Structure and gene–polypeptide relationships of the region encoding glycerol diffusion facilitator (*glpF*) and glycerol kinase (*glpK*) of *Pseudomonas aeruginosa*

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The glycerol facilitator is one of the few known examples of bacterial solute transport proteins that catalyse facilitated diffusion across the cytoplasmic membrane. A second protein, glycerol kinase, is involved in entry of external glycerol into cellular metabolism by trapping glycerol in the cytoplasm as *sn*-glycerol 3-phosphate. Evidence is presented that glycerol transport in *Pseudomonas aeruginosa* is mediated by a similar transport system. The genes encoding the glycerol facilitator, *glpF*, and glycerol kinase, *glpK*, were isolated on a 4.5 kb *EcoRI* fragment from a chromosomal mini-library by functional complementation of an *Escherichia coli glpK* mutant after establishing a map of the chromosomal *glpFK* region with the help of a PCR-amplified *glpK* segment. The nucleotide sequence revealed that *glpF* is the promoter-proximal gene of the *glpFK* operon. The glycerol facilitator and glycerol kinase were identified in a T7 expression system as proteins with apparent molecular masses of 25 and 56 kDa, respectively. The identities of the glycerol facilitator and glycerol kinase amino acid sequences with their counterparts from *Escherichia coli* were 70 and 81 %, respectively; this similarity extended to two homologues in the genome sequence of *Haemophilus influenzae*. A chromosomal $\Delta glpFK$ mutant was isolated by gene replacement. This mutant no longer transported glycerol and could no longer utilize it as sole carbon and energy source. Two ORFs, *orfX* and *orfY*, encoding a putative regulatory protein and a carbohydrate kinase of unknown function, were located upstream of the *glpFK* operon.

Keywords: *Pseudomonas aeruginosa*, glycerol transport, glycerol facilitator/glycerol kinase operon, *glp* regulon

INTRODUCTION

Among the various bacterial solute transport systems, transport by facilitated diffusion across the inner membrane is the least common (Nikaido & Saier, 1992). Until very recently, the only well-studied examples were the glycerol diffusion facilitator (GlpF) of *Escherichia coli* (Sweet *et al.*, 1990; Voegelé *et al.*, 1993;

Weissenborn & Larson, 1992) and the glucose facilitator of *Zymomonas mobilis* (Glf) (Parker *et al.*, 1995; Weisser *et al.*, 1995). Although glycerol, like many other small solutes, can enter the cytoplasm of *E. coli* via passive diffusion, effective glycerol uptake was shown to be protein-mediated (Sanno *et al.*, 1968) and dependent on phosphorylation to *sn*-glycerol 3-phosphate (G3P) (Voegelé *et al.*, 1993). Phosphorylation of glycerol by glycerol kinase (GlpK) prevents glycerol from exiting the cell and is the first step in glycerol metabolism (Hayashi & Lin, 1965; Lin, 1996). GlpF and GlpK interact very closely, with the phosphorylation reaction being stimulated by this interaction (Voegelé *et al.*, 1993), a situation analogous to the hexokinase– and glycerol kinase–porin interactions in mitochondria, in

Abbreviations: CIP, calf intestinal phosphatase; DHAP, dihydroxyacetone phosphate; GlpF, glycerol diffusion facilitator; GlpK, glycerol kinase; G3P, *sn*-glycerol 3-phosphate; MIP, major intrinsic protein.

The GenBank accession number for the sequence reported in this paper is U49666.

which kinase is activated upon interaction with mitochondrial porin (Brdiczka, 1990).

In *E. coli*, the *glpF* and *glpK* genes encoding GlpF and GlpK, respectively, are part of the *glpFKX* operon, of which the function of the *glpX* product remains unknown (Truniger *et al.*, 1992). Transcription of the *glpFKX* operon, which is a member of the *glp* regulon, is negatively regulated by the *glp* repressor and sensitive to catabolite repression (Cozzarelli *et al.*, 1968; Weissenborn & Larson, 1992). The GlpF protein has been identified as a membrane protein with an apparent molecular mass of 25 kDa (Sweet *et al.*, 1990), which is smaller than the calculated molecular mass of 29727 Da (Weissenborn & Larson, 1992). *E. coli* GlpF has a high degree of homology to the family of major intrinsic membrane proteins (MIP), of bacterial, plant and animal origin (Baker & Saier, 1990; Nikaido & Saier, 1992; Weissenborn & Larson, 1992).

Glycerol kinase of *E. coli* is considered to be the pacemaker for the dissimilation of glycerol (Zwaig *et al.*, 1970). The active enzyme is a tetramer (Thorner & Paulus, 1971) of identical 56106 Da (Pettigrew *et al.*, 1988) subunits whose catalytic properties (Thorner & Paulus, 1971) and crystal structure (Hurley *et al.*, 1993) have been investigated.

Like *E. coli*, *Pseudomonas aeruginosa* is also able to utilize glycerol as a carbon source which is primarily metabolized through the Entner-Doudoroff pathway (Cuskey & Phibbs, 1985; Siegel & Phibbs, 1979). However, in contrast to *E. coli*, previous experimentation suggested that in *P. aeruginosa* glycerol transport may be mediated by a high-affinity, binding-protein-dependent transport system (Siegel & Phibbs, 1979; Tsay *et al.*, 1971). Intracellular glycerol is then phosphorylated to G3P by GlpK, and subsequently oxidized to DHAP by G3P dehydrogenase. Besides glycerol, G3P can be transported and utilized as sole carbon source, although growth is much poorer than with glycerol (McCowen *et al.*, 1981).

All of the enzymes involved in transport and metabolism of glycerol and G3P are inducible by both of these substrates, suggesting that their structural genes form a *glp* regulon (McCowen *et al.*, 1981). More recently it was shown that *glp* gene expression in *P. aeruginosa* is negatively regulated by the action of the *glpR*-encoded Glp repressor (Schweizer & Po, 1996), in an analogous manner to *glp* expression in *E. coli*.

Although the discovery of a number of GlpF and GlpK homologues in Gram-negative (Fleischmann *et al.*, 1995; Sweet *et al.*, 1990; Weissenborn & Larson, 1992) and Gram-positive (Claiborne *et al.*, 1995; Holmberg *et al.*, 1990) bacteria suggested the existence of a common mechanism for bacterial glycerol uptake, the nature of transport of this important nutrient in *P. aeruginosa* hitherto remained unclear. The initial models for glycerol transport and metabolism were largely based on comparisons of patterns of enzyme induction in wild-type and a few mutants. Subsequently, several observations were made concerning the transport of glycerol

and G3P, respectively, which were in contrast to published results. Specifically, mutational analyses indicated that the co-induced transport of glycerol and G3P is not due to existence of a single transport system that is considerably more specific for glycerol than G3P, as previously suggested (Siegel & Phibbs, 1979), but is due to two separate transporters (Schweizer & Po, 1994). In addition, despite repeated attempts no binding-protein-dependency for glycerol could ever be demonstrated although previous studies had indicated such a mechanism (Tsay *et al.*, 1971). More recent experiments by Williams *et al.* (1994) suggested that glycerol is probably taken up via facilitated diffusion, involving a mechanism similar to that described for *E. coli*.

To further our understanding of the biology of glycerol metabolism in *P. aeruginosa*, we set out to characterize at the molecular level the region responsible for transport of this important carbon source. Our results suggest the existence of a single glycerol transport system, consisting of a glycerol facilitator and a closely associated GlpK whose overall genetic, biochemical and functional properties are very similar to the corresponding proteins of *E. coli* GlpF. Furthermore, identification of related sequences in other Gram-negative bacteria reveals conservation of the molecular architecture of bacterial glycerol uptake systems.

METHODS

Materials. Chemicals were generally of molecular biology grade and were purchased from Sigma, Fisher or BDH. [^{14}C]Glycerol, [^{35}S]methionine and [^{14}C]methyl-labelled molecular mass markers were purchased from Amersham. DNA sequencing kits for fluorescent sequencing were obtained from Applied Biosystems. Calf intestinal phosphatase (CIP), restriction enzymes, large fragment of DNA polymerase (Klenow enzyme), T4 DNA polymerase, and T4 DNA ligase were purchased from Gibco-BRL, Boehringer Mannheim or Pharmacia. *Taq* DNA polymerase and pGEM-T cloning vector were purchased from Promega. Gibco-BRL and Difco supplied the growth media. Carbenicillin and gentamicin were purchased from the hospital pharmacy at Foothills Hospital (Calgary, Alberta, Canada).

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. LB medium (Sambrook *et al.*, 1989) was used as the rich medium for both *E. coli* and *P. aeruginosa*. M9 medium (Miller, 1992) was used as minimal medium for *E. coli* and *P. aeruginosa*. Carbon sources were incorporated into minimal media at a final concentration of 10 mM. Glycerol phenotypes were also screened on MacConkey medium (Difco) supplemented with 0.5% glycerol. Antibiotics were used in selection media at the following concentrations: *E. coli*, 100 µg ampicillin ml⁻¹ and 15 µg gentamicin ml⁻¹; *P. aeruginosa*, 500 µg carbenicillin ml⁻¹ and 200 µg gentamicin ml⁻¹. Lactose phenotypes were screened on LB plates containing 40 µg X-Gal ml⁻¹.

Isolation, manipulation and transformation of DNA. Restriction enzymes, CIP, T4 DNA polymerase, and T4 DNA ligase were used as per the supplier's instructions. Small scale isolations of plasmid DNA from 1.5 ml cultures of *E. coli* and transformations were done as previously described (Schweizer & Po, 1994). DNA used for automated DNA sequence analysis was prepared according to a protocol provided by Applied Biosystems. DNA restriction fragments were eluted from low-

Table 1. Strains and plasmid vectors of *P. aeruginosa* and *E. coli* K-12

Strain	Relevant characteristics	Reference/origin
<i>P. aeruginosa</i>		
PAO1	Prototrophic	Holloway <i>et al.</i> (1994)
PAO162	PAO1 Δ glpFK:: Ω Gm ^r	This study
PG201	Also known as DSM659; prototrophic	Ochsner <i>et al.</i> (1994)
UO212, UO357, UO606	glpK::Tn5Gm mutants of PG201	U. Ochsner
<i>E. coli</i>		
DH5 α F'	[F ⁺ ϕ 80 Δ lacZ Δ M15] Δ (lacZYA-argF)U169 recA1 endA1 hsdR17 (r _K ⁻ m _K ⁺) supE44 thi-1 gyrA relA	Liss (1987)
TL300	F ⁻ glpK glpR2 araD139 Δ (argF-lac)U169 rpsL150 relA1 thiA1 deoC1 ptsF25 flbB5301 rbsR	Weissenborn & Larson (1992)
Plasmid vectors		
pBS-KS	Ap ^r ; cloning and expression vector	Stratagene
pEX100T	Ap ^r , oriT ⁺ sacB ⁺ ; mobilizable gene replacement vector	Schweizer & Hoang (1995)
pGEM-T	Ap ^r ; T-tailed cloning vector	Promega
pT7-5/pT7-6	Ap ^r ; T7 promoter/T7 polymerase expression vectors	Tabor (1994)
pUC18/19	Ap ^r ; cloning and expression vectors	Yanisch-Perron <i>et al.</i> (1985)
pUCP20/21	Ap ^r ; pUC18/19-based broad-host-range cloning and expression vectors	Schweizer <i>et al.</i> (1996)
pUCP20T/21T	Ap ^r oriT ⁺ ; pUCP20/21-based, mobilizable broad-host-range cloning and expression vectors	Schweizer <i>et al.</i> (1996)
pPS539	Ap ^r ; pGEM-T with 1039 bp chromosomal PCR fragment	This study
pPS544	Ap ^r , glpF ⁺ K ⁺ R ⁺ ; 4495 bp PAO1 chromosomal EcoRI fragment cloned into EcoRI site of pUC18 (glpFK orientation same as Plac*)	This study
pPS585	Ap ^r , glpF ⁺ K ⁺ ; 2678 bp AflIII-SphI fragment from pPS544 cloned between SmaI/SphI sites of pUCP21 (glpFK orientation opposite to Plac)	This study
pPS586	Ap ^r ; 2708 bp EcoRI-HindIII fragment from pPS585 cloned between EcoRI/HindIII sites of pT7-5 (glpFK orientation same as T7 promoter)	This study
pPS587	Ap ^r ; 2708 bp EcoRI-HindIII fragment from pPS585 cloned between EcoRI/HindIII sites of pT7-6 (glpFK orientation opposite to T7 promoter)	This study
pPS592	Ap ^r ; 2708 bp EcoRI-HindIII fragment from pPS585 cloned between EcoRI/HindIII sites of pUCP20T (glpFK orientation same as Plac)	This study

* Plac, *E. coli* lac operon promoter.

gelling-temperature agarose gels as described by Wieslander (1979) (fragments <500 bp) or with the GeneClean kit (Bio101) (fragments >500 bp).

Genetic procedures. Recombinant plasmids were mobilized from *E. coli* strain BW19851 (Metcalf *et al.*, 1994) to *P. aeruginosa* as described by Simon *et al.* (1986). A Δ glpFK mutant was isolated by subcloning the blunt-ended 4.5 kb EcoRI fragment from pPS544 into the SmaI site of pEX100T (Schweizer & Hoang, 1995). The glpFK genes were inactivated by insertion of a 1032 bp SmaI fragment containing the gentamicin resistance (Gm^r) encoding Ω element from pAC Ω Gm (Schweizer & Hoang, 1995) between the Eco47III site (within glpF) and the blunt-ended NcoI site (within glpK) (Fig. 1b). The previously described sacB-based strategy (Schweizer & Hoang, 1995) was then employed to achieve gene replacement of the glpFK region in wild-type strain PAO1 with the Δ glpFK:: Ω Gm insertion.

PCR from chromosomal DNA and cloning of PCR products. For PCR amplification from chromosomal DNA, two oligo-

nucleotide primers PK1 (5'-ATGGAAATCTGGGCCACG-CAG-3') and PK2 (5'-TGCATGGCGTCGAGCACGTC-3'), which were designed from partial glpK sequence information generously provided by Dr Urs Ochsner (University of Colorado Health Sciences Center, CO, USA) were used to prime synthesis from PAO1 chromosomal DNA. A 50 μ l PCR reaction mixture contained 1 $\times$ PCR buffer [50 mM Tris/HCl (pH 8.0), 50% glycerol, 100 mM NaCl, 0.1 mM EDTA, 5 mM DTT, 1% Triton X-100], 200 μ M each dNTP, 1.5 mM MgCl₂, 25 pmol each primer, 150 ng PAO1 chromosomal DNA and 5% DMSO. DNA was denatured at 97 °C for 10 min and the mixture was cooled to 90 °C prior to addition of 2.5 U Taq DNA polymerase. This was followed by 30 cycles (94 °C for 1.5 min; 55 °C for 30 s; 72 °C for 2 min) and a final extension at 72 °C for 10 min. The desired 1039 bp PCR fragment was eluted from a low-gelling-temperature agarose gel and ligated to the pGEM-T (Promega) cloning vector (Table 1) following the supplier's protocol. The resulting plasmid was designated pPS539 (Table 1).

Genomic Southern analysis. For Southern analysis, chromosomal PAO1 DNA was isolated (Schweizer & Hoang, 1995), digested with various restriction endonucleases, electrophoresed on a 1% agarose gel in 0.5×TBE [25 mM Tris (pH 8.3), 25 mM boric acid, 0.5 mM EDTA] and transferred to Photogene nylon membranes (Gibco-BRL) as described by Sambrook *et al.* (1989). Plasmid DNA was biotinylated by random hexamer priming following the NEBlot protocol (New England Biolabs). Following transfer and UV fixation (Sambrook *et al.*, 1989), the membranes were probed with the biotinylated DNA fragment according to the Phototype protocol.

Establishment of chromosomal mini-libraries and cloning of *glpK*. For cloning of the 2.4–6 kb genomic *EcoRI* DNA fragments, *EcoRI*-digested PAO1 chromosomal DNA was electrophoresed on a 1% (w/v) low-melting-point agarose gel in 0.5×TBE and the fragments from the 2.4–6 kb region of the gel were eluted. After ligation into *EcoRI*-digested and CIP-treated pUC18 (Yanisch-Perron *et al.*, 1985), ampicillin-resistant DH5 α F' colonies were selected. Plasmid DNA was isolated from the pooled transformants and used to transform the *glpK* *E. coli* strain TL300 to ampicillin resistance on M9 glycerol medium. Plasmid DNA was isolated from colonies growing on these plates and the presence of *glpK* was verified by re-transformation of strain TL300.

Sequencing of DNA. Double-stranded plasmids based on the cloning vectors pUC18 or pUC19 (Yanisch-Perron *et al.*, 1985) were used as templates. Nucleotide sequences were determined with the Taq DyeDeoxy Terminator Cycle Sequencing kit and the protocol provided by the supplier (Applied Biosystems). Extensions were primed with the commercially available 24 nt pUC/M13 reverse and forward sequencing primers, respectively. Labelled samples were analysed in the University of Calgary DNA Analysis Core Facility on an Applied Biosystems model 373A DNA sequencer. Computer-assisted sequence analyses were performed with the MacDNAsis (Hitachi), SeqEd (Applied Biosystems) and SeqVu (Garvan Medical Institute, Sidney, Australia) programs. GenBank homology searches were performed with the on-line BLAST facilities of the National Center for Biotechnology Information at the National Library of Medicine (Bethesda, MD). PROSITE searches, including TMbase searches, were performed via the ExPASy WorldWide Web molecular biology server from the Geneva University Hospital and the University of Geneva, Geneva, Switzerland.

Transport assays. For assays of glycerol transport in wild-type PAO1 and Δ *glpFK* mutant strains, cells were grown overnight in 4 ml M9 medium containing 0.2% Casamino acids (un-induced) or in the same medium supplemented with 5 mM G3P (induced). One millilitre of these cultures was harvested in a microfuge and washed three times in 1 ml carbon-free 0.5×M9. Glycerol transport was initiated at room temperature by addition of [14 C]glycerol (> 120 mCi mmol $^{-1}$ or $> 4.4 \times 10^9$ Bq mmol $^{-1}$) to the washed cells to a final concentration of 0.3 μ M. Two hundred microlitre aliquots were taken at 20 s intervals, immediately filtered through 0.45 μ m cellulose acetate filters, and washed with approximately 10 ml 0.5×M9 without added carbon source. The filters were dried and the radioactivity remaining on them was determined by liquid scintillation counting. Uptake values are given as pmol glycerol transported by 10^8 cells (assuming that an OD $_{540}$ of 1 corresponds to 8×10^8 cells) (Beckman DU640).

Expression of plasmid-encoded proteins. Plasmid-encoded polypeptides were overexpressed and identified with the bacteriophage T7 RNA polymerase–promoter system (Studier

et al., 1990). The host strain for T7 promoter-containing plasmids was BL21(DE3), which contains the gene for T7 RNA polymerase under the control of the IPTG-inducible *lacUV* promoter (Studier *et al.*, 1990). The products of the cloned genes were selectively labelled with [35 S]methionine as previously described (Schweizer, 1991). Proteins were separated on 0.1% SDS/10% polyacrylamide gels (pH 9.2) (Makowski & Ramsby, 1993). The de-stained gels were soaked in 1 M sodium salicylate (Chamberlain, 1979), dried and labelled proteins visualized by autoradiography.

RESULTS

PCR amplification and cloning of *glpK*

During their studies on rhamnolipid biosynthesis in *P. aeruginosa* strain PG201, Ochsner *et al.* (1994) isolated three Tn5Gm-induced mutants, UO212, UO357 and UO606 (Table 1) that produced reduced levels of rhamnolipids and contained transposon insertions that were localized to the *glpK* gene by nucleotide sequence analysis and GenBank searches (Ochsner, 1993). Based on their sequence information, the two primers PK1 and PK2 (underlined in Fig. 1b) were synthesized and used to amplify a 1039 bp DNA fragment from chromosomal PAO1 DNA. The nucleotide sequence of this fragment was determined and found to contain most of the *glpK* coding sequence (Fig. 1b; sequence located between the converging broken arrows marked PK1 and PK2, respectively). The PCR-amplified fragment was used as the probe in genomic Southern experiments. These analyses established a partial restriction map of the PAO1 *glpK* region (Fig. 1a) and identified a 4.5 kb *EcoRI* fragment which was shown to be located adjacent to previously cloned and sequenced DNA containing the *glpD* gene (Schweizer & Po, 1994). Plasmid clones containing the 4.5 kb *EcoRI* fragment in pUC18 were identified in a chromosomal mini-library by complementation of the *glpK* mutation of *E. coli* strain TL300. All positive clones contained the 4.5 kb *EcoRI* fragment in the same orientation as the *lac* promoter and one representative plasmid, pPS544 (Table 1), was retained for further studies. By subcloning, and complementation of *E. coli* TL300, the *glpK* gene was localized to a 2.7 kb *AflIII*–*SphI* fragment carried by pPS585 and pPS592 (Table 1).

Nucleotide sequence of *glpF* and *glpK*

To further define the ORFs and their products contained on the cloned 2.7 kb *AflIII*–*SphI* fragment, its nucleotide sequence was determined from both strands, utilizing various subclones and deletion derivatives of pPS544. The sequence of the 2678 bp fragment (Fig. 1b) contains two ORFs, and BLASTX searches of GenBank readily identified the *glpF* and *glpK* homologues. The two genes are separated by a short (38 nt) intergenic region whose sequence contains neither apparent transcription initiation nor termination signals. The *glpF* gene is preceded by sequences with homology to the *E. coli* –10 and –35 σ_{70} promoter consensus sequences (underlined in Fig. 1b) and the *glpK* gene is followed by sequences

Fig. 1. Organization (a) and nucleotide sequence (b) of the *glpFK*-containing region of the *P. aeruginosa* chromosome. Abbreviations: D, *glpD* (*sn*-glycerol-3-phosphate dehydrogenase; Schweizer & Po, 1994); F and K, *glpF* and *glpK* (encoding glycerol diffusion facilitator and glycerol kinase, respectively; this study); M, *glpM* (membrane protein affecting alginate synthesis; Schweizer *et al.*, 1995); R, *glpR* (*glp* regulatory gene; Schweizer & Po, 1996); X and Y, *glpX* and *glpY*, encoding a transcriptional regulator and carbohydrate kinase, respectively, of unknown function; this study); Af, *AfIII*; Ba, *BamHI*; Cl, *ClaI*; Ec, *EcoRI*; Ps, *PstI*; Sp, *SphI*. (b) Nucleotide sequence of an *AfIII*-*SphI* fragment containing *glpF* and *glpK* is shown. Listed below the sequence are the deduced amino acid sequences of GlpF and of GlpK. The proposed -35 and -10 promoter sequences and consensus Shine-Dalgarno (SD) sequences are underlined. An inverted repeat sequence that may act as a transcriptional terminator is indicated by convergent arrows. A proposed *glp* operator sequence, O_p , is underlined with asterisks. The priming sites used to amplify a *glpK* fragment from chromosomal DNA with primers PK1 and PK2 are underlined and the directions of synthesis from the primers are indicated by broken arrows. Vertical lines labelled UO212, UO357 and UO606, respectively, indicate nucleotides flanking the insertion points of previously characterized *glpK*::Tn5Gm insertions (Ochsner, 1993).

capable of forming a stable ($\Delta G = -131 \text{ kJ mol}^{-1}$) secondary structure, which may serve as a transcription termination signal (Rosenberg & Court, 1979). Completion of the entire *glpK* sequence also allowed for precise location of the previously isolated *glpK*::Tn5Gm insertions contained in strains UO212, UO357 and UO606 (Fig. 1b; Ochsner, 1993).

Amino acid sequence of GlpF

The *glpF* gene encodes a protein of 279 amino acids with a combined calculated molecular mass of 28897 Da. The extremely low polarity of the GlpF protein is typical

of that found for other membrane transporters (74% nonpolar, 26% polar residues).

P. aeruginosa GlpF is very similar to its homologue from *E. coli* (70% identity; 90% similarity) (Weissenborn & Larson, 1992) and the putative GlpF homologue from *Haemophilus influenzae* (71% identity, 93% similarity) (Fleischmann *et al.*, 1995) (Fig. 2). This similarity also extends to the propanediol facilitator PduF from *Salmonella typhimurium* (65% identity, 91% similarity; GenBank accession no. L31414), and to the glycerol facilitator protein from *Bacillus subtilis* (accession no. M34393) (Holmberg *et al.*, 1990), although to a lesser degree (data not shown). A PROSITE analysis

P.a. GlpF	1	MTTAA	TP	SL	FG	QC	LS	EFLG	TALL	IFFG	AGCVA	33
E.c. GlpF	1	---	MS	Q	T	S	L	KG	QC	I	A	30
H.i. GlpF	1	---	MD	K	S	L	K	AN	C	I	G	28
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P.a. GlpF	34	AL	KV	AG	AS	F	G	L	WE	I	S	66
E.c. GlpF	31	AL	KV	AG	AS	F	G	WE	I	S	63	
H.i. GlpF	29	AL	KV	AG	AS	F	G	L	WE	I	S	61
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P.a. GlpF	67	GA	HL	NP	AV	S	I	AL	WL	F	AG	99
E.c. GlpF	64	GA	HL	NP	AV	S	I	AL	WL	F	AG	96
H.i. GlpF	62	GA	HL	NP	AV	S	I	AL	WL	F	AG	94
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P.a. GlpF	100	AF	CA	AA	LV	Y	L	V	S	L	F	132
E.c. GlpF	97	AF	CA	AA	LV	Y	L	V	S	L	F	129
H.i. GlpF	95	AF	CA	AA	LV	Y	L	V	S	L	F	127
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P.a. GlpF	133	AL	AS	V	F	S	T	Y	P	H	P	165
E.c. GlpF	130	DL	AG	T	F	S	T	Y	P	H	P	162
H.i. GlpF	128	SL	AG	T	F	S	T	Y	P	H	P	160
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P.a. GlpF	166	I	M	A	L	T	D	D	G	N	L	198
E.c. GlpF	163	I	M	A	L	T	D	D	G	N	L	195
H.i. GlpF	161	I	M	A	L	T	D	D	G	N	L	193
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P.a. GlpF	199	P	L	T	G	F	A	M	N	P	A	231
E.c. GlpF	196	P	L	T	G	F	A	M	N	P	A	228
H.i. GlpF	194	P	L	T	G	F	A	M	N	P	A	226
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P.a. GlpF	232	E	I	P	Y	L	V	P	I	F	A	264
E.c. GlpF	229	D	I	P	Y	L	V	P	I	F	A	261
H.i. GlpF	227	E	I	P	Y	L	V	P	I	F	A	259
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P.a. GlpF	265	A	A	A	P	A	E	A	E	P	K	279
E.c. GlpF	262	D	I	C	V	E	E	K	T	T	T	281
H.i. GlpF	260	N	C	G	C	E						264

Fig. 2. Alignments of Gram-negative GlpF proteins. Alignments of *P. aeruginosa* (P.a.) GlpF with GlpF from *E. coli* (E.c.) (GenBank accession no. M55990) and a putative GlpF from *H. influenzae* (H.i.) (accession no. L42023) are shown. Identical residues are boxed and similar residues are shaded in grey. The conserved amino acids constituting the MIP signature are underlined with the black bar. The homologies over the entire lengths of the GlpF proteins shown are 70% (*P. aeruginosa* vs. *E. coli*) and 71% (*P. aeruginosa* vs. *H. influenzae*).

supported the earlier notions that GlpF belongs to the family of MIPs (Nikaido & Saier, 1992; Weissenborn & Larson, 1992) and all GlpF proteins analysed have the signature motif that is typical of this family (Fig. 2).

Amino acid sequence of GlpK

The sequence for *glpK* indicates an ORF of 505 codons. The calculated molecular mass of GlpK was 56063 Da. *P. aeruginosa* GlpK is very similar in size and amino acid composition to the corresponding proteins from *E. coli* (81% identity, 88% similarity; Pettigrew *et al.*, 1988) and *H. influenzae* (72% identity, 86% similarity; Fleischmann *et al.*, 1995) (Fig. 3). This high degree of similarity was also found with GlpK from *Bacillus subtilis* (57% identity, 78% similarity; Holmberg *et al.*, 1990) and *Enterococcus casseliflavus* (32% identity, 54% similarity; A. Claiborne, personal communication), as well as other prokaryotic and eukaryotic GlpKs (data not shown). PROSITE analyses indicated that GlpK belongs to the FGGY family of carbohydrate kinases. Both signatures, signature 1 corresponding to amino acids 139–151 and signature 2 corresponding to amino acids 370–387 (Fig. 3), are found in the central and C-terminal sections, respectively. In addition, GlpK contained the conserved N-terminal DQGTSSR ATP-binding motif (residues 14–21) (Flaherty *et al.*, 1991; Hurley *et al.*, 1993) as well as residues (R87E88–Y139–D249) that are equivalent to residues R83E84–

P.a. GlpK	1	M T D K H N K K Y V V A L D Q G T T S S R A L V F D L D N A N V Y S	33
E.c. GlpK	1	M T I E L K K Y V V A L D Q G T T S S R A V V M D H D A N T I S	30
H.i. GlpK	1	M T D L K K Y V V A L D Q G T T S S R A V L D H N H A N V V E	30
ATP			
P.a. GlpK	34	Q A O R E F F T Q A D E Y P P R A G W H E D P M E I W A T Q S S T L V E	66
E.c. GlpK	31	V S O R E F T Q A D E Y P P R A G W H E D P M E I W A T Q S S T L V E	63
H.i. GlpK	31	A C R E E F T Q I Y P R A G W H E H P M E I W A T Q S S T L N E	63
P.a. GlpK	67	A L A Q A S T I E R D O V J A A I G I T N Q R E T T I V W O R H S G H R	99
E.c. GlpK	64	V L A K A D I S S D O T A A I G I T N Q R E T T I V W X E I T G K	96
H.i. GlpK	64	V L A K A G I T S D E L A A I G I T N Q R E T T I V W X E I T G T	96
P.a. GlpK	100	P L H N V I V W Q H R S A A I C A Q L K H D G L E D Y T R E I T Y	132
E.c. GlpK	97	P I Y N A I V W Q C R R A T A E C H L K R D G L E D Y I R S N T	129
H.i. GlpK	97	P V Y N A I V W Q C R R A T A D I T D K L K A D G H E E L Y I A N T	129
P.a. GlpK	133	G L V I T D P Y F S G T K L W K L D N V E G A R E R A R K A R G E L L	165
E.c. GlpK	130	G L V I D P Y F S G T K W K L D H V E G A R E R A R R G E L L	162
H.i. GlpK	130	G L V I D P Y F S G T K V K W L D N V E G A R E R A R G E L L	162
1			
P.a. GlpK	166	F G T V D T W L W K L T I E R K I V H V T D Y T N A S R T M L F N I	198
E.c. GlpK	163	F G T V T D T W L W K M T O G R V H V T D Y T N A S R T M L F N I	195
H.i. GlpK	163	E G T V D T W L V W K L T O G R V H V T D Y T N A S R T M L F N I	195
P.a. GlpK	199	H T L R D W D A R M L E V L D I P R S M L P E V A R N S S E V Y G N A	231
E.c. GlpK	196	H T L D W D D K M L E V L D I P R E M L P E V A R N S S E V Y G O T	228
H.i. GlpK	196	H T K Q W D D K M L E F L L N I P R S M L P E V A R N S S E V Y G O T	228
P.a. GlpK	232	N I G G V G G G F L P I A G I A G D Q Q A A L F G O M C V F E G Q	264
E.c. GlpK	229	N I G G K G G G T R I P I P S I G I A G D Q Q A A L F G O L C V K E G M	261
H.i. GlpK	229	N I G G K G G V R I P I P S I A G I A G D Q Q A A L Y G H L C V I A G Q	261
P.a. GlpK	265	A K N T Y G T G C F M L M H T G D K A V K S T H G L L T T I A C G	297
E.c. GlpK	262	A K N T Y G T G C F M L M H T G D K A V K S S E N G L L T T I A C G	294
H.i. GlpK	262	A K N T Y G T G C F M L L H T G N K A T T S K N G L L T T I A C N	294
P.a. GlpK	298	P R I G E V G I A L E G A V F N G S T V I O W L R D E L K V I N D S	330
E.c. GlpK	295	P T G E V I N Y A L E G A V F M A G A S T O W L R D E M K I L I N D A	327
H.i. GlpK	295	A K G E F P E Y A L E G S V F I A G A S T Q W L R D E L K I V H D S	327
P.a. GlpK	331	F D S E Y F A T K V N D S N G V V Y V P A F T G L G A P Y W D P Y	363
E.c. GlpK	328	Y D S E Y F A K V Q N D N G V V Y V P A F T G L G A P Y W D P Y	360
H.i. GlpK	328	F D S E Y F A K V T D S N G V V Y V P A F T G L G A P Y W D P Y	360
P.a. GlpK	364	A R G A I F G L T R G V K A L H I R A T L E S I A Y O T R D V L	396
E.c. GlpK	361	A R G A I F G L T R G V N A N H I R A T L E S I A Y O T R D V L	393
H.i. GlpK	361	A R G A I F G L S I R G A N R N H I R A T L E S I A Y O T R D V L	393
2			
P.a. GlpK	397	D A M O R D A G E R L A L R V D G G A V A N N F L M O F Q A D I	429
E.c. GlpK	394	E A M O S D S G E R L O L A L R V D G G A V A N N F L M O F Q A D I	426
H.i. GlpK	394	E A M O S D S G E R L O L A L R V D G G A V A N N F L M O F Q A D I	426
P.a. GlpK	430	L G T R V E R P I V R E T T A L G A A Y L A G L A I G F W S S L D	462
E.c. GlpK	427	L G T R V E R P I V R E T T A L G A A Y L A G L A I G F W S D N L D	459
H.i. GlpK	427	L D V N V E R P V R E T V T A L G A A Y L A G L A I G F W K D L D	459
P.a. GlpK	463	E L K S K A V I E R V F R P E C D E F R A K I Y A G W K K A V E	495
E.c. GlpK	460	E L O E K A V I E R E F R P E C D E F R A K I Y A G W K K A V K	492
H.i. GlpK	460	E L R D K A R V I E R E F R P D S D N E K R E F R Y K G W K K A V K	492
P.a. GlpK	496	R T R G W D D G E L	505
E.c. GlpK	493	R A M A W E E H D E I	502
H.i. GlpK	493	R S L E W A K E D E E	503

Fig. 3. Alignments of Gram-negative GlpK proteins. Alignments of *P. aeruginosa* (P.a.) GlpK with GlpK from *E. coli* (E.c.) (GenBank accession no. M18393) and a putative GlpK from *H. influenzae* (H.i.) (accession no. L42023) are shown. Identical residues are boxed and similar residues are shaded in grey. Amino acids that are part of the FGGY signature motifs 1 and 2, respectively, are underlined with black bars and residues constituting the N-terminal ATP-binding motif are underlined with the cross-hatched bar. Asterisks above amino acid symbols mark residues that are involved in glycerol binding. The homologies over the entire lengths of the GlpK proteins shown are 81% (*P. aeruginosa* vs. *E. coli*) and 86% (*P. aeruginosa* vs. *H. influenzae*).

Y136–D245 of *E. coli* GlpK, which are involved in glycerol binding as determined by crystal structure analyses (Hurley *et al.*, 1993). Note that the crystal structure coordinates are different from those of the deduced amino acid sequence (Fig. 3) since *E. coli* GlpK undergoes post-translational processing so that the N-terminal amino acid of the mature protein is threonine (Pettigrew *et al.*, 1988).

Expression of GlpF and GlpK in *E. coli*

To demonstrate expression of GlpF and GlpK, the 2.7 kb fragment containing their coding sequences was cloned into the expression vectors pT7-5 and pT7-6 (Tabor, 1994) in both orientations with respect to the T7 promoter. Following induction and expression, the radiolabelled proteins were analysed by SDS-PAGE

Fig. 4. Expression of GlpF and GlpK in *E. coli*. Proteins were pulse-labelled with [35 S]methionine, separated by SDS-PAGE, and visualized by autoradiography as described in Methods. Lanes: 1, pT7-5; 2, pPS586; 3, pT7-6; 4, pPS587; M, [14 C]methyl-labelled molecular mass markers (top to bottom) myosin (200 kDa), phosphorylase *b* (97.4 kDa), BSA (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa) and lysozyme (14.3 kDa).

which was followed by autoradiography (Fig. 4). Only pPS586, containing *glpF* and *glpK* in the same orientation as the T7 promoter, expressed two proteins with estimated molecular masses of 25 and 56 kDa (lane 2). These polypeptides were not expressed from pPS587 carrying *glpFK* in the opposite orientation (lane 4), and were also absent from vector-containing cells (pT7-5, lane 1 and pT7-6, lane 3). The observed size (56 kDa) for the *P. aeruginosa* GlpK polypeptide compares very favourably with the size deduced from the nucleotide sequence (56063 Da), as well as the predicted molecular masses of 56228 Da for the *E. coli* GlpK protein (Pettigrew *et al.*, 1988) and 56138 Da for the putative *H. influenzae* GlpK protein (Fleischmann *et al.*, 1995). As previously reported for *E. coli* GlpF (Sweet *et al.*, 1990), the observed size (~ 25 kDa) for *P. aeruginosa* GlpF is smaller than the calculated size (28897 Da).

Isolation of a *P. aeruginosa glpFK* deletion mutant

To confirm that *glpF* and *glpK* are required for glycerol transport and growth on glycerol, a chromosomal $\Delta glpFK$ mutation was introduced into wild-type strain PAO1. This was achieved by replacing the sequences located between an *Eco*47III site located early in *glpF* and a *Nco*I site located in *glpK* with a Gm^r -conferring Ω element, and by returning the mutant construct into the chromosome by allelic exchange. The $\Delta glpFK::\Omega Gm$ insertions were confirmed by genomic Southern analyses (data not shown). The $\Delta glpFK$ mutant no longer grew on 10 mM glycerol and failed to transport glycerol (Fig. 5a). Growth on G3P and its transport were normal (data not shown). The plasmid pPS592 harbouring *glpF*⁺*K*⁺

Fig. 5. Glycerol transport in (a) *P. aeruginosa* wild-type strain PAO1 (squares) and $\Delta glpFK$ strain PAO162 (circles) and (b) $\Delta glpFK$ strain PAO162 complemented with pPS592 (squares) and pUCP20T (circles). Glycerol uptake by washed uninduced (open symbols) and G3P-induced (filled symbols) cells was determined as described in Methods.

restored growth on glycerol as well as glycerol transport (Fig. 5b).

Nucleotide sequence of *orfX* and *orfY*

To test for the presence of other putative *glp* genes upstream of the *glpFK* operon, the nucleotide sequences located between the *Eco*RI and *Afl*III sites (Fig. 1a) were determined (GenBank accession no. U49666). This segment contained one complete ORF, *orfX*, and one partial ORF, *orfY*'. The *orfX* gene encoded a protein of 156 amino acids with a calculated molecular mass of 16363 Da. BLAST searches revealed that its gene product showed significant similarity with a putative regulatory protein, Orf1, from *S. typhimurium* (Burns & Beacham, 1986), protein 1434 of unknown function from *H. influenzae* (Fleischmann *et al.*, 1995), and with the proposed binding-substance regulator EbsC from *Enterococcus faecalis* (Bensing & Dunne, 1993). In the T7 RNA polymerase system, *orfX* expressed a 17 kDa protein (data not shown).

Although only limited sequence was available for *orfY*', the N-terminus of its gene product showed striking homology to carbohydrate kinases, especially GlpKs from various Gram-negative and Gram-positive bacteria. In particular, the N-terminal DQGTSSRA ATP-binding motif, which is conserved in GlpKs, was found. Since the *orfX*–*orfY* intergenic region contained neither apparent transcription initiation nor termination signals, *orfX* and *orfY* may also form a transcriptional unit.

DISCUSSION

The *P. aeruginosa* chromosomal region containing the genes encoding the glycerol facilitator, *glpF*, and GlpK, *glpK*, was cloned by functional complementation of an *E. coli glpK* mutant. Nucleotide sequence analysis revealed that *glpF* and *glpK* are organized in an operon with *glpF* being the promoter-proximal gene, as described for *E. coli* (Sweet *et al.*, 1990; Weissenborn & Larson, 1992) and *H. influenzae* (Fleischmann *et al.*, 1995). The presence of a putative transcription terminator downstream of *glpK*, and the immediately following *glpR* gene (Fig. 1a) (Schweizer & Po, 1996) suggest that *glpFK* are the sole genes of the operon and that *glpX* (Truniger *et al.*, 1992), if present in *P. aeruginosa*, is not a member of the *glpFK* operon. Previous studies showed that *glpFK* expression is negatively regulated by the action of a *glp* repressor and that the putative *glpF* promoter region contained a palindromic sequence that showed a 70% match with the *E. coli glp* operator consensus sequence (Schweizer & Po, 1996). Thus, the *glpFK* operon is a member of a *glp* regulon.

The glycerol facilitator is a protein of extremely low polarity which explains its aberrant mobility on SDS-PAGE (observed molecular mass of 25 kDa versus a calculated mass of 28 897 Da) due to the binding of more SDS by the abundant hydrophobic amino acids as has previously been noted for *E. coli* GlpF (Sweet *et al.*, 1990). Hydropathy analyses of the GlpF primary sequence and predictions of possible transmembrane segments (Hofmann & Stoffel, 1993) further indicated that it probably is an integral membrane protein with six potential membrane-spanning helices. It has previously been pointed out that this design is unique among bacterial plasma membrane transporters (Weissenborn & Larson, 1992), which usually contain 12 transmembrane helices (6+6 design) (Nikaido & Saier, 1992). Signature analysis placed the MIP motif in the second cytoplasmatic loop and confirmed the previous conclusions that GlpF belongs to the MIP family of proteins (Weissenborn & Larson, 1992), which includes the MIP of the bovine lens junction (Gorin *et al.*, 1984). The latter protein produces large, open, aqueous channels presumably at the interface of four subunits (Aerts *et al.*, 1990). Although some properties of GlpF are similar to those of channel-type transporters (Heller *et al.*, 1980), its function must be somewhat different to avoid leakage of solutes from the cytoplasm. As is true with other plasma membrane transporters, the channel is likely to be gated so that it opens only when the proper ligand binds to its binding site (Nikaido & Saier, 1992). Alternatively, gating could also be achieved by a close physical interaction with GlpK, which is required for efficient glycerol phosphorylation (Voegelé *et al.*, 1993).

The analysis of the GlpK amino acid sequence revealed the conserved nature of this protein. When compared to other bacterial GlpKs, the *P. aeruginosa* protein contains all of the conserved motifs including the N-terminal ATP-binding site, the two signatures of the FGYY

family of carbohydrate kinases and the amino acid residues involved in glycerol binding. As in *E. coli* GlpK (Hurley *et al.*, 1993), the functional domains, i.e. the domains involved in ATP and glycerol binding, are located in the N-terminal half of the protein. This may explain why *glpK* insertion mutants that synthesize truncated GlpK proteins retain the ability to grow slowly on glycerol and still exhibit low levels of glycerol transport (see below).

A plasmid-borne *glpFK* deletion was constructed and returned into the chromosome. The resulting mutant failed to grow on 10 mM glycerol and completely lacked glycerol transport activity but transported and grew on G3P normally. Growth on glycerol and glycerol transport was efficiently complemented by a plasmid expressing the cloned *glpFK* operon from the constitutive plasmid-borne *lac* promoter. Due to the increased copy number and constitutive expression of GlpF and GlpK, the maximum velocity of glycerol transport in the complemented, uninduced mutant was threefold higher than that observed in induced wild-type strain PAO1. For unknown reasons, glycerol transport activity was inhibited when the plasmid-containing cells were grown in G3P- or glycerol-containing medium.

Interestingly, although some of the *glpK*::Tn5Gm insertion mutants (Table 1 and Fig. 1b) have insertions, e.g. UO357 and UO212, that lie early within *glpK*, all of these mutants still grew on 10 mM glycerol, albeit at a reduced rate when compared to their parent PG201, and showed significant levels of inducible glycerol transport (2.2–2.9 pmol min⁻¹ per 10⁸ cells on average in all of the mutants versus 11.9 pmol min⁻¹ per 10⁸ cells in their parental strain PG201). Thus, the truncated GlpKs synthesized by these strains seem to be able to phosphorylate the glycerol passing through the facilitator channel and trap it as G3P in the cytoplasm. However, since PG201 is a different *P. aeruginosa* isolate than PAO1, its genetic background may be different from PAO1 and thus these results have to be interpreted with caution. For proper comparison, these experiments will have to be repeated by transferring the *glpK*::Tn5Gm mutations into the PAO1 genetic background. Alternatively, glycerol entering the cell independent of GlpF could be metabolized via a glycerol dehydrogenase and dihydroxyacetone kinase pathway (Sprenger *et al.*, 1989), although this seems unlikely since (i) these activities have never been demonstrated in *P. aeruginosa* and (ii) if they existed the $\Delta glpFK$ mutant described above should show some growth on glycerol – this is not the case.

Since the two reading frames located upstream of the *glpFK* operon did not show any homologies to *glp*-associated functions known to exist in *P. aeruginosa*, e.g. G3P transport, it can be concluded that their structural genes must be located outside the *glpF*–*glpK*–*glpR*–*glpD*–*glpM* region, whose complete sequence has now been assembled, and which has recently been mapped to the 31 min region of the chromosome (Liao *et al.*, 1996). Thus, the architecture

Fig. 6. Model for phospholipid metabolism in *P. aeruginosa*. Abbreviations: Ald, fructose-1,6-bisphosphate aldolase; Alg, alginate biosynthetic enzymes; AP, alkaline phosphatase; Chu, choline-utilization enzymes; CM, cytoplasmic membrane; DHAP, dihydroxyacetone phosphate; Est, esterase; Fad, fatty acid oxidase complex; Fdp, fructose diphosphatase; GAP, glyceraldehyde 3-phosphate; *glpD*, G3P dehydrogenase; *glpF*, glycerol facilitator; *glpK*, glycerol kinase; *glpT*, G3P permease; GPDEst, glycerophosphodiester phosphodiesterase; Lip, lipase; OM, outer membrane; OprB, glucose- and glycerol-specific porin; OprX, unidentified postulated porin; P_i , inorganic phosphate; PL, phospholipids; PLC, phospholipase C; PP, periplasm; Tpi, triosephosphate isomerase.

of the *P. aeruginosa* *glp* regulon may be similar to the *glp* regulon of *E. coli* and contain operons that are located in different regions of the genome.

In *E. coli*, the products of the *glp* regulon are responsible for salvaging the glycerol moiety of degradation products of phospholipids and triglycerides (Lin, 1996). In *P. aeruginosa*, phospholipids have been implicated as important carbon sources for synthesis of alginate (Terry *et al.*, 1991, 1992; Williams *et al.*, 1994), a viscous exopolysaccharide and an important virulence factor found in mucoid cystic fibrosis lung isolates (May *et al.*, 1991).

Phosphatidylcholine and phosphorylcholine can be used by *P. aeruginosa* as sole sources of carbon, nitrogen and phosphorous (Terry *et al.*, 1991, 1992). Although the G3P moiety of the phospholipid backbone is metabolized only very slowly (McCowen *et al.*, 1981), glycerol has been shown to be a particularly good carbon source for energy metabolism (McCowen *et al.*, 1981), for alginate synthesis in bronchial and cystic fibrosis isolates of *P. aeruginosa* (Marty *et al.*, 1992), and readily promotes the appearance of mucoid colonies when supplied in high enough concentrations (Terry *et al.*, 1991).

Fig. 6 illustrates how *P. aeruginosa* may acquire carbon from phospholipids for energy metabolism and for synthesis of the importance virulence factor alginate. Phosphatidylcholine, a major component of lung surfactant (Girod *et al.*, 1992), is degraded to 1,2-diacyl-

glycerol and phosphorylcholine by the action of phospholipase C (Berka & Vasil, 1982; Terry *et al.*, 1992), an enzyme that is also considered an important virulence factor (Vasil *et al.*, 1991). Phosphorylcholine can then serve as carbon and phosphorous source, presumably by action of alkaline phosphatase, which is co-induced with phospholipase C (Shortridge *et al.*, 1992), and the choline-utilization enzymes (cited in Holloway *et al.*, 1994). Diacylglycerol is hydrolysed to glycerol and fatty acids by extracellular lipases or esterases (Jaeger *et al.*, 1992).

Glycerol entering the periplasm via the OprB porin (Williams *et al.*, 1994) is transported into the cytoplasm via the glycerol diffusion facilitator and trapped intracellularly by the action of GlpK. Intracellular G3P is then funnelled into phospholipid biosynthesis, into energy metabolism and/or the alginate biosynthetic pathway after conversion to DHAP by action of membrane-associated G3P dehydrogenase. Although the derivation of G3P and its transport have not been studied in detail in *P. aeruginosa*, it may proceed as previously described for *E. coli*. In *E. coli*, extracellular G3P, or G3P derived in the periplasm from glycerophosphodiester by action of a phosphodiesterase, is transported into the cytoplasm via a G3P permease (Larson *et al.*, 1983).

Even though fatty acid uptake and metabolism have been extensively studied in *E. coli* (Black & DiRusso, 1994), the corresponding enzymes have not been identified in *P. aeruginosa*. As in *E. coli*, fatty acids are probably transported and metabolized by the combined action of a specific outer-membrane protein and a fatty acid oxidase complex (Black & DiRusso, 1994).

Although experimental evidence directly or indirectly indicates the presence of most of these enzymic activities in *P. aeruginosa*, the characterization of many of them awaits cloning and characterization of their structural genes.

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