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Glucose- and Glucokinase-Controlled *mal* Gene Expression in *Escherichia coli*[▽]

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MalT is the central transcriptional activator of all *mal* genes in *Escherichia coli*. Its activity is controlled by the inducer maltotriose. It can be inhibited by the interaction with certain proteins, and its expression can be controlled. We report here a novel aspect of *mal* gene regulation: the effect of cytoplasmic glucose and glucokinase (Glk) on the activity and the expression of MalT. Amylomaltase (MalQ) is essential for the metabolism of maltose. It forms maltodextrins and glucose from maltose or maltodextrins. We found that glucose above a concentration of 0.1 mM blocked the activity of the enzyme. *malQ* mutants when grown in the absence of maltodextrins are endogenously induced by maltotriose that is derived from the degradation of glycogen. Therefore, the fact that *glk malQ*$^+$ mutants showed elevated *mal* gene expression finds its explanation in the reduced ability to remove glucose from MalQ-catalyzed maltodextrin formation and is caused by a metabolically induced MalQ$^-$ phenotype. However, even in mutants lacking glycogen, Glk controls endogenous induction. We found that overexpressed Glk due to its structural similarity with Mlc, the repressor of *malT*, binds to the glucose transporter (PtsG), releasing Mlc and thus increasing *malT* repression. In addition, even in mutants lacking Mlc (and glycogen), the overexpression of *glk* leads to a reduction in *mal* gene expression. We interpret this repression by a direct interaction of Glk with MalT concomitant with MalT inhibition. This repression was dependent on the presence of either maltodextrin phosphorylase or amylomaltase and led to the inactivation of MalT.

The *Escherichia coli* maltose system (4, 52) is geared for the efficient utilization of maltose and maltodextrins. Ten *mal* genes encode proteins found in all compartments of the cell. The lambda receptor in the outer membrane (43, 49) facilitates the diffusion of maltodextrins into the periplasmic space, where they are taken up into the cytoplasm via a binding-protein-dependent ABC transporter (32, 55). There are two main enzymes catalyzing the degradation of maltose and maltodextrins to glucose and α-glucose-1-phosphate. Amylomaltase (MalQ) (29), a maltodextrin glucanotransferase (41, 59), forms from any maltodextrin, including maltose, larger maltodextrins, and glucose (16, 34, 60). Maltotetraose and longer maltodextrins are substrates of the maltodextrin phosphorylase (MalP) (53, 58), yielding by phosphorolysis α-glucose-1-phosphate and smaller maltodextrins. Two other enzymes are a periplasmic amylase (MalS) (20, 51) and a cytoplasmic maltodextrin glucosidase (MalZ) that are not essential for maltose or maltodextrin utilization (44, 51, 57). While MalS produces preferentially maltohexaose from longer maltodextrins in the periplasm, MalZ degrades longer maltodextrins by cleaving glucose from the reducing end of the dextrins in the cytoplasm. The smallest substrate of MalZ is maltotriose, producing maltose and glucose. All *mal* genes are under the positive control of MalT (45), which in turn is activated by the inducer maltotriose (42). In addition to being controlled by MalT, MalZ is also induced under osmoregulation even in a *malT* mutant (15). The control of *mal* gene expression is surprisingly complex. Aside from the basic inducer-dependent activation of MalT as a specific transcriptional activator for all *mal* genes, there are additional regulatory circuits at work. The Phosphotransferase (PTS)-mediated uptake of glucose, controlling the level of the cyclic AMP (cAMP)/CAP complex, subjects *malT*, as well as the genes encoding the ABC transporter, to catabolite repression (9, 10, 46); Mlc, a global repressor of sugar metabolism, also controls *malT* expression in a glucose transport-dependent fashion (14, 48). This mechanism is unusual since Mlc is inactivated as a repressor by sequestration to a transporting and dephosphorylated cytoplasmic domain of PtsG ($EIIB^{Glc}$) (25, 30, 31, 39, 54, 56). The global regulators H-NS and StpA have also been reported to act on *malT* expression (21). The activity of MalT as a transcriptional activator can be modulated (reduced) by interaction with several proteins; this is dramatically seen when they are overproduced: a cytoplasmic esterase (Aes) (23, 36) and a cytoplasmic cysthathionase (MalY) (11, 50, 62). The physiological connection of these enzymes to the maltose system remains unclear. However, the most significant regulating protein is MalK, the ATP-hydrolyzing subunit of the maltodextrin ABC transporter (22, 35). The maltose transport system in nontransporting state binds, via its MalK subunit, MalT, leading to the inactivation of MalT activity (1, 2).

The *mal* system shows the phenomenon of endogenous induction. Thus, significant basal expression of the system does occur in the absence of external maltodextrins and is caused by endogenously produced inducer (13). The formation of the inducer maltotriose occurs by degradation of glycogen. Glycogen phosphorylase (GlgP) produces phosphorylase-limited-glycogen (see Fig. 6), harboring maltotetraose and maltotriose chains α(1-6) glucosidically linked to the main glycogen chain. Maltotetraosyl and maltotriosyl residues are cleaved by the

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glycogen-debranching enzyme (GlgX) to form linear maltotetraose and maltotriose. The former is converted to the inducer maltotriose by MalP, the maltodextrin phosphorylase. The level of maltotriose (and thus endogenous induction) is reduced by MalZ forming glucose and maltose from maltotriose (15). The role of MalQ in endogenous induction is more difficult to understand. On the one hand, it reduces the amount of maltotriose by repolymerizing it to higher maltodextrins plus glucose, which is removed by glucokinase-dependent phosphorylation. On the other hand, and particularly in the presence of MalZ when maltose is ultimately formed from glycogen, MalQ is able to reform maltotriose from maltose, maintaining sufficient levels of maltotriose for induction (15). This is also the mode by which exogenous maltose is transformed into maltotriose after entering the cell. However, when MalQ is lacking, glycogen-dependent endogenous induction becomes high (17), particularly in the absence of MalZ, demonstrating that glycogen-derived inducer is indeed maltotriose.

We demonstrate here that MalQ is strongly feedback inhibited by low concentrations of glucose, pointing to the function of glucose in controlling the activity of MalQ and thus in glycogen-derived endogenous induction. At a high internal glucose concentration, for instance when glucokinase is lacking (as in a *glk* mutant), a *malQ*$^+$ strain becomes phenotypically partially MalQ$^-$, exhibiting increased endogenous induction. In contrast, the removal of glucose activates MalQ and will therefore reduce endogenous induction (see Fig. 6). However, endogenous induction is still observed in mutants lacking glycogen synthase (GlgA), MalP, MalQ, and MalZ (15). In the past we have proposed a mechanism in which glucose is involved in the formation of endogenous inducer, be it maltotriose or an alternative, as-yet-unknown inducer. This view was corroborated by the observation that overexpressed glucokinase (by removing glucose via ATP-dependent phosphorylation) would reduce *mal* gene expression, whereas the addition of glucose to a glucokinase mutant and after transport into the cytoplasm in an unphosphorylated form (in a PtsG mutant via the glucose/galactose ABC transporter) would induce *mal* gene expression (13, 27). We now have made the observation that, in a strain lacking glycogen, MalP, MalQ, and MalZ, the action of glucokinase was dependent on Mlc and acted by binding to PtsG releasing Mlc as transcriptional repressor for *malT* (see Fig. 8). In addition, we found that even in the absence of Mlc, overexpressed glucokinase was able to strongly reduce *mal* gene expression. This Mlc-independent repression by glucokinase could only be observed in the presence of an intact *malP* or an intact *malQ* gene. We propose that glucokinase, together with MalP or MalQ, forms a complex that inhibits MalT (see Fig. 7).

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used are listed in Table 1. They were grown under aerobic conditions in tryptone broth (TB) or minimal medium A (MMA) supplemented with 1% (wt/vol) Casamino Acids (CAA) (28). When the activity of *lacZ* fusions was assayed, precultures were grown in TB and subcultured into minimal medium for assays. Maintenance of plasmids was ensured by the addition of the appropriate antibiotics (ampicillin, 100 μg ml^{-1}; kanamycin, 25 μg ml^{-1}).

Strain constructions. The strains of the present study were constructed via P1 transduction as described by Miller (28). Deletions in *malP*, *malQ*, and *malZ* were done by the method of Datsenko and Wanner (12) in combination with the heat-inducible λRED recombination system in strain DY330 (61). For multiple deletions, the antibiotic resistance cassette was removed with plasmid pCP20 harboring the *Saccharomyces cerevisiae* FLP recombinase (12). All strains were derivatives of Bre1162 (5), which in turn is a derivative of the standard laboratory *lac* deletion strain MC4100 (8). Bre1162 carries a transcriptional *malK-lacZ* fusion. All *mal* gene expression data were obtained with this *malK-lacZ* fusion. The mutation in *glgA* was a *glgA*::Tn*10* insertion. Strains carrying this insertion lack glycogen. The mutation in *glk* initially routinely used for a standard Glk$^-$ phenotype was an insertion of the chloramphenicol cassette, Tn*10*(*cam*), after amino acid 305 in Glk (27). In the course of this project it became clear that this mutation had resulted in the loss of glucokinase activity, but the C-terminally truncated protein still showed the ability to interact with PtsG. We termed this mutation *glk15*::Tn*10* (Cam) (27). A complete *glk* deletion was subsequently obtained by the Datsenko-Wanner technique. The strains GW26, GW27, and GW28 were obtained by transducing a P1 lysate of strain TB48 (Δ*glgA*::*cam* *malT*::Tn*5*) into AS40, AS54, and AS53, selecting for chloramphenicol resistance and screening for the loss of kanamycin resistance and dark blue color on X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) plates. This assured the presence of the *malT* alleles from AS40, AS53, and AS54. The strains retained the *opmR*::Tn*10* marker and were auxotrophic for tryptophan.

Cloning of *malQ* (pMAD145). Plasmid pMAD145 carries the *malQ* gene fused C-terminally to a His_6 tag encoding sequence under the control of the IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible T5 promoter-*lac* operator system. It was constructed by ligating a BamHI/SphI-restricted 2,319-bp DNA fragment into BamHI/SphI-restricted plasmid pQE-70. The fragment was obtained by PCR using the primers malQ_7up (5′-CAC CGG GCT ACT ACC TGG CGA AGA AT-3′) and malQ_BamHI (5′-CAA CCG CGG ATC CCT TCT TCT TCG CTG CAG-3′), introducing a BamHI site instead of the *malQ* stop codon. Chromosomal DNA of *E. coli* MC4100 was used as a template. Final sequencing revealed a silent mutation in the *malQ*-His_6 open reading frame (cytosine at position 1692 was exchanged by thymine). Strain M15 carrying plasmid pREP4 (harboring *lacI*^{q}) was used for the transformation with pMAD145 and IPTG-inducible expression of *malQ*-His_6.

Cloning of TTC1688 of *Thermus thermophilus* encoding glucokinase (GlK_{Tth} protein) yielding pIA1. pIA1 was constructed by ligating a HindIII/SphI-restricted DNA fragment into HindIII/SphI-restricted plasmid pGDR11. The fragment was obtained by PCR using primers *Tth*_Glk_fw (5′-GGACG**GCATGC**GA AGGTGGTGGGGCCTGGACCTGGG-3′) and *Tth*_Glk_rv (5′-GACC**AAGCTT**TT ACCCGCTTCCATCCTTCACCTCCAGGTAGGCGGTG-3′). Chromosomal DNA of *T. thermophilus* HB27 was used as a template. Cloned in pGRD11 GlK_{Tth} can be heterologously expressed in *E. coli* in soluble form. It carries an N-terminal His_6 tag. The protein can be induced by IPTG. Even though derived from a thermophilic bacterium, the purified enzyme still showed considerable activity at 37°C and phosphorylated glucose and mannose, with a K_m of 0.16 mM.

Overexpression and purification of MalQ. *E. coli* strain M15 harboring plasmids pREP4 and pMAD145 was grown in NZA medium (0.5% [wt/vol] yeast extract, 0.75% [wt/vol] NaCl, 1% [wt/vol] N-Z-Amine A [Sigma Aldrich, Munich, Germany]) containing 100 μg of ampicillin/ml and 25 μg of kanamycin/ml at 37°C. When the cells reached an optical density at 578 nm of 0.8, IPTG was added to a final concentration of 1 mM. Cells were grown for an additional 1.5 h and then harvested by centrifugation. The pellet was resuspended in 3 ml of buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, and 17 mM mercaptoethanol. Cells were disrupted by passing them three times at 12,000 lb/in^2 through a French pressure cell, followed by centrifugation at 18,000 × *g* for 45 min at 4°C. The supernatant was loaded onto a Ni affinity column (HiTrap chelating HP 1-ml column) equilibrated with buffer. Bound protein was eluted with a linear gradient of 0 to 500 mM imidazole within 20 column volumes. The protein was extensively dialyzed against 50 mM Tris-HCl (pH 7.5) containing 10 mM $MgCl_2$. The solution was sterilized by filtration and stored at 4°C.

Thin-layer chromatography. [^{14}C]maltose and [^{14}C]glucose were purchased from Amersham with specific radioactivities of 630 and 268 mCi mmol^{-1}, respectively. Both sugars were uniformly labeled. [^{14}C]maltotriose was synthesized and purified as described previously (16, 33). Thin-layer chromatography (silica gel 60 from Merck of 0.25-mm thickness on glass support) was developed with butanol-ethanol-water (5:3:2) and visualized by autoradiography.

β-Galactosidase assay. β-Galactosidase was assayed as described by Miller (28) with the following alterations. We omitted mercaptoethanol from the Z-buffer. Hydrolysis of *ortho*-nitrophenyl-β-galactoside (ONPGal) was done at a constant temperature of 28°C. After stopping the reaction with sodium carbonate, we clarified the suspension by centrifugation before measuring the optical density at 405 nm. We used an extinction coefficient of 4,860 M^{-1} cm^{-1}. The specific activity is given in micromoles of ONPGal hydrolyzed per minute per milligram of protein at 28°C. The protein concentration was taken from the

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or description[a]	Source or reference
E. coli strains		
AS40	MC4100 Δ*malB107 trp*::[Kan^r-*malE*Δ*p92-lacZ*]$_{op}$ *malT*$^+$ *opmR*::Tn*10*	50
AS54	MC4100 Δ*malB107 trp*::[Kan^r-*malE*Δ*p92-lacZ*]$_{op}$ *malT*(T38R) *opmR*::Tn*10*	50
AS53	MC4100 Δ*malB107 trp*::[Kan^r-*malE*Δ*p92-lacZ*]$_{op}$ *malT*(D65E) *opmR*::Tn*10*	50
BRE1162	MC4100 ϕ(*malK-lacZ*)*1113*(λp*lac*Mu50)	5
CL19	Bre1162 Δ*glgA*::*frt glk15*::Tn*10* (Cam) *mlc*::*tet*	This study
CL27	Bre1162 *glgA*::Tn*10* Δ*malZ*::*frt* Δ*glk*::*kan*	This study
CL28	Bre1162 Δ*malZ*::*frt* Δ*glk*::*kan*	This study
GW11	Bre1162 Δ*malZ*::*frt glgA*::Tn*10* Δ(*malQ malP*)::*frt glk15*::Tn*10* (Cam)	This study
GW12	Bre1162 Δ*malZ*::*frt glgA*::Tn*10* Δ(*malQ malP*)::*frt glk15*::Tn*10* (Cam) *mlc*::*kan*	This study
GW15	Bre1162 Δ*malZ*::*frt glgA*::Tn*10* Δ*malP*::*kan glk15*::Tn*10* (Cam)	This study
GW26	MC4100 Δ*malB107 trp*::[Kan^r-*malE*Δ*p92-lacZ*]$_{op}$ *malT*$^+$ *opmR*::Tn*10* Δ*glgA*::*cam*	This study
GW27	MC4100 Δ*malB107 trp*::[Kan^r-*malE*Δ*p92-lacZ*]$_{op}$ *malT*(T38R) *opmR*::Tn*10* Δ*glgA*::*cam*	This study
GW28	MC4100 Δ*malB107 trp*::[Kan^r-*malE*Δ*p92-lacZ*]$_{op}$ *malT*(D65E) *opmR*::Tn*10* Δ*glgA*::*cam*	This study
JM101	Δ(*lac-proAB*) *thi-1 supE* F′[*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]	37
JM-G77	JM101 Δ*ptsG*::*cat* Δ*ptsHI crr*::*kan ptsG-lacZ* in the λ attachment site	37
JW2385	Δ*glk*::*kan*, derivative of MG1655	NBRP (NIG, Japan): *E. coli*
M15	F$^-$ *nalS strS rifS thi lac mtl*	Qiagen
MAD103	Bre1162 Δ*malZ*::*frt glgA*::Tn*10*	This study
MAD104	Bre1162 Δ*malZ*::*frt glk15*::Tn*10* (Cam)	This study
MAD106	Bre1162 Δ*malZ*::*frt glgA*::Tn*10 glk15*::Tn*10* (Cam)	This study
MAD107	Bre1162 Δ*malZ*::*frt glk15*::Tn*10* (Cam) Δ*malQ*::*frt*	This study
MAD108	Bre1162 Δ*malZ*::*frt glgA*::Tn*10 glk15*::Tn*10* (Cam) Δ*malQ*::*kan*	This study
MC4100	*araD139 deoC1 flbB5301 ptsF25 rbsR relA1 rpsL150* Δ(*argF-lac*)*U169*	8
MG1655	Laboratory wild-type strain	
RD38	Bre1162 Δ*malZ*::*kan glgA*::Tn*10*	15
RD103	Bre1162 Δ*malZ*::*kan* Δ*glgA*::*frt malP*::Tn*10*	This study
RD105	Bre1162 Δ*malZ*::*kan* Δ*glgA*::*frt glk15*::Tn*10* (Cam) *malP*::Tn*10*	This study
RD126	Bre1162 Δ*malZ*::*frt glgA*::Tn*10* Δ(*malQ malP*)::*kan*	This study
RD129	Bre1162 Δ*malZ*::*frt* Δ(*malQ malP*)::*kan*	This study
TB04	Bre1162 Δ*malZ*::*kan*	15
TB48	Bre1162 Δ*glgA*::*cam malT*::Tn*5*	This study
Plasmids		
pREP4	pACYC derivative containing the p15A replicon and *lacI*^{q}; Kan^r	Qiagen
pMAD145	pQE70 (Qiagen) C-terminal His_6-tagged MalQ	This study
pBK1	Derivative of pGDR11 *lacI*^{q} N-terminal His_6 tag Glk; Amp^r	Laboratory collection
pGDR11	pQE31 (Qiagen) *lacI*^{q} N-terminal His_6 tag vector; Amp^r	36
pTSG11	Expresses ptsG under *lacI*^{q} and under its own promoter	6
pCSF2	Derivative of pACYC, *glk*$^+$ under natural promoter; Kan^r	27
pCSF34	Derivative of pACYC, *glk15* (Δ305-321) under natural promoter; Kan^r	27
pIA1	TTC1688 (encoding glucokinase of *T. thermophilus*) cloned into pGDR11; Amp^r	This study

[a] Amp^r, ampicillin resistance; Kan^r, kanamycin resistance.

optical density at 578 nm of the bacterial culture. For the correlation, it was assumed that the optical density at 578 nm of 1.0 is equivalent to 107 μg of protein per ml (28). A specific activity of 1 corresponds to about 1,000 Miller units.

Membrane preparation and binding of Glk to PtsG. The protocol described by Lee et al. (25) was followed with several alterations. JM-G77 (lacking *ptsG*) and JM-G77 transformed with *ptsG11* (encoding EIIBC^{Glc}) were used for membrane preparations. JM-G77 carries Δ*ptsG*::*cam* and Δ*ptsHI crr*::*kan* insertions that eliminate all PTS phosphorylation (37, 38). Both strains were grown overnight in 500 ml of MMA with 0.4% (vol/vol) glycerol as the carbon source. The cells were harvested by centrifugation, washed twice with 0.9% (wt/vol) NaCl, and resuspended in 3 ml of binding buffer (20 mM HEPES, 0.8 mM Na_2HPO_4, 5 mM KCl, 137 mM NaCl [pH 7.0]). Cells were broken in a French pressure cell at 16,000 lb/in^2. Unbroken cell fragments were removed by centrifugation (at 25,000 × *g* for 15 min at 4°C), and the supernatant was kept on ice (crude cellular extract). The protein concentration was determined. A volume containing 2 mg of total cellular protein was centrifuged at 100,000 × *g* for 15 min. The supernatant was discarded. The pellet (membrane fraction) was resuspended in 100 μl of binding buffer. Then, 2 μg of purified Glk-His_6 was added, followed by incubation at 37°C for 20 min. The membrane suspension was centrifuged for 15 min at 100,000 × *g* at 4°C. The supernatant was removed and designated SN1 (50 μl was used for further sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE] analysis). The pellet resuspended in 100 μl of binding buffer and washed once was designated P1 (50 μl was used for further SDS-PAGE analysis). The remaining 50 μl of P1 was centrifuged again at 100,000 × *g* at 4°C for 15 min. The supernatant was removed and used for further SDS-PAGE analysis, and this was named SN2. The pellet was resuspended in 50 μl of binding buffer named P2. Equal samples of supernatant and resuspended membranes were subjected to SDS-PAGE analysis, followed by Western blotting with anti-His tag antibodies. The antibodies were obtained from Qiagen. Western blotting was done according to the supplier's protocol. Detection was performed with alkaline phosphatase.

Comparison of the amounts of Glk in different strains. Strains were grown in 100 ml of LB medium at 37°C overnight. Cells were harvested by centrifugation, washed once in phosphate-buffered saline containing 10 mM $MgSO_4$, and resuspended in 2 ml of the same buffer. After sonication (three times for 30 s) unbroken cells were removed by centrifugation (17,000 × *g*, 4°C, 1 h), and the supernatant was finally centrifuged for 1 h at 100,000 × *g* and 4°C. The protein concentration of the supernatant was determined, and a volume containing 90 μg of total cellular protein was precipitated with 7.5% (vol/vol) trichloroacetic acid on ice for 20 min. The precipitate was washed once with ice-cold acetone, and 50 μg was analyzed by SDS-PAGE, followed by Western blotting with anti-Glk

antibodies. Western blotting was done according to the supplier's protocol. Detection was performed with alkaline phosphatase.

RESULTS

Overexpression and purification of MalQ. The *malQ* gene was amplified by PCR from the chromosome of MC4100 and cloned as a C-terminal His_6 version in an IPTG-inducible expression vector. The encoded protein was well expressed and could readily be detected in crude cellular extracts by SDS-PAGE as a prominent band of about 75 kDa (apparent molecular mass). Induction of the enzyme by 1 mM IPTG caused retardation of growth and cessation of growth after 2 h of induction. Nevertheless, the enzyme remained soluble in extracts of overexpressing cells and could easily be purified to apparent homogeneity by nickel-NTA affinity chromatography. The purified enzyme was extensively dialyzed against 50 mM Tris-HCl (pH 7.6) containing 10 mM $MgCl_2$. The solution was sterilized by filtration and kept at 4°C. From a 1-liter culture more than 5 mg of purified MalQ was routinely obtained.

Inhibition of MalQ activity by glucose. MalQ is able to act on maltose, forming glucose and a series of maltodextrins. We used uniformly ^{14}C-labeled maltose at 75 μM as a substrate and analyzed the formation of labeled maltodextrins (and glucose) after 10 min of incubation at 30°C by thin-layer chromatography, followed by autoradiography (Fig. 1A). The presence of unlabeled glucose slowed the reaction visibly at concentrations higher than 20 μM; at 100 μM unlabeled glucose a reaction product was no longer formed (Fig. 1A). When glucokinase plus 24 mM ATP and 50 mM $MgCl_2$ was added to a reaction mixture that was blocked by 100 μM unlabeled glucose, the activity of MalQ resumed. After 1 min the time-dependent formation of labeled maltodextrins plus glucose-6-phosphate was again observed. After 20 min, all maltodextrins had been transformed into long nonmigrating oligosaccharides and glucose-6-phosphate (Fig. 1B). This is caused by the effective removal of glucose from the reaction mixture. The MalQ inhibiting effect of glucose is counteracted by increasing amounts of maltose. These findings demonstrate that under in vivo conditions where free glucose is continuously removed, MalQ will remain highly active and will lead to the polymerization of maltodextrins (including the inducer maltotriose) to long oligosaccharides, resulting in the loss of induction.

Effect of glucose on the endogenous induction of *mal* gene expression in strains synthesizing glycogen. As a measure of *mal* gene expression, we used a transcriptional *lacZ* fusion to *malK* encoding the ATP-hydrolyzing subunit of the maltose/maltodextrin ABC transporter. Thus, the tester strains lacked maltose/maltodextrin transport and, due to the lack of MalK, they contained MalT, the transcriptional regulator, in a MalK-uninhibited state (24). In addition, we deleted the *malZ* gene to avoid complications due to the degradation of the inducer maltotriose by MalZ (15, 57). As a carbon source we used 1% (wt/vol) CAA in MMA. The use of glycerol was avoided since glycerol exerts a considerable catabolite repression on the maltose system (19). The expression of uninduced maltose transport of cells grown on glycerol is about 10 times lower than when grown on CAA (16). We used cells that had been grown overnight. The specific galactosidase activity of a *malK-lacZ* fusion (in strain GW12) decreased after inoculation about twofold in the early logarithmic growth phase. It increased and peaked (30% higher than the overnight culture) in the late log phase and leveled off in stationary phase. Thus, the most reproducible values were obtained in stationary-phase cells. Therefore, expression assays were done in stationary cultures. They were repeated at least four times. They showed routinely a variation of ±5%.

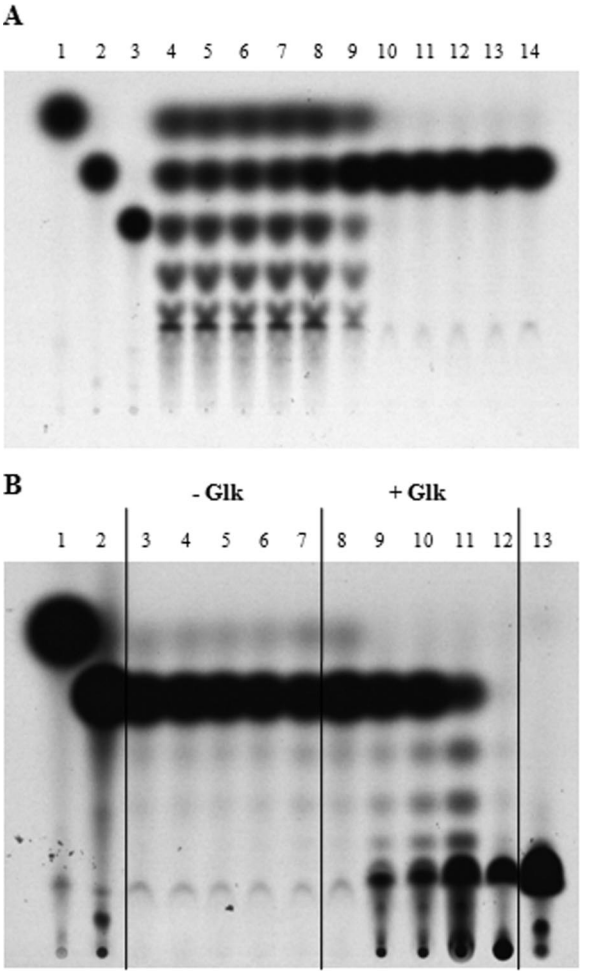

FIG. 1. In vitro MalQ activity with maltose as substrate. (A) The reaction was performed with 4.4 μg of C-terminally His_6-tagged MalQ in a total volume of 16 μl containing 250 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, and different concentrations of unlabeled glucose. After 10 min of incubation at room temperature, [^{14}C]maltose (final concentration, 75 μM) was added to the mixture, followed by incubation for another 20 min at 30°C. Portions (5 μl) of the reaction were spotted. The figure represents the autoradiogram of the TLC plate. The standards were as follows: lane 1, [^{14}C]glucose; lane 2, [^{14}C]maltose; lane 3, [^{14}C]maltotriose. Glucose additions to the enzymatic assays were as follows: lane 4, no glucose; lane 5, 0.0002 mM; lane 6, 0.001 mM; lane 7, 0.002 mM; lane 8, 0.01 mM; lane 9, 0.02 mM; lane 10, 0.1 mM; lane 11, 0.2 mM; lane 12, 1.0 mM; lane 13, 2.0 mM; lane 14, 10 mM. (B) The reaction was performed as in panel A with the additional 50 mM $MgCl_2$, 25 mM ATP, and 100 μM glucose without (lane 3 to 7) and with 1.5 μg of Glk (lanes 8 to 12). After 0, 1, 2, 5, and 20 min, 4 μl of the reaction was spotted. The standards were as follows: lane 1, [^{14}C]glucose; lane 2, [^{14}C]maltose; lane 13, [^{14}C]glucose-6-phosphate.

TABLE 2. Role of MalQ in controlling endogenous glycogen-derived maltotriose-dependent *mal* gene expression

Strain[a]	Additional genotype	MalK-LacZ activity
TB04	*malZ*	2.51
MAD104	*malZ glk15*::Tn*10* (Cam) vector	4.21
CL28	*malZ* Δ*glk*	4.17
MAD104	*malZ glk15*::Tn*10* (Cam) *pglk*$^+$	0.08
CL28	*malZ* Δ*glk* vector	4.35
CL28	*malZ* Δ*glk pglk*$^+$	0.07
MAD107	*malZ glk15*::Tn*10* (Cam) *malQ* vector	6.73
MAD107	*malZ glk15*::Tn*10* (Cam) *malQ pglk*$^+$	4.18
RD129	*malZ malP malQ* vector	6.90
RD129	*malZ malP malQ pglk*$^+$	3.39

[a] All strains are derivatives of BRE1162 (*malK-lacZ malQ*$^+$ *malP*$^+$ *glgA*$^+$). The strains were grown in MMA containing 1% (wt/vol) CAA.

In the first set of experiments we tested *malK-lacZ* expression in a *glgA*$^+$ (glycogen synthase) *malQ*$^+$ strain in the presence or absence of chromosomally or plasmid-encoded and overexpressed glucokinase. Table 2 shows that the presence of chromosomally encoded glucokinase reduced the expression in comparison to the strain lacking glucokinase activity. When glucokinase was overproduced, the expression dropped to near zero. In contrast, in a strain lacking MalQ or both MalQ and MalP (but still producing glycogen and thus maltotriose) the overproduction of glucokinase was less dramatic than in a *malQ*$^+$ strain but was still recognizable (Table 2). We conclude that one part of the "glucose effect" on *mal* gene expression is that with reduced glucose phosphorylating capabilities, MalQ becomes increasingly inhibited by glucose, leading to a partial MalQ minus phenotype, which in turn increases *mal* gene expression by the glycogen-derived formation of maltotriose (that is no longer effectively removed by MalQ). However, it became clear that, in addition to controlling MalQ activity, the overproduction of glucokinase must have additional effects on *mal* gene expression that would reveal themselves more clearly only in the absence of glycogen synthesis.

Role of glucokinase for endogenous induction in the absence of glycogen. As demonstrated above, the presence of glucose controls glycogen-derived endogenous induction by affecting the enzymatic activity of MalQ. According to Fig. 1, by removing glucose via Glk, MalQ is no longer inhibited and repolymerizes the glycogen-derived inducer maltotriose to larger dextrins. These are substrates of maltodextrin phosphorylase (MalP), which in turn forms α-glucose-1-phosphate that enters glycolysis via phosphoglucomutase. However, even in the absence of glycogen- and maltodextrin-metabolizing enzymes, basal *mal* gene expression can still be observed. The origin of this induction, in particular the nature of the "glycogen-independent" inducer, remained unclear. Previously, we had argued that glucose was involved in the formation of an as-yet-unidentified sugar with properties similar to those of maltotriose that would activate MalT. Here, we demonstrate that glycogen-independent induction is due to two mechanisms: one controls the activity of MalT, and the other controls, via Mlc, the basal expression of *malT*. Both mechanisms do not require the existence of an alternative inducer.

TABLE 3. Role of glucokinase in controlling MalT activity

Strain[a]	Additional genotype	MalK-LacZ activity
RD38	*glk*$^+$	1.97
MAD103	*glk*$^+$	2.16
MAD106	*glk15*::Tn*10* (Cam)	2.16
MAD106	*glk15*::Tn*10* (Cam) vector	2.28
MAD106	*glk15*::Tn*10* (Cam) *pglk*$^+$	0.09
CL27	Δ*glk*::kan	2.20
CL27	Δ*glk* vector	1.92
CL27	Δ*glk pglk*$^+$	0.05
MAD 108	*glk15*::Tn*10* (Cam) *malQ*	2.12
MAD108	*glk15*::Tn*10* (Cam) *malQ* vector	2.0
MAD108	*glk15*::Tn*10* (Cam) *malQ pglk*$^+$	0.13
RD103	*malP*	2.19
RD105	*glk15*::Tn*10* (Cam) *malP*	1.95
RD105	*glk15*::Tn*10* (Cam) *malP* vector	2.02
RD105	*glk15*::Tn*10* (Cam) *malP pglk*$^+$	0.09
GW15	*malP glk15*::Tn*10* (Cam) *vector*	1.97
GW15	*malP glk15*::Tn*10* (Cam) *pglk*$^+$	0.06
RD126	*malP malQ*	1.87
GW11	*glk15*::Tn*10* (Cam) *malP malQ*	1.82
GW11	*glk15*::Tn*10* (Cam) *malP malQ* vector	1.87
GW11	*glk15*::Tn*10* (Cam) *malP malQ pglk*$^+$	0.85
CL19 (*malZ*$^+$)	*glk15*::Tn*10* (Cam) *mlc* vector	5.90
CL19 (*malZ*$^+$)	*glk15*::Tn*10* (Cam) *mlc pglk*$^+$	0.46

[a] All strains harbor *malK-lacZ* and lack *glgA* and *malZ*.

Glucokinase interacts with MalT in the presence of MalP or MalQ. The second set of experiments probing *malK-lacZ* expression were done in strains lacking glycogen (*glgA* mutation), as well as either *malP* or *malQ* alone or both *malQ* and *malP*. Table 3 shows that the basal *mal* gene expression in a *malP*$^+$ *malQ*$^+$ background is not noticeably altered by the absence of glucokinase (RD38 or MAD103 versus MAD106). However, it can be reduced 20-fold by the overproduction of glucokinase. This dramatic reduction in *mal* gene expression is dependent on the additional presence of MalP or MalQ. Strains lacking MalP (RD105 and GW15) or MalQ (MAD108) still showed strong reduction of *mal* gene expression upon overexpression of glucokinase, whereas a strain lacking both *malQ* and *malP* (GW11) only showed a twofold reduction upon overexpression of glucokinase (Table 3). The strong reduction in *mal* gene expression by overproduced glucokinase in the presence of MalP and MalQ is also observed in a strain lacking Mlc (Table 3, strain CL19), where the expression of *malT* is increased due to the loss of the repressor for *malT* (14).

In a background of *glgA*$^+$ (Table 2), this MalP/MalQ-dependent reduction of *mal* gene expression by glucokinase overproduction can only be observed in a strain that is MalP$^+$ and MalQ$^+$. There, the MalP/MalQ/glucokinase effect is compounded by the effect of the high activity of MalQ in the presence of glucokinase that increases repression by the removal of the inducer maltotriose. The MalP/glucokinase-dependent repression in a strain containing glycogen is not observed when MalQ is lacking (Table 2, strain MAD107). The explanation for this difference is the production of large amounts of the inducer maltotriose from glycogen (that is not curbed in the absence of MalQ). Thus, maltotriose apparently counteracts the combined effect of MalP and glucokinase. We propose that the combination of MalP/MalQ and glucokinase acts directly on MalT competing with maltotriose, similar to

TABLE 4. The D65E mutation in MalT is resistant to inhibition by Glk

Strain[a]	Additional genotype	MalE-LacZ activity
GW26	*malT*$^+$	0.38
GW26	*malT*$^+$ *pglk*$^+$	0.11
GW27	*malT*(T38R)	0.64
GW27	*malT*(T38R) *pglk*$^+$	0.27
GW28	*malT*(D65E)	2.60
GW28	*malT*(D65E) *pglk*$^+$	2.65

[a] All strains lack *malK*, are Δ*glgA ompR*::Tn*10* harboring *malE-lacZ* in the *tryp* gene cluster.

TABLE 5. Role of glucokinase in controlling the expression of *malT*

Strain[a]	Additional genotype	MalK-LacZ activity
GW11	*glk15*::Tn*10* (Cam) *malP malQ*	1.82
GW11	*glk15*::Tn*10* (Cam) *malP malQ* vector	1.87
GW11	*glk15*::Tn*10* (Cam) *malP malQ pglk*$^+$	0.85
GW11	*glk15*::Tn*10* (Cam) *malP malQ pptsG*$^+$	3.75
GW12	*glk15*::Tn*10* (Cam) *malP malQ mlc* vector	4.27
GW12	*glk15*::Tn*10* (Cam) *malP malQ mlc pglk*$^+$	3.94
GW12	*glk15*::Tn*10* (Cam) *malP malQ mlc pptsG*$^+$	4.25
CL19 (*malZ*$^+$)	*glk15*::Tn*10* (Cam) *mlc* vector	5.90
CL19 (*malZ*$^+$)	*glk15*::Tn*10* (Cam) *mlc pglk*$^+$	0.72

[a] All strains harbor *malK-lacZ* and lack *glgA* and *malZ*.

what has been observed with MalK (22), Aes (36), or MalY (62), proteins that interact directly with MalT (23). Thus, the simplest explanation for this MalP/MalQ/glucokinase-dependent repression would likewise be a direct interaction between MalT and glucokinase that is stimulated by MalP or MalQ.

Evidence for MalT being the target for the inhibition by Glk. In previous work studying the interaction of MalK, MalY, and Aes with MalT (50), we had tested 26 MalT mutants that exhibited a constitutive phenotype being partially independent of the inducer maltotriose. These mutants show elevated *mal* gene expression as tested by a *malE-lacZ* fusion in a Δ*malK* background. All of the mutations are in the first third of MalT. None of the mutants are resistant against all MalT inhibitors, but some are nearly resistant to Aes, indicating the interaction site in MalT with Aes. We transduced the Δ*glgA*::*cam* allele into three of these constructs harboring the wild type and the T38R and D65E mutations in MalT and tested the resulting strains for their *malE-lacZ* expression in the presence or absence of plasmid-encoded Glk (Table 4). In this setup the overexpression of Glk reduced the *malE* expression in a *malT*$^+$ background threefold (GW26), a result clearly less dramatic than the *malK* expression in a similar background (MAD106, Table 3). Nevertheless, this value allowed us to test whether the two mutations in MalT have altered sensitivity toward Glk. GW27 harboring the T38R mutation showed a twofold increase in basal activity in respect to the wild type and nearly the same reduction by overexpressed Glk (Table 4). However, strain GW28 harboring the D65E mutation was entirely resistant to overproduction of Glk and showed nearly tenfold increase in basal activity compared to the wild type (Table 4). This demonstrates that MalT is the target for the interaction with Glk.

Glucokinase regulates the expression of *malT* via the release of Mlc from PtsG. Even in the absence of MalP or MalQ (conditions where the interaction of Glk with MalT is minimal) and with no glycogen present, endogenous induction (or basal *mal* gene expression) is still observed and significantly (>2-fold) reduced by overproduction of glucokinase (Table 5, strain GW11). We observed that the presence of Mlc was required for this reduction. Strain GW12 lacking Mlc in addition to glycogen, MalP, and MalQ, showed, as expected, high *mal* gene expression that was only insignificantly reduced by glucokinase overproduction (Table 5). Thus, it was likely that Mlc is involved in this MalP/MalQ-independent effect of glucokinase. Since glucokinase shows a structure (26) that is very similar to the structure of Mlc (48), we concluded that glucokinase at high concentrations will compete with Mlc for binding PtsG. Mlc released by glucokinase from PtsG would then be more effective in repressing *malT* transcription.

One may argue that in the absence of glucose transport the EIIB domain of PtsG should be predominantly present in its phosphorylated form, preventing the binding of Mlc or of glucokinase for that matter. However, Mlc can obviously be bound and inactivated as a repressor even in the absence of glucose transport. This can be seen by the effect of overproduction of PtsG on *mal* gene expression (Table 5). The overproduction of PtsG in an *mlc*$^+$ background (GW11) in contrast to its *mlc* derivative (GW12) titrates Mlc and increases *mal* gene expression even though no glucose transport occurs and the EIIB domain of PtsG should be predominantly in the phosphorylated state (Table 5). Thus, competition of Mlc binding to PtsG by glucokinase is a valid proposition for a mechanism in which glucokinase can affect glycogen-independent endogenous *mal* gene expression.

To demonstrate that glucokinase is indeed able to interact with PtsG, we prepared inverted membrane vesicles of a *pstG* deletion strain producing plasmid-encoded and IPTG-induced PtsG. In this setup, the EIIB domain of PtsG is exposed on the outside of the inverted vesicles. PtsG-dependent binding of glucokinase was demonstrated by ultracentrifugation and recovery of glucokinase in the washed pellet fraction. In contrast, the membranes of the same *ptsG* deletion strain without PtsG-encoding plasmid did not bind glucokinase (Fig. 2).

Recently, the crystal structure of Mlc in complex with the EIIB domain of PtsG has been determined (31). Since the crystal structure of glucokinase (aside from the lacking helix-turn-helix motif) is very similar to the Mlc structure (48), we

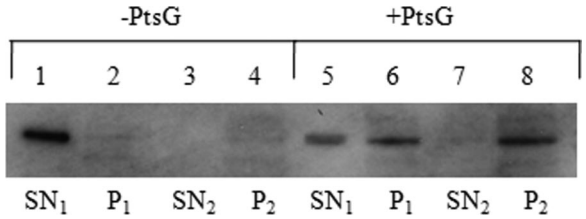

FIG. 2. Interaction of Glk with PtsG. Portions (50 μg) of crude extract of strain JMG77(Δ*ptsG*) (lanes 1 to 4) and JMG77 harboring plasmid-encoded PtsG (lanes 5 to 8) were analyzed by SDS-PAGE followed by Western blotting with anti-His tag antibodies to detect Glk. Lanes: 1 and 5, first membrane-free supernatants (SN1); lanes 2 and 6, first pellets (P1); lanes 3 and 7, second supernatants (SN2); lanes 4 and 8, second pellets (P2).

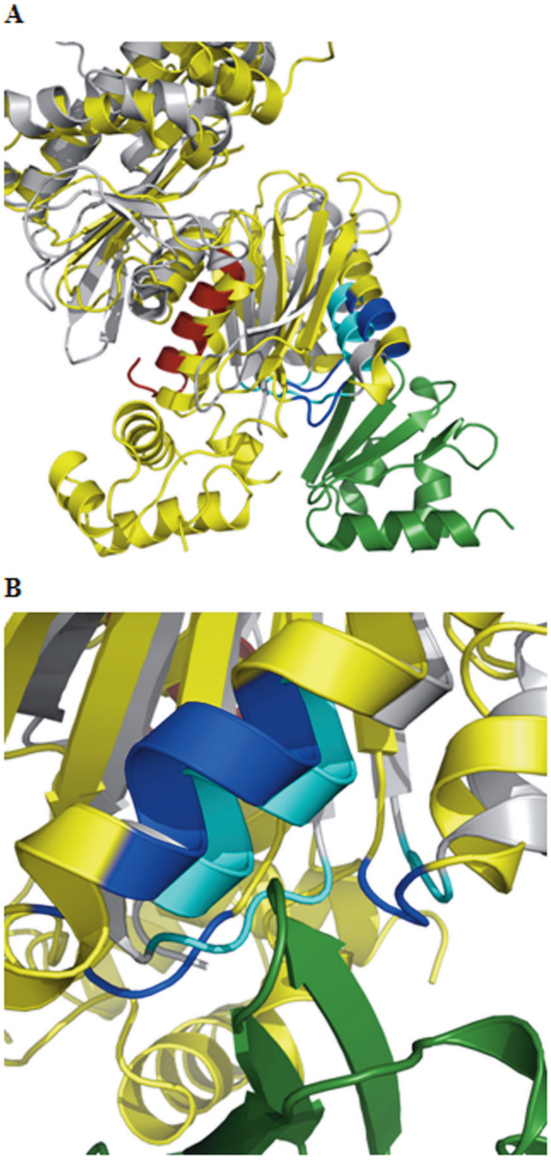

FIG. 3. Superimposition of GlK on interaction between Mlc and PtsG. (A) Partial view of Mlc (yellow) in its interaction with the EIIB domain of PtsG (green) (31). The interacting amino acids between the two proteins are shown in blue. Glk (in gray) (26) has been superimposed onto the Mlc structure (48) by using the Coot program (http://www.ysbl.york.ac.uk/~emsley/coot/). The amino acids contacting the EIIB domain are in cyan. The C-terminal helix harboring the 16 amino acids lacking in Glk15 (Δ305-321) are shown in red. The helix-turn-helix domain of Mlc (lacking in Glk) is on the lower left-hand corner. (B) Detailed view of the interaction site seen from the side opposite that shown in panel A.

tried to manually superimpose the glucokinase structure with the Mlc structure when in complex with EIIB (Fig. 3). Glucokinase could easily be brought into a position where the contact sites to EIIB are nearly identical to the contact sites between Mlc and EIIB. This suggests that both Mlc and glucokinase can compete at the same position of $EIIB^{Glc}$.

Differentiating the three functions of glucokinase by using *glk* deletions and glucokinase from *T. thermophilus*. Throughout the construction of strains used in the present study we had considered the *glk*::Tn*10*(*cam*) mutation as a null mutation of *glk* because of its complete loss in glucokinase activity (27). Since we had this mutation also present on plasmid pCSF34, where *glk*::Tn*10*(*cam*) is under its natural promoter control, we wanted to use it as a control for the effect of *glk*-overexpressing plasmids. To our surprise, this plasmid had the same effect on *mal* gene expression in MAD106 as had pCSF2, a corresponding plasmid expressing the wild-type *glk* under natural promoter control (Table 6). Since the protein encoded by pCSF34 did not show glucokinase activity, it could not act on the level of controlling MalQ activity (via removal of glucose). However, the C-terminally truncated protein (16 C-terminal amino acids are missing) could be interacting with PtsG (releasing Mlc and reducing *malT* expression) or with MalT (affecting MalT activity) or both. Looking at the structure of glucokinase, one can recognize that the 16 C-terminal amino acids form a helix that is not part of the interaction site with EIIB of PtsG (Fig. 3). We tested for the presence of such a truncated protein in the 100,000 × *g* supernatant of cellular extracts. Indeed, pCSF34 caused the synthesis of a protein not present in a strain with *glk* deleted (Fig. 4). Thus, an interaction of this truncated protein at least with PtsG appeared possible. To differentiate between an effect of the truncated Glk on PtsG and MalT, we used strain CL19. This strain lacks GlgA and Mlc, and therefore any reduction in *mal* gene expression by pCSF34 could only be due to the interaction with MalT. However, there was no significant reduction of *mal* gene expression in strain CL19 by pCSF34 (*glk15* Δ306-321) (Table 6). Thus, Glk lacking the 16 C-terminal amino acids was able to interact with PtsG but not with MalT. In Fig. 5, the structure of Glk is shown. The C-terminal 16 amino acids form a prominent alpha-helix which must rep-

TABLE 6. Differentiating the function of Glk in controlling *mal* gene expression

Strain[a]	Additional genotype	MalK-LacZ activity
MAD104 (*glgA*$^+$)	*glk15*::Tn*10* (Cam) vector	4.21
MAD104 (*glgA*$^+$)	*glk15*::Tn*10* (Cam) *pglk*$^+$	0.08
MAD104 (*glgA*$^+$)	*glk15*::Tn*10* (Cam) p*Ttglk*$^+$	1.59
MAD106	*glk15*::Tn*10* (Cam)	2.16
MAD106	*glk15*::Tn*10* (Cam) *pglk*$^+$	0.09
MAD106	*glk15*::Tn*10* (Cam) pCSF34 (*glk15* Δ306-321)	0.88
MAD106	*glk15*::Tn*10* (Cam) pCSF2*glk*$^+$	0.58
MAD106	*glk15*::Tn*10* (Cam) p*Ttglk*$^+$	1.20
RD105	*glk15*::Tn*10* (Cam) *malP*	1.95
RD105	*glk15*::Tn*10* (Cam) *malP* vector	2.02
RD105	*glk15*::Tn*10* (Cam) *malP pglk*$^+$	0.09
RD105	*glk15*::Tn*10* (Cam) *malP pTtglk*$^+$	1.05
GW12	*glk15*::Tn*10* (Cam) *malP malQ mlc* vector	4.27
GW12	*glk15*::Tn*10* (Cam) *malP malQ mlc pglk*$^+$	3.94
GW12	*glk15*::Tn*10* (Cam) *malP malQ mlc pTtglk*$^+$	4.39
CL19 (*malZ*$^+$)	*glk15*::Tn*10* (Cam) *mlc* vector	6.30
CL19 (*malZ*$^+$)	*glk15*::Tn*10* (Cam) *mlc pglk*$^+$	0.71
CL19 (*malZ*$^+$)	*glk15*::Tn*10* (Cam) *mlc* pCFS2 (*glk*$^+$)	3.90
CL19 (*malZ*$^+$)	*glk15*::Tn*10* (Cam) *mlc* pCSF34 (*glk15* Δ306-321)	4.75
CL19 (*malZ*$^+$)	*glk15*::Tn*10* (Cam) *mlc pTtglk*$^+$	6.37

[a] All strains harbor *malK-lacZ* and lack *glgA* and *malZ* unless specified otherwise.

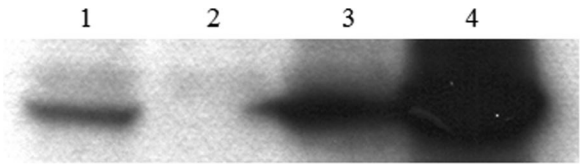

FIG. 4. Size of the truncated version of Glk. Proteins were analyzed by SDS-PAGE, followed by Western blotting with anti-Glk antibodies. Lane 1, wild-type MG1655; lane 2, JW2385 (Δ*glk*); lane 3, JW2385 harboring *glk15* (Δ305-321); lane 4, JW2385 harboring plasmid-encoded N-terminal His_6-tagged wild-type Glk under IPTG induction.

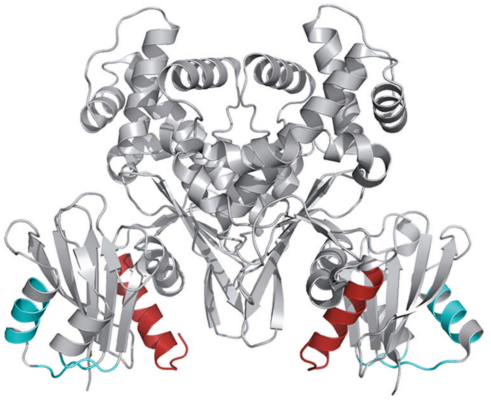

FIG. 5. Structure of Glk. The structure of the Glk dimer (gray) (26) is shown. The C-terminal helix harboring the 16 amino acids lacking in Glk15 (Δ305-321) are shown in red. They most likely constitute the interaction domain with MalT. The putative interaction site with EIIB of PtsG is indicated in cyan.

resent the interaction site with MalT. It is clearly separated from the interaction site with PtsG.

The amount of chromosomally *glk15*::Tn*10* (Cam)-encoded truncated glucokinase just as the wild-type Glk had no significant effect on the activity of MalT or PtsG. This is clear from the data in Table 3. The introduction of a complete deletion in *glk* (strain CL27) did not increase *mal* gene expression in the *glgA malP*$^+$ *malQ*$^+$ background in comparison to the *glk15*::Tn*10* (Cam) mutation. Thus, where the amount of chromosomally encoded Glk endowed with glucokinase activity is concerned it makes itself apparent on *mal* gene expression only in a background of *glgA*$^+$ *malP*$^+$ *malQ*$^+$ (Table 2, compare TB4 to MAD104).

As another control for the function of overexpressed *E. coli* glucokinase, we cloned TTC1688, a gene that is annotated to encode glucokinase from *T. thermophilus*, heterologously expressed it in *E. coli*, and purified it. The protein, GlK_{Tth}, consists of a 303-amino-acid polypeptide chain with only 22% sequence identity with *E. coli* Glk. It is able to phosphorylate glucose and mannose in a ATP-dependent fashion with a K_m of 0.16 mM. At ambient temperature it represents an equilibrium of dimeric and tetrameric forms, as tested by molecular sieve chromatography. Even though the protein has a temperature optimum of 70°C, it is still active at 37°C. We noticed that, during the purification of GlK_{Tth} from *E. coli* extracts, the protein formed a tight complex with MalP from *E. coli* that could be dissociated by 1.5 M NaCl. *E. coli* Glk does not show this tight complex formation.

When the overexpression of p*Ttglk*$^+$ was tested in strain MAD104, a strain that produces glycogen ($GlgA^+$) and does contain MalP and MalQ, *mal* gene expression was reduced 2.6-fold (Table 6). It was much less effective than the overproduction of *E. coli* Glk. Since GlK_{Tth} is still able to phosphorylate glucose, a controlling effect on MalQ activity (reducing maltotriose levels) was expected. When expressed in MAD106 or RD105 (lacking glycogen and therefore no longer producing endogenous maltotriose) the reduction in *mal* gene expression was <2-fold (Table 6). Thus, GlK_{Tth} not only affected MalQ activity but may also be able to interact with PtsG or with MalT or both. However, the overexpression of GlK_{Tth} in strains GW12 or CL19, strains lacking Mlc in addition to GlgA, there was no longer an effect of GlK_{Tth} overexpression (Table 6). This demonstrated that GlK_{Tth} is able to interact with PtsG to release Mlc as a *malT* repressor, but it is not able to interact with MalT to affect its activity.

DISCUSSION

We describe here a complex regulatory network connected to glucose and glucokinase that affects the *mal* gene expression in *E. coli*. In the wild type under laboratory growth conditions these regulatory inputs are hardly recognizable. They may become important under the real-life conditions where varying carbon sources and stress conditions are normal. The effect of uptake and metabolism of glucose on the utilization of alternative carbon sources (mostly carbohydrates) is a well-known phenomenon. Catabolite repression and inducer exclusion in *E. coli* are connected to the uptake of glucose via the glucose-specific PTS, and the state of phosphorylation of the $EIIA^{Glc}$ has been identified as a major player in this global signal transduction pathway controlling the activity of the cAMP/CAP complex to stimulate the expression of catabolite sensitive genes, as well as the activity of the cognate transporter (40). Certainly, the *E. coli* maltose system is prominently affected by both catabolite repression (10) and inducer exclusion (24). However, the phenomena connected to glucose described in this publication are not related to catabolite repression even though the $EIIBC^{Glc}$ (PtsG), the specific PTS for glucose, and Mlc, the regulator for *ptsG* (as well as for *malT*), are integral parts in this regulation. Here, it is not the PtsG-mediated transport of glucose but cytoplasmic glucose released by the metabolism of maltose and maltodextrins (and by glucose containing disaccharides, such as trehalose and lactose) that has regulatory functions. In addition, it is glucokinase itself that exerts regulatory effects on *malT* expression, as well as on MalT activity.

Regulation of MalQ activity by glucose. In a strain wild type for the *mal* genes, as well as glycogen-synthesizing and -degrading enzymes, the *mal* genes exhibit an "uninduced" level of expression when grown on a nonmaltodextrin carbon source (16, 18). This situation reflects endogenous induction by glycogen-derived maltotriose, the identified inducer of MalT, the transcriptional regulator of all *mal* genes (42). We now understand that the concentration of maltotriose is balanced on the one hand by its synthesis from glycogen and on the other hand by its MalQ-mediated removal (formation of glucose plus

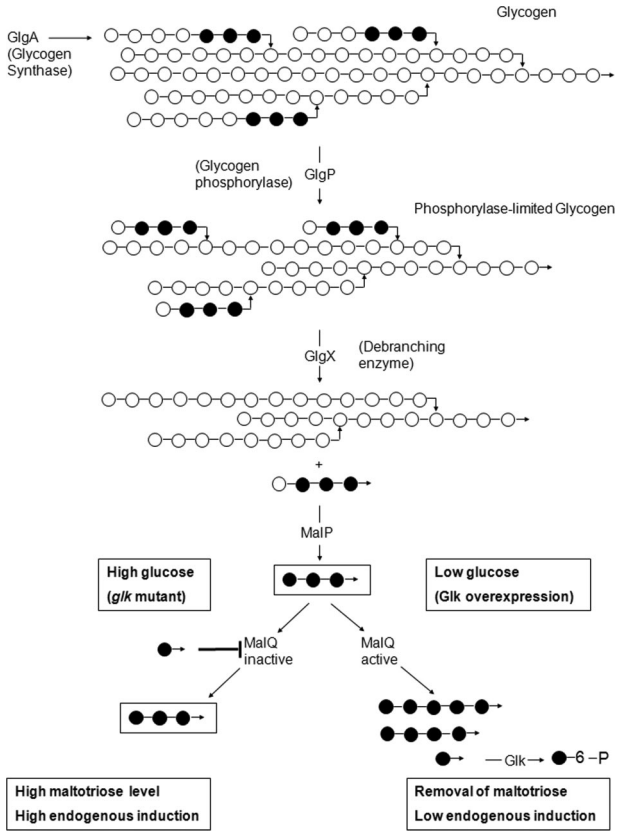

FIG. 6. Effect of cytoplasmic glucose on the activity of MalQ and on glycogen-dependent endogenous induction. Shown is the pathway of glycogen degradation producing maltotriose, the inducer of MalT, the transcriptional activator of all *mal* genes. Glucosyl residues are indicated by small circles, horizontal lines between the circles indicate α(1-4) linkages, bent arrows indicate α(1-6) linkages. Unlinked arrows indicate the reducing end of the maltodextrins and glucose. Solid circles indicate the origin of maltotriose from glycogen and its further metabolism. (Lower left branch) In a *glk* mutant, glucose formed by the action of MalQ or by hydrolysis of glucose containing substrates (not shown) inhibit MalQ, preventing the removal of maltotriose, thus establishing high endogenous induction. (Lower right branch) Overexpression of Glk removes free glucose, allowing high MalQ activity. This leads to the removal of maltotriose by the formation of larger maltodextrins, thus causing the loss of MalT activation and a reduction in *mal* gene expression. Not shown here is the action of MalZ or the synthesis of glycogen.

larger maltodextrins; Fig. 1). It is the removal of maltotriose by MalQ that is controlled by glucose. Thus, excess cytoplasmic glucose will lead to a partial MalQ$^-$ phenotype and therefore to an increase of the inducer maltotriose. In contrast, removal of glucose by glucokinase will activate MalQ and lead to the removal of the inducer maltotriose (Fig. 6). These effects have only become apparent by applying nonphysiological test conditions. Thus, *mal* gene expression was assayed by following the activity of a *malK-lacZ* fusion. The loss of MalK activity renders MalT more responsive to inducer (24) for easier measurement of *mal* gene expression. Similarly, the plasmid-encoded *glk* overexpression was used for more dramatic effects.

One could argue that with the loss of glucose phosphorylating activity in a Glk mutant (and therefore reduced MalQ activity) but wild type for all *mal* genes one should observe a reduced ability to grow on maltose. This is not the case. Only by the introduction of a second mutation removing PtsG, a strong reduction of growth on maltose is observed (13). Indeed, the inability to grow on maltose in strains lacking glucokinase and PtsG (which apparently is also able to phosphorylate cytoplasmic glucose) has been observed previously (6). We propose that it is the strong inhibition of MalQ by internally accumulated glucose (which can no longer be removed by phosphorylation) that is responsible for the growth defect on maltose.

Glucokinase affects MalT activity. The situation becomes more complicated by the observation that even a strain that lacks glycogen and can therefore no longer produce endogenous maltotriose still showed considerable *mal* gene expression (strain RD38, Table 3). Here, too, the overexpression of glucokinase reduced *mal* gene expression ~20-fold. For this to happen, either MalQ (MAD108) or MalP (RD105, GW15) or both (MAD106) had to be present (Table 3). In a mutant lacking both MalQ and MalP the overexpression of glucokinase only reduced *mal* gene expression by ca. 50% (strain GW11, Table 3). Note that a similar phenomenon was observed in a background producing glycogen (Table 3). The decisive difference there was the behavior of the MalQ$^-$ MalP$^+$ strain (strain MAD107). Whereas in a *glgA* background overexpression of glucokinase reduced *mal* gene expression nearly 20-fold (MAD108, Table 3), in the corresponding MalQ$^-$ MalP$^+$ GlgA$^+$ strain the overexpression only led to ca. 30% reduction (strain MAD107, Table 2). We interpret the difference in the two strains by the massive production of maltotriose from glycogen in strain MAD107 which apparently conteracts the effect of glucokinase overproduction. Therefore, the effect of glucokinase on *mal* gene expression must be caused at a level that is competed by the inducer maltotriose. The obvious target for glucokinase action must therefore be MalT, the central regulator of *mal* gene expression (Fig. 7). In the past, MalT has been shown to interact with several proteins (3). The interaction of these enzymes with MalT results in a strong inhibition of its activity as transcriptional regulator and in each case the inhibition is counteracted by maltotriose. In addition, The D65E mutations in *malT* that render MalT largely independent of maltotriose and resistant to the inhibition by Aes (50) also became completely resistant to the inhibition by overexpressed Glk (Table 4). Thus, we conclude that the effect of Glk overproduction falls into the same category as the other known MalT protein inhibitors. When the C-terminally truncated form of Glk (Δ305-321) was used in a strain lacking GlgA, as well as lacking Mlc, the repression was minimal (Table 6, CL19), in contrast to a strain lacking GlgA but producing Mlc (Table 6, MAD106). This demonstrates that it is the C-terminal helix of Glk that is interacting with MalT, whereas this helix is not needed for the interaction with PtsG (Fig. 3 and 5). The additional requirement of either MalQ or MalP in the effect of Glk on MalT is at present not understood. Possibly, a tripartite complex has to be formed for the inhibition to occur. It will take a major effort to biochemically elucidate this interaction with MalT. The interaction of Glk with MalT must be weak, and high concentrations of Glk must be necessary to observe the effect. pCSF2, the plasmid harboring *glk* under its natural promoter, is much less effective than pBK1 where *glk* is under the IPTG-inducible promoter. The

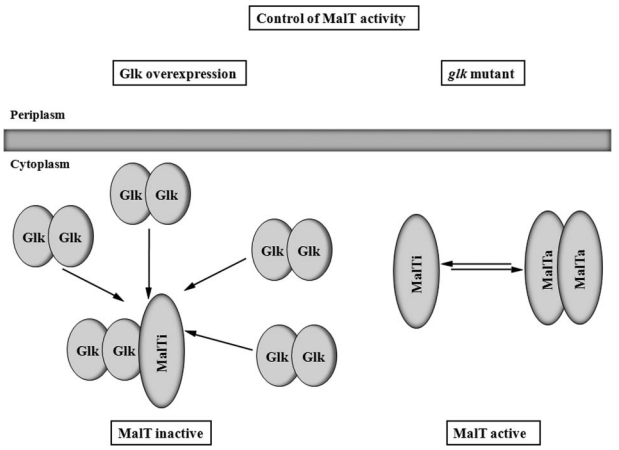

FIG. 7. Binding of Glk to MalT inhibits MalT activity. Two situations are shown. On the right-hand side, the "normal" situation is shown. MalT exists in an equilibrium of the inactive monomer and the active dimer (multimer). On the left-hand side, the overproduction of glucokinase is shown. In the proposed model, high concentrations of Glk result in the binding of Glk dimers to monomeric and inactive MalT, preventing *mal* gene expression. Not shown in this scheme is the essential role of MalP or MalQ in Glk-dependent MalT inhibition. Also not shown is the role of maltotriose in preventing the inhibition by Glk. The established inhibition of MalT by other proteins such as Aes, MalK, or MalY is not shown but was the basis for this model. Also novel is the proposal that even in the absence of the inducer maltotriose MalT can form a transcriptionally active species.

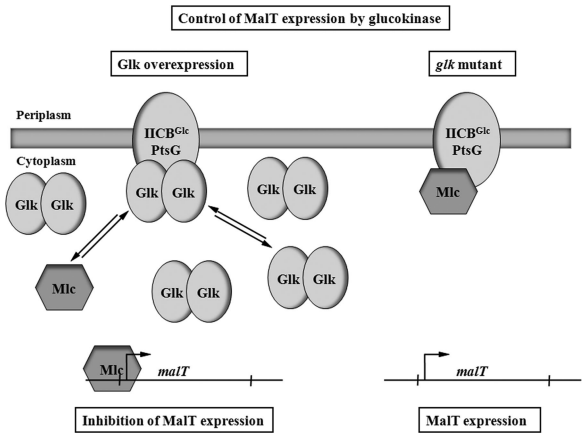

FIG. 8. The competition between Mlc and glucokinase for PtsG binding affects the expression of *malT*. Two situations are shown. On the right-hand side, the "normal" situation is shown. A certain portion of Mlc, the repressor of *malT*, is bound by PtsG and is not available for the inhibition of *malT* transcription. On the left-hand side, the overexpression of Glk is shown. The high concentration of Glk replaces Mlc on PtsG. The increased concentration of Mlc leads to the inhibition of *malT* transcription and therefore to a reduced *mal* gene expression. Not reflected in this scheme is the dependence of *malT* expression on the cAMP/CAP complex and the state of PtsG phosphorylation on binding Mlc (or Glk).

corresponding relative amounts of Glk can be estimated in Fig. 4. Preliminary attempts to coelute His-tagged MalT or MalT fragments during molecular sieve chromatography with Glk (in the presence of MalP and MalQ) have failed, presumably due to the low affinity between the two proteins.

Coming back to the effect of Glk overproduction on *mal* gene expression in a strain that is producing glycogen (Table 2), we realized that there must be additional effects concerning MalT expression and activity. We had discussed the effect of glucose on the enzymatic activity of MalQ. Removal of glucose by overproduced Glk would activate MalQ, which in turn would remove maltotriose as an inducer. After we discovered the effect of Glk overproduction in a strain lacking glycogen (Table 3), it became obvious that the effects seen in Table 2 are not only due to MalQ activation but also due to the effects of Glk on MalT activity and on the interaction with PtsG releasing Mlc as a *malT* repressor. The data of Table 2 and 3 obtained with the *glk* mutation clearly differentiate the two phenomena; whereas in the *glgA*$^+$ strain the loss of glucokinase activity has a significantly stimulating effect (MAD104 versus TB04), the *glk* mutation in the *glgA* background had hardly any effect (MAD106 versus RD38, Table 3).

Glucokinase binding to PtsG affects *malT* expression via the release of Mlc. The third level of *mal* gene regulation became visible by the effect of Glk overproduction in a strain that lacks glycogen, as well as MalP and MalQ (GW11, Table 3). We found that *mal* gene expression was reduced ca. 50% by Glk overproduction. We reasoned that Glk, due to its similar structure to Mlc, could be bound by PtsG and release Mlc that might be partially bound by PtsG (Fig. 8). Since Mlc is the transcriptional repressor of *malT*, increased Mlc concentration would lead to a reduction in *malT* transcription and therefore to a reduction in *mal* gene expression. To demonstrate the validity of this scheme, we used GW12, an *mlc* derivative of strain GW11 (Table 3). Indeed, Glk overproduction in GW12 had hardly any effect on *mal* gene expression. The effect of Glk to release Mlc from PtsG was surprising. In previous work we had shown that the state of PtsG phosphorylation (EIIB^{Glc}) is the major signaling for the binding and inactivation of Mlc. Transport of glucose would lead to EIIB^{Glc}-P dephosphorylation, the state that would be recognized by Mlc, and lead to its inactivation as a specific repressor. In the absence of glucose transport EIIB^{Glc} should be fully phosphorylated and should be unable to bind Mlc. Obviously, this picture is not quite correct. Even in the absence of glucose transport, Mlc must be able to be bound by PtsG at least to some extent since it can be competed for by binding by Glk. This conclusion is corroborated by the observation that overproduction of PtsG led to an increase in *mal* gene expression in an Mlc$^+$ strain but not in an Mlc$^-$ strain.

Previously, we had concluded that the effect of glucokinase was primarily to abolish internal glucose assumed to be involved in the formation of an alternative endogenous inducer, different from maltotriose (13). The observation that glucokinase activity is not needed for the interaction with PtsG renders this possibility no longer valid. The 16-amino-acid C-terminally truncated Glk protein encoded by *glk15*::Tn*10* (Cam) which lacks glucokinase activity is as effective for the release of Mlc from PtsG as is the wild-type Glk, whereas it is no longer able to interact with MalT.

Inducer-dependent and inducer-independent endogenous induction. When glycogen is present, there is continuous synthesis and degradation to small maltodextrins, including maltotriose, the inducer of the maltose system (42). Therefore,

glycogen-dependent endogenous induction occurs predominantly at the level of inducer concentration. In the absence of glycogen there is still considerable *mal* gene expression, but the causative relation to a distinct inducer is lacking. As we have demonstrated, glucokinase itself is controlling *mal* gene expression either by controlling MalT expression or activity. Therefore, the most likely explanation is that in vivo MalT, even in the absence of maltotriose, exhibits basal transcriptional activity. The latter is subject to various protein inhibitors. The postulation for an alternative inducer is no longer necessary.

These conclusions in regard to the regulatory role of glucokinase in *mal* gene expression were obtained under conditions of severe imbalance by the use of mutations, as well as protein overproduction, that are hardly considered physiological. One may argue that they are the product of artificial situations never occurring in vivo. However, the level of glucokinase is not constant in different carbon sources, and it is under the control of the Cra global regulator known to affect genes encoding glycolytic and gluconeogenic enzymes (27, 47). This opens a link of central metabolism to the modulation of *mal* gene expression.

There is still another level of *mal* gene expression that thus far has escaped a reasonable explanation. It is the sensitivity of glycogen-independent endogenous *mal* gene expression to the osmolarity of the medium (7, 15). As with Glk/MalP/MalQ effects on MalT activity, the osmolarity-controlled regulation is competed for by maltotriose (7). The obvious explanation that glucokinase might be either strongly induced or activated by high osmolarity could be excluded. Possibly, thus-far-unrecognized proteins induced by high medium osmolarity might interact with MalT to affect its activity in the same way as Glk/MalP/MalQ shown here.

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