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Regulatory Elements of the *Staphylococcus aureus* Protein A (Spa) Promoter†

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Staphylococcal protein A (Spa) is an important virulence factor of *Staphylococcus aureus*. Transcription of the *spa* determinant occurs during the exponential growth phase and is repressed when the cells enter the postexponential growth phase. Regulation of *spa* expression has been found to be complicated, with regulation involving multiple factors, including Agr, SarA, SarS, SarT, Rot, and MgrA. Our understanding of how these factors work on the *spa* promoter to regulate *spa* expression is incomplete. To identify regulatory sites within the *spa* promoter, analysis of deletion derivatives of the promoter in host strains deficient in one or more of the regulatory factors was undertaken, and several critical features of *spa* regulation were revealed. The transcriptional start sites of *spa* were determined by primer extension. The *spa* promoter sequences were subcloned in front of a promoterless chloramphenicol acetyltransferase reporter gene. Various lengths of *spa* truncations with the same 3′ end were constructed, and the resultant plasmids were transduced into strains with different regulatory genetic backgrounds. Our results identified upstream promoter sequences necessary for Agr system regulation of *spa* expression. The *cis* elements for SarS activity, an activator of *spa* expression, and for SarA activity, a repressor of *spa* expression, were identified. The well-characterized SarA consensus sequence on the *spa* promoter was found to be insufficient for SarA repression of the *spa* promoter. Full repression required the presence of a second consensus site adjacent to the SarS binding site. Sequences directly upstream of the core promoter sequence were found to stimulate transcription.

Staphylococcus aureus is a significant human pathogen that causes a wide range of infections, including skin and wound infections, toxic shock syndrome, arthritis, endocarditis, osteomyelitis, and food poisoning. The bacterium produces in a regulated fashion a number of potential virulence factors, including a variety of exotoxins and cell surface-associated proteins (12, 20, 22, 30). One of the major surface proteins is staphylococcal protein A (Spa), which has been shown to comprise 7% of the cell wall (13). Spa binds the Fc fragment of immunoglobulins from several mammalian species and may be important in phagocytosis avoidance (11). Studies utilizing *spa* mutants have shown that protein A is an important virulence factor in a murine septic arthritis model and has a modest effect on virulence with subcutaneous infections in mice (32, 34).

Spa, like many of the surface matrix binding proteins of *S. aureus*, is expressed during the exponential phase of growth and then is transcriptionally down-regulated during the postexponential phase of growth. This process involves the Agr (accessory gene regulator) quorum-sensing global regulatory system of *S. aureus* (2, 21, 22). The Agr locus is comprised of two divergent transcriptional units, the first being a two-component regulatory system (*agrBDCA*). AgrB is a transmembrane protein that is responsible for the transport and processing of AgrD (44). AgrD is exported as a cyclic peptide, termed the autoinducing peptide (AIP) (21). When AIP accumulates to a threshold concentration, it binds to AgrC, the sensor of the two-component system. AIP binding induces autophosphorylation of AgrC, with the phosphate moiety subsequently transferred to AgrA (26). Activated AgrA is a transcription factor that stimulates transcription from both of the Agr locus promoters. The second Agr locus transcript is a 514-nucleotide RNA, called RNAIII. Although RNAIII encodes delta toxin, it is the RNA species itself that is the effector of the Agr system (20, 31). Accumulation of RNAIII results in the postexponential-phase activation of transcription of many exoprotein genes and inhibition of transcription of many cell wall-associated protein genes, such as *spa*.

Recent studies have shown that several additional regulatory factors are involved in the regulation of *spa* expression, such as SarA (staphylococcal accessory regulator), SarS (initially designated SarH1), Rot (repressor of toxins), SarT, and the ArlR-ArlS two-component system (1, 5–8, 10, 14, 37, 38, 42). SarA is a pleiotropic regulator for multiple genes (10). Gene chip analysis has shown that SarA represses transcription of a number of genes, including *spa*, and stimulates transcription of other genes, including the *agr* promoters (9, 12). Regulation by SarA is thought to be accomplished by both Agr-dependent and Agr-independent mechanisms (9). SarA was demonstrated to bind multiple gene promoters in vitro, and SarA binding sequences have been identified (10, 41). There is a SarA recognition sequence immediately upstream of the −35 promoter element sequence in the *spa* determinant (10). The mechanism(s) by which SarA differentially regulates promoter activity is unknown.

SarS, initially designated SarH1, is one of the Sar family of transcriptional regulators. The *sarS* determinant is located im-

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TABLE 1. Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Relevant characteristic(s)	Source or reference
E. coli strains		
DH5α	ϕ80d*lacZ*ΔM15 *recA1 endA1 gyrA96 thi-1 hsdR17* (r_K^- m_K^-) *supE44 relA1 deoR* Δ(*lacZYA-argF*)*U169*	Gibco-BRL
TOP10F′	F′[*lacI*^{q} Tn*10* (Tet^r)] *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 deoR recA1 ara*Δ*139* Δ(*ara-leu*)*7697 galU galK rpsL endA1 nupG*	Invitrogen
S. aureus strains		
KSI2054	8325-4 *agr*$^+$	45
KSS5186	Δ*agr rot*::*tetM*	43
KSS5130	*agr*$^+$ *sarA*::*kan*	This study
KSS5523	Δ*agr sarA*::*kan*	43
KSS5527	*agr*$^+$ *sarA*::*kan sarS*::*erm*	This study
KSS5528	Δ*agr sarA*::*kan sarS*::*erm*	This study
KT201	*sarS*::pKT200 (*erm*)	42
PM466	Δ*agr*	29
PM783	*rot*::*tetM*	29
RN4220	Accepts foreign DNA (r−)	24
UAMS-957	*cna sarA*::*kan*	4
Plasmids		
pJG2984	*spa* −146 to +7 in pMH109	This study
pJG2993	*spa* −259 to +7 in pMH109	This study
pJG3020	*spa* −89 to +7 in pMH109	This study
pJG3045	*spa* −137 to +7 in pMH109	This study
pJG3046	*spa* −125 to +7 in pMH109	This study
pJG3047	*spa* −110 to +7 in pMH109	This study
pJG3048	*spa* −97 to +7 in pMH109	This study
pJG3049	*spa* −76 to +7 in pMH109	This study
pJG3123	*spa* −61 to +7 in pMH109	This study
pJG3234	*spa* −52 to +7 in pMH109	This study
pJG3314	*spa* −42 to +7 in pMH109	This study
pJG3317	*spa* −38 to +7 in pMH109	This study
pJG3318	*spa* −32 to +7 in pMH109	This study
pJG3394	*spa* −259 to +7 with −91 to −100 deletion in pMH109	This study
pJG3480	*spa* −259 to +7 with SarA box replaced by *lacC* sequences in pMH109	This study
pJG3482	*spa* −89 to +7 with SarA box replaced by *lacC* sequences in pMH109	This study
pJG3483	*spa* −110 to +7 with −91 to −100 deletion in pMH109	This study
pMH109	Shuttle expression plasmid	19

mediately upstream of *spa* and is a positive regulator of *spa* expression (8, 42). SarS was shown to be a DNA binding protein and, thus, may up-regulate *spa* expression by direct binding to the *spa* promoter. Another member of the Sar family of proteins, SarT, has been shown to be a positive regulator of SarS (38). SarT was shown to bind to the *sarS* promoter. Expression of *sarT* results in *sarS*, and subsequently *spa*, expression (38).

An additional member of the Sar family, Rot, was also shown to be a positive regulator of *sarS* expression and thus, indirectly, *spa* expression (37). A gene chip analysis indicated a 15.6-fold enhancement of *spa* transcription by Rot. It has not been determined if the activity of Rot on the *sarS* promoter is a direct or an indirect effect.

The ArlRS two-component regulatory system has been reported to affect *spa* expression (14). Inactivation of either the *arlR* or *arlS* determinant resulted in elevation of *spa* transcription. The *arl* mutations did not change *spa* transcription in an *agrA* or *sarA* mutant background, suggesting that the effect of the Arl proteins on *spa* expression is indirect, mediated through these global regulators (14). The *mgrA*-encoded protein has been shown to be responsible for the regulation of a number of genes in *S. aureus*, including acting as a repressor of protein A production (28). MgrA inactivation results in elevation of both protein A and alpha toxin, products whose genes are oppositely regulated by the Agr system.

A model has been proposed to explain the regulated expression of *spa* (38). In this model, the SarA protein binds to the *spa* promoter to repress transcription. Production of SarS activates transcription of *spa*. SarS production is indirectly regulated by the SarA and Agr systems. When the Agr system is activated, *sarT* transcription is repressed, with the reduction in SarT production leading to a reduction in SarS levels and consequently a loss of Spa production. The SarA protein also represses *sarT* transcription (38).

Although several factors have been reported to influence *spa* expression, it is unclear how these factors work on the *spa* promoter. Here we report our studies on characterization of the *spa* promoter and its regulation. The *spa* promoter has two transcriptional start sites and contains at least three upstream *cis* elements that are required for its regulation.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. The *agr*, *sarA*, and *sarS* mutant alleles were moved by transduction into KSI2054 to create the isogenic strains.

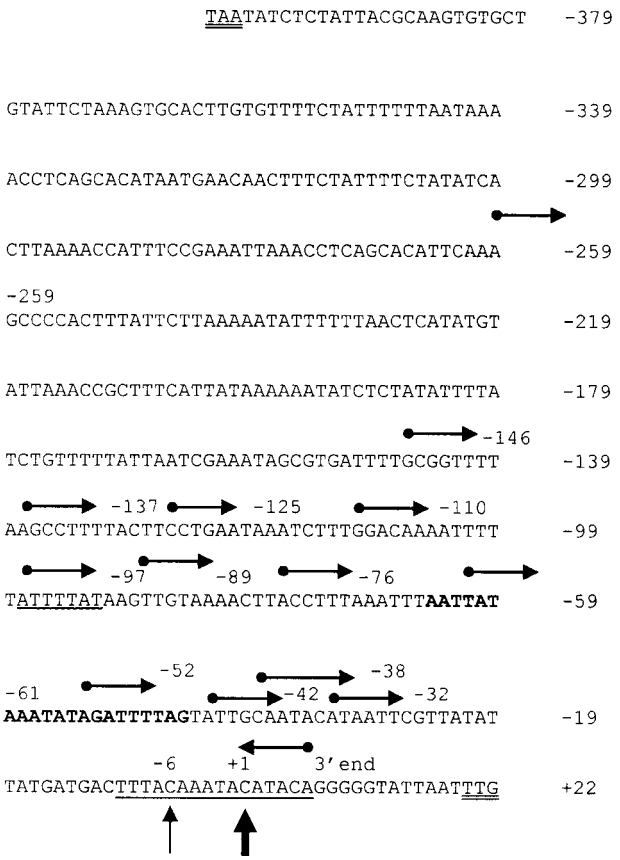


FIG. 1. The *spa* promoter region. The filled circles of the rightward-pointing arrows indicate the start point of the subcloned promoter fragments (all clones end at +7). The fragments are identified by the 5′ end location of the fragments, which are listed to the right of the arrows. The *spa* start codon and the stop codon of the upstream *sarS* determinant are double underlined, the SarA binding sequence of Chien et al. (10) is in bold, and the range of fragments identified by Patel et al. (33) by an S1 protection assay is indicated by the underlining. The double wavy underlined sequence represents a SarA consensus binding site identified by SELEX (41). The upward-facing arrows indicate the start sites of transcription indicated by primer extension.

Trypticase soy broth (Difco) and Trypticase soy agar (Difco) were used for culturing *S. aureus*. Luria-Bertani agar and broth were used to culture *Escherichia coli.* All incubations were performed at 37°C. Antibiotic concentrations used were as follows: tetracycline, 20 μg/ml; kanamycin, 20 μg/ml; chloramphenicol, 10 μg/ml; erythromycin, 5 μg/ml; ampicillin, 100 μg/ml; zeocin, 20 μg/ml.

Primer extension. Total RNA was isolated from *S. aureus* cultures (A_{540} = 2.0) by the RNAzol B (Tel-Test, Inc.) procedure described elsewhere (16). Two primers were used. One primer, Spa1 (5′-CCTACACCTAGTTTACGAATTG-3′), is complementary to the *spa* structural gene (bases +42 to +63, based on the numbering scheme of Fig. 1). The other primer, cat3 (5′-GGTTATACTAAAA GTCGTTTGTTGGTTC-3′), is complementary to the *cat* open reading frame (ORF; 81 to 109 bp downstream of the SacI site) on the pMH109 vector. Each primer was labeled with [^{32}P]ATP (Amersham Biosciences) with T4 polynucleotide kinase (Promega). Primer extension was performed using avian myeloblastosis virus reverse transcriptase (Primer Extension system; Promega). Briefly, 30 μg of RNA was coprecipitated with the ^{32}P-end-labeled primer. The RNA-DNA pellet was resuspended in 7 μl of 250 mM KCl, heated to 94°C for 2 min, and then incubated at 53°C for 20 min. Avian myeloblastosis virus reverse transcriptase was then added to the reaction mixture and incubated at 42°C for 30 min. The reaction was stopped by addition of 20 μl of loading dye. A sequencing reaction using the same primer and the *fmol* DNA cycle sequencing system (Promega) was conducted simultaneously to provide the molecular size markers. The reaction mixtures were resolved on a 6% (7 M urea) polyacrylamide gel. After electrophoresis, the gels were transferred onto 3MM filters, dried, and subjected to radioautography.

DNA manipulations. *S. aureus* chromosomal DNA was isolated from 5-ml overnight cultures as follows. The cells were harvested by centrifugation (2,000 × *g*, 10 min), washed with 5 ml of TE buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA), and resuspended in 0.1 ml of TE. Fifty micrograms of lysostaphin was added, and the sample was incubated at 37°C for 30 min. Following this incubation, equal volumes of 1.6% Sarkosyl and 25 μg of proteinase K were added and the samples were incubated at 60°C for 60 min. The samples were then placed on ice, and the DNA was extracted with TE-saturated phenol followed by extraction with chloroform. The DNA samples were then precipitated with ethanol and resuspended in distilled water.

Plasmid DNA was isolated from *E. coli* by the procedure of Birnboim and Doly (3) and from *S. aureus* as described elsewhere (46).

Electroporation and transduction. Introduction of plasmid DNA into *S. aureus* RN4220 by electroporation was conducted as described previously (23), except that a 0.1-cm electrode gap cuvette was utilized. Transduction of *S. aureus* strains was performed as described previously, utilizing phage 80α (36). Transformants and transductants were selected by growth at 37°C on Trypticase soy agar containing 20 μg of kanamycin/ml.

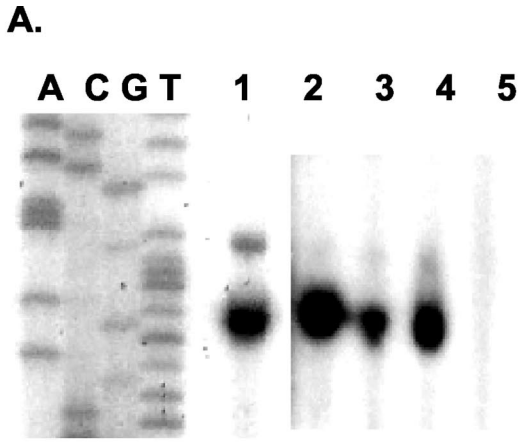
Cloning of the *spa* promoter and deletion derivatives. The *spa* promoter (−259 to +7) and the 5′ series promoter deletion fragments were amplified by PCR (46). An XbaI recognition sequence was incorporated at the 5′ end of the upstream primers, and a SacI recognition sequence was incorporated into the downstream primers. The PCR products were cloned into PCR2.1 TOPO (Invitrogen) and then subcloned into the relatively low-copy-number promoter cloning shuttle vector pMH109 (19) following XbaI and SacI digestion. All constructs were sequenced to confirm they had the correct inserts. The plasmids were isolated from *E. coli* and electroporated into RN4220 (23). Plasmids in RN4220 were transduced into strains of various genetic backgrounds by transduction. Replacement mutagenesis was carried out to substitute an *S. aureus lacC* sequence internal to the ORF (5′-TTCGCTAAGCACAATCATACAT-3′ [35]) for the promoter-proximal SarA box sequence (5′-AATTATAAATATAG ATTTTAGTA-3′ [10]).

Internal promoter deletion fragments were constructed with splicing by overlap extension (17, 18). PCR products were manipulated as described above.

CAT assays. The activities of various *spa* promoter regions were measured with a chloramphenicol acetyltransferase (CAT) assay (40). The CAT assay was modified to a microformula suitable for an enzyme-linked immunosorbent assay plate reader (43). Overnight cultures were diluted to an A_{540} of 0.1 in Trypticase soy broth (for *E. coli*, Luria-Bertani broth and A_{600}) and incubated until the A_{540} reached 2.0. Cells were harvested and washed once with WL buffer (25 mM Tris-HCl [pH 8], 25 mM EDTA). Pellets were resuspended in 1.0 ml of WL buffer, and 1.5 g of 0.1-mm glass beads was added. Cells were lysed using a Beadbeater 8 (Biospec Products) twice for 1 min each at 4°C. The lysed bacterial samples were centrifuged (2,500 × *g*, 10 min) at 4°C, and the supernatants were saved and stored at −80°C. Three microliters of the cell lysate was mixed with 37.5 μl of 10 mM 5,5′-dithiobis-2-nitrobenzoic acid (Sigma), 7.5 μl of 5 mM acetyl coenzyme A (Pharmacia), 206 μl of distilled deionized H_2O, and 6.5 μl of 5 mM chloramphenicol (Sigma). Reactions were carried out in wells of a 96-well plate (Corning), and absorbance at 412 nm was determined with a Spectramax 190 microplate spectrophotometer (Molecular Devices). All strains were cultured in triplicate. The data analyses were done with Microsoft Excel. CAT activity was expressed as nanomoles of chloramphenicol acetylated per milligram of cells (dry weight) per minute at 37°C.

RESULTS

Determination of the *spa* transcriptional start sites. The transcriptional start site for *spa* was identified by an S1 nuclease assay described by Patel et al. (33). The assay yielded a series of DNA bands that corresponded to potential transcription start sites 13 to 28 bases upstream of the TTG initiation codon (Fig. 1). The multiple bands could have resulted from nibbling by S1 nuclease, although the data did not rule out the possibility of there being >1 actual transcription start sites. To more precisely define the start site of transcription, primer extension analysis was carried out. Utilizing the Spa1 primer internal to the *spa* ORF resulted in the appearance of two



B.

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                                                                rbs
chrom.  TTAGTATTGCAATACATAATTCGTTATATTATGATGACTTTACAAATACATACAGGGGG
-42     TCTAGATTGCAATACATAATTCGTTATATTATGATGACTTTACAAATACATACAGAGCTC
-38        TCTAGACAATACATAATTCGTTATATTATGATGACTTTACAAATACATACAGAGCTC
-32              TCTAGATAATTCGTTATATTATGATGACTTTACAAATACATACAGAGCTC
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FIG. 2. Primer extension localization of the *spa* transcription start sites. (A) The Spa1 primer used was complementary to the 5′ end of the *spa* ORF, and RNA was isolated from an *agr*-negative mutant of *S. aureus*. The primer was used to generate the sequencing ladder size standards (lanes A, C, G, and T). Lane 1, primer extension with the Spa1 primer and RNA from strain PM466; lanes 2 to 5, primer extension with the *cat3* primer and RNA isolated from strain PM466 bearing the −259 plasmid (lane 2), the −42 plasmid (lane 3), the −38 plasmid (lane 4), and the −32 plasmid (lane 5). The film for lanes 2 to 5 was positioned to align the bands with those of lane 1. (B) Sequence alignments of the core (−42) and truncated forms of the *spa* promoter used in the primer extension reactions. The sequences incorporated into the primers to provide recognition sequences for the restriction endonucleases XbaI and SacI are indicated in italics. The putative −35 and −10 promoter elements are underlined, and the putative −35 element contributed in part by the inclusion of the XbaI linker sequence in the −38 promoter construct is denoted in bold font.

distinct bands, 6 nucleotides apart (Fig. 2A). The lower band was more intense than the upper band, and this position was designated +1. These two start sites correspond to nucleotides 19 and 25 bases upstream of the start codon, within the range of fragment sizes obtained by S1 mapping by Patel et al. (33). Examination of the sequence upstream of the two start sites revealed typical consensus −35 and −10 sequences, TTGCAA for −35 (−41 to −36) and TATGAT for −10 (−18 to −13), separated by 17 bp. To determine if these sequences constituted the *spa* promoter and if both transcripts arose from a single promoter element, primer extension was carried out using cloned promoter sequences containing the intact putative promoter (−42 to +7; pJG3314) and a construct lacking the first 3 nucleotides of the putative −35 element (−38 to +7; pJG3317). A positive control plasmid bearing the sequences from −259 to +7 (pJG2993) and a negative control containing an incomplete promoter (−32 to +7; pJG3318) were also tested (Fig. 2B). The DNA fragments were positioned upstream of a CAT reporter gene, and primer extension was carried out using RNA isolated from the plasmid-bearing cells (Fig. 2A). The plasmids bearing the intact putative promoter element (pJG3314 and pJG2993) resulted in the same start sites of transcription as identified with the *spa* ORF-specific primer, demonstrating that transcription from the plasmids faithfully reproduced the transcript pattern of the chromosomal protein A gene promoter. Deletion of the first three bases of the putative −35 element did not affect the production or location of the two transcripts. The DNA fragment with a deletion to the −32 position (pJG3318) lacked promoter activity, as expected.

The results indicated that critical features of the *spa* promoter, either as a −35 element or a *cis* element for a required transcriptional activator, lie between positions −42 and −32. It is possible that the putative −35 element is correct and that the deletion and introduction of the XbaI recognition sequence restored a functional −35 element, tcTAGACAaa, which would be an imperfect match to the consensus −35 element sequence and is shifted one nucleotide further upstream relative to the −10 element sequence.

Upstream region sequences of the *spa* promoter required for Agr-mediated regulation. A series of nested fragments with a common downstream end (immediately upstream of the ribosome binding site) and variable upstream ends (indicated in Fig. 1) were cloned into the promoter cloning shuttle vector, pMH109, which bears a *cat* gene that lacks its own promoter but possesses a GGAGG ribosome binding site sequence. The reporter plasmids were transduced into *agr*-positive (KSI2054) and *agr*-null (PM466) strains. The promoter activity of each construct was then determined (Fig. 3A). Results are shown for samples taken at the postexponential phase of growth (A_{540} = 2), a time at which the Agr/Sar system represses *spa* transcription. With the largest promoter fragment (−259), there was approximately a 14-fold difference between the promoter activity from the *agr*-null host relative to that of the *agr*-positive host. This is consistent with the reported Agr effect on protein A production in 8325-derived strains (33) and indicates that

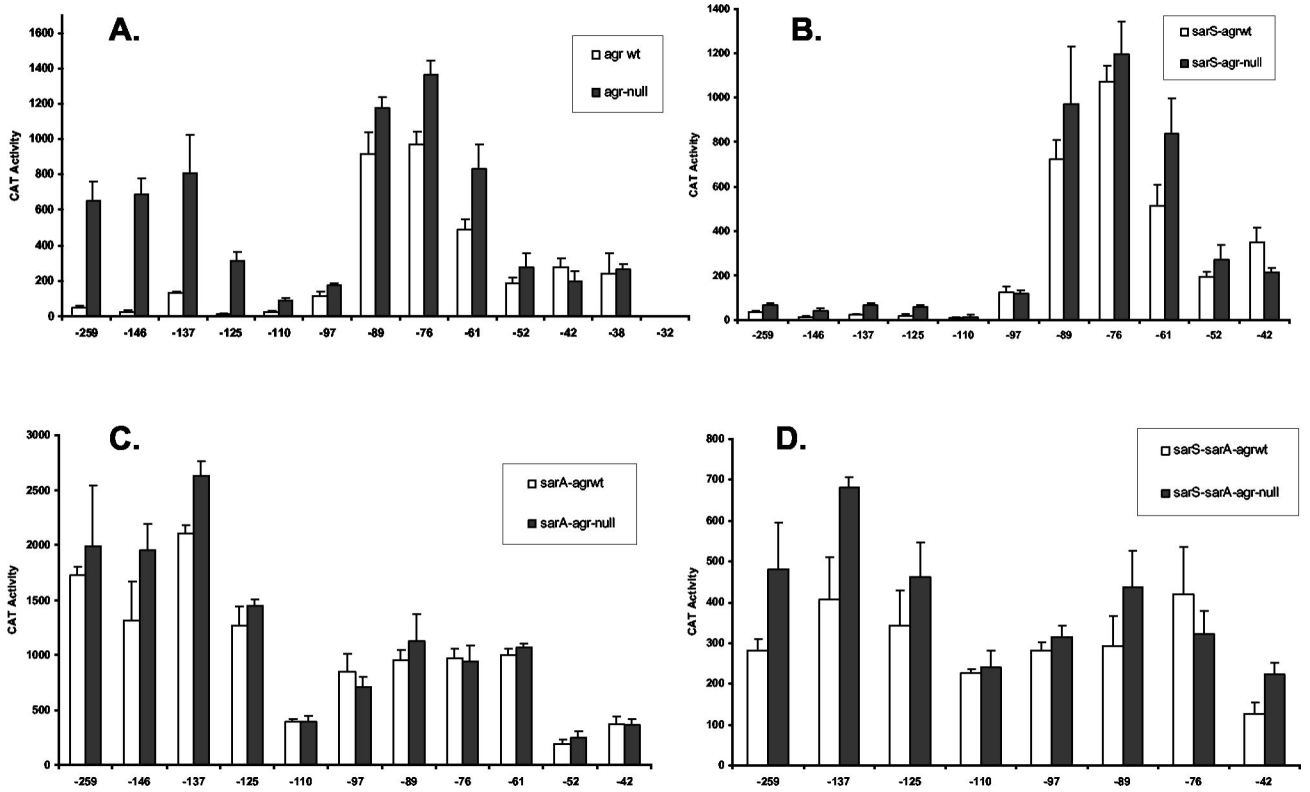

FIG. 3. Effect of *agr*, SarS, and SarA on *spa* promoter activities. The *x* axes indicate the upstream boundary of the promoter fragments. Samples were from postexponential-phase culture lysates. CAT activity is expressed as nanomoles of chloramphenicol acetylated per minute per milligram of cells (dry weight). Values plotted are the means of at least three determinations with the standard deviations. Host strains were agr^+ (white bars) and *agr*-negative (black bars) (A); agr^+/*sarS*-negative (white bars) and Δ*agr*/*sarS*-negative (black bars) (B); agr^+/*sarA*-negative (white bars) and Δ*agr*/*sarA*-negative (black bars) (C); and agr^+/*sarA*-negative/*sarS*-negative (white bars) and Δ*agr*/*sarA*-negative/*sarS*-negative (black bars) (D).

this system accurately reflects the regulatory pattern of the *spa* promoter. The results of the deletion studies revealed the presence of three distinct regulatory sites within the *spa* promoter.

The minimal promoter element (clone −42) displays unregulated expression. The promoter strength does not change with extension to −52. However, extension of the upstream sequence to −61 resulted in an approximately twofold stimulation of the transcriptional activity, and inclusion of sequences to −76 gave an additional twofold increase. However, these clones were not regulated by the Agr system. The sequence between −52 and −76 is very AT rich and may act as a UP element to increase transcriptional efficiency rather than represent a *cis* element for a transcriptional factor (15). To provide an indication of the nature of the elevated transcription associated with the −76 construct, CAT assays were performed on *E. coli* strain DH5α cells bearing the *spa* promoter plasmids (Fig. 4). The *spa* core promoter was found to be active in *E. coli*, and extension of the upstream sequences to −76 resulted in an approximately threefold increase in activity. It is likely that the elevated transcription results from an UP element-like sequence, rather than from a specific transcription factor. It is improbable that *E. coli* and the evolutionarily distinct *S. aureus* would share such a transcriptional factor to up-regulate expression of this promoter.

Inclusion of the sequence to −97 resulted in a marked (eightfold) reduction in promoter activity in *S. aureus*, giving a promoter strength lower than that of the core promoter (Fig. 3A). This is suggestive of a *cis* element for binding a negative transcription factor. Because an additional degree of repression was observed with the −110 promoter fragment, the functional upstream boundary of this putative element is likely to be between −110 and −97.

The constructs with an upstream boundary greater than or equal to −110 had substantially higher CAT values in the *agr* mutant host relative to those in the agr^+ host, reflecting the presence of Agr regulation. Specifically, the host ratios of CAT values of the constructs shorter than −110 were closer to 1, implying this region lacks an Agr-responsive *cis* element. Constructs longer than −110 gave rise to Agr^-/Agr^+ ratio values ranging from 10 to approximately 27, in contrast to the values of 0.7 to 1.8 for the constructs shorter than −110. The −110 construct, although displaying substantially reduced overall CAT values in both the *agr* mutant and in agr^+ hosts, gave a ratio of approximately 4. Using a ratio of 4 as the cutoff value for determination of the presence of Agr regulation allowed us to conclude that the constructs from −110 and larger contain a *cis* element that is involved in Agr regulation. Full Agr system regulation became evident with the promoters that contained sequences to −125, but promoter strength with Agr regulation increased with the construct extending to −137. Therefore, the functional upstream boundary of this element lies between

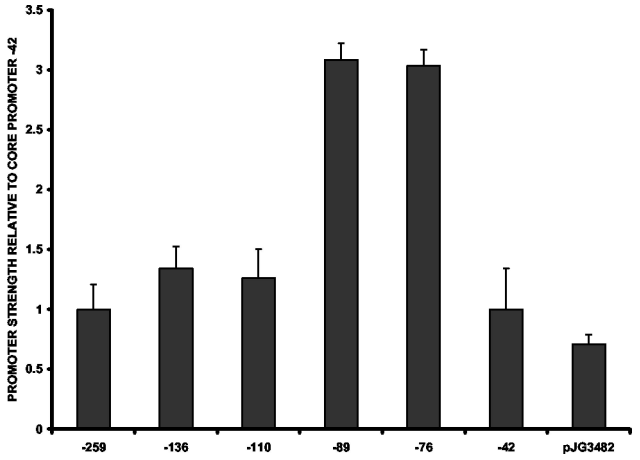

FIG. 4. *spa* promoter activity in *E. coli*, as shown by relative expression of the *spa* promoter fragments. The CAT activity of the −42 core promoter was set as 1.0, and the activities of the other promoter fragments were divided by the core promoter activity. The *x* axis gives the upstream boundary of the promoter fragments cloned into pMH109. The pJG3482 plasmid contains the *spa* −89 to +7 fragment, with the SarA box replaced by *lacC* sequences. Values plotted are the means of at least three determinations with the standard deviations.

positions −137 and −125. With all of these *spa* promoter constructs, the promoter activity in the *agr*-positive host was less than that observed with the unregulated core promoter, suggesting that in a wild-type strain repression of promoter activity, and not simply loss of a positive regulator such as SarS, occurs.

The CAT value was reduced in the *agr*-deficient host with the −110 promoter fragment, relative to that in the larger constructs. This implies that the −110 construct lacks the transcriptional up-regulating element that is present on the longer constructs. The correlation between reduced promoter strength in *agr*-negative hosts and the appearance of an Agr effect implies that the high promoter activity with the constructs larger than the −110 promoter fragment is due to an Agr system-down-regulated transcriptional activator. The CAT values of the −110 and −97 constructs were lower than that of the core promoter construct (−42). This finding is inconsistent with a simple loss of a transcriptional activator binding site. The results suggest that the region upstream of −89 contains a regulatory element associated with reduced transcriptional activity. The evidence that this is a sequence-inherent effect, rather than representing the binding site for a negative transcriptional factor, is presented below.

SarS regulatory domain of the *spa* promoter. The *sarS* determinant encodes a positive regulator of *spa* transcription (8, 42). The nested series of *spa* promoter fragments were introduced into *sarS*-negative *agr*$^+$ and mutant *agr* isogenic strains of *S. aureus* and the promoter activities were determined from postexponential-phase cultures. The results are shown in Fig. 3B. Comparison of these results with the values presented in Fig. 3A with the *sarS*$^+$ strains allows for a determination of the SarS element(s) within the *spa* promoter. With the *agr*$^+$ host, there was minimal overall CAT activity with promoter fragments of −110 and greater, and there was no significant difference between the activities observed in the presence or absence of functional SarS in these postexponential-phase cultures. The results obtained with the mutant *agr* host strain indicated that the −110 clone was the shortest promoter fragment to be substantially affected by SarS. The −97 clone had a very modest reduction in activity, with 68% of the activity seen in the mutant *agr*/*sarS*$^+$ host. The −110 and the larger clones in the mutant *sarS* hosts had activities that ranged from 6 to 18% of those seen when functional SarS protein was produced. In the absence of a functional SarS protein, the promoter activities obtained for the −97 and larger clones were less than the activity obtained with the core promoter, again indicative of active repression occurring in the *sarS*-negative strains. Furthermore, an Agr effect was still demonstrable in the *sarS*-negative strains. For example, there was a two- to threefold reduction in promoter strength with the promoter fragments −259 through −125 in the *agr*$^+$/*sarS*-negative host relative to those in the *agr*-negative/*sarS*-negative host. Therefore, the Agr effect on *spa* transcription is not entirely mediated by the Agr system's effects on *sarS* expression, in agreement with the findings of Tegmark et al. (42).

SarS has been reported to be a DNA binding protein, although its binding site on the *spa* promoter has not been specifically identified (8, 25, 42). When the results of the study of the effect of Agr on the promoter constructs (Fig. 3A) and the effect of loss of SarS production (Fig. 3B) were compared, it was concluded that the upstream boundary for the fully functional SarS *cis* element is between −137 and −125. The smaller fragments (−97, −110, and −125) displayed reduced responses to SarS and consequently to the Agr system. The SarS protein was first identified through its binding to a fragment corresponding to −15 to −110 of the *spa* and other promoters (42). Our results are consistent with their finding that the −110 fragment would bind SarS. Electrophoretic mobility shift results suggest that multiple copies of SarS may be bound to the *spa* promoter, and this may be important for the proper function of this protein as a transcriptional regulator (8, 25).

The *cis* element required for SarA repression of *spa* expression. SarA has been reported to be a negative regulator of *spa* transcription. DNase I footprinting revealed that SarA binding protected the sequence between −188 and −44, which included a 21-bp SarA box sequence 2 bp upstream of the −35 element of the *spa* promoter (10). Using a plasmid-based reporter system, it was determined that deletion of the SarA box sequence resulted in increased promoter activity (10). The SarA binding site identified by Cheung and coworkers is the −64 to −44 sequence shown in Fig. 1. Within this DNase I footprinting-protected sequence is the ATTTTAG (−50 to −44) imperfect SarA binding sequence (ATTTTAT) identified by Stebra et al. (41) using a SELEX procedure.

The nested series of *spa* promoter fragments was introduced into *sarA*-negative *agr*$^+$ and *agr*-negative isogenic strains of *S. aureus*, and the promoter activities were determined from postexponential-phase cultures. The results are shown in Fig. 3C. Comparison of these results with the values presented in Fig. 3A with the *sarA*$^+$ strains allows for a demonstration of SarA repression on the *spa* promoter. The previously identified SarA binding sequence is present within the −76 promoter fragment. However, this promoter fusion construct does not show a reduction in promoter expression, which would be expected

if SarA were bound (Fig. 3A, compare the −76 and −61 activities). When the values of the promoter strengths in the *sarA*$^+$ and *sarA*-negative hosts were compared, no SarA effects were observed until the upstream sequence was extended to −97. The activities of the −97 and larger clones were markedly elevated (i.e., approximately fourfold for the −97 clone) in the *sarA*-negative host (Fig. 3C; note that the graphs in panels C and A have different *y*-axis scales). The −97 promoter clone contains an ATTTTAT sequence (−97 to −91), which is a perfect match to the SarA consensus sequence identified by Stebra et al. (41) using the SELEX technique. These results suggest that this SarA site is functional. The promoter constructs from −97 and larger had elevated CAT values in *sarA*-negative hosts, regardless of *agr* status. The values were approximately 40-fold higher than those of the *sarA*$^+$ *agr*$^+$ host bearing the same constructs and 2- to 3-fold higher than those in the *sarA*$^+$ *agr*-negative host. It should be noted, however, that the −110 promoter fusion construct has a lower promoter strength than the slightly larger and smaller constructs. The increased promoter strength of the larger constructs can be explained by SarS activity. The lower value relative to the smaller constructs is suggestive of an additional repression that is SarA independent. It is important to note that the promoter activity of the −110 and larger *spa* promoter fragments, relative to the activities of the −89 promoter fragment, were reduced in the *E. coli* hosts as well (Fig. 4). This again is suggestive of a sequence-inherent effect rather than a binding site for a transcriptional factor. This regulatory sequence lies between −89 and −110. Thus, two distinct mechanisms to reduce the activity of the *spa* promoter are found with the −97 and larger promoter fragments, one being SarA dependent while the other is SarA independent.

Although deletion of the promoter-proximal SarA box sequence gave rise to an increase in promoter strength (10), our studies where this sequence was included with the core promoter did not result in the expected reduction in promoter strength. Resolution of these conflicting results may involve the contribution of the upstream putative SarA box sequence. To evaluate the contributions of the upstream and downstream putative SarA boxes on SarA-mediated repression of *spa* expression, specific deletions of these elements were created. A 10-bp deletion was made on pJG2993 (the −259 promoter fragment) from −91 to −100, to produce pJG3394. A 10-bp deletion was utilized to remove one turn of the helix and thus minimize potential sidedness concerns. To determine if the −64-to-−44 sequence contributes to SarA-mediated repression of the *spa* promoter, the −259 promoter fragment was mutated such that the *spa* promoter sequence from −64 to −42, containing the promoter-proximal SarA binding sequence, was replaced with the same number of bases from a sequence internal to the *S. aureus lacC* ORF. This replacement approach was taken to avoid altering the spacing of elements within the *spa* promoter, and the *lacC* sequences are unlikely to contain regulatory sequences. These constructs, and the parental plasmid pJG2993, were introduced into the *agr*$^+$ and *agr*-negative host strains. The CAT values were determined and are shown in Fig. 5. In the *agr*-positive wild-type host, removal of the promoter-proximal SarA box sequence gave rise to a substantial increase in promoter activity, whereas loss of the upstream element had a more modest stimulatory effect.

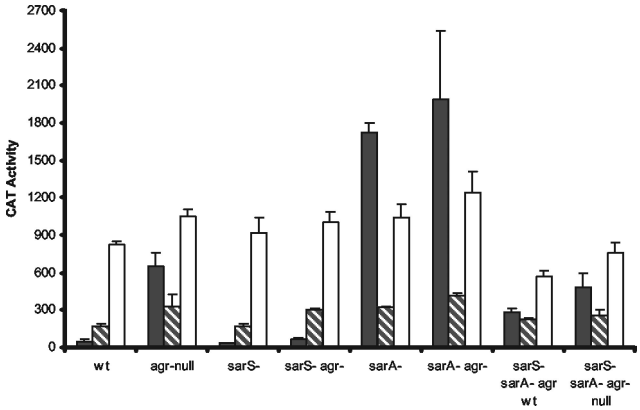

FIG. 5. Effect of the putative SarA binding sites on *spa* promoter activity. Shown are the CAT activities promoted by the −259 promoter fragment (black bars), the −259 promoter fragment with the promoter-proximal SarA box (10) replaced by *lacC* sequences (white bars), and the −259 fragment with the upstream putative SarA binding site deleted (hatched bars). Host strains are indicated along the *y* axis. CAT activity is expressed as nanomoles of chloramphenicol acetylated per minute per milligram of cells (dry weight). Values plotted are the means of at least three determinations with the standard deviations.

In the *agr*-null host strain, the promoter activity of the mutant lacking the promoter-proximal element was elevated, but the activity of the wild-type promoter was actually higher than that of the mutant without the upstream element. Both mutants were impaired in Agr-mediated repression of transcription. The activities of the mutant promoters were independent of SarS, unlike the situation with the wild-type promoter. Similarly, the activities of the mutant promoters were only modestly influenced by the absence of SarA, with only slight increases in activity observed with the isogenic *sarA*-negative strains. Transcription of the wild-type promoter was markedly enhanced in the absence of SarA. With the *sarA*-negative/*sarS*-negative double mutant hosts, the mutant promoter fragments displayed a pattern of expression comparable to that of the wild-type promoter.

The promoter-proximal SarA box replacement mutation was introduced into the −89 promoter fragment to produce pJG3482. The −89 promoter lacks the putative upstream SarA box and was not affected by SarA (Fig. 3C). We examined the effect of loss of the remaining SarA box sequence on the activity of this promoter fragment. The results are shown in Fig. 6. Removal of the SarA box sequence actually resulted in a reduction of promoter activity in the *agr*$^+$ and *agr*-negative hosts. Expression of these promoters in the *sarS*-negative/*sarA*-negative hosts reduced promoter activity with both the wild-type and mutant promoters, although the effect was smaller with the mutant promoter and the result was higher promoter activity for the mutant promoter in *sarS*-negative/*sarA*-negative/*agr*$^+$ host cells.

Extension of the upstream promoter sequences from −89 to −110 resulted in a substantial reduction in promoter activity (Fig. 3A). The −110 fragment contains the upstream SarA box consensus sequence, raising the possibility that SarA binding to this site is responsible for the decrease. However, although the activity of the −97 promoter was elevated in the *sarA*-deficient host (Fig. 3C), the activity remained much lower than that of

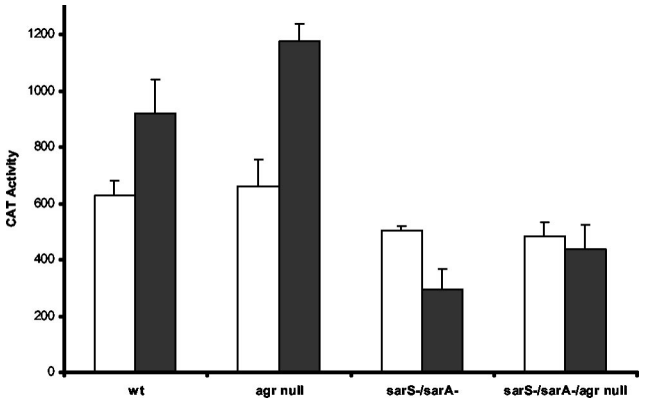

FIG. 6. Effect of the SarA binding site on *spa* promoter activity in the absence of the putative upstream site. Results shown are CAT activities promoted by the −89 promoter fragment (black bars) and the −89 promoter fragment with the promoter proximal SarA box (10) replaced by *lacC* sequences (white bars). Host strains are indicated along the *y* axis. CAT activity is expressed as nanomoles of chloramphenicol acetylated per minute per milligram of cells (dry weight). Values plotted are the means of at least three determinations with the standard deviations.

the −89 promoter clone. To determine if the sequence containing the upstream SarA box were associated with the reduced transcriptional activity, perhaps in a SarA-independent fashion, the 10-bp deletion of the SarA box consensus sequence was introduced into the −110 promoter fragment. The resulting plasmid, pJG3483, was then introduced into the *agr*$^+$ and *agr*-null strains, and promoter activity was measured (Fig. 7). Deletion of the SarA box sequence resulted in a four- to sixfold elevation of promoter activity, although the activity of

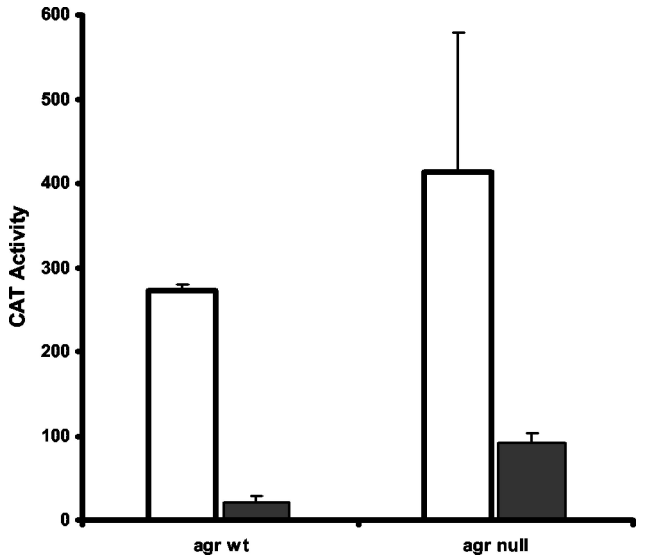

FIG. 7. Effect of the upstream SarA box sequence on expression of the −110 promoter fragment. Results shown are CAT activities promoted by the −110 promoter fragment (black bars) and the −110 promoter fragment with the −91 to −100 deletion (white bars). Host strains are indicated along the *y* axis. CAT activity is expressed as nanomoles of chloramphenicol acetylated per minute per milligram of cells (dry weight). Values plotted are the means of at least three determinations with the standard deviations.

the mutant was only about one-third of the activity seen with the −89 wild-type promoter fragment (compare Fig. 6 and 7). The −110 fragment, therefore, contains sequences responsible for reducing *spa* promoter strength that are independent of SarA-SarA box interactions. This sequence is able to exert its dampening effect on transcription in the heterologous host (*E. coli*) as well (Fig. 4).

The above results indicate that loss of either putative SarA binding site results in a loss of SarA responsiveness. The slight increase in promoter activity of pJG3480 in the *sarA*-negative host is consistent with a minimal SarA effect with only the upstream SarA box element present. The promoter strength remains high with the mutant promoter in a *sarS*-minus host, indicating that the requirement of SarS to promote *spa* transcription is lost when SarA binding is prevented. Interestingly, however, there was a modest decline in promoter activity in the *sarA*-negative/*sarS*-negative double mutant hosts. A slight, residual, Agr effect could still be observed with each of the mutant promoters, an activity that is independent of SarS for the upstream deletion mutant (pJG3394) but SarS dependent for the downstream replacement mutation promoter (pJG3480).

The lack of responsiveness to SarS with the SarA box deletion mutants suggests that the primary function of SarS in promoting transcription of *spa* is to relieve SarA-mediated repression, rather than stimulating transcription directly.

SarS-dependent and SarS-independent effects of SarA on *spa* expression. SarA has been shown to affect the expression of *spa* directly, but it also has been shown to be a repressor of SarS expression (8, 42). Therefore, the elevated CAT values observed in the *sarA*-deficient host with the longer *spa* promoter constructs may have been an indirect result from derepression of *sarS* expression. In order to determine if the effects observed with the *sarA*-negative strain of *S. aureus* were due to a direct effect on the *spa* promoter or were due to an indirect effect resulting from elevated SarS levels, we introduced the deletions of the *spa* promoter region into the *sarS*-negative/*sarA*-negative/*agr*$^+$ (KSS5527) and *sarS*-negative/*sarA*-negative/*agr*-negative (KSS5528) hosts. The resulting CAT values are shown in Fig. 3D. The promoter constructs longer than −110 produced an approximately three- to fourfold reduction in CAT values relative to those obtained in the isogenic *sarS*$^+$ host (compare Fig. 3C and D). Thus, *sarS* derepression contributes substantially to the *spa* promoter activity in *sarA*-negative host cells. However, the constructs longer than the −110 still produced approximately fivefold higher values in cells lacking both SarA and SarS than from those hosts in which SarS was absent while SarA was present. The SarA-mediated repression of *spa* expression thus occurs through two pathways, SarS dependent and SarS independent.

An interesting finding was that the promoters containing the promoter activity enhancing sequences (putative UP element) upstream of the core promoter showed a reduced (approximately twofold) promoter strength in the *sarS*-negative/*sarA*-negative double mutant host cells relative to the values obtained with the single mutant host cells (compare the values for the −76 and −89 fragments in Fig. 3D with the corresponding values in B and C). This suggests that SarS can bind to this core promoter-proximal sequence and act directly as a transcriptional activator or better position the UP element.

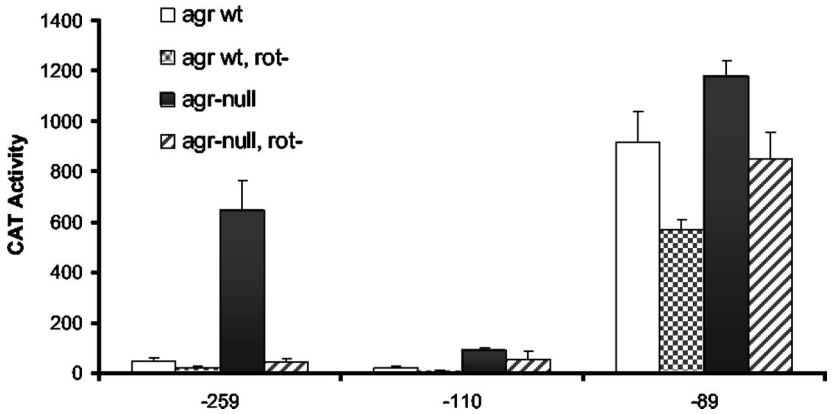

FIG. 8. Effect of Rot on *spa* promoter activities. The *x* axis indicates the upstream boundary of the promoter fragments. Host strains were *agr*$^+$ (white bars), *agr*$^+$/*rot*-negative (stippled bars), *agr*-null (black bars), and *agr*-null/*rot*-negative (hatched bars). Samples were from postexponential-phase culture lysates. CAT activity is expressed as nanomoles of chloramphenicol acetylated per minute per milligram of cells (dry weight). Values plotted are the means of at least three determinations with the standard deviations.

Rot is involved in up-regulation of *spa* expression. The transcriptional regulator, Rot, has been reported to regulate *spa* expression, likely indirectly through control of SarS expression (37). We determined the effect of Rot on our various *spa* promoter constructs. The −259, −110, and −89 constructs were chosen as representatives. If Rot exerts its effect through SarS production, the expectation was that Rot would affect the transcription of the −110 and −259 promoters, because these constructs possessed the SarS binding site. The data (Fig. 8) were consistent with this expectation. The loss of Rot had no appreciable effect on the promoter activity observed with the −89 promoter fragment, but the activities of the −259 and −110 promoters were comparable to those observed with the *sarS*-deficient strains. These results are consistent with Rot acting indirectly on the *spa* promoter through its effects on SarS expression.

DISCUSSION

Protein A is an important virulence determinant in *S. aureus* and is thought to be an important component of the immune evasion machinery of this pathogen. Protein A is expressed during the logarithmic phase of growth in vitro, and it is transcriptionally down-regulated as the cells progress into the postexponential phase of growth. A number of transcriptional regulatory systems impact expression of *spa*, including the Agr and ArlR-ArlS two-component systems, MgrA, SarA, SarS, and SarT. Only SarS and SarA have been shown to directly bind to the *spa* promoter (8, 10, 41, 42). In this study, we have more precisely defined the start site of transcription and characterized the regulatory elements within the *spa* promoter.

We have confirmed the SarA box findings of Chien et al. (10), but we have shown that this site is insufficient for complete repression of *spa* expression by SarA. A second, upstream SarA element (−97 to −91) is required for full repression. The SarS protein, a positive regulator of *spa* transcription, was initially shown to bind to a *spa* promoter-containing fragment extending from −110 to −15 (42). Our functional studies did reveal an effect on *spa* expression on the −110 promoter fragment, but full enhancement of *spa* transcription required additional upstream sequences to −137, indicating that the −110 fragment possesses an incomplete SarS *cis* element.

The requirement for the two SarA boxes in order to demonstrate SarS-mediated activation of transcription indicates that the primary role of SarS is to displace the SarA repressor complex from the promoter. The reduced *spa* promoter strength in the absence of both SarS and SarA argues for an additional role of SarS, namely, directly enhancing transcription from the *spa* promoter, perhaps through a direct interaction with RNA polymerase to facilitate transcription. The activity of SarS to remove SarA from the promoter and the capacity of SarA to suppress SarS production (8) make for a tightly controlled regulatory loop for *spa* expression.

There appear to be two sequence-inherent regulatory sites in the *spa* promoter. The first is a site whose upstream boundary is between −76 and −61 that increases promoter strength. The second site, whose upstream boundary is between −110 and −97, reduces promoter strength. Both elements possess the same activity in *E. coli* host cells. Interestingly, the promoter strength of the fragments possessing the former element displayed reduced activity when the host cells lacked both SarA and SarS, but not with either single mutant host. Binding of Sar proteins can alter the shape of the DNA, inducing bending or overwinding of the DNA (27, 39). The altered DNA topology may have a substantial impact on the functional positioning of the UP element.

The activity of the unregulated core promoter, relative to that of the larger constructs, indicated that the *spa* promoter is under constant regulation, regardless of whether protein A is being maximally produced during exponential growth or maximally repressed during the postexponential phase of growth. The existing model for growth phase regulation of the *spa* promoter suggests that the Agr system acts through a regulation of SarS expression. SarS production is thought to be regulated by the SarA and Agr systems. When the Agr system is activated, *sarT* transcription is repressed, leading to a reduction in SarT levels (38). SarT is a positive regulator of *sarS* transcription. As a consequence of the reduction in SarT activity when the Agr system is activated, SarS levels fall, which

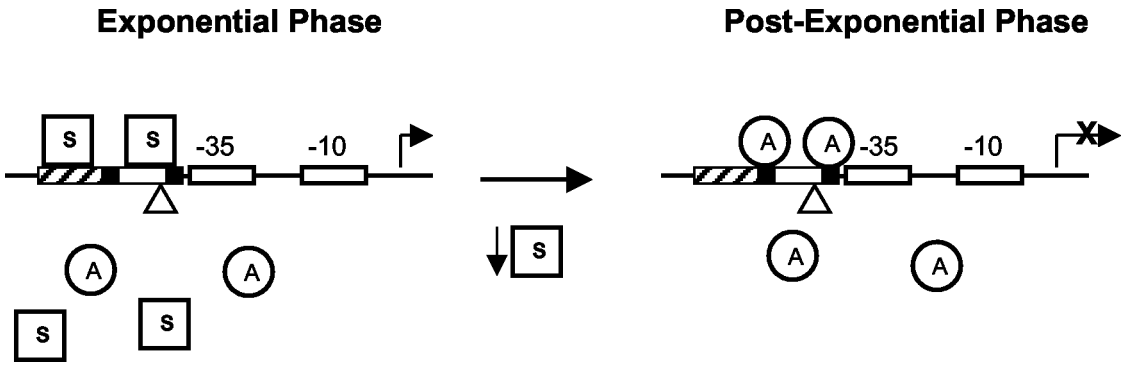

FIG. 9. Model for *spa* promoter regulation. During the exponential phase of growth when the protein A gene is maximally expressed, SarS (squares) is bound to its site (upstream boundary, approximately −137). More than one copy of SarS is likely bound to the promoter (8, 25), and one site of binding appears from this study to be immediately upstream of the core promoter. SarA (circles) is present in the cell (cytoplasmic proteins are depicted below the promoter diagram), but it is displaced from the *spa* promoter by SarS. As the cells enter the postexponential growth phase, activation of the Agr system results in a reduction in SarS production. This frees up the SarA binding sites. SarA binding to the two recognition sites forms the active repression complex. When the cells transition back into the exponential growth phase, SarS is produced and displaces SarA on the *spa* promoter to again form the transcriptionally active complex. The triangle represents the putative UP element sequence. The open boxes represent the *spa* −35 and −10 promoter elements (the core promoter), the black boxes denote the SarA box consensus sequences (−64 to −44 and −97 to −91), and the hatched box represents the SarS binding domain.

leads to a reduction in *spa* transcription. The SarA protein, which is constitutively expressed in *S. aureus*, is thought to affect *spa* transcription directly by binding to the *spa* promoter at the SarA box sequence and indirectly by acting as a repressor of *sarT* transcription (10, 38). The Rot protein is thought to affect *spa* transcription indirectly through its action as a stimulator of *sarS* expression (37).

The work described herein provides for a modification of the *spa* regulatory model (Fig. 9). During the exponential phase of growth, the Agr system is off and SarT and Rot are active. These conditions lead to optimal expression of SarS, which then binds to the SarS binding site in the *spa* promoter. SarS-mediated activation of *spa* transcription is accomplished by preventing the binding of the SarA protein, a repressor of *spa* transcription. The requirement for SarA to occupy two binding sites for repression to occur fits this model. The upstream SarA box, adjacent to the SarS binding region, would be unavailable for SarA binding when SarS was present. SarS may bind in multiple copies to the *spa* promoter, further occluding the SarA binding sites (8). Multiple bound copies of SarS may then lead to either a direct interaction with RNA polymerase to further enhance transcription or a change in the curvature of the DNA, leading to a more active promoter.

When the cells progress into the postexponential phase of growth, activation of the Agr system leads to a reduction in SarT levels and an inactivation of the Rot protein (29, 38). This results in a reduction in SarS production. As the SarS levels in the cell fall, SarA more successfully competes for binding to the *spa* promoter. When SarA occupies both binding sites, *spa* transcription is inhibited and protein A production declines. When favorable growth conditions return, exponential growth is accompanied by the reduction of the Agr RNAIII levels and the increase in SarS production. SarS displaces SarA, and transcription of *spa* ensues.

The use of an interplay between positive and negative regulatory proteins to control *spa* transcription allows for a finer control of the expression of this important protein. Despite what we have learned, gaps in our understanding of *spa* regulation still remain. It is not known how the ArlRS two-component regulatory system or MgrA affects protein A production. Furthermore, the residual Agr effect seen in SarS-negative hosts suggests an additional layer of regulation exists with this promoter.

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REFERENCES

1. **Bayer, M. G., J. H. Heinrichs, and A. L. Cheung.** 1996. The molecular architecture of the *sar* locus in *Staphylococcus aureus.* J. Bacteriol. **178:**4563–4570.
2. **Benito, Y., F. A. Kolb, P. Romby, G. Lina, J. Etienne, and F. Vandenesch.** 2000. Probing the structure of RNAIII, the *Staphylococcus aureus agr* regulatory RNA, and identification of the RNA domain involved in repression of protein A expression. RNA **6:**668–679.
3. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7:**1513–1523.
4. **Blevins, J. S., K. E. Beenken, M. O. Elasri, B. K. Hurlburt, and M. S. Smeltzer.** 2002. Strain-dependent differences in the regulatory roles of *sarA* and *agr* in *Staphylococcus aureus.* Infect. Immun. **70:**470–480.
5. **Cheung, A. L., Y.-T. Chien, and A. S. Bayer.** 1999. Hyperproduction of alpha-hemolysin in a *sigB* mutant is associated with elevated SarA expression in *Staphylococcus aureus.* Infect. Immun. **67:**1331–1337.
6. **Cheung, A. L., A. S. Bayer, G. Zhang, H. Gresham, and Y.-Q. Xiong.** 2004. Regulation of virulence determinants in vitro and in vivo in *Staphylococcus aureus.* FEMS Immunol. Med. Microbiol. **40:**1–9.
7. **Cheung, A. L., K. Eberhardt, and J. H. Henrichs.** 1997. Regulation of protein A synthesis by the *sar* and *agr* loci of *Staphylococcus aureus.* Infect. Immun. **65:**2243–2249.
8. **Cheung, A. L., K. Schmidt, B. Bateman, and A. C. Manna.** 2001. SarS, a SarA homolog repressible by *agr*, is an activator of protein A synthesis in *Staphylococcus aureus.* Infect. Immun. **69:**2448–2455.
9. **Chien, Y., and A. L. Cheung.** 1998. Molecular interactions between two global regulators, *sar* and *agr*, in *Staphylococcus aureus.* J. Biol. Chem. **273:**2645–2652.
10. **Chien, Y., A. C. Manna, S. J. Projan, and A. L. Cheung.** 1999. SarA, a global regulator of virulence determinants in *Staphylococcus aureus*, binds to a conserved motif essential for *sar*-dependent gene regulation. J. Biol. Chem. **274:**37169–37176.

11. **Dossett, J. H., G. Kronvall, R. C. Williams, Jr., and P. G. Quie.** 1969. Antiphagocytic effects of staphylococcal protein A. J. Immunol. **103:**1405–1410.
12. **Dunman, P. M., E. Murphy, S. Haney, D. Palacios, G. Tucker-Kellogg, S. Wu, E. L. Brown, R. J. Zagursky, D. Shlaes, and S. J. Projan.** 2001. Transcriptional profiling-based identification of *Staphylococcus aureus* genes regulated by the *agr* and/or *sarA* loci. J. Bacteriol. **183:**7341–7353.
13. **Forsgren, A.** 1969. Protein A from *Staphylococcus aureus.* Production of protein A by bacterial and L forms of *S. aureus.* Acta Pathol. Microbiol. Scand. **75:**481–490.
14. **Fournier, B., A. Klier, and G. Rapoport.** 2001. The two-component system ArlS-ArlR is a regulator of virulence gene expression in *Staphylococcus aureus.* Mol. Microbiol. **41:**247–261.
15. **Gourse, R. L., W. Ross, and T. Gaal.** 2000. Ups and downs in bacterial transcription initiation: the role of the alpha subunit of RNA polymerase in promoter recognition. Mol. Microbiol. **37:**687–695.
16. **Hart, M. E., M. S. Smeltzer, and J. J. Iandolo.** 1993. The extracellular protein regulator (*xpr*) affects exoprotein and *agr* mRNA levels in *Staphylococcus aureus.* J. Bacteriol. **175:**7875–7879.
17. **Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease.** 1989. Site-directed mutagenesis by overlapping extension using the polymerase chain reaction. Gene **77:**51–59.
18. **Horton, R. M., H. D. Hunt, S. N. Ho, J. K. Pullen, and L. R. Pease.** 1989. Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. Gene **77:**61–68.
19. **Hudson, M. C., and G. C. Stewart.** 1986. Differential utilization of *Staphylococcus aureus* promoter sequences by *Escherichia coli* and *Bacillus subtilis.* Gene **48:**93–100.
20. **Janzon, L., and S. Arvidson.** 1990. The role of the δ-lysin gene (*hld*) in the regulation of virulence genes by the accessory gene regulator (*agr*) in *Staphylococcus aureus.* EMBO J. **9:**1391–1399.
21. **Ji, G., R. C. Beavis, and R. P. Novick.** 1995. Cell density control of staphylococcal virulence mediated by an octapeptide pheromone. Proc. Natl. Acad. Sci. USA **92:**12055–12059.
22. **Kornblum, J., B. N. Kreiswirth, S. J. Projan, H. Ross, and R. P. Novick.** 1990. Agr: a polycistronic locus regulating exoproteins synthesis in *Staphylococcus aureus*, p. 370–402. *In* R. P. Novick (ed.), Molecular biology of the staphylococci. VCH Publishers, New York, N.Y.
23. **Kraemer, G. R., and J. J. Iandolo.** 1990. High-frequency transformation of *Staphylococcus aureus* by electroporation. Curr. Microbiol. **21:**373–376.
24. **Kreiswirth, B. N., S. Lofdahl, M. J. Betley, M. O'Reilly, P. M. Schlievert, M. S. Bergdoll, and R. P. Novick.** 1983. The toxic shock syndrome exotoxin structural gene is not detectably transmitted by a prophage. Nature **305:**709–712.
25. **Li, R., A. C. Manna, S. Dai, A. L. Cheung, and G. Zhang.** 2003. Crystal structure of the SarS protein from *Staphylococcus aureus.* J. Bacteriol. **185:**4219–4225.
26. **Lina, G., S. Jarraud, G. Ji, T. Greenland, A. Pedraza, J. Etienne, R. P. Novick, and F. Vandenesch.** 1998. Transmembrane topology and histidine protein kinase activity of AgrC, the *agr* signal receptor in *Staphylococcus aureus.* Mol. Microbiol. **28:**655–662.
27. **Liu, Y., A. Manna, R. Li, W. E. Martin, R. C. Murphy, A. L. Cheung, and G. Zhang.** 2001. Crystal structure of the SarR protein from *Staphylococcus aureus.* Proc. Natl. Acad. Sci. USA **98:**6877–6882.
28. **Luong, T. T., S. W. Newell, and C. Y. Lee.** 2003. *mgr*, a novel global regulator in *Staphylococcus aureus.* J. Bacteriol. **185:**3703–3710.
29. **McNamara, P., K. C. Milligan-Monroe, S. Khalili, and R. A. Proctor.** 2000. Identification, cloning, and initial characterization of *rot*, a locus encoding a regulatory of virulence factor expression in S*taphylococcus aureus.* J. Bacteriol. **182:**3197–3203.
30. **Novick, R. P.** 2003. Autoinduction and signal transduction in the regulation of staphylococcal virulence. Mol. Microbiol. **48:**1429–1449.
31. **Novick, R. P., H. F. Ross, S. J. Projan, J. Kornblum, B. Kreiswirth, and S. L. Moghazeh.** 1993. Synthesis of staphylococcal virulence factors is controlled by a regulatory RNA molecule. EMBO J. **12:**3967–3975.
32. **Palmqvist, N., T. Foster, A. Tarkowski, and E. Josefsson.** 2002. Protein A is a virulence factor in *Staphylococcus aureus* arthritis and septic death. Microb. Pathol. **33:**239–249.
33. **Patel, A. H., J. Kornblum, B. Kreiswirth, R. Novick, and T. J. Foster.** 1992. Regulation of the protein A-encoding gene in *Staphylococcus aureus.* Gene **114:**25–34.
34. **Patel, A. H., P. Nowlan, E. D. Weavers, and T. Foster.** 1987. Virulence of protein A-deficient and alpha-toxin-deficient mutants of *Staphylococcus aureus* isolated by allele replacement. Infect. Immun. **55:**3103–3110.
35. **Rosey, E. L., and G. C. Stewart.** 1989. The nucleotide sequence of the *lacC* and *lacD* genes of *Staphylococcus aureus.* Nucleic Acids Res. **17:**3980.
36. **Rubin, S. J., and E. D. Rosenblum.** 1971. Effects of recipient strain and ultraviolet irradiation on transduction kinetics of the penicillinase plasmid of *Staphylococcus aureus.* J. Bacteriol. **108:**1192–1199.
37. **Saïd-Salim, B., P. M. Duman, F. M. McAleese, D. Macapagal, E. Murphy, P. J. McNamara, S. Arvidson, T. J. Foster, S. J. Projan, and B. N. Kreiswirth.** 2003. Global regulation of *Staphylococcus aureus* genes by Rot. J. Bacteriol. **185:**610–619.
38. **Schmidt, K. A., A. C. Manna, and A. L. Cheung.** 2003. SarT influences *sarS* expression in *Staphylococcus aureus.* Infect. Immun. **71:**5139–5148.
39. **Schumacher, M. A., B. K. Hurlburt, and R. G. Brennan.** 2001. Crystal structures of SarA, a pleiotropic regulator of virulence genes in *S. aureus.* Nature **409:**215–219.
40. **Shaw, W. V.** 1975. Chloramphenicol acetyltransferase from chloramphenicol-resistant bacteria. Methods Enzymol. **43:**737–755.
41. **Stebra, K. M., S. G. Mackintosh, J. S. Blevins, B. K. Hurlburt, and M. S. Smeltzer.** 2003. Characterization of *Staphylococcus aureus* SarA binding sites. J. Bacteriol. **185:**4410–4417.
42. **Tegmark, K., A. Karlsson, and S. Arvidson.** 2000. Identification and characterization of SarH1, a new global regulator of virulence gene expression in *Staphylococcus aureus.* Mol. Microbiol. **37:**398–409.
43. **Tseng, C.-W., S. Zhang, and G. C. Stewart.** 2004. Accessory gene regulator (Agr) control of staphylococcal enterotoxin D gene expression. J. Bacteriol. **186:**1793–1801.
44. **Zhang, L., L. Gray, R. P. Novick, and G. Ji.** 2002. Transmembrane topology of AgrB, the protein involved in the post-translational modification of AgrD in *Staphylococcus aureus.* J. Biol. Chem. **277:**34736–34742.
45. **Zhang, S., J. J. Iandolo, and G. C. Stewart.** 1998. The enterotoxin D plasmid of *Staphylococcus aureus* encodes a second enterotoxin determinant (*sej*). FEMS Microbiol. Lett. **168:**227–233.
46. **Zhang, S., and G. C. Stewart.** 2000. Characterization of the promoter elements for the staphylococcal enterotoxin D gene. J. Bacteriol. **182:**2321–2325.