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Substrate Specificity and Transport Properties of the Glycerol Facilitator of *Escherichia coli*

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The specificity of the glycerol facilitator (*glpF*) of *Escherichia coli* was studied with an osmotic method. This transport system allowed the entry of polyols (glycerol and erythritol), pentitols, and hexitols. The analogous sugars were not transported. However, urea, glycine, and DL-glyceraldehyde could use this pathway to enter the cell. The *glpF* protein allowed the rapid efflux of preequilibrated xylitol. Glycerol surprisingly did not inhibit the uptake of xylitol, and xylitol only slightly reduced the uptake of glycerol. This observation and the insensitivity of the xylitol transport to low temperature suggest that the facilitator behaves as a membrane channel.

Although most of the specific transport systems of bacteria are energy dependent, a few do not require metabolic energy. In this latter category are the porin channel (4) and the maltose channel (lambda receptor) (10, 17), both located in the outer membrane of *Escherichia coli*. The porin channel is quite nonspecific and admits compounds with molecular weight of 600 or smaller (4). Recent evidence suggests that the interaction of the maltose-binding protein with the channel protein makes the channel normally available only to maltose and maltose dextrins (6, 17).

The cytoplasmic membrane of *Escherichia coli* possesses at least one energy-independent transport system, a glycerol facilitator, that catalyzes the equilibration of the substrate between extra- and intracellular space (15). This system is part of the *glp* regulon which is inducible by glycerol 3-phosphate and provides strong growth advantage on glycerol at low external concentrations (13). However, the rate of glycerol entry into the cells, with or without the membrane carrier, is so rapid that it is difficult to quantitate the transport activity by conventional techniques (1). Thus, the characterization of this transport system has been considerably hampered.

We present here an investigation of the substrate specificity of this transport process with both osmotic and direct chemical techniques. Xylitol was shown to be a convenient substrate for the study of this transport system. A variety of observations suggest that the glycerol facilitator is a channel rather than a carrier.

MATERIALS AND METHODS

Bacterial strains. *E. coli* K-12 strains 1 (*glpF*$^+$*K*$^+$*D*$^+$*R*$^+$), 282 (*glpFK*$^+$*D*$^+$*R*$^+$), and 7 (*glpF*$^+$*K*$^+$*D*$^+$*R*) were used in these experiments (13).

Growth conditions. In most experiments cells were grown aerobically at 37°C in M63 (3) supplemented with 1% tryptone (Difco Laboratories, Detroit, Mich.). In one experiment (see Fig. 2), cells were grown in a synthetic amino acid mixture (14) to avoid induction of the *glp* system by contaminating traces of glycerol and glycerol 3-phosphate found in the commercial tryptone. To induce the *glp* system, glycerol 3-phosphate was added at 0.5 mM. Harvested cells were washed twice with a volume of 50 mM morpholinepropanesulfonate buffer (MOPS) (pH 7.0) equal to that of the culture medium and resuspended in that buffer to give a final density at 600 nm of about 3.0 (approximately 30 mg [dry weight] per ml).

Transport measured by the optical method. Permeation of the carbohydrates was monitored by a photometric method that is based upon changes in light scattering resulting from plasmolysis (shrinkage) and deplasmolysis (reswelling) at 25°C (5, 15). One ml of a 250 mM carbohydrate solution in 50 mM MOPS (pH 7) was placed in a small cuvette and read in a Gilford spectrophotometer. A 0.10-ml sample of a concentrated cell suspension was added to the cuvette to give a final optical density at 600 nm of about 1.0 by aspirating and expelling the contents several times with a Pasteur pipette. The optical density was continuously monitored with a recorder attached to the spectrophotometer. In such an assay, the cytoplasmic volume initially shrinks (plasmolysis) due to the high external osmotic pressure. As the substrate enters, the original volume is restored. The time required for 50% reswelling due to substrate permeation is expressed as $T_{1/2}$ (15).

Uptake measured by the microfuge method. The intracellular concentration of [^{14}C]xylitol or [^{14}C]glycerol was determined after separation of the cells from the incubation medium by centrifugation through silicone oil. Samples (1 ml) were taken in duplicate at various intervals and layered on the top of 0.5 ml of a silicone oil mixture (75% no. 550 fluid plus 25% no. 510 fluid, Dow Corning Corp., Midland, Mich.) in 1.5-ml plastic microfuge tubes. After centrif-

ugation for 1 min (12,000 × *g*), the supernatant aqueous medium and most of the silicone oil were carefully removed; the tip of the tube containing the cell pellet was cut off with a razor blade and placed in a scintillation vial containing 1 ml of 1 N NaOH to dissolve the pellet. After neutralization with 2 N HCl, 9 ml of scintillation fluid (7.2 g of 2,5-diphenyloxazole, 1,200 ml of toluene, and 600 ml of Triton X-100) were added (11). Extracellular, periplasmic, and intracellular spaces were estimated by determining the volume of distribution of ^3H_2O, [^{14}C]sucrose, and [^{14}C]inulin. We assumed that the distribution of inulin and water, or sucrose and water, in the pellet reflects the distribution in the test suspension and that there is no complication by nonspecific binding. Inulin cannot cross the outer membrane of *E. coli*, and therefore its distribution was taken as a measure of extracellular space. Sucrose can cross the outer but not the inner membrane, since the cells were not induced for the *lacY* which has a fortuitous activity on the disaccharide (5). The difference between the sucrose space and the inulin space was taken as a measure of the periplasmic space.

Efflux experiments. A thick suspension of cells induced in the glycerol facilitator was preloaded with 100 mM [^{14}C]xylitol in MOPS for 15 (strain 1) or 60 min (strain 282). One sample was additionally supplemented with ^3H_2O to determine the zero time concentration of xylitol in the cytosol. The cells were separated from the incubation medium by centrifugation through silicone oil. The pellet was processed as described above (microfuge method). The total volume of the cell pellet was calculated from the radioactivity of ^3H_2O. The concentration of [^{14}C]xylitol in the cytoplasm was estimated by using an average intracellular volume obtained from the uptake experiments; 45% of the pellet volume was contributed by the cytosol. Another sample was diluted 100 times into rapidly stirred 50 mM MOPS. At various intervals, 1-ml samples were taken, filtered (0.65-μm pore size membrane, Millipore Corp., Bedford, Mass.), and washed with 3 ml of MOPS buffer. The filter bearing the collected cells was placed in scintillation fluid (11) and counted.

Chemicals. All chemicals were of the highest purity commercially available. ^3H_2O, [^{14}C]sucrose, [^{14}C]-glycerol, and [^{14}C]inulin were purchased from New England Nuclear Corp., Boston, Mass., and [^{14}C]xylitol was obtained from Amersham Corp., Arlington Heights, Ill. [^{14}C]sucrose, [^{14}C]inulin, and [^{14}C]xylitol were purified by chromatography before use.

RESULTS

Specificity of the glycerol facilitator. The presence of an inducible glycerol facilitator was first demonstrated by Sanno et al. (15) who utilized a photometric technique to measure the rate of solute entry. In these experiments, suspended cells were exposed to a hypertonic solution of glycerol. The rapid plasmolysis due to osmotic water movement out of the cytoplasm resulted in an increase in optical density. Entry of glycerol (and reentry of water) led to reswelling to the original cytoplasmic volume which resulted in a decrease in optical density. Since solute entry is rate limiting in this reswelling osmotic process, the decrease in optical density was taken as a measure of substrate permeability.

The equilibration of a glycerol gradient across the cytoplasmic membrane was extraordinarily fast, the half-time being approximately 0.5 min in uninduced cells and much less than 0.1 min in induced or constitutive cells (1, 15). An example of this phenomenon is given in Fig. 1A. In the case of the facilitator-positive strain induced with glycerol 3-phosphate, glycerol entry was so rapid that equilibrium was reached within the 10 s required for mixing and obtaining the first optical reading. The mutant lacking the facilitator showed a clear reswelling curve with a half time of approximately 0.5 min. When the experiment was repeated with xylitol, uninduced cells showed practically no polyol entry in 5 min, while induced cells showed a half-time for equilibration of approximately 0.7 min (Fig. 1B). A number of other compounds were also tested with this technique. Several of the polyols were substrates for the facilitator, but none of the tested sugars was affected (Table 1). The uptake rate of the polyols in general was related to the molecular size of the substrate, the hexitols being taken up considerably more slowly than polyols with lower molecular weights. There is little evidence for stereospecificity of the carrier; D- and L-arabitol differed only slightly in their rates of entry. Among the pentitols, the entry of ribitol was the most rapid.

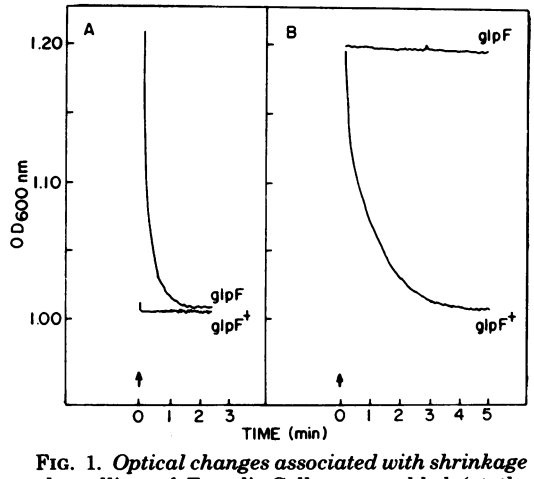

FIG. 1. *Optical changes associated with shrinkage and swelling of E. coli. Cells were added (at the arrow) to a solution of 250 mM glycerol (A) and xylitol (B). In the figure, a reading of 1.2 indicates shrinkage of the cell. The fall in optical density to 1.0 was due to reswelling of the cells as a result of penetration of glycerol or xylitol.*

It came as a surprise that small compounds unrelated to glycerol, such as urea, glycine, and DL-glyceraldehyde, were transported via the glycerol facilitator. Thiourea and imidazole entered the cells too rapidly, even in uninduced cells, for any quantitative measurement. Glycerol 3-phosphate and glycerol 1-phosphate (added as a racemic mixture) were not transported (Table 1).

Xylitol transport by the glycerol facilitator. The rate of xylitol entry was sufficiently slow to allow measurements of the time course of [^{14}C]xylitol uptake with a centrifugation technique. After exposure for 5 min to 10 mM xylitol, the concentration of radioactivity in the cell was observed to be two- to threefold greater than that in the medium. Most of this radioactivity was bound to an anion exchange column (data not shown) and was presumed to be a phosphorylated form of the alcohol. Reiner had shown earlier that xylitol can enter the cell via a phosphoenolpyruvate sugar phosphotransferase system (12). At an external concentration of 100 mM xylitol, however, the contribution to the uptake process by the phosphotransferase system became negligible in comparison with that by the facilitator.

TABLE 1. *Specificity of the glycerol facilitator of E. coli K-12*

Compound transported by the glycerol facilitator[a]	Half time of equilibration (min)[b]	
	glpF$^+$	*glpF*
Glycerol	≪0.1	0.4
DL-Glyceraldehyde	≪0.1	0.4
Glycine	5.0	∞
Urea	0.1	0.2
Erythritol	0.3	2.6
D-Arabitol	3.4	~10.0
L-Arabitol	4.6	~10.0
Ribitol	0.1	∞
Xylitol	0.7	∞
D-galactitol (dulcitol)	7.0	∞
D-Mannitol	~25.0	∞
D-Sorbitol (glucitol)	8.8	∞

[a] Not transported by the glycerol facilitator were: glycerol 1-phosphate, glycerol 3-phosphate, glycylglycine, inositol (cyclohexitol), erythrose, D-arabinose, L-arabinose, D-ribose, D-xylose, D-galactose, D-mannose, and D-sorbose.

The uptake of [^{14}C]xylitol was largely dependent on the expression of the *glpF* gene (Fig. 2). In wild-type cells inducible for the *glpF* (strain 1), induction increased the uptake rate, and growth on glucose reduced the transport rate. In *glpF* negative cells (strain 282), the growth conditions did not affect the residual uptake rate. Moreover, the uptake rate in *glpF* cells was similar to that in catabolite-repressed wild-type cells. In cells constitutive for the glycerol facilitator (strain 7), no induction could be demonstrated, although growth in the presence of glucose resulted in the diminution of the uptake rate. These results provide strong evidence that xylitol indeed is transported by the glycerol facilitator.

When the glycerol facilitator was present, cells equilibrated with [^{14}C]xylitol showed rapid substrate efflux after dilution (Fig. 3). The half-time

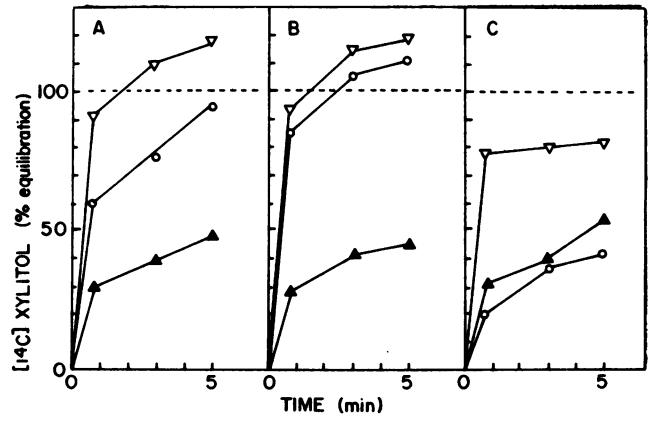

FIG. 2. *Uptake of labeled xylitol into various strains of E. coli grown under different conditions. Entry of 100 mM [^{14}C]xylitol was measured in strain 1 (inducible, ○), strain 7 (constitutive, ▽), and strain 282 (facilitator-negative, ▲). The cells were grown in M63 supplemented with a synthetic amino acid mixture (14) (A, uninduced cells); in M63 supplemented with 1% tryptone, and 1 mM* DL-*α-glycerophosphate (B, fully induced cells); and M63 supplemented with 1% tryptone and 0.2% glucose (C, catabolite-repressed cells). Equilibration of 100% indicates equal concentrations in the intracellular and extracellular compartments.*

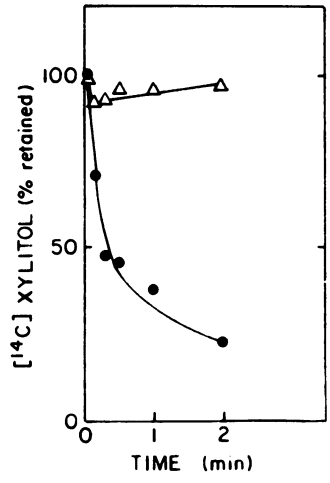

FIG. 3. *Efflux of xylitol. Cells of strains 1 (●) and 282 (△) preloaded with 100 mM [^{14}C]xylitol were diluted into MOPS buffer. The 100% value represents the intracellular concentration of xylitol before dilution (105 mM in strain 1 and 90 mM in strain 282). Cells were grown on 1% tryptone-0.5 mM glycerophosphate.*

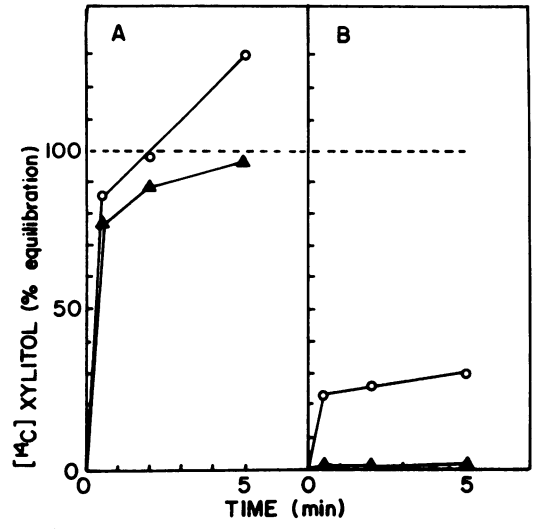

FIG. 4. *Temperature dependence of xylitol entry. Uptake of 100 mM [^{14}C]xylitol was measured into strains 7 (A) and 282 (B) at 25 (○) and 2°C (▲). The cells were grown in M63 supplemented with 1% tryptone. Equilibration of 100% indicates equal concentrations in the intracellular and extracellular compartments.*

of efflux was about 0.3 min. In the glycerol facilitator deleted cells, there was only slow efflux of xylitol (half-time about 10 min).

Competition between glycerol and xylitol. The uptake of 5 mM [^{14}C]xylitol by induced wild-type cells was studied in the presence of 10 to 500 mM glycerol. At none of the concentrations did glycerol significantly inhibit xylitol uptake.

When the accumulation of 20 μM [^{14}C]glycerol was measured in the presence of xylitol (up to 20 mM), the maximal inhibition observed was about 40%; the transport rate was reduced from 2.9 mmol of glycerol per min per mg (dry weight) in the absence of xylitol to 1.7 mmol of glycerol per min per mg (dry weight) in the presence of 20 mM xylitol.

Uptake of xylitol at low temperature. The very rapid entry of substrates through the glycerol facilitator and the weak mutual inhibition between xylitol and glycerol suggests that the glycerol facilitator might serve as a channel rather than a carrier. One possible way to test this hypothesis is to measure the xylitol entry at low temperatures, since channels would be relatively insensitive to temperature changes. It was found that the uptake rate of [^{14}C]xylitol in strain 7 (glycerol facilitator constitutive) was reduced only slightly by lowering the temperature from 25 to 2°C (Fig. 4A). In contrast, the basal entry of xylitol into strain 282 (glycerol facilitator-deleted) was completely inhibited by lowering the temperature (Fig. 4B). This again indicates that in addition to the glycerol facilitator there were membrane carriers with low substrate specificity that allowed xylitol to enter the cells.

DISCUSSION

A variety of observations lead us to postulate that the glycerol facilitator belongs to that group of transport proteins classified as a channel. Diffusion through pores should be relatively temperature insensitive. Substrate specificity of a channel would be determined more by molecular size than by chemical structure; therefore, competitive inhibition between substrates would be minimal. Finally, channels would also be expected to allow rapid substrate entry because the rate tends to be diffusion limited.

Although pores with low transport specificity are well known in the outer membrane of Gram-negative bacteria (2, 10), the glycerol facilitator is the first example of a channel across the cytoplasmic membrane. A passageway with a diameter of about 0.4 nm (as calculated from the atomic radii) should be sufficient to allow the permeation of glycerol. This is to be compared with 1.2 nm for the pore of the outer membrane major proteins (2) and 1.6 nm for the λ receptor (2).

A variety of small straight-chain molecules are admitted by the glycerol facilitator. These transported compounds include several polyhydric alcohols, as well as glyceraldehyde, glycine, and urea. Two substrates, glycerol and xylitol,

were shown to exhibit little or no mutual competition. On the other hand, four-, five- and six-carbon sugars, inositol (cyclohexitol), glycylglycine, and glycerol 3-phosphate do not have ready access through the system. We assume that the entry of ions and ionic compounds are impeded by their charge.

It is of interest to compare the turnover number of the glycerol facilitator with that of the well known transport carrier for lactose. From the data of Alemohammad and Knowles (one-half equilibration of 500 mM glycerol occurred in 0.2 s [1]), the rate is calculated to be 10^9 molecules per s per cell. This is more than three orders of magnitude greater than the rate of lactose entry, which is 6×10^5 molecules per s per cell (7). The turnover number of the facilitator is about 2×10^5 glycerol molecules per s under the assumption that each facilitator molecule forms a separate pore. This is to be compared with 2×10^2 lactose molecules turned over per s by the M protein. The estimations of the turnover numbers of the two transport systems are made as follows. Since glycerol facilitator and glycerol kinase are encoded in the same operon, the molecular ratio of the two proteins synthesized is assumed to be unity, as a first approximation. The specific activity of the kinase in cell extracts is 10 nmol/s per mg of protein (9). Since 5×10^9 cells contain 1 mg of protein, the specific activity of the kinase can also be expressed as 1.2×10^6 glycerol molecules per s per cell. The turnover number of the enzyme is 200 glycerol molecules per s per molecule of kinase (8). This gives a cellular enzyme content of 6×10^3 molecules per cell. The V_{max} for lactose entry is 1 μmol/s per 10^{12} cells (7). The concentration of lactose carrier is 0.2 μmol of M protein per g of cytoplasmic membrane (16). One gram of cytoplasmic membrane is equivalent to 3.3×10^{13} cells. This gives 3,600 M protein molecules per cell. All of the above data were obtained at about 25°C.

If the glycerol facilitator behaves essentially as a nonsaturable transport system, it may be estimated that a flow rate of about 2×10^6 molecules per s per cell is assured when there is a 1 mM concentration gradient across the cell membrane. This would be equivalent to a rate of 1 μmol of glycerol transported per min per mg of cell protein. A glycerol consumption rate of about 0.4 μmol/min per mg of protein is expected to support growth with a 90-min doubling time (9). Thus, the transport rate measured by the osmotic method is not far from the value expected on a physiological basis.

ACKNOWLEDGMENTS

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