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Genetics

Formation of pilin in *Pseudomonas aeruginosa* requires the alternative σ factor (RpoN) of RNA polymerase

(*Pseudomonas* pili/adhesion/transcriptional regulation)

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ABSTRACT **The promoter region of the *Pseudomonas aeruginosa* pilin gene has a high degree of similarity to the nitrogen-regulated promoters of enteric bacteria. These promoters are recognized by the alternative σ factor of RNA polymerase, termed RpoN (NtrA or GlnF). This observation suggested that the *P. aeruginosa* pilin gene may be transcribed by the RpoN-containing RNA polymerase. We, therefore, cloned the RpoN gene from *P. aeruginosa* into *Escherichia coli* (where it formed a functional product) and used that cloned gene to construct a mutant of *P. aeruginosa* that was insertionally inactivated in its RpoN gene. This mutant failed to synthesize pilin, indicating that the RpoN σ factor is required for transcription of the pilin gene.**

With many bacteria that colonize mucosal surfaces, including *Pseudomonas aeruginosa* (1, 2), adhesion by means of pili is a common step during the initial stages of pathogenesis. Although the expression of pili mediates bacterial attachment and colonization, it can also be detrimental to the pathogen. Direct attachment of pili to receptors on a phagocytic cell can facilitate killing of the bacteria when such host defense mechanisms are encountered (3). Bacteria have, therefore, evolved elaborate genetic regulatory mechanisms that limit the expression of pili to conditions where tissue adhesion is essential for bacterial colonization. For example, *Escherichia coli* (4) and *Neisseria gonorrhoeae* (5) use a phase variation mechanism to turn the expression of pili on or off.

The pilin gene of *P. aeruginosa* has been sequenced and its promoter has been identified (6). The pilin promoter region is similar to a class of promoters recognized by RNA polymerase containing an alternative σ factor, termed RpoN (7, 8). This factor is encoded by the RpoN gene in *E. coli* or by the analogous genes in several other bacterial genera. RpoN allows RNA polymerase to recognize the promoters of a number of genes required for diverse metabolic functions, including genes for nitrogen fixation (9), dicarboxylate transport (10), assimilation of poor nitrogen sources (11), and catabolism of aromatic compounds (12).

In this communication we report the cloning of the *P. aeruginosa* RpoN gene and engineering of a strain unable to express a functional RpoN. This strain does not express the pilin polypeptide, thus demonstrating that the pilin gene in *P. aeruginosa* is transcribed by the class of RNA polymerase containing the RpoN gene product. This finding suggests that bacterial virulence factors may be regulated at a molecular level by mechanisms that are responsible for global nutritional regulation.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. *P. aeruginosa* PAK and the pilus-specific bacteriophage PO4 were obtained from David Bradley (Memorial University of Newfoundland, St. John's, Newfoundland). PAK-SR is a streptomycin-resistant mutant of PAK and was isolated after selection on plates containing streptomycin at 200 μg/ml. The *E. coli* DH5 α [*endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1* Δ(*lacZYA-argF*)U169 λ-ϕ80 d*lacZ* ΔM15] and *E. coli* HB101 [*hsd-20 recA13 ara-14 proA2 lac-41 galK2 mtl-1 xyl-5 supE44 rpsL2*] were the recipient strains for recombinant plasmids. *E. coli* YMC10 [*endA1 thi-1 hsdR17 supE44* Δ*lacU169 hutCk*] and *E. coli* TH1 [same as YMC10, with the RpoN gene region deleted) were provided by Boris Magasanik (MIT, Cambridge, MA). The plasmid pNA18, which carries the cloned RpoN gene of *Azotobacter vinelandii* (13), was provided by Christina Kennedy (University of Sussex, Brighton, U.K.). The plasmids pCR12 containing a *lacZ* fused to the dicarboxylate transport (DctA) gene of *Rhizobium leguminosarum*, and pLA114, containing the cloned *R. leguminosarum* DctB and DctD genes, were provided by Lisa Albright and Fred Ausubel (Harvard Medical School, Boston, MA). The DNA specifying tetracycline (*tet*) resistance was excised from plasmid pBR322 (14) and linkers specifying the *Pst* I recognition site were added to allow insertion of this cassette into recombinant plasmids after digestion with *Pst* I. Plasmid pKI10 contains the 10.2-kilobase (kb) *Bam*HI fragment from pKS1 (for description of pKS1, see *Results*), ligated into the *Bam*HI site of pUC18 (15). The plasmid vector pKI100 contains a *Nar* I–*Bgl* II fragment from the replicative form of M13mp18 (16) ligated into *Hin*cII–*Bam*HI restricted pACYC177 (17). Thus, pKI100 has the polylinker–*lacZ* region from M13mp18 and both the origin of replication and kanamycin resistance gene from pACYC177. Plasmid pKI4 was constructed by cloning the 10.2-kb *Bam*HI fragment from pKS1 into the pKI100 vector. The *tet* cassette was cloned into the *Pst* I site of pKI10, which resulted in the construction of pKI10T. The DNA fragment containing the *mob* region of the broad host range plasmid pRP4 was isolated from plasmid pJM703.1 (18), then cloned into the *Eco*RI site of pKI10T resulting in plasmid pKI11. Plasmid pKI5 was constructed by cloning the 11.6-kb *Bam*HI fragment from pKI10T into the *Bam*HI site of pKI100.

DNA Preparation and Analysis. Total DNA was prepared from *P. aeruginosa* PAK as described by Strom and Lory (19). DNA was digested with appropriate restriction enzymes (Bethesda Research Laboratories, Gaithersburg, MD) and electrophoretically separated on 0.8% agarose gel in Tris/acetic acid/EDTA buffer (20). DNA was transferred to nitrocellulose sheets (Schleicher & Schuell) by the method of Southern (21) and probed with nick-translated fragments at 37°C in 50% (vol/vol) formamide. Plasmid DNA was isolated by the method of Holmes and Quigley (22).

Cloning of the *P. aeruginosa* RpoN Gene. A library of *P. aeruginosa* DNA in the cosmid cloning vector pVK102 (23) was prepared by limited digestion of chromosomal DNA with

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Abbreviations: RpoN, alternative σ factor for RNA polymerase; Dct (prefix), dicarboxylate transport.

Sal I, size selection of 15- to 25-kb fragments, and ligation of these fragments into the *Sal* I site of pVK102. The recombinant plasmids were introduced into *E. coli* HB101 after *in vitro* packaging into λ phage. This library was plated onto nitrocellulose filters and screened with the *A. vinelandii* RpoN gene probe by colony hybridization as described (20).

For subcloning of the RpoN gene region from the cosmid, plasmid DNA was extracted, and the probe-reactive 10.2-kb fragment was ligated into the *Bam*HI site of pUC18 and pKI100.

Isolation of Mutations in the RpoN Gene of *P. aeruginosa* PAK. The *P. aeruginosa* PAK mutant lacking a functional RpoN gene was isolated after homologous recombination between the wild-type chromosomal gene and a mutant (*tet* cassette containing) copy from pKI11. Triparental spot matings were performed as described (24), with donor strain *E. coli* DH5α (pKI11), recipient strain *P. aeruginosa* PAK-SR, and *E. coli* DH5α containing the mobilizing plasmid pRK2013. Selection for *P. aeruginosa* containing the insertionally inactivated RpoN gene was accomplished by plating the transconjugates on agar containing streptomycin and tetracycline. Bacteria containing the chromosomally integrated pKI11 were identified by their resistance to carbenicillin.

RESULTS

Identification of a RpoN Gene Homologue in *P. aeruginosa* Strain PAK. The alternative σ factors encoded by RpoN genes from several bacterial species have been shown to be highly conserved in their amino acid and DNA sequence (25). To determine whether this DNA conservation of the RpoN gene coding sequences extends to *P. aeruginosa*, a Southern hybridization analysis of restriction fragments of *P. aeruginosa* PAK chromosomal DNA was performed using the cloned RpoN gene from *A. vinelandii* as a probe. As shown in Fig. 1, the heterologous RpoN gene probe hybridized to a 10.2-kb *Bam*HI fragment, a 13.5-kb *Eco*RI fragment, and a 4.3-kb *Xho* I fragment. Two probe-reactive genomic fragments of 1.5 kb and 1.7 kb were detected in the *Pst* I-digested sample, indicating the presence of a *Pst* I site within the sequence hybridizing with the RpoN probe. The Southern hybridizations were performed under conditions of high stringency, thus the strong reaction of the *A. vinelandii* heterologous probe with *P. aeruginosa* DNA sequences suggests that sequence homology between the RpoN genes of these two organisms is extensive.

Cloning the RpoN Gene from *P. aeruginosa* PAK. Using the same *A. vinelandii* RpoN gene probe, a cosmid library of *P. aeruginosa* PAK DNA was screened for the presence of RpoN sequence-containing clones by colony hybridization. Three colonies contained sequences that hybridized with the probe. Plasmid DNA was isolated from these bacteria and digested with restriction enzymes; hybridization of specific fragments to the *A. vinelandii* RpoN gene probe was confirmed by Southern analysis as shown in Fig. 2. *Eco*RI and *Bam*HI digests of two cosmids (pKS1 and pKS2) and of chromosomal DNA yielded the same probe-reactive fragments of 13.5 and 10.2 kb, respectively, suggesting that the DNA insert in these cosmids contains the RpoN gene homologue as well as additional flanking DNA. Similar digests of pKS3 yielded larger fragments suggesting that the RpoN gene in this insert is located near the vector cloning site. The 10.2-kb *Bam*HI fragment of pKS1 was isolated, subcloned into pUC18 and pKI100 cloning vectors, and used in all subsequent studies.

Characterization of the *P. aeruginosa* RpoN Gene. To determine whether the subcloned 10.2-kb *Bam*HI fragment expressed a functional RpoN, genetic complementation of *E. coli* TH1, an RpoN⁻ mutant, was attempted. To detect whether complementation occurred, TH1 was transformed with two plasmids: pCR12, which encodes a *lacZ* transcriptional fusion gene fused to *R. leguminosarum* DctA gene, and pLA114, which encodes genes for two transcriptional activators DctB and DctD. [Transcription of the DctA gene requires the RpoN-containing RNA polymerase as well as two other positive regulators, DctB and DctD (10).] When plated on medium containing the β-galactosidase chromogenic substrate 5-bromo-4-chloro-3-indolyl β-D-galactoside, these transformed *E. coli* TH1 colonies are white. The inability to synthesize β-galactosidase in *E. coli* TH1 was due to the absence of the RpoN gene product, which is required for initiation of transcription at the DctA promoter. Introduction of the third plasmid pKI4 containing the putative *P. aeruginosa* RpoN gene homologue resulted in blue colonies on medium containing 5-bromo-4-chloro-3-indolyl β-D-galactoside indicating expression of the DctA–*lacZ* fusion gene.

Results of β-galactosidase assays on bacteria grown in liquid medium are shown in Table 1. Negligible levels of β-galactosidase activity were detected in *E. coli* TH1, an RpoN deletion strain, when the vector pKI100 was present. However, significant activation of the DctA–*lacZ* fusion gene was observed in *E. coli* TH1 when pKI4 was present. *E. coli* YMC10, a strain containing a wild-type chromosomal copy of the RpoN gene was also transformed with pCR12, pLA114, and pKI100, and, as expected, *E. coli* YMC10 expressed a high level of β-galactosidase activity. The results from these experiments suggest that the 10.2-kb *P. aeruginosa* DNA

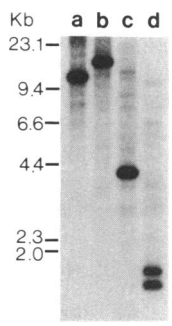

FIG. 1. Southern hybridization analysis of chromosomal DNA from *P. aeruginosa* PAK. DNA (5 μg) was digested with *Bam*HI (lane a), *Eco*RI (lane b), *Xho* I (lane c), or *Pst* I (lane d). The digests were fractionated by agarose gel electrophoresis, blotted, and hybridized with the *A. vinelandii* RpoN gene probe from plasmid pNA18. Locations of molecular size standards are indicated on the left.

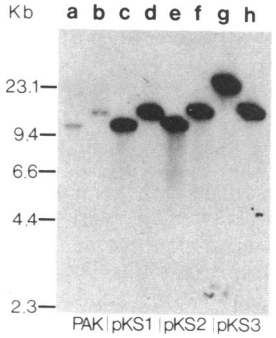

FIG. 2. Southern hybridization analysis of cosmids. Plasmid DNA from bacteria containing cosmids pKS1 (lanes c and d), pKS2 (lanes e and f), pKS3 (lanes g and h) and total DNA from *P. aeruginosa* PAK (lanes a and b) were extracted from bacteria and digested with *Bam*HI (lanes a, c, e, and g) or *Eco*RI (lanes b, d, f, and h). The digests were fractionated, blotted, and hybridized to the radiolabeled *A. vinelandii* RpoN gene probe.

Table 1. Activation of DctA–*lacZ* fusion gene by RpoN

E. coli strain	β-Galactosidase activity, arbitrary units
TH1 (pCR12, pLA114, pKI100)	8.0
TH1 (pCR12, pLA114, pKI4)	230.0
TH1 (pCR12, pLA114, pKI5)	2.1
YMC10 (pCR12, pLA114, pKI100)	950.0

β-Galactosidase assays of *E. coli* strains containing a plasmid-encoded DctA–*lacZ* fusion gene. β-Galactosidase assays were performed as described by Ishikawa and Kato (26) except for the following modifications. Cells (2.5 OD_{600} units) were lysed with chloroform, incubated with 1 mM 4-methylumbelliferyl β-D-galactoside for 5 min at 37°C, followed by determination of the fluorescence of the cleaved substrate (4-methylumbelliferone).

insert in pKI4 encodes a functional RpoN polypeptide that can substitute (albeit less efficiently) for the *E. coli* counterpart in the activation of an *R. leguminosarum* promoter. These findings confirm that the various RpoN gene-encoded σ factors are not only homologous in their amino acid sequence but are similar in their active conformations as well.

An insertionally inactivated RpoN gene was constructed as described in the section below. Plasmid pKI5 containing this insertionally inactivated RpoN gene was unable to complement the RpoN⁻ *E. coli* TH1 mutant as shown by the background levels of β-galactosidase activity (see Table 1). These results indicate that the RpoN gene can be functionally inactivated by the insertion of the tetracycline-resistance gene into a specific site within the 10.2-kb fragment of *P. aeruginosa* DNA.

Construction of an RpoN⁻ PAK Mutant by Gene Replacement. To determine whether the RpoN gene product is essential for transcription of the pilin gene in *P. aeruginosa*, a strain of this organism was engineered such that its chromosomal RpoN gene was insertionally inactivated. Because the heterologous RpoN gene probe hybridized to two *Pst* I restriction fragments (Fig. 1), inserting a tetracycline-resistance (*tet*) determinant into the correct *Pst* I site within the 10.2-kb RpoN sequence-containing *Bam*HI fragment would interrupt the coding sequence for RpoN. In this manner, a mutant RpoN was constructed and insertion of the *tet* cassette into the *Pst* I site within the RpoN gene was confirmed by Southern hybridization analysis. To allow mobilization of this plasmid into *P. aeruginosa*, a 1.9-kb *Eco*RI fragment specifying the *mob* region of plasmid pRP4 was also cloned into this plasmid. The resultant plasmid pKI11 can be mobilized by the helper plasmid pRK2013 but cannot replicate in *P. aeruginosa* due to its ColE1 origin of replication, thus providing a suicide plasmid system for gene replacement.

To replace the chromosomal copy of the RpoN gene with the mutant variant containing the *tet* cassette, pKI11 was mobilized into *P. aeruginosa*, and tetracycline-resistant recipients were isolated. Colonies of tetracycline-resistant *P. aeruginosa* were screened for the presence of the vector by replica plating onto medium containing carbenicillin. Tetracycline-resistant but carbenicillin-sensitive isolates were obtained, and these were presumed to have replaced the wild-type chromosomal RpoN gene with the mutant variant by a double-crossover event. To verify that gene replacement at the RpoN region had taken place, chromosomal DNA was extracted from a representative strain (N1), digested with *Xho* I, and, after agarose gel electrophoresis of fragments, blots were probed with a nick-translated *A. vinelandii* RpoN gene probe, as well as with a similarly labeled *tet* cassette. Fig. 3 shows hybridization of the RpoN gene probe to a 4.3-kb *Xho* I fragment from wild-type *P. aeruginosa* PAK strain and to a 5.7-kb fragment in the digest of DNA from *P. aeruginosa* N1, containing the mutated RpoN gene in its chromosome. The size of the 5.7-kb fragment is the sum of the 4.3-kb *Xho* I fragment containing the wild-type and the 1.4-kb *tet*

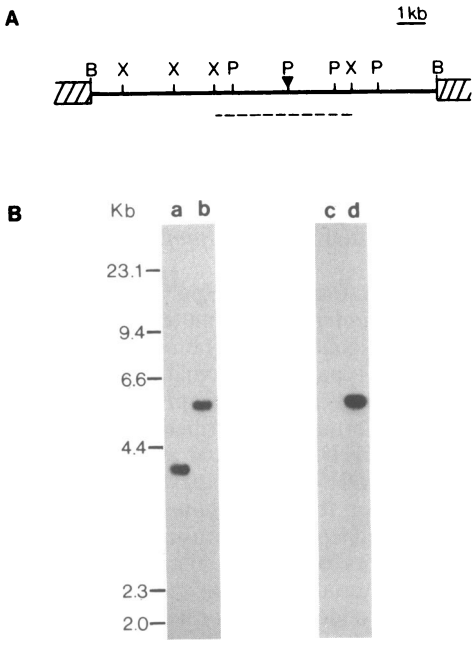

FIG. 3. (*A*) Partial restriction map of pKI11. B, *Bam*HI; P, *Pst* I; X, *Xho* I. The triangle indicates the location of the *tet* cassette used in insertional inactivation of the RpoN gene. The hatched boxes represent the pUC18 vector sequences. The region of insert hybridizing with the *A. vinelandii* RpoN gene probe is indicated by dashed line. (*B*) Southern hybridization analysis of *Xho*I-digested DNA from the wild-type *P. aeruginosa* PAK (lanes a and c) and from the RpoN⁻ mutant N1 (lanes b and d). Blots shown in lanes a and b were probed with the nick-translated *A. vinelandii* RpoN gene from pNA18; blots in lanes c and d were probed with the radiolabeled *tet* cassette.

cassette. The *tet* cassette probe hybridized only to the 5.7-kb *Xho* I fragment from *P. aeruginosa* N1 DNA.

Synthesis of Pilin in RpoN⁻ Mutant of *P. aeruginosa*. The RpoN⁻ mutation resulted in *P. aeruginosa* N1 becoming a glutamine auxotroph, which is characteristic of RpoN⁻ mutations in enteric bacteria (11). To examine the effect of a defective RpoN gene on pilin expression, *P. aeruginosa* N1 was tested for the presence of assembled pili by determining the sensitivity of this mutant strain to the pilus-specific bacteriophage PO4 (27). Killing of wild-type and mutant *P. aeruginosa* was estimated by plating an aliquot of a bacterial suspension on an L-agar plate densely seeded with the phage. Mutant strain *P. aeruginosa* N1 gave rise to colonies after overnight incubation. Resistance to PO4 infection suggests that this mutant does not express pili on its surface.

To determine whether phage resistance of RpoN⁻ mutants might be attributed to the inability of such strains to synthesize the monomeric subunits of pili, lysates of each mutant were examined for the presence of pilin antigen by protein-immunoblot analysis using polyclonal antibody to purified pilin. No detectable pilin polypeptide was found in the RpoN⁻ strain N1 (Fig. 4, lane B). A *P. aeruginosa* PAK strain containing the *tet* cassette at a different location in the chromosome expressed levels of pilin comparable to that observed in the wild-type PAK strain (data not shown), indicating that expression of tetracycline resistance *per se* does not inhibit pilin expression. These results strongly suggest that pilin gene expression requires a functional RpoN product.

DISCUSSION

In this report we provide evidence that pilin gene transcription requires a functional RpoN product. This finding is consistent with our previous DNA sequence data that sug-

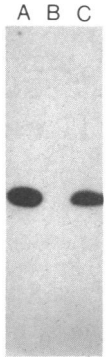

FIG. 4. Immunoblot analysis with pilin antibody of *P. aeruginosa* PAK and N1 whole-cell lysates. Preparation of antisera to pilin and immunoblot analysis of bacterial extract was as described (19), except that the whole-cell lysates were prepared by resuspending cells in 6 M urea/1% $NaDodSO_4$/1% 2-mercaptoethanol followed by boiling for 5 min. Lanes: A, standard purified pilin; B, extract of *P. aeruginosa* N1; C, extract of *P. aeruginosa* PAK-SR.

gested that the pilin promoter is recognized by an RNA polymerase containing the alternative σ factor RpoN. This finding also provides further evidence that a relatively broad spectrum of genes are transcribed by this specialized RNA polymerase. Further, we have demonstrated that the RpoN product of *P. aeruginosa* can specifically activate RpoN-dependent transcription in *E. coli*.

The cloned *P. aeruginosa* pilin gene is poorly expressed in *E. coli* even though a functional RpoN is present (19). Although it remains possible that the *E. coli* RpoN is unable to recognize the *Pseudomonas* pilin promoter, the fact that the *Pseudomonas* RpoN functions in *E. coli* indicates that the two RpoN products are functionally interchangeable. Thus, the above result suggests that pilin expression in *P. aeruginosa* is positively regulated by transcriptional factors that are absent in *E. coli*. This hypothesis is consistent with the general model for RpoN-dependent transcription (7–10). Studies in several organisms have demonstrated that RpoN-containing RNA polymerase cannot initiate transcription in the absence of additional regulatory factors. Such regulatory proteins include: (*i*) NtrC, which transcriptionally activates a wide ranger of nitrogen-regulated promoters in enteric bacteria, (*ii*) NifA, which activates promoters of nitrogen fixation genes, and (*iii*) DctD, which activates the dicarboxylate transport gene DctA in *R. leguminosarum*. Based on this pattern of regulation, it is highly probable that transcription of the pilin gene also requires one or more transcriptional regulatory polypeptides.

Several of the RpoN-regulated genes studied so far share a common mechanism of transcriptional activation that responds to environmental stimuli by a two-component signal-transducing system (28). These regulatory systems consist of a sensory polypeptide that first responds to an environmental stimulus and then modifies and/or interacts with a regulatory protein that binds to DNA sites upstream from promoters and facilitates transcription of regulated genes. The nitrogen regulatory protein NtrC and dicarboxylate transport activator DctD are the regulatory components of this system that coordinately function with RpoN to activate gene expression, while NtrB and DctB are the respective sensory elements. Determining whether pilin expression is regulated by a similar two component system will require identification of additional regulatory factors and elucidation of the precise nature of the regulatory stimulus.

The pilin promoters of *N. gonorrhoeae*, *Moraxella bovis*, *Bacteriodes nodosus*, and *P. aeruginosa* share a high degree of DNA homology to the RpoN consensus promoter (6). Since we have demonstrated that RpoN is absolutely required for pilin expression in *P. aeruginosa*, it is likely the RpoN-containing RNA polymerase transcribes the pilin genes in the other microorganisms as well. Furthermore, the overall pilin regulatory mechanism, including requirement for additional transcriptional factors, may also be similar in these otherwise different organisms. Alternatively, because of the diverse environments in which these pathogens live, it is possible that the sensory components of any one regulatory system will be unique.

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