in epithelial cells, we carried out invasion assays using human lung epithelial cell and microvascular endothelial cell lines. Compared with the wild-type RN6390, the *tet38* mutant QT7 survived in both cell types in 6-fold-lower numbers and was unable to replicate intracellularly. Both defects were restored following complementation of the QT7 strain with a plasmid-cloned wild-type

*tet38*. Notably, both the *mgrA* and the *tetR21* mutant showed similar enhancement of delayed intracellular replication. Since both of these mutants had similarly increased *tet38* expression, the difference in the two mutants in enhanced initial survival (the *tetR21* mutant had enhanced initial survival, and the *mgrA* mutant did not) indicates that additional factors that contribute to initial internalization and survival are affected differently by the two regulators. Further evidence for their distinct regulatory profiles is the difference in chloramphenicol and erythromycin resistance seen between the *tetR21* and *mgrA* mutants. The factors that determine the delay in intracellular replication in the parental strain and regulatory mutants are not known, and it is possible that delays may vary with different regulatory mutants and that this could explain our finding of differences between the *tetR21* mutant and the parental strain RN6390 at the initial time point in the short-incubation assay (45-min bacterium-cell incubation) (Fig. 3B) and the initial time point in the long-incubation assay (120-min bacterium-cell incubation) (Fig. 3A) (41).

Our data indicated that Tet38 contributed to the process of early cell internalization and survival, as well as intracellular replication of *S. aureus* in nonprofessional phagocytes, such as epithelial and endothelial cells. Because initial intracellular survival was stable over several hours and because differences between the *tet38* mutant and parental strain were seen as early as 45 min, we think it is likely that Tet38 contributed directly to internalization within host cells, as it did for delayed intracellular replication. Although both Tet38 and NorB contributed to bacterial survival in a murine abscess model (11), only Tet38 contributed to survival on mouse skin (7), and only Tet38 contributed to survival in epithelial and endothelial cells. The mechanism by which Tet38 facilitates intracellular survival and replication is not known but may relate to an ability to protect from as-yet-undefined intracellular toxins, which could include antibacterial fatty acids.

A number of efflux pumps have been shown to be important for bacterial survival in host environments. For example, the lack of a functional tripartite transperiplasmic efflux pump can lead to the accumulation of xenobiotic compounds inside the *E. coli* cells, most notably bile salts, which are present in high concentrations in the native intestinal environment of this organism. Toxin exposure can trigger stress response systems of bacteria (42). Efflux pumps of *Salmonella*, *Campylobacter*, *Pseudomonas*, and *E. coli* have been shown to facilitate the invasion of and replication within host cells and escape from host defense mechanisms (3, 43). The MacAB transporter of *Salmonella enterica* also serves to protect the bacterium against reactive oxygen species produced by the host cells (44).

In summary, Tet38 contributes to the ability of *S. aureus* to survive and replicate within epithelial and endothelial cells, in addition to its contributions to bacterial colonization of skin and survival in an abscess environment, highlighting its important roles in the adaptation of *S. aureus* to multiple environments. The exact mechanisms by which Tet38 facilitates internalization and intracellular survival are the subject of ongoing investigation. The central regulation of *tet38* expression by regulators such as MgrA, which also regulates the expression of other efflux pumps and multiple other cellular functions, further implies an integral role for Tet38 in *S. aureus* physiology and virulence. TetR21 represents a novel component of the MgrA regulon that also affects other resistance determinants, possibly including additional ef-

flux pumps, thereby adding to the substantial resistance portfolio of this highly successful human pathogen.

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