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Ambrose L. Cheung
Dartmouth College

Adhar C. Manna
Dartmouth College

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Role of the Distal *sarA* Promoters in SarA Expression in *Staphylococcus aureus*

Ambrose L. Cheung and Adhar C. Manna*

Department of Microbiology and Immunology, Dartmouth Medical School, Hanover, New Hampshire 03755

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The global regulatory locus *sarA* comprises a 375-bp open reading frame that is driven by three promoters, the proximal P1 and distal P3 and P2 promoters. We mutated the weaker P3 and P2 promoters to ascertain the effect of the change on SarA protein and target gene expression. Our results indicated that the solely active P1 promoter led to a lower SarA protein level, which has an effect on *agr* transcription and subsequently had corresponding effects on *hla*, *sspA*, and *spa* transcription, probably in both *agr*-independent and *agr*-dependent manners.

Temporal regulation of virulence determinants in *Staphylococcus aureus* has been shown to be under the control of global regulators such as the two component regulatory systems and the SarA protein family (2). The prototypic member of the SarA protein family is the SarA protein, a 14.5-kDa DNA-binding protein that modulates the transcription of specific regulatory loci (e.g., *agr*) (16), as well as downstream target genes (e.g., the alpha-hemolysin and protein A genes) (5). Indeed, transcriptional profiling studies by Dunman et al. (8) demonstrated that the *sarA* locus probably controls target genes both directly and indirectly (i.e., via other regulatory loci). Accordingly, the expression of *sarA* and its effect on target genes likely entail significant metabolic expenditure by the bacteria. It is therefore not surprising that *sarA* expression is tightly controlled during the growth cycle, presumably via regulatory inputs to the *sarA* promoter (2).

Contrary to the terse promoters of many prokaryotic genes, the *sarA* promoter region is extensive (~800 bp), comprising three promoters (P2, P3, and P1) that yield three distinct overlapping transcripts (*sarA* P2, P3, and P1 transcripts), each encoding the SarA protein (Fig. 1) (1). The *sarA* P1 and P2 promoters are σ^A dependent and are activated mostly during the exponential phase, while the P3 promoter is σ^B dependent and is active postexponentially, coinciding with the maximal predicted activity of σ^B during periods of metabolic stress (4). Embedded within the triple *sarA* promoter are both direct and indirect repeats (13), thus leading us to initially theorize that this region may constitute binding sites for regulatory proteins. A subsequent search for DNA-binding proteins with a solid-phase column containing the *sarA* promoter yielded SarR, a homolog of SarA that binds predominantly to the *sarA* P1 promoter region (14). Transcriptional and immunoblot analyses disclosed that SarR likely represses the *sarA* promoter to down-regulate SarA protein expression (14). Thus, SigB and SarR can modulate SarA expression by differential binding to the *sarA* promoter during growth (2).

Transcriptional analysis with XylE fusions divulged that the *sarA* P1 promoter and the native P2-P3-P1 promoters are most predominant (13). However, the contribution of the P2 and P3 promoters to the proximal P1 promoter has not been defined (Fig. 1A). Interestingly, a potential smaller coding region nested within the P3-P1 promoter (previously designated open reading frame 3 [ORF3]) has been shown to modulate *sarA* expression (Fig. 1) (3, 7). To dissect the contribution of the P2 and P3 promoters and to ascertain the role of the putative ORF3 sequence in *sarA* expression (13), we undertook in this study a mutational analysis of the *sarA* promoter. We introduced single copies into the staphylococcal chromosome of an *orf3 sarA* deletion mutant, focusing in particular on the contribution of the P2 and P3 promoters in modulating the activities of the stronger P1 promoter.

Characterization of a *sarA* deletion mutant construct containing a *sarA* fragment with an active *sarA* P1 promoter but inactive P2 and P3 promoters. In our previous studies, we found that a *sarA* fragment containing the native triple promoter together with the *sarA* coding region was able to complement a *sarA* transposon mutant, ALC136 (3). However, deletion analysis indicated that the *sarA* P3-P1 promoter and the intact P2-P3-P1 promoters are more active than the P1 promoter alone (13). We subsequently found that a *sarA* fragment containing the P2-P3-P1 promoters, but lacking the putative ORF3 sequence (Fig. 1A), on a shuttle plasmid resulted in a significant decrease in the SarA protein level compared with the non-deletion-containing control while the effect of deletion of putative ORF4 nested within the P2-P3 promoter was much less (7). To differentiate the effect of the putative ORF3 sequence from those of the P3 and P2 promoters on SarA expression and to avoid the issue of gene copy number, we undertook a site-directed mutagenesis approach in which we mutated the −10 and −35 promoter boxes of the *sarA* P2 and P3 promoters while leaving the P1 promoter intact. We also separately introduced stop codons and a deletion of the putative ORF3 sequence (nucleotides 582 to 678) (1) into a similarly sized *sarA* fragment (Fig. 1A) to examine the effect of a single copy of putative ORF3 on

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Dartmouth Medical School, Vail 205, Hanover, NH 03755. Phone: (603) 650-1310. Fax: (603) 650-1362. E-mail: Adhar.C.Manna@Dartmouth.edu.

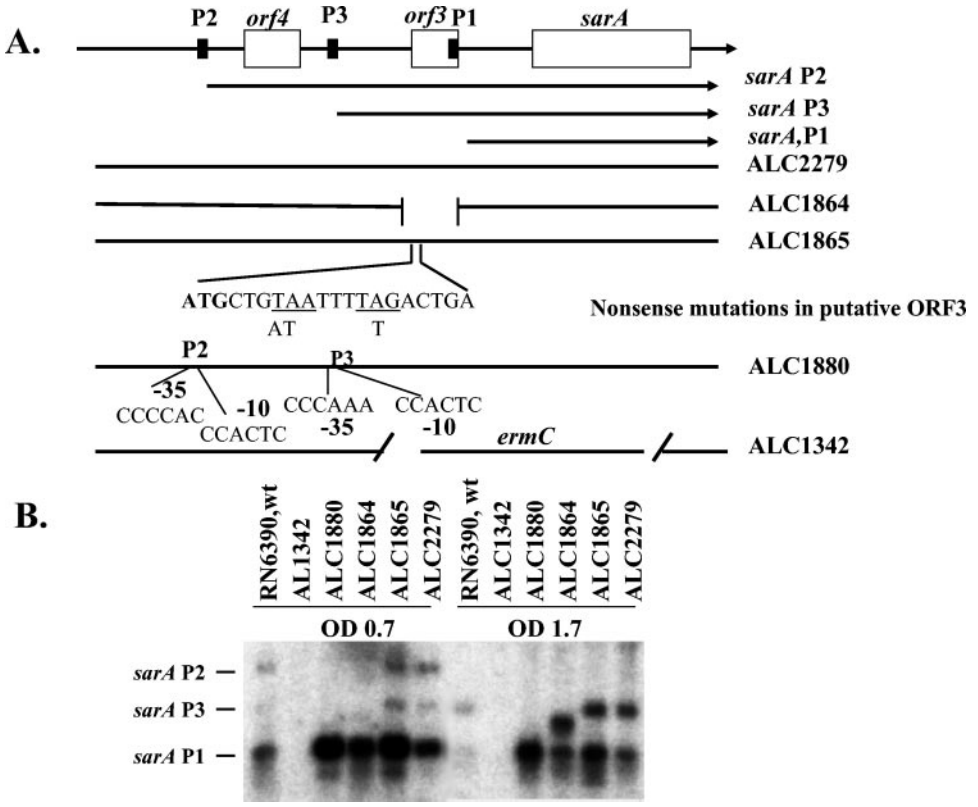

FIG. 1. Construction of *sarA* deletion mutants complemented with various *sarA* fragments in single copy via integration into the lipase gene (*geh*). (A) Schematic representation of the mutated *sarA* fragment constructs. (B) Northern blot assays of various mutant constructs at mid-log (optical density [OD] at 650 nm = 0.7) and early stationary (optical density at 650 nm = 1.7) phases probed with the *sarA* gene probe. wt, wild type.

sarA expression. As the recipient for these mutated fragments, we constructed a *sarA* mutant (ALC1342 [Δ*orf3sarA*::*ermC*]) with a deletion of ORF3 and the *sarA* coding region to ensure that the genetic background was null for *sarA* and *orf3*. Accordingly, the mutated fragments were cloned into integration vector pCL84 (Tet^r) and electroporated into *S. aureus* strain CYL316, a derivative of RN4220 with the integrase gene provided in *trans*. Lipase-negative transformants, resulting from integration of recombinant pCL84 into the lipase gene (12), were selected on tetracycline and egg yolk agar plates. After Southern confirmation, the correct transformants were infected with phage φ11 and the lysate used to infect deletion mutant ALC1342 (Δ*orf3sarA*::*ermC*) to yield transductants. The authenticity of the transductants was confirmed by Southern blot assays with *tetK* and lipase gene probes. As shown in Fig. 1B, the deletion mutant complemented with a single copy of the native *sarA* fragment with mutated P2 and P3 promoters (ALC1880) expressed only the *sarA* P1 transcript while the normally complemented mutant (ALC2279) was able to express all three *sarA* transcripts. As expected, the complemented mutant carrying a stop codon of the putative ORF3 yielded all three *sarA* transcripts (ALC1865) while the corresponding mutant lacking the putative ORF3 sequence had a smaller P3 but an intact P1 transcript (ALC1864).

Effect of the *sarA* fragment with an active P1 promoter but inactive P2 and P3 promoters. To determine if activation of *sarA* P1 but not the P2 and P3 promoters would alter SarA protein expression, we probed an immunoblot of the whole-cell lysate of the mutant complemented with a *sarA* fragment with an active P1 promoter but inactive P2 and P3 promoters (ALC1880) using monoclonal anti-SarA antibody 1D1 (7). As shown in Fig. 2, the mutant construct ALC1880 exhibited a

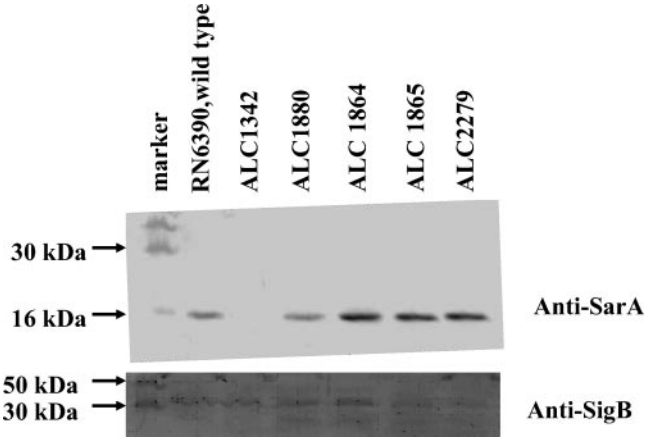

FIG. 2. Immunoblot assay of cell extracts of various *sarA* mutant constructs probed with a 1:1,000 dilution of anti-SarA monoclonal antibody 1D1 and anti-SigB monoclonal antibody 1D1. Densitometric analysis with the Sigma Gel software revealed the following: wild type, 394 densitometric units; ALC1880, 362 densitometric units; ALC2279, 710 densitometric units. Anti-SigB blotting was done to ensure that equal amounts of extracts were applied to all of the lanes.

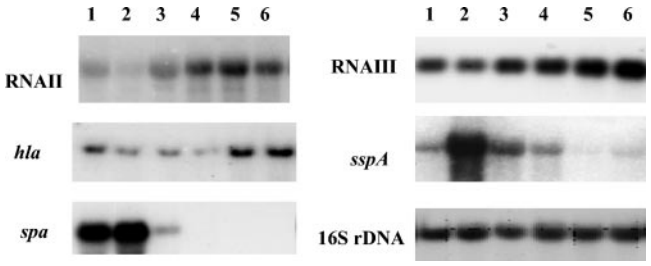

FIG. 3. Northern blot assays of various *sarA* mutant constructs with *agr* RNAII and RNAIII, *hla*, *sspA*, *spa*, and 16S rRNA gene probes. Ten micrograms of total RNA, determined by optical density at 260 nm, was applied to each lane of the blot. Lanes 1 to 6: RN6390 (wild type), ALC1342 (Δ*orf3sarA*::*ermC*), ALC1880 (ALC1342 complemented with *sarA* with mutated P2, mutated P3, and wild-type P1), ALC1864 (ALC1342 complemented with *sarA* with wild-type P2, wild-type P3, and wild-type Δ*orf3* P1), ALC1865 (ALC1342 complemented with *sarA* with wild-type P2, P3 stop, and wild-type *orf3* P1), and ALC2279 (ALC1342 complemented with *sarA* with wild-type P2, wild-type P3, and wild-type P1), respectively. The RNAs for the blots for *agr* RNAII, RNAIII, and *spa* were obtained at late log phase, while those for *hla* and *sspA* were harvested during the early stationary phase.

lower SarA level (362 densitometric units) versus the complemented mutant carrying an identically sized fragment with intact P1, P2, and P3 promoters (ALC2279) expressed SarA protein at a higher level (710 U). The elevated expression of SarA, at either the transcription or the protein level in the complemented strain (ALC2279) compared to wild-type RN6390, is possibly due to the positional effect of the integration of the *sarA* P2-P3-P1 fragment, or the *sarA* gene could be expressed from an exogenous promoter. However, in the mutant constructs in which the promoters were maintained, but the putative ORF3 between the P1-P3 promoters was either deleted (ALC1864) or mutated with nonsense mutations (ALC1865) (Fig. 1A), the expression of SarA was not significantly altered compared with the complemented mutant ALC2279. Collectively, these data indicated that the P3 and P2 promoters rather than the sequence encoding putative ORF3 likely contributed to SarA protein expression.

The *agr* promoters represent some of the target promoters to which SarA binds (6). As the *agr* locus comprises two divergent promoters that yield two distinct transcripts designated RNAII and RNAIII, the expression of these two transcripts in our mutant constructs was examined. As expected for the *sarA* deletion mutant ALC1342, the transcription of RNAII was reduced compared with complemented *sarA* mutant strain ALC2279 or the wild-type strain. Interestingly, in the mutant ALC1880 with the solely active P1 promoter, *agr* RNAII transcription was also reduced compared with the complemented mutant (ALC2279). The complemented strains with those constructs with nonsense mutations or a deletion of the putative ORF3 sequence (ALC1865 and ALC1864) appeared to enhance RNAII transcription compared with the parent or the complemented strain (Fig. 3), in concordance with higher levels of SarA expression in these strains (Fig. 2). The effect of the *sarA* locus on *agr* RNAIII was more or less the same as that on RNAII, with less than the complemented level in ALC1880. The higher expression of *agr* RNAIII in the construct with nonsense mutations or a deletion of the putative ORF3 sequence (ALC1865 and ALC1864) was found compared with wild-type RN6390.

The regulatory molecule of the *sarA* locus is the SarA protein. Transcription and DNA-binding studies indicated that SarA can modulate target gene transcription directly by binding to the target promoter (e.g., *hla* promoter), as well as indirectly via impact on an intermediary regulator such as *agr* (5, 6, 17). Given that an intact *sarA* fragment with inactive P2 and P3 promoters (i.e., only P1 is active) in ALC1880 was able to restore RNAII transcription of the *sarA* deletion mutant to near the parental level or less than the complemented strain. It is likely that the major effect of the P3 and P2 promoters, as divulged by the consistently lower SarA protein expression in the mutant ALC1880 versus complemented strain ALC2279, may be direct on target genes or indirect via *agr*. To verify this, we examined the transcription of three divergent target genes, including *hla* (alpha-toxin gene), *sspA* (V8 protease gene), and *spa* (protein A gene). The transcription of *hla* during late log to early stationary phase, normally diminished in a *sarA* deletion mutant, was also reduced in the ALC1880 mutant with the solely active P1 promoter compared with the parent or the complemented strain but was restored in the complemented mutant (ALC2279) and its counterpart with a nonsense mutation in the putative ORF3 sequence (ALC1865). Interestingly, the mutant construct containing a similar *sarA* fragment but lacking the sequence for putative ORF3 (ALC1864) expressed *hla* poorly compared with the parent and the complemented mutant. The expression of *sspA* encoding V8 protease is normally repressed by *sarA* (8, 11). As anticipated, the expression of *sspA* in the *sarA* mutant was higher than the parent and the complemented mutant (ALC2279). However, the mutant carrying the native *sarA* fragment with only the P1 promoter (ALC1880) was only partially effective in repressing *sspA* expression compared with the complemented mutant ALC2279. Of interest is the finding that the mutant carrying a *sarA* fragment with a deletion in the putative ORF3 sequence (ALC1864) was more effective in *sspA* repression than the mutant construct ALC1880 but less so than the complemented mutant (ALC2279) and the mutant containing nonsense mutations in putative ORF3 (ALC1865). Likewise, the expression of *spa*, a gene normally repressed by *sarA*, was elevated in the *sarA* deletion mutant but partially repressed by a native *sarA* fragment with P1 as the only active promoter (ALC1880). In contrast, the complemented mutant (ALC2279) and mutants with a *sarA* fragment lacking the putative ORF3 sequence (ALC1864) or containing nonsense mutations in ORF3 (ALC1865) were capable of repressing *spa* transcription to almost undetectable levels. These finding are consistent with the observed SarA protein levels in these strains.

The *sarA* promoter region is complex, with an extended promoter region (~800 bp) comprising three promoters (P1, P2, and P3), two putative small coding regions (ORF3 and ORF4), and multiple direct and indirect repeats within (1). In previous studies of *sarA* complementation with multicopy plasmids (4, 10), we determined that the putative ORF3 sequence, but not that of ORF4, within the *sarA* promoter is likely to be important for *sarA* function. To clarify the role of putative ORF3 and the importance of the P3 and P2 promoters and the region upstream of the predominant P1 promoter (13), we constructed strains with single-copy *sarA* fragments carrying

mutations in the P2 and P3 promoter and deletions (97-bp region containing the ribosome-binding site and the N-terminal 24 residues of ORF3) and nonsense mutations in putative ORF3 for complementation in a *sarA* deletion mutant (ALC1342). Based on our results, several themes emanated from this study. First, activation of the *sarA* P2 and P3 promoters serves to augment SarA protein expression (Fig. 2). In particular, this enhancement effect is not mediated via the *sarA* P1 promoter since the P1 promoter activity appeared to be increased in the mutant ALC1880. As the P2 and P3 transcripts also encode SarA, it is plausible that loss of both transcripts would lead to lower SarA expression. Another weak alternative explanation may be that the P2 and P3 transcripts, which contain many inverted repeats, may play a role in SarA translation. Second, the finding that nonsense mutations of the putative ORF3 region in a single-copy *sarA* fragment with intact P1, P2, and P3 promoters (ALC1865) did not affect SarA protein expression or expression of the *hla*, *sspA*, and *spa* genes compared with the complemented mutant indicated that the ORF3 sequence is unlikely to be expressed or is inconsequential in *S. aureus* cells. The finding that deletion of this sequence (ALC1864) led to dysregulation of *hla* and, to a lesser extent, *sspA* (Fig. 3), but we do not know the exact reason. Third, there was a reduction in the SarA protein level in the mutant construct ALC1880 with inactive P2 and P3 promoters. Therefore, there was a significant effect on the expression of *agr* RNAII and RNAIII compared with the complemented strain, thus implying that the modest changes in the SarA protein level in ALC1880 did impact the intermediary regulator *agr* and altered the expression of target genes such as *hla*, *spa*, and *sspA*. Fourth, the effect of a modest reduction in the SarA protein level in ALC1880, due to the inactive P2 and P3 promoters, could result in significant but divergent modulations of various target genes. For instance, *sspA*, a *sarA*-repressible gene, continued to be expressed in ALC1880 while the remaining mutants were more successful in down-regulating *sspA* expression. A similar effect was also observed in the case of *spa* transcription. Of interest is the finding that *hla* expression was down-regulated to comparable degrees in the *sarA* deletion mutant (ALC1342) and the ALC1880 mutant. Although the complemented ALC2279 mutant was able to reestablish *hla* expression, the mutant with a *sarA* fragment lacking the putative ORF3 sequence (ALC1864) expressed *hla* poorly. This discrepancy may be due to some unknown factor. In addition, it has also been shown that besides *sarA* and *agr*, multiple regulatory systems, including *saeRS*, *sarT*, and *rot*, may also contribute to *hla* regulation (9, 15, 18). Nevertheless, the regulatory relationship among *sae*, *sarT*, and *rot* and the effect of the interaction of SarR with these regulators on *hla* remain poorly defined.

In a previous study with multicopy plasmids (4), we have shown that the sequence encoding putative ORF3 is likely required for *agr* expression. However, the major drawback of that study was the increased gene dosage and the limitation that the P2 and P3 promoters were active in the *sarA* background. The present study, designed to resolve these issues, clearly showed that the *sarA* P2 and P3 promoters have some effect, whereas putative ORF3 has a minimal effect, on *agr* expression. This alteration in *agr* expression is clearly due to the effect of the SarA protein level and the ensuing *sspA*, *spa*, and *hla* expression, probably in both *agr*-independent and *agr*-dependent manners. Based on our studies of nonsense mutations, we conclude that the putative ORF3 sequence is unlikely to be translated but may modulate *hla*, but not *sspA* and *spa*, expression, probably by virtue of its role as a binding site for regulatory proteins (e.g., SarR).

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