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The *agmR* Gene, an Environmentally Responsive Gene, Complements Defective *glpR*, Which Encodes the Putative Activator for Glycerol Metabolism in *Pseudomonas aeruginosa*

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The genes for the peripheral glycerol carbon metabolic pathway (*glp*) in *Pseudomonas aeruginosa* are postulated to be positively regulated by GlpR. A gene complementing the *glpR2* allele, affecting expression of the putative activator, was cloned by a bacteriophage mini-D3112-based in vivo cloning method. Mini-D3112 replicons were isolated by transfecting *glpR2* strain PRP406 and selecting clones able to grow on minimal medium containing glycerol as the sole carbon and energy source. Preliminary biochemical characterization indicated that the cloned activator gene for glycerol metabolism (*agmR*) may not be allelic to *glpR*. Restriction analysis and recloning of DNA fragments located the *agmR* gene to a 2.3-kb *Eco*RV-*Sst*I DNA fragment. In a T7 RNA polymerase expression system, a single 26,000-Da protein was expressed from this DNA fragment. The amino acid sequence of this protein, deduced from the nucleotide sequence reported here, demonstrates its homology to the effector (or regulator) proteins of the environmentally responsive two-component regulators. The carboxy-terminal region of AgmR contains a possible helix-turn-helix DNA-binding motif and resembles sequences found in transcriptional regulators of the LuxR family.

Mucoid strains of the opportunistic pathogen *Pseudomonas aeruginosa* isolated from cystic fibrosis patients with chronic pulmonary infections secrete copious amounts of the extracellular polysaccharide alginate (for a comprehensive review, see reference 33). Alginate is biosynthesized from carbohydrates such as fructose, mannitol, glucose, gluconate, and glycerol, with the true precursor being fructose-6-phosphate (2). Since fructose-1,6-bisphosphate aldolase activity is essential for synthesis of alginate from glucose, gluconate, and glycerol, it has been suggested that these substrates must be converted to triose phosphates via the Entner-Doudoroff pathway in order to serve as precursors (2). Although the importance of triose phosphates in alginate biosynthesis has been recognized, little is known about their metabolic origin and even less is known about the regulation of their pathways at the molecular level. One of the regulated pathways able to provide precursors for alginate biosynthesis is the peripheral glycerol carbon metabolic pathway.

In *P. aeruginosa*, glycerol is primarily metabolized through the Entner-Doudoroff pathway (6, 25). Glycerol is transported into the cell by a specific, inducible transport system (*glpT*). Unlike *Escherichia coli*, in *P. aeruginosa* glycerol transport is mediated by a high-affinity, binding protein-dependent transport system (41, 47). Intracellular glycerol is phosphorylated to *sn*-glycerol-3-phosphate (glycerol-P) by glycerol kinase (*glpK*) and subsequently oxidized to dihydroxyacetone phosphate by glycerol-P dehydrogenase (*glpD*). Evidence for this pathway rests primarily on the observed specificity of enzyme induction and on the analysis of mutants defective in specific *glp* genes. Besides glycerol, glycerol-P can be transported and utilized as the sole carbon source, although it supports growth much more poorly than glycerol (25). A mutation has been isolated in a putative regulatory gene (*glpR*) that abolished expression of all known components of the pathway for glycerol catabolism (6). Thus, in contrast to *E. coli*, *glp* gene expression in *P. aeruginosa* seems to be positively regulated. As a first step in elucidating the molecular organization and mode(s) of regulation of the *glp* genes of *P. aeruginosa*, a gene complementing the *glpR2* allele has been cloned.

MATERIALS AND METHODS

Bacterial strains and plasmids. The following *P. aeruginosa* strains were used in this study: PAO1 wild type (B. Holloway via P. Phibbs [6]); PRP406 *glpR2 zwf* (6); and CD10 FP$^-$ *met-9020 pro-9024 blaP9202 blaJ9111 aph-9001* (D3112cts)/pADD948 (7). *E. coli* DH5αF′ [F′ Φ80 *lacZ* ΔM15]Δ(*lacZYA-argF*)U169 *recA1 endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 gyrA relA1* (23) or HB101 *supE44 hsdS20* (r_B^- m_B^-) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1* (34) was used for selection and maintenance of plasmids. For subcloning experiments, the broad-host-range plasmids pUCP18 and pUCP19 (36) were used, unless indicated otherwise.

Growth media. LB medium (34) was used as the rich medium for both *E. coli* and *P. aeruginosa*. As the minimal medium, M9 medium (34), VB medium (48), or LVM medium (21) was used. VBMM medium is VB medium containing 0.3% trisodium citrate. Carbon sources were incorporated into minimal media at a final concentration of 10 mM, and amino acid requirements were satisfied by addition of these components to a final concentration of 0.5 mM. Antibiotics were used in selection media at the following concentrations (per milliliter): ampicillin (Ap), 100 μg for *E. coli*; and carbenicillin (Cb), 500 μg for *P. aeruginosa*. Lactose phenotypes were screened on LB plates containing 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) per ml.

DNA methods. Restriction enzymes, a large fragment of DNA polymerase (Klenow enzyme), T4 DNA polymerase,

and T4 DNA ligase were purchased from Bethesda Research Laboratories, Bethesda, Md., or Pharmacia Canada and used as recommended by the suppliers. Small-scale isolation of *E. coli* and *P. aeruginosa* plasmid DNA was done as described previously (28) with the modification that *P. aeruginosa* DNA was phenol extracted prior to ethanol precipitation. Chromosomal DNA was isolated by the procedure described by Silhavy et al. (42). Plasmid DNA was labeled with biotin-14-dATP, using the BioNick labeling kit (Bethesda Research Laboratories) and following the instructions of the supplier. DNA fragments were transferred from agarose gels to nylon membranes as described by Sambrook et al. (34). After UV fixation, the membranes were treated and probed with biotinylated probes in accordance with the instructions provided with the PhotoGene nucleic acid detection system (Bethesda Research Laboratories). DNA restriction fragments were eluted from low-gelling-temperature agarose gels as described by Wieslander (49) or electroeluted from regular agarose gels as described by Sambrook et al. (34). *E. coli* cells were made competent and transformed by the $CaCl_2$ procedure (34) with the following modifications. After the cells (40 ml) were harvested, they were suspended in 20 ml of ice-cold 0.1 M $MgCl_2$ and immediately centrifuged at 4°C. The cells were then resuspended in 10 ml of TG medium (75 mM $CaCl_2$, 6 mM $MgCl_2$, 15% [wt/vol] glycerol) and kept on ice for 30 min. After centrifugation at 4°C, the competent cells were suspended in 1 ml of TG medium and either used directly or frozen in small aliquots at −70°C. *P. aeruginosa* was transformed by the procedure of Olsen et al. (30). An ordered set of nested exonuclease III (ExoIII)-S1 nuclease deletions was generated by the procedure of Henikoff (14). Briefly, pPS102 DNA was digested to completion with *Bam*HI (leaving ExoIII-sensitive 3′ recessed ends) and *Pst*I (leaving ExoIII-resistant 3′ protruding ends). The restriction sites for both of these enzymes are located at the same junction of chromosomal and vector DNAs, with *Pst*I cleaving closer to the vector than *Bam*HI. Thus, the deletions will progress from the *Bam*HI site into the cloned segment of chromosomal DNA. DNA sequence analysis by the dideoxy-chain termination method was performed on single-stranded M13mp18/19 DNA subclones, using a T7 DNA polymerase kit and the protocol provided by the supplier (Bio/Can Scientific, Mississauga, Ontario, Canada). Labeled DNA fragments were separated on electrolyte gradient gels as described previously (39). The IBI MacVector program was used for computer-assisted sequence analyses.

In vivo cloning of DNA complementing *glpR*. For the cloning of DNA fragments complementing the PAO *glpR2* allele, the bacteriophage mini-D3112-based in vivo cloning method of Darzins and Casadaban (7) was used with the following modifications. A phage D3112 lysate was prepared by heat induction from CD10 containing the pADD948 replicon. Cells of recipient strain PRP406 were grown overnight in LB medium. To each 0.15 ml of these cells, 0 (control), 0.15, or 0.5 ml of lysate (4×10^8 PFU/ml) was added at room temperature. After addition of 3 ml of LVM top agar to each sample, samples were spread on LVM plates containing glycerol and carbenicillin and incubated at 30°C. Colonies growing on these plates after 48 to 60 h were purified on VBMM-carbenicillin medium and analyzed for the presence of recombinant plasmids.

Construction of LacZ gene fusions. AgmR′-′LacZ gene fusions were constructed as follows. First, a 2.3-kb *Eco*RV-*Sst*I fragment from pPS102 (see Fig. 2) was subcloned into pUC12 (26) to yield pUC12-2.2. Next, a 1,364-bp fragment (1,242 bp of DNA of the *agmR* region from the *Pvu*II site at nucleotide [nt] 1,385 [see Fig. 4] plus 122 nt of pUC12 DNA) was isolated from this plasmid and ligated to *Sma*I-cleaved pPZ1, pPZ2, and pPZ3 DNAs. These broad-host-range vectors are based on the pNM480 to -482 vector series and allow the isolation of LacZ protein fusions in all three translational reading frames (37). When transformed into DH5αF′ followed by selection on LB medium containing ampicillin and X-Gal, only the ligations performed with pPZ1 yielded a high number of $LacZ^+$ transformants (32% of all ampicillin-resistant transformants). By comparison, no Lac^+ transformants were obtained when pPZ2 was used, and only 2% were Lac^+ when pPZ3 was used. Restriction analyses indicated that the Lac^+ transformants obtained with pPZ3 had the *Pvu*II fragment inserted in the opposite direction of the one observed in pPZ1. All of the isolates obtained with pPZ1 showed the expected restriction pattern, and one representative clone containing a Φ(*agmR′-′lacZ*)(Hyb) construct (pGZ1) was retained.

Mobilization of recombinant plasmids. When applicable, recombinant plasmids were mobilized from *E. coli* HB101 to *P. aeruginosa* by using the conjugative helper functions of *E. coli* HB101(pRK2013), as described by Goldberg and Ohman (12).

Polypeptide analysis. Plasmid-encoded polypeptides were overexpressed and identified with the bacteriophage T7 RNA polymerase-promoter system (46). The host strain was BL21(DE3) (46), which contains the gene for T7 RNA polymerase under the control of the IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible *lacUV* promoter. Plasmids analyzed were pT7-5, pT7-5(2.2), pT7-6, and pT7-6(2.2). Plasmids pT7-5(2.2) and pT7-6(2.2) were constructed by subcloning a 2.3-kb *Eco*RI-*Bam*HI fragment from pEVS2.2 (see Fig. 2) into *Eco*RI plus *Bam*HI-cleaved pT7-5 and pT7-6 DNA, respectively. Thus, in pT7-5(2.2) transcription from the Φ10 promoter proceeds from the *Sst*I site towards the *Eco*RV site, as indicated for pPS102 in Fig. 2, and in pT7-6 (2.2) transcription from the Φ10 promoter proceeds from the *Eco*RV site towards the *Sst*I site. The product of the cloned gene was preferentially labeled by using the procedure of Kornacki et al. (17) with the following modifications. The M9 medium used for induction, preincubation, and labeling was supplemented with methionine assay medium (final concentration, 10%; Difco Laboratories, Detroit, Mich.). One-milliliter samples were pulse-labeled for 5 min at 37°C with 15 μCi of L-^{35}S-methionine (1,285 mCi/mmol; Amersham Corp., Arlington Heights, Ill.). The labeled cells were pelleted in a microcentrifuge and resuspended in 100 μl of 10 mM Tris HCl (pH 7.3)–1 mM EDTA. After addition of 100 μl of 2× sample loading buffer (0.125 M Tris, 4% sodium dodecyl sulfate, 20% glycerol, 5% β-mercaptoethanol, pH 6.8), the samples were boiled for 2 min and 15-μl aliquots were analyzed on 12.5% sodium dedecyl sulfate-polyacrylamide gels, using the discontinuous buffer system of Laemmli (18).

Nucleotide sequence accession number. The sequence data reported here have been submitted to GenBank and assigned accession number M60805.

RESULTS

Cloning of DNA sequences complementing *glpR2*. As described in Materials and Methods, a gene complementing the *glpR2* allele carried by *P. aeruginosa* PRP406 (6) was cloned from strain CD10 by infecting strain PRP406 with a D3112 lysate containing random fragments of chromosomal DNA.

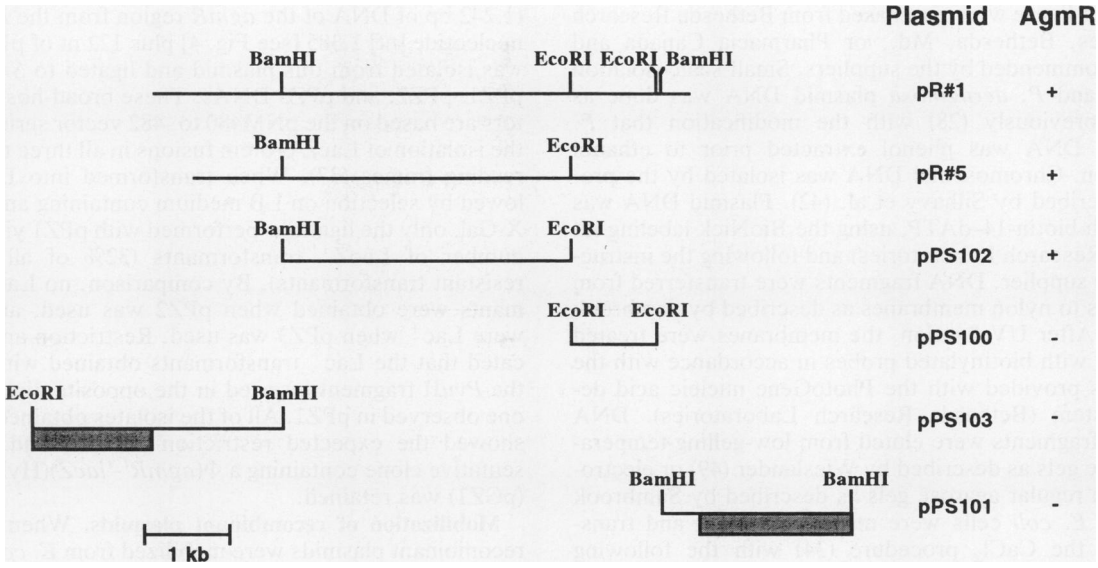

FIG. 1. Physical structure of recombinant plasmids harboring the *agmR* region of *P. aeruginosa*. Clones pR#1 and pR#5 were obtained by using the pADD948 replicon, as described in Materials and Methods. Clones pPS100 through pPS103 were obtained by subcloning the indicated fragments into appropriately cleaved pUCP19. The extent of pADD948 DNA carried by individual clones is shown by the shaded area. The ability of the plasmids to complement the resident *glpR2* mutation of strain PRP406 (AgmR phenotype) is indicated on the right-hand side. Complementation was tested by growth on LVM-glycerol-carbenicillin minimal medium.

With this method, between 50 and 100 putative $glpR^+$ clones were obtained per plate. No colonies were obtained without the addition of CD10 lysate. Due to the presence of the *zwf* marker, the colonies obtained on the selection medium grew very slowly on glycerol minimal medium and did not grow on mannitol minimal medium. Plasmid DNA was isolated from two of these clones (designated pR#1 and pR#5) and analyzed by digestion with *Bam*HI and *Eco*RI. The result of this analysis is shown in Fig. 1. The analyses indicated that pR#1 contained ca. 6.3 kb and pR#5 contained ca. 5.5 kb of chromosomal DNA. Both clones carried a common 3.4-kb *Bam*HI-*Eco*RI fragment.

To demonstrate that the cloned DNA was of *P. aeruginosa* origin, genomic DNA from PAO1 was digested with *Eco*RI and *Eco*RI plus *Bam*HI and probed with nick-translated, biotin-labeled pPS102 DNA (pUCP19 containing the 3.4-kb chromosomal insert of pR#5 [Fig. 1]). The probe hybridized with an expected 3.4-kb *Eco*RI-*Bam*HI fragment and with a 7.8-kb *Eco*RI fragment (data not shown). The results proved that the cloned DNA is of PAO origin.

To assess whether the various clones isolated by the in vivo cloning procedure were indeed related, plasmid DNA was isolated from three additional clones (pR#2, pR#3, and pR#4) and restricted with *Bam*HI, *Bam*HI plus *Eco*RI, and *Eco*RI. When these digests were probed with the gel-purified, 3.4-kb *Bam*HI-*Eco*RI fragment from pR#5, hybridization was observed with pR#3 and pR#4 but not with pR#2 (data not shown). Thus, four of five clones tested carry DNA from the same region of the chromosome, and pR#2 probably represents a random clone conferring Cb^r on a revertant (*glpR2* reverts to $glpR^+$ at a frequency of 10^{-7} [6]). These results prove that a specific gene which is able to complement *glpR2* has been cloned.

To ascertain whether the clones carried DNA complementing *glpR2*, pR#1 and pR#5 were transformed into *E. coli* HB101, along with the vector pADD948. The plasmids were then transferred back to PRP406 by triparental mating, using VBMM medium containing carbenicillin as the selection medium. Colonies growing on the selection medium were then tested for growth on LVM-glycerol or LVM-mannitol medium in the presence of carbenicillin. The transconjugants containing pR#1 or pR#5 grew on glycerol minimal medium but not on mannitol minimal medium. Transconjugants containing the vector pADD948 grew on neither medium. These results again indicated that the isolated plasmids contain a gene able to complement the *glpR2* allele. However, when glycerol transport and glycerol-P dehydrogenase activities were assayed under standard induction conditions (4 h in LVM minimal medium with 10 mM glycerol [47]), no significant induction of either activity was observed compared with cells containing pADD948 (data not shown). The reasons for these observations are not understood at present (see Discussion). Although additional experiments are clearly needed to analyze this phenomenon, the clear restoration of growth on glycerol was judged to be sufficient for initial characterization of the gene complementing *glpR2*, which was tentatively named *agmR* (activator of glycerol metabolism). Additional experiments will have to be performed to assess whether *agmR* is allelic to *glpR*.

Subcloning of the *glpR*-complementing activity of *agmR*. To localize *agmR* further, various gel-purified DNA fragments generated by *Bam*HI, *Eco*RI, and *Bam*HI plus *Eco*RI digestion of pR#1 were subcloned into similarly cleaved pUCP19 DNA (36). After verification of the constructs, plasmid DNA was used to transform PRP406 to carbenicillin resistance on VBMM medium, and complementation of the resident *glpR* mutation was tested as described in the preceding paragraph. The results of the complementation experiments are summarized in Fig. 1. Only pPS102 containing a 3.4-kb chromosomal *Bam*HI-*Eco*RI fragment was able to complement the *glpR* mutation of PRP406. As observed with the larger clones, when glycerol transport and glycerol-P dehydrogenase activities were assayed under standard induction conditions, no significant induction of either activity was ob-

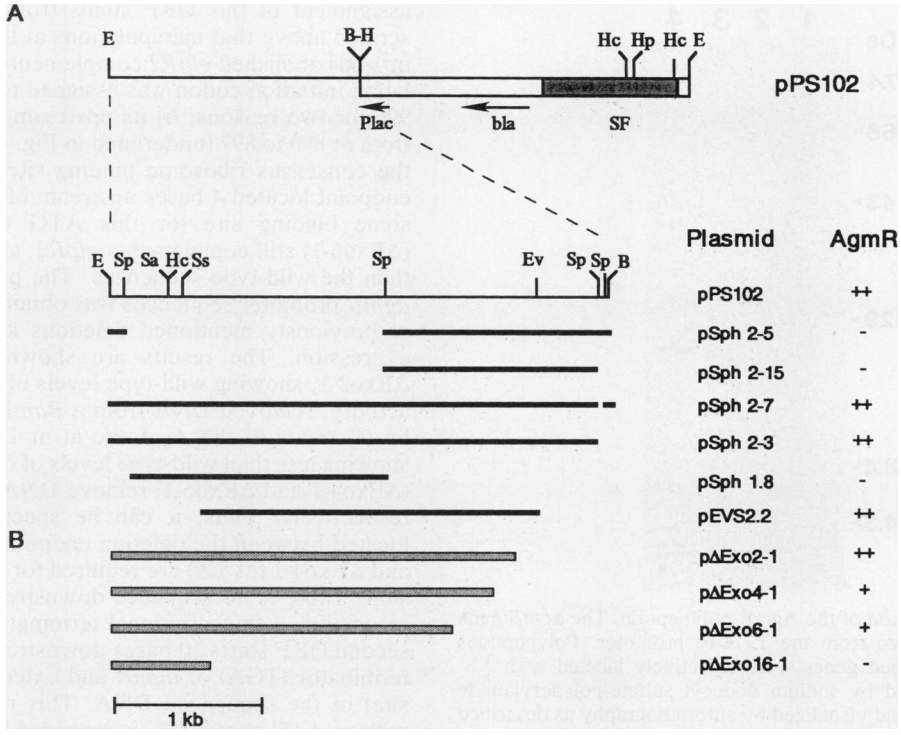

FIG. 2. Restriction map of chromosomal DNA carried by pPS102. (A) Subcloning and deletion analysis. The following restriction endonucleases were used: Ac, *Acc*I; B, *Bam*HI; E, *Eco*RI; Ev, *Eco*RV; H, *Hin*dIII; Hc, *Hin*cII; Hp, *Hpa*I; Sa, *Sal*I; Sp, *Sph*I; Ss, *Sst*I. B–H indicates the extent of the residual sites of the pUCP19 polylinker (*Bam*HI-*Xba*I-*Sal*I[*Acc*I-*Hin*cII]-*Pst*I-*Sph*I-*Hin*dIII). Restriction enzymes *Bgl*II, *Nsi*I, and *Xho*I do not cleave pPS102. The extent of DNA carried by individual plasmids derived from pPS102 is indicated by the solid bars. Clones pSph2-3 to pSph2-15 were obtained by partial *Sph*I digestion of pPS102 DNA. Plasmid pEVS2.2 was obtained by subcloning a 2.3-kb *Eco*RV-*Sst*I fragment from pPS102 into *Sma*I-*Sst*I-cleaved pUCP18 DNA. Similarly, pSph1.8 was obtained by subcloning a 1.8-kb *Sph*I fragment from pPS102 into *Sph*I-cleaved pUCP19 DNA. (B) ExoIII deletion analysis of pPS102. Plasmid pPS102 was subjected to a combined treatment with ExoIII-S1 as described in Materials and Methods. The extent of DNA carried by individual plasmids is indicated by the grey bars. The approximate extent of the deletions in the individual clones is as follows: pΔExo2-1, 600 bp; pΔExo4-1, 800 bp; pΔExo6-1, 1,100 bp; pΔExo16-1, 2,500 bp. The AgmR phenotype (*glpR* complementation activity) of the individual clones was determined as described in the legend to Fig. 1.

served compared with cells containing pUCP19 (data not shown).

A restriction map of pPS102 was constructed to facilitate further subcloning of the *agmR* gene (Fig. 2).

Localization of *agmR* on pPS102. For further localization of *agmR* on the subcloned DNA, plasmid pPS102 DNA was subjected to limited *Sph*I digestion. The *Sph*I fragments generated in this manner were gel purified, religated, and transformed into DH5αF′. The resulting plasmids (Sph1.8 to Sph 2-15; Fig. 2) were transformed into PRP406 to test for complementation of the *glpR* mutation. The results are summarized in Fig. 2. It is evident that cleavage at the *Sph*I site located ca. 1.9 kb from the *Eco*RI site leads to inactivation of *agmR* function. Thus, this site is either located within the *agmR* structural gene or in a region necessary for *agmR* expression. To test this hypothesis, three experiments were performed. First, a 2.3-kb *Eco*RV-*Sst*I fragment encompassing this *Sph*I site was subcloned into *Sst*I-*Sma*I-cleaved pUCP18 (36) and tested for *glpR* complementation. As can be seen from Fig. 2, the resulting plasmid, pEVS2.2, was able to complement the *glpR* mutation of PRP406. In the second experiment, pPS102 DNA was subjected to an ExoIII-S1 nuclease deletion analysis as described by Henikoff (14). After the extent of deletions of individual clones was assessed, the plasmids were used to transform PRP406. The results of this experiment for four representative deletion clones (pΔExo2-1 to pΔExo16-1) are shown in the bottom part of Fig. 2. Removal of 600 bp of DNA from the *Bam*HI end had no effect on the complementation activity of the cloned DNA (clone pΔExo2-1), whereas removal of 800 bp (clone pΔExo4-1) or 1,100 bp (clone pΔExo6-1) still allowed complementation, but to a significantly lesser degree than the pPS102 fragment. As expected from the results described above, a deletion of 2,500 bp (pΔExo16-1), which removed the *Sph*I site plus flanking DNA sequences, abolished complementation activity. In the third experiment, transcription at the *Sph*I site was interrupted by the insertion of a chloramphenicol resistance (Cm^r) cassette (35). When the resulting construct was transformed into strain PRP406, no complementation of the resident *glpR2* allele was observed (data not shown).

Polypeptide product of *agmR*. To overexpress and identify the putative AgmR polypeptide, the wild-type *agmR* allele was expressed in the T7 RNA polymerase-promoter expression system (Fig. 3). Plasmids pT7-5(2.2) and pT7-6(2.2) carry the wild-type *agmR* region on a 2.3-kb *Eco*RV-*Sst*I fragment in opposite orientations relative to the T7 Φ10 promoter in vectors pT7-5 and pT7-6. Only plasmid pT7-6(2.2) expressed a single protein with an estimated molecular mass of 26 kDa (lane 4). This polypeptide was not observed from the plasmid carrying *agmR* in the opposite orientation [pT7-5(2.2); lane 2] and was absent from vectors

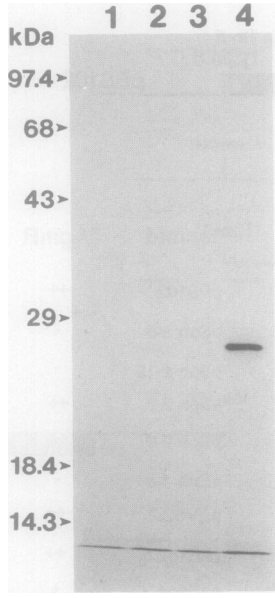

FIG. 3. Identification of the AgmR polypeptide. The *agmR* gene was expressed in vivo from the T7 Φ10 promoter. Polypeptides specified by the cloned genes were selectively labeled with ^{35}S-methionine, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and visualized by autoradiography as described in Materials and Methods. Lanes: 1, pT7-5; 2, pT7-5(2.2); 3, pT7-6; 4, pT7-6(2.2). The positions of the molecular weight (10^3) markers (Bethesda Research Laboratories) (top to bottom) phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, β-lactoglobulin, and lysozyme are indicated on the left.

pT7-5 (lane 1) and pT7-6 (lane 3). The results strongly indicate that the 26-kDa protein is the AgmR protein. Since in pT7-5(2.2) transcription from the Φ10 promoter is proceeding from the *Sst*I site towards the *Eco*RV site, as located on pPS102 (Fig. 2), and in pT7-6(2.2) transcription from the Φ10 promoter is proceeding from the *Eco*RV site towards the *Sst*I site, the presumed transcriptional orientation of the *agmR* gene on pPS102 is from the *Eco*RV site towards the *Sst*I site (Fig. 2). When the 4.4-kb *Bam*HI fragment of pR#1 (Fig. 1) was analyzed in the T7 expression system, an additional weakly expressed ca. 20-kDa protein with the same transcriptional orientation as *agmR* was observed (data not shown).

DNA sequence analysis of the *agmR* region. Subcloning analyses confined the *agmR* coding region to two *Acc*I fragments of ca. 500 and 1,200 bp, respectively (data not shown). The DNA sequence of these two fragments was determined from both strands by subcloning *Rsa*I and other suitable restriction fragments into M13mp18 or mp19 or both (26). When necessary, gaps in the sequence were closed by using synthetic primers. The DNA sequence thus determined is presented in Fig. 4. An open reading frame (ORF) of 662 bases defines a protein with a molecular mass of 24,422 Da (pI = 5.7), which is close to the 26,000-Da mass observed in the T7 polymerase expression system (Fig. 3), and its location is on the strand predicted from these expression analyses. This ORF was confirmed by sequencing across the *agmR'-'lacZ* junction of the Φ(*agmR'-'lacZ*)(Hyb) construct pGZ1, resulting in a LacZ$^+$ fusion protein. The Lac$^+$ phenotype in both *E. coli* and *P. aeruginosa* indicates that the reading frames of *agmR* and *lacZ* must be identical. Further support for the correct assignment of this ORF stems from the observations described above that manipulations at the *Sph*I site located at nt 1,084 abolished *glpR2* complementing activity. The translation initiation codon was assigned to the ATG codon at nt 906 for two reasons: (i) its upstream region (TAACGAGG) from nt 890 to 897 (underlined in Fig. 4) had a good match to the consensus ribosome binding site (40); (ii) the deletion endpoint located 4 bases upstream of the presumptive ribosome binding site for this ATG of a deletion mutant (ΔExo6-1) still complements *glpR2*, albeit to a lesser degree than the wild-type sequences. The putative location of the *agmR* promoter sequences was obtained by further analysis of previously mentioned deletions known to affect *agmR* expression. The results are shown in Fig. 4. Deletion ΔExo2-1, showing wild-type levels of *glpR2* complementing activity, removed DNA from a *Bam*HI site located ca. 400 bp upstream of the *Acc*I site at nt 1 to 259. The deletions showing less than wild-type levels of complementing activity (ΔExo4-1 and ΔExo6-1) remove DNA up to nt 529 and 885, respectively. Thus, it can be speculated that sequences located between the deletion endpoints of ΔExo2-1 (nt 259) and ΔExo4-1 (nt 529) are required for efficient *agmR* expression. There is no sequence downstream of the *agmR* gene resembling a transcriptional terminator. Instead, a possible second ORF starts 10 bases downstream of the translational terminator (TGA) of *agmR* and extends past the end (*Acc*I site) of the sequenced DNA. This reading frame, starting with an ATG at nt 1,581, is preceded by a putative ribosome binding site (GAGA) extending from nt 1,570 to 1,573 (Fig. 4), but no obvious promoterlike sequences are present upstream of this ribosome binding site. Therefore, if *orfX* indeed constitutes an ORF, it is most likely cotranscribed with *agmR*.

Homology of AgmR to two-component regulators. The predicted amino acid sequence of AgmR was used to search the National Biomedical Research Foundation data base with the FASTP program (22). From this search, AgmR was found to share homology with a number of bacterial regulatory proteins: *E. coli* OmpR (5), *E. coli* UhpA (11), *Bacillus subtilis* PhoP (38), *Rhizobium meliloti* NtrC (32), and *Agrobacterium tumefaciens* VirG (32). All of these proteins are effectors (or regulators) of two-component regulatory systems. A more detailed analysis revealed that the N-terminal half of AgmR shared homology with other effectors. Among these are *P. aeruginosa* AlgR (8), AlgB (50), and PhoB (1). A sequence alignment of AgmR with the *E. coli* OmpR and UhpA and *P. aeruginosa* AlgR and PhoB effector proteins (Fig. 5) shows that they all share homology in their first 130 amino acids. These include two conserved aspartic acid residues (amino acids 9 and 55 of AgmR) and a conserved lysine (amino acid 105 of AgmR). In addition, conserved clusters of four hydrophobic residues thought to form the three internal β-strands of the response regulatory proteins are found (amino acids 4-7, 51-54, and 79-82 of AgmR) (43). Moreover, characteristically spaced hydrophobic residues corresponding to the internal faces of amphipathic α-helices that flank the β-sheet are present.

A comparison of the C terminus of AgmR with those of other regulatory proteins shows that AgmR shows extensive similarities to the LuxR family of proteins (13, 45) (Fig. 6). Of the consensus sequence established for a 55-residue region of these proteins (45), a region of AgmR starting with residue 154 possesses 52% identical and 78% conserved amino acids. Within this segment of AgmR shown in Fig. 6, a region corresponding to amino acid residues 176 to 190 was identified with significant homology to the areas of DNA-

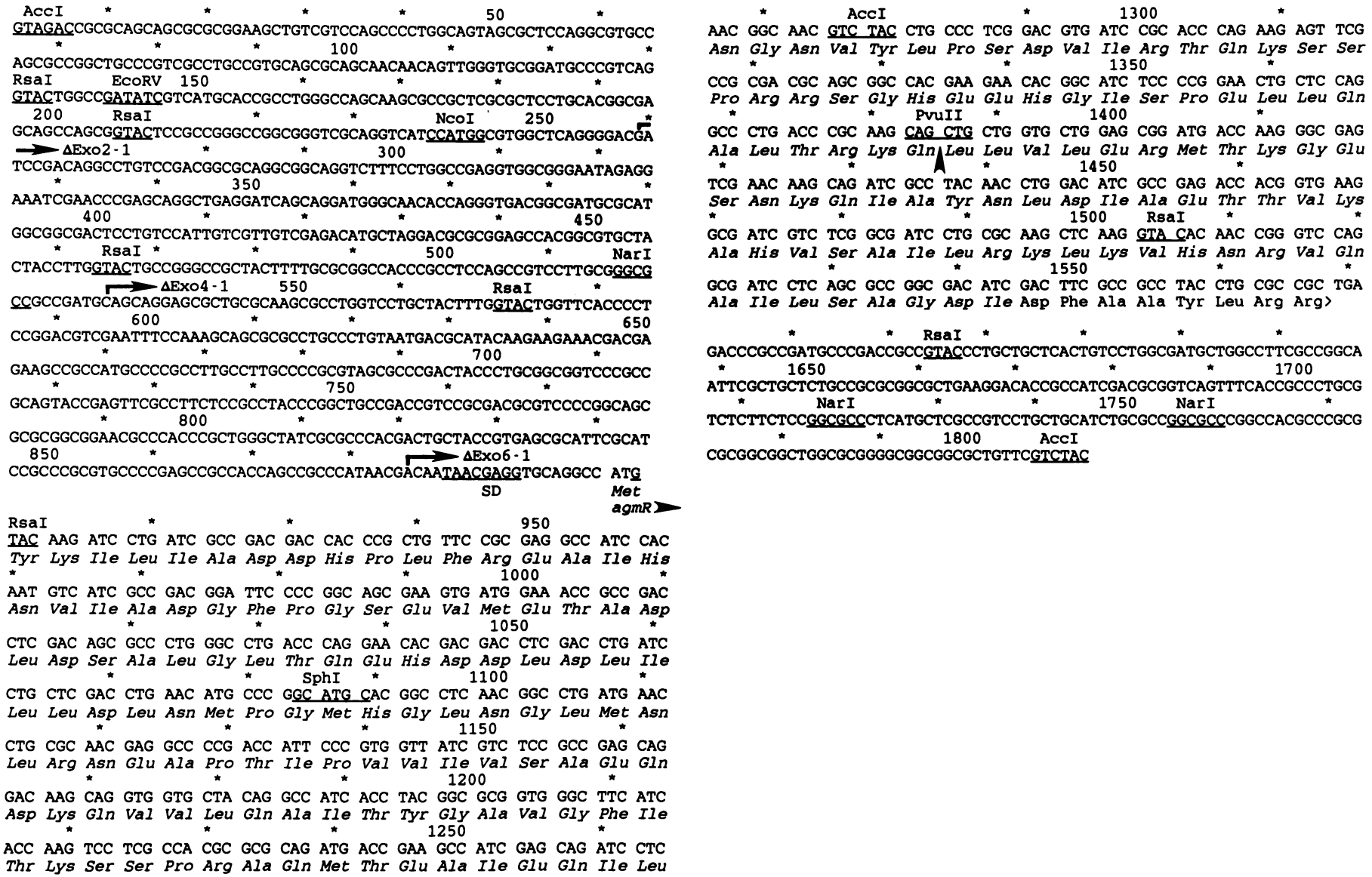

FIG. 4. Nucleotide sequence of the *agmR* region. The predicted amino acid sequence of the *agmR* reading frame is indicated below the appropriate codons. The respective putative ribosome binding (Shine-Dalgarno [SD]) site is underlined. Also indicated are the endpoints of the ExoIII deletions described in the text. The fusion junction of the *agmR'-'lacZ* fusion (pGZ1) is indicated by the vertical arrowhead.

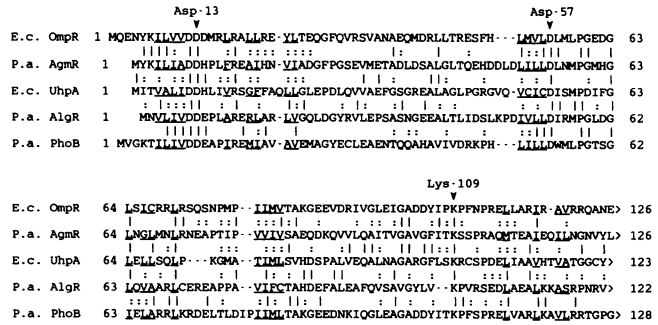

FIG. 5. Sequence alignments of the N-terminal domain of AgmR with other response regulator proteins (*E. coli* [E.c.] OmpR, *E. coli* UhpA, *P. aeruginosa* [P.a.] AlgR, and *P. aeruginosa* PhoB). Residues that are identical (|) and residues that belong to the same family (:) are indicated. The families of amino acids are ILMVFYW, AGPST, QNED, and HKR (29). Clusters of four hydrophobic residues (underlined) correspond to the three internal β-strands demonstrated for CheY, and characteristically spaced hydrophobic residues (also underlined) correspond to the internal faces of amphipathic α-helices that flank the β-sheet (43). Highly conserved amino acid residues (Asp-13, Asp-57, and Lys-109, numbered according to their position in CheY [43]) are indicated.

binding regulatory proteins (helix-turn-helix motifs) that are responsible for the sequence-specific interactions between the protein and its target (10, 31). AgmR has the appropriate amino acids (Ala and Val) at the two most conserved residues (positions 2 and 12) and the conserved nonpolar residues at the predicted positions. Since all members of the LuxR family are positive regulators of transcription, it appears likely that AgmR could function as a specific DNA-binding protein.

DISCUSSION

A gene complementing *glpR2*, an allele abolishing expression of the putative activator protein for glycerol metabolism

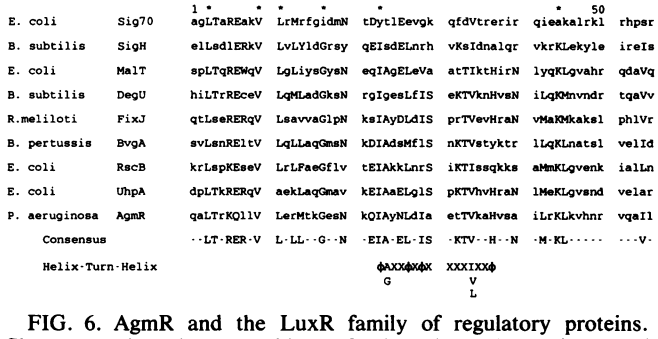

FIG. 6. AgmR and the LuxR family of regulatory proteins. Shown are the relevant residues of selected members of a set of proteins identified by Henikoff et al. (13) and Stout et al. (45). The AgmR sequence shown starts with residue 154. The single-letter code for amino acids is used, and the families of amino acids are assigned as described in the legend to Fig. 5. Asterisks above the alignment indicate residues which are identical in all but one or two members of the set. Conserved amino acids are capitalized and shown on the consensus line. The helix-turn-helix line shows residues expected for proteins with a helix-turn-helix motif (3, 10, 31). Φ, hydrophobic amino acids (Ile, Leu, Val, and Met); X, unspecified amino acids. Amino acids of the motif most strongly conserved (Ala or Gly in position 2 and Ile, Leu, or Val in position 12) are also indicated. The sequences are taken from Stout et al. (45).

in *P. aeruginosa*, has been cloned. Several lines of evidence presented in this report strongly support the conclusion that the cloned gene encodes an activator protein for glycerol metabolism, although it remains unclear whether *agmR* is allelic with *glpR*: (i) several independently isolated clones specifically restored growth on glycerol but not on mannitol; (ii) all but one of the complementing clones analyzed were physically related, suggesting that a specific chromosomal fragment was required for *glpR* complementation; and (iii) the deduced amino acid sequence of AgmR suggested that it is a DNA-binding protein. However, since no induction of glycerol transport could be demonstrated, the possibility of the cloned gene being a GlpR suppressor cannot be entirely ruled out. The most puzzling aspect of these studies was that, although *glpR* mutants containing various complementing plasmids clearly grew on glycerol, significant induction of glycerol-P dehydrogenase and glycerol transport activities could not be shown under standard induction conditions (4 h in LVM-glycerol minimal medium [47]). In agreement with the hypothesis put forward in this study that AgmR is the effector (or regulatory) component of a multicomponent regulatory system and since attempts to identify a corresponding sensor have failed so far, the possibility that the clones analyzed in this study contain only the *agmR* gene and not the corresponding sensory-encoding gene cannot be ruled out. Although most of these systems are organized in a single transcriptional unit, exceptions such as ArcA-ArcB (16) and RcsB-RcsC (44) of *E. coli* have been documented. In addition, several effector proteins of *P. aeruginosa* signal transducing systems have been described in which the sensory components seem to be physically unlinked (8, 50). As a consequence, sensor-independent low-level induction of the glycerol catabolic genes mediated by AgmR could occur, and this low-level induction presumably would be enough for growth. Sensor-independent expression of the respective target genes by the regulatory gene on multicopy plasmids has been demonstrated previously in the *uhp* system of *E. coli* (11). In strain PRP406 the situation is further complicated by the presence of the *zwf* mutation. It has been shown previously that in *zwf* mutants glycerol is metabolized very slowly even in *glp* wild-type strains (6). This slow metabolism, although enough to restore growth, might not allow full induction under standard assay conditions. Alternatively, it is possible that the presence of AgmR (especially when expressed from multiple-copy-number plasmids) provides an alternative pathway for glycerol metabolism, thus bypassing the "normal" *glp* catabolic pathway. In a fashion similar to OmpR, AgmR might somehow cause perturbations of the cytoplasmic membrane, thus allowing glycerol at high (10 mM) concentrations to diffuse passively into the cell. Intracellular glycerol could then be metabolized via action of a glycerol dehydrogenase activity, although such an enzymatic activity has yet to be demonstrated in *P. aeruginosa*.

Expression and DNA sequence analyses identified AgmR, the protein responsible for *glpR2* complementation, as a 24.5-kDa protein composed of 221 amino acids encoded by a 662-base ORF. Preliminary analysis of the *agmR* upstream region revealed that sequences located between nt 259 (Fig. 4, deletion endpoint of pΔExo2-1) and 529 (deletion endpoint of pΔExo4-1) were required for efficient *agmR* expression. Clone pΔExo2-1 showed wild-type (pPS102) levels of complementation activity, whereas clone pΔExo4-1 still allowed complementation, but to a significantly lesser degree than the pPS102 fragment. The reason for the reduced complementation activity observed with clone pΔExo4-1 is not yet

fully understood. The most likely explanation is that the DNA between nt 259 and 529 affected by the deletion in these clones represents DNA involved in the expression of *agmR* and that, in the deletion constructs, *agmR* is no longer expressed from its own promoter but rather from the *lac* promoter, which is less efficient in *P. aeruginosa* (Fig. 2). Thus, since the AgmR initiation codon is located at nt 906, these results indicate that between 377 and 647 bases of DNA are required for efficient *agmR* expression. However, it is unknown whether these sequences are simply required for transcription initiation (i.e., contain promoter sequences) or whether they contain sequences involved in the regulation of *agmR* transcription.

Analysis of the deduced AgmR amino acid sequence revealed its homology to the effector (or regulatory) components of environmentally responsive two-component regulatory systems. Among the effector proteins analyzed, OmpR of *E. coli* was most homologous to AgmR. A comparison of the amino-terminal domain of AgmR with other response regulators and transcriptional activators (Fig. 5) reveals that AgmR contains the highly conserved amino acid residues Asp-13, Asp-57, and Lys-109 found in CheY. According to the proposal of Stock et al. (43), the corresponding residues of AgmR (Asp-9, Asp-55, and Lys-105) would be involved in phosphorylation by the unidentified sensory component. A domain containing a DNA-binding motif is found near the carboxy terminus of AgmR (Fig. 6, residues 176 to 190). Although it remains to be shown, it seems reasonable that this helix-turn-helix motif may enable AgmR to bind DNA and to promote transcription of its target genes. Whether these target genes are the *glp* genes or some other suppressor gene(s) will remain unsolved until experiments aimed at elucidation of the allelic nature of *agmR* and *glpR* can be performed. The signal responsible for induction of the *glp* genes is unknown, although glycerol might be the inducing molecule. It is possible that induction might be mediated by the periplasmic glycerol binding in concert with a transmembrane signal protein, in analogy to the mechanism of virulence gene induction described recently in *Agrobacterium* spp. (4).

In summary, the results presented in this study indicate that, if *agmR* is allelic with *glpR* and if GlpR is indeed the activator responsible for regulation of glycerol metabolism in *P. aeruginosa*, its mode of action is clearly more complex than the mode of action of the *glp* repressor of *E. coli* (19). Unlike *E. coli*, in *P. aeruginosa* growth on glycerol induces not just the *glp*-specific enzymes but also enzymes of the Entner-Doudoroff pathway including fructose-1,6-bisphosphate aldolase, a key enzyme required for alginate biosynthesis from various carbohydrate precursors (2, 20). Alginate biosynthesis itself is regulated by at least two unique response regulators (8, 50). Therefore, the regulation of pathways providing the precursors for alginate biosynthesis and the alginate biosynthetic pathway may be interfaced and coordinated by cross-talk between different response regulatory systems. Cross-talk could be mediated by (i) physical interaction of membrane regulatory proteins, as described recently for ToxR and ToxS (9), or (ii) by sharing a common sensory component, as indicated by in vitro experiments in which phosphorylation of OmpR and transcriptional activation of the *ompF* promoter was obtained by using CheA or NR_{II} in place of EnvZ (15). Genes and operons that encode bacterial virulence factors are often subject to coordinate regulation (27). These regulatory systems are capable of responding to environmental signals that may be encountered during the infectious cycle. Understanding the molecular mechanisms governing global regulation of pathogenicity is essential for understanding bacterial infectious disease.

Using the cloned gene, we are constructing a defined *agmR* mutant in the wild-type PAO1 genetic background to enable testing of its phenotype and a comparison to the previously described *glpR2* mutation. In addition, cloning and physical characterization of the *glpR2* allele itself will establish whether *glpR* and *agmR* are physically related.

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