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, SH1008, 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 (asp23 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 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′-GCTGGAGATTCGCCGATATATTAC-3′ and 5′-TTCCGCCGTTTTCCATTAACATGC-3′ (EcoRI and SmaI sites, respectively, are underlined) and then digested with EcoRI and Smal. The fragment was cloned into the EcoRI-Smal sites of the temperature-sensitive shuttle vector pCL2.2 (24). A region downstream of rsbW was amplified with the following primers with flanking PstI and HindIII restriction sites (underlined), respectively: 5′-TAAACTGCGAGCAGGGCGGAAATAAT-3′ and 5′-TGGTACAGCTTTGTTCTGCGG-3′. The downstream fragment was digested with PstI and HindIII and cloned into the PstI-HindIII sites of pCL2.2, already containing the upstream fragment. The ermC gene was excised from
pALC552 and introduced into the BamHI-Sall site of pCL52.2 (24), thus resulting in divergent transcription of the emrC 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 d11. Transductants were selected on tryptic soy agar plates containing tetracycline at 32°C. One green fluorescent protein (GFP) reporter and Northern blot analyses in the tetracycline-resistant colonies (3
transcripts were selected on tryptic soy agar plates containing tetracycline at 32°C. One
gene (i) GFP reporter assays. To monitor

TABLE 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strains/Promoter Assays</th>
<th>Description or construction</th>
<th>Source or Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli XL-1 Blue</td>
<td>Highly transformable strain</td>
<td>Stratagene</td>
</tr>
<tr>
<td>S. aureus</td>
<td>Wild-type strain with intact rsbU gene</td>
<td>FDA486</td>
</tr>
<tr>
<td></td>
<td>Restriction-deficient derivative of RN4220</td>
<td>22</td>
</tr>
<tr>
<td>RN4220</td>
<td>Restriction-deficient transformable strain of FDA486</td>
<td>24</td>
</tr>
<tr>
<td>ALC2128</td>
<td>rsbU mutant of FDA486</td>
<td>This study</td>
</tr>
<tr>
<td>ALC2129</td>
<td>rsbV mutant of FDA486</td>
<td>This study</td>
</tr>
<tr>
<td>ALC2130</td>
<td>rsbW mutant of FDA486</td>
<td>This study</td>
</tr>
</tbody>
</table>
| FDA486 sarA P3-gfp      | FDA486 with sarA P3 promoter driving gfp

<i>Salicylic acid and sigma factor B</i> 5897

Vol. 188, 2006
Phenotypic studies. (i) Fibrinogen and fibronectin binding assays. We have previously reported that SAL mitigates \textit{S. aureus} binding to solid-phase fibrinogen and fibronectin biomatrices in vitro (17). To assess the effects of various \textit{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 \textmu g/ml). Following pelleting, washing, and bovine serum albumin blocking steps (17), 5 \times 10^5 CFU of each construct were added to six-well polystyrene plates precoated with 50 \mu g 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 (\pm 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 \textit{rsb} mutants, we employed a well-established phenotypic assay in which the ability of alpha- and bovine serum albumin blocking steps (17), 5 \times 10^5 \text{CFU} of each construct were added to six-well polystyrene plates precoated with 50 \mu g 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 (\pm standard deviation [SD]) of three independent runs and expressed as the mean percent reductions of fibrinogen or fibronectin binding under various assay conditions.

The transcription of SigB, encoded by the last gene in the operon, inactivation of \textit{sigB} leads to null expression of SigB (Fig. 1B). Indeed, the SigB protein levels were clearly demonstrable, albeit at lower levels in the \textit{rsbU} and \textit{rsbV} mutants than in the parental strain FDA486 (24). This is consistent with the observation that \textit{rsbU} and \textit{rsbV} are driven by separate promoters and that activation of \textit{rsbU} can occur independently of \textit{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 \textit{rsbU} and \textit{rsbV} mutations is probably minimal. We also examined SigB expression in the \textit{rsbW} mutant. Similar to the \textit{sigB} mutant, the \textit{rsbW} mutant did not express any SigB as detected by immunoblotting; this finding was expected because the expression of RsbW and SigB are translationally coupled (17, 27).

**RESULTS**

The impact of \textit{rsbU}, \textit{rsbV}, and \textit{rsbW} mutations on \textit{sigB} expression. The transcription of \textit{sigB} has been shown to be dependent on at least two promoters, one originating upstream of \textit{rsbU} and another upstream of \textit{rsbV}. A third promoter further upstream of \textit{rsbU} has also been described (23) (Fig. 1A).

To ensure that our mutation on \textit{rsbU} and \textit{rsbV} did not impact on SigB expression, we probed an immunoblot containing equivalent amounts of cell extracts from overnight cultures of \textit{FDA486} and its isogenic mutants were immunoblotted onto nitrocellulose. The blot was then probed with anti-SigB monoclonal antibody ID1 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 \textit{rsbW} and \textit{sigB} mutants of \textit{FDA486}. In both cases, no SigB protein expression was detected. These immunoblot data have been previously published by one of our laboratories (24).

**Induction of \textit{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 \mu g/ml) encompassing both clinically achievable human serum concentrations as well as SAL concentrations previously documented to activate \textit{sigB} expression (17). Using \textit{sarA} P3 promoter activation as a surrogate marker for \textit{sigB} expression, the lowest SAL concentration that clearly increased \textit{sigB} expression compared to untreated cells was 50 \mu g/ml (Fig. 2). We subsequently used the range of 25 to 50 \mu g/ml of SAL for the remaining phenotypic and genotypic studies. SAL-induced \textit{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 \textit{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 \textit{rsb} mutants exhibited substantial increases in \textit{sarA} P3 promoter activity in the presence or absence of SAL. In contrast, at postexponential phase (12 h), the parental strain, but not the \textit{rsbU}, \textit{V}, or \textit{W} mutants, exhibited increases in \textit{sarA} P3 expression in the presence of SAL, although this difference (\textasciitilde 3.5-fold) did not reach statis-
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. 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.

FIG. 3. Effect of SAL (50 $\mu$g/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: $[\text{fluorescence with SAL/fluorescence without SAL} - 1.0] \times 100$. Results are the means ($\pm$ SD) of triplicates from a representative experiment that was repeated three times.
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

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**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.

**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 three times.
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 fibronectin 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 (spaA), 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 regulators 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 hyper-expression (6, 24), as confirmed in the rsbU and rsbV mutants in the current investigation. Because rsbW and sigB are transcriptionally 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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