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Selective Activation of *sar* Promoters with the Use of Green Fluorescent Protein Transcriptional Fusions as the Detection System in the Rabbit Endocarditis Model

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The global regulatory locus *sar* is composed of three overlapping transcripts initiated from a triple-promoter system (designated P1, P3, and P2). To explore if the individual *sar* promoters are differentially expressed in vitro and in vivo, we constructed a shuttle plasmid (pALC1434) containing a promoterless gfp_{UV} gene (a *gfp* derivative [Clontech]) preceded by a polylinker region. Recombinant shuttle vectors containing individual *sar* promoters upstream of the gfp_{UV} reporter gene were then introduced into *Staphylococcus aureus* RN6390. Northern and immunoblot analysis revealed that P1 is stronger than the P2 and P3 promoters in vitro. Additionally, the levels of the gfp_{UV} transcript driven by individual *sar* promoters also correlated with the growth cycle dependency of these promoters in liquid cultures, thus suggesting the utility of pALC1434 as a vehicle for reporter fusion. Using the rabbit endocarditis model, we examined the expression of these three GFP_{UV} fusions in vivo by fluorescence microscopy of infected cardiac vegetations 24 h after initial intravenous challenge. Similar to the in vitro findings, P1 was activated both in the center and on the surface of the vegetations. In contrast, the P3 promoter was silent both in vivo and in vitro as determined by fluorescence microscopy. Remarkably, P2 was silent in vitro but became highly activated in vivo. In particular, the *sar* P2 promoter was activated on the surface of the vegetation but not in the center of the lesion. These data imply that in vivo promoter activation of *sar* differed from that observed in vitro. Moreover, the individual *sar* promoters may be differentially expressed in different areas within the same anatomic niche, presumably reflecting the microbial physiological response to distinct host microenvironments. As the *sar* locus controls the synthesis of both extracellular and cell wall virulence determinants, these promoter-gfp_{UV} constructs should be useful to characterize many aspects of *S. aureus* gene regulation in vivo.

Staphylococcus aureus, a major pathogen both in the community and in hospitals (27), has a highly invasive nature. Once the organism gains access to the bloodstream, patients are at risk of developing serious diseases such as endocarditis and other metastatic complications (4). Despite the use of newer antimicrobial agents, the morbidity and mortality from serious *S. aureus* infections remain high (11). The recent emergence of vancomycin-resistant strains in Japan and subsequently in the United States further underscores the importance of identifying alternative strategies for the development of novel antimicrobial strategies to manage invasive *S. aureus* infections.

It is generally recognized that the pathogenesis of *S. aureus* infections is complex and involves the coordinate expression of multiple gene products (22). However, the majority of the data on *S. aureus* virulence have evolved from in vitro studies of bacterial cells at a particular growth phase, usually in nutrient-rich liquid medium. A major impediment to directly applying these data to in vivo conditions is the finding that this organism can significantly alter its phenotypes in response to changing microenvironments (22, 24). These in vitro studies also ignore the interplay of the organism with important tissues and host defense mechanisms, including host proteins and phagocytes to which the organism is exposed. Recognizing that host factors likely modulate the expression of microbial virulence determinants, we wanted to characterize the in vivo expression of *sar*, a global regulatory locus of *S. aureus* that up-regulates the expression of both extracellular virulence determinants (e.g., hemolysins) and cell wall-associated virulence determinants (e.g., fibronectin-binding proteins) in vitro. In this study, we describe the use of a green fluorescence protein (GFP) reporter gene system to examine *sar* promoter activation in vivo, using a model of invasive *S. aureus* infection (rabbit endocarditis).

Genetic analyses have indicated that the *sar* locus is composed of three overlapping transcripts, with a common 3′ end but initiated from three distinct promoters designated P1, P3, and P2 (2). In broth cultures, the promoters P1 and P2 are activated during the exponential phase and expressed less as cells move toward the stationary phase. In contrast, the P3 promoter, being dependent on the alternative sigma factor, SigB (13, 20), is maximally expressed during the postexponential phase. Using XylE transcriptional fusions, we recently showed that the P1 promoter is the strongest, with ~30-fold more activity than P2 and P3 (20). To examine and compare the activation of individual *sar* promoters in vitro and in vivo, we recently constructed an *Escherichia coli-S. aureus* shuttle vector containing a promoterless gfp_{UV} reporter gene (a *gfp*

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TABLE 1. Strains and plasmids used

Strain or plasmid	Reference or source	Description
Strains		
RN6390	18	Laboratory strain that maintains its hemolytic pattern when propagated on sheep erythrocytes and has a genetic background similar to that of 8325-4
RN4220	21	Mutant of 8325-4 that accepts foreign DNA
ALC1435	This study	RN6390 with pALC1420 (*sar* P1 promoter)
ALC1436	This study	RN6390 with pALC1421 (*sar* P3 promoter)
ALC1437	This study	RN6390 with pALC1422 (*sar* P2 promoter)
ALC1440	This study	RN6390 with pALC1434 (vector)
Plasmids		
pCRII	Invitrogen	*E. coli* PCR cloning vector
pSK236	15	Shuttle vector containing pUC19 cloned into the *Hin*dIII site of pC194
pSPT181	17	Shuttle vector
pALC70	16	pSPT181 containing the entire *sar* locus with *sarA*, *sarC*, and *sarB* transcriptional units
pALC1434	This study	Derivative of pSK236 containing a promoterless gfp_{UV} gene preceded by a polylinker region
pLC4	23	Transcriptional fusion vector containing a promoterless *xylE* reporter gene
pALC669	This study	pCRII containing a 240-bp *sar* P1 promoter fragment (nucleotides 620 to 859) (2)
pALC669	This study	pCRII containing a 162-bp *sar* P3 promoter fragment (nucleotides 364 to 525) (2)
pALC678	This study	pCRII containing a 382-bp *sar* P2 promoter fragment (nucleotides 1 to 197 plus 185 bp upstream) (2)
pALC1420	This study	pSK236 containing gfp_{UV} preceded by the 240-bp *sar* P1 promoter fragment
pALC1421	This study	pSK236 containing gfp_{UV} preceded by the 162-bp *sar* P3 promoter fragment
pALC1422	This study	pSK236 containing gfp_{UV} preceded by the 382-bp *sar* P2 promoter fragment

derivative [Clontech, Palo Alto, Calif.] optimized for expression in prokaryotes) preceded by a polylinker region. By linking individual *sar* promoters to gfp_{UV}, we found that the expression of GFP_{UV} in this vector system is dependent on the strength of the upstream promoter. To examine the activation of the *sar* promoters in vivo, *S. aureus* strains containing individual *sar* promoter-gfp_{UV} transcriptional fusions were injected into rabbits that had been catheterized to induce endocarditis. Upon sacrifice, fluorescence microscopy revealed that the *sar* P1 promoter was active both in vivo and in vitro (on agar plates), while the P3 promoter was silent in both scenarios. Remarkably, the P2 promoter was silent in vitro but became active in vivo, and it appeared to be differentially expressed within different parts of the infected tissues.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids are listed in Table 1. Phage ϕ11 was used as the transducing phage for *S. aureus* strains. CYGP, 0.3GL media (21) and tryptic soy broth were used for the growth of *S. aureus* strains, while Luria-Bertani medium was used for growing *E. coli*. Antibiotics were used at the following concentrations: tetracycline, 5 μg/ml; chloramphenicol, 10 μg/ml; and ampicillin, 50 μg/ml.

Cloning strategies. The plasmid pGFPuv (Clontech) contains a *gfp* derivative that has been optimized for expression in an *E. coli* host. To optimize expression of GFP_{UV} in an *S. aureus* host, we introduced a *sarA* ribosomal binding site (9) upstream of gfp_{UV}, thereby enhancing translation in *S. aureus*. Expermentally, we amplified by PCR a ~750-bp fragment encompassing the gfp_{UV} gene preceded by the *sarA* ribosomal binding site with the following primers containing restriction sites: 5′-ACGCGTCGAC(*Sal*I)-**TAGGGAGAGGTTTTAAAC**-^{289}ATGAGT AAAGGAGAAGAACTT^{309}-3′ (the fragment containing the *sarA* ribosomal binding site is in boldface) and 5′-AACTGCAC(*Pst*I)-^{1005}TTATTTGTAGAG CTCATCCAT^{985}-3′ (numbers indicate nucleotide positions in gfp_{UV}). This fragment was first cloned into the PCR cloning vector pCR2.1 (Invitrogen, Carlsbad, Calif.). The recombinant vector was cut with *Sal*I, blunted with T4 polymerase, cleaved with *Pst*I, and analyzed in a 1% SeaPlaque gel (FMC Inc., Rockland, Maine). The ~750-bp fragment was gel purified and ligated to the *Hin*cII/*Pst*I site of the polylinker region of the *E. coli-S. aureus* shuttle vector pSK236, a chimera of pUC19 and pC194 (12). The recombinant plasmid containing the promoterless gfp_{UV} reporter gene, designated pALC1434, was amplified in *E. coli* and verified by restriction analysis and DNA sequencing.

To assess the utility of pALC1434 and to examine *sar* promoter activation in vitro and in vivo, we employed the triple-promoter system of the *sar* locus to drive the expression of gfp_{UV} with an optimized ribosomal binding site derived from *sarA*. Fragments representing the P1 (240 bp), P3 (162 bp), and P2 (382 bp) promoter regions (20) (Table 1) were amplified by PCR with pALC70 as the plasmid template containing the entire *sar* locus (16). These fragments were ligated into pCR2.1 and amplified in *E. coli*. The inserts within the recombinant pCR2.1 were then cloned into the *Kpn*I/*Xba*I site, a region upstream of the gfp_{UV} within the polylinker site of pALC1434. The inserts were then authenticated by DNA sequencing.

Genetic manipulation of *S. aureus*. Shuttle plasmids were transformed into *S. aureus* RN4220 by electroporation as described by Schenk and Laddaga (25). Transformants were selected on tryptic soy agar containing chloramphenicol. For transduction, phage ϕ11 was used to produce a phage lysate of strain RN4220 containing the recombinant shuttle plasmids. The phage lysate was then used to infect the parental strain RN6390 as described previously (8). Transductants were selected on chloramphenicol-containing agar.

Isolation of RNA and Northern analysis. Total cellular RNAs of *S. aureus* strains were obtained from bacterial cultures grown at 37°C with the FastPrep system (BIO101, Vista, Calif.) as previously described (6). Ten micrograms of RNA was electrophoresed through a 1.2% agarose–0.66 M formaldehyde gel in MOPS (morpholinepropanesulfonic acid) running buffer (20 mM MOPS, 10 mM sodium acetate, 2 mM EDTA, pH 7.0). RNA was transferred onto a Hybond N+ membrane (Amersham) under mild alkaline conditions by using a Turboblotter system (Schleicher and Scheull, Keene, N.H.), fixed to the membrane by baking (80°C for 1 h), hybridized under aqueous conditions at 65°C with α-^{32}P-labeled gel-purified DNA fragments, washed, and autoradiographed (8). Band intensities were quantitated by densitometric scanning with SigmaGel software (Jandel Scientific, San Rafael, Calif.); these values are presented as integrated area units.

Immunoblot analysis of GFP expression. Cell extracts were prepared from *S. aureus* strains. In brief, the bacterial pellet was resuspended in 1 ml of TEG buffer (25 mM Tris, 5 mM EGTA, pH 8), and cell extracts were prepared from lysostaphin-treated cells as described by Mahmood and Khan (19). Proteins from cell extracts of *S. aureus* strains were resolved and transferred onto nitrocellulose membranes as described previously (26). Rabbit anti-GFP polyclonal antibodies (Clontech), diluted 1:5,000, were allowed to incubate with the membrane for 1 h, followed by an additional hour of incubation with a 1:10,000 dilution of goat anti-rabbit–alkaline phosphatase conjugate (Jackson ImmunoResearch, West Grove, Pa.). Immunoreactive bands were detected as previously described (3). SeeBlue prestained protein standards (Novex, San Diego, Calif.) were used for molecular weight estimation.

Spectrofluorimetry. Spectrofluorimetry was conducted to quantitate in vitro expression of GFP_{UV} by various *sar* promoter-gfp_{UV} constructs. Protein concentrations in cell extracts from *S. aureus* strains were assayed by the Bradford dye-binding procedure with bovine serum albumin as the standard (5). The emission and excitation spectra of diluted cell lysates (6.66 μg/ml) were analyzed in an Aminco-Bowman fluorescent spectrophotometer (SLM Instruments, Rochester, N.Y.).

Transcriptional fusion assay with the *xylE* reporter gene. We have previously constructed transcriptional fusions of P1, P3, and P2 promoters of *sar* with *xylE* as a reporter gene in shuttle vector pLC4 (20). To determine the XylE (catechol 2,3-dioxygenase) activities of these *sar* promoter fragments, 10 to 50 ml of cell culture grown overnight was pelleted. The cells were washed twice with 1 ml of ice-cold 20 mM potassium phosphate buffer (pH 7.2), resuspended in 500 μl of

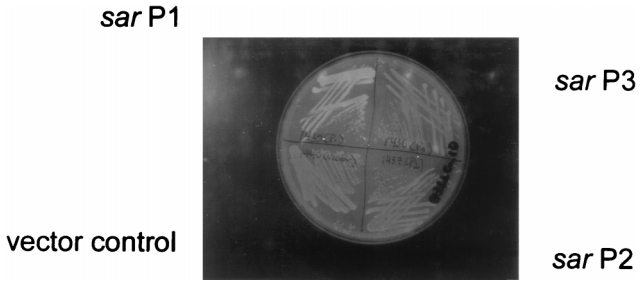

FIG. 1. Excitation of RN6390-derived clones containing *sar* promoters with a long-range UV light (maximal excitation at 365 nm). Strains ALC1435, ALC1436, and ALC1437 contain pALC1434 with the *sar* P1, P3, and P2 promoters, respectively. The control strain ALC1440 contains only the vector pALC1434.

100 mM potassium phosphate buffer (pH 8.0) containing 10% acetone and 25 μg of lysostaphin per ml, incubated for 15 min at 37°C, and then iced for 5 min. Extracts were centrifuged at 20,000 × *g* for 50 min at 4°C to pellet cell debris. The XylE assays were determined spectrophotometrically at 30°C in a total volume of 3 ml of 100 mM potassium phosphate buffer (pH 8.0) containing 100 μl of cell extract and 0.2 mM catechol as described previously (28), with readings of optical density at 375 nm taken at 2, 5, 15, and 25 min. One milliunit is equivalent to the formation of 1.0 nmol of 2-hydroxymuconic semialdehyde per min at 30°C. Specific activity is defined as milliunits per milligram of cellular protein (28).

Rabbit model of endocarditis. To assess the activation of these individual *sar* promoters in vivo, *S. aureus* RN6390 strains containing various *sar* promoter-gfp_{UV} constructs were tested in the rabbit endocarditis model. Briefly, overnight bacterial plate cultures were harvested by centrifugation (2,000 × *g* for 10 min), washed twice in sterile normal saline, and resuspended to an optical density at 620 nm of 1.6 (≈10^9 CFU/ml). Dilutions of the bacterial suspension in phosphate-buffered saline were prepared, and the final infecting inoculum was confirmed by plate counting. For in vivo gene expression, RN6390-derived strains containing recombinant pALC1434 with individual *sar* promoters (P1, P3, and P2) were used to induce endocarditis on the aortic valve of New Zealand White rabbits as described previously (7). In brief, rabbits were anesthetized by intramuscular injections of ketamine chloride at 35 mg/kg and xylazine at 1.5 mg/kg. Forty-eight hours after the introduction of a transaortic valve polyethylene catheter (inner diameter, 0.86 mm) (to induce sterile thrombotic lesions on the valve), animals (three each) were challenged intravenously with 3×10^8 CFU. This inoculum was chosen, based on pilot studies, to ensure adequate numbers of bacteria within vegetations for routine and fluorescence microscopic visualization. Catheters remained in place until animals were sacrificed by lethal injection of sodium pentobarbital (100 mg/kg) at 24 h after bacterial challenge. Aortic valve vegetations were removed and placed into O.C.T.-Tek holding solution for subsequent tissue processing for routine and fluorescence microscopy. Several vegetations were also removed, homogenized, and quantitatively cultured to ensure induction of infection.

Fluorescence microscopy. To evaluate the fluorescence status of various *sar*-gfp_{UV} constructs prior to animal challenge, direct fluorescence of bacterial colonies on overnight agar plates was performed with hand-held long range UV light (365 nm). Additionally, an aliquot of each of the challenge inocula was applied to a slide, air dried, and examined by epifluorescence microscopy.

For bacterial expression in tissues (in vivo), infected vegetations were placed into fixative solutions (Zeus Scientific Inc., Raritan, N.J.). Samples were then washed in citrate buffer, embedded in O.C.T. compound (Sakura Finetek USA Inc., Torrance, Calif.), and frozen. Four-micrometer frozen sections were placed on slides, air dried, and covered with coverslips with aqueous mounting media. Slides were examined with an Olympus DH2RFCA fluorescence microscope with a filter preset at a wavelength of 340 to 400 nm. The slides were also stained with Giemsa stain for localization of bacteria within the vegetative lesion.

RESULTS

In vitro expression of GFP with transcriptional fusions containing *sar* promoters. To evaluate the feasibility of GFP as a reporter system in vitro and to assess in vivo activation of individual *sar* promoters, we constructed a pSK236-derived shuttle vector containing the gfp_{UV} gene preceded by an *S. aureus* ribosomal binding site (of *sarA*) and a polylinker region. This recombinant vector, pALC1434, was then used to test activation of the *sar* promoters in vitro and in vivo. Using the multiple cloning site within the pUC19 portion of pALC1434, we first cloned individual *sar* promoters into the vector in *E. coli*. These recombinant shuttle vectors were then introduced into *S. aureus* RN6390.

TABLE 2. *sar* promoter activity as assayed by XylE assays, Northern analysis, and fluorescence spectroscopy

Promoter	XyE activity[a]	Northern blotting[b]	Emission fluorescence[c]
sar P1	312	12,000	44.0
sar P3	3.0	2,404	12.0
sar P2	4.0	1,005	14.0
Vector	1.0	0	1.0

[a] XylE activity was determined by an enzymatic assay with catechol 2,3-dioxygenase (20) in RN6390-derived clones containing the recombinant transcriptional vector pLC4 in which the respective *sar* promoters were cloned upstream of the *xylE* reporter gene. The results are given as milliunits per milligram of cellular proteins (late-log-phase cells).

[b] Band intensities of the *gfp* transcript at late log phase on Northern blots were quantitated by densitometric scanning with SigmaGel software (Jandel Scientific) and are presented as integrated area units.

[c] For emission fluorescence, cell lysates (20 μg each) containing GFP_{UV} were excited at 395 nm, and the emission fluorescence was measured at 515 nm. The data are presented as fluorescence units compared to the standard curve. The control is the vector pALC1434 alone without any promoter.

In exciting RN6390-derived colonies containing *sar* P1, P3, and P2 promoters with a long-range UV light source (365 nm), colonies grown overnight (37°C) on an agar plate and containing the P1 promoter exhibited the brightest fluorescence, while those of P2 and P3 were much weaker, with slightly higher fluorescence than the vector control (Fig. 1). To quantitate these fluorescences, cell-free lysates of these clones were prepared. Analysis of equivalent amounts of cell extract (20 μg in 3 ml) in a fluorescence spectrophotometer revealed that the excitation maxima of these constructs remained at 395 nm as expected. Quantitative emission fluorescence of the cell lysate derived from the clone containing the *sar* P1 promoter was three- to fourfold higher than those of P2 and P3 promoter fusions (Table 2). As a control, the parental strain RN6390 did not emit any fluorescence.

As another indicator of the *sar* transcriptional activity, we also quantitated the transcription of the gfp_{UV} gene in a Northern blot. Recognizing that individual *sar* promoters are growth cycle dependent, with P1 and P2 most active in the exponential phase and P3 transcribed primarily in the postexponential phase (2), the transcription of gfp_{UV} initiated from each of the *sar* promoters during the growth cycle was examined (Fig. 2 and Table 2). As anticipated, the strongest promoter, P1, and the weaker P2 promoter were most active at mid- to late log phase, with expression tapering during the postexponential phase (Fig. 2). In contrast, the P3 promoter was transcribed

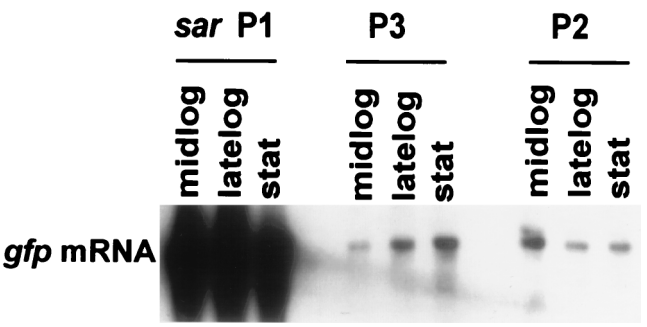

FIG. 2. Northern blot of *gfp* transcripts of pALC1434 driven by the *sar* P1, P3, and P2 promoters. The control vector with no promoter upstream yielded no transcripts (data not shown). stat, stationary.

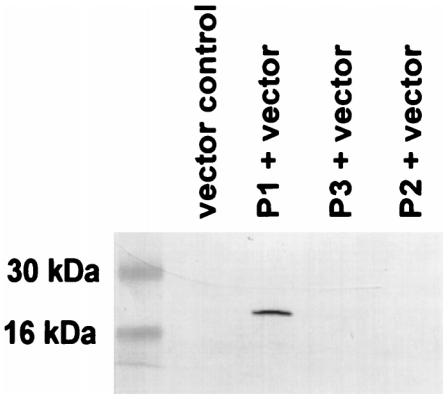

FIG. 3. Immunoblot of cell lysates of RN6390 clones with GFP_{UV} driven by *sar* promoters. The anti-GFP antibody was used at a 1:5,000 dilution.

weakly during the exponential phase and peaked at the postexponential phase. Predictably, gfp_{UV} was not transcribed in the vector control lacking a promoter (data not shown).

As the fluorescence activity relies on the translation of the GFP_{UV} protein, the expression of GFP_{UV} was evaluated on immunoblots. Using rabbit polyclonal antibody against GFP (1:5,000 dilution), we were able to detect GFP expression only in the cell lysate of the RN6390-derived clone containing the *sar* P1 promoter and not in those containing either the weaker P2 or P3 promoter or in the vector control (Fig. 3).

We also compared the gfp_{UV} fusions of these *sar* promoters to those obtained with *xylE* as a reporter gene, using the shuttle vector pLC4 (20). A direct quantitative comparison between the two constructs was not feasible because the plasmid copy numbers of the recombinant pLC4 and pALC1434 may differ in identical *S. aureus* backgrounds (Table 2). Nevertheless, the data with both recombinant pLC4 and pALC1434, containing distinct *sar* promoters, were consistent with the observation that P1 is the strongest promoter, while P2 and P3 promoters revealed significantly lower but equivalent activity (Table 2). Depending on the assay method, the P1 promoter was ≈3 to 80 times stronger than P2 and P3 (Table 2). Collectively, these in vitro data demonstrated the utility of the vector pALC1434 in assessing transcriptional activity with GFP_{UV}.

Expression of GFP in vivo in the rabbit endocarditis model. To explore whether the observed in vitro patterns of *sar* promoter activation were similar in vivo, we examined vegetation tissues of animals with experimental infective endocarditis induced by each of the *sar*-gfp_{UV} constructs by routine and fluorescence microscopy. In animals challenged with the three *sar*-gfp_{UV} constructs, there were no significant differences in bacterial vegetation densities achieved at 24 h after infection (data not shown). The *sar* P1 promoter, as seen with fluorescence microscopy in vitro (Fig. 1), was activated in infected vegetations both on the lesion surface (Fig. 4B) and deep within the vegetations (Fig. 4A). The location of these fluorescent bacteria in the vegetations was confirmed by Giemsa staining (Fig. 4C). To avoid the possibility that the fluorescence activity associated with the P1 promoter was a carryover from the bacterial culture (in which P1 was activated), we conducted a parallel study in which harvested bacteria of the *sar* P1 construct in vitro were resuspended in RPMI 1640 at 4°C for 6 days to completely bleach the fluorescence activity. At the end of this incubation period, epifluorescence microscopy revealed that >90% of the bacteria were nonfluorescent. These bleached bacteria, upon injection into the rabbits, were able to fluoresce within 24-h-old vegetations in a manner similar to that of the nonbleached control, thus suggesting that the fluorescence activity was directly attributed to in vivo activation of P1. In contrast to the case for the *sar* P1 promoter, fluorescence activities were not detected in vegetations containing bacteria with the P3 promoter fusion even though numerous colonies could easily be demonstrated with Giemsa stain throughout the vegetations (data not shown). Despite its quiescent activity in vitro (Fig. 1), the *sar* P2 promoter was highly activated in vivo as revealed by fluorescence microscopy. In particular, the P2 promoter was active in bacterial cells located at the periphery of the vegetative lesion (Fig. 4E) while remaining inactive in cells in the center of the vegetation (Fig. 4D). These results supported the notion that individual *sar* promoters can be differentially activated in vivo within the animal host. More importantly, the pattern of activation in vivo can be different from that in vitro.

DISCUSSION

Temporal expression of many of the extracellular and cell wall virulence determinants in *S. aureus* has been shown to be under the control of the global regulatory locus *sar*. The *sar* locus is composed of three overlapping transcripts in a parallel array, with the *sarA* open reading frame (ORF) present in all three transcripts (2). As documented previously (20), transcriptional assays with an *xylE* reporter gene in vitro revealed that the P1 promoter is at least 30-fold more active than P2 and P3. Activation of these promoters is also growth cycle dependent (2), with the P1 and P2 promoters more active in mid- to late log phase and P3 maximally expressed in the postexponential phase (20).

Recent studies disclosed that the SarA protein level is critical to *agr* activation (10a). In particular, SarA protein levels are positively influenced by the smaller ORFs, designated ORF3 and ORF4, encoded within the largest *sarB* transcript initiated from the *sar* P2 promoter. Activation of the P2 promoter, in conjunction with the P1 promoter, will thus likely lead to a SarA expression level higher than that from the P1 promoter alone (20). Recognizing that SarA binds directly to the *agr* promoter region (10), we also found that increased SarA levels correlated with a higher degree of *agr* activation in vitro (10a).

As the *sar* locus controls an assortment of virulence determinants in *S. aureus* (7) that might be influenced by microenvironmental factors in vivo which are absent in vitro (e.g., host proteins and phagocytic cells), we wanted to evaluate if the pattern of distinct *sar* promoter activations in vitro paralleled those in vivo. For this purpose, we constructed a shuttle vector containing a promoterless gfp_{UV} gene preceded by a polylinker site. By inserting a ribosomal binding site of *S. aureus* upstream of the gfp_{UV} gene, we attempted to optimize the translation of the GFP_{UV} reporter protein of pALC1434 upon activation by an appropriate promoter in an *S. aureus* host. Transcription and immunoblot analyses of the *gfp* gene product (Fig. 2 and 3) driven by different *sar* promoters paralleled *sar* activation data obtained by utilizing a XylE reporter fusion assay (20). Accordingly, P1 was the most active promoter in both assays, with significantly higher activity than either P2 or P3. The growth phase dependency of the *sar* promoters in liquid culture was also evident in the *sar* promoter-gfp_{UV} fusion. More specifically, the gfp_{UV} transcript initiated from the P1 and P2 promoters was maximally transcribed during the mid- to late log phase and activity tapered during the stationary phase, whereas the P3-initiated gfp_{UV} transcript began in the late log phase, with activity peaking during the stationary phase (Fig. 2). These data are consistent with those obtained with the XylE

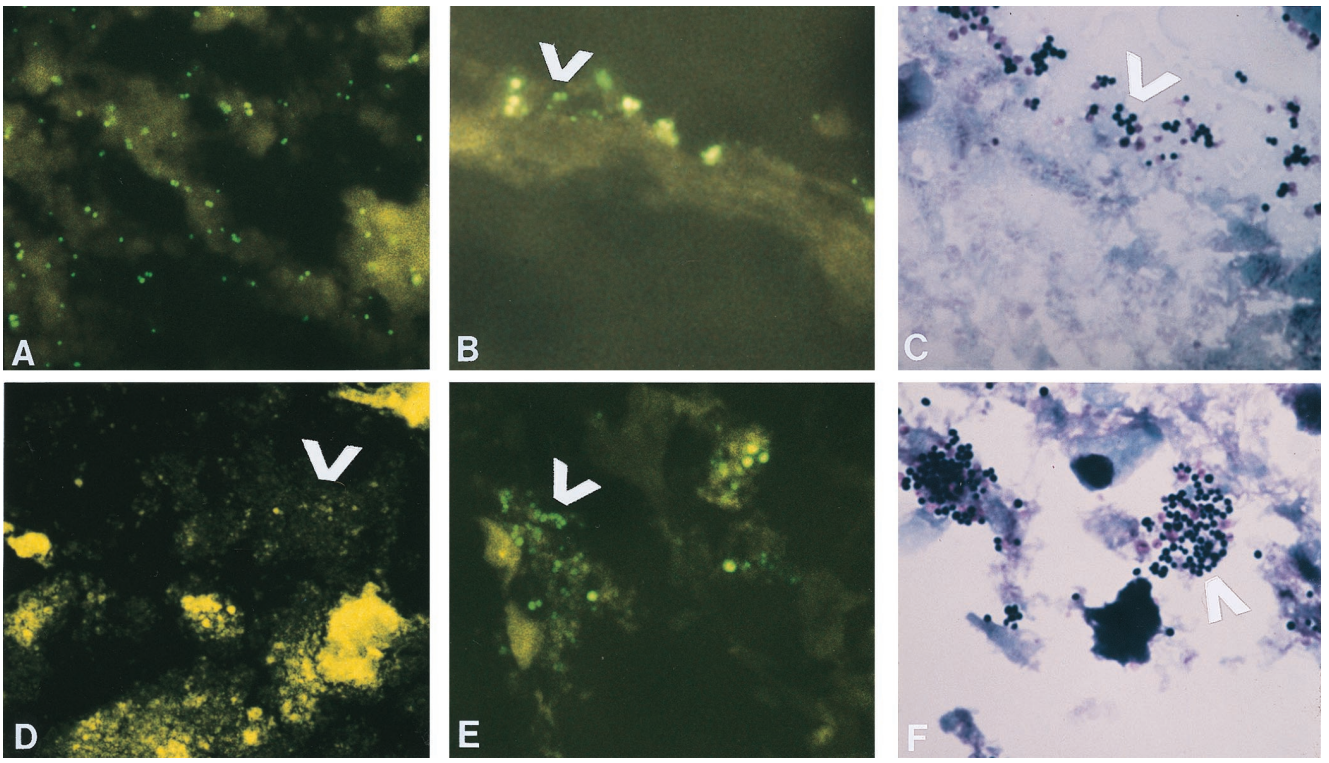

FIG. 4. (A) Twenty-four-hour-old vegetations infected with ALC1435 (RN6390 containing the *sar* P1-gfp$_{UV}$ construct), in which the infecting strain was preincubated in RPMI 1640 medium for 6 days at 4°C to turn off fluorescence (the half-life of GFP$_{UV}$ is 24 h [10b]). These bacteria were then injected into catheterized rabbits to induce endocarditis. As seen, bacterial colonies deep within the vegetation now exhibit uniformly bright green fluorescence. Magnification, ~×300. The area deep within the vegetation is farther from the area of blood flow. (B) Same experiment as in panel A, except that bacterial colonies are on the surfaces of the vegetations. Note brightly fluorescent bacterial colonies (arrowhead). Magnification, ~×300. This area is proximal to the region of blood flow. (C) Same preparation as in panel B stained with Giemsa stain to show bacterial colonies on the vegetation surface. Magnification, ~×225. (D) Twenty-four-hour-old vegetations infected with ALC1437 (*sar* P2-*gfp*$_{UV}$ construct). The arrowhead denotes a colony of nonfluorescent bacteria upon excitation with a UV light source. Note that the yellow-stained background represents infected tissue, not green fluorescent bacteria. Magnification, ~×200. (E) Same experiment as in panel D, except that bacterial colonies are found on the vegetation surface. Note fluorescent P2-*gfp*$_{UV}$-expressing cells (arrowhead) attached to the vegetation surface. Magnification, ~×300. (F) Same preparation as in panel E stained with Giemsa stain to show bacterial colonies on the vegetation surface. Magnification, ~×225.

reporter fusion in vitro (20). Although both GFP and the XylE gene products are relatively stable, the GFP reporter system offers unique advantages because quantitative fluorescence of GFP does not require developing substrate, nor does it necessitate cell lysis. Taken together, our data indicate that the shuttle vector pALC1434 containing a promoterless *gfp*$_{UV}$ gene is highly useful for assaying in vivo promoter activity of *S. aureus*.

To assess the activation of *sar* promoters in vivo, we cloned various *sar-gfp*$_{UV}$ fusions into *S. aureus* RN6390, a prototypic isolate which we have previously used in virulence studies in the rabbit endocarditis model (7). This animal model has several advantages for studying in vivo gene activation, including (i) high achievable bacterial densities within the vegetation for routine and fluorescence microscopy; (ii) wide bacterial distribution throughout the vegetations, thus allowing assessment of specific gene activation in distinct anatomic regions within the same lesion; and (iii) the presence of host proteins (e.g., fibrinogen and fibronectin) and host cells (e.g., platelets) within the vegetations. By harvesting infected cardiac vegetations 24 h after intravenous injection, the activity from individual *sar* promoters can be detected directly in situ by UV fluorescence in infected tissue. Predictably, the P1 promoter was active both in vitro and in vivo. By bleaching the fluorescence activity of the P1 promoter construct prior to intravenous challenge, we were able to show that the fluorescence activity associated with the P1 promoter within the vegetation was not a carryover from in vitro promoter activation in culture. In contrast to the P1 promoter, the P3 promoter was not activated under either in vitro or in vivo conditions. However, since the *sar* P3 promoter is SigB dependent (13, 20) and is activated late in the growth cycle in vitro, we cannot rule out the possibility that the P3 promoter may be activated much later than P1 as the infected vegetation matures (e.g., at 4 to 6 days postinfection). Most remarkably, the P2 promoter, which was not activated under in vitro conditions, became highly active in vivo (Fig. 4). The P2 promoter was active in the periphery of the vegetative lesion, where the bacteria are likely to be active metabolically (14), but not in the center of the lesion, where the organisms are more metabolically quiescent (14). Presumably, the bacteria at the periphery of the vegetations are more rapidly dividing and hence require additional nutrients. One potential means to acquire more nutrients is to lyse host cells with hemolytic enzymes (22), thus requiring activation of specific genes involved in such a cytolytic pathway (e.g., α-hemolysin gene activation via *sar* or *agr*) (15).

We have recently shown that the elaboration of normal levels of α-hemolysin (*hla* gene) is essential for *S. aureus* persistence within vegetations of animals with infective endocarditis (1). One plausible mechanism that the organism can deploy to maximize activation of *hla* is to optimize SarA expression via simultaneous activation of P1 and P2. In a recent study (20), we demonstrated by transcriptional analysis with an XylE reporter system that a combined P2-P3-P1 pro-

moter was more potent in its activity than the P1 promoter alone. As the P3 promoter has a down-regulatory effect on the P1 promoter activity (i.e., the P3-P1 promoter is weaker than P1) (20), it is likely that combined P2 and P1 promoter activities of the *sar* locus would result in higher transcriptional activity. As the *sarA* gene is the major ORF within the *sar* locus, it is reasonable to presume that a higher level of SarA protein expression would ensue. An increased amount of the SarA protein, by virtue of its binding to the *agr* promoter, would in turn activate RNAII and RNAIII transcription to a higher level than that from the P1 promoter alone, thereby leading to increased α-hemolysin production (8). Accordingly, the promoter analysis in vitro, combined with our in vivo finding of simultaneous activation of *sar* P1 and P2 promoters at the periphery (but not at the center) of the lesion, implied that such selective gene activations may provide the organism with a distinct survival advantage (perhaps via enhanced but tightly controlled α-hemolysin production). To verify this scenario in vivo, experiments are planned to examine activation of *hla* promoter-gfp_{UV} fusions in different areas of the vegetations in the rabbit endocarditis model.

Utilizing the *sar* promoter-gfp_{UV} reporter fusion, our studies here clearly demonstrate the selective and differential gene activation of *S. aureus* in vivo in a relevant animal model system. More importantly, gene expression in vivo, as revealed by the *sar* promoter systems, is likely to differ from that in vitro. By delineating promoter activation of specific virulence genes at particular anatomic sites within infectious lesions (e.g., peripheries versus centers of lesions in different target sites, such as kidneys and spleens) in a relevant animal system (e.g., rabbit infective endocarditis), we will have a unique opportunity to scrutinize serially over time the complex pathogenic process of *S. aureus* infections with respect to specific gene activation.

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