

Reverse Genetics of *Escherichia coli* Glycerol Kinase Allosteric Regulation and Glucose Control of Glycerol Utilization In Vivo

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Reverse genetics is used to evaluate the roles in vivo of allosteric regulation of *Escherichia coli* glycerol kinase by the glucose-specific phosphocarrier of the phosphoenolpyruvate:glycose phosphotransferase system, IIA^{Glc} (formerly known as III^{Glc}), and by fructose 1,6-bisphosphate. Roles have been postulated for these allosteric effectors in glucose control of both glycerol utilization and expression of the *glpK* gene. Genetics methods based on homologous recombination are used to place *glpK* alleles with known specific mutations into the chromosomal context of the *glpK* gene in three different genetic backgrounds. The alleles encode glycerol kinases with normal catalytic properties and specific alterations of allosteric regulatory properties, as determined by in vitro characterization of the purified enzymes. The *E. coli* strains with these alleles display the glycerol kinase regulatory phenotypes that are expected on the basis of the in vitro characterizations. Strains with different *glpR* alleles are used to assess the relationships between allosteric regulation of glycerol kinase and specific repression in glucose control of the expression of the *glpK* gene. Results of these studies show that glucose control of glycerol utilization and glycerol kinase expression is not affected by the loss of IIA^{Glc} inhibition of glycerol kinase. In contrast, fructose 1,6-bisphosphate inhibition of glycerol kinase is the dominant allosteric control mechanism, and glucose is unable to control glycerol utilization in its absence. Specific repression is not required for glucose control of glycerol utilization, and the relative roles of various mechanisms for glucose control (catabolite repression, specific repression, and inducer exclusion) are different for glycerol utilization than for lactose utilization.

In *Escherichia coli*, glucose controls utilization of several other carbon sources, including lactose, melibiose, maltose, and glycerol (14, 27, 29, 30, 32). Effects of glucose on the expression of genes needed for metabolism of other sugars, e.g., lactose, formed the foundation for much of the initial understanding of molecular genetic control mechanisms. Glucose effects were found to involve both positive and negative control aspects. At the level of transcriptional control, these two opposing aspects for expression of the *lac* operon are mediated by the cyclic AMP (cAMP)-cAMP receptor protein complex (for catabolite repression) and by the *lac* repressor (for specific repression), respectively. The specific repression is relieved by binding of an inducer. Subsequent studies have revealed that glucose acts to modulate the level of cAMP and the level of the inducer. These controls are exerted by two different forms of IIA^{Glc}, the glucose-specific phosphocarrier of the phosphoenolpyruvate:glycose phosphotransferase system (PTS). The form of IIA^{Glc} that is phosphorylated at an active-site histidine residue participates in the increase of cAMP by activation of adenylate cyclase, and the form of IIA^{Glc} that is unphosphorylated binds to lactose permease and prevents lactose uptake. Because the latter process prevents uptake of the inducer, this mechanism is termed inducer exclusion. IIA^{Glc}-dependent PTS-mediated inducer exclusion is an important regulatory concept that unifies several aspects of

genetic, allosteric, and metabolic controls. The finding of both positive and negative control mechanisms raises the issue of their relative roles in glucose control. In the case of the *lac* operon, recent studies show that specific repression coupled to inducer exclusion is the dominant mechanism for glucose control of lactose utilization (6, 11, 36). In *lacI* strains, glucose control is abolished, which is seen as loss of the repression of β -galactosidase and elimination of the plateau during diauxic growth on glucose-lactose (11). A similar phenotype is seen for strain PPA586, an MG1655 derivative with *lacY*(S209I), in which the lactose permease is thought to be resistant to IIA^{Glc} inhibition (6, 8, 33).

Glycerol kinase (EC 2.7.1.30; ATP-glycerol 3-phosphotransferase) is a component of a regulatory network in *E. coli* by which glucose and other carbon sources control the utilization of glycerol and the gene expression that is needed for glycerol metabolism (14, 27, 29, 32). The proteins involved in glycerol metabolism are encoded by the elements of the *glp* regulon, which displays a complex genetic structure (3, 5, 37, 39). It contains five operons, which are located at three different chromosomal loci. Glucose modulation of glycerol utilization involves both regulation of transcription and posttranslational control of glycerol kinase catalytic activity. Control of transcription of the regulon elements is analogous to the *lac* operon and involves both positive control by cAMP-cAMP receptor protein and negative control by a specific repressor that is encoded by the *glpR* gene. DNA-binding sites for the specific repressor in the *glpFKX* operon have been identified both in the 5' upstream region and internally within the *glpK* coding sequence (37). The inducer for expression of the *glp*

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elements is *sn*-glycerol 3-phosphate (15), which is both the product of the reaction that is catalyzed by glycerol kinase during glycerol catabolism and an important metabolite in phospholipid biosynthesis under all growth conditions. The catalytic activity of glycerol kinase is controlled posttranslationally by the allosteric inhibitors fructose 1,6-bisphosphate (FBP) and IIA^{Glc} (19, 26, 42). These allosteric effectors display V-system regulation (19, 23) that allows efficient control that is not dependent on changes in the concentrations of the substrates. The unusual existence of two very different allosteric effectors that are involved in glucose control has been noted and postulated to be the basis for the extreme effectiveness of glucose in preventing glycerol consumption (14).

Several years ago, Zwaig and Lin identified the mutant *E. coli* strain 43 on the basis of its loss of glucose control of glycerol utilization (42). They showed that the glycerol kinase from strain 43 had lost sensitivity to inhibition by FBP; the role of IIA^{Glc} was unknown at that time. We isolated the *glpK22* allele from strain 43 and showed that it contains a mutation that results in a single amino acid substitution in glycerol kinase, G-304-S (21). The variant enzyme encoded by the *glpK22* allele was purified and characterized. It was found to show greatly reduced sensitivity to FBP inhibition, in agreement with the earlier work, and to show weak activation by IIA^{Glc} with greatly reduced apparent affinity for binding IIA^{Glc}. Thus, this variant glycerol kinase has lost sensitivity to inhibition by both allosteric effectors. This finding raises the question of the relative roles of the regulation by each glycerol kinase allosteric effector in glucose inhibition of glycerol utilization. Results of a study with *Salmonella* suggest that both allosteric inhibitors may be required for glucose control in that organism (19).

In other investigations of the novel allosteric regulation of glycerol kinase, we identified, purified, and characterized *in vitro* variant enzymes that are insensitive to one of the allosteric inhibitors while retaining normal sensitivity to the other inhibitor as well as normal catalytic behavior. Glycerol kinase A-65-T is not sensitive to inhibition by FBP but displays normal regulation by IIA^{Glc} and normal catalytic properties (16). Glycerol kinase T-477-N is not sensitive to inhibition by IIA^{Glc} but displays normal regulation by FBP and normal catalytic properties (see below). These variants provide the basis for reverse genetic investigations of the quantitative contributions of each of the allosteric inhibitors to glucose inhibition of glycerol utilization and control of expression of the *glp* regulon *in vivo*.

Because of the complex genetic structure of the *glp* regulon and the many-faceted control network, it is necessary to place the *glpK* alleles encoding the variant enzymes into the normal chromosomal context. This report describes the construction and characterization of strains with chromosomal *glpK* alleles. Three different genetic backgrounds were utilized for the constructions. Initially, strain DG1 was used, because it is a $\Delta glpK$ (16) strain, to facilitate the constructions. Unexpectedly, however, strain DG1 and its derivatives were found to carry the *glpR208* allele (9). This allele is functionally identical to the *glpR2* allele, and these strains were used here as GlpR⁻ strains. Strain MC4100 was used as a *glpR*⁺ strain because other investigators have performed extensive studies of the *glp* operator structures and transcriptional control in this strain and some of its derivatives (13, 38, 40). Strain MG1655 was used as

a second *glpR*⁺ strain because it is a prototrophic strain and has been used as the background for extensive investigations of the mechanisms of glucose control of how other carbon sources are used (6, 7). The effects of the specific alterations in glycerol kinase allosteric regulatory properties on diauxic growth on glucose-glycerol, glycerol utilization, and levels of glycerol kinase specific activity are described. The results reveal that, in *E. coli*, FBP inhibition of glycerol kinase is quantitatively dominant in glucose control of glycerol utilization, which is independent of inducer exclusion mediated by specific repression.

MATERIALS AND METHODS

Materials. Chemicals and enzymes were purchased from Sigma Chemical Co. (St. Louis, Mo.) unless otherwise indicated. Purified IIA^{Glc} was generously provided by Saul Roseman, Department of Biology, Johns Hopkins University, Baltimore, Md.

Strains and culture methods. Biological materials that were used in these studies are listed in Table 1. All strains were grown in Luria-Bertani (LB) medium or minimal M9 (18) medium with carbohydrate added as indicated. MacConkey glycerol agar was prepared according to the instructions of the manufacturer (Difco). Antibiotics were added when indicated to obtain final concentrations of 75 μ g/ml for ampicillin, 8 μ g/ml for tetracycline, 200 μ g/ml for streptomycin, and 40 μ g/ml for kanamycin.

Enzyme assays and protein determinations. Glycerol kinase enzyme activity was measured by using the continuous ADP-coupled spectrophotometric assay at pH 7.0 and 25°C (24). Other additions are as indicated in the tables and figures. One unit of glycerol kinase catalyzes the formation of 1 μ mol of ADP per min in this assay. For studies with purified glycerol kinase, the enzyme concentration was determined from the A_{280} (16) and varied from 0.1 to 0.5 μ g/ml. Kinetic data were analyzed for initial-velocity or inhibition parameters as previously described (23). For studies with cellular extracts, the protein concentration was determined using the Bio-Rad assay with bovine serum albumin as a standard.

***glpK* alleles.** Alleles that encode the A-65-T (*glpK203*) and G-304-S (*glpK22*) glycerol kinases were derivatives of plasmid pHG165 (35) and have been described previously (16, 21). The allele for the T-477-N glycerol kinase (*glpK204*) was constructed by using the Kunkel method of site-directed mutagenesis, as described previously for the construction of other site-directed variants of glycerol kinase (23). The variant enzyme was purified to >95% homogeneity and characterized *in vitro* by using previously described protocols (23). The catalytic properties that are reported are the average of three experiments using enzyme purified from two independent isolates of the variant enzyme. No significant differences were found in the properties of the different isolates.

Integration of variant genes into the genome. The *glpK* alleles were moved from the pHG165-derived plasmids into the chromosome of strain KH10 by conjugation. Strain KH5, transformed with a plasmid carrying the *glpK* allele, was mated with strain KH10 (18). Exconjugants with a functional *glpK* gene were selected on minimal-glycerol plates with streptomycin. Individual colonies were purified on selection medium several times. Purified colonies were screened on LB and LB-AMP plates to identify AMP^r exconjugants to ensure loss of the plasmid. AMP^r exconjugants were plated on MacConkey glycerol agar to screen for those able to ferment glycerol, i.e., those putatively bearing a chromosomal copy of a functional allele. Strains bearing mutations in *glpK* were identified by determining the glycerol kinase regulatory phenotype with enzyme activity and inhibition assays of cell extracts prepared as follows. AMP^r colonies that fermented glycerol on MacConkey glycerol agar were grown overnight in 2 ml of LB, 1.5 ml of the culture was transferred to a microcentrifuge tube, and the cells were pelleted by microcentrifugation. The cell pellet was resuspended in the same volume of standard buffer (0.1 M triethanolamine HCl, 2 mM glycerol, 1 mM EDTA, 1 mM β -mercaptoethanol, adjusted to pH 7.0 at room temperature by using NaOH) and disrupted by sonication. Cellular debris was removed using centrifugation, and the supernatant was transferred to a new tube. Glycerol kinase activity and the total protein concentration of the extract were determined as described above. The reported specific activities were corrected for glycerol-independent ATPase activity in the cellular extracts by subtracting the rate obtained in assays of extracts from $\Delta glpK$ strains. The correction was 0.1 to 0.3 U/mg for different strains and growth conditions. Extracts were diluted at least 50-fold into the assays, thus reducing the glycerol concentration from the extract to a negligible level. Glycerol kinase activity in the solution was then dependent on addition of glycerol to the assay. The same reaction rate was observed for the respective $\Delta glpK$ strains and for the *glpK*⁺ strains assayed without added glycerol.

TABLE 1. Strains and plasmids used in these studies

Strain or plasmid	Genotype	Source or reference ^a
<i>E. coli</i> strain		
DG1	TB1 Δ <i>glpK202</i>	16
HfrH	F ⁺	Ry Young
HfrPK3	F ⁺ <i>ayi-15 lacY1 leuB6 supE44 thi-1 thr-1 tonA21</i>	Ry Young
JM83	F ⁺ <i>ara glpR208</i> Δ (<i>lac-proAB</i>) <i>rpsL</i> (Str ^r) [ϕ 80 <i>dlac</i> Δ (<i>lacZ</i>)M15] <i>thi</i>	Ry Young; 17
JW383	<i>thr-1 ara-14 eda-50 fhuA31</i> Δ (<i>galK-att</i>)99 <i>hisG4</i> (Oc) <i>lacY1</i> λ^- <i>leuB6 metF159</i> (Am) <i>mtl-1 rpsL136</i> (Str ^r) <i>thi-1 tsx-78 xylA5 zii-510::Tn10</i>	CGSC; 34
Lin 43	HfrC <i>fadL701 glpK22 glpR2 phoA8 pit-10 relA1 sopT1 tonA22 T2^r</i>	CGSC; 42
MC4100	F ⁻ <i>araD139</i> Δ (<i>argF-lac</i>)U169 <i>deoC1 flbB5301 ptsF25 rbsR relA1 rpsL150</i> (Str ^r)	CGSC
MG1655	λ^- <i>rph-1</i>	CGSC
TB1	JM83 <i>glpR208 hsdR</i> (r _K ⁻ m _K ⁺)	1
ts1962	F ⁻ <i>lac-28 proA23 pisI19</i> (Ts) <i>rpsL101</i> (Str ^r) <i>trp-30</i>	CGSC; 12
AP100	<i>ptsI19</i> (Ts) <i>rpsL101</i> (Str ^r)	ts1962 \times HfrH
AP110	AP100 Δ <i>glpK202 zii-510::Tn10</i>	AP100 \times P1.KH10
KH1	DG1 <i>zii-510::Tn10</i>	DG1 \times P1.JW383
KH5	HfrPK3 Δ <i>glpK202 zii-510::Tn10</i>	Hfr PK3 \times P1.KH1
KH10	KH1 <i>pro</i> ⁺	KH1 \times HfrH
KH11	DG1 <i>glpK</i> ⁺ <i>pro</i> ⁺	DG1 \times Hfr PK3
KH12	KH10 <i>glpK203</i>	KH10 \times KH5/pA65T
KH15	KH10 <i>glpK204</i>	KH10 \times KH5/pT477N
KH18	MC4100 Δ <i>glpK202 zii-510::Tn10</i>	MC4100 \times P1.KH1
KH24	MC4100 <i>glpK204 zii-510::Tn10</i>	MC4100 \times P1.KH15
KH34	MC4100 <i>glpK203 zii-510::Tn10</i>	MC4100 \times P1.KH12
KH37	KH10 <i>glpK22</i>	KH10 \times KH5/pCH5
KH38	MC4100 <i>glpK22 zii-510::Tn10</i>	MC4100 \times P1.KH37
KH51	MG1655 Δ <i>glpK202 zii-510::Tn10</i>	MG1655 \times P1.KH1
KH52	MG1655 <i>zii-510::Tn10</i>	MG1655 \times P1.KH1
KH58	MG1655 <i>glpK203 zii-510::Tn10</i>	MG1655 \times P1.KH12
KH59	MG1655 <i>glpK204 zii-510::Tn10</i>	MG1655 \times P1.KH15
KH61	MG1655 <i>glpK22 zii-510::Tn10</i>	MG1655 \times P1.KH38
Plasmids		
pA65T	pHG165 + <i>Hind</i> III fragment <i>glpK203</i>	16
pCH5	pHG165 + <i>Hind</i> III fragment <i>glpK22</i>	21
pCJ102	pBR322 + <i>Hind</i> III fragment <i>glpK</i>	23
pCJ102/T477N	pBR322 + <i>Hind</i> III fragment <i>glpK204</i>	This study
pHG165	Cloning vector; <i>amp</i> ^r	35
pT477N	pHG165 + <i>Hind</i> III fragment <i>glpK204</i>	This study

^a CGSC, *E. coli* Genetic Stock Center, Yale University, New Haven, Conn.

Strains derived by this procedure carry *glpK* alleles in the chromosome in a *glpR208* background and have a tetracycline marker near the *glpK* gene. This marker was used for P1 transduction (18) with P1_{vir} to move the *glpK* alleles into the MC4100 and MG1655 genetic backgrounds. Transductants were selected on LB-tetracycline plates and screened on MacConkey glycerol agar to confirm the presence of glycerol kinase activity. The glycerol kinase regulatory phenotypes of these strains were determined as described above after overnight growth in minimal-glycerol (0.2%) medium.

Diauxic growth curves. Strains were incubated at 37°C overnight in 4 ml of M9 minimal-glucose (0.2%) medium. Three milliliters of the overnight culture was transferred to a sterile microcentrifuge tube, and the cells were collected by centrifugation. The cell pellet was resuspended in 1 ml of 1 \times M9 salts and washed. The washed cells were resuspended in 1 ml of 1 \times M9 salts, and the optical density at 600 nm (OD₆₀₀) was determined. Aliquots of these cells were used to inoculate 80 ml of M9 medium containing either 2.5 mM glucose or 2.5 mM glucose plus 5 mM glycerol to an OD₆₀₀ of ~0.02. Growth at 37°C in a rotary shaker (250 rpm) was monitored by determining the OD₆₀₀. Periodically, 5-ml aliquots were removed from the growing culture and placed on ice. Cells and medium were separated by centrifugation. The cells were resuspended in 1 ml of standard buffer and disrupted with sonication. After clarification by centrifugation, the resulting cellular extracts were assayed for glycerol kinase activity and protein concentration. The concentration of glycerol in the medium was determined by using end-point assays with glycerol kinase in the ADP-coupled assay. The concentration of glucose was determined at the Texas Veterinary Medical Diagnostic Laboratory using endpoint assays with hexokinase and glucose-6-phosphate dehydrogenase in a Hitachi 911 analyzer.

Sedimentation velocity experiments. Glycerol kinases and IIA^{Glc} were exhaustively dialyzed in the same beaker against 0.1 M triethanolamine-HCl (pH 7.0), which also contained 2 mM glycerol, 1 mM β -mercaptoethanol, and 10 μ M

ZnCl₂. Protein samples were diluted with filtered dialysate to 0.3 mg of glycerol kinase per ml (5 μ M [subunits]) and to 0.54 mg of IIA^{Glc} per ml (30 μ M) when present. All samples were clarified by centrifugation prior to ultracentrifugation and then loaded into cells assembled with 12-mm-optical-pathlength double-sector Epon charcoal-filled centerpieces and sapphire windows. Approximately 0.2 ml of sample and 0.25 ml of buffer were loaded into the sample and reference channels, respectively. Samples were run in a Beckman model XL-A analytical ultracentrifuge at 35,000 rpm and 25°C for 90 min. Scans were performed at 280 nm and collected without pausing, allowing 4 min to elapse between scans. Because IIA^{Glc} lacks tryptophans or tyrosines (31), it is transparent at the 280-nm wavelength. Each run was conducted either with all cells containing glycerol kinase or with all cells containing glycerol kinase and IIA^{Glc}, giving three independent measurements. Data were analyzed using the SVEDBERG program (version 6.38) from J. Philo (25).

Rescue of growth in lactose medium. Strains were grown to saturation at 30°C in 5 ml of M9 minimal-glucose (0.2%) medium with ampicillin for plasmid-harboring strains. Cells from 3 ml of the cultures were collected by centrifugation in a sterile tube, then washed and resuspended in 1 \times M9 medium, and the OD₆₀₀ was determined. Aliquots of these cells were used to inoculate 25 ml of pre-warmed M9 minimal-lactose (0.2%) medium with ampicillin when appropriate, at an OD₆₀₀ of 0.025. Cells were incubated in a rotary shaker (250 rpm) at 42°C, and the OD₆₀₀ was determined to monitor growth.

RESULTS AND DISCUSSION

Construction and characterization of allosteric regulatory variant glycerol kinase T-477-N. Two of the allosteric variant glycerol kinases used in these studies, A-65-T and G-304-S,

were described previously, and their catalytic and regulatory properties have been determined by *in vitro* studies with the purified enzymes (16, 21). The T-477-N glycerol kinase was constructed, purified, and characterized as described in Materials and Methods. The substituted amino acid position is in the IIA^{Glc} binding site on glycerol kinase and is >25 Å from the active site and >65 Å from the FBP-binding site (10, 20). Examination of the structure of the glycerol kinase-IIA^{Glc} complex suggests that the variant T-477-N enzyme will prevent IIA^{Glc} binding because of increased side-chain volume and polarity; the methyl group of T-477 is in a nonpolar environment. Previous work has shown that the catalytic activity of glycerol kinase is reduced by substitutions to negatively charged amino acid side chains in this region (4), so the neutral polar amino acid asparagine was used for substitution of T-477. Initial-velocity studies of the substrate dependence of the catalytic properties of the T-477-N variant enzyme yielded the following kinetic parameters for this enzyme: V_{\max} , 13 ± 2 U/mg; K_{ATP} , 7 ± 2 μM; K_{gol} , 6 ± 3 μM; and $K_{i\text{ATP}}$, 53 ± 45 μM. The values of these parameters were not significantly different from those obtained for the normal enzyme: V_{\max} , 15 ± 1 U/mg; K_{ATP} , 9 ± 2 μM; K_{gol} , 5 ± 2 μM; $K_{i\text{ATP}}$, 54 ± 23 μM. The kinetic properties showed apparent negative cooperativity with respect to ATP that was similar to the response obtained for the normal enzyme, showing that this aspect of the regulatory behavior of the enzyme was not altered qualitatively by the substitution.

Effects of the substitution on allosteric regulation by FBP and IIA^{Glc} were assessed by initial-velocity studies. In assays at pH 7.0, 25°C, and 0.4 μg of glycerol kinase per ml, FBP inhibition showed positive cooperativity and yielded the following parameters for the T-477-N glycerol kinase: I_{\max} , $89 \pm 1\%$; Hill coefficient (n_H), 1.8 ± 0.1 ; and K_{app} , 0.3 ± 0.02 mM. The values were not significantly different from the parameters obtained for the normal enzyme: I_{\max} , $93 \pm 1\%$; n_H , 1.7 ± 0.1 ; K_{app} , 0.26 ± 0.02 mM. Figure 1 shows IIA^{Glc} inhibition for normal and T-477-N glycerol kinases. The highest concentration of IIA^{Glc} that was used corresponded approximately to the total concentration of IIA^{Glc} (phosphorylated and unphosphorylated) estimated to occur *in vivo* (27). Inhibition parameters of the normal glycerol kinase agreed with previous results (23). Because of the small extent of inhibition of the T-477-N glycerol kinase, the fitting algorithm could not estimate both I_{\max} and K_{app} ; consequently, the value for I_{\max} was fixed at the value obtained for normal glycerol kinase. The resultant fit to the data obtained for the T-477-N glycerol kinase showed that the substitution decreased the apparent affinity for IIA^{Glc} binding by about 400-fold if the substitution did not affect the extent of inhibition. This result is consistent with the expected effect of increasing the size and polarity of the amino acid at this position, on the basis of the crystal structure of the complex of IIA^{Glc} with the normal glycerol kinase (10).

We utilized two methods to examine binding of IIA^{Glc} to the T-477-N variant glycerol kinase. In the first method, sedimentation velocity measurements were performed as described in Materials and Methods to assess formation of a complex between IIA^{Glc} and glycerol kinase. For the normal glycerol kinase, the apparent sedimentation coefficient was increased from 11 ± 0.06 S to 13.3 ± 0.06 S when 30 μM IIA^{Glc} was added to the ultracentrifuge cell. This concentration of IIA^{Glc}

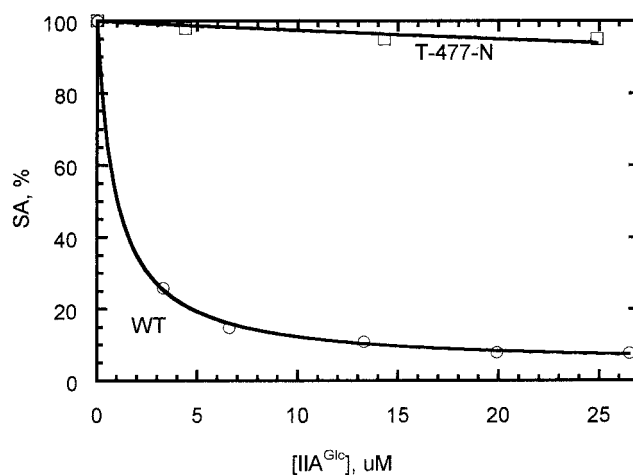


FIG. 1. Effect of the T-477-N substitution on IIA^{Glc} inhibition of glycerol kinase. Initial velocities of glycerol kinase catalyzed reactions were determined at pH 7.0 and 25°C as described in Materials and Methods. The points show the data that were obtained for the normal or T-477-N glycerol kinase and the lines show the fits of the data to the following equation:

$$\text{SA, \%} = 100 - [I_{\max} \cdot (\text{IIA}^{\text{Glc}})] / (K_{\text{app}} + (\text{IIA}^{\text{Glc}})),$$

where SA is specific activity, I_{\max} is the maximum extent of inhibition and K_{app} is the apparent dissociation constant for IIA^{Glc} binding to glycerol kinase. The fits yield the following parameters for normal glycerol kinase: I_{\max} , $96 \pm 1\%$; K_{app} , 0.95 ± 0.06 μM. Those for T-477-N glycerol kinase are as follows: I_{\max} , 96% (fixed); K_{app} , 370 ± 70 μM. Conditions: 2 mM glycerol, 2.5 mM ATP, 0.1 mM ZnCl₂, and IIA^{Glc} as indicated on the x axis. Enzyme concentration, 0.5 μg/ml. WT, wild type.

showed saturation behavior for inhibition of normal glycerol kinase in kinetics assays (Fig. 1), and the increased value of the sedimentation coefficient with IIA^{Glc} indicated its binding to glycerol kinase. The sedimentation coefficient of the T-477-N variant glycerol kinase in the absence of IIA^{Glc} was 10.6 ± 0.1 S and was 10.5 ± 0.06 S with 30 μM IIA^{Glc} included in the cell. Thus, addition of IIA^{Glc} did not alter the sedimentation coefficient of the T-477-N variant, indicating that IIA^{Glc} does not bind to this variant under these conditions. We estimated that this result indicates a reduction of at least 200-fold for the IIA^{Glc} binding affinity for the variant relative to the normal glycerol kinase. This result is consistent with that from the inhibition kinetics studies and supports the assumption that was made in fitting the inhibition data, namely that the substitution decreases the binding affinity but does not alter the extent of inhibition.

The second method provided a measure of IIA^{Glc} binding to glycerol kinase *in vivo*. Strain AP110 did not grow on lactose at the restrictive temperature (42°C). This is believed to be a consequence of the *ts* variant enzyme I in this strain (12), resulting in inability of the PTS to phosphorylate IIA^{Glc} to relieve its inhibition of the lactose permease. Figure 2 shows that growth of AP110 cells on lactose could be rescued by transformation with the plasmid pCJ102, which constitutively expresses the normal glycerol kinase (22). Growth of cells that harbor the plasmid vector pBR322 alone was not rescued. In contrast, growth of cells that express the T-477-N variant glycerol kinase from the same plasmid was not rescued significantly

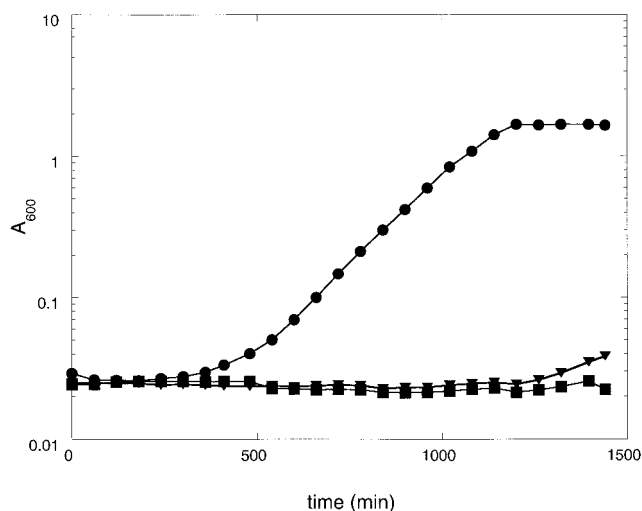


FIG. 2. Glycerol kinase-dependent rescue of growth on lactose. Cells of strain AP110 that harbor plasmids with different *glpK* alleles were inoculated into minimal-lactose medium and incubated with shaking at 42°C. Growth was monitored by determining the OD₆₀₀. ●, pCJ102 (normal glycerol kinase); ■, pBR322 (vector); ▼, pCJ102-T477N (T-477-N glycerol kinase).

in the same time period. Analogous results were obtained on minimal-lactose plates on which just-visible colonies appeared after 5 days of growth at the restrictive temperature for AP110 cells in which the T-477-N variant glycerol kinase was expressed. Cells that contained the vector alone showed no growth after 5 days, and cells in which the normal glycerol kinase was overexpressed produced large colonies after 2 days. Addition of exogenous glycerol was not required for these rescue experiments, and the expression of the glycerol kinases did not affect discernibly the growth rate of the strains on minimal glucose (0.1%) plates at the permissive temperature (30°C). The absence of an effect on the rate of growth on glucose is consistent with simulations indicating that the amount of IIA^{Glc} greatly exceeds the amount required for growth (28). Rescue of growth on lactose by overexpression of normal glycerol kinase is consistent with binding of the glycerol kinase to the unphosphorylated form of IIA^{Glc}, thus titrating the inhibitor away from the lactose permease and allowing entry of lactose into the cell. Both of the glycerol kinases were greatly overexpressed constitutively from the pBR322 construct, and the specific activity in crude extracts was the same for the normal and T-477-N glycerol kinases: 10 U/mg for liquid cultures that were grown overnight at 42°C in LB. Thus, the lack of efficient rescue by the variant glycerol kinase was not due to differences in the level of expression or protein stability. The very high level of glycerol kinase may account for the binding of IIA^{Glc} in the absence of added glycerol; alternatively, ATP or *sn*-glycerol 3-phosphate binding to the glycerol kinase may promote IIA^{Glc} binding. The level of specific activity was >10-fold higher than the level observed for expression from the chromosome during growth on glycerol (see below); yet, no effective rescue was observed with the T-477-N variant glycerol kinase, leading to the expectation that the level of binding to IIA^{Glc} was even lower at one-tenth the glycerol kinase concentration.

Results of the sedimentation velocity experiments and the in vivo rescue experiments indicated that the binding of IIA^{Glc} to the T-477-N variant glycerol kinase is very weak relative to the normal glycerol kinase. Thus, the T-477-N substitution practically abolished binding and inhibition by IIA^{Glc} at these concentrations, and the T-477-N glycerol kinase provided the desired altered allosteric regulatory phenotype of greatly reduced inhibition by IIA^{Glc} with no significant change for the catalytic or other regulatory properties of the enzyme.

Construction and characterization of mutant strains with chromosomal alleles for allosteric regulatory variant glycerol kinases. Strains with chromosomal *glpK* alleles that encode the allosteric regulatory variants of glycerol kinase, T-477-N, A-65-T, and G-304-S, were constructed in the three genetic backgrounds as described under Materials and Methods. The glycerol kinase regulatory phenotype of each strain was verified by assays of enzyme activity in cell extracts. Results of those assays are summarized in Table 2. In each case, the allosteric regulatory phenotype was the same as that observed for the respective purified glycerol kinase. The extract from the strain with the normal glycerol kinase displayed inhibition of glycerol kinase by both FBP and IIA^{Glc} (K⁺ glycerol kinase) that, with the putative glycerol kinase A-65-T, showed inhibition by IIA^{Glc} but was insensitive to FBP (Kⁱ glycerol kinase); that, with the putative glycerol kinase T-477-N, showed inhibition by FBP but was resistant to IIA^{Glc} inhibition (K^r glycerol kinase); and that with the putative glycerol kinase G-304-S was insensitive to FBP and resistant to IIA^{Glc} inhibition (K^{i,r} glycerol kinase). The designations of the regulatory defect (Kⁱ, K^r) follow earlier nomenclature (19). The same results were obtained for all three genetic backgrounds.

Growth rates and glycerol kinase specific activity after overnight growth in minimal glucose, minimal glycerol, and LB media were determined for all of the strains. Differences were seen for growth rates of strains KH11, MC4100, and MG1655 in each of the different media (data not shown). However, in

TABLE 2. Glycerol kinase regulatory phenotypes^a

Strain	FBP inhibition	IIA ^{Glc} inhibition	GlpR/GlpK phenotype ^b
KH11	0.4	0.4	R ⁻ /K ⁺
KH12	1.0	0.5	R ⁻ /K ⁱ
KH15	0.4	1.0	R ⁻ /K ^r
KH37	0.9	1.1	R ⁻ /K ^{i,r}
MC4100	0.3	0.2	R ⁺ /K ⁺
KH34	1.1	0.6	R ⁺ /K ⁱ
KH24	0.4	1.1	R ⁺ /K ^r
KH38	1.1	1.1	R ⁺ /K ^{i,r}
MG1655	0.5	0.3	R ⁺ /K ⁺
KH58	1.1	0.4	R ⁺ /K ⁱ
KH59	0.5	1.0	R ⁺ /K ^r
KH61	1.0	1.0	R ⁺ /K ^{i,r}

^a Extracts from overnight cultures of the indicated strains were prepared, and the specific activity of glycerol kinase was determined as described in Materials and Methods. Strains KH11, KH12, KH15, and KH37 were grown in LB, and the remaining strains were grown in minimal glycerol (0.2%) medium. For evaluation of the regulatory phenotype, FBP or IIA^{Glc} was added to the assay to a final concentration of 5 mM or 3 μM, respectively. The inhibition is expressed as the ratio of the specific activity with added inhibitor to the specific activity without the inhibitor. Specific activities for the uninhibited enzymes are shown in Table 3.

^b Abbreviations: K⁺, normal glycerol kinase; Kⁱ, FBP-insensitive glycerol kinase; K^r, IIA^{Glc}-resistant glycerol kinase; K^{i,r}, FBP-insensitive and IIA^{Glc}-resistant glycerol kinase; R⁻, *glpR208*; R⁺, *glpR*⁺.

TABLE 3. Dependence of glycerol kinase specific activity on growth medium and genetic background

Strain	GlpR/GlpK phenotype	Avg sp act \pm SD (U/mg) ^a on growth medium (no. determinations)		
		Minimal glucose	Minimal glycerol	LB medium
KH11	R ⁻ /K ⁺	0.1 \pm 0.2 (4)	0.8 \pm 0.4 (6)	2.1 \pm 0.6 (9)
KH12	R ⁻ /K ⁱ	0.1 \pm 0.1 (4)	0.2 \pm 0.1 (6)	2.1 \pm 0.9 (7)
KH15	R ⁻ /K ^r	0 \pm 0.02 (4)	0.7 \pm 0.1 (6)	1.9 \pm 0.6 (6)
KH37	R ⁻ /K ^{i,r}	0 \pm 0.1 (4)	0.2 \pm 0.1 (6)	1.2 \pm 0.3 (7)
MC4100	R ⁺ /K ⁺	0 \pm 0.1 (5)	1.1 \pm 0.7 (8)	0.1 \pm 0.2 (7)
KH34	R ⁺ /K ⁱ	0 \pm 0.1 (5)	0.2 \pm 0.1 (7)	0 \pm 0.02 (3)
KH24	R ⁺ /K ^r	0 \pm 0.1 (5)	1.3 \pm 0.7 (7)	0 \pm 0.2 (3)
KH38	R ⁺ /K ^{i,r}	0 \pm 0.1 (4)	0.1 \pm 0.1 (5)	0 \pm 0.2 (4)
MG1655	R ⁺ /K ⁺	0 \pm 0.04 (6)	1.0 \pm 0.2 (7)	0 \pm 0.1 (5)
KH58	R ⁺ /K ⁱ	0 \pm 0.1 (6)	0.2 \pm 0.1 (3)	0 \pm 0.1 (3)
KH59	R ⁺ /K ^r	0 \pm 0.1 (5)	0.6 \pm 0.3 (4)	0 \pm 0.04 (3)
KH61	R ⁺ /K ^{i,r}	0 \pm 0.04 (5)	0.2 \pm 0.1 (6)	0 \pm 0.02 (3)

^a Specific activities were determined as described in Materials and Methods. Numbers in parenthesis indicate the number of independent determinations, and the uncertainties are shown as the sample standard deviation. The values shown were corrected by subtracting the apparent specific activities obtained for the respective Δ *glpK* strains: KH18, 0.3 \pm 0.1 U/mg; KH10 and KH51, 0.2 \pm 0.1 U/mg. The apparent specific activities obtained with the Δ *gkpK* strains are not dependent on the addition of glycerol to the enzyme assay; i.e., they are not glycerol kinase activity but reflect other sources of ADP that are measured by the coupled assay.

strains with variants of glycerol kinase, the growth rate was sensitive to the allosteric regulatory phenotype of glycerol kinase only for cells expressing Kⁱ or K^{i,r} glycerol kinase, for which the growth rate in glycerol was increased in all genetic backgrounds. Strains with Kⁱ or K^{i,r} glycerol kinase also showed a strong fermentation phenotype on MacConkey glycerol agar in all genetic backgrounds. This phenotype was displayed as a large red disk in the agar surrounding the colonies and was seen for both plasmid-borne and chromosomal copies of these alleles. The phenotype has segregated with the mutations following conjugation or transduction in all the strains that have been examined. Strains with K^r glycerol kinase showed a normal fermentation phenotype.

Table 3 shows the specific activity of glycerol kinase in extracts prepared after overnight growth in several media. In glucose-grown cells, the apparent levels of enzyme activity were not significantly different from the apparent activity obtained for the respective Δ *glpK* strains. This apparent activity was not dependent on addition of glycerol to the assay and thus reflected sources of ADP other than glycerol kinase. The basal level of glycerol kinase specific activity in glucose-grown cells was too low to be measured by using the ADP-coupled assay (<0.1 U/mg). Glycerol kinase enzyme activity was repressed by glucose in both *glpR208* and *glpR*⁺ strains, in agreement with an earlier report (41).

The level of enzyme activity of glycerol kinase in cells that were grown on glycerol showed little dependence on the glycerol 3-phosphate repressor phenotype; the levels were the same for KH11, MC4100, and MG1655. However, the expression level did depend on the glycerol kinase regulatory phenotype. The level was reduced in strains with Kⁱ or K^{i,r} glycerol kinases but not in strains with K⁺ or K^r glycerol kinases. Despite this lower level of glycerol kinase activity, the strains with Kⁱ or K^{i,r} glycerol kinases grew more rapidly on glycerol

and displayed the enhanced glycerol fermentation phenotype on MacConkey glycerol plates.

In LB, the glycerol kinase specific activity level showed little dependence on the glycerol kinase regulatory phenotype but was dependent on the glycerol 3-phosphate repressor phenotype. In the *glpR208* strains, high levels of glycerol kinase specific activity were obtained. The levels of specific activity were considerably reduced for the *glpR*⁺ strains and were not significantly above the apparent level observed for the respective Δ *glpK* strains. The low levels of expression of the Kⁱ or K^{i,r} glycerol kinases in minimal glycerol medium did not appear to be related to instability of the enzymes or inherent differences in expressibility because the same high level of specific activity was seen for the normal and variant glycerol kinases in the *glpR208* genetic background during growth on LB.

The *glpK22* allele was used in these experiments to verify that the effects of K^{i,r} glycerol kinase on glucose control were not changed by the different genetic backgrounds used here relative to the initial report of those effects (41). The properties of the strains which expressed this variant that were constructed in this work, including the reduced level of glycerol kinase specific activity following growth in minimal glycerol and the *glpR* allele dependence of the level of glycerol kinase specific activity during growth on LB, agree with those properties described in the earlier work. Diauxic growth curves (data not shown) also agreed with earlier work: a normal plateau for a *glpR*⁺ strain and elimination of the plateau in a *glpR* strain. Thus, the effects of the *glpK22* allele on mechanisms of glucose control in strains used here were not distinguishable from the effects that were observed earlier in a different strain, and the present strains provided suitable genetic backgrounds for assessing the contribution of each of the glycerol kinase allosteric control mechanisms to regulation of glycerol metabolism.

Roles of glycerol kinase allosteric regulation in glucose control of glycerol utilization and glycerol kinase expression. The fundamental observation of glucose control of utilization of other carbon sources is diauxic growth, which is exhibited as a biphasic growth curve. Figure 3A displays growth curves obtained in minimal medium with 2.5 mM glucose plus 5 mM glycerol for strain MC4100. This growth showed the expected two phases, which are separated by a plateau. Figure 3A also shows the basis for the diauxic growth. During the first phase, glucose is utilized while glycerol utilization is inhibited. During the second growth phase, which occurred after the glucose was consumed, glycerol was utilized. Figure 3B shows that the level of glycerol kinase specific activity was barely detectable during the first phase and was induced to higher levels for the phase of glycerol utilization. Thus, glucose prevented both utilization of glycerol and expression of glycerol kinase.

The consequences of the specific changes in the allosteric regulatory properties of glycerol kinase for glucose control of glycerol utilization in derivatives of strain MC4100 also are shown in Fig. 3A and B. The curves for strain 24 (R⁺/K^r) were practically identical to those obtained for strain MC4100 (R⁺/K⁺). Thus, loss of IIA^{Glc} inhibition of glycerol kinase, i.e., inducer exclusion, did not affect the diauxic growth, glycerol utilization, or expression of glycerol kinase under these conditions. In contrast, the curves obtained for strain 34 (R⁺/Kⁱ) differed greatly from those of strain MC4100. Loss of FBP

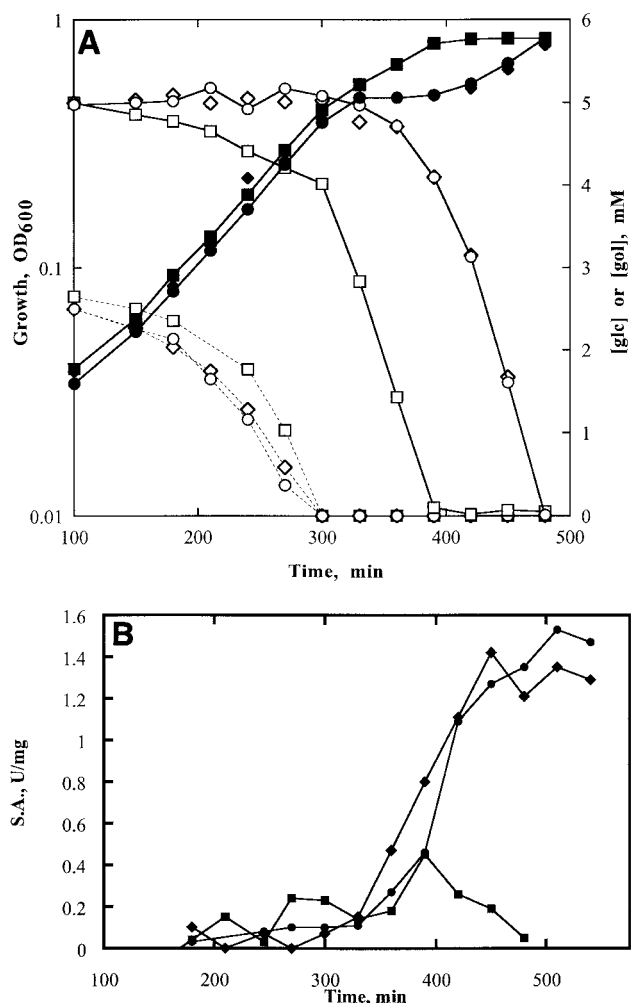


FIG. 3. Roles of glycerol kinase allosteric regulation in diauxic growth, glucose-glycerol utilization, and expression of glycerol kinase in the MC4100 genetic background. Cultures were prepared as described in Materials and Methods. (A) Filled symbols show growth of strains 34 (R^+/K^+) (■), 24 (R^+/K^+) (◆), and MC4100 (R^+/K^+) (●), which were measured as the change in OD_{600} following inoculation. Open symbols connected by dashed lines show the concentration of glucose, and open symbols connected by solid lines show the concentration of glycerol in the medium. (B) Specific activity (S.A.) of glycerol kinase. ●, MC4100 (R^+/K^+); ■, strain 34 (R^+/K^+); ◆, strain 24 (R^+/K^+).

inhibition of glycerol kinase affected all three aspects of glucose control: (i) the plateau in the growth curve was eliminated, (ii) glycerol was consumed throughout the growth and the rate of glucose utilization during the first phase was reduced, and (iii) glycerol kinase specific activity remained at a low level throughout the growth. Thus, FBP inhibition was the quantitatively dominant allosteric regulatory mechanism for control of glycerol kinase in vivo in *E. coli*. A dominant role for FBP inhibition was consistent with the observation that 16 of 18 glycerol-specific revertants of a *ptsI* strain expressed glycerol kinase that was no longer inhibited by FBP (2). For the other two genetic backgrounds, the effects of the altered glycerol kinase allosteric regulatory properties on the diauxic growth curves, carbon source utilization, and glycerol kinase specific

activity were the same as those shown for the MC4100 genetic background (data not shown). Thus, the role of allosteric regulation of glycerol kinase was not dependent on the genetic background.

For each of the strains described here, growth rates and glycerol kinase specific activities in minimal glucose (2.5 mM) medium alone and the first phase in minimal glucose (2.5 mM) plus glycerol (5 mM) medium were indistinguishable (data not shown). Thus, growth on glucose was not affected by the different glycerol kinase regulatory phenotypes, suggesting that the PTS was not altered. The behaviors of strains MG1655 and KH52 with respect to growth rates, diauxic growth, and glycerol kinase specific activities were identical; thus the presence of the *Tn10* element did not affect the properties of the strains under these conditions.

The unexpected lack of effect of loss of IIA^{Glc} inhibition of glycerol kinase, i.e., inducer exclusion, on glucose control of glycerol utilization raises questions about the role of specific repression by the glycerol phosphate repressor. We showed previously that glucose represses expression of glycerol kinase in *glpR* strains, including strain KH11, during growth on glucose plus glycerol (9). Figure 4 compares diauxic growth curves and glycerol utilization for strains KH11 (R^-/K^+) and MC4100 (R^+/K^+). Diauxic growth and glycerol kinase specific activities in strains with the *glpR2* or *glpR208* allele are indistinguishable from those of $\Delta glpR$ strains, indicating that these alleles encode nonfunctional repressors (9). The results (Fig. 4) show that glucose control of glycerol utilization during the first phase of growth also was not dependent on a functional glycerol phosphate repressor.

In all three genetic backgrounds, strains that expressed FBP-

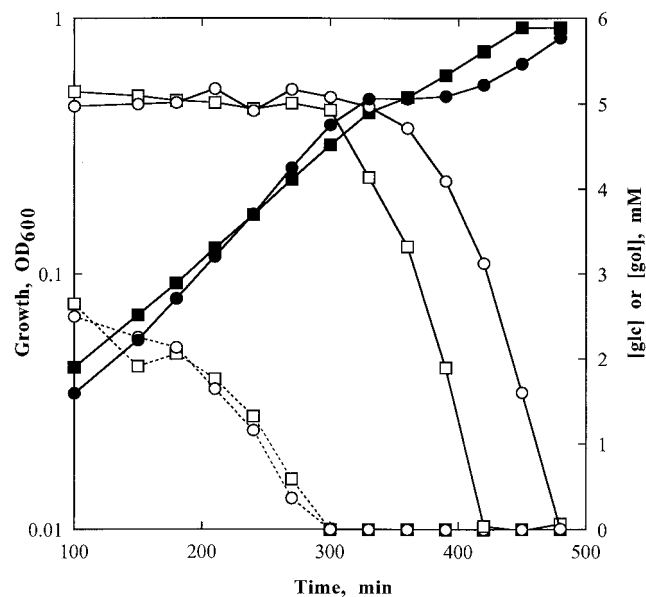


FIG. 4. Growth and glucose and glycerol utilization in the *glpR208* and *glpR*⁺ genetic backgrounds. Cultures were prepared as described in Materials and Methods. The filled symbols show growth measured as the change in OD_{600} following inoculation. Open symbols connected by dashed lines show the concentration of glucose, and open symbols connected by solid lines show the concentration of glycerol in the medium. ○/●, MC4100 (R^+/K^+); □/■, KH11 (R^-/K^+).

insensitive glycerol kinase showed loss of glucose control of glycerol utilization, diauxic growth, and glycerol kinase expression. The behavior of these strains during growth on glycerol was very similar to that of *lacI* (11) or *lacY*(S209I) (6, 8, 33) strains during growth on lactose. Allosteric inhibition of glycerol kinase by IIA^{Glc} alone provided no discernible glucose control in the FBP-insensitive glycerol kinase strains described here. The low level of glycerol kinase in these strains might be expected to contribute to loss of IIA^{Glc} inhibition by favoring dissociation of the glycerol kinase-IIA^{Glc} complex. However, the initial identification of FBP-insensitive glycerol kinase was based on a screen for loss of glucose control of glycerol utilization, and the enzyme was expressed at higher levels from a plasmid-borne copy of the *glpK203* allele lacking the upstream genetic control elements (16). Cells that expressed the normal glycerol kinase from the same plasmid background showed glucose control at the higher level of expression. Glucose control was abolished for the strain with FBP-insensitive glycerol kinase at even the higher level of expression, which suggests that the absence of effective IIA^{Glc} control observed here was not due to the lower level of expression of the chromosomal copy of the *glpK203* allele. The absence of effective IIA^{Glc} control in strains with FBP-insensitive glycerol kinase could reflect synergy in the binding of FBP and IIA^{Glc}, in which FBP enhances the binding of IIA^{Glc}. However, results of isothermal titration calorimetry experiments are consistent with independent binding of these allosteric effectors to the normal glycerol kinase in vitro (I. Luque, D. W. Pettigrew, and E. Freire, unpublished data).

In contrast to the complete loss of glucose control of glycerol utilization that is associated with the FBP-insensitive glycerol kinase, changes in glucose control are not associated with the IIA^{Glc}-resistant glycerol kinase under these conditions. All of the strains with the IIA^{Glc}-resistant glycerol kinase show diauxic growth curves, inhibition of glycerol utilization, and repression of glycerol kinase specific activity that are not discernibly different from those responses in strains with the normal glycerol kinase. Glucose control of glycerol utilization and repression of glycerol kinase activity also are not dependent on a functional glycerol 3-phosphate repressor, i.e., specific repression. The lack of dependence of glucose control on IIA^{Glc} inhibition of glycerol kinase is completely consistent with the lack of dependence on specific repression. Independence from specific repression is implicit in the earlier publications in which the *glpK22* (42) and *glpK203* (16) alleles were identified; in both cases, *glpR* strains were used, and the *glpR glpK*⁺ control strains showed normal glucose control. The consequences of this observation for PTS regulation of carbon source utilization appear to have been unrecognized previously. The lack of dependence of glucose control of glycerol utilization on specific repression suggests that cAMP-dependent catabolite repression may be the dominant mechanism of glucose repression of glycerol kinase activity levels during diauxic growth. Thus, the mechanisms by which glucose controls glycerol utilization by *E. coli* differ significantly from those by which it controls lactose utilization (6, 11, 36). The differences in the relative roles of catabolite repression, specific repression, and inducer exclusion in regulation of use of these two carbon sources may reflect the differences in genetic structure (operon versus regulon) and/or the differences with respect to

the nature of the inducer (unusual disaccharide versus normal metabolite in lipid metabolism).

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