SagS and its unorthodox contributions to *Pseudomonas aeruginosa* biofilm development

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A B S T R A C T

The *Pseudomonas aeruginosa* orphan sensor SagS (PA2824) was initially reported as one of three orphan sensor kinases capable of activating HptB, a component of the HptB signaling pathway that intersects with the Gac/Rsm signaling pathway and fine-tunes *P. aeruginosa* motility and pathogenesis. Since then, this orphan sensor has been reported to be involved in other, unorthodox signaling pathways serving additional functions. The present review is aimed at summarizing the various functions of SagS, with an emphasis on its toggle or dual switch functions, and highlighting the role of SagS as a hub at which the various signaling pathways intersect, to regulate the transition from the planktonic to the sessile mode of growth, as well as the transition of surface-associated cells to a drug tolerant state.

1. Introduction

Pseudomonas aeruginosa is a Gram-negative, facultatively aerobic, rod-shaped bacterium. *P. aeruginosa* may not be a typical pseudomonad in natural environments given its optimum temperature for growth is 37 °C but this bacterium is ubiquitous in moist environments, having been isolated from surface water, soil and vegetation, including vegetables and salads [1,2]. *P. aeruginosa* has simple nutritional needs but can tolerate/is resistant to high concentrations of salts and dyes, weak antiseptics, and many commonly used antibiotics. These properties of *P. aeruginosa* are critical factors for its ecological success, which also help explain the ubiquitous nature of the organism and its prominence as an opportunistic pathogen, capable of causing disease in plants and animals, including humans.

In these various niches, *P. aeruginosa* exists in two main modes of growth, planktonic and as biofilm. During planktonic growth, bacteria appear as single, independent free-floating cells (planktonic), while during the biofilm mode of growth, bacteria are organized in aggregates and surface attached sessile communities encased in a hydrated polymeric matrix of their own synthesis [3–7]. The ability to form biofilms has also been recognized as the dominant mode of bacterial growth in nature and a common trait of various microorganisms, including lower-order eukaryotes [3,4,7,8], likely because the biofilm mode of growth provides protection from environmental challenges and outside threats including antimicrobial compounds and host immune responses [6,9,10]. The mode of growth has additional consequences on the

behaviour of the bacteria. Key characteristics relating to the sessile mode of growth include loss of flagella gene expression, production of biofilm matrix components and increased levels of some virulence determinants [11–13]. Moreover, biofilms demonstrate increased innate resistance to host immune defenses and tolerance to stresses, including starvation, dehydration and antimicrobials. Biofilms have been reported to be 10–1000-times more tolerant to various antibiotics compared to their planktonic counterparts [14–16]. Various factors contribute to the observed tolerance of biofilms including restricted penetration of antimicrobial agents, starvation-induced growth arrest, reduced cellular metabolic and divisional rates, and the distinct biofilm phenotype associated with increased expression of several multidrug efflux pumps and ABC transporters [17–24]. These biofilm characteristics pose severe consequences in industrial and medical settings, with biofilms being refractory to antimicrobial treatment, enabled by low-grade inflammation, and causing chronic state infections [25]. In general, chronic biofilm infections are observed in patients with implants [26,27], patients being immunocompromised or otherwise have a predisposing condition such as cystic fibrosis (CF) causing viscous mucus in their lungs or diabetics or edema impairing the vascularization of the lower limps resulting in chronic wounds [28,29]. Biofilm infections, such as chronic pneumonia in CF patients and ventilator-associated pneumonia, affect 17 million Americans each year, causing an estimated 268,000 deaths 550,000 patients in the USA alone, with over $18 billion in direct costs spent on the treatment of these infections [30].

Although it is now widely accepted that most if not all bacterial

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species form biofilms in a cyclic process, the first biofilm developmental life cycle has been reported for *P. aeruginosa* [31,32]. Biofilm formation of *P. aeruginosa* has been described as a stage-specific process that is initiated by free-living, planktonic bacteria attaching to a surface [31, 32]. Once attached, they multiply and produce biofilm matrix components to form mushroom-like, differentiated, structures called micro-colonies. The biofilm developmental cycle comes full circle when cells disperse from the mature biofilm to restart a planktonic mode of growth [33–35]. It has been reported that *P. aeruginosa* exhibits distinct pattern of protein phosphorylation, one of the well-characterized bacterial regulatory signals, at each stage of biofilm development [36]. Biofilm formation is governed by a sophisticated regulatory network that co-ordinates the temporal alterations in gene expression in response to inter- and intracellular signaling molecules and environmental stimuli as well as contribute to observable phenotypic changes such as differential productions of appendages (flagella and type IV pili) and biofilm polymeric matrix [32,36–46].

In *P. aeruginosa,* several factors have been reported to contribute to the formation of biofilms [47–49]. For example, transition from a planktonic to a surface-associated lifestyle, specifically the transition to the irreversible attachment stage, is regulated by two membrane-bound proteins, the diguanylate cyclase SadC (PA4332) and the phosphodies-terase BifA (PA4367), that inversely regulate the levels of second messenger cyclic-di-GMP (c-di-GMP) [50,51]. The status of this SadC- and BifA-modulated c-di-GMP pool in turn acts as a signal to the downstream constituents, reciprocally impacting Pel polysaccharide production and flagellar functions. Additional factors contributing to the progression toward irreversible attachment include the *sadB*, *pelA*, and *motAB* genes, and components of the CheIV chemotaxis-like cluster [52–55]. Additionally, surface behavior is regulated by the Wsp (wrinkly spreader) chemosensory system, that is homologous to the enteric chemotaxis signal transduction system (Che) [56,57]. The pro-teins involved in the pathway are a methyl-accepting protein (WspA), CheW homologues (WspB and WspD), a CheA homologue (WspE), a diguanylate cyclase response regulator (WspR), a methylesterase response regulator (WspF) and a methyltransferase (WspC), encoded by the *wspABCDEFR* operon (PA3708-3702). The Wsp system responds to mechanical pressure associated with surface growth [58]. Association with surfaces leads to the activation of WspA which in turn promotes the autophosphorylation of WspE. WspE phosphorylates its two response regulators, WspR and WspF. Phosphorylated WspR catalyzes the syn-thesis of c-di-GMP that in turn, induces the production of the exopoly-saccharide Pel and Psl, and thus, enhances attachment. In contrast, phosphorylated WspF acts to reset the system by removing methyl groups from WspA, reducing its ability to activate WspE. The methyl-esterase activity of WspF is opposed by the constitutive methyl-transferase activity of WspC [56]. Another regulatory layer is provided by components of the two-component regulatory systems (TCSs) [48, 59], SagS, BfiRS, BfmRS, and MifRS. These TCSs work in concert to coordinate the sequential progression of biofilm formation by *P. aeruginosa,* with SagS and BfiRS regulating initial attachment, BfmRS biofilm maturation, and MifRS microcolony formation [36,60–63].

Classical TCSs, where a sensor kinase recognizes a signal by modi-fying the phosphorylation state of a cognate response regulator, are generally encoded in operons that include both the sensor kinase and response regulator genes [64]. This supports the co-expression of the corresponding proteins and the formation of an exclusively one-to-one phosphotransfer pair. The genome of *P. aeruginosa* encodes 127 TCS genes, of which 107 are adjacent or closely located each other, while the remainder genes are not adjacent to another TCS gene are referred to as "orphan" TCSs [65]. While BfiRS, BfmRS, and MifRS are classical TCSs, SagS (Surface-attached growth Sensor, PA2824) is an orphan TCS. An interesting aspect of orphan TCSs is that they have been reported to be involved in unorthodox or branched pathways involving more than one partner via multistep phosphorelays (also referred to as "one-to-many" and "many-to-one" phosphotransfer relationships) [64] and as a result,

they participate in cross-communication to modify gene expression and physiological change [65,66].

The findings indicate that SagS is only one of few orphan sensors by *P. aeruginosa* Moreover, SagS also contributes to more than one biofilm-related pathway. Indeed, SagS has been shown to not only contribute to biofilm formation, with inactivation arresting biofilm formation at an early attachment stage [36,61], but also to contribute to two distinct pools of c-di-GMP present in biofilms [46,67], biofilms being rendered tolerant to antimicrobial agents [68], and biofilm formation and drug tolerance in *in vivo* models of infection [69].

Given the orphan sensor kinase SagS' unusual and multifaceted contributions to the *P. aeruginosa* biofilm phenotype, this review will focus on its involvement in unorthodox signaling pathways to enable the transition from the planktonic to the biofilm mode of growth and the switch by biofilm cells to a highly tolerant state. Specifically, we will focus on the phosphorelay by SagS under planktonic conditions with the Gac (global activator of antibiotic and cyanide synthesis) and HptB (histidine phosphotransfer protein B) pathways, the phosphorelay with the TCS BfiSR to modulate c-di-GMP and the small regulatory RNA (sRNA) RsmZ post-attachment to enable biofilm formation, as well as its phosphotransfer-independent role in activating the c-di-GMP responsive transcriptional regulator BrlR (biofilm resistance locus regulator, PA4878) to enable biofilm drug tolerance. Overall, we aim at discussing signal transduction mechanisms by which SagS acts as a regulatory hub for both biofilm development and biofilm tolerance.

1.1. SagS and the Gac system network

The orphan sensor SagS (PA2824) was first described by Hsu et al. [70] as a component of the Gac system network. The major components of the Gac system are GacA and GacS. GacS (LemA, lesion manifestation) was initially identified in *Pseudomonas syringae* in 1992 [71], while GacA (global antibiotic and cyanide control) was identified in *P. fluorescens* CHAO in the same year [72]. Rich et al. [73] provided the first evidence that the orphans GacS (LemA) and GacA form a TCS in *Pseudomonas syringae*, by demonstrating that *gacA* encodes the cognate response regulator for the *lemA* (*gacS*) sensor. The system has since then gained special notoriety in the genus *Pseudomonas* due to its involvement in bacterial virulence, by regulating the reversible transition from acute to chronic infections by the opportunistic pathogen *P. aeruginosa* [74,75]. Acute virulence is associated with twitching and swimming motility, expression of a type III secretion system (T3SS), and the absence of alginate, Psl, or Pel polysaccharide production. Traits associated with chronic infection include growth as a biofilm, reduced motility, and expression of a type VI secretion system (T6SS). In *P. aeruginosa,* the Gac system consists at its core of a transmembrane sensor kinase, GacS (LemA [PA0928]), and its cognate regulator, GacA (PA2586) [73] (Fig. 1). Upon receiving a signal, GacS phosphorylates its cognate response regulator GacA which in turn upregulates the expression of the sRNAs RsmZ (PA3621.1) and RsmY (PA0527.1). Two additional sRNAs have been reported as RsmV and RsmW of which expression is Gac-independent [76,77]. RsmY, RsmZ, RsmV and RsmW sRNAs bind to sRNA-binding proteins of the CsrA family, namely RsmA and RsmF, with sRNA binding post-transcriptionally regulating and fine-tune gene RsmA/RsmF-dependent expression [78] (Fig. 1). For example, at low sRNA concentrations, RsmA and RsmF bind to specific mRNA targets and alter their stability by preventing translation of mRNA transcripts required for the establishment of chronic infections and blocking accessibility to the ribosome-binding site, thus enhancing the expression of genes encoding several acute virulence factors and those involved in bacterial motility while repressing the production of virulence factors associated with chronic infections [79,80]. At high sRNA concentra-tions, sRNAs bind to sRNA-binding proteins, by functioning as decoys to sequester CsrA-family proteins from target mRNAs, and thus, release the repression of virulence factors associated with chronic infections but repress the production of acute infection-associated factors. Therefore,
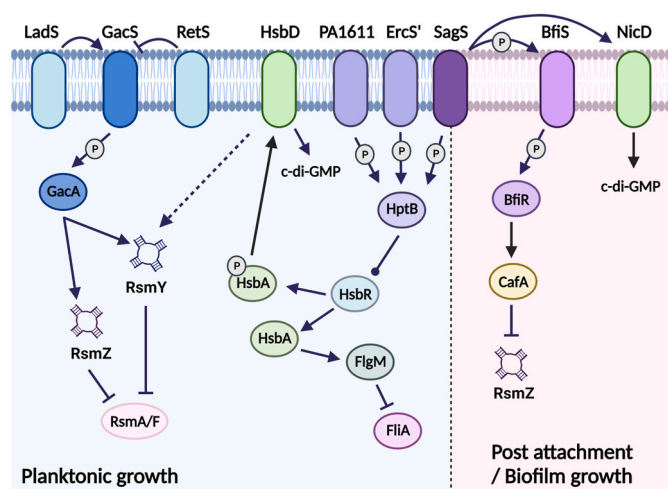
Fig. 1. Schematic representation of the signal transduction system in which SagS participates to modulate biofilm development of *P. aeruginosa*. Under planktonic conditions, SagS regulates small regulatory RNA (sRNA) levels in an HptB-dependent manner. HptB and GacAS contribute to the regulation of the sRNAs, RsmY and both RsmY and RsmZ, respectively [70,86]. These sRNAs sequester the sRNA-binding proteins RsmA and RsmF from target mRNAs [78, 79]. Upon transition to surface associated growth (post-attachment and biofilm growth), SagS promotes biofilm formation via hierarchical phosphotransfer to BfiSR [61], which in turn contributes to the suppression of the sRNA RsmZ level [60]. Arrows and blunt-ended lines indicate stimulatory interactions and inhibitory interactions, respectively. Bulb-ended lines show interactions that can differ depending on conditions. P, protein phosphorylation or phosphotransfer reaction. The image was created using biorender.com.

sequestered RsmA/RsmF has been linked to the expression of genes involved in biofilm formation but the repression of genes involved in acute virulence and motility [79–81].

Two additional transmembrane sensor kinases, LadS (PA3974) and RetS (PA4856), reciprocally modulate gene expression via GacA [82] (Fig. 1). LadS (lost adherence sensor) works in parallel with GacS in response to calcium [38,75,83], while RetS (regulator of exopolysaccharide and type III secretion) hinders GacS/GacA signaling by controlling GacA in an opposite manner to GacS and LadS, thus blocking RsmY and RsmZ production and thus, promoting acute infection and repressing the expression of genes associated with biofilm production [82] (Fig. 1). The role of RetS is supported by the finding of a *P. aeruginosa retS* mutant being unable to establish an infection in a mouse model of acute pneumonia relative to its parental strain [74] (Fig. 1).

An additional component of the Gac-Rsm system is the HptB pathway (Fig. 1). The pathway directly modulates flagellar gene expression as well as fine-tunes sRNA levels, by intersecting with the Gac-Rsm system. The pathway is composed of the histidine phosphotransfer protein HptB (PA3345), the response regulator HsbR (PA3346), the anti-anti sigma factor HsbA, and the diguanylate cyclase HsbD. When HptB is phosphorylated, it activates the phosphatase domain of HsbR, which in turn dephosphorylates the anti-anti-sigma factor HsbA [70,84]. Dephosphorylated HsbA sequesters the anti-sigma factor FlgM from the sigma factor FliA, with free FliA inducing flagellar gene expression and thereby motility. In contrast, when HptB is not phosphorylated, HsbR functions as a kinase, phosphorylates HsbA, which in turn interacts with the diguanylate cyclase HsbD [85]. The switch in HsbA interaction partners from FlgM to HsbD coincides with increased levels of c-di-GMP, decreased swarming motility and increased of biofilm formation. In addition, phosphorylated HptB antagonizes GacA in a manner similar to RetS, likely via phosphorylated HsbA. However, while RetS affects the expression of both RsmY and RsmZ, HptB exclusively regulates RsmY expression [86] (Fig. 1). Input by HptB thus enables finetuning of sRNA

levels and thus, biofilm formation, twitching, swimming, swarming motility and chemotaxis in *P. aeruginosa* in response to environmental cues. These inputs are provided by three orphan sensor kinase hybrids, namely PA1611, PA1976 (ErcS), and SagS, capable of activating HptB [70,87] (Fig. 1). Therefore, SagS indirectly contributes to the reversible transition from acute to chronic infections and the switch from the planktonic to the sessile lifestyle, by finetuning RsmY and RsmZ levels and the modulation of c-di-GMP levels in *P. aeruginosa*.

1.2. SagS is part of a dedicated signaling pathway to regulate biofilm development in a stage-specific manner

Biofilms by *P. aeruginosa* display multiple phenotypes with distinct physiological characteristics (e.g. 3D-biofilm structure, cell-cell communication, and metabolic changes) that can be correlated to distinct episodes or stages of biofilm development [31]. These stages were referred to as reversible and irreversible attachment, maturation, and dispersion (Fig. 2). Each biofilm developmental stage corresponds to unique patterns of protein production and gene expression [31,32]. Several regulators have been identified to affect and contribute to biofilm formation [50,51,56,57]. However, only one regulatory signaling network has been identified that links the regulation of biofilm formation to committed biofilm developmental steps by *P. aeruginosa*. This signaling network is composed of several TCSs named BfiSR, BfmRS, and MifRS that are sequentially activated/phosphorylated (BfiSR<BfmSR<MifSR) over the course of biofilm formation [36]. This is supported by the finding that inactivation of either TCSs arrested biofilm formation at distinct developmental stages. For example, ΔbfiS biofilms have been demonstrated to be arrested at the irreversible attachment stage, while biofilms formed by ΔbfmR and ΔmifR were found to be arrested at the maturation-1 and -2 stages of biofilm development, respectively [36,60–63]. An additional component of this signaling network is the orphan sensor SagS. Moreover, similar to ΔbfiS, ΔsagS biofilms have been demonstrated to be arrested at the irreversible attachment stage [61]. Several lines of evidence suggest that SagS is functioning prior to BfiSR to enable biofilm formation (Fig. 2). For one, t SagS activates BfiS [61]. Secondly, multicopy expression of *bfiR* restored biofilm formation by ΔsagS to wild-type levels, but not *vice versa* [61]. Additionally, despite *P. aeruginosa* mutant strains ΔsagS and ΔbfiS forming impaired biofilms [36,60,61], phosphoproteome analysis indicated ΔsagS biofilms to be arrested prior to ΔbfiS biofilms, at the transition from the planktonic to the initial attachment stage [61]. And lastly, biofilms by ΔsagS and ΔbfiS differed in c-di-GMP levels. Biofilms formed by ΔbfiS harbored wild-type levels of c-di-GMP, approximately 75–78 pmol/mg c-di-GMP, while biofilms formed by ΔsagS exhibited significantly reduced c-di-GMP levels when compared to wild-type biofilms. On average, ΔsagS biofilms only harbored 33 ± 2 pmol/mg c-di-GMP [88,89]. Despite these low levels, ΔsagS biofilms harbor 2–3 times higher c-di-GMP levels relative to planktonic cells [88,89]. Recent findings suggest SagS to likely affect two diguanylate cyclases, PA3177 and NicD (Nutrient-induced cyclase D). SagS interacts with the membrane bound diguanylate cyclase NicD(45) (Fig. 2). Although evidence is lacking that NicD functions in a SagS-dependent manner, it is likely that NicD activity contributes to the c-di-GMP levels present in biofilms. However, inactivation of *nicD* has been shown to coincide with biofilms being similar in architecture to ΔsagS [90]. PA3177 will be discussed below.

It is thus likely that SagS contributes to the cellular c-di-GMP pool of biofilm cells in two different ways, upon the transition to the surface via the HptB pathway (Fig. 1), and post-attachment via NicD (Fig. 2) and PA3177. Additionally, the findings further support SagS to be part of a genetic pathway that regulates the stage-specific biofilm developmental transitions in a hierarchical ordered manner in response to environmental cues, with SagS functioning as an early switch between the planktonic and sessile mode of growth.
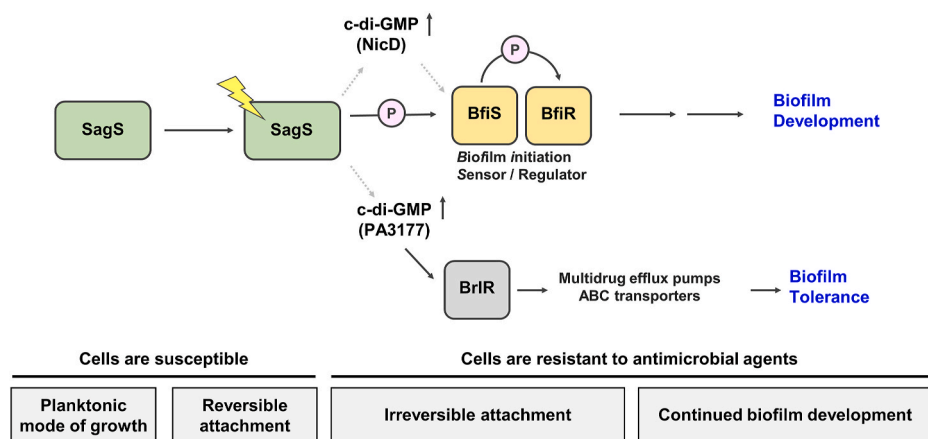
Fig. 2. Contribution of SagS to the motile-sessile and susceptible-resistance switch in *P. aeruginosa*. Transition to the irreversible attachment stage, in which SagS directly interacts with and phosphorylates the TCS BfiSR [60,61], marks the timing when surface-associated cells start to be resistant to antimicrobial agents [68]. SagS regulates the switch from an antimicrobial susceptible to a highly tolerant state via regulation of c-di-GMP levels [88], which are generated by the diguanylate cyclase PA3177 [67], and subsequent activation of BrlR in a phosphotransfer-independent manner [67,68,95]. BrlR, in turn, modulates the expression of multidrug efflux pumps and ABC transporter [12,22]. Gray arrows with dashed lines indicate unidentified mechanism(s).

1.3. SagS affects RsmZ levels in biofilms via BfiSR in a manner independent of HptB

Given that SagS affects the cellular level of c-di-GMP upon surface associated growth, it is not surprising that SagS not only contributes to biofilm formation but also the maintenance of the mature biofilm architecture, as loss of *sagS* expression in mature *P. aeruginosa* biofilms coincided with the collapse of the biofilm architecture [46,60,61,91]. But c-di-GMP is not the only reason for SagS contributing to biofilm formation, as SagS also affects biofilm formation in a manner dependent to sRNAs. In planktonic cells, SagS suppresses RsmY and RsmZ sRNAs accumulation and sequestration of RsmA in an HptB-dependent manner (Fig. 1), with inactivation of *sagS* resulting in an HptB-dependent increase in RsmY and RsmZ expression, increased Psl polysaccharide production, and increased virulence in planktonic cells. Under biofilm growth conditions, however, SagS suppresses sRNA accumulation in an HptB-dependent manner [61] (Fig. 1). Instead, RsmZ levels were found to be dependent on the SagS interaction partners BfiSR (biofilm initiation sensor/regulator) [61]. Post-attachment, SagS phosphorylates the transmembrane sensory protein BfiS which in turn phosphorylates its cognate response regulator BfiR (Figs. 1–2). Phosphorylated BfiR then activates the expression of *cafA* encoding ribonuclease G [60] capable of selectively degrading RsmZ. Activation of BfiR thus effectively results in the reduction of RsmZ levels, without affecting RsmY, under biofilm growth conditions (Fig. 1).

The findings suggested SagS to indirectly affect RsmZ and RsmY levels via the HptB signaling pathway under planktonic growth conditions, while upon surface associated growth, SagS was found to contribute to the modulation of sRNA levels in a manner independent of HptB, but dependent on BfiSR (Fig. 1). Thus, SagS acts as a switch by linking the GacA-dependent sensory system under planktonic conditions to the suppression of sRNAs, particularly RsmZ, post-attachment.

1.4. Antimicrobial tolerance requires SagS

Biofilms have been reported to be difficult to eradicate by conventional antibiotic treatment [3,15,92] This biofilm drug tolerance has been reported to be distinct from mechanisms commonly associated with planktonic cells, such as the acquisition of resistance via uptake of plasmid-borne resistance markers or random mutation and be multifactorial as requiring a combination of several mechanisms [15,19,93]. Instead, biofilm drug tolerance has been reported to be multifactorial, and based on starvation-induced growth arrest, reduced metabolic rates, increased stress tolerance, reduced diffusion rates, and the presence of extracellular polymeric matrices [17–24].

In an effort to understand the timing of induction of this biofilm drug tolerance, Gupta et al. [68] determined when over the course of their

development *P. aeruginosa* biofilms gain their tolerance to antimicrobial agents. The study made use of wild-type cells grown to the reversible, irreversible, early and late maturation biofilm developmental stages under flowing conditions. Additionally, the study made use of mutant strains (Δ*sagS*, Δ*bfiS* and Δ*bfmR*) that had previously been demonstrated to be arrested at distinct stages of biofilm development. Specifically, Δ*sagS* has been reported to be arrested at the transition from the reversible to the irreversible attachment stage, Δ*bfiS* at the irreversible attachment stage, and Δ*bfmR* at the biofilm maturation [36,60–63]. Relative to wild-type biofilms, Δ*sagS* biofilms were significantly more susceptible to various antimicrobial agents when exposed for 1 h compared to wild-type biofilms. Moreover, biofilms by Δ*sagS* were fully eradicated when exposed for 24 h to tobramycin concentrations exceeding 100 ug/ml while biofilms by *P. aeruginosa* wild-type and Δ*bfiS* remained viable at tobramycin concentrations exceeding 400 ug/ml [46,68,88]. The findings suggested that in *P. aeruginosa,* transition to the irreversible attachment stage, regulated by the orphan sensor SagS, marks the timing when biofilms switch to the high-level tolerance phenotype [68]. The findings furthermore suggested that additional factors than biofilm biomass accumulation contribute to biofilm drug tolerance. This was supported by the findings of biofilms formed by Δ*sagS* and Δ*bfiS* differing in drug tolerance despite featuring similar biofilm architectural features [68]. One of such factors is SagS. Several lines of evidence suggest SagS-dependent biofilm drug tolerance is linked to c-di-GMP and the c-di-GMP responsive transcriptional regulator BrlR (Fig. 2). BrlR has been found to contribute to the high-level antimicrobial tolerance of *P. aeruginosa* biofilms by activating the expression of the multidrug efflux pump operons, *mexAB-oprM* and *mexEF-oprN*, several ABC transport systems including PA1874-77, and by repressing the expression of the *oprH-phoPQ* operon [12,22,94–96]. For one, expression of *brlR* is SagS-dependent [68]. Little is known how *brlR* expression is regulated. However, inactivation of *sagS* coincided with decreased levels of BrlR production, with BrlR derived from Δ*sagS* biofilm cells being impaired in binding to the *brlR* promoter and promoters of the multidrug efflux pump operons, *mexAB-oprM* and *mexEF-oprN* [68]. Restoring c-di-GMP to wild-type biofilm-like levels in Δ*sagS* biofilms restored *brlR* expression, DNA binding by BrlR, and recalcitrance to killing by antimicrobial agents of Δ*sagS* biofilm cells [88]. In agreement with c-di-GMP levels contributing to drug tolerance, Gupta et al. [88] demonstrated that reducing cellular c-di-GMP levels of biofilm cells to ≤40 pmol/mg correlated with increased susceptibility and reduced *brlR* expression. Conversely, the authors demonstrated that planktonic cells are rendered significantly more resistant to antimicrobial agents upon increasing c-di-GMP levels present in planktonic cells to biofilm-like levels (≥55 pmol/mg), with increased resistance correlating with increased *brlR*, *mexA*, and *mexE* expression and BrlR production. It is of interest to note that Δ*sagS* biofilms harbored on average 33 ± 2

pmol/mg c-di-GMP [88,89].

Together, these data led to suggest that SagS directly or indirectly modulates c-di-GMP levels to activate BrlR and thus, biofilm drug tolerance (Fig. 2). Moreover, the findings suggested the presence of a SagS-dependent c-di-GMP pool that contributes to the resistance of *P. aeruginosa* biofilms. The c-di-GMP modulating enzyme to provide the necessary pool of c-di-GMP was identified as PA3177 encoding an active diguanylate cyclase (Fig. 2). PA3177 was found to contribute to the cellular c-di-GMP levels in biofilms cells, but not planktonic cells [67]. Moreover, inactivation of PA3177 did not affect biofilm formation but rendered ΔPA3177 mutant biofilms susceptible to antimicrobial agents such as tobramycin and hydrogen peroxide. Similar to Δ*sagS* biofilms, biofilms formed by ΔPA3177 demonstrated reduced BrlR abundance, with ΔPA3177 biofilm cell-derived BrlR unable to bind its target promoters [67]. The findings suggested the presence of a local pool of PA3177-generated c-di-GMP necessary to modulate BrlR activity and in turn, biofilm drug tolerance. Although *P. aeruginosa* biofilm tolerance is linked to PA3177-generated c-di-GMP and BrlR activity, the mechanism (s) of how SagS regulates PA3177 activity still remains to be elucidated. However, the findings strongly suggest SagS to induce the switch of attached cells to transition from a susceptible to a tolerant phenotype.

Given the link between SagS and BrlR, induction of *brlR* gene expression has been used to determine the timing of SagS switch function. Using a *brlR* promoter reporter, Chambers et al. [95] demonstrated *brlR* expression to be detectable under flowing conditions as early as 6 h post initial attachment. Activation of *brlR* expression as early as 6 h post attachment is in agreement with SagS functioning as a switch to regulate the transition to the irreversible attachment stage under flowing conditions. These data suggest that SagS has dual switch functions, regulating the transition from the planktonic to the sessile mode of growth as well as the transition to a heightened drug tolerant phenotype (Fig. 2).

1.5. Role of distinct amino acid residues and domains in the dual switch functions of SagS

SagS regulates both, (i) the transition to biofilm formation and (ii) the transition to cells gaining their enhanced tolerance to antimicrobials. The two functions are associated with distinct pathways or interactions, including the HptB pathway, the interactions with BfiSR, and the modulation with BrlR (Figs. 1 and 2). Much research has focused on elucidating the role of each of the SagS domains in order to determine how SagS carries out its multiple functions [46,91]. SagS harbors multiple domains [46,91] (Fig. 3). Its N-terminus is composed of a periplasmic HmsP sensory domain, flanked by transmembrane helices, followed by a cytoplasmic portion composed of a HAMP (Histidine kinases, Adenylyl cyclases, Methyl binding proteins, Phosphatases) domain, a histidine kinase (HisKA) domain, and a C-terminal receiver (Rec) domain [46,91] (Fig. 3). SagS activation leads to auto-phosphorylation at the histidine residue H315 at the HisKA domain, and subsequent phosphoryl-group transfer to the aspartate residue D713 located in the Rec domain (Fig. 3). The HmsP domain of SagS is homologous to the N-terminal domains of the c-di-GMP phosphodiesterases BifA and HmsP in *P. aeruginosa* and *Yersinia pestis*, respectively [50,97,98]. As sensory domains, HmsP is a likely control point for SagS switch functions. Indeed, a mutant analysis revealed 32 residues located within the HmsP domain that only contribute to blocking one of the two SagS switch functions [91]. Specifically, the study revealed amino acid residue mutations affecting only attachment and subsequent biofilm formation but not biofilm tolerance. The same residues impaired histidine kinase signaling via BfiS. In turn, phosphorylated BfiS activates its cognate response regulator BfiR to transcribe CafA to modulate RsmZ levels [46,61]. These included residues F131 and L154 [91] (Fig. 3). In contrast, residues affecting biofilm drug tolerance, but not attachment and subsequent biofilm formation, negatively impacted BrlR transcription factor levels. These included residues D105, R116 and L126 [91] (Fig. 3). Structural prediction suggested the two sets of residues affecting sensory functions to be located in distinct areas that were previously described to be involved in ligand binding interactions [91].

The role of two select amino acid resides, L154 and D105, was analyzed using a mouse models of chronic pneumonia [69]. Relative to wild-type *P. aeruginosa*, Δ*sagS* mutants demonstrated significantly reduced bacterial colonization and increased susceptibility to the antibiotic tobramycin. Recombinant *P. aeruginosa* Δ*sagS* strains chromosomally expressing the SagS variant SagS_L154A showed reduced bacterial loads in the chronic pneumonia model but were as susceptible as wild-type biofilms. In contrast, interference with the D105 residue had no effect on bacterial colonization but enhanced the susceptibility of *P. aeruginosa* biofilms during tobramycin treatment [69]. The findings supported the notion that interference with the distinct regulatory circuits of SagS affected *P. aeruginosa* pathogenicity and drug tolerance independently in chronic infections in a manner similar to *in vitro* conditions. Additionally, the findings indicated SagS to affect two distinct regulatory pathways, with division of signal relay occurring via the HmsP domain.

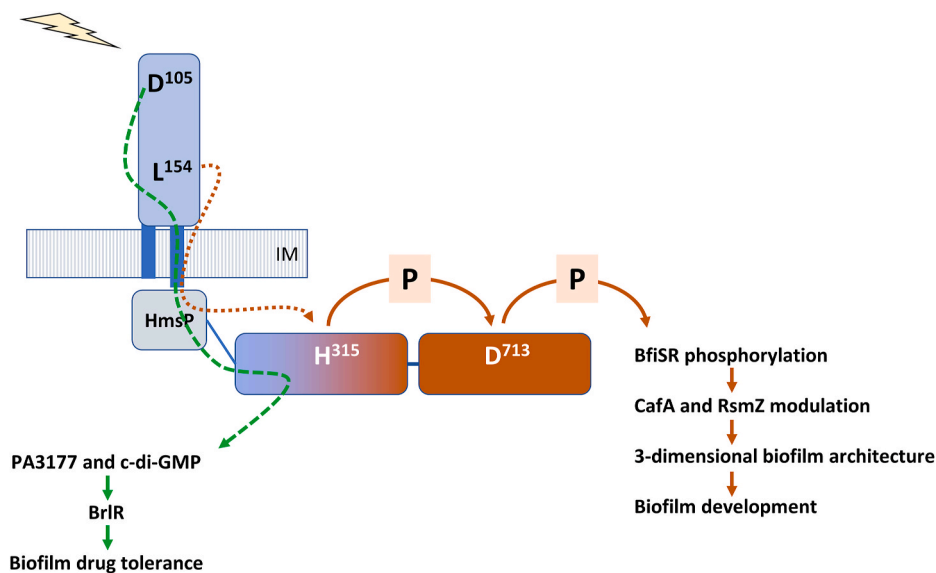Overall, the findings suggested that the periplasmic HmsP domain of

Fig. 3. Domain-dependent modular functions of SagS. Upon sensing signal or cue such as glucose-6-phosphate [45], a signal is transduced from the periplasmic HmsP sensory domain of SagS to its HisKA domain via HAMP domain, followed by phosphotransfer between the histidine residue H315 in the HisKA domain and the aspartate residue D713 in the Rec domain, resulting in subsequent phosphorylation of BfiSR and biofilm development [46]. Residue L154 of HmsP domain contributes to biofilm development via signal cascade from SagS to BfiSR [91]. In contrast, HmsP-HisKA domain of SagS, in which residue D105 of HmsP domain is involved, contributes to biofilm tolerance via regulation of c-di-GMP levels and activation of BrlR [46,91]. Arrows indicate modulatory effect of domains. P, protein phosphorylation or phosphotransfer reaction.

SagS to be a control point in the regulation of biofilm formation and biofilm cells transitioning to a drug-tolerant state. However, if the HmsP domain is central, the distinct regulatory pathways that are activated by SagS, are likely enabled via the modular composition of SagS [46] (Fig. 3). Using SagS constructs lacking one or more domains, Petrova et al. [46] demonstrated that activation of the biofilm formation pathway via BfiSR only requires the HisKA-Rec domain and its His^{315}-Asp^{713}-phosphorelay. Moreover, the two domains were sufficient to restore c-di-GMP levels and biofilm formation by ∆*sagS* to wild-type levels [46] (Fig. 3). In contrast, regulation of the antimicrobial tolerant state was independent of the HisKA-Rec mediated His^{315}-Asp^{713}-phosphorelay. Instead, only the HmsP-HisKA domains were found to be necessary to restore drug tolerance by ∆*sagS* biofilms [46] (Fig. 3). Expression of the HmsP-HisKA domains coincided with restoration of *brlR* expression, BrlR activation, and c-di-GMP to wild-type levels [46]. Interestingly, however, while expression of both SagS domain constructs, HisKA-Rec and HmsP-HisKA, restored c-di-GMP to wild-type levels, only HmsP-HisKA and this variant-derived c-di-GMP rescued the drug tolerance phenotype of ∆*sagS* biofilms. It is likely that the HmsP-HisKA-derived pool of c-di-GMP is generated by the diguanylate cyclase PA3177 [67] (Figs. 2–3). In addition to pointing at the presence of distinct pools of c-di-GMP that serve distinct functions, e. g. to support biofilm formation or enable biofilm drug tolerance, the findings also strongly suggest that biofilm drug tolerance is not a function of biofilm biomass accumulation but instead, that biofilm formation and biofilm drug tolerance are regulated independently (Figs. 2–3).

1.6. Signals and SagS activation

The periplasmic sensory HmsP domain of SagS has been described as the control point of the dual functions of SagS [91]. In addition, several essential amino acid residues contributing to SagS functions were predicted to be located in an areas of the SagS HmsP domain that were previously suggested to be involved in ligand binding interactions [91]. The findings suggested HmsP to also contributes to signal perception (Fig. 3). Using a modified Biolog phenotypic microarray assays, glucose-6-phosphate, one of many host-derive hexose phosphates, has recently been identified as an extracellular signal of SagS [45]. Exposure to glucose-6-phosphate stimulated attachment and biofilm formation in a manner dependent SagS. The increase in surface attached biomass accumulation in response to glucose-6-phosphate coincided with enhanced intracellular c-di-GMP levels by biofilms formed by *P. aeruginosa* wild type and a complemented ∆*sagS* strain. However, no difference in c-di-GMP levels was noted in biofilms formed by ∆*sagS* in the absence or presence of glucose-6-phosphate. The findings indicated SagS modulates the c-di-GMP pool in response to glucose-6-phosphate under biofilm growth conditions. Specifically, SagS was found to likely modulates cellular c-di-GMP levels in a manner dependent on the diguanylate cyclase NicD [45]. While NicD has been previously reported to directly perceive sugars and amino acids such as glutamate, with cue perception resulting in NicD dephosphorylation and increased diguanylate cyclase activity [90], modulation of c-di-GMP levels in response to glucose-6-phosphate was independent of NicD sensing [45]. Instead, NicD activity appeared to be dependent on its interaction with SagS [45]. While these findings indicate that SagS-mediated biofilm formation and c-di-GMP regulation are stimulated by glucose-6-phosphate, it remains to be elucidated whether glucose-6-phosphate directly binds to the sensory domain HmsP of SagS, whether other signals and cues are perceived by SagS, and how SagS modulates NicD (or other diguanylate cyclases) upon signal perception to affect the cellular levels of c-di-GMP present in biofilms.

2. Conclusion and future work

The transition from a planktonic to a sessile lifestyle is regulated by multiple overlapping signaling system. A key player in this transition is

SagS. In this review, we highlighted the roles of SagS in three distinct pathways under planktonic and surface associated growth conditions. A summary of these functions with SagS functioning as a regulatory hub connecting these multiple overlapping signaling system is shown in Fig. 4. Specifically, we focused on the role of SagS under planktonic conditions in which SagS functions as part of the Gac-Rsm and HptB pathways to modulate RsmY and RsmZ sRNAs, c-di-GMP and thus, modulation of virulence motility, polysaccharide production. Thus, in addition to regulates expression of genes encoding several acute virulence factors and those involved in bacterial motility, the system prepares *P. aeruginosa* for the transition from the planktonic to the surface associated mode of growth (Fig. 4). Transition to the surface associated lifestyle induces a switch in SagS, with SagS ceasing to function within the Gac-Rsm and HptB pathways but instead interacting with the TCS BfiSR. This switch leads to SagS partly countering the Gac-Rsm/HptB-dependent sRNA modulation, by suppressing the sRNA RsmZ post-attachment to enable continued biofilm development post-attachment (Fig. 4). SagS-mediated RsmZ degradation and subsequent biofilm formation depends on the phosphotransfer functions of SagS as well as c-di-GMP regulation [46,60,61]. Additionally, the transition coincides with a switch from acute virulence factors to the production of virulence factors associated with chronic infections.

But this is not the only switch that occurs post-attachment. SagS furthermore contributes to the transition of surface-associated cells to a drug tolerant state (Fig. 4). Activation of biofilm drug tolerance is independent of the phosphotransfer functions of SagS and involves the coordination of a distinct pool of c-di-GMP, likely derived by the diguanylate cyclase PA3177, and the c-di-GMP responsive transcriptional regulator BrlR [67,68,88] (Fig. 4). Activation of biofilm-associated antimicrobial tolerance by SagS contributes to the drug tolerance of chronic *P. aeruginosa* infections [46,69,91]. While the modulation of c-di-GMP levels upon the transition to the surface-associated growth are likely derived by HptB and other c-di-GMP modulating pathways, subsequently increased c-di-GMP levels detected in *P. aeruginosa* cells post attachment are dependent on SagS. In fact, SagS contributes to two distinct pools of c-di-GMP, one required for biofilm formation, and what appears to be a local PA3177-derived c-di-GMP pool necessary for the activation of biofilm drug tolerance.

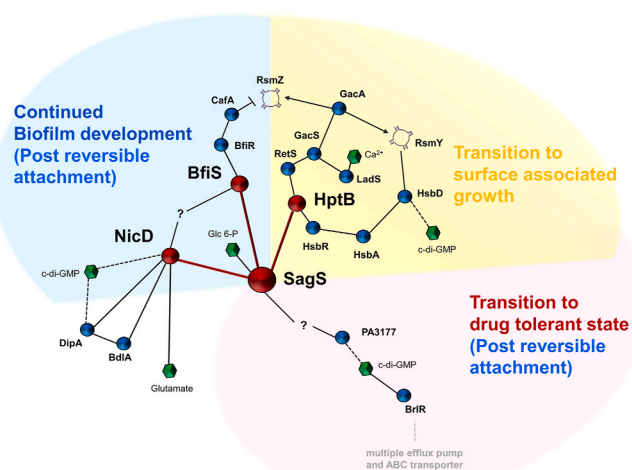SagS contributing to distinct but overlapping signaling system to

Fig. 4. SagS is a hub protein, connecting multiple overlapping signaling system, to enable the transition from a planktonic to a sessile, drug tolerant mode of growth.?, unknown, Arrows and blunt-ended lines indicate stimulatory interactions and inhibitory interactions, respectively. Dashes lines indicate modulation of c-di-GMP levels. Confirmed SagS interaction partners are shown in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

overall affect distinct phenotypes has several implications. For one, SagS functions as a toggle switch to enable the transition from the planktonic to the surface associated mode of growth. The transition coincides with SagS switching from enabling RsmY and RsmZ sRNAs accumulation to the suppression of sRNA RsmZ, and a shift from an acute to a chronic virulence phenotype (Fig. 4). Moreover, SagS is modular in its function, capable of separately regulating biofilm development and biofilm drug tolerance post-attachment (Fig. 4). As interference with the biofilm or tolerance regulatory circuits of SagS affects *P. aeruginosa* pathogenicity in chronic models of infections, the findings furthermore reveal SagS to be a promising new target to treat *P. aeruginosa* biofilm infections.

However, certain gaps in our understanding of SagS remain. For examples, it is not known how SagS switches from interaction with the Gac/Rsm/HptB pathway to initiate a phosphor-signaling relay with BfiSR. It is likely that cues or signals perceived by SagS contribute to this switch. However, so far, only glucose-6-phosphate has been identified as a signal capable of activating SagS [45]. It is of interest to note that the same study also identified several other compounds that appeared to suppress SagS and biofilm formation [45]. While the study did not elucidate whether these compounds shift SagS from interacting with BfiSR to affecting the Gac/Rsm/HptB pathway, it is thus likely that an as of yet unknown compound may contribute to SagS toggling between its pre- and post-attachment functions (Fig. 4). Likewise, the specific mechanism of how SagS modulates the diguanylate cyclases PA3177 and NicD to stimulate distinct c-di-GMP pools is not known. As NicD activity has previously been shown to be modulated upon phosphorylation, it is possible that SagS affects the c-di-GMP pool via protein-protein interactions and subsequent phosphotransfer. However, direct evidence is lacking. Likewise, it is unclear whether additional proteins interact with SagS to regulate its hub functions and/or the two distinct biofilm phenotypic switches.

Declaration of competing interest

Acknowledgments

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