JOURNAL OF BACTERIOLOGY, June 2011, p. 2948–2958 Vol. 193, No. 12
0021-9193/11/$12.00 doi:10.1128/JB.01517-10

Defining the Strain-Dependent Impact of the Staphylococcal Accessory Regulator (*sarA*) on the Alpha-Toxin Phenotype of *Staphylococcus aureus*▽†

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Received 17 December 2010/Accepted 4 April 2011

We demonstrate that mutation of the staphylococcal accessory regulator (*sarA*) limits the accumulation of alpha-toxin and phenol-soluble modulins (PSMs) in *Staphylococcus aureus* isolates of the USA300 clonal lineage. Degradation assays and experiments done with protease inhibitors suggested that this was due to the increased production of extracellular proteases rather than differences associated with the impact of *sarA* on transcription of the target gene (*hla*) or the accessory gene regulator (*agr*). This was confirmed by demonstrating that concomitant mutation of the gene encoding aureolysin (*aur*) reversed the alpha-toxin and PSM-deficient phenotypes of a USA300 *sarA* mutant. Mutation of *sarA* had little impact on the alpha-toxin or PSM phenotypes of the commonly studied strain Newman, which is known to have a mutation in *saeS* that results in constitutive activation of the *saeRS* regulatory system, and we also demonstrate that repair of this defect resulted in the increased production of extracellular proteases and reversed both the alpha-toxin and PSM-positive phenotypes of a Newman *sarA* mutant.

Staphylococcus aureus is an adaptive bacterial pathogen capable of causing both chronic, biofilm-associated infection and acute, life-threatening toxemia. Based on a specific interest in musculoskeletal infection, much of our research has been directed toward defining the mechanistic basis for *S. aureus* biofilm formation, and these studies have led us to conclude that the staphylococcal accessory regulator (*sarA*) plays a primary role in this regard. Specifically, we have demonstrated that mutation of *sarA* in diverse clinical isolates, including those of the USA300 lineage of community-associated methicillin-resistant *S. aureus* (CA-MRSA), limits biofilm formation to a degree that can be correlated with increased susceptibility to functionally distinct classes of antibiotics under both *in vitro* and *in vivo* conditions (1, 2, 45, 47, 48). This suggests that *sarA* would be a viable target for the development of therapeutic agents capable of overcoming the intrinsic resistance of biofilm-associated infections. However, *sarA* has a global regulatory impact in *S. aureus* (7, 15), and this makes it imperative to fully define the role of *sarA* in other clinically relevant contexts as a necessary prelude to the development of such agents.

One of the most important of these contexts is toxin production, a primary example being alpha-toxin. Indeed, alpha-toxin has been shown to be an important virulence factor in many forms of *S. aureus* infection (20, 21, 27, 28, 30, 32, 36). This is potentially problematic in that we previously demonstrated that mutation of *sarA* also results in an apparent increase in the production of alpha-toxin in many strains of *S. aureus* (3). In fact, the only exception among the strains we examined in our earlier study was the 8325-4 strain RN6390. It was subsequently suggested that this discrepancy was due to the combined effect of the *rsbU* and/or *tcaR* mutations that are present in all strains derived from NCTC8325 and the impact of these mutations on the expression of *sigB* and *sarS*, respectively (35). This would suggest that the targeted inhibition of *sarA* in isolates that lack these mutations would result in the increased production of alpha-toxin. This would be particularly important given the increasing clinical prominence USA300 isolates (43), many of which produce alpha-toxin at high levels owing to their high-level expression of the accessory gene regulator (*agr*) (22). Indeed, this is one factor thought to contribute to the enhanced virulence of these isolates (13).

At the same time, we recently demonstrated that mutation of *sarA* in a USA300 strain (UAMS-1625) isolated from a patient with a fatal brain abscess (42) not only resulted in a reduced capacity to form a biofilm but also reduced production of alpha-toxin and a reduced capacity to cause skin lesions (48). This was surprising in that, while isolates of the USA300 clonal lineage are closely related to 8325 strains (14), this isolate does not have the *sigB* or *tcaR* defects that are characteristic of 8325 strains (2). This suggests that other factors contribute to the strain-dependent impact of *sarA* on the production of alpha-toxin. However, these experiments were limited to a single isolate (48), and at present, it is unclear whether the same *sarA*-dependent alpha-toxin phenotype is conserved among other USA300 isolates and, if so, whether this distinguishes these isolates from those of other contemporary clonal

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† Supplemental material for this article may be found at http://jb.asm.org/.

▽ Published ahead of print on 8 April 2011.

lineages of *S. aureus*. We examined all of these issues by generating *sarA* mutants in diverse clinical isolates of *S. aureus* and examining the impact on alpha-toxin and other critical virulence factors, including phenol-soluble modulins.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *S. aureus* strains examined in this study are listed in Table 1. To facilitate the generation of certain mutants and their complemented derivatives, the plasmid conferring resistance to both erythromycin (Erm) and kanamycin (Kan)/neomycin (Neo) was cured from each of three USA300 isolates by growth in half-strength tryptic soy broth (TSB) at 42°C followed by plating on selective and nonselective media to assess loss of the plasmid and to ensure a level of hemolytic activity in the cured derivative comparable to the isogenic parent strain. Generation of *sarA*, *agr*, *hla*, and protease mutants was done by Φ11-mediated transduction from existing mutants (2, 3, 38, 41). Complementation of the *sarA* mutation was done as previously described (3). The Newman *saeRS* mutant (Δ*saeRS*) and *saeS*-repaired Newman derivative [*saeS*(*P18L*)] were constructed by using the pKOR1 system as previously described (25).

All strains were maintained as stock cultures at −80°C in tryptic soy broth (TSB) containing 25% (vol/vol) glycerol. For each experiment, each strain was retrieved from cold storage by plating on tryptic soy agar (TSA) with appropriate antibiotic selection. Antibiotics were used at the following concentrations: erythromycin (Erm; 10 μg per ml), tetracycline (Tet; 5 μg per ml), kanamycin (Kan; 50 μg per ml), and neomycin (Neo; 50 μg per ml). Kanamycin and neomycin were always used together to avoid the spontaneous generation of resistant strains. For phenotypic assays, each strain was grown in TSB without antibiotic selection at 37°C with constant aeration and a medium-to-flask volume ratio of 0.40. Where appropriate, the postexponential growth phase was defined as an optical density at 560 nm (OD_{560}) of 3.0, while stationary-phase samples were defined by overnight (16-h) growth.

In experiments employing the protease inhibitors E-64, 1-10-phenanthroline, and dichloroisocoumarin (DIC), which are specific inhibitors of cysteine, serine, and metalloproteases, respectively, each inhibitor was dissolved in dimethyl sulfoxide (DMSO) and added to TSB at final concentrations of 1 mM, 10 μM, and 0.1 mM (protease inhibitor formulation 1 [PI]) or 1 mM, 10 μM and 0.2 mM (protease inhibitor formulation 2 [PI2]), respectively. As a control, cultures were also grown in TSB containing the equivalent concentration of DMSO (0.7% [vol/vol]) without inhibitors. With the exception of RN6390, which did not grow in the presence of PI2, growth was unaffected by the inclusion of DMSO with or without protease inhibitors (data not shown).

Western blotting. The production of alpha-toxin was assessed by Western blotting of standardized cell-free supernatants using rabbit polyclonal anti-alpha-toxin IgG antibody (Sigma Chemical Co., St. Louis, MO) as previously described (48). For quantitative comparisons, 50 ng of purified alpha-toxin (List Biological Laboratories, Campbell, CA) was included as an internal standard on each gel. The amount of signal observed with this standard was assessed with an Alpha Innotech Flourochem FC2 gel documentation system (Cell Biosciences, Santa Clara, CA) and Image J software analysis. Results observed with this standard were then set to 1.0, with all other results shown relative to this value. Western blots were blocked with 0.5% skim milk containing 0.1 mg/ml human IgG (Sigma Chemical Co., St. Louis, MO).

Transcriptional analysis. To assess the levels of *hla* and RNAIII expression, total bacterial RNA was isolated using the Qiagen RNeasy minikit as previously described (3). Quantitative, real-time reverse transcription-PCR (qRT-PCR) was then performed using *hla*- or RNAIII-specific primers and a corresponding TaqMan probe (Table 2). Results were calibrated by comparison to the results obtained with the same RNA samples using primers and a TaqMan probe corresponding to a 16S rRNA gene (Table 2). Results are reported as relative units by comparison to the results observed with the lowest sample in any given experiment, with the latter being set to a value of 1.0.

Production of extracellular proteases. Protease activity was assessed by zymography as previously described (2) using both 4 to 16% Zymogram Blue casein and 10% Zymogram gelatin gels (Invitrogen, Carlsbad, CA).

Analysis of phenol-soluble modulins. High-pressure liquid chromatography–mass spectrometry (HPLC-MS) was used to detect and quantify phenol-soluble modulins (PSMs) in bacterial culture supernatants as previously described (46). Briefly, 100-μl samples from stationary-phase cultures were injected onto an analytical reversed-phase column and eluted with a gradient from 0.1% trifluoroacetic acid (TFA) in 50% acetonitrile–50% water to 0.1% TFA in 90% acetonitrile–10% water. Using this method, all PSMs can be detected in both their formylated and deformylated forms, but not with equal efficiency (46). Thus, quantitative comparisons can only be made within each class of PSM. Unless otherwise noted, results are presented as the amounts relative to those observed for each class of PSM in a designated parent strain included in each experiment.

RESULTS

Impact of *sarA* on the alpha-toxin phenotype of USA300 isolates. When supernatants from stationary-phase cultures were examined by Western blotting with anti-alpha-toxin antibody, mutation of *sarA* was found to result in an apparent decrease in the production of alpha-toxin in each of three isolates of the USA300 clonal lineage (Fig. 1). In contrast, mutation of *sarA* resulted in an apparent increase in the production of alpha-toxin in the commonly studied strain Newman (Fig. 1). The level of alpha-toxin in early postexponential-phase supernatants (OD_{560} of 3.0) was low (generally less than 10% of that observed in stationary-phase cultures), and mutation of *sarA* had little impact on the alpha-toxin phenotype of any strain (Fig. 2A). This is consistent with the current *S. aureus* regulatory paradigm concluding that production of most exotoxins increases as cultures transition from the exponential to stationary growth phases. This transition was apparent when comparing supernatants from postexponential and stationary-phase cultures from each of the wild-type strains, including Newman (Fig. 2B). However, when the same comparison was done with the isogenic *sarA* mutants, this increase was not apparent in any of the USA300 isolates but remained apparent in Newman (Fig. 2B). This demonstrates that *sarA* function is required for the growth-phase strain-dependent transition to alpha-toxin accumulation in USA300 isolates but not in Newman.

Impact of *sarA* on transcription of *agr* and *hla*. *sarA* is known to modulate gene expression in *S. aureus* via both *agr*-dependent and *agr*-independent pathways (7, 10–12, 15). To address these alternative possibilities, we examined the impact of mutating *sarA* on transcription of both *hla*- and the *agr*-associated regulatory molecule RNAIII, and the results confirmed that mutation of *sarA* had an impact in both contexts. However, these changes were not consistent with the alpha-toxin phenotype. Specifically, the level of both RNAIII and *hla* mRNA was decreased in stationary-phase cultures of a Newman *sarA* mutant (Fig. 3), while the amount of alpha-toxin was increased (Fig. 1). Conversely, the level of *hla* mRNA was increased in USA300 *sarA* mutants, while the amount of both RNAIII (Fig. 3) and alpha-toxin was decreased (Fig. 1). While these results are consistent with a scenario in which *sarA* modulates the phenotype of *S. aureus* at the transcriptional level via both *agr*-dependent and *agr*-independent pathways, the disparity between the transcriptional impact of mutating *sarA* on *agr* and/or *hla* and its impact on the alpha-toxin phenotype suggests the existence of a posttranscriptional component that ultimately defines the *sarA*-dependent alpha-toxin phenotype.

Impact of *sarA* in the context of *agr*. In USA300 isolates, mutation of *sarA* resulted in increased expression of *hla* but decreased expression of *agr* at least as defined by relative levels of RNAIII (Fig. 3). Because RNAIII is required for efficient translation of *hla* mRNA (31), one possible explanation for the disparity between the increased levels of the *hla* transcript and decreased levels of alpha-toxin is reduced translation of *hla*

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid[a]	Description	Source or reference
Strains		
UAMS-1	Methicillin-susceptible *S. aureus*, osteomyelitis isolate	16
UAMS-929	*sarA*::*kan*	3
UAMS-155	*agr*::*tetM*	3
UAMS-930	*agr*::*tetM sarA*::*kan*	3
UAMS-969	*sarA*::*kan* (pSARA)	3
UAMS-970	*agr*::*tetM sarA*::*kan* (pSARA)	3
UAMS-7	S6C	3
UAMS-985	*sarA*::*kan*	3
UAMS-128	RN6390 (8325-4)	34
UAMS-983	*sarA*::*kan*	3
UAMS-240	*sarA*::*tetK*	3
UAMS-982	*agr*::*tetM*	3
UAMS-959	*sarA*::*kan agr*::*tetM*	3
UAMS-979	*sarA*::*kan* (pSARA)	3
UAMS-980	*sarA*::*kan agr*::*tetM* (pSARA)	3
UAMS-1639	*scpA*::*erm*	41
UAMS-1640	*aur*::*erm*	41
UAMS-1974	*spl*::*erm*	38
UAMS-1975	*sspABC*::*erm*	41
UAMS-200	Newman	29
UAMS-988	*sarA*::*kan*	3
UAMS-974	*agr*::*tetM*	3
UAMS-975	*sarA*::*kan agr*::*tetM*	3
UAMS-1907	*sarA*::*kan* (pSARA)	This study
UAMS-1909	*sarA*::*kan agr*::*tetM* (pSARA)	This study
UAMS-2189	*hla*::*erm*	This study
UAMS-2190	*sarA*::*kan hla*::*erm*	This study
UAMS-2167	*saeS*(*P18L*) (CYL11481)	25
UAMS-2170	*saeS*(*P18L*) *sarA*::*kan*	This study
UAMS-2200	*saeS*(*P18L*) *hla*::*erm*	This study
UAMS-2212	*saeS*(*P18L*) *hla*::*erm sarA*::*kan*	This study
UAMS-2225	*saeS*(*P18L*) *aur*::*erm*	This study
UAMS-2226	*saeS*(*P18L*) *aur*::*erm sarA*::*kan*	This study
UAMS-2166	Δ*saeRS* (CYL11771)	25
UAMS-2169	Δ*saeRS sarA*::*kan*	This study
UAMS-261	SH1000	18
UAMS-1762	*sarA*::*kan*	This study
UAMS-732	SC-01	44
UAMS-310	*sarA*::*tetK*	3
UAMS-1625	USA300 isolate	42
UAMS-1653	*sarA*::*tetK*	48
UAMS-1782	USA300 isolate FPR3757	2
UAMS-1804	*sarA*::*kan*	2
UAMS-1819	*agr*::*tetM*	2
UAMS-1837	*agr*::*tetM sarA*::*kan*	2
UAMS-1901	*sarA*::*kan* (pSARA)	2
UAMS-1904	*agr*::*tetM sarA*::*kan* (pSARA)	2
UAMS-1790	USA300 isolate	2
UAMS-1796	*sarA*::*tetK*	2
UAMS-1794	UAMS-1782, Erm-sensitive	This study
UAMS-1802	*sarA*::*kan*	This study
UAMS-2192	*hla*::*erm*	This study
UAMS-2193	*sarA*::*kan hla*::*erm*	This study
UAMS-2205	*scpA*::*erm*	This study
UAMS-2206	*scpA*::*erm sarA*::*kan*	This study
UAMS-2218	*spl*::*erm*	This study
UAMS-2219	*spl*::*erm sarA*::*kan*	This study
UAMS-2220	*aur*::*erm*	This study

Continued on following page

TABLE 1—*Continued*

Strain or plasmid[a]	Description	Source or reference
UAMS-2221	*aur*::*erm sarA*::*kan*	This study
UAMS-2222	*sspABC*::*erm*	This study
UAMS-2223	*sspABC*::*erm sarA*::*kan*	This study
UAMS-1893	USA100 isolate, NRS642	2
UAMS-1941	*sarA*::*tetK*	2
UAMS-1898	USA800 isolate, NRS653	2
UAMS-1944	*sarA*::*tetK*	2
UAMS-1899	USA1000 isolate, NRS676	2
UAMS-1930	*sarA*::*kan*	2
UAMS-1900	USA1100 isolate, NRS484	2
UAMS-1931	*sarA*::*kan*	2
UAMS-2172	USA500, BD02-25	6
UAMS-2174	*sarA*::*kan*	This study
Plasmid		
pSARA		4

[a] The prefix "UAMS" refers to strain designations in the corresponding author's culture collection. All UAMS strains are designated with the prefix "U" in the figures and figure legends.

mRNA owing to the relative absence of RNAIII. To further investigate this issue, we generated *sarA*, *agr*, and *sarA agr* mutants and examined the impact on the production of alpha-toxin with and without complementation of the *sarA* defect. In RN6390 and the USA300 isolate UAMS-1782, mutation of *sarA* or *agr* resulted in an apparent decrease in the production of alpha-toxin, and complementation of the *sarA* defect restored alpha-toxin production in a *sarA* mutant but not in the isogenic *sarA agr* mutant (Fig. 4). This is consistent with the hypothesis that the impact of *sarA* on the production of alpha-toxin is mediated through posttranscriptional changes associated with the reduced production of RNAIII. However, these results must be interpreted with caution because mutation of *sarA* or *agr* had the same impact on the alpha-toxin phenotype, and it is possible that in these strains the impact of any *sarA*-dependent, *agr*-independent effect would be masked by the impact of mutating *agr* on the transcription and/or translation of *hla*. Further support for this hypothesis comes from the observation that the same complementation results were observed in Newman despite the fact that mutation of *sarA* and *agr* had opposite effects on the alpha-toxin phenotype (Fig. 4). This is consistent with the hypothesis that *sarA* has an impact on the alpha-toxin phenotype that is independent of both *agr*-mediated transcriptional regulation and RNAIII-mediated posttranscriptional effects.

Role of extracellular proteases in defining the *sarA*-dependent alpha-toxin phenotype. In previous reports, we demonstrated that mutation of *sarA* results in a dramatic increase in the production of extracellular proteases and that this plays an important role in defining the biofilm-deficient phenotype of *S. aureus sarA* mutants (2, 45). Additionally, Newman is one of the few strains we have examined in which mutation of *sarA*

TABLE 2. PCR primers and probes used in this study

Primer or probe	Oligonucleotide sequence (5′→3′)
RNAIII-F	AGC CAT CCC AAC TTA ATA ACC ATG T
RNAIII-R	GAT GTT GTT TAC GAT AGC TTA CAT GCT AGA
RNAIII-Probe	AGT AGA GTT AGT TTC CTT GGA CTC AGT GCT
hla-F	ACA ACA CTA TTG CTA GGT TCT ATA
hla-R	TGT AGT ATT GCT TCC TAT ATC TGT A
hla-Probe	ATG AAT CCT GTC GCT AAT GCC GCA
16S-F	TGA GAT GTT GGG TTA AGT CCC GCA
16S-R	CGG TTT CGC TGC CCT TTG TAT TGT
16S-Probe	AGC GCA ACC CTT AAG CTT AGT TGC CA
*sae*R7	GGG GAC AAG TTT GTA CAA AAA AGC AGG CTC ACG TCA TCT TCG GCG GCG CT
*sae*R8-1	TCC AGA ACC ACC CGG CGG CCG CTT CAT CAT CCA CGA TCA GT
*sae*S9KO	ATC GTG GAT GAT GAA GCG GCC GCC GGG TGG TTC TGG ATT AGG C
*sae*S8	GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC AAA GCG TGA TAG AAG TGG TGA
*sae*S1	GGG GAC AAG TTT GTA CAA AAA AGC AGG CTA ACG ACA ACT AGC GGT AAA GAA G
*sae*S2	GGG GAC CAC TTT GTA CAA GAA AGC TGG GTT ACG CTC AAG TGG CGT TCG ATA
*sae*S3	ATT GGC GTC GTA TCG AGT ATA CC
*sae*S4	GCA ATT GCT AAA ATA GTT GAA GTT AAT A

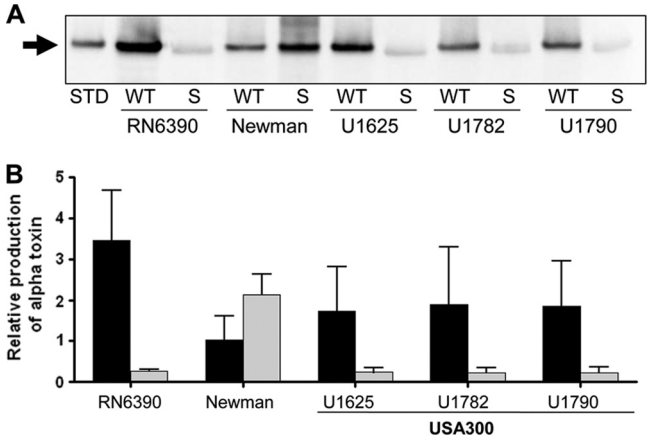

FIG. 1. Impact of *sarA* on the alpha-toxin phenotype of stationary-phase cultures. (A) Western blot using alpha-toxin antibody and stationary-phase supernatants from the indicated wild-type (WT) strains and their isogenic *sarA* (S) mutants. UAMS-1625 (U1625), U1782, and U1790 are isolates of the USA300 clonal lineage (Table 1). STD, alpha-toxin standard. (B) Production of alpha-toxin was assessed by Western blotting of stationary-phase supernatants. Results obtained with each wild-type strain (black) and its isogenic *sarA* mutant (gray) were quantified with Image J and are shown relative to the signal observed with 50 ng of purified alpha-toxin, the amount of which was set to a value of 1.0. Results are shown as the average ± standard deviation from three independent experiments.

had relatively little impact on biofilm formation (2). Based on this, we next examined whether the impact of *sarA* on extracellular proteases may also play a role in defining the *sarA*-dependent alpha-toxin phenotype. The inclusion of a protease

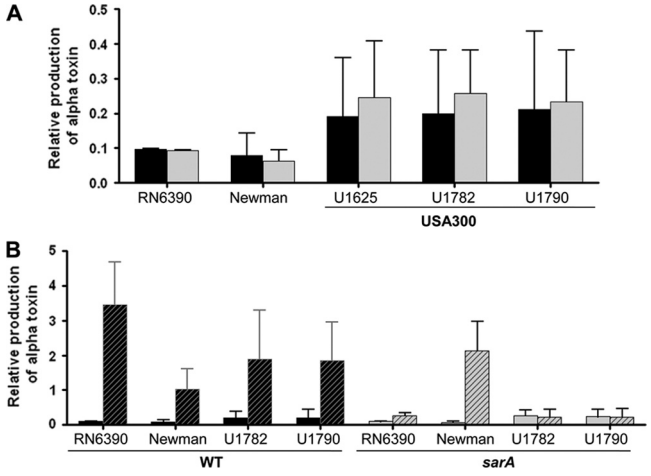

FIG. 2. Impact of *sarA* on the alpha-toxin phenotype of post-exponential-phase cultures. (A) Alpha-toxin production was assessed by Western blotting of supernatants from post-exponential-phase cultures (OD_{560} of 3.0) and quantified by using Image J. Results are shown for the wild-type strains (black) and their isogenic *sarA* mutants (gray) relative to the signal observed with 50 ng of a purified alpha-toxin standard. Results are shown as the average ± standard deviation from three independent experiments. (B) Results of Western blots using supernatants from post-exponential-phase (solid bars [data from panel A]) and stationary-phase supernatants (cross-hatched bars [data from Fig. 1]) supernatants were replotted to illustrate the impact of mutating *sarA* on growth-phase-dependent changes in the alpha-toxin phenotype in different strains of *S. aureus*.

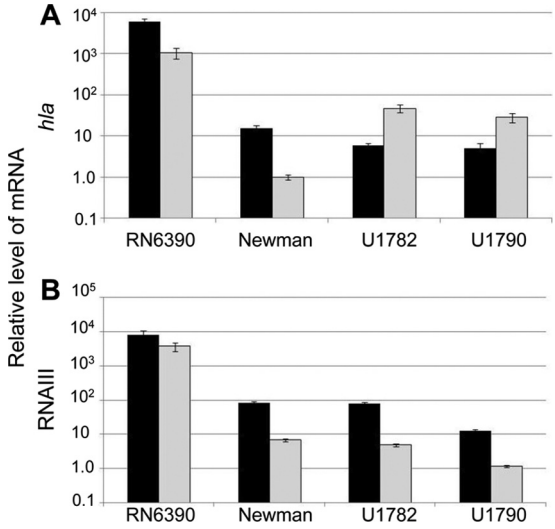

FIG. 3. Impact of *sarA* on the production of RNAIII and transcription of *hla*. Relative levels of the *hla* (A) and RNAIII (B) transcripts in the stationary growth phase were determined for the indicated wild-type (WT) strains (black) and their isogenic *sarA* mutants (gray) by qRT-PCR. Results are shown as the mean ± standard deviation of triplicate samples.

inhibitor cocktail in the growth medium restored the alpha-toxin phenotype in a UAMS-1782 *sarA* mutant (Fig. 5A), but it was difficult to assess the comparative effect in Newman owing to the differential impact of mutating *sarA* on the production and/or accumulation of endogenous alpha-toxin. To overcome this limitation, we repeated the experiment by adding exogenous alpha-toxin to growing cultures of *sarA hla* double mutants generated in both Newman and the USA300 isolate erythromycin-sensitive UAMS-1782 derivative UAMS-1794, and the results confirmed the degradation of purified alpha-toxin in a growing culture of the UAMS-1794 *sarA hla* mutant but not in a Newman *sarA hla* mutant (Fig. 5B).

All of the results discussed above are consistent with the hypothesis that the strain-dependent impact of *sarA* on the

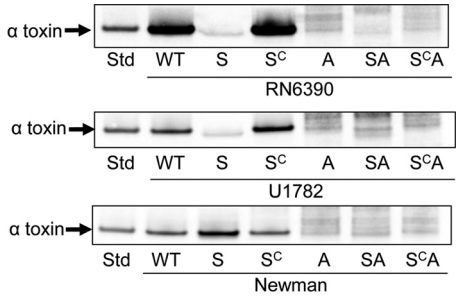

FIG. 4. Production of alpha-toxin in *sarA* and *agr* mutants. Western blots of culture supernatants from stationary-phase cultures were done using anti-alpha-toxin antibody after blocking with skim milk alone. Alpha-toxin was identified by comparison to a purified alpha-toxin standard (Std). Parent strains are indicated below each panel. U1782 is the designation in the corresponding authors' culture collection for the USA300 isolate FPR3757. Lane designations: WT, wild-type strain; S, *sarA* mutant; S^C, complemented *sarA* mutant; A, *agr* mutant; SA, *sarA agr* double mutant; S^CA, *sarA agr* double mutant complemented for *sarA*.

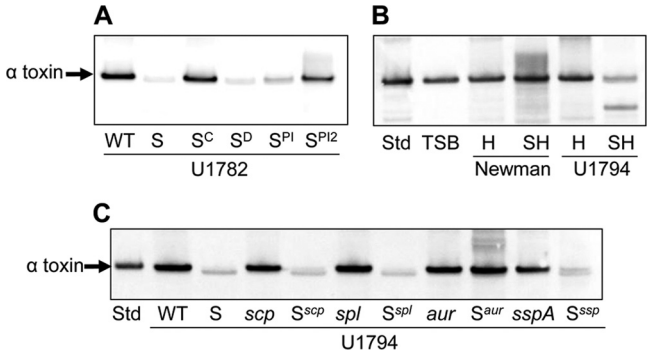

FIG. 5. Impact of extracellular proteases on the alpha-toxin phenotype of *sarA* mutants. Western blots were done with anti-alpha-toxin antibody after blocking with both skim milk and human IgG. (A) Blots were done with stationary-phase supernatants from the USA300 isolate U1782 (WT) and its isogenic *sarA* mutant (S) complemented for the *sarA* defect (S^C) or grown in the presence of DMSO (S^D) or DMSO containing increasing concentrations of protease inhibitor cocktail (S^{PI} versus S^{PI2}). (B) Blots were done after adding purified alpha-toxin (8 μg per ml) to growing cultures of *hla* (H) and *sarA hla* (SH) mutants generated in strain Newman and the USA300 isolate U1794, the latter being derived from U1782 by curing the plasmid conferring resistance to erythromycin. This was necessary to allow generation of the *sarA hla* double mutant. After overnight incubation, supernatants were harvested for Western blotting using anti-alpha-toxin antibody. Controls included an equivalent amount of purified alpha-toxin standard (Std) incubated at 37°C overnight in sterile tryptic soy broth (TSB). (C) Blots were done with stationary-phase supernatants from U1794 (WT) and isogenic derivatives carrying mutations in *sarA* (S) with or without the indicated genes encoding extracellular proteases.

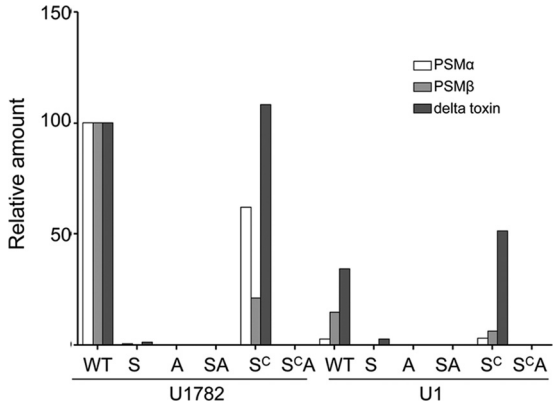

FIG. 6. Impact of *sarA* on phenol-soluble modulins. The PSM phenotype was assessed in stationary-phase cultures by HPLC as previously described (46). Strain designations: WT, wild-type strain; S, *sarA* mutant; S^C, complemented *sarA* mutant; A, *agr* mutant; SA, *sarA agr* double mutant; S^CA, *sarA agr* double mutant complemented for *sarA*. Results for each individual class (alpha- and beta-toxins versus delta-toxin) of PSM in both their formylated and unformylated forms were combined, and the amounts of each class observed in the USA300 isolate U1782 were set to 100%. Results observed in each of the indicated U1782 mutants and in U1 and its derivatives are shown relative to these amounts.

alpha-toxin phenotype of *S. aureus* is defined by the impact of *sarA* on the production of specific extracellular proteases. However, these results do not prove a cause-and-effect relationship. To address this issue, we generated a USA300 *sarA* mutant of UAMS-1794 (UAMS-1802) that carries mutations in the genes encoding different extracellular proteases (*scpAB*, *splABCDEF*, *sspABC*, or *aur*) and examined the impact on the alpha-toxin phenotype. As assessed by casein and gelatin zymography, mutation of *scpAB* or *splABCDEF* had no impact on the protease phenotype (see Fig. S1 in the supplemental material). In contrast, mutation of *sspABC* essentially eliminated all proteases, while mutation of *aur* resulted in the appearance of multiple proteolytic bands in gelatin (but not casein) zymograms. Because aureolysin is an activator of other proteases, including those encoded within the *sspABC* operon (41), we attribute these additional bands to alternative forms of SspA and/or SspB. More importantly, while mutation of *scpAB*, *splABCDEF*, or *sspABC* had no impact on the alpha-toxin phenotype, mutation of the gene encoding aureolysin restored the alpha-toxin-deficient phenotype of a UAMS-1794 *sarA* mutant (Fig. 5C). This confirms that the *sarA*-dependent alpha-toxin phenotype of a USA300 *sarA* mutant is defined by the increased production of extracellular proteases rather than transcriptional changes associated with *hla* or *agr*.

To the extent that mutation of *scpAB*, *splABCDEF*, or *sspABC* individually had no discernible impact on the *sarA*-dependent alpha-toxin phenotype, these results also suggest that the impact of aureolysin is mediated through something other than its role as an activator of other extracellular proteases. However, we did observe a greater effect with the PI2 inhibitor cocktail than the PI formulation (Fig. 5A). These two formulations differ only in the concentration of DIC, which was higher in PI2 than in PI. To the extent that DIC is a specific inhibitor of serine proteases, including *sspA*, and the *spl* proteases, these results suggest a role for proteases other than aureolysin itself.

Impact of *sarA* on phenol-soluble modulins. Although we placed a primary focus in this report on alpha-toxin, the important role of phenol-soluble modulins (PSMs) in defining the virulence of contemporary CA-MRSA led us to assess the impact of the increased production of extracellular proteases in *sarA* mutants in this context. PSMs are not easily detectable by SDS-PAGE, but HPLC analysis demonstrated that both the alpha and beta classes of PSMs were almost undetectable in *sarA* mutants generated in isolates of the USA300 clonal lineage (Fig. 6). This was also true in *agr* mutants, and it was not possible to complement the *sarA* defect in a *sarA agr* double mutant. This suggests that the impact of *sarA* on PSM production occurs in an *agr*-dependent manner. However, while mutation of *sarA* also limited the PSM phenotype in Newman, as with alpha-toxin, the impact was limited by comparison to USA300 isolates (Fig. 7A). Moreover, while the inclusion of protease inhibitors had no impact on the PSM-negative phenotype of a USA300 *sarA* mutant, it partially restored the PSM-positive phenotype of a Newman *sarA* mutant. This suggests that, as with alpha-toxin, the differential impact of *sarA* on the production of extracellular proteases may also contribute to the *sarA*-dependent PSM phenotype. This was also confirmed by demonstrating that concomitant mutation of *aur* partially restored the PSM-deficient phenotype of a USA300 *sarA* mutant (Fig. 7B).

Impact of *saeRS* on the *sarA*-dependent alpha-toxin and PSM phenotypes. Although Newman is one of the *S. aureus* strains most frequently used in studies focusing on both patho-

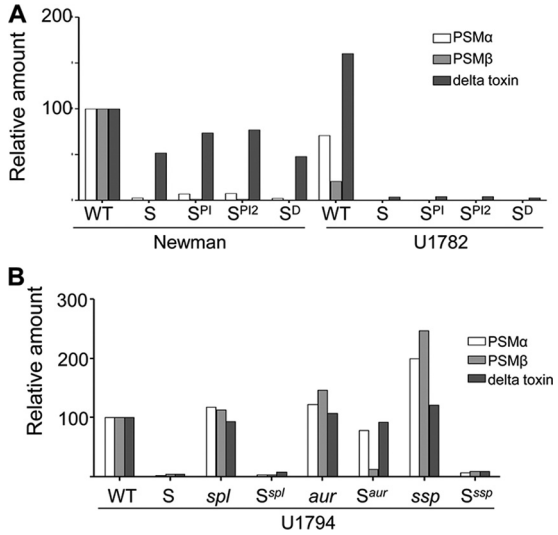

FIG. 7. Role of *sarA* and extracellular proteases in the *agr*-dependent PSM phenotype of USA300 isolates. The PSM phenotype was assessed in stationary-phase cultures by HPLC. (A) The amount of each individual class (alpha- and beta-toxins versus delta-toxin) of PSM in stationary-phase cultures was assessed in both their formylated and unformylated forms by HPLC. The results observed with strain Newman were set to 100%, with the amounts observed in U1782 and isogenic *sarA* mutants generated in both strains (S) and grown with (S^{PI} versus S^{PI2}) and without protease inhibitors shown relative to these amounts. (B) The amounts of each individual class (alpha- and beta-toxin versus delta-toxin) of PSM in both their formylated and unformylated forms were combined, and the amount of each class observed in the USA300 isolate U1794 (WT) was set to 100%. The results observed in each of the indicated U1794 mutants are shown relative to these amounts.

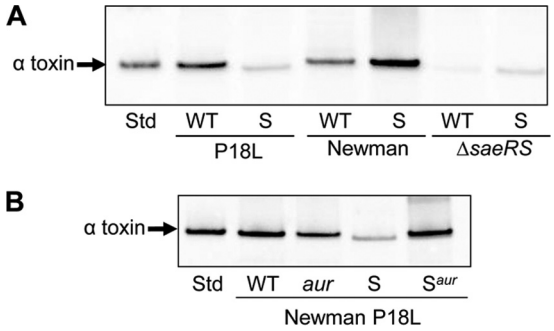

FIG. 8. Impact of *saeRS* on the *sarA*-dependent alpha-toxin phenotype of strain Newman. (A) Western blot with alpha-toxin antibody examining the impact of mutating *sarA* in Newman, its *saeS*-repaired [*saeS*(*P18L*)] derivative (P18L), and an isogenic *saeRS* mutant on the alpha-toxin phenotype. (B) Impact of mutating *aur* and *sarA* alone and in combination with each other in the *saeS*-repaired derivative of strain Newman (P18L).

genesis and regulation of gene expression, it is known to carry a defect in the *saeS* sensor kinase that results in constitutive expression of the *saeRS* regulatory locus (40). To determine whether this might contribute to the *sarA*-dependent alpha-toxin and PSM phenotypes of Newman, we both repaired the *saeS* defect to restore the regulated expression of *saeRS* and generated an *saeRS* null mutant. It was difficult to detect any extracellular proteases by zymography in strains derived from Newman itself, but it was possible to detect altered protease production in the isogenic *sarA* mutants. Specifically, both repair and deletion of *saeRS* in isogenic *sarA* mutants resulted in the increased production of multiple extracellular proteases (see Fig. S2 in the supplemental material). This is consistent with a previous report demonstrating that mutation of *saeRS* results in the increased expression of multiple protease genes, including *aur* and *sspA* (39). The observation that these changes were apparent only in *sarA* mutants also suggests that the impact of *saeRS* on protease production is independent of *sarA*.

As expected based on a previous report demonstrating that transcription of *hla* is dramatically reduced in an *saeRS* mutant (33), mutation of *saeRS* eliminated alpha-toxin production in both strain Newman and its *sarA* mutant (Fig. 8A). However, repair of the *saeS* defect reversed the alpha-toxin-positive phenotype of a Newman *sarA* mutant. We also demonstrated that the alpha-toxin-deficient phenotype of a *sarA* mutant generated in the *saeS*-repaired derivative of Newman was reversed by concomitant mutation of *aur* (Fig. 8B). Finally, both repair of the *saeS* defect or deletion of *saeRS* in Newman resulted in a *sarA*-dependent PSM-deficient phenotype comparable to that observed in USA300 (Fig. 9A). Taken together, these results confirm that the impact of *sarA* on the production of extracellular proteases is responsible for the strain-dependent impact on both the alpha-toxin and PSM phenotypes of *S. aureus* and that, at least in Newman, this is a function of the *saeS* defect and its impact on the production of these proteases. Although we have not yet defined the mechanism by which the constitutive activation of *saeRS* impacts the *sarA*-defined alpha-toxin and PSM phenotypes, neither repair nor mutation of *saeRS* had an impact on the production of SarA itself (Fig. 9B), and this also suggests that the constitutive activation of *saeRS* either represses protease production in a *sarA*-independent manner or alters the functional status rather than the production of SarA.

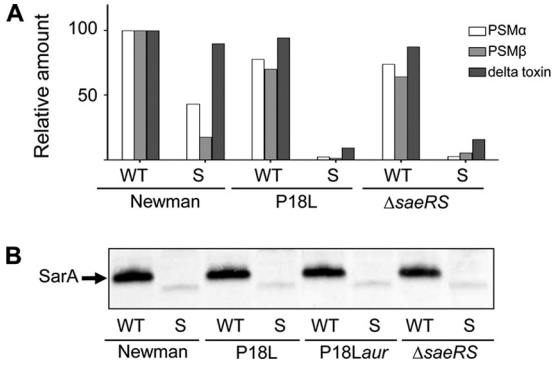

FIG. 9. Impact of *saeS* on the *sarA*-dependent PSM phenotype of strain Newman. (A) The PSM phenotype was assessed by HPLC in Newman, its *saeS*-repaired [*saeS*(*P18L*)] derivative (P18L), and a Newman *saeRS* mutant. Results observed with strain Newman for each class of PSM were set to 100%, with results observed in each of the other strains shown relative to this amount. (B) The amount of SarA was assessed in Newman, its *saeRS* mutant, an *saeS*-repaired derivative (P18L), and an *saeS*(*P18L*) aureolysin mutant (P18L*aur*) by Western blotting as previously described (4).

Impact of *sarA* on the alpha-toxin phenotype of other clinical isolates. To determine whether differences like those observed between USA300 isolates and Newman exist among other clinical isolates, we carried out quantitative Western blot analysis using supernatants from isolates of other clonal lineages. Alpha-toxin was undetectable in isolates of the USA200 (UAMS-1 and UAMS-601), USA400 (MW2), and USA600 clonal lineages irrespective of the functional status of *sarA*. This is consistent with the observations that many USA200 isolates, including UAMS-1 and UAMS-601, have a nonsense mutation in *hla* (6), while MW2 expresses *hla* at very low levels (46). Mutation of *sarA* also had comparatively little impact on the alpha-toxin phenotype of S6C, SC-01, or a single isolate of the USA100 clonal lineage (see Fig. S3 in the supplemental material). In all other strains tested, mutation of *sarA* resulted in an alpha-toxin-deficient phenotype comparable to that observed in USA300. These included RN6390, its *rsbU*-repaired derivative SH1000, and individual isolates of the USA500, USA800, USA1000, and USA1100 clonal lineages (Fig. S3). Thus, the phenotype observed in USA300 *sarA* mutants appears to be representative of most contemporary clonal lineages of *S. aureus*.

DISCUSSION

The *sarA* locus was originally identified in the *S. aureus* strain DB based in part on its increased hemolytic activity owing to its increased production of alpha-toxin (9). This report also noted the reduced production of extracellular protein A in a strain DB *sarA* mutant, and it was suggested that one possible explanation for this was the increased production of extracellular proteases. However, it was concluded that this could not account for the alpha-toxin phenotype due to its increased rather than decreased production. Given its apparently opposing regulatory role in comparison to *agr*, it was also proposed that *sarA* may serve as a "counterregulatory system" to *agr*. However, the *sarA* mutation was subsequently generated in the commonly studied 8325-4 laboratory strain RN6390, and in this background, the alpha-toxin phenotype was opposite to that observed in DB (10, 11). Based on this, the current regulatory paradigm in *S. aureus* is that mutation of *sarA* results in the reduced production of alpha-toxin due to reduced transcription of both *hla* and *agr* (3, 12).

Given the contrasting reports describing DB and RN6390 *sarA* mutants, we examined in an earlier report (3) the phenotypic impact of mutating *sarA* on hemolytic activity in each of seven strains of *S. aureus* (UAMS-1, UAMS-601, DB, SC-01, S6C, Newman, and the 8325-4 strain RN6390). In six of these strains, hemolytic activity was increased, the only exception being RN6390 (3). On the basis of Northern blots examining transcription of *hla* in these strains and Western blots examining the production of alpha-toxin in DB, we concluded that the increased hemolytic activity observed in strains other than RN6390 was a function of the increased transcription of *hla* and consequently the increased production of alpha-toxin (3). More directly, we concluded that the strain-dependent difference in alpha-toxin phenotype was mediated at the transcriptional level and that the phenotype observed in RN6390 was not representative of clinical isolates of *S. aureus*.

A subsequent report concurred with this conclusion and suggested that the phenotypic disparity between 8325-4 strains like RN6390 and other *S. aureus* isolates was due to the combined effects of the *rsbU* and *tcaR* mutations that are present in all 8325-derived strains (35). Specifically, mutation of *sarA* was found to result in increased rather than decreased hemolytic activity in the *rsbU*-repaired 8325-4 strain SH1000, which suggests that the *rsbU* defect in RN6390 plays an important role in defining the *sarA*-dependent alpha-toxin phenotype in *S. aureus*. However, it was also concluded that this was not the only relevant factor since mutation of *sarA* in strain V8 resulted in increased hemolytic activity and increased transcription of *hla* despite the fact that this strain is also an *rsbU* mutant (35). Because SH1000 contains a defect in *tcaR* while V8 does not, and because *tcaR* modulates the expression of *sarS* (26), a model was proposed in which SarA and SarS act cooperatively to repress transcription of *hla*, with the end result of mutating *sarA* being a function of the impact on the total amount of repressor (e.g., SarA plus SarS) (35). Specifically, it was proposed that *sarA* represses the transcription of both *hla* and *sarS*, with SarS in turn acting as a repressor of *hla* transcription. In 8325-4 strains, which already express *sarS* at low levels due to the *tcaR* mutation, mutation of *sarA* results in a paradoxical increase in the overall amount of repressor because of the increased expression of *sarS*, and this would presumably account for the reduced production of alpha-toxin in these strains. In contrast, mutation of *sarA* in strains that do not have the *tcaR* mutation would result in a net decrease in the total amount of repressor due to the loss of SarA and a comparatively modest effect on expression of *sarS*, and this would presumably account for the increased transcription of *hla* and increased production of alpha-toxin (35).

While we have begun to investigate this model directly, the more important consideration is that, based on this model, it would be anticipated that mutation of *sarA* in contemporary clinical isolates that lack both the *rsbU* and *tcaR* mutations would result in the increased production of alpha-toxin. However, we found that mutation of *sarA* in the USA300 isolate UAMS-1625 resulted in reduced hemolytic activity and an apparent reduction in the production of alpha-toxin (48), despite the fact that this isolate expresses both *asp23* and *sarS*, which are indicators of the functional status of *sigB* and *tcaR*, respectively, at levels that are comparable to those of other clinical isolates and significantly higher than those observed in RN6390 (2). In fact, of the strains included in the experiments reported here, all but one expressed both *asp23* and *sarS* at levels that were comparable to each other and considerably higher than those observed in RN6390 (data not shown). The exception was S6C, which expressed *sarS* at levels comparable to those of other clinical isolates but *asp23* at levels comparable to those of RN6390.

This suggests that S6C also has a deficiency in the *sigB* regulatory pathway, and in this respect, it is important to note that S6C and RN6390 exhibited disparate *sarA*-dependent alpha-toxin phenotypes, despite the fact that they share this common defect. Similarly, different *sarA*-dependent alpha-toxin phenotypes were observed among strains that expressed *sarS* at comparable levels. In fact, our SH1000 isolate expressed both *asp23* and *sarS* at levels that exceeded those observed in RN6390, although the latter was lower than the levels observed in contemporary clinical isolates, but in con-

trast to the earlier report cited above (35), we found that this did not alter the *sarA*-dependent alpha-toxin phenotype. More directly, mutation of *sarA* in both RN6390 and SH1000 resulted in an alpha-toxin-deficient phenotype, the only difference being the overall levels in the parent strains and their isogenic *sarA* mutants. While indirect, all of these results collectively provide support for the hypothesis that the strain-dependent impact of *sarA* on the alpha-toxin phenotype of *S. aureus* involves something other than the *rsbU* and/or *tcaR* defects present in 8325-4 strains.

This brings up two important questions, the first being whether there is in fact a predominant *sarA*-dependent alpha-toxin phenotype among *S. aureus* clinical isolates and the second being the mechanistic basis for the strain-dependent phenotypes that do exist. With respect to the first, we conclude based on the results presented here that the most common phenotype of an *S. aureus sarA* mutant, particularly among contemporary clinical isolates, is a reduction in the level of alpha-toxin. In this respect, it is important to note that of the six strains that exhibited the opposite phenotype in our earlier study (3), two (UAMS-1 and UAMS-601) do not produce alpha-toxin due to a nonsense mutation in *hla* (7, 46), one (Newman) is known to carry a defect in *saeS* (17, 40), and, based on transcription levels of *asp23*, another (S6C) appears to carry a defect in the *sigB* pathway. SC-01 was described in a report comparing different strain typing techniques (44), but to date neither this strain nor DB (9) has been extensively characterized. Whether any of these strains also have defects related to *saeRS* that contribute to their *sarA*-dependent alpha-toxin phenotypes remains unknown. Nevertheless, these results collectively suggest that it is in fact these strains that represent the anomaly by comparison to contemporary clinical isolates and that the most prominent and clinically relevant phenotype of *S. aureus sarA* mutants is a decrease in the production and/or accumulation of alpha-toxin. In this respect, it is also worth noting that, in all of the strains other than Newman in which mutation of *sarA* did not result in an alpha-toxin-deficient phenotype, the overall impact was modest by comparison to that in strains that exhibited the opposite phenotype.

With respect to the mechanistic basis for these differences, the current *S. aureus* regulatory paradigm indicates that the *sarA*-encoded DNA binding protein SarA induces the production of alpha-toxin at a transcriptional level via both *agr*-dependent and *agr*-independent pathways (8, 11, 12), and our results demonstrate that the impact of *sarA* on the transcription of both RNAIII and *hla* is a strain-dependent phenotype. In most strains, mutation of *sarA* generally resulted in reduced transcription of *agr*, as measured by the levels of RNAIII, and this is consistent with the hypothesis that *sarA* is required for maximum activation of *agr* transcription (10, 11). However, with the exception of RN6390, SH1000, and Newman, mutation of *sarA* resulted in decreased rather than increased transcription of *hla*, and this suggests that the more important consideration in the context of alpha-toxin is the *agr*-independent impact of *sarA* on *hla* transcription. However, even in this context, a disparity exists between *hla* transcription and the resulting alpha-toxin phenotype, and our results demonstrate that the strain-dependent impact of *sarA* on the production of extracellular proteases is a primary reason for this disparity. This is consistent with a previous report concluding that the primary impact of *sarA* in defining the alpha-toxin-deficient phenotype even in 8325-4 strains is in fact the impact of *sarA* on the production of extracellular proteases (24). Interestingly, while they did not consider *sarA*, there is also a report demonstrating that the strain-dependent production of both alpha-toxin and protein A is generally consistent with the level of RNAIII but that the amount of alpha-toxin produced by one clinical isolate was unexpectedly high given its relatively low levels of *agr* expression, and it was suggested that this may be a function of the reduced production of extracellular proteases (23).

Our results are also the first to demonstrate that *sarA* is required for maximum PSM production and confirm that strain-dependent differences in protease production are also relevant in this context. Whether this is true with other exoproteins has not yet been assessed. It is known that *sarA* mutants produce increased amounts of extracellular nuclease and that this contributes to some degree to their reduced capacity to form a biofilm (2, 45). Additionally, the results we present do not preclude other regulatory roles for *sarA*; indeed, genome-scale transcriptional profiling experiments have confirmed that mutation of *sarA* limits the production and/or stability of multiple transcripts, important examples including all of the genes encoding extracellular proteases (7, 15). Nevertheless, our results emphasize the need to consider the phenotype of *S. aureus sarA* mutants rather than focus on transcriptional changes alone.

Based on all of these considerations, we propose that the primary phenotypic impact of *sarA* on the phenotype of at least some critical exotoxins produced by *S. aureus* is not transcriptional but rather is mediated by the impact of *sarA* on the production of extracellular proteases. In fact, taken together with our previous reports demonstrating that the increased production of extracellular proteases is a primary factor in the biofilm-deficient phenotype of *S. aureus sarA* mutants (2, 45), we would propose a model in which the *sarA*-mediated repression of protease production serves the important purposes of both promoting biofilm formation (2, 45) and allowing *agr* to "fine-tune" gene expression patterns in a manner that simultaneously promotes dispersal from an established biofilm (5) and the production of the extracellular toxins, including alpha-toxin and PSMs, that promote the ability of *S. aureus* to survive outside the protective environment of the biofilm. By analogy, we would propose that *sarA* is the "dam" that represses the "flow" of proteases and thereby allows the "lake" to develop in the form of a biofilm and that *agr* is the "outlet" that allows the controlled release of *S. aureus* and induction of the virulence factors necessary to survive the downstream trip through the bloodstream to a new site of colonization. In this scenario, mutation of *sarA* would represent a failure of the dam such that transcriptional events within defined regions of the biofilm, including those associated with *agr*, are essentially washed away.

In this context it should be noted that a primary motivation for the experiments we describe was the observation that mutation of *sarA* results in a reduced capacity to form a biofilm due to the increased production of extracellular proteases and that this limitation can be correlated with increased antibiotic susceptibility in the context of a biofilm-associated infection (2,

45, 47, 48). This suggests that inhibitors of *sarA* could be used to limit *S. aureus* biofilm formation to a therapeutically relevant degree. However, our earlier results suggested that any therapeutic benefit derived from such inhibitors would potentially be compromised by the adverse consequence of increasing the production of alpha-toxin (3). Thus, the results we report not only demonstrate that this is not the case in most clonal lineages of contemporary *S. aureus* clinical isolates but also that an inhibitor of *sarA* production and/or function would in several important respects be an inhibitor of *agr*-mediated phenotypes, including the "production" of at least some critical exotoxins.

Whether this effect is evident under *in vivo* conditions and therefore therapeutically relevant remains to be determined, but many serious *S. aureus* infections do occur in the relatively localized environment of a biofilm and/or abscess, and in this context, it might be expected that extracellular products, including proteases and exotoxins, would remain in close proximity to each other. In this respect, we would also note that a second important motivation behind the experiments we describe was our demonstration that mutation of *sarA* in the USA300 isolate UAMS-1625 limited the development of skin lesions in our *in vivo* model of biofilm-associated infection (48). To the extent that the results reported here confirm that the alpha-toxin phenotype observed in this isolate is not unique among contemporary clinical isolates of *S. aureus* suggests that *sarA* may in fact be a viable therapeutic target in the context of both chronic, biofilm-associated *S. aureus* infection and acute, toxin-mediated infections, including those caused by CA-MRSA isolates of the USA300 clonal lineage.

ACKNOWLEDGMENTS

This work was supported by grants AI074935 (M.S.S.), AI090250 (L.N.S.), and AI37027 (C.Y.L. and T.T.L.) and by The Intramural Research Program of the National lnstitute of Allergy and Infectious Diseases (M.O.). Support was also obtained from resources provided through the Clinical and Translational Sciences Award (RR0298884) to the University of Arkansas for Medical Sciences. Bacterial isolates were obtained from the NIAID-Supported Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA).

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