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Salicylic Acid Activates Sigma Factor B by *rsbU*-Dependent and -Independent Mechanisms

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Salicylic acid (SAL) may impact *Staphylococcus aureus* virulence by activating the *sigB* operon (*rsbU-V-W-sigB*), thus leading to reductions in alpha-toxin production and decreased fibronectin binding (L. I. Kupferwasser et al., J. Clin. Investig. 112:222–233, 2003). As these prior studies were performed in strain RN6390 (an *rsbU* mutant) and its *rsbU*-repaired variant, SH1000, the current investigation was designed to determine if the SAL effect occurs via *rsbU*- and/or *rsbV*-dependent pathways in an *rsbU*-intact *S. aureus* strain (FDA486). We thus quantified the transcription from two *sigB*-dependent promoters (*asp*23 and *sarA* P3) in FDA486 in response to SAL exposure in vitro, using isogenic single-knockout constructs of *rsbU*, *rsbV*, or *rsbW* and a green fluorescent protein reporter system. SAL induced *sarA* P3 and *asp23* promoter activities in a dose-dependent manner in the parental strain. In contrast, *sigB* activation by SAL was progressively more mitigated in the *rsbU* and *rsbV* mutants. As predicted, SAL caused significant reductions in both alpha-toxin production and fibrinogen and fibronectin binding in the parental strain. The extent of these reductions, compared with the parent, was reduced in the *rsb* mutants (*rsbV* > *rsbU*), especially at low SAL concentrations. Since generation of the free SigB protein usually requires a sequential *rsbU-V-W-sigB* activation cascade, the present phenotypic and genotypic data suggest key roles for both *rsbU* and *rsbV* in SAL-mediated activation of *sigB* in strains with a fully intact *sigB* operon.

The ability of *Staphylococcus aureus* to survive stress conditions is attributable to activation of stress-response regulatory elements, particularly sigma factor B (*sigB*) (10, 11). Such stress conditions include extremes of temperature, pH, and osmolarity, as well as nutrient limitations, ethanol exposures, and oxygen depletion in vitro (10, 11). The response regulator *sigB* can control a number of downstream gene promoters that possess a SigB recognition motif (12). Thus, during in vitro growth in routine laboratory medium, *sigB* activation can modulate the expression of the global regulator, *sarA*, by enhancing activation of the *sarA* P3 promoter (4, 6). We recently showed that salicylic acid (SAL), the major in vivo biometabolite of aspirin in humans, down-modulates the virulence of *S. aureus* in experimental infection models (e.g., infective endocarditis [IE]) by mechanisms that are largely independent of the antiplatelet effects of aspirin (17). These studies indicated that SAL was a potent activator of the *sigB* operon (*rsbU-V-W-sigB*) both in vitro and in vivo. In turn, such activation was associated with a reduction in SarA protein expression and mitigation of *agr*- and *sarA*-dependent gene expression (e.g., *fnbA* and *hla*) (3, 6). The phenotypic consequences of these genotypic effects were reduced production of alpha-toxin and reduced binding to fibronectin and fibrinogen (17). Reductions in these phenotypes have been demonstrated to be critically involved in decreased *S. aureus* virulence in experimental IE (17).

An interesting aspect of the above investigations was that SAL could activate *sigB* in strains containing either an intact or defective *rsbU* locus within the *sigB* operon, thus implicating both *rsbU*-dependent and *rsbU*-independent pathways for activation (17). The current study was designed to further examine the role of *rsbU*, *rsbV*, and *rsbW* in mediating the impact of SAL on *sigB* activation as well as upon *sigB*-modulated downstream virulence phenotypes. For these investigations, we utilized strategic single-knockout constructs within the *sigB* operon of a *sigB*-intact parental strain.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. FDA486 is an *rsbU* $^{+}$ *S. aureus* strain previously used to study expression of fibrinogen-binding proteins and *sigB*-dependent transcription of selected target genes (24). RN4220 is a restriction-deficient *S. aureus* host strain used as the initial recipient for transformation of plasmids (22). *Escherichia coli* XL1-Blue was used for cloning isolated DNA fragments.

Allelic replacement in *S. aureus* strain FDA486. The construction of *rsbU* and *rsbV* mutants in FDA486 has been described in a previous study (24). We also constructed an *rsbW* mutant of FDA486. Briefly, a region upstream of *rsbW* was amplified with the primers 5′-GCTGGAATTCCGCCTGGATATATTTATC-3′ and 5′-TTCGCCCGGGGTTCCTTCATTAACATGC-3′ (EcoRI and SmaI sites, respectively, are underlined) and then digested with EcoRI and SmaI. The fragment was cloned into the EcoRI-SmaI sites of the temperature-sensitive shuttle vector pCL52.2 (24). A region downstream of *rsbW* was amplified with the following primers with flanking PstI and HindIII restriction sites (underlined), respectively: 5′-TAAACTGCAGGAGCAGGTGCGAAATAAT-3′ and 5′-TGCCAAGCTTTGTAATTTCTTAATTGCC-3′. The downstream fragment was digested with PstI and HindIII and cloned into the PstI-HindIII sites of pCL52.2, already containing the upstream fragment. The *ermC* gene was excised from

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description or construction	Source or reference
Strains		
E. coli XL-1 Blue	Highly transformable strain	Stratagene
S. aureus		
FDA486	Wild-type strain with intact *rsbU* gene	24
RN4220	Restriction-deficient derivate of 8325-4	22
ALC2128	*rsbU* mutant of FDA486	24
ALC2129	*rsbV* mutant of FDA486	24
ALC2130	*rsbW* mutant of FDA486	This study
FDA486 *sar*A P3-*gfp*	FDA486 with *sar*A P3 promoter driving gfp_{uvr} in pSK236	This study
ALC2128 *sar*A P3-*gfp*	*rsbU* mutant with *sar*A P3 promoter driving gfp_{uvr} in pSK236	This study
ALC2129 *sar*A P3-*gfp*	*rsbV* mutant with *sar*A P3 promoter driving gfp_{uvr} in pSK236	This study
ALC2130 *sar*A P3-*gfp*	*rsbW* mutant with *sar*A P3 promoter driving gfp_{uvr} in pSK236	This study
ALC2322	FDA486 with *asp23* promoter driving gfp_{uvr} in pSK236	17
ALC2128 *asp23-gfp*	*rsbU* mutant with *asp23* promoter driving gfp_{uvr} in pSK236	This study
ALC2129 *asp23-gfp*	*rsbV* mutant with *asp23* promoter driving gfp_{uvr} in pSK236	This study
ALC2130 *asp23-gfp*	*rsbW* mutant with *asp23* promoter driving gfp_{uvr} in pSK236	This study
Plasmids		
pCL52.2	Shuttle vector with temperature-sensitive origin of replication for *S. aureus*	19
pALC552	pBluescript containing the *ermC* gene	24
pΔ*rsbW*		This study
pSK236	Shuttle vector containing pUC19 cloned into the HindIII site of pC194	20
pALC1484	pSK236 containing gfp_{uvr}	
pALC1672	pSK236 containing *sarA* P3::gfp_{uvr}	This study
pALC2201	pSK236 containing *asp23*::gfp_{uvr}	This study

pALC552 and introduced into the BamHI-SalI site of pCL52.2 (24), thus resulting in divergent transcription of the *ermC* gene from the rest of the *sigB* operon. The constructed plasmid was electroporated into *S. aureus* RN4220 to select for tetracycline-resistant colonies (3 μg/ml) at 32°C. The recombinant pCL52.2 was then transduced from RN4220 into strain FDA486 with phage ϕ11. Transductants were selected on tryptic soy agar plates containing tetracycline at 32°C. One clone containing the plasmid was grown in 0.3 GL broth (0.3 M glycerol-lactate/BHI broth) (21, 23a) with tetracycline (3 $\mu g\ ml^{-1}$) and erythromycin (5 $\mu g\ ml^{-1}$) at 32°C overnight, followed by 12 h of growth in medium with erythromycin at 42°C, the nonpermissive temperature for pCL52.2 replication. The culture was diluted 1:100 into fresh 0.3 GL broth and incubated at 32°C without antibiotics for 12 h; this step was repeated several times. Erythromycin-resistant but tetracycline-sensitive colonies were selected for further analysis. To validate the authenticity of the *rsbW* mutant, we used a primer (5′-ATGGTCTATTTCAAT GGCAGTTAC-3′) corresponding to bases 331 to 335 of the *ermC* gene (FASTA search; GenBank accession no. Y17294) in combination with a primer downstream of the *sigB* gene (5′-AATATCCTTCTTTAATTCCTCAGTA-3′) to confirm replacement of *rsbW* with Δ*rsbW*::*ermC* by PCR. The resultant PCR fragment was sequenced with identical primers. As an additional confirmation, chromosomal DNA from parental strain FDA486 and the *rsbW* deletion mutant strains was digested with EcoRV and probed in Southern hybridization experiments with labeled PCR fragments of individual genes within the *sigB* operon. To confirm that the *rsbU* and *rsbV* mutations did not lead to polar mutations downstream in the operon, we conducted Western blot analysis of cell extracts of FDA486 and its isogenic *rsbU*, *rsbV*, and *rsbW* mutants with anti-SigB monoclonal antibody 1D1 as previously described (6).

Gene expression studies and assay for *sigB* activation by SAL. The function of the *sigB* operon depends on the generation of free SigB protein, i.e., upon the release of SigB from the normally inactive RsbW-SigB complex. This release is facilitated by the competitive binding of RsbW by the dephosphorylated form of RsbV upon activation by RsbU (11, 14, 21). Since the total pool of the SigB protein is relatively constant (free plus bound SigB), the extent of the level of free SigB is typically assessed by quantifying expression of one or more *sigB*-dependent promoters (6, 9). For this reason, we determined the expression of the *sigB*-dependent *sarA* P3 and the alkaline shock protein (*asp23*) promoters by green fluorescent protein (GFP) reporter and Northern blot analyses in the presence or absence of SAL. The *asp23* promoter has been previously used as a faithful surrogate for *sigB* activation (9, 17).

(i) GFP reporter assays. To monitor *sarA* P3 promoter activation in the presence or absence of SAL, we used a promoterless red-shifted variant of the gfp_{uv} gene (gfp_{uvr}), as previously detailed (15). This gene was cloned downstream of the *sarA* P3 promoter in plasmid pALC1484, which was then electroporated into the parental strain FDA486, as well as into various *rsb* mutants described above. The gfp_{uvr} fusion constructs allow detection and quantification of the upstream promoter activity by fluorometric techniques (28). All *rsb* mutant constructs grew at the same rate as the wild-type parental strain. Also, the *rsb* mutant constructs bearing the plasmid containing gfp_{uvr} grew at similar rates in the presence of chloramphenicol (10 μg/ml). Moreover, ethidium bromide-stained gels of plasmids among the wild-type strain and *rsb* mutant constructs revealed similar intensities when equivalent numbers of bacterial cells were analyzed. These latter two observations underscore the equivalence of plasmid copy numbers in the parental strain and mutant constructs. To monitor *sigB* activation of the *sarA* P3 promoter (a relatively weak promoter) in the presence or absence of SAL, we utilized an FL600 microplate fluorescence reader (Bio-Tek Instrument, Winooski, VT) with 485/516 nm filters. To monitor *sigB* activation of the *asp23* promoter (a strong promoter) in the presence or absence of SAL, we utilized a previously published, standard fluorometric assay (Turner Fluorometer; Dubuque, IA) (28). As with the *sarA* P3 construct above, the *asp23* promoter was cloned upstream of the *gfpuvr* gene on plasmid pALC1484 and introduced into the FDA486 strain set as described. For the *sarA* P3 construct, GFP expression was quantified at 0, 2, 12, and 28 h of growth at 37°C with shaking (200 rpm). For the *asp23* construct, aliquots were obtained at 0, 6, 16, and 24 h. Extensive pilot studies had indicated that these distinct sampling times provided the maximal separation in promoter expression profiles between untreated versus SAL-treated cells (data not shown). No antibiotics were included in the growth medium for *gfp* constructs.

(ii) Northern blot hybridization. To verify the putative importance of *rsbU* in activation of the *sigB* operon by SAL, we performed Northern blotting to monitor *sarA* P3 and *asp*23 transcriptions. RNA samples were obtained during early postexponential phase, at an optical density at 650 nm (OD_{650}) nm of 1.7 (~6 h of growth), using an 18-mm borosilicate glass tube in a Spectronic 20 spectrophotometer. Twenty micrograms of total cellular RNA from FDA486 and its isogenic mutants (strains carrying *rsbU*, *rsbV* and *rsbW* mutations) were electrophoresed through a 1.5% agarose–0.66 M formaldehyde gel in running buffer (20 mM morpholinepropanesulfonic acid, 10 mM sodium acetate, 2 mM EDTA, pH 7.0). Blotting of RNA onto Hybond N^+ membranes (Amersham, Arlington Heights, IL) was performed with a Turbo-blotter alkaline transfer system (Scheicher and Schuell, Inc., Keene, NH). The intensities of the 23S and 16S rRNA bands stained by ethidium bromide were verified to be equivalent among samples prior to transfer. A [^{32}P]dCTP-labeled *sarA* fragment was used to detect *sarA* transcript as described previously (2). We utilized specific primers (5′-TA GGTATTGGGTATATGAAAGA-3′ and 5′-TTGTCTTTCTTGGTTATTGTT

T-3′) to amplify a 624-bp fragment to be used as a probe to detect *asp23* expression. All the probes were radiolabeled by the random primer method (Ready-To-Go labeling kit; Amersham). The blots were hybridized under high-stringency conditions, washed, and autoradiographed as previously described (7).

Phenotypic studies. (i) Fibrinogen and fibronectin binding assays. We have previously reported that SAL mitigates *S. aureus* binding to solid-phase fibrinogen and fibronectin biomatrices in vitro (17). To assess the effects of various *rsb* mutations on this SAL-mediated phenotype, the parent and all mutant constructs were grown to postexponential phase for maximal *sigB* expression (9) in the presence or absence of SAL (at 25 or 50 μg/ml). Following pelleting, washing, and bovine serum albumin blocking steps (17), 5×10^3 CFU of each construct were added to six-well polystyrene plates precoated with 50 μg/ml of either fibrinogen or fibronectin. Prior to the addition, the bacterial inoculum was briefly sonicated to ensure singlet cells and then allowed to bind to the above biomatrices for 1 h at 37°C on a rotating platform. After unbound cells were removed by three washes with phosphate-buffered saline, 2 ml of tryptic soy agar was overlaid in all wells. Plates were incubated at 37°C for 24 h, when all visible colonies were counted. Bacterial binding was quantified as the percentage of the initial inoculum bound in the presence or absence of SAL. Data were calculated as the means (± standard deviation [SD]) of three independent runs and expressed as the mean percent reductions of fibrinogen or fibronectin binding under various assay conditions.

(ii) Alpha-toxin activity in the presence of SAL. To monitor the impact of SAL on the production of alpha-toxin in the parental strain versus various *rsb* mutants, we employed a well-established phenotypic assay in which the ability of alpha-toxin to lyse rabbit erythrocytes was measured (17). *S. aureus* strains were grown at 37°C in tryptic soy broth (control cells) or in medium containing 25 or 50 μg/ml SAL for 18 h to stationary phase on a rotary shaker. After cells were pelleted at $5{,}000 \times g$ for 10 min, the number of cells in each tube was standardized by spectrophotometry, and aliquots of serial dilutions of culture supernatants were added to a 1% suspension of washed rabbit erythrocytes in 0.01 M phosphate-buffered saline (pH 7.2) containing 0.1% bovine serum albumin. Purified alpha-toxin (1 μg/ml; Toxin Technology, Sarasota, FL) was used as a positive control. Data were expressed as mean units of hemolytic activity (± SD) per ml of culture supernatant from six separate runs. The hemolytic units were defined as the reciprocal of the highest dilution of the culture supernatant causing ≥50% erythrocyte lysis as measured by optical densitometry (17).

Statistics. Continuous data were statistically analyzed by a Kruskal-Wallis analysis of variance, with corrections for multiple comparisons where appropriate. A *P* value of <0.05 was considered significant.

RESULTS

The impact of *rsbU*, *rsbV*, and *rsbW* mutations on SigB expression. The transcription of *sigB* has been shown to be dependent on at least two promoters, one originating upstream of *rsbU* and another upstream of *rsbV*. A third promoter further upstream of *rsbU* has also been described (23) (Fig. 1A). To ensure that our mutation on *rsbU* and *rsbV* did not impact on SigB expression, we probed an immunoblot containing equivalent amounts of cell extracts from overnight cultures of the mutants with the anti-SigB monoclonal antibody 1D1. As the replacement *ermC* gene is transcribed divergently from the rest of the *sigB* operon, inactivation of *rsbU* and *rsbV* did not lead to null expression of SigB (Fig. 1B). Indeed, the SigB protein levels were clearly demonstrable, albeit at lower levels in the *rsbU* and *rsbV* mutants than in the parental strain FDA486 (24). This is consistent with the observation that *rsbU* and *rsbV* are driven by separate promoters and that activation of *rsbU* can occur independently of *rsbV* (24). The expression of SigB, encoded by the last gene in the operon, in both mutants also suggests that any potential polar effect as a result of the *rsbU* and *rsbV* mutations is probably minimal. We also examined SigB expression in the *rsbW* mutant. Similar to the *sigB* mutant, the *rsbW* mutant did not express any SigB as detected by immunoblotting; this finding was expected because

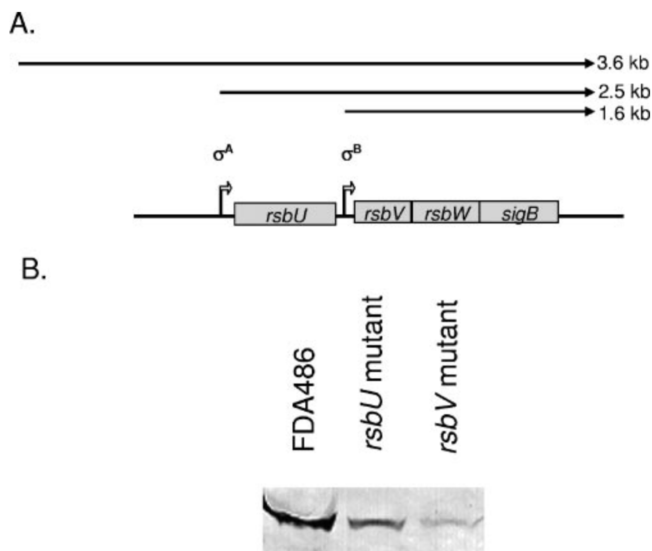

FIG. 1. The effect of *rsbU* and *rsbV* mutations on SigB expression in the FDA486 background. (A) Organization of the *sigB* operon in *S. aureus*. There are two well-described transcripts, one originating from the σ^A promoter upstream of *rsbU* and the other from the σ^B promoter upstream of *rsbV*. A third 3.6-kb transcript was recently described by Senn et al. (25). (B) Equivalent amounts of cell extracts (50 μg each) from FDA486 and its isogenic mutants were immunoblotted onto nitrocellulose. The blot was then probed with anti-SigB monoclonal antibody 1D1 at a 1:2,000 dilution. The protein band was then detected with goat anti-mouse antibody conjugated to alkaline phosphatase and developing substrate as previously described (6). We also examined expression of SigB in *rsbW* and *sigB* mutants of FDA486. In both cases, no SigB protein expression was detected. These immunoblot data have been previously published by one of our laboratories (24).

the expression of RsbW and SigB are translationally coupled (17, 27).

Induction of *sarA* P3 activity by SAL. In pilot screening studies, we sought to establish a single effective SAL concentration range to utilize in the detailed phenotypic and genotypic investigations. We thus exposed the parental strain to a range of SAL concentrations (0 to 100 μg/ml) encompassing both clinically achievable human serum concentrations as well as SAL concentrations previously documented to activate *sigB* expression (17). Using *sarA* P3 promoter activation as a surrogate marker for *sigB* expression, the lowest SAL concentration that clearly increased *sigB* expression compared to untreated cells was 50 μg/ml (Fig. 2). We subsequently used the range of 25 to 50 μg/ml of SAL for the remaining phenotypic and genotypic studies. SAL-induced *sarA* P3 activation in this assay was apparent by 10 h of growth, reflecting both the late-logarithmic-phase maxima of this promoter as well as the time required for maturation of the GFP (13).

We then compared the relative, time-dependent expression of the *sarA* P3 promoter, using GFP reporter assays in the presence or absence of SAL (Fig. 3). Early in log-phase growth, neither the parent nor any of the *rsb* mutants exhibited substantial increases in *sarA* P3 promoter activity in the presence or absence of SAL. In contrast, at postexponential phase (12 h), the parental strain, but not the *rsbU*, *V*, or *W* mutants, exhibited increases in *sarA* P3 expression in the presence of SAL, although this difference (~3.5-fold) did not reach statis-

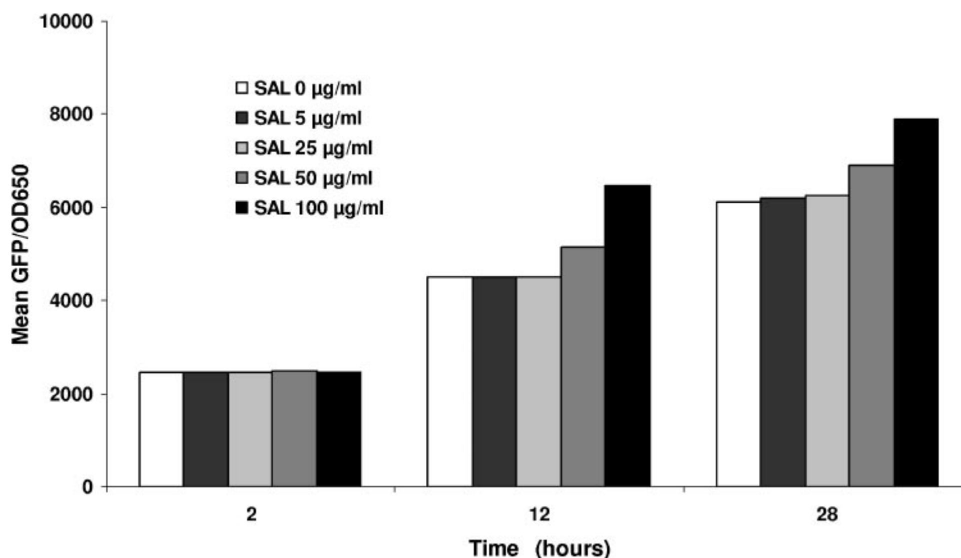

FIG. 2. Effect of SAL at various drug concentrations upon *sarA* P3 promoter activity. Expression of *gfp* driven by the *sarA* P3 promoter was measured during the growth cycle, and fluorescence values were expressed as total GFP fluorescence/OD_{650} to minimize variations in fluorescence due to differing cell densities. These data represent the mean of three independent runs.

tical significance. At 28 h of growth (late stationary phase), the impact of SAL on parental strain FDA486 expression of *sarA* P3 was more pronounced than at 12 h, with an observed 7.5-fold increase compared to uninduced cells ($P < 0.05$). Interestingly, the upregulation in *sarA* P3 expression by SAL at 28 h was higher in the *rsbU* mutant than in the *rsbV* and *rsbW* mutants. This pattern of data shows that augmentation of *sigB* activity due to SAL (albeit relatively small) can still occur with an *rsbU* mutant at late stationary phase. To confirm these observations, we conducted Northern analyses with a *sarA* probe to ascertain *sarA* P3 transcription. At stationary growth phase (Fig. 4A) and also at late exponential phase (not shown), the expression level of the *sarA* P3 transcript was substantially increased in the parental strain with SAL exposure compared to the uninduced control (2,656 versus 1,088 densitometry units, using SigmaGel software) (Fig. 4A). Interestingly, the *rsbU* mutant was also able to modestly increase *sarA* P3 transcription in the presence of SAL (2,069 versus 1,724 densitom-

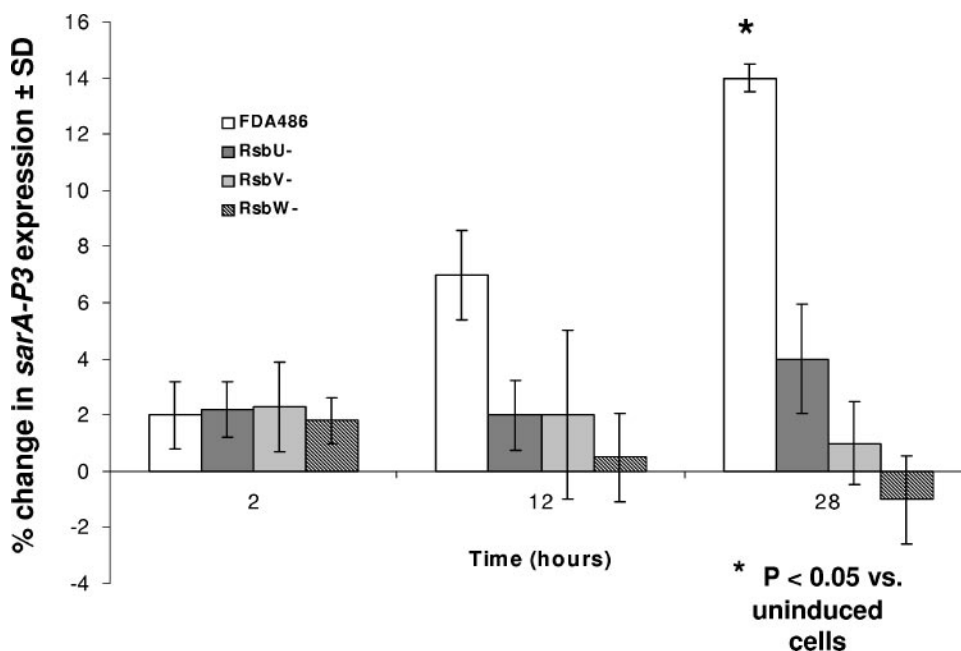

FIG. 3. Effect of SAL (50 µg/ml) on *sarA* P3 promoter activation in the FDA486 parental strains versus the corresponding *rsbU*, *rsbV*, and *rsbW* mutants. The percent differences were calculated by the following formula: [(fluorescence with SAL/fluorescence without SAL) − 1.0] × 100. Results are the means (± SD) of triplicates from a representative experiment that was repeated thee times.

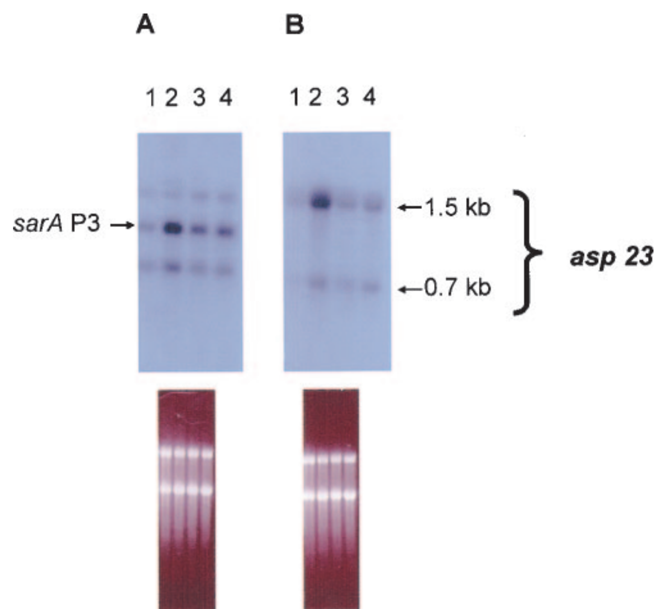

FIG. 4. Transcription of the *sarA* P3 (A) and *asp23* (B) promoter in response to SAL (50 μg/ml) by Northern blotting of *S. aureus* FDA486 (parental) and its corresponding *rsbU* deletion mutants at the postexponential phase of bacterial growth. Lane 1, wild-type strain FDA486; lane 2, wild-type strain FDA486 with SAL; lane 3, *rsbU* mutant; lane 4, *rsbU* mutant with SAL. The figures underneath the blots indicate equivalent loading as reflected by similar ethidium bromide staining of the 16S and 23S rRNA bands.

etry units in the uninduced control). As an additional marker for *sigB* activation, we evaluated *asp23* transcription in the parent strain FDA486 and its isogenic *rsbU* mutant. As shown in Fig. 4B and mirroring data in Fig. 4A, the parental strain displayed an increase in *asp23* transcription with SAL exposure compared to the uninduced control, while the increase in the *rsbU* mutant was more modest.

We next compared the relative, time-dependent expression from the *asp23* promoter, using GFP as a reporter, in the presence or absence of SAL (Fig. 5). Similar to the data for *sarA* P3, the parental strain exhibited a substantial increase in *asp23* expression in the presence of SAL versus uninduced control over the 28-h growth cycle (range, mean percentage change of 1 to 28% at 25 μg/ml versus uninduced controls [data not shown] and 36 to 66% at 50 μg/ml). These differences reached statistical significance at the 28-h time point of SAL exposure ($P < 0.05$ versus both uninduced parental cells and induced *rsb* mutant cells). In contrast, *asp23* expression in the three *rsb* mutants was only modestly induced by SAL at the same drug concentrations. However, these differences (versus uninduced cells) did not reach statistical significance for the mutants.

Phenotypic assays. (i) Fibrinogen and fibronectin binding. In previous studies, we have shown that SAL can reduce the fibrinogen- and fibronectin-binding capacity of wild-type *S. aureus* cells (17). Interestingly, baseline fibrinogen binding, as a reflection of intrinsic bacterial adhesion capacity, was similar between untreated parental cells and the three *rsb* mutants (range, 4.2 to 4.9% of the initial inoculum) (Table 2). Parallel

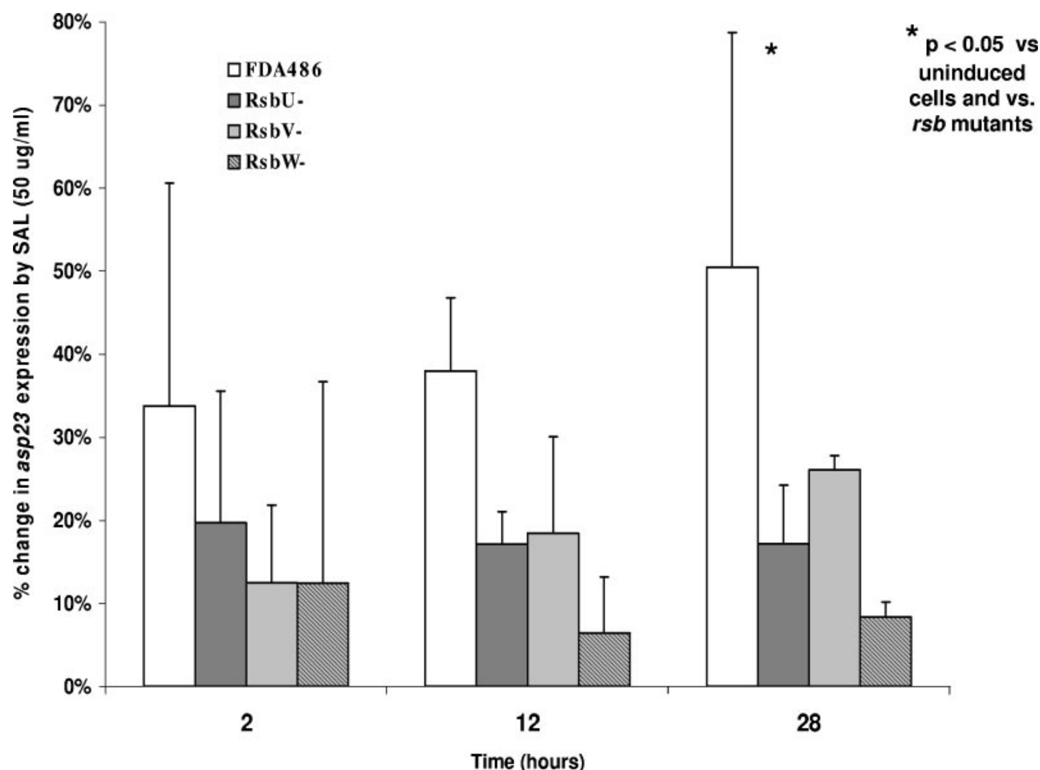

FIG. 5. Effect of SAL (50 μg/ml) on *asp23* promoter activation in the FDA486 parental strain versus the corresponding *rsbU*, *rsbV*, and *rsbW* mutants. The percent differences were calculated by the following formula: [(fluorescence with SAL/fluorescence without SAL) − 1.0] × 100. Results are the means (± SD) of triplicates from a representative experiment that was repeated thee times.

TABLE 2. Adhesion of *S. aureus* strain FDA486 and corresponding *rsbU*, *rsbV*, and *rsbW* mutants to immobilized fibrinogen and fibronectin in the presence or absence of SAL

Strain or mutant	Fibrinogen binding			Fibronectin binding		
	Control[a]	SAL (%)[b]		Control[a]	SAL (%)[b]	
		25 μg/ml	50 μg/ml		25 μg/ml	50 μg/ml
FDA486	4.2 ± 1.1	−25.40	−47.10[c]	3.8 ± 1.1	−20.90	−45.50[c]
rsbU mutant	4.5 ± 1.2	−14.20	−23.30	4.8 ± 1.3	−15.40	−24.30
rsbV mutant	4.5 ± 1.1	−8.90	−22.10	4.8 ± 1.0	−13.90	−25.30
rsbW mutant	4.9 ± 0.8	−11.80	−21.10	4.5 ± 1.4	−8.30	−13.10

[a] Percent binding of initial inoculum to ligand in the absence of SAL.
[b] Mean percent reduction in binding comparing SAL-treated versus untreated (control) *S. aureus* cells. These data are derived from the means of three independent runs and are expressed as mean percent reductions of fibrinogen or fibronectin binding under various assay conditions.
[c] $P < 0.05$ versus untreated control cells.

TABLE 3. α-Toxin hemolytic activity in *S. aureus* strain FDA486 and its isogenic mutants in the presence or absence of SAL

SAL concn (μg/ml)	Hemolytic activity (mean ± SD)[a]			
	FDA486	*rsbU* mutant	*rsbV* mutant	*rsbW* mutant
Control	658 ± 38	825 ± 69	983 ± 104	992 ± 38
25	383 ± 52[b]	700 ± 71	933 ± 58	917 ± 41
50	333 ± 41[b]	658 ± 31	867 ± 29	883 ± 26

[a] Results are expressed in hemolytic units. See text for details.
[b] $P < 0.05$ versus untreated control cells.

data were also observed for fibronectin binding, showing similar binding among all constructs (range, 3.8 to 4.8% of initial inoculum) (Table 2). Upon SAL exposure, the parental cells showed substantial reductions in binding to fibrinogen in a dose-dependent manner, with more reduction at 50 μg/ml ($P < 0.05$ versus control cells) than at 25 μg/ml. Similarly, the SAL-mediated reduction in fibronectin binding for parental cells was greater at 50 μg/ml SAL than the untreated control ($P < 0.05$) (Table 2). For both assays, the reductions in ligand-binding capacity seen in the parental strain were blunted in all *rsb* mutants. Of note, there was somewhat greater dampening of the SAL impact in the *rsbV* (versus uninduced cells) compared to the *rsbU* mutant at 25 μg/ml SAL (but not at 50 μg/ml), although this did not reach statistical significance.

(ii) Alpha-toxin assays. In an earlier study (17), we found that preexposure of wild-type *S. aureus* cells to SAL reduced their capacity to express alpha-toxin, presumably by up-regulating *sigB* activity. To investigate the role of specific genes within the *sigB* operon in mediating this effect, *rsb* mutants were assayed for hemolytic activity (attributable to alpha-toxin) following SAL exposures. As expected, supernatants from stationary-phase parental cells treated with SAL exhibited a significantly diminished capacity to lyse erythrocytes, compared to supernatants from untreated cells, in a dose-dependent manner (~35 to 50% reductions; $P < 0.05$ versus untreated control cells) (Table 3). Of interest, the stationary-phase supernatants from all untreated *rsb* mutant cells exhibited higher baseline hemolytic activity than parental cells, with the *rsbV* and *rsbW* mutants showing a more prominent increase than the *rsbU* mutant. Remarkably, the capacity of SAL to reduce hemolytic activity, as seen in parental cells, was blunted in the *rsbU* mutant. The *rsbV* and *rsbW* mutants also displayed minimal decreases in hemolytic titer in the presence of 25 or 50 μg/ml of SAL compared with nontreated controls. For the *rsbU* mutant, the hemolytic titer at 50 μg/ml of SAL was equal to the parental strain without SAL exposure.

DISCUSSION

The *sigB* operon of *S. aureus* represents a global regulatory system that enables the organism to deal with environmental stresses. However, it differs from the well-characterized *sigB* operon of *Bacillus subtilis* in that it is smaller (four genes versus eight genes) and lacks distinct environmental and energy-sensing modules (27) (Fig. 1A). The *sigB* operon of *S. aureus* consists of a putative sensor (*rsbU*) which responds to a broad range of microenvironmental cues via autophosphorylation to activate the next gene in this operon (*rsbV*). Phosphorylated RsbU acts as a phosphatase (or an anti-anti-sigma factor) to dephosphorylate RsbV. Dephosphorylated RsbV binds competitively to the anti-sigma factor, RsbW, thus displacing the normally inhibitory RsbW from the RsbW-SigB complex (21). The collective result of this activation cascade is the release of free SigB to activate genes with a SigB recognition motif within their promoter region (e.g., *sarA* P3 and *asp23*) (3) via recruitment of RNA polymerase.

Likely related to its role in countering environmental stresses, the *sigB* operon has been shown to be intimately involved in biofilm formation (1), as well as in the regulation of virulence factors. As an example, the activation of the *sarA* P3 promoter impacts the expression of *sarA*-dependent structural genes, including the genes for alpha-toxin (*hla*), V8 protease (*sspA*), and fibronectin-binding proteins (e.g., *fnbA*) (3). SigB may also repress expression of the two-component regulatory system, *saeRS*, which itself positively regulates *hla* and *fnbA* expression (26). Importantly, during in vitro growth, *sigB* is activated at early stationary growth phase, corresponding to its activation of the *sarA* P3 promoter. Since SarA production is the net result of activation of the three *sarA* promoters (*sarA* P1, P2, and P3), *sarA* P3 activation normally leads to enhanced SarA production. However, Karlsson et al. (16) have shown recent data suggesting that clinical *S. aureus* strains differ substantially in intrinsic "tone levels" of *sigB* and SigB-dependent gene expression. This concept was exemplified by the strain-to-strain variability in the production of V8 protease (a SigB-SarA repressible event) (16). Similarly, we have shown that *sigB* can be exogenously stimulated by SAL to a high "tone level," resulting in augmented transcription from the *sarA* P3 promoter. Contrary to growth phase-related effects on SarA expression (i.e., upregulation), hyperactivation of *sigB* due to exogenous SAL leads to reductions in net *sarA* activation (manifested by both reduced SarA protein levels and enhanced V8 protease and lipase production) (16, 17). The mechanism(s) by which excess *sarA* P3 activation by SAL mitigates overall *sarA* expression is not understood but may involve promoter occlusion of the proximal, but more prominent, *sarA* P1 promoter. Alternatively, a direct effect of SAL on the *sarA* promoter complex cannot be ruled out.

We previously demonstrated that the major biometabolite of aspirin, SAL, exerts potent antivirulence effects in vitro and in vivo against a number of well-characterized *S. aureus* strains,

including RN6390, SH1000, ISP479, COL, and Newman (17). These antivirulence effects include reduction in binding to a variety of matrix ligands involved in tissue colonization by *S. aureus* (i.e., fibrinogen, fibronectin, and fibrin), reduction in binding to endothelial cells and platelets (18), and reduction in alpha-toxin production (17). All these phenotypic traits have been linked to the virulence of *S. aureus* in endovascular and other infection models (5). These in vitro phenotypic effects were mirrored in vivo in experimental endocarditis (IE) models as exemplified by the reduced capacity of SAL-treated *S. aureus* cells to bind to sterile aortic valve vegetations in vivo (17). Treatment of animals with established *S. aureus* IE by aspirin or SAL also mitigated virulence, as manifested by reductions in bacterial densities in cardiac vegetations and kidneys, decreases in vegetation size and weight, and prevention of embolic renal infarcts (17, 18). Importantly, the fact that SAL (which is devoid of antiplatelet activities) demonstrated antivirulence properties virtually identical to those of aspirin argued against the idea that the antiplatelet property of aspirin is the principal abating factor and, instead, indicated the possibility that an antibacterial pathway is at work. This hypothesis was validated by our genetic analyses in vitro and in vivo, clearly showing that activation of *sigB* is a critical event in initiating the antivirulence properties of aspirin and SAL (17). This mechanism then leads to down-modulation of global regulons downstream of *sigB* (e.g., *sarA* and *agr*), as well as of key structural genes involved in matrix ligand binding and alpha-toxin production (3). Of note, a recent investigation by Entenza et al. (8) has confirmed that *sigB*-hyperexpressing strains of *S. aureus* exhibit reduced virulence during well-established stages of experimental endocarditis compared to wild-type strains.

Despite the unambiguous role of *sigB* activation by aspirin or SAL in antivirulence properties, the contribution of each gene within the *sigB* operon (i.e., *rsbU*, *rsbV*, or *rsbW*) to this impact is not known. As *sigB* activation by aspirin or SAL occurred in strains RN6390 and ISP479 (*rsbU*-deficient lineage strains of 8325-4) (14) as well as in the *rsbU*-intact strains SH1000, COL, and Newman, this suggested that activation of *sigB* by these compounds could proceed via both *rsbU*-dependent and *rsbU*-independent pathways.

The current study was designed to establish the relative roles of individual genes within the *sigB* operon in mediating the in vitro activation by SAL and in impacting two representative phenotypes (ligand binding and alpha-toxin production). Several interesting findings emerged from this investigation. (i) In parental strain FDA486 (with an intact *sigB* operon), deletion of *rsbU* eliminated a major portion of the capability of the strain to respond to SAL, corresponding to a lesser capacity to activate *sigB* in the *rsbU* mutant. This relationship was evidenced by a much lower level of *sigB*-dependent promoter activation (e.g., *sarA* P3) in the *rsbU* mutant as confirmed by Northern blotting and transcriptional fusions. (ii) The influences of SAL on *sigB* activation were concentration dependent, and the differences between untreated cells and SAL-treated cells were greatest during stationary phases of growth (when *sigB* expression is maximal). (iii) Our data suggested that both *rsbU* and *rsbV* can be targets for SAL. This notion was supported by a hierarchy in the reduction of ligand-binding capacity between the parent and these two latter mutants at 25 μg/ml of SAL (Table 2). This hierarchy was recapitulated in the reduction in hemolytic titers (Table 3) at both SAL concentrations (i.e., parent > *rsbU* mutant > *rsbV* mutant). Based on our previous studies, we recognized that even in the *rsbU* mutant (the putative stress-sensing locus within *sigB*), *sigB* could still be partially activated by energy-dependent stresses (24). This observation and the data from the present study underscore the notion that *sigB* can be activated by *rsbU*-dependent and *rsbV*-dependent pathways. (iv) SigB is normally a repressor of alpha-toxin gene (*hla*) expression; thus, deletion of genes within the *sigB* operon normally results in alpha-toxin hyperexpression (6, 24), as confirmed in the *rsbU* and *rsbV* mutants in the current investigation. Because *rsbW* and *sigB* are translationally coupled (21), the *rsbW* mutant behaves essentially like a *sigB* mutant. Whether SigB represses alpha-toxin production in the presence of SAL via inhibition of *sarA*, *agr*, and/or *sae* remains to be defined. (v) As noted above, SAL exposure in parental strain FDA486 resulted in a reduction in ligand-binding phenotypes; this effect was blunted in the *rsbU* mutant and more so in the *rsbV* and *rsbW* mutants at 25 μg/ml. The basis for the disappearance of this differential effect between *rsbU* and *rsbV* mutants at 50 μg/ml of SAL is not immediately evident. It is plausible that SAL at higher concentrations may affect the baseline phosphorylation of RsbV.

In summary, we have confirmed that SAL exerts substantial effects on phenotypes involved in endovascular virulence via activation of the *sigB* operon; interruption of the *sigB* gene cascade by mutating loci within the operon will blunt this response. Further, the phenotypic effects of SAL appear to proceed via both *rsbU*-dependent and *rsbV*-dependent pathways. Whether SAL has direct influences upon downstream structural genes (e.g., *hla*) or if SAL can upregulate pathways outside of *sigB* to impact the above phenotypes remains to be defined.

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