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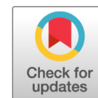


# Transcriptional Regulator TetR21 Controls the Expression of the *Staphylococcus aureus* LmrS Efflux Pump

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**ABSTRACT** TetR21 controls the expression of Tet38 and LmrS efflux pumps. A *tetR21* mutant, QT21, exhibited a 4-fold increase in the transcription level of *lmrS*. *Staphylococcus aureus lmrS* overexpressor showed increases of 4-fold and 2-fold, respectively, in the MICs of chloramphenicol and erythromycin, while the MICs of *lmrS* mutant QT18 and *lmrS-tetR21* mutant QT1821 remained similar to those of parental strain RN6390. TetR21 does not bind to the promoter of *lmrS*, suggesting indirect regulation of *lmrS*.

**KEYWORDS** *S. aureus*, transporter, TetR21, LmrS, antibiotics

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TetR21, a member of the TetR family, represses the expression of the Tet38 efflux pump (1). The *tetR21* mutant QT21 shows resistance to chloramphenicol and erythromycin that is not attributable to increased expression of Tet38 (1). These additional phenotypes suggested that TetR21 also controlled expression of other efflux pumps. Several staphylococcal efflux pumps, such as NorA, NorB, NorC, Tet38, MdeA, SepA, LmrS, and SdrM, contribute to multiple drug resistance (MDR) (2–9). LmrS, a major facilitator superfamily (MFS) efflux pump that showed homology with the lincomycin resistance efflux pump of *Bacillus* and *Lactobacillus* species, was found to confer resistance to chloramphenicol and erythromycin (2, 10). In this study, we selected and evaluated seven open reading frames (ORFs) with similarity to staphylococcal MDR pumps from the *Staphylococcus aureus* strain NCTC8325 genome for their transcription and drug resistance levels in mutant QT21.

We report here the role of LmrS as an additional TetR21-regulated transporter contributing to the chloramphenicol and erythromycin resistance phenotypes in mutant QT21.

All strains and plasmids used in this study are listed in Table 1. *S. aureus* was cultivated in Luria-Bertani (LB) broth (Difco, Sparks, MD) at 37°C unless otherwise stated. All antibiotics and compounds were purchased from Sigma Chemical Co. (St. Louis, MO). Each 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 (8). All primers in this study were synthesized at Massachusetts General Hospital (MGH) by the MGH Core Facility (Boston, MA).

**Construction of mutant strains QT18 (*lmrS*) and QT1821 (*lmrS*, *tetR21*).** The procedure was carried out as previously described (1) using the following *lmrS* primers designed from regions 1 kb upstream and downstream of SAOUHSC_02418 of *S. aureus* NCTC8325: 5′-TTAATGCATTGGTACCAACAACAGCCATCT-3′ (18AF/KpnI) (KpnI is underlined), 5′-GAAGGAAATTTTAAAATAATTATAGTAGTT-3′ (18BR), 5′-AACTACTATAATTATTTTAAAATTTCCTTCATCATAATTCCTCCTTTTT-3′ (18CF) (the complementary sequence of 18BR is underlined), and 5′-ATATCTGAAGTTGAGCTCGGCACATATGTG-3′ (18DR/SacI) (SacI is underlined). The in-frame deletion mutant was named QT18. The *lmrS-tetR21* double mutant was created using the same technique as described above with the

**TABLE 1** Bacterial strains and plasmids used in this study

| Strain, plasmid, or primer | Genotype or relevant characteristic(s) | Reference or source |
|---|---|---|
| *S. aureus* | | |
| RN6390 | 8325-4 wild type | 14 |
| QT21 | 8325-4 Δ*tetR21* | 1 |
| QT18 | 8325-4 Δ*lmrS* | This study |
| QT1821 | 8325-4 Δ*lmrS* Δ*tetR21* | This study |
| RN6390 (pSK950-*lmrS*) | *lmrS* overexpressor | This study |
| *E. coli* | | |
| DH10B | *E. coli* for cloning | 15 |
| Top10 | *F-mcrA* Δ*(mrr-hsdRMS-mcrBC)* *φ80lacZ*Δ*M15* Δ*lacX74 nupG recA1 araD139* Δ*(ara-leu)7697 galE15 galK16 rpsL*(Str$^r$)[a] *endA1* $\lambda^-$ | Invitrogen |
| DH5α (pLZ113-*lmrS*) | *lmrS* overexpressor | This study |
| DH5α (pLZ113-*tetR21*) | *tetR21* overexpressor | This study |
| Plasmid | | |
| pSK950 | *E. coli/S. aureus* temp-sensitive plasmid | 16 |
| pIMAY | *E. coli/S. aureus* temp-sensitive plasmid | 15 |

[a]Str$^r$, streptomycin resistant.

*tetR21* mutant strain QT21 as the recipient strain and the same set of *lmrS* primers. The double mutant *lmrS-tetR21* was named QT1821.

**Construction of *lmrS* overexpressor.** Primers with inserted EcoRI and BamHI sites (underlined) were 5′-GAGGAATTGAATTCATGATGGCTAAAGTTG-3′ (18F-EcoRI) and 5′-ACTATAATTATTGGATCCTTAAAATTTCCT-3′ (18R-BamHI). The procedure was done as previously described (7). Transformants were selected on LB agar with tetracycline 5 μg/ml and grown at 30°C. Plasmid pLZ113 was used to generate pLZ113-*tetR21* and pLZ113-*lmrS* for complementation of mutants QT21, QT18, and QT1821.

**Real-time reverse transcription-PCR assays.** The procedure was performed as previously described with the primers listed in Table 2 and with the housekeeping gene *gmk* as an internal control (1). Three independent biological samples were analyzed in triplicate and normalized against *gmk* gene expression. Statistical analyses were performed based on the Student *t* test to determine the significance in gene expression values.

**TetR21 affects *lmrS* expression.** *tetR21* mutant QT21 showed increases of 4-fold and 2-fold, respectively, in the MICs of tetracycline and palmitoleic acid, both of which are Tet38 pump substrates, and a 2-fold increase in the MICs of chloramphenicol and erythromycin (11). The *tet38-tetR21* double mutant showed increases in the MICs of only chloramphenicol and erythromycin, suggesting the involvement of one or more

**TABLE 2** Relevant real-time PCR primers used in this study

| Gene (bp) | Primer |
|---|---|
| *gmk* (108) | |
| Forward | 5′-TCAGGACCATCTGGAGTAGGTAAAG-3′ |
| Reverse | 5′-TTCACGGATTTGACGTGTTG-3′ |
| *tet38* (106) | |
| Forward | 5′-ATGAATGTTGAATATTCTAA-3′ |
| Reverse | 5′-TGGCTACAGAAATCAAT-3′ |
| *lmrS* (120) (SAOUHSC_02418) | |
| Forward | 5′-CAGTATAAATCAATGGTCTA-3′ |
| Reverse | 5′-CTTTATCTGCCTTGTTATCA-3′ |
| *sdrM* (110) (SAOUHSC_02420) | |
| Forward | 5′-AGCGATTGAATCATCTATTA-3′ |
| Reverse | 5′-TTACTAATGCGATAAAATA-3′ |

**TABLE 3** Relative expression of efflux pump and candidate efflux pump genes in the *tetR21* mutant

| | | Changes in expression level (QT21 vs RN6390)[a] | |
|---|---|---|---|
| ***S. aureus* ORF** | **Gene** | **2 h** | **5 h** |
| SAOUHSC_00099 | *tet38* | 4.0 ± 0.2[b] | 3.9 ± 0.2[b] |
| SAOUHSC_02762 | *norD* | 0.86 ± 0.2 | 1.3 ± 0.1 |
| SAOUHSC_02700 | *mdeA* | 0.75 ± 0.1 | 0.80 ± 0.1 |
| SAOUHSC_02418 | *lmrS* | 4.0 ± 0.1[b] | 3.9 ± 0.1[b] |
| SAOUHSC_02420 | *sdrM* | 3.2 ± 0.1[b] | 3.0 ± 0.1[b] |
| SAOUHSC_02419 | *sepA* | 0.9 ± 0.1 | 0.8 ± 0.1 |
| SAOUHSC_00246 | HP[c] | 0.9 ± 0.1 | 1.3 ± 0.1 |
| SAOUHSC_02740 | HP | 0.8 ± 0.1 | 1.0 ± 0.1 |

[a]Normalized values of the *tetR21* mutant QT21 over values of the wild-type RN6390 strain. The housekeeping gene *gmk* was used as the internal control. Each assay was done in triplicate with three separate biological samples.
[b]The differences in the gene expression levels between QT21 and RN6390 are statistically significant as determined by a Student's *t* test ($P < 0.05$).
[c]HP, hypothetical protein.

other transporters. We evaluated seven ORFs from *S. aureus* NCTC8325 with similarity to staphylococcal multidrug transporters.

Three adjacent ORFs, SAOUHSC_02418 (LmrS) (conferring resistance to chloramphenicol, erythromycin, and other compounds) (2), SAOUHSC_02419 (SepA) (conferring resistance to acriflavine and ethidium bromide) (5), and SAOUHSC_02420 (SdrM) (conferring resistance to norfloxacin, acriflavine, and ethidium bromide) (9) were chosen together with SAOUHSC_02762 (NorD) (12), SAOUHSC_02700 (MdeA) (3, 13), and SAOUHSC_02740 and SAOUHSC_00246 (which showed 58% and 79% similarity with NorB, respectively).

The *tet38* transcripts were 4-fold higher in QT21 than in RN6390. The *lmrS* and *sdrM* transcripts were, respectively, 4-fold and 3.2-fold higher at 2 h and 3.9-fold and 3.0-fold higher at 5 h of growth in mutant QT21 than in RN6390 (Table 3). No significant differences were found for the expression of the other ORFs.

**LmrS confers resistance to chloramphenicol and erythromycin.** QT21 MICs showed a 2-fold increase for chloramphenicol, erythromycin, rhodamine, chlorhexidine, tetraphenylphosphonium chloride (TPP-Cl), and norfloxacin and an increase of 4-fold for tetracycline. All of these compounds are thought to be substrates of LmrS, except tetracycline (Tet38 substrate) (2, 10) and norfloxacin (SdrM substrate) (9) (Table 4).

The overexpressor RN6390 (pSK950-*lmrS*) showed a 4-fold increase in the MICs of chloramphenicol and TPP-Cl, a 2-fold increase in the MICs of erythromycin, rhodamine, and chlorhexidine. RN6390 (pSK950-*sdrM*), however, showed no change in the MICs of norfloxacin (Table 4) or ethidium bromide (data not shown).

The in-frame deletion mutant *lmrS* (QT18) showed an MIC profile similar to that of RN6390 regarding all substrates of LmrS (Table 4). The in-frame deletion double mutant *lmrS-tetR21* (QT1821) showed susceptibilities to chloramphenicol, erythromycin, rhodamine, chlorhexidine, and TPP-Cl similar to those of RN6390 and QT18 but retained a 4-fold increase in the tetracycline MIC similar to that of QT21. QT1821 showed a 2-fold increase in the norfloxacin MIC, as was observed with QT21, suggesting additional effects of TetR21 on other resistance determinants. Complementation of QT21, QT18, and QT1821 with cloned plasmids pLZ113-*tetR21* and pLZ113-*lmrS* reversed their sensitivities to tetracycline, chloramphenicol, and erythromycin to the parental levels.

**Conclusion.** TetR21 was initially identified for its ability to regulate *tet38* expression, but a *tetR21* mutant had additional resistances not attributable to Tet38. Thus, we tested the *tetR21* mutant for effects on the expression of other known efflux pump genes and found increased expression of *lmrS* and *sdrM*. Overexpression of *lmrS* but not *sdrM* from a plasmid generated resistance to chloramphenicol and other LmrS substrates previously reported (2, 10), and a *tetR21-lmrS* double mutant eliminated the non–tetracycline resistance phenotypes found in QT21 with the exception of the low-level norfloxacin resistance. Thus, *tetR21* regulates *tet38* and *lmrS* in *S. aureus* and

**TABLE 4** Susceptibility of *S. aureus* strains to antibiotics and chemical compounds

| | MIC (μg/ml) for[a]: | | | | | | |
|---|---|---|---|---|---|---|---|
| ***S. aureus* strain** | **NOR** | **TET** | **CHL** | **ERY** | **Rh** | **CHG** | **TPP-Cl** |
| RN6390 | 0.5 | 0.06 | 4 | 0.06 | 8 | 0.06 | 0.5 |
| QT7 (Δ*tet38*) | 0.5 | 0.03 | 4 | 0.06 | 8 | 0.06 | 0.5 |
| QT21 (Δ*tetR21*) | 1.0 | 0.25 | 8 | 0.12 | 16 | 0.12 | 1.0 |
| QT18 (Δ*lmrS*) | 0.5 | 0.06 | 4 | 0.06 | 8 | 0.06 | 0.5 |
| QT1821 (Δ*lmrS*-Δ*tetR21*) | 1.0 | 0.25 | 4 | 0.06 | 8 | 0.06 | 0.5 |
| RN6390 (pSK950)[b] | 0.5 | | 4 | 0.06 | 8 | 0.06 | 0.5 |
| RN6390 (pSK950-*lmrS*) | 0.5 | | 16 | 0.12 | 16 | 0.12 | 2.0 |
| RN6390 (pSK950-*sdrM*) | 0.5 | | 4 | 0.06 | 8 | 0.06 | 0.5 |
| RN6390 (pLZ113) | 0.5 | 0.06 | 4 | 0.06 | 8 | 0.06 | 0.5 |
| QT21 (pLZ113-*tetR21*) | 0.5 | 0.06 | 4 | 0.06 | 8 | 0.06 | 0.5 |
| QT18 (pLZ113-*lmrS*) | 0.5 | 0.06 | 4 | 0.06 | 8 | 0.06 | 0.5 |
| QT1821 (pLZ113-*tetR21*, pLZ113-*lmrS*) | 0.5 | 0.06 | 4 | 0.06 | 8 | 0.06 | 0.5 |

[a]NOR, norfloxacin; TET, tetracycline; CHL, chloramphenicol; ERY, erythromycin; Rh, rhodamine 6G; CHG, chlorhexidine; TPP-Cl, tetraphenylphosphonium chloride.
[b]All strains harboring plasmid pSK950 were grown in the presence of tetracycline, 5 μg/ml, and at 30°C.

contributes to multiple low-level resistances. As yet, the mechanism underlying the low-level resistance to norfloxacin has not been identified. TetR21, which bound directly to the putative *tet38* promoter (1), did not demonstrate binding to the putative promoter of *lmrS* (data not shown). TetR21 is an additional regulator, like MgrA, that modulates expression of multiple efflux pumps.

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