

sn-Glycerol-3-Phosphate Transport in *Salmonella typhimurium*

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Salmonella typhimurium contains a transport system for *sn*-glycerol-3-phosphate that is inducible by growth on glycerol and *sn*-glycerol-3-phosphate. In fully induced cells, the system exhibited an apparent K_m of 50 μ M and a V_{max} of 2.2 nmol/min \cdot 10⁸ cells. The corresponding system in *Escherichia coli* exhibits, under comparable conditions, a K_m of 14 μ M and a V_{max} of 2.2 nmol/min \cdot 10⁸ cells. Transport-defective mutants were isolated by selecting for resistance against the antibiotic fosfomycin. They mapped in *glpT* at 47 min in the *S. typhimurium* linkage map, 37% cotransducible with *gyrA*. In addition to the *glpT*-dependent system, *S. typhimurium* LT2 contains, like *E. coli*, a second, *ugp*-dependent transport system for *sn*-glycerol-3-phosphate that was derepressed by phosphate starvation. A *S. typhimurium* DNA bank containing *EcoRI* restriction fragments in phage λ gt7 was used to clone the *glpT* gene in *E. coli*. Lysogens that were fully active in the transport of *sn*-glycerol-3-phosphate with a K_m of 33 μ M and a V_{max} of 2.0 nmol/min \cdot 10⁸ cells were isolated in a Δ *glpT* mutant of *E. coli*. The *EcoRI* fragment harboring *glpT* was 3.5 kilobases long and carried only part of *glpQ*, a gene distal to *glpT* but on the same operon. The fragment was subcloned in multicopy plasmid pACYC184. Strains carrying this hybrid plasmid produced large amounts of cytoplasmic membrane protein with an apparent molecular weight of 33,000, which was identified as the *sn*-glycerol-3-phosphate permease. Its properties were similar to the corresponding *E. coli* permease. The presence of the multicopy *glpT* hybrid plasmid had a strong influence on the synthesis or assembly of other cell envelope proteins of *E. coli*. For instance, the periplasmic ribose-binding protein was nearly absent. On the other hand, the quantity of an unidentified *E. coli* outer membrane protein usually present only in small amounts increased.

The *glp* regulon-dependent transport system for *sn*-glycerol-3-phosphate (G3P) in *Escherichia coli* (22) is a proton motive force-dependent transport system (8) comparable to the *E. coli* lactose system (34). It is the product of one gene (24), and membrane vesicles are still active in G3P transport (8). Recently, the *E. coli glpT* gene product, the G3P permease, was identified as an oligomeric cytoplasmic membrane protein (21). The *E. coli* operon containing *glpT* has one other gene, *glpQ*, which is distal to *glpT*. *glpQ* codes for a periplasmic phosphodiesterase that produces G3P by hydrolysis of glycerophosphodiester, which are degradation products of phospholipids (20). The *glpQ* product is identical to the formerly described periplasmic GLPT protein (32).

Even though several reports on the use of the *Salmonella typhimurium* G3P transport system in isolating pleiotropic mutants defective in the phosphotransferase system (10) or in the cyclic

AMP-dependent gene activator system have been published (2, 3), the physiological and biochemical parameters of the transport system have not been determined in detail. The pleiotropic mutants were isolated by selecting for resistance to the cell wall antibiotic fosfomycin (14), which is transported by catabolite-repressible G3P transport systems (18). Thus, *glpT* mutants can easily be isolated by this selection procedure (33).

The present paper reports the kinetic properties of the *S. typhimurium* transport system and the cloning of its gene, as well as the identification of the permease protein on polyacrylamide gels.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used are listed in Table 1. Overnight cultures were grown in minimal medium A (MMA) (25) or E-medium (11) containing 0.2% of a carbon source and the appropriate supplements. Plasmid-containing strains were grown in the presence of tetracycline. The sugars used were of the D-configuration. For dere-

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TABLE 1. Bacterial strains, phages, and plasmids

Strain	Known genotype ^a	Source/reference
<i>S. typhimurium</i> LT2		
RH1	<i>glpT</i>	This study
TR5903	<i>his-203 gyrA271</i>	(11)
TT5345	<i>his-203 zeh-775::Tn10</i>	(11)
RH3	<i>his-203 gyrA271 glpT</i>	This study
RH8	<i>his-203 glpT zeh-775::Tn10</i>	This study
<i>E. coli</i> K-12		
MC4100	F ⁻ <i>araD139 Δ(argF-lac)U169 relA1 rpsL150 ffbB5301 deoC1 ptsF25</i>	(29)
TS100	F ⁻ <i>glpR</i> , otherwise as MC4100	(24)
DL39	F ⁻ <i>gyrA Δ(glpT-glpA)593 zei-724::Tn10</i> , otherwise as TS100	(24)
DL291	F ⁻ <i>gyrA Δ(glpT-glpA)593 recA</i> , otherwise as TS100	(24)
SH173	F ⁻ <i>gyrA Δ(glpT-glpA)593</i> , otherwise as MC4100	H. Schweizer
TL11	F ⁻ <i>metB1 leu(Am) trp(Am) lacZ(Am) galK(Am) galE sueA sueC tsx relA supD43,74 rpsL gyrA Δ(glpT-glpA)593</i>	(21)
Phage		
λgt7- <i>glpT</i>	<i>glpT</i> (from <i>S. typhimurium</i> LT2)	This study
λgt4- <i>lac-5</i>	<i>lacZ lacY</i>	(11)
Plasmid		
pACYC184	<i>tet</i> Cm ^r	(9)
pRH100	<i>tet glpT</i> (<i>S. typhimurium</i>)	This study
pRH103	<i>tet glpT</i> (Am)	This study
pGS31	<i>tet glpT,Q</i> (<i>E. coli</i>)	(21)

^a The gene symbol of the phages and plasmids stands for the wild-type allele.

pressing the *pho* system, the strains were grown at limiting phosphate concentrations as described elsewhere (4).

Selection of *glpT* mutants. About 10⁸ cells of a growing culture of *S. typhimurium* LT2 (in E-medium with 0.2% glycerol) were plated on the same medium containing 0.1 mM fosfomycin. Colonies were picked after 2 days and tested for growth on maltose and glycerol and for fosfomycin sensitivity after induction of the hexose-phosphate transport system with 0.2 mM glucose-6-phosphate (35). We generated amber mutations in plasmid-encoded *glpT* by in vitro hydroxylamine mutagenesis and identified them using the temperature-sensitive amber-suppressor strain TL11 (21).

Transport assay. For the determination of the initial rate of G3P uptake, the cells were washed twice and suspended at room temperature in 2.5 ml of phosphate-free G plus L medium (13) at an optical density at 578 nm of 1.0. [¹⁴C]G3P (150 mCi/mmol; final concentration, 0.13 μM) was added, and 200-μl samples were withdrawn at the times indicated, filtered through membrane filters (0.45-μm pore size; Sartorius), and washed with 0.9% NaCl. The dried filters were counted in a toluene-based scintillation fluid.

Enzyme activities. Anaerobic G3P-dehydrogenase was tested by growing the strains anaerobically on MMA plates containing 0.2% glycerol, 20 mM fumarate, and 0.03% Casamino Acids. The expression of β-galactosidase was tested by plating in top agar containing 0.5 mg of 5-bromo-4-chloro-3-indolyl-β-galactoside (X-Gal).

The expression of alkaline phosphatase activity on plates was tested by overlaying colonies with a solution of 5-bromo-4-chloro-3-indolylphosphate (1 mg/ml) in dimethylformamide.

glpQ-dependent phosphodiesterase was measured

in 0.5 ml of 1 M hydrazine buffer containing 0.2 M glycine (pH 9.5) and 2 mM MgCl₂. First, 10 mM CaCl₂, 0.5 mM NAD⁺, and G3P dehydrogenase (20 U/ml) were added. Then, 0.5 mM glycerophosphocholine was added, and the reaction was started by the addition of 0.01 to 0.05 ml of the enzyme solution (osmotic shock fluid). The absorbance at 334 nm was followed (20).

Preparation of periplasmic and membrane proteins. Periplasmic proteins were isolated by the cold osmotic shock procedure of Neu and Heppel (26), which for *S. typhimurium* strains was modified as described by Aksamit and Koshland (1).

For the isolation of membrane proteins, strains were grown overnight in 100 ml of L-broth (LB) (25) containing 10 μg of tetracycline per ml. The sedimented cells were washed once with 20 mM potassium phosphate buffer (pH 7.5), containing 50 mM KCl. They were suspended in 1.5 ml of the same buffer containing 5 mM EDTA. After the cells were passed three times through a French pressure cell, DNA was digested by the addition of 10 μg of DNase per ml and 20 mM MgSO₄, and unbroken cells were removed by centrifugation. The supernatant was centrifuged on a sucrose step gradient (15, 53, and 70%) for 4 h in an SW41 rotor at 35,000 rpm. Inner membrane proteins formed a yellow band on the top of the 53% layer, whereas outer membrane proteins could be collected from a band on the top of the 70% layer. Protein concentrations were determined by the method of Lowry et al. (23).

Binding tests. For testing the binding activity of ribose-binding protein, 50 μl of shock fluid (0.2 mg/ml of protein) was mixed with 5 μl of [¹⁴C]ribose (60 mCi/mmol; final concentration, 60 μM). After 30 s of incubation at room temperature, the whole samples were poured into 0.8 ml of ice-cold saturated (NH₄)₂SO₄, filtered through membrane filters (0.45-

μm pore size; Millipore Corp.), and washed with the same $(\text{NH}_4)_2\text{SO}_4$ solution. The filters were dried and counted (27).

Analytical techniques. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% gels was done by the method of Laemmli (19). The electrophoresis buffer contained 25 mM Tris, 0.2 M glycine, and 0.1% SDS. Protein samples were treated for 3 min at 95°C with 2% SDS and 3% mercaptoethanol in 44 mM Tris buffer (pH 8.8) containing 10% glycerol and 0.001% bromphenol blue. Inner membrane samples, however, were treated with 1% SDS and 12 mM dithiothreitol for 15 min at 50°C. Samples were put on the gel either then or after additional boiling for 3 min (21).

Agarose gel electrophoresis for analyzing DNA preparations was performed as described by Davis et al. (11) with Tris-acetate buffer.

Genetic techniques. P22 transductions, the preparation of λ lysates, and the subsequent λ DNA isolation were done as described elsewhere (11). For cloning *glpT*, lysogenic complementation (11) was used. After simultaneous infection with λgt7 pool phage and $\lambda\text{gt4-lac-5}$, strain DL39 was plated on G3P as the only carbon source. The colonies were screened for the presence of β -galactosidase and the repressed state of alkaline phosphatase. Phage lysates were obtained by heat induction, and single plaques were tested on indicator plates containing X-Gal. Colorless plaques were purified and retested for the lysogenic complementation of strain DL39 on G3P.

The digestion of DNA with restriction nucleases (Boehringer Mannheim) was done by the method of Berman et al. (6). The religation of digested DNA fragments was performed as described elsewhere (11). Plasmid-DNA was isolated by the technique of Birnboim and Doly (7), and transformation was done by the procedure described by Davis et al. (11).

RESULTS

Transport of G3P in *S. typhimurium* LT2.

Figure 1 shows the ability of strain LT2 to take up G3P at an external concentration of 0.13 μM after growth on glucose, galactose, glycerol, or G3P as the carbon source. As can be seen, G3P uptake was induced by glycerol and G3P. Like the corresponding system in *E. coli*, G3P uptake was inhibited by P_i with a K_i of 8 mM (data not shown). The kinetic parameters of G3P uptake were measured in fully induced cells and in the absence of P_i (Fig. 2). For comparison, the corresponding uptake in *E. coli* was also measured. The *S. typhimurium* system exhibited a K_m of 50 μM versus 14 μM in *E. coli*, whereas the V_{max} was identical ($2.2 \text{ nmol/min} \cdot 10^8 \text{ cells}$) in both organisms.

***glpT* mutations of *S. typhimurium* LT2 and their genetic locations.** Fosfomycin-resistant clones were isolated from a LT2 derivative that carries the tetracycline resistance transposon Tn10 at 47 min (*zeh-775::Tn10*) on the *S. typhimurium* linkage map, supposedly in the vicinity of the *glpT* region. During the preliminary experiments, we

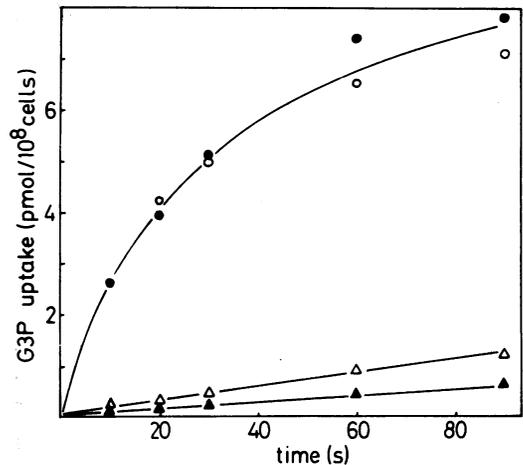


FIG. 1. Transport of G3P of strain LT2 grown on E-medium with different carbon sources. Symbols: ●, G3P; ○, glycerol; △, galactose; and ▲, glucose. The initial G3P concentration was 0.13 μM .

noticed that with *S. typhimurium* a 100-fold-higher concentration of fosfomycin (100 μM) was needed than with *E. coli* to successfully kill *glpT*⁺ strains on plates containing glycerol as the carbon source. This is probably due to a more resistant target enzyme and not to an inefficient transport of fosfomycin through the *glpT*-dependent transport system. After the induction of the hexose-phosphate transport system, *glpT* strains became sensitive to fosfomycin. However, unlike *E. coli*, again 100 μM fosfomycin was necessary to successfully kill the cells.

The fosfomycin-resistant mutant RH3 was chosen for further studies. No uptake of G3P at a 0.13 μM concentration could be detected with this strain (Fig. 3). Also, it was unable to grow on G3P as a sole source of carbon. Revertants were isolated at a frequency of 10^{-8} by selecting for growth on G3P. To map the mutation in RH3, we performed P22-mediated transductions with TR5903 as the donor ($\text{Tet}^s \text{gyrA } glpT^+$). The results of the three-factor crosses are shown in Table 2. Accordingly, *glpT* is 37% linked to *gyrA* and 4% linked to *zeh-775::Tn10* with the relative sequence *glpT*, *gyrA*, *zeh-775::Tn10*.

***glpQ*-dependent phosphodiesterase activity.** Periplasmic proteins were isolated by cold osmotic shock from strain LT2 grown either on glycerol or galactose and in the presence of fucose and analyzed on SDS-PAGE. For comparison, the shock proteins of DL291 carrying *glpQ* from *E. coli* on a multicopy plasmid (pGS31) are also shown in Fig. 4. No protein of the same apparent size as the *E. coli glpQ* product was detected in LT2, but we observed two other proteins (apparent molecular weights,

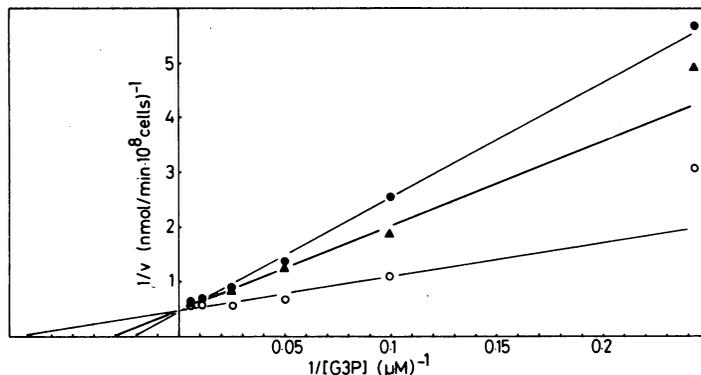


FIG. 2. Determination of K_m and V_{max} of *glpT*-dependent G3P uptake in *S. typhimurium* LT2 (●), *E. coli* TS100 (○), and *E. coli* DL39 (λ gt7-*glpT*/ λ gt4-*lac-5*) (▲). The cells were grown on E-medium with glycerol (*S. typhimurium*) or MMA with glycerol (*E. coli*).

38,000 and 30,000) induced by glycerol (or repressed by galactose). Although the *glpQ*-encoded protein could not be identified in osmotic shock fluids by analytical gel techniques, its phosphodiesterase activity was demonstrated (Table 3). Its specific activity was comparable to that of the corresponding enzyme in *E. coli*.

To establish whether or not *glpQ* is located, as in *E. coli*, on the same operon and distal to *glpT*, we screened 10 independent fosfomycin-resistant *glpT* mutants for phosphodiesterase activity. Three mutations had a polar effect on *glpQ* expression. RH1 carried one of them. Its activity was about 10% of the wild type (Table 3). The position of the *glpT* mutation in RH1 was confirmed by cotransduction with *zeh-775::Tn10*. These results show that *glpQ* is, indeed, located distal to *glpT* on the same operon.

Second *S. typhimurium* G3P transport system under *pho* control. Recently, a second transport system (*ugp*) for G3P that is under *pho* control has been characterized in *E. coli* (30, 31). Thus, conditions that lead to the derepression of alkaline phosphatase also derepress a periplasmic binding protein-dependent transport system for G3P (4). *S. typhimurium* does not contain alkaline phosphatase but does have the regulatory outfit for its synthesis (36). Therefore, it was of interest to determine whether *S. typhimurium* contains the *pho* regulon-dependent *ugp* transport system for G3P. Figure 3 shows a comparison of the uptake rates of G3P for the *glpT*⁺ strain LT2 grown with glycerol and high P_i , the *glpT* strain RH3 with glycerol or glucose and high P_i , as well as RH3 with glucose and a P_i concentration that is known to derepress alkaline phosphatase in *E. coli*. As can be seen, phosphate-limiting growth conditions led to the induction of a G3P transport system in RH3.

Cloning of the *glpT* region of *S. typhimurium* LT2 in *E. coli*. The availability of an *S. typhimurium*

ium DNA bank in the form of *EcoRI* restriction fragments in phage λ gt7 (11) prompted an attempt to clone the *glpT* region of *S. typhimurium* into *E. coli*. λ gt7 contains only one *EcoRI* restriction site in the nonessential region of the phage and can accommodate up to 16 kilobases (kb) of foreign DNA for packaging. Since λ gt7 is *cI att int* and therefore cannot lysogenize by itself, λ gt4-*lac-5* (11) was used as the helper

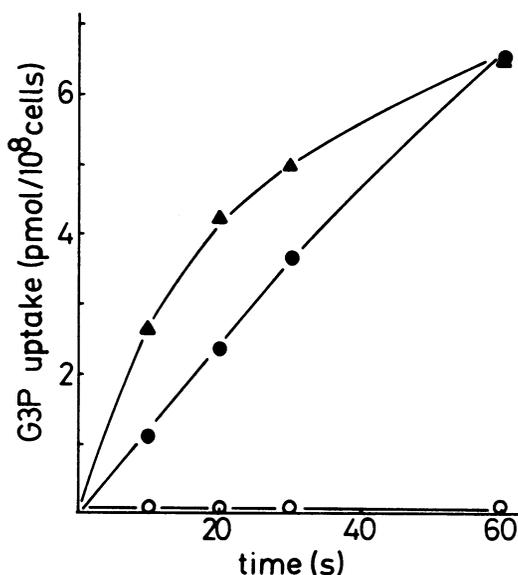


FIG. 3. *ugp*-dependent G3P uptake of *S. typhimurium glpT* strain RH3, grown on G plus L medium plus glucose containing 60 μ M P_i (●) or 1 mM P_i (○). The G3P uptake after growth in E-medium plus glycerol containing 74 mM P_i was identical to that in cells grown in G plus L medium plus glycerol containing 1 mM P_i (○). For comparison, *glpT*-dependent G3P uptake of LT2 grown on E-medium plus glycerol is shown (▲).

TABLE 2. P22-mediated cotransduction between *glpT*, *gyrA*, and *zeh-775::Tn10*

<i>S. typhimurium</i> donor	<i>S. typhimurium</i> recipient	Selected marker	Recombinant class	% of total ^a
RH8 (Tet ^r <i>gyrA glpT</i>)	TR5903 (Tet ^s <i>gyrA glpT</i> ⁺)	Tet ^r	<i>gyrA glpT</i> ⁺	50.7
			<i>gyrA</i> ⁺ <i>glpT</i> ⁺	46.7
			<i>gyrA</i> ⁺ <i>glpT</i>	2
			<i>gyrA glpT</i>	0.7
TR5903 (Tet ^s <i>gyrA glpT</i> ⁺)	RH8 (Tet ^r <i>gyrA</i> ⁺ <i>glpT</i>)	<i>glpT</i> ⁺	<i>gyrA</i> ⁺ Tet ^r	61
			<i>gyrA</i> Tet ^r	34
			<i>gyrA</i> Tet ^s	3
			<i>gyrA</i> ⁺ Tet ^s	2
			<i>gyrA</i> ⁺	88
TT5345 (Tet ^r <i>gyrA</i> ⁺ <i>glpT</i> ⁺)	RH3 (Tet ^s <i>gyrA glpT</i>)	Tet ^r <i>glpT</i> ⁺	<i>gyrA</i> ⁺	88
			<i>gyrA</i>	12

^a Out of 150 selected recombinants in the first cross, 100 in the second cross, and 50 in the third cross.

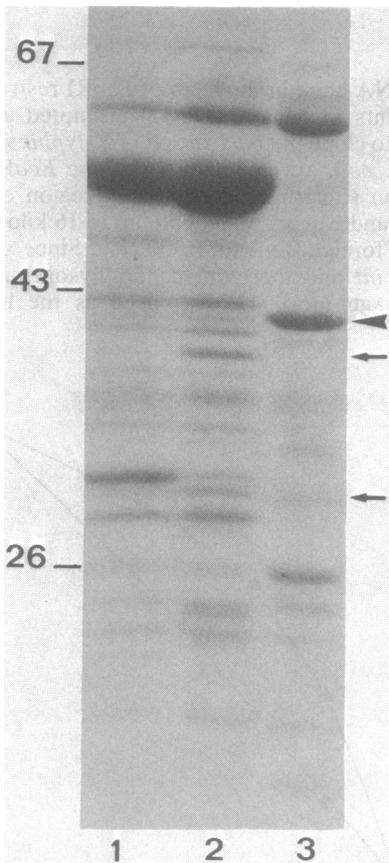


FIG. 4. SDS-PAGE analysis of cold osmotic shock fractions (Coomassie blue staining) from DL291(pGS31) grown on LB-glycerol containing 10 µg of tetracycline per ml (lane 3), LT2 grown on E-medium plus glycerol (lane 2), or LT2 grown on galactose and 0.2 mM fucose (lane 1). Glycerol-inducible (or galactose-repressible) proteins are indicated by arrows. The *E. coli glpQ* product is indicated by the arrowhead. The standard marker proteins were bovine serum albumin, ovalbumin, α -chymotrypsinogen, and lysozyme. Molecular weights of the protein standards, $\times 10^3$.

phage for lysogenic complementation. This phage carries the temperature-sensitive repressor *cI857* and the gene for β -galactosidase. As the cloning recipient, *E. coli* DL39 [Δ (*glpT-glpA*)593 Δ (*argF-lac*)U169] was used. One phage (λ gt7-*glpT*), isolated by lysogenic complementation, was chosen for DNA preparation, restriction analysis, and subcloning into an appropriate plasmid vector.

Figure 5 shows the G3P transport activities of the recipient DL39, DL39 carrying λ gt7-*glpT* and λ gt4-*lac-5*, and the wild-type strain TS100. As can be seen, transport activity has been restored in the lysogenic strain. The kinetic analysis of G3P uptake in this strain (carrying a single copy of *S. typhimurium glpT*) gave a K_m of 33 μ M and a V_{max} of 2.0 nmol/min \cdot 10⁸ cells (Fig. 2). The cloned *S. typhimurium glpT* region (present as a single copy) was repressed by the *E. coli glpR* product. G3P transport rates were inducible by a factor of 5 in the λ gt7-*glpT*/ λ gt4-

TABLE 3. Phosphodiesterase activity

Strain	Growth conditions	Phosphodiesterase activity ^a (μ mol/min \cdot mg of protein)
<i>S. typhimurium</i>		
LT2	E-medium, glycerol	1.5
LT2	E-medium, glucose	<0.01
RH1	MMA, glycerol	0.15
RH3	MMA, glycerol	1.70
<i>E. coli</i>		
MC4100	MMA, glycerol	1.0
MC4100	MMA, glucose	0.05
DL291(pGS31)	LB glycerol, tetracycline (10 μ g/ml)	23.0
DL39	MMA, glycerol	<0.01
DL39(pRH100)	MMA, G3P	<0.01

^a Measured in crude osmotic shock fluid with glycerophosphocholine as the substrate.

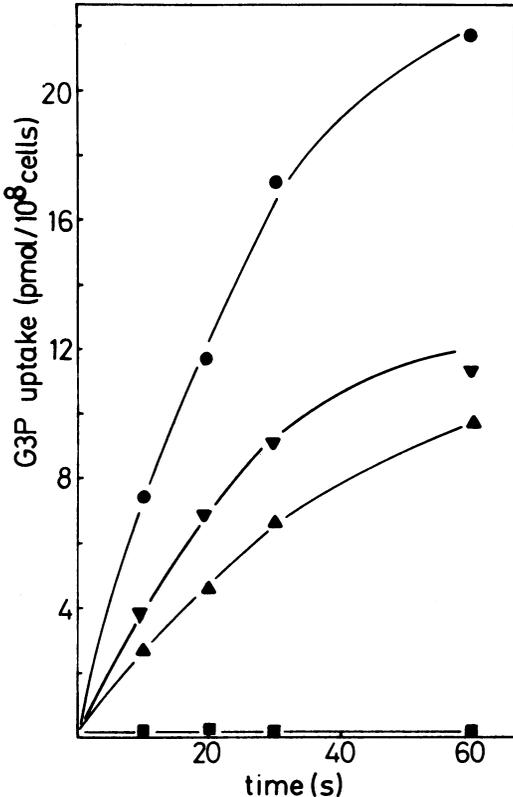


FIG. 5. *glpT*-dependent G3P transport after cloning the *S. typhimurium glpT* region into a $\Delta glpT$ recipient *E. coli* strain. Symbols: ●, DL39(pRH100) grown on MMA plus G3P; ▲, DL39 ($\lambda gt7$ -*glpT*/ $\lambda gt4$ -*lac-5*); ▼, TS100; and ■, DL39. The last three strains were grown on MMA plus glycerol.

lac-5 lysogenic strain SH173 [*glpR*⁺ Δ (*glpT*-*glpA*) 5931] when the growth medium of the cells was changed from succinate to glycerol (data not shown).

Subcloning of the *glpT* region into a multicopy plasmid vector. The DNA of $\lambda gt7$ -*glpT* was prepared and analyzed by agarose gel electrophoresis after cleavage with *EcoRI*. Besides the two λ arms of 19.6 and 15.2 kb, two fragments of approximately 5.5 and 3.5 kb were found. For subcloning, plasmid pACYC184 was chosen (9). It contains one *EcoRI* cleavage site in the chloramphenicol resistance gene. $\lambda gt7$ -*glpT* DNA and pACYC184 were digested with *EcoRI* and religated. Transformants of DL39 were selected on G3P minimal medium. Colonies were screened for chloramphenicol sensitivity and for derepressed alkaline phosphatase. This was necessary, since strains exhibiting constitutive synthesis of this enzyme could, at least at P_i concentrations below 1 mM, grow on G3P without containing a transport system for G3P. One

strain, DL39(pRH100), was chosen for further studies. The G3P transport activity of this strain was about twice that found in the wild type (Fig. 5). However, this strain did not express *glpQ*-encoded phosphodiesterase activity (Table 3).

glpT hybrid plasmid pRH100 contained (besides the vector DNA of 4.05 kb in length) the *EcoRI* fragment of 3.5 kb which was identical to one of the fragments present in the $\lambda gt7$ -*glpT* DNA. A restriction map of pRH100 is shown in Fig. 6. The removal of the 0.2-kb *BglII*-*BamHI* fragment followed by religation resulted in a loss of G3P transport activity. In addition, recloning the 3-kb *EcoRI*-*PstI* fragment into pBR322 still allowed full expression of *glpT*. This defines the position of the *glpT* gene on the plasmid: it starts to the right of the *BamHI* site and ends to the right of the *PstI* site. It also defines the direction of transcription: the entire 3.5-kb *EcoRI* fragment (pRH100) carries the intact *glpT* gene but lacks part of *glpQ*, which has to be adjacent and distal to *glpT*. Since there would be ample space for *glpQ* to the right of the *BglII*-*BamHI* fragment, the remainder of *glpQ* can only be located to the left of the *BglII* site; therefore, the transcription of the *glpT*-*glpQ* operon is from right to left on Fig. 6.

***S. typhimurium glpT* gene product.** We were unable to express the *glpT* gene of pRH100 by cell-free *in vitro* synthesis or in minicells, using [³⁵S]methionine as a protein label. Therefore, attempts were made to identify the plasmid-encoded proteins directly by SDS-PAGE analysis of subcellular fractions.

Cytoplasmic membrane proteins. Cytoplasmic membrane proteins of strain DL291 carrying the plasmids pACYC184, pRH100, pGS31, and pRH103 are shown in Fig. 7. pGS31 carries the entire *E. coli glpT, Q* region on a 7.3-kb *PstI* fragment (20). pRH103 is a derivative of pRH100, which carries an amber mutation in *glpT*. As can be seen, DL291(pRH100) and DL291(pGS31) both expressed a protein with a molecular weight of 33,000 that is absent in



FIG. 6. Restriction map of pRH100, the hybrid plasmid harboring *glpT* in pACYC184. The linear form is shown, opened at the *EcoRI* site between the vector and the cloned DNA. The restriction endonucleases were *AvaI* (A), *BamHI* (B), *BglII* (Bg), *EcoRI* (E), *HindIII* (H), *PstI* (P), *SstII* (S), and *SalI* (SI). The left *EcoRI* fragment represents chromosomal DNA; the right *EcoRI* fragment represents pACYC184 DNA.

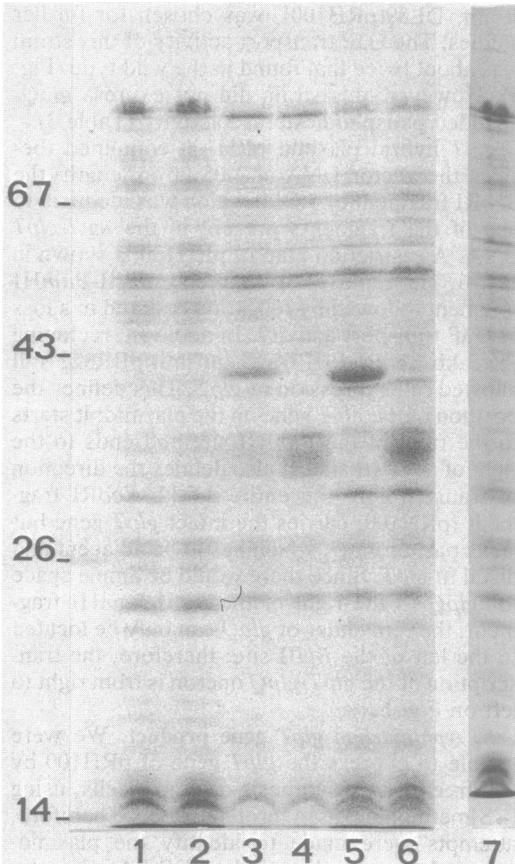


FIG. 7. SDS-PAGE analysis of cytoplasmic membrane proteins (Coomassie blue staining). The samples were treated with 1% SDS and 12 mM dithiothreitol for 15 min at 50°C and either directly loaded onto the gel (lanes 1, 3, and 5) or first heated to 100°C for 3 min (lanes 2, 4, 6, and 7). The inner membrane proteins shown are from *E. coli* DL291 harboring pACYC184 (lanes 1 and 2), pRH100 (lanes 3 and 4), pGS31 (lanes 5 and 6), or pRH103 (lane 7). The strains were grown at 37°C on LB containing 10 μ g of tetracycline per ml. Molecular weights of the protein standards, $\times 10^3$.

DL291(pACYC184) and in DL291(pRH103). The protein exhibited the same characteristic behavior on SDS-PAGE as does the G3P permease protein from *E. coli* (21): a sharp band with an apparent molecular weight of 42,000 was seen in preparations treated in SDS at 50°C, whereas a somewhat diffuse band with an apparent molecular weight of 33,000 was seen in preparations that were heated to 95°C in SDS after the membranes were dissolved at 50°C. The absence of this protein in the *glpT* amber mutant and the similarity of its behavior with that of the *E. coli* protein establish its identity with the G3P permease protein. DL291 containing pRH100 or pGS31 also synthesized a protein

of 62,000 daltons that was absent in DL291 containing the vector only. This protein is most likely the larger subunit of the anaerobic G3P dehydrogenase (21, 28).

Outer membrane proteins. An analysis of outer membrane proteins on SDS-PAGE shows one protein with an apparent molecular weight of 45,000 expressed more strongly in DL291 (pRH100) as compared with the same strain harboring pGS31 or the *glpT* amber plasmid pRH103 (Fig. 8).

Periplasmic proteins. Among the periplasmic proteins (Fig. 9) of DL39 carrying λ gt7-*glpT* and λ gt4-*lac-5* or pRH100, no new protein was found that was missing in the shock proteins of the recipient strain alone, whereas in the case of pGS31, the periplasmic *glpQ* product (with an apparent molecular weight of 40,000) was easily identified (Fig. 4). The glycerol-inducible periplasmic proteins of *S. typhimurium* wild-type strain LT2 (Fig. 4) were absent in the *E. coli* strains carrying the *S. typhimurium glpT* gene. DL39(pRH100) also did not exhibit periplasmic phosphodiesterase activity (Table 3).

pRH100 did not complement the anaerobic G3P dehydrogenase-negative phenotype of DL39. Thus, it does not carry the complete information of *glpQ* and *glpA*. However, a pro-

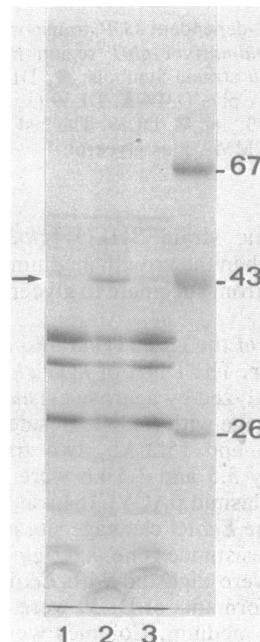


FIG. 8. SDS-PAGE analysis of outer membrane proteins (Coomassie blue staining) from DL291 harboring plasmids pGS31 (lane 3), pRH100 (lane 2), or the *glpT* amber plasmid pRH103 (lane 1). The cells were grown in LB and 10 μ g of tetracycline per ml. Molecular weights of the protein standards, $\times 10^3$.

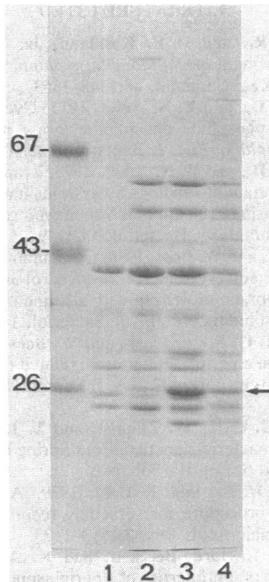


FIG. 9. SDS-PAGE analysis of cold osmotic shock fractions (Coomassie blue staining) of the following strains: DL39 ($\Delta glpT$) (lane 3) and DL39 ($\lambda gt7-glpT/\lambda gt4-lac-5$) (lane 2), both grown on MMA plus glycerol; DL39(pRH100) grown on MMA plus G3P (lane 1); and DL291(pRH103) grown on MMA plus glycerol (lane 4). The growth media of the last two strains contained 10 μ g of tetracycline per ml. The position of ribose-binding protein is indicated by the arrow. Molecular weights of the protein standards, $\times 10^3$.

tein with a molecular weight of 62,000 could be seen on SDS-PAGE when plasmid-encoded proteins were radioactively labeled in UV-irradiated cells of the *recA* strain DL291(pRH100). This protein was also present in cytoplasmic membranes from this strain (see above). It is not the product of the *glpT* gene, since *glpT* amber mutants still synthesized it normally. This protein is also not encoded by pACYC184 (Fig. 7). These data, together with the fact that the DNA insert is only 3.5 kb long, suggest that the 62,000-dalton protein is identical to one subunit of the anaerobic G3P dehydrogenase, the product of the *glpA* gene. This gene has been located in *E. coli* adjacent to *glpT* (28).

Influence of cloned *S. typhimurium glpT* region on other periplasmic systems in *E. coli*. As shown in Fig. 9, some periplasmic proteins of *E. coli* were present in smaller amounts or nearly absent when strain DL39 carried $\lambda gt7-glpT$ or the multicopy plasmid pRH100. In the latter case, the effect is much stronger. Ribose-binding protein, which in MC4100 derivatives is expressed constitutively (J. Beckwith, personal communication), is one example. Concomitant with the loss of the ribose-binding protein band on SDS-PAGE, ribose-binding activity in the osmotic

shock fluid of DL39(pRH100) disappeared (Table 4). In addition, strains harboring pRH100 do not grow on ribose. In preliminary studies, it was found that maltose-binding protein (after maltose induction) was also strongly depressed in strains harboring pRH100. pRH103, which carries a hydroxylamine-induced amber mutation in the plasmid-borne *glpT*, did not exhibit these effects. Therefore, they must be caused by the transcription of the truncated *glpT-glpQ* operon on pRH100. Expressing the intact *E. coli glpT-glpQ* operon (pGS31) in *E. coli* does not show these effects (21).

DISCUSSION

The *glp* regulon-dependent G3P transport system of *S. typhimurium* closely resembles the corresponding system of *E. coli*. Thus, fosfomycin and P_i are recognized as substrates, and G3P is transported with similar kinetic parameters, even though the *E. coli* system exhibits a three- to fourfold-higher apparent affinity for G3P.

Genetically, the *glpT* region responsible for G3P transport is located in the vicinity of *gyrA*, as is the case in *E. coli*. The *glpT* operon of *E. coli* contains *glpQ* (distal to *glpT*), coding for a periplasmic phosphodiesterase (22). In *S. typhimurium* LT2, such a glycerol/G3P-inducible activity is also found in osmotic shock fluids. However, no protein resembling the *E. coli glpQ* product, formerly called GLPT protein (5), could be identified among LT2 shock proteins either by SDS-PAGE (Fig. 4) or by two-dimensional PAGE (17; data not shown). However, it is clear that *glpQ*, the gene coding for this enzyme, is located in the same operon and distal to *glpT*.

As *E. coli*, *S. typhimurium* expresses a second transport system for G3P under conditions of P_i starvation. Therefore, besides the *phoS/phoT*-specified phosphate transport system (36), a *ugp*-like system under *pho* control is in existence, whereas alkaline phosphatase, the *phoA* product in *E. coli*, is absent in *S. typhimurium*.

The *glpT* region of strain LT2 was cloned and established in an *E. coli* mutant deleted for *glpT*. This was done with a single copy contained in a

TABLE 4. Ribose-binding capacity of cold osmotic shock fractions

<i>E. coli</i> strain	Growth conditions	Binding of [14 C]ribose (μ mol/g of protein)
DL39	MMA, glycerol	5.3
DL39 ($\lambda gt7-glpT/\lambda gt4-lac-5$)	MMA, glycerol	0.88
DL39(pRH100)	MMA, G3P	0.23
DL291(pRH103)	MMA, glycerol	2.5

λ prophage or after insertion into a multicopy plasmid. The *glpT* gene of *S. typhimurium* was contained in a 3.5-kb *EcoRI* fragment. It codes for the G3P permease protein, which is located in the cytoplasmic membrane and resembles the corresponding *E. coli glpT* product (21). The cloned region does not carry the information for the entire anaerobic G3P dehydrogenase, but still expresses the gene for its larger subunit (62,000 daltons). It does not confer phosphodiesterase activity on *glpQ*-deficient recipient strains. Therefore, with regard to the position of *glpT*, we conclude that the two *EcoRI* restriction sites are located just beyond *glpA* (coding for the 62,000-dalton subunit) on the one side and within *glpQ* on the other.

Surprisingly, the cloned *S. typhimurium* DNA fragment exhibited a strong effect on other systems, particularly when it was present in multiple copies. The proper synthesis or assembly of the periplasmic ribose- and maltose-binding proteins was affected, and the outer membrane also seemed to be perturbed. A 45,000-dalton protein usually present only in small amounts became a major component, in addition to the OmpC, OmpF, and OmpA proteins. A reasonable explanation for this phenomenon could be that the synthesis of a residual fragment of the periplasmic phosphodiesterase with a normal signal sequence initiates the secretion process, but since the second part of the protein is missing, it is conceivable that further secretion steps could not take place and, therefore, that secretion sites are blocked. This would be reminiscent of the situation where the N-terminal part of a periplasmic protein is fused to a cytoplasmic protein (e.g., *malE-lacZ* fusion), and in some cases, the hybrid protein sticks to the membrane tightly, interfering with the secretion of other periplasmic and outer membrane proteins (15, 16). However, in the present situation, no accumulation of the precursor of any secretory protein is apparent. The alternative possibility—that the synthesis of the foreign G3P permease itself may cause the problems—seems unlikely: the *S. typhimurium* protein appears to be similar to the *E. coli* permease protein, but even a single copy of the cloned *EcoRI* fragment (contained in the λ prophage) resulted in a severe reduction of ribose-binding protein.

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