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A Novel MATE Family Efflux Pump Contributes to the Reduced Susceptibility of Laboratory-Derived *Staphylococcus aureus* Mutants to Tigecycline

Fionnuala McAleese,[1]* Peter Petersen,[1] Alexey Ruzin,[1] Paul M. Dunman,[1]† Ellen Murphy,[1] Steven J. Projan,[2] and Patricia A. Bradford[1]

Wyeth Research, Pearl River, New York,[1] and Cambridge, Massachusetts[2]

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Tigecycline, an expanded-broad-spectrum glycylcycline antibiotic is not affected by the classical tetracycline resistance determinants found in *Staphylococcus aureus*. The in vitro selection of mutants with reduced susceptibility to tigecycline was evaluated for two methicillin-resistant *S. aureus* strains by serial passage in increasing concentrations of tigecycline. Both strains showed a stepwise elevation in tigecycline MIC over a period of 16 days, resulting in an increase in tigecycline MIC of 16- and 32-fold for N315 and Mu3, respectively. Transcriptional profiling revealed that both mutants exhibited over 100-fold increased expression of a gene cluster, *mepRAB* (multidrug export protein), encoding a MarR-like transcriptional regulator (*mepR*), a novel MATE family efflux pump (*mepA*), and a hypothetical protein of unknown function (*mepB*). Sequencing of the *mepR* gene in the mutant strains identified changes that presumably inactivated the MepR protein, which suggested that MepR functions as a repressor of *mepA*. Overexpression of *mepA* in a wild-type background caused a decrease in susceptibility to tigecycline and other substrates for MATE-type efflux pumps, although it was not sufficient to confer high-level resistance to tigecycline. Complementation of the *mepR* defect by overexpressing a wild-type *mepR* gene reduced *mepA* transcription and lowered the tigecycline MIC in the mutants. Transcription of *tet*(M) also increased by over 40-fold in the Mu3 mutant. This was attributed to a deletion in the promoter region of the gene that removed a stem-loop responsible for transcriptional attenuation. However, overexpression of the *tet*(M) transcript in a tigecycline-susceptible strain was not enough to significantly increase the MIC of tigecycline. These results suggest that the overexpression of *mepA* but not *tet*(M) may contribute to decreased susceptibility of tigecycline in *S. aureus*.

Staphylococcus aureus is an important human pathogen causing infections that range in severity from superficial skin abscesses to more serious invasive diseases (32). Methicillin-resistant *S. aureus* (MRSA) is a problem in hospitals worldwide (1). The transfer of vancomycin resistance from *Enterococcus* species into *S. aureus* (8) and the emerging problem of MRSA infections in the community setting (27) are of particular concern. Tigecycline, a novel glycylcycline antibiotic, exhibits good antimicrobial activity against a broad spectrum of gram-positive and gram-negative pathogens including MRSA and *S. aureus* strains with intermediate and high levels of resistance to vancomycin (7, 23).

Tigecycline belongs to the glycylcycline class of antibiotics, which are not affected by either of the classical mechanisms of resistance to tetracyclines, specific efflux pumps or ribosomal protection (24). Glycylcyclines have a higher binding affinity for the ribosome than do classical tetracyclines (3, 5), and recent studies have suggested that steric hindrance due to a bulky side group may enable tigecycline to overcome most of the known tetracycline resistance mechanisms (3).

Tigecycline exhibits potent antimicrobial activity against gram-positive and most gram-negative species. However, *Pseudomonas aeruginosa* and *Proteus mirabilis* species have demonstrated intrinsic reduced susceptibility to tigecycline, which has been attributed to the resistance nodulation cell division family AcrAB and MexAB-OprM pumps (9, 31). Surveillance studies carried out have not identified any naturally occurring *S. aureus* isolates with decreased susceptibility to tigecycline to date. However, the ability of *S. aureus* to develop reduced susceptibility to tigecycline when grown by serial passaging under selective pressure in vitro was demonstrated previously (K. Kuwahara-Arai, H. Hanaki, K. Ohkuma, and K. Hiramatsu, Abstr. 42nd Intersci. Conf. Antimicrob. Agents Chemother., abstr. E-1137, 2002).

In this study, the isolation of mutants with reduced susceptibility to tigecycline was evaluated for two MRSA strains by serial passage in increasing concentrations of this antibiotic. The mechanisms of reduced susceptibility were investigated by transcription profile analysis.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *S. aureus* strains were routinely propagated in Trypticase soy broth (BBL, Cockeysville, Md.) or in Mueller-Hinton II broth (MHB II; BBL). *Escherichia coli* cultures were grown in Luria-Bertani medium (LB; Difco, Detroit, Mich.). Gradient plates were prepared using brain heart infusion agar (Difco). The following antibiotics were incorporated into the medium when appropriate: ampicillin, 50 μg/ml; chloramphenicol, 7.5 or 15 μg/ml; kanamycin, 50 μg/ml.

Antibiotic susceptibility testing. The MICs of various antibiotics and substrates were determined by broth microdilution or broth macrodilution as indicated, using twofold serial dilutions in MHB II using standard NCCLS procedures (21). Microdilution MIC testing for tigecycline was performed by using the referenced method (using fresh MHB II, <12 h old) (22). The following antibi-

* Corresponding author. Mailing address: Wyeth Research, Room 3219, Bldg. 200, 401 N. Middletown Rd., Pearl River, NY 10965. Phone: (845) 602-2432. Fax: (845) 602-5671. E-mail: mcaleef@wyeth.com.

† Present address: 986495, Department of Microbiology and Pathology, University of Nebraska Medical Center, Omaha, NE 68198.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Reference
S. aureus strains		
Mu3	Clinical MRSA isolate with heterogeneous resistance to vancomycin	15
N315	Clinical MRSA isolate	17
RN4220	Restriction deficient derivative of 8325-4	16
Plasmids		
pALC2073	pSK236 containing the *tet*(R) gene and the tetracycline-inducible *xyl*/*tetO* promoter region cloned into the PstI and SmaI sites	2
pCLL3432	pALC2073 with *tet*(M) cloned into the EcoRI site in the sense direction	This study
pCLL3433	pALC2073 with *tet*(M) cloned into the EcoRI site in the antisense direction	This study
pCLL3434	pALC2073 with *mepR* cloned into the SacI site in the sense direction	This study
pCLL3435	pALC2073 with *mepR* cloned into the SacI site in the antisense direction	This study
pCLL3436	pALC2073 with *mepA* cloned into the EcoRI site in the sense direction	This study
pCLL3437	pALC2073 with *mepA* cloned into the EcoRI site in the antisense direction	This study

otics and substrates were used in this study and prepared fresh on the day of testing: minocycline, vancomycin, tobramycin, oxacillin, tetracycline, ciprofloxacin, norfloxacin, ethidium bromide (EtBr), tetraphenylphosphonium bromide (TPP), and erythromycin (Sigma Chemical Co., St. Louis, Mo.); imipenem (United States Pharmacopoeia, Rockville, Md.); levofloxacin (Johnson & Johnson, Spring House, Pa.); and tigecycline, piperacillin-tazobactam, DMG-MINO (9-*N*,*N*-dimethylglycylamido-minocycline), and DMG-DMDOT (9-*N*,*N*-dimethylglycylamido-6-demethyl-6-deoxytetracycline) (Wyeth Research, Pearl River, N.Y.). DMG-MINO and DMG-DMDOT belong to the glycylcycline family of antibiotics (30). Gradient plates were prepared using brain heart infusion agar and contained gradients of tigecycline, EtBr, TPP, DMG-MINO, DMG-DMDOT, or imipenem as appropriate. Paper strips were soaked in 0.5 McFarland suspensions of overnight cultures grown on agar plates, applied to gradient plates, and removed after 5 min. Plates were incubated for 20 to 22 h at 35°C.

Selection of decreased susceptibility to tigecycline. Mutants with decreased susceptibility to tigecycline were isolated following serial passage of *S. aureus* strains Mu3 and N315 in increasing concentrations of this drug. Briefly, tubes containing 2 ml of MHB II with twofold-increasing concentrations of tigecycline were inoculated with 5×10^5 CFU/ml of Mu3 or N315. Following overnight incubation at 35°C without shaking, the MIC was determined by the macrodilution method as the lowest drug concentration to inhibit bacterial growth. The tubes with the highest drug concentration that permitted growth were used to inoculate a series of tubes containing fresh MHB II with twofold-increasing concentrations of tigecycline adjusted to a starting concentration of 5×10^5 CFU/ml. These were incubated overnight at 35°C without shaking as before. Again, the lowest drug concentration that inhibited growth was determined by the macrodilution method to be the MIC, and the culture growing at the highest drug concentration was used to prepare the inoculum for the next passage. This process was repeated for 16 days. The inoculating cultures were saved for each of the 16 passages, and the ribotype of each was determined to confirm the integrity of the selected mutant using the RiboPrinter system (Qualicon, Wilmington, Del.) according to the manufacturer's instructions. Each strain was analyzed using two restriction enzymes, EcoRI and PvuII.

DNA manipulations. Standard nucleic acid techniques were performed according to the methods of Sambrook et al. (28). The following kits and reagents were used where appropriate and in accordance with the manufacturers' instructions: FailSafe PCR system and Fast-Link DNA ligation kit (Epicentre, Madison, Wis.), QIAquick gel extraction kit (QIAGEN, Inc., Valencia, Calif.), pCR4-TOPO cloning vector (Invitrogen), Spin mini-prep kit (QIAGEN), and Big Dye, version 3.1, sequencing kit (Applied Biosystems, Foster City, Calif.). Oligonucleotide primers (GeneLink, Hawthorne, N.Y.) used in the study are listed in Table 2. For PCRs, DNA was denatured for 1 min at 94°C, primers were annealed for 1 min at a primer-specific temperature, and a 1-min extension at 72°C was allowed per kb of DNA. This was repeated for 30 cycles followed by a final 10-min extension at 72°C. To detect point mutations, gel-purified DNA fragments were cloned into pCR4-TOPO and inserts from three independent PCRs and multiple pCR4-TOPO clones were sequenced in both directions using the M13 forward and reverse primers. For purification of plasmids from *S. aureus*, lysostaphin was added to buffer P1 at a final concentration of 10 μg per ml of starting culture followed by a 30-min incubation at 37°C.

TABLE 2. Primers and probes used in this study

Primer/probe name	Sequence (5′ to 3′)[b]	5′ Restriction site[a]	Expected size of product (bp)
Tet MF1	CCGGAATTCCTGGGATTTTTATGCCCTTTTG	EcoRI	
Tet MR1	CCGGAATTCTCCTTTCCACTTTAATTCAAATC	EcoRI	2,053
MepAF1	CCGGAATTCGCTAATTATTGGAAAAGACAAGG	EcoRI	
MepAR1	CCGGAATTCATATACTCAGCCAGAAGTGTAC	EcoRI	1,419
MepRF1	CCCGAGCTCTAGACATCTAACGAAATGGTGG	SacI	
MepRR1	CCCGAGCTCCAGTCACACTTACCATTTATTG	SacI	466
pTet MF2	CTCGCAATTTGAGTTGATATTAG	NA	
pTet MR1	TTCATGTGATTTTCCTCCATTC	NA	436
pMepRF1	TTGAAACAGGATCGGTTGTTTC	NA	
pMepRR1	CCATTTATTGCACCACCATTTC	NA	534
MepRF2	CAATAAATGGAATTCACTTATTCG	NA	
MepRR2	CTTTCATTGTTCAATACTCCTTG	NA	539
RT-PCR MepA fwd	TTATGGAAACTTCGCGATTGC	NA	
RT-PCR MepA rev	AACACCTTCACATAATCCCATGATAAT	NA	91
RT-PCR MepA probe	AGTTATGGTATCTCATTTAGACTTGTGCAATTTCCAGAA	NA	
RT-PCR Tet M fwd	AATGGGCTTAGTGTTTGTTTAGCA	NA	
RT-PCR Tet M rev	CGGGTCTGGCAAACAGGTT	NA	74
RT-PCR Tet M probe	AGCTAAAAGGATATCAGGTTACCACTGGC	NA	
RT-PCR 16SrRNA fwd	CCAGCAGCCGCGGTAAT	NA	
RT-PCR 16SrRNA rev	CGCGCTTTACGCCCAATA	NA	62
RT-PCR 16SrRNA probe	CGTAGGTGGCAAGCGTTATCCGGA	NA	

[a] NA, not applicable.
[b] Restriction sites are underlined.

Transcription profile analysis of RNA and DNA samples. Chromosomal DNA was prepared and labeled as described by Dunman et al. (10). For RNA preparation, *S. aureus* cultures grown overnight in 5 ml of Trypticase soy broth were used to inoculate 50 ml of MHB II in a 250-ml Erlenmeyer flask to a starting optical density at 650 nm of 0.05. Cells were grown at 37°C with shaking at 200 rpm for approximately 3.5 h to mid-exponential phase (corresponding to an optical density at 650 nm of 0.6 to 1.0 under the growth conditions described above). An equal volume of an ice-cold acetone-alcohol (1:1) solution was added, and cells were harvested by centrifugation. Cell pellets were resuspended in 500 μl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8) and lysed using the FastPrep system (Qbiogene, Carlsbad, Calif.). RNA was isolated using the RNeasy mini kit (QIAGEN), and on-column DNase treatment was performed using the RNase-free DNase kit (QIAGEN) according to the manufacturers recommendations. Reverse transcription, cDNA fragmentation, and terminal labeling of cDNA fragments with biotin were carried out in accordance with the manufacturer's protocol for antisense prokaryotic arrays (Affymetrix, Inc., Santa Clara, Calif.). Custom-designed *S. aureus* GeneChips (Affymetrix) are described in detail by Dunman et al. (10). Hybridization and detection of labeled material and analysis of data has been described previously (4, 11).

Construction of plasmids overexpressing *tet*(M), *mepR*, or *mepA*. *tet*(M), *mepR*, and *mepA* gene fragments were PCR amplified using Mu3 chromosomal DNA template and primers TetMF1, TetMR1, MepRF1, MepRR1, MepAF1, and MepAR1, respectively (as detailed in Table 2). PCR fragments were gel purified, digested with either EcoRI or SacI where appropriate and cloned into pALC2073 cut with either EcoRI or SacI, thus placing these genes under the control of a tetracycline-inducible promoter. Ligation mixtures were transformed into chemically competent *Escherichia coli* DH5α cells (Invitrogen) as specified by the manufacturer. The orientation and integrity of the inserts were verified by restriction mapping and sequencing. Plasmid constructs containing inserts of the *tet*(M), *mepR*, or *mepA* gene in either the sense or antisense direction are listed in Table 1. The antisense constructs were used as negative controls. Plasmids were electroporated into *S. aureus* strain RN4220 (12) with selection on chloramphenicol (7.5 μg/ml) and subsequently transduced into the relevant N315 or Mu3 strains using ϕ80α by standard methods (12). Real-time (RT)-PCR analysis indicated that the *mepA*, *mepR*, and *tet*(M) genes were highly expressed even in the absence of tetracycline inducer, suggesting that the regulation of these constructs was leaky.

RT-PCR. Oligonucleotide primers and probes used for RT-PCR (Table 2) were designed with Primer Express software, version 2.0 (Applied Biosystems), and purchased from QIAGEN. The probes were labeled by the manufacturer with the reporter dye 6-carboxyfluorescein at the 5′ end and the quencher dye 6-carboxytetramethylrhodamine at the 3′ end. DNase-treated RNA samples were prepared as described above. RT-PCR was performed using the *Taq*man one-step RT-PCR master mix reagents kit (Applied Biosystems) with a BioRad iCycler detection system (BioRad, Hercules, Calif.). A typical RT-PCR sample (25 μl) contained 5 μl of a serial dilution of RNA template (range from 0.02 ng/ml to 200 ng/ml) and was run in duplicate. Critical threshold cycle (C_t) numbers were defined by the detection system software. Relative quantification of target gene expression was performed by normalization to an endogenous reference (16S rRNA gene) as recommended by the manufacturer.

RESULTS AND DISCUSSION

Isolation of mutants with decreased susceptibility to tigecycline. Previous attempts to select for spontaneous mutants of *S. aureus* strain 8325-4 by growth on 4× the MIC of several glycylcyclines were unsuccessful (S. Projan, unpublished results). In the present study, *S. aureus* strains Mu3 and N315 were grown in increasing concentrations of tigecycline over a period of 16 days to facilitate the selection of mutants with decreased susceptibility to this drug. N315 and Mu3 are closely related MRSA isolates belonging to a clinically prevalent clonotype, and Mu3 exhibits a heterogeneous vancomycin-resistant phenotype (15, 17). Neither strain carries the efflux pump-encoding *tet*(K) gene, but Mu3 has a copy of *tet*(M), which encodes a ribosome protection protein. The tigecycline MIC for strain Mu3 increased from 1 to 16 μg/ml over the course of the serial passage experiment as determined by the macrodilution method. This increase in MIC occurred in a stepwise fashion with twofold incremental increases in MIC observed at passage numbers 6, 10, 12, and 15. Similarly, the tigecycline MIC for strain N315 increased from 0.5 to 4 μg/ml in three steps observed at passage numbers 9, 13, and 15. Stepwise mutants for both strains were selected for further study and termed Mu3_mut1 to Mu3_mut4 and N315_mut1 to N315_mut3, respectively. Tigecycline MICs were subsequently redetermined using the reference broth microdilution method and found to be 0.5, 1, 4, 8, and 16 μg/ml for Mu3 and Mu3_mut1 through Mu3_mut4, respectively. Tigecycline MICs for N315 strains were 0.25, 0.5, 2, and 4 μg/ml for the parent and mutant strains N315_mut1 through N315_mut3 (Table 3). Mu3_mut4 and N315_mut3 were serially passaged daily in drug-free media for a period of 15 days. The decreased susceptibility of these strains to tigecycline was maintained, suggesting that any mutations that may have occurred were stable.

Susceptibility profiles of Mu3 and N315 parent and mutant strain pairs. The antibiotic susceptibility profile of the Mu3/Mu3_mut4 and N315/N315_mut3 strain pairs was determined to ascertain whether the phenotype of the mutants had changed with respect to agents other than tigecycline (Table 3). Elevated MICs of two additional glycylcycline compounds, DMG-MINO and DMG-DMDOT, were observed for both mutants, and the MICs of EtBr and TPP increased in the N315 mutant, suggesting that a more general resistance mechanism may be involved. Interestingly, the imipenem MIC for N315_mut3 decreased. In general, MIC changes occurred in a stepwise manner in the intermediate mutants (Table 3). Several twofold changes in MIC were observed for ciprofloxacin, norfloxacin, minocycline, and tetracycline (data not shown). The MICs of oxacillin, piperacillin-tazobactam, vancomycin, tobramycin, levofloxacin, and erythromycin did not change in the mutant strains (data not shown).

GeneChip analysis of Mu3 and N315 parent and mutant strains. *S. aureus* GeneChips were used to perform a global-scale comparison of the DNA and RNA profiles of parent strains Mu3 and N315 and their respective mutants to characterize any changes in the DNA content or gene expression profile that might be responsible for decreased susceptibility to tigecycline in the mutants. No differences were observed between the DNA profiles of Mu3 and Mu3_mut4 or N315 and N315_mut3, indicating that the mutants had not lost any of the genes represented on the GeneChip during the serial passage experiment that may have altered their susceptibility to this antibiotic (data not shown). However, this approach is not sensitive enough to detect any point mutations or small deletions that may have had an impact on gene expression.

To identify any changes in gene expression that might be responsible for the altered susceptibility of the Mu3 and N315 mutants to tigecycline, the transcription profiles of the parent and each of the mutant strains were compared using RNA harvested from the mid-exponential phase of growth. Table 4 lists the genes whose expression is altered by at least eightfold in the Mu3 or N315 mutants compared to their respective parent strains. An efflux pump-encoding gene, *mepA*, exhibited dramatically altered expression in all mutants, and the *tet*(M) gene was overexpressed in Mu3_mut3 and Mu3_mut4. Both seemed likely candidates to play a role in the development of resistance to tigecycline and were investigated further.

TABLE 3. Microdilution MIC values for N315 and Mu3 strains

Strain	Plasmid	Chromosomal phenotype[a,b]	Plasmid phenotype	MIC (μg/ml)[c] of:					
				TGC	EtBr	TPP	DMG-MINO	DMG-DMDOT	IPM
N315		MepR MepA		0.25	4	16	0.125	0.125	2
N315	pCLL3436	MepR MepA	$MepA^{++}$	1	16	64	0.25	0.125	1
N315	pCLL3432	MepR MepA	Tet M^{++}	0.5	4	16	0.125	0.125	2
N315_mut1		$MepR_T^{++}$ $MepA^{++}$		0.5	16	64	0.5	0.25	0.25
N315_mut1	pCLL3434	$MepR_T^{++}$ $MepA^{++}$	$MepR^{++}$	0.125	8	32	0.125	0.125	2
N315_mut2		$MepR_T^{++}$ $MepA^{++}$		2	32	64	1	0.25	0.125
N315_mut3		$MepR_T^{++}$ $MepA^{++}$		4	32	64	2	0.5	0.125
N315_mut3	pCLL3434	$MepR_T^{++}$ $MepA^{++}$	$MepR^{++}$	2	8	32	1	0.25	0.5
N315_mut3	pCLL3432	$MepR_T^{++}$ $MepA^{++}$	Tet M^{++}	4	32	64	2	0.5	0.125
Mu3		MepR MepA		0.5	128	512	0.5	1	128
Mu3	pCLL3436	MepR MepA	$MepA^{++}$	2	128	512	1	1	128
Mu3	pCLL3432	MepR MepA	Tet M^{++}	1	128	512	0.5	1	128
Mu3_mut1		$MepR_T^{++}$ $MepA^{++}$		1	128	512	1	2	128
Mu3_mut1	pCLL3434	$MepR_T^{++}$ $MepA^{++}$	$MepR^{++}$	0.125	128	512	0.5	1	128
Mu3_mut2		$MepR_T^{++}$ $MepA^{++}$		4	128	512	2	2	64
Mu3_mut2	pCLL3434	$MepR_T^{++}$ $MepA^{++}$	$MepR^{++}$	2	128	512	1	0.5	128
Mu3_mut2	pCLL3432	$MepR_T^{++}$ $MepA^{++}$	Tet M^{++}	4	128	512	2	2	64
Mu3_mut3		$MepR_T^{++}$ $MepA^{++}$ Tet M^{++}		8	128	512	2	4	64
Mu3_mut4		$MepR_T^{++}$ $MepA^{++}$ Tet M^{++}		16	128	512	4	4	64
Mu3_mut4	pCLL3434	$MepR_T^{++}$ $MepA^{++}$ Tet M^{++}	$MepR^{++}$	8	128	512	2	2	128

[a] $MepR_T$ refers to the truncated MepR product resulting from the introduction of a premature stop codon in the *mepR* gene of N315 and Mu3 mutants.
[b] The MepA phenotype refers to wild-type levels of *mepA* expression. $MepA^{++}$, Tet M^{++}, and $MepR^{++}$ describe the phenotypes when *mepA*, *tet*(M), or *mepR*, respectively, is overexpressed from the chromosome or plasmid as indicated.
[c] TGC, tigecycline; EtBr, ethidium bromide; TPP, tetraphenylphosphonium bromide; DMG-MINO, 9-*N,N*-dimethylglycylamido-minocycline; DMG-DMDOT, 9-*N,N*-dimethylglycylamido-6-demethyl-6-deoxytetracycline; IPM, imipenem.

Overexpression of an efflux pump-encoding gene by N315 and Mu3 mutants. A previously uncharacterized gene cluster now designated *mepRAB* (multidrug export protein) (N315 open reading frame numbers SA0322 [*mepR*], SA0323 [*mepA*], and SA0324 [*mepB*]) (17) was dramatically overexpressed (~100-fold) in the Mu3 and N315 mutants (Table 4) and represents the only genes whose expression was commonly altered by a factor of >8 in both strains. SA0323 was previously identified by Garvis et al. as *svrA* (staphylococcal virulence regulator); however, it was not identified as a putative efflux pump (13). BLAST analysis revealed that *mepR* encodes a MarR-like transcriptional regulator, *mepA* encodes a novel efflux pump belonging to the multidrug and toxin extrusion (MATE) family of efflux pumps (20), and *mepB* encodes a hypothetical protein of unknown function. MATE pumps are best characterized by the NorM efflux pump or *Vibrio parahaemolyticus* and *Neisseria* species (19, 26). These proteins function as Na^+-driven efflux pumps. Kyte-Doolittle hydropathy analysis of MepA predicts 12 transmembrane domains, which is typical of this family of pumps (18). The *mepRAB* genes are closely linked, and it is likely that they are coexpressed and/or coregulated. Transcription of the *mepA* gene in

TABLE 4. Genes with expression changes of >8-fold between Mu3 and N315 parent and mutant strains ($P < 0.05$)

N315 ORF[a]	Gene name	Product or putative function	Fold change vs[b]:						
			Mu3				N315		
			Mu3_mut1	Mu3_mut2	Mu3_mut3	Mu3_mut4	N315_mut1	N315_mut2	N315_mut3
SA0129		LPXTG protein	NC	−2.4	−5.0	−9.1	NC	NC	NC
SA0214	*uhpT*	Hexose phosphate transport protein	NC	NC	NC	NC	−16	−34	−21
SA0322	***mepR***	**MarR-like transcriptional regulator**	**103**	**91**	**66**	**83**	**71**	**71**	**78**
SA0323	***mepA***	**MATE family efflux pump**	**97**	**100**	**104**	**111**	**102**	**89**	**106**
SA0324	***mepB***	**Hypothetical protein**	**85**	**115**	**114**	**142**	**100**	**96**	**106**
SAS016		Hypothetical protein	NC	NC	NC	NC	5.3	9.5	8.6
SA0889		Hypothetical protein	NC	NC	NC	2.3	10.7	5.8	11.3
SA1048	*pyrE*	Orotate phosphoribosyltransferase	NC	−2.6	−3.4	−8.4	NC	NC	NC
SA1203	*trpF*	Anthranilate isomerase	NC	NC	NC	NC	−6.3	−10.5	−7.1
SA1204	*trpB*	Tryptophan synthase	NC	NC	2.0	2.1	−6.4	−8.2	−7.9
SA1205	*trpA*	Tryptophan synthase	NC	NC	NC	2.3	−6.3	−8.0	−8.3
SA1898		Hypothetical protein	NC	NC	NC	NC	16	9.0	19
SA2405	*betA*	Choline dehydrogenase	16.2	16.2	24.5	7.6	2.1	NC	NC
SA2406	*gbsA*	Glycine betaine aldehyde dehydrogenase	69	63	104	36	2.8	NC	NC
SAV0398[c]	***tet*(M)**	**Tetracycline resistance protein**	NC	NC	**44**	**44**	NC	NC	NC

[a] Genes investigated in this report are highlighted in boldface type. ORF, open reading frame.
[b] NC, no change (<2-fold change in gene expression). Changes for genes with decreased expression are indicated with a minus sign.
[c] Mu50 open reading frame number.

the N315 and Mu3 mutants was examined by RT-PCR. These results confirm that expression of *mepA* is increased in each of the stepwise mutants of N315 (198-, 83-, and 155-fold for mut1 to mut3, respectively) and Mu3 (16-, 62-, 82-, and 66-fold for mut1 to mut4, respectively) (data not shown). A fourfold increase in *mepA* expression between Mu3_mut1 and Mu3_mut2 was detected by RT-PCR analysis that was not observed by transcription profile analysis. This may be related to saturation of the signal detected on the GeneChip.

It was considered that *mepR* might play a role in regulation of the *mepRAB* locus. The *mepR* gene including ~500 bp of the region upstream and the *mepRA* intergenic region were sequenced for each of the Mu3 and N315 mutants using primers pMepRF1, pMepRR1, MepRF2, and MepRR2. No mutations were identified in the *mepR* upstream region, but a single T→A transition at base position 123 in the *mepR* coding sequence was found to occur in N315_mut1 and was maintained in the N315_mut2 and N315_mut3 mutants. This mutation introduces a premature stop codon in the *mepR* gene, resulting in a predicted truncation of MepR from 139 to 40 amino acid residues. A 4-bp deletion was identified in the *mepR* gene of Mu3_mut1, Mu3_mut2, Mu3_mut3, and Mu3_mut4. This deletion removes bases 313 to 316 and causes a frameshift that introduces a premature stop codon at position 380, resulting in a MepR truncation from 139 to 125 amino acid residues. These results suggest that MepR may function as a repressor of the *mepRAB* locus. This is similar to the *acrAB* pump system in gram-negative bacteria, which is repressed by AcrR and the multiple antibiotic resistance *marRAB* locus of *E. coli* which shares a similar organization to *mepRAB* (14).

Effect of *mepA* overexpression on the susceptibility profile of parent and mutant strains. The importance of efflux pumps in the reduced susceptibility of gram-negative organisms such as *P. mirabilis* and *P. aeruginosa* to tigecycline has previously been reported (9, 31). The increased expression of *mepA* detected in strains N315_mut1 and Mu3_mut1 is potentially linked to the two- to fourfold increases in the MIC of tigecycline, EtBr, TPP, DMG-MINO, and DMG-DMDOT observed for these strains (Table 3). To confirm this, the *mepA* gene was overexpressed from a multicopy plasmid in the parent strains Mu3 and N315. This caused a fourfold increase in the MIC of tigecycline (Table 3). A similar result was observed on tigecycline gradient plates (3.2- and 3.5-fold increases for Mu3 and N315, respectively) (data not shown). This suggests that the MepA MATE efflux pump can export tigecycline from the cell. Overexpression of *mepA* also caused a fourfold increase in N315 resistance to EtBr and TPP and twofold increases in DMG-MINO MICs for both N315 and Mu3, suggesting that this pump can export multiple substrates (Table 3). These results were confirmed by gradient plate analysis (data not shown). Despite the increased MICs of DMG-DMDOT in the Mu3 and N315 mutants, overexpression of *mepA* in the parent strains was not sufficient to increase the MIC of this glycylcycline, suggesting that the MepA pump does not alter DMG-DMDOT susceptibility. Overexpression of *mepA* did not promote any changes in the EtBr, TPP, or imipenem MIC in strain Mu3, which already exhibits high MICs for these compounds (Table 3). A plasmid overexpressing the *mepA* gene in the antisense direction was used as a negative control and did not cause any changes in MIC compared to the parent strain (data not shown).

Complementation of the *mepR* defect. The presence of mutations in the *mepR* gene and overexpression of the *mepRAB* locus in the Mu3 and N315 mutants suggest that MepR may function to repress transcription of these genes. If this is the case, then overexpression of the wild-type *mepR* gene from pCLL3434 in the mutant strains should complement the defect in the chromosomal *mepR* copy and cause a reduction in *mepA* expression. RT-PCR experiments showed that transcription of *mepA* decreased by 57-fold in Mu3_mut1 and by 87-fold in N315_mut1 when the wild-type *mepR* gene was overexpressed in these strains (data not shown). This supports the hypothesis that MepR can function as a repressor of *mepA* expression, but it is not clear whether this is a direct or indirect effect.

The effect of overexpression of a wild-type copy of the *mepR* gene was of principle interest for N315_mut1, Mu3_mut1, and Mu3_mut2 strains. These are the only strains where *mepA* expression can potentially be associated with the MIC increases described in the previous section, as *mepA* expression does not increase further in later-stage mutants, despite continuing increases in tigecycline MICs. Complementation of the *mepR* defect in N315_mut1 caused a decrease in the MICs of tigecycline (fourfold for N315_mut1 and eightfold for Mu3_mut1) and DMG-MINO (fourfold for N315_mut1 and twofold for Mu3_mut1) and a twofold decrease in the MICs of EtBr and TPP for N315_mut1. This suggests that the elevated MICs observed in these first-step mutants are likely due to the derepression of *mepA* expression caused by the *mepR* mutation. These effects were also observed on gradient plates, but the presence of single colonies growing at the high end of the gradient suggests that spontaneous mutants can arise and grow at higher concentrations (data not shown). Interestingly, overexpression of the wild-type *mepR* gene in N315_mut1 restored the imipenem MIC to the level of the parent strain and caused a two- to fourfold decrease in the MIC of DMG-DMDOT for N315_mut1, Mu3_mut1, and Mu3_mut2 despite the fact that *mepA* overexpression alone did not impact on the MICs of these compounds in the parent strains. This suggests that *mepR* can have an effect on antibiotic susceptibility independent of *mepA*. Complementation of the *mepR* defect in Mu3_mut2 decreased the MICs of tigecycline and DMG-MINO by only twofold but did not result in parental strain levels, suggesting that other mutations may have accumulated in this strain. Similarly, overexpression of the wild-type *mepR* gene in the final-step mutants, N315_mut3 and Mu3_mut4, was not sufficient to restore the MICs of tigecycline, DMG-MINO, DMG-DMDOT, EtBr, TPP, or imipenem to the level of the parent strain, which suggests that the altered antibiotic susceptibility profile of the final step mutants cannot be explained solely either by the defect in *mepR* or the resulting overexpression of the MepA efflux pump and that multiple mutations may be involved. Further experiments such as disruption of *mepA* are required to define the exact contribution of the *mep* locus to the susceptibility phenotype of these mutants.

Overexpression of *tet*(M) by Mu3 mutants. Expression of the *tet*(M) gene, which encodes a tetracycline resistance determinant, increased >40-fold in Mu3_mut3 and Mu3_mut4 but did not change in Mu3_mut1 or Mu3_mut2 compared to the parent strain (Table 4). RT-PCR analysis was used to confirm these results, and increases in *tet*(M) expression were only

observed for Mu3_mut3 and Mu3_mut4 (66- and 64-fold, respectively) (data not shown). The *tet*(M) gene is regulated by a mechanism of transcription attenuation attributed to the presence of stem-loops in the region 5′ to the start codon (29). Sequencing of the *tet*(M) promoter region of Mu3_mut3 and Mu3_mut4 using primers pTet MF1 and pTet MR1 revealed an 87-bp deletion (54 to 140 bases upstream of the start codon inclusively) that would remove this stem-loop-forming region and probably explains why more *tet*(M) transcript was detected in these strains. No changes were detected in the *tet*(M) promoter regions of Mu3_mut1 and Mu3_mut2.

Effect of *tet*(M) overexpression on tigecycline susceptibility. It has previously been shown that tigecycline can overcome TetM-mediated resistance (24) and that this may be due to tighter binding of this antibiotic class to the ribosome (3, 5). To date, no glycylcycline-resistant *tet*(M) mutants have been generated in the laboratory (25). Although *tet*(M) is not sufficient to mediate resistance to tigecycline when expressed from its native promoter, we hypothesized that increased transcription of this gene, as detected in Mu3_mut3 and Mu3_mut4, might produce enough Tet M to dissociate tigecycline from the ribosome. Overexpression of *tet*(M) by strain Mu3 or N315 only mediated a twofold increase in tigecycline MIC (Table 3), but no change was detected on gradient plates (data not shown). A plasmid overexpressing *tet*(M) in the antisense direction was used as a negative control and did not alter the tigecycline MICs in the parent strain (data not shown). As expected, overexpression of *tet*(M) did not alter the MICs of EtBr, TPP, DMG-MINO, DMG-DMDOT, or imipenem. It was considered that the effects of *tet*(M) overexpression were only evident when the bacteria were also overproducing the MepA efflux pump. However, overexpression of *tet*(M) by strains Mu3_mut2 and N315_mut3 (which also overexpress *mepA*) did not lead to any additional increases in tigecycline MIC (Table 3). These results suggest that the two mechanisms do not act synergistically despite the evolution of mutants with decreased susceptibility that have developed mutations at both loci in a stepwise fashion. Therefore it appears that the overexpression of Tet M plays no role in decreased susceptibility to tigecycline.

Other gene expression changes in mutant strains. In addition to *mepRAB* and *tet*(M), a number of other genes, of which some are involved in metabolic functions and stress adaptation, had altered expression in the Mu3 and N315 mutant strains (Table 4 and data not shown). For example, homologs of the *betA* and *gbsA* genes, which showed increased expression in the Mu3 mutants, are important in the adaptation to osmotic stress (6). The Mu3 mutants were found to grow slower than the wild-type strain and did not reach the same optical density in the stationary phase. Mu3_mut4 took 1.75-fold longer to double in A_{650} units during the exponential phase, and this effect was reproducible during three independent experiments (data not shown). It is likely that the changes in gene expression mentioned above contribute to the decreased tigecycline susceptibility of the mutants and the altered growth characteristics of the Mu3 mutants, but the mechanism is unclear.

Concluding remarks. Mutation leading to increased transcription of the *mepRAB* locus occurred during the first stepwise increase in tigecycline MIC for both N315 and Mu3 and suggests that such mutations can occur in a single step. However, despite an ~100-fold increase in transcription of *mepA* in Mu3_mut1 and N315_mut1, only a twofold increase in tigecycline MIC was observed. Furthermore, overexpression of *mepA* alone could only promote a fourfold increase in tigecycline MIC. This suggests that tigecycline can function as a substrate for MepA but that this efflux pump alone is unlikely to result in high-level resistance to tigecycline by *S. aureus*. Experiments presented here also showed that overexpression of *tet*(M) alone, or in combination with the increased transcription of *mepRAB* in Mu3_mut2 and N315_mut3, did not contribute to the decreased susceptibility to tigecycline. The reason for selection of the mutation leading to overexpression is unclear. The 16- and 32-fold increases in tigecycline MIC observed here are most likely due to the combination of a number of effects that include MepA and alterations in the expression of other as yet uncharacterized genetic loci. To date, no naturally occurring *S. aureus* isolates with decreased susceptibility to tigecycline have been identified, and it seems unlikely that clinically significant resistance to tigecycline will emerge in a single step via mutations in either the *mepRAB* or *tet*(M) locus.

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REFERENCES

1. **American Journal of Infection Control.** 2001. CDC NNIS System: National Nosocomial Infections Surveillance (NNIS) system report, data summary from January 1992-April 2001, issued August 2001. Am. J. Infect. Control **29:**404–421.
2. **Bateman, B. T., N. P. Donegan, T. M. Jarry, M. Palma, and A. L. Cheung.** 2001. Evaluation of a tetracycline-inducible promoter in *Staphylococcus aureus* in vitro and in vivo and its application in demonstrating the role of *sigB* in microcolony formation. Infect. Immun. **69:**7851–7857.
3. **Bauer, G., C. Berens, S. J. Projan, and W. Hillen.** 2004. Comparison of tetracycline and tigecycline binding to ribosomes mapped by dimethylsulphate and drug-directed Fe2+ cleavage of 16S rRNA. J. Antimicrob. Chemother. **53:**592–599.
4. **Beenken, K. E., P. M. Dunman, F. McAleese, D. Macapagal, E. Murphy, S. J. Projan, J. S. Blevins, and M. S. Smeltzer.** 2004. Global gene expression in *Staphylococcus aureus* biofilms. J. Bacteriol. **186:**4665–4684.
5. **Bergeron, J., M. Ammirati, D. Danley, L. James, M. Norcia, J. Retsema, C. A. Strick, W. G. Su, J. Sutcliffe, and L. Wondrack.** 1996. Glycylcyclines bind to the high-affinity tetracycline ribosomal binding site and evade Tet(M)- and Tet(O)-mediated ribosomal protection. Antimicrob. Agents Chemother. **40:**2226–2228.
6. **Boch, J., G. Nau-Wagner, S. Kneip, and E. Bremer.** 1997. Glycine betaine aldehyde dehydrogenase from *Bacillus subtilis*: characterization of an enzyme required for the synthesis of the osmoprotectant glycine betaine. Arch. Microbiol. **168:**282–289.
7. **Bozdogan, B., D. Esel, C. Whitener, F. A. Browne, and P. C. Appelbaum.** 2003. Antibacterial susceptibility of a vancomycin-resistant *Staphylococcus aureus* strain isolated at the Hershey Medical Center. J. Antimicrob. Chemother. **52:**864–868.
8. **Centers for Disease Control and Prevention.** 2002. *Staphylococcus aureus* resistant to vancomycin-United States. Morb. Mortal. Wkly. Rep. **51:**565–567.
9. **Dean, C. R., M. A. Visalli, S. J. Projan, P. E. Sum, and P. A. Bradford.** 2003. Efflux-mediated resistance to tigecycline (GAR-936) in *Pseudomonas aeruginosa* PAO1. Antimicrob. Agents Chemother. **47:**972–978.
10. **Dunman, P. M., W. Mounts, F. McAleese, F. Immermann, D. Macapagal, E. Marsilio, L. McDougal, F. C. Tenover, P. A. Bradford, P. J. Petersen, S. J. Projan, and E. Murphy.** 2004. Uses of *Staphylococcus aureus* GeneChips in genotyping and genetic composition analysis. J. Clin. Microbiol. **42:**4275–4283.
11. **Dunman, P. M., E. Murphy, S. Haney, D. Palacios, G. Tucker-Kellogg, S. Wu, E. L. Brown, R. J. Zagursky, D. Shlaes, and S. J. Projan.** 2001. Transcription profiling-based identification of *Staphylococcus aureus* genes regulated by the *agr* and/or *sarA* loci. J. Bacteriol. **183:**7341–7353.
12. **Foster, T. J.** 1998. Molecular genetic analysis of staphylococcal virulence. Methods Microbiol. **27:**433–454.
13. **Garvis, S., J. M. Mei, J. Ruiz-Albert, and D. W. Holden.** 2002. *Staphylococcus*

aureus svrA: a gene required for virulence and expression of the agr locus. Microbiology **148:**3235–3243.

14. **Grkovic, S., M. H. Brown, and R. A. Skurray.** 2002. Regulation of bacterial drug export systems. Microbiol. Mol. Biol. Rev. **66:**671–701.
15. **Hiramatsu, K., N. Aritaka, H. Hanaki, S. Kawasaki, Y. Hosoda, S. Hori, Y. Fukuchi, and I. Kobayashi.** 1997. Dissemination in Japanese hospitals of strains of *Staphylococcus aureus* heterogeneously resistant to vancomycin. Lancet **350:**1670–1673.
16. **Kreiswirth, B. N., S. Lofdahl, M. J. Betley, M. O'Reilly, P. M. Schlievert, M. S. Bergdoll, and R. P. Novick.** 1983. The toxic shock syndrome exotoxin structural gene is not detectably transmitted by a prophage. Nature **305:**709–712.
17. **Kuroda, M., T. Ohta, I. Uchiyama, T. Baba, H. Yuzawa, I. Kobayashi, L. Cui, A. Oguchi, K. Aoki, Y. Nagai, J. Lian, T. Ito, M. Kanamori, H. Matsumaru, A. Maruyama, H. Murakami, A. Hosoyama, Y. Mizutani-Ui, N. K. Takahashi, T. Sawano, R. Inoue, C. Kaito, K. Sekimizu, H. Hirakawa, S. Kuhara, S. Goto, J. Yabuzaki, M. Kanehisa, A. Yamashita, K. Oshima, K. Furuya, C. Yoshino, T. Shiba, M. Hattori, N. Ogasawara, H. Hayashi, and K. Hiramatsu.** 2001. Whole genome sequencing of methicillin-resistant *Staphylococcus aureus*. Lancet **357:**1225–1240.
18. **Kyte, J., and R. F. Doolittle.** 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157:**105–132.
19. **Morita, Y., A. Kataoka, S. Shiota, T. Mizushima, and T. Tsuchiya.** 2000. NorM of *Vibrio parahaemolyticus* is an Na^+-driven multidrug efflux pump. J. Bacteriol. **182:**6694–6697.
20. **Morita, Y., K. Kodama, S. Shiota, T. Mine, A. Kataoka, T. Mizushima, and T. Tsuchiya.** 1998. NorM, a putative multidrug efflux protein, of *Vibrio parahaemolyticus* and its homolog in *Escherichia coli*. Antimicrob. Agents Chemother. **42:**1778–1782.
21. **National Committee for Clinical Laboratory Standards.** 2000. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard M7-A5, 5 ed., vol. 17. National Committee for Clinical Laboratory Standards, Wayne, Pa.
22. **National Committee for Clinical Laboratory Standards.** 2005. Performance standards for antimicrobial susceptibility testing. Informational supplement M100-S15, vol. 15. National Committee for Clinical Laboratory Standards, Wayne, Pa.
23. **Petersen, P. J., P. A. Bradford, W. J. Weiss, T. M. Murphy, P. E. Sum, and S. J. Projan.** 2002. In vitro and in vivo activities of tigecycline (GAR-936), daptomycin, and comparative antimicrobial agents against glycopeptide-intermediate *Staphylococcus aureus* and other resistant gram-positive pathogens. Antimicrob. Agents Chemother. **46:**2595–2601.
24. **Petersen, P. J., N. V. Jacobus, W. J. Weiss, P. E. Sum, and R. T. Testa.** 1999. In vitro and in vivo antibacterial activities of a novel glycylcycline, the 9-*t*-butylglycylamido derivative of minocycline (GAR-936). Antimicrob. Agents Chemother. **43:**738–744.
25. **Projan, S. J.** 2000. Preclinical pharmacology of GAR-936, a novel glycylcycline antibacterial agent. Pharmacotherapy **20:**224S–228S.
26. **Rouquette-Loughlin, C., S. A. Dunham, M. Kuhn, J. T. Balthazar, and W. M. Shafer.** 2003. The NorM efflux pump of *Neisseria gonorrhoeae* and *Neisseria meningitidis* recognizes antimicrobial cationic compounds. J. Bacteriol. **185:**1101–1106.
27. **Said-Salim, B., B. Mathema, and B. N. Kreiswirth.** 2003. Community-acquired methicillin-resistant *Staphylococcus aureus*: an emerging pathogen. Infect. Control Hosp. Epidemiol. **24:**451–455.
28. **Sambrook, J. E., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
29. **Su, Y. A., P. He, and D. B. Clewell.** 1992. Characterization of the *tet*(M) determinant of Tn916: evidence for regulation by transcription attenuation. Antimicrob. Agents Chemother. **36:**769–778.
30. **Sum, P. E., and P. Petersen.** 1999. Synthesis and structure-activity relationship of novel glycylcycline derivatives leading to the discovery of GAR-936. Bioorg. Med. Chem. Lett. **9:**1459–1462.
31. **Visalli, M. A., E. Murphy, S. J. Projan, and P. A. Bradford.** 2003. AcrAB multidrug efflux pump is associated with reduced levels of susceptibility to tigecycline (GAR-936) in *Proteus mirabilis*. Antimicrob. Agents Chemother. **47:**665–669.
32. **Waldvogel, F. A.** 2000. *In* G. L. Mandel, J. E. Bennett, and R. Dolin (ed.), Principle and practice of infectious diseases, p. 2069–2092. Churchill Livingstone, Philadelphia, Pa.