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Coordinated Regulation by AgrA, SarA, and SarR To Control *agr* Expression in *Staphylococcus aureus*▽†

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The *agr* locus of *Staphylococcus aureus* is composed of two divergent transcripts (RNAII and RNAIII) driven by the P2 and P3 promoters. The P2-P3 intergenic region comprises the SarA/SarR binding sites and the four AgrA boxes to which AgrA binds. We reported here the role of AgrA, SarA, and SarR on *agr* P2 and P3 transcription. Using real-time reverse transcription (RT)-PCR and promoter fusion studies with selected single, double, triple, and complemented mutants, we showed that AgrA is indispensable to *agr* P2 and P3 transcription, whereas SarA activates and SarR represses P2 transcription. *In vitro* runoff transcription assays revealed that AgrA alone promoted transcription from the *agr* P2 promoter, with SarA enhancing it and SarR inhibiting *agr* P2 transcription in the presence of AgrA or with SarA and AgrA. Electrophoretic mobility shift assay (EMSA) analysis disclosed that SarR binds more avidly to the *agr* promoter than SarA and displaces SarA from the *agr* promoter. Additionally, SarA and AgrA bend the *agr* P2 promoter, whereas SarR does not. Collectively, these data indicated that AgrA activates *agr* P2 and P3 promoters while SarA activates the P2 promoter, presumably via bending of promoter DNA to bring together AgrA dimers to facilitate engagement of RNA polymerase (RNAP) to initiate transcription.

Staphylococcus aureus is an opportunistic pathogen that causes a broad range of human infections in both community and hospital settings (17, 22). In most cases, infections begin as a localized lesion and then spread to the bloodstream. Once the infection is in the bloodstream, patients are at risk for developing endocarditis and other metastatic complications (11).

The pathogenicity of *S. aureus* is a complex process that involves the coordinated expression of many virulence factors, which can be divided into two main categories, based on their functions either as surface protein adhesins or as secreted toxins and enzymes. Typically, surface protein adhesins are produced during the exponential phase of growth. The second stage of infection, analogous to the post-exponential phase of growth, is characterized by enhanced toxin and enzyme production, eventually leading to tissue destruction and bacterial spread (22). The regulatory events controlling the transition from the exponential phase to the post-exponential phase of growth are mediated in part by *agr*, which, upon activation, represses surface protein expression and promotes secretion of extracellular toxins (25).

The *agr* locus is composed of two divergent transcripts called RNAII and RNAIII. RNAII encodes four genes (*agrDBCA*), with AgrD encoding a 46-residue peptide which is processed into a cyclic autoinducing peptide (AIP) and then exported by AgrB (25). Upon accumulation of AIP with increasing cell density, it induces phosphorylation of AgrC, a membrane sensor within a two-component regulatory system, followed by a second-step phosphorylation of the response regulator AgrA (25). AgrA can then bind to specific direct repeats in the intergenic region between the RNAII and RNAIII promoters to modulate *agr* transcription (18). Activation of RNAIII, the *agr* effector molecule, results in repression of many surface-associated proteins while promoting exoprotein gene transcription and, to a lesser extent, translation (13, 25).

Besides AgrA, a number of regulators controlling *agr* expression have been described (5, 7). Among these is SarA, a 14.7-kDa DNA binding protein that is a prototypic member of a family called the SarA protein family (6, 7). Most, if not all, members of the SarA protein family are winged helix proteins that bind to target promoters to alter virulence gene expression (7, 21). Indeed, DNA binding studies revealed that SarA can bind to the *agr* P2 and P3 promoters (8, 30). Transcription studies of *S. aureus* cells *in vivo* have consistently shown that SarA upregulates RNAII expression (8, 9); however, *in vitro* transcription analysis of the *agr* P2 promoter in the presence of SarA alone revealed repression rather than activation (3). This discrepancy between *in vivo* activation and *in vitro* repression of the *agr* P2 promoter by SarA has not been explained until now.

SarR is a 13.6-kDa protein that is also a member of the SarA protein family. *In vitro*, SarR can bind to the *agr* promoter to influence *agr* transcription in *S. aureus* strain RN6390 (23), a strain with a defective *rsbU* gene which is required for the optimal expression of the stress-induced alternative sigma factor called SigB (19, 28). Using real-time reverse transcription (RT)-PCR and transcriptional fusion analyses of a deletion mutant (without an antibiotic marker) in strain SH1000 (*rsbU*-

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FIG. 1. The intergenic sequence between the *agr* P2 and P3 promoters. There are 186 bp between the two transcription start sites (labeled with bent arrows). The −10 and −35 promoter boxes for P2 and P3 promoters are depicted on top of the sequence. The AgrA tandem repeats are indicated by long bold arrows (the set below is complementary to the top set). The SarA and SarR binding sites, located between the two sets of tandem repeats, share a partial overlap, with the SarA binding site (palindromic sequence) highlighted by dotted lines and the SarR binding site boxed. The −10 promoter box for the P3 promoter has an extended −10 sequence (TGT), which may reduce the requirement for the canonical −35 promoter motif (14). The intergenic *agr* P2-P3 sequence here is conserved in 6 published *S. aureus* genomes. The difference between this sequence and divergent ones in other *S. aureus* genomes resides primarily in one base change (marked with an asterisk) downstream of the −10 promoter box of the *agr* P2 promoter, with a C replaced by a T.

restored variant of RN6390), we have recently shown that SarR can downregulate *agr* RNAII expression in strain SH1000 (34), different from what we have discerned with the *sarR* mutant of strain RN6390 (*rsbU* mutant) in which RNAII transcription was slightly elevated compared with that of the parent. The binding site of SarR on the *agr* promoter, located between the P2 and P3 promoters, shares an overlap with that of SarA (8, 24) (Fig. 1).

Given the temporal regulation of *agr* by SarR and SarA and the effect of AgrA on its cognate promoter, we wanted to investigate the role of AgrA, SarA, and SarR on transcription from the *agr* P2 and P3 promoters, using deletion mutants generated without any replacement antibiotic marker but containing intact promoter and transcription termination signals. Our data showed that AgrA is essential to transcriptions from the *agr* P2 and P3 promoters. More specifically, the AgrA promoter boxes and the overlapping SarA and SarR binding sites between the P2 and P3 promoters (Fig. 1) are critical to *agr* P2 transcription but not P3 transcription. Using *in vitro* runoff transcription assays, we found that SarA, in the presence of AgrA, promotes transcription from the *agr* P2 promoter *in vitro*, while SarR acts as a repressor. Electrophoretic mobility shift assay (EMSA) and promoter DNA bending assays suggest that SarA and AgrA bind and bend the *agr* P2 promoter, whereas SarR displaces SarA and binds but does not bend the promoter, thus providing a mechanism by which SarA and SarR modulates the *agr* P2 promoter activity via DNA bending in the presence of AgrA.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *S. aureus* strains used in this study, listed in Table 1, are derivatives of SH1000, an *rsbU*-restored strain of the 8325-4 strain (15). Growth of *S. aureus* was conducted in tryptic soy broth (TSB; Difco) supplemented with erythromycin (3 or 10 μg/ml, depending on the copy number of the erythromycin cassette) as needed. Gene deletions of strain SH1000 for *sarA* and *sarR* were carried out using the temperature-sensitive pMAD plasmid as described previously (1, 36). For the *agrA* mutant, we introduced translation stops (TAATGA) right next to the start codon to ensure that AgrA was not translated without any interruption of RNAII transcription, using pMAD. A similar technique was used to replace the deleted gene with a wild-type copy. *Escherichia coli* DH5α was the vehicle for routine DNA manipulation. Shuttle vector pALC1484, a derivative of pSK236 containing the gfp_{uvr} reporter gene (16), was used to construct promoter DNA-green fluorescent protein (GFP) reporter fusions. For growth in *E. coli*, Luria-Bertani (LB) broth was routinely used; ampicillin was added to a final concentration of 50 μg/ml as needed. For growth in *S. aureus*, chloramphenicol at 10 μg/ml was used in TSB. Growth was monitored at 37°C at an optical density at 650 nm (OD_{650}) with a Spectronic 20D+ spectrophotometer (Spectronic Analytical Instruments, Garforth, England), using 18-mm borosilicate glass tubes.

Promoter DNA-GFP reporter assay. Promoter-GFP reporter fusions were constructed by inserting the *agr* promoter fragments into the EcoRI and XbaI sites of pALC1484 (16). Recombinant plasmids were then transformed first into *S. aureus* strain RN4220 for proper methylation followed by introduction into the wild-type, mutant, and restored strains of SH1000 derivatives as described previously (33). Overnight cultures were diluted 1:500 into 10 ml of TSB containing chloramphenicol (10 μg/ml) to yield an OD_{650} of ~0.01 and grown at 37°C with constant shaking at 250 rpm. Fluorescence (excitation/emission = 485/515 nm) was then monitored with an FL600 fluorescence spectrophotometer (Biotek, Winooski, VT) every 2 h and reported as fluorescence units/OD_{650} values.

RNAP purification. For purification of native RNA polymerase (RNAP) from *S. aureus*, a DNA insert bearing the His_{10} tag at the C terminus of *rpoC* was cloned into the pMAD vector and introduced into the chromosome of a *sarA agrA sarR* triple mutant of SH1000. RNAP was then purified from 3 liters of cells grown in TSB to an OD_{600} of ~1.0. Briefly, cells were harvested by centrifugation, followed by freezing overnight at −80°C. Cells were then suspended in 30 ml lysis buffer (50 mM Tris-HCl [pH 8.0], 100 mM NaCl, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride [PMSF], and 20% glycerol) and lysed with four passages through a French press followed by clarification of the lysate with centrifugation (14,000 rpm, 30 min). RNAP was then purified by passaging the clear lysate through 10 ml of Ni-nitrilotriacetic acid (Ni-NTA) in a column (Qiagen), washing the resin with 10× volume of lysis buffer with 50 mM imidazole, followed by elution with 50 ml of elution buffer (similar to lysis buffer but with 250 mM NaCl) containing 500 mM imidazole. The eluted RNAP was concentrated with Amicon Ultracel-10K (Millipore), dialyzed with buffer containing 10 mM Tris-HCl (pH 8.0), 100 mM KCl, 5 mM $MgCl_2$, and 20% glycerol using a Slide-A-Lyzer dialysis cassette (Pierce), and stored at −20°C. The purities of various subunits within RNAP were confirmed by SDS-gel analysis.

Purification of SarA, SarR, and AgrA proteins. Both *sarA* and *sarR* coding regions are cloned into pET11b and pET14b, respectively, in *E. coli* BL21(DE3)pLysS. Protein expression and purification were conducted as described previously (8, 23). For AgrA protein purification, the *agrA* coding region was cloned into the NdeI and SmaI sites of pTYB2 (New England BioLabs), followed by transformation into BL21(DE3)pLysS cells. Cells grown in 1 liter of LB and induced with 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 5 h were harvested by centrifugation, passed through a French press thrice, and clarified by a second centrifugation step. The clarified lysate was loaded on a chitin bead (NEB) column, and the AgrA protein was purified according to the manufacturer's protocol. For additional purification, the fractions of interest were applied to a Bio-Rad High Q column and eluted with an NaCl gradient of 0.1 to 0.5 M. Fractions containing the AgrA were concentrated with Amicon Ultracel-10K (Millipore) and dialyzed with 10 mM Tris-HCl (pH 8.0), 50 mM NaCl, and 5% glycerol using a Slide-A-Lyzer dialysis cassette (Pierce).

***In vitro* runoff transcription assay.** Linear DNA templates of *rpsD* (control template) and *agr* P2 and P3 promoters were generated by PCR. *In vitro* transcription experiments were carried out as follows: the promoter DNA template (20 nM) was incubated with 50 nM RNAP with or without AgrA, SarA, or SarR (as indicated) in buffer containing 10 mM Tris-HCl (pH 8.0), 50 mM NaCl, 5 mM $MgCl_2$, and 50 μg/ml of bovine serum albumin (BSA) for 10 min at room temperature. To initiate the reaction, a nucleotide mixture containing 200 μM (each) ATP, GTP, and CTP, 10 μM UTP, and 10 μCi [α-^{32}P]UTP was added to the reaction mix and incubated at 37°C, and then the reaction was terminated after 15 min with the addition of 10 μl of stop solution (1 M NH_4CH_3COO, 30 mM EDTA, and 100 μg/ml yeast tRNA). The reaction mixture was ethanol precipitated and resolved in an 8% urea-PAGE gel. Images were scanned using

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Reference
S. aureus strains		
RN4220	A mutagenized strain that accepts foreign DNA	
RN6390	Laboratory strain related to strain 8325-4, *rsbU* mutant	
SH1000	Strain 8325-4 with *rsbU* restored	
ALC7075	SH1000 Δ*sarA*	This study
ALC7076	SH1000 *agrA* mutant with $agrA^{TAATGA}$ (denoted as $agrA^{TAATGA}$)	This study
ALC7077	SH1000 Δ*sarR*	This study
ALC7078	SH1000 $agrA^{TAATGA}$ Δ*sarA*	This study
ALC7079	SH1000 $agrA^{TAATGA}$ Δ*sarR*	This study
ALC7080	SH1000 Δ*sarA* Δ*sarR*	This study
ALC7081	SH1000 $agrA^{TAATGA}$ Δ*sarA* Δ*sarR*	This study
ALC7082	SH1000 $agrA^{TAATGA}$ Δ*sarA* Δ*sarR* $rpoC_{His10}$	This study
ALC7083	SH1000 Δ*sarA*/*sarA* complement	This study
ALC7084	SH1000 $agrA^{TAATGA}$/*agrA* complement	This study
ALC7085	SH1000 Δ*sarR*/*sarR* complement	This study
ALC7086	SH1000 Δ*sarR* $agrA^{TAATGA}$/*agrA* complement	This study
ALC7087	SH1000 Δ*sarR* Δ*sarA*/*sarA* complement	This study
E. coli strains		
DH5a	F^- *endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG* ϕ80*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169* *hsdR*17(r_K^- m_K^+) λ^-	
BL21(DE3)/pLysS	F^- *ompT gal dcm lon* $hsdS_B$(r_B^- m_B^-) λ(DE3) pLysS(Cm^r)	
Plasmids		
pTYB2	Expression vector with a self-cleavable intein tag	NEB
pMAD	*E. coli-S. aureus* shuttle vector containing temp-sensitive origin of replication *bgaB*, Erm^r Amp^r	1
pALC1484	*E. coli-S. aureus* shuttle vector derived from pSK236-containing multiple cloning site upstream of the gfp_{uvr} gene (a *gfp* variant), Amp^r Cm^r	21
pALC1742-2	*agr* P2 promoter driving the expression of gfp_{uvr} in pALC1484	This study
pALC1743-2	*agr* P3 promoter driving the expression of gfp_{uvr} in pALC1484	This study
pCY7	Bending vector	31
pAM1163	pCY7 containing the SarA/SarR binding site	This study
pAM1847	pCY7 containing the AgrA P2 tandem site	This study

Molecular Dynamics Typhoon 8600 (GE Healthcare) and processed using ImageQuant version 5.2.

RNA extraction and real-time RT-PCR. Cells of wild-type or mutant strains grown in TSB to mid-, late, and post-exponential phases were harvested at 4,000 rpm for 10 min (4°C) and stored overnight at −80°C. Cells were then suspended in 350 μl of TRIzol (Invitrogen), mixed with glass/zirconia beads, and lysed with a reciprocating shaker (BioSpec). Final steps of RNA extraction and purification were carried out using a RiboPure-bacteria RNA purification kit (Ambion/Applied Biosystems, Austin, TX) as described in the manufacturer's insert. To remove residual DNA, RNAs were treated with RNase-free DNase I, provided in the kit.

For real-time RT-PCR, 1 μg each of RNA was used to generate cDNA via reverse transcription using a Roche first-strand transcriptor kit (Roche, Mannheim, Germany). Using a SYBR green I master kit (Roche, Mannheim, Germany), cDNAs were analyzed and quantified in a LightCycler 480 instrument (Roche) according to the manufacturer's instructions, using gene-specific primers. The list of primers is available from the authors upon request. Data were reported as *agrB* and RNAIII expression levels normalized to those of *gyrB*.

EMSA analysis. The *agr* promoter fragment (102 bp), generated by PCR, was labeled with [γ-^{32}P]ATP (Perkin Elmer) by end labeling one of the PCR primers using T4 polynucleotide kinase (NEB) prior to PCR. The PCR product was then resolved in a 6% nondenaturing gel and purified using an Elutip-d column (Schleicher and Schuell). Binding reaction was carried out by mixing the labeled probe (~10,000 cpm) with increasing concentrations of the protein and incubating for 20 min at room temperature. For displacement analysis, the labeled probe was allowed to bind initially either SarA or SarR prior to the addition of different concentrations of the competing protein and incubated for another 20 min. Complexes were resolved in a denaturing 6% urea-PAGE gel, and images were scanned using Molecular Dynamics Typhoon 8600 (GE Healthcare).

DNA bending assays. Hybridized oligonucleotides containing the SarA and SarR binding sites in the *agr* promoter or the AgrA P2 tandem site were cloned into the SacI-BglII site of the bending vector pCY7 (31) to yield plasmids pAM1163 and pAM1847, respectively. Plasmids were digested with EcoRI, HindIII, BstNI, EcoRV, NheI, and BamHI, and the fragments were purified and labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. Labeled fragments (~5,000 cpm) were then mixed with and without SarA (0.5 μmol), SarR (0.5 μmol), or AgrA (25 nmol) in binding buffer containing 25 mM Tris (pH 7.5), 0.1 mM EDTA, 75 mM NaCl, 10% glycerol, 1 mM dithiothreitol (DTT), and 0.5 μg of sonicated calf thymus at room temperature for 20 min. The reaction mixtures were then resolved in a 5% acrylamide gel, dried, and autoradiographed.

RESULTS

RNAII transcription in *agrA*, *sarA*, and *sarR* mutant strains. Previous studies on transcriptional control of the *agr* locus in *S. aureus* revealed that AgrA, SarA, and SarR likely play important roles in regulating RNAII and RNAIII transcription. However, the detailed events on how these proteins exert control on the *agr* P2 and P3 promoters (yielding RNAII and RNAIII, respectively) and subsequently on downstream effector genes are not well understood. To address this question, we constructed, using pMAD (1), single, double, and triple mutants of *agrA*, *sarA*, and *sarR* in strain SH1000, a derivative of strain 8325-4 with restored *rsbU* activity (15), by deleting the respective coding regions without any antibiotic marker and leaving the promoter and the transcription termination signal intact. As for the *agrA* mutant, we introduced nonsense mutations into Lys and Ile at positions 2 and 3 in the AgrA coding region. We then analyzed by quantitative real-time RT-PCR,

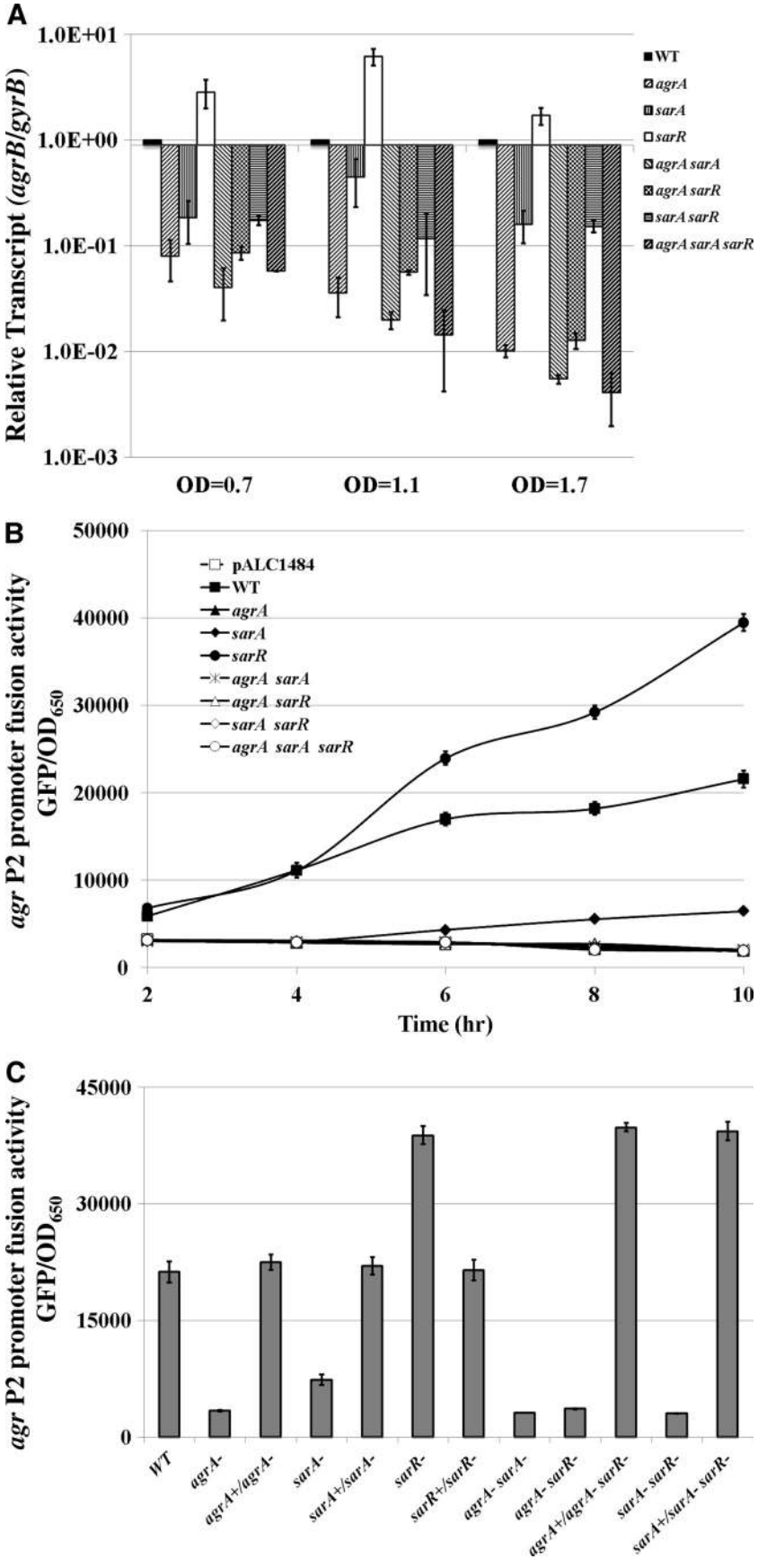
using *agrB* and the sequence coding RNAIII as probes, the transcription of RNAII and RNAIII in TSB-grown cells during exponential, late exponential, and stationary phases (OD_{650} of 0.7, 1.1, and 1.7, respectively, as determined with 18-mm borosilicate glass tubes in a Spectronic 20D+ spectrophotometer [Garforth, England]). As illustrated in Fig. 2A, the deletion of *agrA* or *sarA* alone resulted in a significant decrease in RNAII transcription in all three growth phases compared with that of the parent. However, the effect was more dramatic with the *agrA* mutant than with the *sarA* mutant, especially during the late exponential and post-exponential phases (OD_{650} of 1.1 and 1.7). This finding is consistent with the notion that AgrA is an important factor in expression from the *agr* P2 promoter. The fact that deletion of *sarA* alone did not render the *agr* P2 promoter completely inactive implies additional genetic requirement besides SarA for the optimal transcription from the P2 promoter (37). Notably, the downregulatory effect of the double *sarA agrA* mutation on RNAII transcription was similar to that of the single *agrA* mutant, suggesting that the major effect of *sarA* on RNAII expression is likely to be mediated via AgrA. Interestingly, deletion of *sarR* triggered an upregulation in RNAII transcription, with the effect more pronounced during the late exponential phase (OD_{650} of 1.1), thus suggesting that SarR acts as a repressor of the *agr* locus in the SH1000 background. However, strains bearing the *sarR* deletion in combination with the *agrA* and/or *sarA* deletions did not exhibit the aforementioned derepression, especially during the late exponential phase, in contrast to what has been found with the single *sarR* mutant, thus implying that derepression of RNAII in the *sarR* mutant strain is likely dependent on intact *agrA* and/or *sarA* loci.

We also confirmed these data by analyzing *agr* P2 promoter fusion with GFP_{uvr} (16). For the promoter fusion construct, we have cloned a 235-bp P2 promoter fragment encompassing the previously reported 4 putative AgrA boxes, the putative SarA and SarR binding sites, and the core promoter elements (Fig. 1) upstream of gfp_{uvr} in pALC1484. The recombinant plasmid was introduced into various isogenic mutants of SH1000. To minimize the variation in growth density, we normalized these data to fluorescence units/OD_{650} values. As shown in Fig. 2B, the activity of the *agr* P2 promoter was elevated in the *sarR* mutant but was reduced in the isogenic *sarA* mutant compared with that of the parental strain. In contrast, the *agrA* mutant, the double *agrA sarA*, *agrA sarR*, and *sarA sarR* mutants, as well as the triple mutant, all exhibited low levels of *agr* P2 promoter activity, similar to that of the empty vector control.

To verify these data, we complemented *agrA*, *sarA*, and *sarR* single mutants as well as the double *agrA sarR* and *sarA sarR* mutants by replacing the deleted gene with a native copy using pMAD and analyzing the ensuing *agr* P2 promoter activity with GFP-mediated fluorescence. Our data showed that the proportionately reduced P2 promoter activity levels in *agrA* and *sarA* mutants were restored to wild-type levels upon complementation, while that of the *sarR* mutant was reduced to the parental level (Fig. 2C). More importantly, both the *agrA sarR* and *sarA sarR* double mutants, upon respective complementation with *agrA* and *sarA*, restored *agr* P2 promoter activity level to that of the *sarR* mutant, at a level much higher than the SH1000 parental strain. Taken together, the above results concur with those of the quantitative RT-PCR data, strongly suggesting that AgrA and, to a lesser extent, SarA activate *agr* RNAII transcription, whereas SarR acts as a repressor, exerting a greater impact on the later stage of growth. It should be clarified that our data here on RNAII expression with the *sarR* mutant of SH1000 ($rsbU^+$) differed from that reported for RN6390 (*rsbU* mutant), where the *sarR* mutant exhibited slightly lower RNAII expression than the isogenic parent (24). Indeed, a direct comparison of P2 promoter activities using promoter DNA-reporter fusions confirmed the discrepancy in RNAII expression between *sarR* mutants of RN6390 and SH1000 (data not shown). We speculate that reduced SigB activity in strain RN6390 may conceivably impact RNAII expression and hence becomes a contributing factor to slightly lower *agr* P2 activity in the *sarR* mutant in RN6390. In contrast to RN6390, we have repeatedly found that RNAII transcription in strain SH1000 was consistently elevated in the *sarR* mutant compared with that of the isogenic parent.

SarR-mediated repression impacts the *agr* P2 but not the *agr* P3 promoter. We also examined the effect of AgrA, SarA, and SarR on the *agr* P3 promoter driving transcription of RNAIII, which is the *agr* effector molecule (27). As with the *agr* P2 promoter fusion constructs, the 219-bp *agr* P3 promoter fragment encompassing the AgrA, SarA, and SarR binding sites was fused upstream of the GFP_{uvr} reporter gene in pALC1484; the recombinant plasmid was then introduced into various mutant strains of SH1000. Surprisingly, a mutation in *sarR*, unlike the effect on the *agr* P2 promoter, did not lead to any significant increase in *agr* P3 promoter activity compared with that of the parent (Fig. 3A). A deletion of *sarA* resulted in a notable decrease in *agr* P3 promoter activity in comparison to the parent, whereas an *agrA* mutation rendered the same promoter completely inactive. Reconstitution of the mutated *sarA* or *agrA* gene with a wild-type copy in the chromosome restored P3 promoter activity to the wild-type level, while complementation of the *sarR* mutant at the chromosomal level had no effect, as determined by GFP-mediated fluorescence (Fig. 3B). When *agrA* and *sarA* were restored in the respective *agrA sarR* and *sarA sarR* double mutants, the *agr* P3 promoter exhibited wild-type promoter activity similar to that found in the *sarR* mutant, strongly implying that a deletion in *sarR* has no direct role in influencing RNAIII transcription. RT-PCR of RNAIII expression in these mutants and complemented mutants also supported results of the promotion fusion studies (data not shown). Collectively, these results clearly imply that AgrA positively regulates both *agr* P2 and P3 promoters, whereas SarR impacts on P2 but not P3 transcription. While SarA also upregulates the P2 promoter, it is likely that the effect on the *agr* P3 promoter may be indirect (i.e., via upregulation of RNAII and subsequently AgrA), because the double *sarA agr* mutant, similar to the single *agr* mutant, had little, if any, P3 promoter activity.

The effect of AgrA box and SarA/SarR binding sites on *agr* P2 and P3 transcription. To establish the crucial promoter elements relevant to *agr* transcription, we focused on four AgrA promoter boxes to which AgrA binds (32) as well as the *sarA* and *sarR* binding sites shown to be important for *agr* transcription (Fig. 1) (8, 24). As AgrA exists as a dimer (32) and hence binds to two tandem AgrA promoter boxes (18), we introduced transversion mutations on individual AgrA boxes within each of the tandem AgrA boxes as well as truncation


A
Relative Transcript (agrB/gyrB)
1.0E+01
1.0E+00
1.0E-01
1.0E-02
1.0E-03
OD=0.7
OD=1.1
OD=1.7
WT
agrA
sarA
sarR
agrA sarA
agrA sarR
sarA sarR
agrA sarA sarR
B
agr P2 promoter fusion activity GFP/OD650
50000
40000
30000
20000
10000
0
2
4
6
8
10
Time (hr)
pALC1484
WT
agrA
sarA
sarR
agrA sarA
agrA sarR
sarA sarR
agrA sarA sarR
C
agr P2 promoter fusion activity GFP/OD650
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30000
15000
0
WT
agrA-
agrA+/agrA-
sarA-
sarA+/sarA-
sarR-
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sarA- sarR-
sarA+/sarA- sarR-

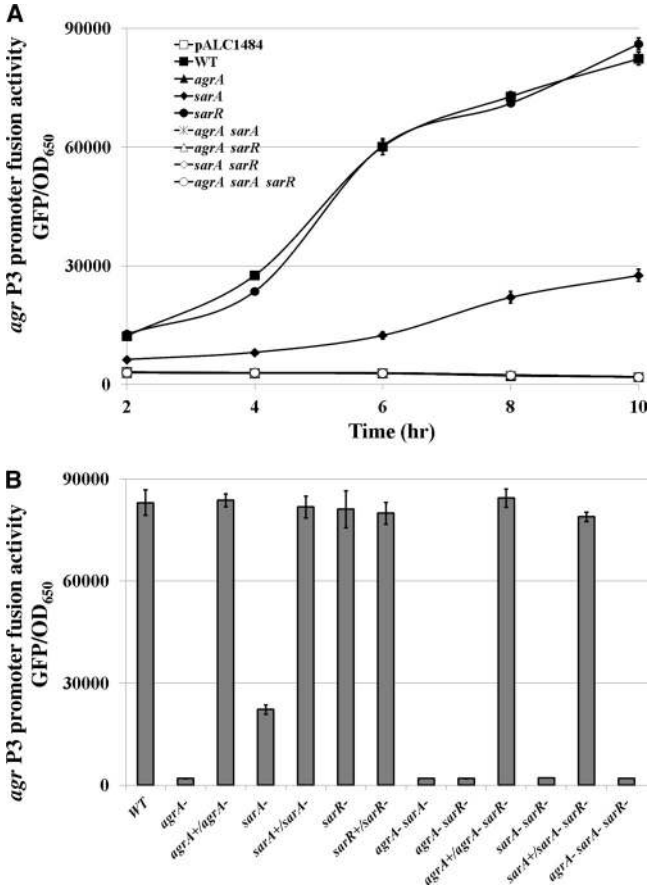
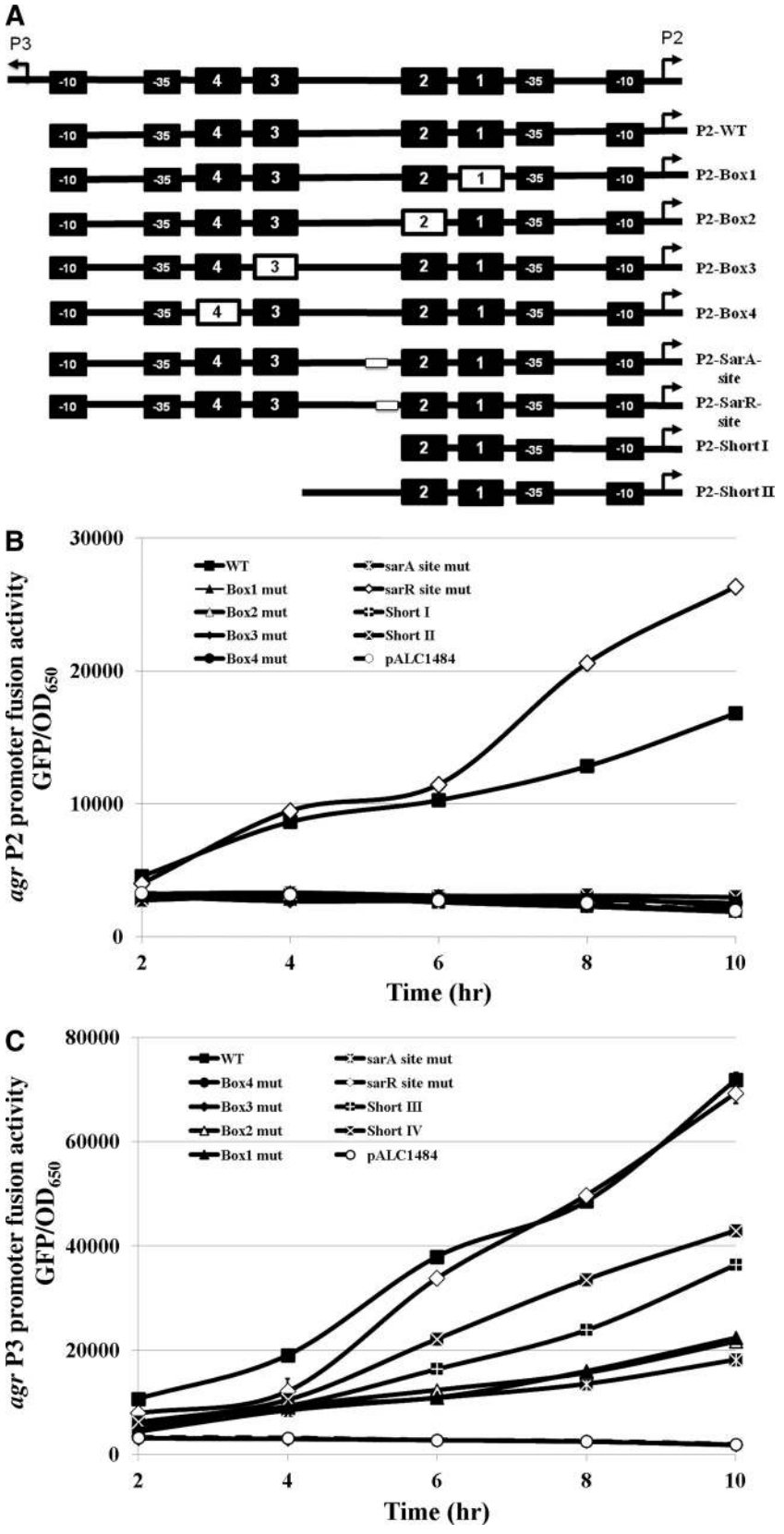


FIG. 3. Promoter fusion analysis of the *agr* P3 promoter in assorted *agrA*, *sarA*, and *sarR* mutants during growth. (A) The 219-bp *agr* P3 promoter was fused to the GFP_{uvr} reporter gene in pALC1484 in assorted mutants. The data are presented as fluorescence units/OD_{650} values. The results are presented as the means from three independent experiments, with the error bars representing standard deviations. (B) Promoter GFP fusion of the *agr* P3 promoter in selected complemented mutants during the postexponential phase. The data are given as mean fluorescence units/OD_{650} values. The experiments have been repeated at least three times, with the data presented as the means from three independent experiments. The error bars represent standard deviations.

and transition mutations to the putative SarA and SarR binding sites between the P2 and P3 promoters (Fig. 4A). These mutated and truncated promoters were then used to generate modified *agr* P2 or P3 GFP reporter fusions in pALC1484 followed by introduction of these recombinant plasmids into parental strain SH1000. We found that transversion mutations to one of the proximal (box 1 or box 2) or distal (box 3 or box 4) AgrA boxes resulted in almost complete abolishment of the *agr* P2 promoter activity, similar to what has been found with the vector alone (Fig. 4B). A similar trend on the *agr* P2 promoter activity was also observed with truncation of the putative SarA/SarR binding sites and the distal AgrA promoter boxes, while the proximal AgrA boxes (short I) were left intact. The transition mutation of the SarA binding sites (P2-SarA site) also exhibited significantly reduced *agr* P2 activity, while that of the SarR binding site mutation (P2-SarR site) led to a higher level of *agr* P2 promoter activity than that of the parent (Fig. 4B). Deletion of the distal AgrA promoter boxes (boxes 3 and 4 [short II]) also has a profound effect on diminishing the *agr* P2 promoter activity. Collectively, these data suggest that the regulatory control of the *agr* P2 promoter is more stringent in that it requires multiple regulatory promoter elements, including AgrA and SarA binding sites, to exhibit optimal *agr* P2 promoter activity.

We also studied the effects of these mutations on *agr* P3 promoter activity as determined by GFP fusions in strain SH1000 (Fig. 4C). As has been reported previously (2, 4, 26), the *agr* P3 promoter activity was significantly higher than the *agr* P2 counterpart (Fig. 4C versus B). Mutation in one of the proximal AgrA boxes (box 4 or box 3) resulted in an inactive promoter, whereas a similar mutation in one of the distal AgrA promoter boxes (box 2 or box 1) led to a moderate reduction in *agr* P3 promoter activity compared with that of the parental strain. Interestingly, elimination of both distal AgrA boxes (boxes 1 and 2) as seen in the short IV promoter fragment resulted in a reduction in *agr* P3 promoter activity, while mutations in one of the distal AgrA boxes (box 2 or box 1) also exhibited reduced *agr* P3 activity, but this activity level was still higher than that of the *agr* mutant with the vector control. Truncation of the SarA/SarR binding sites and the distal tandem AgrA promoter boxes (short III) rendered the P3 promoter less active than the short IV promoter fragment encompassing the SarA/SarR binding site but closer in activity to the box 1 or box 2 mutant, thus indicative of the importance of the SarA binding site in the presence of proximal tandem AgrA boxes (short III versus short IV). Transition mutation of the SarR binding site led to a pattern of P3 promoter activity similar to that of the wild type, quite different from the effect of this site on the P2 promoter (Fig. 4B and C). In contrast, the transition mutation of the SarA binding site resulted in lower P3 activity. Taken together, these results strongly suggest that during the late stage of growth, active transcription of RNAIII tends to overcome the requirement for SarA and is likely dependent solely on AgrA activity, as evidenced by the absence

FIG. 2. Transcriptional analysis of the *agr* P2 promoter in assorted *agrA*, *sarA*, and *sarR* mutants during growth. (A) Real-time RT-PCR analysis of the RNAII transcript during exponential (OD_{650} = 0.7), late exponential (OD_{650} = 1.1), and post-exponential (OD_{650} = 1.7) phases. Transcription levels of the mutants are normalized to the *gyrB* transcript and are reported relative to transcriptions in the parental strain SH1000 (set to 1.0 at each of the ODs) at the identical ODs. The data are presented as the averages from three independent, real-time RT-PCR experiments. The error bars represent standard deviations. (B) Transcriptional fusions of the 235-bp *agr* P2 promoter fused to the GFP_{uvr} reporter gene in pALC1484 in assorted mutants. The data are presented as fluorescence units (obtained at multiple time points) divided by OD_{650} values as described in Materials and Methods. The error bars represent standard deviations. (C) Promoter GFP fusion of the *agr* P2 promoter in selected complemented mutants during the post-exponential phase. The data are given as mean fluorescence units/OD_{650} values obtained from triplicate samples. The experiments have been repeated at least three times, with the data presented as the means from three independent experiments. The error bars represent standard deviations.


A
P3
P2
-10
-35
4
3
2
1
P2-WT
P2-Box1
P2-Box2
P2-Box3
P2-Box4
P2-SarA-site
P2-SarR-site
P2-Short I
P2-Short II
B
agr P2 promoter fusion activity GFP/OD650
WT
Box1 mut
Box2 mut
Box3 mut
Box4 mut
sarA site mut
sarR site mut
Short I
Short II
pALC1484
Time (hr)
C
agr P3 promoter fusion activity GFP/OD650
WT
Box4 mut
Box3 mut
Box2 mut
Box1 mut
sarA site mut
sarR site mut
Short III
Short IV
pALC1484
Time (hr)

of *agr* P3 promoter activity when the proximal AgrA box (box 3 or box 4) was mutated, even with the SarA binding site intact.

The binding affinity of SarR and SarA to the *agr* promoter element. In previous studies, we have shown that the SarA protein level reached its peak during the late exponential phase and tapered toward the post-exponential phase (10) (see Fig. SA in the supplemental material). In contrast, SarR protein expression tended to peak postexponentially, which we implicated to be involved in the repression of *sarA* transcription during the later stage of bacterial growth (23). Given this discrepancy in peak expression profiles, we consider the plausibility that SarA may act early on the *agr* P2 promoter during the exponential phase to increase AgrA expression, while SarR could downregulate the *agr* P2 promoter postexponentially. A corollary for this scheme is that SarR must have a higher binding affinity than SarA to the *agr* promoter, thus enabling displacement of SarA from the *agr* P2 promoter as the intracellular concentration of SarR increases. Accordingly, we conducted gel shift assays of purified SarA or SarR with a radiolabeled 102-bp *agr* promoter that encompasses the four AgrA boxes and the SarA/SarR binding sites. As shown in Fig. 5A, the gel shift pattern of SarR differed from that of SarA, with multiple retarded complexes consistent with dimer-dimer interactions (20, 24). Additionally, the K_d (dissociation constant) of SarR to the *agr* promoter, as determined by the concentration of protein that shifted 50% of the probe, is ~50 nM, much lower than the K_d of SarA at ~200 nM. This implies higher SarR binding affinity to the *agr* promoter than SarA. To determine if SarR can efficiently displace SarA from the *agr* promoter fragment, we preincubated the radiolabeled *agr* promoter fragment with SarA at a concentration close to the K_d (150 nM) at room temperature for 20 min, followed by the addition of increasing concentrations of SarR. As displayed in Fig. 5B (left), the addition of 25 nM SarR to the reaction mixture of SarA and the *agr* promoter fragment was able to alter the gel shift pattern from that of SarA to SarR (see Fig. 5A). We also found that SarA could displace SarR from the *agr* promoter in a similar fashion; however, this dislodgement of SarR required a much higher concentration of SarA, beginning at ~150 nM (Fig. 5B, right). These data are consistent with the notion that SarR binds with higher affinity to the *agr* promoter than SarA and in all likelihood efficiently displaces SarA from the *agr* promoter during the post-exponential phase, at a time when the concentration of SarR is highest (23).

***In vitro* runoff transcription from the *agr* P2 promoter with AgrA, SarA, and SarR.** To assess the effect of AgrA, SarA, and SarR on *agr* transcription *in vitro*, we first purified RNA polymerase (RNAP) from the triple *agrA sarA sarR* mutant of SH1000 to eliminate any potential contamination of the core RNAP with the above three transcription factors. Core RNAP

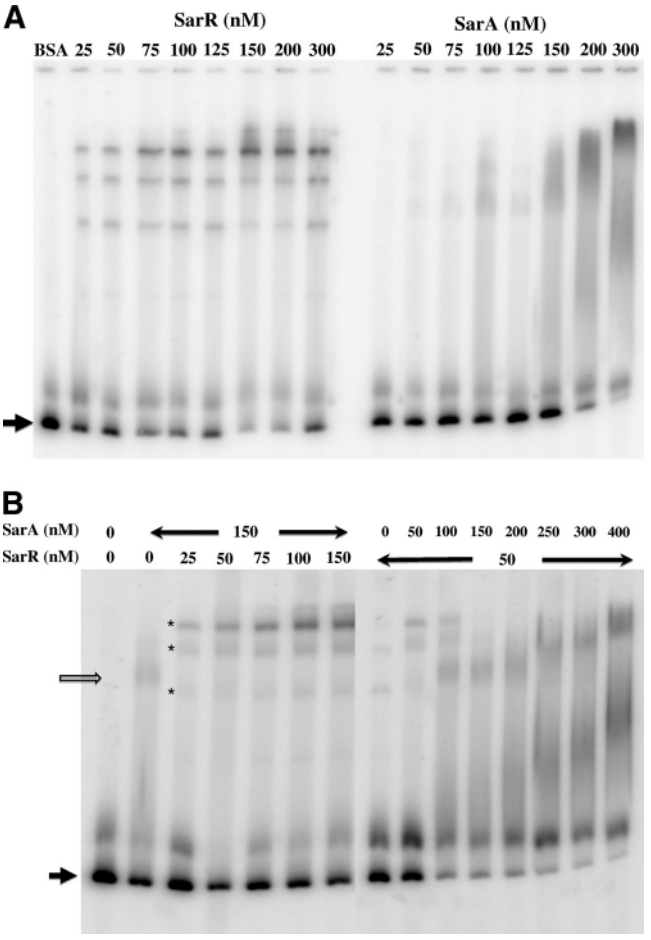


FIG. 5. Gel shift assays (EMSA) of the *agr* promoter with SarA and SarR. (A) EMSA with an end-labeled *agr* promoter fragment (10,000 cpm) in increasing concentrations of SarA or SarR. The *agr* promoter fragment is 102 bp in length and comprises four AgrA boxes together with the intervening sequence that contains the overlapping SarA/SarR binding sites. The bold arrow points to the unbound *agr* probe. (B, left) EMSA of the end-labeled 102-bp *agr* promoter fragment (10,000 cpm) preincubated with 150 nM SarA for 20 min at room temperature followed by the addition of increasing concentrations of SarR; (right) EMSA of the *agr* promoter fragment preincubated with 50 nM SarR for 20 min at room temperature followed by the addition of increasing concentrations of SarA. The filled arrow indicates unbound *agr* probe, while the empty arrow highlights the SarA promoter complex; the three asterisks designate SarR-promoter complexes. Multiple complexes of SarR with the *agr* promoter have been seen in gel shift assays (23), presumably due to SarR dimer-dimer interactions. EMSA of SarA and SarR with the control promoter fragment can be found in Fig. SB in the supplemental material.

FIG. 4. The roles of AgrA promoter boxes and SarA/SarR binding sites in transcription from the *agr* P2 and P3 promoters in strain SH1000 as determined by transcriptional fusions. (A) Schematics of the mutations and truncation of the *agr* P2 promoters in pALC1484 in strain SH1000. An analogous scheme of the mutated and truncated P3 promoters was also constructed, except that the P3 promoter replaces the P2 promoter, with the elements in reverse orientation (see Fig. SC in the supplemental material). In addition, the P3-short III is analogous to P2-short I, and P3-short IV is similar to P2-short II. (B) Transcriptional fusions of the native, mutated, and truncated *agr* P2 promoter linked to GFP_{uvr} in pALC1484 in SH1000. (C) Transcriptional fusions of the native, mutated, and truncated *agr* P3 promoter linked to GFP_{uvr} in pALC1484 in SH1000. The data in panels B and C are presented as the mean results from three independent experiments. The error bars represent standard deviations.

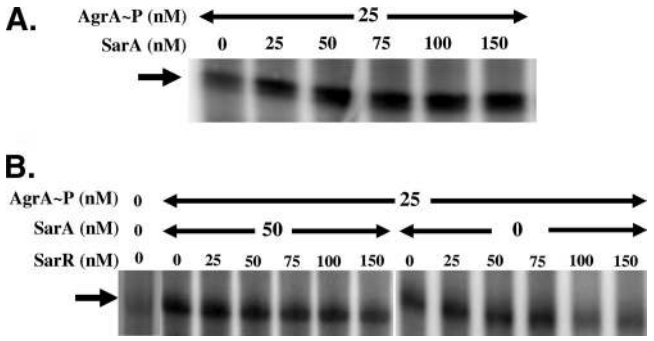


FIG. 6. *In vitro* runoff transcription assays of the *agr* P2 promoter with AgrA, SarA, and SarR. (A) The *agr* P2 promoter fragment was incubated with AgrA (25 nM in acetyl phosphate) in the absence or presence of SarA at increasing concentrations for 10 min at room temperature. The amount of AgrA used in this assay was determined by prior titration, showing increased *agr* P2 transcription with rising concentrations of AgrA. RNAP, nucleotides, and [α-^{32}P]UTP were added to the above reaction mixtures and allowed to incubate at 37°C for 15 min, followed by the addition of stop solution as described in Materials and Methods. The reaction mixtures were then resolved in 8% urea-PAGE gels, which were then dried and exposed to a phosphorimaging cassette. A single *agr* P2 transcript of the expected size was generated with this assay. (B) Runoff transcription assay of the *agr* P2 promoter with acetyl phosphate-treated AgrA with SarR and SarA (left) or with SarR (right).

was purified with a nickel column from the cell lysate of the triple mutant containing a chromosomal copy of *rpoC* bearing a C-terminal His tag as described in the experimental procedures. SDS-PAGE analysis had confirmed the purity of the core RNAP. Runoff transcription assays were then conducted with a linearized DNA template of the *agr* P2 promoter containing four AgrA boxes and the SarA/SarR binding sites. As shown in Fig. 6A, AgrA alone, preincubated with 1 mM acetyl phosphate, could promote transcription from the *agr* P2 promoter, whereas its absence resulted in a very low level of transcription (Fig. 6B, far left lane). The addition of increasing concentrations of SarA to a fixed amount of AgrA resulted in an increase in *agr* P2 transcription (Fig. 6A), thus indicating that SarA can augment P2 transcription in the presence of AgrA, whereas SarA alone led to a mild repression in *agr* P2 transcription (data not shown). Of interest, transcription from the *agr* P2 promoter was inhibited by rising concentrations of SarR, even in the presence of AgrA (Fig. 6B, right). More importantly, P2 transcription, even in the presence of both AgrA and SarA, was significantly inhibited by SarR in a dose-dependent fashion (Fig. 6B, left).

DNA bending assays with SarA, SarR, and AgrA. One possibility by which SarA and/or SarR could modulate *agr* transcription is with DNA bending (20, 21). Accordingly, recombinant pCY7, the bending vector (29) containing the fragment with the SarA/SarR binding sites, was digested with EcoRI, HindIII, BstNI, EcoRV, NheI, and BamHI to release the insert (Fig. 7A). All fragments, 432 bp in length, were then end labeled with [γ-^{32}P]ATP and evaluated by EMSA with SarA or SarR (0.5 μM). As shown in Fig. 7B, SarA was able to bend the *agr* promoter fragment, with the mobility of the *agr* promoter element being more retarded when the bend occurs at the center than at the edge of the fragment. The relative mobility of the complex with SarA bound in the middle (μ_M) and near

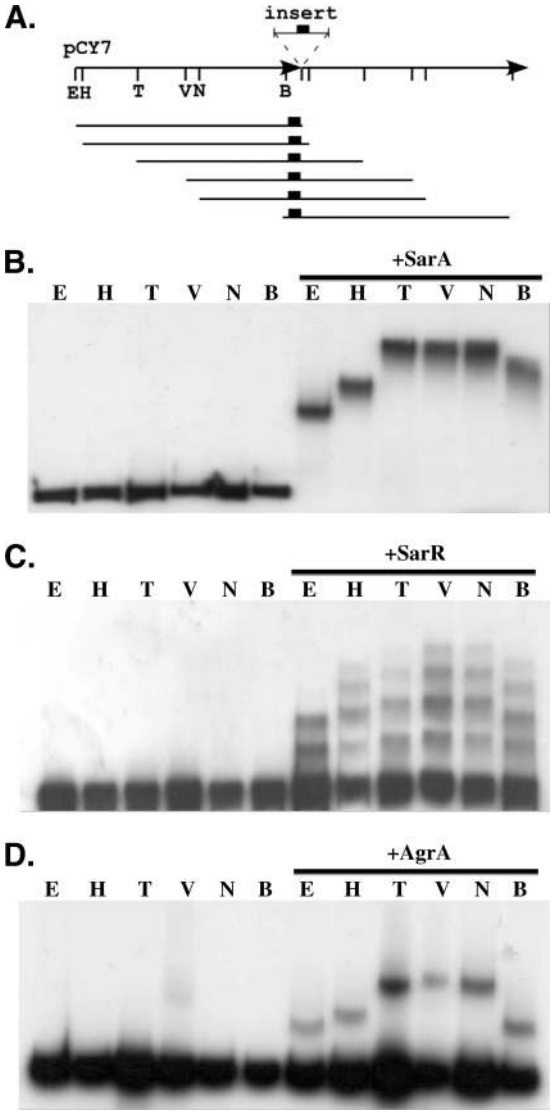


FIG. 7. DNA bending assays of the various *agr* promoter fragments encompassing SarA/SarR or AgrA P2 tandem sites. (A) The bending vector pCY7 (31) containing a duplicated region (arrows) separated by a multicloning site into which paired oligonucleotides were cloned (see Materials and Methods). Digestion of the recombinant vector with various restriction enzymes results in fragments of identical lengths but with the insert permutated with respect to the fragment ends. (B) Bending assay performed with 0.5 nmol SarA and purified 5′ end-labeled promoter fragments derived from pAM1163 digested with EcoRI (E), HindIII (H), BstNI (T), EcoRV (V), NheI (N), or BamHI (B). (C) Bending assay performed with 0.5 nmol SarR using the same fragments as those described in panel A. Depending upon SarR concentration, EMSA of the *agr* promoter fragment with SarR can yield three or four complexes, akin to what has been described in Fig. 5B; however, the relative mobility of a specific complex was little changed across the spectrum of restriction digests. (D) Bending assay performed with 25 nmol AgrA on the purified 5′ end-labeled fragments derived from pAM1847 containing the AgrA tandem P2 site. The addition of acetyl phosphate did not detectably alter the relative mobility of the AgrA-promoter complex. Protein concentrations used in each of the above-described assays (panels B to D) were determined using pilot titrations.

the edge of the DNA fragment (μ_E) was determined and used to calculate the bending angle (α) based on the following equation: $\mu_M/\mu_E = \cos(\alpha/2)$ (38). With this method, we determined the bending angle of the *agr* promoter to be ~79° when SarA is bound. In contrast to SarA, there was little or no bending of the *agr* promoter fragment containing the SarA/SarR binding site with SarR (Fig. 7C), even at a concentration of SarR that was 5-fold higher than that of SarA (data not shown). We also determined the bending effect of AgrA on the proximal tandem AgrA boxes of the *agr* P2 promoter (Fig. 7D). In this scenario, AgrA also induced bending of the *agr* P2 fragment containing the proximal AgrA boxes, with the bending angle estimated to be ~81°. The addition of acetyl phosphate to AgrA prior to incubation with the promoter fragment did not alter the bending activity, thus implying that the effect of phosphorylated AgrA is likely to activate the transcription complex rather than altering the bending angle of the *agr* P2 promoter DNA.

DISCUSSION

Some of the well-described intracellular signaling molecules that bind the *agr* promoter are AgrA, SarA, and SarR (25, 26). AgrA, once activated via phosphorylation, binds as a dimer to each of two 9-bp direct repeats within the P2-P3 promoter region (see Fig. 1) (18, 32). SarA and SarR, on the other hand, are dimers (20, 21) that recognize and bind to an overlapping site between two AgrA binding direct repeats (Fig. 1) (9, 24). To understand how these three signaling molecules interact at the *agr* promoter, we undertook the current study to evaluate the individual and combined effects of AgrA, SarA, and SarR on *agr* P2 and P3 promoter activities.

To avoid any issues with polar effects and to differentiate this study from prior studies, we have constructed all of our mutants in SH1000 using pMAD (1), carefully deleting only the coding regions without any antibiotic marker replacement and leaving the promoters and transcription termination signals intact. The use of SH1000, a strain with a restored copy of *rsbU* and hence an intact *sigB* operon, is notable in this study, because previous studies on *agr* regulation by *agrA*, *sarA*, and *sarR* have been conducted with strains 8325-4 or RN6390, both of which are defective in *rsbU*. In addition, various complemented mutants in this study were constructed by replacing the deleted gene with a wild-type copy on the chromosome. We have also verified by Northern blots that truncated transcripts of appropriate sizes were transcribed in the mutants. Using *agrA*, *sarA*, *sarR*, double and triple mutants, and complemented mutants in the SH1000 background, we have unequivocally shown that AgrA is absolutely required for *agr* P2 and P3 transcription. In distinction to previous data, we demonstrated here that the transcriptional regulators SarR and SarA primarily affect the *agr* P2 promoter to alter RNAII transcription (Fig. 2 and 3). As RNAII encodes AgrA, which binds to the AgrA boxes to influence P3 transcription (18, 32), it is likely that the effect of SarA and SarR on the P3 promoter is indirect and is mediated via AgrA encoded by RNAII. This was confirmed by promoter fusion studies of the *agrA sarR* mutant wherein the double mutant exhibited little P2 and P3 activity, similar to that of the *agrA* mutant (Fig. 2 and 3). Additionally, the *sarA agr* mutant also displayed an almost negligible level of P2 and P3 promoter activities, whereas the single *sarA* mutant exhibited an intermediate level of P2 and P3 transcription between the parent and the *sarA agr* double mutant. Restoration of *agrA* to the *agrA sarR* double mutant and *sarA* to the *sarA sarR* double mutant returned *agr* P2 transcription to the *sarR* mutant level, which was much higher than that of the parental strain (Fig. 2C). In contrast to the *agr* P2 transcription, reconstitution of *agrA* to the *agrA sarR* double mutant returned the P3 promoter activity only to the wild-type level (Fig. 3B), thus implicating AgrA, but not SarR, to be the crucial element in *agr* P3 transcription. Accordingly, the major effect of AgrA on RNAII and RNAIII promoters and the salient effect of SarA and SarR on RNAII but not the RNAIII promoter are prominent features of *agr* regulation in *S. aureus.*

Previous studies have identified two sets of direct repeats within the *agr* P2-P3 promoter region where AgrA binds (18). Nestled between the two sets of direct repeats are the palindrome and the overlapping inverted repeats to which winged helix structures such as SarA and SarR bind, respectively (Fig. 1) (20, 21, 31). To dissect the contribution of these binding sites to *agr* P2 and P3 transcription, we have mutated the AgrA boxes and truncated and mutated the SarA/SarR binding site of an *agr* promoter fragment on a plasmid carrying the GFP_{uvr} fusion in strain SH1000 (Fig. 4A). Our data clearly showed distinct P2 and P3 activation profiles in response to these mutations in the AgrA boxes. In particular, mutations of the proximal (box 1 or box 2) and distal (box 3 or box 4) AgrA boxes showed that all of the AgrA boxes where activated AgrA binds are essential to transcription from the *agr* P2 promoter. Mutation of the SarA binding site also reduced the P2 promoter activity to a very low level, while a similar mutation in the SarR binding site elevated P2 promoter activity to a degree higher than that of the parental strain (Fig. 4B). As can be observed in the *sarR sarA* mutant, this effect of *sarR* on the *agr* P2 promoter is dependent on an intact *agrA* (Fig. 2B and C). The scenario with the *agr* P3 promoter is very much different, with the proximal AgrA box (box 3 or box 4) being more important than the distal box (box 2 or box 1) in *agr* P3 transcription (Fig. 4C). These data are in agreement with our assertion that AgrA is the major effector of *agr* P3 transcription.

Our results with *agrA*, *sarA*, and *sarR* mutants, coupled with those of the mutated AgrA boxes, reinforced the notion that the *agr* P2 and P3 promoters are regulated differentially, with AgrA being the critical element in P3 transcription and SarA and SarR, in conjunction with AgrA, being important to P2 transcription. This notion of differential regulation of *agr* P2 and P3 promoters was also supported by promoter activation studies in the rabbit endocarditis model, where P3 promoter activation appears to differ from P2 activation (37).

We have previously found that SarA and SarR protein expression varies during growth, with SarA expressed maximally during the late exponential phase (10) and SarR peaking postexponentially (23). The growth-phase-dependent expression of SarA described here differed from the data of Fujimoto et al., wherein they described SarA as being constant during all stages of growth (12). This discrepancy may be due to their use of rabbit anti-SarA polyclonal antibodies, which may have other native anti-*S. aureus* antibodies; in contrast, our Western blot was developed with a murine anti-SarA monoclonal anti-

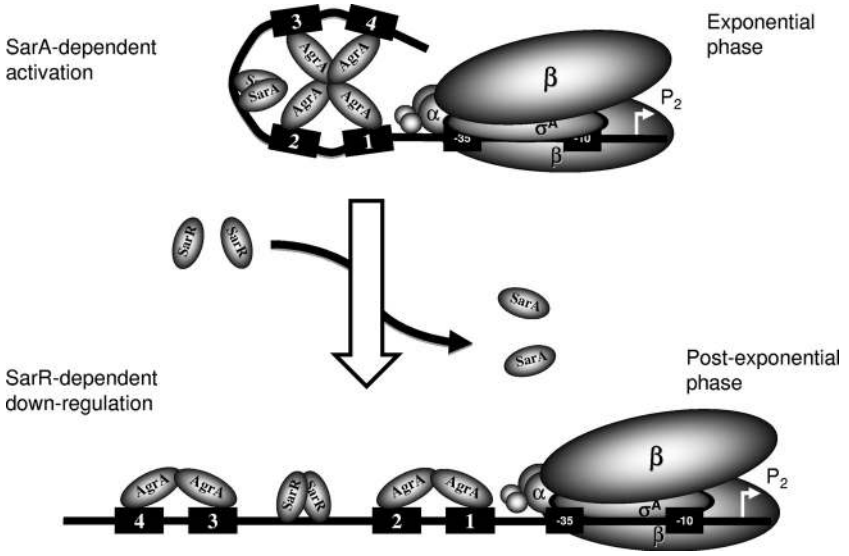


FIG. 8. A proposed model for the regulation of the *agr* P2 promoter by AgrA, SarA, and SarR. For the P2 promoter, the major transcription factor is the AgrA dimer, which binds to the tandem AgrA boxes to induce ~80° bending (Fig. 7). Binding of SarA will introduce additional bending (~80° by SarA alone) to that induced by AgrA to allow interaction of two AgrA dimers anchored at two tandem AgrA boxes, thus enhancing efficient recruitment of RNAP to initiate transcription. SarR binds with a higher affinity than SarA to an overlapping site on the *agr* promoter site. Accordingly, SarR, upon accumulation during the transition from late exponential to post-exponential phase, displaces SarA from the *agr* promoter. However, in contrast to SarA, SarR binds but does not bend significantly, thus precluding more active engagement of RNAP. As a result, there is downregulation of transcription from the *agr* P2 promoter due to SarR. In the case of the *agr* P3 promoter, the sole and required transcription factor is AgrA. SarA and SarR can contribute to this effect by modulating *agrA* expression via activation or repression of RNAII.

body (see the supplemental data). As SarR and SarA bind to overlapping sequences on the *agr* promoter, we conceptualized that SarR may have a higher binding affinity to the *agr* promoter than SarA, thus allowing displacement of SarA from the *agr* promoter in transition from the late exponential phase to the post-exponential phase. For EMSA analysis with the *agr* P2 promoter, we have elected to use the unphosphorylated form of SarA, because we have found that a *pknB* mutant of SH1000 exhibited a higher level of RNAII expression than the parent (35), thus implying that the unphosphorylated form of SarA may be more active on the *agr* P2 promoter than the phosphorylated counterpart. Using these proteins, we have found that SarR indeed has a lower K_d for the *agr* P2 promoter than SarA, thus implying higher binding affinity of SarR to the *agr* promoter than that of SarA. Competition assays with SarR added to a preincubated mixture of SarA with an *agr* promoter fragment also validated successful displacement of SarA by SarR at ~50 nM, while the corresponding assay with SarA displacing SarR necessitated a much higher concentration of ~150 nM (Fig. 5B).

In contrast to *agr* P3 transcription, we have shown that both SarA and SarR required AgrA to exert its activation and repression of the *agr* P2 promoter, respectively. To validate this finding, we purified core RNAP from a triple *agrA sarA sarR* mutant of SH1000 to steer clear of any prior contamination of RNAP with these three transcription factors. Using this RNAP, we found that acetyl phosphate-treated AgrA can activate transcription from the *agr* P2 promoter (Fig. 6). This activation can be further enhanced by SarA in a dose-dependent fashion. Importantly, this finding contrasts with the data from Chakrabarti and Misra, who reported that SarA represses *agr* P2 transcription (3). This discrepancy can be explained by the fact that SarA likely activates *agr* P2 transcription only in the presence of AgrA. In the absence of AgrA, we also found that SarA can repress *agr* P2 transcription in a dose-dependent fashion. Interestingly, SarR can repress *agr* P2 transcription in the presence of AgrA alone or a combination of AgrA and SarA (Fig. 6B). These data, together with those from the *sarR* mutant, highlighted the repressive role of SarR in the presence of AgrA and SarA under conditions that more likely reflect the scenario *in vivo*.

We have also conducted the *agr* promoter bending assay with these proteins. Interestingly, SarA, but not SarR, was able to induce bending of the *agr* promoter fragment comprising the SarA/SarR binding sites (Fig. 7), with the estimated bending angle of the *agr* promoter fragment induced by SarA alone to be ~80°. We also observed that AgrA could induce ~80° bending of the tandem AgrA boxes (box 1 and box 2) proximal to the *agr* P2 promoter. Notably, Sidote et al. observed in crystallization studies that the C-terminal DNA binding domain of AgrA, as a monomer, bends a single 9-bp *agrA* promoter fragment ~40° (32). Our data here suggest that AgrA, as a dimer, can provoke additional bending upon binding to the tandem repeat of AgrA boxes. Conceivably, induced bending of the *agr* P2 promoter comprising the tandem AgrA boxes and the SarA/SarR binding sites would be close to ~160° or higher, given the limits of approximation in these assays. These data, together with those from EMSA and *in vitro* transcription assays, suggest a model whereby SarA and SarR can modulate transcription from the *agr* P2 promoter via interaction with AgrA on the *agr* promoter (Fig. 8). AgrA dimer, by virtue of its binding to each of the tandem AgrA boxes to induce DNA bending (32), is the main driving force behind *agr* P2 and P3 transcription. While AgrA is the requisite factor for *agr* P3

transcription, the scenario with *agr* P2 transcription is quite different, with two AgrA dimers first binding to two sets of tandem AgrA boxes to induce bending of the *agr* promoter (Fig. 7) (18, 32) to facilitate P2 transcription. During the exponential phase, the SarA protein level increases (10), leading to binding of the SarA dimer to the SarA binding site situated between the two tandem AgrA boxes (21). Binding of SarA induces additional bending of the *agr* P2 promoter (Fig. 7 and 8), presumably more than that of AgrA alone, thus further enhancing transcriptional activation of the *agr* P2 promoter by RNA polymerase (Fig. 6A). We surmise that this enhancement may be due to additional interaction of the two AgrA dimers anchored on two sets of tandem AgrA boxes (Fig. 8), facilitated by the ~160° bending of the *agr* promoter by SarA and AgrA. In transition from the late exponential phase to the post-exponential phase, the SarR protein accumulates (23). As the SarR protein binds to the *agr* promoter with higher affinity than SarA, it would displace the SarA bound on the *agr* promoter (see Fig. 5B). In contrast to SarA, SarR binds but does not induce bending of the *agr* promoter, possibly unwrapping the transcription competent complex, thus leading to down-regulation of transcription from the *agr* P2 promoter.

While our model does not support direct modulation of the *agr* P3 promoter by SarR and SarA, it does not preclude indirect effects whereby SarA and SarR would activate and repress the *agr* P2 promoter, respectively, to modulate AgrA expression, which in turn would dictate transcription from the P3 promoter.

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