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Periplasmic Protein Related to the *sn*-Glycerol-3-Phosphate Transport System of *Escherichia coli*

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Two-dimensional gel electrophoresis of shock fluids of *Escherichia coli* K-12 revealed the presence of a periplasmic protein related to *sn*-glycerol-3-phosphate transport (GLPT) that is under the regulation of *glpR*, the regulatory gene of the *glp* regulon. Mutants selected for their resistance to phosphonomycin and found to be defective in *sn*-glycerol-3-phosphate transport either did not produce GLPT or produced it in reduced amounts. Other mutations exhibited no apparent effect on GLPT. Transductions of *glpT*$^+$ *nalA* by phage P1 into these mutants and selection for growth on *sn*-glycerol-3-phosphate revealed a 50% cotransduction frequency to *nalA*. Reversion of mutants that did not produce GLPT to growth on *sn*-glycerol-3-phosphate resulted in strains that produce GLPT. This suggests a close relationship of GLPT to the *glpT* gene and to *sn*-glycerol-3-phosphate transport. Attempts to demonstrate binding activity of GLPT in crude shock fluid towards *sn*-glycerol-3-phosphate have failed so far. However, all shock fluids, independent of their GLPT content, exhibited an enzymatic activity that hydrolyzes, under the conditions of the binding assay, 30 to 60% of the *sn*-glycerol-3-phosphate to glycerol and inorganic orthophosphate.

The *sn*-glycerol-3-phosphate (GLP) transport system in *E. coli* appears to be ideally suited for a combined genetic-biochemical analysis. There is an easy selection procedure for transport-negative *(glpT)* strains that utilizes selection for resistance against phosphonomycin (9, 15, 22). Transport-positive revertants can be obtained by growth on GLP (16). Also, constitutive *(glpR)* strains can be selected (5). *glpT* strains have been found to be 50% cotransducible by phage P1 to the *nalA* marker, and the map position of 43 min on the *E. coli* chromosome has been proposed for the *glpT* mutation (5, 14). From these studies it cannot be determined whether the *glpT* gene consists of one or more cistrons. Unlike most transport systems in *E. coli*, the GLP transport system is homogeneous, i.e., the substrate is transported by only one system. This system has been characterized as an energy-dependent active transport system (K_m, 12 μM) translocating the substrate without chemical alteration (8).

Despite these advantages, no attempts have been made to study the system with biochemical methods. In particular, it is not clear whether the system is mediated via a periplasmic binding protein (2) or a typical membrane-bound system active in membrane vesicles of the Kabackosome type (12). In the present publication, we demonstrate the existence of a protein in the osmotic shock fluid of glycerol-induced or -constitutive strains that appears intimately involved in GLP transport and is likely to be the gene product of *glpT*.

MATERIALS AND METHODS

The bacterial strains used are listed in Table 1. Strain LA 3400 was constructed by P1 transduction according to the procedure of Miller (18); selection was by growth on maltose. Strains LA 3401 to 3405 were isolated as spontaneous phosphonomycin-resistant clones of strain 72 as described (22). All phosphonomycin-resistant strains were tested for growth on glycerol and GLP in a low-phosphate medium (7). Growth on glycerol and no growth on GLP was scored as defective in the GLP transport system. P1 transduction was used to map the *glpT* mutations. The procedure used was that of Miller (18). Selection was by growth on GLP in low-phosphate medium (7). To screen for the presence or absence of the anaerobic GLP dehydrogenase, the mutants were streaked on plates containing minimal medium A (18), 0.2% glycerol, 0.03% Casamino Acids, and 0.02 M fumarate (14). The plates were incubated at 37 C in a GasPak anaerobic tank (BBL). Growth under these conditions was scored as positive for anaerobic GLP dehydrogenase (14). For the transport assay, the strains were grown under aeration at 37 C overnight in low-phosphate me-

TABLE 1. *Bacterial strains used*

Strain no.	Parent	Sex	Isolation procedure	Genotype	Reference
72		Hfr		Δ(*glpR-malA*), *phoA*	Lin (5)
W3092cy⁻		F⁻		*galK*, *lacY*	Wu (24)
LA 3399	JK 114 R 3	F⁻	Resistance against nalidixic acid (100 μg/ml) spontaneous mutant	*argH*, *ara*, *cir*, *lac*, *gal*, *ura*, *trp*, *his*, *thi*, *mal*, *man*, *mgl*, *nalA*, *xyl*, *purC*, *tonA*, *str-r*	Konisky (4)
LA 3400	72	Hfr	P1 transduction with P1 grown on W3092cy⁻	*glpR*⁺, *mal*⁺, *phoA*	
LA 3401	72	Hfr	Resistance against phosphonomycin, spontaneous mutants	Δ(*galpR-malA*), *phoA*, *glpT*	
LA 3402	72	Hfr	"	"	
LA 3403	72	Hfr	"	"	
LA 3404	72	Hfr	"	"	
LA 3405	72	Hfr	"	"	
LA 3406	LA 3401	Hfr	Growth on GLP, spontaneous revertant	Δ(*glpR-malA*), *phoA*, *glpT*⁺	
LA 3407	LA 3402	Hfr	"	"	
206		Hfr		*lac*, *mtl*, *xyl*, *mal*, *pdl*, *gal*, *phoA*, *phoR*, *glpA*, *thr*, *leu*, *arg*, *met*, *ilv*, *his*, *thi*, *str*	Ken Andrews[a]

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dium (7) with either 0.4% glycerol or 0.4% succinate as a carbon source. The cells were washed twice with fresh growth medium minus the carbon source and resuspended in the same medium to an optical density of 0.5 units at 576 nm.

Transport assays for GLP are unusually sensitive in respect to the ionic strength of the uptake medium. For instance, after growth in low-phosphate growth medium that contains 240 mM tris(hydroxymethyl)aminomethane (Tris) (pH 7.0) and subsequent resuspension to 10 mM Tris (pH 7.0), transport activity is reduced to less than 10% as compared with the same assay in fresh growth medium minus carbon source. This reduction in transport appears reversible and can at least partially be restored by adding 240 mM Tris or 240 mM NaCl. Therefore, all transport assays were performed with cells washed and resuspended in growth medium minus carbon source.

To isolate periplasmic proteins, the cold osmotic shock procedure was performed according to Neu and Heppel (19). Five hundred-milliliter cultures were grown under aeration at 37 C in minimal medium A with 0.4% of the corresponding carbon source. Anaerobic cultures were grown with 0.4% glycerol as carbon source and 0.02 M fumarate as electron acceptor, as described (14). The obtained shock fluids were lyophilized, resuspended in 3 ml of 0.01 M Tris-hydrochloride (pH 7.0), and dialyzed overnight against the same buffer. The somewhat turbid solution was then centrifuged for 30 min at 100,000 × g. The resulting solution was applied to the two-dimensional polyacrylamide gel electrophoresis system (11).

Transport of GLP. To 3 ml of cells prepared in the previously described manner, 50 μl of [^{14}C]GLP (130 mCi/mmol, New England Nuclear Corp.) was added to a final concentration of 0.3 μM. After various time intervals, 0.5 ml was removed and filtered through a membrane filter (Millipore Corp.) of 0.65-μm pore size. The filters were then washed with 10 ml of growth medium. All operations were carried out at room temperature. The filters were dried at 120 C for 10 min and counted in toluene-based scintillation fluid.

Analytical techniques. Two-dimensional gel electrophoresis was performed as described (11). Shock fluid, 100 to 200 μl (about 300 μg of protein) prepared in the manner described above, was applied on the first dimension, without previous dialysis against 8 M urea that had been recrystallized from ethanol. This method proved superior to prior treatment with urea (dialysis overnight), since long exposure to 8 M urea resulted in multiple spots of some proteins in the direction of the first dimension.

Binding of crude shock fluids for [^{14}C]GLP was performed similarly, as described (20). To 300 μl of shock fluid (about 200 μg of protein) of the respective strains prepared in the described fashion, 10 μl of [^{14}C]GLP was added to a final concentration of 0.6 μM. The preparations were filtered under 40 lb/in^2 of nitrogen pressure through small filters (Amicon UM 10). The filters were counted after the addition of 0.2 ml of water in naphthalin-dioxan-based scintillation fluid.

To detect hydrolytic activity to GLP in the crude shock fluid, 20 μl of the above-described shock fluid was incubated with 10 μl of [^{14}C]GLP at a final concentration of 6 μM. The mixture was incubated for 1 h at room temperature. Then, 20 μl was spotted on chromatography paper (Whatman 3 MM). Descending chromatography was applied

with a mixture of *n*-butanol, pyridine, glacial acetic acid, and water (15:10:3:12) as solvent for 12 h. Radioactivity was determined in dried paper strips cut in small equal pieces using toluene-based scintillation fluid.

RESULTS

Two-dimensional gel electrophoresis of shock fluid from *E. coli*. Figure 1 shows the pattern of a Coomassie blue-stained two-dimensional polyacrylamide gel slab of the periplasmic proteins obtained by osmotic shock from strain LA-3400, after growth with 0.4% glycerol as carbon source. Separation of the proteins occurs in the first dimension (left to right) in 8 M urea, predominantly according to their electrical charge. The second dimension (top to bottom) occurs in sodium dodecyl sulfate, emphasizing differences in molecular weight. With this technique nearly all of the proteins in the osmotic shock fluid of *E. coli* can be separated. Therefore, if the synthesis of a yet unknown protein belonging to this compartment can be influenced by induction, repression, or mutation, it should easily be recognized by this analytical technique.

On the basis of this rationale, we observed a protein among the periplasmic proteins that appeared to be inducible during growth in the presence of glycerol.

The GLP transport protein (GLPT). Figures 2A and B show the detailed area of the two-dimensional gels from shock fluids of strain LA-3400 grown with trehalose and glycerol as carbon source. One protein spot (no. 1) appears inducible by glycerol. Under the denaturing conditions of the first dimension, the protein exhibits an unusual high negative charge. In the second dimension (sodium dodecyl sulfate), an approximate molecular weight of 40,000 can be estimated. The same analysis of the shock protein of a glycerol-constitutive *glpR* strain (Fig. 2C) grown in the presence of succinate demonstrates that the protein in question is under the control of the *glpR* regulatory

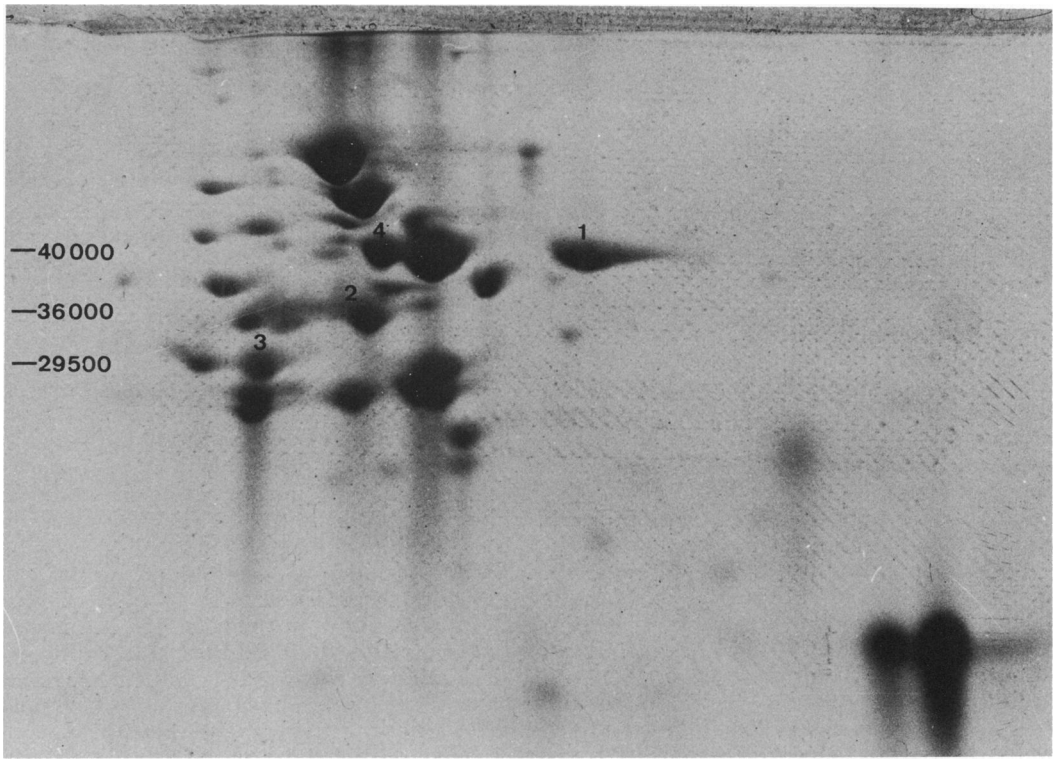

FIG. 1. *Two-dimensional polyacrylamide gel electrophoresis of shock fluid of strain LA 3400 (wild type) grown in the presence of glycerol. The first dimension consists of electrophoresis in 8 M urea (pH 8.4), followed by electrophoresis in 0.2% sodium dodecyl sulfate (pH 6.48), as described (11). About 300 μg of protein was applied. The numbers and molecular weights correspond to the following proteins: 1, GLPT; 2, galactose-binding protein (3); 3, ribose-binding protein (23); 4, maltose-binding protein (13).*

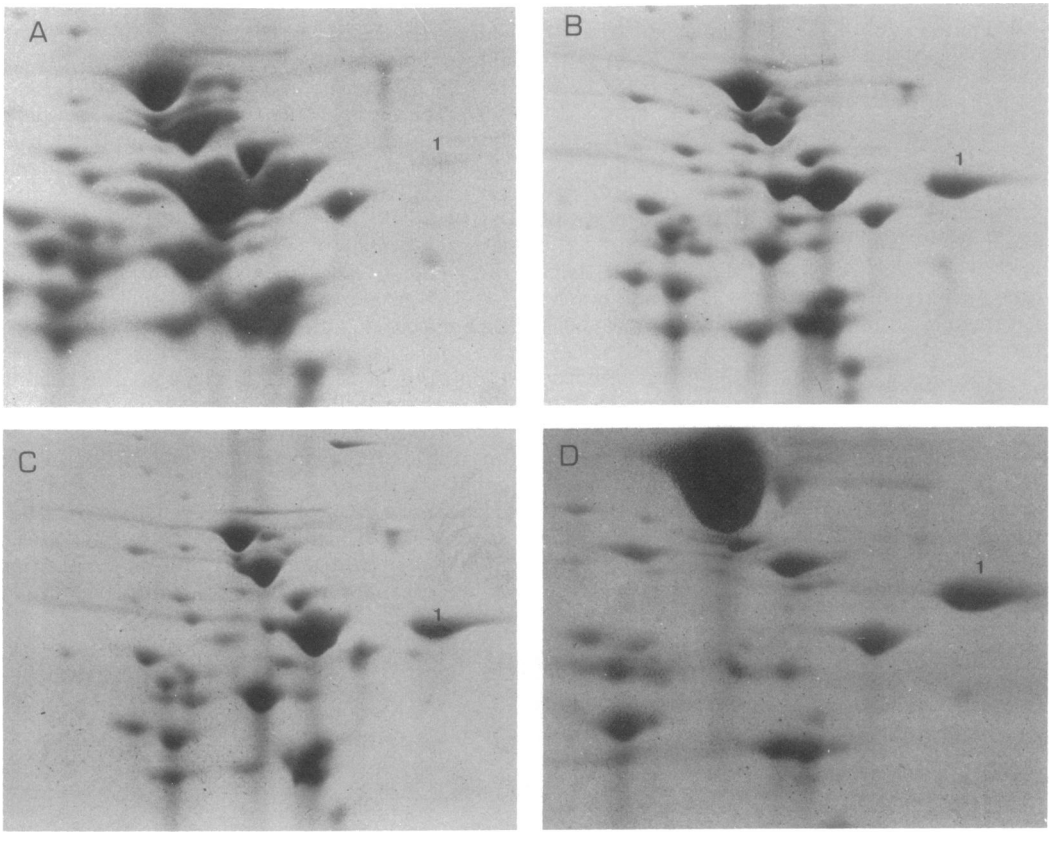

FIG. 2. *Detailed pictures of two-dimensional polyacrylamide gel slabs of shock fluids from several strains: (A) LA 3400 (wild type) grown on trehalose; (B) LA 3400 grown on glycerol; (C) strain 72 (glpR) grown on succinate; (D) strain 72 grown on glycerol in the presence of fumarate under anaerobic conditions. Experimental conditions as in Fig. 1.*

gene. Furthermore, GLPT is fully induced under conditions of anaerobic growth (Fig. 2D). This is consistent with the differential respiratory control observed with the different operons of the *glp* regulon. In contrast to gene *glpD*, which is maximally inducible under aerobic conditions, the genes *glpT* and *glpA* were reported to be fully inducible under anaerobic growth conditions (6).

From the availability of this protein in the periplasmic shock fluid, it seemed likely that it might be either the gene product of the *glpF* (glycerol facilitator) (21) or the *glpT* gene, known to be involved in GLP transport (5).

***glpT* mutant analysis.** By growth in the presence of phosphonomycin, an inhibitor of cell wall synthesis and substrate of the GLP transport system, we isolated spontaneous *glpT* mutants (22). The two-dimensional polyacrylamide gel electrophoretic analysis of the shock proteins of two independently isolated *glpT* strains, derivatives of the constitutive *glpR* strain 72, are shown in Fig. 3A and C. Both mutant preparations lack the protein entirely. Simultaneously, both strains fail to grow on GLP but grow normally on glycerol. Their defect must therefore be confined to the transport of GLP. Measurements of GLP uptake in these mutants in comparison to its wild-type parent is shown in Fig. 4. Using a substrate concentration of 0.3 μM, far below 12 μM, the K_m of the system (8), the mutants exhibit less than 5% transport activity of their parent. Transport of GLP in the wild type is competitively inhibited by orthophosphate with a K_i of 20 mM (not shown). This value agrees well with the K_i determined previously (8), and indeed characterizes the measured GLP uptake as being mediated by the GLP transport system. Application of the cold osmotic shock procedure of Neu and Heppel (19) to the wild-type strain drastically reduces transport activity (Fig. 4A). This is consistent with the notion that the GLP transport system is dependent

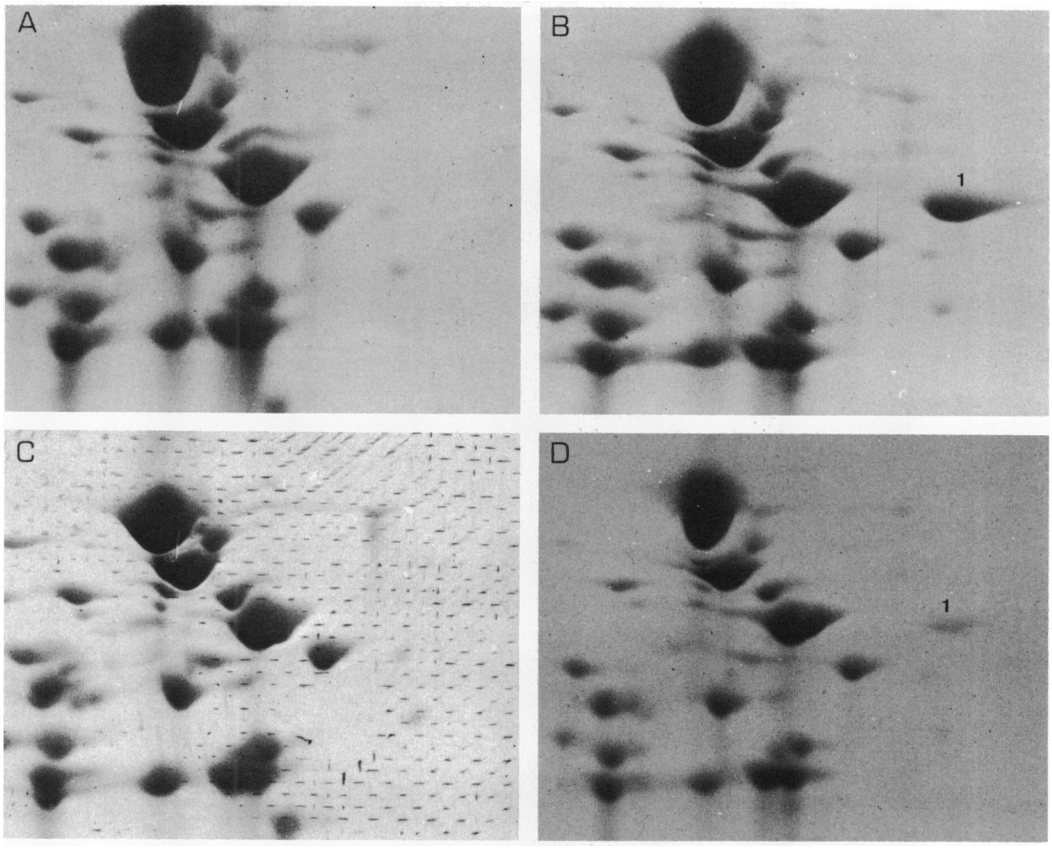

FIG. 3. *Detailed pictures of two-dimensional polyacrylamide gel slabs of shock fluids from the following glpT mutants and their revertants. (A) LA 3402 (glpT) grown on glycerol; (B) LA 3407 (glpT$^+$ revertant of LA 3402) grown on glycerol; (C) LA 3401 (glpT) grown on glycerol; (D) LA 3406 (glpT$^+$ revertant of LA 3401) grown on glycerol. The number 1 designates GLPT. Experimental conditions as in Fig. 1.*

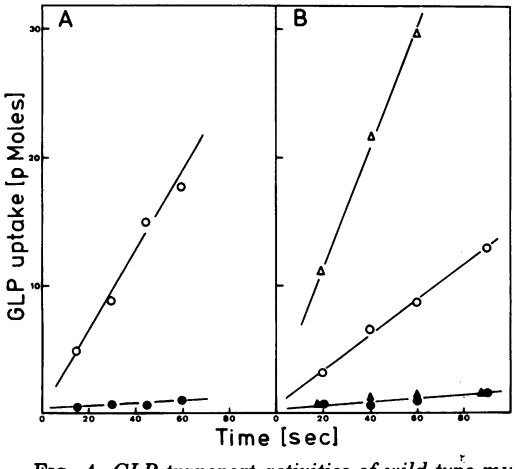

FIG. 4. *GLP transport activities of wild-type mutant and revertant strains. The bacterial cultures were resuspended in growth medium minus carbon source at an optical density of 0.5 units (576 nm). [^{14}C]GLP was added at an initial concentration of 0.3 μM. The data are expressed in terms of the amount GLP taken up per 0.5-ml sample. All operations were done at room temperature. (A) Strain LA 3400 before (○) and after (●) osmotic shock (19); (B) mutant strain LA 3401 (●), revertant LA 3406 (○); mutant strain LA 3402 (▲); revertant strain LA 3407 (△).*

on a periplasmic component. Spontaneous revertants of mutants LA 3401 and LA 3402 to growth on GLP were isolated and measured for transport activity, as well as the capability of producing GLPT. Figures 3B and D show that one strain (LA 3407) has fully regained the capability of producing GLPT, whereas strain LA 3406 shows only a partial reversion. The extent to which GLPT reappears in these revertants is reflected in their transport activity for GLP (Fig. 4B).

Shock fluids of several other mutants that fail to grow on GLP after isolation for resistance against phosphonomycin were analyzed

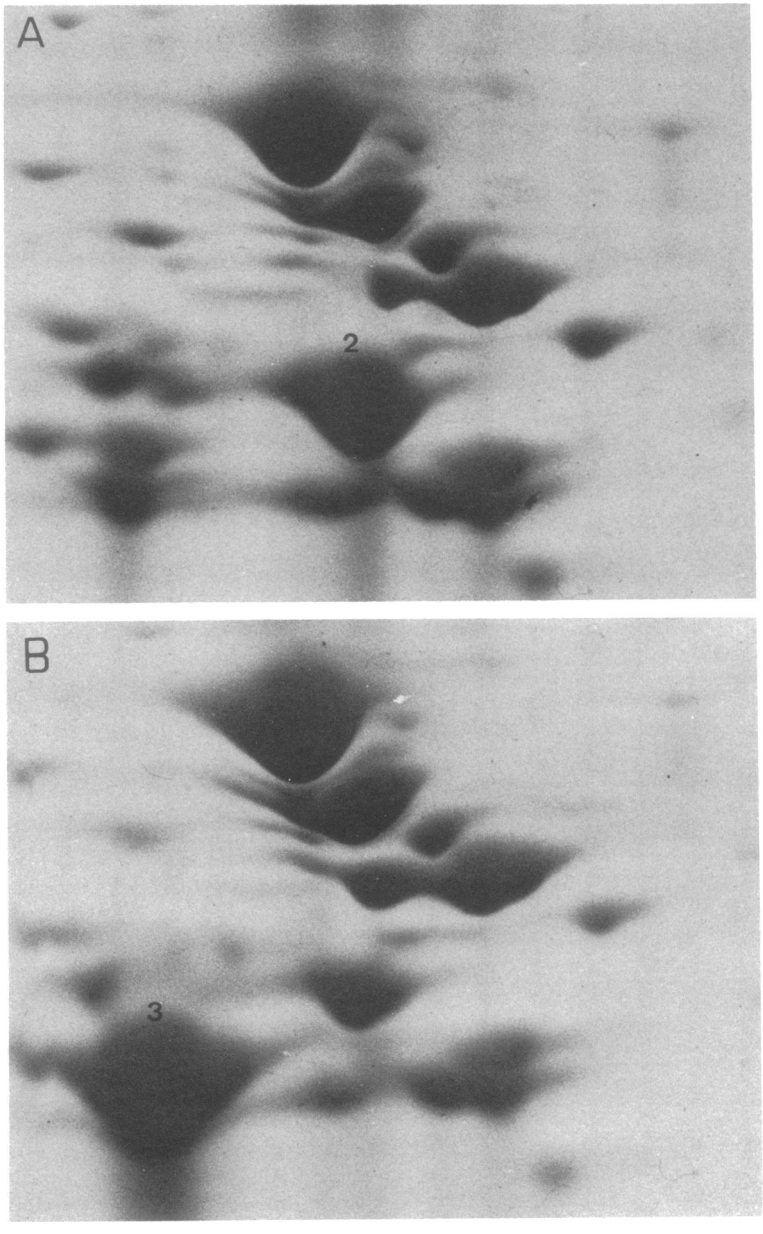

FIG. 5. *Detailed pictures of two-dimensional polyacrylamide gel slabs of shock fluids from strain LA 3400 (wild type) grown in the presence of galactose (A) and ribose (B). Experimental conditions as in Fig. 1.*

by the two-dimensional gel electrophoresis (not shown). Some of these mutants (LA 3405) exhibit a reduced amount of GLPT; others (LA 3404) show no apparent alteration either in the amount of GLPT or in its position on the gel slab. At this point, it cannot be decided whether the mutation of this type resides in GLPT in such a way as not to alter its electrophoretic mobility or that the mutation occurs in an additional component of the GLP transport system.

Genetic analysis of the isolated *glpT* mutants. Since phosphonomycin-resistant mutants have previously been reported to arise from mutation of the hexose-phosphate transport system as well as in the phosphoenol-

pyruvate uridine diphospho-*N*-acetylglucosamine enolpyruvyl transferase (22), it was necessary to genetically characterize our GLP transport mutants.

A lysate of phage P1 grown on a *nalA* strain was introduced into two GLP transport-negative strains selecting for growth on GLP. The transductants were scored for *nalA*. In the first transduction with strain LA 3403, of 42 transductants, 18 (43%) were *nalA*. In the second transduction with strain LA 3401, of 79 transductants, 49 (62%) were *nalA*. These results are similar to the published co-transduction frequency of 50% between *glpT* and *nalA* (5, 14) and establishes the mutation of these strains as *glpT*. The shock fluids of four *glpT*$^+$ transductions were examined by two-dimensional electrophoresis and found to contain GLPT (not shown).

Two-dimensional map of periplasmic proteins of *E. coli*. The two-dimensional polyacrylamide gel electrophoretic technique can be performed in a highly reproducible fashion and exhibits an astonishing separation power. We were therefore tempted to use this technique to identify a few of the known periplasmic binding proteins on such a two-dimensional polyacrylamide gel map.

Figures 5A and B show, as an example, the detailed pictures of the corresponding gels of strain LA 3400 grown in the presence of galactose and ribose, respectively. The induction of two protein spots, presumably the corresponding substrate-binding proteins, are obvious. In the same way, the maltose-binding protein can be detected (not shown). Figure 1 summarizes the map positions of some binding proteins: 1, GLPT; 2, galactose-binding protein; 3, ribose-binding protein; and 4, maltose-binding protein.

DISCUSSION

We describe here a novel periplasmic protein, GLPT, that is under the control of the *glpR* gene and appears intimately involved in GLP transport. This conclusion is based on the following points: (i) Wild-type strains produce GLPT only when grown in the presence of glycerol, whereas *glpR* strains also produce it when grown on succinate. (ii) Some *glpT* mutants do not produce GLPT; the corresponding genetic lesions are co-transducible by phage P1 with *nalA*, a marker previously found to be co-transducible with *glpT* (5, 14). (iii) Reversion to growth on GLP of *glpT* strains that do not produce GLPT results in strains that transport GLP and again produce GLPT. The extent of transport activity in these revertants is reflected in the amount of GLPT they are able to produce.

The possibility that GLPT might be identical with the flavin-linked anaerobic GLP dehydrogenase (14), the gene locus of which is closely linked to *glpT* (14), appears unlikely for several reasons: (i) This soluble enzyme is involved in the energy production of the cell under anaerobic condition and has therefore no function outside the cytoplasmic membrane. (ii) Selection for phosphonomycin resistance, the method by which our *glpT* mutants are isolated, has not been reported to affect the anaerobic dehydrogenase. (iii) All mutants that lack GLPT are unable to transport GLP. (iv) All *glpT* mutants reported here grow anaerobically on glycerol in the presence of fumarate, conditions requiring the presence of a functional anaerobic dehydrogenase (14).

By analogy with other transport systems that are mediated via periplasmic components (2), it seems likely that GLPT is a binding protein specific for GLP. Yet, all attempts to measure binding activity for GLP in crude shock fluids, even with the most sensitive method of ultrafiltration (20), have so far failed to give positive results. However, with an expected binding affinity of 12 μM (the K_m of GLP transport in whole cells [8]) and the relatively small amount of GLPT in crude shock fluids, binding activity might be difficult to detect. Another complication arises from the observation that all shock fluids, independent of their content of GLPT, contain an enzymatic activity that splits GLP in glycerol and inorganic orthophosphate. This is surprising in view of the fact that all strains used in this study are *phoA* and therefore do not produce the periplasmic alkaline phosphatase. By paper chromatographic analysis of samples incubated under conditions similar to those used for the binding assay by ultrafiltration, it was found that 30 to 60% of the GLP is hydrolyzed in 1 h. Therefore, a meaningful test for binding activity or any enzymatic activity of GLPT has to await the purification of GLPT.

Since transport of GLP appears to depend on the presence of the periplasmic GLPT, one would not expect that membrane vesicles of the Kabackosome-type are able to transport GLP. However, stimulation of membrane-bound transport systems by GLP, as well as its own transport, has been reported (1, 10). Measurement of possible residual amounts of GLPT in membrane vesicles might explain this discrepancy. Alternatively, periplasmic transport components might be essential for transport in whole cells but not in membrane vesicles, a

phenomenon reported for the *E. coli* dicarboxylic transport system (17). This might indicate that GLPT belongs, at least functionally, to the outer membrane and facilitates the diffusion of GLP through this layer similarly as the λ receptor does for maltose (S. Szmelcman, M. Schwartz, T. J. Silhavy, and W. Boos, Eur. J. Biochem., in press).

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