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Impacts of *sarA* and *agr* in *Staphylococcus aureus* Strain Newman on Fibronectin-Binding Protein A Gene Expression and Fibronectin Adherence Capacity In Vitro and in Experimental Infective Endocarditis

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We investigated the impacts of *sarA* and *agr* on *fnbA* expression and fibronectin-binding capacity in *Staphylococcus aureus* in vitro and in experimental endocarditis. Although *sarA* up-regulated and *agr* down-regulated both *fnbA* expression and fibronectin binding in vitro and in vivo, *fnbA* expression was positively regulated in the absence of both global regulators. Thus, additional regulatory loci contribute to *fnbA* regulation and fibronectin-binding capacities in *S. aureus*.

Staphylococcus aureus is the most common cause of endovascular infections (2). The capacity of *S. aureus* to cause human diseases involves a variety of cell surface-associated and extracellular virulence factors (5, 7, 11, 15). Two fibronectin-binding proteins (FnBPA and FnBPB), have been ascribed multiple functions, including cell-specific binding (e.g., epithelial and endothelial cells), invasion and persistence within such cells, and triggering of host cell apoptosis (1, 14, 21, 26). Additionally, FnBPA has been shown to be involved in adherence to damaged heart valves (23). Moreover, FnBPs expressed on the *S. aureus* surface may be degraded by extracellular proteases (17, 18), suggesting that such enzymes participate in the transition of *S. aureus* cells from an adhesive to invasive phenotype.

Classically, the expression of FnBPs and the synthesis of extracellular proteases are controlled in vitro by at least two global regulatory loci: the accessory gene regulator (*agr*) and the staphylococcal accessory regulator (*sarA*) in *S. aureus* (3, 22, 24). There is a complex interaction between *sarA* and *agr* to coordinately regulate *S. aureus* virulence factor expression, including selected adhesins and extracellular proteases (7, 9, 22). In the present study, we have characterized the impacts of the *sarA* and *agr* loci upon *fnbA* expression, fibronectin-binding capacity, and protease activity in a set of isogenic *S. aureus* Newman strains in vitro and in an experimental rabbit endocarditis model.

***fnbA* promoter expression in vitro.** The *S. aureus* strains and plasmids used in this study are listed in Table 1 (strain Newman is *agr* type 1). Flow cytometry (FACScalibur; Becton-Dickinson, San Jose, Calif.) was utilized for quantification of *fnbA* promoter expression, employing a promoter-green fluorescent protein (GFP) reporter fusion, as previously described (28, 30). As expected, *fnbA* promoter expression was maximal during exponential growth of the parental strain and then plateaued (Fig. 1). In addition, the anticipated positive and negative regulatory effects of *sarA* and *agr*, respectively, on *fnbA* promoter expression were observed (Fig. 1) (3, 24, 29). Interestingly, the percentage of *fnbA*-expressing cells in the *sarA agr* double mutant paralleled that of the *sarA* single knockout mutant during exponential and early postexponential growth phases but increased to near-parental levels in late stationary growth phase (Fig. 1). These data suggest that environmental cues (e.g., low pH, nutrient limitation) or other regulatory loci contribute to *fnbA* expression during the stationary growth phase in the absence of *sarA* and *agr* in vitro (e.g., *sae*) (27).

Northern blot analysis of *fnbA* transcription. RNA isolation and Northern blot analysis were performed as described previously (29). The transcription of *fnbA* in the parental strain was maximal during mid-log phase (Fig. 2). As expected, in the *agr* mutant, there was substantial up-regulation in *fnbA* transcription during the late log phase, while *fnbA* transcription in the *sarA* mutant was markedly reduced compared to that in the parental strain (Fig. 2) (24, 29). It is noteworthy that the level of *fnbA* transcription in the *sarA agr* double mutant was between the levels of the single *agr* and *sarA* mutants. Interestingly, we also observed a bimodal increase in *fnbA* transcription in the double mutant, with the first peak occurring during the mid-log phase and a smaller but noticeable peak occurring during the late stationary phase (overnight culture). Therefore, these in vitro transcriptional data concurred with those of the GFP reporter gene fusion data sets above.

Protease activity in vitro. To quantify overall protease activity, a microplate assay kit (Molecular Probes, Eugene, Oreg.) was utilized as previously described (4). Protease activ-

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TABLE 1. *S. aureus* strains and plasmids used in this study

Strain or plasmid	Description	Reference
Strains		
Newman	Wild type	29
ALC637	Newman, *sarA*::Tn*917*LTV1	29
ALC355	Newman Δ*agr*::*tetM*	29
ALC638	Newman Δ*agr*::*tetM sarA*::Tn*917*LTV1	29
ALC1829	Newman with recombinant pALC1484	This work
ALC1827	Newman with recombinant pALC1484 containing the *fnbA* promoter	29
ALC1825	ALC637 with recombinant pALC1484 containing the *fnbA* promoter	29
ALC1835	ALC355 with recombinant pLAC1484 containing the *fnbA* promoter	29
ALC1838	ALC638 with recombinant pALC1484 containing the *fnbA* promoter	29
ALC1645	RN6390 with Δ*spa*::*Etbr* mutation	30
Plasmid		
pALC1484	pSK236 with a promoterless gfp_{uvr} gene	29

ity was slightly decreased in the *agr* mutant (~0.8-fold) but significantly increased in the *sarA* mutant (three- to fivefold; $P < 0.05$) compared to the parental strain (Fig. 3). These data are consistent with the documented repression of protease production by *sarA* (17, 18). The *sarA agr* double mutant had a quantitative protease phenotype that was intermediate between those of the *sarA* and *agr* single mutants. No protease activity was observed from any study strain after 4 h of incubation (data not shown).

To evaluate the effect of the global protease inhibitor, α2-macroglobulin (Boehringer-Mannheim, San Diego, Calif.), the above experiments were repeated with cultures preexposed to the inhibitor (range, 0.4 to 1.6 U/ml). Protease activity was inhibited (>80% for all study strains) in the presence of α2-macroglobulin (1.2 U/ml) (data not shown).

Fibronectin adherence in vitro. To quantitate correlation between *fnbA* expression and fibronectin adherence phenotypes, we evaluated the fibronectin-binding capacities of *S. aureus* by direct binding to immobilized human fibronectin as previously described (17, 19). It is noteworthy that fibronectin-binding properties of the set of strains paralleled the individual *fnbA* promoter expression profiles of the strains in vitro (Fig. 4). For example, fibronectin binding was higher in the *agr* mutant than in the parental strain, but it was lower in the *sarA* mutant (binding of the parental strain to fibronectin reached ~5% of the inoculum after 24 h of incubation). In addition, the fibronectin-binding capacity of the *sarA agr* double mutant exceeded that of the *sarA* mutant during the stationary growth phase (16 to 24 h).

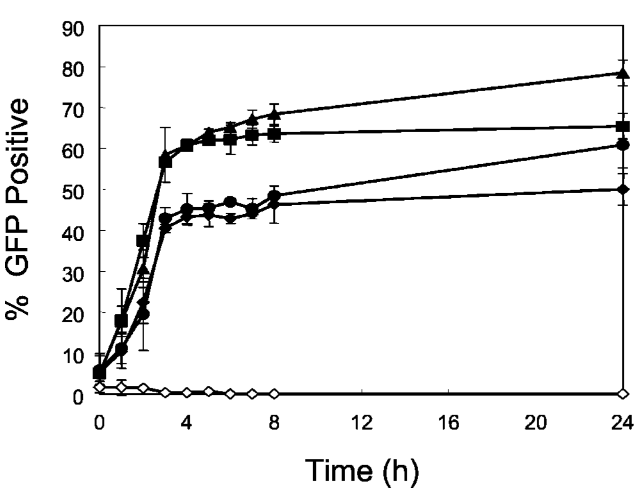

FIG. 1. Expression of the *fnbA* promoter in the parental and mutant strains in vitro. The percentage of GFP-positive *S. aureus* cells during 24 h of incubation in vitro is shown for the *fnbA*::gfp_{uvr} parental strain (■), *agr* mutant (▲), *sarA* mutant (◆), *sarA agr* double mutant (●), and promoterless gfp_{uvr} strain (◇).

Since the production of proteases is down-regulated in the *agr* mutant but up-regulated in the *sarA* mutant and since FnBPs can be degraded by such proteases, it was conceivable that alterations in fibronectin-binding phenotypes might be related to variations in their individual protease production profiles. To test this hypothesis, all study strains were preexposed to α2-macroglobulin (1.2 U/ml), and the fibronectin adherence properties were then determined. For all study strains grown in the presence of α2-macroglobulin, there was a ≥30% increase in fibronectin-binding activities throughout the growth cycle compared to strains grown in the absence of the inhibitor. These data suggest that extracellular protease production contributes modestly to the overall fibronectin-binding capacity of *S. aureus* strains. However, the fact that all strains were equally affected by protease inhibition and only to a modest extent indicates that the predominant mechanisms dictating phenotypic fibronectin binding probably occur at the level of *fnbA* transcription.

Experimental rabbit endocarditis model. Recent studies have demonstrated that *S. aureus* virulence gene regulation profiles defined in vitro are often not precisely mirrored in vivo (6, 8, 28, 30). These data imply that host environmental cues play a major role in the activation of key *S. aureus* virulence genes. Thus, we sought to correlate *fnbA* promoter activation profiles defined in vitro with those delineated in experimental rabbit endocarditis. A well-characterized rabbit endocarditis model was used in these studies as previously described (28, 30).

(i) Microbiologic evaluation. *S. aureus* densities achieved in vegetations were significantly higher than in kidney and spleen for all study constructs at 48 h postinfection ($P < 0.05$) (Table 2). In addition, all the mutants had lower target tissue bacterial densities than the parental strain, although these differences did not reach statistical significance. By comparison, *S. aureus*

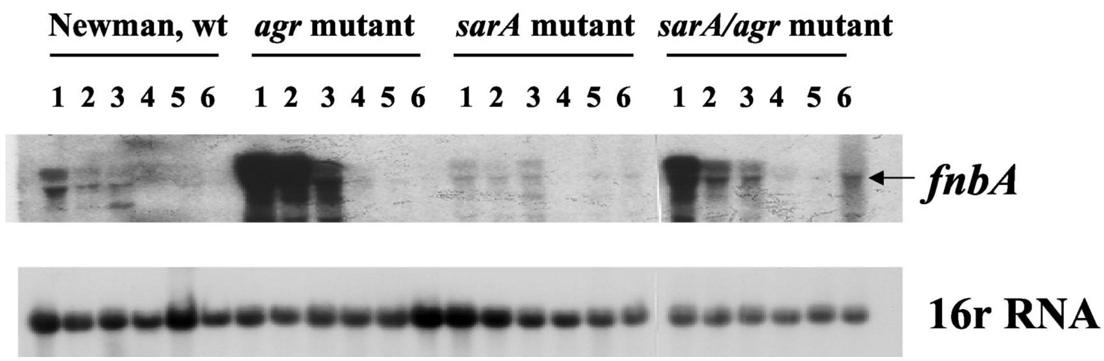

FIG. 2. Northern blots of *fnbA* transcripts from the wild-type (wt) Newman strain and its isogenic *agr*, *sarA*, and *sarA agr* double mutants. RNAs were harvested from mid-log phase (lanes 1 and 2), late log phase (lane 3), early stationary phase (lane 4), and late stationary phase (lanes 5 and 6). We have included transcription of 16S rRNA as a loading control.

densities in vegetations were comparable in all study strains at 8 h postinfection (data not shown).

(ii) *fnbA* promoter expression in vivo. Flow cytometry and a protein A-based immunodetection system were used for detection of *fnbA* promoter expression in the endocarditis model as previously detailed (28, 30). Interestingly, *fnbA* promoter expression profiles defined in vivo in all target tissues for the various constructs paralleled in vitro *fnbA* expression profiles (Fig. 5). Compared to the parental cells, we observed increased *fnbA* expression in the *agr* mutant and decreased *fnbA* expression in the *sarA* mutant, with expression in the double mutant being greater than that of the *sarA* mutant in all target tissues (Fig. 5) ($P < 0.05$ for the parent versus the *agr* mutant in vegetations at 48 h). As in prior studies (28, 30), there were target tissue-specific differences in gene expression, with maximal *fnbA* expression seen in vegetations and kidneys, with reduced expression in the spleen. It is interesting that the extent of GFP expression for the various constructs paralleled the percentage of GFP expression in the target tissues studied (data not shown).

(iii) Fibronectin adherence ex vivo. To determine the relative ability of the study strains (obtained directly from vegetations) to adhere to fibronectin, a modification of the above in vitro adherence assay was performed. Briefly, *S. aureus* cells ($\sim 5 \times 10^3$ CFU based on anticipated vegetation densities) from each vegetation sample (at 24 h after infection [10^7 CFU/animal]) were directly assessed ex vivo for fibronectin-binding capacity by the in vitro assay detailed above. The in vivo *fnbA* expression profiles noted above roughly paralleled the fibronectin-binding capacities of the various constructs isolated directly from cardiac vegetations (data not shown). For example, compared to parental cells, the *agr* mutant cells adhered slightly more to fibronectin, but the *sarA* mutant adhered significantly less to fibronectin ($P < 0.05$). Remarkably, the *sarA agr* double mutant adhered to fibronectin to a higher extent than the *sarA* single mutant ($P < 0.05$ for *sarA* single mutant

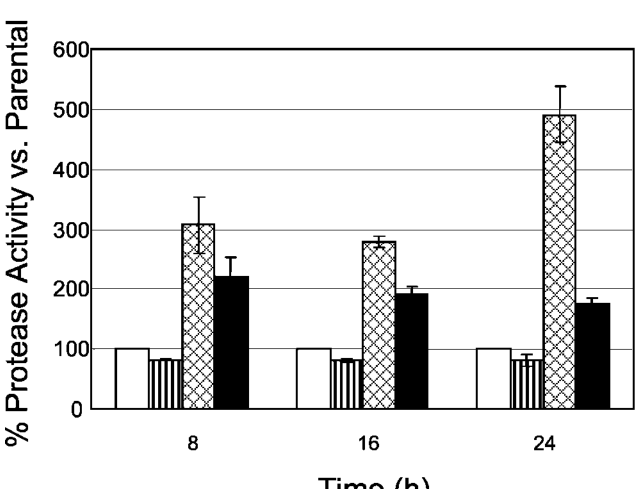

FIG. 3. Protease activity in *S. aureus fnbA*::gfp_{uvr} in the parental strain (□) and *agr* (▥), *sarA* (▩), and *sarA agr* (■) mutants during 24 h of incubation in vitro. The total protease activity in *sarA* and/or *agr* mutants is shown as the percent activity relative to that of the corresponding parent strain, which was normalized at 100%.

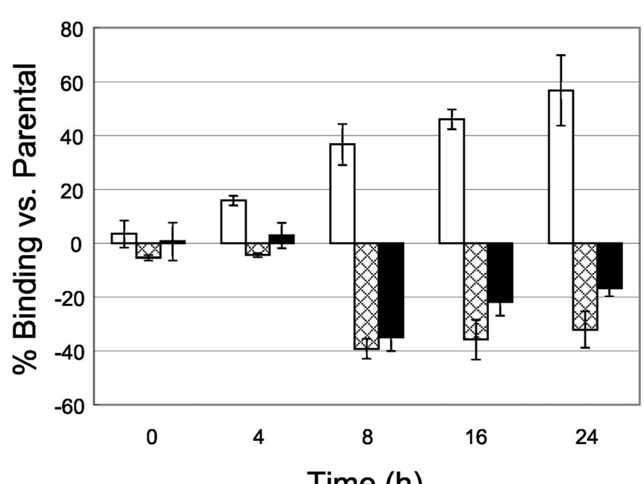

FIG. 4. Adherence of *S. aureus* Newman *fnbA*::gfp_{uvr} in *agr* (□), *sarA* (▩), and *sarA agr* (■) mutants to fibronectin versus the parental strain in vitro. The results are presented as the mean percentage (± standard deviation [error bar]) fibronectin binding compared to that of the parental strain.

TABLE 2. *S. aureus fnbA*::gfp_{uvr} Newman parental strain and its isogenic *agr* and/or *sarA* mutant densities in target tissues of animals with endocarditis challenged with 10^7 CFU/animal

Strain (no. of rabbits)	Log_{10} CFU/g of tissue (mean ± SD)		
	Vegetation	Kidney	Spleen
Parental (10)	8.62 ± 0.32	7.67 ± 0.42[a]	6.62 ± 0.56[a]
agr mutant (12)	8.30 ± 0.37	7.06 ± 0.80[a]	6.11 ± 0.55[a]
sarA mutant (10)	7.95 ± 0.88	6.72 ± 1.19[a]	5.62 ± 0.75[a]
sarA agr double mutant (10)	7.87 ± 0.52	6.66 ± 0.87[a]	5.89 ± 0.46[a]

[a] Significantly different from the value obtained for vegetation ($P < 0.05$).

versus parental strain), mirroring the in vitro and in vivo (intravegetation) profiles of *fnbA* expression.

Several interesting observations emanated from this investigation. As expected, *fnbA* promoter expression in vitro was maximal during exponential growth of the parental strain and then plateaued. In addition, using single knockout mutants, the anticipated positive and negative regulatory effects of *sarA* and *agr*, respectively, on *fnbA* promoter expression in vitro were confirmed (3, 24, 29). Surprisingly, the extent of *fnbA* promoter expression in the *sarA agr* double mutant paralleled that of the *sarA* single knockout mutant during exponential and early post-exponential growth phases but increased to near-parental levels in late stationary growth phase. Similarly, utilizing Northern blot analysis, Blevins et al. (3) noted *fnbA* transcription in *sarA agr* double mutants to be at or above the levels observed for the *sarA* single mutants in two clinical *S. aureus* strains. Collectively, these data suggest that during the stationary growth phase, environmental cues (e.g., low pH, nutrient limitation) or other regulatory loci that influence *fnbA* expression contribute to *fnbA* regulation in the absence of *sarA* and *agr* in vitro (e.g., the *sae* regulon seems to be required for *fnbA* activation in the Newman strain [27]). Further, delineation of the regulatory functions of the growing family of *sarA* homologs may also yield relevant information in this context (10, 12, 20, 25).

To correlate the above differences in *fnbA* expression profiles in vitro with a key functional phenotype, we compared the temporal fibronectin-binding capacities of this set of strains. It is noteworthy that the growth phase-related fibronectin-binding properties of this set of strains in our solid-phase assay paralleled their individual *fnbA* promoter expression profiles in vitro (results which are consistent with those reported by Blevins et al. [3], who utilized a liquid-phase fibronectin-binding assay). As with our *sarA agr* double mutant, in six of the seven strains Blevins et al. (3) studied (including the Newman strain), the fibronectin-binding capacity of the *sarA agr* double mutant exceeded that of the *sarA* single mutant by as much as twofold. Importantly, *fnbA* promoter expression profiles and fibronectin-binding phenotypes defined in vitro for the various constructs in the current study roughly paralleled the profiles and phenotypes defined in vivo in all target tissues in early and well-established infections.

It has been reported that *sarA* and *agr* mediate their effects on *fnbA* at the transcriptional level; this same regulatory pattern has been demonstrated in the Newman strain by Western blot analysis (29). These data indicate that the fibronectin-binding activities of *S. aureus* strains are at least partially due to direct regulation of *fnbA* transcription by *sarA* and *agr*. However, the overall *S. aureus* fibronectin-binding capacity is likely multifactorial, including extracellular protease production as well as production of a cadre of other FnBPs (e.g., FnbB, Ebh [host extracellular matrix binding protein homologue], and Emp [extracellular matrix protein binding protein]) (13, 16). Taken together, it is likely that the collective fibronectin-binding capacity of a given *S. aureus* strain reflects a composite of the activation and regulation of these various loci. Future studies will be required to evaluate the in vitro and in vivo expression paradigms of these other FnBPs, using animal models and gene reporter systems similar to those used in this study, to determine their relative contributions to net fibronectin binding in vitro and in vivo.

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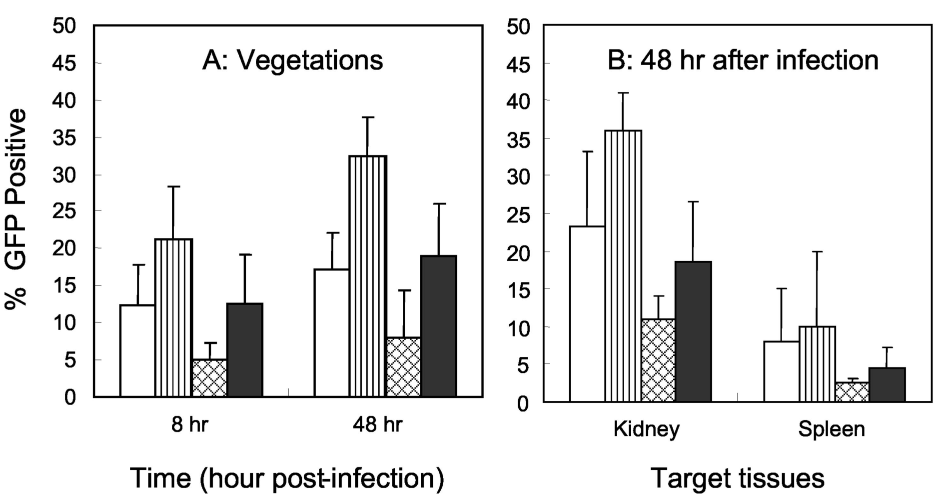

FIG. 5. *S. aureus fnbA* promoter expression in vegetation, kidney, and spleen during the course of experimental endocarditis. (A) Percent GFP-positive *S. aureus* cells in vegetations at 8 and 48 h infection. (B) Percent GFP-positive *S. aureus* cells in kidney and spleen at 48 h infection. Symbols: □, parental strain; ▥, *agr* mutant; ▩, *sarA* mutant; ■, *sarA agr* double mutant.

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