Identification of an *Escherichia coli pepA* Homolog and Its Involvement in Suppression of the *algB* Phenotype in Mucoid *Pseudomonas aeruginosa*

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Strains of *Pseudomonas aeruginosa* isolated from the respiratory tracts of patients with cystic fibrosis often display a mucoid morphology due to high levels of expression of the exopolysaccharide alginate. The response regulator AlgB is required for full transcription of the alginate biosynthetic operon. Repeated attempts to demonstrate a direct interaction between AlgB and the promoter region of *algD*, the first gene in the alginate operon, have thus far been unsuccessful. The possibility that AlgB exerts its effect on *algD* indirectly exists. To identify putative genes under the control of AlgB which affect *algD* transcription, transposon mutagenesis of nonmucoid *algB* derivatives of the mucoid strain FRD1 was employed. Of approximately 3,000 transposon mutants screened, 6 were found to display phenotypes which were mucoid relative to the phenotype of the parental *algB* strain. The phenotypes of these mutants ranged from being only slightly mucoid to being indistinguishable from that of the original FRD1 strain. One of the particularly mucoid transposon mutants was chosen for further study. This strain was found to be disrupted in a previously uncharacterized open reading frame with 56% amino acid identity to PepA of *Escherichia coli*. PepA is classified as a leucine aminopeptidase, and homologs have been detected in a number of bacterial, plant, and animal species. This novel gene has been designated *phpA* (*P. aeruginosa* homolog of *pepA*). The insertional inactivation of *phpA* was found to correlate with the mucoid phenotype and an increase in *algD* transcription in the *algB* strain. Expression of *phpA* from an ectopic chromosomal locus compensated for the transposon insertion in the native *phpA* gene, restoring *algD* transcription to levels similar to those observed in the parental *algB* strain. While *phpA* expression did not appear to be under the control of AlgB at the transcriptional level, this study demonstrates that loss of *phpA* in an *algB* genetic background had a positive effect on alginate expression and, more specifically, on transcription of the alginate biosynthetic operon.

Pseudomonas aeruginosa has been and continues to be a formidable opportunistic pathogen. A ubiquitous gram-negative rod in soil and water environments, *P. aeruginosa* poses little threat to most healthy individuals. In certain clinical scenarios, however, *P. aeruginosa* becomes a pathogen of dire consequence. Among the patients for whom *P. aeruginosa* has life-threatening implications are individuals afflicted with the genetic disease cystic fibrosis (CF) (24).

A unique characteristic of many *P. aeruginosa* isolates from CF patients is a distinctive mucoid colony morphology. This mucoid morphology results from the production of the exopolysaccharide alginate, a linear polymer of L-guluronic and D-mannuronic acids (24). Alginate is an important virulence factor associated with chronic respiratory infections in CF patients (24). These patients are initially colonized with nonmucoid strains of *P. aeruginosa*, but over time mucoid strains emerge and predominate. This conversion occurs in vivo and seems to result from outgrowth of variants often harboring mutations in one of the *muc* genes (34). The mucoid clinical CF isolate FRD1 (*mucA22*) represents such a variant (34) and is the source of the strains used in this study. *mucA* encodes a product antagonistic to the activity of the alternative σ factor σ^{22}, also referred to as AlgT or AlgU (40, 52). The dysregulation of σ^{22} activity leads to increased expression of the reg-

ulatory proteins AlgB, AlgR, and AlgZ (2, 51). Each of these factors has been shown to contribute positively to transcription of *algD*, the first gene in the alginate biosynthetic operon (2, 22, 24, 51).

AlgB is a member of the NtrC family of response regulators (50). As such, AlgB is expected to interact directly with DNA in order to regulate gene expression. However, repeated attempts to demonstrate binding of AlgB in the region extending from −570 to +1000 relative to the *algD* promoter have been unsuccessful. Despite this fact, transcriptional fusion studies indicate that levels of *algD* transcription are at least 20-fold lower in an *algB*::Tn*501* insertion mutant than in the parental strain FRD1 (50, 51).

To reconcile this apparent discrepancy, we hypothesized that AlgB exerts its effect on *algD* transcription indirectly by altering the expression of an intermediate gene (Fig. 1). We reasoned that AlgB either activates or represses transcription of its direct target and that this regulation has a consequent positive effect on *algD* transcription. Since neither *algR* transcription nor AlgZ DNA-binding activity is affected in an *algB* mutant (2, 51), these factors are not likely to be the proposed intermediate of AlgB. To investigate our hypothesis and identify potential AlgB targets relevant to alginate expression, we performed transposon mutagenesis on nonmucoid *algB* mutants derived from the mucoid CF isolate FRD1. This approach has led to the discovery of a previously uncharacterized *P. aeruginosa* gene that affects transcription of *algD* and the consequent expression of alginate.

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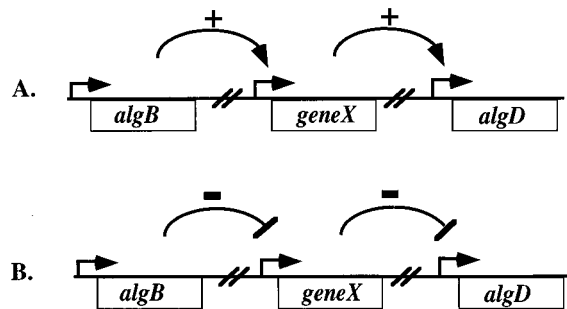
FIG. 1. Two hypotheses for the mechanism of AlgB transcriptional activation of *algD*. (A) AlgB activates transcription of its direct target, gene X. The product of gene X then contributes to transcriptional activation of *algD*. (B) AlgB represses transcription of gene X. If expressed, the product of gene X inhibits transcription of *algD*.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. All *P. aeruginosa* strains except PA01 are ultimately derived from the mucoid clinical CF isolate FRD1. All enzymes used for construction of recombinant DNA were purchased from Promega unless otherwise noted. All plasmids were constructed by standard cloning techniques (32).

Media, antibiotics, and growth conditions. Luria-Bertani (LB) broth (10.0 g of tryptone, 5.0 g of yeast extract, 5.0 g of NaCl per liter [pH 7.5]) and LB agar (Difco) were used for growth of all *Escherichia coli* strains. For growth of *P. aeruginosa*, modified LB broth and agar with no exogenous NaCl (LBNS and LANS, respectively) were used. Antibiotics were used at the following concentrations: tetracycline, 15 μg/ml (*E. coli*) or 100 μg/ml (*P. aeruginosa*); kanamycin, 30 μg/ml; ampicillin, 100 μg/ml; carbenicillin, 300 μg/ml; gentamicin, 15 μg/ml (*E. coli*) or 100 μg/ml (*P. aeruginosa*); and mercuric chloride, 18 μg/ml. Incubation was carried out at 37°C except for sucrose counterselection (see below), which was carried out at 30°C. All chemicals were purchased from Sigma unless otherwise specified.

Bacterial conjugation, allelic exchange, and transposon mutagenesis. Triparental conjugation was carried out as described previously (51). Transconjugants were selected on LANS with appropriate antibiotics. Irgasan DP300 (Ciba Geigy) was included at 25 μg/ml to select against the *E. coli* donor strain. For allelic exchange, pEX100T (41) or pDJW525 (31) was used for construction of allele replacement vectors. These vectors contain an origin of transfer that allows for conjugation, a ColE1 origin of replication that allows episomal propagation in *E. coli* but not in *P. aeruginosa*, the selectable β-lactamase gene, and the counterselectable *sacB* gene, which is lethal in the presence of sucrose. Following conjugation of an allele replacement vector into *P. aeruginosa*, recombinants resulting from a single homologous recombination (merodiploids) were selected for on LANS containing carbenicillin and Irgasan at 37°C. To force a second recombination event resulting in excision of the appropriate allele and vector backbone, merodiploid strains were cultured overnight at 37°C in LBNS without selection and then plated at 30°C on LANS containing 5% (wt/vol) sucrose and, where appropriate, other antibiotics. Sucrose-resistant recombinants were screened for loss of carbenicillin resistance (Cb^r) and either loss or acquisition of the appropriate selectable markers. Transposon mutagenesis was performed by conjugation of pSUP::Tn5-B50 or pSUP::Tn5-B30 into the nonmucoid *algB* strains FRD444 and FRD840 followed by selection on LANS containing tetracycline and Irgasan at 37°C. Tetracycline-resistant (Tc^r) colonies were screened for a mucoid phenotype after 24 to 36 h of incubation.

Cloning of the transposon insertions. Southern blot analysis (43) was used to determine suitable restriction fragments on which to recover the transposon-inactivated loci. Digested genomic DNAs from the transposon mutants were then size fractionated by agarose gel electrophoresis, purified from the gel with the Qiaquick gel purification system (Qiagen), and ligated into either pUC18 (48) or pBluescript KS(−) (Stratagene). Ligations were transformed into *E. coli* JM109, and the transformations were plated on LB containing tetracycline.

DNA sequence analysis, colony hybridization, and physical mapping. DNA sequencing was performed on an ABI Prism 377 DNA sequencer (Perkin-Elmer). To determine the precise site of transposition in each of the cloned restriction fragments described in the paragraph above, each fragment was subcloned (by using unique restriction sites in the transposons) as two smaller fragments, each of which was comprised of one end of the transposon along with the adjacent *P. aeruginosa* chromosomal DNA. Each of these subclones was then sequenced with the oligonucleotide Tn5-OUT (5′CGGGAAAGGTTCCGTTC AGG3′), which hybridizes to either end of the Tn5 derivatives. Analysis and assembly of the final sequence shown in Fig. 2 were performed with Factura and AutoAssembler software (Perkin-Elmer). Homology searches were performed with the Gapped BLAST program (1) on the National Center for Biotechnology

Information website (www.ncbi.nlm.nih.gov). The GAP program of the Wisconsin Package (version 9.1; Genetics Computer Group, Madison, Wis.) was used to determine the percentages of amino acid identity described in Table 2. The PILEUP and PRETTY programs of the Wisconsin Package were used to create the alignment depicted in Fig. 3. The sequences of the *E. coli*, *Haemophilus influenzae*, *Rickettsia prowazekii*, and *Mycobacterium tuberculosis* PepA homologs were obtained from the SwissProt database (accession no. P11648, P45334, P27288, and Q10401, respectively). Additional sequence information was obtained from the Pseudomonas Genome Project (www.pseudomonas.com, 15 June 1998 release). Colony hybridization was performed as described by Grunstein and Hogness (25). Probes for both Southern blot and colony hybridization were synthesized with the Prime-a-Gene system (Promega). Physical mapping experiments were conducted as described elsewhere (39) by pulsed-field gel electrophoresis of *Dpn*I- and *Spe*I-cleaved PA01 chromosomal DNA followed by Southern hybridizations with the 6.8-kb *Bam*HI-*Hin*dIII fragment from pSW109 as a probe.

Construction of the allele replacement vector pSW134. The construction of pSW134, which was used for complementation studies, was accomplished as follows. First, pSW109 was digested with *Xho*I and religated to delete all sequence between the *Xho*I site shown in Fig. 2 and the *Xho*I site in the pKS$^−$ multiple-cloning site. The resulting construct, pSW127, was digested with *Bam*HI, and the *Bam*HI fragment from pGmΩ1 (H. Schweizer) bearing the gene encoding resistance to gentamicin (*aacC1*) was inserted to create pSW128. This plasmid was treated with *Xho*I, blunt ended, and ligated to a *Sma*I fragment from pHP45Ω-Tc (16), which bears the gene that encodes tetracycline resistance (*tet*). The resulting plasmid, pSW133, contained the 2,134-bp *Bam*HI-*Xho*I fragment shown in Fig. 2 flanked at the 5′ end by an Ω*aacC1* cassette and at the 3′ end by Ω*tet*. To create pSW134, pSW133 was digested with *Ase*I (New England Biolabs) and blunt ended and the approximately 2.3-kb fragment containing *phpA* flanked by the transcription termination signals from the Ω elements was gel purified and ligated into *Bgl*II-digested Klenow fragment-treated pUS68. pUS68 is a pEX100T-based vector providing the sequence required for homologous recombination at *algB* (31). The original transcriptional terminators from the Ω elements are oriented so as to terminate transcription into *phpA* from either direction. Therefore, *phpA* should be expressed only from a promoter 3′ of the *Bam*HI site shown (Fig. 2).

***algD-xylE* fusions.** The construction of pDJW530, which was used to generate chromosomal *algD-xylE* fusions, was performed as follows. An approximately 10-kb *Hin*dIII fragment containing *algD* plus flanking sequences was cloned into the gene replacement vector pDJW525 (31), resulting in pDJW482. A 2.2-kb *Sma*I fragment of pX1918G which contained a promoterless *xylE* gene and the *aacC1* gene, which encodes resistance to gentamicin, was subcloned into *Xho*I-digested, Klenow fragment-treated pDJW482, thus placing *xylE* transcription under the control of the *algD* promoter. The resulting plasmid, pDJW530, was mobilized into *P. aeruginosa* strains, and gene replacements were performed as described above. For *algD-xylE* transcriptional fusion experiments, overnight shaken LBNS cultures were diluted 1:1,000 in fresh prewarmed LBNS and incubated in a 37°C shaker (250 rpm). Culture growth was monitored with 1-cm-path-length cuvettes in a Spectronic Genesys 5 spectrophotometer (Spectronic Instruments). Cultures were harvested at a final optical density at 540 nm (OD$_{540}$) between 0.3 and 0.7. A 1.0-ml sample was pelleted at 20,800 × g for 5 min. Pellets were resuspended in 1 ml of assay buffer (50 mM potassium phosphate buffer [pH 7.5], 10% acetone), and samples were kept on ice until they were assayed. A 100-μl aliquot of each sample was mixed with 900 μl of assay buffer containing 1 mM catechol (Sigma), and the increase in OD$_{375}$ was monitored at ambient temperature for 2 min against a 1-ml blank of the assay buffer-catechol solution. The rate of formation of the reaction product, 2-hydroxymuconic semialdehyde, was calculated from the rate of increase in OD$_{375}$ and the molar extinction coefficient of the product, 4.4×10^4 M^{-1}. Values were normalized by dividing them by the OD$_{540}$ of the culture at the time of harvest.

Nucleotide sequence accession number. The nucleotide sequence shown in Fig. 2 has been deposited in the GenBank database under accession no. AF054622.

RESULTS

Isolation of mucoid transposon mutants of nonmucoid *algB* strains. We have hypothesized that AlgB exerts transcriptional control over *algD* indirectly (Fig. 1). This hypothesis can be reduced to two basic scenarios. The first possibility is that AlgB activates expression of an intermediate gene (gene X) (Fig. 1A), resulting in an overall increase in *algD* transcription. The second possibility is that AlgB increases *algD* transcription by repressing expression of the intermediate gene (Fig. 1B). In order to investigate either of these possibilities and identify potential AlgB targets, we performed transposon mutagenesis on the nonmucoid *algB* mutants FRD444 and FRD840, derivatives of the mucoid CF isolate FRD1. For this procedure, we

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristic(s)[a]	Reference or source
Strains		
Escherichia coli		
HB101	*proAB leuB6 thi-1 lacY1 hsdR hsdM recA13 supE44 rpsL20*	7
JM109	*endA1 recA1 gyrA96 thi hsdR17* (r_K^- m_K^+) *relA1 supE44* Δ(*lac-proAB* F′ *traD36 proAB lacI*^{q}ZΔM15	53
Pseudomonas aeruginosa		
PA01	Prototrophic wild-type strain	28
FRD1	CF isolate, Alg$^+$ (due to *mucA22* mutation)	37
FRD440	*mucA22 algT*::Tn*501*	19
FRD444	*mucA22 algB*::Tn*501*	23
FRD831	*mucA22* Δ*algR*::Ω*aacC1*	31
FRD840	*mucA22* Δ*algB*::Ω*aacC1*	31
FRD444.1	*mucA22 algB*::Tn*501 phpA*::Tn*5*-B50 (Tc^r)	This study
FRD444.2	Mucoid Tn*5*-B50 mutant of FRD444	This study
FRD444.3	Mucoid Tn*5*-B50 mutant of FRD444	This study
FRD875	*mucA22 algD*::*xylE aacC1*	This study
FRD879	*mucA22 algD*::*xylE aacC1 algB*::Tn*501*	This study
FRD906	*mucA22 algB*::Tn*501 phpA*::Tn*5*-B50 (Tc^s)	This study
FRD909	*mucA22 algB*::Tn*501 phpA*::*xylE aacC1*, tandem transcription of *phpA* and *xylE aacC1*	This study
FRD910	*mucA22 algB*::Tn*501 phpA*::*xylE* Ω*aacC1*Ω, tandem transcription of *phpA* and *xylE aacC1*	This study
FRD911	*mucA22 algB*::Tn*501 phpA*::*xylE aacC1*, opposing transcription of *phpA* and *xylE aacC1*	This study
FRD912	*mucA22 algB*::Tn*501 phpA*::*xylE* Ω*aacC1*Ω, opposing transcription of *phpA* and *xylE aacC1*	This study
FRD913	*mucA22 algB*$^+$ *phpA*::*xylE aacC1*, tandem transcription of *phpA* and *xylE aacC1*	This study
FRD918	Mucoid Tn*5*-B50 mutant of FRD840	This study
FRD920	*mucA22 algD*::*xylE aacC1 algB*::Tn*501 phpA*::Tn*5*-B50	This study
FRD925	Mucoid Tn*5*-B50 mutant of FRD840	This study
FRD927	*mucA22 algD*::*xylE aacC1 algB*:*phpA phpA*::Tn*5*-B50	This study
FRD940	*mucA22 algB*::Tn*501 mucD*::Tn*5*-B30 (Tc^r)	This study
Plasmids		
pDJW530	pDJW482 + 2.2-kb *Sma*I fragment of pX1918G *algD*::*xylE aacC1* (Materials and Methods)	This study
pEX100T	ColE1 Ap^r *oriT sacB*	41
pLAFR3	IncP1 Tc^r λcos *oriT*	44
pMMB67HE	IncQ Ap^r *oriT*	20
pRK2013	ColE1-Tra(RK2)$^+$ Km^r	17
pSUP::Tn*5*-B50	P15A Cm^r Tc^r RP4 *mob*	42
pSUP::Tn*5*-B30	P15A Cm^r Tc^r RP4 *mob*	42
pSW40	pEX100T + 7.2-kb *Bam*HI-*Sph*I fragment of pSW50, *phpA*::Tn*5*-B50 (Tc^r)	This study
pSW40.1	pSW40 cut with *Hin*dIII and religated to delete a 3.5-kb fragment from the middle of Tn*5*-B50 (Tc^s)	This study
pSW50	7.3-kb *Bam*HI-*Xho*I fragment in pUC18 *phpA*::Tn*5*-B50 (Tc^r)	This study
pSW90	pEX100T + 1.95-kb *Bam*HI-*Sph*I fragment of pSW109, wild-type *phpA*	This study
pSW109	pKS$^-$ + 6.8-kb *Bam*HI-*Hin*dIII fragment of wild-type *phpA*	This study
pSW110	pLAFR3 + 6.8-kb *Bam*HI-*Hin*dIII fragment of pSW109, wild-type *phpA*	This study
pSW134	pUS68 + 2.3-kb *Ase*I fragment from pSW133, *algB*::*phpA* (Materials and Methods)	This study
pX1918G	ColE1 Ap^r Gm^r	41
pX1918GT	ColE1 Ap^r ΩGm^r	41

[a] Ap^r, Km^r, Cm^r, and Gm^r, ampicillin, kanamycin, chloramphenicol, and gentamicin resistance, respectively.

used a set of Tn*5* derivatives (42). One of these, Tn*5*-B50, contains an outwardly directed constitutive promoter of the *nptII* gene and has been found to be active in *Pseudomonas* (42). Therefore, this transposon should be able to activate transcription of genes normally requiring AlgB if it inserts upstream of this gene in the proper orientation (Fig. 1A). Alternatively, Tn*5*-B50 may insertionally inactivate AlgB-repressed genes (Fig. 1B). In either case, the requirement for AlgB may be bypassed and the original mucoid phenotype may be restored. A second Tn*5* derivative used in this study was Tn*5*-B30, which contains a promoterless *nptII* gene. While Tn*5*-B30 is not likely to activate gene expression, it might still inactivate putative targets of AlgB. In addition, the promoter-

less *nptII* gene, conferring resistance to kanamycin when expressed, may be used to determine if the inactivated locus is regulated by AlgB, provided that the transposon has inserted in the correct orientation.

Transposon mutagenesis was performed on the nonmucoid *algB* strains FRD444 and FRD840 as described in Materials and Methods, and the resulting Tc^r colonies were screened for the mucoid phenotype at 37°C. Of approximately 3,000 Tn*5*-B50 mutants screened, five isolates which displayed a mucoid phenotype relative to that of the parental strains were obtained. Interestingly, this phenotypic difference was apparent only in areas of heavy growth on LANS plates (i.e., in the first and second quadrants of streak plates, where growth was con-
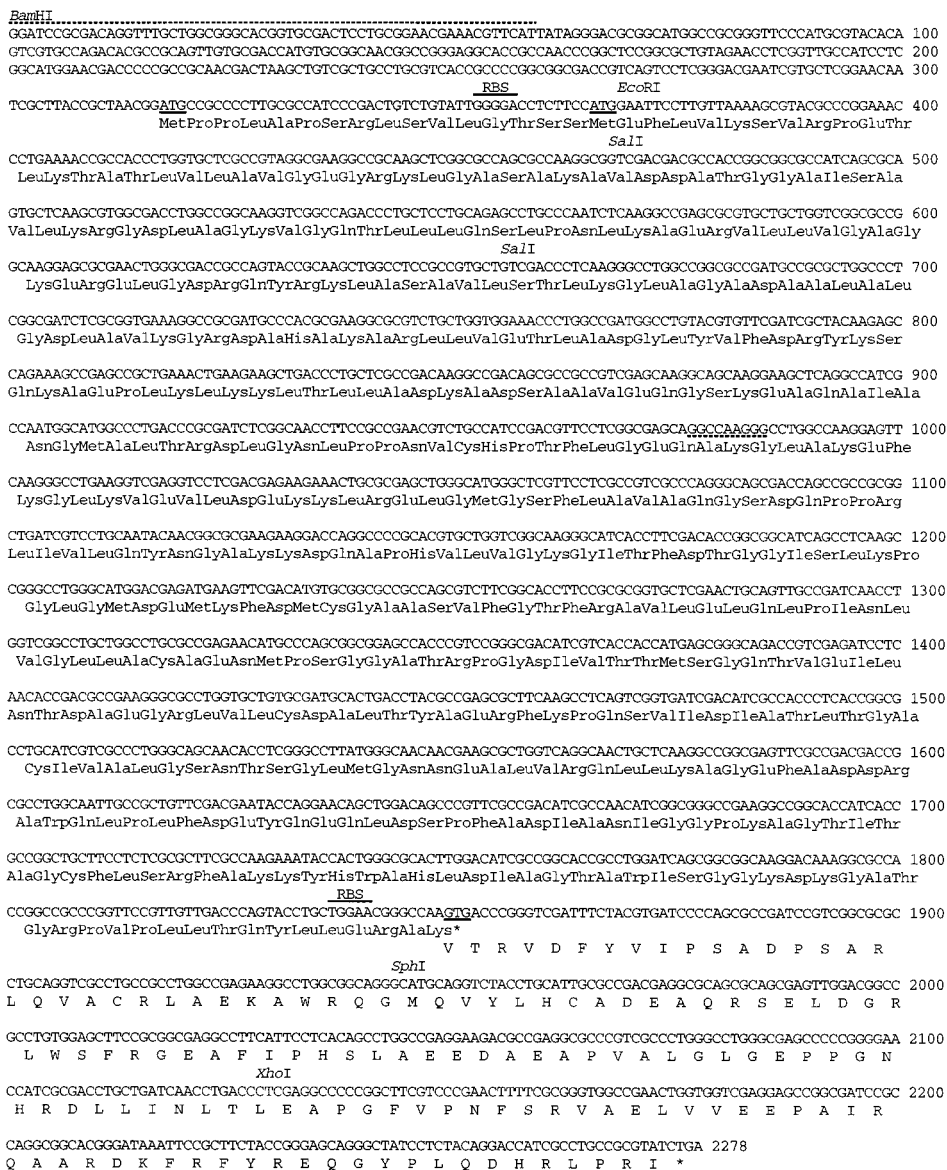
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BamHI
GGATCCGCGACAGGTTTGCTGGCGGGCACGGTGCGACTCCTGCGGAACGAAACGTTCATTATAGGGACGCGGCATGGCCGCGGGTTCCCATGCGTACACA  100
GTCGTGCCAGACACGCCGCAGTTGTGCGGACCATGTGCGGCAACGGCCGGGAGGCACCGCCAACCCGGCTCCGGCGCTGTAGAACCTCGGTTGCCATCCTC  200
GGCATGGAACGACCCCCGCGCAACGACTAAGCTGTCGCTGCCTGCGTCACCGCCCCGGCGGCGACCGTCAGTCCTCGGGACGAATCGTGCTCGGAACAA  300
                                                                          RBS            EcoRI
TCGCTTACCGCTAACGGATGCCGCCCCTTGCGCCATCCCGACTGTCTGTATTGGGGACCTCTTCCATGGAATTCCTTGTTAAAAGCGTACGCCCGGAAAC  400
                MetProProLeuAlaProSerArgLeuSerValLeuGlyThrSerSerMetGluPheLeuValLysSerValArgProGluThr
                                                                                              SalI
CCTGAAAACCGCCACCCTGGTGCTCGCCGTAGGCGAAGGCCGCAAGCTCGGCGCCAGCGCCAAGGCGGTCGACGACGCCACCGGCGGCGCCATCAGCGCA  500
LeuLysThrAlaThrLeuValLeuAlaAlaValGlyGluGlyArgLysLeuGlyAlaSerAlaLysAlaValAspAspAlaThrGlyGlyAlaIleSerAla
GTGCTCAAGCGTGGCGACCTGGCCGGCAAGGTCGGCCAGACCCTGCTCCTGCAGAGCCTGCCCAATCTCAAGGCCGAGCGCGTGCTGCTGGTCGGCGCCG  600
ValLeuLysArgGlyAspLeuAlaGlyLysValGlyGlnThrLeuLeuLeuGlnSerLeuProAsnLeuLysAlaGluArgValLeuLeuValGlyAlaGly
                                                                       SalI
GCAAGGAGCGCGAACTGGGCGACCGCCAGTACCGCCAAGCTGGCCTCCGCCGTGCTGTCGACCCTCAAGGGCCTGGCCGGCGCCGATGCCGCGCTGGCCCT  700
LysGluArgGluLeuGlyAspArgGlnTyrArgLysLeuAlaSerAlaValLeuSerThrLeuLysGlyLeuAlaGlyAlaAspAlaAlaLeuAlaLeu
CGGCGATCTCGCGGTGAAAGGCCGCGATGCCCACGCGAAGGCGCGTCTGCTGGTGGAAACCCTGGCCGATGGCCTGTACGTGTTCGATCGCTACAAGAGC  800
GlyAspLeuAlaValLysGlyArgAspAlaHisAlaLysAlaArgLeuLeuValGluThrLeuAlaAspGlyLeuTyrValPheAspArgTyrLysSer
CAGAAAGCCGAGCCGCTGAAACTGAAGAAGCTGACCCTGCTCGCCGACAAGGCCGACAGCGCCGCCGTCGAGCAAGGCAGCAAGGAAGCTCAGGCCATCG  900
GlnLysAlaGluProLeuLysLeuLysLysLeuThrLeuLeuAlaAspLysAlaAspSerAlaAlaValGluGlnGlySerLysGluAlaGlnAlaIleAla
CCAATGGCATGGCCCTGACCCGCGATCTCGGCAACCTTCCGCCGAACGTCTGCCATCCGACGTTCCTCGGCGAGCAGGCCAAGGGCCTGGCCAAGGAGTT  1000
AsnGlyMetAlaLeuThrArgAspLeuGlyAsnLeuProProAsnValCysHisProThrPheLeuGlyGluGlnAlaLysGlyLeuAlaLysGluPhe
CAAGGGCCTGAAGGTCGAGGTCCTCGACGAGAAGAAACTGCGCGAGCTGGGCATGGGCTCGTTCCTCGCCGTCGCCCAGGGCAGCGCGACCAGCCGCCGCGG  1100
LysGlyLeuLysValGluValLeuAspGluLysLysLeuArgGluLeuGlyMetGlySerPheLeuAlaValAlaGlnGlySerAspGlnProProArg
CTGATCGTCCTGCAATACAACGGCGCGAAGAAGGACCAGGCCCCGCACGTGCTGGTCGGCAAGGGCATCACCTTCGACACCGGCGGCATCAGCCTCAAGC  1200
LeuIleValLeuGlnTyrAsnGlyAlaLysLysAspGlnAlaProHisValLeuValGlyLysGlyIleThrPheAspThrGlyGlyIleSerLeuLysPro
CGGGCCTGGGCATGGACGAGATGAAGTTCGACATGTGCGGCGCCGCCAGCGTCTTCGGCACCTTCCGCGCGGTGCTCGAACTGCAGTTGCCGATCAACCT  1300
GlyLeuGlyMetAspGluMetLysPheAspMetCysGlyAlaAlaSerValPheGlyThrPheArgAlaValLeuGluLeuGlnLeuProIleAsnLeu
GGTCGGCCTGCTGGCCTGCGCGCGAGAACATGCCCAGCGGCGGAGCCACCCGTCCGGGCGACATCGTCACCACCATGAGCGGGCAGACCGTCGAGATCCTC  1400
ValGlyLeuLeuAlaCysAlaGluAsnMetProSerGlyGlyAlaThrArgProGlyAspIleValThrThrMetSerGlyGlnThrValGluIleLeu
AACACCGACGCCGAAGGGCGCCTGGTGCTGTGCGATGCACTGACCTACGCCGAGCGCTTCAAGCCTCAGTCGGTGATCGACATCGCCACCCTCACCGGCA  1500
AsnThrAspAlaGluGlyArgLeuValLeuCysAspAlaLeuThrTyrAlaGluArgPheLysProGlnSerValIleAspIleAlaThrLeuThrGlyAla
CCTGCATCGTCGCCCTGGGCAGCAACACCTCGGGCCTTATGGGCAACAACGAAGCGCTGGTCAGGCAACTGCTCAAGGCCGGCGAGTTCGCCGACGACCG  1600
CysIleValAlaLeuGlySerAsnThrSerGlyLeuMetGlyAsnAsnGluAlaLeuValArgGlnLeuLeuLysAlaGlyGluPheAlaAspAspArg
CGCCTGGCAATTGCCGCTGTTCGACGAATACCAGGAACAGCTGGACAGCCCGTTCGCCGACATCGCCAACATCGGCGGGCCGAAGGCCGGCACCATCACC  1700
AlaTrpGlnLeuProLeuPheAspGluTyrGlnGluGlnLeuAspSerProPheAlaAspIleAlaAsnIleGlyGlyProLysAlaGlyThrIleThr
GCCGGCTGCTTCCTCTCGCGCGTTCGCCAAGAAATACCACTGGGCGCACTTGGACATCGCCGGCACCGCCTGGATCAGCGGCGGCAAGGACAAAGGCGCCA  1800
AlaGlyCysPheLeuSerArgPheAlaLysLysTyrHisTrpAlaHisLeuAspIleAlaGlyThrAlaTrpIleSerGlyGlyLysAspLysGlyAlaThr
                                                                         RBS
CCGGCCGCCCGGTTCCGTTGTTGACCCAGTACCTGCTGGAACGGGCCAAGTGACCCGGGTCGATTTCTACGTGATCCCCAGCGCCGATCCGTCGGCGCGC  1900
GlyArgProValProLeuLeuThrGlnTyrLeuLeuGluArgAlaLys*    V  T  R  V  D  F  Y  V  I  P  S  A  D  P  S  A  R
                                  SphI
CTGCAGGTCGCCTGCCGCCTGGCCGAGAAGGCCTGGCGGCAGGGCATGCAGGTCTACCTGCATTGCGCCGACGAGGCGCAGCGCAGCGAGTTGGACGGCC  2000
L  Q  V  A  C  R  L  A  E  K  A  W  R  Q  G  M  Q  V  Y  L  H  C  A  D  E  A  Q  R  S  E  L  D  G  R
GCCTGTGGAGCTTCCGCGGCGAGGCCTTCATTCCTCACAGCCTGGCCGAGGAAGACGCCGAGGCGCCCGTCGCCCTGGGCCTGGGCGAGCCCCCGGGGAA  2100
 L  W  S  F  R  G  E  A  F  I  P  H  S  L  A  E  E  D  A  E  A  P  V  A  L  G  L  G  E  P  P  G  N
                                                  XhoI
CCATCGCGACCTGCTGATCAACCTGACCCTCGAGGCCCCCGGCTTCGTCCCGAACTTTTCGCGGGTGGCCGAACTGGTGGTCGAGGAGCCGGCGATCCGC  2200
 H  R  D  L  L  I  N  L  T  L  E  A  P  G  F  V  P  N  F  S  R  V  A  E  L  V  V  E  E  P  A  I  R
CAGGCGGCACGGGATAAAATTCCGCTTCTACCGGGAGCAGGGCTATCCTCTACAGGACCATCGCCTGCCGCGTATCTGA  2278
 Q  A  A  R  D  K  F  R  F  Y  R  E  Q  G  Y  P  L  Q  D  H  R  L  P  R  I  *
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FIG. 2. Nucleotide and derived amino acid sequences for the *P. aeruginosa* homologs of *pepA* (*phpA*) and *holC*. The peptide sequence of PhpA is indicated with three-letter amino acid abbreviations, while the sequence of HolC is displayed with one-letter abbreviations. Potential start codons are underscored with solid lines. Stop codons are indicated by asterisks. Potential ribosome binding sites (RBS) are shown. The site of insertion of Tn5-B50 in *phpA* is shown by a dashed line, indicating the direct repeat generated by the transposition. ORF280 (see the text) is indicated by a dashed overline and extends 5′ of the sequence shown.

fluent). In addition, the phenotypes of these mutants ranged from only slightly mucoid to a degree of mucoid morphology indistinguishable from that of the original mucoid strain, FRD1. When grown on normal LB agar (containing 5 g of NaCl per liter), none of these mutants displayed a mucoid phenotype whereas FRD1 was still mucoid. A number of studies have suggested that alginate expression is enhanced by high osmolarity (3, 47), which seems to be in conflict with our observations. Whether the mucoid appearance of the transposon mutants on LANS versus LB agar results from differential gene expression is presently unknown. Of the approximately 250 Tn5-B30 mutants isolated from the only experiment using this transposon, one displayed a particularly mucoid phenotype on LANS and was even slightly mucoid on LB agar.

Data obtained from Southern blot analysis (data not shown)

were used to clone the transposon-disrupted loci from the mutants. The cloned transposon insertions were subjected to sequence analysis, and similarity searches were performed as described in Materials and Methods. Table 2 summarizes the nature of the mutants isolated in this study in terms of that of the parental strain, the transposon used, the phenotype, and the similarity profiles of the inactivated loci to known sequences. While quantitative determination of the growth rates and levels of alginate have not been performed for these mutants, some qualitative observations bear mention. FRD444.1 and FRD940 appeared the most mucoid and produced colonies similar in size to the parental *algB* mutants. FRD444.2 was moderately mucoid but produced much smaller colonies. FRD444.3, FRD918, and FRD925 were the least mucoid and also produced smaller colonies. The mucoid phenotypes of

TABLE 2. Mucoid transposon mutants isolated in this study

Mutant	Parental strain	Transposon	Phenotype[a]	Tn-disrupted ORF similar to:	% Amino acid identity[b]
FRD444.1	FRD444	Tn5-B50	Mucoid	*E. coli* PepA (P11648)[c]	56
FRD444.2	FRD444	Tn5-B50	Mucoid	*E. coli* TrmD (P07020)[c]	65
FRD444.3	FRD444	Tn5-B50	Slightly mucoid	*E. coli* Hnr (P37055)[c]	25
FRD918	FRD840	Tn5-B50	Slightly mucoid	*E. coli* Orn (P39287)[c]	67
FRD925	FRD840	Tn5-B50	Slightly mucoid	ND[d]	
FRD940	FRD444	Tn5-B30	Mucoid	*P. aeruginosa* MucD (U32853)[e]	100

[a] Phenotype on LANS at 37°C compared to that of the parental strain.
[b] Determined as described in Materials and Methods.
[c] SwissProt database accession number.
[d] ND, not determined.
[e] GenBank database accession number.

these last three strains were particularly unstable, often reverting to the nonmucoid phenotype after a single passage. Although this article focuses on only one of these mutants, FRD444.1, a brief mention of the sequence information obtained for the other mutants is germane to our discussion.

FRD444.2 was found to be disrupted in an open reading frame (ORF) with 65% amino acid identity to TrmD of *E. coli*. *trmD* encodes the tRNA (m1G37) methyltransferase of *E. coli* and *Salmonella typhimurium*. This enzyme is required for methylation of guanosine at position 37 in all tRNAs that recognize codon CCN, CGN, or CUN (4). This base modification has been implicated in prevention of frameshifting during translation (4). *trmD* of *E. coli* is part of a four-gene operon containing *rpsP*, *rimM*, *trmD*, and *rplS* (10). This same arrangement is found in the *P. aeruginosa* PA01 chromosome as reported in the 15 June 1998 release of the *P. aeruginosa* Genome Sequencing Project.

The transposon in FRD444.3 was discovered to lie in an uncharacterized ORF with similarity to several members of the response regulator class of proteins. The highest degree of amino acid identity observed (25%) was to the predicted product of *hnr* of *E. coli* (5). Like most response regulators, the *E. coli* Hnr protein and the ORF inactivated in FRD444.3 contained an amino-terminal domain with conserved aspartates and lysine residues in the predicted phosphorylation acid pocket active site (46). The carboxy-terminal output domain of this ORF was not conserved with any other proteins in the database.

FRD918 contained a Tn5-B50 insertion in an ORF with 67% amino acid identity to *orn* of *E. coli*. *orn*, previously known as ORF *o204a* until it was renamed by Zhang et al. (54), encodes an oligoribonuclease which is highly conserved and highly specific for small oligoribonucleotides. Zhang et al. reported that they have been unsuccessful in preliminary attempts to inactivate *orn*, suggesting that this gene may be essential in *E. coli* (54). Our data suggest that, at least for *P. aeruginosa*, this gene is not absolutely essential. The insertion occurred approximately one-fifth of the way into the ORF. Although FRD918 and FRD925 were isolated independently in separate experiments, Southern blot analysis with three single-enzyme digests as well as three double-enzyme digests revealed the restriction pattern of FRD925 to be identical to that of FRD918. The alginate phenotypes of these strains were also similar, suggesting that *orn* expression is disrupted in both of these strains.

FRD940, the single mucoid Tn5-B30 mutant obtained in this study, was discovered to harbor the transposon in the intergenic region between *mucC* and *mucD* in the *algT*(*algU*) *mucABCD* cluster. The transposition occurred 12 bp upstream of the initiation codon for *mucD* and thus likely prevents the expression of *mucD*. Inactivation of *mucD* has been shown to cause conversion to the mucoid phenotype (6). MucD has significant amino acid identity (39%) to *E. coli* HtrA (DegP), a periplasmic serine protease. The orientation of Tn5-B30 was such that transcription of the promoterless *nptII* gene occurred in the same direction as the *algT*(*algU*)*mucABCD* cluster. FRD940 demonstrated growth on LB agar containing up to 700 μg of kanamycin per ml, while neither FRD1 nor FRD444 exhibited growth even on 300 μg/ml, the lowest concentration tested (data not shown). This indicated that a promoter upstream of the coding sequence of *mucD* drove expression of *nptII*. By allelic exchange, a wild-type copy of *algB* was provided to FRD940. Preliminary experiments revealed no discernible differences between the MICs of kanamycin for the *algB*⁺ and *algB* mutant *mucD*::*nptII* strains (data not shown). Since transcription of *mucD* did not appear to be AlgB dependent, we focused the remainder of this study on FRD444.1.

FRD444.1 harbors an insertion in a previously uncharacterized ORF with 56% amino acid identity to PepA of *E. coli*. PepA is classified as a leucine aminopeptidase (45, 49), and similarity searches revealed *pepA* to be a highly conserved gene in a variety of bacteria (Fig. 3) as well as plants and animals. PepA (also known as XerB) is also an accessory factor in the Xer-mediated site-specific recombination system, which acts to monomerize multimers of multicopy plasmids formed by homologous recombination (45). In addition, PepA is involved in transcriptional repression of the *carAB* operon, which encodes the subunits for carbamoylphosphate synthetase, as well as in repression of its own gene, *pepA* (13). Recently, Hauser et al. reported the identification of a secreted protein from *P. aeruginosa* which they designated PepA, for *Pseudomonas* exoprotein A (26). The sequence of the gene encoding this protein, *pepA*, and the predicted amino acid sequence showed no homology with known sequences and should not be confused with true homologs of the PepA aminopeptidase of *E. coli*. To prevent any confusion between *pepA* described by Hauser et al. and the gene described here, we have chosen the designation *phpA* (for *P. aeruginosa* homolog of *pepA*).

Nucleotide and predicted amino acid sequences of *phpA*. In order to clone the wild-type *phpA* gene, genomic DNA from FRD1 was analyzed by a Southern blot analysis with *Xho*I-treated pSW50 as a probe template. The probe hybridized to an approximately 6.8-kb *Bam*HI-*Hin*dIII fragment (data not shown). Size-fractionated *Bam*HI- and *Hin*dIII-treated FRD1 genomic DNA was ligated into *Bam*HI- and *Hin*dIII-treated pKS⁻, and the ligated plasmid was transformed into *E. coli* JM109. From this library, a clone designated pSW109 which contained an approximately-6.8-kb *Bam*HI-*Hin*dIII fragment was identified by colony hybridization. The sequence of the first 2,278 bp of the insert in pSW109 was determined (Fig. 2).

```
        1                                                                                                   100
E. col  ~~~~~~~~~~~~~~~~~MEFSVKSGSPEKQRSACIVVGVFEPRRLSPIAEQLDKISDGYISALLRRG.ELEGKPGQ.TLLLHHVPNVLSERILLIGCGKER
H. inf  ~~~~~~~~~~~~~~~~~MKYQAKN.TALSQATDCIVLGVYENNKFSKSFNEIDQLTQGYLNDLVKSG.ELTGKLAQ.TVLLRDLQGLSAKRLLIVGCGKKG
P. aer  MPPLAPSRLSVLGTSSMEFLVKSVRPETLKTATLVLAVGEGRKLGASAKAVDDATGGAISAVLKRG.DLAGKVGQ.TLLLQSLPLKAERVLLVGAGKER
R. pro  ~~~~~~~~~~~~~~~~~MLNINFVNEESSTNQGLIVFIDEQLKLNNNLIALDQQHYELISKTIQNKLQFSGNYGQITVVPSVIKSCAVKYLIIVGLGNVE
M. tub  ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Con     ----------------M----K----E-------V--V-E--KL-------D-----G-IS-----G--L-GK-GQ-T-LL---------R-L-VG-GK--

        101                                                                                                 200
E. col  ELDERQYKQVIQKTINTLNDTGSMEAVCFLTELHVKGRNNYWKVRQAVETAKETLYSFDQLKTNKSEPRRPLRKMVFNVPTRRELTSGERAIQHGLAIAA
H. inf  ELTERQYKQIIQAVLKTLKETNTREVISYLTEIELKDRDLYWNIRFAIETIEHTNYQFDHFKSQKAE.TSVLESFIFNT....DCAQAQQAISHANAISS
P. aer  ELGDRQYRKLASAVLSTLKGLAGADAALALGDLAVKGRDAHAKARLLVETLADGLYVFDRYKSQKAEPLK.LKKLTL.LADKADSAAVEQGSKEAQAIAN
R. pro  KLTEAKIEELGGKILQHATCAKIATIGLKIINRINRFTSPTFTSLIASGAFLASYRFHKYKTTLKEVEKFAVESIEILTDNNSEAMKLFEVKK...LIAE
M. tub  ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~MQRTPRSARCTVR...PSLT
Con     EL-ERQY-------L-TL-----------L------K-R------R-A-ET-----Y-FD--K--K-E----L----------------------AIA-

        201                                                                                                 300
E. col  GIKAAKDLGNMPPNICNAAYLASQARQLADSYSKNVITRVIGEQQMKELGMHSYLAVGQGSQONESLMSVIEYKGNA....SEDARPIVLVGKGLTFDSGG
H. inf  GIKAARDIANMPPNICNPAYLAEQAKNLAEN.STALSLKVVDEEEMAKLGMNAYLAVSKGSENRAYMSVLTFNNAP....DKNAKPIVLVGKGLTFDAGG
P. aer  GMALTRDLGNLPPNVCHPTFLGEQAKGLAKEF.KGLKVEVLDEKKLRELGMGSFLAVAQGSDQPPRLIVLQYNG.A....KKDQAPHVLVGKGITFDTGG
R. pro  AVFFTRDISNEPSNIKTPQVYAERIVEILEPL..GVNIDVIGEHDIKNLGMGALLGVGQGSQONESKLVVMEYKG.....GSRDDSTIALVGKGVIFDTGG
M. tub  AVATARDLVNTPPSHLFPAEFAKRAKTLSESV..GLDVEIDEKALKKAGYGGVIGVGQGSSRPPRLVRLIHRGSRLAKNPQKAKKVALVGKGITFDTGG
Con     G---ARDL-N-PPNIC-PA-LAEQAK-LAE----GL---VIDE----K-LGMG--LAVGQGS-N---L-VL-Y-G--------DA-P-VLVGKG-TFDTGG

        301                                                                                                 400
E. col  ISIKPSEGMDEMKYDMCGAAAVYGVMRMVAELQLPINVIGVLAGCENMPGGRAYRPGDVLTTMSGQTVEVLNTDAEGRLVLCDVLTYV.ERFEPEAVIDV
H. inf  ISLKPAADMDEMKYDMCGAASVFGTMKTIAQLNLPLNVIGVLAGCENLPDGNAYRPGDILTTMNGLITVEVLNTDAEGRLVLCDTLTYV.ERFEPELVIDV
P. aer  ISLKPGLGMDEMKFDMCGAASVFGTFRAVLELQLPINLVGLLACAENMPSGGATRPGDIVTTMSGQTVEILNTDAEGRLVLCDALTYA.ERFKPQSVIDI
R. pro  ISLKPSSNMHLMRYDMAGSAAVVGTIIALASQKVPVNVVGVGLVENMQSGNAQRPGDVVVTMSGQTAEVLNTDAEGRLVLADTVWYVQEKFNPKCVIDV
M. tub  ISIKPAASMHHMTSDMGGAAAVIATVTLAARLRLPIDVIATVPMAENMPSATAQRPGDVLTQYGGTTVEVLNTDAEGRLILADAIVRACED.KPDYLIET
Con     ISLKP---MDEMKYDMCGAAAV-GT----A-L-LPINVIGVLA--ENMPSG-A-RPGDVLTTMSGQTVEVLNTDAEGRLVLCD-LTYV-ERF-P--VIDV

        401                                                                                                 500
E. col  ATLTGACVIALGHHITGLMANHNPLAHELIAASEQSGDRAWRLPLGDEYQEQLESNFADMANIGGRP..GGAITAGCFLSRFTRK.YNWAHLDIAGTAWR
H. inf  ATLTGACVVALGQHNSGLVSTDNNLANALLQAATETTDKAWRLPLSEEYQEQLKSPFADLANIGGRW..GGAITAGAFLSNFTKK.YRWAHLDIAGTAWL
P. aer  ATLTGACIVALGSNTSGLMGNNEALVRQLLKAGEFADDRAWQLPLFDEYEQLDSPFADIANIGGPK..AGTITAGCFLSRFAKK.YHWAHLDIAGTAWI
R. pro  ATLTGAITVALGSTYAGCFSNNDELADKLIKAGEAVNEKLWRMPLHDDYDAMINSDIADIANTGNVPGAAGSCTAAHFIKRFIKDGVDWAHLDIAGVA.N
M. tub  STLTGAQTVALGTRIPGVM.GSDEFRDRVAAISQRVGENGWPMPLPDDLKDDLKSTVADLANVSG.QRFAGMLVAGVFLREFVAESVDWAHIDVAGPAYN
Con     ATLTGAC-VALG----GLM-N---LA--L--A-E---D-AWRLPL-DEYQEQL-S-FAD-ANIGG----AG-ITAG-FLSRF-KK-Y-WAHLDIAGTAW-

        501              54
E. col  S.GK....AKGATGRPVALLAQFLLNRAGFNGEE
H. inf  Q.GA....NKGATGRPVSLLTQFLINQVK~~~~~
P. aer  SGGK....DKGATGRPVPLLTQYLLERAK~~~~~
R. pro  SNNASALCPKGAVGYGVRLLEKFIKEYN~~~~~
M. tub  TGSAWGYTPKGATGVPTRTMFAVLEDIAKNG~~~
Con     S-GA-----KGATGRPV-LL-QFL---AK-----
```

FIG. 3. Alignment of the peptide sequences of the PepA homologs from *E. coli* (*E. col*), *H. influenzae* (*H. inf*), *P. aeruginosa* (*P. aer*), *R. prowazekii* (*R. pro*), and *M. tuberculosis* (*M. tub*). Conserved residues (Con) are indicated where three or more sequences agree. The absolutely conserved (underscored) residues are believed to be involved in the active site and/or metal ion binding based on X-ray crystallographic studies of bovine lens leucine aminopeptidase (9).

There are two potential initiation codons for *phpA*, resulting in respective predicted products of 495 and 511 amino acids (Fig. 2). The second AUG codon is downstream of a potential ribosome binding site and best aligns with the initiation codons of the majority of the PepA homologs shown in Fig. 3. The predicted molecular mass of *P. aeruginosa* PhpA has been calculated to be either 52,298 or 53,892 Da, depending on which initiation codon is used. These values correlate well with the 54.8- and 53.5-kDa molecular masses predicted for the PepA homologs of *E. coli* and *H. influenzae*, respectively. There appears to be very strong amino acid conservation among the various PepA homologs depicted in Fig. 3, including the absolute conservation of seven amino acid residues shown to be in the active site or involved in metal binding of bovine lens leucine aminopeptidase (9) (Fig. 3).

Immediately 3′ and partially overlapping the PhpA ORF, a second ORF encoding a product with 31% amino acid identity to HolC of *E. coli* (11) and 33% identity to the HolC homolog from *H. influenzae* (18) was detected. HolC is the *chi* subunit of the DNA polymerase holoenzyme. Interestingly, the same genomic arrangement, *pepA* followed by *holC*, is also conserved in *E. coli* (11). Although there was no AUG initiation codon detected for *P. aeruginosa holC*, based on the alignment with the *H. influenzae holC* homolog, there was a potential GUG start codon (Fig. 2).

We used our sequence to scan the database of the Pseudomonas Genome Project (www.pseudomonas.com). We found a sequence containing *phpA* which revealed additional sequence 5′ of *phpA*. This 5′ region contained an uncharacterized ORF (encoding a potential product of 280 amino acids) which would be transcribed in the opposite direction from *phpA* (Fig. 2). Sequence comparison analysis indicated that the predicted product of this ORF (designated here ORF280) is 34% identical to a hypothetical protein from *M. tuberculosis* (data not shown). The genomic organization of the elements described above are represented schematically in Fig. 4.

Physical mapping of *phpA* on the *P. aeruginosa* PA01 chromosome. Pulsed-field gel electrophoresis was used for chromosomal mapping of *phpA*. The approximately 6.8-kb *Bam*HI-*Hin*dIII fragment from pSW109 hybridized to the 302-kb *Dpn*I (H) fragment and the 517-kb *Spe*I (A) fragment of the *P. aeruginosa* PA01 chromosome. This places *phpA* on the approximately 100-kb linking fragment and localizes *phpA* to approximately 27 to 28.5 min on the PA01 chromosome. The
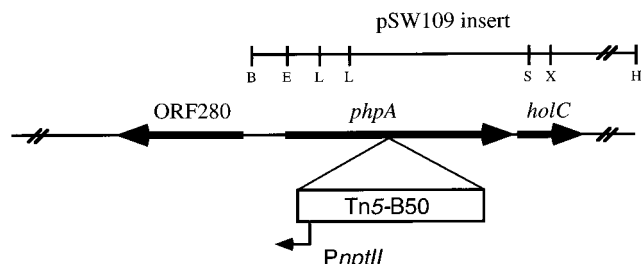

FIG. 4. Genome organization of the *P. aeruginosa phpA* region. The region contained within the insert of pSW109 is indicated. Additional 5′ sequence information was obtained from the *P. aeruginosa* Genome Sequencing Project (15 June 1998 release). ORF280 displays 34% amino acid identity to an ORF of unknown function in *M. tuberculosis*. The orientation of Tn5-B50 enables transcription of ORF280 while at the same time inactivating *phpA* and possibly exerting a polar effect on *holC*. Restriction endonuclease sites correspond to those shown in Fig. 2. Abbreviations: B, *Bam*HI; E, *Eco*RI; L, *Sal*I; S, *Sph*I; X, *Xho*I; H, *Hin*dIII.

gene *exoS* and the insertion sequence IS-PA-1 also map in this region of the chromosome (29).

The Tn5-B50 insertion in *phpA* causes conversion to the mucoid phenotype in an *algB* mutant but not in an *algT* or *algR* mutant. Although FRD444.1 displayed a mucoid phenotype, it was not clear whether this was due to the transposon insertion or to secondary mutations which were Tn5-B50 independent. In addition, we wanted to determine if the Tn5-B50 insertion demonstrated gene specificity and restored the mucoid phenotype only in an *algB* mutant. To address these issues, it was necessary to create identical Tn5-B50 mutations in other *P. aeruginosa* strains and in a "clean" *algB* mutant background. We took advantage of the *P. aeruginosa* DNA flanking Tn5-B50 in pSW50 to construct an allele replacement vector, pSW40. This plasmid was conjugated into the mucoid CF isolate FRD1 and into the nonmucoid strains FRD444 (*algB*::Tn*501*), FRD440 (*algT*::Tn*501*), and FRD831 (Δ*algR*::Ω*aacC1*) (Table 1). Recombinants were selected as described in Materials and Methods. The alginate phenotypes of the FRD1, FRD440, and FRD831 recombinants were not visibly altered, whereas the phenotype of the FRD444 recombinant had changed from nonmucoid to mucoid (data not shown). This confirmed that the mucoid phenotype of FRD444.1 was due to the Tn5-B50 insertion. In addition, this result indicated that the same insertion did not affect the visible alginate phenotype in FRD1 or the *algR* or *algT* genetic background.

Polar and nonpolar insertions in *phpA* result in a mucoid phenotype in an *algB* mutant background. Due to the orientation of Tn5-B50 in *phpA* (Fig. 4) we postulated three possibilities for the cause of the mucoid phenotype of FRD444.1. The phenotype may result from Tn5-B50 driving transcription of ORF280 or some other gene 5' of *phpA* (Fig. 4). Alternatively, the phenotype of FRD444.1 may result from the loss of *phpA* or a polar effect on the *holC* gene. In order to determine the cause of the mucoid phenotype of FRD444.1, we constructed a series of insertions in *phpA* in the chromosome of FRD444 (*algB* mutant). To accomplish this, pSW90 (Table 1) was digested with *Sal*I, which removed an approximately 190-bp fragment from the coding region of *phpA*. The ends of the digested plasmid were rendered blunt and ligated to the *xylE*-*aacC1*-containing *Sma*I fragments of either pX1918GT or pX1918G (41). These fragments created polar and nonpolar insertions, respectively, in the direction of *aacC1* transcription. Each insertion was obtained in both orientations in *phpA*, and the resulting plasmids were used to perform allelic exchange (Fig. 5). We reasoned that if expression of ORF280 5' of *phpA* were the cause of the phenotype of FRD444.1, then only the nonpolar insertion in FRD911 would result in a mucoid phenotype (Fig. 5). However, if the phenotype of FRD444.1 resulted from a polar effect on *holC*, then all insertions shown in Fig. 5 except the nonpolar insertion in FRD909 would result in a mucoid phenotype. We observed that all insertions in *phpA* resulted in conversion to the mucoid phenotype (Fig. 5). These results are consistent with the inactivation of *phpA* as the cause of conversion.

To confirm that it was indeed inactivation of *phpA* which was responsible for the mucoid phenotype, we attempted complementation of the lesion in *phpA* using both IncP (pLAFR3)- and IncQ (pMMB67HE)-type vectors. However, the results proved ambiguous. We repeatedly failed to obtain any transconjugants with pMMB67HE, despite the fact that the parental strain FRD444 maintained this plasmid quite well (data not shown). Using FRD906, a tetracycline-sensitive (Tc^s) version of FRD444.1 generated by allelic exchange, we were able to obtain transconjugants with pLAFR3. However, the FRD906 strain formed colonies much smaller than those of the
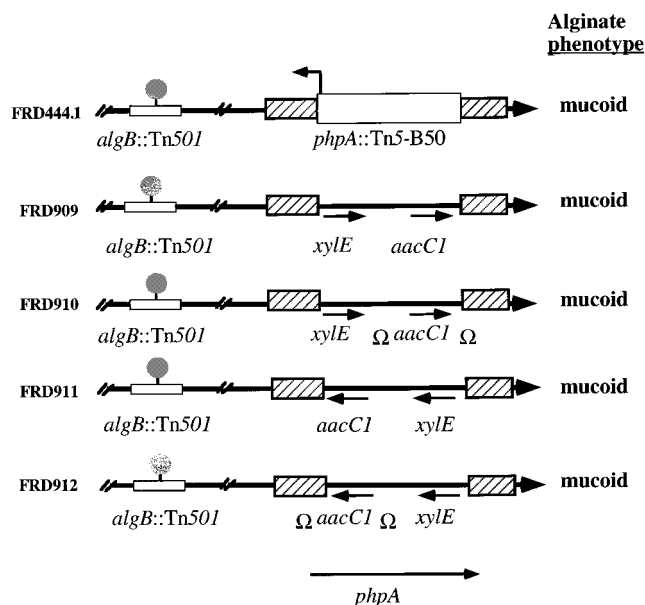
FIG. 5. Polar and nonpolar insertions in *phpA* result in a mucoid phenotype in *algB*::Tn*501* *P. aeruginosa*. Allelic exchange was performed on the nonmucoid *algB*::Tn*501* strain FRD444, replacing the wild-type *phpA* with the *phpA*::*xylE aacC1* alleles shown. The alginate phenotypes of the resulting strains were then compared to the mucoid phenotype of the *phpA*::Tn5-B50 strain FRD444.1. Ω designates the original Ω element described by Prentki and Krisch (38), which contains the factor-independent transcriptional terminators of bacteriophage T4D gene *32* as well as translational stop codons in all six reading frames. The presence of Ω therefore generates an insertion polar on transcription and translation in either direction.

parental strain FRD444 harboring the same plasmid. The mucoid status of these colonies was impossible to determine due to their apparent growth defect. Interestingly, when pSW110 (pLAFR3 containing *phpA*) was conjugated into FRD906, colonies appeared similar in size and in nonmucoid morphology to FRD444 harboring pLAFR3.

Expression of *phpA* is not regulated by AlgB at the transcriptional level. The *xylE* genes from pX1918GT and pX1918G are promoterless and thereby establish transcriptional fusions when they are inserted downstream of a promoter. In order to determine whether *phpA* was transcriptionally regulated by AlgB, we compared the XylE activity of FRD909 (Fig. 5) to that of the isogenic *algB*$^+$ strain FRD913 (not shown). There was no discernible difference in the XylE activities between these two strains, indicating that *phpA* expression was not affected by AlgB at the transcriptional level (data not shown). Nevertheless the visible increase in alginate production in FRD444.1 (mutated *algB* and *phpA*) compared to the level of production in the parental *algB* mutant FRD444 warranted further investigation.

Inactivation of *phpA* in an *algB*::Tn*501* strain results in increased *algD* transcription. Data described above indicated that inactivation of *phpA* circumvented an *algB* mutation, restoring alginate production to the normally nonmucoid strain FRD444. Mutations in *algB* result in an approximately 20-fold reduction in *algD* transcription (51). To quantify the effect that inactivation of *phpA* had on *algD* expression, we used pDJW530 (Table 1) to construct *xylE* transcriptional fusions to the chromosomal *algD* promoters of FRD1 (*mucA22*), FRD444 (*mucA22 algB*::Tn*501*), and FRD444.1 (*mucA22 algB*::Tn*501* *phpA*::Tn5-B50), which resulted in the strains FRD875, FRD879, and FRD920, respectively (Table 1; Fig. 6). The levels of XylE
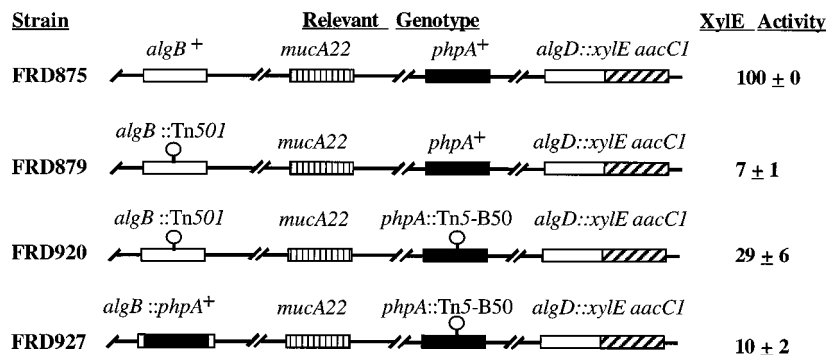
FIG. 6. Inactivation of *phpA* in an *algB*::Tn*501* strain results in increased *algD* expression. The four strains shown were constructed by replacing the chromosomal wild-type *algD* with *algD*::*xylE* by allelic exchange. XylE activity was assayed as detailed in Materials and Methods. Activity was normalized by dividing by the OD_{540} at the time of harvest. For each experiment, the activity of strain FRD875 was set at 100%. The activities of the remaining strains were then expressed as percentages of the activity of FRD875. The results of six independent experiments were used to determine the means and standard deviations shown.

activity in these strains were measured, and the data from six independent experiments are summarized in Fig. 6. The *algB*::Tn*501* strain FRD879 exhibited XylE activity equal to approximately 7% of that of the *algB*+ strain FRD875. However, the *algB*::Tn*501 phpA*::Tn*5*-B50 strain FRD920 expressed an approximately fourfold higher level of XylE activity than FRD879, reaching 29% of the value of FRD875 (Fig. 6). These data are in agreement with the visible alginate phenotypes of the corresponding *algD*+ strains (data not shown).

Expression of *phpA* from an ectopic chromosomal locus complements the Tn*5*-B50 insertion in the native *phpA* gene. Due to the effect of the *phpA*::Tn*5*-B50 insertion on the ability of the organism to acquire and/or properly maintain plasmids (see above), it was impossible to perform standard complementation analyses with plasmid-borne *phpA* alleles. To be certain that inactivation of *phpA* was the cause of the increase in *algD* transcription in the *algB* genetic background, we developed a strategy to complement the lesion in *phpA* by providing a wild-type copy of *phpA* elsewhere in the *P. aeruginosa* chromosome. This complementation would avoid any effects of *phpA* mutations on plasmid biology as well as the effects of any proteins encoded by plasmid-borne genes, antibiotic selection, or gene dosage. Since we were investigating the effects of *phpA* in an *algB* mutant background, the Tn*501*-marked *algB* allele provided a convenient locus for integration of a wild-type *phpA* allele. To carry out this experiment, we first constructed pSW134, an allele replacement vector containing the wild-type *phpA* gene. This copy of *phpA* was flanked at either end by factor-independent transcriptional terminators (38). These terminators were in turn bordered by sequences flanking the *algB* gene, thus providing the necessary homology for recombination of the vector with the chromosome at the *algB* locus (Materials and Methods). To isolate *phpA*+ gene replacements at the *algB*::Tn*501* locus, sucrose-resistant colonies were screened for loss of mercuric chloride resistance (Hg^r) (the marker on Tn*501*). The level of XylE activity of the resulting strain, FRD927 (*phpA*::Tn*5*-B50 *algB*::*phpA*), was found to be significantly lower than that observed in the parental strain FRD920 and was similar to that in FRD879 at 10% of wild-type XylE levels (Fig. 6). This result confirmed that loss of *phpA* was responsible for the phenotype of the original *phpA*::Tn*5*-B50 strain FRD444.1. In addition, the transcriptional terminators flanking *phpA* at the *algB* locus were oriented so as to block any incoming transcription from the adjacent chromosomal sequences. The observation of reduced *algD* transcription in FRD927 indicated that at least one pro-

moter capable of expressing *phpA* was located between the start of *phpA* and the *Bam*HI site depicted in Fig. 2.

DISCUSSION

In this study we have identified the *P. aeruginosa* homolog of *pepA*, which encodes leucine aminopeptidase in *E. coli* and a variety of other species. We have shown that insertional inactivation of this gene in the nonmucoid *algB* mutant FRD444 results in conversion to the mucoid phenotype. This is accompanied by a fourfold increase in *algD* transcription. We have designated this novel *pepA* homolog *phpA* to avoid confusion with *P. aeruginosa pepA*, recently described by Hauser et al. (26). Hermes et al. described the purification of an aminopeptidase from *Pseudomonas putida* which demonstrated activity toward a select group of L amino acid amides, particularly the L-leucine derivative, as well as all four dipeptide combinations of leucine and phenylalanine (27). Those authors determined the subunit molecular mass to be 53 kDa, which is in agreement with the predicted molecular mass of PhpA. However, the pI of the *P. putida* aminopeptidase was estimated to be 10.5 (27) whereas the predicted pI of PhpA is either 8.9 (first start codon) or 8.7 (second start codon). Whether *phpA* encodes the enzyme described by Hermes et al. remains to be determined, but there is little doubt that *phpA* is in fact the homolog of *E. coli pepA*. The extensive sequence similarity at the amino acid level, the conservation of predicted active-site residues, and the genomic clustering with *holC* in both organisms speak to the authenticity of the relationship between *E. coli pepA* and *P. aeruginosa phpA*.

A possible connection between an aminopeptidase activity of PhpA and transcriptional activation of *algD* involves σ^{22} (AlgT and AlgU). σ^{22} is a member of the ECF (extracytoplasmic function) family of alternative σ factors (14, 33, 35) and is homologous to σ^E of *E. coli* (15, 35). This family of σ factors is believed to play an important role in stress response. σ^E of *E. coli* is involved in the transcription of the extreme heat shock σ factor σ^H (σ^{32}) (15). σ^E is also required for expression of HtrA (DegP), a periplasmic serine protease thought to function in the degradation of abnormal or denatured periplasmic proteins which may arise as a result of thermal or chemical insult (15). Consistent with this role, *algT* mutants of *P. aeruginosa* were found to be more susceptible to killing by heat and paraquat (35). Recently, Boucher et al. described two *P. aeruginosa* genes displaying similarity to *htrA*, namely, *mucD* and *algW* (6). MucD is encoded within the *algTmucABCD* cluster

mapping near 67.5 min on the *P. aeruginosa* chromosome and is suggested to be subject to positive regulation by σ²² (6). MucD is believed to be a periplasmic protein and thus may play a role analogous to that of HtrA (6). AlgW, encoded at 69 min, lacks a conserved signal peptide and was postulated to localize to the cytoplasm, where it too may play a role in degrading denatured proteins (6). Accumulation of abnormal proteins has been shown to be a signal for σ³² activation in *E. coli* (8, 21, 30). If denatured proteins are indeed a signal for induction of stress response, loss of PhpA aminopeptidase function might be expected to further stimulate the σ²² regulon and partially overcome the requirement for AlgB by leading to the incomplete breakdown of senescent and misfolded proteins in the cytosol. The finding that a transposon insertion in *mucD* also suppressed the *algB* phenotype is entirely consistent with a hypothesis that increased expression or activity of AlgT overcomes the lack of AlgB and restores the mucoid phenotype. A similar argument could be made for the *trmD* mutant, i.e., that loss of TrmD leads to an increase in frameshifting during translation (4), in turn leading to abnormal or misfolded proteins which stimulate AlgT activity or expression. It should be noted, however, that only in the case of the *phpA* mutant have we ruled out the possibility that the phenotypes of the transposon mutants listed in Table 2 are due to polar effects. Nevertheless, according to this scenario many mutations may lead to partial suppression of *algB* as long as such mutations ultimately induce the stress response.

The effect of the *phpA*::Tn*5*-B50 insertion on *algD* transcription may or may not be due to the mechanism described above. In fact, PepA of *E. coli* is a remarkably multifunctional protein. In addition to the aminopeptidase activity of PepA, there at least two systems in which *E. coli* PepA participates as a site-specific DNA-binding protein involved in negative transcriptional regulation. PepA interacts with the promoter region of the *pepA* gene, repressing transcription from one of its three promoters (13). PepA has also been shown to bind within the promoter region of the *carAB* operon (13). The *carAB* genes encode the subunits of carbamoylphosphate synthetase. The upstream promoter of *carAB*, P1, is repressed in the presence of high concentrations of pyrimidines, a repression which requires site-specific DNA binding of PepA to the *carAB* control region (12). Therefore, by analogy, either PhpA may directly repress *algD* transcription or it may repress expression of a positive regulator of *algD*. *E. coli* PepA also plays a structural role in the Xer-mediated resolution of multimeric forms of multicopy plasmids such as ColE1 and pSC101 (45). Whether a site-specific recombination is involved in *algD* regulation is not known. It is also unknown whether PhpA plays a role in resolving plasmid multimers in *P. aeruginosa*, although this function would be consistent with our observations on the effects of *phpA* lesions on plasmid maintenance. Interestingly, the aminopeptidase activity of PepA is not required for its role in site-specific recombination or pyrimidine-specific regulation of *carAB* (13, 36). Experiments are under way to determine whether PhpA possesses aminopeptidase activity and whether such activity is required for the negative effect that PhpA has on *algD* transcription.

The original goal of these investigations was to identify any genes under AlgB control which participate in *algD* transcription. The fact that expression of *phpA* does not appear to be affected by AlgB at the transcriptional level suggests that *phpA* is not a true intermediate between AlgB and *algD*. However, since in *E. coli*, PepA represses transcription of its own gene (13), it remains a possibility that AlgB regulates *phpA* expression by a mechanism requiring the *phpA* gene product. Another indication that *phpA* is not the target of AlgB relevant to

alginate expression is the observation that insertional inactivation of *phpA* in an *algB* mutant does not restore *algD* transcription to wild-type levels. In fact, the finding that transposon insertions in a variety of genes (Table 2) can at least partially suppress the *algB* phenotype raises new questions as to the mechanism whereby AlgB activates transcription of *algD*. Although transcription of *phpA* does not appear to be regulated by AlgB, it is clear from these studies that loss of *phpA* has a pronounced effect on *algD* transcription in the *algB* genetic background. Investigation of the mechanism of this effect as well as analysis of other *algB*-suppressing mutants should provide valuable insight into the basic physiological and genetic regulation of alginate expression.

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