RetS inhibits *Pseudomonas aeruginosa* biofilm formation by disrupting the canonical histidine kinase dimerization interface of GacS

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Bacterial signaling histidine kinases (HKs) have long been postulated to function exclusively through linear signal transduction chains. However, several HKs have recently been shown to form complex multikinase networks (MKNs). The most prominent MKN, involving the enzymes RetS and GacS, controls the switch between the motile and biofilm lifestyles in the pathogenic bacterium *Pseudomonas aeruginosa*. While GacS promotes biofilm formation, RetS counteracts GacS using three distinct mechanisms. Two are dephosphorylating mechanisms. The third, a direct binding between the RetS and GacS HK regions, blocks GacS autophosphorylation. Focusing on the third mechanism, we determined the crystal structure of a cocomplex between the HK region of RetS and the dimerization domain of GacS. This is the first reported structure of a complex between two distinct bacterial signaling HKs. In the complex, the canonical HK homodimerization interface is replaced by a strikingly similar heterodimeric interface between RetS and GacS. We further demonstrate that GacS autophosphorylates in trans, thus explaining why the formation of a RetS-GacS complex inhibits GacS autophosphorylation. Using mutational analysis in conjunction with bacterial two-hybrid and biofilm assays, we not only corroborate the biological role of the observed RetS-GacS interactions, but also identify a residue critical for the equilibrium between the RetS-GacS complex and the respective RetS and GacS homodimers. Collectively, our findings suggest that RetS and GacS form a domain-swapped hetero-oligomer during the planktonic growth phase of *P. aeruginosa* before unknown signals cause its dissociation and a relief of GacS inhibition to promote biofilm formation.

Sensor histidine kinase (HK)-linked signal transduction systems are the primary means whereby bacteria sense extracellular signals to shape an adaptive response (1–3). The classic two-component signaling system consists of autophosphorylation of the HK followed by phosphate transfer to a cognate response regulator (RR). In the closely related phosphorelay systems, there are two additional transfer steps. Here, the phosphate moves from the HK region to a receiver domain with no coupled output domain, then to a histidine phosphotransfer (HPt) protein, and from there finally to an RR (4, 5). The additional phosphotransfers allow for finer-tuned output regulation (2, 6). Hybrid signaling HKs contain a sensory domain, HK region, a receiver domain, and an HPt domain within a single polypeptide chain (5). Because the tethering of the HK region to the receiver domain confers specificity to the associated phosphotransfer step, the otherwise stringent evolutionary requirement for HK-RR complementarity is more relaxed in hybrid HKs (7). Cross talk between distinct phosphorelay chains was long thought to be undesirable and therefore forbidden (8–10). However, mounting evidence suggests the presence of intricately webbed multikinase networks (MKNs) (11). At this point, we have gained a reasonably clear understanding of how the linear phosphotransfer events are facilitated within a single relay, and we have only a cursory understanding of how such cross talk occurs and is regulated. To date, the best studied example of interactions within an MKN is perhaps the multilayered interplay between the HK family enzymes RetS and GacS in the opportunistic pathogen *Pseudomonas aeruginosa*. The hybrid HK GacS and its cognate RR GacA sit at the heart of the Gac/Rsm signal transduction pathway (12). This pathway allows *P. aeruginosa* to switch between a motile, invasive lifestyle—which causes an acute infection in a human host—and a sessile, biofilm-associated lifestyle—which often results in a chronic infection in a human host (13, 14). Once phosphorylated, GacA acts as a transcriptional activator, indirectly upregulating genes associated with the sessile biofilm lifestyle (15–17). Conversely, GacA indirectly downregulates genes associated with a motile, invasive lifestyle, such as the expression of flagella-mediated motility-related genes and Type Three Secretion System–related genes necessary for producing the observed cytotoxic effects in an acute infection (15, 16).

GacS is reciprocally regulated by two HK family proteins, LadS and RetS (11). LadS enhances the phosphotransfer activity of GacS via phosphorylation of the HPt domain of GacS (18, 19). RetS, on the other hand, inhibits GacS via three distinct mechanisms (summarized in Fig. S1) (11, 12, 20). RetS has an unusual architecture consisting of a periplasmic sensor domain, an HK region, and two receiver domains. RetS uses its...

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Crystal structure of a RetS-GacS complex

HK region and C-terminal receiver domain to siphon phosphate groups from the receiver and DHp domains of GacS, respectively (11, 20). Mediated by direct interactions between the HK regions of the two enzymes, RetS also interferes with the initial autophosphorylation of GacS (11, 12, 21). This mode of inhibition is not well understood and is the focus of the present study. Initial models suggested that RetS might form a heterodimeric complex with GacS (12). However, our recent work demonstrated that the GacS dimer remains intact upon RetS binding (21), suggesting the formation of a larger heteromeric assembly, perhaps a tetramer (21). In the same study we also demonstrated that a structurally dynamic region of the RetS DHp domain is important for GacS binding and might be involved in regulating the interaction.

In the present study, we report the crystal structure of a complex between the RetS HK region and the GacS DHp domain. The RetS-GacS interface closely resembles the canonical interface in homodimeric enzymes. Consistent with the proposed role of helix cracking in the regulation of the interaction, the structurally dynamic helix of RetS DHp is fully formed and involved in GacS binding. We experimentally determined that GacS auto-phosphorylates in trans. Thus, the RetS<sub>HK</sub>-GacS<sub>DHp</sub> structure also answers the question how RetS prevents GacS autophosphorylation, because RetS binding disrupts the spatial arrangements needed for trans-autophosphorylation.

Results

RetS and GacS form a DHp-DHp interface that closely resembles the dimerization interface in canonical signaling histidine kinases

Cocrystallization of the HK region of RetS (RetS<sub>HK</sub>, amino acid residues 413–649) and the DHp domain of GacS (GacS<sub>DHp</sub>, amino acid residues 270–349) yielded crystals that gave X-ray diffraction data up to 2.3 Å resolution using a CC<sub>1/2</sub> threshold of 0.3 as cutoff (data collection and structure refinement statistics are provided in Table 1) (22). The structure was solved via molecular replacement using a single molecule of the previously solved RetS<sub>HK</sub> homodimer (21). The complex consists of a 1:1 heterodimer wherein the DHp domain of RetS and GacS forms an extensive interface (Fig. 1). The final model contains residues 414–573, 604–639 of RetS and GacS residues 285–344. RetS residues 413, 574–603, and 640–649 appear to be structurally dynamic in the complex because no electron density was observed for these sections of the molecule. Similarly, there was no interpretable electron density for GacS residues 270–284 and 345–349. RetS<sub>HK</sub> consists of a CA and a DHp domain. The overall folds of the individual domains mirror those observed in the crystal structure of the RetS<sub>HK</sub> homodimer (21). The RetS-DHp domain assumes the canonical helix-loop-helix fold wherein the conserved histidine residue H424 is located on the α1 helix and solvent-exposed. The RetS-CA domain assumes the expected α/β sandwich fold comprising the α3–α4 helices, the α7 helix, and strands β1–β7. The residues that formed helices α5 and α6 helices in the RetS<sub>HK</sub> homodimer show no electron density in the RetS-GacS complex (residues 574–603) (Fig. S2). The DHp domain of GacS forms the anticipated helix-loop-helix structure. The conserved catalytic histidine residue H293 is also solvent-exposed.

The DHp domains of RetS and GacS form a four-helix bundle, closely resembling the canonical interface observed in homodimeric bacterial HKs (1, 2, 23) (Fig. 2A). The same section of RetS<sub>HK</sub> also partakes in RetS homodimerization or, in the case of GacS, would also be predicted to form the binding surface in a GacS homodimer. Altogether 25 GacS residues correspond to a surface area of 1351 Å<sup>2</sup> and 23 RetS residues that cover 1348 Å<sup>2</sup> of surface area form the extensive interface. The 89 nonbonded contacts are largely hydrophobic, containing only four hydrogen bonds (Fig. 2B).

In HKs the α1 helix is kinked N-terminal at highly conserved threonine and proline residues. The kinking provides the plasticity needed for autophosphorylation, phosphotransfer, and phosphatase activity (24). This kink is also observable N-terminal to Thr<sub>428</sub> and Pro<sub>429</sub> of RetS and N-terminal to Thr<sub>297</sub> of GacS (Fig. 3A). The kink angle for GacS is 28.7º and the angle for RetS is 22.8º (Fig. 3A). In the RetS<sub>HK</sub> homodimer, the corresponding section is unfolded in one molecule and displays a kink angle of 33.3º in the other molecule (Fig. 3). While RetS is not a functional kinase, the kink does play a role in regulating the equilibrium between a domain-swapped RetS-GacS oligomer and the individual homodimers (21). We previously demonstrated that this section is critical for GacS binding but not RetS homodimerization and predicted that it would be helical in the heteromeric complex (21). Consistent with these predictions, the dynamic N-terminal section of RetS α1 helix is now helical and forms part of the interface in the complex with GacS (Fig. 3B).

GacS binding forces conformational changes in RetS<sub>HK</sub>

In the RetS-GacS complex, RetS<sub>HK</sub> assumes a distinct conformation compared to those observed in the RetS<sub>HK</sub> homodimer. Overall, it closely resembles Chain A (PDB code 6dk7) with an RMSD of 0.359 Å between the DHp domains. Here, the positions of the CA domains are very similar as demonstrated by the 5.3º angle of rotation between the two CA domains (Fig. 4). A larger angle of rotation between the

Table 1

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Outer shell statistics are provided in parentheses.
two CA domains of 35.4° accounts for the larger overall RMSD of 1.53 Å between the RetSHK molecule in the RetS-GacS complex and Chain B of the RetS homodimer (Fig. 4).

Beyond the relative movements of the CA and DHp domains, GacS binding significantly impacts regions that were previously implicated in the regulation of the RetS-GacS interaction. In the RetSHK-GacSDHp complex, residues 574–603 encompassing a section of the molecule containing the so-called ATP lid loop are not structured. In the asymmetric RetSHK homodimer, the ATP lid loop regions assume two distinct but well-defined conformations consisting of a short N-terminal helix and the lid loop (21) (Fig. S2). The ATP lid loop of other HKs also displays conformational plasticity, such as in the HK from Bacillus subtilis DesK (25). However, RetS has lost the ability to bind ATP (21). Instead, the lid region from one RetSHK molecule forms a short helix that displaces an unfolded section of α1 helix from the other molecule at the DHp-DHp interface (21).

The biological significance of these interactions was corroborated in vitro and in vivo (21). In the RetS-GacS complex, the α1 helix of GacS DHp is fully folded, while the lid region is now dislodged from the interface and apparently unstructured.

GacS autophosphorylates in trans

Because RetS binding disrupts the DHp-DHp interface of the GacS dimer, we reasoned, this interaction should interfere with GacS autophosphorylation if GacS actually autophosphorylates in trans. Many HKs autophosphorylate in trans, although some have been demonstrated to autophosphorylate in cis (26, 27). BarA, the GacS homolog in Escherichia coli autophosphorylates in trans (28). The handedness of the loop between the α1 and α2 helices of the DHp domain may also be used to predict whether an HK will autophosphorylate in trans or in cis (26). Given that the GacS homolog autophosphorylates in trans and, in the structure, GacS has a right-handed loop between the α1 and α2 helices of the DHp domain, GacS was predicted to autophosphorylate in trans as well. To test this prediction, an autophosphorylation assay followed by Zn2+-Phos-tag SDS-PAGE was performed (Fig. 5). GacScyt (GacS 219–925) and the GacScyt variants H293A and G472A G474A were examined in the assay. Variation of the conserved HK region histidine residue (H293) to an alanine inhibits GacS autophosphorylation (28). The variation of conserved G2 box residues G472 and G474 to alanines inhibits the ability of GacScyt to bind ATP. The G2 box is a conserved region in HKs located within the CA domain, which binds ATP (28). Individual variant constructs (GacScyt H293A and GacScyt G472A G474A) are unable to autophosphorylate in cis, but when both variant constructs are introduced into the autophosphorylation assay, they can autophosphorylate in trans. The observed mobility shift when both GacScyt H293A and GacScyt G472A G474A were present in the
autophosphorylation assay demonstrated that GacS autophosphorylates in trans, thus providing evidence supporting the proposed mechanism by which RetS inhibits GacS autophosphorylation (Fig. 5).

**GacS L309 and I302 are critical for promoting complex formation with RetS**

In order to experimentally corroborate the RetS-GacS interface found in the crystal structure, a number of interface residues were mutated and the variants examined via the bacterial adenylate cyclase two-hybrid (BACTH) assay. The DHp domains of RetS and GacS share a 56.9% amino acid sequence identity (Fig. 1), whereas the entire HK regions of RetS and GacS share 40.4% sequence identity. The high degree of conservation in the DHp domain might explain the overall complementarity of their molecular surfaces. Yet, we also sought to identify distinctive residues that promote the formation of a RetS-GacS complex at the DHp-DHp interface over the formation of the typical homodimers. The interactions of the cytoplasmic region of GacS (GacS\textsubscript{cyt}) with itself and with the cytoplasmic region of RetS (RetS\textsubscript{cyt}) were used as positive controls and reference points (Fig. 6). The GacS homodimer is expected to contain multiple dimerization interfaces including HAMP-HAMP and DHp-DHp domains (29). Therefore, the RetS-GacS interactions are expected to be more sensitive to mutations disrupting the interactions between the DHp domains than the more extensively paired GacS homodimer. A complicating factor in this analysis was the observation we made in prior experiments that monomeric RetS and GacS are not stable proteins. A variant that has completely lost the ability to dimerize could either be misfolded or simply be unstable because it can no longer dimerize. Therefore, we were particularly interested in identifying GacS residues that are critical for RetS binding but not for GacS dimerization. A number of residues were probed. The GacS L309R mutation attenuated homodimerization but completely disrupted the RetS\textsubscript{cyt}-GacS\textsubscript{cyt} interaction (Figs. 6 and S3) confirming that L309 is important in the RetS-GacS complex. The GacS\textsubscript{cyt} I325R mutation abrogated both GacS\textsubscript{cyt} homodimerization and the RetS\textsubscript{cyt}-GacS\textsubscript{cyt} interaction (Figs. 6 and S3). GacS\textsubscript{cyt} I302V formed a stable homodimer, while binding
to RetS was partially disrupted (Figs. 6 and S3). This suggests that the GacScytI302V construct is stable and, as predicted by the crystal structure, that I302 is involved in RetS binding. Two RetScyt variant constructs were also examined in the BACTH assay. RetScyt L463R could no longer form homodimers or bind to GacS (Fig. S4). The RetScyt V433I mutation was too subtle as the substitution had no significant impact on RetScyt homodimerization or the RetScyt-GacScyt interface (Fig. S4). Because the GacS L309R and GacS I302V mutations showed differential binding profiles in the BACTH assays, we decided to examine their impact on P. aeruginosa biofilm formation.

Discussion

Initially, the Gac/Rsm pathway was discovered as a central signal transduction pathway in Pseudomonads that regulates the production of secondary metabolites (e.g., antimicrobials, hydrogen cyanide, siderophores) and also the switch between a motile, invasive lifestyle and a sessile biofilm-associated lifestyle (16, 30–33). However, beyond the biological significance of this particular signaling pathway, the GacS-GacA system has become the model for studying crosswise interactions between multiple signaling kinases. HKs have been demonstrated to maintain a high degree of fidelity for their cognate RR s and vice versa, but we are beginning to recognize that MKNs are often necessary to control complex outputs (9, 34). Such MKNs were once postulated to be prohibited, but it now appears many bacterial species use them to integrate diverse extracellular signals to regulate adaptive responses (34). MKNs control transitions associated with virulence, response to switching from aerobic to anaerobic conditions, the integration of diverse quorum sensing signals, as well as sporulation and fruiting body formation (34). However, none is more complex than the MKN associated with the regulation of the
GacS-GacA system. At least seven HKs coordinate their signaling to fine-tune *P. aeruginosa* gene expression. LadS and RetS do so through direct interactions with GacS (11, 12, 18, 20), while PA1611 appears to sequester RetS to promote GacS signaling (35, 36). However, RetS, PA1611, ErcS, and SagS all appear to also interact with HptB to modulate RsmY levels (37–40). SagS interacts with BfiS to integrate the BfiS/BfiR system, which promotes biofilm formation into the MKN (39).

The mechanism whereby these signaling pathways integrate are varied and, in some cases, multifaceted as the interactions can be both activating and suppressing. Often, the molecular mechanisms underlying the MKNs can be readily understood as they involve well-characterized protein–protein interactions mirroring canonical signaling pathways. The basis for the direct pairwise interactions of the HK regions observed for RetS-GacS but also RetS-PA1611 and SagS-BfiS in *P. aeruginosa*, as well as the DivL-CckA interactions in *Caulobacter crescentus* are less well understood (41). The present evidence of domain-swapping between DHp domains in the RetS-GacS complex suggests that once again MKN signaling evolved from known contact interfaces of regular linear HK systems. Although, earlier work suggests that the interface formed between PA1611 and RetS involves the DHp domain of PA1611 and the beta-sheet of the CA domain of RetS, suggesting an interface that does not have an equivalent in known HK contacts (36). The present structure may broadly represent the basis for how heteromeric HK-HK interactions inhibit autophosphorylation in MKNs.

The *RetSHK-GacSDHp* complex structure answers the question of how binding between the *RetS* and GacS DHp domains prevents GacS autophosphorylation because the formation of a heterodimeric DHp-DHp interface should inhibit GacS trans-autophosphorylation (11, 12). However, perhaps this inhibition is not complete, thus explaining why RetS uses not one but three distinct mechanisms to inhibit GacS signaling (Fig. S1). Potential trans-autophosphorylation of GacS in a heteromeric *RetS-GacS* complex is at this point only speculative; however, the siphoning of phosphates from the catalytic histidine in GacS-HK by the second receiver domain of RetS would otherwise appear to be redundant. Yet, Francis *et al.* (11) demonstrated that this phosphatase activity is critical for inhibiting GacS signaling in *vivo*. The RetS HK region also

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**Figure 4. RetS CA domain movement.** Alignment of the DHp domain of RetS with the DHp domain of the RetS homodimer Chain A (PDB 6dk7) (RMSD = 0.359) visualizes slight movement of the CA domain as demonstrated by the angle of rotation between the CA domains of 5.3° (top image). Alignment of the DHp domain of RetS with the DHp domain of the RetS homodimer Chain B (PDB 6dk7) (RMSD = 1.533) visualizes the greater movement of the CA domain as demonstrated by the angle of rotation between the CA domains of 35.4° (bottom image). RetS is shown in light pink. RetS homodimer Chain A is shown in blue. RetS homodimer Chain B is shown in cyan.
dephosphorylates the receiver domain of GacS in a manner similar to transmitter phosphatase activity (2, 11, 42). It is not known if RetS-GacS binding through the DHp-DHp increases the efficiency or is in fact a prerequisite for the efficient working of the two other inhibitory mechanisms. In recent years, some progress has been made toward elucidating the roles of periplasmic sensory domains of RetS and GacS in regulating their interplay. Remarkably, the sensory domain of RetS appears to promote the inhibition of GacS when exposed to host cell-derived mucins, while P. aeruginosa lysis releases a molecular signal, also recognized by the RetS sensory domain that causes GacS activation (43, 44). The sensory domain of GacS, on the other hand, is required for GacS activation, but the longstanding hunt for the elusive ligand is ongoing (32).

Overall, the present study has uncovered the novel heterodimeric DHp-DHp interface in the RetS-GacS complex, which readily explains how direct binding of RetS-HK to GacS-HK interferes with GacS trans-autophosphorylation. The observed RetSHK-GacSDHp structure is also consistent with the proposed model for regulation of RetS-GacS binding via RetS helix-cracking, which predicted that a structurally dynamic section of RetS would form the N-terminal end of the DHp α1 helix and interact with GacS (21). Another structurally dynamic feature of the RetS-HK dimer, the so-called ATP lid loop region and a short a helix N-terminal to the ATP lid loop region of the RetS CA domain are unstructured in the RetSHK-GacSDHp complex. Consistent with this prediction, the ATP lid loop region and a short a helix N-terminal to the ATP lid loop region of the RetS CA domain are unstructured in the RetSHK-GacSDHp complex. The mutational analysis of the DHp-DHp interface offered additional insight into which residues might be critical in providing specificity for the unusual heteromeric RetS-GacS interactions in favor of the RetS-RetS and GacS-GacS interfaces. We demonstrated upon variation of select residues (GacS I302, GacS L309) an inhibition to binding in the heterodimeric interface of the cytoplasmic regions, but not an equivalent inhibition to binding in the homodimeric interface of the cytoplasmic regions. We also demonstrated a phenotype comparable to the hyperbiofilm retS mutant for the GacS I302V strain in an in vivo assay, demonstrating the importance of I302 in RetS binding (13).

There is a disparity between the observation that RetS disrupts the GacS DHp-DHp dimerization interface and our previous finding that RetS overall does not disrupt the GacS homodimer (20). This apparent contradiction may be explained by the fact that the GacS protein construct used in the original FRET measurements included not only the histidine kinase region but also the HAMP domain of GacS (21). HAMP domains are ubiquitous signaling domains of signaling HKs and methyl accepting chemotaxis proteins and facilitate homodimerization and signal transduction by forming structurally dynamic intermolecular four-helix
The GacS HAMP domain appears to maintain GacS-GacS association even in the presence of RetS (Fig. 8). This observation is also consistent with the finding that the HAMP domain is required for GacS homodimerization in Pseudomonas fluorescens (29). Similarly, the periplasmic domain of RetS has also been demonstrated to dimerize in vitro (46, 47). If and how this interaction is affected by GacS binding is unknown.

Figure 6. Interface variants in the BACTH assay. A, examination of interface variants in the BACTH β-galactosidase assay. Interface variants were examined in the BACTH β-galactosidase assay after 24 h incubation at 30 °C. Assay was performed in triplicate. Statistical significance was determined by a two-tailed, nonpaired Student’s t test. *p < 0.01, **p < 0.001, ***p < 0.0001. B, examination of interface variants in the BACTH MacConkey agar assay. Interface variants were examined in the BACTH MacConkey agar assay after 24 h incubation at 32 °C. Assay was performed in triplicate.

Figure 7. In vivo biofilm assay assessing GacS variants. Relative biofilm production assessed by the crystal violet biofilm assay. PAKΔgacS + pHERD20T-gacS I302V was demonstrated to have a phenotype comparable to that of PAKΔretS pHERD20T in the crystal violet biofilm assay after incubation at 37 °C for 6 h. The complemented strains and PAKΔgacS + pHERD20T-gacS L309R were demonstrated not to be significantly different from PAK pHERD20T. Assay was performed in triplicate. * indicates that the strain demonstrated significantly more biofilm production than PAK pHERD20T. Statistical significance was determined by a two-tailed, nonpaired Student’s t test. *p < 0.01.
Collectively, the present work and previous results support a model in which RetS and GacS form a domain-swapped complex (Fig. 8). The exact stoichiometry and size of this complex remain to be determined. While the presence of additional GacS-GacS and RetS-RetS interfaces might make it tempting to propose the formation of a symmetric heterotetramer (Fig. 8, Model 2), steric factors may create an asymmetric complex, cause dissociation of the RetS dimer, or may even facilitate the formation of a larger polymeric structure consisting of alternating RetS and GacS dimers.

Experimental procedures

Cloning and site-directed mutagenesis

The plasmid construct for the expression of RetSHK (residues 413–649) from the pDEST-HisMBP plasmid was created previously (21). For the expression of the GacS_HK protein, the section of the gacS gene that encodes residues 270–349 was amplified via PCR from pDEST-HisMBP-GacS_HK using GacS_350_stop_F and GacS_350_stop_R primers. The PCR product was cloned into pDONR221 and from there into pDEST-HisMBP using Gateway recombinational cloning (Thermo Fisher) to create pDEST-HisMBP-GacSDHp. Constructs for the BACTH assay were generated using standard cloning protocols. pKT25-RetS cyt-GFP, encoding RetS residues 387–942 in frame with the gfp gene, was generated previously (21). pKT25-RetS cyt-GFP L463R and V433I variants were created using Agilent QuickChange XL site-directed mutagenesis kit (Agilent) following the manufacturer’s protocol. A pUT18c-GacSHK plasmid was created by introducing a stop codon after gacS codon 509 into the previously generated pUT18c-GacS_HK (GacS_HK includes residues 219–925) plasmid using the Agilent QuickChange XL site-directed mutagenesis kit (Agilent) and GacS509STPx2_F and GacS509STPx2_R primers following the manufacturer’s protocol (21). Agilent QuickChange XL site-directed mutagenesis kit (Agilent) was also used to create the pUT18c-GacS_HK constructs expressing variants L309R, I325R, and I302V following the manufacturer’s protocol. Ultimately, the pUT18c-GacS_HK constructs expressing the original gacS sequence and the three mutated genes were only used in this study as templates to cut-and-paste the L309R, I325R, and I302V associated mutations into the pUT18c-GacS cyt vector via internal restriction sites (XbaI and Stul) using standard restriction cloning protocols.

GacS cyt variants GacS cyt H293A and GacS cyt G472A G474A used for the in vitro autophosphorylation assays were also generated with the Agilent QuickChange XL site-directed mutagenesis kit (Agilent) using the pQE60-GacS cyt vector as template, which was previously generated and generously shared with us by Dr Steven Porter’s group (University of Exeter) (11).

The Agilent QuickChange XL site-directed mutagenesis kit (Agilent) was also used to create pHERD20T-gacS L309R and pHERD20T-gacS I302V variants using the previously generated pHERD20T-gacS vector as a template following the manufacturer’s protocol (48, 49). Cloning and site-directed mutagenesis primers are listed in Table S1. Recombinant DNA used in this study is listed in Table S2.

Recombinant protein expression and purification

BL-21(DE3)(RIL) pDEST-HisMBP-RetS_HK was grown in 6 L Lysogeny broth (LB) with 100 μg/ml ampicillin, 30 μg/ml chloramphenicol, and 10 g glucose/l at 37 °C, shaking at 250 rpm. The cultures were induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) after the OD600 reached 0.6, and incubated at 18 °C for 18 h, again shaking at 250 rpm. A 35.5 g cell pellet was resuspended in 200 ml NiNTA A buffer (buffer compositions are listed in Table S3) and 0.3 mM phenylmethanesulfonyl fluoride (PMSF) and lysed via sonication (21). The soluble fraction was collected via centrifugation for 1 h at 100,000g, 4 °C. HisMBP-RetS_HK was purified via Ni-NTA affinity chromatography using a 30 ml Ni-NTA Superflow column (Qiagen) and a 150 ml linear gradient elution with Ni-NTA B
buffer. SDS-PAGE was used to assess purification of HisMBP- RetS_HK. To remove the HisMBP-tag, the collected protein was incubated with 1 mg His6_TEV protease per 50 mg protein, as estimated by the UV280 absorption and extinction coefficient of the fusion protein, and dialyzed into Ni-NTA A buffer. A second 30 ml Ni-NTA Superflow column (Qiagen) was used to separate RetS_HK from the HisMBP tag and the His6_TEV protease. SDS-PAGE was used to assess purification of RetS_HK. RetS_HK was further purified using a HiTrap Q HP column (GE Life Sciences) with a 150 ml linear gradient of anion exchange buffer A and anion exchange buffer B. SDS-PAGE was used to assess purification of RetS_HK. The eluted sample was pooled on a 26/60 Superdex 200 column (GE Life Sciences) pre-equilibrated in RetS_HK gel filtration buffer. SDS-PAGE was used to assess purification of RetS_HK. Expression and purification protocols for GacSDHp from the pDEST-HisMBP-GacSDHp plasmid followed the same protocol as that for RetS_HK.

Selenomethionine-substituted RetS_HK was produced by altering the growth conditions prior to protein purification. BL-21 (DE3)(RIL) pDEST-HisMBP-RetS_HK was grown in 1 L Lysogeny broth (LB) with 100 μg/ml ampicillin, 30 μg/ml chloramphenicol and 10 g glucose at 37°C, shaking at 250 rpm until the OD600 was 0.5. The culture was incubated on ice for 30 min, after which the cells were pelleted via centrifugation at 4000g, 4°C for 15 min. The cell pellet was resuspended in 100 ml of M9 salts plus Medicilon noninhibitory amino acid cocktail (NIAAC), pelleted a second time at 4000g, 4°C for 15 min, and then resuspended in 100 ml of M9 salts plus Medicilon NIAAC (Medicilon). Mediclon selenomethionine M9 medium (Mediclon) with 100 μg/ml ampicillin and 30 μg/ml chloramphenicol was inoculated with the resuspended pellet. The cultures were incubated at 37°C for 30 min, after which time protein expression was induced with 1 mM IPTG, and the cultures were incubated at 18°C for 18 h. Protein purification of selenomethionine-substituted RetS_HK followed the same protocol as for RetS_HK.

To produce GacScyt, the pQE60-GacScyt plasmid was transformed into the E. coli JM109 cell line. Cells were grown in LB medium with 100 μg/ml ampicillin at 37°C for approximately 3 h until the OD600 was 0.6. The temperature was reduced to 18°C and expression was induced with the addition of 1 mM IPTG. Induction continued for 18 h, after which cell pellets were harvested. Cell pellets were resuspended in 10 ml GacScyt NiNTA A buffer per gram cell pellet with 0.3 mM PMSF and then lysed via sonication. After centrifugation at 100,000g, 4°C for 1 h, the supernatant was applied to a Ni-NTA Superflow column (Qiagen) for FPLC and eluted using a 150 ml linear gradient of GacScyt Ni-NTA A buffer and GacScyt Ni-NTA B buffer. SDS-PAGE was used to assess purification of GacScyt. The sample was then applied to a 26/60 Superdex 200 gel filtration column (GE Life Sciences) for the final purification step using GacScyt gel filtration buffer. SDS-PAGE was used to assess GacScyt purification. (Buffer compositions are listed in Table S3.)

GacScyt pQE60 variants GacScyt H293A and GacScyt G472A G474A were expressed in E. coli JM109 cell line following the same protocol as for GacScyt. The purification followed the same protocol as for GacScyt.

**RetS_HK–GacSDHp crystallization and structure determination**

Crystals containing the complex of seleno-methionine-substituted RetS_HK with GacSDHp were grown by vapor diffusion in a 6 μl hanging drop containing a 1:5 volume ratio of protein:mother liquor, with 440 μM of each RetS_HK and GacSDHp in gel filtration buffer. The mother liquor was composed of 30% PEG3350, 0.2 M Li2SO4, 0.1 M Tris-HCl, pH 8.5. The reservoir contained 0.4 ml of mother liquor. Crystals grew over a 2-week period at room temperature. The crystals were loop-mounted and flash frozen in liquid nitrogen. X-ray diffraction data were collected at Advanced Light Source Beamline 4.2.2 at Lawrence Berkeley National Laboratory. The diffraction images were processed and integrated with XDS and intensities converted to amplitudes using Aimless (50–52). The resolution was cut off at 2.3 Å using a CC1/2 threshold of 0.3 (22). The anomalous signal of the selenium atoms was not strong enough to facilitate structure solution. Therefore, the structure was solved with the Phaser molecular replacement tool within the Phenix suite using the structure of RetS_HK (PDB 6DK8) as the search model (21, 53, 54). Iterative cycles of model building in Coot and automated refinements in Phenix and RefMac were used to build the RetS_HK–GacSDHp structure (53, 55–57). The degree of rotation between the CA domain of RetS and the CA domains of the RetS homodimer (PDB 6dk7) was estimated via PyMOL (58).

**Bacterial adenylate cyclase two-hybrid (BACTH) assay**

Interface variants were examined in the BACTH MacConkey agar assay and the BACTH β-galactosidase assay (59, 60). For the BACTH MacConkey agar assay, LB cultures containing 100 μg/ml ampicillin and 50 μg/ml kanamycin were incubated for 18 h at 37°C. The OD600 was adjusted to 1.0. Two microliter of culture was dispensed onto MacConkey agar containing 0.5 mM IPTG, 1 (w/v) % maltose, 100 μg/ml ampicillin, and 50 μg/ml kanamycin. The MacConkey agar plates were incubated at 32°C for 24 h. The BACTH MacConkey agar assay was performed in triplicate. For the BACTH β-galactosidase assay, LB cultures with 100 μg/ml ampicillin, 50 μg/ml kanamycin, and 0.5 mM IPTG were incubated for 24 h at 30°C. OD600 was measured for each culture. Twenty microliter of culture was added to 80 μl permeabilization solution (100 mM Na2HPO4, 20 mM KCl, 2 mM MgSO4, 0.8 mg/ml cetrimonium bromide, 0.4 mg/ml sodium deoxycholate, 5.4 μl β-mercaptoethanol) (60). Samples were incubated for 30 min at 30°C, after which, 0.6 ml of substrate solution (60 mM Na2PO4, 40 mM NaH2PO4, 1 mg/ml o-nitrophenyl β-D-galactoside, 2.7 μl β-mercaptoethanol) was added to the samples with the time noted (60). The reaction was stopped via the addition of 1 M Na2CO3 and the time was recorded (60). The samples were centrifuged at 16,000g for 10 min and absorbance at 420 nm was recorded (60). Miller Units were calculated using the following formula: 1000 × (Abs420/OD600 × volume of sample in ml × reaction time in
minutes) (60). The BACTH β-galactosidase assay was performed in triplicate.

**Autophosphorylation assay and Zn^{2+} Phos-tag SDS-PAGE**

The Zn^{2+}-Phos-tag assay closely followed the steps provided in the manufacturer’s protocol, and all solutions were prepared according to the manufacturer’s protocol (FUJIFILM Wako Chemicals). Autophosphorylation was performed via the incubation of 5 μM GacScyt or GacScyt variant proteins GacScyt (Pseudomonas aeruginosa) H293A and GacScyt (P. aeruginosa) G472A G474A with 2 mM ATP for 30 min at 21 °C (11). The reaction was stopped via the addition of 3× loading buffer to a final concentration of 1× following manufacturer’s protocol (FUJIFILM Wako Chemicals). The samples were analyzed via Zn^{2+}-Phos-tag SDS-PAGE following manufacturer’s protocol using 100 μM Phos-tag in a 10% acrylamide Zn^{2+}-Phos-tag SDS-PAGE gel (FUJIFILM Wako Chemicals) (28). The autophosphorylation assay and Zn^{2+}-Phos-tag SDS-PAGE were performed in triplicate.

**Crystal violet biofilm assay**

*P. aeruginosa* PAK strains were examined in the crystal violet biofilm assay. The strains were plated to LB agar containing 300 μg/ml carbenicillin. Individual colonies were used to inoculate LB containing 300 μg/ml carbenicillin, which was then incubated at 37 °C overnight while shaking at 250 rpm. The strains were subcultured into modified M63 media containing 0.5% arabinose and 300 μg/ml carbenicillin, which were incubated at 37 °C overnight while shaking at 250 rpm (61). The OD_{600} of the cultures was adjusted to 0.05 using modified M63 media containing 0.5% arabinose and 300 μg/ml carbenicillin. Hundred microliter of each culture was dispensed into 96-well plates (Corning # 2797) (61). Plates were covered with aluminum foil and incubated at 37 °C for 6 h. Following incubation, the media were removed via pipette and the wells were washed with water via pipette. The wells were stained with 0.1% crystal violet for 10 min and then washed with water via pipette (61). The remaining crystal violet-stained cells were solubilized in 125 μl of 30% acetic acid for 15 min (61). Hundred microliter of the solution was transferred to a 96-well plate (Corning # 3370) and absorbance at 600 nm was measured using a Tecan M200 plate reader.

**Data availability**

All data are contained within the manuscript.

**Supporting information**—This article contains supporting information (10, 11, 19).

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**Conflict of interest**—The authors declare that they have no conflicts of interest with the contents of this article.

**Abbreviations**—The abbreviations used are: BACTH, bacterial adenylate cyclase two-hybrid; DHp, dimerization and histidine phosphotransfer; HK, histidine kinase; HPt, histidine phosphotransfer; MKN, multikinase network; RR, response regulator.

**Crystal structure of a RetS-GacS complex**

References


Crystal structure of a RetS-GacS complex


Crystal structure of a RetS-GacS complex


