Direct interaction between sensor kinase proteins mediates acute and chronic disease phenotypes in a bacterial pathogen

Andrew L. Goodman,[1,4,5] Massimo Merighi,[1,4] Mamoru Hyodo,[1] Isabelle Ventre,[1,2] Alain Filloux,[2,3] and Stephen Lory[1,6]

[1]Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115, USA; [2]LISM-IBSM-CNRS, Marseille Cedex 20, France; [3]Imperial College London, Centre for Molecular Microbiology and Infection, London SW7 2AZ, United Kingdom

The genome of the opportunistic pathogen *Pseudomonas aeruginosa* encodes over 60 two-component sensor kinases and uses several (including RetS and GacS) to reciprocally regulate the production of virulence factors involved in the development of acute or chronic infections. We demonstrate that RetS modulates the phosphorylation state of GacS by a direct and specific interaction between these two membrane-bound sensors. The RetS–GacS interaction can be observed in vitro, in heterologous systems in vivo, and in *P. aeruginosa*. This function does not require the predicted RetS phosphorelay residues and provides a mechanism for integrating multiple signals without cross-phosphorylation from sensors to noncognate response regulators. These results suggest that multiple two-component systems found in a single bacterium can form multisensor signaling networks while maintaining specific phosphorelay pathways that remain insulated from detrimental cross-talk.

Two-component system (TCS) signaling pathways are a major signaling mechanism in bacteria and archaea, and are also found in simple eukaryota and higher plants (Wolanin et al. 2002). These diverse organisms capitalize on TCS pathways to monitor critical external and internal stimuli (including levels of nutrients, concentration of ions and gases, temperature, redox states, and cell density) and translate these signals into adaptive responses. Classical TCS pathways share a conserved core architecture: a homodimerizing histidine kinase protein domain (the "sensor") and a cognate receiver domain (the "response regulator"), coupled mechanistically through a histidine–aspartic acid phosphorelay (Stock et al. 2000). Most cognate sensor response regulator pairs are also linked genetically, encoded by adjacent loci in the chromosome (Alm et al. 2006). Although a single bacterial species can encode up to hundreds of genes specifying TCS pathways, it appears that these systems are insulated against detrimental cross-phosphorylation between sensors and noncognate response regulators (Bijlsma and Groisman 2003; Baker and Stock 2007; Laub and Goulian 2007). The identification of multistep phosphorelays (with intermediary proteins between sensor and regulator) and branched pathways (phosphotransfer between one sensor and multiple response regulators and vice versa) (Laub and Goulian 2007) suggests that TCS pathways have the capacity to form sensitive and complex signaling networks.

In contrast to microorganisms with restricted habitats, the genomes of bacteria capable of occupying a number of diverse environments typically contain a disproportionately large number of genes encoding signal transduction and regulatory systems, including TCSs that allow them to sense and respond to a wide range of environmental signals. For opportunistic bacterial pathogens, a number of these systems regulate the expression of genes necessary for transitioning from the environmental reservoir to the host, overcoming innate defense mechanisms and initiating the disease process. The bacterial pathogen *Pseudomonas aeruginosa* dedicates a large portion (~8%) of its genome toward transcriptional regulation that includes coding sequences for the components of ~70 TCS pathways (Stover et al. 2000). This organism draws on a broad arsenal of virulence factors to shift from its primary reservoir in the environment to a habitat inside its human host, where it causes acute infections in

[4]These authors contributed equally to this work.
[5]Present address: Center for Genome Sciences, Washington University School of Medicine, St. Louis, MO 63110, USA.
[6]Corresponding author.
E-MAIL stephen_lory@hms.harvard.edu; FAX (617) 738-7664.
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sites compromised by injury or lacking adequate immune surveillance. Animal models have identified specific virulence mechanisms, including flagellar motility and the Type III secretion system (TTSS) that are required for acute infection (Fleiszig et al. 1997; Feldman et al. 1998). In addition to acute infections, changes in lung pathology resulting from cystic fibrosis (CF) lead to chronic *P. aeruginosa* infections that are the primary cause of mortality for patients with this common hereditary disease. Isolates of *P. aeruginosa* from CF patients, in contrast to those from acutely infected patients, are characterized by acquisition of stable mutations leading to derepression of alginate production (mucoidy), auxotrophy, and loss of motility, O-antigen production, and TTSS function (Hancock et al. 1983; Mahenthiralingam et al. 1994; Jain et al. 2004; Li et al. 2005). Comparative genomic analyses of clonal isolates from acute and chronic infections suggest a high frequency of mutations in regulatory genes (Smith et al. 2006). These observations suggest that some virulence factors associated with acute infections may be dispensable in chronic disease.

Recent studies suggest that *P. aeruginosa* coordinates the activities of multiple orphan TCS sensor kinases as a genetic "switch" that reciprocally regulates genes involved in acute and chronic infections. The unusual hybrid sensor kinase RetS (Fig. 1A) is required for TTSS activation, concomitant repression of biofilm formation, and colonization/dissemination in murine acute infection models (Goodman et al. 2004; Laskowski et al. 2004; Zolfaghar et al. 2005). Genes under RetS control are inversely regulated by two other sensor kinases: GacS and LadS. Microarray studies have shown that the regulatory activity of RetS appears to be channeled through a GacS and GacA TCS (A. Brencic and S. Lory, in prep.), found in a number of different microorganisms. Genetic studies in *P. aeruginosa* suggest that in analogy with the homologous BarA/UvrY system in *Escherichia coli*, the sensor kinase GacS may phosphorylate the response regulator GacA to initiate downstream signaling (Lapouge et al. 2008). In turn, phosphorylated GacA activates the transcription of the two small RNAs, RsmZ and RsmY, which are antagonists of the translational regulator RsmA. RsmA binds to specific mRNA targets, stabilizing some and inducing the degradation of others. In addition to GacS, another sensor kinase, LadS, functions as an activator of expression of RsmZ and RsmY (Ventre et al. 2006). It therefore appears that the signaling network consisting of RetS, LadS, and GacS/GacA controls the expression of a significant number of *P. aeruginosa* virulence genes primarily (and perhaps exclusively) at the level of mRNA translation and/or stability. Taken together, these genetic approaches identify multiple components in a "switch" between acute and chronic infection phenotypes and suggest that these TCS proteins may function outside of the classical sensor kinase response regulator signaling paradigm.

Here we report that RetS modulates the phosphorylation state of GacS through a direct and specific protein interaction. This interaction determines the rate of
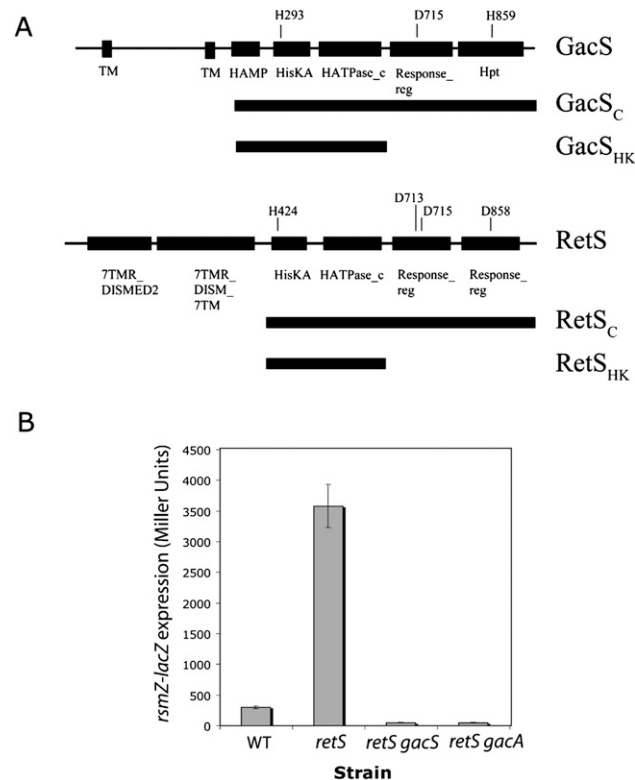

Figure 1. RetS suppresses *rmsZ* expression via the TCS GacS/GacA. (*A*) Domain organization of the *Pseudomonas aeruginosa* GacS and RetS sensor kinases and the map of the deletion constructs used for protein purification and two-hybrid analysis (GacS$_c$, GacS$_{HK}$, RetS$_c$, and RetS$_{HK}$). The locations of conserved phosphorelay residues are marked above each protein (amino acid positions); Pfam domain designations and construct boundaries are indicated (domain sizes not to scale). (*B*) RetS function is dependent on GacS and GacA. Wild-type *P. aeruginosa* and various deletion strains carrying chromosomal *rsmZ-lacZ* fusions were grown in LB for 8 h and assayed for β-galactosidase activity. Error bars represent one standard deviation from the mean.

phosphotransfer between GacS and its cognate response regulator GacA. The observation that two sensor kinases can modulate downstream signaling by direct interaction suggests that higher-order TCS signaling networks can be achieved without cross-phosphorylation between sensors and noncognate response regulators.

Results

GacS and GacA are required for RetS function

Previous work suggested that three physically unlinked sensor kinases (*gacS*, *retS*, and *ladS*) and the response regulator *gacA* regulate virulence factor expression via the small RNA *rsmZ* (Supplemental Fig. S1). The observation that *rsmZ* promoter activity is similarly derepressed in *retS* and *retS ladS* strain backgrounds indicates that LadS is not directly required for RetS function but instead functions independently (Ventre et al. 2006). In order to establish the epistatic relationships

between RetS, GacS, and GacA in regulating *rsmZ* expression, we measured the activity of an *rsmZ-lacZ* fusion in varying genetic backgrounds. In agreement with previous reports, transcription from the *rsmZ* promoter is activated by GacS or GacA and repressed by RetS (Fig. 1B; data not shown). Introduction of the *retS* gene tagged with the coding sequence for the VSV-G epitope into the *retS* mutant strain returns *rsmZ-lacZ* activity to wild-type levels (Supplemental Fig. S2) with a concomitant restoration of other phenotypes disregulated in a *retS* mutant, including abolishment of hyperbiofilm formation and of Type III secretion (data not shown). Derepression of *rsmZ* promoter activity in the *retS* mutant is abolished by in-frame deletion of *gacS* or *gacA*, indicating that RetS function requires the presence of GacS and GacA (Fig. 1B).

One possible explanation for the observation that the activation of the *rsmZ* promoter activity in the *retS* mutant requires *gacS* and *gacA* is RetS-dependent repression of transcription of one or both of these genes. This explanation for RetS function, however, seems very unlikely. Genomewide transcriptional profiling indicates that *gacS* and *gacA* transcript levels are not altered in a *retS* mutant, suggesting that RetS does not influence the expression of either one of these genes (Goodman et al. 2004). To assess whether other regulatory factors are required for RetS-mediated *rsmZ* regulation, we screened an ~100,000-colony transposon insertion library for mutants that induce or repress *rsmZ-lacZ* transcription. Repeated identification of *retS* as the strongest negative regulator (approximately fivefold to 10-fold effects; eight independent insertions) and *gacS*, *gacA*, and *rsmA* as the strongest positive regulators (~50- to 500-fold effects; three, five, and four independent insertions, respectively) of *rsmZ* transcription is consistent with the hypothesis that proteins encoded by these genes are the central components of the regulatory network and could interact at some level other than transcription.

GacS interacts with RetS in P. aeruginosa

The observation that RetS function requires GacS, the initial signaling element in the Gac/Rsm pathway, suggests that these proteins may function through direct interaction. To test this hypothesis, we assessed whether RetS forms a complex with GacS in vivo and they can be copurifed from detergent solubilized *P. aeruginosa* membrane preparations. We coexpressed GacS-His6 with RetS-VSV-G in a *P. aeruginosa gacS gacA retS* mutant and used Ni-NTA affinity chromatography to capture complexes with GacS-His6 (Fig. 2). Pull-down of a complex of RetS-VSV-G and GacS-His6 could be clearly demonstrated, while two inner membrane proteins XcpT or XcpY were not detected in a complex with GacS-His6, even after sevenfold concentration. To further test the avidity of the GacS:RetS interaction, we treated the cells with the chemical cross-linker dithiobis[succinimidyl-propionate] (DSP) prior to solubilization of membranes and Ni-NTA affinity chromatography. No enhancement in the recovery of RetS bound to GacS was noted, suggesting that the two proteins form highly stable, detergent resistant complexes.
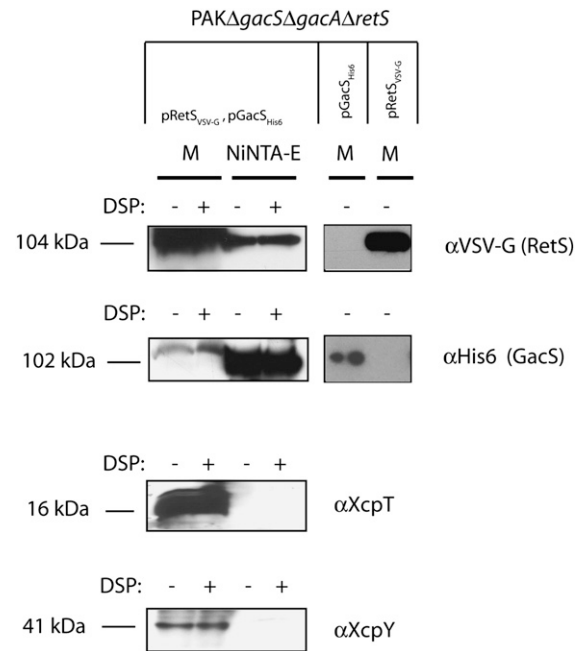

Figure 2. Specific pull-down of GacS:RetS multiprotein complexes in vivo. A *gacS retS gacA* mutant of *P. aeruginosa* was transformed with plasmids expressing $gacS_{His6}$ and/or $retS_{VSV-G}$ genes and grown to OD ~3.0. Cultures were split into two aliquots of 500 mL, washed with PBS, and treated with 1 mM of the membrane permeable cross-linker DSP ("DSP+") or DMSO ("DSP−") for 30 min. Cells were harvested, lysed, divided into two aliquots for total membrane purification ("M") or for pull-down of $GacS_{His6}$ and GacS-associated proteins by Ni(II)-NTA metal affinity chromatography ("NiNTA-E") in the presence of CHAPS as a membrane-solubilizing detergent. Total membranes (M) and Ni(II)-NTA eluate ("NiNTA-E") fractions were analyzed by SDS-PAGE and probed by Western immunoblotting with antibodies against the VSV-G and His6 tags, XcpT, and XcpY. NiNTA-E lanes were loaded at a sevenfold higher cell equivalent per lane.

The cytoplasmic domains of RetS and GacS interact directly in vitro

In order to assess whether RetS and GacS interaction is direct, we determined the binding affinity of the purified cytoplasmic (soluble) domains of these proteins by isothermal titration calorimetry. We cloned, expressed, and purified the cytoplasmic portions of RetS and GacS (and of the unrelated sensor kinase PilS) as N-terminal His6 fusions (Fig. 1A). The resulting proteins ($RetS_c$, $GacS_c$, $PilS_c$) were soluble, expressed at high levels, and were purified to >95% purity by a combination of metal affinity chromatography and gel filtration (Supplemental Fig. S3). Interaction of $GacS_c$ or $PilS_c$ with $RetS_c$ was assessed by isothermal titration microcalorimetry. We used an ~100-fold molar excess of $RetS_c$ relative to $GacS_c$ or $PilS_c$ over a maximum of 30 microinjections. These calorimetric measurements and subsequent modeling of the binding isotherm using a single-binding-site model provide a characterization of $GacS_c$–$RetS_c$ protein–protein-binding thermodynamics (Fig. 3A), revealing
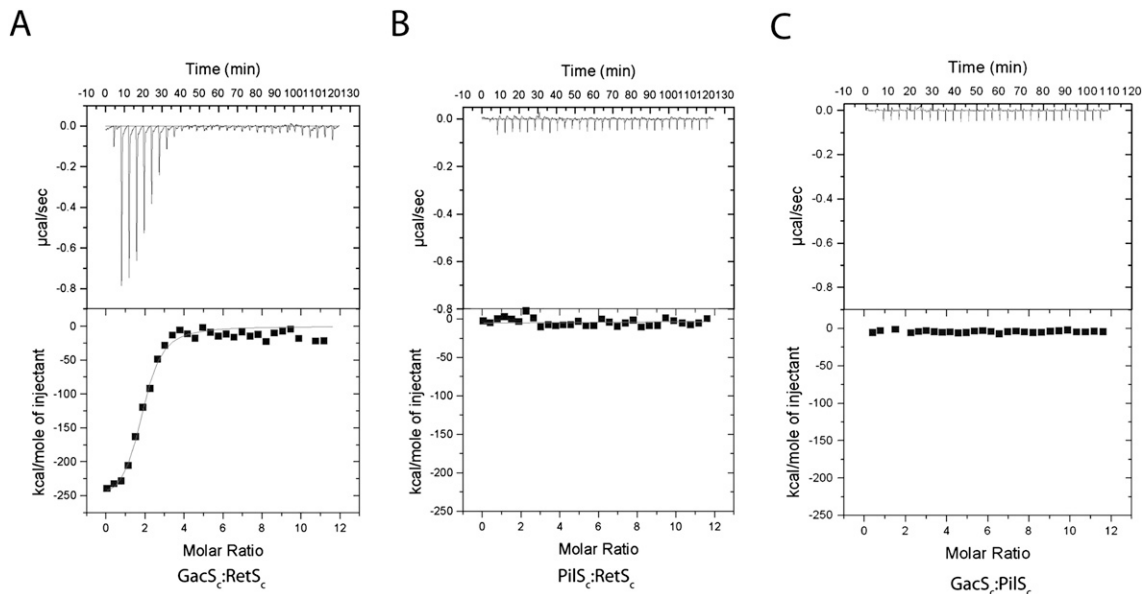
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Figure 3. Isothermal titration calorimetric analysis of RetS$_c$ binding to GacS$_c$. Titration of RetS$_c$ (0.1 µM) with 10 µM GacS$_c$ (*A*) or PilS$_c$ (*B*) or titration of PilSc (0.1 µM) with 10 µM GacS$_c$ (*C*). Heat of binding was measured by isothermal titration calorimetry in a VP-ITC microcalorimeter. The *top* panels show the baseline corrected titration data, and the *bottom* panels show the binding isotherm fitted using nonlinear binding models.

a large entropic loss counteracted by a large binding enthalpy (−855 kcal/mol). The estimated dissociation constant (K_d) was 33 nM. These results imply significant and extensive structural rearrangements upon protein–protein interaction. In contrast, similar experiments using the unrelated sensor kinase PilS$_c$ as the titrated protein did not show any measurable interaction with RetS$_c$ above background (Fig. 3B), further supporting the hypothesis that GacS$_c$–RetS$_c$ complex formation is direct and specific, and does not require the transmembrane domains. Isothermal titration calorimetry was also performed with GacS$_c$ as the titrant and PilS$_c$ as the receptor, and the results showed also no signal above the background dilution heat (Fig. 3C).

The kinase core domains of GacS and RetS are sufficient for in vivo interaction

TCS sensor kinases have been shown to form homodimers via their kinase core HisKA-H$^+$ATPase domains of each monomer (Tomomori et al. 1999). We used a bacterial two-hybrid system to determine whether these domains also mediate the GacS–RetS interaction in vivo. The kinase core domains of RetS, GacS, and PilS were cloned in bacterial two-hybrid expression vectors to create C-terminal fusions to two complementary fragments of the *Bordetella pertussis* adenylate cyclase (CyaA). The GacS fusion also included most of the HAMP domain except for the first 18 amino acids. When introduced into a reporter strain of *E. coli*, interacting protein fusions reconstitute a functional adenylate cyclase, increasing the cellular pool of cAMP (Karimova et al. 1998). As expected for this family of proteins, strong signals indicative of interactions were detected between

homodimers of RetS, GacS, and PilS (Fig. 4). Strains of *E. coli* coexpressing the HisKA/H$^+$ATPase domain of RetS and the corresponding domain of GacS, as CyaA fusions, also resulted in a significant increase in cAMP levels, suggesting that these domains mediate heterodimer formation between RetS and GacS monomers in vivo. In contrast, the fusions containing the HisKA/H$^+$ATPase domains of RetS and PilS or PilS and GacS were unable to reconstitute significant levels of active CyaA when coexpressed in *E. coli*. Reciprocal exchange of the fusion domains resulted in the detection of both the homo- and heterodimeric interactions.

In vitro reconstitution of the GacS–GacA phosphorelay

The observations that GacS and GacA are required for RetS function in *P. aeruginosa* and that RetS and GacS interact directly in vivo and in vitro raised the possibility that RetS could function by acting as a phosphatase of GacS~P or by interfering with GacS autophosphorylation. Since sensor kinases posses an intrinsic ATPase activity, we examined the abilities of RetS and GacS to hydrolyze ATP. Purified GacS$_c$ and RetS$_c$ proteins were incubated with γ[^{32}P]ATP or α[^{32}P]ATP followed by an analysis of the reaction products by PEI-cellulose thin-layer chromatography. Depending on the location of the label in the substrate ATP, [^{32}P]P$_i$ and α[^{32}P]ADP were detected in the presence of GacS$_c$ but not with RetS$_c$ (Fig. 5A), implying a lack of autophosphorylation/ATP-phosphatase activity of the latter protein. For some hybrid sensor kinases, fast rates of phosphotransfer to the conserved aspartic acid residues in the receiver domain and subsequent loss of the phosphate group prevent the accumulation of phosphorylated protein; this autophosphatase
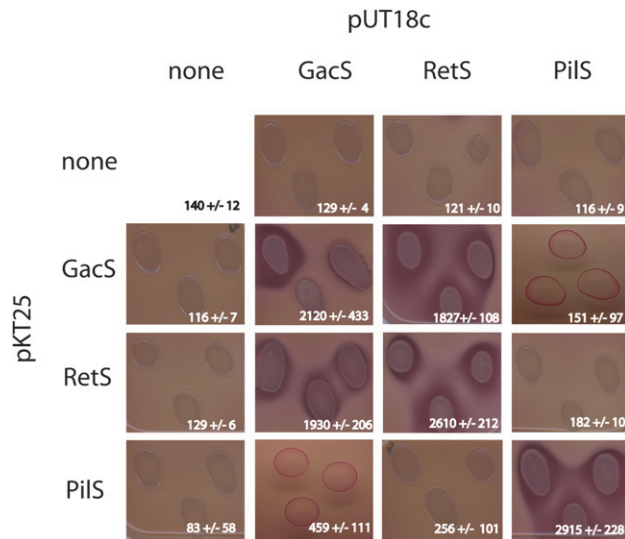
Figure 4. Cytoplasmic domains of RetS and GacS interact in vivo. DNA fragments encoding the HisKA/H⁺ATPase domains of GacS, RetS, and PilS (as a control) were expressed as fusions with one of two fragments of *Bordetella* adenylate cyclase from vectors pKT25 and pUT18c. Reconstitution of adenylate cyclase in the *E. coli* strain DHM1 was detected by the ability of the cells to ferment maltose when grown on MacConkey agar plates. The interactions were also quantified by β-galactosidase assays using liquid cultures of the same cells (average Miller units and standard deviations are shown in each panel).

activity can be blocked by mutation of these aspartic acid residues (Rasmussen et al. 2006). We therefore mutated each of the conserved residues—H424, D713, and D858—of $RetS_c$ (Fig. 1A). No autophosphorylation or phosphatase activity was observed in any of these proteins (Fig. 5A), suggesting that $RetS_c$ very likely lacks this intrinsic enzymatic activity.

In order to define the consequences of RetS/GacS interaction on GacA phosphorylation, we first developed an in vitro phosphotransfer system between GacS and GacA. $GacS_c$ was incubated in the presence of $\gamma[^{32}P]ATP$ and the labeled reaction products were visualized following SDS-PAGE and autoradiography (Fig. 5B). $GacS_c$ autophosphorylation reached saturation between 45 and 60 min and was Mg^{2+}-dependent, as is common for proteins of this class. The half-life of $GacS_c{\sim}P$ was determined to be ~120 min after separation of unincorporated ATP (Fig. 5C). Mn^{2+} could also substitute for Mg^{2+} as cofactor (data not shown). When $GacS_c$ was incubated in the presence of GacA (purified as a maltose-binding protein [MBP] fusion protein), phosphotransfer to the response regulator was observed after as early as 5 min (Fig. 5B). No accumulation of phosphorylated $RetS_c$ was observed over a 120-min time course, in the presence of either Mg^{2+} or Mn^{2+} (data not shown).

RetS suppresses GacS autophosphorylation in vitro

In order to determine whether the purified $RetS_c$ protein affects the autophosphorylation reaction of $GacS_c$, we incubated these two proteins in the presence of

$\gamma[^{32}P]ATP$. $RetS_c$ was also incubated with $\gamma[^{32}P]ATP$ and $PilS$ (Fig. 6). Incubation of $RetS_c$ and $GacS_c$ at increasing molar ratios (from 1:1 to 20:1) lead to a significant decrease in autophosphorylation of $GacS_c$. In contrast, $RetS_c$ had no effect on the ability of $PilS_c$ to autophosphorylate (Fig. 6A).

We next determined whether the $RetS_c$-mediated inhibition of $GacS_c$ autophosphorylation is dependent on the canonical residues involved in TCS phosphorelay. We expressed and purified three $RetS_c$ variants with substitutions in the conserved histidine and/or aspartic acid residues ($RetS_c$ [H], $RetS_c$ [DDD], and $RetS_c$ [HDDD]). As observed for $RetS_c$, each mutant protein was able to reduce phosphorylation of $GacS_c$ but not $PilS_c$, suggesting that the putative phosphorelay residues in RetS are not required for its interaction with GacS (Fig. 6B). To determine whether the phosphate on GacS can be removed by RetS, we first purified $GacS_c{\sim}P$ by gel filtration on a G-25 column followed by incubation with $RetS_c$. $RetS_c$ was able to reduce the phosphorylation level of $GacS_c{\sim}P$ under these conditions, suggesting that RetS can modulate the steady-state phosphorylation level of GacS homodimers in the absence of free ATP (Fig. 6C). To test whether the RetS core kinase domain (shown to be sufficient for in vivo homo- and heterodimer formation in the two-hybrid system) is also sufficient for the suppression of GacS autophosphorylation, we purified the HisKA-H⁺ATPase kinase core fragment of RetS ($RetS_{HK}$) and its derivative with a null mutation in the H424 residue (Fig. 1A). Both of these proteins were also able to reduce $GacS{\sim}P$ levels (Fig. 6C). In none of these experiments we were able to detect accumulation of $RetS_c{\sim}P$.

RetS does not require its classical phosphorelay residues for regulation of *rsmZ* in vivo

The observation that the mutant alleles of $RetS_c$ still function to prevent $GacS_c$ phosphorylation in vitro suggests that these mutant proteins should also be functional in regulating the transcription of *rsmZ* in *P. aeruginosa*. To test this hypothesis, we introduced these mutations into the native chromosomal copy of the *retS* gene in an *rsmZ-lacZ* parental strain and assessed their activity by their ability to repress transcription from the *rsmZ* promoter (Fig. 7). Unlike the *retS* deletion strain, each mutant showed wild-type levels of *rsmZ* transcription, suggesting that the canonical phosphorelay residues are not required for RetS function in vivo. Low-copy plasmids carrying these alleles were similarly able to restore RetS activity to a *retS* mutant strain (Supplemental Fig. S4). Analogous results were obtained when using the inversely regulated *exoS-lacZ* fusion (data not shown).

Discussion

Bacteria use TCS as the primary mechanism for environmental adaptation. Pathogenic microorganisms make extensive use of TCS phosphorelays to assimilate signals in infected hosts and coordinate the expression of virulence determinants. Genomes of most bacteria contain
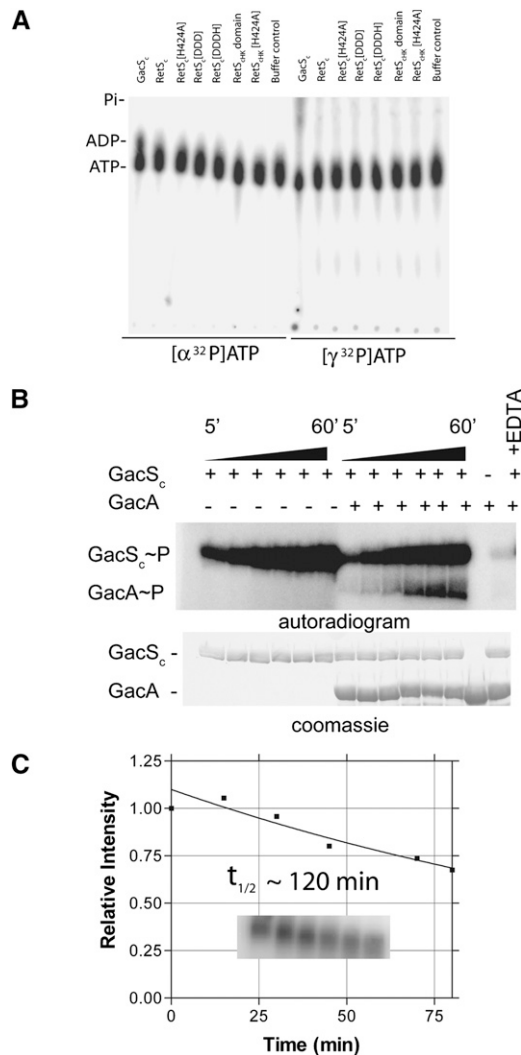
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Figure 5. GacS autophosphorylation and phosphotransfer to GacA in vitro. (A) ATPase activity of GacS$_c$. GacS$_c$, and allelic variants of RetS$_c$ were incubated with [α^{32}P] or [γ^{32}P]ATP for 60 min and reactions were spotted on PEI-cellulose TLC plates for nucleotide analysis. In the presence of [α^{32}P]ATP, GacS$_c$ produced labeled ADP, consistent with γ-phosphatase activity. Incubation of GacS$_c$ with [γ^{32}P]ATP released [^{32}P]Pi and produced a distinct radioactive signal at the origin, implying the formation of GacS$_c$~P. (B) Autophosphorylation of GacS and phosphotransfer to GacA. GacS$_c$ was incubated with [γ^{32}P]ATP for up to 60 min in the presence or absence of the response regulator GacA (purified as an MBP fusion) and reaction products were analyzed by SDS-PAGE. Phosphorylation was determined by storage phosphorimaging followed by staining with Coomassie Brilliant Blue. (C) GacS$_c$~P half-life. GacS$_c$ was incubated with [γ^{32}P]ATP for 30 min and unincorporated nucleotides were removed by G-25 spin chromatography. At different time intervals, the phosphoprotein preparation was treated with SDS sample buffer and analyzed by SDS-PAGE. Phosphorylation was determined by storage phosphorimaging and data were fitted into an exponential decay model.

multiple copies of genes encoding TCS proteins ranging from a few to 278 in *Myxococus xanthus* (Whitworth and Cock 2008). Branched pathways, where multiple sensors target the same response regulator or a single sensor phosphorylates several response regulators, capitalize on the conserved domain organization in these proteins. However, despite similarities between the components of distinct pathways, phosphorelays are well-insulated from cross-talk between systems, and cells have evolved mechanisms to avoid potentially deleterious interference between signals transmitted through specific pathways (Laub and Goulian 2007).

Previous genetic studies of the opportunistic pathogen *P. aeruginosa* implicated at least three orphan sensor kinases (GacS, RetS, and LadS) in coordinating the expression of virulence factors associated with the transition between acute and chronic infections (Goodman et al. 2004; Laskowski and Kazmierczak 2006; Ventre et al. 2006). A comparison of transcriptomes of wild-type *P. aeruginosa* with those of a *retS* and a *retS gacS* double mutant shows a significant overlap. In total, 84% of the 397 genes of the *retS* regulon were also reciprocally controlled by GacS, including type III secretion, type VI secretion, pili and biofilm-related genes (A. Brencic and S. Lory, in prep.). Based on genetic data in various *Pseudomonas* species, and on biochemical data in *E. coli* and *Salmonella*, GacS is the likely cognate sensor kinase for the response regulator GacA, which is the transcription factor controlling the expression of the Rsm family of small RNAs. Additionally, neither RetS nor LadS are encoded by genes linked to specific response regulators; however, they also regulate the transcription of the *P. aeruginosa* small RNA RsmZ (Ventre et al. 2006). The findings described in this study indicate that *Pseudomonas* GacS can transphosphorylate GacA, and suggest that the sensor kinase RetS exerts its regulatory activity through a direct and specific interaction with GacS, interfering with its phosphorylation and presumably, limiting phosphorylation of GacA. In this way, the signals from two sensor kinases are integrated not at the level of cross-phosphorylation of a shared response regulator, but instead, by the formation of a heteromeric complex in the bacterial membrane.

Several lines of evidence suggest that the interaction between GacS and RetS is direct. GacS bound to RetS can be isolated from *P. aeruginosa* membranes as a detergent-stable complex. Fusion proteins of putative interactive domains of the proteins expressed in *E. coli* strongly activate a two-hybrid reporter system that detects heterodimer formation. In vitro, the purified soluble portion of RetS directly binds the corresponding domain of GacS with high affinity. Reconstituted phospho-transfer reactions, consisting of purified components, further suggest that the RetS/GacS interaction modulates the phosphorylation state of GacS. Interestingly, the conserved histidine residue in the histidine phosphotransferase domain (H424) and the aspartate resides in the adjacent two receiver domains (D713 and D858) are not required for RetS activity in regulating *rsmZ* expression in vivo or for its ability to interfere with GacS phosphorylation in vitro.
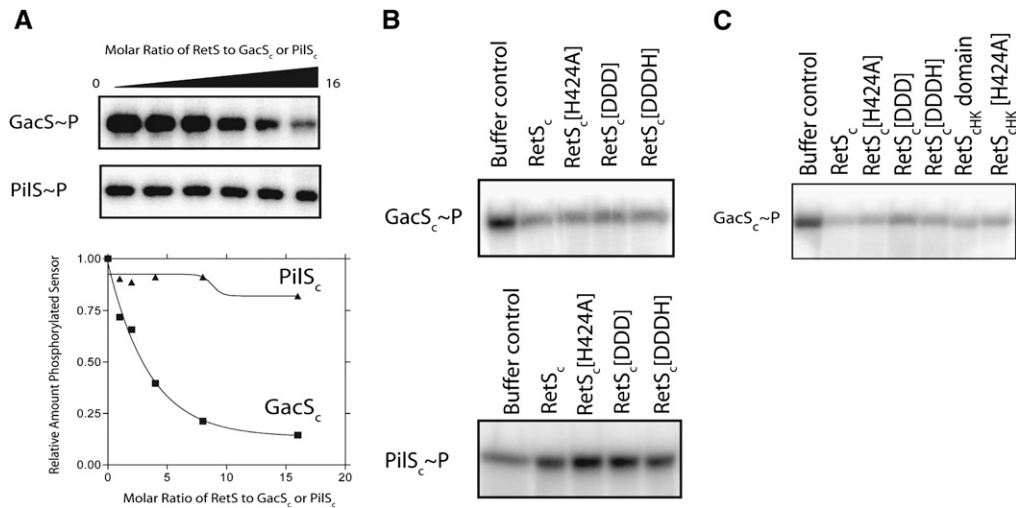
Figure 6. RetS suppresses GacS autotophosphorylation in a phosphorelay-independent fashion. (*A*) Dose-dependent inhibition of $GacS_c$ phosphorylation by $RetS_c$. Purified $GacS_c$ and the unrelated sensor kinase $PilS_c$ were incubated in the presence of increasing amounts (0–32 μM) of $RetS_c$ for 5 min, followed by addition of 500 μM [γ^{32}P]ATP at 0.6 Ci/mmol for 15 min. Reaction products were analyzed by SDS-PAGE and storage phosphorimaging. Signal intensities are quantified in the *bottom* panel. (*B*) The conserved phosphorelay residues of $RetS_c$ are not required for its ability to inhibit $GacS_c$ phosphorylation. Purified $GacS_c$ (or $PilS_c$) were incubated with 500 μM [γ^{32}P]ATP for 15 min in the presence of wild-type $RetS_c$ and variants with alanine or asparagine substitutions for the canonical histidine and aspartate residues, respectively. The extent of phosphorylation was determined following SDS-PAGE by storage phosphorimaging. (*C*) $RetS_c$ reduces the phosphorylation level of prephosphorylated $GacS_c$. $GacS_c$ was preincubated with [γ^{32}P]ATP and subjected to size-exclusion chromatography on a G-25 column to remove unincorporated [γ^{32}P]. Phospho-$GacS_c$ was incubated with wild-type $RetS_c$ or its allelic variants for 30 min.

Recent studies in the *P. aeruginosa* strain PA103 have shown that a mutant RetS allele missing the conserved residue H424 is partially able to complement a *retS* deletion for TTSS expression, although this phenotype
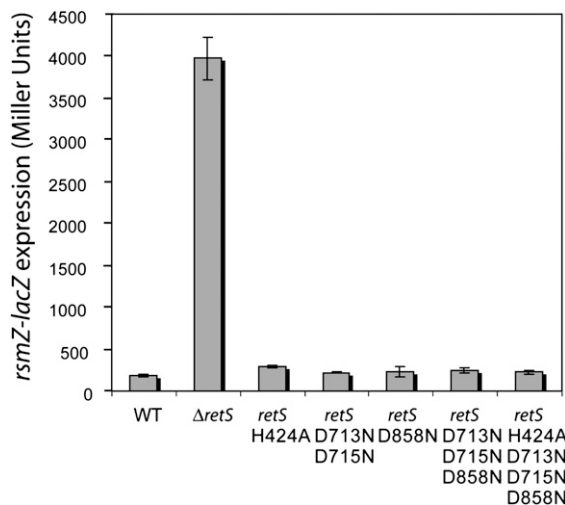

Figure 7. RetS-dependent regulation of *rsmZ* is independent of conserved phosphorelay residues in vivo. A *P. aeruginosa* carrying a chromosomal *rsmZ-lacZ* fusion and various chromosomal alleles of *retS* (expressing wild-type RetS and variants with single or multiple point mutations inactivating predicted phosphorelay residues) were grown in LB for 8 h and assayed for β-galactosidase activity. The conserved residues tested are all dispensable for RetS function in this assay.

is reported to be temperature-dependent (Laskowski and Kazmierczak 2006). This group also reports that the residues D713 and D858 are dispensable and required, respectively, for RetS function. The basis of these discrepancies is unclear. Additionally, work by Hsu et al. (2008) has shown that RetS, and three additional *P. aeruginosa* sensor histidine kinases, can phosphorylate the histidine phosphotransfer protein HptB in vitro. The same four kinases were shown to interact with HptB in a bacterial two-hybrid assay. Hsu et al. (2008) further showed that although RetS lacks autophosphorylation activity as we have observed, it can receive phosphate from phosphorylated HptB. Whether this reaction occurs in vivo, requires the conserved RetS phosphorelay residues, or has a role in the downstream *rsmZ*-dependent signaling pathway examined here is not known. Nevertheless, this work raises the intriguing possibility that in addition to interfering with GacS autophosphorylation, RetS may participate in additional phosphotransfer reactions, which may explain the evolutionary conservation of essential residues in its histidine kinase and receiver domains.

Our findings reported here provide evidence for a model in which the unorthodox sensor kinase RetS regulates the expression of its downstream targets by a novel mechanism, involving interference with another sensor kinase through the formation of heteromeric complexes. This activity is consistent with the dispensability of its conserved phosphorelay residues. Sensor kinases involved in multistep phosphorelay reactions, such as GacS, share a conserved modular architecture and a common mech-
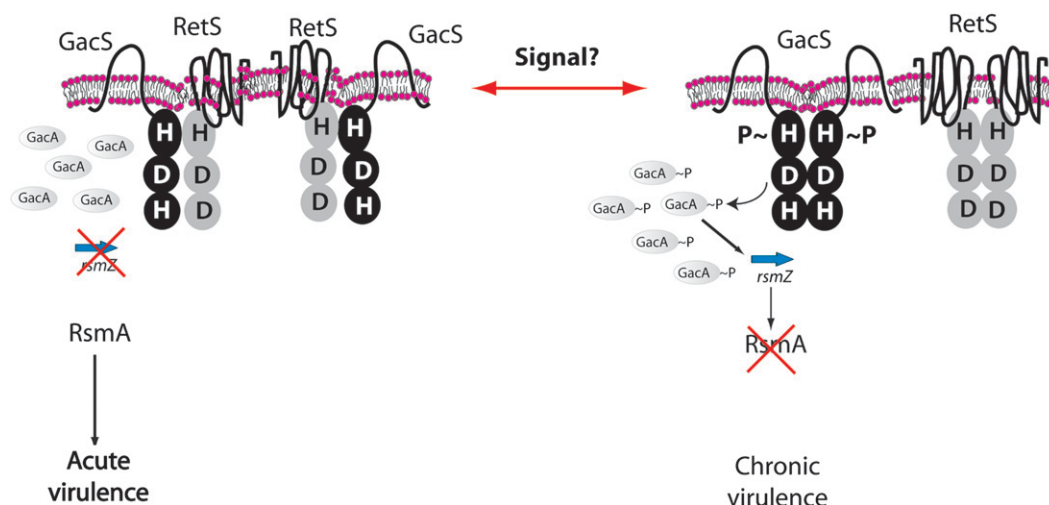
Figure 8. Model for regulation of GacS/GacA signal transduction by RetS. *P. aeruginosa* reciprocally regulates genes involved in acute and chronic infection through the interaction between the two membrane-bound sensors RetS and GacS. During the acute virulence phase, RetS forms heterodimers with GacS, blocking GacS autophosphorylation (and subsequent phosphotransfer to the response regulator GacA) leading to reduction in *rsmZ* expression. RsmA lacking this regulatory RNA promotes the translation of genes for acute virulence factors, while destabilizing transcripts of factors important in chronic infections. Upon perception of unknown signals, GacS and RetS each form homodimers, allowing autophosphorylation of GacS and phosphorylation of GacA. The Gac-dependent small RNA RsmZ sequesters the mRNA-binding protein RsmA, resulting in the expression of genes involved in chronic infection.

anism of phosphate transfer. They function as homodimers in which the γ-phosphate from ATP bound by one monomer is transferred intermolecularly to the conserved histidine on the histidine kinase domain of the opposing monomer. The experiments described here are consistent with a model in which RetS blocks GacS phosphorylation at this very early step of the signal transduction pathway, by disrupting the formation of GacS homodimers and instead forming a nonproductive GacS: RetS heterodimer or a higher-order structure of GacS and RetS homodimer (Fig. 8). Several biophysical approaches from structure determination, to dynamic light scattering following size-exclusion chromatography may be used in future studies to test these two hypotheses in detail.

Analysis of global gene expression in *P. aeruginosa* has shown that RetS and GacS are constitutively coexpressed under most conditions. What signals trigger the activation of RetS is unclear. RetS (and LadS) contain a putative periplasm-facing signal receiver domain, referred to by PFAM designation 7TMR-DISMED2, located between a single N-terminal transmembrane segment on one side and seven transmembrane segments on the other. It is conceivable that RetS forms a homodimer where it remains sequestered from GacS. Binding of an external signal to the 7TMR-DISMED2 could dissociate the homodimer and make RetS monomers available for inhibitory interactions with GacS. Identification of the ligands that activate RetS would provide a critical piece of information toward testing this and perhaps other models of RetS activity.

The observation that heterologous sensor kinases can interact directly adds an additional level of signaling potential to this very broadly conserved protein family. By modulating signal transduction at the level of sensor kinase phosphorylation, multiple inputs can be integrated without the need for cross-phosphorylation between sensors and noncognate response regulators. For orphan senor kinases, which are quite abundant in a number of microorganisms, this mechanism could provide a simple means for controlling the expression of genes that respond to multiple signals.

Materials and methods

Bacterial strains growth conditions

Bacterial strains and plasmids are described in Supplemental Table S1. Unless otherwise stated, bacteria were grown at 37°C in LB medium or ECPM1 (Coligan et al. 2007). McConkey-maltose media was prepared as described (Ausubel et al. 1987). Antibiotics were added at the following concentrations: carbenicillin 100 µg mL^{-1} (for *E. coli*) or 150 µg mL^{-1} (for *P. aeruginosa*); gentamicin 15 µg mL^{-1} (*E. coli*) or 75 µg mL^{-1} (for *P. aeruginosa*); chloramphenicol 34 µg mL^{-1}; kanamycin 50 µg mL^{-1}. For *Pseudomonas* selection media, Irgasan at 25 µg mL^{-1} was used.

Molecular and genetic techniques

DNA purification, molecular cloning, and PCR were performed following standard procedures as described (Ausubel et al. 1987). Deletion strains were constructed by SOE-PCR of deletion alleles, ligation into plasmid pEXG2 (Rietsch et al. 2005), and allelic exchange by sucrose selection on LB agar containing 6% sucrose as described (Horton et al. 1989). Plasmids were introduced into recipient cells by electroporation or conjugation by triparental mating using pRK2013 as the helper plasmid (Ditta et al. 1980). All mutations were verified by PCR and sequencing

using a Big Dye fluorescent terminator and an ABI3770 capillary sequencer at the Dana Farber Cancer Center Core Laboratory. Transposon mutagenesis was performed as previously described with a *mariner* element derivative of Mar2xT7 (Liberati et al. 2006).

Plasmid construction

Genes and deletion alleles were amplified from *P. aeruginosa* PAK genomic DNA using the primers described in Supplemental Table S1. *P. aeruginosa* expression constructs were appended with the C-terminal epitopes VSV-G (YTDIEMNRLGK) or His$_6$ by PCR and cloned into expression vectors described in Supplemental Table S1. Plasmid pET15b (Novagen) was used to engineer N-terminally tagged RetS and GacS proteins for expression in *E. coli*. For expression of GacA fused to the MBP, the gene was amplified from genomic DNA, and ligated into the expression vector pMalc2X.

Site-directed mutagenesis

Oligonucleotide-directed mutagenesis of the pPSV35 and pET15b plasmids carrying a *retS* allele was performed using the primer pairs described in Supplemental Table S1 and the Quick-Change protocol (Clontech) modified by a two-step procedure (Wang and Malcolm 2002). Mutant clones were screened by PCR followed by digestion at restriction sites engineered with silent mutations next to the substituted codons or by direct sequencing.

Bacterial two-hybrid assay

Bacterial two-hybrid experiments were conducted as described (Karimova et al. 1998). PCR fragments corresponding to *gacS*, *retS*, and *pilS* were cloned into the pUT18c and pKT25 vectors to create chimeric proteins with 18-kDa C-terminal adenylate cyclase (CyaA) fragment (amino acids 225–399) or 25-kDa N-terminal adenylate cyclase fragment (amino acids 1–224) fusions, respectively. All fusion proteins were confirmed by DNA sequencing. cAMP production by reconstituted CyaA was measured indirectly by maltose or lactose metabolism in an *E. coli cya* mutant (DHM1) transformed with the pKT25- and pUT18-derived plasmids. Maltose metabolism was assayed on McConkey plates after incubation 2–4 d at 30°C. Red colonies appeared when reconstitution of active CyaA was obtained. The strength of complementation was quantified by measuring β-galactosidase assays in cells grown overnight at 30°C in LB medium supplemented with 1.0 mM IPTG (Miller 1992).

Protein expression and purification

For His-tagged protein expression and purification, *E. coli* BL21 carrying pET15b plasmids described in Supplemental Table S1 were grown in 1 L LB or ECPM1 until the culture reached an OD (at 600 nm) of 0.6 (LB) or 5.0 (ECPM1). IPTG was added to a final concentration of 0.3 mM and the cultures were grown at 15°C for 15 h or at 30°C for 5 h. Cells were harvested by centrifugation and resuspended in 40 mL of ice-cold lysis buffer (5 mM imidazole, 500 mM NaCl, 10% glycerol, 0.1% Triton X-100, 1 mM PMSF, 20 mM HEPES at pH 8.0 at 4°C), containing 500 μL of EDTA-free protease inhibitor cocktail (Sigma). The cell suspension was sonicated with a Branson Sonic disruptor at 50% duty, 10 sec on/30 sec off for 25 cycles. The lysate was first centrifuged at 6000*g* for 10 min, then at 45,000*g* for 60 min at 4°C. Column chromatography was performed at 4°C. The supernatant was loaded with a Pharmacia P-1 peristaltic pump onto a 1-mL His-

Trap FF (fast flow Ni^{2+}-Sepharose 6B; Amershan/GE) column equilibrated with 20 column volumes (CV) of buffer A (5 mM imidazole, 500 mM NaCl, 10% glycerol, 0.1% reduced Triton X-100, 20 mM HEPES at pH 8.0 at 4°C). Chromatography was performed in an AKTA-Prime FPLC apparatus (Amershan/GE). The column was washed with 20 CV of buffer A or until the UV absorption readings stabilized, then weakly bound proteins were washed with a 0%–12% gradient of buffer B (500 mM imidazole, 500 mM NaCl, 10% glycerol, reduced 0.1% Triton X-100, 20 mM HEPES at pH 8.0 at 4°C) until UV absorbance returned to background. His6-tagged proteins were eluted with a 12%–100% gradient of buffer B in 20 CV. Fractions containing the UV-absorbing peak were analyzed by SDS-PAGE, pooled and buffer-exchanged in a G-25 HiLoad column (Amersham/GE) equilibrated with storage buffer (200 mM KCl, 2 mM DTT, 0.2 mM EDTA, 20 mM HEPES at pH 8.0). Proteins were concentrated in Vivaspin 15R (Viva Science) ultrafiltration devices (10 kDa molecular weight cutoff) and stored at −80°C after addition of sterile glycerol to 50% final concentration. His6-GacS$_c$ was further purified by size exclusion chromatography using a Sephacryl S-200 HR column (Amersham/GE) equilibrated with storage buffer.

For purification of MBP-tagged GacA, the protein was expressed from the pMalc2X in *E. coli* T7Express LacI^q (New England Biolabs), a BL21(DE3) derivative. Cultures were grown in 1 L of EPCM1 medium with induction at 15°C for 14 h with 0.5 mM IPTG when OD$_{600}$ reached 1.0. Cleared cell lysates were obtained as described above. Low-pressure chromatography using a high-flow amylose resin (New England Biolabs) packed on a 25-mL column was performed with buffer C (20 mM HEPES at pH 8.0, 250 mM NaCl, 0.2% NP-40, 1 mM EDTA) as the eluant. The bound protein was eluted with 10 mM maltose in buffer C, desalted on a HiLoad G-25 column, and further purified by anion exchange chromatography on a mono-Q HiTrap column. Purified fractions were concentrated by ultrafiltration as described above. Protein concentration was determined by UV spectroscopy using extinction coefficients calculated as described (Gill and von Hippel 1989). The purity of proteins was evaluated by SDS-PAGE followed by Coomassie Brilliant Blue staining (Laemmli 1970).

Isothermal titration calorimetry

Isothermal titration calorimetry experiments were performed using a VP-ITC microcalorimeter (MicroCal). Recombinant proteins (GacS$_c$, RetS$_c$, and PilS$_c$) were buffer-exchanged into 20 mM HEPES (pH 8.0), 200 mM KCl, 0.2 mM EDTA by G-25 spin-column chromatography; concentration was determined by amino acid analysis using a Beckman Model 7300 instrument. A solution containing 5 μM GacS$_c$ or PilS$_c$ was used as titrant, whereas solutions containing 0.1 μM RetS$_c$ were used in the calorimetry cell. The heat of reaction per injection (microcalories per second) was determined by integration of the peak areas using the Origin version 5.0 software (OriginLab). Heat of binding ($\Delta H°$), the stoichiometry of binding (n), and the dissociation constant (K_d) were calculated from plots of the heat produced per mole of ligand injected versus the molar ratio of ligand to receptor (Ladbury and Chowdhry 1996; Ladbury 2004).

In vitro phosphorylation assay

For autophosphorylation and phosphotransfer assays, 2 μM GacS$_c$ or PilS$_c$ was autophosphorylated with 500 μM [γ^{32}P]ATP (0.08 Ci/mmol). GacS$_c$ experiments were conducted in presence or absence of the response regulator GacA. All phosphorylation

assays were conduced in 10 mM HEPES (pH 8.0), 5 mM MgCl$_2$, 50 mM KCl$_2$, 1 mM DTT, and 0.1 mM EDTA in a final volume of 20 μL. Reactions were stopped by adding Laemli sample buffer (Laemmli 1970) and they were analyzed by SDS-PAGE. The extent of phosphorylation was determined by storage phosphorimaging. When GacS$_c$~P was required, the autophosphorylation reaction was passed over a G-25 column to separate unincorporated nucleotides from the phosphoprotein. The half life of GacS$_c$~P was determined by fitting the experimental data into an exponential decay model using GraphPad Prism (version 3.02).

Thin-layer chromatography

For the analysis of the nucleotides, 0.5- to 1.0-μL products from the phosphorylation reactions were spotted on PEI-cellulose plates (Sigma) predeveloped with water and methanol. The samples were run with 1.5 M KH$_2$PO$_4$ (pH 3.65) as the mobile phase and air-dried, and plates were analyzed by storage phosphorimaging.

In vivo cross-linking and purification of RetS:GacS complexes

For in vivo cross-linking experiments, bacteria were grown overnight and inoculated 1:1,000 in 1 L LB with 1 mM IPTG and appropriate antibiotics until cultures reached OD ~1.0. Cells were pelleted at 5000g at 20°C and resuspended in 100 mL 1× PBS. Where indicated, the cross-linking agent DSP was added to 1 mM final concentration and cells were incubated for 30 min at 37°C with agitation. The cross-linking reaction was quenched with 50 mM Tris-HCl for 20 min and cells were pelleted. Bacterial pellets were resuspended in 35 mL of buffer D (20 mM Na$_2$HPO$_4$ at pH 8.0, 500 mM NaCl, 5 mM imidazole, 2% CHAPS, 0.5% Triton X-100, 1 mM PMSF, 200 μL of Sigma protease inhibitor cocktail, 5 mg/mL lysozyme) and lysed in a Branson sonicator, and the unlysed cells were removed by centrifugation at 20,000g for 45 min. Supernatants were incubated with 2 mL of Ni-NTA slurry for 2 h at 4°C. The Ni^{2+}-IMAC resin was packed into a Bio-Rad Econo-Column and washed with 25 CV of buffer E (20 mM Na$_2$HPO$_4$ at pH 8.0, 500 mM NaCl, 5 mM imidazole, 0.2% CHAPS, 0.1% Triton X-100), followed by 25 CV of buffer E with 50 mM imidazole. The His6-tagged proteins were eluted with buffer E containing 250 mM imidazole. Protein eluates were concentrated in an Amicon ultrafiltration device (10 kDa MWCO) and stored at −80°C until analysis by Western immunoblotting and mass spectrometry. Gel loading was normalized per number of cells. For total membrane purification, bacteria were grown as above, lysed, and total membrane purified as described previously (Merighi et al. 2007).

Western blot analysis

Proteins were separated by SDS-PAGE and transferred to a PVDF membrane. PVDF membranes were blocked overnight with 20 mM Tris-HCl at pH 7.5, 150 mM NaCl (TBS) with 3% nonfat dry milk at 4°C, rinsed in TBST (20 mM Tris-HCl at pH 7.5, 150 mM NaCl, 0.05% Tween-20) and probed with monoclonal antibodies diluted in TBS–1% nonfat dry milk. Anti-His antibody (H3; Santa Cruz Biotechnologies) was diluted 1:1500, monoclonal anti-VSV-G antibodies (Santa Cruz Biotechnologies) was diluted 1:20,000. Antibodies to XcpT and XcpY were described previously (Nunn and Lory 1993; Michel et al. 1998) and were used at a 1:4000 dilution. The PVDF membranes were washed and probed with goat anti-mouse IgG(H + L) conjugated with HRP (KPL). Supersignal West Pico chemiluminescent substrate (Pierce) was used for the detection of the conjugates. Blots were reprobed following

washings with 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 100 mM β-mercaptoethanol for 30 min at 55°C.

Mass spectrometry

Proteins were separated by SDS-PAGE, fixed, and stained with Coomassie Brilliant Blue. Bands of interest were excised and treated with MS-grade trypsin and peptides were extracted follwing treatment with DTT and iodoacetamide alkylation as described (Coligan et al. 2007). Peptides were analyzed using a cyclotron ion trap (FT-ICR) spectrometer at the Harvard Partners Center for Genetics and Genomics.

β-Galactosidase assay

β-Galactosidase assays were carried out using a spectrophotometric method with ortho-nitrophenyl-β-D-galactopyranoside (ONPG) as a substrate (Miller 1992). Assays were performed in triplicate. Specific enzyme activities are reported in Miller units (ONPG assays).

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Direct interaction between sensor kinase proteins mediates acute and chronic disease phenotypes in a bacterial pathogen

Andrew L. Goodman, Massimo Merighi, Mamoru Hyodo, et al.

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