Phosphorylation of MgrA and Its Effect on Expression of the NorA and NorB Efflux Pumps of *Staphylococcus aureus*[▽]

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MgrA is a global regulator in *Staphylococcus aureus* that controls the expression of diverse genes encoding virulence factors and multidrug resistance (MDR) efflux transporters. We identified *pknB*, which encodes the (Ser/Thr) kinase PknB, in the *S. aureus* genome. PknB was able to autophosphorylate as well as phosphorylate purified MgrA. We demonstrated that *rsbU*, which encodes a Ser/Thr phosphatase and is involved in the activation of the SigB regulon, was able to dephosphorylate MgrA-P but not PknB-P. Serines 110 and 113 of MgrA were found to be phosphorylated, and Ala substitutions at these positions resulted in reductions in the level of phosphorylation of MgrA. DNA gel shift binding assays using *norA* and *norB* promoters showed that MgrA-P was able to bind the *norB* promoter but not the *norA* promoter, a pattern which was the reverse of that for unphosphorylated MgrA. The double mutant MgrA_{S110A-S113A} bound to the *norA* promoter but not the *norB* promoter. The double mutant led to a 2-fold decrease in *norA* transcripts and a 2-fold decrease in the MICs of norfloxacin and ciprofloxacin in strain RN6390. Thus, phosphorylation of MgrA results in loss of binding to the *norA* promoter, but with a gain of the ability to bind the *norB* promoter. Loss of the ability to phosphorylate MgrA by Ala substitution resulted in increased repression of *norA* expression and in reductions in susceptibilities to NorA substrates.

Staphylococcus aureus is responsible for a broad range of infections, ranging from skin and soft tissue infections to more serious illnesses, such as bacteremia and endocarditis (2–5, 19). Resistance to antimicrobial compounds in *S. aureus* can result from drug target modifications, drug inactivation, or extrusion of drugs by multidrug resistance (MDR) efflux pumps (9, 11, 17). The *S. aureus* global transcriptional regulator MgrA is a MarR and SarA homolog and is involved in the regulation of expression of virulence genes (α -hemolysin, protein A, lipase, protease, and coagulase genes), autolysins, and type 8 capsular polysaccharide (20, 25). MgrA also modulates the expression of efflux pumps, such as NorA, NorB, NorC, Tet38, and AbcA, which are involved in drug resistance (21, 36, 38). Recent studies on the role of MgrA in the regulation of expression of various genes suggested that *norA* expression may be affected by the regulon responsible for the alternative sigma factor SigB (21, 25). In Gram-positive bacteria, such as *Bacillus subtilis* and *S. aureus*, the *sigB* regulon has been studied intensively and shown to be involved in the expression of various genes, including genes of the general stress response and genes associated with virulence (6, 18, 23). The activation of the *sigB* regulon occurs by a series of phosphorylation and dephosphorylation steps that include the phosphatase RsbU (31, 34). The effects of MgrA on expression of *norA* differ in *rsbU* mutants, and phosphorylation of MgrA affects its ability to bind the *norA* promoter region (35). PknB, a newly identified serine/threonine (Ser/Thr) kinase, may also effect phosphorylation of MgrA, because increased levels of MgrA-P were found after

incubation of MgrA with extracts prepared from cells overexpressing *pknB* (35). The *S. aureus* kinase PknB was identified based on its homology to the PknB protein of *Mycobacterium tuberculosis* and has been shown to be involved in *S. aureus* cell wall metabolism (10, 13). PknB is an autokinase and catalyzes target protein phosphorylation in a time-dependent manner, using Mn²⁺ or Mg²⁺ as a cofactor. The catalytic domain of PknB is localized in the cytoplasm, separated by a putative transmembrane domain and three extracellular PASTA domains (penicillin-binding and Ser/Thr kinase-associated domains). This catalytic domain contains highly conserved residues characteristic of the STK (serine/threonine kinase) family that are required for the kinase activity (13, 30). Thus, we hypothesized that the state of phosphorylation of MgrA can be modulated directly by PknB and RsbU proteins. In this study, we investigated the direct role of PknB in the phosphorylation of MgrA, identified RsbU as a phosphatase capable of dephosphorylating MgrA-P, and determined the role of MgrA/MgrA-P in the regulation of *norA* and *norB* as well as the impact of this regulation on resistance to quinolone antimicrobials.

MATERIALS AND METHODS

Bacterial strains, plasmids, growth media, and other materials. Bacterial strains and plasmids used in this study are listed in Table 1. *S. aureus* strains were cultivated in brain heart infusion broth (BHI) (Difco, Sparks, MD) at 37°C unless stated otherwise. *Escherichia coli* strains were grown in Luria-Bertani (LB) medium. Lysostaphin, IPTG (isopropyl- β -D-thiogalactopyranoside), ampicillin, norfloxacin, ciprofloxacin, sparfloxacin, moxifloxacin, and tetracycline were obtained from Sigma Chemical Co., St. Louis, MO. All primers used in this study were synthesized at the Tufts University Core Facility, Boston, MA, and are listed in Table 2.

Site-directed mutagenesis. Amino acid changes (serine or cysteine to alanine) were introduced into MgrA by means of a QuikChange II-E site-directed mutagenesis kit (Stratagene, La Jolla, CA). Plasmid pQT5 (Table 2) carrying *mgrA*

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or relevant characteristics	Reference or source
Strains		
<i>S. aureus</i> strains		
RN4220	8325-4 <i>r</i> mutant	22
RN6390	8325 wild type (<i>rsbU</i>)	27
SH1000	Functional <i>rsbU</i> ⁺ derivative of 8325-4	18
COL	<i>mec</i> , high-Mc ^r clinical isolate	15
<i>E. coli</i> strains		
DH5 α	F [−] ϕ 80 <i>dlacZ</i> Δ <i>M15</i> Δ(<i>lacZYA-argF</i>) <i>U169 deoR</i> <i>recA1 endA1 phoA</i> <i>hsdR17</i> (r _K [−] m _K [−]) <i>supE44</i> λ <i>thi-1 gyrA96 relA1</i>	Gibco-BRL
BL21	F [−] <i>dcm ompT hsdS</i> (r _B [−] m _B [−]) <i>gal</i> λ	Stratagene
XL Blue	<i>recA1 endA1 gyrA96 thi-1</i> <i>hsdR17 supE44 relA1 lac</i> [F' <i>proAB lacI</i> ^q Δ <i>M15</i> Tn10 (Tet ^r)]	Stratagene
Plasmids		
pSK950	10.5-kb plasmid carrying the <i>attP</i> site of phage L54a, replicon of pE194, Tc ^r Em ^r (<i>S. aureus</i>)	29
pQT4	pSK950- <i>mgrA</i>	39
pQT4a	pSK950- <i>mgrA</i> _{S110A}	This study
pQT4b	pSK950- <i>mgrA</i> _{S113A}	This study
pQT4c	pSK950- <i>mgrA</i> _{S110A-S113A}	This study
pQT5	pTrcHisA- <i>mgrA</i>	39
pQT5a	pTrcHisA- <i>mgrA</i> _{S110A}	This study
pQT5b	pTrcHisA- <i>mgrA</i> _{S113A}	This study
pQT5c	pTrcHisA- <i>mgrA</i> _{S110A-S113A}	This study
pQT5d	pTrcHisA- <i>mgrA</i> _{C12A}	This study
pQT16	pSK950- <i>pknB</i>	35
pQT18	pTrcHisA- <i>pknB</i>	This study
pQT19	pTrcHisA- <i>rsbU</i>	This study

was extracted from *E. coli* DH5 α and used as a template for the procedure. Fifty nanograms of plasmid DNA was mixed with the reaction buffer, primers, deoxynucleoside triphosphates (dNTPs), and *Pfu* Ultra HF DNA polymerase to a final volume of 50 μ l. The PCR cycling parameters for the mutagenesis reaction were 1 cycle of 30 s at 95°C and 16 cycles of 30 s at 95°C, 1 min at 55°C, and 4 min at 68°C, following the manufacturer's recommendations. The reaction mixtures were placed on ice for 2 min to cool down to 37°C before DpnI digestion for 1 h at 37°C. The final reaction mixtures were used to electroporate *E. coli* XL1-Blue cells. Transformants were selected on LB plates with ampicillin (100 μ g/ml). DNA sequencing was performed to verify the presence of mutations.

RT-PCR assays. Total *S. aureus* RNA was prepared by extraction from lyso-staphin-treated cells grown to the exponential phase at 37°C or 30°C, using an RNeasy Mini kit (Qiagen, Valencia, CA). Quantitative real-time reverse transcription-PCR (RT-PCR) assays were carried out using a QPCR SYBR green kit (ABgene, Thermoscientific, United Kingdom) and the Chromo4 system for real-time PCR detection (Bio-Rad Laboratories, Hercules, CA). The primers used in this study are listed in Table 2. The housekeeping gene *gmk* was used as an internal control. All samples were analyzed in triplicate and normalized against *gmk* gene expression.

Purification of MgrA, PknB, and RsbU proteins. MgrA protein was purified as previously described (39). The *pknB* and *rsbU* genes were amplified by PCR from *S. aureus* RN6390 and COL chromosomes, respectively, subcloned into plasmid pTrcHisA (Invitrogen, Carlsbad, CA), and then introduced into *E. coli* BL21. For purification of histidine-tagged PknB and RsbU, *E. coli* BL21 harboring either plasmid was grown to mid-log phase in LB medium, at which time IPTG (1 mM) was added to the culture. After 3 h, the cells were harvested by centrif-

ugation and then resuspended in 20 mM sodium phosphate buffer, pH 7.4. The cells were lysed with lysozyme (0.02%) and then centrifuged (100,000 $\times$ g) for 90 min. The supernatant was applied to a nickel affinity column (iminodiacetic acid [IDA]-Sepharose-Ni) (Amersham Pharmacia Biotech, Uppsala, Sweden) and then washed with buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5% glycerol) supplemented with 10 mM imidazole. RsbU and PknB proteins were eluted with buffer supplemented with 100 mM imidazole. The homogeneity of the eluted protein was verified by SDS-PAGE.

Measurement of RsbU activity. RsbU activity was measured by a colorimetric method developed for studies of the RsbU protein of *B. subtilis* that detects the release of inorganic phosphate in aqueous solution (7, 40). The assays were based on the absorption of a molybdate-malachite green-phosphate complex at 630 nm, using a malachite green phosphate detection kit (R&D Systems, Minneapolis, MN). MgrA-P and PknB-P were used as substrates for RsbU. To monitor the dephosphorylation reaction, 1 nM RsbU was mixed with 20 nM MgrA-P or PknB-P in an assay buffer containing 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 10 mM MgCl₂, and 1 mM MnCl₂ at 25°C. At intervals of 5, 10, and 15 min, the reactions were stopped by adding the malachite green reagent solution, which contained ammonium molybdate, followed by absorbance readings at 630 nm. The green complex formed between malachite green, molybdate, and inorganic phosphates absorbs at 630 nm and was the basis for quantification of inorganic phosphate release.

In vitro kinase assays. *In vitro* phosphorylation of 1 μ g of purified MgrA-His or PknB-His protein was performed at 30°C for 20 min in a kinase buffer containing 5 mg/ml bovine serum albumin (BSA), 150 mM Tris-HCl (pH 7.5), 100 mM MgCl₂, 1 mM dithiothreitol (DTT), 1 mM EDTA, and 200 μ M ATP. The reactions were stopped by the addition of bromophenol blue sample buffer (13).

Immunodetection of phosphorylated proteins. Phosphoproteins were detected using chemiluminescence-labeled phosphoprotein antibodies. After SDS-PAGE electrophoresis, proteins were transferred to a nitrocellulose membrane at 200 mA for 90 min. All washing steps were performed at room temperature. The membrane was washed twice for 10 min each with TBS buffer (10 mM Tris-HCl, 150 mM NaCl, pH 7.5), followed by incubation for 1 h in blocking buffer (5% BSA, 0.1% Tween 20 in TBS buffer). Membranes were washed twice for 10 min each in TBS-Tween/Triton buffer (20 mM Tris-HCl, 500 mM NaCl, 0.05% Tween 20, 0.2% Triton X-100, pH 7.5) and once with TBS buffer. Membranes were incubated with phospho-serine or phospho-threonine antibody solution (Qiagen, Germantown, MD) (1/100 dilution of antibody in TBS-Tween 20 buffer) at 4°C overnight and then washed twice for 10 min in TBS-Tween/Triton buffer and once in TBS buffer. Membranes were incubated with secondary antibody for 1 h at room temperature and washed four times for 10 min each in TBS-Tween/Triton. The chemiluminescence detection reaction was performed and the membrane was exposed to X-ray film according to the manufacturer's recommendations.

DNA mobility shift analyses. Primers designed to amplify the putative promoter regions of *norA* and *norB* are listed in Table 2. One of the primers was biotinylated by the Tufts University Core Facility (Boston, MA). The gel mobility shift assay was carried out using a LightShift chemiluminescence EMSA kit (Pierce, Rockford, IL) as recommended by the manufacturer. Purified MgrA (1 μ g) was mixed with biotin-labeled DNA in 20 μ l of binding buffer (10 mM HEPES, pH 8, 60 mM KCl, 4 mM MgCl₂, 0.1 mM EDTA, 0.1 mg/ml of BSA, 25 mM dithiothreitol) containing 1 μ g of poly(dI-dC), 200 ng of sheared herring sperm DNA, and 10% glycerol for 20 min at room temperature. The quantities of proteins and DNA were previously determined to be sufficient to create a band shift (14, 39). After incubation, the binding mixture was analyzed by 5% non-denaturing polyacrylamide electrophoresis.

RESULTS

Phosphorylation of MgrA by PknB. We purified His-tagged MgrA protein by using a nickel affinity column, followed by incubation of the purified protein with crude cell extracts prepared from strains RN6390, SH1000, and RN6390(pQT16), which expresses *pknB* from plasmid pQT16. The mixtures were applied to nickel columns, and MgrA proteins were repurified using elution buffer containing imidazole. A phosphoprotein purification of the eluates was performed as previously described (35). SDS-PAGE and Western blotting indicated that the highest level of phosphorylation of MgrA occurred follow-

TABLE 2. Primers used in this study

Primer use and target	Sense of primer	Primer sequence (5'–3')	Amplicon DNA length (kb)
Gene amplification ^a			
<i>rsbU</i>	Sense	ACATTAGGGATCCATGTCTTA	1.0
	Antisense	TCAAATTAGAATTCTTATATACC	
<i>pknB</i>	Sense	ATAAAGTGGATCCATGATAG	2.0
	Antisense	ATTATATGAATTCCTAAATA	
Real-time RT-PCR			
<i>gmk</i>	Sense	TATCAGGACCATCTGGAGTAGG	0.1
	Antisense	CATCAACTTCACCTTCACGC	
<i>norA</i>	Sense	TTGGAATCGTTTAACATAAC	0.17
	Antisense	AGAAAGTTGTCATTGAATCA	
<i>norB</i>	Sense	GAAGATAGTTTCAATACAGA	0.23
	Antisense	ATGCTGCTGAAAGTCCTTGA	
DNA-protein gel mobility shift binding assays			
<i>norA</i> promoter	Sense	TGCAATTTTCATATGATCAATCCC	0.15
	Antisense	AGATTGCAATTCATGCTAAATATT	
<i>norB</i> promoter	Sense	ATAAGGTAAGATAACTAGCA	0.15
	Antisense	ATCTCTATTTGCCTCCCTATA	
Site-directed mutagenesis ^b			
S110A mutation	Sense	AAAGTGAACTATTAGACCAGAATTAGCTAATGCATCTGACA AAGTCGCTTC	
	Antisense	GAAGCGACTTTGTCAGATGCATTAGCTAATTCTGGTCTAATA GTTTCACTTT	
S113A mutation	Sense	ACCAGAAATTAAGTAATGCAGCTGACAAAGTCGCTTCAGC	
	Antisense	GCTGAAGCGACTTTGTCAGCTGCATTACTTAATTCTGGT	
S110A-S113A mutation	Sense	AAGTGAACTATTAGACCAGAATTAGCTAATGCAGCTGACA AAGTCGCTTCAGCTTC	
	Antisense	GAAGCTGAAGCGACTTTGTCAGCTGCATTAGCTAATTCTGGT CTAATAGTTTCACTT	
C12A mutation	Sense	GATCAACATAATTTAAAAGAACAGCTAGCCTTTAGTTTGTAC AATGCTCAAAGACA	
	Antisense	TGTCTTTGAGCATTGTACAAACTAAAGGCTAGCTGTTCTTTTA AATTATGTTGATC	

^a Restriction enzyme recognition sites are underlined. EcoRI, GAATTC; BamHI, GGATCC.

^b The point mutations are underlined.

ing incubation with extracts of cells overexpressing *pknB* from a plasmid. Higher relative levels of phosphoprotein were also found for incubation with extracts of strain RN6390 (*rsbU*) than those for incubation with extracts of strain SH1000 (*rsbU*⁺) (Fig. 1A), confirming our previous findings (35).

To evaluate further a possible direct role for PknB in the phosphorylation of MgrA, we purified His-tagged PknB by using the expression plasmid pQT18 and nickel-affinity chromatography. The His tag of PknB was cleaved off by enterokinase prior to the kinase reaction to allow the recovery of MgrA-His-P by the nickel column after phosphorylation (Fig. 1B). After incubation of PknB and MgrA, Western blotting with anti-serine phosphate (anti-Ser-P) antibody produced positive signals with the PknB (autokinase) and MgrA proteins; unincubated proteins gave no signal (Fig. 1B). Thus, PknB itself can phosphorylate MgrA at one or more serine residues. Western blotting using anti-threonine phosphate (anti-Thr-P) antibody was also positive (data not shown), suggesting that PknB also phosphorylated MgrA at one or more threonine residues.

Specificity of MgrA serines phosphorylated by PknB. MgrA has 13 serine residues among 147 amino acids. To determine

those serine residues phosphorylated by PknB, MgrA-P was submitted to mass spectrometry (data not shown). We identified two residues, Ser110 and Ser113, that were phosphorylated. While Thr66 was also found to be phosphorylated, as shown previously, Ser118 was not (35).

To confirm the mass spectrometry data, we replaced Ser110 and Ser113 with alanine by site-directed mutagenesis, creating MgrA proteins with single or double alanine substitutions at these two positions (Fig. 2A). We then analyzed the efficiency of PknB phosphorylation of the mutant MgrA proteins. PknB, treated with enterokinase to eliminate the histidine tag, was incubated with mutant MgrA proteins, and the MgrA-His proteins were repurified using a nickel column to eliminate PknB, which did not bind to the column. A second phospho-purification step was then carried out to separate MgrA-P-His from MgrA-His. We performed Western blotting and immunodetection using anti-Ser-P antibody as previously described. There was a decrease of 3-fold in the intensities of the signals of the MgrA_{S110A} and MgrA_{S113A} proteins, and almost no signal was found for the MgrA_{S110A-S113A} protein (Fig. 2B), indicating that Ser110 and Ser 113 accounted for the majority of the serine phosphorylation of MgrA by PknB.

FIG. 1. Serine phosphorylation of MgrA and PknB. (A) Purified MgrA-His (1 μ g) after incubation with cell extracts from strains RN6390, SH1000, and RN6390 overexpressing *pknB* from plasmid pQT16. Results of SDS-PAGE (upper panel) and Western blotting with anti-Ser-P antibody (lower panel) are shown. (B) Phosphorylation of purified PknB and MgrA by PknB. One microgram of each protein was used for the kinase assays. MgrA and PknB were mixed together for 20 min at 30°C. After repurification with a nickel affinity column, the phosphorylated proteins were submitted to SDS-PAGE and Western blotting (anti-Ser-P antibody). MM, molecular markers.

Binding of MgrA and MgrA-P to *norA* and *norB* promoter DNAs. We previously demonstrated that in contrast to MgrA, MgrA-P binds poorly to *norA* promoter DNA. Expression of *norB* is affected in the opposite manner from that of *norA* by overexpression or knockout of *mgrA*, and MgrA binds little to the *norB* promoter (35, 36). Thus, we sought to determine, using DNA band shift assays, if protein phosphorylation played a role in these reciprocal effects and, specifically, the effect of phosphorylation on MgrA binding to *norB* promoter DNA. For the *norA* promoter, DNA band shifts were seen with MgrA but not MgrA-P, as shown previously (35). In contrast, for the *norB* promoter, DNA band shifts were seen with MgrA-P but not MgrA (Fig. 2C). Thus, phosphorylation of MgrA underlies the reciprocal promoter binding properties for *norA* versus *norB* promoter DNAs.

To determine if substitution of Ala for Ser at positions 110 and 113 itself affected the ability of MgrA and MgrA-P to bind DNA, we performed DNA binding assays using the mutated MgrA proteins and the same target DNAs. Mutant MgrA proteins bound *norA* promoter DNA, indicating that the mutant proteins retained the ability to bind specific DNA. In contrast to MgrA-P, MgrA_{S110A}, MgrA_{S113A}, and MgrA_{S110A-S113A} treated with kinase retained the ability to bind *norA* promoter DNA (Fig. 3A). For *norB*, promoter binding of MgrA proteins after PknB

incubation was detected with MgrA, MgrA_{S110A}, and MgrA_{S113A} but not with MgrA_{S110A-S113A} (Fig. 3B).

Effects of Cys12Ala mutation and oxidation on MgrA binding to *norA* and *norB* DNAs. In previous studies, the oxidation of Cys12 of MgrA led to the dissociation of the MgrA-*sarV* promoter DNA complex. The addition of a reducing agent (DTT) allowed complex formation (8). The 40-bp *sarV* promoter fragment used in this study had MgrA binding sites, as defined by Manna et al. (28). In the two 150-bp DNA fragments used for *norA* and *norB* promoter binding assays, a 6-bp DNA sequence (TGTTGT) located between the -35 and -10 regions of *norA* was identical with the published MgrA binding region of the *sarV* gene (Fig. 4A). No such match was found for the *norB* promoter DNA fragment. To determine if oxidation of Cys12 affected the binding of MgrA to the *norA* promoter DNA, as it does for *sarV* promoter DNA, we performed site-directed mutagenesis to generate a Cys12Ala mutant. The Ala12 MgrA mutant bound *norA* promoter DNA similarly to wild-type MgrA (Fig. 4B).

We also evaluated the effects of the reducing agent DTT in the formation of the MgrA-DNA complex, using the *norA* and *norB* promoters as target DNAs. The binding reactions were done using two different binding buffers, one supplemented with DTT at 25 mM and one without DTT. After 20 min of

FIG. 2. Role of Ser110 and Ser113 in MgrA phosphorylation and promoter binding. (A) Amino acid sequence of the MgrA protein. The cysteine (C), threonine (T), and two serine (S) residues are indicated in bold and underlined. Their respective positions in the sequence are included. Alanines (A) were chosen to replace the cysteine and the two serines, using site-directed mutagenesis. (B) Reductions in phosphorylation levels of mutated MgrA-P proteins. SDS-PAGE (upper panel) and Western blot (anti-Ser-P antibody; lower panel) results are shown. (C) DNA mobility shift assays using MgrA and MgrA-P (1 μ g of each protein) and the *norA* and *norB* promoters (2 ng of each DNA). MgrA bound to the *norA* promoter, while MgrA-P bound to the *norB* promoter. These quantities of protein and DNA were previously determined to be sufficient to create band shifts for the binding assays (36, 39).

incubation at room temperature, the mixtures were analyzed by electrophoresis through a nondenaturing 5% acrylamide gel. The presence or absence of DTT did not affect the protein-DNA binding process between MgrA and the two promoters (Fig. 4C). Similar results were found when the Cys12Ala MgrA mutant was used for the assays (data not shown).

Dephosphorylation of MgrA by RsbU. Prior work has shown that *rsbU* affects the function of MgrA in the regulation of MDR efflux pumps such as NorA (21, 25, 35). When *mgrA* was overexpressed, *norA* transcript levels increased 2-fold in RN6390 (*rsbU* mutant) but decreased 2-fold in SH1000 (*rsbU*⁺) (35, 39). We also demonstrated that the quantity of

MgrA-P was slightly higher in RN6390 than in SH1000, suggesting a role for *rsbU* in the phosphorylation state of MgrA in the cell, but a direct effect of RsbU on MgrA was unclear. Since *rsbU* encodes a serine/threonine phosphatase, we hypothesized that RsbU could also function as a phosphatase for MgrA. Thus, we amplified the wild-type *rsbU* gene from *S. aureus* COL and cloned it into the expression plasmid pTrcHisA to generate plasmid pQT19. pQT19 was introduced into *E. coli* BL21 for protein expression. BL21(pQT19) cells were cultured, and the RsbU protein, with an N-terminal histidine tag, was purified following a procedure similar to that used for MgrA and PknB protein purifications. To assess the

FIG. 3. DNA gel shift binding assays using MgrA and MgrA_{S110A}, MgrA_{S113A}, and MgrA_{S110A-S113A} mutants. (A) DNA gel shift binding assays using the *norA* promoter and MgrA proteins, with and without phosphorylation by PknB *in vitro*. Two nanograms of DNA and 1 μ g of each protein were used in each assay. (B) DNA gel shift binding assays using the *norB* promoter and MgrA proteins, with and without phosphorylation by PknB *in vitro*. Two nanograms of DNA and 1 μ g of each protein were used in each assay.

ability of RsbU to dephosphorylate MgrA, we carried out dephosphorylation reactions, using either MgrA-P or PknB-P as a potential substrate of RsbU in the absence of ATP. We used RsbU-His as a negative control. RsbU mediated a 4-fold increase in the release of inorganic phosphate when incubated with MgrA-P, but not with PknB-P, compared to incubation of RsbU-His alone (Fig. 5A). Western blotting using anti-Ser-P antibody was performed to complement the malachite green assays and showed a decrease of 3-fold in the intensity of the signal generated by MgrA-P over time (Fig. 5B). The same experiment was carried out using PknB-P, which showed no change in the intensity of PknB-P (data not shown). Thus, RsbU has specific phosphatase activity that affects MgrA but

not PknB and may have a direct role in modulating the state of phosphorylation of MgrA *in vivo*.

Effects of MgrA phosphorylation on *norA* and *norB* expression and resistance to quinolones. We subcloned the *mgrA* gene with mutations encoding S110A, S113A, and S110A/S113A into plasmid pSK950, generating pQT4a, pQT4b, and pQT4c, respectively, which were introduced into RN6390 and SH1000 by electroporation (Table 1). RN6390 and SH1000 with plasmid pQT4 (wild-type *mgrA* cloned into pSK950) or pSK950 served as controls. MICs of known substrates of NorA (norfloxacin and ciprofloxacin) and NorB (norfloxacin, ciprofloxacin, moxifloxacin, and sparfloxacin) were determined, and *norA* and *norB* transcript levels were determined by RT-PCR.

In RN6390, an RsbU phosphatase-negative strain with high steady-state levels of MgrA-P, as previously reported (35), the MICs of norfloxacin and ciprofloxacin were increased in the presence of plasmid-encoded wild-type MgrA, as was the level of *norA* expression. In contrast, the MICs of moxifloxacin and sparfloxacin decreased 3-fold and 2-fold, respectively (Table 3). By real-time RT-PCRs, we detected an increase of 2.5-fold of the *norA* transcripts and a decrease of 2-fold of the *norB* transcripts. These effects were both diminished when the plasmid-encoded single Ala mutants of *mgrA* were substituted for wild-type *mgrA* and further diminished with the double Ala mutant of *mgrA*, thereby linking the lack of ability to phosphorylate MgrA with reductions in its effects on *norA* expression and resistance in whole cells. We observed a 5-fold reduction in the transcript level of *norA*, while *norB* transcripts returned to almost the same level of transcripts as that in RN6390(pSK950), when we compared RN6390(pQT4) with RN6390(pQT4c) (Table 3). Notably, these MgrA phosphorylation-dependent changes in norfloxacin and ciprofloxacin susceptibility and increases in *norA* expression were abolished in strain SH1000, which has an intact RsbU phosphatase, suggesting that the RsbU phosphatase activity itself modulates the repressor effects of MgrA on *norA* expression by its effects on the phosphorylation state of MgrA. These findings are consistent with a model in which MgrA is a repressor of *norA* expression and repression may be relieved by phosphorylation of MgrA, which abrogates MgrA binding to the *norA* promoter.

The pattern of effects of *mgrA* expression on MICs of moxifloxacin and sparfloxacin (which are substrates of NorB but not NorA) and on *norB* expression was opposite from that of effects on *norA* expression and on susceptibility to norfloxacin and ciprofloxacin. In the context of binding of MgrA-P but not MgrA to the *norB* promoter, these findings are consistent with a model in which MgrA-P is a repressor of *norB* expression and suggest that the phosphorylation state of MgrA mediates the reciprocal expression patterns of *norA* and *norB*.

DISCUSSION

Protein phosphorylation is an important mechanism in the regulation of cellular functions in both eukaryotes and prokaryotes. The phosphorylation state of regulatory proteins is often modulated by a combination of kinase and phosphatase reactions that in some cases result in a series of phosphate transfers among a relay of proteins in response to internal and environmental signals (31–33). PknB of *S. aureus* is a recently identified Ser/Thr kinase, based on its homology to known

FIG. 4. DNA gel shift binding assays. (A) Comparison of the DNA sequences of the *norA* (top two lines) and *sarV* promoters. The *norA* promoter harbors 4 putative DNA binding sites (14) and the -35 and -10 consensus sequences, as reported by Fournier et al. (14) and verified by DNase I protection footprint analysis (data not shown). Between the -35 and -10 sequences is a short sequence (TGTTGT) (boxed) that matches the *mgrA* binding site identified by Manna et al. in the *sarV* promoter (28). The *norA* and *sarV* promoter sequences are numbered as reported in each reference. MgrA binding sites are indicated in bold and underlined. (B) DNA gel shift binding assays using the *norA* promoter (2 ng) and mutated MgrA proteins (1 μ g), with and without phosphorylation by PknB *in vitro*. (C) DNA shift binding assays using the MgrA and MgrA-P proteins, the *norA* and *norB* promoters, and reaction buffers with or without DTT. The same amounts of protein and DNA were used in all assays.

Ser/Thr kinases (STK family) of other bacterial species, such as PknB of *M. tuberculosis* and PrkC of *B. subtilis*. These proteins share highly conserved residues characteristic of the STK family that are essential for the catalytic activity of the enzymes (13, 30). The gene adjacent to *pknB* encodes a putative phosphatase called Stp1, which belongs to the protein phosphatase M family (10). *pknB* and *stp* are cotranscribed and translationally coupled (10, 30). PknB, also called Stk1, can autophosphorylate as well as phosphorylate the serine and threonine residues of other proteins, including many glycolytic and protein synthetic enzymes, further placing PknB in central metabolic pathways, such as those for nucleotide biosynthesis, glycolysis, amino acid metabolism, and cell wall metabolism (10, 24, 30). The roles played by PknB are many. We initially observed an increase in the amount of MgrA-P after purified MgrA-His was incubated with crude cell extract prepared from cells overexpressing *pknB* from a plasmid. Due to the complexity of the

crude extract composition, we could not assign the increase in the amount of MgrA-P as necessarily due to the kinase activity of PknB itself. To evaluate further the ability of PknB itself to phosphorylate MgrA, we measured the ability of purified PknB protein to phosphorylate MgrA. PknB was demonstrated both to autophosphorylate and to phosphorylate MgrA at specific residues, Ser110, Ser 113, and Thr66 but not Ser118, as reported previously, after incubation with cell extracts (35).

To determine the relevance of phosphorylation of Ser110 and Ser113, we replaced these two serines, singly or together, with alanines and evaluated the effects of the changes in PknB kinase-treated MgrA on binding to *norA* and *norB* putative promoters and the effects of *mgrA* overexpression on *norA* and *norB* expression and quinolone resistance patterns. Progressive decreases in reactivity with phosphoserine antibody occurred with single and double mutations, confirming the mass spectrometry data showing that these residues were specifically

FIG. 5. RsbU phosphatase activity on MgrA-P and PknB-P substrates. (A) Purified RsbU (1 nM) was incubated with a 20 nM concentration of either MgrA-P or PknB-P. RsbU-His was used as a negative control. Release of inorganic phosphate was measured by a change in absorbance at 630 nm (optical density at 630 nm [OD₆₃₀]). Inorganic phosphate was used to create a standard curve for the malachite green assay. (B) Loss of phosphorylation of MgrA after incubation with RsbU. A Western blot made using anti-Ser-P antibody is shown. At 6 min, there was a 50% reduction of the signal generated. The SDS-PAGE gel shows that MgrA-P remained intact during the assays. MM, molecular markers.

phosphorylated by PknB and that they were important for binding to *norA* and *norB* promoter DNAs. Residues Ser110 and Ser113 are both located in a region of the protein that is crucial for the formation of the MgrA dimer (8). Ser113 has been implicated specifically in the dimerization of MgrA via the recognition of Cys12 at the N-terminal domain, and Cys12 is thought to be involved in MgrA binding to the *sarV* promoter (8). Oxidation of Cys12 resulted in dissociation of MgrA from *sarV* promoter DNA. Unlike the *sarV*-MgrA complex, however, the ability of MgrA to bind to *norA* and *norB* promoter DNAs was unaffected by the oxidation state of Cys12 or its replacement with Ala. Comparing the nucleotide sequences of

sarV, *norA*, and *norB* promoters, a six-nucleotide sequence (TGTTGT) was common to the *norA* and *sarV* promoters and was at a site that was shown to be the site of MgrA binding to the *sarV* promoter. No match was detected for the *norB* promoter DNA. Thus, the DNA binding of MgrA is variably dependent on the oxidation and phosphorylation states of the protein, depending specifically on the promoter, thereby adding another level of modulation of its regulatory effects in response to internal, and possibly environmental, signals.

The regulatory functions of MgrA also differ in strains with and without functional RsbU, which is a component of the SigB regulon and is induced by and modulates responses to several stress conditions (21, 25, 26). Since RsbU functions as a specific Ser/Thr phosphatase that initiates activation in the SigB regulon and dephosphorylates RsbV (15, 16, 32), we hypothesized that RsbU could also serve as a phosphatase of MgrA-P. Purified RsbU indeed functioned directly as a phosphatase of MgrA-P, but not PknB-P, *in vitro*. In contrast, the Stp1 Ser/Thr phosphatase encoded by *stp1*, which is adjacent to *pknB* in the *S. aureus* genome, has been shown to dephosphorylate PknB-P (10). Further investigations are under way to determine if Stp1 can dephosphorylate MgrA-P.

Although we cannot exclude the possibility that kinases other than PknB and phosphatases other than RsbU may also affect the state of MgrA phosphorylation in whole cells, our data showing that MgrA regulation of *norA* and *norB* *in vivo* differs in strains with and without functional RsbU in a manner that is dependent on the presence of Ser110 and Ser113 argue that RsbU may function *in vivo* to dephosphorylate MgrA-P, as it can *in vitro*.

MgrA and MgrA-P bind oppositely to the promoters of *norA* and *norB*, consistent with a model in which MgrA functions as a repressor of *norA* and MgrA-P acts as a repressor of *norB*, with the loss of binding of MgrA-P to the *norA* promoter and of MgrA to the *norB* promoter serving to relieve the respective repression. In strain RN6390, in which a functional RsbU phosphatase is absent, thereby favoring a higher ratio of MgrA-P to MgrA, increased expression of *norA* and increased resistance to the NorA substrates norfloxacin and ciprofloxacin occurred when MgrA was expressed from a plasmid. Similarly, in this background, *norB* expression and resistance to the NorB

TABLE 3. Real-time RT-PCR results and antimicrobial MICs^a

Strain	Relative gene transcript level (fold)		MIC			
	<i>norA</i>	<i>norB</i>	Nor	Cip	Moxi	Sparf
RN6390(pSK950)	1	1	0.5	0.25	0.03	0.06
RN6390(pQT4)	2.5	0.5	2	1	0.01	0.03
RN6390(pQT4a)	1.5	1	0.5	0.25	0.03	0.06
RN6390(pQT4b)	1.5	1	0.5	0.25	0.03	0.06
RN6390(pQT4c)	0.5	1.5	0.25	0.125	0.03	0.06
SH1000(pSK950)	1	1	0.5	0.25	0.03	0.06
SH1000(pQT4)	0.5	1	0.25	0.125	0.03	0.06
SH1000(pQT4a)	1	1	0.25	0.125	0.03	0.06
SH1000(pQT4b)	1	1	0.25	0.125	0.03	0.06
SH1000(pQT4c)	0.5	1	0.125	0.125	0.03	0.06

^a Strains harboring plasmids pSK950 and pQT were grown in the presence of tetracycline (5 µg/ml). Nor, norfloxacin; Cip, ciprofloxacin; Moxi, moxifloxacin; Sparf, sparfloxacin.

substrates moxifloxacin and sparfloxacin were decreased. In strain SH1000, in which a functional RsbU phosphatase may favor lower levels of MgrA-P, these effects of MgrA expressed from a plasmid were reversed (i.e., reduced *norA* expression and reduced resistance to NorA substrates) or blunted (i.e., no decrease in *norB* expression or resistance to NorB substrates). Thus, not only the level of MgrA but also its phosphorylation state appears to be important in balancing the levels of expression of the NorA and NorB MDR efflux pumps.

Recently, many studies have shown that environmental stresses, such as stringency conditions, heat shock, oxidative stress, acidity, and heme stress, can affect the expression of *norA*, *norB*, and genes encoding other transporters (1, 8, 12, 41). In addition, an intact *norB* gene was shown to contribute to bacterial fitness in staphylococcal abscesses (12). Thus, efflux pump expression appears to be an integral part of cell adaptation to various environmental stresses. The regulatory networks mediating these effects are complex and layered. Although *arlRS* (a two-component system), *rsbU*, and *pknB* affect *norA* expression indirectly, MgrA and NorG are the direct effectors binding to the *norA* promoter. Although *mgrA* mutants affected the expression of *norB* (25, 36, 37), the direct effectors were not known. We now have demonstrated that MgrA itself, in its phosphorylated form, can serve as a direct regulator of *norB* expression. Thus, posttranslational modification of MgrA plays an important role in its functions to modulate efflux pump expression.

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