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Nature and Properties of Hexitol Transport Systems in *Escherichia coli*

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In *Escherichia coli* K-12 the naturally occurring hexitols D-mannitol, D-glucitol, and galactitol are taken up and phosphorylated via three distinct transport systems by a mechanism called either group translocation or vectorial phosphorylation. For every system, a membrane-bound enzyme II-complex of the phosphoenolpyruvate-dependent phosphotransferase system has been found, each requiring phosphoenolpyruvate, enzyme I, and HPr or alternatively P-HPr as the phosphate donor. Cells with a constitutive synthesis of all hexitol transport systems but with low P-HPr levels have very low transport and phosphorylating activities in vivo, although 40 to 90% of the enzyme II-complex activities are detected in cell extracts of such mutants. No indications for additional hexitol transport systems, especially for systems able to transport and accumulate free hexitols as in *Klebsiella aerogenes*, have been found. Substrate specificity, K_m, and V_{max} of the three transport systems for several hexitols and hexitol analogues have been determined by growth rates, transport activities, and in vitro phosphorylating activities. Each system was found to take up several hexitols, but only one hexitol serves as the inducer. This inducer invariably is the substrate with the highest affinity. Since bacterial transport systems, as a general rule, seem to have a relatively broad substrate specificity, in contrast to a more restricted inducer specificity, we propose to name the system inducible by D-mannitol and coded by the gene *mtlA* the D-mannitol transport system, the system inducible by D-glucitol and coded by *gutA* the D-glucitol transport system, and the system inducible by galactitol and coded by *gatA* the galactitol transport system.

Bacteria frequently will transport an amino acid or a carbohydrate by several uptake systems, for instance the three naturally occurring hexitols. D-Glucitol is taken up by three transport systems, D-mannitol is taken up by two, and galactitol is taken up by one (8). To determine whether each transport is catalyzed by a separate system or whether one system does serve for several substrates, isogenic mutants have been isolated in which all hexitol transport systems except one are eliminated (8). Such mutants then can be used to test the exact specificity of a system, its properties, and its nature.

Carbohydrates seem to be taken up and accumulated in bacteria by facilitated diffusion, active transport, or group translocation (3). In the first two cases, unaltered substrate is translocated through the membrane. Carbohydrates, however, taken up via group translocation, also known as vectorial phosphorylation, are accumulated intracellularly in the phosphorylated form. The high-energy phosphate involved in this phosphorylation, rather than being derived directly from adenosine 5'-triphosphate, is passed from phosphoenolpyruvate (PEP) to two soluble proteins, enzyme I and HPr, before being transferred to a membrane-bound enzyme II-complex and finally to the carbohydrate (3, 7, 15, 20, 21, 24; see Discussion). Since the specificity of the reaction is due to the enzyme II-complex, it is suggested that the name of its inducer, usually also the main substrate, be added as a suffix. The isolation and mapping of mutations affecting the regulation and the activities of three such enzyme II-complexes specific for hexitols have been described in the preceding paper (8).

The soluble protein HPr can possibly be substituted by another soluble protein called K_m factor (23), and enzyme II-complexes have been described that consist of a soluble, substrate-specific, inducible factor III and a membrane-bound protein (18, 21). Carbohydrates accumulated by such a PEP-dependent phosphotransferase system (PTS) will be called PTS carbohydrates in contrast to non-PTS carbohydrates transported by different systems.

Apart from the enzyme II-complex and factor III, the other proteins (enzyme I, HPr) and cofactors are unspecific. Their absence does not inactivate a single enzyme II-complex but blocks the activities of all transport systems of the PTS type. Transport systems of the non-PTS type may also be affected by the lack of enzyme I or HPr (7, 9, 12, 13, 17, 24). For reasons still unclear, full induction of inducible systems is coupled to high levels of these two proteins. Enzyme I and HPr for PTS systems thus have the dual role of providing the high-energy phosphate during uptake and insuring their induction. Low levels of the two proteins can be compensated for in induction by the use of constitutive mutants (2, 9, 17, 24). Therefore, mutants constitutive for the *mtl*, *gut*, and *gat* operons (8) have been used throughout this work. Constitutivity, of course, cannot compensate the lack of P-HPr, the phosphorylated derivative of HPr, during transport.

In this paper, further evidence for the existence of three independent membrane-bound enzyme II-complexes, each requiring P-HPr as the phosphate donor, will be given. Furthermore, data are presented showing substrate specificity and affinity constants of each system as measured by growth, transport, and phosphorylation in vitro.

MATERIALS AND METHODS

Chemicals. Polyols were obtained from Fisher Chemical Co., Boston, Mass., and from E. Merck, Darmstadt, Germany. Since even chromatographically pure polyols contain 0.1 to 0.6% contaminating polyols, they were purified further by the biological scavenging method as follows. Cells of a mutant able to ferment constitutively the contaminating polyols with a high efficiency but unable to ferment the polyol to be purified were pregrown to exponential growth in minimal medium-glycerol to 4×10^8 bacteria per ml. The culture was centrifuged, washed at room temperature, and suspended in minimal medium to 2.5×10^8 bacteria per ml. The cells were starved for 30 min at 37 C under constant aeration before the polyol to be purified was added to 50 or 100 mM final concentration. The cells were incubated further until the increase of the culture density stopped. This was usually achieved after one doubling: 90 to 120 min. The culture was immediately filtered through a membrane filter (0.65-μm pore size). The exact concentration of the remaining purified polyol was determined chemically, by the periodate method according to Korn (6), and biologically, by measuring the growth yield. Purity was also checked by thin-layer chromatography (8). Finally, the amount of contaminating polyol remaining in the purified polyol solution was tested by isotope dilution experiments. In such experiments, the inhibition of the uptake of low concentrations of hexitol (0.5 μM D-[^{3}H]mannitol, 5 μM D-[^{3}H]glucitol, or [^{14}C]galactitol) by added purified polyols was used to calculate the amount of contamination.

The 2 to 5% D-glucitol usually present in commercial D-mannitol can be lowered by repeated recrystallization to 0.1%, whereas one scavenging brings it below 0.01%. Similarly, the amount of D-mannitol in D-glucitol which can only be recrystallized from pyridine is brought below 0.01% by one scavenging, as is the amount of D-glucitol in galactitol.

Bacterial strains. The origin and genotypes of the hexitol transport mutants are given in Table 1. Their isolation was described in the preceding paper (8). Strains 238 and 158 have been described previously (10). A mutant of *Klebsiella* (*Aerobacter*) *aerogenes* 2007 with the genotype *mtlA*$^-$*D*$^+$ *gut*$^+$ was obtained from E. C. C. Lin (22). Strain 2007 is an Mtl-positive revertant that grows on D-mannitol via nicotinamide adenine dinucleotide-dependent D-arabinitol (D-arabitol) dehydrogenase (EC 1.1.1.11).

Culture media and growth conditions. Culture media and growth conditions were exactly as described in the preceding paper (8). To determine growth rates in K_m tests on polyol concentrations too

TABLE 1. *Origin and genotype of the hexitol transport mutants*[a]

Strain	Parent	Mtl	Gut	Gat	Frc	*mtl*			*gut*			*gat*			*pts*	
						C	*A*	*D*	*C*	*A*	*D*	*C*	*A*	*D*	*I*	*H*
L146		−	−	−	+	+	−	+	c	−	+	c	−	+	+	+
L156	L146	+	−	−	+	c	+	+	c	−	+	c	−	−	+	+
L172	L156	+	+	−	+	c	+	+	+	−	+	c	−	+	+	+
L153	L146	−	+	+	+	+	−	+	+	−	+	c	+	+	+	+
L173	L148	+	+	+	+	c	+	+	c	+	+	c	+	+	+	+
L175	L173	−	−	−	−	c	+	+	c	+	+	c	+	+	−	(+)

[a] Mtl, Gut, Gat, and Frc, Positive phenotypes for D-mannitol, D-glucitol, galactitol, and fructose growth, respectively; *mtl*, *gut*, *gat*, and *frc*, corresponding genotypes. *C*, Genes specifying inducibility or constitutivity; *A*, genes coding enzyme II-complexes; *D*, genes coding the dehydrogenases. +, Positive or wild type; −, negative or mutated; c, constitutive. The genes *ptsI* and *ptsH* code for enzyme I and HPr activity, respectively, of the PEP-dependent PTS.

low to give detectable absorbance changes, the cell titer was measured by plating and counting colonies. Overnight cultures were pregrown in media containing 0.2% of the polyol to be tested. The cells were centrifuged, washed, and suspended in prewarmed medium to 100 bacteria per ml. Since the yield of such cultures for, for example, 0.05 μM D-mannitol is 3×10^8 bacteria per ml, reasonable growth curves are obtained.

Uptake assays. Standard uptake assays were described in the preceding paper (8). The final concentrations of the hexitols during the tests were: D-[^{3}H]mannitol (20 Ci/mol), 5 μM; D-[^{3}H]glucitol (10 Ci/mol), 25 μM; [^{14}C]galactitol (5.5 Ci/mol), 25 μM. Uptake activities are expressed as nanomoles per minute per milligram of protein.

To test the K_m values of hexitols for the different transport systems, 4.5 ml of cells at 25 C was mixed at 0 min with 0.5 ml of a mixture of radioactive hexitol and increasing amounts of nonradioactive hexitol. Samples (1 ml) were taken after 10, 20, 30, and 60 s, blown immediately into 5 ml of cold buffer, filtered, and washed. A 0-min value was always included and was obtained by mixing 0.9 ml of cells with 0.1 ml of radioactive hexitol used in the test at 0 C and filtering as fast as possible. The cells were precooled for 5 min at 0 C.

In transport chase experiments, 12.7 ml of strain L156 cells was mixed with 1.3 ml of D-[^{3}H]mannitol (100 Ci/mol) or 12.7 ml of strain 2007 cells was mixed with 5 mM D-[^{3}H]mannitol (2 Ci/mol). After 15, 30, and 60 s, 1-ml samples were blown into 5 ml of minimal medium, filtered immediately, washed, and counted in a scintillation mixture. After 60 s the remaining cells were diluted 1:5 into minimal medium containing 0.5 mM unlabeled D-mannitol, in the case of strain L156, or 0.100 M D-mannitol, for strain 2007. After 10 s and the times indicated in Fig. 1, 5-ml samples were filtered, washed, and treated as described above.

Assay of enzyme I activity in cell extracts. Activity was measured by following the rate of D-mannitol-1-phosphate formation from D-[^{14}C]mannitol (20). The assay mixture contained in a total volume of 0.2 ml: D-[^{14}C]mannitol (2.5 Ci/mol; 30 μM); PEP (5 mM); $MgCl_2$ (50 μM); tris(hydroxymethyl)aminomethane-hydrochloride buffer (0.1 M, pH 7.6); and 0.02 ml of extract of strain L156 for enzyme II-complex^{mtl} activity. From 0.01 to 0.10 ml of the extracts to be tested for enzyme I activity was added, and the amount of P-HPr formed was then estimated from the rate of formation of D-[^{14}C]mannitol-1-phosphate. Activities are expressed as nanomoles per minute per milligram of protein.

Assays of enzyme II-complex activities. The enzyme II-complex assays too were done as described in the preceding paper (8), with the following hexitol concentrations: D-[^{14}C]mannitol (2.5 Ci/mol), 30 μM; D-[^{14}C]glucitol (5 Ci/mol), 0.21 mM; and [^{14}C]galactitol (5.5 Ci/mol), 0.5 mM. All enzyme activities are expressed as nanomoles per minute per milligram of protein.

K_m values of hexitols for the different enzyme II-complexes were obtained by adding increasing amounts of unlabeled hexitol to constant amounts of radioactive hexitol (see Fig. 2 through 4).

Assay of hexitol-phosphate dehydrogenase activities. D-Mannitol-1-phosphate dehydrogenase (EC 1.1.1.17), D-glucitol-6-phosphate dehydrogenase (EC 1.1.1.10), and galactitol-1-phosphate dehydrogenase activities were tested as described previously (8). The activities are expressed as micromoles per minute per milligram of protein.

Protein determinations. The protein determinations were done by the biuret method (11), with bovine serum albumin in 0.1 M tris(hydroxymethyl)aminomethane-hydrochloride buffer as the calibrating protein.

RESULTS

Mutant L146 which is unable to grow on D-mannitol, D-glucitol, or galactitol and is unable to transport or phosphorylate any hexitol has been isolated, as described previously (8), from *Escherichia coli* K-12. Mutations in three different genes, *mtlA*, *gutA*, and *gatA*, were necessary to obtain this mutant from the wild-type parent. Mutants were then reisolated from strain L146, synthesizing alternatively a constitutive and active transport system coded by gene *mtlA* (L156), *gutA* (L148), or *gatA* (L153). The triple-positive and constitutive strain L173 was then constructed and finally its derivative L175 (*ptsI*). The complete genotypes and properties of these isogenic mutants, summarized in Tables 1 and 2, are as follows.

(i) Strain L156 ($mtlC^cA^+D^+$ $gutC^cA^-D^+$ $gatC^cA^-D^+$). By virtue of becoming $mtlC^cA^+D^+$, strain L156 grows rapidly on D-mannitol and has high, constitutive levels of transport and enzyme II-complex^{mtl} activity. It is also able to transport and phosphorylate D-glucitol at low rates and with low affinity (see Table 5). If measured near the K_m of the latter systems for D-glucitol, its transport and phosphorylation are inhibited ≥90% by equimolar D-mannitol concentrations. The generation time of strain L156 on 10 mM D-glucitol is 600 min. Because of a polar mutation in gene *gutA*, this strain has low D-glucitol-6-phosphate dehydrogenase activities (0.01 μmol/min per mg of protein). If a weakly polar *gutA* mutation is transduced into strain L156, the dehydrogenase activity (0.39 μmol/min per mg of protein) rises to 25% of the wild-type activity, and the generation time of mutant L172 on D-glucitol is 215 min. Neither of the two $mtlC^cA^+D^+$ strains will grow on galactitol or accumulate or phosphorylate this hexitol to any appreciable rate.

(ii) Strain L148 ($mtlC^+A^-D^+$ $gutC^cA^+D^+$ $gatC^cA^-D^+$). Strain L148, which is positive and

TABLE 2. *Growth, transport, and enzyme II-complex activities*[a]

Strain	D-Mannitol			D-Glucitol			Galactitol		
	GT	TP	EII^{mtl}	GT	TP	EII^{gut}	GT	TP	EII^{gat}
L146	NG	0.1	0.01	NG	0.1	0.01	NG	0.1	0.01
L156	54	4.7	2.00	600	0.1	1.40	NG	0.1	0.01
L172	54	4.9	1.90	215	0.1	1.30	NG	0.1	0.01
L148	95	0.1	0.90	60	10.0	2.00	NG	0.1	0.25
L153	NG	0.1	0.02	133	2.4	1.70	92	12.0	0.50
L173	60	8.0	2.00	84	9.1	2.80	93	15.0	1.00
L175	NG	0.1	0.70	NG	0.1	1.70	NG	0.1	0.90

[a] NG, No detectable growth; GT, generation time in minutes; TP, transport activity in nanomoles per minute per milligram of protein; EII, enzyme II-complex activity in nanomoles per minute per milligram of protein.

constitutive for the *gut* operon, is also able to grow on D-mannitol although at a lower rate. It does transport and phosphorylate this hexitol with a low affinity (see Table 5). Both activities measured near the corresponding K_m are inhibited ≤85% by equimolar D-glucitol concentrations. The system coded by *gutA* apparently has no affinity for galactitol.

(iii) Strain L153 ($mtlC^+A^-D^+$ $gutC^+A^-D^+$ $gatC^cA^+D^+$). Mutant L153, which is positive and constitutive for the *gat* operon, does not grow on D-mannitol but does grow slowly on D-glucitol. The relatively high transport and enzyme II-complex activity for D-glucitol is inhibited to 80% by equimolar concentrations of galactitol, thus indicating the recognition of D-glucitol by an enzyme II-complex^{gat} in strain L153. No affinity for D-mannitol can be seen.

As a comparison, the growth, transport, and enzyme II-complex activities of the original triple-negative strain L146 and of triple-positive derivative L173 and its *ptsI* mutant L175 are included in Table 2.

Nature of the hexitol transport systems. As long as the enzymes coded by the genes *mtlA*, *gutA*, and *gatA* have not been isolated, only indirect, though strong, evidence as to their nature can be given.

A mutant, L173, positive and constitutive for all three hexitol operons was constructed. This strain has a high growth rate on each hexitol as well as high constitutive transport activities (Table 3). The low transport activities for D-glucitol and galactitol after growth on D-mannitol are due to a strong catabolite repression exerted by this hexitol on the *gut* and *gat* operons, respectively (10).

L175 is a derivative of strain L173, unable to grow or transport hexitols or D-fructose. The mutation of this mutant was shown to map near 46 min (*ptsI, H*) on the *E. coli* linkage map (8). Positive revertants can easily be obtained on all four carbohydrates mentioned. Regardless of the carbohydrate used, all revertants have a positive phenotype on each of the four substrates.

TABLE 3. *Growth rates and transport activities of strain L173 grown on different hexitols*[a]

Growth medium	Generation time (min)	Transport activity		
		D-Mannitol	D-Glucitol	Galactitol
Casein acid hydrolysate	60	3.12	4.45	11.10
D-Mannitol	60	5.65	1.67	4.63
D-Glucitol	84	3.72	6.95	8.30
Galactitol	92	6.64	15.80	10.20

[a] Growth rates were monitored at 37 C on 10 mM hexitols. Transport activities in washed cells were determined as described in the text (nanomoles per minute per milligram of protein).

The levels of P-HPr in strains L146, L173, and L175 have been determined as 80, 100, and 0.4%, respectively (Table 4). Positive revertants of strain L175 regain high P-HPr levels. Tested in vivo the transport activities for D-mannitol, D-glucitol, and galactitol in strain L175 are very low. Phosphorylation activity, however, tested in vitro with external P-HPr added amounts to 35 to 90%. These high enzyme II-complex activities and the corresponding dehydrogenase activities found in extracts of strain L175 are proof that the low transport activity is not due to a lack of induction but to the lack of P-HPr.

If cell extracts prepared by lysozyme-ethylenediaminetetraacetic acid treatment (10) are centrifuged and washed, the cell wall-cell membrane fraction can be separated quantitatively from the supernatant. Similarly, by centrifuging sonic extracts for 2 h at 100,000 × *g* followed by a washing, the membrane-bound enzyme II-complex fraction can be separated

TABLE 4. *Growth and enzyme activities of pts⁺ and ptsI mutants*[a]

Strain	Phenotype				D-Mannitol			D-Glucitol			Galactitol			PTS
	Mtl	Gut	Gat	Frc	TP	EII^{mtl}	DH	TP	EII^{gut}	DH	TP	EII^{gat}	DH	(EI + HPr)
L146	−	−	−	+	0.1	0.01	0.0	0.1	0.01	0.00	0.1	0.01	0.00	0.740
L173	+	+	+	+	8.0	2.00	5.0	9.1	2.80	1.70	15.0	1.00	0.63	0.920
L175	−	−	−	−	0.1	0.70	3.6	0.1	1.70	0.62	0.1	0.90	0.10	0.004

[a] Mtl, Gut, Gat, and Frc, Positive (+) or negative (−) phenotype for growth on D-mannitol, D-glucitol, galactitol, and D-fructose, respectively. TP, transport activity; EII, corresponding enzyme II-complex activity, both in nanomoles per minute per milligram of protein; DH, Hexitol-phosphate dehydrogenase activity in micromoles per minute per milligram of protein; EI + HPr, amount of P-HPr in nanomoles per minute per milligram of protein formed by a cell extract containing PEP, enzyme I, and HPr of the PTS.

from the soluble fraction (21). The three hexitol-specific enzyme II-complexes sediment with the membrane fraction. To restore full activity, PEP, enzyme I, and HPr or, alternatively, purified P-HPr has to be added (unpublished data).

The addition of a 100-fold excess of unlabeled D-mannitol (0.5 mM) to cells of strain L156 accumulating D-[^{3}H]mannitol (5 μM) does not chase the radioactivity for 15 min (Fig. 1). In a similar experiment involving an *mtlC^{c}A$^+$D$^-$* mutant, some radioactivity is lost in 30 min. From the supernatant of such cells, only uncharged radioactive material not binding to diethylaminoethyl anion-exchange paper is isolated. Radioactive D-glucitol and galactitol taken up by the transport systems coded by the genes *gutA* and *gatA* also cannot be chased by a 100-fold excess of unlabeled substrate. If after the accumulation of radioactive hexitols in strains lacking the hexitol-phosphate dehydrogenase the cells are extracted in boiling water, the radioactivity found in the concentrated supernatant cochromatographs on thin-layer cellulose plates (8) with D-mannitol-1-phosphate, D-glucitol-6-phosphate, and galactitol-1-phosphate, respectively. The material binds to diethylaminoethyl anion-exchange paper. This binding is prevented by pretreatment with alkaline phosphatase. Finally, the radioactive material can be used in the corresponding hexitol dehydrogenase tests and gives a positive reaction with the predicted dehydrogenase.

As a control, *K. aerogenes* 2007 was also tested. This mutant has been isolated as an Mtl$^+$ revertant of mutant 2006, with a defect in the enzyme II-complex^{mtl}, the normal transport system for D-mannitol in *Klebsiella*. Mutant 2007 has been shown to grow on D-mannitol by means of a constitutive D-arabinitol dehydrogenase (4, 22). This enzyme is able to dehydrogenate D-arabinitol and catalyze the conversion of free D-mannitol to D-fructose. The generation

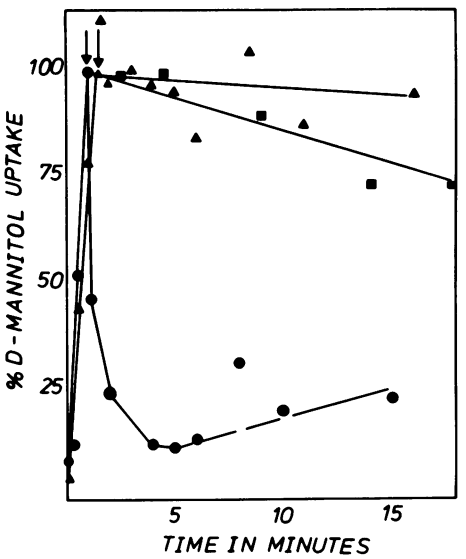

FIG. 1. *D-[^{3}H]mannitol transport chase experiment. To 10 ml of cells prepared as described in the text was added 1 ml of D-[^{3}H]mannitol at 20 Ci/mol and 50 μm for strains L156 and 239 (mtlC^{c}A$^+$D$^-$) or at 2 Ci/mol and 5 mM for strain 2007. After 60 and 90 s, the bacteria were diluted into 50 ml of buffer containing a 100-fold excess of unlabeled D-mannitol. Samples (5 ml) were filtered and washed at the times indicated. In the case of strain L156 (▲), 100% activity amounts to 13.6, for strain 239 (■) it amounts to 5.24, and for strain 2007 (●) it amounts to 19.7 nanomoles per minute per milligram of protein.*

time of strain 2007 on 10 mM D-mannitol is 66 min, and the K_m for growth is near 1 mM. This contrasts well to a K_m of the enzyme II-complex^{mtl} for D-mannitol of 40 μM. Whereas a wild-type strain tested at 30 μM has 27 U (nanomoles per minute per milligram of protein) of this enzyme, strain 2007 tested at 0.5 mM has $\leq$0.1 U. Its uptake activity tested at 5 μM is barely detectable but rises to 24 U at 0.5 mM. As shown in Fig. 1, this radioactivity is

chased to more then 95% in 4 min, and the material extracted from cells does not bind to diethylaminoethyl-paper filters. The system accumulating free D-mannitol in strain 2007 is constitutive and most likely identical to the constitutive transport system for D-arabinitol described in this mutant (21).

Thus, PTS-type transport systems can clearly be distinguished from active transport systems in chase experiments.

Thus, we might conclude that the three transport systems coded by the genes *mtlA*, *gutA*, and *gatA* are three distinct, membrane-bound enzyme II-complexes requiring P-HPr as a phosphate donor and accumulating intracellularly D-mannitol-1-phosphate, D-glucitol-6-phosphate, and galactitol-1-phosphate. No indications for the existence of additional systems transporting free hexitols or for dehydrogenases able to dehydrogenate free hexitols in *E. coli* K-12 have been found.

Properties of the hexitol transport systems. The kinetic properties of the hexitol transport systems (K_m, V_{max}, substrate specificity) are summarized in Fig. 2 to 4 and in Table 5. Measurements were done as described above. All values were obtained by using the substrates purified by scavenging.

(i) System coded by gene mtlA. This system coded by *mtlA* has a high affinity for its inducer D-mannitol as measured by growth, transport, and enzyme II-complex^{mtl} activity, although this value is 10 times higher then the previous ones. With D-glucitol as substrate, the reverse is found, but all K_m values are considerably higher than for D-mannitol. The *mtlA* system has no detectable affinity for galactitol.

(ii) System coded by gene gutA. The affinities of the system coded by *gutA* for D-glucitol and D-mannitol are exactly the opposite of the values for the *mtlA* system, i.e., high for the inducer D-glucitol and low for D-mannitol. No affinity for galactitol was detected. Since the K_m values for D-mannitol as measured by transport (33 mM), by growth (0.5 mM), or phosphorylation in vitro (0.06 mM) differ unusually, the transport activity was retested at 25 and 37 C in phosphate (pH 7.0) and tris(hydroxymethyl)aminomethane (pH 7.5) buffer. The values varied from 20 to 55 mM. This value

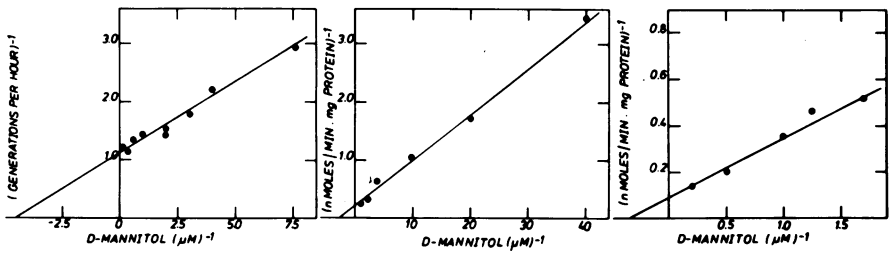

FIG. 2. *K_m and V_{max} values of* D-*mannitol for the transport system coded by gene mtlA. The different values were obtained by determining the growth rates on purified substrates by plating and counting cell colonies (A), measuring initial uptake rates between 0 and 60 s (B), and measuring the rate of formation of* D-[^{14}C]*mannitol-1-phosphate in the cell extracts (C). In every case, purified* D-*mannitol containing ≤0.01%* D-*glucitol was used as the unlabeled substrate.*

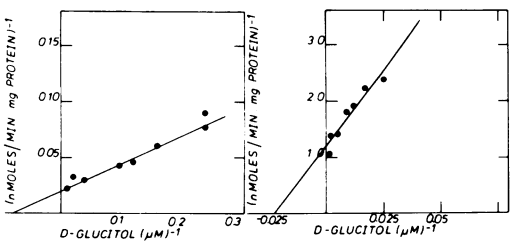

FIG. 3. *K_m and V_{max} values of* D-*glucitol for the transport system coded by gene gutA. The different values were obtained by measuring initial uptake rates (A) or the rate of formation of* D-[^{14}C]*glucitol-6-phosphate (B) in the cell extracts. The* D-*glucitol used contained ≤0.01%* D-*mannitol.*

contrasts especially to the low K_m for the phosphorylation reaction (for a possible explanation see below).

(iii) System coded by gene gatA. The system coded by *gatA* has a high affinity for galactitol as measured by all three reactions. Furthermore, as expected from growth behavior, the system has a good affinity for D-glucitol and no detectable affinity for D-mannitol.

DISCUSSION

The data presented in this and the preceding paper (8) show that the genes *mtlA*, *gutA*, and *gatA* map in three distinct operons. They code for three independant enzyme II-complexes of a

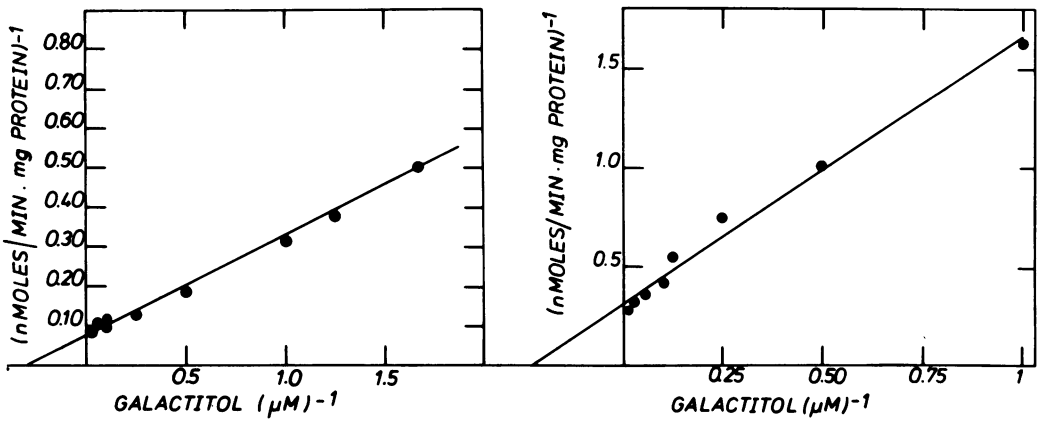

FIG. 4. *K_m and V_{max} values of galactitol for the transport system coded by gene gatA. The different values were obtained by measuring initial uptake rates (A) or the rate of formation of [^{14}C]galactitol-1-phosphate in the cell extracts (B). The galactitol used contained ≤0.01% D-glucitol.*

TABLE 5. *K_m and V_{max} values of the hexitols for hexitol transport systems*[a]

Transport system	Substrate tested	Growth		Transport		Enzyme II-complex	
		K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
mtlA	D-Mannitol	0.22 μM	0.90	0.37 μM	4.5	2.90 μM	11.00
	D-Glucitol	2.30 mM	0.28	2.50 mM	25.0	0.40 mM	9.50
	Galactitol	—	0.00	—	0.0	—	0.00
gutA	D-Mannitol	0.48 mM	0.63	33.0 mM	77.0	0.06 mM	7.20
	D-Glucitol	7.00 μM	1.00	12.0 μM	50.0	44.0 μM	0.83
	Galactitol	—	0.00	—	0.0	—	0.00
gatA	D-Mannitol	—	0.00	—	0.0	—	0.00
	D-Glucitol	0.33 mM	0.65	3.70 mM	68.0	0.80 mM	3.30
	Galactitol	5.00 μM	0.65	3.30 μM	13.4	4.40 μM	3.20

[a] V_{max} values for growth are expressed as generations per hour; those for transport and enzyme II-complex activities are expressed as nanomoles per minute per milligram of protein. The D-mannitol utilized contained ≤0.01% D-glucitol, the D-glucitol contained ≤0.01% D-mannitol, and the galactitol contained ≤0.01% D-glucitol.
[b] —, No detectable affinity.

PTS system. These are apparently of the membrane-bound type and require PEP as phosphate donor (Table 5). Mutants constitutive for the three operons with low P-HPr levels consequent to a mutation in the *pts* operon synthesize nearly normal levels of enzyme II-complex and hexitol-phosphate dehydrogenase activities. Nevertheless, they are unable to grow on, or transport, hexitols. Since among positive revertants no mutants were detected with a K_m factor-like protein substituting for the P-HPr protein (23), the presence of such a factor specific for hexitols seems unlikely. Inducible and soluble FIII-like proteins also have not been found. Instead, it could be shown that induction increases the level of the membrane-bound and substrate-specific proteins (8, 10). In summary, the data indicate the presence of three membrane-bound and P-HPr-dependent enzyme II-complexes of the PEP-dependent PTS. Such transport by group translocation delivering D-mannitol-1-phosphate, D-glucitol-6-phosphate, or galactitol-1-phosphate into the cytoplasm seems best adapted to the hexitol metabolism, the next catabolic enzyme being, in every case, a hexitol-phosphate-specific dehydrogenase (Fig. 5). Dephosphorylation by phosphatases followed by an immediate adenosine 5'-triphosphate-dependent rephosphorylation indeed seems a wasteful procedure. The only PTS carbohydrate clearly shown to be dephosphorylated and chased out of the cell in the free

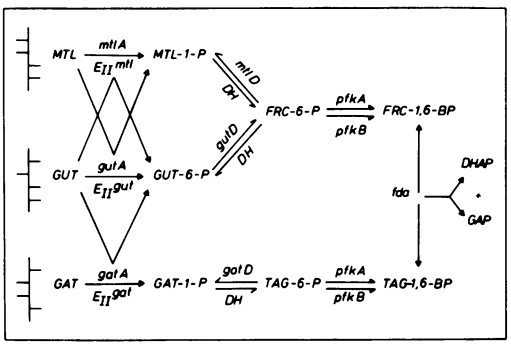

FIG. 5. *Hexitol metabolic pathways in E. coli K-12. The orientation of the hydroxyl groups at carbon atoms C2 through C4 is given schematically for* D-*mannitol (MTL),* D-*glucitol (GUT), and galactitol (GAT), and for the corresponding phosphates at the left of the figure. Further carbohydrates involved are* D-*fructose (FRC),* D-*tagatose (TAG), dihydroxyacetonephosphate (DHAP), and glyceraldehydephosphate (GAP). Genes coding for enzyme II-complexes (EII) are designated by the letter A; genes coding for hexitol-phosphate dehydrogenases (DH) are designated by the letter D preceded by the name of their operon. Genes coding for phosphofructokinase A (EC 2.7.1.11) or B are designated pfkA or pfkB, and the gene coding for the fructose-1,6-biphosphate aldolase (EC 4.1.2.13) is designated fda.*

form is the non-metabolizable and highly toxic α-methyl-glucoside-phosphate. Since the glucoside-specific FIII is involved in this dephosphorylation (W. Kundig, personal communication), the process might conceivably be a detoxification effective at high internal glucose-phosphate levels. The lack of chase of hexitol-phosphates then could be a further indication that no FIII specific for hexitols does occur in *E. coli* K-12. Thus far no indications for the presence of adenosine 5′-triphosphate-dependent hexitol kinases, hexitol dehydrogenases, or transport systems accumulating free hexitols as detected in *Klebsiella* have been found.

If as postulated (15) the enzyme II-complexes are to be considered as a carrier and the first metabolic enzyme at the same time, they would be the pacemaker enzymes in metabolic pathways involving such complexes. For D-mannitol and D-glucitol it has been shown (10) that indeed the level of enzyme II-complex activity determines the rate of catabolism. However the K_m and V_{max} values for the different hexitols summarized in Table 5 and measured by growth rates, transport, and enzyme II-complex activities in vitro show variations to some extent. Similar deviations of K_m and V_{max} values determined in vivo and in vitro have also been reported for the *lac* system in *Staphylococcus aureus* (18) and for the *gptA-gptB* systems in *E. coli* (1, 3, 5, 15). They are most likely to be expected for complex, multistep systems (18). The only major exception seems to be the K_m of D-mannitol for the *gutA* system, which is 500 times higher in vivo than in vitro although the transport data have been repeated under several conditions. One possible explanation is the inhibition of hexitol transport activity by hexose-phosphates (8) not seen in cell extracts. Such an inhibition could simulate high K_m values in vivo. The strong differences could also indicate, however, different binding sites involved in translocation and phosphorylation.

As shown further in Table 5, each hexitol transport system does accumulate its inducer with a high affinity and at least another hexitol with low affinity. Therefore, the apparent presence of six hexitol systems mentioned earlier is, in fact, reduced to three (Fig. 5). 2-Deoxy-arabino-hexitol (also 2-deoxy-D-mannitol or 2-deoxy-D-glucitol at the same time) is transported and phosphorylated with a good affinity by the *gutA* system and with a very low affinity by the *mtlA* system (8). This latter system obviously recognizes the hydroxyl group at C2 and C5. This seems reasonable since D-mannitol (and galactitol) is an open-chained symmetrical molecule (Fig. 5). The terminal carbon atoms can be considered as C1 or C6. Thus, as shown by the subsequent isomerization to fructose-6-phosphate and tagatose-6-phosphate, respectively, all enzyme II-complexes really catalyze the synthesis of hexitol-6-phosphate, the names D-mannitol-1-phosphate and galactitol-1-phosphate being chosen only to comply with carbohydrate nomenclature rules.

Uptake of a carbohydrate or an amino acid by several transport systems seems to be a general rule in bacteria. For such an organism living in a frequently changing environment, a series of transport systems for each substrate might be an advantage. Strains of *E. coli*, e.g., growing on D-mannitol, have an enhanced adaptation lag for growth on D-glucitol due to a severe catabolite and transient repression increased by feedback inhibition caused by D-mannitol. If, however, after the exhaustion of D-mannitol D-glucitol is allowed to enter the cell by the *mtlA* system, this lag is shortened drastically (10).

Besides the hexitol transport systems of *E. coli* and the D-arabinitol system of *Klebsiella* described in the present paper and elsewhere (22), numerous other examples of transport of one substrate by several systems have been described (1, 3, 7, 9, 15–17, 25). The nomencla-

ture of such systems with a broad substrate specificity sometimes is confusing. In contrast to this, the mapping or the analysis of the induction specificity frequently gives clear results. Thus, in the case of the systems coded by *mglP* (9), *galP* (25), *gptA* (1), and *araE* (3) in *E. coli* or the D-arabinitol system of *Klebsiella* and in the case of the hexitol systems discussed, the inducer invariably is the substrate with the highest affinity. Here the structural gene of the transport system always forms an operon with the structural gene of the dehydrogenase specific for the phosphorylated inducer. I propose, therefore, to call the transport system belonging to the *mtl* operon the D-mannitol transport system and similarly the systems belonging to the *gut* and *gat* operons the D-glucitol and the galactitol transport systems.

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