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Glycerol-3-Phosphate-Induced Catabolite Repression in *Escherichia coli*

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The formation of glycerol-3-phosphate (G3P) in cells growing on TB causes catabolite repression, as shown by the reduction in *malT* expression. For this repression to occur, the general proteins of the phosphoenolpyruvate-dependent phosphotransferase system (PTS), in particular EIIA^{Glc}, as well as the adenylate cyclase and the cyclic AMP-catabolite activator protein system, have to be present. We followed the level of EIIA^{Glc} phosphorylation after the addition of glycerol or G3P. In contrast to glucose, which causes a dramatic shift to the dephosphorylated form, glycerol or G3P only slightly increased the amount of dephosphorylated EIIA^{Glc}. Isopropyl-β-D-thiogalactopyranoside-induced overexpression of EIIA^{Glc} did not prevent repression by G3P, excluding the possibility that G3P-mediated catabolite repression is due to the formation of unphosphorylated EIIA^{Glc}. A mutant carrying a C-terminally truncated adenylate cyclase was no longer subject to G3P-mediated repression. We conclude that the stimulation of adenylate cyclase by phosphorylated EIIA^{Glc} is controlled by G3P and other phosphorylated sugars such as D-glucose-6-phosphate and is the basis for catabolite repression by non-PTS compounds. Further metabolism of these compounds is not necessary for repression. Two-dimensional polyacrylamide gel electrophoresis was used to obtain an overview of proteins that are subject to catabolite repression by glycerol. Some of the prominently repressed proteins were identified by peptide mass fingerprinting. Among these were periplasmic binding proteins (glutamine and oligopeptide binding protein, for example), enzymes of the tricarboxylic acid cycle, aldehyde dehydrogenase, Dps (a stress-induced DNA binding protein), and D-tagatose-1,6-bisphosphate aldolase.

Catabolite repression refers to the reduction in transcription of sensitive operons that is caused by certain carbon sources in the medium, most prominently by glucose (glucose effect). In *Escherichia coli*, phosphoenolpyruvate-dependent phosphotransferase system (PTS)-mediated uptake of glucose is crucial for this effect. The model largely accepted for *E. coli* focuses on the level of cyclic AMP (cAMP) synthesized by the membrane-bound adenylate cyclase (29, 30, 38). EIIA^{Glc}, an intermediate in the phosphorylation cascade of the PTS for glucose, in its phosphorylated form is thought to stimulate adenylate cyclase. The basal level of adenylate cyclase activity would be present in the absence of EIIA^{Glc} or in the presence of unphosphorylated EIIA^{Glc}. As a consequence, CAP, the catabolite activator protein (or cAMP receptor protein [CRP]) that is needed for the transcription of sensitive operons (22) is linked in its activity to the PTS (31). The glucose PTS is also responsible for inducer exclusion, i.e., inhibition of the different transport systems by unphosphorylated EIIA^{Glc} (31).

Even though participation of the PTS in catabolite repression and inducer exclusion in *E. coli* has been documented very well, the effects of non-PTS sugars are less clear. Thus, glucose-6-phosphate or gluconate exerts strong catabolite repression although it is not transported by the PTS. Recent studies on catabolite repression caused by non-PTS sugars concluded that it is the amount of CAP, as well as cAMP, that is altered in response to non-PTS sugars (14, 18, 34). In these studies, it was not determined whether or not metabolism is required for catabolite repression to occur.

Glucose-6-phosphate and other non-PTS carbon sources were also shown to cause inducer exclusion by influencing the phosphorylation state of EIIA^{Glc}, indicating that substrates can cause dephosphorylation of EIIA^{Glc} phosphate (EIIA^{Glc}-P) without being transported by the PTS. The dephosphorylation of EIIA^{Glc}-P brought about by glucose-6-phosphate, melibiose, lactose, and arabinose metabolism was nearly as strong as that of the PTS sugars tested. Other carbon sources (like glycerol, galactose, maltose, etc.) caused weaker dephosphorylation (12, 13). For glucose-6-phosphate, the authors showed that metabolism of the sugar phosphate is necessary for the dephosphorylation of EIIA^{Glc}-P and it was concluded that the phosphoenolpyruvate/pyruvate ratio determines the phosphorylation state of EIIA^{Glc}. Thus, inducer exclusion by non-PTS sugars could be explained by the temporary dephosphorylation of EIIA^{Glc}. Consequently, in the absence of metabolism, for instance, in a *pgi* mutant, glucose-6-posphate does not cause inducer exclusion. Thus, one might conclude that when metabolism of the non-PTS sugars is prevented, neither catabolite repression nor inducer exclusion should occur. However, this conclusion does not hold for the catabolite repression exerted by glycerol or glycerol-3-phosphate (G3P), a phenomenon that we recently studied by using the *E. coli* maltose system as a model (11). In that study, it was shown that glycerol has to be phosphorylated to G3P in order to exert catabolite repression but that further metabolism is not necessary.

The maltose system of *E. coli* (2) is a typical catabolite-sensitive regulon. It consists of 10 genes encoding proteins for

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TABLE 1. Strains and plasmids used in this study

Strains and plasmids	Relevant genotype	Source and/or reference
Strains		
Bre1161	MC4100; ϕ(*malT-lacZ*)*1110* (λp*lac* Mu50)	4
ET16	Bre1161; *glpF*::Tn*10 glpK*	11
ET18	Bre1161; *glpD3* glpR *zhe-733*::Tn*10*	11
ET126	Bre1161; Δ*crr*::Kan	Allele Δ*crr*::Kan from strain IT1199; 35
ET160	Bre1161; *glpD3 glpK*::Cam	This work
ET182	Bre1161; *pgm*	*pgm* allele from reference 24
ET187	Bre1161; *pgi*::Tn*10*	Allele *pgi*::Tn*10* from strain RHo53; 16
ET190	Bre1161; *cya*∗ Δ(800–848) *Sph*I Cam	Allele *cya*∗ Δ(*800–848*) *Sph*I Cam from strain IT1142; 35
ET194	Bre1161; *galT*	Allele *galT* from strain LR2-167 (from J. W. Lengeler)
ET220	Bre1161; Δ*cya*::Kan	Allele Δ*cya*::Kan from strain HT28; 19
HI12	Bre1161; *glpK203 zii-510*::Tn*10*	Allele *glpK203* from strain KH12; 15
HI13	Bre1161; *glpK204 zii-510*::Tn*10*	Allele *glpK204* from strain KH59; 15
HS3084	MC4100; ϕ(*malK*$^+$-*lamB-lacZ*)*42-1*	10
MC4100	F$^-$ *araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 deoC1 ptsF25 rbsR flbB5301*	5
WT196	Bre1161; *cya*∗ Δ(*800–848*) *Sph*I Cam; Δ*crr*	35
Plasmid pBCP260	*crr laqI*^{q}	36

the uptake and metabolism of maltose and maltodextrins. These genes are specifically controlled by MalT, the positive activator of all *mal* genes (32). Expression of *malT*, as well as of some, but not all, *mal* genes, is under control of the cAMP-CAP system (7, 8). Since *malT* expression is independent of maltose or maltodextrins as an inducer, its expression is independent of the effects of inducer exclusion, allowing the testing of catabolite expression exclusively. But *malT* expression is controlled not only by cAMP-CAP-dependent catabolite repression but also by Mlc, a global negative regulator of carbohydrate metabolism (9). In this paper, we present evidence that catabolite repression of *malT* exerted by glycerol or G3P involves the cAMP-CAP system, as well as EIIA^{Glc} of the PTS. Metabolism of G3P is not required, and dephosphorylation of EIIA^{Glc} is not the determining factor. Instead, we conclude that it is EIIA^{Glc}-P-dependent stimulation of adenylate cyclase that is inhibited by G3P or other phosphorylated sugars, such as glucose-6-phosphate. Two-dimensional (2D) gel electrophoresis was used to identify some of the proteins that are significantly affected by glycerol-mediated repression.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. P1 *vir* general transductions were done as described by Miller (26). Strain ET160 was constructed by mini-Tn *10*::Cam mutagenesis (21) of strain ET18. Its isolation was based on its insensitivity to repression by glycerol. The *E. coli* K-12 strains were grown at 37°C in Tryptone broth (TB) or TB buffered with phosphate-based minimal medium (26). For inoculation, overnight cultures in TB medium were routinely used. If necessary, kanamycin (100 μg/ml), tetracycline (5 μg/ml), or chloramphenicol (15 μg/ml) was added.

Enzymatic activity assays. For enzymatic activity assays, overnight cultures were permeabilized and β-galactosidase activity was measured as previously described (26). Specific activity is given in micromoles of substrate per minute (units) per milligram of protein. The values given in the tables represent averages of two independent measurements, each done in duplicate. The individual measurements did not vary by more than 10%.

Preparation of cell extract for 2D gel electrophoresis. *E. coli* K-12 strain MC4100 was routinely grown at 37°C in TB with or without 0.4% (vol/vol) glycerol. Cultures were inoculated with an overnight culture grown in TB, and after 18 h of shaking, the bacteria were harvested by centrifugation. After washing with TE (10 mM Tris, 1 mM EDTA, pH 7.5), the pellet was resuspended in TE and cells were disrupted by being passed through a French press. After ultracentrifugation at 4°C and 100,000 × *g* for 60 min, the protein content of the supernatant fraction was determined as described by Bradford (3).

2D gel electrophoresis. For isoelectric focusing (IEF), proteins were solubilized in a rehydration solution containing 8 M urea, 2 M thiourea, 2% (wt/vol) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonic acid (CHAPS), 28 mM dithiothreitol (DTT), 1.3% (vol/vol) Pharmalytes (pH 3 to 10), and bromophenol blue. After rehydration for 24 h under low-viscosity paraffin oil, IPG strips (Amersham Pharmacia Biotech) covering a pH range of 4 to 7 or 3 to 10 were subjected to IEF with the following voltage-time profile: a linear increase from 0 to 500 V for 1,000 Vh, 500 V for 2,000 Vh, a linear increase from 500 to 3,500 V for 10,000 Vh, and a final phase of 3,500 V for 35,000 Vh for the pH range of 4 to 7 and 20,000 Vh for the pH range of 3 to 10. After IEF, the individual strips were consecutively incubated, for 15 min each time, in equilibration solutions A and B (50 mM Tris-HCl [pH 6.8], 6 M urea, 30% [vol/vol] glycerol, and 4% [wt/vol] sodium dodecyl sulfate [SDS] completed with DTT at 3.5 mg/ml [solution A] or iodoacetamide at 45 mg/ml instead of DTT [solution B]). In the second dimension, proteins were separated on SDS–10 to 12.5% polyacrylamide gels with the Investigator System (Genomic Solutions) at 2 W/gel. For routine use, proteins were stained with PhastGel BlueR in accordance with the manufacturer's (Amersham Pharmacia Biotech) instructions. After scanning, analysis of the 2D polyacrylamide gel electrophoresis (PAGE) images was done with the Melanie3 software package (Bio-Rad). Separate gels were analyzed under each condition, and only spots displaying the same pattern under all of the conditions were labeled.

Protein identification by peptide mass fingerprinting. Protein spots were excised from stained 2D gels, destained, and digested with trypsin (Promega). Peptides were extracted as described by Otto et al. (28). They were purified with C_{18} tips in accordance with the manufacturer's (Millipore Inc.) instructions and eluted with 50% acetonitrile–0.1% (vol/vol) trifluoroacetic acid. Peptide solutions were mixed with an equal volume of saturated α-cyano-3-hydroxycinnamic acid solution in 50% acetonitrile–0.1% (vol/vol) trifluoroacetic acid and applied to a sample template for matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Peptide masses were determined in the positive ion reflector mode in a Voyager DE RP mass spectrometer (PerSeptive Biosystems) with internal calibration. Mass accuracy was usually in the range of 10 to 50 ppm. Peptide mass fingerprints were compared to databases by using the program MS-Fit (http://prospector.ucsf.edu). The searches considered oxidation of methionine, pyroglutamic acid formation at the N-terminal glutamine, and modification of cysteine by carbamidomethylation, as well as partial cleavage leaving one internal cleavage site.

Determination of the phosphorylation state of EIIA^{Glc}. The phosphorylation state of EIIA^{Glc} was determined as described by Hogema et al. (13). Cells were grown in phosphate-based minimal medium-buffered TB with or without 0.4% (vol/vol) glycerol or G3P, respectively. A 0.2-ml volume of logarithmic-phase cells (optical density at 600 nm, 0.5) was mixed with 20 μl of 10 M NaOH and vortexed for 10 s. After addition of 1 ml of cold ethanol and 180 μl of 3 M sodium acetate (pH 5.2), the samples were frozen at −70°C. The extracts were thawed

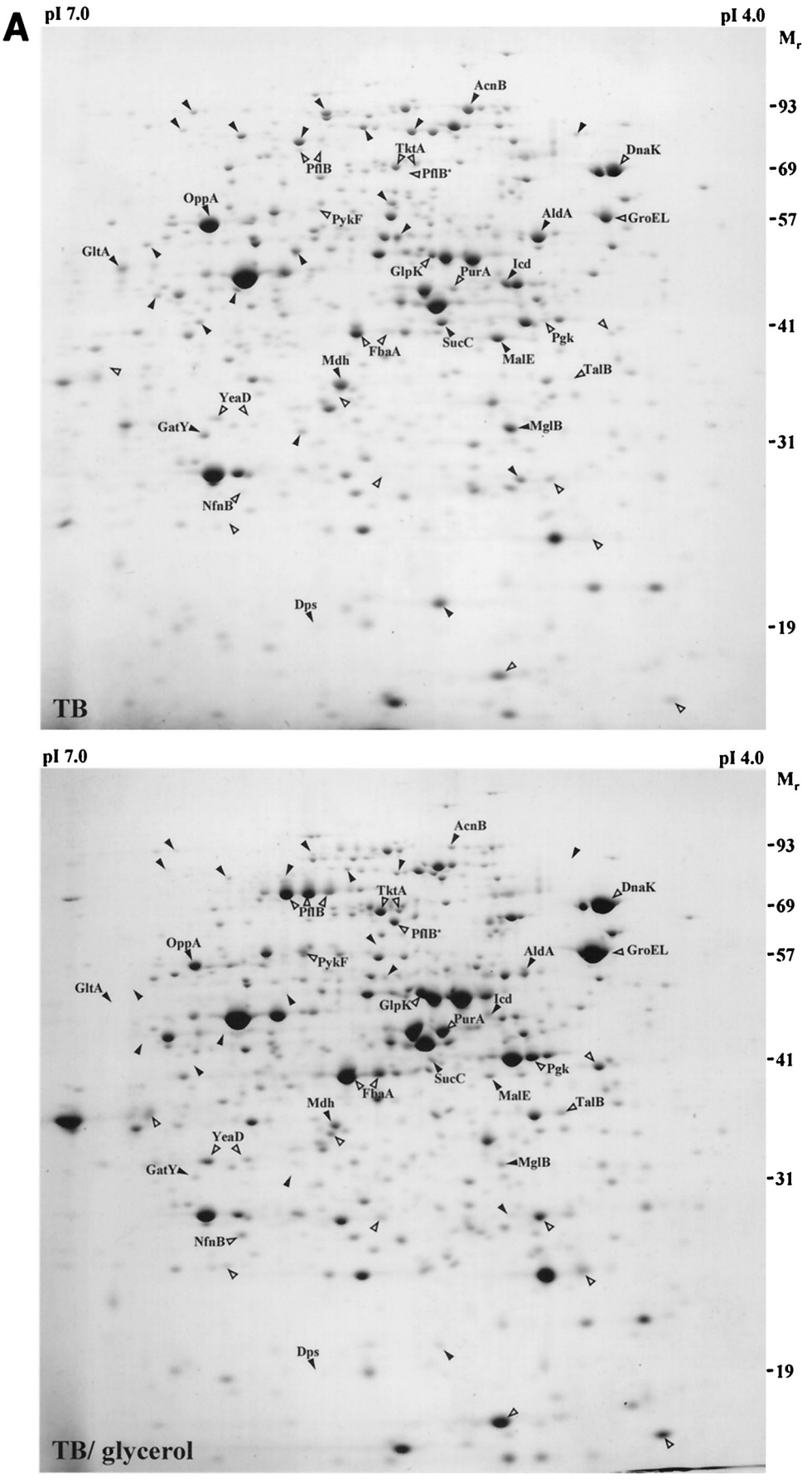

A
pI 7.0
pI 4.0
M$_r$
-93
-69
-57
-41
-31
-19
AcnB
TktA
PflB
PflB*
DnaK
OppA
PykF
AldA
GroEL
GltA
GlpK
PurA
Icd
SucC
Pgk
FbaA
Mdh
MalE
TalB
YeaD
GatY
MglB
NfnB
Dps
TB
pI 7.0
pI 4.0
M$_r$
-93
-69
-57
-41
-31
-19
AcnB
TktA
PflB
PflB*
DnaK
OppA
PykF
AldA
GroEL
GltA
GlpK
Icd
PurA
SucC
Pgk
FbaA
MalE
Mdh
TalB
YeaD
GatY
MglB
NfnB
Dps
TB/ glycerol

FIG. 1.

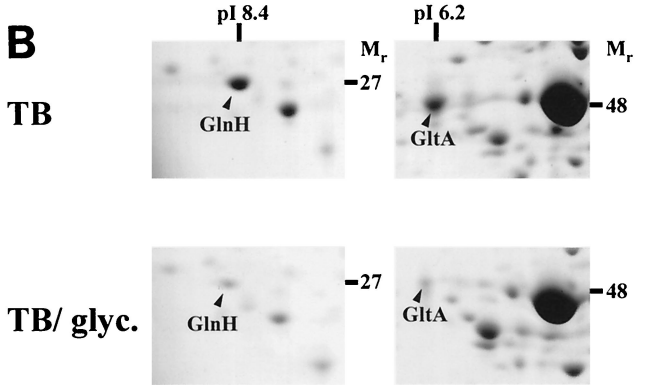


FIG. 1. Influence of glycerol on the protein profile of *E. coli* MC4100. *E. coli* K-12 strain MC4100 was grown in TB or TB with glycerol for 18 h. Crude protein extracts were prepared and separated by 2D gel electrophoresis. Each gel was loaded with 300 μg of protein extract. After staining, the images were recorded and analyzed. Protein spots induced or repressed by glycerol are marked by open and filled triangles, respectively. Proteins identified by peptide mass fingerprinting are indicated. (A) Images of 2D gels covering a pH range of 4 to 7. (B) Sections of the alkaline part of 2D gels covering a pH range of 3 to 10. M_r is given in thousands.

and centrifuged in an Eppendorf microcentrifuge at 4°C. The pellets were washed in ethanol and resuspended in 200 μl of sample buffer. Samples (10 μl) were loaded onto SDS–15% polyacrylamide gels and electrophoresed. The proteins were transferred to nitrocellulose membranes and probed with polyclonal anti-$EIIA^{Glc}$ antibody, followed by a horseradish peroxidase-conjugated secondary antibody. For detection, an ECL kit was used in accordance with the method of the manufacturer (Amersham Pharmacia).

RESULTS

2D gel analysis of cells grown in TB with and without glycerol. To obtain an overview of the impact of glycerol-G3P-mediated catabolite repression, strain MC4100 was grown in TB with and without 0.4% glycerol. Under these conditions, catabolite repression by glycerol on a *malT-lacZ* fusion can be monitored (see Tables 4 to 7). Crude protein extracts of these outgrown cultures were separated by 2D gel electrophoresis (Fig. 1A, pHs 4 to 7, and B, pHs 3 to 10). Thirty protein spots, indicated by filled arrowheads, were produced in smaller amounts when the strain was grown in the presence of glycerol and are therefore subject to glycerol catabolite repression. Of these proteins, 12 prominent spots were identified by peptide mass fingerprinting and are listed in Table 2. Among these, proteins known to be subject to PTS-mediated catabolite repression can be found, including the maltose and galactose binding proteins, as well as enzymes taking part in the tricarboxylic acid (TCA) cycle. More surprising was the finding that the glutamine and oligopeptide binding proteins are also prominently subject to repression. The DNA control region of their operons does not give any hint of a binding site for the cAMP-CAP complex. The above-described analysis demonstrates that glycerol-mediated repression is pleiotropic and not restricted to the peculiarities of the maltose system. Twenty-seven spots were present more prominently in the glycerol-induced culture. Eighteen of those were analyzed and represent 12 different proteins. Ten of these were identified by peptide mass fingerprinting and are listed in Table 3. Two of them were identified as GroEL and DnaK by their correlation to Swiss2D-Page (http://www.expasy.ch/ch2d). Of the 10 proteins identified by peptide mass fingerprinting, TktA, FbaA, YeaD, and PflB appear as at least two isoforms on 2D SDS-PAGE. Of the 12 proteins identified as present in increased amounts after growth in the presence of glycerol, only one, GlpK, is part of the *glp* regulon. Attempts to find operator binding sites typical for GlpR, the negative repressor of the *glp* regulon, in front of the other nine genes only revealed the presence of a "half" palindromic consensus binding site for GlpR (39) in front of the *tktA* gene.

Titration of $EIIA^{Glc}$ by glycerol kinase is not the cause of

TABLE 2. Overview of glycerol-repressed proteins identified by peptide mass fingerprint matching

Protein	pI[a]	Molecular mass (kDa)[a]	Function	SwissProt accession no.	MOWSE score[b]	Sequence coverage (%)[b]
TCA cycle						
AcnB	5.24	93.5	Aconitase B	P36683	1.51e + 12	44
GltA	6.21	48.0	Citrate synthase	P00891	1.92e + 07	34
Icd	5.15	45.8	Isocitrate dehydrogenase, NADP specific	P08200	1.64e + 07	45
Mdh	5.61	32.3	Malate dehydrogenase	P06994	1.92e + 06	56
SucC	5.37	41.4	Succinyl-CoA synthetase, beta subunit	P07460	6.98e + 05	48
Periplasmic binding protein						
GlnH	8.44	27.2	Periplasmic glutamine binding protein	P10344	2.09e + 05	64
MalE	5.53	43.4	Maltose binding protein	P02928	2.21e + 07	64
MglB	5.68	35.7	Galactose binding protein	P02927	9.81e + 04	60
OppA	6.05	60.9	Periplasmic oligopeptide binding protein	P23843	2.01e + 10	49
Other						
AldA	4.88	52.1	Aldehyde dehydrogenase	P25553	3.02e + 05	37
Dps	5.65	18.6	Stress-induced DNA binding protein	P27430	7.23e + 04	71
GatY	5.98	31.1	D-Tagatose-1,6-biphosphate aldolase	P37192	6.34e + 04	33

[a] The theoretical isoelectric point and molecular mass were calculated with the Compute pI/mw tool of the proteomics tool collection at the ExPASy Molecular Biology Server (http://www.expasy.ch/tools/pi_tool.html).

[b] MOWSE (molecular weight search) scores and sequence coverage data were included to facilitate judgement of the reliability of the data.

TABLE 3. Overview of glycerol-induced proteins identified by peptide mass fingerprint matching

Protein	pI[a]	Molecular mass (kDa)[a]	Function	SwissProt accession no.	MOWSE score[b]	Sequence coverage (%)[b]
GlpK (glycerol utilization)	5.36	56.1	Glycerol kinase	P08859	9.67e + 13	50
Glycolysis-gluconeogenesis-pentose phosphate cycle						
FbaA	5.52	39.0	Fructose-bisphosphate aldolase	P11604	7.58e + 08	48
Pgk	5.08	41.0	Phosphoglycerate kinase	P11665	1.70e + 09	54
PykF	5.77	50.7	Pyruvate kinase	P14178	6.67e + 07	47
TalB	5.11	35.1	Transaldolase	P30148	1.17e + 09	69
TktA	5.43	72.2	Transketolase	P27302	7.76e + 05	22
Other						
NfnB	5.80	23.9	Oxygen-insensitive NAD(P)H nitroreductase	P38489	1.74e + 04	48
PflB	5.60	85.2	Pyruvate formate lyase I	P09373	4.59e + 13	45
PurA	5.32	47.2	Adenylosuccinate synthetase	P12283	6.45e + 11	63
YeaD	5.89	32.7	Unknown	P39173	1.89e + 08	64

[a] The theoretical isoelectric point and molecular mass were calculated with the Compute pI/mw tool of the proteomics tool collection at the ExPASy Molecular Biology Server (http://www.expasy.ch/tools/pi_tool.html).
[b] MOWSE scores and sequence coverage data were included to facilitate judgement of the reliability of the data.

glycerol-mediated catabolite repression. In this report, we propose that it is the interference of G3P with the stimulation of adenylate cyclase by $EIIA^{Glc}$-P that causes catabolite repression. A plausible alternative explanation was that glycerol kinase, together with its product, G3P (or with glycerol), could bind to and titrate the unphosphorylated form of $EIIA^{Glc}$, thus reducing the amount of $EIIA^{Glc}$-P available for the stimulation of adenylate cyclase. This phenomenon, called inducer exclusion, occurs in cells induced for the *glp* system (33) when glucose is added. One GlpK tetramer is capable of binding four $EIIA^{Glc}$ molecules, as deduced from the crystal structure (17). To determine whether this titration of $EIIA^{Glc}$ by glycerol kinase could be the reason for glycerol-mediated repression in a *glpD* mutant, we measured the expression of a *malT-lacZ* fusion in a *glpD glpK* double mutant. Table 4 shows that this double mutation does not prevent repression by G3P. Moreover, $EIIA^{Glc}$ overexpression in a *glpFKX*$^+$ strain still allows twofold repression of *malT-lacZ* by G3P in Bre1161 (*glp*$^+$), ET18 (*glpD*), or ET16 (*glpK*). Table 5 shows the β-galactosidase activity of the *malT-lacZ* fusion strains harboring isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible and plasmid-encoded $EIIA^{Glc}$ that were grown in TB and TB-G3P with increasing IPTG concentrations (for the induction of $EIIA^{Glc}$). As judged by Western blot analysis, at 30 μM IPTG, the amount of $EIIA^{Glc}$ produced was about 20 times the chromosomal level. The data in Table 5 show that G3P in the growth medium maintained repression despite high levels of $EIIA^{Glc}$. We also tested two *glpK* alleles that had been characterized as resistant to $EIIA^{Glc}$ and those that are resistant to inhibition by fructose-1,6-diphosphate (15). In each case, repression of *malT-lacZ* expression by glycerol and G3P was unchanged. This demonstrated that glycerol kinase itself is not involved and excluded the possibility that the titration of $EIIA^{Glc}$ by GlpK is the basis of G3P-mediated repression. However, the observation that overexpression of $EIIA^{Glc}$, to some extent, lessened the repression by G3P points to an interaction of $EIIA^{Glc}$-P with adenylate cyclase as a target for G3P inhibition.

G3P causes only weak dephosphorylation of enzyme $EIIA^{Glc}$-P. It has been suggested that non-PTS compounds like glucose-6-phosphate may elicit repression by altering the ratio of phosphorylated to unphosphorylated $EIIA^{Glc}$ and, consequently, would cause catabolite repression by reducing adenyl-

TABLE 4. β-Galactosidase specific activity of a *malT-lacZ* fusion in some glycerol mutants

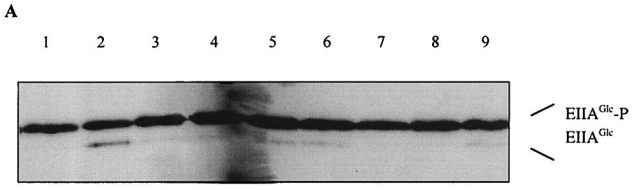
Strain	Relevant genotype	β-Galactosidase sp act[a]		
		TB	TB-glycerol	TB-G3P
Bre1161		0.43	0.12	0.18
ET18	*glpD glpR*	0.39	0.09	0.15
ET160	*glpD glpR glpK*	0.41	0.36	0.16
HI12	*glpK203*	0.45	0.07	0.10
HI13	*glpK204*	0.39	0.10	0.15

[a] Specific activity is given in units per milligram of protein. Strains were grown overnight at 37°C in TB, in TB–0.4% glycerol, or in TB-G3P. *glpK203*, $EIIA^{Glc}$ resistant; *glpK204*, fructose-1,6-diphosphate insensitive.

TABLE 5. Influence of $EIIA^{Glc}$ overexpression on repression of *malT-lacZ* by G3P

Strain	Relevant genotype	IPTG concn [μM]	β-Galactosidase sp act[a]	
			TB	TB-G3P
Bre1161			0.43	0.18
Bre1161/pBCP260	*prrp*$^+$	0	0.43	0.20
		10	0.43	0.22
		30	0.37	0.27
ET18	*glpD glpR*		0.39	0.15
ET18/pBCP260	*glpD glpR prrp*$^+$	0	0.43	0.20
		10	0.41	0.19
		30	0.36	0.19
ET16	*glpK glpF*		0.40	0.15
ET16/pBCP260	*glpK glpF crrp*$^+$	0	0.42	0.21
		10	0.39	0.19
		30	0.38	0.18

[a] Specific activity is given in units per milligram of protein. Strains were grown overnight at 37°C in TB or in TB–0.4% G3P.



B

wt			*glpD*			*glpK*		
	Glyc	G3P		Glyc	G3P		Glyc	G3P
0.43	0.12	0.18	0.39	0.09	0.15	0.40	0.42	0.15

FIG. 2. Determination of the phosphorylation state of the enzyme IIA^{Glc} in the presence of glycerol or G3P. (A) Isogenic strains Bre1161 (lanes 1 to 3), ET18 (*glpD glpR*; lanes 4 to 6), und ET16 (*glpF glpK*; lanes 7 to 9) were grown in TB alone (lanes 1, 4, and 7), in TB with 0.4% glycerol (lanes 2, 5, and 8), or in TB with 0.4% G3P (lanes 3, 6, and 9). Samples were taken during logarithmic growth at an optical density at 600 nm of 0.5. $EIIA^{Glc}$ and $EIIA^{Glc}$-P were detected by Western blotting with $EIIA^{Glc}$-specific antibodies. (B) β-Galactosidase specific activity of MalT-LacZ in micromoles of substrate per minute and milligram of protein. wt, wild type.

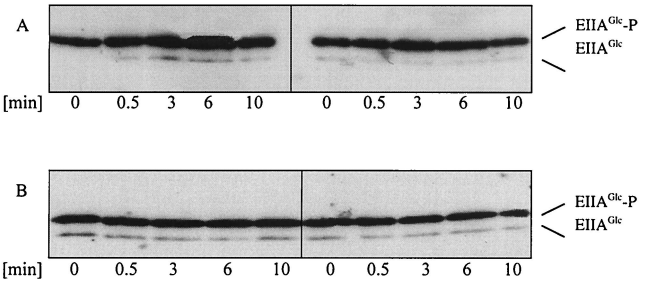


FIG. 3. Time-dependent dephosphorylation of $EIIA^{Glc}$-P by the addition of G3P. Isogenic *malT-lacZ* fusion strains Bre1161 (A) and ET18 (*glpD glpR*) (B) were grown at 37°C in TB (first five lanes) or in TB with 0.4% G3P (last five lanes). At time 0, 0.2% G3P was added. Samples were taken at the indicated time points and analyzed by SDS-PAGE, followed by Western blotting with anti-$EIIA^{Glc}$-P antibodies. The positions of $EIIA^{Glc}$-P and $EIIA^{Glc}$ are indicated.

ate cyclase activity that is no longer stimulated maximally by $EIIA^{Glc}$-P (13). The authors of that report used a method that makes it possible to separate the phosphorylated and unphosphorylated forms of $EIIA^{Glc}$ by SDS-gel electrophoresis (27). In order to test the phosphorylation state of $EIIA^{Glc}$ in response to the presence of glycerol or G3P in the growth medium, we investigated several isogenic mutants defective in glycerol metabolism (Fig. 2A). In wild-type strain Bre1161 grown in TB, $EIIA^{Glc}$ was completely phosphorylated. There was weak dephosphorylation in the presence of glycerol and less in the presence of G3P. The *glpD* mutant, defective in aerobic G3P dehydrogenase, already showed small amounts of unphosphorylated $EIIA^{Glc}$ in the absence of exogenous glycerol or G3P that were not increased by the addition of glycerol or G3P. There was weak dephosphorylation in the TB-G3P culture of the glycerol kinase (*glpK*) mutant by G3P but not by glycerol. Figure 2B shows the corresponding repression of *malT-lacZ* in these strains. The comparison shows that G3P does cause a little dephosphorylation of $EIIA^{Glc}$-P; however, the reduction in the levels of $EIIA^{Glc}$-P (and thus, the reduction in the activity of adenylate cyclase) is too small to account for the repression observed. In particular, in the *glpD* mutant, the proportion of the unphosphorylated to the phosphorylated form of $EIIA^{Glc}$ does not coincide with the degree of repression. The conversion of a small proportion of $EIIA^{Glc}$-P to the unphosphorylated form upon addition of glycerol or G3P was fast, already appearing 0.5 min after the addition of G3P to TB-grown Bre1161 cells (Fig. 3A, first five lanes), with no further significant dephosphorylation thereafter. In TB-G3P-grown cells, further addition of G3P had no effect (Fig. 3A, last five lanes). In a *glpD* mutant, weak dephosphorylation of the enzyme $EIIA^{Glc}$ was observed from the beginning on, whether the cells were grown in the presence of G3P or not, and there was no change after G3P was added (Fig. 3B).

In contrast, the presence of glucose in the growth medium leads to complete dephosphorylation of $EIIA^{Glc}$-P (13). Depletion of $EIIA^{Glc}$-P in cells exposed to glucose has been the basis for the model of glucose-mediated repression in *E. coli* (31). This explanation cannot not be correct for G3P-mediated catabolite repression.

Influence of G3P on the $EIIA^{Glc}$-P–adenylate cyclase interaction. Adenylate cyclase is thought to contain a regulatory C-terminal domain that functions as the interaction site for stimulation by $EIIA^{Glc}$-P. A strain harboring an adenylate cyclase form lacking the last 48 C-terminal amino acids (35) appeared, to some extent, resistant to glucose. *malT-lacZ* expression in this mutant is less sensitive to glucose than in the strain harboring wild-type adenylate cyclase (Table 6). Also, *malT-lacZ* expression is resistant to repression by exogenous G3P. As expected, deletion of $EIIA^{Glc}$-P in this mutant abolishes the sensitivity of *malT-lacZ* expression to G3P, as well as its sensitivity to glucose. This is consistent with the notion that the $EIIA^{Glc}$-P-mediated stimulation of adenylate cyclase is the major target of repression by G3P.

Repression of *malT-lacZ* expression by glucose-6-phosphate and galactose. Catabolite repression by non-PTS compounds has been observed for some time, glucose-6-phosphate being one of the most effective examples. The availability of a *malT-lacZ* fusion offered an opportunity to measure catabolite repression by glucose-6-phosphate free of inducer exclusion. The data are shown in Table 7. Glucose-6-phosphate strongly inhibited *malT-lacZ*. The introduction of a *pgi* mutation preventing the conversion of glucose-6-phosphate to fructose-6-phosphate and further glycolysis did not abolish the strong

TABLE 6. *malT-lacZ* expression in *cya*∗ Δ(*800–848*) strains

Strain	Relevant genotype	β-Galactosidase sp act[a]		
		TB	TB-G3P	TB-Glc
Bre1161		0.56	0.16	0.08
ET126	Δ*crr*	0.09	0.15	0.14
ET190	*cya*∗ Δ(*800–848*)	0.16	0.16	0.05
ET196	*cya*∗ Δ(*800–848*) Δ*crr*	0.08	0.10	0.12
ET220	Δ*cya*	<0.02	<0.02	<0.02

[a] Specific activity is given in units per milligram of protein. Strains were grown overnight at 37°C in TB, TB–0.4% G3P, or TB–0.2% glucose.

TABLE 7. Glucose-6-phosphate mediates repression by galactose

Strain	Relevant genotype	β-Galactosidase sp act[a]		
		TB	TB-Gal	TB-G6P
Bre1161		0.58	0.18	0.05
ET194	*galT*	1.28	1.06	ND
ET182	*pgm*	0.60	0.63	ND
ET187	*pgi*	0.58	0.20	0.08

[a] Specific activity is given in units per milligram of protein. Strains were grown overnight at 37°C in TB, TB–0.4% galactose, or TB–0.2% G6P. ND, not detected.

repression by glucose-6-phosphate. These findings are not in conflict with a recent report by Kimata et al. (20), who reported that a mutation in *pgi* prevented catabolite repression by glucose. In this case, loss of carbon flow through the glycolytic pathway led to the degradation of *ptgG* mRNA, thus preventing glucose uptake.

We also observed that the presence of galactose repressed the expression of *malT-lacZ*. In order to identify the inhibitory compound responsible, we introduced several mutations and tested their effects on the repression of *malT-lacZ* by galactose (Table 7). A mutation in *galT* encoding the enzyme that forms UDP-galactose and glucose-1-phosphate from galactose-1-phosphate and UDP-glucose is insensitive to galactose. Thus, galactose-1-phosphate is unable to exert catabolite repression. Likewise, when the transformation of glucose-1-phosphate to glucose-6-phosphate was prevented by a *pgm* mutation, allowing the accumulation of glucose-1-phosphate derived from external galactose, the strain was still insensitive to galactose. Thus, glucose-1-phosphate also cannot exert catabolite repression. However, when metabolism of glucose-6-phosphate to fructose-6-phosphate was prevented by a *pgi* mutation, repression by galactose was still observed. Thus, galactose-induced catabolite repression must be mediated by glucose-6-phosphate.

DISCUSSION

In this study, non-PTS sugar-mediated catabolite repression was analyzed. Our model system used G3P as the repression-eliciting compound and *malT* as a representative of a catabolite repression-sensitive gene (8). The latter was chosen for its independence of inducer exclusion. We chose TB as a low catabolite repression medium and measured repression upon the addition of glycerol, G3P, and other compounds. Even though pH alterations do occur in this weakly buffered medium, addition of phosphate buffer to keep the pH constant over the entire growth period did not alter the transcriptional repression by G3P (11). It was important to be sure that assaying the effect of carbon sources on *malT* expression does indeed reflect catabolite repression and is not a peculiarity of the maltose system. Thus, we studied the glycerol-mediated repression of the majority of the *E. coli* proteins. The 2D gel electrophoretic analysis shown in Fig. 1 allowed us to conclude that at least 30 of the proteins visualized by this technique were reduced when the cells were grown to stationary phase in the presence of glycerol. Twelve of these proteins were identified by mass fingerprinting. Thus, the glycerol effect is not a peculiarity seen in the regulation of the maltose system but is pleiotropic in nature. As expected, among the proteins subject to this pleiotropic effect, we found those that are typically encoded by catabolite-repressible genes, such as enzymes of the TCA cycle and sugar binding proteins. Surprisingly, genes encoding the glutamine binding protein or the oligopeptide binding protein were also among those repressed, even though the promoters of their genes are not known to contain CAP binding sites. Possibly, the reduction in the amount of these proteins is indirect, due to a cAMP-CAP-dependent regulator. Also surprising was the number of proteins induced by glycerol. The only known glycerol-inducible genes of *E. coli* are the 12 *glp* genes that are under negative control by the GlpR repressor (23). Not all of these *glp* gene products would be seen. Membrane proteins were removed prior to gel electrophoresis by high-speed centrifugation, and proteins produced in small amounts would not be visible. Of the 27 proteins that were induced by glycerol, we could only identify one, GlpK, that genuinely belongs to the *glp* regulon. Nine additional proteins were identified by protein mass fingerprinting. The proteins identified mostly belong to the pathways for glycolysis and gluconeogenesis. It is unlikely that they are regulated by GlpR, the central regulator of the *glp* regulon, since no corresponding operator site was found in the control regions of their genes. They were probably induced by the metabolic products of glycerol metabolism. A surprise was the large amount of PflB, pyruvate formate lyase, present in the glycerol-induced culture. This enzyme is known to be induced only under anaerobic conditions. Possibly, the presence of the glycerol-metabolizing enzymes exerts an effective drain of available oxygen since in *E. coli*, glycerol can only be utilized oxidatively. We also noticed the clear induction of heat shock proteins GroEL and DnaK. We have no explanation for their appearance.

Previously, we had established that $EIIA^{Glc}$ is necessary for glycerol-mediated repression and that glycerol has to be phosphorylated to G3P but that no further metabolism is needed. Since PTS sugar-mediated catabolite repression is based on the dephosphorylation of $EIIA^{Glc}$-P, we analyzed whether or not $EIIA^{Glc}$ would undergo a dramatic change in its degree of phosphorylation. In contrast to the effect of glucose, this was not the case with glycerol or G3P. Clearly, addition of glycerol or G3P did result in the formation of small amounts of dephosphorylated $EIIA^{Glc}$ that was completed within 1 min. However, the small amount of dephosphorylated $EIIA^{Glc}$ was not related to the degree of repression. This was seen very clearly in the *glpD* mutant, which is unable to metabolize G3P. Here, dephosphorylated $EIIA^{Glc}$ is already present prior to the addition of G3P and its amount is not further increased by the addition of exogenous G3P. However, the addition of G3P reduces *malT-lacZ* expression. *glpD* mutants do contain elevated levels of endogenous G3P, even when grown in the absence of glycerol or G3P. This is due to the function of dihydroxyacetone phosphate dehydrogenase providing G3P from dihydroxyacetone phosphate for phospholipid biosynthesis. Endogenous G3P, not removed by GlpD, must be the cause for the small amounts of dephosphorylated $EIIA^{Glc}$ observed. It is unclear how dephosphorylation of $EIIA^{Glc}$ by G3P comes about.

Since the minute reduction in the amount of $EIIA^{Glc}$-P upon addition of glycerol or G3P cannot account for repression (by reduction of stimulation of adenylate cyclase activity), what are

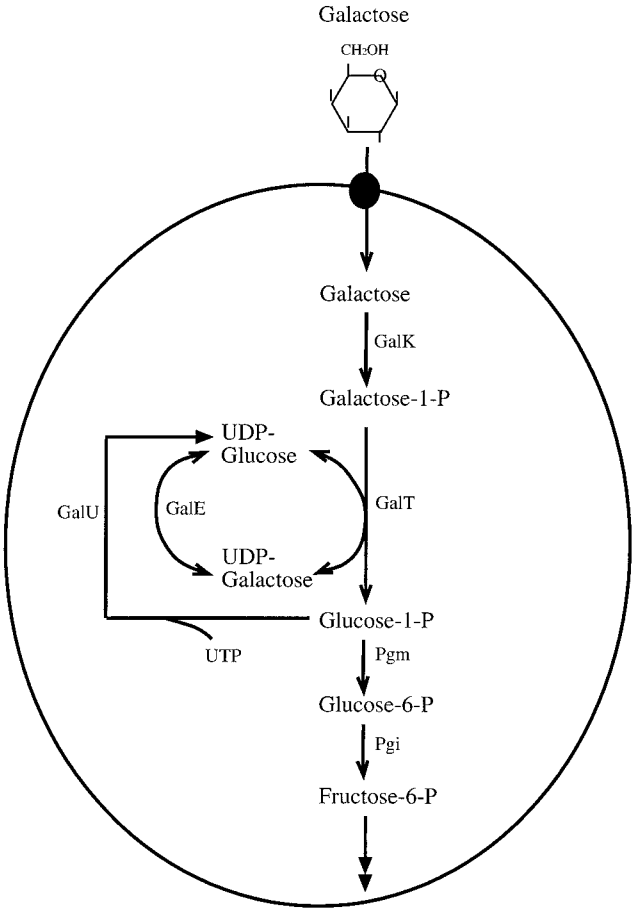


FIG. 4. Galactose mediates repression by the formation of glucose-6-phosphate (Glucose-6-P). The effects of the *galT*, *pgm*, and *pgi* mutations on the formation of glucose-6-phosphate are indicated.

other options to explain repression? Chagneau et al. (6) have recently concluded that the induction of glycerol kinase is responsible for repression. In their scheme, glycerol kinase would interact with dephosphorylated EIIA^{Glc}, removing EIIA^{Glc}-P from the equilibrium and thus reducing EIIA^{Glc}-P-mediated stimulation of adenylate cyclase. They reject the possibility that it is the activity of glycerol kinase (in forming G3P from glycerol) that causes repression, since repression by IPTG-induced glycerol kinase was also observed in the absence of exogenous glycerol. We feel that this mode of action is rather unlikely. As we have shown, the amount of unphosphorylated EIIA^{Glc} that would be able to react with glycerol kinase is very small and the effective concentration (for the stimulation of adenylate cyclase) of EIIA^{Glc}-P is hardly reduced by *glp* induction. Also, in the glycerol kinase-EIIA^{Glc} interaction scheme, overproduction of EIIA^{Glc} should eliminate glycerol-induced repression. We have shown here that this is not the case. Also, the use of a *glpK* allele whose product is insensitive to EIIA^{Glc} (15) still showed the same level of repression in response to glycerol or G3P. Thus, our explanation for the observation of Chagneau et al. (6) is that glycerol kinase-mediated formation of G3P causes repression. Their observation that repression by glycerol kinase also occurs in the absence of exogenous glycerol finds its explanation in the endogenous formation of glycerol that has been established for some time (37). Also, the interaction of glycerol kinase with EIIA^{Glc} that is the basis for their explanation requires binding of glycerol to glycerol kinase (17). Thus, even if interaction between these two proteins does occur to a minor extent under conditions of repression (15), it always necessitates the presence of glycerol that will invariably lead to the formation of G3P. The last argument is that glycerol-mediated repression requires glycerol kinase (for the formation of G3P) but repression by G3P does not and is the same in the wild type, a *glpK* mutant, and a *glpK glpD* mutant.

Our model to explain G3P-mediated catabolite repression is based on the knowledge that adenylate cyclase and EIIA^{Glc}-P, as well as G3P, are necessary players. In addition, we have to conclude that the amount of EIIA^{Glc}-P cannot be the regulating principle, as it is in glucose-mediated repression. Therefore, we propose that the stimulation of adenylate cyclase by EIIA^{Glc}-P is the target of repression by G3P. We postulate that G3P inhibits EIIA^{Glc}-P-mediated stimulation of adenylate cyclase, thus lowering the cAMP concentration. A recent publication described the catabolite repression of citrate fermentation genes in *Klebsiella pneumoniae* by glycerol and gluconate, among other compounds (25). The authors of that report also noted the discrepancy between the state of dephosphorylation of EIIA^{Glc} and the repressing effect of non-PTS sugars while maintaining the importance of the cAMP-CAP system in this type of catabolite repression.

What about other non-PTS sugars that elicit catabolite repression? Using glucose-6-phosphate, we found a strong repression of *malT-lacZ* that appears to be independent of glucose-6-phosphate glycolytic metabolism. Analysis of galactose-mediated repression revealed that the causative agent again was glucose-6-phosphate, whose further metabolism was not required for repression (Fig. 4 shows the galactose pathway).

Where do these sugar phosphates act as inhibitors? They could fit into a binding site in EIIA^{Glc}-P, altering its ability to stimulate adenylate cyclase. Alternatively, these sugar phosphates may mimic the phosphorylated histidine in EIIA^{Glc}-P, preventing the proper interaction with adenylate cyclase. A challenging biochemical approach involving adenylate cyclase and EIIA^{Glc}-P is necessary to elucidate the molecular mechanism of this type of repression.

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