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Dual Function of PilS During Transcriptional Activation of the *Pseudomonas aeruginosa* Pilin Subunit Gene

JESSICA M. BOYD* AND STEPHEN LORY

Department of Microbiology, University of Washington, Seattle, Washington 98195-7242

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The polar pili of *Pseudomonas aeruginosa* are composed of subunits encoded by the *pilA* gene. Expression of *pilA* requires the alternative sigma factor RpoN and a pair of regulatory elements, PilS and PilR. These two proteins are members of the two-component regulatory family, in which PilS is the sensory component and PilR is the response regulator. By using expression and localization analyses, in this work we show that PilS is synthesized as a 59-kDa polypeptide located in the *P. aeruginosa* cytoplasmic membrane. When the *pilS* gene is expressed in *Escherichia coli*, aberrant translational initiation results in a smaller, 40-kDa polypeptide. Unexpectedly, overexpression of *pilS* in *P. aeruginosa* results in decreased transcription of the *pilA* gene. Moreover, fully functional PilS was not required for this inhibitory effect. A mutation in the histidine residue essential for kinase activity resulted in a protein unable to activate transcription, yet when overexpressed in the presence of the wild-type PilS protein, this protein still repressed pilin synthesis. A shorter form of PilS, lacking its transmembrane segments, was active and fully capable of stimulating *pilA* transcription but when overexpressed did not show the inhibitory effect on pilin expression seen with full-length PilS. We also show that overexpression of *pilR* can activate transcription of *pilA* even in the absence of PilS. On the basis of our studies, we propose a complex mechanism of regulation of PilS function, involving other cellular factors that control PilS and its activities during the phosphorelay mechanism of signal transduction.

Pseudomonas aeruginosa is a common gram-negative environmental microorganism that can also be an important opportunistic human pathogen. *P. aeruginosa* infections of mucosal surfaces, such as the respiratory tracts of patients with cystic fibrosis, are very likely initiated by a specific interaction of adhesins on the bacterial surface with epithelial cell or mucin receptors (43, 46). This colonization step is followed by elaboration of a number of extracellular toxic factors which cause tissue damage and elicit a strong inflammatory response. It is apparent that bacterial adhesion to epithelial cells is mediated by the pilus organelle (52), which is composed of a major subunit protein, pilin, encoded by the *pilA* gene, and assembled with the assistance of a number of accessory proteins (reviewed in references 18 and 47).

The mechanism for expression and assembly of *P. aeruginosa* pili is complex. Previous work in this laboratory has shown that *pilA* is transcribed by RNA polymerase containing the alternative sigma factor, σ^{54} (RpoN) (48). As with other genes transcribed with σ^{54}, *pilA* requires an additional transcriptional activator, PilR. This transcriptional activator is a member of a two-component signal transduction system. Such systems contain, in addition to the transcriptional regulator, a sensor protein which transmits environmental signals to the regulator by a phosphorelay mechanism. PilS is the sensory component involved in activation of *pilA* through the regulator PilR. Once activated by a specific environmental signal, the sensor protein autophosphorylates at a conserved histidine residue and subsequently transfers the phosphate to the response regulator. In the absence of continuous signal, dephosphorylation of the response regulator leads to reduced transcription from the regulated promoter. Response regulators seem to have intrinsic autophosphatase activity but are generally aided in this respect by other proteins. In some systems this function is fulfilled by the sensory component itself (19) while other systems use a third protein (16). Neither the nature of the signal which stimulates autophosphorylation of PilS nor the mechanism of the removal of the phosphate from phospho-PilR is known at this time.

The purpose of this study was to characterize the signal transduction system composed of PilS and PilR. We show that PilS is a 58,997-Da inner membrane protein predicted to have six transmembrane helices in its N terminus. The C-terminal portion of PilS is cytoplasmic and contains all the conserved domains indicative of histidine kinase sensors. We have constructed several truncated and full-length variants of PilS and examined their effects on pilin expression. Interestingly, when overexpressed, *pilS* inhibits transcription of *pilA*, and this inhibitory effect is independent of its kinase activity. We also show that overexpression of *pilR* in the absence of PilS can activate pilin transcription. These data provide evidence for a fine modulation of the activity of the sensor, perhaps involving another bacterial component.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are described in Table 1 and diagrammed schematically in Fig. 1. Bacteria were grown in L broth or in minimal medium A (9) supplemented with 1% glycerol, 1% monosodium glutamate, and 1 mM $MgCl_2$. The following antibiotics were used: against *Escherichia coli*, ampicillin (100 μg/ml), tetracycline (20 μg/ml), kanamycin (25 μg/ml), and streptomycin (30 μg/ml); against *P. aeruginosa*, carbenicillin (100 to 150 μg/ml), tetracycline (75 to 200 μg/ml), gentamicin (10 to 80 μg/ml), streptomycin (200 μg/ml), and spectinomycin (200 μg/ml). IPTG (isopropyl-β-D-thiogalactopyranoside) was added at concentrations indicated.

DNA manipulations. All plasmids were isolated by the alkaline lysis method (3). Standard recombinant DNA techniques were used (2). Enzymes were purchased either from Bethesda Research Laboratories (Gaithersburg, Md.) or from New England Biolabs (Beverly, Mass.). Mutations were made with single-stranded template derived from plasmid pJB300 in the *dut ung E. coli* mutator strain, RZ1032, following the method described by Venkitaraman (50). Mutations were made at the conserved histidine residue, His-319, with the following oligonucleotide: 5′ GTTGCGGATCTCAVGGGCGATGCCG 3′ (V = G, C, or A). This method created three mutations, His-319 to Arg (His319Arg),

* Corresponding author. Mailing address: Department of Microbiology, University of Washington, Box 357242, Seattle, WA 98195-7242. Phone: (206) 616-4378. Fax: (206) 543-8297. Electronic mail address: jessieb@u.washington.edu.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant trait(s)[a]	Source or reference
E. coli		
DH5α	*hsdR recA lacZYA ϕ80 lacZ*ΔM15	Bethesda Research Laboratories
XL-1 Blue	*recA1 endA1 gyrA96 thi1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q} ZΔM15 Tn*10* (Tet^r)]	Stratagene
RZ1032	*dut ung Hfr thi1 relA1 supE44* Zbd-279::Tn*10* Tet^r	27
P. aeruginosa		
PAK	Wild-type	D. Bradley
PAK-R1	*pilR*::Tn*5G* Gm^r	21
PAK-RA	*pilS*::Tn*5G* Gm^r	21
PAK-ΔS2	In-frame deletion of *pilS*, Str^r	This study
Plasmids[b]		
pBluescript II SK$^-$	Ap^r phagemid cloning vector	Stratagene
pMMB67EH/HE	Ap^r broad-host-range cloning vector IncQ *lacI*^{q} *ptac*	13
pMSZ5	Tet^r IncP with *ppilA-lacZ* fusion	21
pRK2013	Kan^r mobilizer	10
pJB300	*pilS* and *pilR* in pBluescript II SK$^-$	5
pJB300 GTG445	pJB300 with GTG445 changed to ATC	5
pJB300 TTG1042	pJB300 with TTG1042 changed to GGG	5
pJB300 ATG1057	pJB300 with ATG1057 changed to ATC	5
pJB304	Cytoplasmic domain of *pilS pilR* in pBluescript SK$^-$	This study
pJB307	Cytoplasmic domain of *pilS* with His tag in pBluescript SK$^-$	This study
pJB306ΩpBR	In-frame deletion of *pilS pilR* in Bluescript SK$^-$, fused to pBR322	This study
pJB207	*pilS* and *pilR* with *pilS* in pMMB67EH	5
pJB207 GTG445	pJB207 with GTG445 changed to ATC	5
pJB207 TTG1042	pJB207 with TTG1042 changed to GGG	5
pJB207 ATG1057	pJB207 with ATG1057 changed to ATC	5
pJB210	*pilS* with *ptac ppilS* in pMMB67HE	This study
pJB211	*pilS* with ppilS in pMMB67EH	This study
pJB222	pJB210 with C-terminal His tag	This study
pJB224	Cytoplasmic domain of *pilS* with *ptac* in pMMB67HE	This study
pJB226	*pilS pilR* with *ptac* in pMMB67EH	This study
pJB228	*pilS* with *ptac* in pMMB67EH	This study
pJB228HR	pJB228 with *pilS* His-319 changed to Arg	This study
pJB228HL	pJB228 with *pilS* His-319 changed to Leu	This study
pJB228HP	pJB228 with *pilS* His-319 changed to Pro	This study
pKI23	*pilR* with *ptac* in pMMB67EH	21

[a] Abbreviations: Tet^r, Kan^r, Ap^r, and Gm^r, resistance to tetracycline, kanamycin, ampicillin, and gentamicin, respectively.
[b] The structures of the plasmids constructed in the course of this study are outlined in Fig. 1.

His319Leu, and His319Pro. An *Eco*RI site was introduced between the *pilS* ribosome binding site and start codon with the following oligonucleotide: 5′ GTTGAGCGCGCACGAATTCCCTGGTCCTGGCG 3′. This enabled the positioning of the *pilS* GTG start codon to within 10 bp of the ribosome binding site of the *tac* promoter carried on the pMMB67EH vector. Two plasmids were built this way, pJB226, which carries both *pilS* and *pilR*, and pJB228, which carries only *pilS*. A cytoplasmic version of PilS, PilS-cyt, was constructed in the pMMB67HE vector by deleting the 5′ end of the *pilS* gene up to the *Sph*I site; this construct, pJB224, uses the *tac* promoter and is presumed to initiate at methionine 205 of full-length PilS.

DNA sequencing was performed by the dideoxy-chain termination method (44) with the Sequenase DNA sequencing kit (United States Biochemical Corp., Cleveland, Ohio). Single-stranded template was prepared from the *E. coli* strain XL1-Blue with the helper phage VCSM13 (both from Stratagene, La Jolla, Calif.). Triparental spot matings (29, 39) with the mobilizing plasmid pRK2013 were performed to transfer plasmids from *E. coli* to *P. aeruginosa*.

Immunoblotting procedures. Overnight cultures of *P. aeruginosa* grown in broth with or without IPTG were harvested, resuspended in 2× reducing buffer (3% sodium dodecyl sulfate [SDS], 20% glycerol, 5% β-mercaptoethanol, 100 mM Tris-HCl [pH 8.0], 0.01% bromophenol blue), boiled 5 min, and sonicated 3 s to shear DNA. Cellular fractions were also prepared for electrophoresis by boiling in 2× reducing buffer. All samples were separated by SDS-polyacrylamide gel electrophoresis (PAGE) (28) and electroblotted to Protran nitrocellulose membranes (Schleicher and Schuell, Keene, N.H.) (49). Blots were incubated with appropriate antibodies, and bound immunoglobulins were identified with ^{125}I-labeled protein A (New England Nuclear, Boston, Mass.).

Construction and purification of the PilS-MBP and PilR-MBP fusion proteins and antibody production. Maltose-binding protein (MBP) fusions to both PilS and PilR were made with the MBP system from New England Biolabs. The PilS fusion was made with the pmal-cR1 vector that directs synthesis of the fusion protein to the cytoplasm. Only the cytoplasmic portion of the *pilS* gene was used. The 1,030-bp *Sph*I-*Eco*RV fragment of pJB300 was cloned into the *Stu*I site of the pmal-cR1 vector by blunt-end ligation after treatment with T4 polymerase. The entire *pilR* gene was used to create the PilR-MBP fusion; the construction of this plasmid has been previously described (23). Fusion proteins were expressed in *E. coli* DH5α induced with IPTG and purified on an amylose resin column as described in the New England Biolabs protocol. Attempts to cleave MBP from PilS and PilR were unsuccessful, so the entire fusions were used. Antibodies to the PilS-MBP and PilR-MBP fusions were made in New Zealand White rabbits (Pocono Rabbit Farm, Canadensis, Pa.). The crude sera from these rabbits were purified by ammonium sulfate precipitation (50% saturation) followed by isolation of immunoglobulin G on a protein A affinity column (Pierce, Rockford, Ill.). Specific anti-PilS and anti-PilR antibodies were purified by passage over an *E. coli* lysate column (Pierce) and adsorption to an acetone powder of PAK-RA (pMMB67EH).

Construction and purification of the PilS-His tag fusion and N-terminal sequencing. The purification strategy for full-length PilS involved the addition of a C-terminal hexahistidine tag followed by nickel affinity chromatography. The sequence encoding the His tag was added to the *pilS* gene in plasmid pJB304 by PCR with the following primers: His tag, 5′ GTACGTACGTGAATTCAATGA TGATGATGATGGTGGCTGAGTTTGCGCGG 3′; and KS, 5′ CGAGGTCG ACGGTATCG 3′. The His tag primer inserted six histidines between the last amino acid codon of *pilS* and the stop codon. It also introduced an *Eco*RI site just after the stop codon. The KS primer is a commercially available sequencing primer which hybridizes to the pBluescript II vector (both from Stratagene). The 1-kb PCR fragment generated from this amplification was cloned into pBluescript II KS$^-$ with *Eco*RI and *Hin*dIII, giving pJB307, and the His tag end was sequenced to assure that the tag was complete and in frame. Since pJB307 does not contain the whole *pilS* gene and to obviate the need to sequence the entire PCR product, the His tag was added to *pilS*-containing plasmid pJB210 with a 200-bp *Sst*I-*Eco*RI fragment from pJB307 that contained the last 31 codons of *pilS*, the six histidine codons, and a stop codon, creating pJB222.

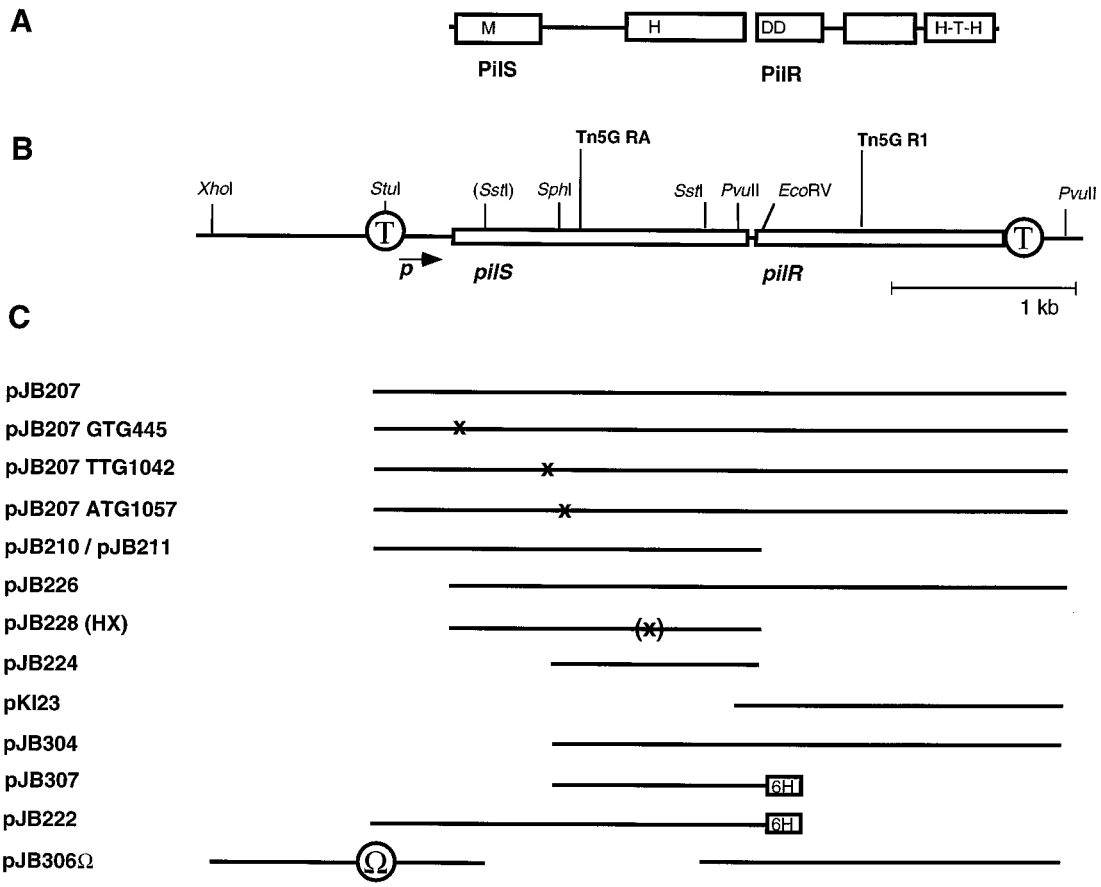

FIG. 1. (A) Schematic representation of PilS and PilR showing major functional domains and some residues conserved among two-component systems. M, membrane-bound domain; H, histidine residue conserved among sensor kinases; DD, aspartate residues conserved among response regulators; H-T-H, helix-turn-helix DNA binding domain. (B) Map of *pilSR* operon. The arrow shows the position of the promoter and the direction of transcription. The position of the Tn*5G* transposons, R1 and RA, are indicated, as are some relevant restriction enzyme sites. (*Sst*1), artificially created restriction site; T, terminator. (C) Plasmids used in this study. Lines delineate inserted fragments and correspond to the maps in panels A and B. X, site of mutations; 6H, hexahistidine tag; Ω, omega cassette.

The PilS-His tag was purified by nickel affinity chromatography with Probond resin (Invitrogen, San Diego, Calif.). Briefly, 2-liter cultures of *P. aeruginosa* PAK-RA(pJB222) were grown overnight in L broth with 1 mM IPTG. Cells were harvested and resuspended in 50 mM Tris (pH 8.0)–10 mM $MgCl_2$. DNase I and RNase I were added to 50 μg/ml each, and the cells were lysed by French press at 1,200 lb/in^2. Phenylmethylsulfonyl fluoride was added to 100 μg/ml, lysozyme was added to 0.5 mg/ml, and the solution was incubated at room temperature for 20 min. After a low-speed centrifugation step was performed (10,000 × *g* for 10 min) to remove unlysed cells, the supernatant was layered onto a 15% sucrose step gradient over a 70% sucrose cushion and centrifuged for 1 h at 35,000 × *g*. The membranes were removed from the 15% sucrose–70% sucrose interface. Total protein was measured by the Bio-Rad DC detergent-compatible protein assay (Bio-Rad, Hercules, Calif.), and the sample was diluted to a protein concentration of 5 mg/ml in TBS (50 mM Tris-HCl (pH 8.0), 150 mM NaCl). PilS-His was solubilized from the inner membrane by a 1-h incubation on ice in the presence of 0.25% Sarkosyl. The insoluble membrane fragments were removed by centrifugation at 100,000 × *g* for 1 h. After the NaCl concentration of the supernatant was increased to 500 mM, the supernatant was loaded onto the Probond resin following the manufacturer's instructions, except that 0.25% Sarkosyl was added to all column buffers. Bound protein was eluted from the column with 200 mM imidazole–0.25% Sarkosyl.

N-terminal sequencing was performed at the University of Washington Molecular Pharmacology Department on PilS-His tag that had been purified by PAGE and transferred to polyvinylidene difluoride membranes.

Cellular fractionation. *P. aeruginosa* cells were separated into membrane and cytosolic-periplasmic fractions. Cells were grown and lysed as described above, except that total membranes were harvested (100,000 × *g* for 1 h) instead of being separated on a sucrose gradient. To remove contaminating proteins from the membrane and cytosolic-periplasmic fractions, the supernatant was collected and the membrane pellet was resuspended in TBS. These two fractions were again centrifuged at 100,000 × *g* for 1 h. This final step was repeated once more before immunoblot analysis. The blots were probed with anti-β-lactamase (5 Prime → 3 Prime Inc., Boulder, Colo.) as the marker for soluble material and anti-OprF (8) as the membrane marker.

Construction of a nonpolar deletion in *pilS*. An *Sst*I site was introduced into plasmid pJB300 at amino acids 38 and 39 of PilS by oligonucleotide mutagenesis with the following oligonucleotide: 5′ GACCTGATCTTCGAGCTCGCTGGA GATC 3′. This restriction site was in frame with a preexisting *Sst*I site at amino acids 498 and 499. The resulting 1,380-bp *Sst*I fragment was deleted, and the plasmid was recircularized, resulting in pJB306. This left the *pilS* promoter and *pilR* gene intact but removed 460 of 531 amino acids from PilS. The region upstream of *pilS* was reconstructed by addition of an 800-bp *Xho*I-*Stu*I fragment from cosmid pKIR2 (21); the streptomycin resistance and transcriptional terminator omega cassette from plasmid pUC19Ω (26) was inserted in the *Stu*I site to make plasmid pJB306Ω (Fig. 1). Plasmid pJB306Ω was fused to pBR322 (4) at the *Sca*I site in the *bla* gene of each plasmid, creating pJB306ΩpBR. Plasmid pJB306ΩpBR has transfer functions but cannot replicate in *P. aeruginosa*. It was mobilized into *P. aeruginosa* PAK-RA which has a Tn*5G* insertion in *pilS* at the region to be deleted. The gentamicin resistance transposon of PAK-RA was replaced with the deletion and the streptomycin-resistant Ω cassette by marker exchange. Southern blots were performed to confirm the success of the marker exchange (data not shown). An individual clone was chosen and designated PAK-ΔS2.

β-Galactosidase assay. β-Galactosidase activity of *P. aeruginosa* grown overnight in L broth with or without the addition of 1 mM IPTG was measured as described by Miller (35).

Accession number. The DNA sequence of the *pilSR* operon has been assigned GenBank accession no. L22436 and NCBI sequence ID 347992. The amino acid sequence of PilS has been assigned NCBI sequence ID 508715.

RESULTS

Expression of *pilS* and its membrane localization in *P. aeruginosa*. We had previously demonstrated that when ex-

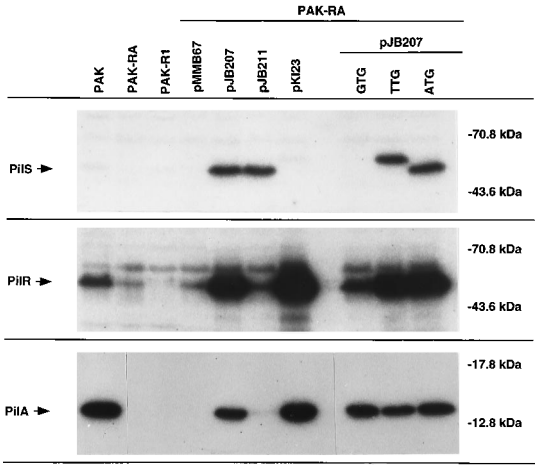

FIG. 2. Western blots of *P. aeruginosa*. Whole-cell extracts were prepared in triplicate and probed with one of three antibodies (anti-PilS, anti-PilR, or anti-PilA). The positions of the antigens are indicated on the left. Molecular mass standards are on the right. The bacterial strain is indicated at the top of each lane. To visualize chromosomal expression of PilS and PilR, twice as much material was loaded into the first three lanes as in the other lanes.

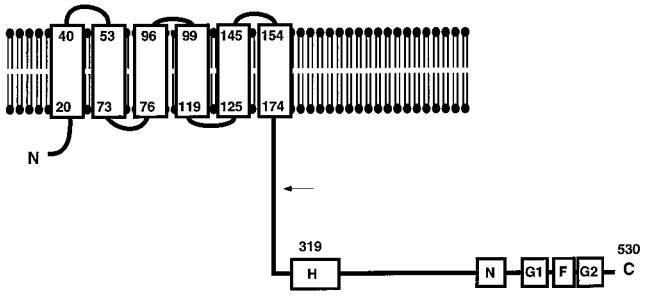

FIG. 3. Schematic representation of PilS showing the six N-terminal transmembrane helices predicted by TopPred II (7). Numbers indicate amino acid residues. Labeled boxes represent the conserved domains (for a review of H, N, G1, F, and G2, see reference 42). The arrow indicates the approximate N terminus of the truncated cytoplasmic derivative of PilS, PilS-cyt, which corresponds to residue 205 of the full-length protein.

pressed in *E. coli* under control of the T7 promoter-RNA polymerase, *pilS* is synthesized as a 40-kDa polypeptide (5). This raised the possibility that PilS, lacking putative transmembrane domains, is cytoplasmic. This result was further supported when a site-directed mutation of the predicted translational start site (TTG at nucleotide position 1042 [TTG1042]) failed to produce the 40-kDa protein in T7 expression experiments with *E. coli*. We have since produced antibody to PilS which has made it possible to examine the expression of *pilS* and the localization of its protein product in *P. aeruginosa*.

Western blot (immunoblot) analysis of *P. aeruginosa* whole-cell lysates with anti-PilS antibody clearly shows that PilS produced in *P. aeruginosa* has a mobility in polyacrylamide gels of about 50-kDa (Fig. 2). The levels of PilS and PilR produced by the single chromosomal copy of the genes are very low and difficult to visualize by Western blot, but when *pilS* and *pilR* are expressed from a multicopy plasmid (pJB207) their protein products are readily detectable. The 50-kDa size of PilS is consistent with a protein beginning at the translational initiation codon GTG445 (Fig. 2 of reference 5), which is 600 bp upstream from the TTG apparently used by *E. coli*. In order to identify the PilS start codon in *P. aeruginosa*, derivatives of plasmid pJB207 with mutations at three candidate PilS start codons (Fig. 1) were created. These plasmids were introduced into PAK-RA (*pilS*::Tn*5G*) and were tested by Western blot for their ability to produce PilS and PilR (Fig. 2). These were the same three mutations used in the *E. coli* T7 expression experiments: GTG445 changed to ATC, TTG1042 changed to GGG, and ATG1057 changed to ATC. As shown in Fig. 2, *P. aeruginosa* PAK-RA carrying pJB207 GTG445 failed to express *pilS*, confirming that GTG445 is the PilS start codon in *P. aeruginosa*. In T7 expression experiments with *E. coli*, this mutant construct produced the 40-kDa PilS-related protein. On the other hand, PAK-RA (pJB207 TTG1042) did produce an anti-PilS-reactive product in *P. aeruginosa*, although its mobility was less than that of wild-type PilS. In *E. coli* this mutant did not produce any detectable PilS-related product. The evidence from these two mutant constructs suggests that the *E. coli* translational machinery fails to recognize the *P. aeruginosa* initiation codon. Thus, in *E. coli* translation is initiated at an internal site, producing a smaller polypeptide. Expression of *pilS* and *pilR* from the third mutant, ATG1057, was normal in both *E. coli* and *P. aeruginosa*. The level of *pilR* expression from the TTG and ATG mutants was normal, and it was slightly reduced in the GTG mutant. Levels of pilin production from all three mutant plasmids were identical to that produced by pJB207. The reason for the decreased electrophoretic mobility of the PilS TTG1042 mutant is not clear, but this decrease probably reflects an alteration in secondary structure.

To verify that translation of *pilS* mRNA initiates at start codon GTG445, the N terminus of PilS was sequenced. To aid in purification, six histidine residues were added to the C terminus of *pilS* by PCR. This PilS-His tag was produced in *P. aeruginosa* and purified on a nickel affinity column. N-terminal sequencing confirmed that the first three amino acids are Met-Arg-Ala, which correspond to the first three amino acids predicted from the DNA sequence starting at GTG445.

On the basis of these data, PilS is a 58,997-Da protein that initiates at a GTG start codon 600 bp upstream of the TTG predicted in the previous paper (5). Examination of the predicted 180 N-terminal amino acid residues of PilS showed them to be highly hydrophobic, suggesting that this region might form membrane-spanning helices. Indeed, analysis of this region with the computer program TopPred II (7), which predicts the topology of proteins in biological membranes, suggested that this region could form six membrane-spanning helices, with the conserved C-terminal kinase domain in the cytosol (Fig. 3). Further proof of the membrane localization of PilS was ascertained by separation of *P. aeruginosa* into total membrane and cytosolic-periplasmic fractions. Western blots of these fractions (Fig. 4) clearly showed that PilS was localized to the membrane fraction and that PilR was in the soluble cytosolic-periplasmic fraction. Since PilS could be solubilized from the total membrane fraction with low concentrations (0.25%) of Sarkosyl, a trait of other *P. aeruginosa* inner-membrane proteins (40), we concluded that PilS is localized to the inner membrane.

Operon organization of *pilSR*. The antibodies to PilS and PilR also allowed investigation of the operon organization of *pilS* and *pilR*. There are only 14 bp between the *pilS* stop codon and the *pilR* start codon, with no apparent promoter sequences; thus, it was likely that *pilS* and *pilR* were expressed as a polycistronic message. This was confirmed by Western blots of PAK and the two transposon mutants PAK-RA (*pilS*::Tn*5G*) and PAK-R1 (*pilR*::Tn*5G*) (Fig. 2). Neither of these strains can produce pilin, and both are resistant to killing by the pilin-specific phage PO4. Strain PAK-RA produced no PilS and very

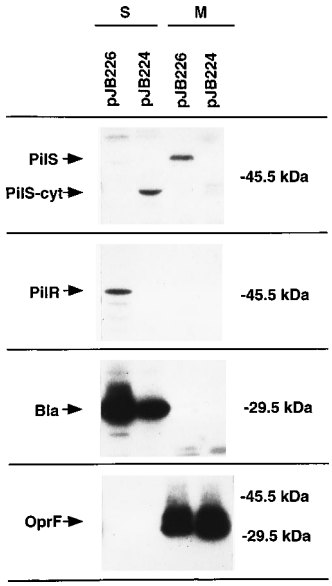

FIG. 4. Cellular localization of PilS, PilR, and PilS-cyt. *P. aeruginosa* PAK-RA carrying either pJB226 (both *pilS* and *pilR*) or pJB224 (*pilS-cyt*) was separated into membrane (M) and soluble (S) fractions. Western blots were prepared in quadruplicate and probed with one of four antibodies (anti-PilS, anti-PilR, anti-β-lactamase, or anti-OprF). The positions of molecular mass standards are indicated on the right. The positions of the relevant antigens are indicated on the left.

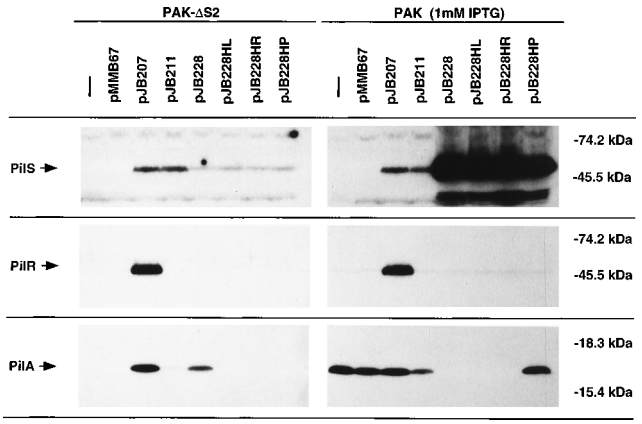

FIG. 5. Complementation of *P. aeruginosa pilS* mutants. The wild-type *P. aeruginosa* PAK or the *pilS* in-frame deletion mutant PAK-ΔS2 carrying various *pilS* plasmids was grown under noninducing or inducing conditions and assayed for the production of pilin. Western blots of whole-cell extracts were prepared in triplicate and probed with one of three antibodies (anti-PilS, anti-pilR, or anti-PilA). The positions of the relevant antigens are indicated on the left. The positions of molecular mass standards are indicated on the right. The bacterial strain and growth condition are indicated at the top of each lane. —, strain without a plasmid.

little PilR (presumably directed from the transposon promoter). Conversely, PAK-R1 (*pilR*::Tn*5G*) produced normal levels of PilS but no PilR. Attempts to complement PAK-RA with *pilS* carried on plasmid pJB211 were unsuccessful. Although this strain produced high levels of PilS, it produced little or no pilin antigen and was partially resistant to killing by the pilus-specific phage PO4. Since plasmid pJB207, carrying both *pilS* and *pilR*, was able to complement the *pilS* mutant strain, we concluded that the Tn*5G* insertion in PAK-RA exerted a polar effect on *pilR* expression.

To further investigate the polar nature of the PAK-RA mutant, a strain with a nonpolar deletion in the *pilS* gene was engineered. This strain, PAK-ΔS2, has the *pilS* promoter and *pilR* gene intact but has 460 of 530 amino acids of PilS deleted. Western blot analysis (Fig. 5) indicated that this strain produced no PilS (even after prolonged exposure of the Western blots) but produced wild-type or slightly reduced levels of PilR. This strain did, however, produce some pilin (visible only after prolonged exposure of the Western blot) and was partly phage PO4 sensitive. Similar to what was observed for the transposon mutant PAK-RA, pilin production of PAK-ΔS2 was not complemented by the *pilS*-expressing plasmid pJB211 (Fig. 5). However, as was the case for the polar mutant, this nonpolar *pilS* mutant could be complemented with plasmid pJB207, which expresses both *pilS* and *pilR* together.

Overexpression of *pilS* has a negative effect on pilin gene expression. The findings described above raised the possibility that overexpression of PilS may have an inhibitory effect on pilin expression. Plasmid pJB211 carries not only *pilS* but also the *pilS* promoter and produces large amounts of PilS. In order to test the effects of various levels of PilS in the cell, a plasmid that more tightly regulates expression of *pilS* was constructed. This construct, pJB228, removes the *pilS* promoter and places the *tac* promoter and ribosome binding site of the vector within 10 bp of the *pilS* GTG start codon. When induced with 1mM IPTG, P. aeruginosa(pJB228) produced much higher levels of PilS (as visualized by Western blot [Fig. 5]) than did the same strain carrying plasmids pJB207 or pJB211. When uninduced, pJB228 produced less PilS than did plasmids pJB207 and pJB211. The low level of expression from the uninduced plasmid is presumably due to leakiness of the *tac* promoter. In contrast to pJB211, the small amount of PilS produced by uninduced pJB228 was sufficient to complement PAK-ΔS2 (Fig. 5). The only difference between these two strains is the amount of PilS, and the strain that produces the most PilS (pJB211) produces the least PilA.

To examine whether the high levels of PilS produced by pJB228 could inhibit pilin production in the wild-type strain, this plasmid was introduced into *P. aeruginosa* PAK and induced with IPTG (Fig. 5). As shown by these Western blots, synthesis of pilin in this strain was drastically reduced compared with that of PAK carrying the vector control. Thus, overexpression of *pilS* has a dominant negative effect on the expression of pilin. When strain PAK(pJB228) is grown without IPTG, very little extra PilS is produced and pilin production is not inhibited (data not shown).

The inhibitory effect of overexpression of *pilS* on pilin synthesis was further confirmed by controlling the extent of *pilS* transcription with different concentrations of IPTG in cultures of PAK-ΔS2(pJB228). As the amount of PilS was increased with IPTG at concentrations from 0.01 to 1 mM, the amount of pilin decreased beyond the limit of detectability (Fig. 6). In all of these samples, the amount of PilR remained at a constant wild-type level (data not shown).

To examine whether the inhibitory effect of high PilS concentrations was due to reduced *pilA* transcription, a *pilA-lacZ* promoter fusion plasmid, pMSZ5, was introduced into relevant *P. aeruginosa* strains. In all cases, the level of pilin seen on a Western blot correlated with the level of β-galactosidase activity of that strain (Table 2). For example, PAK(pMSZ5, pMMB67) produced high levels of β-galactosidase activity, 24,000 U, which corresponded to a readily visible anti-PilA-reactive band on Western blots of PAK(pMMB67) (Fig. 5 and 6). When the vector control was replaced with a plasmid carrying *pilS*, pJB228, both the level of β-galactosidase and the

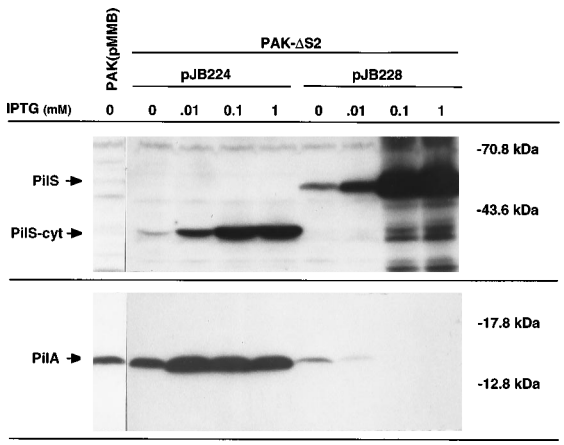

FIG. 6. The effects of various amounts of PilS on pilin expression. The *pilS* deletion strain, ΔS2, carrying either pJB228 (full-length PilS) or pJB224 (cytoplasmic derivative of PilS) was grown with increasing amounts of the inducer IPTG and assayed for production of pilin. Western blots of whole-cell extracts were prepared in duplicate and probed with anti-PilS or anti-PilA. The positions of the antigens are indicated on the left. The positions of molecular mass standards are indicated on the right. The bacterial strain, plasmid, and growth condition are indicated at the top of each lane.

intensity of the pilin band were reduced. When the expression of *pilS* in this strain is increased by induction with IPTG, the level of β-galactosidase and the intensity of the pilin band are reduced even further. Therefore, the overexpression of *pilS* leads to decreased levels of pilin polypeptide by adversely influencing transcription of the *pilA* gene, and this inhibitory effect does not involve alterations in the levels of PilR.

High levels of PilR can activate pilin expression without PilS. While plasmids that overexpressed *pilS* inhibited *pilA* transcription, plasmids with both *pilS* and *pilR* allowed normal pilin expression in all strains. To further investigate the role of PilR in these strains, plasmid pKI23, which carried the *pilR* gene regulated by the *tac* promoter, was introduced into *pilR* and *pilS* mutants of *P. aeruginosa*. As expected, this plasmid was able to restore normal pilin expression to the *pilR*::Tn*5G* strain PAK-R1 (Table 2). Surprisingly, it was also able to restore pilin expression to the *pilSR* mutant strain PAK-RA (Fig. 2). Since PilR could not have been phosphorylated by PilS in PAK-RA, it was either phosphorylated by cross talk with another histidine kinase or it was autophosphorylated with small-molecule phosphodonors as has been shown for other response regulators (12, 32, 34). Alternatively, excess PilR may be able to activate *pilA* transcription in the unphosphorylated state.

TABLE 2. Transcriptional activation of the *pilA* promoter during overexpression of *pilS*

Strain	Plasmid	β-Galactosidase activity (Miller units)	
		Uninduced	1 mM IPTG
PAK(pMSZ5)	pMMB67	23,964	23,574
	pJB228	16,037	1,087
PAK-RA(pMSZ5)	pMMB67	2,209	1,334
	pJB226	30,606	7,370
PAK-ΔS2(pMSZ5)	pMMB67	6,418	3,883
	pJB228	10,949	1,088
	pJB224	26,138	70,840
PAK-R1(pMSZ5)	pMMB67	1,787	1,635
	pKI23	26,085	71,405

Analysis of point mutations in the conserved histidine residue of PilS. Sensor proteins of two-component systems have a conserved histidine residue, which has been shown to be the site of autophosphorylation in all cases studied (15, 38). In the case of PilS, the corresponding residue is at position 319, within a highly conserved region. In order to determine if this conserved histidine is important in signal transduction, site-directed mutagenesis of the codon was used to change the histidine to either arginine, leucine, or proline. Constructs analogous to pJB228 carrying these mutations were created and designated pJB228HR (His319Arg), pJB228HL (His319Leu), and pJB228HP (His319Pro). All of these plasmids directed production of a PilS-related product comparable in size and amount to that produced by pJB228, as determined by immunoblot analysis with anti-PilS antibody (Fig. 5). These plasmids were introduced into strain PAK-ΔS2, grown in the absence of IPTG, and tested for the production of pilin. None of the PilS histidine mutants were able to complement the defect in pilin synthesis caused by the chromosomal deletion of *pilS*, which the wild-type PilS could do. This result confirmed the requirement for the conserved histidine 319 for PilS activity and indicates the use of the well-studied phosphorelay mechanism in PilS-mediated signal transduction for the transcription of *pilA*.

In order to examine whether the inhibition of pilin synthesis, caused by overexpression of *pilS*, depends on the presence of the conserved histidine, pJB228HL, pJB228HR, and pJB228HP were introduced into wild-type PAK and high-level expression of the *pilS* mutants was induced with IPTG (Fig. 5). No pilin was detectable in PAK carrying the PilS-H319L and PilS-H319R mutants while PAK carrying the PilS-H319P construct produced wild-type levels of pilin. Thus, PilS-H319L and PilS-H319R exhibited the same dominant negative effect on pilin expression as did the wild-type PilS while PilS-H319P could not inhibit pilin expression. When these histidine mutants were grown in PAK without IPTG, very little PilS-related product was produced and pilin synthesis was not inhibited (data not shown).

The activities of PilS-H319L and PilS-H319R show that histidine 319 is necessary for activation of pilin gene expression, but it is not essential for inhibition of pilin gene transcription.

Expression of the cytoplasmic portion of PilS results in elevated levels of *pilA* expression. Many membrane-bound histidine kinases have been shown to be restricted to the signal-sensitive form by membrane association through a hydrophobic membrane domain, and when freed of this region these kinases are capable of constitutively activating their cognate response regulators (20, 24, 33). A cytoplasmic derivative of PilS, PilS-cyt, was engineered to examine whether low-level expression of this form could complement a *pilS* mutation by activating *pilA* transcription and also to see if it retained the ability to inhibit pilin expression when highly expressed. PilS-cyt was constructed by deleting the 5′ end of the *pilS* gene up to the *Sph*I site (Fig. 1). This plasmid, pJB224, uses the *tac* promoter of the vector pMMB67HE and produces a 40-kDa protein that reacts with the anti-PilS antibody. The cytoplasmic location of PilS-cyt was confirmed by cellular fractionation experiments (Fig. 4). Like plasmid pJB228, the amount of PilS-cyt produced by pJB224 can be controlled by the amount of IPTG in the culture. In the absence of IPTG, pJB224 was capable of fully complementing PAK-ΔS2 (Fig. 6). Unlike full-length PilS, however, increased amounts of PilS-cyt caused increased amounts of pilin. As shown in Table 2, the increased

level of pilin in this strain was due to increased *pilA* transcription. Also, unlike the full-length PilS, PilS-cyt did not interfere with pilin expression in the wild-type strain PAK (data not shown). These results suggest that PilS-cyt is constitutively active.

DISCUSSION

The sensory components of bacterial two-component regulatory systems often reside in the membrane, where they are the recipients of external signals that initiate the phosphorelay mechanism of signal transduction. The signal stimulates autophosphorylation of the sensor followed by transfer of the phosphate to the regulator protein, which, if it is a transcriptional activator, binds to a specific sequence near the promoter of the target gene, leading to its expression. In this paper we show that PilS, the sensory component of the pilin expression system of *P. aeruginosa*, is also a membrane protein with many features similar to those of other sensors of two-component systems; however, it also has some unique characteristics.

We used antibodies to PilS to show that it is a 58,997-Da, membrane-bound protein. We predict that the N terminus is embedded in the inner membrane and that the C-terminal domain is cytoplasmic. This is in contrast to the study we previously reported, in which T7 expression experiments in *E. coli* led us to conclude that PilS was a much smaller, 40-kDa, hydrophilic protein which was therefore likely to be found in the cytosol (5). Hobbs and coworkers (17), working with PilS in *P. aeruginosa* PAO1, identified two possible translational start positions, one being GTG445 and the other being TTG1042. The *P. aeruginosa* immunoblot analysis described in this work clearly shows that PilS is a 50- to 60-kDa protein. Analysis of the GTG445 mutant coupled with the N-terminal sequence data confirms that PilS starts at the GTG codon, and the cellular fractionation experiments prove that PilS is a membrane protein.

The shorter PilS-related protein seen in the *E. coli* T7 expression experiments was probably caused by the inability of *E. coli* to recognize the authentic initiation codon, leading to errant translational initiation of the *pilS* mRNA. We reported previously that a *pilS* promoter-*lacZ* fusion was not expressed in *E. coli*; thus, it seems that neither the *pilS* promoter nor the *pilS* translational initiation signals are recognized by *E. coli.*

The hallmark of sensor kinases is the conserved histidine residue which is phosphorylated during signal transduction (15). In PilS, this residue is at position 319, within a highly conserved region, and we have shown that it is essential for activation of the *pilA* promoter. PilS mutants with leucine, arginine, or proline substitutions for the histidine cannot complement the *pilS* deletion strain, presumably because these residues cannot be phosphorylated.

On the basis of computer analysis (7), the N-terminal 180-amino-acid segment of PilS is predicted to form six transmembrane helices, with very short periplasmic and cytoplasmic loops (2 to 12 amino acids) (Fig. 3). The C-terminal 350 residues are predicted to be cytoplasmic. The prototype membrane-associated sensors follow the EnvZ structural model and have, near their amino termini, two transmembrane segments bordering a large periplasmic loop. This loop is sometimes referred to as the input domain, because it receives the external signal which initiates the phosphorelay mechanism (reviewed in reference 42). The multiple-transmembrane helix arrangement is an alternative structural organization found not only in PilS but in several other histidine kinases as well (DivJ of *Caulobacter crescentus* [41], FixL of *Rhizobium meliloti* [30], ComP of *Bacillus subtilis* [51], PrrB of *Rhodobacter sphaeroides* [11], and RegB of *Rhodobacter capsulatus* [37]). Another example is the UhpB protein of *E. coli* and *Salmonella typhimurium*, which regulates expression of the hexose phosphate transporter, UhpT, in response to external glucose-6-phosphate. The N terminus of UhpB is predicted to form eight transmembrane segments, with mostly short, connecting loops. UhpB activation requires another membrane-bound protein, UhpC, which is the actual hexose phosphate receptor. UhpB and UhpC act together as a complex, presumably through their membrane-bound domains (22). The functions of these transmembrane helices may simply be to anchor the protein in the membrane, perhaps at a specific place, and to present the kinase domain to the cytosol at the membrane surface. On the other hand, the secondary structure arrangement of these domains in the membrane could have functional significance. It seems clear that at least for UhpB, these transmembrane helices form the input domain.

Unlike EnvZ and UhpB, PilS has a segment of 140 residues between the membrane-spanning domain and the conserved kinase domain (Fig. 3). In some other membrane-bound sensors that also have this extra region, it has been shown to be the input domain. In the *R. meliloti* FixL protein for example, this region binds a heme molecule which is used to sense oxygen concentration (30, 31, 36). The VirA sensor of *Agrobacterium tumefaciens* has two input domains, one periplasmic and another cytosolic (6).

It is possible that PilS also has two input domains, one, comprising the transmembrane helices, which could sense signals localized in the membrane, and another, comprising the region between the last transmembrane helix and the kinase domain, which could sense signals at the cytosolic surface of the membrane. While the signal to which PilS responds is unknown, it may be expected to respond to the cell cycle, since to maintain the unipolar location of the pili the new daughter cells need to produce pili at some controlled point during cell division. In this regard it is exciting that DivJ, a histidine kinase involved in cell cycle regulation in *C. crescentus*, has very high degree of structural homology to PilS. It too is proposed to have six transmembrane helices and a cytosolic domain between the last of these helices and the conserved kinase domain (41). PilS may also respond to external environmental signals. If PilS does respond to two different signals, this may explain why it has been so difficult to identify conditions that repress pilin expression.

We have observed that high concentrations of PilS in *P. aeruginosa* result in inhibition of *pilA* transcription. This inhibitory effect was directly proportional to the amount of PilS in the cell. The dominant negative effect of high concentrations of PilS is a strong indication of protein-protein interaction, and there are at least three possible proteins with which PilS could be interacting: (i) itself, (ii) PilR, and (iii) an unidentified signal protein. All of the sensor kinases tested to date have been shown to act as dimers, and there is no reason to suspect that PilS is any different. This could not be an explanation for the dominant negative effect, however, because dimer formation should not inhibit autophosphorylation or phosphotransfer. Even heterodimers made of one wild-type sensor and one sensor incapable of being phosphorylated have been shown to be capable of transphosphorylation and phosphotransfer, but both the PilS H319L and PilS H319R mutants block the signal transduction cascade when overexpressed in the wild-type background.

We know that PilS and PilR must interact directly in order for phosphate transfer to occur. It is possible that high concentrations of PilS sequester PilR and make it unavailable to bind to the *pilA* promoter. The results with PilS-cyt, however,

counter this argument. We know that PilS-cyt also interacts with PilR because PilS-cyt can complement a *pilS* deletion strain, but this mutant does not demonstrate the dominant negative effect when overexpressed, and instead it has a dominant positive effect on pilin expression. This suggests that the dominant negative effect of overproduced PilS is not due to altered stoichiometry with PilR.

The third possibility is that PilS interacts with another cellular component which controls its stimulatory or inhibitory activities. This cellular component may be the actual PilS activation signal, or it may be the receptor for the signal. We believe that it is the altered stoichiometry of PilS and this putative factor that leads to inhibition of pilin synthesis.

Many sensors of two-component systems, in addition to functioning as kinases, are also phosphatases specific to their cognate regulators (1). These two enzymatic activities determine the steady-state level of the active (phosphorylated) form of the regulators in the cell. We hypothesize that PilS carries out both of these activities: it is a kinase when activated by a signal, and it is a phosphatase in the absence of such a signal. Presumably the amount of signal is limited, so that most of the excess PilS cannot interact with it, leading to higher levels of PilS in the phosphatase state than in the kinase state. This in turn results in accumulation of inactive, unphosphorylated PilR. This hypothesis also explains the unexpected result that *P. aeruginosa* with a nonpolar deletion in *pilS* can still produce low levels of pilin. Like other response regulators, PilR can presumably be phosphorylated by other means (cross talk with other kinases or autophosphorylation with small phosphodonors), and without its cognate phosphatase the phospho-PilR form will accumulate, leading to transcriptional activation. Availability of purified PilS and PilR makes it possible to test this hypothesis biochemically, and such work is in progress.

In support of this model, overexpression of *uhpB*, the sensor kinase of the *E. coli* hexose phosphate uptake system, reduces the level of the hexose transporter, UhpT. This may be a consequence of the altered ratio of UhpB to the glucose-6-phosphate receptor, UhpC (25).

The PilS-mediated inhibition of *pilA* transcription does not depend on the autokinase activity of PilS, since the histidine substitution mutants PilS-H319L and PilS-H319R also inhibit *pilA* transcription when overexpressed in the wild-type background. This effect can be explained if these mutant proteins maintain their phosphatase activity and their ability to interact with the putative signal. In support of this hypothesis, it has been shown that the conserved histidine residue of sensor kinases is not required for phosphatase activity (reviewed in reference 42). PilS-H319P did not demonstrate the dominant negative effect, suggesting either that it cannot interact with the putative signal or that it does not have phosphatase activity. It is likely that the structural changes introduced by the proline substitution would drastically alter its ability to interact with other proteins. The dominant positive activity of the PilS-cyt mutant suggests that it does not require interaction with the signaling component and that it is constitutively a kinase. Indeed, this is the case when other membrane-bound sensor kinases have been freed of their transmembrane domains (20, 24, 33).

We have shown that overexpression of *pilR* in the absence of PilS allows transcription of *pilA*. Although this effect has been seen with a number of other response regulators (14, 45), the reason for it is not clear. Phosphorylation of response regulators modulates transcription, but whether this modulation is due to alteration in DNA binding affinity, oligomerization rates, or other functions is the source of some debate, and the cause of modulation may not be the same for all members of this class. Presumably there is an equilibrium between active and inactive forms that is normally controlled by phosphorylation, but even in the absence of phosphorylation there are still a few active forms in the cell. When response regulator genes are overexpressed, the total number of active forms is increased and transcription can proceed. Alternatively, in the absence of their kinases, regulators may be phosphorylated by other means. Experiments are under way to determine the case for PilR.

In conclusion, this study shows that PilS, the sensor kinase of a two-component system responsible for regulation of pilin gene transcription in *P. aeruginosa*, is a 58,997-Da inner membrane protein. We propose that PilS interacts not only with its cognate regulator, PilR, but also with another cellular component, most likely a protein, located either in the inner membrane or at the cytosolic surface of the inner membrane. This putative component may be responsible for switching PilS from the PilR kinase state to the phospho-PilR-phosphatase state and may be either the actual PilS activation signal or an intermediary signal-binding protein.

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