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Cloning and Nucleotide Sequence of the *glpD* Gene Encoding *sn*-Glycerol-3-Phosphate Dehydrogenase of *Pseudomonas aeruginosa*

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Nitrosoguanidine-induced *Pseudomonas aeruginosa* mutants which were unable to utilize glycerol as a carbon source were isolated. By utilizing PAO104, a mutant defective in glycerol transport and *sn*-glycerol-3-phosphate dehydrogenase (*glpD*), the *glpD* gene was cloned by a phage mini-D3112-based in vivo cloning method. The cloned gene was able to complement an *Escherichia coli glpD* mutant. Restriction analysis and recloning of DNA fragments located the *glpD* gene to a 1.6-kb *Eco*RI-*Sph*I DNA fragment. In *E. coli*, a single 56,000-Da protein was expressed from the cloned DNA fragments. An in-frame *glpD'*-*'lacZ* translational fusion was isolated and used to determine the reading frame of *glpD* by sequencing across the fusion junction. The nucleotide sequence of a 1,792-bp fragment containing the *glpD* region was determined. The *glpD* gene encodes a protein containing 510 amino acids and with a predicted molecular weight of 56,150. Compared with the aerobic *sn*-glycerol-3-phosphate dehydrogenase from *E. coli*, *P. aeruginosa* GlpD is 56% identical and 69% similar. A similar comparison with GlpD from *Bacillus subtilis* reveals 21% identity and 40% similarity. A flavin-binding domain near the amino terminus which shared the consensus sequence reported for other bacterial flavoproteins was identified.

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 comprehensive reviews, see references 17 and 23). Our laboratory is interested in studying the regulated carbohydrate metabolic pathways which are able to provide precursors for alginate biosynthesis. Of particular interest to our studies are the previously reported findings that triose phosphates are obligate intermediates in the biosynthesis of alginate (2) and that fructose 1,6-bisphosphate aldolase is essential for this to occur (3). We are therefore focusing our efforts on one peripheral carbon metabolic pathway which is capable of directly providing these triose phosphate intermediates (3), namely, the glycerol metabolic pathway.

In *P. aeruginosa*, glycerol is primarily metabolized through the Entner-Doudoroff pathway (6, 18). Glycerol is transported into the cell by a specific, inducible transport system (*glpT*). Unlike the case for *Escherichia coli*, in *P. aeruginosa* glycerol transport seems to be mediated by a high-affinity, binding protein-dependent transport system (32, 37). Intracellular glycerol is phosphorylated to *sn*-glycerol-3-phosphate (G3P) by glycerol kinase (*glpK*) and subsequently oxidized to dihydroxyacetone phosphate by G3P 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. Like glycerol, G3P can be transported and utilized as the sole carbon source, although it supports growth much more poorly than glycerol does (18). A mutation in a putative regulatory gene (*glpR*) that abolished expression of all known components of the pathway for glycerol catabolism has been isolated (6). As a first step in elucidating the molecular organization and mode(s) of regulation of the *glp* genes of *P. aeruginosa*, we recently cloned the *agmR* gene by complementation of the *glpR2* allele (26). In our ongoing quest to understand the peripheral glycerol carbohydrate metabolic pathway, this paper describes the cloning, nucleotide sequence, and characterization of the *glpD* structural gene.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. The bacterial strains and plasmids used in this study are listed in Table 1. LB medium (24) was used as the rich medium for both *E. coli* and *P. aeruginosa*. As minimal medium, BSM medium (6), M9 medium (24), VB medium (38), or LVM medium (13) 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 used in selection media were ampicillin at 100 μg/ml for *E. coli* and carbenicillin at 500 μg/ml 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.

Mutant isolation. Nitrosoguanidine (NTG)-induced *glp* mutants were isolated by the procedures recommended by Stinson et al. (36) and Cuskey and Phibbs (6), which were appropriately modified for the purposes of this study. Briefly, NTG-treated and washed cells of strain PAO1 were allowed to recover by growing them for several generations (4 h) in LB medium. After being washed, the cells were suspended in BSM medium (6) containing 20 mM glucose as the sole carbon and energy source and grown overnight at 37°C. This step insured that *eda* mutants, which do not grow on either glucose or glycerol, would not be enriched. From the resulting mutant

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant properties	Reference or origin
P. aeruginosa		
PAO1	Prototroph	6
CD10	FP$^-$ *met-9020 pro-9024 blaP9202 blaJ9111 aph-9001* (D3112cts)	7
PAO104	*glp-1*; derived from PAO1 by NTG mutagenesis	This study
E. coli		
DH5αF′	[F′Φ80 *lacZ*ΔM15] Δ(*lacZYA-argF*)U169 *recA1 endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 gyrA relA1*	16
SM10	*thi-1 thr leu tonA lacY supE recA* RP4-2-Tc::Mu Km^r	34
SH305	F$^-$ *araD139* Δ(*argF-lac*)U169 *rpsL150 relA1 thiA1 deoC1 ptsF25 flbB5301 rbsR* Δ*glpD102 recA1 srl*::Tn*10*	29
ECL707	F$^-$ *gld*::ΔTn*10 glpK*::ΔTn*10 ptsD*::ΔTn*10 araD139* Δ(*argF-lac*)U169 *rpsL150 relA1 thiA1 deoC1 ptsF25 flbB5301 rbsR*	35
Plasmids		
pUCP18/19	Ap$^{r a}$; broad-host-range pUC18/19 derivatives	25
pPZ10/20/30	Ap^r; broad-host-range protein fusion vectors	26
pADD948	Ap^r Cm^r; in vivo cloning vector	7
pG11	Ap^r; *glpD*$^+$ (pADD948 with 2.8-kb PAO1 insert)	This study
pEB22	Ap^r; *glpD*$^+$ (6-kb *Eco*RI-*Bam*HI insert from pG11 subcloned into pUCP18)	This study
pEB22ΔE1	Ap^r; *glpD*$^+$ (deletion of *Eco*RI-1–*Eco*RI-2 fragment from pEB22)	This study
pEB22ΔE2	Ap^r; *glpD* (deletion of *Eco*RI-2–*Eco*RI-3 fragment from pEB22ΔE1)	This study
pEB22ΔE1ΔSp	Ap^r; *glpD*$^+$ (deletion of *Sph*I fragment from pEB22ΔE1)	This study
pPS248	Ap^r; *glpD*$^+$ (1.55-kb *Bsi*CI-*Sph*I fragment from pEB22DE1 subcloned into *Sma*I-*Sph*I sites of pUCP18)	This study
pPS249	Ap^r; *glpD* (1.55-kb *Bsi*CI-*Sph*I fragment from pEB22DE1 subcloned into *Sma*I-*Sph*I sites of pUCP19)	This study
pGZ22	Ap^r; Φ(*glpD′-′lacZ*)(Hyb) (262-bp *Eco*RI-2–*Eco*RI-3 fragment from pEB22 in *Eco*RI site of pPZ20)	This study

[a] The *bla* gene of these broad-host-range vectors confers ampicillin resistance (Ap^r) in *E. coli* and carbenicillin resistance (Cb^r) in *P. aeruginosa*.

pool, *glp* mutants were enriched by growing the cells overnight in BSM medium with 20 mM glycerol supplemented with 100 μg of D-cycloserine and 500 μg of carbenicillin per ml. After cells were washed, dilutions were plated on BSM medium supplemented with 0.5 mM succinate and 10 mM glycerol. Prospective *glp* mutants which formed small colonies on this medium (6) were purified on BSM medium with 10 mM glucose and tested for growth on BSM medium containing 10 mM glucose, gluconate, glycerol, L-α-G3P, mannitol, or succinate. Only mutants which did not grow on glycerol and/or G3P but grew normally on all other sugars tested as sole carbon and energy sources were retained for further studies.

Transport assays. Glycerol transport and G3P transport in wild-type PAO1 and mutant strains were measured as follows. Cells were grown overnight in LVM medium containing 10 mM succinate and 0.2% Casamino Acids (CAA). They were washed three times in the same volume of carbon-free LVM. For induction of glycerol transport, the cells were incubated with shaking in LVM medium in the presence or absence of 10 mM glycerol. For induction of G3P transport, cells were treated exactly the same way except that glycerol was substituted by 10 mM G3P. Before determination of glycerol transport, the cells were again washed and treated as described above. Glycerol transport and G3P transport were initiated at room temperature by addition of [U-^{14}C]glycerol (>120 mCi/mmol; Amersham) or [U-^{14}C]G3P (>120 mCi/mmol; New England Nuclear) to the washed cells at final concentrations of 0.3 and 0.7 μM, respectively. Aliquots were taken at the time intervals indicated below, immediately filtered through 0.2-μm-pore-size cellulose acetate filters, and washed with ca. 10 ml of LVM without an added carbon source. The filters were dried, and the radioactivity remaining on them was determined by liquid scintillation counting. Uptake rates are given as picomoles of substrate transported by 4×10^8 cells (A_{540} = 0.5).

Assay of G3P dehydrogenase. Washed membranes were prepared from cells cultured in 400 ml of LVM containing 0.4% CAA with or without inducer (10 mM glycerol or G3P) as previously described (6), except that the cells were disrupted by passage through a French pressure cell and that the membranes were pelleted by centrifugation at 200,000 × *g* for 1 h at 4°C in a Beckman tabletop ultracentrifuge with a TLA100.2 rotor. G3P dehydrogenase in the washed membrane fractions was then measured by a previously described spectrophotometric assay (18). An extinction coefficient of 17,000 M^{-1} cm^{-1} (570 nm) was used for calculations of specific activities. Protein concentrations were determined by the method of Bradford (4) with bovine serum albumin as the standard.

DNA methods. Restriction enzymes, the large fragment of DNA polymerase (Klenow enzyme), T4 DNA polymerase, and T4 DNA ligase were purchased from Bethesda Research Laboratories (Bethesda, Md.), Boehringer Mannheim Canada, or Pharmacia Canada and used as recommended by the suppliers. Small-scale isolations of plasmid DNA from *E. coli* and *P. aeruginosa* were done as previously described (20) with the modification that DNA from *P. aeruginosa* was phenol extracted prior to ethanol precipitation. DNA restriction fragments were eluted from low-gelling-temperature agarose gels as described by Wieslander (39). *E. coli* cells were made competent and transformed by the $CaCl_2$ procedure (8) with the modifications introduced by Schweizer (26). *P. aeruginosa* was transformed by the procedure of Olsen et al. (21). DNA sequence analysis by the dideoxy-chain termination method was performed on double-stranded DNA templates, using a Sequenase kit and the protocol provided by the supplier (United States Biochemical, Cleveland, Ohio), with the modification that elongated templates were treated with terminal deoxynucleotidyl transferase (Boehringer Mannheim Biochemicals Canada) prior to the termination step to avoid

common DNA sequencing ambiguities (14). Labeled DNA fragments were separated on 7 M urea–6% polyacrylamide electrolyte gradient gels as previously described (30). The IBI MacVector v3.5 program (IBI, New Haven, Conn.) or Hitachi MacDNASIS v2.0 program was used for computer-assisted sequence analyses. Chromosomal DNA from PAO1 was isolated by the procedure described by Meade et al. (19) for *Rhizobium meliloti*. Restriction endonuclease-digested chromosomal DNA fragments were transferred to Photogene nylon membranes (GIBCO BRL, Gaithersburg, Md.) as described by Sambrook et al. (24). A 728-bp DNA *Pst*I fragment was excised from pEB22ΔE1 and cloned into pUC19 (41). The resulting plasmid (pPS227) DNA was used as the DNA probe and labelled by random hexamer priming with dioxygenin-dUTP by the Genius Nucleic Acid Labelling System protocol (Boehringer Mannheim Biochemicals Canada). Following transfer and UV fixation, the membranes were probed with the dioxygenin-labelled DNA according to a published procedure (10).

In vivo cloning of *glp*-complementing DNA. For the cloning of DNA fragments complementing the PAO104 *glp* allele, the phage mini-D3112-based in vivo cloning method of Darzins and Casadaban (7) was employed, with the modifications and procedure previously described (26).

Construction of a *glpD'*-*'lacZ* gene fusion. A *glpD'*-*'lacZ* gene fusion was constructed by ligating a 262-bp *Eco*RI fragment from pEB22 to *Eco*RI-cleaved pPZ10, pPZ20, and pPZ30 DNAs. These broad-host-range vectors allow the isolation of LacZ protein fusions in all three translational reading frames (27). After transformation into DH5αF′ followed by selection on LB medium containing ampicillin and X-Gal, only the ligations performed with pPZ20 yielded a large number of $LacZ^+$ transformants. By comparison, no Lac^+ transformants were obtained when pPZ10 and pPZ30 were used. Restriction analyses indicated that the Lac^+ transformants obtained with pPZ20 showed the expected restriction pattern, and one representative clone containing a Φ(*glpD'*-*'lacZ*)(Hyb) fusion (pGZ22) was retained.

Mobilization of recombinant plasmids. When applicable, recombinant plasmids were mobilized from *E. coli* SM10 (34) to *P. aeruginosa* as described by Simon et al. (33).

Polypeptide analysis. For analysis of whole-cell protein profiles, cells were grown overnight in LB-ampicillin medium and 1-ml samples were withdrawn. After the A_{600} was read, the samples were centrifuged and suspended in the appropriate volumes of 2× sample loading buffer (0.125 M Tris, 4% sodium dodecyl sulfate [SDS], 20% glycerol, 5% β-mercaptoethanol, pH 6.8) according to the following formula: 1 ml of cells in culture × A_{600} × 0.08 = microliters of 2× sample buffer needed for resuspension. The samples were boiled for 5 min, and 3-μl aliquots were analyzed on 0.1% SDS–10% polyacrylamide gels with the discontinous buffer system of Laemmli (12).

Nucleotide sequence accession number. The nucleotide sequence reported in this paper has been assigned GenBank accession number L06231.

RESULTS

Isolation of *glp* mutants. Although various mutants specifically defective in the glycerol metabolic pathway have previously been described (18, 32, 37), they either were no longer available or did not prove useful for our studies, partially since they were D3112 resistant. NTG-induced *glp* mutants were obtained by the procedure described in Materials and Methods. According to their growth patterns and their patterns of induction of the *glp*-specific enzymes, these mutants could be divided into several classes (data not shown). The characteristics of one mutant, PAO104 (*glp-1*), pertinent to this study are summarized in Table 2 and Fig. 1. This mutant shows no significant levels of G3P dehydrogenase activity, lacks glycerol transport, and does not utilize glycerol at high concentrations or grow on G3P. However, since glycerol kinase activity is unaffected (e.g., transformation of this strain with plasmids containing only *glpD* restores growth on glycerol [see below]), it is unlikely that PAO104 is a *glp* regulatory mutant, e.g., carrying *glpR* (6). This notion is corroborated by the finding that pG11 (see below) restores growth of PAO104 on glycerol but fails to restore growth of the *glpR2* mutant PRP406 (6). Thus, either *glp-1* comprises two mutations which exert a polar effect on each other or, alternatively, PAO104 contains two mutations affecting transport and dehydrogenase separately. Despite the lack of its transport, glycerol is still able to induce G3P transport at high (10 mM) concentrations.

TABLE 2. G3P dehydrogenase activity in PAO1, PAO104, and plasmid-containing derivatives

Strain/plasmid	Genotype	G3P dehydrogenase sp act[a]	
		Uninduced	Induced
PAO1	Wild type	0.22	7.10
PAO104	*glp-1*	0.33	0.28
PAO104/pUCP18	*glp-1*	0.17	0.33
PAO104/pPS248	*glp-1*/*glpD*$^+$	9.30	14.62

[a] G3P dehydrogenase activity was measured in washed membranes isolated from cells grown in LVM medium with 0.4% CAA (uninduced) or in the same medium supplemented with 10 mM glycerol (induced). Specific activities are given as nanomoles per minute per milligram of protein.

Figure 1 shows the kinetics of glycerol and G3P transport in PAO104 compared with those in wild-type strain PAO1. It is evident that the *glp-1* mutation totally abolishes glycerol transport but has no effect on G3P transport. Therefore, this mutation might be equivalent to the previously described *glpT* mutations (32, 37).

Cloning of DNA complementing the growth defect of *glp* mutant PAO104. A DNA fragment complementing the growth defect of PAO104 was cloned from strain CD10 by infecting this mutant with a D3112 lysate containing random fragments of chromosomal DNA as previously described (26). After phage infection, the cells were spread on LVM plates containing glycerol and carbenicillin and incubated at 37°C. By this method, several glycerol-positive clones per plate were obtained after 48 to 60 h. After single-colony purification, plasmid DNA was isolated from 10 of these clones and analyzed by digestion with *Bam*HI and *Eco*RI. All clones were found to contain plasmids with chromosomal inserts ranging from <10 to >30 kb.

To ascertain whether the clones carried DNA complementing the *glp-1* mutation(s) of PAO104, they were transformed into the *E. coli* mobilizer strain SM10. The plasmids were then transferred back to PAO104 by mating, using VBMM medium containing carbenicillin as the selection medium. When tested on LVM glycerol medium in the presence of carbenicillin, only one of the transconjugants, containing a plasmid designated pG11, grew. These results indicated that pG11 contains sequences able to complement the growth defect of PAO104. Plasmid DNA was again isolated from the transconjugant and analyzed by digestion with *Eco*RI and *Bam*HI. Surprisingly, after passage through *E. coli* SM10, the original chromosomal insert of >30 kb was reduced to ca. 2.8 kb. When pG11 was isolated from *E. coli* SM10, it was found to be identical in size

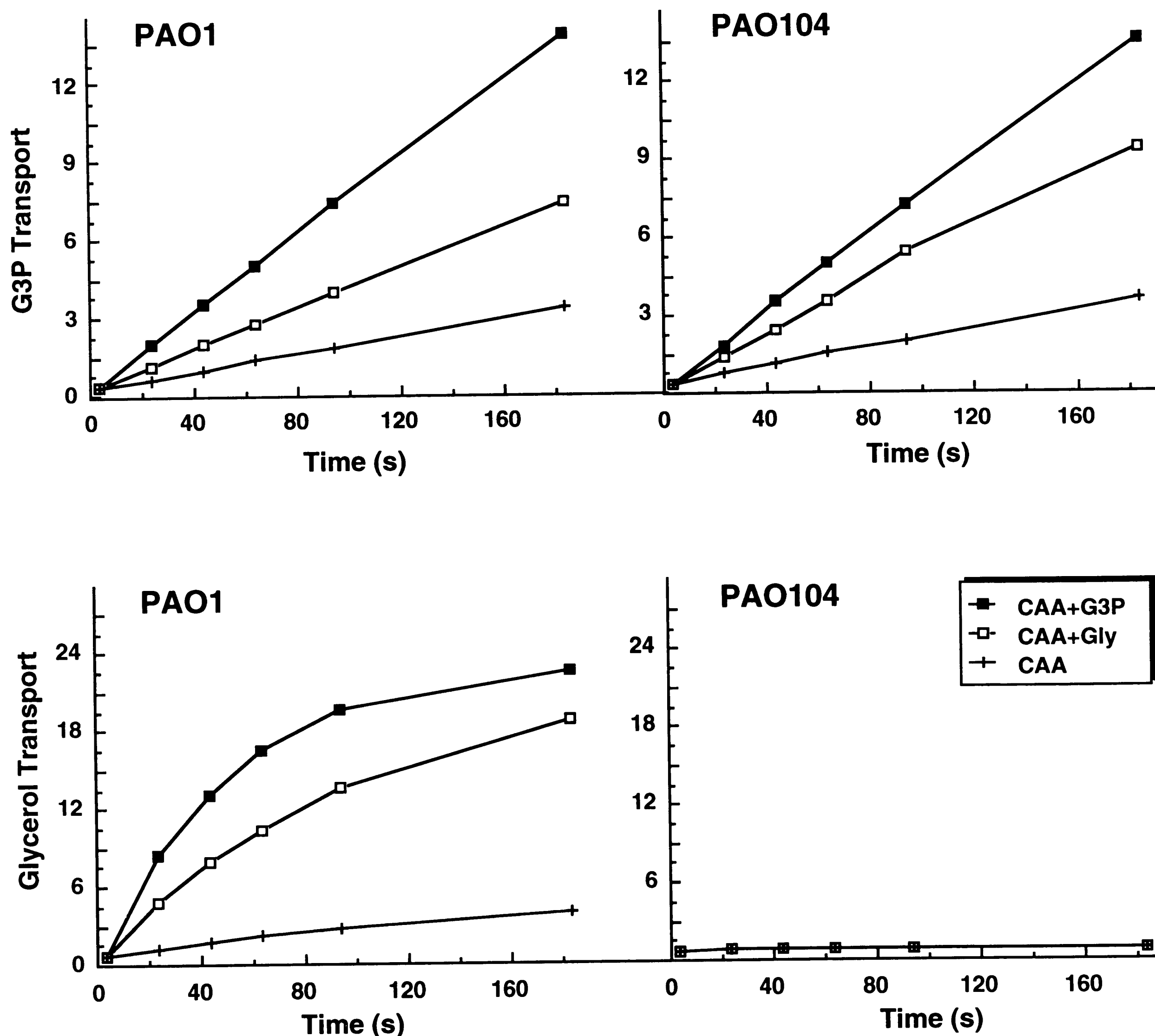

FIG. 1. Glycerol and G3P transport in wild-type PAO1 and *glp-1* mutant PAO104. Cells of strains PAO1 and PAO104 were grown overnight in LVM medium containing 10 mM succinate and 0.2% CAA. Glycerol (Gly) and G3P transport were induced and measured as described in Materials and Methods. Uptake rates are given as picomoles of substrate transported by 4 × 10^8 cells (A_{540} = 0.5).

to that found subsequently in the transconjugant after mobilization, indicating that it underwent a substantial deletion when originally transformed into strain SM10. However, since pG11 was still able to complement the growth defect of PAO104, it was retained for further studies. Although pG11 complements the growth defect of PAO104, it does not restore glycerol transport (data not shown), indicating that (i) it contained sufficient DNA only to complement the *glp-1* mutation affecting G3P dehydrogenase activity and (ii) the *glpD*-containing region was unaffected by this rearrangement (see below). Plasmid pG11 did not restore growth of the *glpR2* mutant strain PRP406 (6) on glycerol.

Subcloning and DNA sequence analysis of the *glpD* gene. The *glpD*-complementing activity was further localized on pG11 by deletion and subcloning analyses. The results are summarized in Fig. 2. The same plasmids were also used to complement various known *E. coli glp* mutations and were found to specifically complement the *glpD* mutation of SH305 but not the *glpK* mutation of ECL707. These results provided further evidence that the *P. aeruginosa glpD* structural gene had been cloned and that this gene was located in its entirety on pEB22ΔE1. The nucleotide sequence of most of the chromosomal DNA contained on this plasmid was determined by subcloning appropriate fragments into pUC18/19 (41), its broad-host-range derivatives pUCP18/19 (25), or M13mp18/19 (41). The sequence was determined for both strands, and the sequence across restriction sites used for subcloning was determined by sequencing overlapping clones. The sequence thus determined (Fig. 3) has a GC content of 67.5%, which is typical for *P. aeruginosa* DNA, and contains an open reading frame of 510 amino acids constituting a protein with a predicted molecular weight of 56,150. This size is in excellent agreement with the molecular weight of 56,000 determined in subsequent expression experiments (see Fig. 5). The *glpD* open reading frame was assigned by virtue of the fact that it is the only open reading frame in frame to the Φ(*glpD'-'lacZ*)(Hyb)

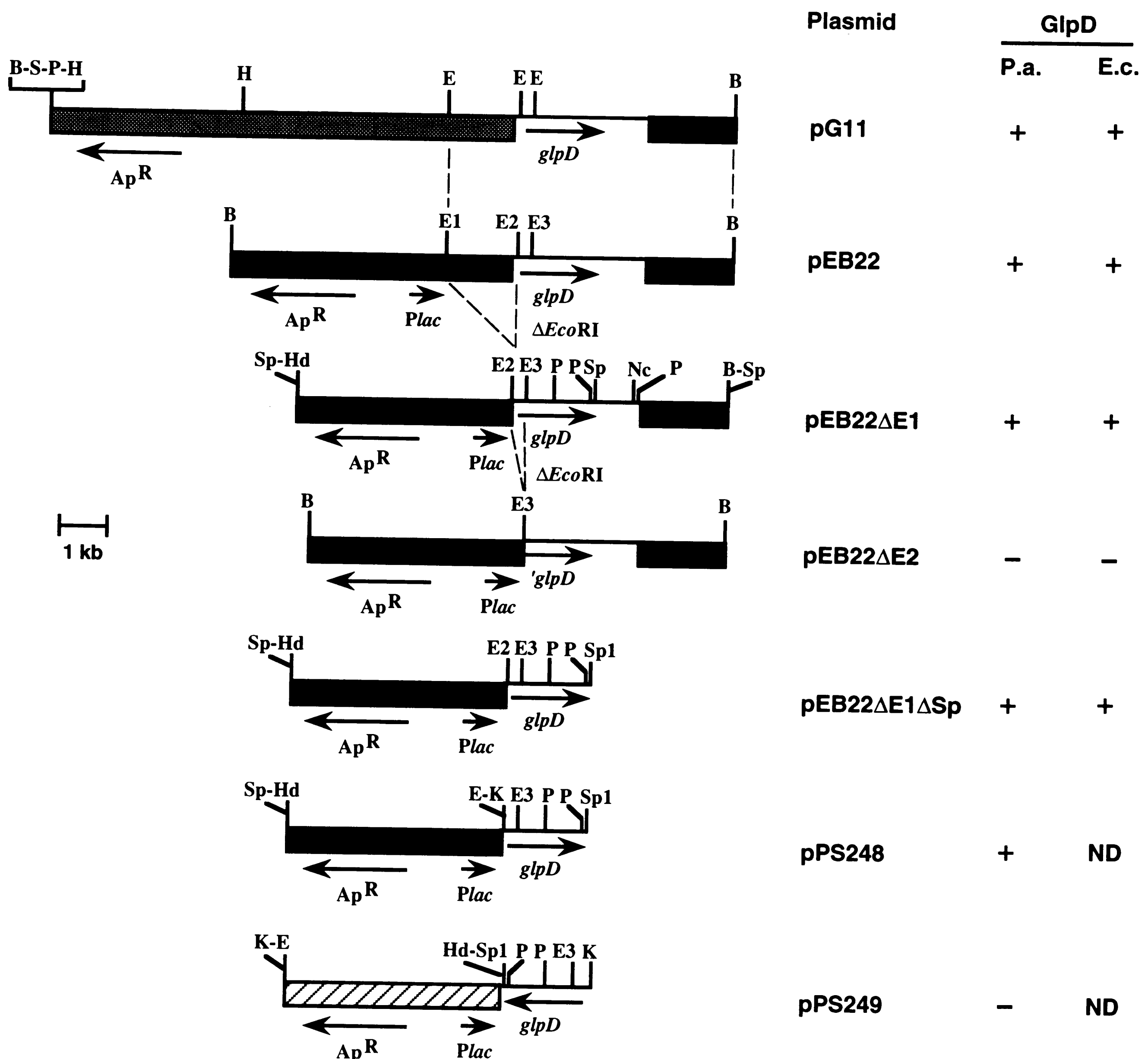

FIG. 2. Restriction maps of recombinant plasmids: subcloning and deletion analysis. The *glpD* complementation activities of the individual clones were determined as described in the text, using the *P. aeruginosa glp-1* mutant PAO104 (P.a.) and the *E. coli glpD* mutant SH305 (E.c.) as the plasmid hosts. Ap^R indicates the *bla* gene, which confers ampicillin resistance in *E. coli* and carbenicillin resistance in *P. aeruginosa*. P*lac* indicates the location of the *lac* promoter. Abbreviations: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; S, *Sal*I; Sp, *Sph*I; Nc, *Nco*I. B-Sp (*Bam*HI-*Xba*I-*Sal*I-*Pst*I-*Sph*I) and Sp-Hd (*Sph*I-*Hin*dIII) indicate the residual sites of the polylinker of pUCP18. Similarly, E-K (*Eco*RI-*Sac*I-*Kpn*I) and Sp-Hd (*Sph*I-*Hin*dIII) mark the residual polylinker sites of pUCP19. The solid line indicates chromosomal DNA, the grey shaded box represents pADD948 DNA, the black box indicates pUCP18 DNA, and the hatched box indicates pUCP19 DNA.

construct pGZ22 (see Materials and Methods), resulting in a $LacZ^+$ fusion protein. The Lac^+ phenotype indicates that the reading frames of *glpD* and *lacZ* must be identical. In addition, this open reading frame is located on the correct strand and is transcribed in the correct direction as deduced from the expression experiments described above. The translation initiation codon was assigned to the ATG codon at nucleotide (nt) 47, since its upstream region (AGGG) from nt 35 to 38 (underlined in Fig. 3) had a reasonable match to the consensus ribosome binding site (31). In contrast, the only other possible initiation codon (GTG at nt 91) is not preceded by a suitable ribosome binding site. No promoter-like sequences were found in the sequences located upstream of the *glpD* gene, indicating that expression of this gene might be from promoters provided by vector sequences. To test this hypothesis, two experiments were performed.

First, the entire *glpD* coding region was excised from pEB22ΔE1 on a *Bsi*CI-*Sph*I fragment and cloned between the *Sma*I and *Sph*I sites of pUCP18 (pPS248) or pUCP19 (pPS249) (25). It should be noted that cleavage with *Sph*I

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EcoRI
GAATTCGAACAAAACTACGTTCACTTTCGACTTCAGGGCCTTTCACATGAGTCAAGCGCA   60
   BsiCI                           SD             M  S  Q  A  H     5

CACCCCCTCCGCTCCACTCGCCGAAGTCTACGATGTCGCCGTGGTCGGCGGTGGCATCAA  120
 T  P  S  A  P  L  A  E  V  Y [D  V  A  V  V  G  G  G  I  N    25

CGGTGTAGGGATCGCCGCCGACGCAGCCGGGCGCGGCCTGTCCGTGTTCCTTTGCGAACA  180
 G  V  G  I  A  A  D  A  A  G  R  G  L  S  V  F  L  C  E  Q]   45

GCACGACCTGGCCCAGCACACTTCCTCGGCCAGCAGCAAGCTGATCCACGGCGGCCTGCG  240
 H  D  L  A  Q  H  T  S  S  A  S  S  K  L  I  H  G  G  L  R    65
                 EcoRI
CTACCTCGAACACTACGAATTCCGCCTGGTCCGCGACACGCTGGCCGAGCGCGAGGTCCT  300
 Y  L  E  H  Y  E  F  R  L  V  R  D  T  L  A  E  R  E  V  L   85
  BalI
GCTGGCCAAGGCGCCGCACATCGTCAAGCCGCTGCGCTTCGTCCTGCCGCACCGTCCGCA  360
 L  A  K  A  P  H  I  V  K  P  L  R  F  V  L  P  H  R  P  H   105
                          NaeI
CCTGCGCCCGGCCTGGATGATCCGCGCCGGCCTGTTCCTCTACGATCACCTCGGCAAGCG  420
 L  R  P  A  W  M  I  R  A  G  L  F  L  Y  D  H  L  G  K  R   125

CGAGAAGCTGCCCGCCTCGCGCGCCTGCGTTCACCGGCAGCAGCCGCTGAAGGCCGAGAT  480
 E  K  L  P  A  S  R  A  C  V  H  R  Q  Q  P  L  K  A  E  I   145

CCGCCGTGGCTTCGAGTACTCCGACTGCGCGGTGGACGATGCGCGCCTGGTGGTGCTCAA  540
 R  R  G  F  E  Y  S  D  C  A  V  D  D  A  R  L  V  V  L  N   165

CGCGATCTCCGCCCGCGAGCACGGCGCCCACGTCCATACCGCACCCGCTGCGTCAGCGCC  600
 A  I  S  A  R  E  H  G  A  H  V  H  T  A  P  A  A  S  A  P   185

CGTCGCAGCAAGGGACTCTGGCACCTGCACCTGGAGCGCAGCGACGGCAGCCTGTATTCG  660
 V  A  A  R  D  S  G  T  C  T  W  S  A  A  T  A  A  C  I  R   205
                          NaeI
ATCCGCCCGCGCCCTGGTGAACGCCGCCGGCCCCTGGGTAGCGCGCTTCATCCAGGACGA  720
 S  A  R  A  L  V  N  A  A  G  P  W  V  A  R  F  I  Q  D  D   225

CCTGAAACAGAAGTCGCCCTACGGTATCCGCCTGATCCAGGGCAGCCACATCATCGTGCC  780
 L  K  Q  K  S  P  Y  G  I  R  L  I  Q  G  S  H  I  I  V  P   245
                              PstI
GAAGCTGTACGAAGGCGAGCACGCCTACATCCTGCAGAACGAAGACCGCCGCATCGTCTT  840
 K  L  Y  E  G  E  H  A  Y  I  L  Q  N  E  D  R  R  I  V  F   265

CGCCATCCCCTATCTGGACCGTTTCACCATGATCGGCACCACCGATCGCGAGTACCAGGG  900
 A  I  P  Y  L  D  R  F  T  M  I  G  T  T  D  R  E  Y  Q  G   285

CGACCCGGCCAAGGTGGCGATCAGCGAAGAGGAAACCGCCTACCTGCTGCAAGTGGTGAA  960
 D  P  A  K  V  A  I  S  E  E  E  T  A  Y  L  L  Q  V  V  N   305
                        NotI                      NaeI
CGCCCATTTCAAGCAGCAACTCGCGGCCGCCGACATCCTGCACAGCTTCGCCGGCGTGCG 1020
 A  H  F  K  Q  Q  L  A  A  A  D  I  L  H  S  F  A  G  V  R   325

TCCGCTGTGCGACGACGAGTCCGACGAGCCTTCGGCGATCACCCGCGACTACACCCTGTC 1080
 P  L  C  D  D  E  S  D  E  P  S  A  I  T  R  D  Y  T  L  S   345
       NaeI
GCTCTCCGCCGGCAACGGCGAGCCGCCGCTGCTCTCGGTGTTCGGCGGCAAGCTGACCAC 1140
 L  S  A  G  N  G  E  P  P  L  L  S  V  F  G  G  K  L  T  T   365

CTACCGCAAGCTGGCCGAATCGGCGCTGACGCAGCTCCAGCCGTTCTTCGCCAATCTCGG 1200
 Y  R  K  L  A  E  S  A  L  T  Q  L  Q  P  F  F  A  N  L  G   385

CCCGGCCTGGACCGCCAAGGCACCGCTGCCGGGCGGCGAGCAGATGCAGAGCGTCGAGGC 1260
 P  A  W  T  A  K  A  P  L  P  G  G  E  Q  M  Q  S  V  E  A   405
             BalI
GCTCACCGAGCAACTGGCCAACCGCTACGCCTGGCTCGACCGCGAACTGGCGCCTGCGCT 1320
 L  T  E  Q  L  A  N  R  Y  A  W  L  D  R  E  L  A  P  A  L   425
                SmaI
GGGCGCGCACTACGGCACCCGGGTGTGGCGCCTGCTGGACGGGGTCAATGGCGAAGCCGA 1380
 G  A  H  Y  G  T  R  V  W  R  L  L  D  G  V  N  G  E  A  D   445

TCTCGGCGAGCACCTGGGCGGCGGCCTGTATGCCCGCGAAGTGGACTACCTGTGCAAGCA 1440
 L  G  E  H  L  G  G  G  L  Y  A  R  E  V  D  Y  L  C  K  H   465
    ApaI
CGAGTGGGCCCAGGACGCCGAGGACATCCTCTGGCGCCGCAGCAAGCTCGGCCTGTTCCT 1500
 E  W  A  Q  D  A  E  D  I  L  W  R  R  S  K  L  G  L  F  L   485
                                 PstI
CTCGCCGAGCCAGCAGGTGCGCCTCGGCCAGTACCTGCAGAGCGAGCATCCGCATCGCCC 1560
 S  P  S  Q  Q  V  R  L  G  Q  Y  L  Q  S  E  H  P  H  R  P   505
SacII  SphI
GCGGGTGCATGCGGCCTGATCGCCAGCCAGAAACGTCGAGGCCCGCTTTTGCGGGCCTCG 1620
 R  V  H  A  A  *     --  ---  ---- --------->    <---------   510

TTCGTTATGGAAGGGTACGGGACGATCCGGGCATAATGCGCGACGCCACCAAGCCGGTGG 1680
 ----  ---  --
CGCCGCCGCAGTCGAGAATCCCGTGGACCTGATGTTCAAGGCGCTGATCGGCGCCGCCGT 1740
                                                NaeI
GGTCGTGCTCCTCGCCGTCCTGTCGAAGACCCGCAACTACTACATCGCCGGC 1792
```

FIG. 3. Nucleotide sequence of the *glpD* region. The predicted amino acid sequence of the *glpD* reading frame is indicated below the appropriate codons. Its putative ribosome binding (Shine-Dalgarno [SD]) site is underlined. The amino acids which constitute a putative flavin-binding domain are bracketed (see Fig. 6 for details). The dashed underlines (between nt 1583 and 1634) mark nucleotides capable of forming putative stem-loop structures.

removes the last codon of *glpD* (Fig. 3) and that ligation into the same site of the pUCP18 vector leads to the formation of an in-frame fusion of GlpD and LacZα. However, this results in expression of an active GlpD protein (Table 2). When the resulting plasmids were transformed into the *glp-1* mutant PAO104, only the pUCP18-based subclone pPS248 containing *glpD* in the same transcriptional orientation as the *lac* promoter (P*lac*) could complement *glp-1* (Fig. 2). Since *glpD* is transcribed from P*lac*, which is constitutively expressed in *P. aeruginosa*, GlpD activity can be detected in uninduced PAO104 cells at levels that exceed the levels observed in induced PAO1 cells. This activity was only slightly (1.5-fold) higher in extracts from glycerol-grown cells compared with the same activity observed in extracts obtained from uninduced cells. Since normal induction ratios range from 20- to 30-fold (e.g., 32-fold for PAO1 [Table 2]), this increase is insignificant and probably does not reflect induction of transcription from a glycerol-inducible promoter. Similar results were obtained when the insert from pEB22 was cloned in the reverse orientation into pUCP19 (data not shown).

Second, the Φ(*glpD′*-*′lacZ*)(Hyb) construct pGZ22 was transformed into PAO1, and β-galactosidase (βGal) activities were assayed in LVM-CAA-glycerol-grown (induced) and LVM-CAA-grown (uninduced) cells. No significant difference in βGal levels was observed (data not shown). The low-level βGal expression in pGZ22 (10 to 20 nmol/min · mg) is probably due to the previously described pPZ20 weak vector promoter (27). These results confirmed that *glpD* cloned in pEB22 and its derivatives is most likely expressed from P*lac* and not from its own promoter.

A search for possible secondary structures in the nucleotide sequence flanking the *glpD* coding region by utilizing the RNA secondary structure prediction feature of the Hitachi MacDNASIS program revealed the presence of palindromic sequences which are located immediately downstream of the *glpD* termination codon (Fig. 3; underlined). These sequences are capable of forming two putative stem-loop structures: (i) a shorter stem-loop with 10 bases pairing without mismatches between nt 1597 and 1620 ($\Delta G = -27.3$ kcal/mol [ca. -114 kJ/mol]) and (ii) a longer-stem loop with 19 bases pairing and several mismatches (bulges) between nt 1583 and 1634 ($\Delta G = -40.2$ kcal/mol [ca. -167 kJ/mol]).

The continuity of the sequenced DNA region was verified by Southern analysis of chromosomal DNA (Fig. 4). PAO1 chromosomal DNA was digested with *Bsi*CI plus *Nco*I (lane 1) and *Pst*I (lane 2) and probed with an internal *Pst*I fragment (nt 812 to 1540) from pEB22ΔE1. The presence of the expected 728-bp *Pst*I fragment and 2,242-bp *Bsi*CI-*Nco*I fragment (the *Nco*I site is located 448 bp downstream of the *Nae*I site as shown in Fig. 3 [cf. Fig. 2]) indicated the presence of these sequences in the PAO1 genome and demonstrates the absence of rearrangements from the sequenced region.

Identification of the *glpD* gene product. Plasmids pEB22, pEB22ΔE1, and pEB22ΔE2 were transformed into *E. coli* DH5αF′, and total cellular proteins were analyzed by SDS-polyacrylamide electrophoresis (Fig. 5). Cells containing

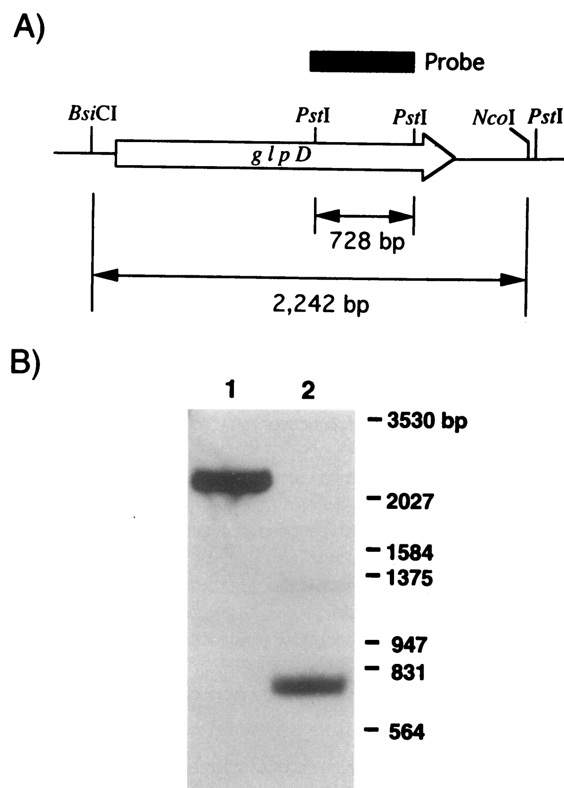

FIG. 4. Genomic Southern analysis of the *glpD* region. The physical map of the PAO1 *glpD* region is shown (A). Genomic DNAs were digested to completion (B) with *Bsi*CI plus *Nco*I (lane 1) and *Pst*I (lane 2) and hybridized to the biotin-labelled 728-bp *Pst*I fragment shown in panel A. The molecular sizes of standards (λ DNA digested with *Eco*RI plus *Hin*dIII) are indicated on the right.

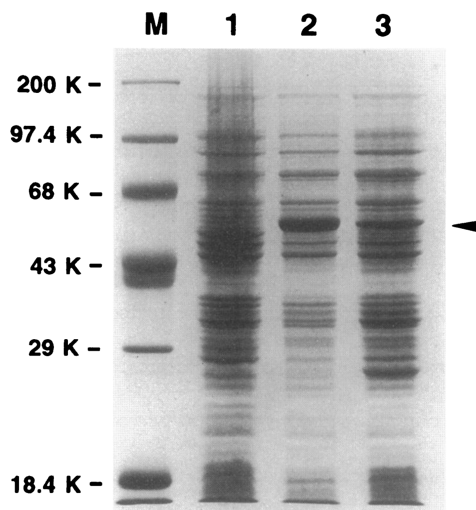

FIG. 5. Overexpression of *P. aeruginosa* GlpD in *E. coli* DH5αF′. Strain DH5αF′ harboring various plasmids was grown overnight at 37°C in LB medium containing 100 μg of ampicillin per ml. Samples were withdrawn, and after the optical density at 600 nm was read, the samples were centrifuged and the cells were suspended in the appropriate volumes of 2× sample loading buffer (26) to adjust for differences in optical densities. Protein fractions were separated on a 0.1% SDS–10% polyacrylamide gel. Lane 1, pEB22ΔE2; lane 2, pEB22ΔE1; lane 3, pEB22. The arrowhead marks the position of the overproduced 56,000-molecular-weight protein. Lane M, molecular weight markers (top to bottom: myosin, phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, and β-lactoglobulin) in thousands (K).

pEB22 (lane 3) and pEB22ΔE1 (lane 2) overproduced a ca. 56,000-molecular weight polypeptide which was absent in extracts from cells containing pEB22ΔE2 (lane 1). This size is in excellent agreement with the molecular weight of 56,000 previously reported for the *E. coli* GlpD protein (1, 29) and with the molecular weight of 56,150 predicted by DNA sequencing (Fig. 3). It is evident that deletion of the *Eco*RI fragment bordered by the *Eco*RI-1 and *Eco*RI-2 sites led to a relative increase in GlpD expression, whereas deletion of the fragment from *Eco*RI-2 to *Eco*RI-3 led to the total loss of expression of the 56,000-molecular-weight protein. The relative increase in expression from pEB22ΔE1 compared with that from pEB22 was probably due to the fact that P*lac* was brought closer to the start of the *glpD* gene by deletion of the fragment from *Eco*RI-1 to *Eco*RI-2. Deletion of the fragment from *Eco*RI-2 to *Eco*RI-3 removed the N terminus of the structural gene (Fig. 3).

Characteristics of the amino acid sequence of GlpD. The predicted amino acid sequence of GlpD was used to search the National Biomedical Research Foundation data base with the FASTP program (15). From this search, this open reading frame was found to have homology with a number of bacterial flavin-binding proteins (Fig. 6). A flavin-binding domain exhibiting 72% identity (or 93% similarity when conserved amino acid substitutions are counted) to the corresponding domain of the *E. coli* GlpD protein can be identified close to the N terminus (residues 16 to 45). In addition, compared with other bacterial flavoproteins, the characteristic residues and spacing of the consensus sequence are conserved.

An alignment of the predicted *P. aeruginosa* GlpD sequence with the previously sequenced *glpD* genes encoding the corresponding GlpD polypeptides from *E. coli* (1) and *Bacillus subtilis* (11) is shown in Fig. 7. A comparison of the *P. aeruginosa* GlpD sequence (510 aa) with the *E. coli* GlpD sequence (501 aa) reveals that the two polypeptides are 56% identical and 69% similar. Although the two polypeptides are most identical in the N-terminal regions, there are periodicities of substantial homology throughout the proteins. A similar comparison of *P. aeruginosa* GlpD with its counterpart from *B.*

		G	X	G	XX	G	XXX	A	XXXXXX	G	consensus		
16	DVAVV	G	G	G	IN	G	VGI	A	ADAAGR	G	LSVFLCEQ	45	GlpD P.a.
5	DLIVI	G	G	G	IN	G	AGI	A	ADAAGR	G	LSVLMLEA	33	GlpD E.c.
22	DLFII	G	G	G	IT	G	AGT	A	LDAASR	G	MKVALSEM	53	GlpD B.s.
10	DVIII	G	G	G	AT	G	AGI	A	RDCALR	G	LRVILVER	39	GlpA E.c.
6	DTVIM	G	G	G	LA	G	LLC	G	LQLQKH	G	LRCAIVTR	33	GlpB E.c.
6	KIVIV	G	G	G	AG	G	LEM	A	TQLGHK	K	AKITLVDR	40	NDH E.c.
8	DAVVI	G	A	G	GA	G	IAR	L	AQISQS	G	QTCALLSK	37	SdhA E.c.

FIG. 6. Amino acid sequences of the flavin-binding sites from various flavoproteins from *B. subtilis* (B.s.), *E. coli* (E.c.), and *P. aeruginosa* (P.a.) GlpD, aerobic G3P dehydrogenase (1, 11); GlpA and GlpB, anaerobic G3P dehydrogenase subunits (5); NDH, NADH dehydrogenase (42); SdhA, succinate dehydrogenase (40).

```
                 METKDLIVIGGGINGAGIAADAAGRGLSVLMLEAQDLACATSSASSKLIHGGLRY    55 E.c.
                     |  |: |||||||| |||||||||||||:: |   |||  |||||||||||||||
          MSQAHTPSAPLAEVYDVAVVGGGINGVGIAADAAGRGLSVFLCEQHDLAQHTSSASSKLIHGGLRY    66 P.a.
            :      :       ||: ::|||| | | | |||:||: | | |   |:|  ||| |:|::||||||
MMNHQFSSLERDRMLTDMTKKTYDLFIIGGGITGAGTALDAASRGMKVALSEMQDFAAGTSSRSTKLVHGGLRY    74 B.s.

LEHYEFRLVSEALAEREVLLKMAPHIAFPMRFRLPHRPHLRPAWMIRI-GLFMYDHLGKRTSLPGST--   121 E.c.
|||||||| ::|||||||| |||| |:|| |||||||||||||| |||:||||||| ||:|
LEHYEFRLVRDTLAEREVLLAKAPHIVKPLRFVLPHRPHLRPAWMIRA-GLFLYDHLGKREKLPASR--   132 P.a.
|  :|:::| :   || :::  :||:  |  ::|| :       : :  : || :|| |:   |
LKQFEVKMVAEVGKERAIVYENGPHVTTPEWMLLPFHKGGTFGSFTTSIGLRVYDFLAGVKKSERRSML   143 B.s.

---GLRFGANSVLKPEIKRGFEYSDCWVDDARLVLANAQMVVRKGGEVLTRTRATSAR---RENGLWIV   184 E.c.
    :  :  ||:||:|||||||| |||||||: ||      :|: | | : | ||     |: |   :
----ACVHRQQPLKAEIRRGFEYSDCAVDDARLVVLNAISAREHGAHVHTAPAA-SAPVAARDSGTCTW   196 P.a.
    :  :     |      | | :  ||||| :      |   ||      :         |     : : :
SAKETLQKEPLVKKDGLKGGGYYVEYRTDDARLTIEVMKEAVKFGAEPVNYSKVKELLYEKGKAVGVLI   212B.s.

EAEDIDTGKKYSWQARGLVNATGPWVKQF-FDDGMHLPSPYGIRLIKGSHIVVPRVHTQKQAYILQNED   249 E.c.
 |       :      ||:||||:|||| :|  ||       |||||||| ||||:||::        ||||||||
SAATAACIRS----ARALVNAAGPWVARFIQDDLKQK-SPYGIRLIQGSHIIVPKLYEGEHAYILQNED   257 P.a.
         :      |: :|||:|||  | : |          :        | |:::   : : :   ::
--EDVLTKKEYKVYAKKIVNATGPWVDQLREKDHSKNGKHLQHTK--GIHLVFDQSVFPLKQAVYF---   274 B.s.

----KRIVFVIPWMDEFSIIGTTDVEYKGDPKAVKIEESEINYLLNVYNTHFKKQLSRD-DIVWTYSGV   315 E.c.
     :|||| ||::| |::||||| || |||  ||| | |  |||:|:|:||| ||:    ||: :::||
----RRIVFAIPYLDRFTMIGTTDREYQGDPAKVAISEEETAYLLQVVNAHFKQQLAA-ADILHSFAGV   323 P.a.
      |:|||||      |::||||  |         :  :: |:  |::  :|   |  :    :   ||  |:||:
DTPDGRMVFAIPREGK-TYVGTTDTVYKEALEHPRMTTEDRDYVIKSINYMFPELNITANDIESSWAGL   342 B.s.

RPLCDDESDSPQAITRDYTLDIHDENGKAPLLSVFGGKLTTYRKLAEHALEKLTPYYQGIGPAWTKESV   381 E.c.
||||||||| | |||||||| :     || :||||||||||||||||||| ||     |:: :|||||  :
RPLCDDESDEPSAITRDYTLSLSAGNGEPPLLSVFGGKLTTYRKLAESALTQLQPFFANLGPAWTAKAP   389 P.a.
|||  :|: :|| |:|    :  | :            |||||:|||:|:  :  :   : : |     :
RPLIHEEGKDPSEISRKDEIWTSDSGLITI----AGGKLTGYRKMADDIVDLVRDRLKEEGEKDFGPCK   407 B.s.

LPGGAIEGDRDDYAARLRRRYPFLTESLARHYARTYGSNSELLLGNAGTVSDLGEDFGHEFYEAELKYL   447 E.c.
||||        : ::  |  ||::|    ||  :     ||:      ||         :||||  :|  :|    |: ||
LPGGEQMQSVEALTEQLANRYAWLDRELAPALGAHYGTRVWRLLDGVNGEADLGEHLGGGLYAREVDYL   455 P.a.
            :  : : :|     ::       |      |  :    :: : :  | | :  :      :      |      ::
TKNMPISGGHVGGSKNLMSFVTAKTKEGIAAGLSEKDAKQLAIRYGSNVDRVFDRVEALKDEAAKRNIP   475 B.s.

VDHEWVRRADDALWRRTKQGMWLNADQQSRVSQWLVEYTQQRLSLAS   501 E.c.
 |||   |:|  ||||:|  |::|  : ||  |::|:|           |   : :
CKHEWAQDAEDILWRRSKLGLFLSPSQQVRLGQYLQSEHPHRPRVHAA   510 P.a.
   :           :           :  : |:         : : :        : : :
VHILAEAEYSIEEEMTATPADFFVRRTGRLFFDINWVRTYKDAVIDFMSERFQWDEQAKNKHTENLNKLLHDAVVPLEQ   554 B.s.
```

FIG. 7. Sequence alignments of *P. aeruginosa* (P.a.) GlpD with GlpD from *B. subtilis* (B.s.) and *E. coli* (E.c.). 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. The flavin-binding sites in the respective proteins are underlined.

subtilis (554 aa) reveals 21% identity and 40% similarity. As seen with *E. coli* GlpD, the two polypeptides are most identical in the N-terminal regions and have substantial homology elsewhere in the proteins, with the least homology in the C-terminal portions.

DISCUSSION

In our quest to understand the peripheral glycerol carbon metabolic pathway in *P. aeruginosa*, various mutants defective in this pathway were isolated by NTG mutagenesis. This method was chosen because repeated attempts to isolate Tn*501*-induced mutants failed. In our hands, the previously described method of enrichment for *glp* mutants (6) yielded unacceptably large numbers of *eda* mutants, which are deficient in glucose 6-phosphate dehydrogenase and do not grow on either glucose or glycerol (for a review, see reference 23). We found that this problem could be avoided by simply outgrowing the NTG-treated cells in minimal medium containing glucose as the sole carbon and energy source before enriching for specific *glp* mutants.

Of particular interest for the purposes of this study was a mutant (PAO104 *glp-1*) which simultaneously became defective in glycerol transport and G3P dehydrogenase. Thus, *glp-1* may confer the same defects as the previously described *glpT* (32, 37) and *glpD* mutations, respectively. From the results presented in this study, it is unlikely that this phenotype is caused by a regulatory mutation similar to *glpR2* (6). If that were the case, transformation of PAO104 with plasmids containing only *glpD* would not restore growth on glycerol, since *glpK* would not be expressed, and one would expect complementation of *glpR2* by pG11. It is presently unclear whether *glp-1* represents a polar mutation or whether it comprises two mutations affecting transport and dehydrogenase separately. As a first step to test the latter hypothesis, a gentamicin

resistance gene cassette was inserted at a *Nae*I site located 210 bp downstream of the end of *glpD* (Fig. 3), and the insertionally inactivated downstream region was then crossed into the chromosome by the previously described gene replacement procedure (28). The resulting mutant grew on glycerol and transported glycerol normally (data not shown). Thus, if the gene(s) encoding the glycerol transport system (*glpT*) and *glpD* constitute an operon, *glpT* would have to be located proximally to *glpD*.

Although the *glp-1* mutation totally abolishes glycerol transport, it has no effect on G3P transport (Fig. 1). Even though the exact nature of this mutation is unknown, this is the first genetic evidence that glycerol and G3P transport might be mediated by two distinct transport systems. Thus, the coinduced permeability of *P. aeruginosa* to glycerol and G3P is probably not due to one uptake system that is considerably more specific for glycerol than for G3P, as previously suggested (32). It is interesting that despite the lack of its transport, glycerol is still able to induce G3P transport at high (millimolar) concentrations. This suggests that at high concentrations glycerol may be diffusing freely through the membrane, which is consistent with the finding that transformation of PAO104 with $glpD^+$-containing plasmids restores its ability to grow on glycerol in the absence of glycerol transport.

A *glpD*-complementing DNA fragment was cloned by a phage D3112-based in vivo cloning method. The original clone (>30 kb) was found to be unstable in *E. coli*. This situation is reminiscent of the previously reported finding that the *B. subtilis glpPKD* region could not be recovered in nonmutated form from *E. coli* (11). However, a single clone containing ca. 2.8 kb of contiguous *P. aeruginosa* chromosomal DNA could be recovered, and Southern analysis proved the presence of the analyzed region in the PAO1 chromosome. Subcloning, expression, and DNA sequence analyses indicated that high-level *glpD* expression required for complementation was not achieved from its own promoter but required plasmid-associated promoters. These results support the hypothesis that like in *B. subtilis* (11), *glpD* may be part of an operon containing an additional *glp* gene(s).

The deduced amino acid sequence of *P. aeruginosa* GlpD (Fig. 7) proved its relatedness to the *glpD*-encoded gene products from *E. coli* and *B. subtilis*. A comparison of the GlpD sequences from these organisms showed a closer evolutionary relatedness between *P. aeruginosa* and *E. coli* (69% similarity) than between *P. aeruginosa* and *B. subtilis* (40% similarity). The region displaying the most sequence similarity between the different GlpD proteins is the flavin adenine dinucleotide-binding site (Fig. 6 and 7), and this similarity extends to other flavin-containing proteins (Fig. 6). The other region displaying a high degree of sequence similarity among all three GlpD proteins is located between aa 258 and 381 (*P. aeruginosa*), 251 and 373 (*E. coli*), and 280 and 399 (*B. subtilis*). From a comparison of this region from *E. coli* GlpD with the triosephosphate isomerase sequences from *E. coli* and *Saccharomyces cerevisiae*, Austin and Larson (1) have proposed that this region might encompass, at least in part, the G3P-binding site. The conservation of this region in all three GlpD sequences determined to this date supports this notion.

Analysis of the nucleotide sequences located downstream of *glpD* revealed the existence of palindromic DNA sequences capable of forming two putative stable stem-loop structures. Despite their potential relatively high stabilities ($\Delta G = -27.3$ to -40.2 kcal/mol), these stem-loop structures have significant homology neither to bacterial repetitive extragenic palindromic sequences (9) nor to Rho-dependent or Rho-independent transcriptional terminators (22). In support of this notion, preliminary data obtained by utilizing *lacZ*-based reporter gene constructs indicated that neither of these putative stem-loop structure indeed functions as an efficient transcriptional terminator (data not shown). However, some seemingly host-specific differences could be observed. Whereas these sequences had no effect on βGal expression in *E. coli*, in *P. aeruginosa* they reduced βGal expression by about 35%. This reduction could reflect a weak host-specific transcriptional terminator, or it might reflect a contribution of a stem-loop structure to mRNA stabilization (9). However, an elucidation of the physiological function of these putative stem-loop structures, if any, clearly awaits further experimentation.

We are currently in the process of isolating defined mutations in *glpD* and its flanking regions to further define the role of this region of the PAO1 chromosome in glycerol metabolism and in providing the metabolic precursor(s) for alginate biosynthesis.

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