SagS contributes to the motile-sessile switch and acts in concert with BfiSR to enable \textit{Pseudomonas aeruginosa} biofilm formation

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ABSTRACT

The interaction of *P. 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, is linking sRNA modulating activities via the Gac/HptB/Rsm system post attachment 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, HptB-dependent increase in *rsmYZ*, increased Psl polysaccharide production and virulence. Moreover, *sagS* inactivation rescued attachment but abrogated biofilm formation by Δ*gacA* and Δ*hptB*. Δ*sagS* was impaired in biofilm formation at a stage similar to 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 BfiS. Our findings indicate that SagS acts as a switch by linking the GacA-dependent sensory system under planktonic conditions to suppression of sRNA post attachment and BfiSR required for the development of *P. aeruginosa* biofilms in a sequential and stage-specific manner.
INTRODUCTION

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 free-swimming to the surface attached mode 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 guanosine 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 P. aeruginosa, to control the transition between a motile and a biofilm lifestyle via its concentration, with high levels fostering the sessile lifestyle, while low cyclic di-GMP concentrations favor motility (e.g. twitching, 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 E. 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 action of the two small non-coding RNAs (sRNAs) CsrB and CsrC (24, 25, 28, 46, 59, 64).

Similarly to the Csr system in E. coli, sRNAs have been shown to play an important role in attachment, polysaccharide production, virulence, and quorum sensing by Pseudomonas aeruginosa (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. 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. Additional components modulating sRNA levels were recently identified as the histidine phosphotransfer protein HptB. HptB is activated via three orphan sensor kinase hybrids (PA2824, PA1611, PA1976) and in turn relays the signal (phosphor group) to the response regulator PA3346.
and 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, 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* has 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 ribonuclease 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, 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 post 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 post attachment to enable biofilm formation. By regulating the motile-sessile switch, SagS links the Gac/HptB/Rsm and the BfiSR signaling transduction systems into a multisensor signaling network.
MATERIAL AND METHODS

Bacterial strains, plasmids, media, and culture conditions. All bacterial strains and plasmids used in this study are listed in Table S1. P. aeruginosa strain 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 VBMM minimal medium (53) in shake flasks at 220 rpm.

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

Biofilm formation. Biofilms were grown using a once-through continuous flow tube reactor system to obtain proteins and RNA and 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 minimal medium in the absence/presence of 0.1% arabinose. Quantitative analysis of CSLM images of flow cell-grown biofilms was performed using COMSTAT (20). Initial biofilm formation was measured using the microtiter dish assay system, with crystal violet staining (40) and by microscopy (12). The number of cells attached to the glass surface of a flow cell covering an area of 400 µm² 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 2D/PAGE, followed by blotting onto PVDF membranes and phosphoprotein detection using anti-Phospho-(Ser/Thr)Phe antibodies (Cell Signaling Technologies, Danvers, MA). Analysis of phosphorylated BfiS-V5/6xHis levels in protein extracts of P. aeruginosa strains inactivated in or overexpressing sagS was accomplished using phosphoprotein purification via MOAC as described previously (43), followed by the detection of BfiS-V5/6xHis by immunoblotting with anti-V5 antibodies (Invitrogen Corp). For phosphotransfer studies, V5/6xHis-tagged BfiS and
SagS purified using Ni-NTA Spin Columns (QIAGEN), were combined and incubated in phosphorylation buffer (50mM Tris-HCl, pH7.5, 50mM KCl, 5mM MgCl\textsubscript{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 prior to incubation were used as controls.

PsI polysaccharide dot blot analysis. PsI polysaccharide was extracted from planktonic and 144-hr-old biofilm cells essentially as described by Byrd et al. (10). Quantitation of PsI production was done by determining the anti-PsI 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).

Quantitative reverse transcriptase PCR (qRT-PCR). qRT-PCR was used to determine the expression levels of pslA and rsmAYZ using 1 µg of total RNA. 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), with oligonucleotides listed in Table S2. Relative transcript quantitation, with mreB used as 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 (4, 36, 56) using heat and ethanol precipitation followed by centrifugation. Supernatants were combined, dried using a Speed-Vac and resuspended in 10 mM ammonium bicarbonate buffer. Samples (10 µl or 20 µl) were analyzed using an Agilent 1100 HPLC equipped with an autosampler, degasser, and detector set to 253 nm, and separated using a reverse-phase C\textsubscript{18} Targa column (2.1 x 40 mm; 5 µm) at a flow rate of 0.2 ml/min with the following gradient: 0 to 9 min, 1% B; 9 to 14 min, 15% B; 14 to 19 min, 25% B; 19 to 26 min, 90% B; 26 to 40 min, 1% B (A, 10 mM ammonium acetate; B, 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 MS/MS using a QStar mass spectrometer (Applied Biosystems) by detecting the following 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 pull-down assays. Pull-down assays were used to determine whether SagS interacts with BfiS and BfiR. HA-tagged SagS was incubated with extracts containing 6xHis-tagged BfiS or BfiR. Subsequently, BfiS or BfiR were immunoprecipitated using immobilized anti-6xHis antibodies, immunoprecipitation eluates separated by SDS/PAGE, and assessed by immunoblot analysis for the presence of SagS using anti-HA antibodies (or vice versa). Pull-down assays were carried out using 200 ug protein from cellular extracts, with HA-tagged proteins used as bait and (V5)6xHis-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 ug/mL and immunoblotting at 0.1 ug/mL. Two V5/6xHis-tagged proteins, the inner membrane protein PA3343 and BdlA were used as controls to determine the specificity of SagS-BfiS complex formation in vitro.

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 at a 25/22°C, 16hr-light/8hr-dark cycle, and analyzed as previously described (44).

Statistical analysis. A Student’s t-test was performed for pair-wise comparisons of groups, and multivariant analyses were performed using a 1-Way ANOVA followed by a post-priori 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, 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 only phosphorylated 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 ΔsagS attaching twice as efficiently as PAO1 within the first two hours as determined by microscopy and CV staining (Fig. 1B-C). The findings are consistent with previous reports by Goodman et al. demonstrating increased attachment capabilities for this mutant (17). However, increased attachment by ΔsagS was only temporary, and the difference in attachment diminished with increasing attachment times. No difference in attachment was observed between PAO1 and ΔsagS following 8 hrs of attachment, while continued incubation resulted in decreased attachment by ΔsagS compared to the wild type (Fig. 1C-D). Complementation (ΔsagS/pJN-sagS) restored the attachment phenotype to wild type levels while overexpression of sagS enhanced attachment after prolonged growth (Fig. 1B-C). Growth curves in liquid media suggested that the increase in attachment was not a result of a general increase in growth rate.

A ΔsagS 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 ΔsagS biofilm formation as compared to PAO1. However, the mutant strain failed to develop the biofilm architecture typically observed in PAO1 biofilms following 144 hr under biofilm growth conditions (Fig. 2A, Table 1). Instead, ΔsagS formed only thin biofilms that lacked
large cellular aggregates and microcolonies and were composed of ≥5-fold less biomass as compared to the wild type (Table 1). Overall, \(\Delta\text{sagS}\) biofilms most closely resembled those of PAO1 following 24 hr of growth. Daily monitoring of \(\Delta\text{sagS}\) biofilms by confocal microscopy over a period of 24-144 hr revealed that \(\Delta\text{sagS}\) biofilms failed to accumulate additional biomass typically seen in wild type biofilms following continued growth under flowing conditions (post 48 hr of growth, Table 1, Fig. 2A (43, 52)) indicating that the observed biofilm architecture at the 144 hr time point was not due to premature disaggregation. In contrast, overexpression of \(\text{sagS}\) resulted in biofilms that were similar in architecture to PAO1 biofilms, but displayed significantly increased biomass, as well as average and maximum thickness when grown for 144 hr (Fig. 2A, Table 1). Complementation restored the biofilm architecture to wild type levels.

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

Loss of \(\text{sagS}\) expression due to arabinose removal resulted in the collapse of the mutant biofilm architecture within three days (Fig. 2B – 72 hr, Table 2). While similar architectural collapse was observed following inactivation of \(\text{bfiS, bfmR,}\) and \(\text{mifR}\) expression (43), the collapse observed following \(\text{sagS}\) 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 reduction of biofilm variables including biofilm biomass and thickness, which further decreased upon continued incubation (Table 2). Under the conditions tested, no reduction of the 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 *AsagS* biofilms indicated that *AsagS* was 
arrested in biofilm formation. This prompted us to determine the biofilm developmental stage at 
which the *AsagS* 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 *AsagS* biofilms grown for 144 hr to *P. aeruginosa* wild type biofilms grown for 8, 24, 72, and 144 hr using anti-phospho-Ser/Thr antibodies 
indicated that *AsagS* biofilms failed to exhibit phosphorylation events typically observed during 
normal biofilm development following 144 hr of growth (Table 3, Table S3). For instance, *AsagS* 
biofilms lacked all phosphorylated proteins typically found in mature, 144-hr-old biofilms, but 
instead exhibited stage-specific phosphorylation events typically detected in 8- and 24-hr-old wild 
type biofilms: the phosphoproteome contained 26 out of 38 phosphorylated proteins and 12 out of 33 
phosphorylated proteins that are specific to 8- and 24-hr-old wild type biofilms, respectively (Table 3, 
Table S3). Furthermore, *AsagS* biofilms lacked evidence of BfiS phosphorylation (Table S3), which 
was previously detected following 8 hr of biofilm growth (43), indicating that *AsagS* biofilms are 
probably arrested prior to Δ*bfiS*, at the transition from the planktonic to the initial attachment stage. 
Comparison of the phosphorylation patterns of both *AsagS* and Δ*bfiS* indicated that while both 
mutant biofilms exhibited stage-specific phosphorylation events typically detected in 8- and 24-hr-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 
the sessile states, mutants inactivated in and overexpressing *sagS* 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 *AsagS* in a planktonic-specific 
manner was detected by immunoblot analysis using anti-Psl antibodies (Fig. 3A-B). Under planktonic 
growth conditions, Psl abundance in *AsagS* 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 *AsagS* 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 inactivated in or overexpressing \( \text{sagS} \) (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 \( \Delta \text{sagS} \) and wild type cells grown planktonically was detected, indicating that increased adhesiveness and Psl production are independent of cyclic di-GMP levels in \( \Delta \text{sagS} \).

SagS controls \( \text{rsmY} \) and \( \text{rsmZ} \) expression under planktonic growth conditions.

The Gac/Rsm network directly modulates the levels of sRNAs \( \text{rsmY} \) and \( \text{rsmZ} \) to regulate the expression of exopolysaccharide components of the \( \text{P. aeruginosa} \) biofilm matrix, attachment, and swarming motility (6-8, 17, 19). SagS has been previously linked to this network via the histidine phosphotransfer protein \( \text{HptB} \), with \( \text{HptB} \) having been shown to be dependent on the GacA response regulator (6, 22), which directly controls \( \text{rsmY} \) and \( \text{rsmZ} \) transcription (6-8, 17, 19). This prompted us to ask whether SagS is involved in the regulation of \( \text{rsmYZ} \) levels. Inactivation of \( \text{sagS} \) correlated with elevated \( \text{rsmA} \) levels and significantly increased \( \text{rsmYZ} \) levels as compared to the wild type under planktonic growth conditions (Fig. 4A). Under biofilm growth conditions, \( \text{rsmAYZ} \) levels were 2-fold increased in \( \Delta \text{sagS} \) biofilms (Fig. 4B). No difference in \( \text{rsmAYZ} \) gene expression was detected when transcript levels of \( \text{PAO1/pJN-sagS, \Delta sagS/pJN-sagS} \) and the wild type were compared. In contrast, analysis of the \( \text{bfiS} \) mutant strain, which is arrested in biofilm formation at a similar stage compared to \( \Delta \text{sagS} \) (43, 44), demonstrated that the RNA levels of \( \text{rsmA, rsmY, and rsmZ} \) were unaltered in \( \Delta \text{bfiS} \) under planktonic growth conditions but significantly increased upon biofilm growth as compared to the wild type (Fig. 4A-B). We also measured the activity of \( \text{rsmY-lacZ and rsmZ-lacZ} \) chromosomal transcriptional fusions in planktonic, 24- and 144-hr-old biofilm cells. In agreement with the expression levels, transcription from \( \text{rsmY} \) and \( \text{rsmZ} \) promoters was found to be repressed by SagS regardless of growth conditions (Fig. 4C-D). This is in contrast to \( \Delta \text{bfiS} \), for which \( \text{rsmYZ} \) transcription levels were comparable to the wild type regardless of growth conditions, while \( \text{rsmYZ} \) RNA levels were significantly increased under biofilm growth conditions (44). These findings strongly suggest that SagS and BfiS reciprocally modulate \( \text{rsmAYZ} \) abundance at distinct levels in a growth mode dependent manner. The mutants further differed with respect to \( \text{pslA} \) expression and Psl production (Fig. 3). While increased \( \text{rsmYZ} \) levels by \( \Delta \text{sagS} \) correlated with increased \( \text{pslA} \) expression and Psl production under planktonic growth conditions, no difference in Psl production was detected in \( \Delta \text{bfiS} \) regardless of the growth conditions (Fig. 3).
Deletion of \textit{sagS} enhances early attachment but abrogates biofilm formation by \textit{gacA} and \textit{hptB} mutant strains.

Given that our findings implicated SagS in the regulation of \textit{rsmYZ} levels (Fig. 4), we next determined whether SagS regulatory function and 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 \textit{gacA} and deletion of its small regulatory RNA targets \textit{rsmYZ} resulting in reduced attachment compared to the parental strain \textit{P. aeruginosa} PAK (8, 41). Inactivation of \textit{hptB} in \textit{P. aeruginosa} PAK resulted in hyperattachment, while the same mutation in \textit{P. aeruginosa} PAO1 only affected attachment in the presence of glucose and casamino acids (6, 33). Here, we demonstrate that in \textit{P. aeruginosa} PAO1, inactivation of \textit{rsmYZ}, \textit{gacA} and \textit{hptB} results in reduced attachment following 1 hr of incubation under static conditions (Fig. 5A). Continued incubation, however, resulted in increased or hyper-attachment by \textit{ΔgacA} and \textit{ΔrsmYZ} (Fig. 5A) indicating that, similarly to \textit{ΔsagS}, mutations in \textit{gacA} and \textit{rsmYZ} only have a temporary effect on attachment. In contrast, \textit{ΔhptB} demonstrated reduced attachment regardless of attachment time (Fig. 5A). Second site mutation in \textit{sagS} rescued the attachment defect of \textit{ΔgacA}, \textit{ΔhptB}, and \textit{ΔrsmYZ}, following 1 hr of attachment while suppressing attachment upon continued incubation (Fig. 5A).

In agreement with the attachment phenotype following 24 hrs of incubation, \textit{ΔgacA} formed hyperbiofilms characterized by significantly increased biofilm biomass and height compared to wild type biofilms. Deletion of \textit{sagS} in \textit{ΔgacA} significantly reduced biofilm biomass accumulation by more than 20-fold with mutant biofilms resembling those of \textit{ΔsagS} biofilms (Fig. 5C, Table 1). \textit{ΔhptB} formed only thin biofilms lacking microcolonies and being on average only 2 µm thick, with secondary site mutation in \textit{sagS} resulting in a further 2-3-fold reduction in the biofilm biomass (Fig. 5C, Table 1). Thus, introducing a \textit{sagS} deletion into the \textit{gacA} and \textit{hptB} mutants abrogates biofilm formation of the parent strain, underscoring the importance of SagS in biofilm formation. Our findings indicate SagS to function upstream of GacA to regulate attachment and biofilm formation. Moreover, the similarity of the \textit{ΔsagS} and \textit{ΔhptB} attachment (post 24 hrs) and biofilm phenotypes of (Fig. 5A, C) suggested HptB and SagS to 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 \textit{rsmAYZ}, respective RNA levels in \textit{AgacA}, \textit{AhptB} and strains harboring a second site mutation in \textit{sagS} were determined under planktonic conditions. In agreement with previous results, inactivation of \textit{gacA} resulted in significantly reduced \textit{rsmYZ} levels under planktonic growth conditions. Deletion of \textit{sagS} in \textit{AgacA} did not significantly alter \textit{rsmAYZ} levels. The observation that \textit{rsmAYZ} RNA levels are
similarly reduced in ΔgacA and ΔgacAΔsagS strain backgrounds indicated that SagS is not directly required for GacA function but 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 ΔhptB PAK strain (not shown), inactivation of hptB in P. aeruginosa PAO1 was correlated with 2-fold reduced rsmA levels, while rsmYZ levels remained comparable to wild type (Fig. 5B). Second site sagS mutation in ΔhptB 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 ΔsagS grown planktonically appeared primarily to be dependent on HptB previously shown to be GacA-dependent (6).

**BfiR is required for SagS to enable biofilm formation.**

Phosphoproteome analysis suggested ΔsagS biofilms to be arrested prior to ΔbfiS, 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 ΔasagS would not result in 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 ΔasagS to wild type levels.

Overexpression of sagS in ΔbfiS had no effect on biofilm formation. Similarly, overexpression of bfiS, encoding the sensor kinase of the BfiSR TCS, in ΔasagS did not affect the overall biofilm architecture (Fig. 6A, Table 1). The findings indicate 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 ΔasagS resulted in restoration of the biofilm architecture (Fig. 6A). Instead of forming thin biofilms lacking large cellular aggregates and microcolonies, ΔasagS/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 ΔasagS biofilms and 3-4-fold increase compared to PAO1 biofilms of comparable age (Table 1).

Restoration of the ΔasagS biofilm architecture to wild type levels by complementation or overexpression of bfiR, correlated with rsmYZ levels that were comparable to wild type and vector only controls (Fig. 6B). Compared to ΔasagS biofilms, complemented biofilms had 3.37-fold reduced rsmZ levels, while overexpression of sagS or bfiR coincided with a ~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 ΔasagS results,
at least in part, from elevated rsmYZ abundance, deletion of rsmYZ in ∆sagS did not restore biofilm architecture to wild type levels. Instead, ArsmYZ∆sagS biofilms were comparable in biofilm modulating sRNA levels, the ∆sagS biofilm phenotype is independent of rsmYZ.

SagS interacts directly with BfiS, but only indirectly with BfiR.

The observation that ∆sagS biofilm formation was restored to wild type levels by bfiR overexpression suggested that SagS and BfiSR either 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 a yet unknown signal from SagS absent in ∆sagS biofilms.

To test these hypotheses, we assessed whether SagS forms a complex with BfiSR in vitro. Pull-down of a complex of 6xHis-tagged BfiS and hemagglutinin-tagged SagS (SagS-HA) as well as a complex of BfiS-V5/6xHis and SagS-HA could be clearly demonstrated when pull-down assays were probed for HA-tagged SagS (Fig. 7A). Two proteins were used as controls to determine the specificity of the SagS-BfiS interaction, which included 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 sp. (65), and BdlA (36).

Neither BdlA nor PA3343 were detected in a complex with SagS-HA (Fig. 7A). Complex formation was also detectable when SagS-HA was used as bait to detect 6xHis-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 ∆bfiS mutant background (Fig. 7B). Complex formation of SagS and BfiS was confirmed by in vivo pull-downs (Fig. 7C) and by using purified enzymes (not shown), thus indicating the two proteins are working in concert.

BfiS phosphorylation is SagS-dependent.

The observations of SagS forming a complex with BfiS to enable biofilm formation (Figs. 7A-C), raised the possibility of SagS being involved in the transfer of a phosphoryl group to BfiS. This was supported by the finding of ∆sagS biofilms lacking evidence of BfiS Ser/Thr-phosphorylation (Table S3). 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 purification of total phosphoproteins via metal oxide affinity chromatography (MOAC) from PAO1, ∆sagS and PAO1/pMJT-sagS biofilm and planktonic cells overexpressing bfiS-V5/6xHis. Under biofilm growth conditions, overexpression of sagS resulted in increased detection of phosphorylated BfiS via immunoblotting using anti-V5 antibodies compared to
the wild type (Fig. 7D). In contrast, deletion of sagS correlated with reduced levels of phosphorylated BfiS (Fig. 7D). 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 lack of sagS resulting in increased levels of phosphorylated BfiS (Fig. 7D). The findings suggested phosphorylation of BfiS to be SagS dependent, with SagS reducing or interfering with BfiS phospho-hylation under planktonic conditions.

To demonstrate transfer of a phosphoryl group between SagS and BfiS in vitro, we made use of the finding that both V5/6xHis-tagged proteins are phosphorylated when overexpressed in E. coli (Fig. 7E). Purified SagS and BfiS were co-incubated and subjected to phosphoprotein enrichment via MOAC, with the resulting protein eluates subjected to SDS/PAGE and immunoblot analysis using anti-V5 antibodies. Co-incubation of SagS with BfiS led to a significant decrease in 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 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 post-attachment via modulation of rsmYZ 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 ΔsagS elicits an earlier onset of initial signs of infection compared to the wild type as indicated by lesions and discoloration (Fig. 8A). Moreover, ΔsagS was hypervirulent and resulted in A. thaliana plant death three days earlier than did the wild type (Fig. 8B). These results suggested a contribution of SagS signaling to virulence of P. aeruginosa. In contrast, AbfiS mutants were avirulent (44) and showed significantly delayed onset of initial signs of infection and significantly reduced virulence compared to the wild type and ΔsagS (Fig. 8A-B). Considering that the main difference observed between the two strains is the growth mode dependence of rsmYZ levels, we determined the virulence properties of ΔsagS in the absence of rsmYZ (Fig. 8C). The triple mutant was found to exhibit a hypervirulent phenotype comparable to that
of the single sagS mutant. Inactivation of sagS in ΔgacA or ΔhptB rendered these strains hypervirulent, significantly increasing the virulence of these strains. These results indicated that the SagS virulence phenotype or the difference in virulence between ΔbfiS and ΔsagS are not dependent on rsmYZ levels.
DISCUSSION

The opportunistic pathogen \textit{P. aeruginosa} is capable of causing a wide variety of human diseases ranging from bacteremia primarily caused 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 \textit{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. 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 \textit{rsmYZ} observed in planktonically growing cells. The planktonic-specific modulation of \textit{rsmYZ} in \textit{AsagS} 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 \textit{sagS} (17, 33, 65). BLAST and BLINK analysis revealed the presence of orthologs primarily in the genomes of Proteobacteria including \textit{P. putida}, \textit{P. fluorescens}, \textit{P. syringae}, \textit{Geobacter sp.}, \textit{Shewanella sp.}, and \textit{Vibrio sp.}. In addition to sensor kinase/response regulator domain structure and genomic context, the two proteins share additional similarities. For instance, inactivation of \textit{sagS} and \textit{retS} coincided with increased Psl production and increased attachment in a microtiter plate assay (Figs. 1, 3, (17)), which is consistent with previous reports indicating that increased levels of \textit{rsmYZ} enhance attachment (17, 18, 63). Modulation of these properties in \textit{AsagS} was independent of motility or cyclic di-GMP. 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 \textit{sagS} inactivation on attachment, however, was only temporary, with reduction of attached biomass being noticeable as soon as 8 hr following initial attachment (Figs. 1-2, Table 1) (43). Moreover, a \textit{AsagS} mutant was impaired at early stages of biofilm development, in a manner similar to the phenotypes observed for \textit{AretS} and \textit{AbfiS} (Fig. 2, (17, 43, 44)). Similar analysis performed with \textit{AbfiS} showed a strictly
antagonistic control of rsmYZ, with elevated levels of sRNAs in biofilm grown cells but no variation 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 post attachment to enable biofilm formation (17, 18, 63).

However, while the rsmYZ levels in ΔsagS appeared to be dependent on HptB, the post-attachment phenotypes of the two mutants were very similar, suggesting similar or convergent regulatory (but not necessarily dependent) roles for the two proteins in biofilm formation. Moreover, the ΔsagS biofilm phenotype was independent of rsmYZ levels, as indicated by the finding that while restoration of ΔsagS biofilm formation by BfiR correlated with reduction in rsmYZ levels, inactivation of rsmYZ in ΔsagS did not restore biofilm formation to wild type levels (Fig. 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 essential for biofilm formation.

Pull-down assays demonstrated SagS to interact directly with BfiS and only indirectly with BfiR via BfiS (Fig. 7). However, overexpression of bfiR but not bfiS restored ΔsagS 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 ΔsagS biofilms (Figs. 6-7).

Considering that SagS is not phosphorylated under biofilm growth conditions post 8 hr of attachment, a time at which BfiSR phosphorylation becomes detectable by anti-phospho-Ser/Thr immunoblot analysis (43), the signal likely consists of phosphoryl group relayed from the hybrid sensor SagS to BfiS. The experiments described here are consistent with a model in which SagS enhances phosphorylation of BfiS under biofilm growth conditions, while blocking phosphorylation or dephosphorylating BfiS under planktonic conditions (Fig. 9).

What signals trigger the opposing activities of SagS with respect to BfiS phosphorylation are unclear. However, SagS-BfiS complex formation and phosphotransfer between these two sensor proteins is reminiscent 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 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 non-cognate response regulators (18). In a manner similar to 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-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 an opposing manner, which is dependent on the mode of growth (Fig. 4). Moreover, SagS and BfiSR differ in their contribution to virulence of *P. aeruginosa* when tested in a non-mammalian virulence model. While the ΔbfiS mutant was avirulent, a ΔasagS mutant strain was hypervirulent (Fig. 8, (44)). Taking into account that both ΔasagS and ΔbfiS biofilms are arrested in biofilm development (with ΔasagS biofilms being arrested prior to ΔbfiS biofilms, Fig. 2, Table S3), the findings suggest a possible role of 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 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.

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**Figure 1.** Attachment is disregulated in the strain inactivated in \textit{sagS}, encoding a hybrid sensor kinase/response regulator, 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. Predicted transmembrane-spanning segments are shown in dark gray. (B) Direct cell count of wild type and mutant strains attached to polystyrene following 1 and 2 hr post initial attachment. Cells attached per 400 $\mu$m$^2$ were quantified using ImageProPlus software. (C) Evaluation of attachment to polystyrene by crystal violet staining 1 and 24 hr post initial attachment relative to \textit{P. aeruginosa} PAO1 in the absence/presence of the empty plasmid (PAO1 ± pJN105). (D) Time course of attachment to polystyrene over the course of 24 hr by \textit{P. aeruginosa} PAO1 and $\Delta$sagS. *Significantly different from respective wild type values ($p < 0.05$) as determined by ANOVA and Sigma Stat. Error bars indicate standard deviation.

**Figure 2.** SagS is required for the formation and maintenance of \textit{P. aeruginosa} biofilms. (A) Biofilms of strains inactivated in or overexpressing sagS, grown for 144 hours, were visualized by CSLM. \textit{P. aeruginosa} PAO1 in the absence/presence of the empty plasmid (PAO1 ± pJN105) were used as controls. (B) Inactivation of sagS expression in mature biofilms results in biofilm architectural collapse and biofilm dispersion. \textit{P. aeruginosa} $\Delta$sagS complemented with sagS ($\Delta$sag/pJN-sagS) 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 hr, after which time the biofilms were visualized by confocal microscopy (0 hr). Arabinose was subsequently eliminated from the growth medium and the biofilm architecture monitored post arabinose removal at the times indicated. 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 $\mu$m.

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

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

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

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

**Figure 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 6xHis-tagged BfiS or BfiR or a vector control (pJN105), followed by anti-6xHis immunoprecipitation. The immunoprecipitation eluates were subsequently assessed by immunoblot analysis for the presence of SagS-HA using anti-HA antibodies (anti-HA Ab). Tagged PA3343 and BdlA were used as negative controls. (B) SagS/BfiR interactions in wild type...
(PAO1/pJN-bfiR-V5/6xHis, PAO1/pJN-sagS-HA) and ∆bfiS (ΔbfiS/pJN-bfiR-V5/6xHis, ΔbfiS/pJN-sagS-HA) mutant backgrounds were assessed via anti-HA pull-downs followed by immunoblot analysis for the presence of BfiR-V5/6xHis using anti-V5 antibodies (anti-V5 Ab). Cell extracts obtained from PAO1/pJN105 were used as controls. (C) SagS and BfiS form a complex in vivo as demonstrated by pull-downs using PAO1/pMJT-sagS-HA/pJN-bfiS-V5/6xHis. PAO1/pJN-bfiS-V5/6xHis 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 immunoblots for BfiS-V5 of phosphoproteins purified by MOAC [MOAC] and total cell protein extracts [TCE]. (E) Under planktonic conditions, SagS dephosphorylates BfiS.

Figure 8. Effect of sagS inactivation on P. aeruginosa virulence. A. thaliana plants, grown with the indicated PA14 strains, were monitored for (A) signs of infections indicated by discoloration of plants and (B) death manifesting as wilting and necrosis spread throughout the entire plant. (C) Inactivation of sagS renders ΔgacA, ΔhptB and ΔrsmYZ mutant strains more virulent. The graphs represent the averages of three experiments, with 8 plants used per replicate per strain. Lines indicate the difference between highest and lowest survival rates observed, while bars represent distribution between the mean and median of the replicates.

Figure 9. Model for the role of SagS in linking Gac/HptB/Rsm and BfiSR signaling system prior to and post the motile-sessile switch. The proposed model of differential SagS activity prior to and post the motile-sessile switch integrates present findings (indicated by black arrows) and previously published data (grey arrows with hatched lines). Under planktonic conditions, SagS is phosphorylated and directly interferes with BfiSR activity, probably via dephosphorylating 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 RNA (sRNA) 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 (post attachment 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 appeared to be independent of HptB (and GacA). Shaded 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.
Table 1. COMSTAT analysis of *P. aeruginosa* wild type and mutant biofilm structure.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Total biomass (µm³/µm²)</th>
<th>Substratum coverage (%)</th>
<th>Average thickness (µm)</th>
<th>Maximum thickness (µm)</th>
<th>Roughness coefficient (dimensionless)</th>
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<tbody>
<tr>
<td><em>P. aeruginosa</em> PAO1</td>
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<td></td>
<td></td>
<td></td>
</tr>
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<td>PAO1 24hr</td>
<td>0.95 (±0.39)*</td>
<td>12.11 (±5.08)*</td>
<td>0.78 (±0.38)*</td>
<td>7.8 (±1.14)*</td>
<td>1.69 (±0.12)*</td>
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<tr>
<td>PAO1 72hr</td>
<td>3.27 (±1.29)*</td>
<td>32.43 (±8.31)*</td>
<td>3.02 (±1.52)*</td>
<td>18.29 (±10.86)*</td>
<td>1.20 (±0.31)*</td>
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<tr>
<td>PAO1 144hr</td>
<td>7.99 (±3.63)</td>
<td>44.44 (±22.58)</td>
<td>7.17 (±4.97)</td>
<td>35.63 (±26.73)</td>
<td>1.24 (±0.37)*</td>
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<tr>
<td><em>P. aeruginosa</em> PAO1 isogenic mutants following 144 hr of growth</td>
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<tr>
<td>ΔsagS</td>
<td>0.82 (±0.63)*</td>
<td>7.07 (±5.14)*</td>
<td>0.83 (±0.62)*</td>
<td>9.00 (±2.83)*</td>
<td>1.74 (±0.20)*</td>
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<td>ΔsagS/pJN-sagS</td>
<td>11.89 (±8.48)</td>
<td>25.34 (±11.36)</td>
<td>11.90 (±8.99)</td>
<td>47.67 (±20.96)</td>
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<td>94.22 (±21.57)*</td>
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<td>ΔdptB</td>
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<td>1.51 (±0.56)</td>
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<td>ΔdptB/sagS</td>
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<td>7.19 (±6.34)</td>
<td>0.57 (±0.69)</td>
<td>6.89 (±2.93)*</td>
<td>1.82 (±0.17)*</td>
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*COMSTAT analysis was carried out from biofilms grown in triplicate using at least 6 images per replicate.

*Significantly different from 144-hr-old PAO1; *p* < 0.05 as determined by ANOVA and SigmaStat.
Table 2. COMSTAT analysis\(^a\) of *P. aeruginosa* wild type and complemented mutant biofilm structure following removal of arabinose and, thus, lack of *sagS* expression.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Time (hr)(^b)</th>
<th>Total biomass (µm(^3)/µm(^2))</th>
<th>Substratum coverage (%)</th>
<th>Average thickness (µm)</th>
<th>Maximum thickness (µm)</th>
<th>Roughness coefficient (dimensionless)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAO1/pJN105</td>
<td>0</td>
<td>11.92 (±9.28)</td>
<td>28.85 (±13.29)</td>
<td>11.97 (±11.48)</td>
<td>37.17 (±26.78)</td>
<td>1.25 (±0.28)</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>31.66 (±10.56)</td>
<td>29.37 (±11.36)</td>
<td>30.99 (±10.58)</td>
<td>66.33 (±13.90)</td>
<td>1.22 (±0.30)</td>
</tr>
<tr>
<td></td>
<td>144</td>
<td>36.92 (±18.00)</td>
<td>45.01 (±19.18)</td>
<td>37.91 (±18.76)</td>
<td>111.33 (±19.06)</td>
<td>0.92 (±0.44)</td>
</tr>
<tr>
<td>ΔsagS/pJN-ΔsagS</td>
<td>0</td>
<td>11.89 (±8.48)</td>
<td>25.34 (±11.36)</td>
<td>11.90 (±8.99)</td>
<td>47.67 (±29.96)</td>
<td>1.38 (±0.23)</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>1.88 (±0.84)*</td>
<td>16.28 (±9.72)*</td>
<td>1.83 (±0.99)*</td>
<td>14.50 (±12.00)*</td>
<td>1.55 (±0.30)</td>
</tr>
<tr>
<td></td>
<td>144</td>
<td>1.40 (±0.84)*</td>
<td>15.55 (±7.77)*</td>
<td>1.21 (±0.85)*</td>
<td>6.33 (±2.53)*</td>
<td>1.61 (±0.20)*</td>
</tr>
</tbody>
</table>

\(^a\) COMSTAT analysis was carried out from biofilms grown in triplicate using at least 6 images per replicate.
\(^b\) Time 0 = 144-hr-old biofilms; Time corresponds to the time following the switch to an arabinose-free medium.
* Significantly different from PAO1/pJN105 at respective time point; *p* ≤ 0.05 as determined by ANOVA and SigmaStat.
Table 3. Phosphorylation patterns of ∆sagS biofilms in comparison to ∆bfiS biofilms.

<table>
<thead>
<tr>
<th>Biofilm developmental stage</th>
<th>Detected phosphorylation events per indicated stage in wild type <em>P. aeruginosa</em> PAO1</th>
<th>Detected phosphorylation events in 144 hr mutant biofilms</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>∆bfiS</td>
</tr>
<tr>
<td>Planktonic</td>
<td>22</td>
<td>2</td>
</tr>
<tr>
<td>Motile-sessile switch*</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>Reversible attachment (8 hr)</td>
<td>38</td>
<td>25</td>
</tr>
<tr>
<td>Irreversible attachment (24 hr)</td>
<td>33</td>
<td>11</td>
</tr>
<tr>
<td>Maturation (144 hr)</td>
<td>39</td>
<td>0</td>
</tr>
<tr>
<td>Dispersion (216 hr)</td>
<td>19</td>
<td>0</td>
</tr>
<tr>
<td>Biofilm-specific**</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>Constitutive</td>
<td>26</td>
<td>26</td>
</tr>
</tbody>
</table>

*, present in both planktonic and initially-attached biofilm (8 and 24 hrs) cells (43).

**, present in all surface-attached cells regardless of biofilm stage or age (43).
**Figure 1**

A) Schematic representation of the genetic loci involved in the attachment process.

B) Graph showing the number of attached cells per 400 µm² over time for PAO1 (±pJN105), ∆sagS, and PAO1/pJN-sagS.

C) Bar chart comparing relative attachment to PAO1 (±pJN105) for ∆sagS, ∆sagS/pJN-sagS, and PAO1/pJN-sagS at 1 hr and 24 hrs.

D) Graph depicting absorbance at 570 nm over attachment time (hrs) for PAO1 and ∆sagS.
Figure 3

A. Psl Production (%)

- ΔsagS
- PAO1/pJN-sagS
- WFPA801

Planktonic

B. Psl Production (%)

- ΔsagS
- PAO1/pJN-sagS
- WFPA801

Biofilm

C. pslA expression (%)

- ΔsagS
- PAO1/pJN-sagS
- WFPA801

Planktonic

Biofilm
Figure 4
Figure 5

A

Relative attachment

1 hr
24 hrs

B

Relative RNA levels (%)

rsmA
rsmY
rsmZ

ΔsagS
ΔgacA
ΔhptB
ΔhptBΔsagS
Figure 5, continued

ΔgacA
ΔhptB
ΔhptBsagS
ΔgacAsagS

C

Figure 5, continued
Figure 6

A

B

C

\[ \text{Relative rsmAYZ RNA levels (\%)} \]

\[ \Delta \text{bfiS/pJN105} \quad \Delta \text{bfiS/pJN-bfiS} \quad \Delta \text{bfiS/pJN-sagS} \]

\[ \Delta \text{sagS/pJN-bfiS} \quad \Delta \text{sagS/pJN-bfiR} \]

\[ \text{PAO1/pJN-105} \quad \Delta \text{sagS/pJN-bfiR} \]

\[ \Delta \text{rsmYZsagS} \]

\[ \text{on July 8, 2017 by guest http://jb.asm.org/ Downloaded from} \]
A  Probed for the presence of HA-tagged SagS

B  Probed for the presence of V5/6xHis-tagged BfiR

C  Probed for the presence of V5/6xHis-tagged BfiS in vivo

Figure 7
Figure 8

A. Graph showing the percentage of infected plants over time post-infection for different strains: PA14, ΔsagS, and ΔbfiS.

B. Bar chart showing the percentage of plant death over time post-infection for PA14, ΔsagS, and ΔbfiS.

C. Box plot showing the distribution of plant death for WT, ΔsagS, ΔrmsYZ, ΔrmsYZ ΔsagS, ΔgacA, ΔgacA ΔsagS, ΔhptB, and ΔhptB ΔsagS.
**Figure 9**

Planktonic growth

- SagS
- BfiS
- BfiR

Dephosphorylation

HtpB

Post Attachment/Biofilm Growth

- SagS
- BfiS
- BfiR

Genes required for Biofilm development

HtpB

sRNA regulation

RetS → GacS → GacA

Page dimensions: 612.0x792.0

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