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Identification of *pilR*, Which Encodes a Transcriptional Activator of the *Pseudomonas aeruginosa* Pilin Gene

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Two regulatory mutants of *Pseudomonas aeruginosa*, R1 and RA, that affect transcription of the pilin gene were isolated. This was done by introducing a plasmid carrying a fusion of the pilin gene's promoter with the *lacZ* gene into a bank of *P. aeruginosa* DNA mutagenized with the transposon Tn*5*G. The block in pilin expression in these mutants was shown to be at the level of transcription, since these mutants did not synthesize either pilin mRNA or pilin antigen. A restriction fragment derived from the R1 mutant that contains the entire transposon plus flanking chromosomal DNA was cloned and used as a probe to screen a cosmid library of *P. aeruginosa* DNA. Cosmids that could complement the pilin expression defect in both R1 and RA were isolated. The gene inactivated in R1 was sequenced. This gene, designated *pilR*, encodes an ~50-kDa polypeptide which exhibits significant similarity to the NtrC family of response regulators of the two-component regulatory system. PilR contains the amino-terminal aspartic acid residues which are conserved among the response regulators, suggesting that pilin gene transcription is regulated via a phosphotransfer mechanism in which PilR is phosphorylated by an as yet unidentified protein kinase.

Pseudomonas aeruginosa is responsible for a wide range of severe and sometimes fatal diseases in immunocompromised individuals. This organism also causes chronic respiratory disease in patients with cystic fibrosis (10). A number of surface and extracellular components synthesized by *P. aeruginosa* contribute to its pathogenicity. One such virulence factor is pili, which have been implicated in playing a key role during the initial stages of colonization of the host by mediating attachment of the bacterium to host cell receptors (36).

Previously we have shown that pilin gene expression requires RpoN, an alternative sigma factor of RNA polymerase (12). A number of different bacterial genes are transcribed by RpoN-containing RNA polymerase, including the genes for glutamine synthetase in a number of bacterial species (8, 9, 15, 16), the genes for the dicarboxylate transport polypeptide in rhizobia (25), the genes for the xylene-catabolic enzymes in *Pseudomonas putida* (4, 13), the nitrogen fixation genes in *Rhizobium meliloti* (11, 25) and *Klebsiella pneumoniae* (11), and the genes for the flagellar components of *Caulobacter crescentus* (19, 20). These genes share several conserved features, including the canonical promoter sequence GG-N_{10}-GC and a requirement for binding of an activator protein to a site near the promoter, prior to open complex formation and initiation of transcription (14).

Here we report the isolation and characterization of transposon mutants that are blocked in their ability to transcribe the pilin gene. The DNA from one mutant, R1, was cloned and sequenced. The predicted amino acid sequence for this regulatory protein, termed PilR, shares homology with a subclass of response regulators that promote transcription of *rpoN*-dependent genes. Thus, PilR is a new member of the two-component sensor-regulator family.

MATERIALS AND METHODS

Bacterial strains and plasmids. A list of all strains and plasmids used in this work is presented in Table 1.

Genetic techniques. A library of *P. aeruginosa* carrying random insertions of the transposon Tn*5*G (Tn*5* encoding a gentamicin resistance gene) was constructed as previously described (22). Triparental spot matings were done as previously described (6). In brief, donor, recipient, and helper strains were mixed together in minimal salts solution, spotted onto L-agar medium, and incubated overnight at 37°C. Transconjugates were selected on L-agar medium containing either tetracycline (150 μg/ml) and gentamicin (50 μg/ml) or ampicillin (100 μg/ml) and neomycin (700 μg/ml). Plasmid pRK2073 was used as the helper plasmid in all the matings.

The mutant N1G contains the *rpoN* gene insertionally inactivated with a gentamicin resistance gene and was constructed as follows. The tetracycline resistance gene from pKI11 (12) was deleted by digestion with *Bgl*II and replaced with a 1.8-kb *Bam*HI fragment containing the gentamicin resistance gene from pPC110 (32). The resulting plasmid was pKI11G, which was then introduced into *P. aeruginosa* PAK, and gentamicin-resistant but carbenicillin (150 μg/ml)-sensitive transconjugates were selected.

DNA and RNA manipulations. *P. aeruginosa* chromosomal DNA was extracted as described by Strom and Lory (31). All plasmid DNA was isolated by the method of Birnboim and Doly (1). A cosmid library of *P. aeruginosa* DNA was prepared in pVK102 as previously described (12). DNA was digested with restriction enzymes (Bethesda Research Laboratories, Gaithersburg, Md.) and analyzed by agarose gel electrophoresis. Specific fragments used in cloning or preparation of probes were purified by electroelution (17). DNA fragments were blotted onto Nytran sheets (Schleicher & Schuell, Keene, N.H.) by the method of Southern (28). DNA probes were radiolabeled by the random-priming method (Bethesda Research Laboratories). Southern blots were hy-

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TABLE 1. Strains, vectors, and plasmids

Strain, vector, or plasmid	Genotype or description	Source or reference
Strains		
Escherichia coli		
DH5α	*endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1* Δ(*lacZYA-argF*)*U169 U169* λ-φ80 d*lacZ* ΔM15; recipient for recombinant plasmids	Bethesda Research Laboratories
HB101	*hsd-20 recA13 ara-14 proA2 lac-41 galK2 mtl-1 xyl-5 supE44 rpsL2*; recipient for recombinant plasmids	2
JC2926(pRK2073)	Helper plasmid	6
Pseudomonas aeruginosa		
PAK	Wild-type clinical isolate	David Bradley
N1	*rpoN* mutant; *rpoN* is insertionally inactivated with a tetracycline resistance gene	12
N1G	*rpoN* mutant; *rpoN* is insertionally inactivated with a gentamicin resistance gene	This study
R1	PAK pilin regulatory mutant with Tn*5*G insertion in *pilR*	This study
RA	PAK pilin regulatory mutant	This study
Cloning vectors		
pUC18cm′	*E. coli* cloning vector; Cm^r	33
pBluescriptIISK−	*E. coli* cloning vector; Ap^r	Stratagene
pDN18	Broad-host-range cloning vector; Tc^r	21
pDN19	Broad-host-range cloning vector; Tc^r	21
Recombinant plasmids		
pMSZ5	Pilin promoter-*lacZ* fusion; 431-bp *Hin*dIII-*Stu*I fragment from pMS27A (31) cloned into the vector pSP329 (32)	Mark Strom
pR1-2	5-kb *Sal*I fragment from the chromosome of R1 cloned into pUC18cm; the *Sal*I fragment contains the Tn*5*G plus flanking chromosomal DNA; used to probe the *P. aeruginosa* cosmid library	This study
pRA-8	7.1-kb *Sal*I fragment from the chromosome of RA cloned into pUC18cm; the *Sal*I fragment contains Tn*5*G plus flanking chromosomal DNA	This study
pKIR2–pKIR5	Cosmids from pVK102 library of PAK chromosomal DNA; identified by hybridization to *pilR* probe	This study
pKI21	1.6-kb *Pvu*II fragment from pKIR2 cloned into *Sma*I site of pBluescriptIISK−	This study
pKI22	*Eco*RI-*Bam*HI fragment from pKI21 cloned into pDN18	This study

bridized at high stringency in 50% formamide (vol/vol) (17) and washed at 50°C.

RNA was extracted by the method of Rubens et al. with minor modifications (26). An RNA slot blot apparatus (Schleicher & Schuell) was used to transfer the RNA to Nytran sheets, and the blots were probed with a radiolabeled pilin gene fragment isolated from pMS27A.

Protein analysis. Levels of β-galactosidase activity in the Tn*5*G mutants containing the pilin promoter-*lacZ* fusion were determined as described by Miller (18). Protein extracts were analyzed by immunoblotting following sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. The blots were incubated with rabbit antiflagellin antibody (33) or mouse antipilin antibody (31), and bound immunoglobulins were identified by using ^{125}I-protein A (New England Nuclear, Boston, Mass.).

DNA sequence analysis. DNA sequencing analysis was performed by the dideoxy-chain termination method (27) using the Sequenase DNA sequencing kit (United States Biochemical Corp., Cleveland, Ohio). The sequences were verified from both strands by using either the universal primer or synthetic oligonucleotide primers. Briefly, the 4.3-kb *Xho*I fragment from cosmid pKIR2 was subcloned into M13mp18 and M13mp19, which were subsequently used as templates for sequencing.

The left half of Tn*5*G and the flanking chromosomal DNA was deleted from pR1-2 by digesting it with *Bam*HI and *Bgl*II and religating the 4.8-kb fragment; this resulted in plasmid pΔR1. The 2.5-kb insert from pΔR1 was then cloned into M13mp18 by using *Eco*RI and *Sal*I. To obtain the opposite strand for sequencing, pΔR1 was digested with *Hin*dIII and cloned into M13mp19. The right half of Tn*5*G and the

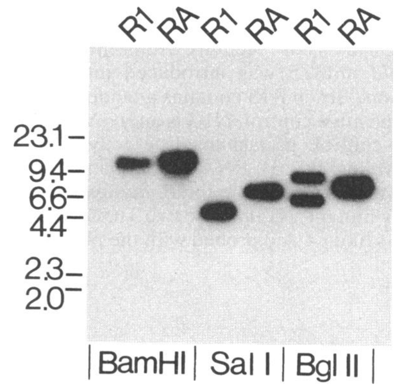

FIG. 1. Southern hybridization analysis of chromosomal DNA from R1 and RA, the Tn*5*G mutants of *P. aeruginosa*. Chromosomal DNA from R1 and RA was extracted and digested with *Bam*HI, *Sal*I, or *Bgl*II. The digests were fractionated by agarose gel electrophoresis, blotted, and probed with the Tn*5*G transposon from plasmid pRK2013::Tn*5*G. The molecular size standards are indicated on the left and are in kilobases.

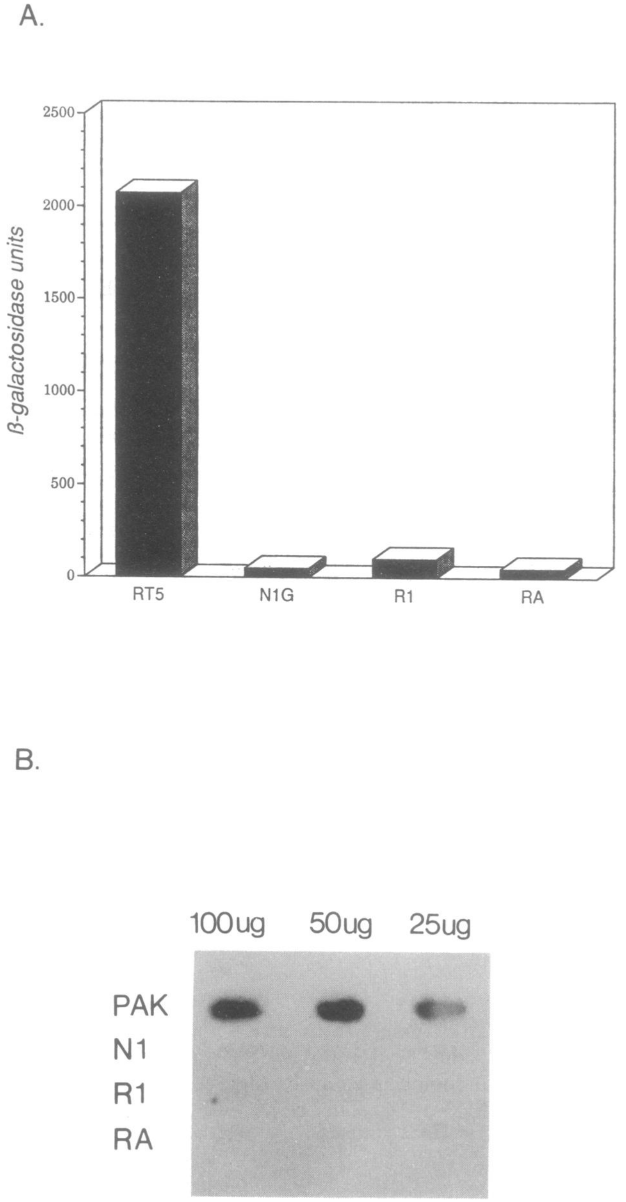

FIG. 2. Transcription of the pilin gene in Tn*5*G mutants. (A) Levels of β-galactosidase activity from the pilin promoter-*lacZ* fusion. Plasmid pMSZ5 was introduced into R1, RA, and two additional strains. Strain RT5 contains a random Tn*5*G insertion and was used as a positive control. N1G is an *rpoN* mutant and was used as a negative control. β-Galactosidase activity was measured as described by Miller (18). (B) RNA slot blot analysis of pilin-specific mRNA. RNA was isolated from the *P. aeruginosa* wild-type strain, PAK; the *rpoN* mutant, N1; and the two Tn*5*G mutants, R1 and RA. Total RNA was blotted and probed with the pilin gene from plasmid pMS27A.

flanking chromosomal DNA from pR1-2 was deleted by digesting with *Hin*dIII and religating the 3.8-kb fragment; this resulted in pΔR1H3. The 1.5-kb *Eco*RI-*Hin*dIII fragment from pΔR1H3 was cloned into M13mp18 and M13mp19. The opposite strand was sequenced by using an oligonucleotide primer complementary to the end of the Tn*5*G transposon.

Nucleotide sequence accession number. The nucleotide sequence of *pilR* reported in this study was submitted to GenBank and assigned accession number M83311.

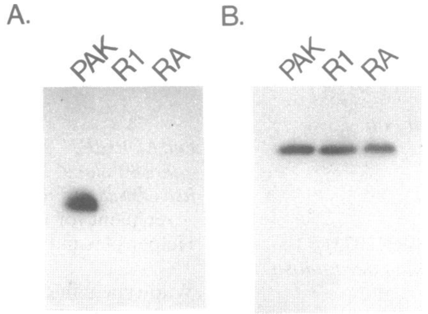

FIG. 3. Immunoblot analysis of whole-cell lysates from *P. aeruginosa* wild-type strain PAK and the R1 and RA mutants. The blot was incubated with antiserum to pilin (A) or antiserum to flagellin (B).

RESULTS

Isolation and characterization of pilin regulatory mutants. Mutants of *P. aeruginosa* that were unable to transcribe the pilin gene were isolated by screening a library of random chromosomal Tn*5*G insertions. In order to limit the analysis to mutants specifically affected in transcription and not in pilus biogenesis, a pilin gene promoter-*lacZ* fusion on plasmid pMSZ5 was introduced into the *P. aeruginosa* Tn*5*G-mutagenized library. Most colonies carrying this plasmid were blue on Luria broth plates with 5-bromo-4-chloryl-3-indolyl-β-D-galactopyranoside (X-Gal), however white colonies at a frequency of ca. 1 in 5,000 were also observed. The inability to activate the pilin gene promoter in *trans* was presumed to be a result of a mutation in a regulatory gene.

DNA from these candidate mutants was extracted, digested with restriction enzymes, and analyzed by Southern blotting using Tn*5*G as a probe. Two different Southern hybridization patterns resulted, as shown by representative isolates R1 and RA (Fig. 1), and all of the mutants exhibited one or the other pattern. In this analysis, enzymes that lack a recognition sequence in Tn*5*G (*Bam*HI and *Sal*I) were used, as was *Bgl*II, which cleaves the transposon approximately in the middle. The insertions in R1 and RA reside in different-size *Sal*I and *Bgl*II fragments; however, both are apparently in the same-size *Bam*HI fragment or possibly in two different, but electrophoretically inseparable, *Bam*HI fragments. The Tn*5*G insertions in R1 and RA are therefore different and are possibly in linked genes.

The mutations in R1 and RA are in regulatory genes that affect transcription of the pilin gene. As predicted from the white colony appearance on plates containing X-Gal, the levels of β-galactosidase activity of the pilin promoter-*lacZ* fusion (pMSZ5) in *P. aeruginosa* R1 and RA were less than 5% of that in the isogenic wild-type *P. aeruginosa* RT5 and comparable to that in the *rpoN* mutant, N1G (Fig. 2A). Mutants R1 and RA were also analyzed for pilin-specific transcripts. A slot blot analysis of total bacterial RNA in which the pilin gene was used as a probe is shown in Fig. 2B. Pilin-specific mRNA was present in wild-type PAK and was absent from the *rpoN* mutant, N1G. Pilin mRNA was not detected in the R1 and RA mutants, indicating that pilin expression is blocked at the transcriptional level in both of these mutants.

Expression of RpoN-controlled genes in *P. aeruginosa* R1

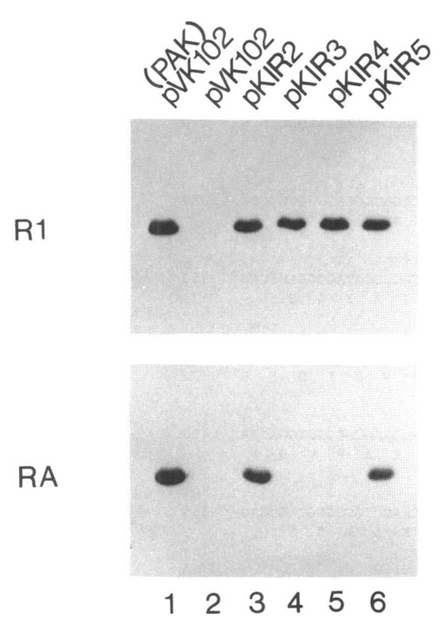

FIG. 4. Expression of pilin in R1 and RA mutants containing different cosmids. Lane 1 in both panels is a whole-cell lysate of PAK containing pVK102. Lanes 2 to 6 are whole-cell lysates from the R1 or RA mutant (as indicated) containing the cosmid that is designated above each lane. All blots were incubated with antiserum to pilin.

and RA. Synthesis of pilin and flagellin was examined in the R1 and RA mutants. As predicted, expression of pilin protein was abolished (Fig. 3A). To assess whether the Tn*5*G insertion in R1 or RA had any effect on the expression of flagellin, another gene indirectly regulated by RpoN (29, 33), the same extracts were probed with antiflagellin antiserum. Equal amounts of flagellin protein were detected in R1 and RA compared to that in the wild-type PAK (Fig. 3B); thus, the Tn*5*G insertions in R1 and RA do not affect flagellin expression. Collectively, these studies show that a chromosomal locus encoding one or possibly two transcription factors has been identified by Tn*5*G insertions in R1 and RA. This locus is different from *rpoN*, because the mutations do not affect flagellin synthesis or result in a glutamine requirement. Moreover, introducing a plasmid containing *rpoN* into R1 or RA does not restore pilin expression (data not shown). These results, taken together, suggest that the mutations in R1 and RA appear to be specific for pilin expression.

Complementation of the pilin synthesis defect in R1 and RA. To clone the regulatory locus, defined by RA and R1, the chromosomal DNA region flanking the Tn*5*G from R1 was isolated in order to be used as a probe to screen a cosmid library of *P. aeruginosa* DNA. Using the 5-kb *Sal*I fragment from pR1-2 as a probe, colony hybridization analysis of the cosmid library identified four clones (pKIR2, pKIR3, pKIR4, and pKIR5) that reacted with this probe. To determine whether any of these cosmids could complement the mutation in R1 or RA, the cosmids were introduced into both of the mutants, and levels of pilin antigen were determined by immunoblot analysis with antibody against pilin (Fig. 4). All four cosmids were able to complement the mutation in R1 and restore pilin expression (Fig. 4, panel R1, lanes 3 to 6). Furthermore, cosmids pKIR2 and pKIR5 were also able to complement RA (Fig. 4, panel RA, lanes 3 and 6); however, cosmids pKIR3 and pKIR4 did not (Fig. 4, lanes 4 and 5). This complementation pattern suggests that insertions in R1 and RA are in separate but linked genes.

To further show physical linkage of the Tn*5*G insertions in R1 and RA, cosmids and chromosomal DNA were digested with various restriction enzymes and were examined by Southern blot analysis using the R1-derived probe (5-kb *Sal*I fragment from pR1-2) and the RA-derived probe (7.1-kb *Sal*I fragment from pRA-8). Based on the hybridization patterns of the cosmid and chromosomal DNAs, a restriction map of the chromosomal region containing the Tn*5*G insertions was generated (Fig. 5). Also shown is the location of the gene, *pilR*, as determined by the nucleotide sequence of the region flanking the Tn*5*G in R1 (see below).

Nucleotide sequence of the pilin gene regulator defined by R1. The insert DNA from pR1-2 (the R1-derived clone) and fragments from cosmid pKIR2 were sequenced. An open reading frame whose initiating ATG is 8 bp downstream from the sequence GGAAG, a possible ribosome binding site, was identified (Fig. 6). This open reading frame predicted a polypeptide of 446 amino acids, which is equivalent to a molecular size of ~50 kDa, and was designated PilR.

Homology searches for the predicted PilR amino acid sequence among entries in GenBank revealed that PilR has similarity to response regulators belonging to the NtrC family, all of which initiate transcription with the RpoN-containing RNA polymerase (Table 2). The similarity was even more dramatic when the conserved central regions were compared (Table 2). Alignment of PilR with two previously identified *P. aeruginosa* response regulators showed 34% identity with AlgR (3) and 41% identity with AlgB (37) (data not shown). This moderate level of sequence

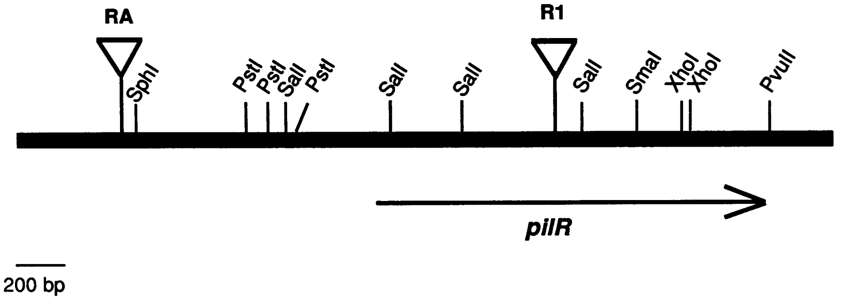

FIG. 5. Restriction enzyme map of the chromosomal region adjacent to the transposon insertion sites in R1 and RA. The open triangle denotes the location of transposon Tn*5*G in either the R1 or RA mutant. The site of transposon insertion in the R1 mutant was defined by DNA sequencing analysis. The location of the transposon insertion in the RA mutant was determined by Southern blot analysis. The arrow represents the location of *pilR* as defined by DNA sequence analysis.

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   1  CGGATCGACTACGCAATCGCGAGGAAGGCGGCGGCCTTCCGCATCACCTTCGCCCACCCGCGCAAACTCA  70
    PvuII      .         .         .         .         .         .         .
  71  GCTGACGGAAGCCGCACGCATGAGCCGACAAAAAGCCCTGATCGTCGACGATGAACCGGATATCCGCGAA  140
                         M  S  R  Q  K  A  L  I  V  D  D  E  P  D  I  R  E
               .         .         .         .         .         .         .
 141  CTGCTGGAAATCACTCTCGGCCGCATGAAGCTGGACACCCGCAGCGCCCGCAACGTCAAGGAAGCCCGCG  210
      L  L  E  I  T  L  G  R  M  K  L  D  T  R  S  A  R  N  V  K  E  A  R  E
               .         .         .         .         .         .         .
 211  AGTTGCTGGCCCGCGAGCCGTTCGACCTGTGCCTCACCGACATGCGCCTGCCGGACGGCAGCGGCCTCGA  280
        L  L  A  R  E  P  F  D  L  C  L  T  D  M  R  L  P  D  G  S  G  L  D
               .         .         .         .         .         .         .
 281  TCTGGTCCAGTACATCCAGCAGCGCCATCCACAGACCCCGGTGGCCATGATCACCGCGTACGGCAGCCTG  350
       L  V  Q  Y  I  Q  Q  R  H  P  Q  T  P  V  A  M  I  T  A  Y  G  S  L
               .         .         .         .         .         .         .
 351  GACACCGCGATCCAGGCGCTCAAGGCCGGTGCCTTCGACTTCCTAACCAAACCGGTCGACTTCGACTTCC  420
      D  T  A  I  Q  A  L  K  A  G  A  F  D  F  L  T  K  P  V  D  F  D  F  R
               .         .         .         .         .         .         .
 421  GCTTGCGGGAGCTGGTGGCAACCGCCCTGCGCTTGCGCAACCCGGAAGCCGAGGAAGCGCCGGTGGACAA  490
        L  R  E  L  V  A  T  A  L  R  L  R  N  P  E  A  E  E  A  P  V  D  N
               .         .         .         .         .         .         .
 491  CCGCCTGCTCGGCGAGTCGCCGCCGATGCGCGCCCTGCGCAACCAGATCGGCAAGCTGGCGCGCAGCCAG  560
       R  L  L  G  E  S  P  P  M  R  A  L  R  N  Q  I  G  K  L  A  R  S  Q
               .         .         .         .         .         .         .
 561  GCGCCGGTCTACATCAGTGGCGAGTCCGGTAGCGGCAAGGAGCTGGTGGCGCGCCTGATCCACGAGCAGG  630
      A  P  V  Y  I  S  G  E  S  G  S  G  K  E  L  V  A  R  L  I  H  E  Q  G
               .         .         .         .        ▽          .         .
 631  GGCCACGTATCGAGCGGCCGTTCGTGCCGGTGAACTGCGGCGCGATTCCCTCCGAACTGATGGAAAGCGA  700
        P  R  I  E  R  P  F  V  P  V  N  C  G  A  I  P  S  E  L  M  E  S  E
               .         .         .         .         .         .         .
 701  GTTCTTCGGCCACAAGAAAGGCAGCTTCACTGGCGCTATCGAAGACAAGCAGGGCCTGTTCCAGGCCGCC  770
       F  F  G  H  K  K  G  S  F  T  G  A  I  E  D  K  Q  G  L  F  Q  A  A
               .         .         .         .         .         .         .
 771  AGCGGCGGCACCCTGTTCCTCGACGAAGTCGCCGACCTGCCGATGGCCATGCAGGTCAAACTGCTCCGGG  840
      S  G  G  T  L  F  L  D  E  V  A  D  L  P  M  A  M  Q  V  K  L  L  R  A
               .         .         .         .         .         .         .
 841  CGATCCAGGAAAAGGCCGTGCGCGCGGTCGGCGGCCAGCAGGAGGTCGCCGTCGACCTGCGCATCCTCTG  910
        I  Q  E  K  A  V  R  A  V  G  G  Q  Q  E  V  A  V  D  L  R  I  L  C
               .         .         .         .         .         .         .
 911  CGCCACCCACAAGGACCTCGCCGCCGAAGTCGGCGCCGGGCGCTTCCGCCAGGACCTCTACTACCGCCTC  980
       A  T  H  K  D  L  A  A  E  V  G  A  G  R  F  R  Q  D  L  Y  Y  R  L
               .         .         .         .         .         .         .
 981  AACGTCATCGAGCTGCGCGTACCGCCGCTGCGCGAACGCCGCGAGGACATCCCGCTGCTCGCCGAACGCA  1050
      N  V  I  E  L  R  V  P  P  L  R  E  R  R  E  D  I  P  L  L  A  E  R  I
               .         .         .         .         .         .         .
1051  TCCTCAAGCGCCTGGCCGGCGACACCGGCCTGCCGGCCGCCAGGCTGACCGGCGACGCACAGGAGAAGCT  1120
        L  K  R  L  A  G  D  T  G  L  P  A  A  R  L  T  G  D  A  Q  E  K  L
               .         .         .         .         .         .         .
1121  GAAGAACTACCGCTTCCCGGGCAACGTGGGCGAGCTGGAAAACATGCTGGAGCGCGCCTACACCCTGTGC  1190
       K  N  Y  R  F  P  G  N  V  G  E  L  E  N  M  L  E  R  A  Y  T  L  C
               .         .         .         .         .         .         .
1191  GAAGACGACCAGATCCAGCCTCACGACCTGCGCCTGGCCGATGCGCCGGGTGCCAGCCAGGAAGGCGCCG  1260
      E  D  D  Q  I  Q  P  H  D  L  R  L  A  D  A  P  G  A  S  Q  E  G  A  A
               .         .         .         .         .         .         .
1261  CGAGCCTGAGCGAAATAGACAACCTCGAGGACTACCTGGAAGACATCGAGCGCAAGCTGATCATGCAGGC  1330
        S  L  S  E  I  D  N  L  E  D  Y  L  E  D  I  E  R  K  L  I  M  Q  A
               .         .         .         .         .         .         .
1331  GCTCGAGGAGACCCGCTGGAACCGCACCGCCGCGGCCCAGCGCCTGGGCCTGACGTTCCGCTCGATGCGC  1400
       L  E  E  T  R  W  N  R  T  A  A  A  Q  R  L  G  L  T  F  R  S  M  R
               .         .         .         .         .         .         .
1401  TACCGCCTGAAAAAGCTGGGCATCGACTGAAAGTGAAAAGGCCTGTCCGAAGACAGGCCTTTTGGTTTTC  1470
      Y  R  L  K  K  L  G  I  D  *
               .         .         .         .         .         .         .
1471  GCTCCTCAGAGGCGACCAGCCGGGGCGTAGGGGACCGGGTCGATGATCGGTTCCCGCCCGCTCATGAGAT  1540
               .         .         .         .         .         .         .
1541  CCGCCAGCAGACGGCACGACGCCGGTGCCAGGACCAGCCGTTGCGGTAGTGCCGGTATTCAGCCAGAGCC  1610
               .         .         .         .         .         .   PvuII  .
1611  GTCGAAGCCAGGCACCGGACCGATATAGGGATGCTTCGGAGAGCCGGCGCACCTGCCAGTGGCACAGCTG  1680
               .
1681  CATGTCGCAGTCGCACAGT  1699
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FIG. 6. Nucleotide sequence of *pilR* and its predicted amino acid sequence. The putative ribosome binding site is underlined. The *Pvu*II sites are boxed. The open triangle denotes the site of the Tn*5*G insertion in the R1 mutant.

identity confirmed that PilR is indeed a newly identified regulatory element in *P. aeruginosa*.

Comparison of PilR with *R. meliloti* NtrC is shown in Fig. 7. PilR contains the invariant amino acids of this family (Asp-11, Asp-54, and Lys-104). Asp-54, conserved among the response regulators, is presumably a site of phosphorylation by the cognate sensory element (30). A helix-turn-helix DNA-binding motif (23) can be also found near the C terminus of PilR; however, this region shows little sequence similarity with NtrC. The most conserved region is located between amino acids 163 and 317. This domain contains two putative nucleotide-binding sites, consistent with the known ATPase activity of NtrC (34).

To demonstrate that the observed pilin expression defect in R1 is due to an insertion in *pilR*, plasmid pKI22, which contains only *pilR* and minimal flanking sequence, was introduced into R1 and tested to see whether complementation occurred. Synthesis of pilin, as detected by immunoblots of whole-cell extracts, occurred in the R1 mutant harboring plasmid pKI22 (Fig. 8). This result indicates that the 1.6-kb *Pvu*II fragment (Fig. 6) in pKI22 contains the entire locus required to complement R1 and that the Tn*5*G insertion in R1 is indeed in *pilR*.

DISCUSSION

Previous studies have indicated that pilin gene transcription requires RNA polymerase containing RpoN, an alternative sigma factor. A common feature of all RpoN transcribed genes is a requirement for regulatory proteins which are necessary for transcriptional initiation (14). This model led us to search for additional genes that may encode regulatory proteins required for pilin gene expression.

Two different pilin regulatory mutants, R1 and RA, were isolated by screening a Tn*5*G library of *P. aeruginosa* DNA for the inability to activate a pilin promoter-*lacZ* fusion. Both of the mutants were unable to synthesize pilin-specific mRNA or pilin antigen; therefore, the insertions are very likely in regulatory genes. In addition, these regulatory elements appear to control expression of only the pilin gene, because the ability to synthesize flagellin or grow in the absence of glutamine was not affected by the mutations in RA and R1.

Cosmids were isolated on the basis of their ability to hybridize to a DNA probe derived from the DNA flanking the Tn*5*G insertion in R1. These cosmids were able to complement the pilin synthesis defect in R1, and some complemented both R1 and RA. Southern blot analysis confirmed that all of the complementing cosmids contained DNA from the same region of the *P. aeruginosa* chromosome. The mutations in R1 and RA are therefore linked.

The DNA region flanking the transposon insertion in R1 was sequenced, and a single open reading frame predicted a protein product of ~50 kDa. The amino acid sequence of this protein, designated PilR, was homologous with the NtrC family of response regulators of the two-component regulatory system (30). The most extensive homology is near the

TABLE 2. Pairwise comparison of sequence similarities between selected response regulators[a]

Comparison type and regulator	% Similarity with: DctD	FlbD	NifA	NtrC	XylR
Overall					
PilR	58.2	59.8	54.2	62.2	58.3
DctD		56.0	52.8	59.9	54.6
FlbD			58.6	61.6	55.5
NifA				55.2	54.7
NtrC					57.1
Central domain (amino acid residues)					
PilR (163–317)	71.4	72.7	69.5	71.4	70.8
DctD (173–326)		70.1	70.8	72.7	70.8
FlbD (148–302)			76.0	77.3	75.3
NifA (205–359)				74.0	77.3
NtrC (167–320)					72.7

[a] The Gap program from University of Wisconsin's Genetics Computer Group was used to determine the overall percent amino acid similarity between the different response regulator proteins. Similarly, the Bestfit program was used to determine the percent amino acid similarity between the different central domains of the response regulator proteins. Protein sources: PilR, *P. aeruginosa*; DctD and NifA, *Rhizobium leguminosarum*; FlbD, *C. crescentus*; NtrC, *R. meliloti*; XylR, *P. putida*.

```
             .         .         .         .         .
  1 MSRQKALIVDDEPDIRELLEITLGRMKLDTRSARNVKEARELLAREPFDL 50
    |. .. |:.||::.||.:|: .|:|  .|.| ..|. . : ::| :. ||
  1 MTGATILVADDDAAIRTVLNQALSRAGYDVRITSNAATLWRWIAAGDGDL 50

             .         .         .         .         .
 51 CLTDMRLPDGSGLDLVQYIQQRHPQTPVAMITAYGSLDTAIQALKAGAFD 100
    .:||: :||:.::||:. |.. :|: || ::.| ..: |||.| . ||:|
 51 VVIDVVMPDENAFDLLPRIKKARPDLPVLVMSAQNTFMTAIKASEKGAYD 100

             .         .         .         .         .
101 FLTKPVDFDFRLRELVATAL....RLRNPEAEEAPVDNRLLGESPPMRAL 146
    :|.||.|:.  | :::: ||    | ... .::.. : .|:| |::|..:
101 YLPKPFDLT.ELIGIIGRALAEPKRRPSKLEDDSQDGMPLVGRSAAMQEI 149

             .         .         .         .         .
147 RNQIGKLARSQAPVYISGESGSGKELVARLIHEQGPRIERPFVPVNCGAI 196
     . :::| ..: .: |.||||.||||||| :|: |.| : |||::| :||
150 YRVLARLMQTDLTLMITGESGTGKELVARALHDYGKRRNGPFVAINMAAI 199

             .         .         .         .         .
197 PSELMESEFFGHKKGSFTGAIEDKQGLFQAASGGTLFLDEVADLPMAMQV 246
    |.:|:|||:|||.||.|||| . . | |:.|.||||||||::|:||. |.
200 PRDLIESELFGHEKGAFTGAQTRSTGRFEQAEGGTLFLDEIGDMPMDAQT 249

             .         .         .         .         .
247 KLLRAIQEKAVRAVGGQQEVAVDLRILCATHKDLAAEVGAGRFRQDLYYR 296
    :|||.:|: .  .|||. .:  |:||:.||:||| ..:..| ||:|||||
250 RLLRVLQQGEYTTVGGRTPIRSDVRIVAATNKDLKQSINQGLFREDLYYR 299

             .         .         .         .         .
297 LNVIELRVPPLRERREDIPLLAERILKRLAGDTGLPAARLTGDAQEKLKN 346
    |||:.||:||||:| |||| |. :::.. |:..||.. |:..:| | :|.
300 LNVVPLRLPPLRDRAEDIPDLVRHFVQQ.AEKEGLDVKRFDQEALELMKA 348

             .         .         .         .         .
347 YRFPGNVGELENMLERAYTLCEDD....QIQPHDLRLADAPGASQEGAA. 391
    ..:|||| ||||:: |  .|:.:|    :| .::|| . :.:: :.:||
349 HPWPGNVRELENLVRRLTALYPQDVITREIIENELRSEIPDSPIEKAAAR 398

             .         .         .         .         .
392 ..SLSEIDNLED.....................YLEDIERKLIMQALEET 418
      |||  :.:|:                      |.::| .||:.||..|
399 SGSLSISQAVEENMRQYFASFGDALPPSGLYDRVLAEMEYPLILAALTAT 448

         H  —  T  — H  .
419 RWNRTAAAQRLGLTFRSMRYRLKKLGID 446
    | |.. ||: |||. ..:| :::.||:.
449 RGNQIKAADLLGLNRNTLRKKIRELGVS 476
```

FIG. 7. Sequence alignment of PilR (top line) and *R. meliloti* NtrC (bottom line). The conserved Asp-11, Asp-54, and Lys-104 are boxed. Potential ATP-binding sites are underlined, and a putative helix-turn-helix motif is indicated by H-T-H. The Gap program from the University of Wisconsin Genetics Computer Group was used to generate this alignment.

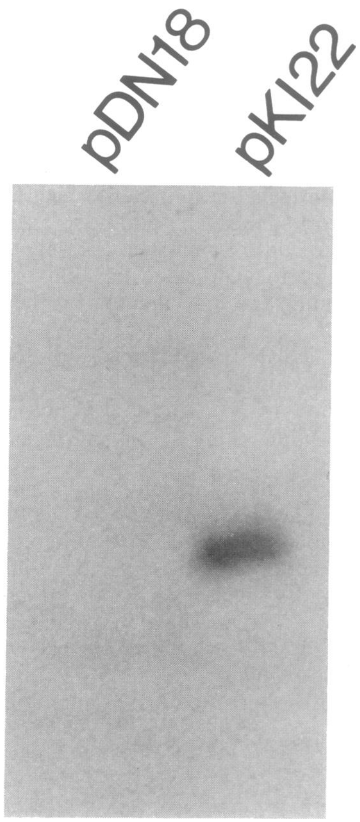

FIG. 8. Immunoblot analysis of R1 containing the cloned *pilR*. Whole-cell lysates of R1 with either pDN18 (vector) or pKI22 (vector plus the *pilR* gene) were analyzed on an SDS–15% polyacrylamide gel and incubated with antipilin antiserum.

amino terminus, the domain that is phosphorylated, and also in the central region. Relatively low levels of similarity are found near the PilR carboxy terminus, which contains the putative DNA-binding domain, as indicated by a helix-turn-helix motif. The amino-terminal region contains the aspartic acids at positions 11 and 54 and the lysine at position 104 which are found in all response regulator proteins (30). The central domain of PilR also contains two regions that are similar to the motif found in most nucleotide-binding proteins (7). The basic arrangement of domains, previously identified in NifA and NtrC (5), appears to be conserved in PilR as well. It is unlikely that PilR is the *P. aeruginosa* equivalent of NtrC, because NtrC mutants require glutamine for growth and the *pilR* mutant does not exhibit this property.

The similarity of PilR with members of the NtrC family suggests that the molecular mechanism of PilR's activity may be similar to that of NtrC's activity. The aspartic acids near the amino terminus of NtrC are presumably phosphorylated (35), and this modification is required for NtrC to facilitate an open-promoter complex formation following its interaction with σ^{54}-containing RNA polymerase (14). It is therefore likely that PilR is phosphorylated at Asp-54 or possibly Asp-11 before it can activate transcription of the pilin gene.

The phosphorylated form of NtrC has an endogenous ATPase activity, as shown by Weiss et al. (34), and they have postulated that it is this ATPase activity which is essential in order for phosphorylated NtrC to promote an open-promoter complex with the σ^{54}-containing holoenzyme. Moreover, this ATPase activity requires the intact nucleotide-binding site located in the central domain of NtrC (34). This region is highly conserved among all members of this regulatory family, including PilR. The extensive similarity at the amino-terminal and central domains between NtrC and PilR suggests that PilR may have the same mechanism of transcriptional activation.

The amino acid sequence at the carboxy terminus of PilR shows little sequence similarity to that of NtrC. However, characteristic of many DNA-binding proteins, including NtrC, PilR contains a short helix-turn-helix motif centered around the glycine at position 430. Protein-DNA interactions involve specific contacts between amino acids of the regulatory protein and a regulatory DNA sequence; thus, it is likely that the DNA recognition sequence for PilR will differ from the enhancer sequence recognized by NtrC or other response regulator proteins.

The mechanism of signal transduction leading to pilin gene transcription is not known; however, the striking similarities between PilR and the members of the NtrC family of regulatory proteins suggest a phosphorelay mechanism. In enteric bacteria, nitrogen limitation induces autophosphorylation of a protein kinase, the product of the NtrB gene. The NtrB-phosphate then modifies NtrC, presumably at one or both of the N-terminal aspartic acid residues. Only the phosphorylated form of NtrC is active and able to promote transcription (14). At this time, the identity of the protein kinase which modifies PilR is not known. One candidate is the gene interrupted by the transposon mutation in *P. aeruginosa* RA. We estimate that the insertion mutation in RA is approximately 1.7 kb from the beginning of *pilR*. This adjacent region could code for a cognate sensory element, similar to the organization of the enterobacterial *ntrB-ntrC* operon (16). Alternatively, the insertion in RA could affect expression of *pilR* by disrupting a regulatory region. A number of proteins of the NtrC family regulate gene expression by binding to enhancer sequences located several hundred base pairs from the promoter (24). Work is currently under way to determine whether the regulatory region disrupted in RA specifies a *cis*-acting regulatory sequence of *pilR* or encodes a gene product which may respond to a regulatory signal for pilin gene expression.

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