JOURNAL OF BACTERIOLOGY, Dec. 2011, p. 6614–6628 Vol. 193, No. 23
0021-9193/11/$12.00 doi:10.1128/JB.00305-11

SagS Contributes to the Motile-Sessile Switch and Acts in Concert with BfiSR To Enable *Pseudomonas aeruginosa* Biofilm Formation▽†

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Received 3 March 2011/Accepted 18 September 2011

The interaction of *Pseudomonas aeruginosa* with surfaces has been described as a two-stage process requiring distinct signaling events and the reciprocal modulation of small RNAs (sRNAs). However, little is known regarding the relationship between sRNA-modulating pathways active under planktonic or surface-associated growth conditions. Here, we demonstrate that SagS (PA2824), the cognate sensor of HptB, links sRNA-modulating activities via the Gac/HptB/Rsm system postattachment to the signal transduction network BfiSR, previously demonstrated to be required for the development of *P. aeruginosa*. Consistent with the role of SagS in the GacA-dependent HtpB signaling pathway, inactivation of *sagS* resulted in hyperattachment, an HptB-dependent increase in *rsmYZ*, increased Psl polysaccharide production, and increased virulence. Moreover, *sagS* inactivation rescued attachment but abrogated biofilm formation by the Δ*gacA* and Δ*hptB* mutant strains. The Δ*sagS* strain was impaired in biofilm formation at a stage similar to that of the previously described two-component system BfiSR. Expression of *bfiR* but not *bfiS* restored Δ*sagS* biofilm formation independently of *rsmYZ*. We demonstrate that SagS interacts directly with BfiS and only indirectly with BfiR, with the direct and specific interaction between these two membrane-bound sensors resulting in the modulation of the phosphorylation state of BfiS in a growth-mode-dependent manner. SagS plays an important role in *P. aeruginosa* virulence in a manner opposite to that of BfiS. Our findings indicate that SagS acts as a switch by linking the GacA-dependent sensory system under planktonic conditions to the suppression of sRNAs post-attachment and to BfiSR, required for the development of *P. aeruginosa* biofilms, in a sequential and stage-specific manner.

Biofilms are complex communities of microorganisms attached to surfaces and embedded in a self-produced extracellular matrix (13), with biofilm formation initiated by bacteria attaching to a surface. The switch from the motile to the sessile mode of growth is an essential step in the formation of biofilms and the modulation of virulence. Several factors have been shown to impact the transition from the free-swimming to the surface-attached mode of growth, including appendages (type IV pili and flagella) (29, 39, 40, 51, 52, 61, 62) and the intracellular signaling molecule bis(3′-5′)-cyclic diguanylic monophosphate (cyclic di-GMP). First described to control extracellular cellulose biosynthesis in *Acetobacter xylinum* (48, 49), cyclic di-GMP has been demonstrated in several microorganisms, including *Pseudomonas aeruginosa*, to control the transition between a motile and a biofilm lifestyle via its concentration, with high levels fostering the sessile lifestyle and low cyclic di-GMP concentrations favoring motility (e.g., twitching and swarming) and the planktonic mode of growth (14, 27, 36, 47, 56, 60). Regulatory systems linking the modulation of cyclic di-GMP levels and attachment capabilities include the genetic pathway composed of BifA, SadB, and SadC, which regulates Pel and Psl exopolysaccharide production as *P. aeruginosa* transitions from a planktonic to a surface-associated lifestyle (11, 30, 34), and the *Escherichia coli* Csr system (28). CsrABCD, originally identified as a system to regulate glycogen biosynthesis, controls carbon and secondary metabolism, biofilm formation, motility, and quorum sensing via destabilization of respective mRNA targets. Regulation of the activity of the RNA binding protein CsrA is mediated in part by the actions of the two noncoding small RNAs (sRNAs) CsrB and CsrC (24, 25, 28, 46, 59, 64).

Similarly to the Csr system in *E. coli*, sRNAs in *Pseudomonas aeruginosa* have been shown to play an important role in attachment, polysaccharide production, virulence, and quorum sensing (6, 9, 23, 37, 42, 44). However, in contrast to the *E. coli* Csr system, the Rsm system in *P. aeruginosa* has not been linked to the modulation of cyclic di-GMP levels. sRNAs *rsmZ* and *rsmY* serve as antagonists of the translational regulator RsmA. The binding of RsmA to specific mRNA targets differentially affects their stability, turnover, and translation rates, thus controlling the expression of a significant number of virulence and attachment genes at the level of mRNA translation and/or stability (17, 25, 45, 63). Expression levels of the sRNAs are directly controlled by GacA/GacS, which are inversely controlled by the two-component hybrids RetS and LadS (18, 63). RetS negatively controls *rsmY* and *rsmZ* gene expression, while LadS positively controls sRNA levels. An additional component modulating sRNA levels was recently identified as the

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

▽ Published ahead of print on 23 September 2011.

histidine phosphotransfer protein HptB. HptB is activated via three orphan sensor kinase hybrids (PA2824, PA1611, PA1976) and in turn relays the signal (a phosphor group) to the response regulator PA3346 and to RetS (6, 22). While HptB also intersects at the GacA response regulator, which directly controls sRNA production to reciprocally regulate the expression of exopolysaccharide components of the *P. aeruginosa* biofilm matrix and the organism's attachment and swarming motility, HptB appears to exclusively regulate *rsmY* expression (6–8, 17, 19, 22).

While attachment is enhanced by increased sRNA levels, subsequent surface-attached growth and biofilm formation by *P. aeruginosa* have recently been demonstrated to be hampered by elevated sRNA levels, in particular those of *rsmZ* (44). Modulation of *rsmZ* levels under biofilm growth conditions is dependent on the novel two-component system (TCS) BfiSR, which regulates the suppression of *rsmZ* via RNase G (CafA) (44). BfiSR was found to be required for biofilm development by *P. aeruginosa*, with inactivation of the biofilm-specific TCS arresting biofilm formation in a manner that coincided with its timing of phosphorylation (43). The interaction of *P. aeruginosa* with surfaces can thus be described as a two-stage process: initially, colonizing bacteria express and modulate genes and proteins required for the increase in sRNA levels (including LadS, GasAS, and HtpB), while the subsequent persistent biofilm developmental stages depend on the production and posttranslational modification of a different set of proteins (BfiSR, CafA) that promote biofilm formation by decreasing sRNAs. However, the coordinate regulation of these two contrasting sets of factors/proteins prior to and following the motile-sessile switch and subsequent biofilm formation remains to be elucidated.

Here, we report the identification of the probable sensor/response regulator hybrid PA2824, which we named SagS (surface attachment and growth sensor hybrid), as a sensor essential for biofilm formation that links, via interaction with and modulation of the phosphorylation state of BfiS, to the regulatory pathways that reciprocally modulate sRNA levels prior to and following attachment to enable biofilm formation. By regulating the motile-sessile switch, SagS links the Gac/HptB/Rsm and BfiSR signaling transduction systems into a multisensor signaling network.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and culture conditions. All bacterial strains and plasmids used in this study are listed in Table S1 in the supplemental material. *P. aeruginosa* strains PAO1 and PA14 were used as parental strains, the latter being required for the *Arabidopsis* virulence studies. All planktonic strains were grown in Lennox broth (LB) or Vogel-Bonner minimal medium (VBMM) (53) in shake flasks at 220 rpm.

Strain construction. Isogenic mutants were constructed by allelic replacement using sucrose counterselection, as previously described (54), with the gene replacement vector pEX18Gm (21). Complementation and overexpression were accomplished by placing the respective genes under the control of an arabinose-inducible promoter in the pJN105 vector (38). C-terminal V5/6×His tagging was accomplished by subcloning into pET101D (Invitrogen), N-terminal 6×His tagging of BfiS was accomplished by subcloning into pQE30Xa (Qiagen), while a C-terminal hemagglutinin (HA) tag was introduced into SagS via PCR using the sequence AGCGTAGTCTGGGACGTCGTATGGGTA. The tagged constructs were introduced into pJN105 and pMJT1. Primers used for strain construction are listed in Table S2 in the supplemental material.

Biofilm formation. Biofilms were grown in a once-through continuous-flow tube reactor system to obtain proteins and RNA or in flow cells to view the biofilm architecture, as previously described (1, 2, 43, 52, 57). Biofilms were grown at 22°C in 1/20-diluted LB or VBMM in the absence or presence of 0.1% arabinose. Quantitative analysis of confocal laser scanning microscopy (CSLM) images of flow cell-grown biofilms was performed using COMSTAT (20). Initial biofilm formation was measured by using the microtiter dish assay system with crystal violet (CV) staining (40) and by microscopy (12). The number of cells attached to the glass surface of a flow cell covering an area of 400 μm^2 was quantified using ImageProPlus software.

Phosphoprotein detection and phosphotransfer analysis. Detection of phosphoproteins via immunoblot analysis was carried out as previously described (43, 57). Briefly, 200 μg of total cell protein was subjected to separation by two-dimensional PAGE (2D-PAGE), followed by blotting onto polyvinylidene difluoride (PVDF) membranes and phosphoprotein detection using anti-phospho-(Ser/Thr)Phe antibodies (Cell Signaling Technologies, Danvers, MA). Analysis of phosphorylated BfiS-V5/6×His levels in protein extracts of *P. aeruginosa* strains in which *sagS* was inactivated or overexpressed was accomplished using phosphoprotein purification via metal oxide affinity chromatography (MOAC) as described previously (43), followed by the detection of BfiS-V5/5×His by immunoblotting with anti-V5 antibodies (Invitrogen Corp.). For phosphotransfer studies, V5/6×His-tagged BfiS and SagS, purified using Ni-nitrilotriacetic acid (NTA) spin columns (Qiagen), were combined and incubated in phosphorylation buffer (50 mM Tris-HCl [pH 7.5], 50 mM KCl, 5 mM $MgCl_2$) for 60 min at room temperature, after which time the samples were subjected to MOAC phosphoprotein purification and anti-V5 immunoblot analysis. Purified BfiS and SagS alone were used as controls.

Psl polysaccharide dot blot analysis. Psl polysaccharide was extracted from planktonic and 144-h-old biofilm cells essentially as described by Byrd et al. (10). Quantitation of Psl production was done by determining the anti-Psl dot blot spot volume (23) using the ImageMaster analysis software (GE Healthcare).

Motility assays. Swimming, swarming, and twitching motilities were assessed in tryptone or LB medium containing 0.3%, 0.5%, and 1.0% agar, respectively, as previously described (39, 55, 57).

qRT-PCR. Quantitative reverse transcriptase PCR (qRT-PCR) was used to determine the expression levels of *pslA* and *rsmAYZ*. Isolation of mRNA and cDNA synthesis were carried out as previously described (2, 3, 43, 57). qRT-PCR was performed using the Eppendorf Mastercycler ep realplex (Eppendorf AG, Germany) and the SYBR Fast qPCR kit (Kapa Biosystems, Woburn, MA) (see Table S2 in the supplemental material for a list of oligonucleotides). Relative transcript quantitation, with *mreB* used as a housekeeper control, was done as previously described (44).

Extraction and quantification of cyclic di-GMP from *P. aeruginosa*. Cyclic di-GMP was extracted in triplicate from wild-type and mutant strains grown planktonically, essentially as described previously (4, 36, 56), using heat and ethanol precipitation followed by centrifugation. Supernatants were combined, dried using a SpeedVac, and resuspended in 10 mM ammonium bicarbonate buffer. Samples (10 μl or 20 μl) were analyzed using an Agilent 1100 high-performance liquid chromatography (HPLC) system equipped with an autosampler, degasser, and detector set to 253 nm and were separated using a reverse-phase C_{18} Targa column (2.1 by 40 mm by 5 μm) at a flow rate of 0.2 ml/min with the following gradients for each time range: for 0 to 9 min, 1% B; for 9 to 14 min, 15% B; for 14 to 19 min, 25% B; for 19 to 26 min, 90% B; and for 26 to 40 min, 1% B (where A is 10 mM ammonium acetate and B is methanol plus 10 mM ammonium acetate). Commercially available cyclic di-GMP was used as a reference for the identification and quantification of cyclic di-GMP in cell extracts. Moreover, the identity of HPLC-eluted cyclic di-GMP was confirmed by tandem mass spectrometry (MS/MS) using a QStar mass spectrometer (Applied Biosystems) by detecting the cyclic di-GMP fragments 691.1→151.9, 691.1→248.0, and 691.1→539.8 *m/z*, as described by Thormann et al. (60).

Determination of SagS protein interactions using pulldown assays. Pulldown assays were used to determine whether SagS interacts with BfiS and BfiR. HA-tagged SagS was incubated with extracts containing 6×His-tagged BfiS or BfiR. Subsequently, BfiS or BfiR was immunoprecipitated using immobilized anti-6×His antibodies, and the immunoprecipitation eluates were separated by SDS-PAGE and assessed by immunoblot analysis for the presence of SagS using anti-HA antibodies (and vice versa). Pulldown assays were carried out using 200 μg protein from cellular extracts, with HA-tagged proteins used as bait and V5/6×His-tagged proteins used as prey (and vice versa). Mouse anti-HA and anti-His or anti-V5 antibodies (Invitrogen) were used for immunoprecipitation at 1 μg/ml and immunoblotting at 0.1 μg/ml. Two V5/6×His-tagged proteins, the inner membrane protein PA3343 and BdlA, were used as controls to determine the specificity of the SagS-BfiS complex formation *in vitro*.

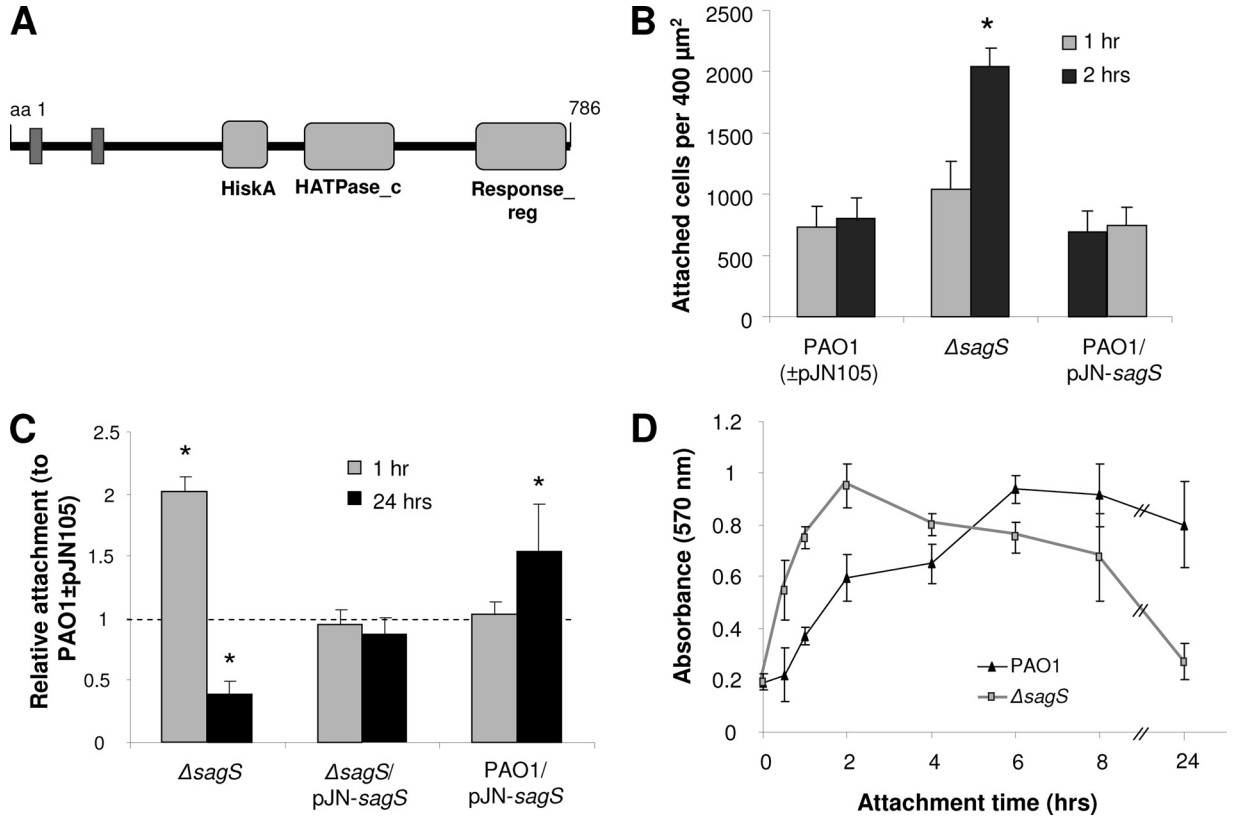

FIG. 1. Attachment is dysregulated in the strain in which *sagS*, encoding a hybrid sensor kinase/response regulator, is inactivated, as determined by microscopy and crystal violet staining. (A) Domain organization of SagS. Domains are predicted by sequence homology using Pfam. HiskA, histidine kinase domain; HATPase_c, histidine ATPase domain C; Response_reg, response regulator domain; aa, amino acid. Predicted transmembrane-spanning segments are shown in dark gray. (B) Direct cell counts of wild-type and mutant strains attached to polystyrene 1 and 2 h after initial attachment. Numbers of cells attached per 400 μm^2 were quantified using ImageProPlus software. *, significantly different from respective wild-type values ($P < 0.05$), as determined by ANOVA and Sigma Stat. (C) Evaluation of attachment to polystyrene by crystal violet staining 1 and 24 h after initial attachment, relative to that of *P. aeruginosa* PAO1 in the absence/presence of the empty plasmid (PAO1 ± pJN105). *, significantly different from respective wild-type values ($P < 0.05$), as determined by ANOVA and Sigma Stat. (D) Time course of attachment to polystyrene over the course of 24 h by *P. aeruginosa* PAO1 and the Δ*sagS* strain. Error bars indicate standard deviations.

Virulence testing. The role of *sagS* in virulence was assessed using the *Arabidopsis thaliana* infection model. Plants were infected essentially as described by Starkey and Rahme (58), incubated for a period of 12 days in a 16-h-light (25°C)/8-h-dark (22°C) cycle, and analyzed as previously described (44).

Statistical analysis. Student's *t* test was performed for pairwise comparisons of groups, and multivariant analyses were performed using a one-way analysis of variance (ANOVA) followed by an *a posteriori* test using Sigma Stat software. All experiments were carried out in triplicate.

RESULTS

The probable sensor/response regulator hybrid SagS. The *sagS* gene encodes a 786-amino-acid polypeptide similar in sequence and domain organization to the sensor kinase/response regulator hybrid family of signal transduction proteins (Fig. 1A). This orphan sensor hybrid was previously identified to phosphorylate the GacA-dependent histidine phosphotransfer protein HptB *in vitro* and to interact with HptB *in vivo* and was thus implicated in playing a role in the GacAS-dependent control of sRNA levels (6, 22). Moreover, while *sagS* expression was found to be independent of the mode of growth (not shown), SagS was found to be phosphorylated only when *P. aeruginosa* was grown planktonically (43), suggesting that SagS might play a role in the motile-sessile transition, possibly via sRNA regulation.

SagS plays a temporal role in attachment. To elucidate the role of SagS in the motile-sessile switch and, thus, the transition to a surface-associated lifestyle, we first determined whether inactivation or overexpression of *sagS* affected attachment. Inactivation of *sagS* resulted in significantly enhanced attachment, with the Δ*sagS* strain attaching twice as efficiently as the PAO1 strain within the first 2 h, as determined by microscopy and CV staining (Fig. 1B and C). The findings are consistent with previous reports by Goodman et al. demonstrating increased attachment capabilities for this mutant (17). However, increased attachment by the Δ*sagS* strain was only temporary, and the difference in attachment diminished with increasing attachment times. No difference in attachment was observed between PAO1 and the Δ*sagS* strain following 8 h of attachment, while continued incubation resulted in decreased attachment by the Δ*sagS* strain compared to that of the wild type (Fig. 1C and D). Complementation of the Δ*sagS* mutant with pJN-*sagS* restored the attachment phenotype to wild-type levels, while overexpression of *sagS* enhanced attachment after prolonged growth (Fig. 1B and C). Growth curves in liquid

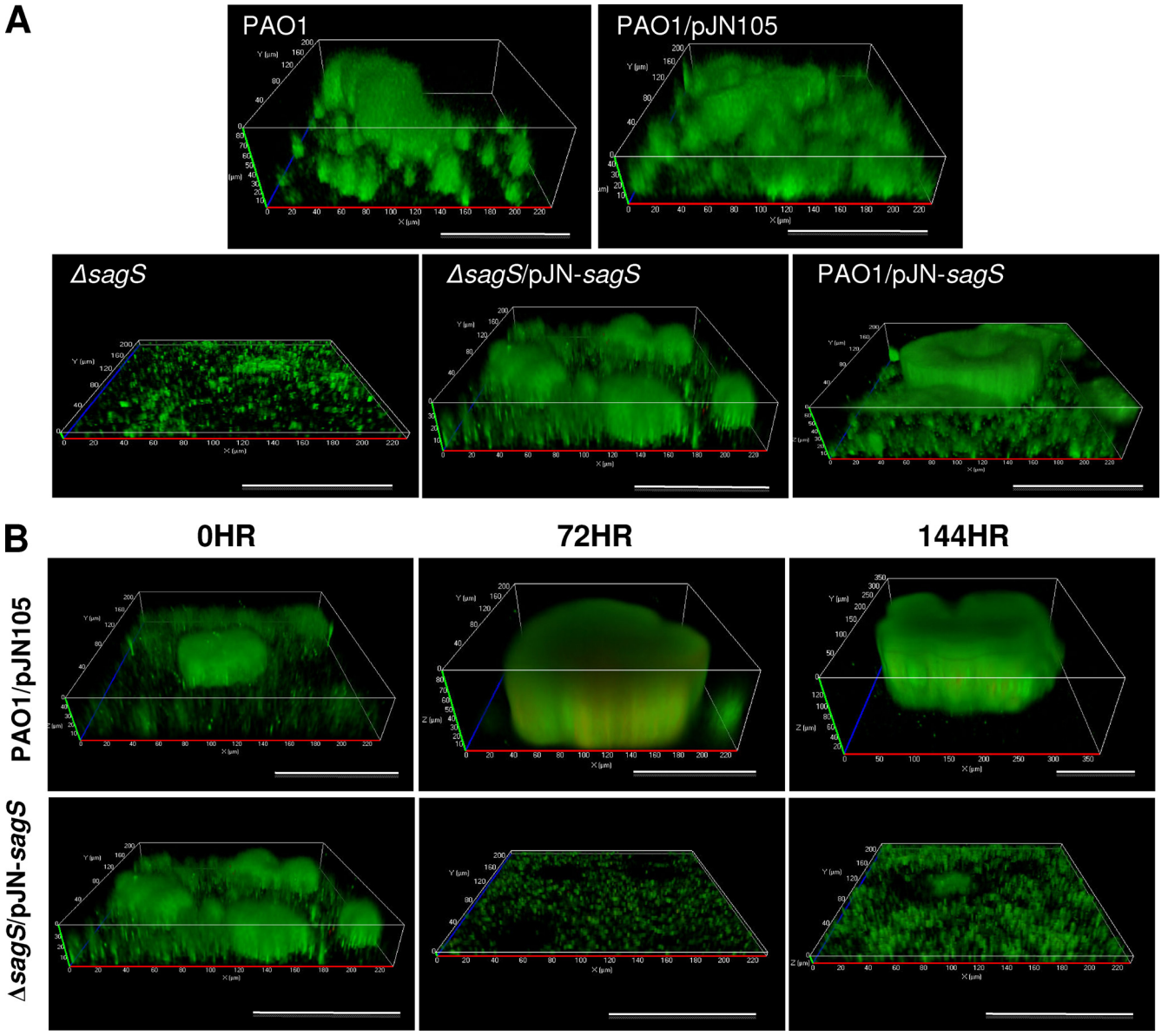

FIG. 2. SagS is required for the formation and maintenance of *P. aeruginosa* biofilms. (A) Biofilms of strains with *sagS* inactivated or overexpressed that were grown for 144 h were visualized by CSLM. *P. aeruginosa* PAO1 strains in the absence and presence of the empty plasmid were used as controls. (B) Inactivation of *sagS* expression in mature biofilms results in biofilm architectural collapse and biofilm dispersion. The *P. aeruginosa* Δ*sagS* strain complemented with *sagS* (the Δ*sag*/pJN-*sagS* strain) and placed under the regulation of the arabinose-inducible P_{BAD} promoter was grown under continuous-flow conditions in VBMM in the presence of 0.1% arabinose for 144 h, after which time the biofilms were visualized by confocal microscopy (0 h). Arabinose was subsequently eliminated from the growth medium, and the biofilm architecture was monitored following arabinose removal at the times indicated. The PAO1 strain harboring the empty pJN105 vector was used as a control. Biofilms were stained with the Live/Dead BacLight viability stain (Invitrogen Corp.). White bars = 100 μm.

media suggested that the increase in attachment was not a result of a general increase in growth rate.

A Δ*sagS* mutant strain of *P. aeruginosa* is impaired in biofilm formation. Since inactivation of *sagS* had only a temporary positive effect on attachment, with the difference in attachment diminishing upon continued surface exposure (Fig. 1), we asked whether inactivation or overexpression of this regulatory protein would alter or affect biofilm formation. We anticipated detecting no difference in biofilm formation between the Δ*sagS* strain and PAO1. However, the mutant strain failed to develop the biofilm architecture typically observed in PAO1 biofilms following 144 h under biofilm growth conditions (Fig. 2A; Table 1). Instead, the Δ*sagS* strain formed only thin biofilms that lacked large cellular aggregates and microcolonies and that were composed of ≥5-fold-less biomass than those of the wild type (Table 1). Overall, Δ*sagS* biofilms most closely resembled those of PAO1 following 24 h of growth. Daily monitoring of Δ*sagS* biofilms by confocal microscopy over a period of 24 to 144 h revealed that Δ*sagS* biofilms failed to accumulate the additional biomass typically seen in wild-type biofilms fol-

TABLE 1. COMSTAT analysis of *P. aeruginosa* wild-type and mutant biofilm structures[a]

P. aeruginosa strain and incubation time (h)	Total biomass ($\mu m^3/\mu m^2$)	Substratum coverage (%)	Avg thickness (μm)	Maximum thickness (μm)	Roughness coefficient (dimensionless)
PAO1					
24	0.95 ± 0.39*	12.11 ± 5.08*	0.78 ± 0.38*	7.8 ± 1.14*	1.69 ± 0.12
72	3.27 ± 1.29*	32.43 ± 8.31	3.02 ± 1.52*	18.29 ± 10.86*	1.20 ± 0.31
144	7.99 ± 3.63	44.44 ± 22.58	7.17 ± 4.97	35.63 ± 26.73	1.24 ± 0.37
Isogenic mutant after 144 h of growth					
PAO1 Δ*sagS*	0.82 ± 0.63*	7.07 ± 5.14*	0.83 ± 0.62*	9.00 ± 2.83*	1.74 ± 0.20
PAO1 Δ*sagS*/pJN-*sagS*	11.89 ± 8.48	25.34 ± 11.36	11.90 ± 8.99	47.67 ± 29.96	1.38 ± 0.23
PAO1/pJN-*sagS*	14.04 ± 9.98*	25.64 ± 11.26	16.97 ± 14.51*	55.49 ± 28.84	1.09 ± 0.37
PAO1 Δ*bfiS*/pJN-*sagS*	2.10 ± 2.54*	8.12 ± 6.98*	1.89 ± 2.48*	27.71 ± 16.19	1.82 ± 0.15
PAO1 Δ*sagS*/pJN-*bfiS*	1.33 ± 1.59*	4.79 ± 2.00*	1.24 ± 1.78*	27.33 ± 13.08	1.89 ± 0.06
PAO1 Δ*sagS*/pJN-*bfiR*	27.76 ± 14.60*	28.11 ± 12.21	29.40 ± 15.79*	92.33 ± 24.98*	1.12 ± 0.35
PAO1 Δ*gacA*	17.49 ± 10.77*	16.14 ± 7.66	21.04 ± 14.45*	94.22 ± 21.57*	1.31 ± 0.40
PAO1 Δ*gacA* Δ*sagS*	0.65 ± 0.73*	3.75 ± 3.18*	0.67 ± 0.79*	13.79 ± 8.61*	1.90 ± 0.08
PAO1 Δ*rsmYZ*	19.00 ± 13.70*	44.07 ± 27.85	20.28 ± 16.59*	84.00 ± 31.59*	1.09 ± 0.40
PAO1 Δ*rsmYZ* Δ*sagS*	0.29 ± 0.64*	2.51 ± 2.74*	0.25 ± 0.59*	8.33 ± 9.44*	1.95 ± 0.06
PAO1 Δ*hptB*	1.64 ± 1.98*	17.44 ± 24.20	1.61 ± 1.88*	8.22 ± 3.99*	1.51 ± 0.56
PAO1 Δ*hptB* Δ*sagS*	0.64 ± 0.69*	7.19 ± 6.34*	0.57 ± 0.69*	6.89 ± 2.93*	1.82 ± 0.17*

[a] The COMSTAT analysis was carried out with biofilms grown in triplicate, using at least 6 images per replicate. *, significantly different from the value for 144-h-old PAO1. *P* was ≤0.05, as determined by ANOVA and SigmaStat.

lowing continued growth under flowing conditions (after 48 h of growth) (Table 1; Fig. 2A) (43, 52), indicating that the observed biofilm architecture at the 144-h time point was not due to premature disaggregation. In contrast, overexpression of *sagS* resulted in biofilms that were similar in architecture to PAO1 biofilms but that displayed significantly increased biomass, as well as increased average and maximum thickness, when grown for 144 h (Fig. 2A; Table 1). Complementation restored the biofilm architecture to wild-type levels.

SagS expression is required for the maintenance of mature biofilm architecture. Our observations indicated that SagS was essential for the stage-specific development of *P. aeruginosa* biofilm formation, as the Δ*sagS* strain was arrested in biofilm formation, while *sagS* overexpression had no effect on continued biofilm development or on biofilm architecture (Fig. 2A). To determine whether *sagS* is also required for the maintenance of biofilm architecture, we made use of a complemented mutant strain (the Δ*sagS*/pJN-*sagS* strain), which harbored *sagS* under the control of the arabinose-inducible P_{BAD} promoter. This allowed mutant biofilms to form wild-type-like biofilms within 144 h of growth in the presence of arabinose (Fig. 2B, 0 h), after which time arabinose was removed from the growth medium to stop the transcription of *sagS*. The resulting biofilm architecture was viewed over a period of 144 h after arabinose removal using confocal microscopy. Wild-type *P. aeruginosa* harboring an empty pJN105 vector was used as a control.

Loss of *sagS* expression due to arabinose removal resulted in the collapse of the mutant biofilm architecture within 3 days (Fig. 2B, 72 h; Table 2). While a similar architectural collapse was observed following inactivation of *bfiS*, *bfmR*, and *mifR* expression (43), the collapse observed following *sagS* inactivation was distinct, coinciding with biofilm dispersion, as evidenced by the formation of large voids (Fig. 2B) indicative of dispersion events. The voids were detectable as soon as 1 day following arabinose removal (not shown). Cells that remained attached at the surface showed increased motility, as determined by microscopy (not shown). The collapse was furthermore apparent by significant reductions in biofilm variables, including biofilm biomass and thickness, which further decreased upon continued incubation (Table 2). Under the conditions tested, no reduction in the biomass and thickness of the

TABLE 2. COMSTAT analysis of *P. aeruginosa* wild-type and complemented mutant biofilm structure following removal of arabinose, which results in a lack of *sagS* expression[a]

P. aeruginosa strain	Time (h)[b]	Total biomass ($\mu m^3/\mu m^2$)	Substratum coverage (%)	Avg thickness (μm)	Maximum thickness (μm)	Roughness coefficient (dimensionless)
PAO1/pJN105	0	11.92 ± 9.28	28.85 ± 13.29	11.97 ± 11.48	37.17 ± 26.78	1.25 ± 0.28
	72	31.66 ± 10.56	29.37 ± 11.36	30.99 ± 10.58	66.33 ± 13.90	1.22 ± 0.30
	144	36.92 ± 18.00	45.01 ± 19.18	37.91 ± 18.76	111.33 ± 19.06	0.92 ± 0.44
PAO1 Δ*sagS*/pJN-*sagS*	0	11.89 ± 8.48	25.34 ± 11.36	11.90 ± 8.99	47.67 ± 29.96	1.38 ± 0.23
	72	1.88 ± 0.84*	16.28 ± 9.72*	1.83 ± 0.99*	14.50 ± 12.00*	1.55 ± 0.30
	144	1.40 ± 0.84*	15.55 ± 7.77*	1.21 ± 0.85*	6.33 ± 2.53*	1.61 ± 0.20*

[a] The COMSTAT analysis was carried out with biofilms grown in triplicate, using at least 6 images per replicate. *, significantly different from the value for PAO1/pJN105 at the respective time point. *P* was ≤0.05, as determined by ANOVA and SigmaStat.

[b] Time zero for biofilms was 144 h. Time corresponds to the time following the switch to an arabinose-free medium.

TABLE 3. Comparison of phosphorylation patterns between Δ*sagS* and Δ*bfiS* biofilms

Biofilm developmental stage (h)	No. of detected phosphorylation events in wild-type *P. aeruginosa* PAO1	No. of detected phosphorylation events in 144-h-old biofilm of:		
		Δ*bfiS* mutant	Δ*sagS* mutant	Δ*bfiS* mutant and Δ*sagS* mutant
Planktonic	22	3	2	1
Motile-sessile switch[a]	8	6	6	5
Reversible attachment (8)	38	25	26	18
Irreversible attachment (24)	33	11	12	7
Maturation (144)	39	0	0	0
Dispersion (216)	19	0	0	0
Biofilm-specific[b]	7	6	7	6
Constitutive	26	26	26	26

[a] Present in both planktonic and initially attached biofilm cells (at 8 and 24 h) (43).
[b] Present in all surface-attached cells regardless of biofilm stage or age (43).

wild-type biofilm architecture was observed (Fig. 2B; Table 2). These findings indicate that the effect of SagS is reversible with respect to biofilm architecture and biofilm development.

Inactivation of *sagS* arrests biofilm development prior to the irreversible attachment stage. Quantitative analysis of confocal microscope images of Δ*sagS* biofilms indicated that the Δ*sagS* strain was arrested in biofilm formation. This prompted us to determine the biofilm developmental stage at which the Δ*sagS* mutant was arrested. We have previously demonstrated that wild-type *P. aeruginosa* biofilms exhibit distinct, stage-specific protein phosphorylation patterns over the course of development and that mutant strains impaired in biofilm formation demonstrate phosphorylation profiles that are indicative/predictive of the developmental stage at which developmental arrest occurs (43). A comparison of the phosphorylation patterns of Δ*sagS* biofilms grown for 144 h to those of *P. aeruginosa* wild-type biofilms grown for 8, 24, 72, and 144 h using anti-phospho-Ser/Thr antibodies indicated that Δ*sagS* biofilms failed to exhibit phosphorylation events typically observed during normal biofilm development following 144 h of growth (Table 3; see also Table S3 in the supplemental material). For instance, Δ*sagS* biofilms lacked all of the phosphorylated proteins typically found in mature, 144-h-old biofilms and instead exhibited stage-specific phosphorylation events typically detected in 8- and 24-h-old wild-type biofilms; the phosphoproteome contained 26 out of 38 and 12 out of 33 phosphorylated proteins that are specific to 8-h-old and 24-h-old wild-type biofilms, respectively (Table 3; see also Table S3 in the supplemental material). Furthermore, Δ*sagS* biofilms lacked evidence of BfiS phosphorylation (see Table S3 in the supplemental material), which was previously detected following 8 h of biofilm growth (43), indicating that Δ*sagS* biofilms are probably arrested prior to Δ*bfiS* biofilms at the transition from the planktonic to the initial attachment stage.

Comparison of the phosphorylation patterns of both the Δ*sagS* and Δ*bfiS* strains indicated that while both mutant biofilms exhibited stage-specific phosphorylation events typically detected in 8-h-old and 24-h-old wild-type biofilms, inactivation of *sagS* and *bfiS* affected distinct sets of phosphorylated proteins under biofilm growth conditions (Table 3).

SagS controls Psl production. To begin elucidating the mechanisms by which SagS affects transitioning between the motile and sessile states, mutants in which *sagS* was inactivated or overexpressed were tested for properties known to affect initial attachment, including cellular motility, cyclic di-GMP levels, and polysaccharide production, which have been shown to play a role in promoting biofilm formation in diverse *P. aeruginosa* strains (15, 16, 26, 50). Increased Psl production by the Δ*sagS* strain in a planktonic-stage-specific manner was detected by immunoblot analysis using anti-Psl antibodies (Fig. 3A and B). Under planktonic growth conditions, Psl abundance in the Δ*sagS* strain was comparable to that observed in the Psl-overproducing strain WFPA801 (Fig. 3A). Moreover, qRT-PCR demonstrated increased expression of *pslA* involved in Psl polysaccharide biosynthesis in the Δ*sagS* strain under free-swimming growth conditions but not in biofilms (Fig. 3C). While cellular motility has been shown to affect initial attachment, no differences with respect to twitching, swimming, and swarming were detected for strains in which *sagS* was inactivated or overexpressed (not shown). Mutant strains demonstrating increased attachment and matrix polymer production have been previously linked to increased cyclic di-GMP levels (5, 56). However, no difference in cyclic di-GMP levels between Δ*sagS* mutant cells and wild-type cells grown planktonically was detected, indicating that increased adhesiveness and Psl production are independent of cyclic di-GMP levels in the Δ*sagS* strain.

SagS controls *rsmY* and *rsmZ* expression under planktonic growth conditions. The Gac/Rsm network directly modulates the levels of sRNAs *rsmY* and *rsmZ* to regulate the expression of exopolysaccharide components of the *P. aeruginosa* biofilm matrix and the organism's attachment and swarming motility (6–8, 17, 19). SagS has been previously linked to this network via the histidine phosphotransfer protein HptB, with HptB having been shown to be dependent on the GacA response regulator (6, 22), which directly controls *rsmY* and *rsmZ* transcription (6–8, 17, 19). This prompted us to ask whether SagS is involved in the regulation of *rsmYZ* levels. Inactivation of *sagS* correlated with elevated *rsmA* levels and significantly increased *rsmYZ* levels compared to those of the wild type under planktonic growth conditions (Fig. 4A). Under biofilm growth conditions, *rsmAYZ* levels were increased 2-fold in Δ*sagS* biofilms (Fig. 4B). No difference in *rsmAYZ* gene expression was detected when transcript levels of PAO1/pJN-*sagS*, Δ*sagS*/pJN-*sagS*, and the wild type were compared. In contrast, analysis of the *bfiS* mutant strain, which is arrested in biofilm formation at a stage similar to that of the Δ*sagS* strain (43, 44), demonstrated that the RNA levels of *rsmA*, *rsmY*, and *rsmZ* were

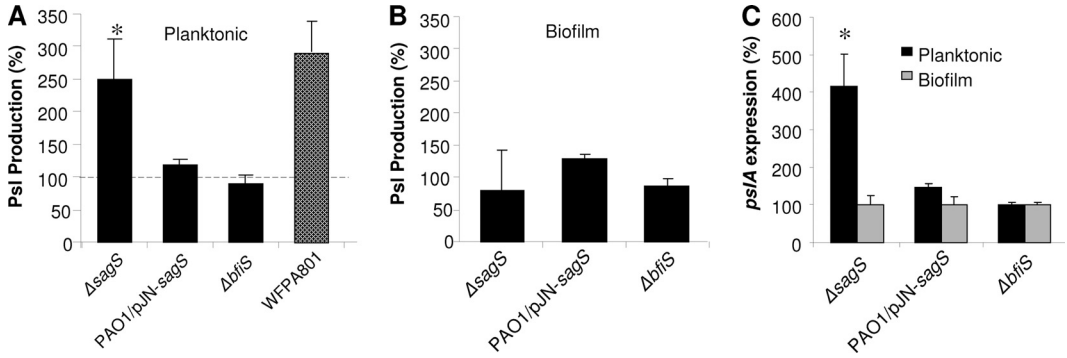

FIG. 3. Inactivation of *sagS* correlates with increased Psl production under planktonic growth conditions. Psl production by *P. aeruginosa* strains grown planktonically (A) and as biofilms (B), relative to that of *P. aeruginosa* PAO1, as determined using dot blot analysis of anti-Psl dot blot spot volumes. The Psl-overproducing strain WFPA801 was grown in the presence of 2% arabinose. (C) *pslA* expression, as determined by qRT-PCR, in *P. aeruginosa* strains grown planktonically and as biofilms, relative to that of *P. aeruginosa* PAO1. Error bars indicate standard deviations. *, significantly different from values for respective wild-type cells grown under identical conditions/modes of growth ($P < 0.05$) as determined by ANOVA and Sigma Stat.

unaltered in the Δ*bfiS* strain under planktonic growth conditions but that they were significantly increased compared to wild-type levels upon biofilm growth (Fig. 4A and B). We also measured the activities of *rsmY-lacZ* and *rsmZ-lacZ* chromosomal transcriptional fusions in planktonic, 24-h-old, and 144-h-old biofilm cells. In agreement with the expression levels, transcription from *rsmY* and *rsmZ* promoters was found to be repressed by SagS regardless of growth conditions (Fig. 4C and D). This is in contrast to what was observed with the Δ*bfiS* strain, for which *rsmYZ* transcription levels were comparable to those of the wild type regardless of growth conditions, while *rsmYZ* RNA levels were significantly increased under biofilm growth conditions (44). These findings strongly suggest that SagS and BfiS reciprocally modulate *rsmAYZ* abundance at distinct levels in a growth-mode-dependent manner. The mutants further differed with respect to *pslA* expression and Psl

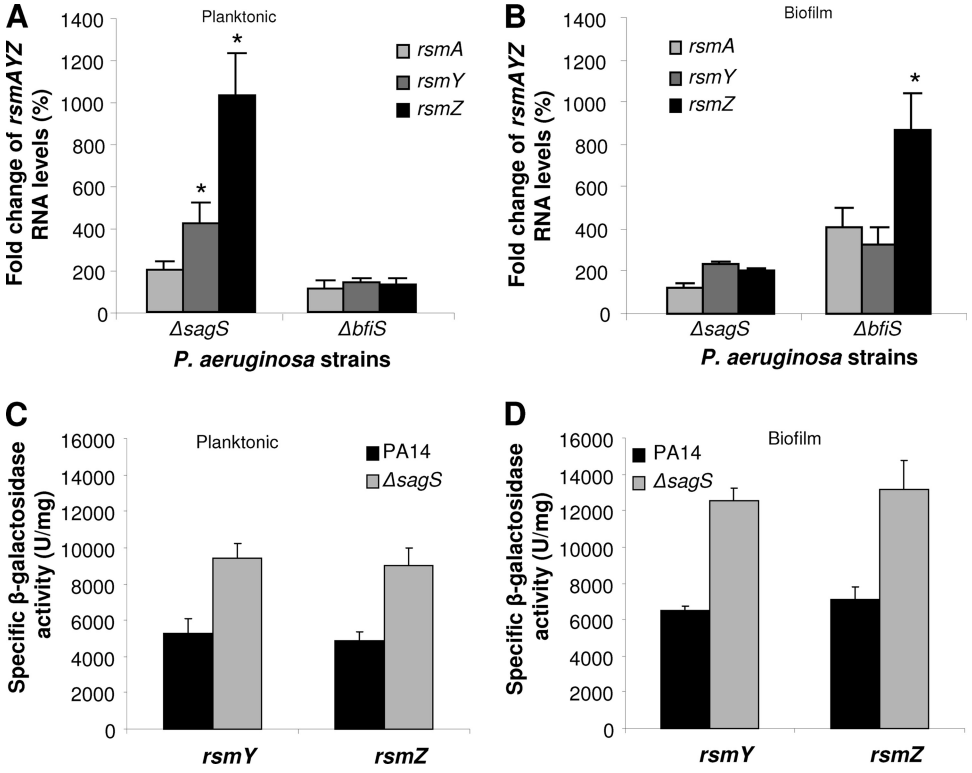

FIG. 4. SagS controls *rsmY* and *rsmZ* expression under planktonic but not biofilm growth conditions. (A and B) *rsmAYZ* RNA levels in *P. aeruginosa* strains grown planktonically (A) and as biofilms (B), relative to those in PAO1, as determined by qRT-PCR. (C and D) Transcriptional reporter fusion assays for *rsmY* and *rsmZ* expression in *P. aeruginosa* and Δ*sagS* mutant strains grown planktonically (C) and as biofilms (144 h) (D). Error bars indicate standard deviations. *, significantly different from respective wild-type cells grown under identical conditions/modes of growth ($P < 0.05$) as determined by ANOVA and Sigma Stat.

production (Fig. 3). While increased *rsmYZ* levels in the Δ*sagS* strain correlated with increased *pslA* expression and Psl production under planktonic growth conditions, no difference in Psl production was detected in the Δ*bfiS* strain regardless of the growth conditions (Fig. 3).

Deletion of *sagS* enhances early attachment but abrogates biofilm formation by the *gacA* and *hptB* mutant strains. Given that our findings implicated SagS in the regulation of *rsmYZ* levels (Fig. 4), we next determined whether SagS regulatory function and its associated phenotypes are dependent on the components of the Gac/Rsm system. Components of this network have been demonstrated to play a role in attachment and biofilm formation, with inactivation of *gacA* and deletion of its small regulatory RNA targets, *rsmYZ*, resulting in reduced attachment compared to that of the parental strain, *P. aeruginosa* PAK (8, 41). Inactivation of *hptB* in *P. aeruginosa* PAK resulted in hyperattachment, while the same mutation in *P. aeruginosa* PAO1 affected attachment only in the presence of glucose and Casamino Acids (6, 33). Here, we demonstrate that in *P. aeruginosa* PAO1, inactivation of *rsmYZ*, *gacA*, and *hptB* results in reduced attachment following 1 h of incubation under static conditions (Fig. 5A). Continued incubation, however, resulted in increased attachment or hyperattachment by the Δ*gacA* and Δ*rsmYZ* strains (Fig. 5A), indicating that, similarly to the Δ*sagS* mutation, mutations in *gacA* and *rsmYZ* have only a temporary effect on attachment. In contrast, the Δ*hptB* strain demonstrated reduced attachment regardless of attachment time (Fig. 5A). A second-site mutation in *sagS* rescued the attachment defect of the Δ*gacA*, Δ*hptB*, and Δ*rsmYZ* strains following 1 h of attachment while also suppressing attachment upon continued incubation (Fig. 5A).

In agreement with the attachment phenotype following 24 h of incubation, the Δ*gacA* strain formed hyperbiofilms characterized by significantly increased biofilm biomass and height compared to those of wild-type biofilms. Deletion of *sagS* in the Δ*gacA* strain significantly reduced biofilm biomass accumulation by more than 20-fold, with mutant biofilms resembling those of Δ*sagS* biofilms (Fig. 5C; Table 1). The Δ*hptB* strain formed only thin biofilms that lacked microcolonies and were, on average, only 2 μm thick, with a secondary-site mutation in *sagS* resulting in a further 2- to 3-fold reduction in the biofilm biomass (Fig. 5C; Table 1). Thus, introducing a *sagS* deletion into the *gacA* and *hptB* mutants abrogates biofilm formation of the parent strain, underscoring the importance of SagS in biofilm formation. Our findings indicate that SagS functions upstream of GacA to regulate attachment and biofilm formation. Moreover, the similarities between the attachment (after 24 h) and biofilm phenotypes (Fig. 5A and C) of the Δ*sagS* and Δ*hptB* strains suggested that HptB and SagS play similar or convergent regulatory roles, with inactivation of both resulting in comparable impairments of biofilm formation.

In order to establish the epistatic relationships between SagS, HptB, and GacA in regulating *rsmAYZ*, respective RNA levels in the Δ*gacA* and Δ*hptB* strains and the strains harboring a second-site mutation in *sagS* were determined under planktonic conditions. In agreement with previous results, inactivation of *gacA* resulted in significantly reduced *rsmYZ* levels under planktonic growth conditions. Deletion of *sagS* in the Δ*gacA* strain did not significantly alter *rsmAYZ* levels. The observation that *rsmAYZ* RNA levels are similarly reduced in Δ*gacA* and Δ*gacA* Δ*sagS* strain backgrounds indicated that SagS is not directly required for GacA function and instead functions independently. HptB has been shown to exclusively regulate *rsmY* expression in *P. aeruginosa* PAK (6, 22). While we were able to demonstrate reduced *rsmY* expression in an

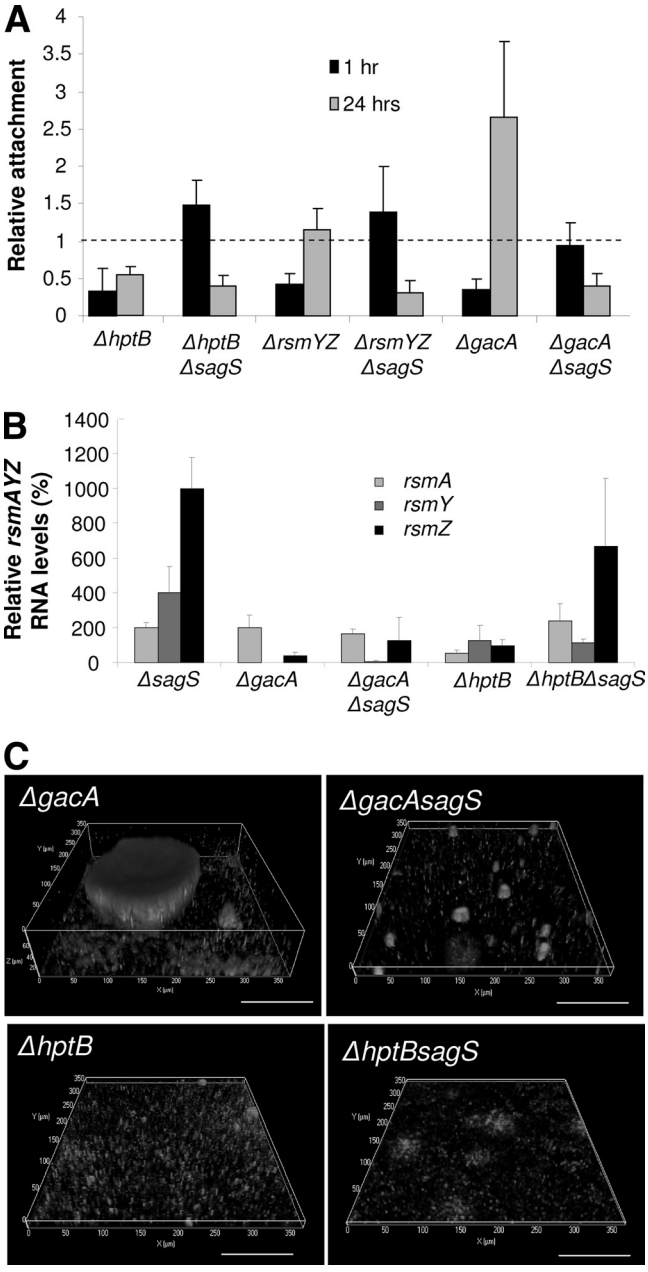

FIG. 5. Second-site mutations in the Δ*sagS* strain implicate three SagS-dependent phenotypes as HptB dependent, linking SagS to the GacS/GacA/RsmZ regulatory pathway. (A) Evaluation of attachment to polystyrene by crystal violet staining 1 and 24 h after initial attachment in the Δ*sagS* strain harboring various second-site mutations, relative to that in *P. aeruginosa* PAO1. (B) *rsmAYZ* RNA levels in various Δ*sagS* strains grown planktonically, relative to those of PAO1, as determined by qRT-PCR. (C) Biofilm architecture of the Δ*sagS* strain harboring various second-site mutations. Biofilms were visualized by CSLM after 144 h. White bars = 100 μm.

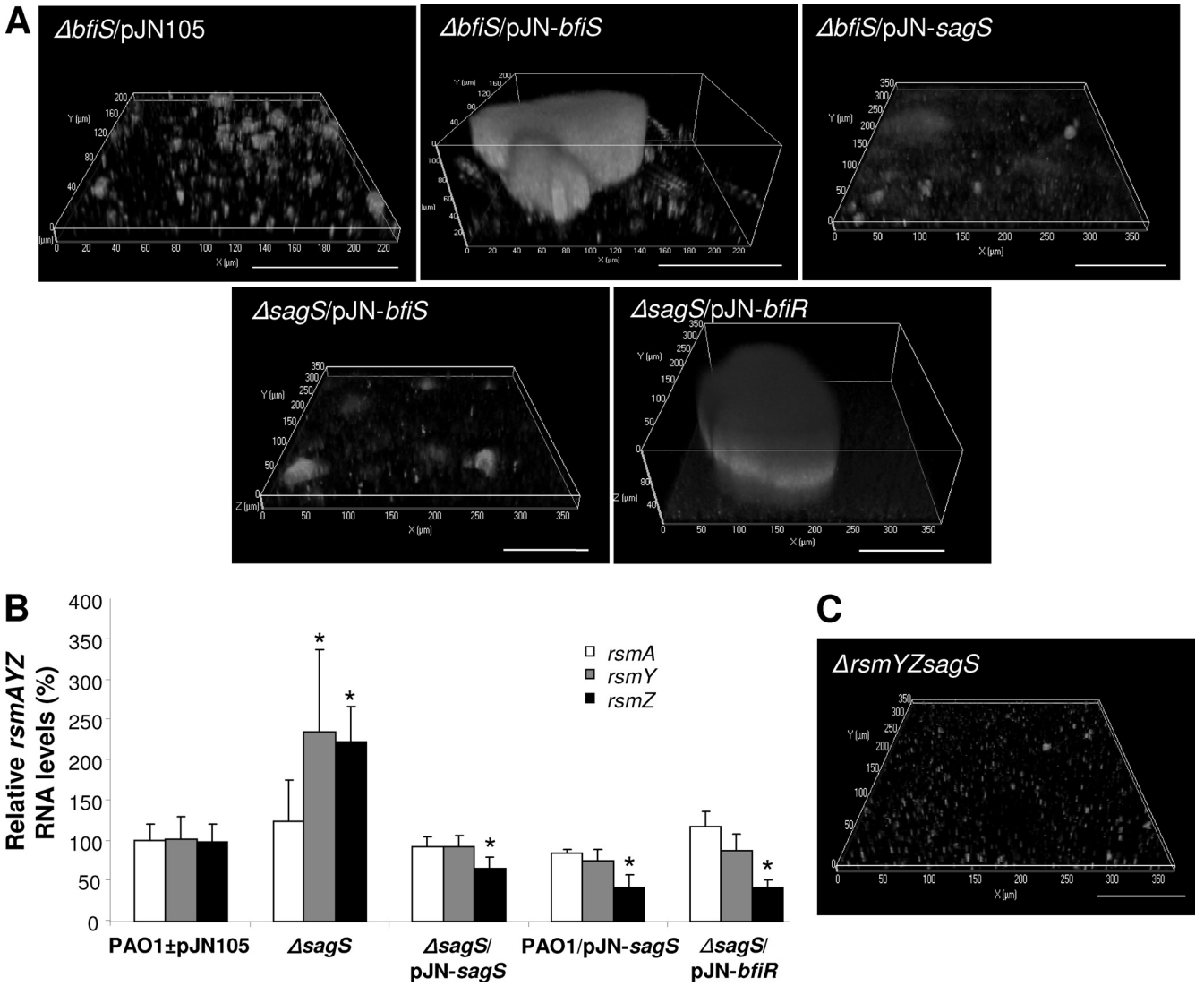

FIG. 6. Biofilm formation of the Δ*sagS* strain is restored by BfiR and correlates with reduced *rsmYZ* levels. (A) Expression of *bfiR*, but not *bfiS*, restores biofilm formation by the Δ*sagS* strain. Confocal images showing the biofilm architecture of Δ*bfiS* mutants harboring an empty plasmid or overexpressing *bfiS* and *sagS* and the biofilm architecture of Δ*sagS* mutants overexpressing *bfiS* and *bfiR.* (B) *rsmAYZ* RNA levels in Δ*sagS* biofilms complemented with or overexpressing *sagS* or *bfiR*, relative to the levels in *P. aeruginosa* PAO1 and PAO1/pJN105 (PAO1±pJN105), as determined by qRT-PCR. (C) The biofilm architecture of the Δ*sagS* strain is not restored to wild-type levels upon inactivation of *rsmYZ*, as revealed by confocal microscopy. White bars = 100 μm.

Δ*hptB* PAK strain (not shown), inactivation of *hptB* in *P. aeruginosa* PAO1 was correlated with 2-fold-reduced *rsmA* levels, and *rsmYZ* levels remained comparable to those of the wild type (Fig. 5B). A second-site *sagS* mutation in the Δ*hptB* strain resulted in significantly increased *rsmA* (4-fold) and *rsmZ* (7-fold) levels without affecting *rsmY* (Fig. 5B). While our findings confirm the dependency of *rsmYZ* levels on GacA, *rsmY* levels in Δ*sagS* mutant cells grown planktonically appeared to be dependent primarily on HptB, previously shown to be GacA dependent (6).

BfiR is required for SagS to enable biofilm formation. Phosphoproteome analysis suggested Δ*sagS* biofilms to be arrested prior to Δ*bfiS* biofilms at the transition from the planktonic to the initial attachment stage, indicating a possible link between these two sensory proteins. We reasoned that if SagS and BfiSR play distinct roles in biofilm formation, overexpression of *bfiS* or *bfiR* in the Δ*sagS* strain would not result in the restoration of wild-type biofilm formation (and vice versa). However, if SagS and BfiSR have overlapping or sequential functions during biofilm development, overexpression of *bfiS* or *bfiR* is anticipated to restore biofilm formation by the Δ*sagS* strain to wild-type levels.

Overexpression of *sagS* in the Δ*bfiS* strain had no effect on biofilm formation. Similarly, overexpression of *bfiS*, encoding the sensor kinase of the BfiSR TCS, in the Δ*sagS* strain did not affect the overall biofilm architecture (Fig. 6A; Table 1). The findings indicate the lack of a signal(s) required for the activation of either sensor kinase. In contrast, overexpression of *bfiR*, encoding the cognate response regulator of BfiS, in the Δ*sagS* strain resulted in the restoration of the biofilm architecture (Fig. 6A). Instead of forming thin biofilms lacking large

cellular aggregates and microcolonies, Δ*sagS*/pJN-*bfiR* biofilms were, on average, 30 μm thick, mostly due to the presence of large microcolonies. Overexpression of *bfiR* furthermore resulted in a 25-fold increase in biofilm biomass compared to those of Δ*sagS* biofilms and a 3- to 4-fold increase compared to those of PAO1 biofilms of comparable age (Table 1).

Restoration of the Δ*sagS* biofilm architecture to wild-type levels by complementation or overexpression of *bfiR* correlated with *rsmYZ* levels that were comparable to those of wild-type and vector-only controls (Fig. 6B). Compared to Δ*sagS* biofilms, complemented biofilms had 3.37-fold-reduced *rsmZ* levels, while overexpression of *sagS* or *bfiR* coincided with an ~5.2-fold reduction in *rsmZ* abundance. It should be noted that *rsmA* levels were unaltered (Fig. 6B). These findings underscored previous observations of a negative correlation between *rsmZ* levels and biofilm formation (44). However, while the findings suggested that the biofilm formation defect observed for the Δ*sagS* strain results, at least in part, from elevated *rsmYZ* abundance, deletion of *rsmYZ* in the Δ*sagS* strain did not restore biofilm formation to wild-type levels. Instead, Δ*rsmYZ* Δ*sagS* biofilms were comparable in biofilm architecture to Δ*sagS* biofilms (Fig. 6C; Table 1), suggesting that while SagS is involved in modulating sRNA levels, the Δ*sagS* biofilm phenotype is independent of *rsmYZ*.

SagS interacts directly with BfiS but only indirectly with BfiR. The observation that Δ*sagS* biofilm formation was restored to wild-type levels by *bfiR* overexpression suggested either that SagS and BfiSR belong to distinct pathways that are short-circuited by *bfiR* overexpression or instead that these proteins may function through direct interaction to enable biofilm formation and to receive an as-yet-unknown signal from SagS that is absent in Δ*sagS* biofilms. To test these hypotheses, we assessed whether SagS forms a complex with BfiSR *in vitro*. A pulldown of a complex of 6×His-tagged BfiS and hemagglutinin-tagged SagS (SagS-HA), as well as a complex of BfiS-V5/6×His and SagS-HA, could be clearly demonstrated when pulldown assays were probed for HA-tagged SagS (Fig. 7A). Two proteins were used as controls to determine the specificity of the SagS-BfiS interaction, namely, the inner membrane protein PA3343, characterized by 5 predicted transmembrane helices harboring a CheY-like receiver domain and a GGDEF domain having 64% similarity to the sensory histidine kinase of *Synechocystis* spp. (65), and BdlA (36). Neither BdlA nor PA3343 was detected in a complex with SagS-HA (Fig. 7A). Complex formation was also detectable when SagS-HA was used as bait to detect 6×His-tagged BfiS (not shown) and BfiR (Fig. 7B). However, the interaction of SagS with BfiR was found to be indirect and to occur via BfiS, as no interaction between BfiR and SagS was detected in a Δ*bfiS* mutant background (Fig. 7B). Complex formation of SagS and BfiS was confirmed by *in vivo* pulldown assays (Fig. 7C) and by using purified enzymes (not shown), thus indicating that the two proteins work in concert.

BfiS phosphorylation is SagS dependent. The observations of SagS forming a complex with BfiS to enable biofilm formation (Fig. 7A to C) raised the possibility of SagS being involved in the transfer of a phosphoryl group to BfiS. This was supported by the finding of Δ*sagS* biofilms lacking evidence of BfiS Ser/Thr phosphorylation (see Table S3 in the supplemental material). We therefore determined the consequences of SagS/BfiS interaction on the phosphorylation status of BfiS under planktonic and biofilm growth conditions *in vivo* by quantifying the amount of phosphorylated BfiS present following the purification of total phosphoproteins via metal oxide affinity chromatography (MOAC) in PAO1, Δ*sagS*, and PAO1/pMJT-*sagS* biofilm and planktonic cells overexpressing *bfiS*-V5/6×His. Under biofilm growth conditions, overexpression of *sagS* resulted in increased detection of phosphorylated BfiS via immunoblotting using anti-V5 antibodies compared to the level detected in the wild type (Fig. 7D). In contrast, deletion of *sagS* correlated with reduced levels of phosphorylated BfiS (Fig. 7D) compared with those found in the wild type. The findings are in strong support of BfiS phosphorylation being SagS dependent under biofilm growth conditions. Interestingly, differential expression of *sagS* had the opposite effect on BfiS phosphorylation under planktonic growth conditions, with overexpression of *sagS* correlating with reduced BfiS phosphorylation levels and a lack of *sagS* resulting in increased levels of phosphorylated BfiS (Fig. 7D). The findings suggested the phosphorylation of BfiS to be SagS dependent, with SagS reducing or interfering with BfiS phosphorylation under planktonic conditions.

To demonstrate the transfer of a phosphoryl group between SagS and BfiS *in vitro*, we made use of the finding that both V5/6×His-tagged proteins are phosphorylated when overexpressed in *E. coli* (Fig. 7E). Purified SagS and BfiS were coincubated and subjected to phosphoprotein enrichment via MOAC, with the resulting protein eluates being subjected to SDS-PAGE and immunoblot analysis using anti-V5 antibodies. Coincubation of SagS with BfiS led to a significant decrease in the phosphorylation of BfiS *in vitro* (Fig. 7E), confirming phosphotransfer between SagS and BfiS, with SagS acting as a phosphatase under planktonic growth conditions.

Inactivation of *sagS* renders *P. aeruginosa* hypervirulent. Transitions between free-swimming and surface-associated modes of growth have been previously linked to global shifts in the virulence mechanisms of *P. aeruginosa* (17, 63). In *P. aeruginosa*, this switch has been shown to depend on sRNA levels and the GacA/Rsm system orchestrating the reciprocal expression of virulence factors required for acute infection and the coordinate repression of genes promoting adaptation to chronic persistence (17, 31, 32, 66, 67). Furthermore, BfiSR, which regulates biofilm formation postattachment via modulation of *rmsYZ* levels, has also been shown to be essential for pathogenesis. As the present findings implicated SagS in the regulation of *rsmYZ* levels, as well as in the modulation of the phosphorylation state of BfiS, we asked whether the function of this hybrid regulator impacts *P. aeruginosa* virulence properties. Using the *A. thaliana* infection model, we observed that the Δ*sagS* strain elicits an earlier onset of initial signs of infection than the wild type, as indicated by lesions and discoloration (Fig. 8A). Moreover, the Δ*sagS* strain was hypervirulent and resulted in *A. thaliana* plant death 3 days earlier than did the wild type (Fig. 8B). These results suggested a contribution of SagS signaling to the virulence of *P. aeruginosa.* In contrast, Δ*bfiS* mutants were avirulent (44) and showed significantly delayed onsets of initial signs of infection and significantly reduced virulence compared to the wild type and the Δ*sagS* mutant (Fig. 8A and B). Considering that the main difference observed between the two strains is the growth mode dependence of *rsmYZ* levels, we determined the virulence properties

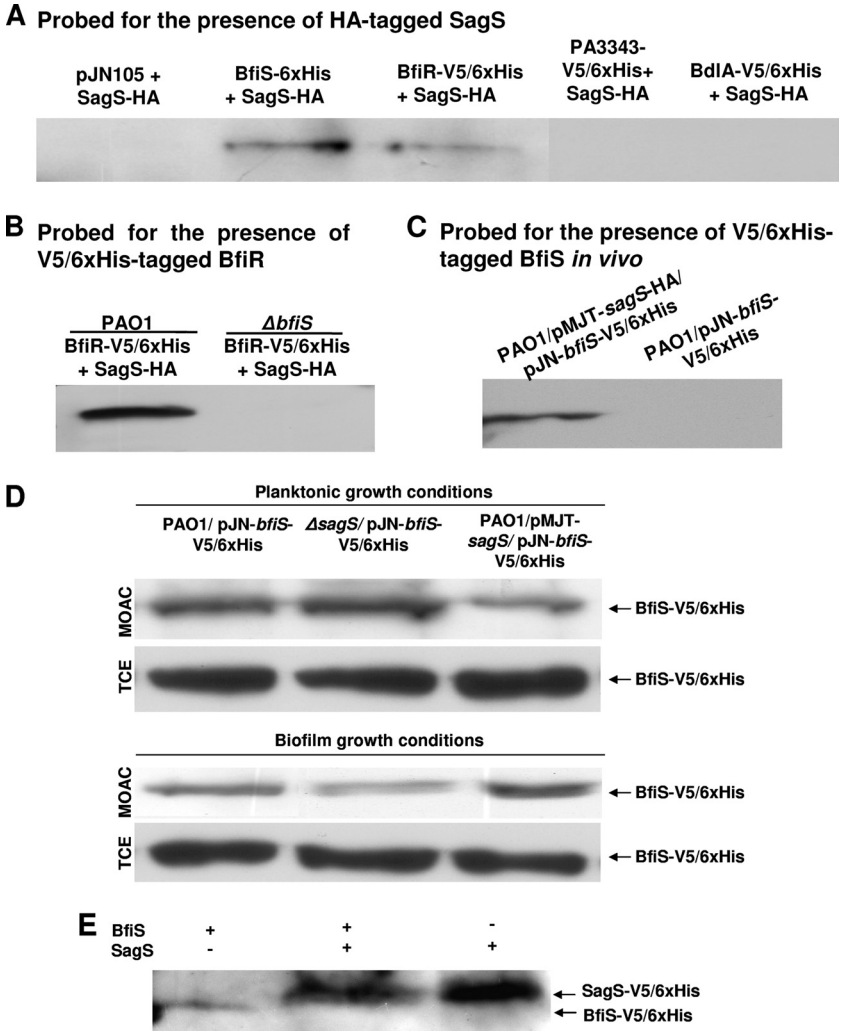

FIG. 7. SagS directly interacts with and modulates the phosphorylation status of BfiS in a growth-mode-dependent manner. (A) Extracts of PAO1 bearing HA-tagged SagS were incubated with extracts containing 6×His-tagged BfiS or BfiR or a vector control (pJN105), and incubation was followed by anti-6×His immunoprecipitation. The immunoprecipitation eluates were subsequently assessed by immunoblot analysis for the presence of SagS-HA using anti-HA antibodies. Tagged PA3343 and BdlA were used as negative controls. (B) SagS/BfiR interactions in the wild-type (PAO1/pJN-*bfiR*-V5/6×His, PAO1/pJN-*sagS*-HA) and Δ*bifS* mutant (Δ*bfiS*/pJN-*bfiR*-V5/6×His, Δ*bfiS*/pJN-*sagS*-HA) backgrounds were assessed via anti-HA pulldown assays followed by immunoblot analysis using anti-V5 antibodies for the presence of BfiR-V5/6×His. Cell extracts obtained from PAO1/pJN105 were used as controls. (C) SagS and BfiS form a complex *in vivo* as demonstrated by pulldown assays using PAO1/pMJT-*sagS*-HA/pJN-*bfiS*-V5/6×His. PAO1/pJN-*bfiS*-V5/6×His was used as a negative control. (D) Phosphorylation of BfiS is modulated by the absence/presence of SagS in a growth-mode-dependent manner, as revealed by anti-V5 immunoblotting for BfiS-V5 in phosphoproteins purified by MOAC and in total cell protein extracts (TCE). (E) Under planktonic conditions, SagS dephosphorylates BfiS.

of the Δ*sagS* strain in the absence of *rsmYZ* (Fig. 8C). The triple mutant was found to exhibit a hypervirulent phenotype comparable to that of the Δ*sagS* single mutant. Inactivation of *sagS* in the Δ*gacA* or Δ*hptB* strain rendered these strains hypervirulent, significantly increasing their virulence. These results indicated that the SagS virulence phenotype and the difference in virulence between the Δ*bfiS* and Δ*sagS* strains are not dependent on *rsmYZ* levels.

DISCUSSION

The opportunistic pathogen *P. aeruginosa* is capable of causing a wide variety of human diseases, ranging from bacteremia caused primarily by free-swimming cells to biofilm-related diseases due to colonization of medical devices and chronic infections of immunocompromised patients and those suffering from cystic fibrosis. The ability of *P. aeruginosa* to persist as free-swimming or biofilm cells is dependent on posttranslational modification and/or the differential expression of genes required for the maintenance of either mode of growth, resulting in the modulation of small regulatory RNA (sRNA) levels (7, 8, 17, 18, 44, 63). Here, we demonstrate that SagS (PA2824) is essential for the transition from the free-swimming to the sessile mode of growth. The transition from the free-swimming to the sessile mode of growth regulated by SagS correlated with the modulation of sRNA levels, with elevated levels of *rsmYZ*

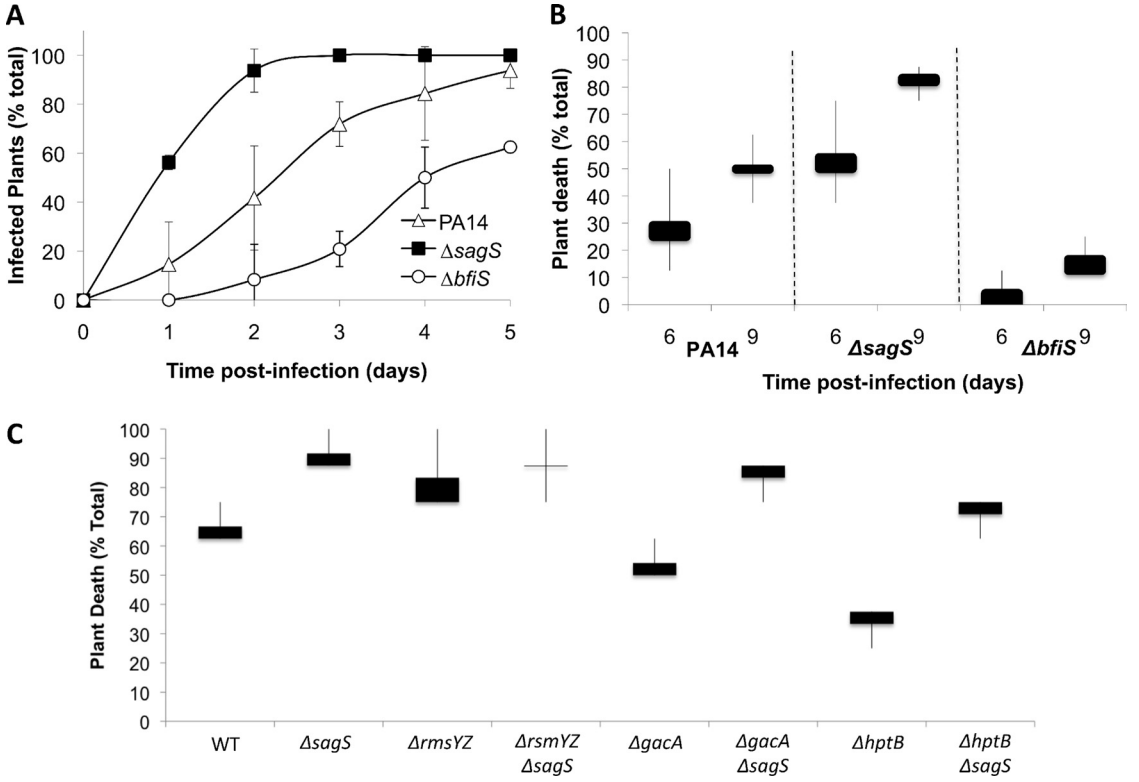

FIG. 8. Effect of *sagS* inactivation on *P. aeruginosa* virulence. *A. thaliana* plants, grown with the indicated PA14 strains, were monitored for signs of infections, indicated by discoloration of plants (A), and for death, manifesting as wilting and necrosis spread throughout the plant (B). (C) Inactivation of *sagS* renders the Δ*gacA*, Δ*hptB*, and Δ*rsmYZ* mutant strains more virulent. The graphs represent the averages of results from three experiments, with 8 plants used per replicate per strain. Lines indicate the differences between the highest and lowest survival rates observed, while bars represent distributions between the means and medians of the replicates. WT, wild type.

being observed in planktonically growing cells. The planktonic-growth-specific modulation of *rsmYZ* in the Δ*sagS* strain was found to be upstream of GacA but dependent on HptB (Fig. 5B). Indeed, SagS has been demonstrated to be one of three sensor kinase hybrids that undergo autophosphorylation and transfer a phosphoryl group specifically to the histidine phosphotransfer protein HptB, which in turn relays the signal to the response regulator PA3346 (6, 22). HptB is also capable of phosphorylating RetS, and both HptB and RetS intersect at the GacA response regulator, which directly controls sRNA production.

Similarly to RetS, SagS is a member of the sensor kinase/response regulator hybrid family of signal transduction proteins (Fig. 1) and is encoded by a single gene operon, with no genes encoding response regulators in the close proximity of *sagS* (17, 33, 65). BLAST and BLINK analysis revealed the presence of orthologs primarily in the genomes of proteobacteria, including *Pseudomonas putida*, *Pseudomonas fluorescens*, *Pseudomonas syringae*, *Geobacter* spp., *Shewanella* spp., and *Vibrio* spp. In addition to sensor kinase/response regulator domain structure and genomic context, the two proteins share other similarities. For instance, inactivation of *sagS* and *retS* coincided with increased Psl production and increased attachment in a microtiter plate assay (Fig. 1 and 3) (17), which is consistent with previous reports indicating that increased levels of *rsmYZ* enhance attachment (17, 18, 63). Modulation of these properties in the Δ*sagS* strain was independent of motility or cyclic di-GMP levels. A similar disconnect between attachment capabilities, Psl production, and motility was recently demonstrated by Merritt et al. (35), indicating that these characteristics are not necessarily linked. The effect of *sagS* inactivation on attachment, however, was only temporary, with the reduction of attached biomass being noticeable as soon as 8 h following initial attachment (Fig. 1 and 2; Table 1) (43). Moreover, a Δ*sagS* mutant was impaired at early stages of biofilm development in a manner similar to that observed for the Δ*retS* and Δ*bfiS* strains (Fig. 2) (17, 43, 44). Similar analysis performed with the Δ*bfiS* strain showed a strictly antagonistic control of *rsmYZ*, with elevated levels of sRNAs in biofilm cells but no variation in the sRNAs of cells in the planktonic growth mode (Fig. 4). The findings identified the probable sensor/response regulator hybrid SagS as part of the regulatory system linking the opposing factors/proteins that reciprocally modulate sRNA levels prior to and after attachment to enable biofilm formation (17, 18, 63). However, while the *rsmYZ* levels in the Δ*sagS* strain appeared to be dependent on HptB, the post-attachment phenotypes of the two mutants were very similar, suggesting similar or convergent (but not necessarily dependent) regulatory roles for the two proteins in biofilm formation. Moreover, the Δ*sagS* biofilm phenotype was independent of *rsmYZ* levels, as indicated by the finding that while restoration of Δ*sagS* biofilm formation by BfiR correlated with a reduction in *rsmYZ* levels, inactivation of *rsmYZ* in the Δ*sagS* strain did not restore biofilm formation to wild-type levels (Fig.

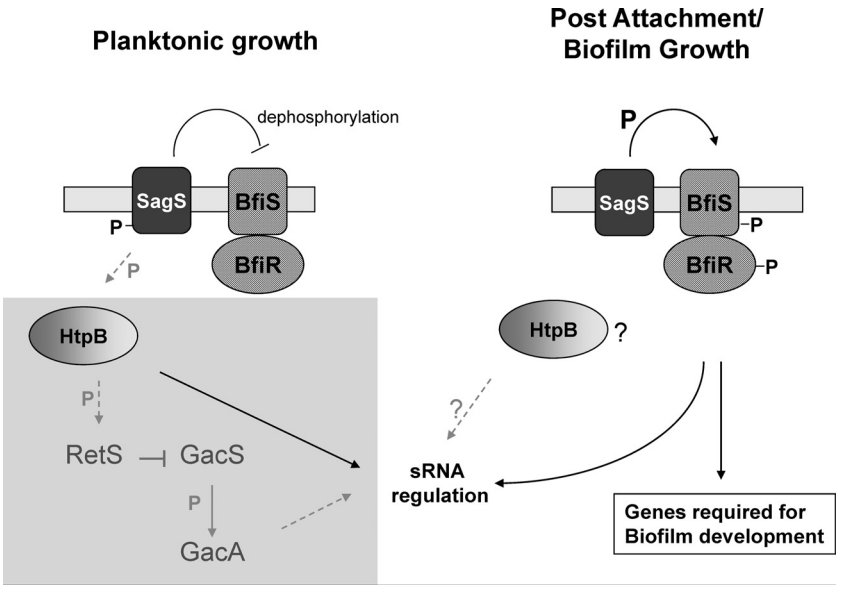

FIG. 9. Model for the role of SagS in linking the Gac/HptB/Rsm and BfiSR signaling systems prior to and following the motile-sessile switch. The proposed model of differential SagS activity prior to and following the motile-sessile switch integrates present findings (indicated by black arrows) and previously published data (gray arrows with dashed lines). Under planktonic conditions, SagS is phosphorylated and directly interferes with BfiSR activity, probably via dephosphorylation of BfiSR. Moreover, SagS modulates sRNA levels under planktonic conditions in an HptB-dependent manner. GacAS and HptB contribute to the regulation of the small regulatory RNAs (sRNAs) *rsmY* and *rsmZ*, which act through RsmA sequestration. RetS participates in a phosphotransfer with HptB and directly interferes with GacS activity (18, 22). Transition to the surface (postattachment and under biofilm growth conditions) coincides with SagS dephosphorylation, presumably via phosphoryl group transfer from SagS to BfiS, resulting in BfiSR activation (43), suppression of sRNA levels (in particular, *rsmZ* levels), and activation of genes and gene products required for biofilm development by *P. aeruginosa* (43, 44). While both SagS and HptB play a role in biofilm formation, SagS function appears to be independent of HptB (and GacA). The lightly shaded square area indicates components belonging to the Gac/HptB/Rsm signaling system (without indicating proper location in the cell). P, phosphoryl group or phosphoryl group transfer; ?, unknown contribution to biofilm formation or sRNA regulation under biofilm growth conditions; IM, inner membrane.

6). The finding suggested that SagS, the potential cognate sensor for HptB, serves an additional function besides its involvement in controlling the levels of sRNAs to enable biofilm formation (Fig. 9). This function, which will be the subject of future studies, appears to be required (in a Gac/Rsm system-independent manner) for the activation of the two-component system BfiSR, which is essential for biofilm formation.

Pulldown assays demonstrated that SagS interacts directly with BfiS and only indirectly with BfiR via BfiS (Fig. 7). However, overexpression of *bfiR* but not *bfiS* restored Δ*sagS* biofilm formation to wild-type levels, suggesting that in order for biofilm formation to occur, SagS is required to interact with BfiS, resulting in the relay of a signal which is lacking in Δ*sagS* biofilms (Fig. 6 and 7). Considering that SagS is not phosphorylated under biofilm growth conditions after 8 h of attachment, a time at which BfiSR phosphorylation becomes detectable by anti-phospho-Ser/Thr immunoblot analysis (43), the signal likely consists of a phosphoryl group relayed from the hybrid sensor SagS to BfiS. The experiments described here are consistent with a model in which SagS enhances the phosphorylation of BfiS under biofilm growth conditions while blocking phosphorylation or dephosphorylating BfiS under planktonic conditions (Fig. 9).

The signals that trigger the opposing activities of SagS with respect to BfiS phosphorylation are unclear. However, SagS-BfiS complex formation and the phosphotransfer between these two sensor proteins are reminiscent of complex formation and the phosphotransfer of the RetS and GacS heterodimer described previously (18), with complex formation blocking GacS autophosphorylation (and subsequent phosphotransfer to the response regulator GacA), leading to a reduction in *rsmYZ* expression. The finding suggested that heterologous sensor kinases can interact directly to form multisensor signaling networks, with RetS interaction acting as a switch in modulating the downstream activity of the GacAS system (18). The observation furthermore suggested that by modulating signal transduction at the level of sensor kinase phosphorylation, multiple inputs can be integrated without the need for cross-phosphorylation between sensors and noncognate response regulators (18). In a manner similar to that of RetS, SagS acts as a switch to regulate the transition from the planktonic to the surface-attached mode of growth by directly interacting with and modulating the phosphorylation status of BfiS. Present findings are consistent with a model in which SagS suppresses sRNA accumulation and sequestration of the mRNA-binding protein RsmA under planktonic growth conditions, probably by acting in concert with HptB (6, 22) while blocking BfiS phosphorylation (Fig. 7D and E). Upon transition to surface-associated growth, however, SagS interacts with BfiSR to promote BfiS phosphorylation (Fig. 7D) and, likely, BfiSR activation, with this TCS playing an essential role in suppressing sRNA levels and enabling *P. aeruginosa* to transition from reversible to irreversible attachment (43, 44). Our findings are summarized in a model shown in Fig. 9. Additional support for SagS acting as a switch is given by the observation that SagS and BfiS modulate *rsmYZ* levels in opposing man-

ners, which are dependent on the mode of growth (Fig. 4). Moreover, SagS and BfiSR differ in their contributions to the virulence of *P. aeruginosa* when tested in a nonmammalian virulence model. While the Δ*bfiS* mutant was avirulent, a Δ*sagS* mutant strain was hypervirulent (Fig. 8) (44). Taking into account that both the Δ*sagS* and Δ*bfiS* biofilms are arrested in biofilm development (with Δ*sagS* biofilms being arrested prior to Δ*bfiS* biofilms) (Fig. 2; see also Table S3 in the supplemental material), the findings suggest possible roles for SagS and BfiSR in contributing to the switch from acute infection to chronic persistence.

In conclusion, our data suggest that the sensor kinase hybrid SagS regulates the transition between the motile and sessile modes of growth by modulating sRNA levels in a growth-mode-dependent manner (Fig. 9). Moreover, SagS interacts with BfiS in a manner reminiscent of the RetS-GacS heterodimer interaction. To our knowledge, this is the first description of a *P. aeruginosa* protein enabling the motile-sessile switch by linking the reciprocal modulation of sRNA levels via the Gac/HptB/Rsm system to the signal transduction network composed of BfiSR, BfmSR, and MifSR, previously demonstrated to be required for the development of *P. aeruginosa* biofilms, in a sequential and stage-specific manner, into multisensor signaling networks.

ACKNOWLEDGMENTS

We thank D. Wozniak for kindly providing the anti-Psl antibody and D. Haas, A. Filloux, and H.-L. Peng for kindly providing the Δ*hptB*, Δ*rsmYZ*, and Δ*gacA* mutant strains and the respective parental strains.

This work was supported by grants from the Army Research Office (W911NF0710604) and the National Institutes of Health (2R15 HL073835-02).

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