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Regulation of Gluconeogenesis by the Glucitol Enzyme III of the Phosphotransferase System in *Escherichia coli*

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The *gut* operon was subcloned into various plasmid vectors (M. Yamada and M. H. Saier, Jr., J. Bacteriol. 169:2990–2994, 1987). Constitutive expression of the plasmid-encoded operon prevented utilization of alanine and Krebs cycle intermediates when they were provided as sole sources of carbon for growth. Expression of the *gutB* gene alone (encoding the glucitol enzyme III), subcloned downstream from either the lactose promoter or the tetracycline resistance promoter, inhibited utilization of the same compounds. On the other hand, overexpression of the *gutA* gene (encoding the glucitol enzyme II) inhibited the utilization of a variety of sugars as well as alanine and Krebs cycle intermediates by an apparently distinct mechanism. Phosphoenolpyruvate carboxykinase activity was greatly reduced in cells expressing high levels of the cloned *gutB* gene but was nearly normal in cells expressing high levels of the *gutA* gene. A chromosomal mutation in the *gutR* gene, which gave rise to constitutive expression of the chromosomal *gut* operon, also gave rise to growth inhibition on gluconeogenic substrates as well as reduced phosphoenolpyruvate carboxykinase activity. Phosphoenolpyruvate synthase activity in general varied in parallel with that of phosphoenolpyruvate carboxykinase. These results suggest that high-level expression of the glucitol enzyme III of the phosphotransferase system can negatively regulate gluconeogenesis by repression or inhibition of the two key gluconeogenic enzymes, phosphoenolpyruvate carboxykinase and phosphoenolpyruvate synthase.

The phosphoenolpyruvate (PEP)-dependent sugar phosphotransferase system (PTS) possesses several regulatory functions as well as the primary capacity to initiate the catabolism of a variety of sugars (25, 27). The PTS regulates the uptake of several carbohydrates (non-PTS sugars) including glycerol, lactose, melibiose, and maltose (23, 26, 27). Regulation seems to be effected by direct protein-protein interactions between the glucose enzyme III (III^{Glc}) and non-PTS sugar permeases or catabolic enzymes. The direct binding of III^{Glc} to the lactose permease of *Escherichia coli* (17, 18, 20, 21) and to the *E. coli* and *Salmonella typhimurium* glycerol kinases (5, 18, 24) have been demonstrated. The PTS also regulates the activity of adenylate cyclase (3, 23, 28), and a mechanism has been proposed in which the phosphorylated form of III^{Glc} activates the enzyme (28). Recently, it has been found that constitutive expression of the fructose regulon prevents gluconeogenesis in *S. typhimurium* and *E. coli* (1, 6). Available evidence suggested an involvement of the fructose-specific enzyme III (III^{Fru}) (1).

In this communication, we present evidence that the glucitol PTS is also capable of regulating gluconeogenesis in *E. coli*. The *gut* operon, encoding the protein components of the glucitol PTS and glucitol-6-phosphate dehydrogenase, is localized at 58 min on the *E. coli* chromosome (11, 14). Recently, we cloned and sequenced the entire operon and its associated regulatory regions (34, 35; M. Yamada and M. H. Saier, Jr., submitted for publication). The operon consists of the *gutA* gene (encoding II^{Gut}), the *gutB* gene (encoding III^{Gut}), the *gutD* gene (encoding glucitol-6-phosphate dehydrogenase), the *gutM* gene (encoding a small protein which functions in the transcriptional activation of the *gut* operon) and the *gutR* gene (encoding the repressor of the *gut* operon) (34, 35; Yamada and Saier, submitted). The results reported in this communication lead to the conclusion that regulation of gluconeogenesis via the glucitol PTS is effected by III^{Gut}.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* strains 236sr (Hfr *leuB6 thi-1 lacZ4 mtlA9 rpsL8 supE44 gut recA*) (10) and JM103 [Δ(*lac-pro*) *thi strA supE endA sbcB15 hsdR4* F′ *traD36 proAB lacI*^{q}ZΔ*M15*) (15) were used as host strains for cloning the *gut* operon and its components. LJ1008 (F$^-$ *thr-1 leu-6 thi-1 supE44 lacY tonA21 Δmtl-29*) (10) was used for Tn*5* mutagenesis. *S. typhimurium* strains LJ530 (*mtlA61 gutC151 gutB152*) and LJ531 (*mtlA61 gutC151 gutA153*) were used in PEP-dependent [^{14}C]glucitol phosphotransferase assays (see below). pUC18 and pUC19 (15) were used as cloning vectors. The construction of pMYS5, pMYS24, pMYS28, pMYS139, pMYS140, and pMYS157 has been described previously (34). T28RT (*lacY* F′ *lacI*^{q}*Y*) and pTE18 [*lac* Δ(*I*)*O*$^+$ *P*$^+$ Δ(*Z*)*Y*$^+$ Δ(*A*)] were obtained from P. Overath (32).

DNA manipulations. Restriction endonuclease cleavage, ligation, transformation, DNA isolation, and gel electrophoresis were performed as described previously (13). Construction of deletion mutants (33) and NH_2OH mutagenesis (8) were also carried out as described previously. Tn*5* mutagenesis was performed as described by Davis et al. (4).

Growth media and selection conditions. Subclones in pUC18 were selected as white colonies on LB plates (16) containing 50 μg of ampicillin per ml, 1 mM isopropyl-β-D-thiogalactopyranoside, and 30 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside per ml. Growth tests were performed on modified medium 63 (29) minimal plates containing 0.2% of a sugar, 0.1% alanine, or 0.2% of a Krebs cycle intermediate. For enzyme and transport assays, cells were grown in LB medium containing the appropriate antibiotics until the late-logarithmic phase of growth. Overproduction of the lactose permease in strain T28RT harboring the plasmid pTE18 (32) was carried out by the addition of 1

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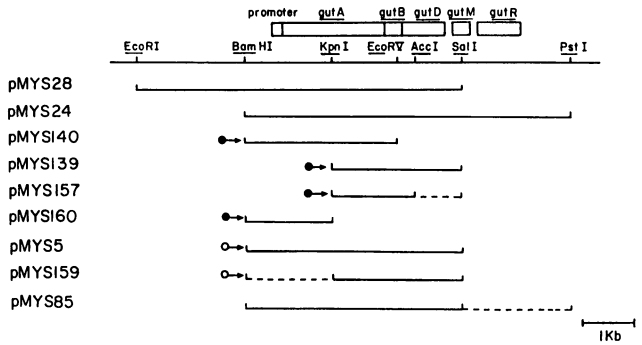

FIG. 1. Clones of the *gut* operon and subcloning of the *gutA* and *gutB* genes. The top diagram shows the *gut* operon and the positions of the promoter and the *gutA*, *gutB*, *gutD*, *gutM*, and *gutR* genes. The bottom diagrams represent subclones of the *gut* operon and its genes. pMYS5, pMYS24, and pMYS28, which carry the *gut* operon, were constructed as described previously (34) by using vectors pACYC184, pACYC177, and pBR322, respectively. pMYS139, pMYS140, and pMYS157 were also constructed previously (34) by using pUC18 as the vector. The construction of pMYS160 was performed by insertion of the *Bam*HI-*Kpn*I fragment into pUC19. pMYS159 was made from pMYS5 by deletion of the region between the *Bam*HI and *Kpn*I sites. pMYS85 was made from pMYS24 by deletion between the *Sal*I and *Pst*I sites. Dotted lines indicate the deleted regions. Arrows show the positions and directions of readthrough from the promoters of the lactose operon (●→mh) and the tetracycline resistance gene (○→mh).

mM isopropyl-β-D-thiogalactopyranoside and 1 mM cAMP to the medium during exponential growth. T28RT harboring pBR322 was used as the control strain.

Enzyme assays. Crude extracts were prepared as described previously (34). Assays for PEP-dependent glucitol phosphorylation in crude extracts (34), glucitol-6-phosphate dehydrogenase in crude extracts (19), PEP carboxykinase in high-speed supernatants (9), PEP synthase in crude extracts (2), and carbohydrate uptake into whole cells (30) have been described. Protein was determined by the method of Lowry et al. (12) with bovine serum albumin as the protein standard.

RESULTS

Effect of the constitutive expression of the *gut* operon on cell growth. We have subcloned the *gut* operon, which consists of the *gutA*, *gutB*, *gutD*, *gutM*, and *gutR* genes (34) (Fig. 1). The *gutM* gene encodes the transcriptional activator of *gut* operon expression (Yamada and Saier, submitted), whereas the *gutR* gene encodes the repressor of the *gut* operon. The entire region has been sequenced (35; Yamada and Saier, submitted). pMYS5 possesses most of the *gut* operon in a 4-kilobase DNA fragment, but it lacks the *gutR* gene so that the plasmid-encoded *gut* operon is constitutively expressed. On the other hand, pMYS24 carries the *gutR* gene as well as the *gut* operon, and it therefore expresses the operon inducibly.

We noticed that cells harboring pMYS5 grew very poorly on minimal plates supplemented with any of a variety of carbon sources (Table 1). The cells grew normally on rich media such as LB medium. Cells harboring pMYS24 grew on all of these plates.

Cells harboring pMYS28 (34), which constitutively expresses the *gut* operon, exhibited defective growth, as did cells harboring pMYS5. Furthermore, cells harboring deletion mutant plasmids (34) from pMYS5, in which about 0.5 kilobase of DNA at the 5′-flanking region or 0.2 kilobase of DNA at the 3′-flanking region was removed, exhibited the same growth phenotype as did cells harboring the parental plasmid. Therefore, flanking regions of the *gut* operon are not related to growth inhibition. These results suggest that the constitutive expression of the *gut* operon prevents growth on minimal plates containing alanine or one of the Krebs cycle intermediates.

Tn*5* mutagenesis for the construction of *gutR* mutants. LJ1008 (10), in which the 5′-flanking and NH_2-terminal regions of the mannitol operon were deleted, was subjected to Tn*5* mutagenesis (4). LJ1008 cannot grow on minimal plates containing mannitol (10). We suspected that mutation of the glucitol operon to constitutivity would allow mannitol utilization, because (i) mannitol can be taken up and phosphorylated by the glucitol PTS (31), and (ii) mannitol-1-phosphate dehydrogenase activity has been demonstrated in this strain (19). Mutants were selected for growth on minimal plates containing 0.2% mannitol and 20 μg of kanamycin per ml. One such mutant, LJ1008 (*gutR*::Tn*5*), showed constitutive expression of the glucitol phosphotransferase activity (see below). The mutation prevented growth on alanine and all Krebs cycle intermediates (Table 1). Thus, high-level expression of the chromosomal *gut* operon seems to cause complete growth inhibition on such carbon sources. Although the Tn*5* insertion mutation was not mapped, it is probably in the *gutR* gene.

Mutagenesis of the plasmid-encoded *gut* operon with NH_2OH. pMYS85 is a pMYS24 derivative that lacks the *Sal*I-*Pst*I fragment encoding the *gutR* gene. Consequently, the *gut* operon is expressed constitutively in cells carrying the plasmid. Cells harboring this plasmid show the same growth phenotype as the cells harboring pMYS5 or pMYS28 described above. pMYS85 treated with NH_2OH (8) was introduced into strain 236sr (*gut mtl*). After cultures were screened on eosin-methylene blue-mannitol plates, eight fermentation-negative mutants were isolated. Enzyme assays revealed that six of these mutants (pMYS142, pMYS143, pMYS145, pMYS146, pMYS154, and pMYS155) were defective in the *gutA* gene (Table 2). The remaining two mutants (pMYS148 and pMYS150) were deletion mutants, as shown by gel electrophoretic analysis after restriction enzyme digestion. The two deletion mutants became capable of growth on all carbon sources. pMYS151 and pMYS153 seemed to be defective in the promoter of the operon because they had equally reduced activities of the glucitol phosphotransferase components as well as glucitol-6-phosphate dehydrogenase. Although the six *gutA* mutants still exhibited poor growth on glycerol, alanine, and Krebs cycle intermediates, they recovered their ability to grow on all sugars. These results suggest that expression of the *gutA* gene brings about growth inhibition on a variety of carbon sources including sugars but that expression of the *gutB* gene specifically causes growth inhibition in minimal medium containing glycerol, alanine, or a Krebs cycle intermediate.

Since it has been reported that *E. coli* and *S. typhimurium* mutants which expressed the fructose PTS constitutively showed reduced PEP carboxykinase and PEP synthase activities (1, 6), PEP carboxykinase activity was measured in these mutants (Table 2). The activity of cells harboring pMYS148 and pMYS150, which showed negligible activity of the glucitol operon products, was the same as in cells harboring only the vector (Table 2). Other mutant plasmids possessing intermediate activities of the glucitol catabolic enzymes showed partial restoration of PEP carboxykinase

TABLE 1. Growth of *E. coli* strains which overproduce specific proteins of the glucitol PTS[a]

Host	Plasmid	Genes[b]	Growth on the following carbon sources[c]													
			Glc	Fru	Gut	Mtl	Gon	Gal	Rib	Gly	Ala	Ace	Pyr	Suc	Mal	Fum	Oxa
236sr			+	+	−	−	+	+	+	+	+	+	+	+	+	+	+
236sr	pMYS5	*gutABD*	±	±	±	±	−	−	−	−	−	−	−	−	−	−	−
236sr	pMYS24	*gutABDMR*	+	+	+	+	+	+	+	+	±	±	+	+	±	+	+
236sr	pMYS28	*gutABD*	±	±	±	±	−	−	−	−	−	−	−	−	−	−	−
236sr	pMYS85	*gutABD*	±	±	±	±	−	−	−	−	−	−	−	−	−	−	−
236sr	pMYS159	*gutBD*	+	+	−	−	+	+	+	±	−	−	±	−	−	−	−
JM103	pUC18		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
JM103	pMYS139	*gutBD*	+	+	+	+	+	+	±	±	−	±	±	±	−	−	−
JM103	pMYS140	*gutA*	±	±	±	±	±	±	±	±	−	−	±	−	−	−	−
JM103	pMYS157	*gutB*	+	+	+	+	+	+	+	±	−	±	±	±	−	−	−
JM103	pMYS162		+	+	+	+	+	+	+	+	+	+	+	+	+	+	±
LJ1008			+	+	+	−	+	+	+	±	±	±	+	+	+	+	−
LJ1008 (*gutR*::Tn*5*)			+	+	+	+	+	+	+	−	−	−	−	±	−	−	−

[a] Growth was measured after 48 h on minimal plates as described in Materials and Methods: +, normal growth; ±, poor growth; −, no growth. All growth responses were compared with those of wild-type cells as a control.

[b] Plasmid-encoded glucitol genes.

[c] Abbreviations: Glc, glucose; Fru, fructose; Gut, glucitol; Mtl, mannitol; Gon, gluconate; Gal, galactose; Rib, ribose; Gly, glycerol; Ala, alanine; Ace, acetate; Pyr, pyruvate, Suc, succinate; Mal, malate, Fum, fumarate; Oxa, oxaloacetate.

activity. Therefore, the reduction of the activity of this enzyme seemed to correlate with expression of enzyme III^{Gut} or glucitol-6-phosphate dehydrogenase activity or both.

Effect of *gutA* and *gutB* gene expression on cell growth. The *Bam*HI-*Eco*RV and *Kpn*I-*Sal*I fragments from pMYS24 were separately subcloned into the *Bam*HI-*Hin*cII and *Kpn*I-*Sal*I sites of pUC18, respectively (34) (Fig. 1). The resultant plasmids, pMYS140 and pMYS139, carried the *gutA* gene (within the *Bam*HI-*Eco*RV fragment) and the *gutBD* genes (within the *Kpn*I-*Sal*I fragment), respectively. In both recombinants, the direction of transcription from the *lac* promoter on pUC18 was the same as that of the *gutA* and *gutBD* genes in the *gut* operon. pMYS157 was constructed by deletion of the 0.8-kilobase *Acc*I fragment from pMYS139 and lacks a part of the *gutD* gene (34). These recombinants were introduced into JM103, and the presence or absence of the *gutA*, *gutB*, and *gutD* genes on the recombinant plasmids was confirmed by PEP-dependent phosphotransferase and glucitol-6-phosphate dehydrogenase assays.

The presence of pMYS140, pMYS139, or pMYS157 prevented cell growth on glycerol, alanine, and Krebs cycle intermediates without causing induction of the *lac* promoter (Table 1). By contrast, cells carrying vector pUC18 grew normally on such media. Unlike pMYS139 and pMYS157, pMYS140 gave rise to cell growth inhibition on a variety of sugars. To examine the effect of the *gut* operator and the 5′-flanking region on cell growth, pMYS162, which carries the *Bam*HI-*Kpn*I fragment inserted between the *Bam*HI and *Kpn*I sites on pUC19, was constructed. The transcriptional direction of the *gut* promoter on the recombinant plasmid was the same as that from the *lac* promoter. Cells carrying the plasmid grew normally on minimal media, as did cells carrying pUC19. Therefore, the *gut* promoter and the 5′-flanking region exerted no effect on growth. When 236sr was used as the host strain instead of JM103, essentially the same results were obtained with all recombinants.

pMYS159 was constructed by deletion of the *Bam*HI-*Kpn*I fragment from pMYS5 (Fig.1). As a result, the *gutBD* genes on the plasmid were under the control of the tetracycline resistance gene promoter. Cells harboring this plasmid also exhibited the same growth inhibition, as did cells harboring pMYS139 and pMYS157. These results suggest that expression of the *gutB* gene inhibited growth on glycerol, alanine, and the Krebs cycle intermediates, whereas expression of the *gutA* gene inhibited growth on the sugars tested.

PEP carboxykinase and PEP synthase activities. PEP carboxykinase and PEP synthase activities were measured in the glucitol-constitutive strains. In cells harboring pMYS5 and pMYS28, the activities were reduced compared with those of cells harboring pMYS24 in which *gut* operon expression is inducible (Table 3). The activities in the Tn*5* insertion mutants were also lower than those of the parental strain. PEP carboxykinase activity of cells harboring the enzyme III^{Gut} clone, pMYS157, was reduced to about one-fourth compared with those of strains carrying the vector.

TABLE 2. Activities of enzymes of the *gut* operon and of PEP carboxykinase in mutants induced with NH_2OH

Plasmid	Relative sp act[a]			
	II^{Gut}[b] (*gutA*)	III^{Gut}[c] (*gutB*)	Glucitol-6-phosphate dehydrogenase (*gutD*)	PEP carboxykinase[d]
pMYS85	100	100	100	100
pMYS142	5	53	40	190
pMYS143	4	22	25	230
pMYS145	3	65	55	120
pMYS146	2	39	36	140
pMYS148	1	4	0	400
pMYS150	2	2	0	400
pMYS151	38	28	40	170
pMYS153	56	58	60	180
pMYS154	3	43	37	150
pMYS155	1	32	27	170

[a] Mutant plasmids obtained by NH_2OH mutagenesis of pMYS85 were introduced into strain 236sr, and enzyme assays were conducted as described in the text. All values represent the averages of two or three experiments and are expressed as specific activities relative to cells harboring pMYS85. Absolute specific activities for cells harboring pMYS85 were as follows: enzyme II^{Gut}, 80 pmol/min per mg of protein; enzyme III^{Gut}, 260 pmol/min per mg of protein; glucitol-6-phosphate dehydrogenase, 8.8 μmol/min per mg of protein; PEP carboxykinase, 40 nmol/min per mg of protein.

[b] Enzyme II^{Gut} activity was measured in crude extracts of LJ531.

[c] Enzyme III^{Gut} activity was measured in crude extracts of LJ530.

[d] The activities of strain 236sr and of strain 236sr carrying plasmid pUC18 were comparable and corresponded to a specific activity of 400 relative to strain 236sr carrying plasmid pMY585. All values represent the averages of duplicate determinations. Duplicates seldom deviated from each other by more than 30%. Absolute specific activities are given in Tables 3 and 4.

TABLE 3. Activities of enzymes of the *gut* operon and of PEP carboxykinase in cells carrying various plasmids and in a mutant strain carrying the *gutR*::Tn*5* chromosomal mutation

Host	Plasmid	Genes[a]	Enzyme activity (nmol/min per mg of protein)			
			PEP-dependent glucitol phosphotransferase	Glucitol-6-phosphate dehydrogenase	PEP carboxykinase[b]	PEP synthase[b]
236sr			2.0	4	160	255
236sr	pMYS5	*gutABD*	780	16,000	38	149
236sr	pMYS24	*gutABDMR*	8.0	100	150	
236sr	pMYS28	*gutABD*	830	17,000	31	
236sr	pMYS159	*gutBD*	320[c]	3,300	67	
JM103	pUC18		3.0	4	140	342
JM103	pMYS139	*gutBD*	870[c]	43,000	26	170
JM103	pMYS140	*gutA*	220[d]	94	120	300
JM103	pMYS157	*gutB*	820[c]	4	20	175
LJ1008			5.0	4	190	310
LJ1008 (*gutR*::Tn*5*)			200	5,500	58	180

[a] Plasmid-encoded glucitol genes.
[b] Values represent an average of two or three experiments. Duplicate determinations seldom deviated from one another by more than 30%.
[c] Activity was measured in crude extracts of LJ530. Consequently III^{Gut} is rate limiting.
[d] Activity was measured in crude extracts of LJ531. Consequently II^{Gut} is rate limiting.

This activity was reduced to about one-eighth by inclusion of the *lac* operon inducer isopropyl-β-D-thiogalactopyranoside. The clone expressing enzyme II^{Gut}, pMYS140, showed an insignificant reduction in activity. The *gutA*$^-$ mutants obtained by NH_2OH mutagenesis of the plasmid also showed reduced activities (Table 2). These results suggest that high-level expression of III^{Gut} is responsible for the reduction in PEP carboxykinase and PEP synthase activities.

Uptake study. Carbon source uptake into the *E. coli* cells was studied after 2 h of induction of the corresponding transport system in LB broth in the presence of the carbon source (0.5%). Cells were harvested during exponential growth (Table 4). Compared with that of the host strain, the rates of uptake of glucose, succinate, and alanine were normal in cells harboring pMYS139 or pMYS143. On the other hand, these uptake rates were reduced by 30 to 50% in cells harboring pMYS28, pMYS85, or pMYS140. These results indicate that the overproduction of enzyme III^{Gut} had no effect on these uptake rates, although enzyme II^{Gut} overproduction inhibited uptake appreciably.

Effect of lactose permease overproduction on cell growth. The lactose permease in T28RT harboring pTE18 was overproduced by induction with isopropyl-β-D-thiogalactopyranoside and cAMP (32). Cell growth was observed on minimal plates containing various carbon sources (Table 5). When lactose permease production was induced, cells harboring pTE18 exhibited growth inhibition on several carbon sources. Although the effect was similar to that of enzyme II^{Gut} overproduction (Table 1), growth inhibition seemed to be weaker than that observed when enzyme II^{Gut} was overproduced. This effect is presumably a quantitative effect which may correlate with the level of production of the membrane protein.

DISCUSSION

In this report we demonstrate an unexpected regulatory function of the glucitol PTS in *E. coli*. High-level constitutive expression of the proteins of the glucitol PTS led to growth inhibition when cells were grown in minimal medium with any one of a variety of carbon sources (Table 1). The chromosomal mutant LJ1008 *gutR*::Tn*5* and cells carrying the *gutB* gene alone on a multicopy plasmid could not utilize alanine or Krebs cycle intermediates on minimal plates. By contrast, cells bearing the *gutABD* or *gutA* clones on multicopy plasmids grew poorly on minimal plates containing any one of a variety of carbon sources, including

TABLE 4. Effect of glucitol catabolic enzyme overproduction on carbon source uptake

Host	Plasmid	Genes[a]	Uptake[b] (nmol/min per mg of protein) of:			
			Glucose	Glycerol	Alanine	Succinate
236sr	pMYS85	*gutABD*	13	50	16	
236sr	pMYS143	*gutBD*	19	69	26	
236sr	pACYC177		18	66	25	
236sr	pMYS5	*gutABD*		35		
236sr	pACYC184			69		
236sr	pMYS28	*gutABD*	9.5	38		
236sr	pBR322		17	74		
JM103	pMYS140	*gutA*			14	50
JM103	pMYS139	*gutBD*			20	75
JM103	pMYS157	*gutB*		31		
JM103	pUC18			63	19	83
LJ1008						101
LJ1008 (*gutR*::Tn*5*)						88

[a] Plasmid-encoded glucitol genes.
[b] The concentration of the ^{14}C-labeled substrate was 1 mM. Values represent averages of two or three experiments. Duplicate values were within 20% of each other.

TABLE 5. Growth phenotype of an *E. coli* strain which overproduces the lactose permease

Host	Plasmid	1 mM IPTG–1mM cAMP	Growth on the following carbon source[a]:										
			Glc	Fru	Gut	Mtl	Gon	Gal	Rib	Ara	Suc	Mal	Fum
T28RT	pTE18	−	+	+	+	+	+	+	+	+	+	+	+
T28RT	pTE18	+	+	±	±	+	+	−	−	−	−	−	−
T28RT	pBR322	−	+	+	+	+	+	+	+	+	+	+	+
T28RT	pBR322	+	+	+	+	+	+	+	+	+	+	+	+

[a] See footnote *c* of Table 1.

carbohydrates. Two distinct regulatory mechanisms appeared to be operative. One involves III^{Gut} as a regulatory protein that controls the gluconeogenic enzymes PEP carboxykinase and PEP synthase. The other involves enzyme II^{Gut} and functions by a completely different mechanism. Thus, defective growth of LJ1008 *gutR*::Tn*5* seems to be due to the constitutive expression of the *gutB* gene, whereas the overexpression of the *gutA* gene on a multicopy plasmid seems to bring about growth inhibition on a wider variety of carbon sources.

Since glucose, alanine, and succinate were transported at normal rates into fully induced *E. coli* cells which carried the *gutB* gene on a multicopy plasmid, it appears that these transport systems are not the regulatory targets of III^{Gut}. On the other hand, the constitutive expression of the *gutA* gene on a multicopy plasmid partially inhibited uptake of these compounds. The transport systems may therefore be directly or indirectly influenced by high-level expression of the *gutA* gene.

We noticed that cells constitutively expressing the *gutB* gene at high levels exhibited reduced PEP carboxykinase and PEP synthase activities (Table 3). The NH_2OH mutagenesis experiment revealed that the levels of these enzyme activities correlated inversely with the amounts of functional III^{Gut} produced (Table 2 and unpublished results). These observations suggest that the repression or inhibition of certain gluconeogenic enzymes, two of which are PEP carboxykinase and PEP synthase (1, 6), is mediated by III^{Gut}.

The growth phenotype of III^{Gut} overproducers of *E. coli* studied here was similar to that of *fru*R mutants of *S. typhimurium* studied previously (1). Further, the degrees of reduction in the PEP carboxykinase and PEP synthase activities were similar in these two studies and in that of Geerse et al. (6). The growth phenotype was also identical to that of an *E. coli* double mutant presumed to be defective in the structural genes of the two gluconeogenic enzymes (7). In the mutant studied, the PEP carboxykinase possessed about 30% of wild-type activity, yet it was unable to utilize lactate or a Krebs cycle intermediate as a sole source of carbon (7). Additionally, in the study of Geerse et al. (6), a *fruR* mutant in *E. coli* was shown to possess 35% of wild-type PEP synthase activity; yet, like those studied by us (1), it was not able to utilize lactate as a source of carbon. It therefore appears that the reduced activities of the two gluconeogenic enzymes in *fruR* mutants of *S. typhimurium* (1, 6) and in III^{Gut} overproducers of *E. coli* (Tables 3 and 4) can quantitatively account for the growth phenotype.

The growth phenotype of III^{Gut}- and III^{Fru}-overproducing strains (Table 1) (1) eliminates the possibility that a gluconeogenic enzyme common to glycolysis is the physiologically relevant target of regulation. Since the cells are capable of glycerol utilization (Table 1), the sole defect can not be fructose 1,6-diphosphatase or a subsequent enzyme involved in the interconversion of the biosynthesized sugars. Because III^{Gut}-overproducing strains grew normally on all sugars, the possibility of a generalized growth defect resulting from overexpression of the *gutB* gene can be eliminated. Since the induced uptakes of citrate, succinate, and alanine were not impaired (1) (Table 4) and the alanine and succinate dehydrogenase activities were present in normal or elevated levels (1; unpublished results), these transport systems and enzymes as well as their transcriptional apparatuses can not be the physiologically relevant targets of regulation. Since several of the compounds which could not be used by *fruR* mutants (1) or III^{Gut} overproducers (Table 1) (i.e., citrate) can be metabolized both aerobically and anaerobically by distinct pathways, a defect in aerobic metabolism cannot account for the growth phenotypes. Recently, we have confirmed this conclusion by showing that D-lactate stimulates the uptake of [^{14}C]proline in III^{Gut}-overproducing strains, showing that the aerobic metabolism of lactate is normal (unpublished results). All of the evidence currently available therefore argues in favor of the postulates that (i) III^{Fru} and III^{Gut} regulate synthesis of PEP carboxykinase and PEP synthase, and (ii) reduced levels of these two enzymes are both necessary and sufficient to account for the growth phenotype. The work described in this communication thus provides an essential link in a chain of evidence defining a novel and important regulatory mechanism whereby the PTS controls gluconeogenesis in enteric bacteria.

We previously reported that the constitutive expression of the fructose PTS in *S. typhimurium* and *E. coli* gave rise to growth inhibition accompanied by decreased expression of PEP carboxykinase activity (1). It was shown that the regulatory effect was not mediated by cyclic AMP or its receptor protein, that the permeases responsible for inducer uptake apparently functioned normally, and that reductions in the activities of the permeases and initial catabolic enzymes could not account for the growth phenotype. Regulation by the fructose system appeared to be effected by the fructose-specific enzyme III (1). Recent evidence suggests that III^{Fru} regulates the transcription rather than the activity of PEP synthase (A. M. Chin and M. H. Saier, Jr., unpublished results). It is interesting in this regard that enzyme III^{Glc}, the central regulatory protein controlling the uptake and utilization of several carbohydrates (27), allosterically regulates the activities of the target permeases and catabolic enzymes and only indirectly controls their synthesis. Distinct regulatory functions for the various enzymes III of the PTS have been proposed (27).

Although the constitutive expression of the *gutA* gene on a multicopy plasmid influenced cell growth on alanine and Krebs cycle intermediates as well as various sugars, the effect on PEP carboxykinase activity was only slight and clearly could not account for the growth phenotype (Table 3). Since essentially no growth inhibition was observed when the chromosomal mutant LJ1008 (*gutR*::Tn*5*) was utilizing various sugars as sole sources of carbon, overproduction of enzyme II^{Gut} could not be responsible for the growth phe-

notype of this strain. Overproduction of enzyme II^{Gut} seems to prevent cell growth by a mechanism analogous to that by which overproduction of lactose permease (22) or mannitol enzyme II (10) inhibits growth. Overproduction of any one of these three integral membrane proteins resulted in the same growth phenotype (Tables 1 and 5) but did not depress the PEP carboxykinase activity (data not shown). Therefore, the growth-inhibitory mechanism mediated by overproduction of enzyme II^{Gut}, enzyme II^{Mtl}, or the lactose permease must be different from that mediated by III^{Gut}.

We found that strains which produced high levels of III^{Gut} grew poorly on glycerol. It is possible that glycerol kinase, which is allosterically inhibited by III^{Glc} (18), is also regulated by III^{Gut}. Our preliminary experiment showed that glycerol kinase activity was reduced about 40% in the glucitol-constitutive strains. This result seems to be coincident with the uptake study (Table 4) in which glycerol uptake was reduced 25 to 50% in the glucitol-constitutive strain and in the enzyme III^{Gut} overproducer. This is the first evidence (albeit preliminary) that an enzyme III other than III^{Glc} can affect regulation of a non-PTS catabolic enzyme or permease. Further studies will be required to define the extent and significance of enzyme III-mediated regulatory processes and to define the mechanisms involved.

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