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Nucleotide Sequence of the *rpoN* Gene and Characterization of Two Downstream Open Reading Frames in *Pseudomonas aeruginosa*

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The *rpoN* gene of *Pseudomonas aeruginosa* is required for the expression of a number of diverse genes, ranging from several classes of bacterial adhesins to enzymes for amino acid biosynthesis. The nucleotide sequence of the *rpoN* gene and its flanking region has been determined. The deduced amino acid sequence of the *rpoN* product is highly homologous to sequences of RpoN proteins of other microorganisms. Moreover, two open reading frames (ORF1 and ORF2) encoding peptides of 103 and 154 amino acids long, respectively, were found downstream of the *rpoN* gene. These two ORF products have a high degree of amino acid sequence homology with products of similar ORFs located adjacent to the *rpoN* genes in other microorganisms. Mutations in either ORF lead to a significant increase in *P. aeruginosa* generation time when propagated on minimal medium. These mutations had no effect on the expression of pilin or flagellin genes, whose expression depends on RpoN. Complementation analysis showed that the two ORFs are in the same transcriptional unit and the growth defects of the two ORF mutants on minimal medium are due to mutational effects on ORF2. The adverse effect of the ORF mutations on the growth of *P. aeruginosa* in minimal media can be suppressed by the addition of glutamine but not arginine, glutamate, histidine, or proline. Since *rpoN* mutants of *P. aeruginosa* display this same amino acid requirement for growth, the ORF2 product very likely functions as a coinducer of some but not all of the RpoN-controlled genes.

Pseudomonas aeruginosa is a gram-negative bacterium which is a major cause of severe infection in immunocompromised patients (2). Pili are important virulence factors, as they mediate adhesion of the microorganisms to epithelial cells. Expression of *pilA*, the structural gene for the pilin subunit, has been well characterized genetically (4, 26, 33). Transcription of the *pilA* gene requires an alternative sigma factor (σ^{54}), the product of the *rpoN* gene, as well as a pair of transcriptional activators, PilS and PilR (9, 12, 13). In addition to the defect in pilin formation, *rpoN* mutants of *P. aeruginosa* are defective in expression of secondary adhesins for epithelial cells and mucin, flagellin, glutamine synthetase, and urease genes (12, 31, 35, 36). The same *rpoN* mutants are unable to use arginine, glutamate, histidine, or proline as a sole nitrogen source (35).

Initial observations that σ^{54} is required for the expression of genes of nitrogen assimilation were followed by the discovery a wide range of genes that are also transcribed by the σ^{54}-containing RNA polymerase (reviewed in references 8 and 34). Products of these genes are not necessarily part of bacterial nitrogen metabolism; they are also involved in functions such as motility, transport of different nutrients, and formation of pili (12, 19, 21, 29, 35). All of these genes have a consensus promoter sequence of -24(GG)/-12(GC), and their expression requires at least one transcriptional activator.

The *rpoN* genes of many microorganisms have been cloned and sequenced, including those of *Klebsiella pneumoniae* (18), *Azobacter vinelandii* (16), *Rhizobium meliloti* (28), *Rhodobacter capsulatus* (14), *Pseudomonas putida* (11), *Thiobacillus ferrooxidans* (1), and *Bradyrhizobium japonicum* (15). In addition to the conserved sequence encoding σ^{54}, a cluster of flanking genes encoding small polypeptides was noted in most of these bacteria (17); some are upstream while some are downstream of the *rpoN* gene. Furthermore, in *K. pneumoniae*, mutations in either of the two open reading frames (ORFs) downstream of the *rpoN* gene resulted in an increase in the expression of σ^{54}-dependent *nif* genes, although no effect on amino acid assimilation was observed (17). This observation lead to a hypothesis that the functions of these two proteins are to interfere with the activity of RpoN and thus modulate expression of certain genes transcribed by this species of RNA polymerase.

In this report, we present the nucleotide sequence of *rpoN* and its flanking regions from *P. aeruginosa*. Mutational analysis was used to establish the roles of the two proteins encoded by the genes downstream of *rpoN*. Our findings suggest that while none of these proteins are essential for the expression of pili and flagella, one of them may be a transcriptional activator of metabolic genes that are transcribed by the σ^{54}-containing RNA polymerase.

MATERIALS AND METHODS

Enzymes and chemicals. All restriction enzymes, T4 DNA ligase, and the Klenow fragment of DNA polymerase I were purchased from Bethesda Research Laboratories, Inc., Gaithersburg, Md. Isotopes [α-^{32}P]dCTP, [α-^{35}S]dATP, and [^{35}S]methionine were purchased from Dupont, NEN Research Products, Boston, Mass. The DNA Sequenase kit was purchased from U.S. Biochemical Corp., Cleveland, Ohio.

Bacterial strains and plasmids. Strains and plasmids used in this study are listed in Table 1. In plasmid constructs pSJ9210, pSJ9211, pSJ9212, and pSJ9213, ORF1 and/or ORF2 is cloned in the same direction as the *tac* promoter of the vector pMMB67EH (Fig. 3B), while in plasmids pSJ9363, pSJ9364, pSJ9365, and pSJ9366, ORF1 and/or ORF2 is cloned in the same direction as the T7 promoter of the vector pDN18.

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TABLE 1. Strains and plasmids

Strain or plasmid	Genotype or description	Source or reference
E. coli DH5α	*endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1* Δ(*lacZYA-argF*)*U169* λ-φ80 d*lacZ*Δ*M15*; recipient for recombinant plasmids	Bethesda Research Laboratories
P. aeruginosa		
PAK	Wild type	David Bradley
PAK-N1	PAK mutant with Tn*5G* insertion in *rpoN* locus	12
PAK-*orf1*	PAK mutant with Ω fragment insertion in ORF1	This study
PAK-*orf2*	PAK mutant with Ω fragment insertion in ORF2	This study
Cloning vectors		
pTZ18R	*E. coli* cloning vector	U.S. Biochemical Corp.
pMMB67EH	IncQ cloning vector with *tac* promoter directing from *Eco*RI to *Hin*dIII site	5
pDN18	IncP cloning vector	22
pUC19Ω	pUC19 plasmid with Ω fragment insertion	36
Recombinant plasmids		
pMMBT7	T7 RNA polymerase gene cloned under control of the *tac* promoter in pMMB67EH	23
pMSZ5	pilin gene::*lacZ* transcriptional fusion in pSP329	Mark Strom
pSJ925	6.5-kb *Eco*RI-*Bam*HI fragment of pKI10 (11) cloned into pTZ18R	This study
pSJ926	6.5-kb *Eco*RI-*Bam*HI fragment of pKI10 (11) cloned into pDN18	This study
pSJ929	1.0-kb *Sty*I-*Pst*I fragment containing ORF1 and ORF2 cloned into *Sma*I-*Pst*I site of pTZ18R	This study
pSJ9210/pSJ9364	1.0-kb *Eco*RI-*Pst*I fragment from pSJ929 cloned into pMMB67EH/pDN18	This study
pSJ9211/pSJ9364	0.5-kb *Sst*I fragment from pSJ929 cloned into *Sst*I site of pMMB67EH/pDN18	This study
pSJ9212/pSJ9365	0.8-kb *Msc*I-*Pst*I fragment from pSJ929 cloned into *Sma*I-*Pst*I site of pMMB67EH/pDN18	This study
pSJ9213/pSJ9366	0.4-kb *Sst*I-*Pst*I fragment from pSJ929 cloned into pMMB67EH/pDN18	This study
pSJ9221	Ω fragment of *Sma*I digest from pUC19Ω inserted into *Msc*I site of pSJ925	This study
pSJ9226	Ω fragment of *Sma*I digest from pUC19Ω inserted into *Mlu*I site of pSJ925	This study
pPT269	flagellin gene::*lacZ* transcriptional fusion in pSP329Gm	36

Bacterial culture conditions. L agar and L broth were used for the growth of *Escherichia coli* and *P. aeruginosa*. For growth tests of *P. aeruginosa* mutants, minimal medium A (3) was used. To assess nutritional requirements, bacteria were first grown overnight on L-agar medium and single colonies were then restreaked on minimal medium A with or without supplementation of amino acids, followed by overnight incubation at 37°C. Bacterial growth was determined after 16 h of incubation. Antibiotics were used as needed at concentrations of 100 μg/ml (ampicillin), 50 μg/ml (spectinomycin), 25 μg/ml (streptomycin), and 10 μg/ml (tetracycline) for *E. coli* and 150 μg/ml (carbenicillin), 200 μg/ml (spectinomycin), 200 μg/ml (streptomycin), 50 μg/ml (gentamicin), and 100 μg/ml (tetracycline) for *P. aeruginosa*.

DNA sequence analysis. DNA sequence analysis was performed by the dideoxy-chain termination method (30) using the DNA Sequenase kit from U.S. Biochemical Corp. A 1.65-kb *Eco*RI-*Pst*I fragment and a 1.45-kb *Pst*I fragment containing 5′ and 3′ halves of the *rpoN* gene, respectively (see Fig. 3A), were cloned from pK110 into M13mp18 and M13mp19, in both orientations. Both strands were sequenced progressively with synthetic oligonucleotide primers in addition to the universal primer.

Generation of chromosomal ORF1 and ORF2 mutations. A 2-kb Ω fragment was isolated from pUC19Ω following *Sma*I digestion and was ligated into unique *Msc*I or *Mlu*I sites of pSJ925, generating Ω insertions in ORF1 or ORF2 (see Fig. 3A). The resulting plasmids, pSJ9221 and pSJ9226, with Ω insertions in ORF1 and ORF2, respectively, were linearized by *Eco*RI digestion and electroporated into wild-type *P. aeruginosa* PAK. Transformants were selected on L-agar plates containing 200 μg each of spectinomycin and streptomycin per ml. Individual colonies were tested for sensitivity to carbenicillin. Carbenicillin-sensitive colonies were picked, and Southern blot analysis was used to confirm the double crossover event.

Electroporation of *P. aeruginosa*. Bacteria were grown overnight on L-broth or L-agar medium. Cells were washed in 10% glycerol at least two times and resuspended in a 2× to 5× pellet volume of 10% glycerol. The cell suspension (50 μl) was mixed with 20 to 200 ng of DNA on ice (circular DNA was used for transformation, and linear DNA was used for marker exchange) and electroporated in a 0.2-cm cuvet at the following setting: voltage, 2.5 kV; resistance, 400 Ω; and capacity, 25 μFd (using the Electroporator model gene pulser from Bio-Rad). Immediately after electroporation, cells were resuspend in 5 ml of L broth and shaken at 37°C for 1 h before being plated on an appropriate selection medium. For marker exchange, the loss of antibiotic resistance encoded on the original vector was tested before further confirmation by Southern hybridization.

Miscellaneous methods. T7 expression of the two ORFs and labeling them with [^{35}S]methionine in *P. aeruginosa* were monitored by the protocol previously described (23). β-Galactosidase activity was measured as described by Miller (20). Immunoblotting was conducted as described elsewhere using mouse antipilin antibody (33).

Nucleotide sequence accession number. The *rpoN*, ORF1, and ORF2 nucleotide sequence has been submitted to GenBank. Its accession number is L26916.

RESULTS

Sequence analysis of the *rpoN* gene and its flanking region. The *rpoN* gene of *P. aeruginosa* has previously been cloned and mapped. Two fragments, a 1.65-kb *Eco*RI-*Pst*I fragment and a 1.45-kb *Pst*I fragment, containing the 5′ and 3′ halves of the *rpoN* gene, respectively, were subcloned and sequenced (Materials and Methods). The *rpoN* coding sequence extends for 1,494 bp (from nucleotides 185 to 1678 in Fig. 1) and encodes a 497-amino-acid polypeptide (Fig. 1). Two ORFs were found downstream of the *rpoN* gene, transcribing in the same direc-

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GTTCCGCTCGCCCGCGGTTTCGCAACCACGAGTGCGCCTGGAGCGGATGATTTATTCAGAGAAACCCTAGGCAAGTACGAAAATTCAGGCATATAATTTGCTTCCTGGCGGTGCTGCCAC   120
                                                                   -35                     -10

CTCGGCACCGTAGTAGTGGATGGTGCCATCCGGTACCAGCGAATAAGGTACTAAGCCCTCAGCCATGAAACCATCGCTAGTCCTCAAGATGGGCCAGCAGCTGACGATGACGCCGCAGCT   240
                                              RBS              M  K  P  S  L  V  L  K  M  G  Q  Q  L  T  M  T  P  Q  L
                                                               (RpoN)

GCAACAGGCCATACGCCTCCTCCAGCTATCCACCCTGGACCTCCAGCAGGAAATCCAGGAAGCGCTGGAATCCAATCCCATGCTCGAACGCCAGGAAGACGGCGACGATTTCGACAACAG   360
 Q  Q  A  I  R  L  L  Q  L  S  T  L  D  L  Q  Q  E  I  Q  E  A  L  E  S  N  P  M  L  E  R  Q  E  D  G  D  D  F  D  N  S

CGACCCTCTCGCCGATGGCGCCGAACAGGCGGCCAGCGCGCCCCAGGAGAGCCCTCTCCAGGAAAGCGCCACGCCCAGCGTCGAAAGCCTCGACGACGACCAGTGGAGCGAACGCATACC   480
 D  P  L  A  D  G  A  E  Q  A  A  S  A  P  Q  E  S  P  L  Q  E  S  A  T  P  S  V  E  S  L  D  D  D  Q  W  S  E  R  I  P

CAGCGAGTTGCCGGTGGATACCGCCTGGGAAGACATCTACCAGACCAGCGCCAGCAGCCTGCCCAGCAACGACGATGACGAATGGGACTTCACCGCGCGCACCAGCAGCGGCGAGAGCCT   600
 S  E  L  P  V  D  T  A  W  E  D  I  Y  Q  T  S  A  S  S  L  P  S  N  D  D  D  E  W  D  F  T  A  R  T  S  S  G  E  S  L

GCACAGCCACCTGCTCTGGCAGGTCAACCTGGCACCGATGTCGGACACCGATCGGATGATCGCGGTGACCATCATCGATAGCATCAACAACGATGGCTACCTCGAAGAGTCCCTCGAAGA   720
 H  S  H  L  L  W  Q  V  N  L  A  P  M  S  D  T  D  R  M  I  A  V  T  I  I  D  S  I  N  N  D  G  Y  L  E  E  S  L  E  E

AATCCTCGCCGCCATCGATCCGGAACTGGACGTCGAGCTCGACGAAGTGGAAGTCGTGCTGCGCCGCATCCAGCAACTGGAACCCGCCGGCATCGGCGCTCGCAACCTGCGCGAGTGCCT   840
 I  L  A  A  I  D  P  E  L  D  V  E  L  D  E  V  E  V  V  L  R  R  I  Q  Q  L  E  P  A  G  I  G  A  R  N  L  R  E  C  L

GCTGCTGCAACTGCGCCAGTTGCCGTCCACCACGCCCTGGCTGAACGAAGCGCTGCGCCTGGTATCCGACTACCTCGACCTGCTCGGCGGCCGCGACTACAGCCAATTGATGCGGCGCAT   960
 L  L  Q  L  R  Q  L  P  S  T  T  P  W  L  N  E  A  L  R  L  V  S  D  Y  L  D  L  L  G  G  R  D  Y  S  Q  L  M  R  R  M

GAAGCTCAAGGAAGACGAACTGCGCCAGGTCATCGAGCTGATCCAGTGCCTGCATCCCCGGCCCGGTTCGCAGATCGAATCGAGCGAAGCCGAATACATCGTCCCCGACGTCATCGTGCG  1080
 K  L  K  E  D  E  L  R  Q  V  I  E  L  I  Q  C  L  H  P  R  P  G  S  Q  I  E  S  S  E  A  E  Y  I  V  P  D  V  I  V  R

CAAGGACAACGAGCGCTGGCTGGTCGAGCTGAACCAGGAGGCGATGCCACGCCTGCGCGTCAACGCCACCTACGCCGGCATGGTCCGCCGCGCCGACAGCAGCGCCGACAATACCTTCAT  1200
 K  D  N  E  R  W  L  V  E  L  N  Q  E  A  M  P  R  L  R  V  N  A  T  Y  A  G  M  V  R  R  A  D  S  S  A  D  N  T  F  M

GCGCAACCAGTTGCAGGAAGCCCGCTGGTTCATCAAGACGCTGCAGAGCCGCAACGAAACGCTGATGAAGGTCGCCACCCAGATCGTCGAGCACCAGCGCGGTTTCCTCGACTACGGCGA  1320
 R  N  Q  L  Q  E  A  R  W  F  I  K  T  L  Q  S  R  N  E  T  L  M  K  V  A  T  Q  I  V  E  H  Q  R  G  F  L  D  Y  G  E

AGAAGCCATGAAGCCGCTGGTCCTGCACGACATCGCCGAAGCGGTGGGCATGCACGAATCGACCATTTCGCGGGTCACCACGCAGAAATACATGCATACGCCGCGCGGCATTTTCGAGCT  1440
 E  A  M  K  P  L  V  L  H  D  I  A  E  A  V  G  M  H  E  S  T  I  S  R  V  T  T  Q  K  Y  M  H  T  P  R  G  I  F  E  L
                   "Helix-turn-Helix"

GAAATATTTCTTTTCAAGTCACGTCAGTACCGCCGAAGGCGGGGAATGCTCGTCCACCGCGATCCGCGCGATCATCAAGAAACTGGTGGCCGCGGAAAATGCGAAAAAGCCATTGAGCGA  1560
 K  Y  F  F  S  S  H  V  S  T  A  E  G  G  E  C  S  S  T  A  I  R  A  I  I  K  K  L  V  A  A  E  N  A  K  K  P  L  S  D

CAGCAAGATCGCTGGTTTACTGGAGGCACAGGGCATTCAAGTGGCTCGCCGCACCGTCGCCAAATACCGCGAATCCCTGGGCATAGCCCCTTCGAGCGAGCGCAAGCGACTGGTGTGACG  1680
 S  K  I  A  G  L  L  E  A  Q  G  I  Q  V  A  R  R  T  V  A  K  Y  R  E  S  L  G  I  A  P  S  S  E  R  K  R  L  V  *
                                              "RpoN Box"

TTGATCCACGCCAAGGTGTTCCGGAGGCAGGTTGCTAACCTGCCACTTACACACGGGCAACAAGGAGAACGCGGTATGCAAGTCAACATCAGTGGCCATCAACTGGATGTGACCGACGCC  1800
                                                              RBS            M  Q  V  N  I  S  G  H  Q  L  D  V  T  D  A
                                                                             (ORF1)

CTGCGCGACTATGTCGGCGAGAAACTGAGCCGTCTGGAGCGCCATTTCGACAAGATCACCAATGTTCAGGTGATCATGGAGGTCGAGAAACTGAAGCAGAAGATCATCGAAGCCACCCTG  1920
 L  R  D  Y  V  G  E  K  L  S  R  L  E  R  H  F  D  K  I  T  N  V  Q  V  I  M  E  V  E  K  L  K  Q  K  I  I  E  A  T  L

CGCATCGCCGGTGGCGAGATCGTAGCCAATGCGGAACATGAGGACATGTACGCGGCCATCGACCTGCTGATCGACAAACTCGACCGTCAACTGATCAAACACAAAGAGAAGTATCTCGAA  2040
 R  I  A  G  G  E  I  V  A  N  A  E  H  E  D  M  Y  A  A  I  D  L  L  I  D  K  L  D  R  Q  L  I  K  H  K  E  K  Y  L  E

CGGCAGCAGGGCGTAGGCGCCCGCTGATCTCCCCTCCCCTATGATCCGACTTGAGCAAATCCTGACCCCCGGCCGTTCCCTCGTGAACGTGCCGGGCGGCAGCAAGAAACGCGTACTCGA  2160
 R  Q  Q  G  V  G  A  R  *              M  I  R  L  E  Q  I  L  T  P  G  R  S  L  V  N  V  P  G  G  S  K  K  R  V  L  E
                                        (ORF2)

ACAAATCGCCAACCTGGTAGCCAGGGAGCTGCCGGAGCTCGACGCCCAGACCATCTTCGAAAACCTGGTGGCCCGGGAAAAACTCGGTTCCACCGGCTTCGGCAATGGCATCGCCATTCC  2280
 Q  I  A  N  L  V  A  R  E  L  P  E  L  D  A  Q  T  I  F  E  N  L  V  A  R  E  K  L  G  S  T  G  F  G  N  G  I  A  I  P

GCACTGCCGCCTGTCTGGCTGCCAGAGCCCCATCAGCGCCGTGCTGCACCTGGATGCTCCCGTGGACTTCGACGCGCTGGACGGCGCGCCGGTCGACCTGCTCTTCGTCCTGCTGGTACC  2400
 H  C  R  L  S  G  C  Q  S  P  I  S  A  V  L  H  L  D  A  P  V  D  F  D  A  L  D  G  A  P  V  D  L  L  F  V  L  L  V  P

GGAGGCCGCGACCGAAGAACATCTCGAACTGCTCCGGCAGATCGCCGCCATGCTCGACCGTGCCGACGTACGCGACCGCCTGCGCAGCGCTCCCACCGCCGAAGCGCTTTATCAGATCGT  2520
 E  A  A  T  E  E  H  L  E  L  L  R  Q  I  A  A  M  L  D  R  A  D  V  R  D  R  L  R  S  A  P  T  A  E  A  L  Y  Q  I  V

GGTAGACGTACAGAACGGGCAATGACATGCGCCTGATCATCGTCAGTGGCCGCTCGGGCTCGGGCAAGAGCACCGCCCTGAACGTCCTCGAAGACAACGGCTTCTATTGCATCGACAACC  2640
 V  D  V  Q  N  G  Q  *

TTCCCGCCAGCCTGCTTCCCGAC*2664
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FIG. 1. Nucleotide sequence of the *rpoN* gene and flanking region. Amino acid sequences deduced from *rpoN*, ORF1, and ORF2 are given under the nucleotide sequences. The putative −35, −10, and ribosome binding sites (RBS) are marked. Amino acids within the RpoN box and helix-turn-helix are underlined.

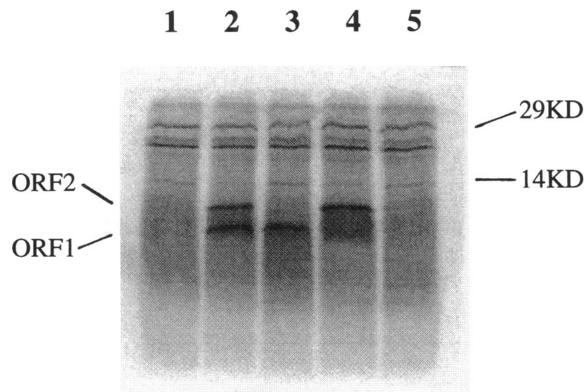

FIG. 2. T7 expression of ORF1 and ORF2 gene products. *P. aeruginosa* PAK(pMMBT7) containing plasmid pDN18 (vector), pSJ9363 (ORF1 and ORF2), pSJ9364 (ORF1), pSJ9365 (ORF2), pSJ9366 (downstream of ORF2) (lanes 1 to 5, respectively) was subjected to a T7 gene expression and [^{35}S]methionine labeling experiment. Labeled total cellular proteins were separated on a sodium dodecyl sulfate–20% polyacrylamide gel.

tion as *rpoN*. The first ORF, ORF1, is separated by 78 bp from the translational stop codon of the *rpoN* gene and has a coding capacity of 103 amino acids. The second ORF, ORF2, separated by 13 bp from ORF1, encodes a 154-amino-acid polypeptide (Fig. 1).

The *rpoN* coding sequence is preceded by an apparent consensus promoter sequence for the *E. coli* σ^{70} RNA polymerase (−35TAGGCA/−10TATAAT) and a Shine-Dalgarno sequence (AGGT), but neither of the two downstream ORFs is preceded by an obvious promoter sequence. However, the start codon of ORF1 is preceded by a typical Shine-Dalgarno sequence (AGGAG), while no obvious ribosome binding site was observed immediately upstream of the start codon of ORF2 (Fig. 1). Despite the absence of an obvious Shine-Dalgarno sequence, ORF2 was translated as well as ORF1 in *P. aeruginosa* when the two ORFs were expressed by the T7 promoter as shown in Fig. 2.

An amino acid sequence homology search of the GenBank data base indicated that the *P. aeruginosa* RpoN has significant homology with RpoN proteins from other bacteria. The similarities are scattered throughout the entire sequence, and the invariant sequence ARRTVAKYR, termed the RpoN box (37), is located near the carboxy terminus of *P. aeruginosa* RpoN between positions 474 and 482 (Fig. 1). A conserved helix-turn-helix DNA-binding sequence of RpoN proteins (10) is also found upstream of the RpoN box between positions 382 and 407.

In addition to conservation of amino acid sequences between RpoN proteins, the adjacent two ORF products also have amino acid homology. The ORF1 product has a high degree of amino acid homology with the products of similar ORFs located next to *rpoN* genes of *A. vinelandii* (ORF107) (16), *P. putida* (ORF102) (11), *K. pneumoniae* (ORF95) (17), *Alcaligenes eutrophus* (ORF130) (38), *Salmonella typhimurium* (27), and *T. ferrooxidans* (ORF3) (1). ORF2 has homology with products of *K. pneumoniae* (ORF162) (17), *B. japonicum* (ORF>90) (15), and *A. eutrophus* (ORF90) (38). Interestingly, the ORF1 and ORF2 products also have amino acid homology with products of ORFs that are not located next to the *rpoN* gene. For example, the ORF1 product has amino acid homology with the product of an ORF adjacent to the *pheA* gene of *E. coli* (10), and the ORF2 product has homology with the phosphotransferase Fpr protein of *S. typhimurium* (6) which is involved in the fructose phosphotransferase system.

Generation of two ORF mutations. In earlier studies with *K. pneumoniae*, Merrick and Coppard (17) have shown that the two ORFs located downstream of the *rpoN* gene exert a negative effect on the expression of certain *rpoN*-regulated genes. To see whether the two ORFs of *P. aeruginosa* have similar functions, we first generated two ORF mutations. Chromosomal insertional mutants, ORF1::Ω and ORF2::Ω, were constructed by electroporating the linearized plasmids pSJ9221 and pSJ9226, respectively, into wild-type PAK and selecting for spectinomycin and streptomycin resistance encoded by the Ω fragment (Materials and Methods). Among all of the recombinants, about 10% were sensitive to carbenicillin, indicating the loss of the vector (carries the carbenicillin resistance gene) through a double crossover event. The remaining 90% retained carbenicillin resistance, and these were integrated, in their entirety, into the chromosome. Insertional mutants were further verified by Southern blot analysis of total genomic DNA. Southern blots of *Pst*I or *Bam*HI DNA digests were probed with a radiolabeled 1.0-kb *Eco*RI-*Hin*dIII fragment of pSJ929 which contains both ORF1 and ORF2. A 1.45-kb probe-reactive fragment, identified in a *Pst*I digest of wild-type DNA, was absent in either of the DNA samples of the two ORF insertional mutants. Instead, the probe hybridized to a new band of 3.45-kb which is the result of 2.0-kb Ω-fragment insertion. Similarly, when DNA samples were digested with *Bam*HI, the probe hybridized to an 11-kb fragment in the wild-type DNA digest, while two bands, 6.5 and 4.5 kb, were probe reactive in the DNA digest of the *orf1* mutant and two bands, 7.0 and 4.0 kb, were seen in DNA digest of the *orf2* mutant (data not shown). Changes in the mobility of the hybridizing fragments correspond to the expected differences resulting from the insertion of a 2-kb Ω fragment and introduction of two new *Bam*HI sites (Fig. 3A).

Effect of two ORF mutations on the activities of *rpoN*-regulated genes. Earlier studies from this laboratory have shown that the *rpoN* gene of *P. aeruginosa* is required not only for pilin gene expression but also for the expression of flagella and genes involved in nitrogen assimilation (12, 32, 35). The *rpoN* mutant strain of *P. aeruginosa* is a glutamine auxotroph and unable to utilize arginine, glutamate, proline, or histidine as a sole nitrogen source (35).

The effect of ORF1 and ORF2 mutations on pilin gene expression was assessed with a pilin gene::*lacZ* fusion carried on plasmid pMSZ5. β-Galactosidase activities were measured in ORF1 and ORF2 mutant backgrounds by using *rpoN* and wild-type PAK strains as negative and positive controls, respectively. As expected, the *rpoN* mutation resulted in background levels of detectable β-galactosidase activity (95 Miller units). Mutations in either of the downstream ORFs had no effect on the expression of *lacZ* from the pilin gene promoter, and the levels of β-galactosidase were comparable to that of the wild type (2,224, 2,378 and 2,024 Miller units for the ORF1 mutant, ORF2 mutant, and wild type, respectively). Similarly, the effect of the two ORF mutations on the activity of the flagellin gene promoter was also examined. A *flaA*::*lacZ* fusion construct, pPT269, was introduced into an isogenic series of wild-type PAK and *rpoN*, ORF1, and ORF2 mutants. Results showed that β-galactosidase activities were comparable to those in the wild type in ORF1 and ORF2 mutant backgrounds, while the same fusion was not expressed in a strain lacking the *rpoN* gene product (675, 551, 488, and 75 Miller units for the wild type and ORF1, ORF2, and *rpoN* mutants, respectively). We conclude from these data that ORF1 and ORF2 do not have any

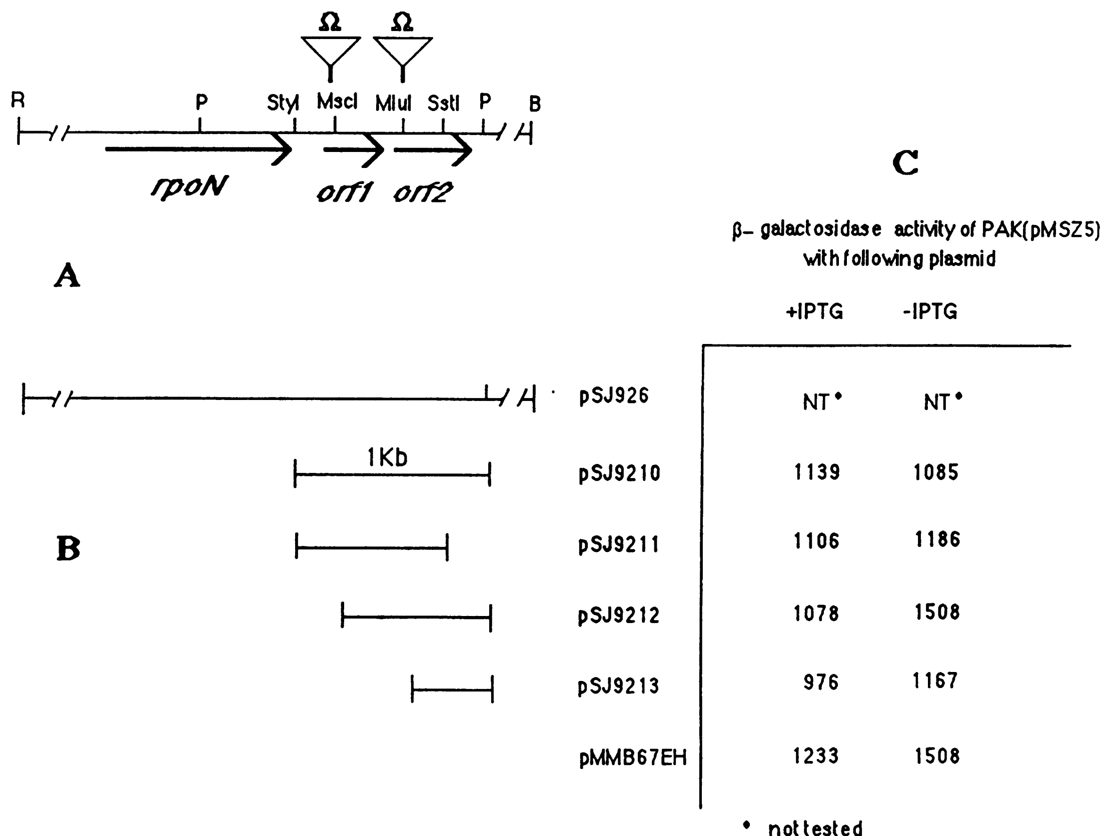

FIG. 3. Effect of elevated expression of ORF1 and/or ORF2 on pilin promoter activity. (A) Map of the *rpoN* region of the *P. aeruginosa* chromosome. Restriction sites, Ω insertion sites, and transcriptional directions of *rpoN*, ORF1, and ORF2 are shown. (B) Plasmid constructs expressing ORF1 and/or ORF2 under control of the *tac* promoter of pMMB67EH. (C) β-galactosidase activities of PAK(pMSZ5) harboring each of the constructs shown in panel B. Log-phase culture cells were grown for an additional 5 h in the presence or absence of 2 mM IPTG. Average numbers from three independent experiments are presented.

observable effect on the expression of the pilin and flagellin genes.

During selection of the two chromosomal Ω insertion mutants (ORF1::Ω and ORF2::Ω), we noticed that they grew slowly on minimal medium A. Compared with wild-type PAK, the two mutants take at least twice as long time to form similarly sized colonies on minimal medium A. When their generation time is measured, the doubling time of wild-type *P. aeruginosa* PAK in liquid minimal medium A is 60 min while those of the two mutants are 120 to 150 min, compared with 180 to 200 min for the isogenic *rpoN* mutant. The abilities of the two mutants to utilize arginine, glutamine, proline, histidine, and glutamate as sole nitrogen sources were also tested. Results indicate that the slow-growth phenotypes cannot be rescued by supplementation of minimal medium A with arginine, histidine, glutamic acid, or proline but the addition of glutamine restored normal growth of the two ORF mutants. This is the same phenotype exhibited by the *rpoN* mutant strain. This was further shown by measuring generation time where the growth rates of all mutants (*rpoN*, ORF1, and ORF2) approached that of the wild type when minimal medium A was supplemented with glutamine. Because this is the identical pattern seen with *rpoN* mutants (35), these results suggested that the two ORFs may affect expression of genes of amino acid assimilation pathways by controlling gene expression in conjunction with RpoN.

ORF2 is required for normal bacterial growth on minimal medium. In order to further examine which of the two ORFs was responsible for the growth defect on minimal medium, plasmids carrying the two ORFs together or individually under the control of the *tac* promoter were constructed (Fig. 3B). These plasmids were introduced into each ORF mutant (ORF1::Ω and ORF2::Ω), and the growth rates of the resulting strains on minimal medium A were examined. As shown in Table 2, plasmids containing ORF2 (pSJ926, pSJ9210, and pSJ9212) are able to complement both ORF1 and ORF2

TABLE 2. Complementation of growth defects of the two ORF mutants

Plasmid	Colony size[a] of:		
	rpoN mutant	ORF1 mutant	ORF2 mutant
pMMB67EH	−	±	±
pSJ926 (*rpoN* ORF1 ORF2)	+	+	+
pSJ9210 (ORF1 ORF2)	−	+	+
pSJ9211 (ORF1)	−	±	±
pSJ9212 (ORF2)	−	+	+
pSJ9213	−	±	±

[a] Bacteria were grown overnight on L-agar plates with antibiotics and restreaked on minimal medium A without antibiotics. Sizes of colonies were compared after growth at 37°C for 16 h. +, large colonies; ±, small colonies; −, no colony. All PAK colonies were large.

mutations while plasmid pSJ9211, which contains ORF1 only, is unable to complement either mutation. The same complementation pattern was observed with plasmid constructs of pSJ9363, pSJ9364, pSJ9365, and pSJ9366. These results indicate that ORF1 and ORF2 are in the same transcriptional unit and the observed low growth rates of the two ORF mutants on minimal medium A are due to the mutational effect on the ORF2. Our visualization of the two ORF gene products by T7 expression system (Fig. 2) further supports the role of the ORF2 protein in vivo. Since the constructs expressing ORF1 and ORF2 are unable to complement the growth defect of the *rpoN* mutant strain on minimal medium A (Table 2), the glutamine auxotroph phenotype of the *rpoN* mutants is not due to a polar effect on ORF2.

Effect of elevated expression of the two ORFs on pilin promoter activities. We further tested the effect of elevated expression of the two ORFs on the expression of RpoN-regulated genes. With the pMMB67EH vector as a control, constructs in which the ORFs were under the control of the *tac* promoter were introduced into a wild-type *P. aeruginosa* strain (PAK) containing pMSZ5 (a pilin gene::*lacZ* fusion construct). The resulting strains were tested for β-galactosidase activities in the presence or absence of 2 mM isopropyl-β-D-thiogalactopyranoside (IPTG). As shown in Fig. 3C, no obvious difference in β-galactosidase activity was observed between strains either with or without the addition of IPTG. Also, the abovementioned strains did not show any changes in generation time when grown in minimal medium A.

DISCUSSION

We have determined the nucleotide sequence of the *P. aeruginosa rpoN* gene and the adjacent region encoding two short polypeptides. Analysis of the deduced amino acid sequences of this region shows similarity in product sequence and genetic organization with the *rpoN* genes of different bacteria. The highest degree of homology is with another member of the genus *Pseudomonas*, *P. putida* (94.2%); however, the similarity with enterobacterial RpoN proteins is still in excess of 70%. On the basis of these levels of sequence conservation, the previously observed functional interchangeability of the *rpoN* genes between *E. coli* and *P. aeruginosa* is not surprising (12).

Two additional ORFs encoding short proteins were identified in the 3′ region of the *P. aeruginosa rpoN* gene, and these are also found at the corresponding locations in other bacteria (17, 38). Complementation analysis of mutations in either ORF suggested that ORF1 and ORF2 are cotranscribed and the slow-growth phenotypes, observed in both mutant strains when grown on minimal medium A, are due to mutational effect on the ORF2. The presence of translated ORF1 and ORF2 gene products in the T7 expression system further supports the role of the ORF2 in vivo. We do not know at this time whether ORF1 and ORF2 are cotranscribed with the *rpoN* gene. But the growth defect of *rpoN* is not due to a polar effect on ORF2, since the *rpoN* mutant strain showed the same defect even when ORF1 and ORF2 are introduced in *trans* (pSJ9210) (Table 2).

In *K. pneumoniae*, the two ORFs in a region 3′ to the *rpoN* gene were shown to modulate RpoN function (17). Mutations in either of these ORFs had no effect on bacterial growth on minimal medium but resulted in an increase in expression of RpoN-regulated *nif* genes, suggesting that these ORFs interfere with the function of σ^{54} for expression of selected genes (17). Similarly, the *flgM* gene product of *S. typhimurium*, a 7.8-kDa protein, functions as a transcriptional inhibitor of flagellin by directly interacting with the flagellin-specific sigma factor FliA (σ^F) and disturbing its ability to form a complex with RNA polymerase core enzyme (7, 24). Recently, Orsini et al. have cloned and partially purified a 10-kDa anti-σ^{70} factor of bacteriophage T4 which has a strong inhibitory activity towards σ^{70}-directed transcription but has no significant effect on σ^{70}-independent transcription (25). In contrast, functional analysis of the two downstream ORFs of *P. aeruginosa* indicated that ORF2 does not function as an anti-sigma factor; instead, it functions as a transcriptional activator of certain RpoN-regulated genes.

Pilin, flagellin, and nitrogen assimilation genes are all regulated by RpoN, but ORF2 affects only genes involved in nitrogen assimilation (positively) and not pilin or flagellin genes. In contrast, ORF1 and ORF2 of *K. pneumoniae* negatively affect the expression of *nif* genes and have no effect on the expression of the nitrogen assimilation genes. Further, the ORF2 mutants of *P. aeruginosa* have growth requirements similar to those of the *rpoN* mutants, suggesting that the ORF2 product exerts its function in conjunction with the RpoN protein, likely in the critical step of open complex formation. It is possible that ORF2 modifies the activity of RpoN protein or affects the level or activity of an as yet unidentified transcriptional regulator of genes for nitrogen assimilation.

The ORF2 product has a high degree of amino acid sequence homology with the Fpr protein of *S. typhimurium* (42% identity and 69% similarity over a 40-amino-acid region) which is involved in the fructose uptake system (6). *S. typhimurium* strains lacking this protein grow more slowly on media in which fructose is the only carbon source. It is possible that the slow-growth phenotype of the ORF2 mutants is due to a defect in carbohydrate uptake, although the slow-growth phenotypes of the two ORF mutants persisted even when bacteria were grown on fructose, glucose, or glycerol (unpublished observation). The precise role of ORF2 in the expression of RpoN-controlled genes, or in activity of RpoN during transcriptional initiation, is currently being investigated.

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