Molecular Interactions between Two Global Regulators, sar and agr, in Staphylococcus aureus*

Yueh-tyng Chien† and Ambrose L. Cheung§

From the Laboratory of Bacterial Pathogenesis and Immunology, The Rockefeller University, New York, New York 10021

The expression of many virulence determinants in Staphylococcus aureus is controlled by regulatory loci such as agr and sar. We have previously shown that the SarA protein is required for optimal transcription of RNAII and RNAIII in the agr locus. To define the specific molecular interaction, we overexpressed SarA as a glutathione S-transferase (GST) fusion protein by cloning the 372-base pair (bp) sarA gene into the vector. The purified GST-SarA as well as cleaved SarA were able to bind specifically to the P2, P3, and the combined P2-P3 promoter fragments of agr in gel shift assays. Using monoclonal antibodies to SarA, we found that SarA is a part of the retarded protein-DNA complex as evidenced by the formation of a supershifted band. The SarA binding site on the agr promoter, mapped by DNase I footprinting assay, covered a 29-bp region between the P2 and P3 promoters devoid of any direct repeats. A synthetic 45-bp fragment encompassing the 29-bp sequence also bound the SarA protein in band shift assays. Serial in-frame deletion analysis of sarA revealed that, with the exception of 15 residues in the N terminus, almost all of SarA (residues 16–124) is essential for agr binding activity. Northern analysis confirmed that only the sar mutant clone containing a truncated sarA gene with a 15-residue deletion in the N terminus (SarA16–124) could activate agr transcription to a level approaching that of the full-length counterpart (SarA1–124). Taken together, these data indicated that SarA is a DNA-binding protein with binding specificity to the P2 and P3 interpromoter region of agr, thereby activating RNAII and RNAIII transcription.

Staphylococcus aureus is a major human pathogen with many clinical manifestations (1). Infections due to this organism can range from minor wound infections to severe sepsis. The capacity of S. aureus to cause various infections is probably related to the fact that this organism has an amazing ability to respond to changing environments. The adaptive response is highly coordinated and is generally modulated by regulatory elements via signal transduction pathways. The regulatory elements, in turn, control the transcription of a wide variety of unlinked genes, many of which are involved in pathogenesis (2, 3).

† Recipient of the Irma T. Hirshl Career Scientist Award as well as AHA-Genentech Established Investigator Award from the American Heart Association and to whom correspondence should be addressed.
‡ Supported by a New York Heart Participatory Laboratory Award.
§ Recipient of the Irma T. Hirshl Career Scientist Award as well as AHA-Genentech Established Investigator Award from the American Heart Association and to whom correspondence should be addressed.

Temporal expression of many of the virulence determinants in S. aureus has been shown to be under the control of several genetic loci, agr (accessory gene regulator), sar (staphylococcal accessory regulator), and sae (3–5). The agr locus consists of two divergent transcripts, RNAII and RNAIII, driven by two distinct promoters, P2 and P3, respectively (3). RNAII is the effector of the agr response that involves up-regulation of genes involved in exoprotein synthesis and down-regulation of genes encoding surface proteins (6, 7).

Another regulatory locus, designated sar, was uncovered in our laboratory (4). Unlike agr, the sar locus activates the synthesis of both extracellular (e.g. hemolysins) and cell wall-associated proteins (e.g. fibronectin-binding protein (8, 23)). The sar locus, encoded within a 1.2-kb DNA fragment, encompasses three overlapping transcripts (9). These transcripts, designated sarA (0.58 kb), sarB (0.8 kb), and sarC (1.2 kb), have common 3' ends but three distinct promoters. A major 372-bp ORF (sarA) together with extensive (~800 bp) upstream sequence is present within the largest transcript, sarB. Phyloitic analysis revealed that the sar locus is necessary for hemolysin production, probably mediated by the interaction of sar gene products with the P2 and, to a lesser extent, P3 promoter of agr (10, 11). The ensuing transcription of RNAII and RNAIII would lead to activation of exoprotein gene transcription (7).

In contrast to the RNAIII-mediated control of exoprotein synthesis in agr, we recently reported that the SarA protein likely modulates α-hemolysin production in S. aureus (10). Transcriptional and deletion analyses indicated that the intact SarA protein is required for full agr expression (i.e. RNAII and RNAIII) in S. aureus (10). In this report, we examined the binding of GST-SarA fusion protein as well as purified SarA to the agr promoters. Our results demonstrated that both of these proteins bind to the P2 and P3 promoters of agr in a dose-dependent fashion. The binding affinity of GST-SarA appears to be higher with the P2 than with the P3 promoter. DNase I footprinting analysis of SarA binding to the agr promoter region revealed that the SarA binding site resides in a 29-bp sequence between the P2 and P3 promoter region (~73 to ~101 upstream of the P2 transcription start). Interestingly, the binding site excludes the 7-bp consensus repeat (AGTGAAG) within the P2-P3 interpromoter region previously reported by Morfeld et al. (11). A labeled 45-bp DNA oligonucleotide probe encompassing the 29-bp binding site also bound the GST-SarA fusion protein in a gel retardation assay. In-frame deletion analysis of the SarA protein revealed that, with the exception of the 15 residues in the N terminus, almost all of the SarA protein (residues 16–124) is essential for binding activity to the agr promoter (P2-P3). Northern analysis also confirmed that only the sar mutant clones containing either the full-length

* Supported by a New York Heart Participatory Laboratory Award.
§ Recipient of the Irma T. Hirshl Career Scientist Award as well as AHA-Genentech Established Investigator Award from the American Heart Association and to whom correspondence should be addressed.

The abbreviations used are: kb, kilobase(s); GST, glutathione S-transferase; bp, base pair(s); PCR, polymerase chain reaction; IPTG, isopropyl-1-thio-D-galactopyranoside; MOPS, 4-morpholinepropane-sulfonic acid; nt, nucleotide(s); ORF, open reading frame.
SarA1-124 or the truncated form (SarA16-124) could activate RNAII and RNAIII transcription of the agr locus. Taken together, our data indicated that SarA is a DNA-binding protein with binding specificity to the interpromoter region between P2 and P3 of agr, thereby leading to activation of P2 (RNAII) and P3 (RNAIII) transcription.

EXPERIMENTAL PROCEDURES

**Bacterial Strains, Plasmids, and Growth Media—**The bacterial strains and plasmids used in this study are listed in Table I. Phase ρ1 was used as the transducing phage for *S. aureus* strains. CYPF 0.3 GL and tryptic soy broth media were used for the growth of *S. aureus* (4, 6) while Luria-Bertani (LB) was used for growing *Escherichia coli*. Antibiotics were used at the following concentrations: erythromycin at 5 μg/ml, chloramphenicol at 10 μg/ml, tetracycline at 5 μg/ml, and ampicillin at 50 μg/ml.

**Construction and Purification of GST-SarA Fusion Proteins—**The intact 372-bp sarA gene (13) was amplified by PCR using a plasmid template (pALC70) and primers containing restriction sites (Xhol and BamHI) to facilitate cloning. Likewise, sarA gene fragments coding for the truncated SarA with in-frame deletions in the N and C termini were amplified by PCR. PCR products representing assorted sarA fragments were digested with Xhol and BamHI and ligated into GST vector pGEX-4T-1 (Pharmacia Biotech Inc., Piscataway, NJ). The resulting GST-fusion proteins were detected by anti-SarA monoclonal antibody (see below) on immunoblots, and the purity was analyzed by SDS-polyacrylamide gel electrophoresis. Purified SarA protein or homogeneous GST-SarA fusion proteins were detected by anti-SarA monoclonal antibody (see below) on immunoblots, and the purity was analyzed by SDS-polyacrylamide gel electrophoresis. Purified SarA protein and GST fusion proteins were detected by anti-SarA monoclonal antibody (see below) on immunoblots, and the purity was analyzed by SDS-polyacrylamide gel electrophoresis. Purified SarA protein or homogeneous GST-SarA fusion proteins were used for the gel shift and footprinting assays.

**Production of Anti-SarA Monoclonal Antibodies—**Purified SarA protein was authenticated by determining the 15 residues at the N terminus of the truncated SarA with in-frame deletions in the N and C termini were digested with Xhol and BamHI and ligated into GST vector pGEX-4T-1 (Pharmacia Biotech Inc., Piscataway, NJ). The resulting GST-fusion proteins were detected by anti-SarA monoclonal antibody (see below) on immunoblots, and the purity was analyzed by SDS-polyacrylamide gel electrophoresis. Purified SarA protein or homogeneous GST-SarA fusion proteins were detected by anti-SarA monoclonal antibody (see below) on immunoblots, and the purity was analyzed by SDS-polyacrylamide gel electrophoresis. Purified SarA protein and GST fusion proteins were detected by anti-SarA monoclonal antibody (see below) on immunoblots, and the purity was analyzed by SDS-polyacrylamide gel electrophoresis. Purified SarA protein or homogeneous GST-SarA fusion proteins were used for the gel shift and footprinting assays.

**DNase I Footprinting—**Binding reactions were performed as described for the gel mobility shift assay except that a total volume of 100 μl was used. DNase I (Boehringer Mannheim) (0.01 unit) was added and incubated for 2 min at room temperature. The reaction was terminated by adding 100 μl of freshly made stop solution (50 mM Tris-HCl (pH 8.0), 2% (w/v) SDS, 10 mM EDTA, proteinase K at 0.4 μg/ml, and glycogen at 100 μg/ml). The reaction mixture was extracted with phenol-chloroform. DNA was ethanol-precipitated, washed with 70% ethanol, and resuspended in loading buffer (98% deionized formamide, 10 mM EDTA, 0.025% (w/v) xylene cyanol FF, and 0.025% (w/v) bromphenol blue). DNA was denatured at 95 °C for 3 min and run on a 6% sequencing gel. Chemical cleavage at purine (A+G) residues were performed by the standard method (19).

**RESULTS**

**Purification of GST-SarA Fusion Proteins Produced in *E. coli*—**The intact 372-bp sarA gene was cloned in-frame in GST fusion vector pGEX-4T-1 and overproduced in *E. coli* DH5α. The expressed recombinant fusion protein contained a 26-kDa GST protein fragment followed by a thrombin cleavage site and the SarA protein. Soluble fraction of the cell lysate from *E. coli* that had been induced with IPTG was prepared and purified on a GST affinity column as described under “Experimental Procedures.” The soluble crude extract and the purified protein were analyzed by SDS-polyacrylamide gel electrophoresis. As shown in Fig. 1A, the purified GST-SarA fusion protein (41.5 kDa) and the SarA protein (14.5 kDa) generated by cleavage with thrombin were essentially homogeneous. The identity of these proteins was confirmed by immunoblots with monoclonal antibody 1D1 raised against SarA (Fig. 1B). These protein preparations were used in subsequent band shift and footprinting assays (see below).

**Binding of SarA to agr P2 and P3 Promoters—**Indirect bio-
chemical evidence with crude extracts from *S. aureus* mutant clones containing *sar* fragments from our laboratory (10, 20) suggested that SarA may interact with the *agr* locus by binding to the P2 promoter. To examine further the interaction between SarA and *agr* promoter, purified recombinant GST-SarA and SarA from *E. coli* were used in band shift assays with *agr* P2 and P3 promoters. As shown in Fig. 2, A and B, the purified GST-SarA fusion protein bound to the *agr* P2 and P3 promoters in a dose-dependent fashion, with \( \geq 50\% \) retardation for P2 and P3 promoter probes by adding 1 and 10 \( \mu \)g of the fusion protein, respectively. With the combined P2-P3 promoter fragment (Fig. 2C), binding activity of GST-SarA appeared to be increased (\( \geq 50\% \) band shift with 0.5 \( \mu \)g of protein). The estimated dissociation constant \( (K_d) \) for the P2 promoter (18 \( \mu \)M) was lower than its P3 (190 \( \mu \)M) counterpart, thus implying that SarA binds with higher affinity to the P2 than to the P3 *agr* promoter. Remarkably, the combined P2-P3 promoter has the highest affinity for the SarA protein \( (K_d = 6 \text{ nM}) \). The addition of unlabeled P2-P3 fragment interfered with the formation of the binding complex between the labeled probe and the GST-SarA fusion protein (Fig. 2C, lanes 7–12) while increasing concentrations of a 165-bp *nifH2* promoter region from *Methanosarcina barkeri* or calf thymus DNA had no effect (data not shown). We also tested the binding of the full-length SarA (GST portion removed by thrombin digestion) to the *agr* P2-P3 promoter (Fig. 2D). As anticipated, purified SarA was able to bind to the P2-P3 promoter, with the binding pattern similar to that of the fusion protein (Fig. 2, C and D).

![Figure 1](https://example.com/image1.png)

**Figure 1.** Coomassie Blue-stained SDS-polyacrylamide gel (A) showing GST-SarA and the full-length SarA generated by the cleavage of thrombin and the corresponding immunoblot (B) with 1D1 anti-SarA monoclonal antibody. Lane 2, crude lysate prepared from *E. coli* (strain ALCl137) as described under “Experimental Procedures”; lane 3, purified GST-SarA eluted from the GST-Sepharose affinity column; lane 4, purified SarA. The molecular mass standards (SeeBlue, Novex) are 250, 98, 64, 50, 36, 30, 16, and 6 kDa (lanes 1 and 5).

![Figure 2](https://example.com/image2.png)

**Figure 2.** Binding of purified GST-SarA to *agr* P2 (A) and P3 (B) promoters. The labeled 152-bp P2 (nt 1622–1773 according to published sequence) (3) or 122-bp P3 (nt 1539–1660) fragment was incubated with 0, 0.5, 1, 3, 5, or 10 \( \mu \)g of purified GST-SarA (lanes 1–6) in the presence of 2 \( \mu \)g of poly(dI-dC)-poly(dI-dC) followed by electrophoresis through a 5\% polyacrylamide gel (see “Experimental Procedures”). C, specificity of SarA binding to the 235-bp P2-P3 promoter fragment (nt 1539–1773) of *agr*. In competition assay, various amounts of unlabeled P2-P3 fragment were added to a tube containing 0.3 \( \mu \)g of labeled *agr* P2-P3 fragment prior to the addition of purified GST-SarA. *Lanes 1–6* show the binding of increasing amounts of GST-SarA (0, 1, 2, 3, 4, and 5 \( \mu \)g) to the P2-P3 promoter fragment; *lanes 7–12* show reactions containing labeled P2-P3 DNA, 2 \( \mu \)g of GST-SarA and 0 ng (lane 8), 20 ng (lane 9), 40 ng (lane 10), 60 ng (lane 11), and 100 ng (lane 12) of unlabeled P2-P3 promoter fragment; *lane 7* contains the labeled probe alone. D, the binding of purified SarA to labeled P2-P3 promoter fragment. In *lanes 1–7*, 0, 0.5, 1, 3, 5, 7, and 10 \( \mu \)g of purified SarA was added per assay.

The Effect of Anti-SarA Antibody on Gel Shift Assays—The data above indicated that recombinant SarA from *S. aureus* is capable of binding to the *agr* promoter. To show that SarA is truly a part of the retarded protein-DNA complex, we used four anti-SarA monoclonal antibodies (1D1, 1F7, 3H2, and 8F3) to detect its presence. By incubating these four anti-SarA monoclonal antibodies with the reaction mixture containing SarA and labeled P2-P3 probe, we discovered that they bound to the retarded SarA-DNA complex (Fig. 3) as evidenced by the formation of a supershifted complex in each instance (lanes 3, 6, 9, and 12). As a control, the addition of antibody alone without SarA did not produce any shifted band, suggesting that the supershifted band is not due to the binding of antibody to the *agr* promoter probe (Fig. 3, lanes 4, 7, 10, and 13).

**Mapping the P2 and P3 Binding Sites of SarA with DNase I**
Protection Assay—Our band shift assays have shown that SarA binds specifically to the P2 and P3 promoter region of agr. To determine the binding site more precisely, we analyzed protein-DNA complexes by the DNase I protection assay (19). Binding to both top and bottom strands was examined using PCR-amplified templates. When ~50 μg of purified GST-SarA was used, a large region of protection was observed on both strands (Fig. 4, A and B). In both instances, the protected regions are identical and correspond to nt −73 to −101 upstream of the P2 transcription start site (−83 to −111 upstream of P3), covering a 29-bp region (Fig. 4C). We also performed the footprinting experiments with purified SarA derived from thrombin-cleaved GST-SarA. The protection pattern is essentially the same (data not shown).

Binding of SarA to a 45-bp DNA Fragment Containing the Protected DNA Region Identified by Footprinting Analysis—The transcription starts between two agr promoters (P2 and P3) are separated by a 186-bp sequence containing several direct and inverted repeats. The upstream regions of P2 and P3 are very similar in that each contains duplications of the heptanucleotides 5′-AGTTAAG-3′ (Fig. 4C). These repeats have been suggested to be the binding site for a common regulatory protein, with SarA being a potential candidate (11). However, our footprinting analysis indicated that the SarA protein binds to a 29-bp region located between the P2 and P3 promoters. This region does not contain the putative repeats. To confirm our footprinting result, we performed band shift assays with a labeled 45-bp oligonucleotide probe spanning the 29-bp SarA binding site. As shown in Fig. 5, the 45-bp DNA fragment was retarded by the GST-SarA fusion protein in a dose-dependent manner (Fig. 5B). In contrast, a 103-bp fragment containing the repeats but not the SarA binding site failed to bind to the SarA protein (Fig. 5C). These data strongly suggest that the direct repeats are not the binding site for SarA. To evaluate the specificity of this binding, we also performed a series of competition assays with combinations of labeled and unlabeled DNA. The results showed that the unlabeled 45-bp DNA was able to compete with labeled P2, P3, or 45-bp DNA fragments for the binding to the SarA protein (data not shown).

Mapping the DNA-binding Domain of the SarA Protein—To map the DNA-binding domain of SarA to the agr P2 and P3 promoters, we constructed an assortment of GST fusion proteins spanning full-length to truncated forms of SarA (Fig. 6A). These constructs were expressed with IPTG induction and purified to homogeneity as described under Experimental Procedures.” Gel shift experiments were used to test interactions of these truncated fusion proteins with the agr promoter seq-
sequence (P2-P3) in an attempt to determine the minimal SarA protein sequence that can function as a DNA-binding protein (Fig. 6B). N-terminal truncations up to 15 residues (ALC1133) still bound the P2-P3 promoter (Fig. 6B, lane 7), but truncations extending to 25 residues or more (ALC1218, ALC1193, ALC1132, and ALC1054) did not yield any protein-DNA complex. A C-terminal truncation of as little as 11 residues (ALC1176) failed to bind to the P2-P3 promoter probe (Fig. 6B).

These results suggested that an extensive region extending from residue 15 to the C terminus of SarA is required for DNA binding to the agr promoter.

Northern Analysis of RNAII and RNAIII Expression in Various sarA-deletion Constructs—To evaluate whether in vitro binding of truncated SarA protein to the agr promoter region (Fig. 6B) correlates with activation of RNAII and RNAIII in S. aureus cells, we assayed for agr-related transcription in sar mutant clones (see Table I, ALC70, ALC1273, and ALC1274).

To serve as the standard against which in-frame deletions of sarA can be compared, we utilized a previously constructed sarA mutant clone carrying a recombinant shuttle plasmid (pSPT181) that contained the entire sarA locus including sarA and extensive upstream sequence (~800 bp). Consequently, activation of RNAII and RNAIII in this clone (ALC70) has been shown to be optimal (10). We constructed two in-frame mutations corresponding to N15 and N25 (Fig. 6A) to yield sarA mutant clones ALC1273 and ALC1274. To ensure translation of the full-length or truncated SarA protein in these deletion clones, we utilized anti-SarA monoclonal antibody to confirm the presence of truncated SarA proteins in cell extracts prepared from these S. aureus clones (Fig. 7A). As expected, clone ALC70 containing the intact sarA gene in a multicopy plasmid expressed the full-length SarA (14.5 kDa) while ALC1273 (15-amino acids deletion) and ALC1274 (25-amino acids deletion) yielded truncated SarA protein of ~13 and 12 kDa, respec-
A lower level of SarA expression in parental strain RN6390 as compared with ALC70 (multicopy plasmid) may be attributable to the single copy effect. Based on our observation that truncated SarA protein with a 15-amino acid truncation in the N terminus (N15) was able to bind the agr P2-P3 promoter (Fig. 6B), we also determined that strain ALC1273, containing this form of truncated SarA, was sufficient for restoring RNAII and RNAIII expression in a sar mutant (Fig. 7B, lane 2), with levels approaching that of the wild type. Northern blots of RNAII and RNAIII (C) in sar mutant clones with in-frame sarA deletion. Lane 1, ALC70; lane 2, ALC1273 with N15 truncated SarA; lane 3, ALC1274 with N25 truncated SarA; lane 4, parent RN6390; lane 5, sar mutant ALC136; lane 6, ALC475, ALC136 with control vector pSPT181.

**DISCUSSION**

Expression of virulence determinants in *S. aureus* is dependent in part on two global regulatory loci, i.e. sar and agr. Recognizing that the sar locus is composed of three overlapping transcriptional units (sarA, C, and B), we have assayed previously the binding of agr promoter fragment to crude cell extracts from sar mutant clones containing a single copy of the respective transcriptional units (10). Coupled with *in vitro* truncation in the N terminus failed to activate transcription from the agr promoter (Fig. 7B, lane 3). Taken together, these data indicated that the minimum sarA sequence expressed in ALC1273 was adequate for agr expression in a sar mutant.
translation studies, these antecedent data provide evidence that the SarA protein likely regulates hemolysin production by modulating RNAII and RNAIII transcription of agr. However, experimental evidence linking direct binding of SarA to the agr promoter region is lacking. In this report, we presented gel shift and footprinting data showing that purified SarA binds specifically to the agr P2-P3 promoter region. Dissociation constant values ($K_d$) derived from dose response analysis imply that the SarA protein binds with higher affinity to the P2 than to the P3 promoter (e.g. 1 µg versus 10 µg of GST-SarA for 50% retardation of the labeled P2 and P3 probes, respectively; Fig. 2, A and B). The differential affinity in promoter binding of SarA concurs with our earlier observation that the effect of a sar mutation was more pronounced on RNAII (P2) than on RNAIII (P3) transcription (10). However, a careful scrutiny of these $K_d$ values (6, 18, and 190 nm for P2-P3, P2, and P3, respectively) reveals relatively low binding affinities between SarA and the agr promoters. Consistent with this finding was our recent observation that a sar mutant clone complemented with a single copy of the sarB transcript (encoding SarA and additional smaller ORFs upstream) produced a higher level of SarA and the agr promoter activity (10). Consequently, we speculate that SarA, in conjunction with additional factors encoded by sequences upstream of sarA, may be required for optimal binding to the agr promoter. In this regard, we have preliminary mutagenesis data to suggest that the 39-aa ORF immediately upstream of sarA may be required for full agr expression. Alternatively, sarA-independent regulatory factors may be involved in this interaction.

DNase I footprinting analysis has uncovered the SarA binding site to be a 29-bp sequence located in the interpromoter region between P2 and P3 (Fig. 4). Several relevant observations can be deduced from this experiment. First, the finding that the binding site is more proximal to the P2 than to the P3 −35 promoter box (Fig. 4C) would be compatible with the hypothesis that the SarA protein probably modulates P2 more than P3 transcription. Alternatively, other factors may be required to bind cooperatively with SarA to the P3 promoter (21). Second, this sequence does not encompass any of the 7-bp repeats (AGTTAAG) that have been suggested by Morfeldt et al. (11) to be the putative binding site for SarA protein. In support of our premise is the finding that a 45-bp labeled fragment encompassing this 29-bp sequence but lacking any of the repeats bound to the SarA protein, whereas a 103-bp fragment containing two of the repeats did not bind (Fig. 5). Third, in divergence to the proposed repeats leading to multiple binding sites and hence a ladder-like pattern of band shift (11), the footprinting data as well as the formation of a single retarded DNA-protein complex (Fig. 2, A, B, and D) presented here strongly substantiate a single binding site when purified SarA protein was used for the binding assay. The reason for the disparate results may arise from the fact that purified protein was used in our assays, whereas crude affinity purified extracts were used in theirs (11). Alternatively, the crude extract employed by Morfeldt et al. (11) may be contaminated with additional DNA binding proteins. In this regard, our group has purified another distinct 13-kDa DNA-binding protein with binding specificity to the agr promoter region. Preliminary N-terminal sequencing revealed some degree of homology to the SarA protein. Amazingly, this protein almost co-migrated with the SarA protein in SDS-gel.2

The identity of a single SarA-agr complex also corroborated with the formation of a supershifted band upon the addition of anti-SarA monoclonal antibodies to the retarded protein-DNA complex (Fig. 3). A careful scrutiny of the supershifted complexes revealed two distinct modes of interactions. The monoclonals 1F7, 3H2, and 8F3 have distinct supershifted bands that migrated more slowly than the SarA-agr promoter complex. In contrast, the antibody 1D1 had a diffuse supershifted complex, consistent with partial disruption of the SarA-DNA complex. Preliminary mapping of these antibodies revealed that the epitope for monoclonals 1F7, 3H2, and 8F3 resides more toward the C-terminal segment, whereas 1D1 is directed toward the middle portion of the SarA molecule. Conceivably, monoclonal 1D1 may interfere with the DNA-binding domain of the SarA molecule, thus resulting in partial disruption of the protein-DNA complex. Clearly, additional experiments need to be performed to validate this hypothesis. In mapping the SarA DNA-binding domain by serial truncations of the sarA gene (Fig. 6), we found that only GST fusions containing the full-length SarA or a minimally truncated form (SarA16–124) could bind to the agr promoter on gel shift assays. Based upon the size (29 bp) of the SarA recognition site (Fig. 4), it is rather unlikely that the actual DNA-binding domain entails almost the entire SarA molecule. This premise leads us to speculate that, with the exception of 15 residues in the N terminus, the remaining residues in SarA must contribute to the proper conformation of the DNA-binding domain of the molecule to facilitate binding to the agr promoter. Clearly, further mutagenesis studies are needed to exactly define the DNA-binding domain of SarA. Of interest is the fact that SarA1–113 (C11 in Fig. 6B), representing an 11-residue truncation in the C terminus, constitutes the previously reported SarA molecule of S. aureus strain RN6390 (9) while the full-length SarA1–124 is identical to that of S. aureus strains DB and RN450. The failure of GST-SarA1–113 (C11) to bind to agr promoter in vitro (Fig. 6B) corroborated with our previous data in which the cell extract of a sar mutant clone containing the truncated sarA gene either as a recombinant multicopy plasmid (20) or as a single copy chromosomal insertion (10) did not bind to the agr promoter fragment in gel shift assays. To resolve this discrepancy, we resequenced the sarA fragment from strain RN6390 and concluded that a spontaneous mutation (CGA to TGA) may have occurred in the E. coli clone containing the sarA gene of strain RN6390. This was confirmed by directly sequencing the sarA PCR product generated with chromosomal template of strain RN6390 and high fidelity Pfu polymerase.

In agreement with our in vitro binding data (Fig. 6), Northern analysis of S. aureus sar mutant clone (ALC1273) containing the minimally truncated SarA16–124 revealed that this clone could activate RNAII and RNAIII transcription to a level approaching that of the full-length SarA counterpart (ALC70). As predicted from the gel shift assay, the clone containing the N25 deletion in sarA (ALC1274) did not activate RNAII and RNAIII transcription as effectively as the intact SarA control (Fig. 7, B and C, lanes 3 and 1). However, unlike the gel shift data, the SarA16–124 protein was still capable of augmenting RNAII transcription at a low level as compared with the sar mutant control (ALC136), indicating that the truncated SarA molecule (Fig. 7A, lane 3) was capable of low level agr activation. This disparity is not surprising because we have previously found that Northern analysis for agr-related transcription is more sensitive than comparable gel-retardation assays (10, 20).

Attributable to its pleiotropic nature, the SarA protein is likely to be a DNA-binding protein to multiple promoters. Our

---

2 Y.-t. Chien and A. L. Cheung, unpublished data.

3 Y.-t. Chien and A. L. Cheung, unpublished observation.
study here has clearly shown that the SarA binding site on the agr promoter resides in a 29-bp sequence that is extremely AT-rich (26/29 or 89.6%). This degree of AT richness is highly unusual even for *S. aureus*. Consequently, it is unlikely that this sequence is found randomly at a high frequency in the staphylococcal chromosome. We speculate that this may be a common domain for SarA binding in *sarA*-dependent promoters. Clearly, a lot of work remains to be done to extend this hypothesis. More recently, we have cloned and sequenced a *sarA* homolog in *Staphylococcus epidermidis* (24). Knowing that an agr homolog exists in coagulase negative species (22, 25), it will be of interest to determine if a similar SarA binding site occurs in *S. epidermidis* and other related pathogenic species.

REFERENCES
1. Crossley, K. B., and Archer, G. L. (1997) *The Staphylococci in Human Disease*, Churchill Livingston, New York
2. Waldvogel, F. A. (1985) in *Principles and Practice of Infectious Diseases* (Mandell, G. L., Douglas, R. G. J., and Bennett, J. E., eds) pp. 1097–1116, John Wiley & Sons, New York
3. Kornblum, J., Kreiswirth, B., Projan, S. J., Ross, H., and Novick, R. P. (1990) in *Molecular Biology of the Staphylococci* (Novick, R. P., ed) pp. 373–402, VCH Publishers, New York
4. Cheung, A. L., Kooney, J. M., Butler, C. A., Projan, S. J., and Fischetti, V. A. (1992) *Proc. Natl. Acad. Sci. U. S. A.* 89, 6462–6466
5. Giraud, A. T., Cheung, A. L., and Nagel, R. (1997) *Arch. Microbiol.* 168, 53–58
6. Novick, R. P., Ross, H. F., Projan, S. J., Kornblum, J., Kreiswirth, B., and Moghazeh, S. (1993) *EMBO J.* 12, 3967–3977
7. Morfeldt, E., Taylor, D., von Gabain, A., and Arvidson, S. (1995) *EMBO J.* 14, 4569–4577
8. Cheung, A. L., Eberhardt, K. J., Chung, E., Yeaman, M. R., Sullam, P. M., Ramos, M., and Bayer, A. S. (1994) *J. Clin. Invest.* 94, 1815–1822
9. Bayer, M. G., Heinrichs, J. H., and Cheung, A. L. (1996) *J. Bacteriol.* 178, 4563–4570
10. Cheung, A. L., Bayer, M. G., and Heinrichs, J. H. (1997) *J. Bacteriol.* 179, 3963–3971
11. Morfeldt, E., Tegmark, K., and Arvidson, S. (1996) *Mol. Microbiol.* 21, 1227–1237
12. Cheung, A. L., Eberhardt, K., and Heinrichs, J. H. (1997) *Infect. Immun.* 65, 2243–2249
13. Cheung, A. L., and Projan, S. J. (1994) *J. Bacteriol.* 176, 4168–4172
14. Jones, K. F., Khan, S. A., Erickson, B. W., Hollingshead, S. A., Scott, J. R., and Fischetti, V. A. (1986) *J. Exp. Med.* 164, 1226–1238
15. Schenk, S., and Laddaga, R. A. (1992) *FEBS Microbiol. Lett.* 94, 133–138
16. Towbin, H., Staehelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U. S. A.* 76, 4350–4354
17. Blake, M. S., Johnston, K. H., Russell-Jones, G. J., and Gotschlich, E. C. (1984) *Anal. Biochem.* 136, 175–179
18. Cheung, A. L., Eberhardt, K., and Fischetti, V. A. (1994) *Anal. Biochem.* 222, 511–514
19. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1989) *Molecular Cloning, a Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
20. Heinrichs, J. H., Bayer, M. G., and Cheung, A. L. (1996) *J. Bacteriol.* 178, 418–423
21. Vandenesch, F., Kornblum, J., and Novick, R. P. (1991) *J. Bacteriol.* 173, 6313–6320
22. Vandenesch, F., Projan, S. J., Kreiswirth, B., Etienne, J., and Novick, R. P. (1993) *FEMS Microbiol. Lett.* 111, 15–22
23. Cheung, A. L., and Wolz, C. (1997) *Abstracts of the 37th ICAAC*, B60, Toronto, Canada
24. Fluckiger, U., and Cheung, A. L. (1997) *Abstracts of the Annual Meeting of the American Society for Microbiology*, B32, Miami, FL
25. Van Wamel, W., Fluit, A. C., and Verhoef, J. (1996) *Abstracts of the Annual Meeting of the American Society for Microbiology*, B338, New Orleans, LA
Molecular Interactions between Two Global Regulators, \textit{sar} and \textit{agr}, in \textit{Staphylococcus aureus}

Yueh-tyng Chien and Ambrose L. Cheung

\textit{J. Biol. Chem.} 1998, 273:2645-2652.
doi: 10.1074/jbc.273.5.2645

Access the most updated version of this article at http://www.jbc.org/content/273/5/2645

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 18 references, 9 of which can be accessed free at http://www.jbc.org/content/273/5/2645.full.html#ref-list-1