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Multidrug Resistance in *Staphylococcus aureus* Due to Overexpression of a Novel Multidrug and Toxin Extrusion (MATE) Transport Protein

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Efflux is an important mechanism of multidrug resistance (MDR) in bacteria. The multidrug and toxin extrusion (MATE) family is the most recently described group of MDR efflux proteins, none of which have previously been identified in *Staphylococcus aureus*. Two independently derived *S. aureus* mutants having efflux-related MDR phenotypes were studied using microarray technology and a marked overexpression of an open reading frame (ORF; *mepA*) encoding a protein homologous with MATE family proteins was observed in both. There was concomitant overexpression of ORFs in close proximity to *mepA* (~100 bp) encoding a MarR-type regulator (*mepR*, upstream of *mepA*) and a protein of unknown function (*mepB*, downstream). Experiments in which *mepA* was overexpressed or disrupted revealed that the encoded protein has a broad substrate profile that includes several monovalent and divalent biocides and the fluoroquinolone antimicrobial agents norfloxacin and ciprofloxacin. The function of MepB is obscure, it does not contribute to the MDR phenotype conferred by MepA. MepR overexpression reversed the MDR phenotypes of both mutants by repressing *mepA* transcription. All three ORFs are preferentially transcribed as a single *mepRAB* unit, suggesting that the three genes form an operon.

Multidrug efflux is an important mechanism of biocide and antimicrobial agent resistance in bacteria (18, 36). Several distinct families of membrane-based proteins mediate this process, with members of the major facilitator superfamily (MFS) constituting the largest number of described examples (4). In *Staphylococcus aureus* multidrug resistance (MDR) efflux proteins, hereafter referred to as MDR pumps, belonging to the MFS and small multidrug resistance families have been described previously (4, 30, 32). The best-characterized *S. aureus* MDR pumps are the MFS proteins QacA/B and NorA, each of which is capable of expelling multiple biocides and, with respect to NorA, selected fluoroquinolone antimicrobial agents (4, 29, 30, 32, 35).

MFS and small multidrug resistance MDR pumps depend on the proton motive force for substrate transport (30). A new family of MDR pumps was recently identified in gram-negative organisms and is referred to as the multidrug and toxin extrusion (MATE) family. The first MATE MDR pump was found in *Vibrio parahaemolyticus* (NorM protein), and homologues have since been found in other gram-negative bacteria, plants, and the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (5, 5a, 11, 13, 14, 24–26, 37). Both the proton and sodium ion gradients have been identified as the energy source for substrate transport for MATE family transporters (11, 26).

Analysis of *S. aureus* genome data in the public domain reveals many possible MDR pumps belonging to the MFS but few candidates in other transporter families. A MATE type MDR pump was recently described for *Clostridium difficile*, but no member of this family has yet been identified in other gram-positive bacteria (6). Recently, our laboratory reported the in vitro selection of two *S. aureus* mutants that have MDR efflux phenotypes unrelated to NorA (15, 16). Herein we show that a MATE family pump is responsible for much of the MDR phenotypes exhibited by both mutants.

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MATERIALS AND METHODS

Bacterial strains, plasmids, media, and reagents. The strains and plasmids employed in this study are listed in Table 1. Exposure of SA-K1712 to 10-fold its norfloxacin MIC on solid media yielded SA-K1748, which, in addition to its MDR phenotype, acquired a mutation resulting in an S80F GrlA substitution (16). SA-K2068 was selected by passing *S. aureus* NCTC 8325-4 on a 0 to 4× MIC concentration gradient of ethidium bromide (EtBr) plus moxifloxacin (15). Unless otherwise noted, all reagents were the highest grade available and were obtained from Sigma Chemical Co. (St. Louis, Mo.), or in the case of some antimicrobial agents, their respective manufacturers. Mueller-Hinton II broth was obtained from BD Biosciences, Inc., Sparks, Md., and Luria-Bertani (LB) broth from Invitrogen, Carlsbad, Calif.

pK434, pK436, pK438, and pK440 were constructed by amplifying the *mepR*, *mepA*, *mepB*, and *mepAB* open reading frames, respectively, including their ribosome binding sites, from *S. aureus* NCTC 8325-4. PCR products were cloned into pALC2073, placing each gene under the control of a *xyl/tetO* tetracycline-inducible promoter (1). Plasmids were electroporated into *S. aureus* RN4220 and then transferred to SA-K1758, which is a *norA* deletion strain, by transduction using phage 85 (9, 31). The use of SA-K1758 as the host strain to characterize the MepA substrate profile was considered essential to eliminate confounding effects of NorA-mediated efflux of common substrates. All strains bearing pALC2073-based constructs were grown in the presence of chloramphenicol (10 μg/ml) to assure plasmid maintenance and, where appropriate, were induced by 50 ng/ml of tetracycline. Strains possessing pALC2073 alone, pK434, pK436, pK438, or pK440 are indicated by the name of the parent strain appended with -P, -R, -A, -B, or -AB, respectively, to indicate that in that strain the vector only (control), *mepR*, *mepA*, *mepB*, or *mepAB* is being expressed.

Genome data and bioinformatic analyses. Genome data was obtained from The Institute for Genome Research (http://www.tigr.org) (*S. aureus* COL), the *Staphylococcus aureus* Genome Sequencing Project at the University of Okla-

TABLE 1. Study strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
S. aureus strains		
NCTC 8325-4	Commonly utilized laboratory strain cured of known prophages	28
RN4220	NCTC 8325-4 r-; capable of stably maintaining recombinant plasmids	19
SA-K1712	NCTC 8325-4 with *norA* disrupted by *tetK*	16
SA-K1748	MDR mutant of SA-K1712; GrlA S80F	16
SA-K1748-P	SA-K1748 + pALC2073	This study
SA-K1748-R	SA-K1748 + pK434	This study
SA-K1748 *mepA*-	*mepA* disrupted by fusion with *lacZ*	This study
SA-K2068	MDR mutant of NCTC 8325-4	15
SA-K2068-P	SA-K2068 + pALC2073	This study
SA-K2068-R	SA-K2068 + pK434	This study
SA-K2068 *mepA*-	*mepA* disrupted by fusion with *lacZ*	This study
SA-K1758	NCTC 8325-4 with *norA* deleted	31
SA-K1758-P	SA-K1758 + pALC2073	This study
SA-K1758-A	SA-K1758 + pK436	This study
SA-K1758-B	SA-K1758 + pK438	This study
SA-K1758-AB	SA-K1758 + pK440	This study
Plasmids		
pALC2073	*S. aureus* vector containing a tetracycline-inducible promoter controlling expression of cloned genes, Cm^r	1
pAZ106	Suicide vector used to disrupt *mepA*	17
pK434	pALC2073-*mepR*	This study
pK436	pALC2073-*mepA*	This study
pK438	pALC2073-*mepB*	This study
pK440	pALC2073-*mepAB*	This study

homa (http://www.genome.ou.edu/staph.html) (*S. aureus* NCTC 8325), and the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/genomes/MICROBES/Complete.html) (*S. aureus* MSSA 476, MRSA 252, Mu50, N315, and MW2). Data from these sources will be referred to collectively as *S. aureus* genome data.

Nucleotide sequence and protein hydropathy analyses were performed using DS Gene, version 1.5 (Accelrys, San Diego, Calif.). Homology analyses of protein sequences were performed using the PSI-BLAST algorithm available from the National Center for Biotechnology Information at http://www.ncbi.nlm.nih.gov/BLAST. Helix-turn-helix predictions were made using the Pôle BioInformatique Lyonnais software suite available at http://pbil.univ-lyon1.fr.

Transcriptional profiling. The genome-wide transcriptional profiles of SA-K1712, SA-K1748, *S. aureus* NCTC 8325-4, and SA-K2068 were determined using previously published methods (2, 7). Briefly, total RNA was extracted from strains grown in LB broth to an optical density at 550 nm of 0.4 using the RNeasy Maxi kit (QIAGEN Inc., Valencia, Calif.). The RNA was converted to cDNA using random primers. The cDNA was fragmented, biotinylated, and then hybridized with custom-designed *S. aureus* GeneChips (Affymetrix, Santa Clara, Calif.). Hybridizing cDNA was detected by reaction with streptavidin and comparatively quantitated and analyzed using a GeneArray scanner (Agilent Technologies, Palo Alto, Calif.) and Genespring, version 6.1, software (Silicon Genetics, Redwood City, Calif.).

Antimicrobial susceptibilities. Susceptibility testing was performed in triplicate using Mueller-Hinton II broth and microdilution techniques according to NCCLS guidelines (27).

EtBr efflux. The loss of EtBr from EtBr-loaded SA-K1758-P and SA-K1758-A was determined fluorometrically as described previously (16). The effect of reserpine (20 μg/ml) or carbonyl cyanide *m*-chlorophenylhydrazone (100 μM) on EtBr efflux also was determined. Assays were performed in triplicate, and data were compared by using the Student *t* test.

Nucleotide sequencing and Southern blots. Nucleotide sequences were determined in both directions by the Center for Molecular Medicine and Genetics, Wayne State University, Detroit, Mich., employing an automated dideoxy chain termination-based method (33).

PCR-generated *mepR*, *mepA*, and *mepB* probes were labeled with [α-^{32}P]dATP (800 Ci/mmol; NEN Life Science Products, Inc., Boston, Mass.) using the RadPrime DNA labeling system as recommended by the manufacturer (Invitrogen). Southern analyses were performed using standard techniques (34). A *gyrA* probe was employed for normalization purposes, and product band intensities were digitally quantitated using a phosphorimaging system (Storm 860; Molecular Dynamics, Sunnyvale, Calif.) and Phoretix one-dimensional advanced software (version 2003.02; Nonlinear Dynamics Ltd., Newcastle upon Tyne, United Kingdom).

Disruption of *mepA*. PCR was used to amplify 550 bp of internal *mepA* sequence near its 5′ end using primers incorporating BamHI and EcoRI sites. The product was cloned into pAZ106, and the resultant construct was transformed into *S. aureus* RN4220 (17, 19). Integration of the plasmid into the chromosome creates a transcriptional fusion between *mepA* and *lacZ*, resulting in a functional *mepA* knockout mutation. The mutation was transduced into SA-K1748 and SA-K2068 using phage 85.

To study the effect of *mepA* disruption in isolation in SA-K2068, it was necessary to preserve its known *mepR* mutation. This was accomplished by screening *mepA*::*lacZ* transductants for their ability to turn blue on media containing 5-bromo-4-chloro-3-indolyl-β-D-thiogalactopyranoside (X-Gal; 100 μg/ml). Strains with wild-type *mepR* should retain a white color under these conditions because in such strains *mepA* expression is quite low (see Results and Discussion). One blue transductant was selected for analysis, and sequencing established that its *mepR* mutation was maintained.

Primer extension and Northern blot analyses. Transcriptional profiling data were confirmed, and transcription start sites determined, by primer extension and Northern blot analyses. Strains were grown in LB broth and cells were collected at an optical density at 550 nm of 1. RNA was recovered using the RNeasy Midi kit (QIAGEN). For primer extension experiments, mRNAs were labeled using the primer extension system-avian myeloblastosis virus reverse transcriptase kit (Promega Corp., Madison, Wis.). The oligonucleotides used for primer extension analyses were end labeled with [γ-^{32}P]ATP (3,000 Ci/mmol; NEN) according to procedures recommended by the manufacturer of the primer extension system, and products were visualized by autoradiography. Transcription start sites were determined by comparing the size of primer extension products with a concomitantly run dideoxy chain-termination sequencing reaction according to a protocol supplied with the primer extension system.

For Northern blotting, strains were grown and RNA extracted as described above for primer extension analyses. RNA (10 μg) from each strain was separated in a formaldehyde-containing agarose gel, transferred to a nylon membrane, and hybridized under high-stringency conditions (42°C, 50% formamide) with the same probes as were used in Southern analyses. Autoradiography was used to detect hybridizing bands.

Nucleotide sequence accession number. The GenBank accession number for the *mepRAB* loci of *S. aureus* NCTC 8325-4 is AY661734.

TABLE 2. Susceptibilities of study strains

Compound[d]	MIC (μg/ml) for strain:									
	SA-K1712	SA-K1748	SA-K1748*mepA*-	SA-K1748-R[a]	NCTC 8325-4	SA-K2068	SA-K2068*mepA*-	SA-K2068-R	SA-K1758-P[a]	SA-K1758-A[a]
Antimicrobial agents										
Norfloxacin	0.31	3.13 (10)[b]	3.13	3.13	1.25	6.25 (5)	1.56	1.56	0.31	1.25 (4)
Ciprofloxacin	0.08	1.25 (16)	0.63	0.63	0.31	5.0 (16)	0.63	0.63	0.08	0.16 (2)
Moxifloxacin	ND[c]	ND	ND	ND	0.08	0.31 (4)	0.31	0.16	0.06	0.06 (1)
Monovalent cations										
Acriflavine	3.13	6.25 (2)	1.56	0.78	6.25	12.5 (2)	3.13	3.13	1.56	6.25 (4)
BAC	0.08	0.63 (8)	0.08	0.08	0.63	1.25 (2)	0.63	0.63	0.16	0.63 (4)
Cetrimide	0.13	0.25 (2)	0.13	0.06	0.25	0.50 (2)	0.25	0.25	0.06	0.25 (4)
Crystal violet	0.04	0.16 (4)	0.08	0.04	0.13	0.25 (2)	0.13	0.06	0.06	0.25 (4)
EtBr	0.39	12.5 (32)	0.39	0.39	3.13	25 (8)	6.25	6.25	0.39	25.0 (64)
Pyronin Y.	0.78	1.56 (2)	0.39	0.39	1.56	6.25 (4)	1.56	0.78	0.39	1.56 (4)
Rhodamine	0.63	1.25 (2)	0.31	0.16	0.63	1.25 (2)	0.63	0.31	0.31	1.25 (4)
TPP	6.25	50 (8)	3.13	3.13	12.5	100 (8)	25.0	12.5	3.13	50.0 (16)
TMA-DPH	0.63	5.0 (8)	0.31	0.31	12.5	25 (2)	12.5	12.5	0.78	12.5 (16)
Divalent cations										
Chlorhexidine	0.08	0.31 (4)	0.04	0.04	0.16	0.63 (4)	0.31	0.31	0.04	0.63 (16)
DAPI	0.31	2.5 (8)	0.31	0.31	0.63	10.0 (16)	2.50	2.50	0.31	5.0 (16)
Dequalinium	0.16	1.25 (8)	0.04	0.04	0.63	2.5 (4)	1.25	0.63	0.04	1.25 (32)
Hoechst 33342	0.31	0.63 (2)	0.31	0.31	0.63	5.0 (8)	0.63	0.63	0.16	0.63 (4)
Pentamidine	0.63	25 (40)	0.31	0.16	12.5	50 (4)	6.25	12.5	0.31	25.0 (80)

[a] -R, -P, and -A denote the presence of pK434, pALC2073, and pK436, respectively.
[b] Values in parentheses indicate the *n*-fold increase in MIC compared to the appropriate parent strain.
[c] ND, not determined.
[d] BAC, benzalkonium chloride; TPP, tetraphenylphosphonium; TMA-DPH, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene p-toluenesulfonate; DAPI, 4′-6-diamidino-2-phenylindole.

RESULTS AND DISCUSSION

SA-K1748 and SA-K2068 have broad drug resistance profiles. Limited MIC data for SA-K1748 and SA-K2068 has been published previously (15, 16). In Table 2, a much broader substrate profile than was formerly appreciated for both mutants is obvious. Not included are data for compounds showing no change between parent (SA-K1712 and NCTC 8325-4) and mutant (SA-K1748 and SA-K2068) strains, which was the case for erythromycin, gentamicin, and nafcillin.

SA-K1748 was derived from a *norA*-disrupted parent that contains a *norA*::*tetK* mutation, which rules out any role of NorA in its MDR phenotype (16). As noted previously, SA-K1748 acquired a mutation in its selection process, resulting in an S80F GrlA substitution, which is certain to contribute to its raised norfloxacin MIC and may obscure MIC changes that may occur as a result of efflux pump activity. Previous work in our laboratory has shown that SA-K2068 has no known topoisomerase mutations and that its MDR phenotype is unrelated to NorA, as the sequence of *norA* and its promoter region are wild type and *norA* expression is unchanged from that of *S. aureus* NCTC 8325-4 (parent strain). The increased fluoroquinolone MICs in SA-K2068 are efflux related based on drug accumulation experiments performed previously (15).

Of particular interest are raised MICs to both mono- and divalent cations in both mutants, including members of the quaternary ammonium (benzalkonium chloride, cetrimide, dequalinium), phenanthridine (EtBr), triphenylmethane (crystal violet), diamidine (4′,6-diamidino-2-phenylindole [DAPI], pentamidine) and biguanidine (chlorhexidine) families. Some of these compounds, as well as other members of these chemical families, are used as biocides/disinfectants in health care settings. This distribution of affected compounds in both mutants is reminiscent of the substrate profile observed for QacA (23).

Both MDR mutants have increased quantities of transcripts from three contiguous ORFs. Genome-wide transcriptional profiling data are given in Table 3. Only open reading frames (ORFs) having eightfold or greater changes in mRNA abundance are shown. Compared to the respective parent strains, the most significant changes observed for both mutants were in three contiguous ORFs corresponding to SA0404, SA0405, and SA0406 (COL numbering scheme). The corresponding ORFs for *S. aureus* N315 are SA0322, SA0323, and SA0324, respectively. Runoff transcripts and Northern blots (see below) verified the increased quantities of these mRNAs in both mutants, confirming transcriptional profiling data. The abundance of mRNAs of genes encoding proteins involved in carbohydrate metabolism and virulence (*spa*) also changed significantly in one or the other mutant strain but not in both. Thus, SA0404, SA0405, and SA0406 were considered likely candidates to be responsible for the MDR phenotypes of SA-K1748 and SA-K2068. Subsequent sequence analysis and experimental data indicated that SA0405 encodes a multidrug export protein, resulting in our naming these ORFs *mepR*, *mepA*, and *mepB*, respectively.

Analysis of *S. aureus* genome data reveals that the *mepRAB* loci are conserved in all sequenced strains, with intergenic distances of 106 bp (*mepR-A*) and 103 to 106 bp (*mepA-B*). This genetic organization is consistent with *mepRAB* forming an operon. The encoded protein products of *mepR* and *mepA* (139 and 451 residues, respectively) are 98 to 100% identical between these strains. The putative *mepB* protein product demonstrates more variability, having a predicted size of 146 residues (*S. aureus* NCTC 8325, COL, MRSA 252, MSSA 476,

TABLE 3. Genome-wide transcriptional profiling data for SA-K1748 and SA-K2068[d]

S. aureus COL ORF	Gene	Product or putative function	Fold change in expression of strain:		Direction
			SA-K1748[a]	SA-K2068[b]	
SA0095	*spa*	Protein A precursor	NC[c]	11.0	Down
SA0404	*mepR*	Transcriptional regulator	14.9	35.5	Up
SA0405	*mepA*	MATE-type multidrug efflux protein	77.0	150.9	Up
SA0406	*mepB*	Unknown function	79.3	147.6	Up
SA0757	*fruR*	Transcriptional regulator, fructose operon	NC	12.4	Down
SA1321	*glpD*	Aerobic glycerol-3-phosphate dehydrogenase	NC	12.6	Up
SA2184	*lacC*	Tagatose-6-phosphate kinase	9.3	NC	Down
SA2185	*lacB*	Galactose-6-phosphate isomerase, LacB subunit	9.5	NC	Down
SA2186	*lacA*	Galactose-6-phosphate isomerase, LacA subunit	8.2	NC	Down
SA2549		Putative esterase	NC	8.7	Up

[a] Compared to *S. aureus* SA-K1712.
[b] Compared to *S. aureus* NCTC 8325-4.
[c] NC, no change.
[d] Only ORFs with changes in expression of eightfold or greater in either strain are shown, with data for both strains given for comparative purposes.

and MW2) or 152 residues (*S. aureus* N315 and Mu50). MepB homology between strains is 81 to 100%.

PSI-BLAST analysis revealed that MepR has strong homology with MarR-type regulatory proteins and MepA has strong homology with MATE family transport proteins. MepR has a putative helix-turn-helix motif (residues 48 to 69), and its sequence is 25% identical and 42% similar to that of MarR. Kyte-Doolittle hydropathy analysis of MepA predicts 12 transmembrane segments, and the protein has modest homology to the MATE proteins NorM of *Vibrio parahaemolyticus* and CdeA of *C. difficile* (21 and 26% identity, respectively) (6, 26). Greater homology with MepA was observed for proteins predicted from nucleotide sequence data available for other gram-positive bacteria including *Listeria innocua* (ORF lin0989, 41% identity), *Listeria monocytogenes* (ORF lmo0990, 40%), *Lactococcus lactis* (hypothetical protein YpbC, 38%), and another *Clostridium difficile* protein (EffD, 37%) (3, 8, 10; http://www.ncbi.nlm.nih.gov/genomes/MICROBES/Complete.html). All of these proteins are predicted to be MATE family transporters, but none have been characterized.

MepB is predicted to be a soluble protein and has no apparent homology with proteins of known function. It is a member of COG4815, which consists of an orthologous group of uncharacterized but conserved proteins of unknown function found in the genomes of many bacteria including *Listeria* and *Bacillus* spp., *Staphylococcus epidermidis*, and *Enterococcus faecalis*. The closest homologues are found in *L. innocua* and *L. monocytogenes* (ORFs lin1093 and lmo1133, respectively; 45% identity). These ORFs are not contiguous with genes encoding MATE proteins, being more than 100 kb away in both instances. However, this arrangement does not eliminate a possible interaction between these MepB homologues and MATE proteins.

MATE transporters that have been characterized include NorM and VmrA of *V. parahaemolyticus*, NorM of *Erwinia amylovora*, VcmA and VcrM of *Vibrio cholerae*, BexA of *Bacteroides thetaiotaomicron*, HmrM of *Haemophilus influenzae*, and most recently PmpM of *Pseudomonas aeruginosa* (5, 11, 13, 14, 24–26, 37). These proteins confer variable degrees of multidrug resistance with substrates including some fluoroquinolones and biocide/disinfectant agents, EtBr, acriflavine, rhodamine, and occasionally aminoglycosides. Drug susceptibility data for SA-K1748 and SA-K2068 reveal raised MICs to many of the same compounds.

The expression of *mepRAB* is augmented in both MDR mutants. Southern analyses revealed that gene amplification of *mepRAB* had not occurred in either mutant (data not shown). Thus, augmented *mepRAB* expression is the basis for the amplified signals observed for these ORFs in genome-wide transcriptional profiling.

SA-K2068 has both *mepR* and *mepA* mutations. The nucleotide sequences of *mepRAB* of both parent strains (*S. aureus* NCTC 8325-4 and SA-K1712) were identical to that of *S. aureus* COL, as was that of SA-K1748. However, in SA-K2068, a 2-bp deletion in *mepR* (nucleotides 239 to 240) and a 4-bp tandem duplication in *mepA* (nucleotides 1299 to 1302) were found. These mutations result in the introduction of premature stop codons and predicted truncations of MepR from residues 139 to 97 and MepA from residues 451 to 439. The deleted residues of MepA (residues 440 to 451) lie in its predicted carboxy-terminal cytoplasmic tail, and their loss causes only a slight alteration in the predicted secondary structure of the protein as predicted by Kyte-Doolittle analysis. If MepA is responsible for the MDR phenotype of SA-K2068, this change has little to no impact on protein function. However, the *mepR* mutation in SA-K2068 is likely to result in an inactive MepR and suggests that it may be a repressor of *mepAB*, and perhaps *mepRAB*, transcription. The augmented expression of *mepRAB* observed in transcriptional profiling of SA-K2068 can be explained by the loss of a functional MepR and, consequently, its repressive effect. However, augmented expression of *mepRAB* in SA-K1748 is more difficult to explain because in that strain *mepR* (and, presumably, MepR) is intact. A trans-acting regulatory factor(s) must exist, the expression or activity of which is altered in SA-K1748, which is capable of overriding the presumed MepR repressive effect. A candidate protein(s) having this function was not obvious from analysis of transcriptional profiling data.

There is a large inverted repeat region extending from 4 to 70 bp downstream of the *mepB* stop codon, which is likely to be a transcription termination signal (Fig. 1A). No other repeats were identified following the *mepR* or *mepA* coding regions.

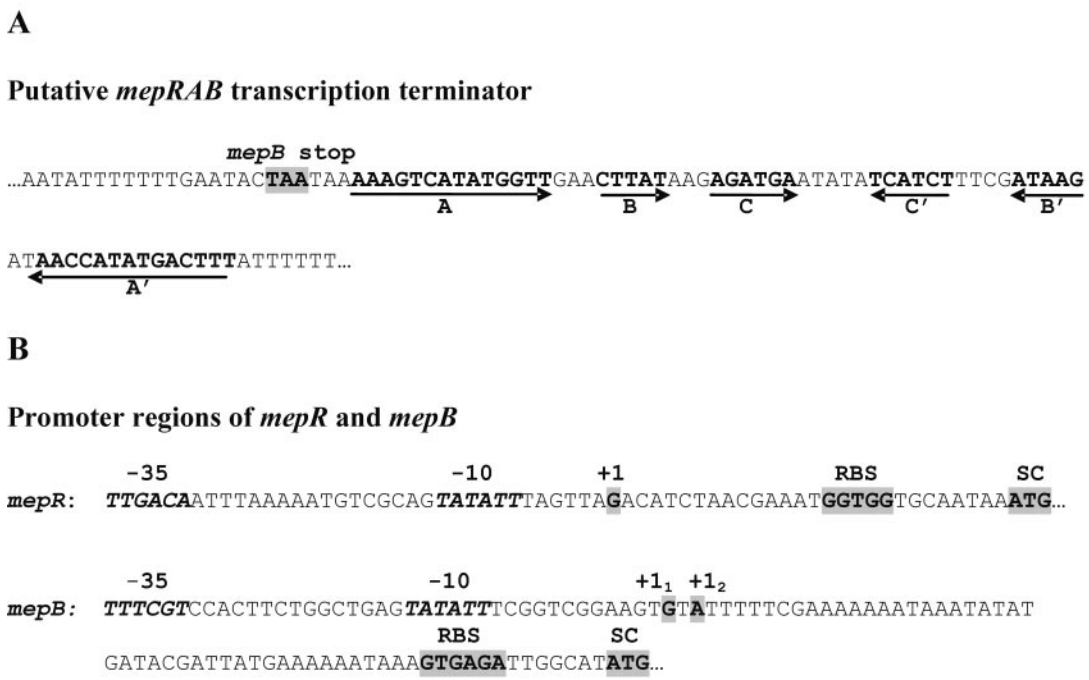

FIG. 1. Selected nucleotide sequences of the *mepRAB* locus. (A) Terminal portion of *mepB* and flanking DNA. The *mepB* stop codon is indicated, followed by inverted repeats (A and A', B and B', C and C') that may function as a *mepRAB* transcription terminator (arrows). (B) Promoter regions of *mepR* and *mepB*. Transcription start sites (+1), ribosome binding sites (RBS), and start codons (SC) are shown in boldface type and highlighted with a grey background. Possible −35 and −10 motifs are shown in boldface type and italicized. Two transcription start sites were identified for *mepB* ($+1_1$ and $+1_2$).

The positioning of this presumptive transcription termination signal, which has the characteristics of a typical rho-independent terminator (stem-loop followed by several U residues in an mRNA) provides additional evidence that *mepR*, *mepA*, and *mepB* are likely to form an operon (20).

Expression of *mepA* verifies it as an MDR pump. The effect of expression of *mepA* from the heterologous tetracycline-inducible promoter of pALC2073 in a *norA* deletion background is illustrated in Table 2. Compared to the presence of the empty vector (SA-K1758-P), the expression of *mepA* (SA-K1758-A) resulted in 2- to 80-fold MIC increases for all tested compounds with the exception of moxifloxacin. We have shown previously that SA-K2068 effluxes moxifloxacin, but the involved pump(s) clearly do not include MepA and were not evident in transcriptional profiling data. Independent expression of *mepB* had no effect on drug susceptibility, and concomitant expression of *mepAB* resulted in susceptibilities identical to those observed for expression of *mepA* alone (data not shown). These results indicate that MepB does not contribute to the MDR phenotypes of SA-K1748 or SA-K2068.

SA-K1758-A exhibited a 10-fold increase in EtBr efflux compared to SA-K1758-P ($P = 0.001$) (Fig. 2). When reserpine (20 μg/ml) was included in the assay, no significant difference was observed between SA-K1758-P and SA-K1758-A ($P = 0.30$). Carbonyl cyanide *m*-chlorophenylhydrazone (100 μM) reduced the EtBr efflux of SA-K1758-A by 67% (data not shown). These data demonstrate the complete reserpine sensitivity of MepA as well as its dependence on intact membrane energetics for function.

The best MepA substrates are pentamidine, EtBr, and the biocide dequalinium, but reasonably good substrates also include tetraphenylphosphonium, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene ρ-toluenesulfonate, DAPI, and the clinically relevant biocide chlorhexidine. Relatively poor substrates include the fluoroquinolone antibacterial agents norfloxacin and ciprofloxacin. The small MIC increases observed for these compounds probably are not clinically relevant by themselves, but combined with other resistance mecha-

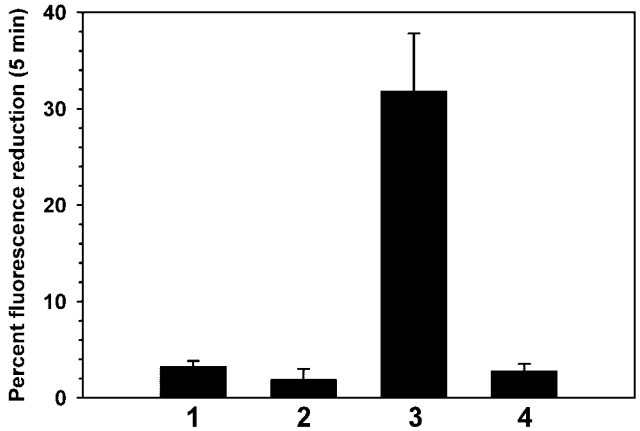

FIG. 2. MepA-mediated efflux of EtBr. Bars: 1, SA-K1758-P; 2, SA-K1758-P with reserpine (20 μg/ml); 3, SA-K1758-A; 4, SA-K1758-A with reserpine. Results are expressed as percent reduction in fluorescence (means ± standard deviations), which correlates with EtBr efflux, over 5 min.

nisms, they may become so. In addition, the activity of efflux pumps may lower intracellular drug concentrations to subinhibitory levels favoring the emergence of high-level resistance by way of target mutations. This hypothesis is supported by earlier work showing that inhibition of efflux pump activity in *S. aureus* by reserpine dramatically reduces the frequency at which mutants resistant to norfloxacin arise (22).

The structural diversity between MepA substrates is considerable, which is a well-known characteristic of MDR efflux pumps. The single commonality between all of them is that each carries a positive charge or has at least one nitrogen atom that could become protonated, resulting in a positive charge. It is likely that MepA substrates interact electrostatically with the negatively charged amino acid residue(s) within the drug binding site(s). Testing this hypothesis will require crystal structure analysis of MepA in the presence and absence of substrate.

MepA of SA-K2068 is fully functional. We have proposed that the minor truncation of *mepA* in SA-K2068 is of no functional consequence. This fact was established by cloning $mepA_{\text{SA-K2068}}$ into pALC2073 and expressing this construct in SA-K1758. An effect identical to that of expression of intact *mepA* was observed, establishing the fully functional nature of MepA in SA-K2068 (data not shown).

Disruption of *mepA* in SA-K1748 and SA-K2068 establishes the contribution of MepA to their MDR phenotypes. Disruption of *mepA* resulted in a complete reversion of the MDR phenotype of SA-K1748 for all nonfluoroquinolone substrates, indicating that MepA activity is the basis for the raised MICs of all other tested compounds in this mutant (Table 2). Disruption of *mepA* in SA-K2068 had a similar effect, indicating that MepA is also the basis for the majority of the MDR expressed by this mutant. However, the incomplete effect of *mepA* disruption on DAPI resistance in SA-K2068 indicates that this mutant has a MepA-independent resistance mechanism for this compound. The responsible ORFs, which were not identified in existing microarray data, may be discovered by additional transcriptional profiling experiments of SA-K2068, its *mepA*-disrupted derivative, and perhaps SA-K2068 expressing pK434 (overexpressing *mepR*) (see below).

MepR is a repressor of *mepA* transcription. The effect of overexpression of *mepR* in SA-K1748 (SA-K1748-R) and SA-K2068 (SA-K2068-R) is shown in Table 2. For SA-K1748, excess MepR resulted in a reversion of all MICs except for those of norfloxacin and ciprofloxacin to values at or below those observed for the parent strain (SA-K1712). The presence of the S80F GrlA mutation in SA-K1748 likely obscures the effect that MepA, and thus also MepR, might have on its fluoroquinolone MICs. The reduction in MICs of a few compounds (acriflavine, rhodamine, dequalinium, and pentamidine) to values fourfold below those observed in SA-K1712 raises the possibility that, at least in SA-K1748, MepR may have global regulatory effects on other pumps with substrate profiles that overlap that of MepA. A comparison of the genome-wide transcriptional profile of SA-K1748 and SA-K1748-R is the next logical step to test this hypothesis.

With respect to SA-K2068, overexpression of *mepR* also reversed MIC increases of all substrates except DAPI, the MIC of which remained fourfold above that of NCTC 8325-4. The probable existence of a MepA-independent resistance mechanism for this compound has already been discussed.

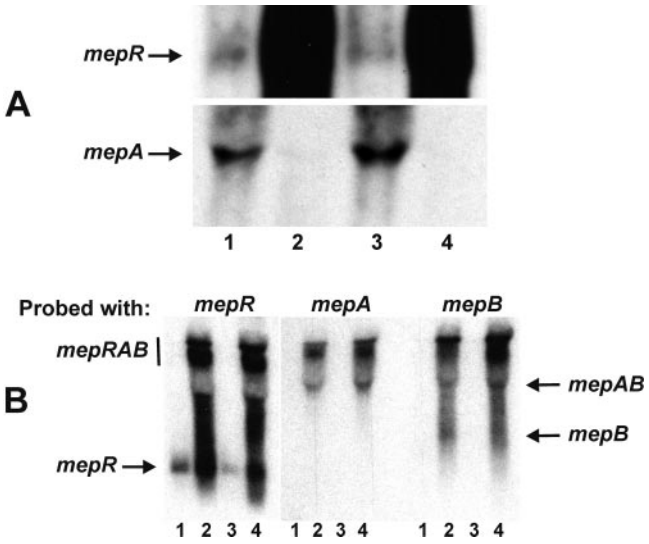

FIG. 3. (A) Effect of overexpression of *mepR* on *mepA* expression. Lane 1, SA-K2068-P; lane 2, SA-K2068-R; lane 3, SA-K1748-P; lane 4, SA-K1748-R. The negative effect of MepR on *mepA* expression is clearly evident. (B) Expression of *mepRAB*. Lane 1, *S. aureus* NCTC 8325-4; lane 2, SA-K2068; lane 3, SA-K1712; lane 4, SA-K1748.

The MepR effect in both mutants is supportive of the conclusion that it is a negative regulator of *mepA* expression. The MIC-reducing effect of MepR in SA-K2068 likely results from the replacement of a dysfunctional MepR (see below) with a functional one. This presumption was tested by cloning $mepR_{\text{SA-K2068}}$ into pALC2073 and determining the effect of expressing this construct in SA-K1748 and SA-K2068. No effects on MICs were observed, verifying that the truncated protein is inactive (data not shown). However, the MepR effect in SA-K1748 is intriguing in that its MepR is predicted to be intact. We presume that the abundant MepR resulting from its expression from pALC2073 overwhelms the *trans*-acting regulatory factor(s) stimulating *mepRAB* expression that we predict to exist in this strain.

Northern blotting using *mepR* and *mepA* probes was performed employing RNA isolated from SA-K1748 and SA-K2068 expressing either pALC2073 or pK434 (Fig. 3A). Overexpression of *mepR* in both mutant backgrounds essentially eliminated *mepA* transcripts. These data confirm MepR as a repressor of *mepA* transcription. Whether this effect is direct or indirect is not established by these data, but it is most likely to be direct because of the operon-like genetic organization of *mepRAB*. The demonstration of binding of MepR to either the *mepR* or *mepA* promoter regions would be strong evidence in favor of a direct effect.

***mepRAB* is the favored transcript in both mutants.** Northern blotting using probes for each ORF detected only *mepR* transcripts in parent strains (Fig. 3B). Since MepR is a repressor of *mepA* transcription, the detection of only *mepR* transcripts in parent strains is the expected result. Consistent with transcriptional profiling data and runoff transcript analyses, both mutants had marked increases in *mepR* and *mepRAB* transcripts, with small amounts of *mepAB* and *mepB* transcripts detectable. It is apparent that the favored transcript in both mutants is *mepRAB*, further favoring the conclusion that the *mepRAB* loci

form an operon. The well-defined hybridization signal having the highest molecular weight in each mutant is probably the intact *mepRAB* transcript, and the less distinct signal at a slightly lower molecular weight may consist of *mepRAB* degradation products. A transcript originating upstream of *mepR* including all or some of *mepR*, but none of *mepA*, plus degradation products of the same may explain the diffuse region of hybridization at higher molecular weights than the *mepR* signal in mutant strains.

Independent promoters exist for *mepR* and *mepB*. Because *mepA* and *mepB* are not detectably transcribed in wild-type strains (Fig. 3B), RNA from SA-K1748 and SA-K2068 was used to identify transcription start sites for *mepR*, *mepA*, and *mepB*. A single start site (+1 nucleotide) was found for *mepR*, none was clearly identified for *mepA*, and two were apparent for *mepB* ($+1_1$ and $+1_2$) (Fig. 1B). Multiple transcripts for a single ORF are not unusual in *S. aureus*; the *sarA* locus has three (21). Small differences in transcript size may make their resolution problematic in Northern blots, which was the case with respect to *mepB*. The presence of *mepAB* transcripts on Northern blots indicates that a *mepA* promoter must exist despite our inability to identify a transcription start site for this gene. The comparatively small quantities of *mepAB* and *mepB* transcripts observed in Northern blots suggest that the *mepB* and presumed *mepA* promoters are inefficient compared to that of *mepR*. Alternatively, compared to *mepRAB* transcripts, *mepAB* and *mepB* transcripts may be quite short-lived.

Concluding remarks. MepA is the first *S. aureus* MATE family MDR transporter to be identified and is capable of transporting several clinically relevant biocides and antimicrobial agents. Our data indicate that MepR represses *mepA* expression and probably expression of the entire *mepRAB* operon based on the visualization of *mepR* but no *mepRAB*, *mepAB*, or *mepB* transcripts in the parent strains examined herein (Fig. 3B). MepB does not contribute to the function of MepA, nor does it have any susceptibility-modifying effects of its own. Its function is not clear, but its presence within the *mepRAB* operon suggests that it plays some role in the natural function of MepA.

The purpose of this study was to characterize novel efflux-related MDR phenotypes in *S. aureus* using two non-NorA-mediated MDR mutants isolated at different times by independent selection methods. In both instances, parent strains were exposed to compounds that were subsequently identified as MepA substrates (i.e., norfloxacin or EtBr). This raises the possibility that substrate exposure may select for *mepA* upregulated mutants in clinical strains, which may have therapeutic implications. Reduced biocide susceptibility may allow *S. aureus* to persist in the environment, putting patients who later enter affected areas at risk of nosocomial acquisition of the organism. The contribution of efflux pumps capable of biocide or antimicrobial agent efflux to the increasing problem of MDR in clinical *S. aureus* strains already may be significant, and an analysis of the expression of *mepA*, *norA*, and *mdeA* (12) in a selection of strains possessing reserpine-reversible MDR is needed to test this hypothesis.

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