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Mutational Analysis of RetS, an Unusual Sensor Kinase-Response Regulator Hybrid Required for *Pseudomonas aeruginosa* Virulence†

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***Pseudomonas aeruginosa* is an opportunistic pathogen capable of causing both acute and chronic infections in a wide range of hosts. Expression of the type III secretion system (T3SS) proteins is correlated with virulence in models of acute infection, while downregulation of the T3SS and upregulation of genes important for biofilm formation are observed during chronic infections. RetS, a hybrid sensor kinase-response regulator protein of *P. aeruginosa*, plays a key role in the reciprocal regulation of virulence factors required for acute versus chronic infection and is postulated to act in concert with two other sensor kinase-response regulator hybrids, GacS and LadS. This work examines the roles of the putative sensing and signal transduction domains of RetS in induction of the T3SS in vitro and in a murine model of acute pneumonia. We identify distinct signaling roles for the tandem receiver domains of RetS and present evidence suggesting that RetS may serve as a substrate for another sensor kinase. Phenotypes associated with RetS alleles lacking periplasmic and/or transmembrane domains further indicate that the periplasmic domain of RetS may transmit a signal that inhibits RetS activity during acute infections.**

Pseudomonas aeruginosa is a highly virulent, gram-negative, opportunistic pathogen that causes both chronic and acute infections in cystic fibrosis patients, burn victims, and other immunocompromised individuals (32). Many virulence factors have been implicated in *P. aeruginosa* pathogenesis, including a type III secretion system (T3SS) that has been closely linked to morbidity and mortality in animal models as well as humans (16, 37). *P. aeruginosa* translocates four effectors through its type III secretion (T3S) apparatus: ExoU, ExoS, ExoT, and ExoY (11, 17, 53–55). Effector translocation inhibits bacterial phagocytosis, causes extensive tissue damage, and elicits a host inflammatory response.

Expression of and secretion by the T3SS are induced by contact with host cells but can also be elicited in vitro by growing bacteria in the presence of calcium chelators, such as nitriloacetate (NTA) or EGTA (11, 23). We recently identified RetS/RtsM as a hybrid sensor kinase-response regulator protein required for expression of the T3SS in response to in vitro inducing conditions and host cell contact and for virulence in a murine model of acute pneumonia (29). RetS was independently identified by Goodman et al. and Zolfaghar et al., who found that RetS also regulates type IV pilus expression and exopolysaccharide synthesis (15, 56). A suppressor screen and subsequent studies indicate that two other hybrid sensor kinase proteins, GacS and LadS, also regulate the same groups of genes as RetS does by directly or indirectly controlling levels of the small regulatory RNA *rsmZ* (15, 49). *rsmZ* titrates the amount of free, or active, RsmA in the cell; RsmA is a global regulator which appears to control gene expression at the post-transcriptional level (8, 19). Recent microarray analysis of a PAO1 *rsmA* mutant has revealed that 53 genes whose expression is altered in this background also show altered expression in the *retS* mutant background, supporting the hypothesis that the *rsmZ*/RsmA system is regulated by RetS (9).

The RetS gene encodes a hybrid two-component signaling protein with a sensor kinase domain followed by two response regulator receiver domains in tandem (Fig. 1A) (6). Hybrid proteins containing both sensor kinase and response regulator receiver domains are common among bacteria; however, the presence of two tandem response regulator receiver domains is unusual. Signaling through two-component systems begins with the autophosphorylation of a sensor kinase at a conserved histidine (His) residue, usually in response to an extracellular signal. This phosphate is ultimately the substrate of a phosphotransfer reaction to a conserved aspartate (Asp) residue in a cognate response regulator domain/protein. Phosphorylation of the response regulator results in a change in activity or function of the protein (43). The phosphotransfer reaction can occur directly or, in the case of phosphorelays, may involve protein intermediates with response regulator or Hpt domains. However, the phosphotransfer always occurs from His to Asp to His to Asp residues (5), suggesting either that an unidentified Hpt intermediate interacts with RetS or that the two receiver domains are not functionally equivalent. Two other proteins possessing tandem response regulator receiver domains have been described: PleD, a protein required for polar development during the swarmer-to-stalked-cell transition of *Caulobacter crescentus* (1, 2, 18), and FrzZ, a protein required for swarming motility and fruiting-body formation in *Myxococcus xanthus* (47). The second receiver domain of PleD does not appear to be functional, as it lacks many of the conserved residues characteristic of response regulator domains, including the invariant aspartate residue that is the site of phosphorylation (18). The two receiver domains of FrzZ both contain

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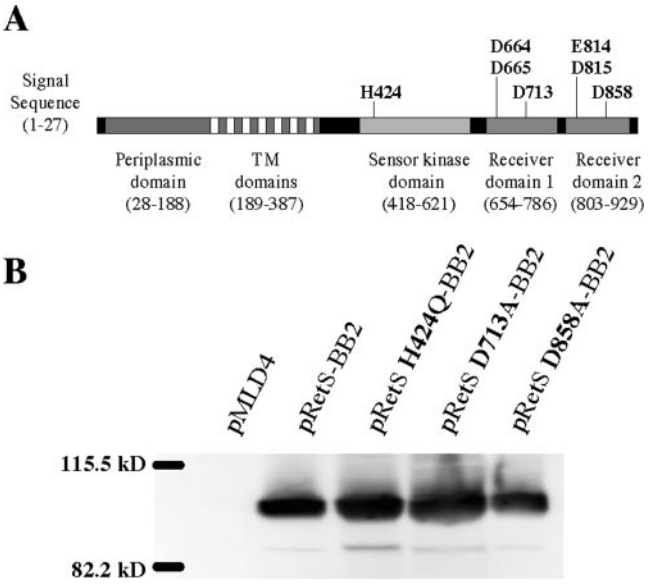

FIG. 1. (A) Schematic representation of RetS domain structure and mutagenesis targets. *retS* encodes a 942-amino-acid protein predicted to contain an N-terminal signal sequence, a large periplasmic region, seven transmembrane domains, a sensor kinase domain, and two response regulator receiver domains in tandem. Residues targeted for mutagenesis are shown, with the predicted sites of phosphorylation being H424, D713, and D858. (B) Steady-state levels of RetS variants containing point mutations at the predicted sites of phosphorylation. Δ*retS* strains expressing untagged RetS (pMLD4), epitope-tagged wild-type RetS (pRetS-BB2), and epitope-tagged RetS containing various point mutations (pRetS H424Q-BB2, pRetS D713A-BB2, and pRetS D858A-BB2) were grown in LB-carbenicillin overnight. Bacterial pellets were lysed, and 15 μg of total protein was separated by SDS-PAGE. Proteins were transferred to a PVDF membrane and detected by Western blotting using a monoclonal anti-BB2 antibody.

the conserved residues characteristic of response regulator domains but do not appear to be functionally equivalent, as demonstrated by distinct patterns of protein-protein interaction in a yeast two-hybrid screen (51).

In this study, we used a mutational approach to analyze the role of the putative signaling domains in RetS-mediated regulation of T3S. We also examined the roles of the periplasmic and transmembrane regions of RetS in sensing and responding to signals that induce T3SS expression. Our work, which is the first mutational analysis of a hybrid sensor kinase-response regulator protein with this domain architecture, reveals that the tandem receiver domains have distinct roles in controlling RetS activity. Phenotypes associated with point mutation of the RetS sensor kinase histidine indicate that RetS may serve as a substrate for another sensor kinase. Lastly, results of in vivo assays suggest that the periplasmic domain of RetS may transmit a signal that inhibits RetS activity during acute infection.

MATERIALS AND METHODS

Bacterial strains and plasmids. All bacterial strains and plasmids used in this study used in this study are listed in Tables 1 and 2, respectively. *P. aeruginosa* strains were maintained on Vogel-Bonner minimal medium or cultured in Luria broth (LB) with antibiotics as required (200 μg/ml carbenicillin, 100 μg/ml tetracycline) (50). *Escherichia coli* strains were cultured in LB with antibiotics as required (100 μg/ml ampicillin, 20 μg/ml tetracycline). All strains were maintained at −80°C as 15% glycerol stocks. To induce T3S, *P. aeruginosa* strains were grown in MinS, a minimal medium containing 10 mM of the calcium chelator NTA (35), at 30°C or 37°C, as noted, with aeration. This medium was rendered noninducing by the omission of NTA and the addition of 2.5 mM $CaCl_2$.

Construction of RetS mutations. All PCR primers employed in this study are listed in Table 3 and were obtained from either Yale University Pathology Department, Invitrogen, or Integrated DNA Technologies. Primer sequences are based on the PAO1 genome sequence (www.pseudomonas.com) (44). All amplifications were carried out with *Pfu* Turbo polymerase (Stratagene), with various plasmids as template, as listed. For cloning purposes, we generated a derivative of pMLD4 (29) lacking the restriction sites between EcoRI and XhoI, inclusive, in the vector backbone. Briefly, pMLD4 was digested with EcoRI and

TABLE 1. Bacterial strains used in this study

Strain	Description[a]	Reference or source
E. coli		
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q} *ZΔM15* Tn*10* (Tc^r)]	Stratagene
XL2-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q} *ZΔM15* Tn*10* (Tc^r) Amy Cm^r]	Stratagene
S17-1	Used for inserting mating constructs into *P. aeruginosa*; *thi pro hsdR recA RP4-2* (Tc::Mu) (Km::Tn*7*)	42
P. aeruginosa		
PA103	Virulent lung isolate; known T3SS effectors are ExoT and ExoU	31
Δ*retS* (Δ*rtsM*)	PA103 with in-frame deletion of aa 37 to 924 of *retS* (*rtsM*)	29
Δ*retS attB*::*retS*	*retS* (expressed from its own promoter) integrated at the *attB* site	29
Δ*retS attB*::*retS* H424Q	*retS* H424Q integrated at the *attB* site	This study
Δ*retS attB*::*retS* D664A D665A	*retS* D664A D665A integrated at the *attB* site	This study
Δ*retS attB*::*retS* D713A	*retS* D713A integrated at the *attB* site	This study
Δ*retS attB*::*retS* E814A D815A	*retS* E814A D815A integrated at the *attB* site	This study
Δ*retS attB*::*retS* D858A	*retS* D858A integrated at the *attB* site	This study
Δ*retS attB*::*retS* H424Q D713A	*retS* H424Q D713A integrated at the *attB* site	This study
Δ*retS attB*::*retS* H424Q D858A	*retS* H424Q D858A integrated at the *attB* site	This study
Δ*retS attB*::*retS* D713A D858A	*retS* D713A D858A integrated at the *attB* site	This study
Δ*retS attB*::*retS* H424Q D713A D858A	*retS* H424Q D713A D858A integrated at the *attB* site	This study
Δ*retS attB*::*retS* Δ37-185	*retS* Δ37-185 integrated at the *attB* site	This study
Δ*retS attB*::*retS* Δ37-360	*retS* Δ37-360 integrated at the *attB* site	This study

[a] Abbreviations: Ap, ampicillin; Cb, carbenicillin; Cm, chloramphenicol; Gm, gentamicin; Km, kanamycin; Tc, tetracycline; aa, amino acids.

TABLE 2. Plasmids used in this study

Plasmid	Description[a]	Source or reference
pGEM-T	Cloning vector; Ap^r	Promega
pBS-SK (pBluescriptSK)	Cloning vector; Ap^r	Stratagene
pUCP-SK	*P. aeruginosa-E. coli* shuttle vector; Ap^r (Cb^r)	52
mini-CTX-2	Contains *attP* site for integration at chromosomal *attB site*; Tc^r	21
pFLP2	Source of inducible *flp* recombinase; Ap^r (Cb^r)	20
pMLD4	3.5-kb NotI-NsiI fragment containing *retS* under control of its own promoter from cosmid pM0013207 into NotI-PstI of pUCP-SK; Ap^r (Cb^r)	29
pRetS	pMLD4 derivative in which the EcoRI-XhoI region of the multiple cloning site has been removed; Ap^r (Cb^r)	This study
pRetS-BB2	pMLD4 derivative carrying C-terminal BB2-tagged *retS* under the control of its own promoter; Ap^r (Cb^r)	29
Mini-CTX-2-*retS*	3.5-kb NotI-EcoRI fragment from pMLD4 in NotI-EcoRI of mini-CTX-2; Tc^r	29
pRetS-SK	1.0-kb SalI-EcoRV fragment of pRetS containing aa 152 to 500 of RetS into SalI-EcoRV of pBS-SK; Ap^r	This study
pRetS-SK H424Q	pRetS-SK containing H424Q point mutation; Ap^r	This study
pRetS-R1	2.7-kb NotI-BamHI fragment of pRetS containing 259 bp upstream and aa 1 to 810 of RetS into NotI-BamHI of pBS-SK; Ap^r	This study
pRetS-R1 D664A D665A	pRetS-R1 containing D664A D665A point mutations; Ap^r	This study
pRetS-R1 D713A	pRetS-R1 containing D713A point mutation; Ap^r	This study
pRetS-R2	1.8-kb EcoRV-KpnI fragment from pRetS containing aa 498 to 942 and 457 bp downstream of RetS into EcoRV-KpnI of pBS-SK; Ap^r	This study
pRetS-R2 E814A D815A	pRetS-R2 containing E814A D815A point mutations; Ap^r	This study
pRetS-R2 D858A	pRetS-R2 containing D858A point mutation; Ap^r	This study
pRetS H424Q	1.0-kb SalI-EcoRV fragment of pRetS-SK H424Q into SalI-EcoRV of pRetS; Ap^r (Cb^r)	This study
pRetS D664A D665A	2.7-kb NotI-BamHI fragment of pRetS-R1 D664A D665A into NotI-BamHI of pRetS; Ap^r (Cb^r)	This study
pRetS D713A	2.7-kb NotI-BamHI fragment of pRetS-R1 D713A into NotI-BamHI of pRetS; Ap^r (Cb^r)	This study
pRetS E814A D815A	1.8-kb EcoRV-KpnI fragment of pRetS-R2 E814A D815A into EcoRV-KpnI of pRetS; Ap^r (Cb^r)	This study
pRetS D858A	1.8-kb EcoRV-KpnI fragment of pRetS-R2 D858A into EcoRV-KpnI of pRetS; Ap^r (Cb^r)	This study
pRetS H424Q D713A	1.0-kb SalI-EcoRV fragment of pRetS H424Q into SalI-EcoRV of pRetS D713A; Ap^r (Cb^r)	This study
pRetS H424Q D858A	1.0-kb SalI-EcoRV fragment of pRetS H424Q into SalI-EcoRV of pRetS D858A; Ap^r (Cb^r)	This study
pRetS D713A D858A	855-bp BamHI-KpnI fragment of pRetS D858A into BamHI-KpnI of pRetS D713A; Ap^r (Cb^r)	This study
pRetS H424Q D713A D858A	1.0-kb SalI-EcoRV fragment of pRetS H424Q into SalI-EcoRV of pRetS D713A D858A; Ap^r (Cb^r)	This study
pRetS H424Q-BB2	2.7-kb NotI-BamHI fragment of pRetS H424Q into NotI-BamHI of pRetS-BB2; Ap^r (Cb^r)	This study
pRetS D713A-BB2	2.7-kb NotI-BamHI fragment of pRetS D713A into NotI-BamHI of pRetS-BB2; Ap^r (Cb^r)	This study
pRetS D858A-BB2	330-bp BamHI-MfeI fragment of pRetS D858A into BamHI-MfeI of pRetS-BB2; Ap^r (Cb^r)	This study
mini-CTX-2-*retS* H424Q	3.5-kb NotI-KpnI fragment of pRetS H424Q into NotI-KpnI of mini-CTX-2; Tc^r	This study
mini-CTX-2-*retS* D664A D665A	3.5-kb NotI-KpnI fragment of pRetS D664A D665A into NotI-KpnI of mini-CTX-2; Tc^r	This study
mini-CTX-2-*retS* D713A	3.5-kb NotI-KpnI fragment of pRetS D713A into NotI-KpnI of mini-CTX-2; Tc^r	This study
mini-CTX-2-*retS* E814A D815A	3.5-kb NotI-KpnI fragment of pRetS E814A D815A into NotI-KpnI of mini-CTX-2; Tc^r	This study
mini-CTX-2-*retS* D858A	3.5-kb NotI-KpnI fragment of pRetS D858A into NotI-KpnI of mini-CTX-2; Tc^r	This study
mini-CTX-2-*retS* H424Q D713A	3.5-kb NotI-KpnI fragment of pRetS H424Q D713A into NotI-KpnI of mini-CTX-2; Tc^r	This study
mini-CTX-2-*retS* H424Q D858A	3.5-kb NotI-KpnI fragment of pRetS H424Q D858A into NotI-KpnI of mini-CTX-2; Tc^r	This study
mini-CTX-2-*retS* D713A D858A	3.5-kb NotI-KpnI fragment of pRetS D713A D858A into NotI-KpnI of mini-CTX-2; Tc^r	This study
mini-CTX-2-*retS* H424A D713A D858A	3.5-kb NotI-KpnI fragment of pRetS H424Q D713A D858A into NotI-KpnI of mini-CTX-2; Tc^r	This study
pKO-37-185	367-bp NotI-SpeI fragment (promoter and aa 1 to 36) and 939-bp SpeI-EcoRV fragment (aa 186 to 500) of RetS cloned in tandem into NotI-EcoRV of pBS-SK; Ap^r	This study
pKO-37-360	367-bp NotI-SpeI fragment (promoter and aa 1 to 36) and 414-bp SpeI-EcoRV fragment (aa 361 to 500) of RetS cloned in tandem into NotI-EcoRV of pBS-SK; Ap^r	This study
pRetS Δ37-185	1.3-kb NotI-EcoRV fragment of pKO-37-185 into NotI-EcoRV of pRetS; Ap^r (Cb^r)	This study
pRetS Δ37-360	780-bp NotI-EcoRV fragment of pKO-37-360 into NotI-EcoRV of pRetS; Ap^r (Cb^r)	This study
pRetS Δ37-185-BB2	160-bp MfeI-KpnI fragment of pRetS-BB2 into MfeI-KpnI of pRetS Δ37-185; Ap^r (Cb^r)	This study
pRetS Δ37-360-BB2	160-bp MfeI-KpnI fragment of pRetS-BB2 into MfeI-KpnI of pRetS Δ37-360; Ap^r (Cb^r)	This study
mini-CTX-2-*retS* Δ37-185	3.0-kb NotI-KpnI fragment of pRetS Δ37-185 into NotI-KpnI of mini-CTX-2; Tc^r	This study
mini-CTX-2-*retS* Δ37-360	2.5-kb NotI-KpnI fragment of pRetS Δ37-360 into NotI-KpnI of mini-CTX-2; Tc^r	This study

[a] Abbreviations: Ap, ampicillin; Cb, carbenicillin; Cm, chloramphenicol; Gm, gentamicin; Km, kanamycin; Tc, tetracycline; aa, amino acids.

XhoI, treated with T4 DNA polymerase (New England Biolabs), and ligated with T4 DNA ligase (New England Biolabs) to generate pRetS.

All point mutations were constructed by site-directed mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene) using the primer pairs listed in Table 3. For ease of construction of the mutations, small regions of RetS were cloned into pBS-SK and used as the template for site-directed mutagenesis as indicated below. The presence of desired mutations and absence of additional undesired mutations were confirmed by sequencing (Keck DNA Sequencing

TABLE 3. Primer sequences used in this study

Primer	Sequence[a]
exoT-RT-PCR-1	5′-CCGCCAGCCAGCATGTACTC-3′
exoT-RT-PCR-2	5′-GGGTGTTCGCCGAGGTACTG-3′
pelA-RT-PCR-1	5′-GGCGCTCACCGTACTCGATC-3′
pelA-PCR-2	5′-GATGCGCCGGCCATTCTCG-3′
pslD-RT-PCR-1	5′-CGCACGCCGCAGGAAATCG-3′
pslD-RT-PCR-2	5′-CTTCCGAGTCCTCGCGCATG-3′
rsmZ-RT-PCR-1	5′-CGTACAGGGAACACGCAAC-3′
rsmZ-RT-PCR-2	5′-AAAAAGGGGCGGGGTAATAC-3′
rplU-RT-PCR-1	5′-CGCAGTGATTGTTACCGGTG-3′
rplU-RT-PCR-2	5′-AGGCCTGAATGCCGGTGATC-3′
H424Q-1	5′-GCCAAGATCAGCCAGGAGATCCGCACGCCC-3′
H424Q-2	5′-GGGCGTGCGGATCTCCTGGCTGATCTTGGC-3′
D664A D665A-1	5′-GCTGCTGGTGGTGGCCGCCAACGAAACCTGCCGC-3′
D664A D665A-2	5′-GCGGCAGGTGGCGTTGGCGGCCACCACCAGCAGC-3′
D713A-1	5′-GTGGTCCTGCTCGCCCAGGACATGCCC-3′
D713A-2	5′-GGGCATGTCCTGGGCGAGCAGGACCAC-3′
E814A D815A-1	5′-GATCCTCGTCGCCGCGGCCAACAGCATCTCC-3′
E814A D815A-2	5′-GGAGATGCTGTTGGCCGCGGCGACGAGGATC-3′
D858A-1	5′-CTGGTGCTGATGCGGTGCGAGATGCCG-3′
D858A-2	5′-CGGCATCTCGCAGGCCATCAGCACCAG-3′
RetS 1-36-1	5′-GCGCGGCCGCGTGCTCTGTG-3′
RetS 1-36-2	5′-GCACTAGTGGGAACGCTGGAAACGCCG-3′
RetS 186-500-1	5′-GCACTAGTAAGCCCGCCTACGCCTTC-3′
RetS 186-500-2	5′-GCGATATCCAGGCAGTCCTCG-3′
RetS 361-500-1	5′-GCACTAGTCAACTCGATCCGGGCTG-3′
RetS 361-500-2	5′-GCGATATCCAGGCAGTCCTCG-3′

[a] Restriction enzyme sites are underlined.

Facility, Yale University). To construct the H424Q mutation in the sensor kinase domain, a 1.0-kb SalI-EcoRV fragment from pRetS was cloned into the SalI-EcoRV sites of pBS-SK to generate pRetS-SK. This plasmid was used as the template for site-directed mutagenesis using primers H424Q-1 and H424Q-2 to generate pRetS-SK H424Q. The SalI-EcoRV fragment containing the point mutation from pRetS-SK H424Q was then cloned into pRetS using the same sites (replacing the wild-type sequence) to generate pRetS H424Q. To express the point mutation as a single-copy integrate, the entire RetS open reading frame including 259 bp upstream and 457 bp downstream was cloned as a 3.5-kb NotI-KpnI fragment from pRetS H424Q into mini-CTX-2 (21) to generate mini-CTX-2-*retS* H424Q.

For mutations in the first receiver domain, a 2.7-kb NotI-BamHI fragment from pRetS was cloned into the NotI-BamHI sites of pBS-SK to generate pRetS-R1. This plasmid was used as the template for site-directed mutagenesis using primers D664A D665A-1 and D664A D665A-2 or D713A-1 and D713A-2 to generate pRetS-R1 D664A D665A and pRetS-R1 D713A, respectively. A strategy analogous to that described above was used to reintroduce these point mutations into pRetS and mini-CTX-2-*retS*. For mutations in the second receiver domain, a 1.8-kb NotI-BamHI fragment from pRetS was cloned into the EcoRV-KpnI sites of pBS-SK to generate pRetS-R2. This plasmid was used as the template for site-directed mutagenesis using primers E814A D815A-1 and E814A D815A-2 or D858A-1 and D858A-2 to generate pRetS-R2 E814A D815A and pRetS-R2 D858A, respectively. Again, a subcloning strategy was used to reintroduce these point mutations into pRetS and mini-CTX-2-*retS*. Mutations in the sensor kinase and receiver one and receiver two domains were further combined to generate a series of constructs with mutations in multiple domains as indicated in Table 2. A subset of the point mutations were tagged with the BB2 epitope tag as outlined in Table 2.

To construct RetS Δ37-185, a region containing the *retS* promoter and amino acids 1 to 36 of RetS (367 bp) was amplified from pMLD4 using primer pairs RetS 1-36-1 and RetS 1-36-2. Additionally, a region spanning amino acids 186 to 500 of RetS (939 bp) was amplified using primer pairs RetS 186-500-1 and RetS 186-500-2. The two regions were subcloned in tandem into pBS-SK via a SpeI site introduced by PCR to generate pKO-37-185. The entire 1.3-kb NotI-EcoRV site was cloned from pKO-37-185 into pRetS via the same sites to generate pRetS Δ37-185. To construct RetS Δ37-360, a region including amino acids 361 to 500 of RetS (414 bp) was amplified from pMLD4 using primer pair RetS 361-500-1 and RetS 361-500-2. This region was subcloned in tandem into pBS-SK with the region containing the *retS* promoter and amino acids 1 to 36 of RetS (367 bp) via a SpeI site introduced by PCR to generate pKO-37-360. The entire 780-bp NotI-EcoRV site was cloned from pKO-37-360 into pRetS via the same sites to generate pRetS Δ37-360. The deletions were cloned into mini-CTX-2 as described above for the point mutations to generate mini-CTX-2-*retS* Δ37-185 and CTX-2-*retS* Δ37-360. The RetS Δ37-185 and RetS Δ37-360 constructs were tagged with the BB2 epitope tag as outlined in Table 2.

All plasmids were introduced into *E. coli* strains by electroporation (39). Plasmids derived from mini-CTX-2 were mobilized into *P. aeruginosa* by mating. After integration into the *attB* site, vector backbone sequences were excised by Flp recombinase as previously described (20). All other plasmids were transformed into chemically competent *P. aeruginosa* (34).

Reverse transcription-PCR and quantitative real-time PCR (qRT-PCR). To isolate RNA from bacteria grown in vitro, bacterial strains were grown overnight at 30°C with aeration in MinS without NTA, supplemented with 2.5 mM $CaCl_2$, and then subcultured 1:25 into fresh medium and grown at 30°C with aeration to mid-logarithmic phase (optical density at 600 nm [OD_{600}] of 0.4 to 0.8). Bacteria were then pelleted, washed twice with MinS with 10 mM NTA, and subcultured into MinS with 10 mM NTA (normalized to an OD_{600} of 0.05). This second culture was grown at 30°C with aeration to mid-logarithmic phase (OD_{600} of 0.4 to 0.8), at which time a volume of culture containing approximately 2×10^9 bacteria was collected and used for RNA isolation as described previously (41). The RNA preparation was checked for DNA contamination by PCR amplification of the 50S ribosomal protein RplU using *Taq* DNA polymerase (Invitrogen) and primer pairs *rplU*-RT-PCR 1 and *rplU*-RT-PCR 2. cDNA was synthesized using 2 μg purified RNA, random primer hexamers (Invitrogen), and SuperScript II reverse transcriptase (Invitrogen).

To isolate RNA from bacteria grown in vivo, mouse infections were carried out as described previously (29) and below. Two mice per strain were infected with equal numbers of either PA103 or Δ*retS attB*::*retS* H424Q bacteria. At 18 h postinfection (hpi), the mice were sacrificed and their lungs were removed, homogenized, and resuspended in 4 ml phosphate-buffered saline/gram of tissue. The suspensions collected from each strain were pooled, and serial dilutions were plated to determine the number of bacteria recovered. Two volumes of RNA Protect reagent (QIAGEN) was added to the lung suspensions, and RNA isolation and cDNA synthesis were carried out as described previously (41) and above, with the modification that cDNA was synthesized using 1 μg purified RNA.

For quantitative real-time PCR, 1 μl of cDNA synthesized from RNA isolated from each bacterial strain was added to the iQ SYBR Green Supermix (Bio-Rad) and either 200 nM of each of the primers *exoT*-RT-PCR 1 and *exoT*-RT-PCR 2, 62.5 nM of each of the primers *pelA*-RT-PCR 1 and *pelA*-RT-PCR 2, 125 nM of each of the primers *pslD*-RT-PCR 1 and *pslD*-RT-PCR 2, or 125 nM of each of the primers *rsmZ*-RT-PCR 1 and *rsmZ*-RT-PCR 2 was added (Table 3). To control for the amount of total RNA, the same reaction was carried out using 250 nM each of the primers *rplU*-RT-PCR 1 and *rplU*-RT-PCR 2 (Table 3). The reactions were run in a DNA Engine Opticon2 Continuous Fluorescence detection system (MJ Research) and analyzed using Opticon Monitor 2.02.24 software (MJ Research).

SDS-PAGE and Western blot analysis. For detection of T3S proteins, bacteria were grown overnight with aeration at 30°C or 37°C as indicated in MinS containing 10 mM NTA and then centrifuged to separate cell-associated (pellet) and secreted (supernatant) proteins. Samples were prepared as previously described (17) and normalized to total protein (15 μg/lane) for cell-associated proteins (determined using the Pierce bicinchoninic acid assay) and to colony counts (5×10^8 CFU/lane) for secreted proteins. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore). ExoU and ExoT were detected by Western blotting with polyclonal rabbit antiserum (1:20,000) as previously described (30).

To detect expression of BB2-tagged constructs, bacteria were grown overnight with aeration at 37°C in LB-carbenicillin. Bacteria were centrifuged, lysed in 4% SDS, and normalized to total protein (15 μg/lane). SDS-PAGE and Western blotting were carried out as described above. Blots were probed with anti-BB2 monoclonal antibody (kindly provided by C. Tschudi, Yale University), followed by a goat anti-mouse immunoglobulin G–horseradish peroxidase conjugate (1:2,000; Bio-Rad). Detection and analysis were performed as previously described (30).

Mouse model of acute pneumonia. Mouse infections were carried out as described previously (29), using 8- to 10-week-old female C57BL/6 mice obtained from the National Cancer Institute and housed under specific-pathogen-free conditions. All studies were approved by the Yale University Institutional Ani-

mal Care and Use Committee. Statistical analysis was performed using Prism software (GraphPad).

Cellular fractionation. Bacterial strains were cultured in LB-carbenicillin at 37°C with aeration for 16 to 18 h, subcultured 1:50 into fresh LB-carbenicillin, and grown at 37°C with aeration to mid-logarithmic phase (OD_{600} of 0.4 to 0.8). Cells were fractionated into soluble (cytoplasmic plus periplasmic), inner membrane, and outer membrane fractions as previously described (38). To extract peripherally associated membrane proteins, the membrane pellet generated by lysing spheroplasts was incubated in 50 mM Tris, pH 8.0, and 0.3 M NaCl for 10 min at 37°C prior to extraction of the inner and outer membrane proteins. Sample volumes representing equivalent cell numbers were separated by SDS-PAGE, transferred to a PVDF membrane (Millipore), and probed for the BB2 epitope to detect RetS and the truncations as described above. Localization of β-lactamase was also determined using rabbit anti-β-lactamase (1:2,000; Chemicon International) followed by a goat anti-rabbit immunoglobulin G–horseradish peroxidase conjugate (1:2,000; Bio-Rad). Detection and analysis were carried out as previously described (30). As a control, a sample of the culture used to perform the fractionation was lysed in 4% SDS and analyzed in parallel (whole-cell lysate).

RESULTS

Identification and mutation of predicted phosphoacceptor residues within RetS. RetS is a member of the subtype 1B family of sensor histidine kinases (28). The region of RetS corresponding to the sensor kinase domain (amino acids 418 to 621) was aligned with the sensor kinase domains of two other subtype 1B family proteins, ArcB and BarA (both from *E. coli*) (see Fig. S1A in the supplemental material) (46). The amino acid sequence of the sensor kinase domain of RetS is 31% identical and 56% similar to that of ArcB and 35% identical and 59% similar to that of BarA, similar to what is observed when ArcB and BarA are compared (38% identical, 58% similar) (45). The invariant histidine residue that is the site of autophosphorylation in ArcB and BarA (14, 25, 26) corresponds to H424 in RetS. Using site-directed mutagenesis, we replaced H424 with glutamine (RetS H424Q). Such a change has been shown elsewhere to eliminate the kinase activity of other sensor kinase proteins (13, 24, 48).

We also aligned the protein sequences of the two tandem C-terminal receiver domains of RetS (R1 and R2, respectively) with one another and with the receiver domains of CheY from *E. coli* and PleD from *C. crescentus* (see Fig. S1B in the supplemental material) (46). The two domains of RetS show 27% identity and 46% similarity at the amino acid level. Their sequences show a similar degree of relatedness to both CheY (R1, 28% identical, 47% similar; R2, 29% identical, 49% similar) and PleD (R1, 33% identical, 50% similar; R2, 26% identical, 45% similar) (45). The alignment of RetS R1 and R2 domains with other response regulator proteins highlights the conservation of an invariant aspartate residue in both R1 (D713) and R2 (D858). This aspartate corresponds to the site of phosphorylation in both CheY and the first receiver domain of PleD (10, 18, 40). Furthermore, two acidic residues that are important for Mg^{2+} binding and the formation of an acidic pocket required for phosphotransfer are conserved in both receiver domains of RetS (22). These correspond to D664 and D665 in RetS R1 and E814 and D815 in R2. To assess the contribution of R1 or R2 domains to RetS function, aspartate residues that are predicted sites of phosphorylation as well as residues forming the acidic pocket in R1 and R2 were mutated to alanines (RetS D664A D665A, RetS D713A, RetS E814A D815A, and RetS D858A). We also constructed versions of RetS in which these point mutations were combined, resulting in double mutants targeting both receiver domains (RetS D713A D858A) or the sensor kinase plus either R1 or R2 (RetS H424Q D713A and RetS H424Q D858A). We also constructed a triple mutant lacking all predicted sites of phosphorylation (RetS H424Q D713A D858A; see Materials and Methods).

In order to examine the effect of point mutations at the histidine kinase or R1 or R2 domains on protein expression and stability, BB2 epitope-tagged alleles of RetS H424Q, RetS D713A, and RetS D858A were also constructed (7). Although expression of *retS* as a single-copy integrant in the *attB* site of the chromosome is sufficient to complement the phenotype of a Δ*retS* strain (29), epitope-tagged RetS expressed in this manner could not be detected by Western blotting (data not shown). Therefore, the tagged proteins were expressed in a Δ*retS* strain from a high-copy-number plasmid under the control of the native *retS* promoter. As seen in Fig. 1B, the steady-state levels of RetS H424Q-BB2, RetS D713A-BB2, and RetS D858A-BB2 were similar to that seen for RetS-BB2, indicating that the mutations did not affect steady-state protein levels (Fig. 1B). Therefore, gross alterations in protein levels or stability are unlikely to account for the phenotypes described below.

Changes in ExoT expression are mirrored by alterations in *exoT* transcription. RetS regulates transcription of genes encoding the type III apparatus, regulators, translocators, and effectors, either directly or indirectly (15, 29, 56). We therefore assessed the effects of point mutations in the sensor kinase, R1, and R2 domains of RetS on transcription of a T3SS effector by measuring *exoT* expression using qRT-PCR. In these and subsequent experiments, wild-type RetS and the various point mutants were expressed as single-copy constructs integrated into the chromosomal *attB* site of a Δ*retS* strain under control of the native *retS* promoter (see Materials and Methods). As the growth curves of bacterial strains expressing either wild-type or mutant alleles of RetS were identical in conditions used to induce T3S (data not shown), we controlled for total mRNA levels by normalizing *exoT* mRNA to *rplU* mRNA, which encodes a 50S ribosomal protein. As seen in Table 4, the wild-type strain PA103 expresses 0.981 ± 0.067 pg of *exoT* mRNA per pg *rplU* mRNA under T3S-inducing conditions. *exoT* mRNA levels in the Δ*retS* strain are 1.7% of wild-type levels, while complementation with the wild-type gene restores *exoT* mRNA levels to 71% of that observed in PA103.

Table 4 clearly shows that alleles carrying mutations in R2 did not restore *exoT* gene expression to Δ*retS*. RetS alleles in which an R2 mutation was combined with a sensor kinase and/or R1 mutation also failed to complement Δ*retS*. Likewise, bacteria expressing the sensor kinase mutant RetS H424Q showed markedly decreased *exoT* mRNA levels (11.5% of PA103 level). In contrast, complementation with an R1 mutant allele (RetS D713A or RetS D664A D665A) restored *exoT* mRNA to levels equal to or greater than those observed with PA103 or the wild-type complemented strain. Of note, a double mutant carrying sensor kinase and R1 point mutations (RetS H424Q D713A) showed higher levels of *exoT* expression than those observed for the sensor kinase mutant alone. This finding, as well as the phenotypes of the R1 mutant alleles,

TABLE 4. Transcription of the T3S effector ExoT in strains containing point mutations in RetS

Bacterial strain	pg *exoT* mRNA/pg *rplU* mRNA[a]	Expression relative to PA103 (% ± SD)
PA103	0.981 ± 0.067	100 ± 6.8
Δ*retS*	0.017 ± 0.005	1.70 ± 0.46
Δ*retS attB*::*retS*	0.700 ± 0.135	71.4 ± 13.8
Δ*retS attB*::*retS* H424Q	0.113 ± 0.004	11.5 ± 0.4
Δ*retS attB*::*retS* D664A D665A	0.779 ± 0.116	79.4 ± 11.8
Δ*retS attB*::*retS* D713A	1.078 ± 0.131	109.9 ± 13.4
Δ*retS attB*::*retS* E814A D815A	0.033 ± 0.004	3.35 ± 0.39
Δ*retS attB*::*retS* D858A	0.074 ± 0.009	7.59 ± 0.89
Δ*retS attB*::*retS* H424Q D713A	0.267 ± 0.053	27.2 ± 5.4
Δ*retS attB*::*retS* H424Q D858A	0.055 ± 0.006	5.56 ± 0.58
Δ*retS attB*::*retS* D713A D858A	0.048 ± 0.004	4.87 ± 0.44
Δ*retS attB*::*retS* H424Q D713A D858A	0.048 ± 0.007	4.84 ± 0.69

[a] The values are expressed as the average of the ratio of pg of *exoT* mRNA per pg of *rplU* mRNA ± standard deviation ($n = 3$). The results are representative of three independent experiments.

TABLE 5. Transcription of the small regulatory RNA *rsmZ* in strains containing point mutations in RetS

Bacterial strain	pg *rsmZ* mRNA/pg *rplU* mRNA[a]	Expression relative to PA103 (% ± SD)
PA103	0.033 ± 0.009	100 ± 27
Δ*retS*	0.593 ± 0.077	1,810 ± 230
Δ*retS attB*::*retS*	0.090 ± 0.049	274 ± 149
Δ*retS attB*::*retS* H424Q	0.469 ± 0.082	1,430 ± 250
Δ*retS attB*::*retS* D713A	0.078 ± 0.009	237 ± 27
Δ*retS attB*::*retS* D858A	0.493 ± 0.177	1,500 ± 540
Δ*retS attB*::*retS* H424Q D713A	0.226 ± 0.049	688 ± 149
Δ*retS attB*::*retS* H424Q D858A	0.455 ± 0.164	1,380 ± 500
Δ*retS attB*::*retS* D713A D858A	0.740 ± 0.218	2,250 ± 660
Δ*retS attB*::*retS* H424Q D713A D858A	0.689 ± 0.118	2,100 ± 360

[a] The values are expressed as the average of the ratio of pg of *rsmZ* mRNA per pg of *rplU* mRNA ± standard deviation ($n = 3$). The results are representative of three independent experiments.

suggests that the function of the R1 domain might be to inhibit RetS-dependent induction of T3SS expression.

Induction of the T3SS in vitro results in secretion of the effectors ExoU and ExoT. We confirmed that changes in *exoT* expression were mirrored by changes in ExoU and ExoT production and secretion, as detected by Western blotting of bacterial lysates and supernatants, respectively. As seen in Fig. 2, mutation of the predicted active-site aspartate in R2 (D858A) resulted in the loss of ExoU and ExoT expression and secretion in response to in vitro T3S-inducing conditions. Complete loss of protein expression was also observed when the acidic pocket of receiver domain 2 was mutated (RetS E814A D815A; data not shown) and when the D858A mutation was combined with point mutations in receiver domain 1 and/or the sensor kinase domain (Fig. 2). Bacteria expressing the sensor kinase mutant (RetS H424Q) also did not produce or secrete detectable amounts of ExoU or ExoT. Lastly, mutation of R1 residues (either D664A D665A or D713A) had no apparent effect on RetS function, as these bacteria produced and secreted ExoU and ExoT at wild-type levels (Fig. 2 and data not shown). Again, the combination of the sensor kinase and R1 domain point mutations (RetS H424Q D713A) resulted in intermediate levels of ExoU and ExoT production and secretion, greater than those seen for RetS H424Q. All bacterial strains were also assayed under noninducing conditions, i.e., in MinS lacking NTA and supplemented with 2.5 mM $CaCl_2$. In no case did we observe the production or secretion of ExoU or ExoT (data not shown), indicating that none of the RetS mutations confers a "constitutively active" phenotype.

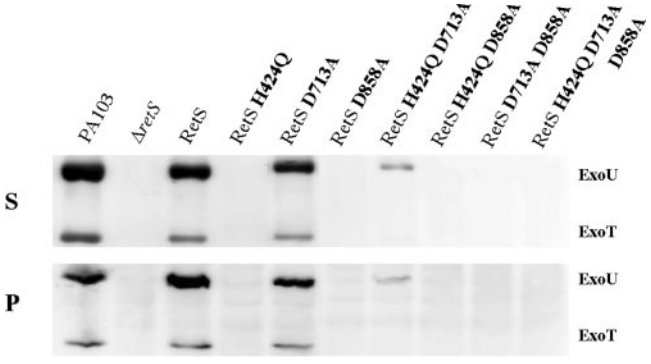

FIG. 2. In vitro T3S of ExoU and ExoT from strains expressing point mutations in RetS. The figure shows Western immunoblots of culture supernatants (S) and cell pellet lysates (P) of bacteria grown overnight in T3S-inducing medium at 30°C. Samples were normalized to bacterial counts (5×10^8/lane) or total protein (15 μg/lane) for supernatant and lysate samples, respectively. ExoU and ExoT were detected by Western blotting using a polyclonal antibody to the T3S effectors as described in Materials and Methods. The positions of ExoU and ExoT are indicated. The wild-type copy of *retS* and *retS* alleles containing point mutations are expressed as single-copy chromosomal integrates under the control of the native *retS* promoter in a Δ*retS* strain.

Effects of point mutants on non-T3SS targets of RetS. The microarray analysis published by Goodman et al. showed that RetS inhibited the expression of genes within two operons implicated in exopolysaccharide biosynthesis in PAK, *pel* and *psl* (15). We predicted that our series of RetS point mutants would show inverse patterns of regulation for *pel* and *psl* compared to T3SS genes. We therefore designed primers to amplify *pelA* and *pslD* mRNAs but found that we were unable to detect these mRNAs above background in our PA103 samples (data not shown). Another target of RetS regulation is the small regulatory RNA *rsmZ*, which is postulated to affect gene expression by titrating the concentration of free RsmA available in the cell (8, 15, 19). In agreement with the results of Ventre et al. (49), we found that *rsmZ* levels were markedly elevated in Δ*retS* bacteria compared to PA103 grown under T3SS-inducing conditions (Table 5). Likewise, bacteria expressing RetS alleles mutated at the predicted R2 phosphoacceptor residue, D858, also showed very high levels of *rsmZ* (Table 5). The RetS H424Q allele, which did not support expression of *exoT* in response to in vitro inducing conditions, showed elevated *rsmZ* levels. Conversely, the R1 mutant allele

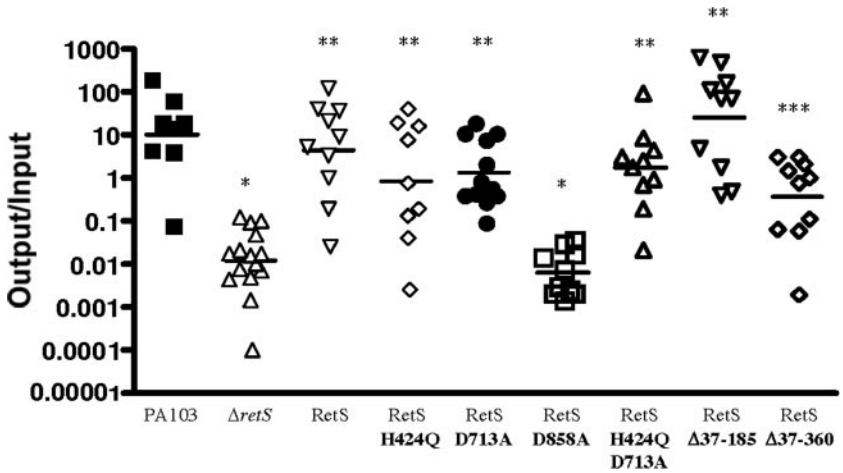

FIG. 3. Recovery of RetS mutant derivatives in a mouse model of acute pneumonia. Lightly anesthetized 8- to 10-week-old female C57BL/6 mice were infected with approximately 5×10^5 CFU of each bacterial strain. The mice were euthanized 18 h postinfection, and the lungs were removed, homogenized, and resuspended in Hanks' balanced salt solution plus 0.25% Triton X. Serial dilutions of organ suspensions were plated on Vogel-Bonner minimal medium to determine CFU/gram of tissue. Results are expressed as the ratio of CFU recovered/gram of tissue (output) to CFU present in the initial inoculum (input) and represent results from $n = 10$ to 15 mice per strain; the line shows the geometric mean for each group. The Mann-Whitney test was used to calculate P values (two-tailed) for each pairwise comparison. *, $P < 0.0001$ compared to the Δ*retS attB*::*retS* strain; **, $P \leq 0.003$ compared to the Δ*retS* strain; ***, $P = 0.0021$ compared to the Δ*retS* strain and $P = 0.0232$ compared to the Δ*retS attB*::*retS* strain. The wild-type and mutant alleles of *retS* are expressed as single-copy chromosomal integrates under the control of the native *retS* promoter in a Δ*retS* strain.

RetS D713A showed low levels of *rsmZ* mRNA, comparable to those observed in PA103 and the Δ*retS attB*::*retS* strain. Thus, the effects of RetS point mutations on T3SS gene expression likely result from the ability of these mutant proteins to directly or indirectly repress *rsmZ* transcription.

Only the receiver domain 2 of RetS is required for virulence in a mouse model of acute pneumonia. The in vitro results presented above suggest that residues predicted to play roles in phosphorylation of the sensor kinase and R2 domains are required for RetS to induce expression of T3SS genes under in vitro inducing conditions. Conversely, mutation of the predicted site of phosphorylation in R1 appears to enhance RetS activity, suggesting that phosphorylation of this domain might inhibit RetS function. We tested whether these results also held true in a mouse model of acute pneumonia, in which RetS function is required for virulence (15, 29). Although virulence in this model is strongly associated with the expression of the T3SS effector ExoU (3, 11, 17), non-T3SS factors are also likely to play roles in establishing murine infection. Age- and sex-matched C57BL/6 mice were infected intranasally with approximately 5×10^5 wild-type bacteria or bacteria expressing mutant variants of RetS. Figure 3 shows the ratio of bacteria recovered from the lungs relative to the initial inoculum at 18 hpi, with geometric means indicated for each group. In this assay, wild-type PA103 is recovered in numbers approximately 10-fold higher than the initial inoculum dose, whereas the Δ*retS* strain is recovered at 1% of the initial dose (29). Bacteria expressing the R2 mutant allele RetS D858A were avirulent in this murine model, as seen in Fig. 3. Mice infected with this strain did not appear ill at 18 hpi, and bacteria were recovered in numbers less than 1% of the initial dose. Moreover, bacteria expressing RetS D858A were not recovered from liver or spleen, indicating that the bacteria were not able to disseminate systemically (data not shown). This was similar to what we had observed for the Δ*retS* strain (29). In contrast, bacteria expressing RetS D713A appeared to retain virulence: bacteria were recovered from the lung in a 1.5-fold excess over the inoculum and were able to disseminate to liver and spleen (Fig. 3 and data not shown). For comparison, the Δ*retS* strain complemented with wild-type RetS was recovered from the lung in numbers ca. fivefold greater than the inoculum, which is not a statistically significant difference from that observed with RetS D713A ($P = 0.2775$). Thus, the virulence of bacteria expressing R1 or R2 mutant alleles of RetS mirrors their ability to induce T3SS expression in vitro.

Bacteria expressing the RetS H424Q allele, however, appeared more virulent in the murine acute pneumonia model than would be predicted from their phenotype in vitro. Δ*retS attB*::*retS* H424Q bacteria were recovered from mouse lungs in numbers slightly less than the inoculum size (ratio, 0.87), which was approximately 100 times greater than the recovery observed for either the Δ*retS* strain or the strain complemented with the R2 mutant allele, the Δ*retS attB*::*retS* D858A strain. Indeed, the difference between the recovery of the Δ*retS attB*::*retS* H424Q strain and that of the Δ*retS attB*::*retS* strain was not statistically significant ($P = 0.1785$). RetS H424Q was also able to support bacterial dissemination to the liver and spleen (data not shown). Thus, the predicted phosphorylation site of R2, but not of the sensor kinase domain or R1, appeared essential for the function of RetS in vivo. This conclusion was reinforced by the phenotype of the double mutant RetS H424Q D713A. This strain, which lacks the predicted phosphorylation sites within the sensor kinase and R1 domains, was recovered from the lungs in numbers 1.8-fold higher than the initial dose and was able to disseminate to liver and spleen, as observed for the Δ*retS attB*::*retS* strain (Fig. 3 and data not shown). These results suggest that the R2 domain

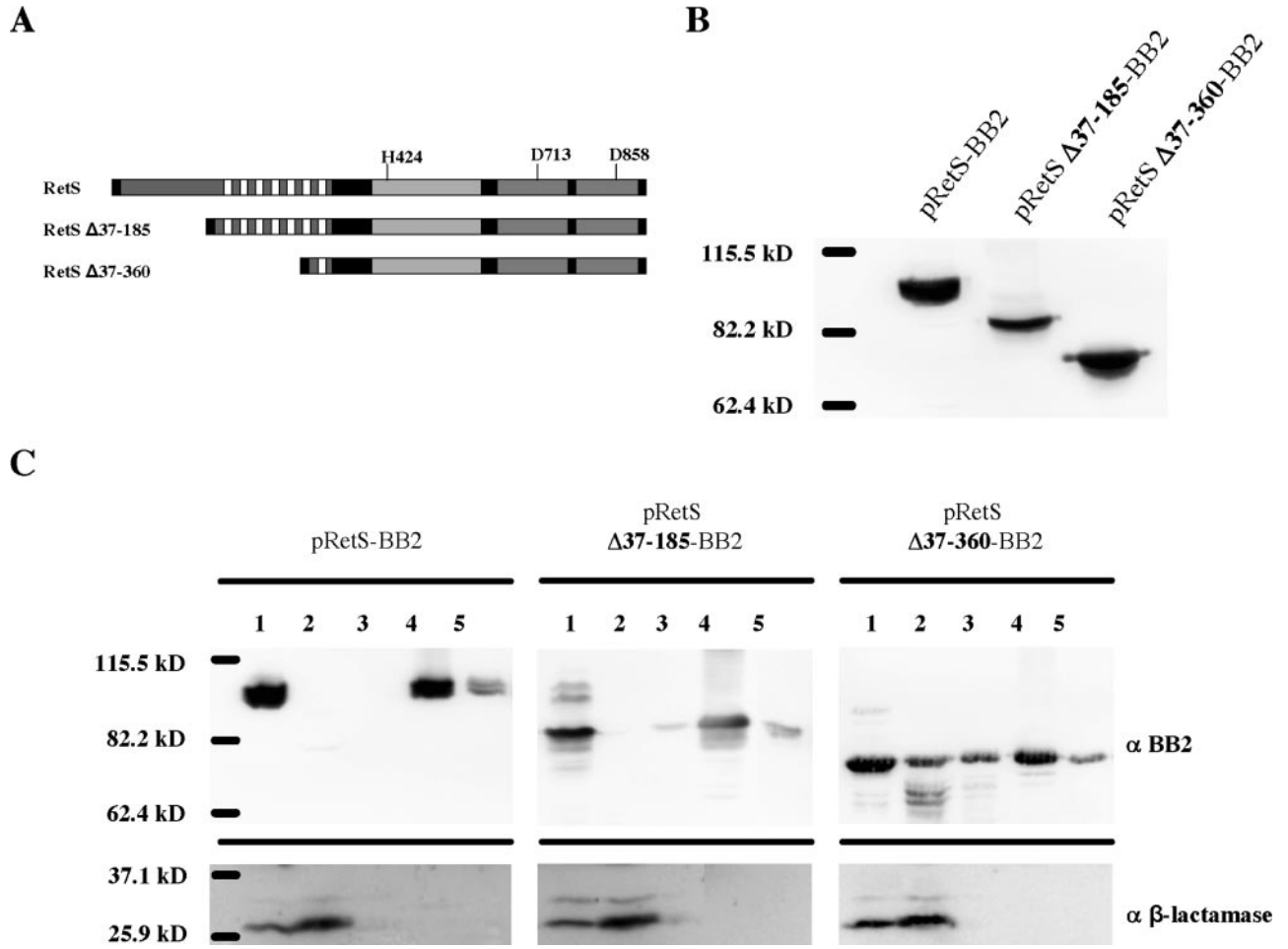

FIG. 4. Construction of RetS truncations. (A) RetS deletion schematic. Truncations of RetS lacking either the periplasmic domain (RetS Δ37-185) or the periplasmic and six of the seven transmembrane domains (RetS Δ37-360) were constructed as described in Materials and Methods. (B) Steady-state levels of epitope-tagged RetS truncations. Δ*retS* strains expressing epitope-tagged alleles of wild-type RetS (pRetS-BB2), RetS Δ37-185 (pRetS Δ37-185-BB2), and RetS Δ37-360 (pRetS Δ37-360-BB2) were grown in LB-carbenicillin overnight. Bacterial pellets were lysed, and 15 μg of total protein was separated by SDS-PAGE. Proteins were transferred to a PVDF membrane and detected by Western blotting using a monoclonal anti-BB2 antibody. (C) Subcellular localization of epitope-tagged RetS truncations. Δ*retS* strains expressing epitope-tagged wild-type RetS (pRetS-BB2), RetS Δ37-185 (pRetS Δ37-185-BB2), and RetS Δ37-360 (pRetS Δ37-360-BB2) were grown in LB-carbenicillin to mid-logarithmic phase and fractionated as described in Materials and Methods. The lanes correspond to the indicated fractions: 1, whole-cell lysate; 2, cytoplasmic and periplasmic proteins; 3, peripheral (high-salt-extracted) membrane proteins; 4, inner membrane proteins; 5, outer membrane proteins. The three top panels were probed with anti-BB2 monoclonal antibody and show the distribution of the epitope-tagged RetS alleles; the bottom panels were probed with a polyclonal anti-β-lactamase antibody.

of RetS might serve as a substrate for another sensor kinase besides RetS.

RetS H424Q is able to induce T3SS genes in vivo. The observation that the Δ*retS attB*::*retS* H424Q strain was significantly more virulent in the murine acute pneumonia model than either the Δ*retS* or the Δ*retS attB*::*retS* D858A strain raised the question of whether the H424Q mutant bacteria still induced T3SS genes in vivo to promote replication and dissemination in mice. We tested whether RetS H424Q supported induction of the T3SS during murine infection by preparing RNA from the lungs of mice infected with either PA103 or the Δ*retS attB*::*retS* H424Q strain and assaying the amount of *exoT* mRNA present in each sample using qRT-PCR. We recovered 0.0343 ± 0.011 pg of *exoT* mRNA per pg *rplU* mRNA from the RetS H424Q-expressing bacteria, compared to 0.106 ± 0.009 pg *exoT* mRNA per pg *rplU* mRNA from PA103. Thus, the H424Q mutant expressed 32.3% ± 9.9% as much *exoT* as did PA103 in the mouse lung. We also assayed *rsmZ* mRNA levels in wild-type and Δ*retS attB*::*retS* H424Q bacteria harvested from mouse lungs. Since *rsmZ* is predicted to be repressed under conditions of acute infection, we expected to find relatively low levels of mRNA. Indeed, we were unable to detect *rsmZ* mRNA in either of these samples, suggesting that the regulatory circuit which suppresses *rsmZ* transcription was fully functional in bacteria expressing RetS H424Q.

Although the stimuli that trigger T3SS expression in the mouse lung are not completely understood, temperature is likely to be important (23). We therefore grew bacteria in vitro at 37°C in MinS and again assayed *exoT* mRNA levels using qRT-PCR. *exoT* mRNA levels in bacteria expressing the sensor

kinase mutant RetS H424Q were 48.0% ± 13.3% of those measured for PA103 under these conditions. Thus, RetS H424Q appears to support induction of T3SS genes at 37°C, in vitro or in vivo. We can also observe ExoU and ExoT production and secretion by Western blotting in the Δ*retS attB*::*retS* H424Q strain grown at 37°C in MinS (data not shown), suggesting that these bacteria are likely using the T3SS during murine infection.

The predicted periplasmic domain is not required for the ability of RetS to induce expression of the T3S effectors. Two-component systems often respond to extracellular signals. PSORT analysis predicts that RetS has an amino-terminal signal sequence, followed by a large periplasmic domain (amino acids 28 to 188) and seven transmembrane domains (Fig. 4A). We constructed a mutant which lacks the periplasmic region but retains the putative signal sequence and seven transmembrane domains (RetS Δ37-185). We also constructed a truncation of RetS lacking both the periplasmic domain and six of the seven transmembrane domains (RetS Δ37-360). Both constructs were tagged with the BB2 epitope, allowing us to measure steady-state protein levels and subcellular localization of each mutant (7). The tagged proteins were expressed in a Δ*retS* strain from a high-copy-number plasmid under the control of the native *retS* promoter. The steady-state levels of RetS Δ37-185-BB2 and RetS Δ37-360-BB2 were similar to that seen for RetS-BB2, indicating that the truncations did not alter the expression or steady-state stability of RetS (Fig. 4B).

We also examined the subcellular localization of wild-type and mutant RetS alleles (Fig. 4C). As predicted from its structure, RetS fractionated as an integral inner membrane protein. The protein lacking the periplasmic domain (RetS Δ37-185-BB2) showed a similar pattern of localization to the inner membrane fraction. Truncation of the periplasmic domain and six of the seven transmembrane domains (RetS Δ37-360-BB2) still allowed localization to the inner membrane (45% of total protein fractionated), although protein was now detected in the soluble (cytoplasm plus periplasm) and high-salt-extracted fractions. To ensure that the observed localization to the inner membrane was not an artifact of the fractionation protocol, we examined the localization of the periplasmic protein β-lactamase. As seen in Fig. 4C, β-lactamase was found in only the whole-cell lysate and the fraction containing cytoplasmic and periplasmic proteins. Therefore, it appears that a significant proportion of RetS Δ37-360-BB2 is indeed targeted to the inner membrane even when it is overexpressed. For functional assays, RetS Δ37-185 and RetS Δ37-360 were both expressed from single-copy constructs integrated into the chromosomal *attB* site of a Δ*retS* strain under the control of the native *retS* promoter as described for the RetS point mutants.

To investigate the effect of these truncations on the ability of RetS to induce T3S, we examined in vitro secretion of the effectors ExoU and ExoT by Western blotting from cultures grown at 30°C (Fig. 5). Deletion of the periplasmic domain (RetS Δ37-185) did not affect the function of RetS: ExoU and ExoT were produced and secreted at wild-type levels. However, the mutant lacking the periplasmic domain plus six of seven transmembrane domains (RetS Δ37-360) did not complement Δ*retS* bacteria for production of ExoU and ExoT. Identical results were observed when ExoU and ExoT production and secretion were assayed at 37°C (data not shown). We also tested whether the RetS Δ37-185 allele could support T3S in the absence of an in vitro inducing signal. However, when bacteria expressing RetS Δ37-185 were cultured under noninducing conditions, no production or secretion of ExoU or ExoT was noted (data not shown). These results demonstrate that the periplasmic domain is not required for RetS to induce expression of the T3SS but that the transmembrane domains are necessary for RetS activity.

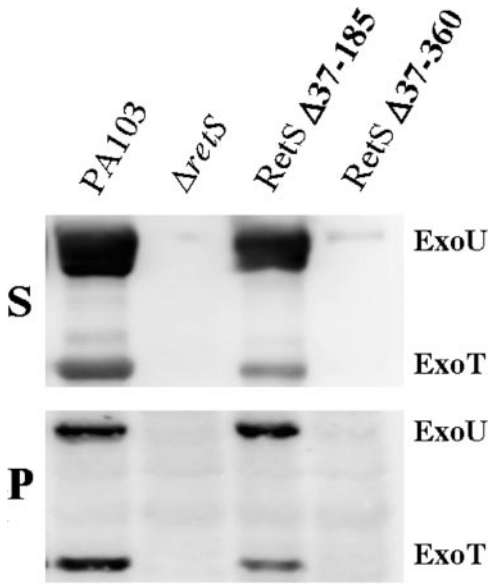

FIG. 5. In vitro T3S of ExoU and ExoT does not require the periplasmic domain of RetS. Bacteria were grown overnight in T3S-inducing medium at 30°C. Samples were normalized to bacterial counts (5×10^8/lane) or total protein (15 μg/lane) for supernatant (S) and lysate (P) samples, respectively. ExoU and ExoT were detected by Western blotting using a polyclonal antibody to the T3S effectors as described in Materials and Methods. The positions of ExoU and ExoT are indicated. The wild-type copy of *retS* and truncations of *retS* are expressed as single-copy chromosomal integrates under the control of the native *retS* promoter in a Δ*retS* strain.

Virulence phenotypes of periplasmic and transmembrane domain deletion mutants in vivo. Strains expressing the truncated alleles of RetS were also assayed in the mouse model of acute pneumonia. As shown in Fig. 3, the strain expressing RetS Δ37-185 was recovered from mouse lungs in numbers approximately 30-fold greater than the inoculum. (Although the mean level of recovery of the mutant was higher than that of bacteria expressing full-length RetS, this difference did not achieve statistical significance [$P = 0.1685$].) Surprisingly, a strain expressing RetS Δ37-360 was not as attenuated as we would have predicted. Mice infected with RetS Δ37-360 appeared moderately ill at 18 hpi, and bacteria were recovered in numbers approximately equal to the initial dose (Fig. 3). The number of bacteria recovered from the lungs differed from that of a Δ*retS* strain ($P = 0.0021$) and from that of a strain expressing wild-type RetS ($P = 0.0232$). Furthermore, this strain was able to spread systemically to the spleen and liver (data not shown). These results suggest that the periplasmic domain of RetS might serve to inhibit RetS function in vivo. Deletion of the transmembrane domain of RetS clearly attenuates RetS function but not to the same degree as point mutations within

R2. Thus, the only indispensable residues within RetS appear to be those predicted to form the phosphoacceptor site of receiver domain 2: E814, D815, and D858.

DISCUSSION

RetS is an unusual hybrid two-component signaling protein that is required for *P. aeruginosa* virulence. Deletion of *retS* results in the failure to induce expression of the T3SS in response to host cell contact (15, 29). The Δ*retS* phenotype is also characterized by accelerated biofilm formation: this is likely the result of increased transcription of the *pel* and *psl* operons (as demonstrated by microarray analysis), which are involved in exopolysaccharide synthesis (15). Genes involved in type IV pilus assembly are also downregulated in the Δ*retS* background, though the Δ*retS* mutant shows increased binding to epithelial cells compared to the isogenic wild-type parent strain in both the PA103 and PAK backgrounds (15, 56). Recent work has demonstrated that the reciprocal regulation of genes involved in T3S and exopolysaccharide synthesis likely involves two additional sensor kinases, GacS and LadS (15, 49). Signals transduced by these three sensor kinase proteins are hypothesized to ultimately control the levels of a small RNA, *rsmZ*, which in turn regulates the reciprocal expression of genes involved in T3S and biofilm formation by titrating the amount of free RsmA available in the cell. Such a system would allow for *P. aeruginosa* to switch between patterns of gene expression that characterize acute infection versus chronic colonization; however, both the signals that are sensed by this system as well as the biochemical relationships between these various regulators remain uncharacterized.

RetS contains one sensor kinase domain followed by two tandem response regulator receiver domains. In this study we have shown that the active-site aspartates in receiver domain 1 are not required for RetS to induce expression of the T3SS in vitro or for virulence in a mouse model of acute pneumonia. Indeed, mutation of these residues results in increased expression of the T3SS, allowing us to speculate that phosphorylation of receiver domain 1 may inhibit RetS activation. Phosphorylation of the R1 domain could allosterically inhibit RetS; alternatively, the R1 phosphoacceptor site could compete with the R2 domain site for phosphorylation. R1 mutations do not result in a "constitutively active" phenotype, however, as expression of T3SS genes still requires an inducing signal, such as growth in MinS. In contrast, an intact phosphoacceptor site within receiver domain 2 is absolutely required for RetS activity. Strains containing mutations in receiver domain 2, alone or in combination with mutations in other domains, behaved identically to Δ*retS* bacteria in vitro and in vivo, highlighting the importance of this predicted phosphoacceptor site.

PA103 is not flagellated and does not form biofilms; thus, we have been unable to assess the effects of RetS point mutations on the hyperbiofilm phenotype observed in PAKΔ*retS*. We attempted to measure transcription of genes in the *pel* and *psl* operons, which are inhibited by RetS, using qRT-PCR (15, 49). *pel* and *psl* mRNA levels could not be detected above background, however, when bacteria were grown under T3SS-inducing conditions in vitro. We were able to detect *rsmZ* RNA, however, using qRT-PCR. A *rsmZ-lacZ* transcriptional reporter construct was previously shown to be strongly activated by deletion of *retS* in the PAK background (49); we likewise found that *rsmZ* RNA levels were 18-fold increased in PA103Δ*retS* compared to PA103. RetS, GacS, and LadS are postulated to control T3SS expression by regulating *rsmZ* transcription (15, 49). The results of our experiments are in agreement with this hypothesis, as RetS alleles which did not support induction of T3SS expression, such as those containing point mutants in the R2 domain, showed very high levels of *rsmZ* RNA (15- to 22-fold greater than those detected in PA103). Conversely, the strain complemented with the R1 mutant (Δ*retS attB*::*retS* D713A), which was able to express and secrete T3SS effectors at wild-type levels, expressed *rsmZ* at levels comparable to those observed in PA103.

Mutations within the sensor kinase domain that replaced the active-site histidine did not abolish RetS function, as we would have predicted, and allowed for intermediate levels of T3SS expression. Recombination of the H424Q mutation into the *retS* locus of PAK likewise resulted in diminished but not absent levels of ExoS and ExoT production and secretion in response to in vitro inducing conditions, suggesting that this phenotype is not strain specific (M. A. Laskowski and B. I. Kazmierczak, unpublished results). Bacteria expressing RetS H424Q were significantly more virulent in the mouse model of acute pneumonia than Δ*retS* or Δ*retS attB*::*retS* D858A bacteria. These observations are not compatible with a model in which phosphorylation of R2 can occur only by the RetS sensor kinase domain and instead suggest that phosphotransfer to RetS receiver domain 2 can be catalyzed by another sensor kinase. Indeed, Lory and colleagues have speculated that cross talk may occur at some level between RetS, GacS, and/or LadS, which would allow bacteria to integrate multiple signals governing the T3SS at the level of *rsmZ* expression (49). To our knowledge, no examples of cross talk between hybrid sensor kinase-response regulator proteins have been described. However, cross talk is described between noncognate sensor kinases and response regulators. For example, the response regulator OmpR is phosphorylated by the sensor kinase EnvZ, resulting in changes in the expression of two porins, OmpF and OmpC, in response to osmolarity (12, 33, 36). However, OmpR can also be phosphorylated by the noncognate sensor kinase ArcB, which allows for the regulation of porin expression by anaerobic conditions (33).

An alternative explanation for the absence of a null phenotype for RetS H424Q lies in the fact that sensor kinase domains also exhibit phosphatase activity. Mutations that alter the conserved histidine residue consistently disrupt the kinase activity of sensor kinases (13, 24, 48); however, these same mutations have a less predictable effect on the phosphatase activity of these proteins. For example, substitution of the catalytic histidine in EnvZ by glutamine, aspartate, valine, or arginine disrupts both the kinase and phosphatase activities of the protein (24, 27). Other substitutions tested at this position, such as tyrosine, cysteine, serine, glutamate, and alanine, disrupt only the kinase activity and leave the phosphatase activity intact (24). The effects of any particular histidine substitution on the phosphatase activity of a sensor kinase are not predictable, however. For instance, all of the substitutions reported to disrupt EnvZ phosphatase activity leave the phosphatase activity of the *Vibrio harveyi* sensor kinase LuxN intact (13). It is therefore possible that the H424Q mutation disrupts only the

kinase activity of RetS and that it is RetS phosphatase activity which is critical for its function.

One of the most interesting and significant attributes of *P. aeruginosa* is its ability to alternate between the expression of virulence factors associated with acute infection and of the expression of those required for chronic colonization of a host. This alteration is thought to occur in response to various environmental signals, and their identification might allow for the therapeutic manipulation of such signals to attenuate virulence. As RetS, along with GacS and LadS, is a membrane-associated protein whose activity as a sensor kinase-response regulator hybrid is expected to be regulated in response to extracellular signals, we examined the contribution of the periplasmic and transmembrane domains to RetS function. The periplasmic domain of RetS belongs to a class of bacterial periplasmic sensor modules (7TMR-DISMED2); these domains are predicted to adopt an all-beta-fold secondary structure with structural characteristics reminiscent of certain carbohydrate-binding domains (4). LadS also contains a 7TMR-DISMED2 domain, suggesting that these two proteins may respond to carbohydrates of host or bacterial origin (49). Deletion of the periplasmic domain of RetS did not affect the ability of bacteria to induce the T3SS in response to growth in MinS. Interestingly, there was a trend toward increased virulence of RetS Δ37-185-expressing bacteria in the murine acute pneumonia model, suggesting that this domain might inhibit RetS function in vivo. Deletion of the periplasmic domain did not permit T3SS expression in the absence of in vitro inducing stimuli, however. A RetS allele lacking the periplasmic domain plus six of the seven transmembrane domains was severely attenuated in vitro, much like the Δ*retS* mutant. This argues either that the transmembrane domains have an independent sensing function or that they are required for robust interactions of RetS with other proteins that ultimately lead to activation of RetS by cross talk. The RetS Δ37-360 allele did not recapitulate the in vivo phenotype of a Δ*retS* strain, however, and bacteria expressing this allele were recovered in slightly lower numbers than those expressing RetS H424Q. This may again suggest that a significant aspect of RetS function is to participate in cross talk with other two-component signal transduction proteins and that mutant forms of RetS which cannot efficiently respond to external signals by autophosphorylation can nonetheless function as substrates for other sensor kinases. Biochemical verification of these hypotheses is currently under way.

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REFERENCES

1. **Aldridge, P., and U. Jenal.** 1999. Cell cycle-dependent degradation of a flagellar motor component requires a novel-type response regulator. Mol. Microbiol. **32:**379–391.
2. **Aldridge, P., R. Paul, P. Goymer, P. Rainey, and U. Jenal.** 2003. Role of the GGDEF regulator PleD in polar development of *Caulobacter crescentus*. Mol. Microbiol. **47:**1695–1708.
3. **Allewelt, M., F. T. Coleman, M. Grout, G. P. Priebe, and G. B. Pier.** 2000. Acquisition of expression of the *Pseudomonas aeruginosa* ExoU cytotoxin leads to increased bacterial virulence in a murine model of acute pneumonia and systemic spread. Infect. Immun. **68:**3998–4004.
4. **Anantharaman, V., and L. Aravind.** 2003. Application of comparative genomics in the identification and analysis of novel families of membrane-associated receptors in bacteria. BCM Genomics **12:**34–54.
5. **Appleby, J. L., J. S. Parkinson, and R. B. Bourret.** 1996. Signal transduction via the multi-step phosphorelay: not necessarily a road less traveled. Cell **86:**845–848.
6. **Bateman, A., E. Birney, L. Cerruti, R. Durbin, L. Etwiller, S. R. Eddy, S. Griffiths-Jones, K. L. Howe, M. Marshall, and E. L. Sonnhammer.** 2002. The Pfam protein families database. Nucleic Acids Res. **30:**276–280.
7. **Brookman, J. L., A. J. Stott, P. J. Cheeseman, N. R. Burns, S. E. Adams, A. J. Kingsman, and K. Gull.** 1995. An immunological analysis of Ty1 virus-like particle structure. Virology **207:**59–67.
8. **Burrowes, E., A. Aggas, A. O'Neill, C. Adams, and F. O'Gara.** 2005. Characterisation of the regulatory RNA RsmB from *Pseudomonas aeruginosa* PAO1. Res. Microbiol. **156:**7–16.
9. **Burrowes, E., C. Baysse, C. Adams, and F. O'Gara.** 2006. Influence of the regulatory protein RsmA on cellular functions in *Pseudomonas aeruginosa* PAO1, as revealed by transcriptome analysis. Microbiology **152:**405–418.
10. **Djordjevic, S., P. N. Goudreau, Q. Xu, A. M. Stock, and A. H. West.** 1998. Structural basis for methylesterase CheB regulation by a phosphorylation-activated domain. Proc. Natl. Acad. Sci. USA **95:**1381–1386.
11. **Finck-Barbancon, V., J. Goranson, L. Zhu, T. Sawa, J. P. Weiner-Kronish, S. M. J. Fleiszig, C. Wu, L. Mende-Mueller, and D. W. Frank.** 1997. ExoU expression by *Pseudomonas aeruginosa* correlates with acute cytotoxicity and epithelial injury. Mol. Microbiol. **25:**547–557.
12. **Forst, S., J. Delgado, and M. Inouye.** 1989. Phosphorylation of OmpR by the osmosensor EnvZ modulates expression of the *ompF* and *ompC* genes in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **86:**6052–6056.
13. **Freeman, J. A., B. N. Lilley, and B. L. Bassler.** 2000. A genetic analysis of the functions of LuxN: a two-component hybrid sensor kinase that regulates quorum sensing in *Vibrio harveyi*. Mol. Microbiol. **35:**139–149.
14. **Georgellis, D., A. S. Lynch, and E. C. C. Lin.** 1997. In vitro phosphorylation study of the Arc two-component signal transduction system of *Escherichia coli*. J. Bacteriol. **179:**5429–5435.
15. **Goodman, A. L., B. Kulasekara, A. Rietsch, D. Boyd, R. S. Smith, and S. Lory.** 2004. A signaling network reciprocally regulates genes associated with acute infection and chronic persistence in *Pseudomonas aeruginosa*. Dev. Cell **7:**745–754.
16. **Hauser, A. R., E. Cobb, M. Bodi, D. Mariscal, J. Valles, J. Engel, and J. Rello.** 2002. Type III protein secretion is associated with poor clinical outcomes in patients with ventilator-associated pneumonia caused by *Pseudomonas aeruginosa*. Crit. Care Med. **30:**521–528.
17. **Hauser, A. R., P. J. Kang, and J. Engel.** 1998. PepA, a novel secreted protein of *Pseudomonas aeruginosa*, is necessary for cytotoxicity and virulence. Mol. Microbiol. **27:**807–818.
18. **Hecht, G. B., and A. Newton.** 1995. Identification of a novel response regulator required for the swarmer-to-stalked-cell transition in *Caulobacter crescentus*. J. Bacteriol. **177:**6223–6229.
19. **Heurlier, K., F. Williams, S. Heeb, C. Dormond, G. Pessi, D. Singer, M. Camara, P. Williams, and D. Haas.** 2004. Positive control of swarming, rhamnolipid synthesis, and lipase production by the posttranscriptional RsmA/RsmZ system in *Pseudomonas aeruginosa* PAO1. J. Bacteriol. **186:**2936–2945.
20. **Hoang, T. T., R. R. Karkhoff-Schweizer, A. J. Kutchma, and H. P. Schweizer.** 1998. A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. Gene **212:**77–86.
21. **Hoang, T. T., A. J. Kutchma, A. Becher, and H. P. Schweizer.** 2000. Integration-proficient plasmids for *Pseudomonas aeruginosa*: site-specific integration and use for engineering of reporter and expression strains. Plasmid **43:**59–72.
22. **Hoch, J. A., and T. J. Silhavy (ed.).** 1995. Two-component signal transduction. American Society for Microbiology, Washington, D.C.
23. **Hornef, M. W., A. Roggenkamp, A. M. Geiger, M. Hogardt, C. A. Jacobi, and J. Heesemann.** 2000. Triggering the ExoS regulon of *Pseudomonas aeruginosa*: a GFP-reporter analysis of exoenzyme (Exo) S, ExoT and ExoU synthesis. Microb. Pathog. **29:**329–343.
24. **Hsing, W., and T. J. Silhavy.** 1997. Function of conserved histidine-243 in phosphatase activity of EnvZ, the sensor for porin osmoregulation in *Escherichia coli*. J. Bacteriol. **179:**3729–3735.
25. **Ishige, K., S. Nagasawa, S. Tokishita, and T. Mizuno.** 1994. A novel device of bacterial signal transducers. EMBO J. **13:**5195–5202.
26. **Iuchi, S.** 1993. Phosphorylation/dephosphorylation of the receiver module at the conserved aspartate residue controls transphosphorylation activity of histidine kinase in sensor protein ArcB of *Escherichia coli*. J. Biol. Chem. **263:**23972–23980.
27. **Kanamaru, K., H. Aiba, and T. Mizuno.** 1990. Transmembrane signal transduction and osmoregulation in *Escherichia coli*. I. Analysis by site-directed mutagenesis of the amino acid residues involved in phosphotransfer between

the two regulatory components, EnvZ and OmpR. J. Biochem. (Tokyo) **108:**483–487.
28. **Kim, D.-J., and S. Forst.** 2001. Genomic analysis of the histidine kinase family in bacteria and archaea. Microbiology **147:**1197–1212.
29. **Laskowski, M. A., E. Osborn, and B. I. Kazmierczak.** 2004. A novel sensor kinase-response regulator hybrid regulates type III secretion and is required for virulence in *Pseudomonas aeruginosa*. Mol. Microbiol. **54:**1090–1103.
30. **Li, L., M. Ledizet, K. Kar, R. A. Koski, and B. I. Kazmierczak.** 2005. An indirect enzyme-linked immunosorbent assay for rapid and quantitative assessment of type III virulence phenotypes of *Pseudomonas aeruginosa* isolates. Ann. Clin. Microbiol. Antimicrob. **4:**22–36.
31. **Liu, P. V.** 1966. The roles of various fractions of *Pseudomonas aeruginosa* in its pathogenesis: identity of the lethal toxins produced *in vitro* and *in vivo*. J. Infect. Dis. **116:**481–489.
32. **Lyczak, J. B., C. L. Cannon, and G. B. Pier.** 2000. Establishment of *Pseudomonas aeruginosa* infection: lessons from a versatile opportunist. Microbes Infect. **2:**1051–1060.
33. **Matsubara, M., S. I. Kitaoka, S. I. Takeda, and T. Mizuno.** 2000. Tuning of the porin expression under anaerobic growth conditions by His-to-Asp cross-phosphorelay through both the EnvZ-osmosensor and ArcB-anaerosensor in *Escherichia coli*. Genes Cells **5:**555–563.
34. **Mattick, J. S., M. M. Bills, B. J. Anderson, B. Dalrymple, M. R. Mott, and J. R. Egerton.** 1987. Morphogenetic expression of *Bacteroides nodosus* fimbriae in *Pseudomonas aeruginosa*. J. Bacteriol. **169:**33–41.
35. **Nicas, T. I., and B. H. Iglewski.** 1984. Isolation and characterization of transposon-induced mutants of *Pseudomonas aeruginosa* deficient in production of exoenzyme S. Infect. Immun. **45:**470–474.
36. **Pratt, L. A., W. Hsing, K. E. Gibson, and T. J. Silhavy.** 1996. From acids to *osmZ*: multiple factors influence synthesis of the OmpF and OmpC porins in *Escherichia coli*. Mol. Microbiol. **20:**911–917.
37. **Roy-Burman, A., R. H. Savel, S. Racine, B. L. Swanson, N. S. Revadigar, J. Fujimoto, T. Sawa, D. W. Frank, and J. P. Wiener-Kronish.** 2001. Type III protein secretion is associated with death in lower respiratory and systemic *Pseudomonas aeruginosa* infections. J. Infect. Dis. **183:**1767–1774.
38. **Russel, M., and B. Kazmierczak.** 1993. Analysis of the structure and subcellular location of filamentous phage pIV. J. Bacteriol. **175:**3998–4007.
39. **Sambrook, J., and D. W. Russel.** 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
40. **Sanders, D. A., B. L. Gillece-Castro, A. M. Stock, A. L. Burlingame, and J. D. E. Koshland.** 1989. Identification of the site of phosphorylation of the chemotaxis response regulator protein, CheY. J. Biol. Chem. **264:**21770–21778.
41. **Schuster, M., C. P. Lostroh, T. Ogi, and E. P. Greenberg.** 2003. Identification, timing, and signal specificity of *Pseudomonas aeruginosa* quorum-controlled genes: a transcriptome analysis. J. Bacteriol. **185:**2066–2079.
42. **Simon, R., U. Priefer, and A. Puhler.** 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram negative bacteria. Bio/Technology **1:**784–791.
43. **Stock, A. M., V. L. Robinson, and P. N. Goudreau.** 2000. Two-component signal transduction. Annu. Rev. Biochem. **69:**183–215.
44. **Stover, C., X. Pham, A. Erwin, S. Mizoguchi, P. Warrener, M. Hickey, and F. S. Brinkman.** 2000. Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. Nature **406:**959–964.
45. **Tatusova, T. A., and T. L. Madden.** 1999. BLAST 2 Sequences, a new tool for comparing protein and nucleotide sequences. FEMS Microbiol. Lett. **174:**247–250.
46. **Thompson, J. D., D. G. Higgins, and T. J. Gibson.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighing, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:**4673–4680.
47. **Trudeau, K. G., M. J. Ward, and D. R. Zusman.** 1996. Identification and characterization of FrzZ, a novel response regulator necessary for swarming and fruiting-body formation in *Myxococcus xanthus*. Mol. Microbiol. **20:**645–655.
48. **Uhl, M. A., and J. F. Miller.** 1994. Autophosphorylation and phosphotransfer in the *Bordetella pertussis* BvgAS signal transduction cascade. Proc. Natl. Acad. Sci. USA **91:**1163–1167.
49. **Ventre, I., A. L. Goodman, I. Vallet-Gely, P. Vasseur, C. Soscia, S. Molin, S. Bleves, A. Lazdunski, S. Lory, and A. Filloux.** 2006. Multiple sensors control reciprocal expression of *Pseudomonas aeruginosa* regulatory RNA and virulence genes. Proc. Natl. Acad. Sci. USA **103:**171–176.
50. **Vogel, H. J., and D. M. Bonner.** 1956. Acetylornithinase of *Escherichia coli*: partial purification and some properties. J. Biol. Chem. **218:**97–106.
51. **Ward, M. J., K. C. Mok, D. P. Astling, H. Lew, and D. R. Zusman.** 1998. An ABC transporter plays a developmental aggregation role in *Myxococcus xanthus*. J. Bacteriol. **180:**5697–5703.
52. **Watson, A. A., R. A. Alm, and J. S. Mattick.** 1996. Construction of improved vectors for protein production in *Pseudomonas aeruginosa*. Gene **172:**163–164.
53. **Yahr, T. L., J. Goranson, and D. W. Frank.** 1996. Exoenzyme S of *Pseudomonas aeruginosa* is secreted by a type III pathway. Mol. Microbiol. **22:**991–1003.
54. **Yahr, T. L., L. M. Mende-Mueller, M. B. Friese, and D. W. Frank.** 1997. Identification of type III secreted products of the *Pseudomonas aeruginosa* exoenzyme S regulon. J. Bacteriol. **179:**7165–7168.
55. **Yahr, T. L., A. J. Vallis, M. K. Hancock, J. T. Barbieri, and D. W. Frank.** 1998. ExoY, an adenylate cyclase secreted by the *Pseudomonas aeruginosa* type III secretion system. Proc. Natl. Acad. Sci. USA **95:**13899–13904.
56. **Zolfaghar, I., A. A. Angus, P. J. Kang, A. To, D. J. Evans, and S. M. J. Fleiszig.** 2005. Mutation of *retS*, encoding a putative hybrid two-component regulatory protein in *Pseudomonas aeruginosa* attenuates multiple virulence mechanisms. Microbes Infect. **7:**1305–1316.

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