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Mode of Action and Biochemical Characterization of REP8839, a Novel Inhibitor of Methionyl-tRNA Synthetase

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Aminoacyl-tRNA synthetases have attracted interest as essential and novel targets involved in bacterial protein synthesis. REP8839 is a potent inhibitor of MetS, the methionyl-tRNA synthetase in *Staphylococcus aureus*, including methicillin-resistant *S. aureus* (MRSA), and in *Streptococcus pyogenes*. The biochemical activity of REP8839 was shown by specific inhibition of purified *S. aureus* MetS (50% inhibitory concentration, <1.9 nM). Target specificity was confirmed by overexpression of the *metS* gene in *S. aureus*, resulting in an eightfold increase in the MIC for REP8839. Macromolecular synthesis assays in the presence of REP8839 demonstrated a dose-dependent inhibition of protein synthesis and RNA synthesis in *S. pneumoniae* R6, but only protein synthesis was affected in an isogenic *rel* mutant deficient in the stringent response. Strains with reduced susceptibility to REP8839 were generated by selection of strains with spontaneous mutations and through serial passages. Point mutations within the *metS* gene were mapped, leading to a total of 23 different amino acid substitutions within MetS that were located around the modeled active site. The most frequent MetS mutations were I57N, leading to a shift in the MIC from 0.06 μg/ml to 4 μg/ml, and G54S, resulting in a MIC of 32 μg/ml that was associated with a reduced growth rate. The mutation prevention concentration was 32 μg/ml in four *S. aureus* strains (methicillin-sensitive *S. aureus* and MRSA), which is well below the drug concentration of 2% (20,000 μg/ml) in a topical formulation. In conclusion, we demonstrate by biochemical, physiologic, and genetic mode-of-action studies that REP8839 exerts its antibacterial activity through specific inhibition of MetS, a novel target.

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major cause of nosocomial infections with severe morbidity and mortality worldwide. According to a recent surveillance study, the methicillin (oxacillin) resistance rates among intensive care unit *S. aureus* isolates from the United States, Canada, and Europe ranged from 19.7% to 59.4% (29). In the United States, 42.8% of all *S. aureus* isolates in 2003 were methicillin resistant. In the United Kingdom, the MRSA rate has increased by 5% from 2003 to 2004 to reach 40% (50). MRSA outbreaks in the community are also on a sharp incline. This rising global health and socioeconomic problem demands new measures for prevention and control of MRSA (11, 41, 46). Efforts have been made to decrease nosocomial MRSA outbreaks through identification of MRSA colonization at hospital admission followed by adequate interventions (9). Since the elimination of nasal carriage could prevent infection, decolonization of carriers, especially in high-risk groups, is an important goal and warrants the discovery and development of new remedies (33). Many different topical agents have the potential to eliminate MRSA from the anterior nares or from the skin of carriers and thereby decrease the risk of subsequent infection. These include mupirocin, known as Bactroban ointment (32, 39), fusidic acid (36), indolmycin (23), silver sulfadiazine with cerium nitrate (43), chlorhexidine (51), tea tree oil (4), garlic-derived allicin extract (8), and autolysins, such as lysostaphin (31). Mupirocin, an inhibitor of isoleucyl-tRNA synthetase (IRS), has been used for many years as a topical agent to eliminate nasal carriage of *S. aureus* and to treat community-acquired skin infections, such as impetigo and secondarily infected traumatic skin lesions due to staphylococci and streptococci (21). However, resistance to mupirocin is on the rise, and its effectiveness is compromised in areas where clinical use is high, leading to persistence or recolonization (44, 47). According to the SENTRY antimicrobial surveillance program in 2000, 1.9 to 5.6% of *S. aureus* isolates and 12.8 to 39.9% of coagulase-negative staphylococci isolated in the United States, Canada, Latin America, and Europe were mupirocin resistant (10). Both low-level mupirocin resistance (MIC = 8 to 256 μg/ml) and high-level resistance (MIC > 256 μg/ml) have been described elsewhere (13, 15). Low-level mupirocin resistance is caused by point mutations which have been mapped within the *ileS* gene encoding the isoleucyl-tRNA synthetase (1). High-level mupirocin resistance is mediated through acquisition of a second and phylogenetically distinct isoleucyl-tRNA synthetase gene, *mupA* (18).

Aminoacyl-tRNA synthetases carry out the condensation of a specific amino acid with its cognate tRNA in a reaction that is dependent on ATP. Methionyl-tRNA synthetase (MRS) represents a novel target that is essential and well conserved among gram-positive microbes. Most bacteria, including *S. aureus*, contain a type 1 MRS, although a significant proportion of *Streptococcus pneumoniae* strains contain a second gene, *metS2*, which encodes a type 2 MRS that is more closely related to archaeal synthetases (3, 14). Both types of MRS belong to class I tRNA synthetases that harbor an ATP-binding Rossman fold with the conserved H(M/I)GH and KMSKS motifs (1, 12). A potent MRS type 1 inhibitor with antibacterial activity against staphylococci and enterococci has recently been

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identified as a high-throughput screening hit (26). Subsequent medicinal chemistry efforts resulted in REP8839, which is a fluorovinylthiophene-containing diaryldiamine with a promising antibacterial profile and spectrum of activity (7, 17, 25, 27, 28). Here, we report on the mode of action of REP8839, including the characterization of laboratory-generated mutants with decreased susceptibility to this novel synthetic compound.

MATERIALS AND METHODS

Strains, plasmids, media, and chemicals. *S. aureus* and *Streptococcus pyogenes* strains were from the American Type Culture Collection (ATCC) (Manassas, VA), from Focus Bio-Inova (Herndon, VA) and NARSA (Network on Antimicrobial Resistance in *S. aureus*; Focus Bio-Inova). *S. aureus* strain RN4220 containing pYH4-*metS* and the *S. pneumoniae* strains R6 and R6 *rel* (16) were from GlaxoSmithKline (Collegeville, PA). Mueller-Hinton broth (MHB), Mueller-Hinton agar (MHA), and blood agar plates were from Remel (Lenexa, KS). Streptococci were grown in MHB containing 3% lysed horse blood. Purified preparations of *S. aureus* MetS, *Haemophilus influenzae* MetG, *Escherichia coli* MetG, and *S. pneumoniae* MetS2, as well as a rat liver lysate, were provided by GlaxoSmithKline (Collegeville, PA). *E. coli* MRE 600 tRNA was from Roche Applied Science (Indianapolis, IN).

REP8839 was synthesized at Replidyne, and mupirocin was from Pliva (Zagreb, Croatia). Novobiocin (USP), gentamicin (Sigma), and vancomycin (USP) were used as control agents. Anhydrotetracycline was from BD Biosciences (Palo Alto, CA). The radiolabeled compounds [5-^{3}H]uridine, L-[4,5-^{3}H]leucine, and L-[*methyl*-^{3}H]methionine were from Amersham Biosciences Corp. (Piscataway, NJ).

MRS enzymatic assay. Assays for inhibition of $tRNA^{Met}$ aminoacylation were carried out much as described previously (34). Reaction mixtures (50 μl) contained 65 mM Tris-HCl, pH 8.0, 80 mM KCl, 10 mM magnesium acetate, 2.5 mM dithiothreitol, 5 mM ATP, 1 mg/ml *E. coli* tRNA, 0.25 mg/ml bovine serum albumin, and 7 μM methionine (1.4 μCi/nmol) in round-bottom 96-well Costar plates (Corning, NY). MRS enzymes were diluted in 50 mM Tris-HCl, pH 8.0, 2 mM dithiothreitol, and 0.3 mg/ml bovine serum albumin and added (26 μl) to wells containing REP8839 (4 μl) in dimethyl sulfoxide or to control wells containing 4 μl dimethyl sulfoxide or 0.5 M EDTA. A cofactor mix containing the remaining reaction mixture components (20 μl) was added, and plates were incubated for 15 min at room temperature (23°C). Reactions were terminated, and the tRNA was precipitated by the addition of 150 μl 5% trichloroacetic acid (TCA). Reaction mixtures were transferred to 96-well filter plates (Durapore) (catalog no. MVHBN4550; Millipore, Bedford, MA) and filtered using a Manifold (Innovative Microplate, Chicopee, MA). Samples were washed with 300 μl 10% trichloroacetic acid, followed by 300 μl 95% ethanol, and air dried overnight. Reaction products were counted by liquid scintillation using MicroScint (50 μl) and a TopCount-NXT (Packard BioScience, Boston, MA).

Microbiological assays. Broth microdilution MIC testing was performed in 96-well microtiter plates according to CLSI (formerly NCCLS) document M7-A6 (6). MIC testing of the *metS* overexpressor strain *S. aureus* RN4220(pYH4-*metS*) occurred in the presence or absence of anhydrotetracycline (10 ng/ml) to regulate *metS* expression from the TetR-dependent promoter. Macromolecular synthesis assays were performed in *S. pneumoniae* R6 and an isogenic *rel* mutant (16). The cells were grown statically for 6 h at 35°C in 10 ml MHB containing 3% lysed horse blood, and the cell density was adjusted to match the 0.5 McFarland standard (approximately 10^8 CFU/ml). A 96-well microtiter plate containing 50 μl REP8839 (0.008 to 16 μg/ml) was inoculated with 50 μl of cells and incubated for 10 min at 35°C, and the radiolabeled precursors [5-^{3}H]uridine and L-[4,5-^{3}H]leucine were added. Incorporation was stopped after 10 min by the addition of 100 μl of 20% ice-cold TCA. The plates were refrigerated for 1 h to allow cell lysis and precipitation of macromolecules, followed by vacuum filtration of the samples through 96-well Durapore plates (Millipore, Bedford, MA). The filter plates were washed twice with 200 μl of 10% ice-cold TCA and then once with 200 μl of cold ethanol and air dried. MicroScint O (Perkin-Elmer, Boston, MA) was added (50 μl), the plates were sealed and counted in a Packard TopCount NXT, and the data were normalized to the data for untreated controls. Growth competition assays between *S. aureus* wild-type cells and MetS mutant cells were performed in cocultures with a starting inoculum of roughly 10^5 CFU/ml of each strain. The cultures were grown at 35°C with shaking (200 rpm), and samples were removed at hourly intervals. Serial 10-fold dilutions in saline were prepared in a 96-well plate, and 10 μl of each dilution was spotted on blood agar (total CFU) and on selective agar containing 1 μg/ml of REP8839 (MetS mutant CFU). Colonies were enumerated, and wild-type CFU were calculated. Additionally, the ratios of wild-type to mutant cells were calculated in late exponential growth phase (9.5 h) and in stationary phase (24 h).

Spontaneous resistance rates and MPC determination. *S. aureus* cells were grown in MHB for 4 to 6 h at 35°C, harvested by centrifugation (4,000 rpm, 10 min) and resuspended in MHB at about 10^{10} CFU/ml. Agar plates containing REP8839 or mupirocin (1 to 32 μg/ml) were seeded with 0.1 ml of this cell suspension and incubated at 35°C. Colonies were counted after 48 h, and the spontaneous resistance rates were calculated on the basis of the exact cell number in the inoculum, as determined on drug-free plates. These first-step mutants were purified on MHA containing REP8839 or mupirocin at half the original concentration. Second-step mutants were isolated by repeating the procedure above with first-step isolates. Mutant stability was assessed by MIC testing after serial propagation on five drug-free plates. To determine the mutation prevention concentration (MPC), five agar plates at each drug concentration were seeded each with 2×10^9 CFU to achieve an inoculum of 10^{10} CFU, and colonies were counted after 5 days.

Serial passages. Changes in the susceptibilities of bacteria to REP8839 were monitored during serial passages in drug-containing broth. The first passage was a broth microdilution MIC test. For the subsequent passages, the cells growing in the well with the highest inhibitor concentration, typically 0.5× MIC, were resuspended, diluted 1,000-fold into broth, and used to inoculate a fresh 96-well MIC plate. The MICs of a total of 20 daily passages were recorded. After passage 20, the isolate was cultured on a blood agar purity plate before determination of the final MIC.

Characterization of mutants with reduced susceptibility to REP8839. Genomic DNA for molecular analysis was isolated using the DNeasy tissue kit (QIAGEN, Inc., Valencia, CA). A 1,067-bp fragment comprising the 5′ portion of the *metS* gene was amplified by PCR with high-fidelity PCR Supermix (Invitrogen, Carlsbad, CA) using primers *metS1* (5′-ACATTACGAGGAGGAACAG) and *metS2* (5′-GGTGTAAATACGCCATCTG). The 3′ portion of *metS* was amplified with primers *metS3* (5′-GTCTTTGCACATGGTTGGA) and *metS4* (5′-TGCTTCTC TAGCACGTGTA), yielding a 1,203-bp product. The PCR protocol for both fragments consisted of initial denaturation (5 min at 94°C); 30 cycles, with 1 cycle consisting of denaturation (1.5 min at 94°C), annealing (1 min at 55°C), and extension (1.5 min at 72°C); followed by incubation for 10 min at 72°C in a Techgene thermal cycler (Techne, Princeton, NJ). PCR products were analyzed on a 0.8% agarose–Tris-borate-EDTA gel, cloned into pCR4-TOPO (Invitrogen, Carlsbad, CA), and sequenced with T7 and T3 primers (Molecular Biology Core Facility, University of Colorado Health Sciences Center, Denver, CO). Duplicate PCR fragments were processed to minimize PCR and DNA sequencing errors. The *metS* DNA sequences were assembled and aligned using Vector NTI (InforMax, Bethesda, MD).

Modeling of *S. aureus* MetS. Amino acid sequences for *E. coli* MetG apo form (Protein Data Bank identification code [PDB ID] 1QQT) and cocrystallized form with methionine (PDB ID 1F4L) were derived from the Protein Data Bank database (2). The WhatCheck (20) protein verification tool was used to assess the overall quality of the PDB files. Global pairwise amino acid sequence alignments for *E. coli* and *S. aureus* were generated with NCBI's Cn3D/MMDB sequence alignment tool (19, 48). The Needleman-Wunsch algorithm (38) and the BLOSUM62 amino acid substitution scoring matrix were used for alignments. Homology models for *S. aureus* used *E. coli* MetG apo form (PDB ID 1QQT) as the template and were created with MODELLER (35) by the satisfaction of spatial restraints (42) and the optimization of three-dimensional (3D) structure using CHARMM (Chemistry at Harvard Macromolecular Mechanics) force field energy terms. Root mean square deviation metrics were computed for the final 3D *S. aureus* structure relative to the *E. coli* template structure.

RESULTS

Biochemical activity of REP8839. Novel diaryldiamine compounds that are potent inhibitors of *S. aureus* MetS have been identified (25–28). Structure-activity relationship studies led to the identification of REP8839, containing a fluorovinyl-substituted thiophene ring on the left-hand side (17). REP8839 is a highly potent (nanomolar) inhibitor of *S. aureus* MetS (Table 1). The 50% inhibitory concentration (IC_{50}) was as low as the concentration of enzyme present in the assay, and the correlation between IC_{50} and enzyme concentration remained consistent as the enzyme concentration was varied, indicating that

TABLE 1. Inhibition of MetS activity by REP8839

MRS enzyme activity	IC_{50} (nM)[a]
S. aureus MetS	<1.9
H. influenzae MetG	25
E. coli MetG	307
S. pneumoniae MetS2	>500
Rat liver lysate[b]	>500

[a] The specific activity of individual enzyme preparations varied. Enzyme concentrations were adjusted to achieve charging of 15 to 25 pmol of $tRNA^{Met}$ in 15 min at 23°C. The enzyme concentrations were 1.5 nM for *S. aureus*, 3.0 nM for *H. influenzae*, 2.0 nM for *E. coli*, and 12 nM for *S. pneumoniae* MRS.

[b] Rat MRS concentration was not determined; the rat liver lysate catalyzed the charging of 5 pmol of $tRNA^{Met}$ in 15 min at 23°C.

measurement of a true inhibitory constant is limited by the enzyme concentrations (27). It was concluded that the IC_{50} for REP8839 inhibition of *S. aureus* MetS is <1.9 nM.

REP8839 also inhibited two gram-negative homologs of *S. aureus* MetS, *H. influenzae* MetG and *E. coli* MetG, albeit with at least 13-fold- and 160-fold-lower potency, respectively (Table 1). These studies were carried out with enzyme concentrations that yielded activity within the linear range of the assay. Due to various specific activities for the purified proteins from each organism, a somewhat different enzyme concentration was required for each preparation (Table 1). However, in contrast to the result for *S. aureus* MetS, the IC_{50} for REP8839 inhibition was significantly higher than the enzyme concentrations used in the assay for both the *H. influenzae* MetG (8-fold) and *E. coli* MetG (100-fold) enzymes, which allows determination of reliable IC_{50} values.

A recent study identified a second methionyl-tRNA synthetase gene, *metS2*, in 46% of 315 clinical isolates of *S. pneumoniae* (14). Interestingly, the *S. pneumoniae* MetS2 protein was not inhibited by potent *S. aureus* MetS inhibitors (14), including REP8839 (Table 1). The inhibition of a mammalian MRS activity by REP8839 was also determined, and since purified eukaryotic MRS was unavailable, we used MRS activity present in a rat liver lysate, which was not inhibited by up to 500 nM REP8839 (Table 1).

Target specificity of REP8839 in bacterial cells. The mode of action of REP8839 in bacterial cells was evaluated to confirm the target specificity of this novel compound. First, the MetS target was overexpressed in *S. aureus* using a plasmid-borne copy of *metS* under the control of an inducible promoter, and the MICs of REP8839 and control agents are shown in Table 2. Induction of MetS expression in *S. aureus*

TABLE 2. MIC changes in *S. aureus* upon overexpression of the MetS target

Antimicrobial agent	*S. aureus* RN4220 MIC (μg/ml)			
	pYH4 vector control[a]		pYH4-*metS*[a]	
	− aTc inducer	+ aTc inducer	− aTc inducer	+ aTc inducer
REP8839	0.008	0.008	0.12	1
Mupirocin	0.06	0.06	0.06	0.06
Novobiocin	0.25	0.25	0.25	0.25
Vancomycin	1	1	0.5	0.5

[a] In the presence (+) or absence (−) of anhydrotetracycline (aTc) inducer.

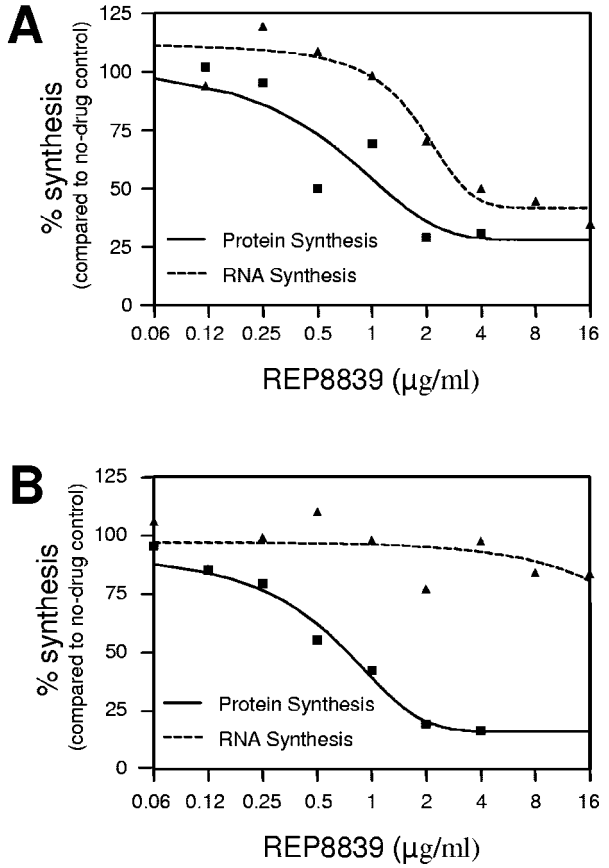

FIG. 1. Macromolecular synthesis assay in *S. pneumoniae* R6 (A) and an isogenic *rel* mutant in which the stringent response was affected (B). Cells were treated with REP8839 for 10 min, and the incorporation of [5-^{3}H]uridine and L-[4,5-^{3}H]leucine was determined to measure protein synthesis and RNA synthesis.

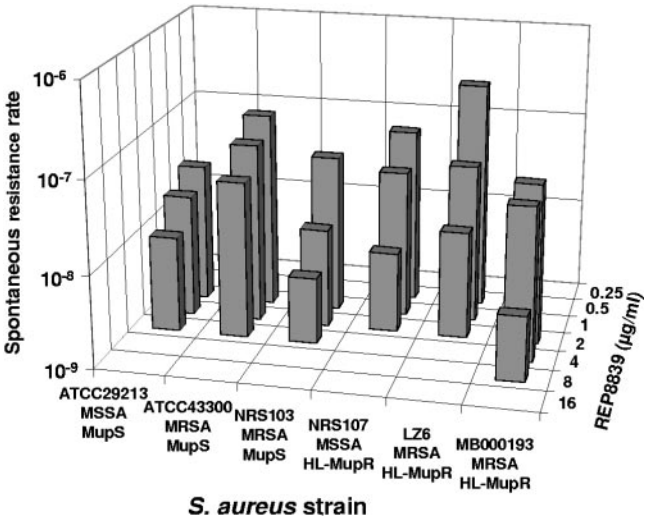

FIG. 2. Spontaneous resistance rates to REP8839 in different *S. aureus* strains. Approximately 10^9 cells were plated on agar containing REP8839 at concentrations ranging from 0.25 to 16 μg/ml, and colonies were enumerated after 48 h. MupS, mupirocin susceptible; HL-MupR, high-level mupirocin resistant.

TABLE 3. Mutation prevention concentration for REP8839 and mupirocin in *S. aureus*

S. aureus strain	MIC (μg/ml)		MPC (μg/ml)	
	REP8839	Mupirocin	REP8839	Mupirocin
ATCC 29213 (MSSA)	0.03	0.12	32	32
ATCC 43300 (MRSA)	0.06	0.12	32	32
LZ10 (MRSA)	0.03	0.12	32	>32
1079077 (MRSA)	0.25	0.25	32	>32

RN4220(pYH4-*metS*) resulted in an eightfold shift in the MIC for REP8839 from 0.12 μg/ml to 1 μg/ml. Interestingly, even the uninduced *S. aureus* RN4220(pYH4-*metS*) was significantly less susceptible to REP8839 than the *S. aureus* RN4220(pYH4) vector control strain (MIC of 0.12 μg/ml versus MIC of 0.008 μg/ml, respectively), indicating that plasmid pYH4-*metS* caused some MetS expression even in the absence of inducer. The MICs for the control drugs mupirocin, novobiocin, and vancomycin remained unchanged upon MetS overexpression. These data provide good evidence that REP8839 exerts its antibacterial activity through specific inhibition of MetS in *S. aureus*.

Second, macromolecular synthesis assays in *S. pneumoniae* in the presence of REP8839 demonstrated a dose-dependent inhibition of protein and RNA synthesis, as expected with compounds that elicit the stringent response (Fig. 1A). However, only protein synthesis was affected in a *rel* mutant deficient in the stringent response (Fig. 1B). These data provide direct evidence that REP8839 is a specific inhibitor of protein synthesis.

Development of resistance to REP8839. Several *S. aureus* strains were evaluated for their propensity to become spontaneously resistant to REP8839 when exposed to drug concentrations above the MIC. All strains tested, including MRSA and mupirocin-resistant isolates, were susceptible to REP8839 with an initial MIC range of 0.03 to 0.5 μg/ml, but most strains gave rise to first-step resistant colonies on agar containing 4 μg/ml of REP8839. *S. aureus* MB000193, which was initially the least susceptible strain (MIC = 0.5 μg/ml), was capable of forming colonies on agar with 8 μg/ml of REP8839. The calculated resistance rates from a population of approximately 10^9 cells depended on the concentration of REP8839 and were in the order of 10^{-7} to 10^{-8} after 48 h (Fig. 2). To further investigate the emergence of resistance, the MPC was determined, which is defined as the minimal drug concentration that prevents the spontaneous emergence of resistant subpopulations from 10^{10} cells within 5 days. For *S. aureus*, including methicillin-susceptible *S. aureus* (MSSA) and MRSA strains, the MPC was 32 μg/ml for REP8839 and greater or equal to 32 μg/ml for mupirocin (Table 3). The generation of second-step mutants was investigated by exposure of first-step mutants to even higher concentrations of REP8839. Three first-step mutants that contained different point mutations in *metS* and had elevated MICs of 4 to 8 μg/ml were capable of producing second-step mutants with a new MIC of 32 μg/ml at rates of 2.4×10^{-8} to 2.2×10^{-9}.

The propensity of staphylococci and streptococci to develop resistance to REP8839 was also evaluated in 20 serial passages of a total of 19 strains. The presence of subinhibitory concentrations of REP8839 caused the MIC for REP8839 to shift from an initial range of 0.015 to 0.06 μg/ml to a range of 0.06 to 16 μg/ml, and five strains had a MIC of 16 μg/ml after 20 passages (Table 4). There was no correlation between the decrease in susceptibility to REP8839 and the oxacillin or mupirocin resistance phenotype of the individual staphylococcal strains. In *S. pyogenes* the effect of passaging was less dramatic than in staphylococci, with an MIC increase from 0.12 μg/ml to 1 μg/ml. Passages with mupirocin produced a similar shift of the MIC range from 0.06 to 0.5 μg/ml to a range of 0.5

TABLE 4. MIC changes in staphylococci and streptococci upon serial passages in broth containing REP8839 or mupirocin

Strain[a]	Phenotype[b]	REP8839 MIC (μg/ml)		Mupirocin MIC (μg/ml)	
		Initial	After 20 passages	Initial	After 20 passages
S. aureus ATCC 29213	MSSA, MupS	0.06	4	0.06	0.5
S. aureus ATCC 43300	MRSA, MupS	0.06	1	0.06	8
S. aureus LZ10	MRSA, MupS	0.03	0.5	0.25	2
S. aureus NRS103	MRSA, MupS	0.12	1	0.25	1
S. aureus 1079077	MRSA, MupS	0.25	16	0.5	1
S. aureus 31-1334	MRSA, LL-MupR	0.03	8	32	128
S. aureus 1079101	MSSA, HL-MupR	0.06	16	4,096	4,096
S. aureus NRS107	MSSA, HL-MupR	0.015	0.5	4,096	4,096
S. aureus LZ1	MRSA, HL-MupR	0.06	0.06	4,096	4,096
S. aureus LZ6	MRSA, HL-MupR	0.06	2	4,096	4,096
S. aureus 10-420	MRSA, HL-MupR	0.06	8	4,096	4,096
S. aureus 87-2797	MRSA, HL-MupR	0.03	16	2,048	4,096
S. aureus 25-670	MRSA, HL-MupR	0.06	8	2,048	4,096
S. epidermidis NRS8	MRSE, LL-MupR	0.06	0.12	32	128
S. epidermidis 936528	MRSE, HL-MupR	0.03	0.5	2,048	4,096
S. epidermidis 936606	MRSE, MupS	0.06	16	0.12	2
S. haemolyticus NRS116	MRSH, MupS	0.12	16	0.12	1
S. pyogenes ATCC 19615	EryS	0.12	1	0.06	4
S. pyogenes MB000143	EryR	0.12	1	0.06	0.5

[a] Strains of *S. aureus, Staphylococcus epidermidis, Staphylococcus haemolyticus*, and *S. pyogenes.*

[b] MupS, mupirocin susceptible; LL-MupR, low-level mupirocin resistant; HL-MupR, high-level mupirocin resistant; EryS, erythromycin susceptible; EryR, erythromycin resistant.

TABLE 5. Molecular genetic characterization of laboratory-generated *S. aureus* mutants with decreased susceptibility to REP8839

Isolation method	Isolate(s)	Parental strain (phenotype[a])	MIC (μg/ml)	MetS mutation(s)
Spontaneous resistance	SSM5, others (25 isolates)	ATCC 29213	4–8	157N
	SR5-1; SR5-2	ATCC 43300 (MRSA)	8	157N
	FSM 9, others (6 isolates)	87-2797 (MRSA, HL-MupR)	4–16	I57N
	SR23-1	LZ10 (MRSA)	8	I57N
	SR59-1, SR59-2	1079077 (MRSA)	8	I57N
	SR74-2	MB000193 (MRSA)	8	I57N
	SR3, SR18, SR21	ATCC 29213	16–32	G54S
	FSM7	87-2797 (MRSA, HL-MupR)	16	G54S
	SR74-1	MB000193 (MRSA)	16	G54S
	SR84-1, SR84-2	MB000057 (MRSA)	16	G54S
	SR23-2	LZ10 (MRSA)	32	G54S
	SR19, SR23	ATCC 29213	0.5–1	None
	FSM8	87-2797 (MRSA, HL-MupR)	1	None
Serial passage	SP-1B5, SP-2D4, others (8 isolates)	ATCC 29213, ATCC 43300, others	8–16	I57N
	SP-21C	31-1334 (MRSA, LL-MupR)	8	I57T
	SP-27H	10-420 (MRSA, HL-MupR)	4	E52D
	SP-27D	14-354 (MRSA, LL-MupR)	2	A61V
	SP-21A	ATCC 29213	4	A77V
	SP-25G	LZ6 (MRSA, HL-MupR)	8	V108M
	SP-2C4	ATCC 43300 (MRSA)	2	L213W
	SP-28B	25-670 (MRSA, HL-MupR)	4	V215A
	SP-4A2	87-2797 (MRSA, HL-MupR)	8	G223C
	SP-11A3	31-1334 (MRSA, LL-MupR)	8	1238F
	SP-1A2	ATCC 29213	4	A247E
	SP-9B5	ATCC 29213	4	I57N V296F
	SP-2B5	ATCC 43300 (MRSA)	32	I57N R100S
	SP-4B5	87-2797 (MRSA, HL-MupR)	32	I57N V242F
	SP-25C	31-1334 (MRSA, LL-MupR)	2	T50A V242I
	SP-26C	1079101 (HL-MupR)	32	G54A A64P
	SP-25F	LZ1 (MRSA, HL-MupR)	16	A61T A64S
	SP-22B	25-670 (MRSA, HL-MupR)	8	A61T V108L
	SP-21G	LZ6 (MRSA, HL-MupR)	2	I94N V215A
	SP-21H	10-420 (MRSA, HL-MupR)	8	V215I V242F
	SP-22C	1079101 (HL-MupR)	16	L19F Q58L A64P
	SP-25D	14-354 (MRSA, LL-MupR)	8	A61T E98G M269I
	SP-26B	25-670 (MRSA, HL-MupR)	4	P230T A247E L257P
	SP-21B	ATCC 43300 (MRSA)	2	None
	SP-3A5	31-1334 (MRSA, LL-MupR)	2	None
	SP-4D5	87-2797 (MRSA, HL-MupR)	0.25	None
Second-step mutations	SSM 1-01, SSM 1-02, SSM 1-03	ATCC 29213	32	I57N A247E
	SSM 6-01	ATCC 29213	32	I57N G54S
	SSM 6-02, 6-03	ATCC 29213	32	I57N I238F
	SSM 14-01, 14-02	LZ6 (MRSA, HL-MupR)	32	G54S V108M

[a] HL-MupR, high-level mupirocin resistant; LL-MupR, low-level mupirocin resistant.

to 8 μg/ml in mupirocin-susceptible strains and from 32 μg/ml to 128 μg/ml in low-level mupirocin-resistant strains.

All of the laboratory-generated mutants with reduced susceptibility to REP8839 that were derived from mupirocin-susceptible strains remained susceptible to mupirocin (data not shown). This observation, together with the finding that all low- and high-level mupirocin-resistant strains are susceptible to REP8839 (7), indicates that there is no cross-resistance between REP8839 and mupirocin.

Molecular analysis of MetS and physiology of *S. aureus* with reduced susceptibility to REP8839. The 1,974-bp *metS* gene that encodes the 75-kDa MetS enzyme appears to be very well conserved among different *S. aureus* strains. Our analysis of MetS amino acid sequences obtained from 18 *S. aureus* strains, including 16 clinical isolates, with an MIC range for REP8839 of 0.008 to 0.5 μg/ml revealed only a single variation. The residue at position 260 was arginine in 12 strains and lysine in 6 strains, but this was not linked to the MIC for REP8839 or to the oxacillin resistance status. A total of 89 strains obtained through spontaneous resistance to REP8839 or serial passages were characterized by DNA sequencing of the *metS* gene to reveal point mutations that lead to amino acid substitutions within MetS (Table 5). The majority of isolates obtained from spontaneous resistance experiments possessed an asparagine residue at position 57 instead of an isoleucine, and these I57N mutants had elevated MICs ranging from 4 to 16 μg/ml. The second type of spontaneous mutants harbored a G54S substitution in MetS, leading to MICs ranging from 16 to 32 μg/ml. These G54S mutants formed tiny colonies on agar containing REP8839 but gave rise to a subpopulation that formed large colonies on subsequent blood agar purity plates that were no longer resistant to REP8839 and were in fact true revertants in

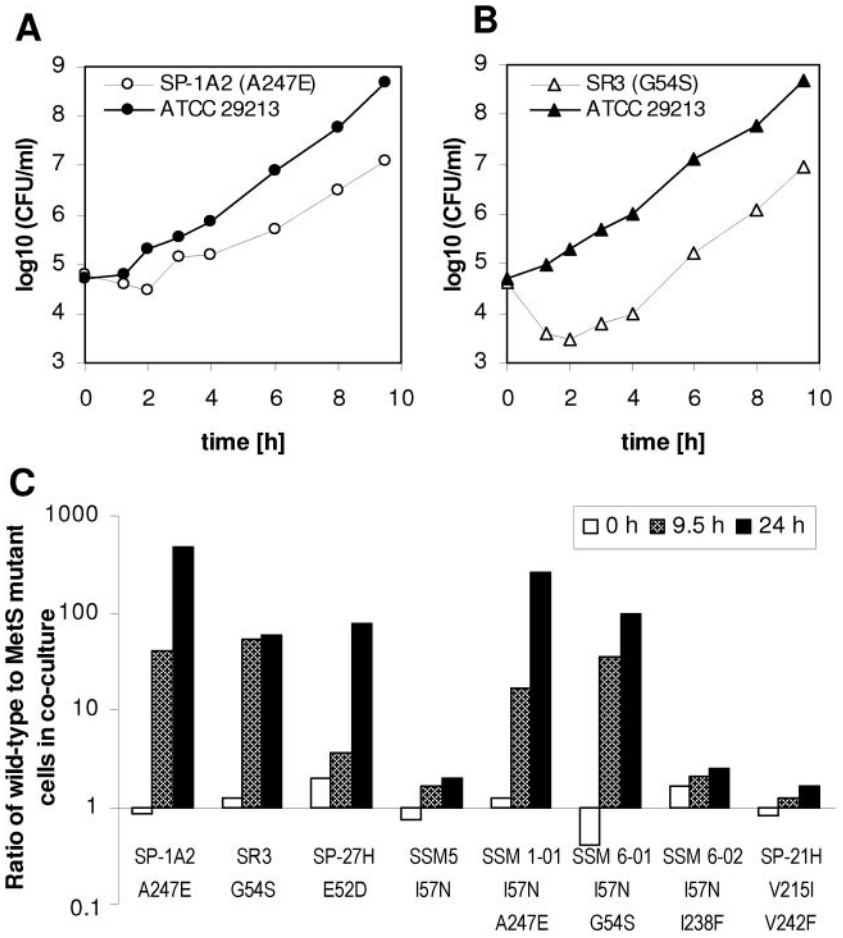

FIG. 3. Fitness burden due to MetS mutations. Wild-type cells were grown in competition cocultures with SP-1A2 cells (MetS with A247E) (A) or with SR3 cells (MetS with G54S) (B). Diluted samples were plated on blood agar to enumerate total cells or on selective agar containing REP8839 to enumerate MetS mutant cells. Increased ratios of wild-type cells to MetS mutant cells in the mixed cultures illustrate the varying cost of fitness (C).

which G54 had been restored. The serial passages produced a larger variety of MetS mutations, including double and triple mutations (Table 5). I57N mutants with MICs of 8 to 16 μg/ml were still predominant, and a I57T mutant (MIC = 8 μg/ml) was also isolated. Other mutants containing a single MetS mutation leading to a MIC of 2 to 8 μg/ml were the mutants containing E52D, A61V, A77V, V108M, L213W, G223C, I238F, A247E, and V215 mutations. Nine mutants harbored two changes in MetS, and three strains contained triple mutations, which often were combinations of individual mutations found in the single mutants. All second-step mutants contained combinations of I57N or G54S with another key mutation described above, and these were the most resistant strains, with MIC of 32 μg/ml for REP8839. No mutants with MICs of >32 μg/ml were observed.

The characterization of MetS mutants with decreased susceptibility to REP8839 provides further evidence that REP8839 is a specific inhibitor of MetS in *S. aureus*. Only a few spontaneous mutants or serial passage isolates did not have any alterations in MetS (e.g., SR19, SR23, FSM8, SP-21B, SP-3A, and SP-4D), however, their susceptibilities to REP8839 were only slightly decreased (MIC = 0.25 to 2 μg/ml).

The effects of MetS mutations on fitness were assessed in competition cocultures of *S. aureus* MetS mutants with wild-type cells. The most dramatic growth reduction was detected in strain SP-1A2 containing an A247E mutation (Fig. 3A) and in strain SR3 containing G54S (Fig. 3B). The cost-of-fitness index, which we defined as the ratio of wild-type cells to MetS mutant cells after exponential growth, was between 50 and 500 for all mutants that harbored A247E or G54S substitutions, meaning that they were unable to compete with wild-type cells (Fig. 3C). SP-27H cells containing an E52D substitution in MetS were outnumbered by wild-type cells 4-fold at 9.5 h, but 78-fold at 24 h (Fig. 3C), suggesting that competition continued during stationary phase in an equilibrium between dying and growing cells. Other mutants, including SSM5 (I57N), SSM 6-02 (I57N I238F), and SP-21H (V215I V242F) remained relatively fit, with cost-of-fitness indices of 1.6 to 2.5 (Fig. 3C).

Comparison of *S. aureus* MetS and *E. coli* MetG and molecular modeling. All mutations leading to a higher MIC for REP8839 were located within the amino-terminal half of the MetS enzyme, which is in agreement with the fact that the carboxy-terminal portion contains the tRNA binding domain and thus seems unlikely to be affected by binding of REP8839 (12). An alignment of type 1 and type 2 MRS amino acid sequences of representative species is depicted in Fig. 4 with

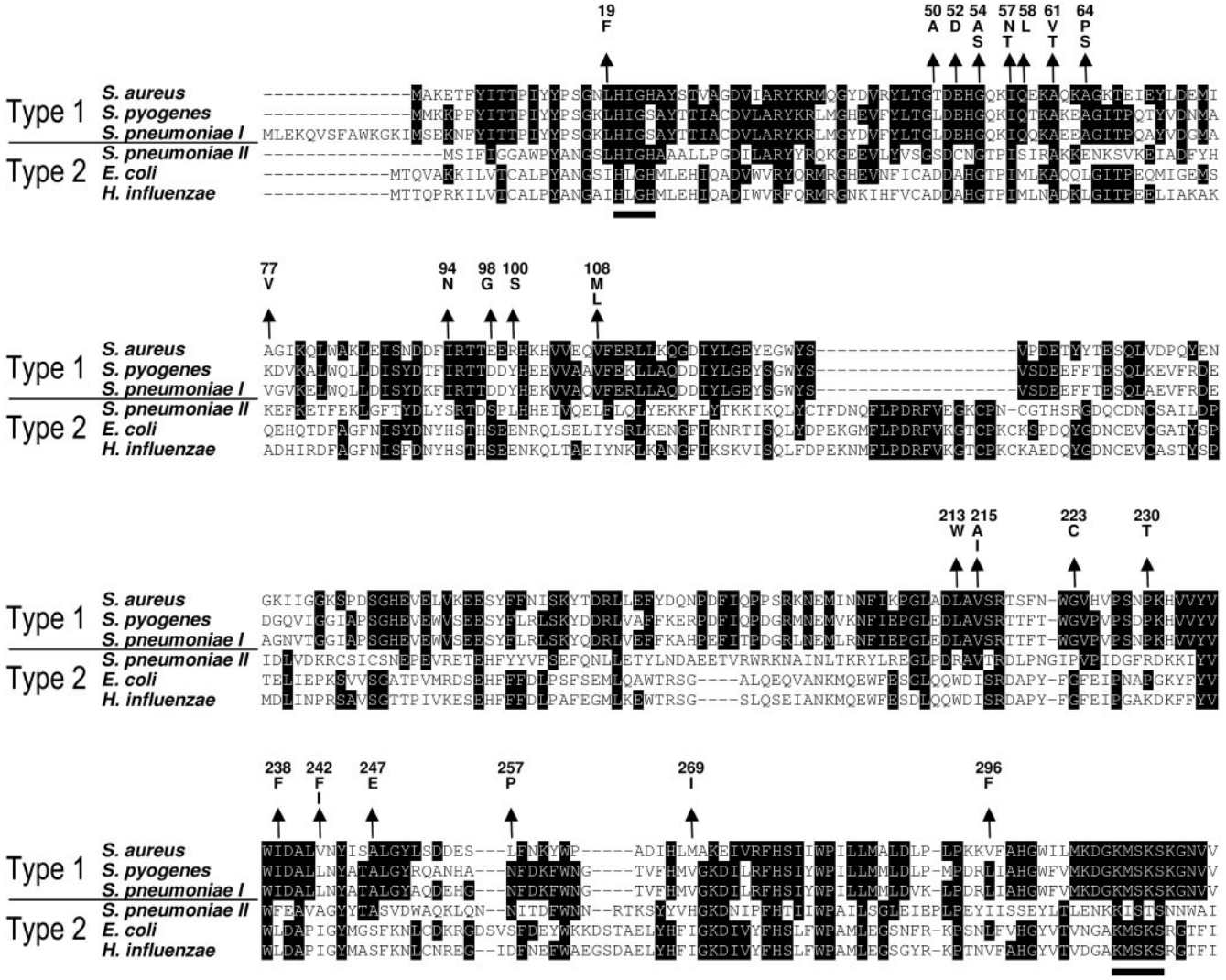

FIG. 4. Alignment of MRS type 1 and type 2 amino acid sequences. Conserved residues are indicated by white letters on black background, and the H(M/I)GH and KMSKS motifs of the ATP-binding Rossman fold that are typically found in all class I tRNA synthetases are underlined. Arrows indicate the positions and point to amino acid changes in *S. aureus* mutants with decreased susceptibility to REP8839. Gaps introduced to maximize alignment are indicated by dashes.

the locations of all characterized mutations. The region from amino acid residues 50 to 64 was most frequently affected in MetS mutants and contains G54, I57, and A61, all of which are conserved within the six bacterial strains, and A64, which is conserved among strains harboring type 1 MRS. Interestingly, several residues of altered *S. aureus* MetS had changed to the corresponding amino acids found at these positions in type 2 MRS, for which REP8839 has a much lower affinity, such as 213W, 215I, 238F, and 269I. None of the mutations were located within the well-conserved ATP-binding Rossman fold that contains the motifs H(M/I)GH and KMSKS, which characterize the class I tRNA synthetases.

A structural model of *S. aureus* MetS was generated on the basis of the known *E. coli* MetG apo form (PDB ID 1QQT) and the amino acid sequence alignment that showed 38% similarity. The accuracy of homology models with 40% sequence similarity is typically as good as nuclear magnetic resonance-derived structures (5). *E. coli* MetG and *S. aureus* MetS appear to possess a considerably conserved 3D structure, as depicted in Fig. 5. All 23 amino acid substitutions found within mutant MetS proteins were clustered around the active site where the ligand methionine is bound. The amino acid substitutions in mutant MetS proteins from strain SSM 6-01 (I57N G54S) and SP-25F (A61T A64S) are located in close proximity to active-site residues (Fig. 6). Their larger size and their hydrophilic nature may impair MetS function considerably and may cause the observed fitness burden of strain SSM 6-01.

DISCUSSION

Aminoacyl-tRNA synthetases represent novel molecular targets for the development of new classes of antibacterial agents. Pathogen-specific inhibition of this family of universal and essential targets has been validated for mupirocin, which selectively inhibits the bacterial isoleucyl-tRNA synthetase without affecting the mammalian counterpart (22). Many years of

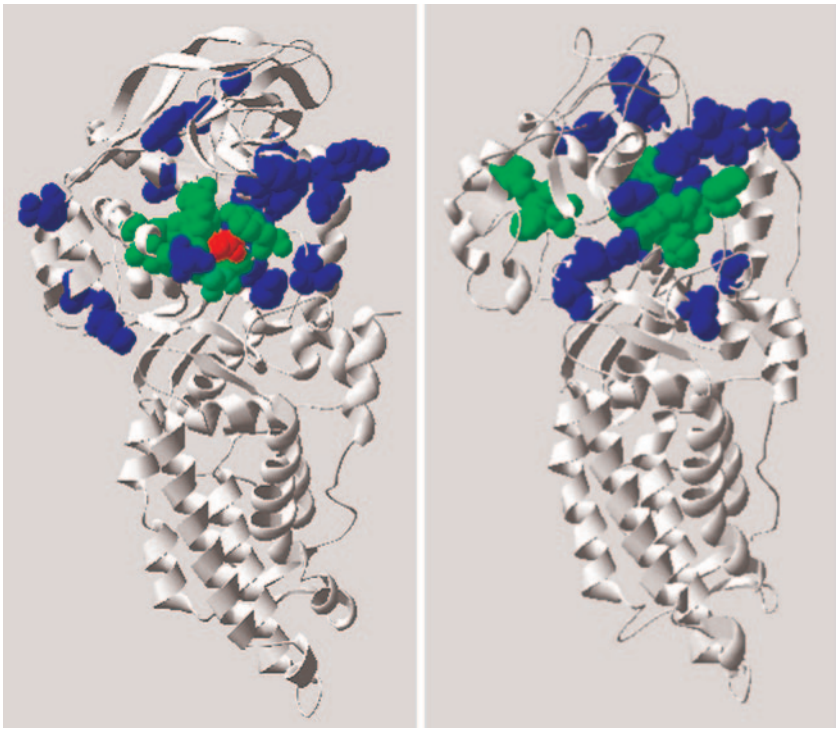

FIG. 5. Model of the *S. aureus* MetS apo form. The *E. coli* MetG cocrystallized form (PDB ID 1F4L) is shown in the left panel, with the methionine substrate highlighted in red. The right panel depicts a 3D model of the *S. aureus* MetS apo form that was generated on the basis of the *E. coli* MetG apo form as a template (PDB ID 1QQT). The 10 active-site residues within MRS are shown in green, and the positions of the residues affected in *S. aureus* MetS mutants with decreased susceptibility to REP8839 are shown in blue.

using topical formulations containing 2% mupirocin to eradicate *S. aureus* from the anterior nares and to prevent infections in carriers have resulted in a substantial increase in resistance rates. It is believed that low drug concentrations in the pharynx posttreatment may increase the risk of emergence of resistance, and this has been proposed as a mechanism for the development of mupirocin-resistant strains (49). A novel agent, REP8839, is being developed as a topical agent targeting the methionyl-tRNA synthetase. REP8839 is fully synthetic, and thus the lack of preexisting resistance is not surprising. However, natural resistance to REP8839 occurs in *S. pneumoniae*, which harbors a second gene (*metS2*) that may have been acquired through horizontal transfer (3). The distribution of *metS2* appears to be limited to *S. pneumoniae*, *Bacillus anthracis*, *Bacillus cereus*, and *Clostridium perfringens* (3). In fact, all strains of *S. pyogenes* ($n > 50$) and *S. aureus* ($n > 130$) examined so far contained only *metS1*, not *metS2*, and were susceptible to REP8839 (data not shown).

The binding of REP8839 to *S. aureus* MetS was so potent that determinations of an IC_{50} essentially resulted in enzyme titration. This was also observed previously for other similarly active MRS inhibitors (26, 27) and is reminiscent of the effects seen for the inhibition of IRS by mupirocin, which is a two-step process in which the initial enzyme-inhibitor complex undergoes an isomerization to form a tightened enzyme-inhibitor complex (40). The dissociation constant for mupirocin is approximately 20 pM, with a half-life of 140 min (40).

The mode of action of REP8839 was examined in whole-cell macromolecular synthesis assays. Inhibition of a tRNA synthetase essentially mimics starvation for amino acids by lowering the ratio of charged to uncharged tRNA within the cell. All known inhibitors of tRNA synthetases with whole-cell activity induce a stringent response, leading to a rapid decrease in the incorporation of radiolabeled uridine and (p)ppGpp levels. In contrast, "relaxed" strains with mutations in the (p)ppGpp synthetase gene do not exhibit a decrease in RNA synthesis. REP8839 inhibited both RNA and protein synthesis in *S. pneumoniae* R6, while only protein synthesis was reduced in an isogenic *rel* mutant strain, providing evidence for tRNA synthetase inhibition by REP8839.

Target specificity of REP8839 was also demonstrated through resistance studies. REP8839 was essentially equal to mupirocin regarding the MPC and was superior to mupirocin regarding the level of resistance in stable spontaneous mutants. The highest MIC for REP8839 was 32 μg/ml, which is several orders of magnitude below the drug concentration of 2% (20,000 μg/ml) in a typical topical agent. However, the local drug concentration at the site of infection may vary, and it is a known fact that exposure to topical agents containing 2% mupirocin or fusidic acid can select for low-level resistance. Reduced susceptibility to REP8839 was associated with varying cost of fitness. In a report on mupirocin resistance, first-step mutations (V588F and V631F) in IRS were generally not associated with fitness costs, but second-step mutants were unfit and produced compensatory mutations to restore fitness (24).

Our collection of MetS mutants may prove useful for gaining further insight into the drug-target interactions. Attempts to obtain crystals of the *S. aureus* MetS enzyme have failed, but crystallographic studies on *E. coli* MetG have been more successful, both in native form and as a complex with methionine

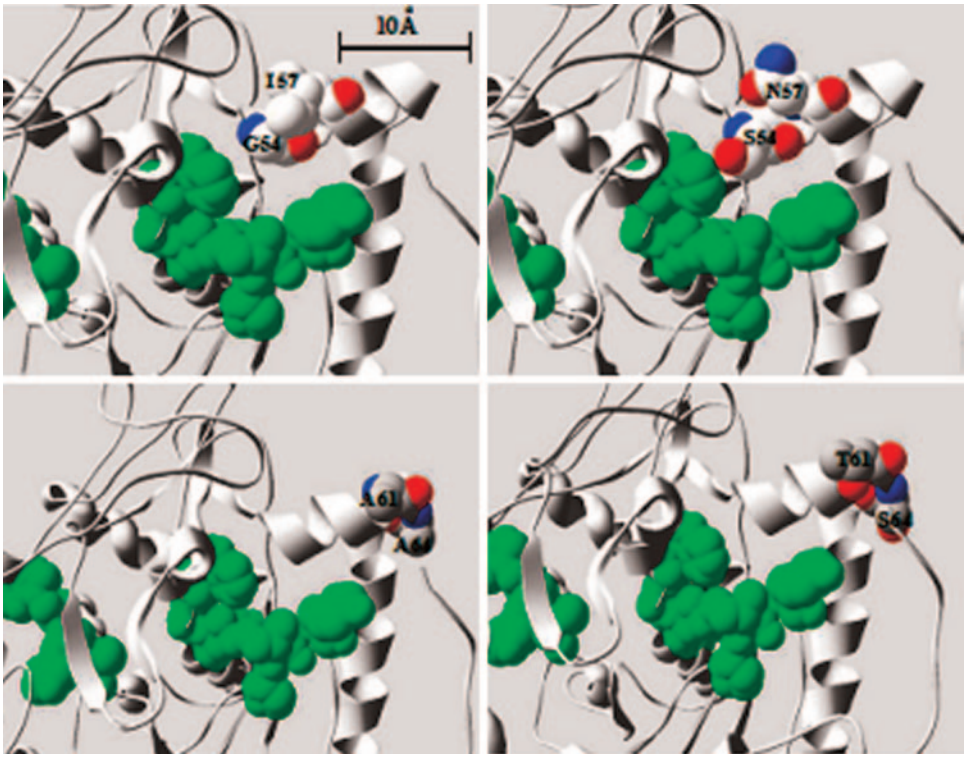

FIG. 6. Comparison of modeled active sites of *S. aureus* wild-type and mutant MetS. The key residues (colored) proximal to the active-site residues that outline the methionine/ATP binding pocket (green) caused the largest shift in MIC for REP8839 when altered. (Top) Wild-type MetS (I57 G54) and mutant MetS (N57 S54). (Bottom) Wild-type MetS (A61 A64) and mutant MetS (T61 S64).

(37, 45). The hydrophobic binding pocket consists of 10 amino acids surrounding the L-methionine, whose amine group is hydrogen bonded to the carboxyl group of Asp52 and the carbonyl oxygen atom of Leu13 (45). In a recent study, two analogs of REP8839 were docked into the binding pocket of *E. coli* MetG using the comparative molecular field analysis method and were found to form hydrogen bonds to Asp296 and to a water molecule (30). Although the overall identity between *S. aureus* MetS and *E. coli* MetG is only 26%, the two proteins are similar in length (658 versus 677 residues, respectively) and share key residues implicated in substrate binding. Interestingly, G54 and I57 that were identified in our study as the most frequent sites of substitutions leading to reduced REP8839 susceptibility are conserved in *E. coli* and *S. aureus* and are very near the Asp52 residue that forms a hydrogen bond to methionine in *E. coli* MetG. Moreover, many MetS mutations occur in the region from amino acids 213 to 296, which is located near the binding pocket and centered around the Val252 residue that is conformationally constrained in the *E. coli* enzyme upon inhibitor binding.

In the energy minimization model for one of the representatives of this class (*E. coli* MetG), the REP8839 binding site overlaps the known binding site of methionine (30). Our model of the *S. aureus* MetS active site was created using the known crystal structure of the *E. coli* MetG apo form (PDB ID 1QQT) as the template. Strikingly, all of the amino acid substitutions in our collection of *S. aureus* MetS mutants with decreased susceptibility to REP8839 are located around the active site. The amino acid changes I57N G54S (strain SSM 6-01, MIC = 32 μg/ml) and A61T A64S (strain SP-25F, MIC = 16 μg/ml) clearly extend into the active site. Furthermore, the substitution of the small nonpolar side chains found in wild-type MetS with the bulkier and polar side chains in these mutants likely affects the hydrophobic pocket, leading to lower affinity for REP8839. Studies are under way to investigate the precise roles of individual mutant protein residues in substrate binding, inhibitor binding, and enzyme kinetics.

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