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External-pH-Dependent Expression of the Maltose Regulon and *ompF* Gene in *Escherichia coli* Is Affected by the Level of Glycerol Kinase, Encoded by *glpK*

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The expression of the maltose system in *Escherichia coli* is regulated at both transcriptional and translational levels by the pH of the growth medium (pHo). With glycerol as the carbon source, transcription of *malT*, encoding the transcriptional activator of the maltose regulon, is weaker in acidic medium than in alkaline medium. *malT* transcription became high, regardless of the pHo, when glycerol-3-phosphate or succinate was used as the carbon source. Conversely, *malT* expression was low, regardless of the pHo, when maltose was used as the carbon source. The increase in *malT* transcription, associated with the pHo, requires the presence of glycerol in the growth medium and the expression of the glycerol kinase (GlpK). Changes in the level of *glpK* transcription had a great effect on *malT* transcription. Indeed, a *glpFKX* promoter-down mutation has been isolated, and in the presence of this mutation, *malT* expression was increased. When *glpK* was expressed from a high-copy-number plasmid, the *glpK*-dependent reduced expression of the maltose system became effective regardless of the pHo. Analysis of this repression showed that a *malTp1 malTp10* promoter, which is independent of the cyclic AMP (cAMP)-cAMP receptor protein (CRP) complex, was no longer repressed by *glpFKX* amplification. Thus, GlpK-dependent repression of the maltose system requires the cAMP-CRP complex. We propose that the pHo may affect a complex interplay between GlpK, the phosphotransferase-mediated uptake of glucose, and the adenylate cyclase.

In *Escherichia coli*, the level of expression of many genes is influenced by the pH of the growth medium (pHo) (11, 19). *E. coli* has a constitutive homeostatic mechanism which allows cells to maintain their internal pH between 7.4 and 7.8 over a pHo range of 5 to 8.5 (35). Within this pHo range, cells are able to sense a stimulus, triggered by the pHo, and transmit it to the target genes. Among these target genes are the maltose and the porin regulons (1, 12, 14).

ompF and *ompC* porin genes are controlled by the cognate sensor kinase EnvZ and the response regulator OmpR (22). A higher OmpR phosphate level at low pHo than at high pHo could be responsible for the pHo regulation of porin genes (14). In addition, the alternate phosphodonor acetyl phosphate may play a crucial role in the modulation of the OmpR-phosphate level and the subsequent pHo regulation of porin genes (14).

The maltose regulon of *E. coli* consists of genes encoding proteins involved in the uptake and metabolism of maltose and maltodextrins (4, 5). These genes are clustered in five transcriptional units controlled by the transcriptional activator MalT. *malT* expression is subjected to catabolite repression and then requires the presence of the cyclic AMP (cAMP)-cAMP receptor protein (CRP) complex. Recently, Mlc, a global negative regulator for the transcription of several genes whose products are involved in carbon utilization, has been found to be a repressor for *malT* (6). The expression of some operons and genes of the maltose regulon is also directly under the control of the cAMP-CRP complex. Lowering the pHo from 8 to 5 decreases *malTp* activity. This pHo effect relies on the cAMP-CRP binding (1). Indeed, with a cAMP-CRP-independent *malT* promoter, the pHo effect is not observed. *malT* pHo regulation triggers the pHo regulation of all the MalT-dependent promoters. In the absence of Mlc, the pHo regulation of *malT* is still effective (1). Recently, Eppler and Boos demonstrated that growth in tryptone broth containing glycerol reduced *malT* expression two- to threefold compared to tryptone broth without glycerol (10). To establish this repression, the enzyme IIA^{Glc}, the cAMP-CRP complex, and the phosphorylation of glycerol to *sn*-glycerol-3-phosphate (G3P) by glycerol kinase (GlpK) are all necessary, but further metabolism to dihydroxyacetone by glycerol phosphate dehydrogenase is not (10).

The glycerol regulon is organized in multiple loci around the chromosome, and its expression is negatively controlled by GlpR (17). The *glpTQ* and *glpACB* operons located near 51 min encode the G3P permease/glycerophosphodiesterase and the subunits of the anaerobic G3P dehydrogenase, respectively (9). The *glpD* gene, encoding the aerobic G3P dehydrogenase, is located near 77 min and is transcribed divergently from the *glpEGR* operon (27). GlpE is a sulfur transferase (23). *glpG* encodes a protein of unknown function, and *glpR* encodes the repressor (28, 34). The *glpFKX* operon located near 88 min encodes the glycerol diffusion facilitator (GlpF), the glycerol kinase (GlpK), and a fructose

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype[a]	Source or reference
Strains		
CGSC5023	*fhuA22 relAl T2R*	B. Bachmann, Yale University
DH5α	F$^-$ *endAl* (r_K^- m_K^+) *supE44 thi-l hsdR17 gyrA* (Nal^r) *recAl* Δ(*lacZYA-argF*)*U169 deoR relAl* [ϕ*80*Δ*lac*(*lacZ*)*M15*]	BRL, Inc
GPH1768	MC4100/pGPH1768	1
GPH8549	MC4100 Δ*cya zie296*::Tn*10 crp*∗	1
GPH8804	MC4100 *lac*$^+$	Lac$^+$ with PI/CGSC5023
GPH8805	MC4100 ϕ(*ompA-lacZ*) (Hyb)	1
GPH8818	MC4100 *malTp1 malT p10*/pGPH1768	1
GPH8840	MC4100 *iea18* (*glpFp18*)/pGPH1768	This work
GPH8881	MC4100/pGPH8881	1
GPH9244	Cured GPH8840	This work
GPH9401	GPH9244(*iea18*)/pGPH8881	This work
GPH9463	MC4100/pGPH9463	1
GPH9473	GPH9244(*iea18*)/pGPH9463	This work
GPH9604	MC4100 Φ(*ompF′-lacZ*$^+$)*7.14*	Lac$^+$ with Pl on MA2946
GPH9605	GPH9244(*iea18*) Φ(*ompF′-lacZ*$^+$)*7.14*	Lac$^+$ with Pl on MA2946
GPH9909	GPH9244(*iea18*) Φ(*ompC′-lacZ*$^+$)*10.21*	Lac$^+$ with Pl on MA2948
GPH9910	MC4100 Φ(*ompC′-lacZ*$^+$)*10.21*	Lac$^+$ with Pl on MA2948
GPH11296	MC4100 *an*λ::(*malK′-lacZ*$^+$)*1*	This laboratory
JC2296	HFr P4X *thi metB1 relA1 spoT1* Δ(*lac*)*U169*	This laboratory
MA2946	JC2296 Φ(*ompF′-lacZ*$^+$)*7.14*	12
MA2948	JC2296 Φ(*ompC′-lacZ*$^+$)*10.21*	12
MC4100	F$^-$ *araD139* Δ(*argF-lac*)*205* λ$^-$ *fthD5301 fruA25 relA1 rpsL150 rbsR22 deoC1*	M. Casadaban, University of Chicago
Plasmids		
pACT3	*tac* promoter expression vector, 5.3 kb, Cam^r (20 μg ml^{-1})	8
pACYC177	Cloning vector, 3.9 kb, Amp^r (50 μg ml^{-1}) Kan^r (50 μg ml^{-1})	26
pBR322	Cloning vector, 4.4 kb, Amp^r (50 μg ml^{-1}) Tet^r (15 μg ml^{-1})	3
pCJ102	*glpK* constitutively expressed in pBR322	20
pGPH1768	Φ(*malK′-lacZ*$^+$)*1* in pJEL250	1
pGPH8881	Φ(*malT′-lacZ*$^+$)*1* in pJEL250	1
pGPH9260	Φ(*malTpl malTp10′-lacZ*$^+$)*1* in pJEL250	1
pGPH9463	Φ(*malP′-lacZ*$^+$)*1* in pJEL250	1
pGPH9925	4,374-bp PCR product from MC4100 containing *glpFKX* cloned into *Hin*cII-cut pACYC177	This work
pGPH9928	4,374-bp PCR product from GPH8840 containing *glpFKXp18* cloned into *Hin*cII-cut pACYC177	This work
pGPH9993	*glpF*$^+$ (2,707-bp *Bst*EII deletion of pGPH11250)	This work
pGPH11247	Φ(*glpF′-lacZ*$^+$)*1* in pJEL250	This work
pGPH11248	Φ(*glpFp18′-lacZ*$^+$)*1* in pJEL250	This work
pGPH11249	*glpX*$^+$ (1,033-bp *Eco*RI/*Eco*RV deletion of pGPH9925)	This work
pGPH11250	*glpF*$^+$ *glpK*$^+$ (805-bp *Aat*II deletion of pGPH9925)	This work
pGPH11264	*glpK*$^+$ (505-bp *Eco*RI/*Rsr*II deletion of pGPH11250)	This work
pGPH11542	*glpFKX* cloned into *Sma*I/*Sac*I-cut pACT3	This work
pJEL250	Temperature-sensitive copy number; *lacZYA* operon fusion vector, 13 kb, Amp^r (30 μg ml^{-1})	29
pUC18	Cloning vector, 2.7 kb, Amp^r (50 μg ml^{-1})	33

[a] Genetic nomenclature is from the work of Berlyn (2). Kan^r, Cam^r, Amp^r, and Tet^r, resistance to kanamycin, chloramphenicol, ampicillin, and tetracycline, respectively.

1,6-biphosphatase (GlpX) (7). This operon is subjected to multiple controls, including catabolite repression mediated by cAMP-CRP and repression by cooperative binding of GlpR to tandem operator sites which overlap the promoter. G3P, the product of the reaction catalyzed by glycerol kinase, is the inducer of the *glp* regulon (17). GlpK, as well as MalK, is involved in interactions with the unphosphorylated form of enzyme IIA^{Glc}, which is an intermediate in the phosphorylation cascade of the phosphotransferase (PTS)-mediated uptake and concomitant phosphorylation of glucose. This interaction inhibits the metabolism of non-PTS-carbohydrates, such as glycerol and maltose, by preventing the induction of their respective catabolic operons (21). This process is known as inducer exclusion (15). Rohwer et al. have shown that a high level of glycerol kinase could result in IIA^{Glc} sequestration into an inactive complex (25). The phosphorylated form of IIA^{Glc} is considered to be involved in the activation of adenylate cyclase, and this leads to increased intracellular levels of cAMP, which binds to CRP and elaborates the cAMP-CRP complex (24).

As the involvement of the cAMP-CRP complex in the glycerol-dependent repression of *malT* transcription in rich medium (10) and in the pHo regulation of *malT* transcription during growth with glycerol as a carbon source (1) has been clearly established, we were interested in studying the connections between the glycerol effect and the pHo regulation of maltose regulon. This study shows that the phosphoenolpyruvate:carbohydrate PTS system and adenylate cyclase are involved in the complex molecular interplay between the different regulatory mechanisms that regulate gene expression according to the pHo.

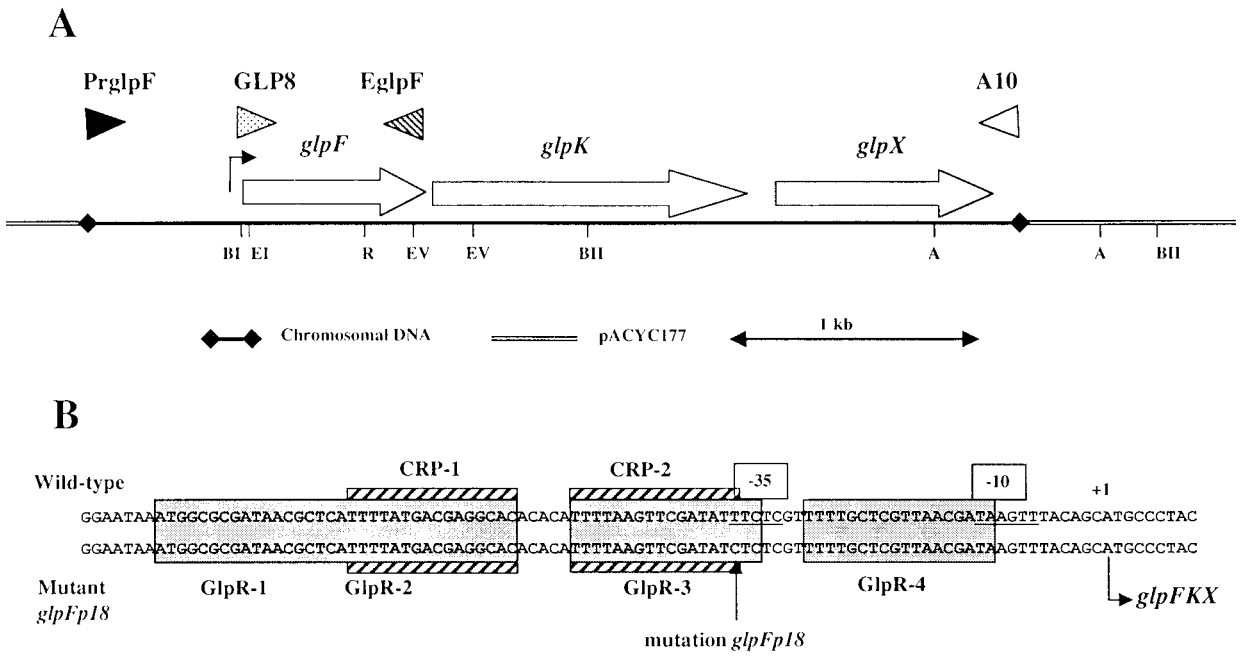

FIG. 1. Schematic representation of the *glpFKX* operon cloned in pACYC177 and sequence of the parental and mutant *glp* promoter regions. (A) Open reading frames within the *glpFKX* operon, with the direction of transcription, are indicated by open arrows. Restriction sites: EI, *Eco*RI; BI, *Bam*HI; R, *Rsr*II; EV, *Eco*RV; BII, *Bst*EII; A, *Aat*II. The *glp* transcriptional start site is indicated with an arrow upstream of *glpF* (32). The primers used in PCR amplification are indicated with triangles. (B) Grey boxes indicate the binding site of the GlpR repressor. Striped boxes indicate the cAMP-CRP binding site. The transcriptional start point is at position +1 (32). Putative −10 and −35 sequences are underlined.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Bacterial strains and plasmids used in this study are listed in Table 1. Standard media were used in this study, including Luria-Bertani (LB) broth (18), MacConkey medium with lactose (final concentration, 2% [wt/vol]) added (Difco Laboratories, Detroit, Mich.), and M63 minimal medium (18) supplemented with 3 μM thiamine hydrochloride, the appropriate amino acids, and sugars as the carbon source. Buffered minimal medium (MM) was as previously described (13). It was adjusted with 1 N NaOH to pH 4.75, 5, 5.25, 5.5, 5.75, 6.5, 7, 7.5, and 8. MM-MES is a solid MM containing 100 mM MES [2-(*N*-morpholino)ethane sulfonic acid; pK_a, 6.1], instead of 50 mM in MM and no TAPS [tris(hydroxymethyl)methylaminopropane sulfonic acid; pK_a, 8.4]. This buffered medium was adjusted to pH 5 with 1 N NaOH. MM and MM-MES were supplemented with 3 μM thiamine hydrochloride. Carbon sources used in these media were 44 mM glycerol, 12 mM G3P, 21 mM succinate, 22 mM glucose, and 58 mM maltose. When required, 40 μg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) ml^{-1} or the following antibiotics were added: 30 μg (pJEL derivatives) or 100 μg of ampicillin ml^{-1}, 50 μg of kanamycin ml^{-1}, 15 μg of tetracycline ml^{-1}, and 20 μg of chloramphenicol ml^{-1}.

Growth conditions. Overnight subcultures, grown at 30°C in LB broth supplemented with the appropriate antibiotics, were used to inoculate MM adjusted to different pHo values and supplemented with various carbon sources and antibiotics. These cultures were incubated at 30°C until the stationary phase was reached. At this point, the different buffered cultures were diluted with the same fresh medium to an optical density at 600 nm of 0.02 (path length, 1 cm; Jouan spectrophotometer). These new cultures were incubated at 30°C up to an optical density at 600 nm between 0.2 and 0.3. Up to this cell density, the pHo remains constant.

Enzyme assays. β-Galactosidase activities associated with operon or gene fusions were measured on toluenized cells, as previously described (12). One unit of β-galactosidase activity was defined as the amount of enzyme that hydrolyzed 1 nmol of substrate min^{-1}. Results from at least three independent experiments were averaged to obtain the values presented below.

PCR amplification. PCRs were performed using standard conditions (16) and *Pfu* enzyme (Promega Corp, Madison, Wis.). Primers were phosphorylated by the T4 polynucleotide kinase prior to amplification. The amplified fragments were separated by agarose gel electrophoresis, extracted (QiaEx gel purification kit; Qiagen), and ligated into the appropriate vector. The ligated DNA was transformed into *E. coli* DH5α, and antibiotic-resistant clones were isolated by growth at 37°C in LB broth containing the appropriate antibiotics.

In vitro plasmid construction. Fragments of 4,374 bp, including the *glp* promoter, were amplified from MC4100 and GPH8840 chromosomes by PCR (Fig. 1A). Primers used to amplify this fragment hybridized to a region 615 bases upstream of the transcriptional start point (+1) of *glpFKX* (PrGlpF, 5′-AGAT GAAGCGTAATCAGACC-3′) and to a region 152 bases downstream of the *glpX* last codon (A10, 5′-TGAACGGTGAAGACTAAACAG-3′). The 4,374-bp fragments were ligated into *Hin*cII-cut and dephosphorylated pACYC177. Kanamycin-resistant clones were isolated. pGPH9925 corresponds to the PCR product from MC4100, and pGPH9928 corresponds to that from GPH8840.

The *glp* promoter region was amplified from MC4100 and GPH8840 chromosomes by PCR. The primers used were PrGlpF and EGlpF (5′-GTAGTCATA TTACAGCGAAGCTT-3′) (Fig. 1A), which hybridized to a region encompassing the last codon of *glpF*. These primers gave 1,577-bp products which were digested by *Bam*HI. The 718-bp promoter fragments were separated by agarose gel electrophoresis, extracted, and ligated into *Sma*I- and *Bam*HI-digested pUC18. The 723-bp promoter fragments were excised from pUC18 with *Eco*RI and *Bam*HI and ligated into *Eco*RI- and *Bam*HI-digested pJEL250 to create pGPH11247, with the parental *glpFp* operon fusion, and pGPH11248, with the mutant *glpFp18* operon fusion.

A 3,751-bp fragment, including the *glp* operon without its promoter, was amplified from the MC4100 chromosome by PCR. Primer A10 was used in combination with primer GLP8 (5′-CATCGTGGAGCTCCGTGACTTTC-3′) (Fig. 1A). This primer hybridized to a region extending from +22 to +44 relative to the transcriptional start point. At position +32, a C was introduced instead of a G in order to create a *Sac*I site for subcloning. The 3,751-bp fragment was *Sac*I digested, and a fragment containing a promoterless *glp* operon, but with an intact Shine-Dalgarno site, was obtained. The 3,741-bp fragment was purified and ligated into *Sma*I- and *Sac*I-digested pACT3 to create pGPH11542.

In order to test the effect of each *glpFKX* gene, plasmid constructs from pGPH9925 were generated (Fig. 1A). pGPH9925 was digested with *Eco*RI and *Eco*RV, end filled with a Klenow fragment, and self-ligated to create pGPH11249, which expresses only *glpX*. pGPH9925 was digested with *Aat*II and self-ligated to create pGPH11250, which keeps only *glpF* and *glpK* intact. pGPH11250 was digested with *Eco*RI and *Rsr*II, end filled with a Klenow fragment, and self-ligated to create pGPH11264, which contains only *glpK*.

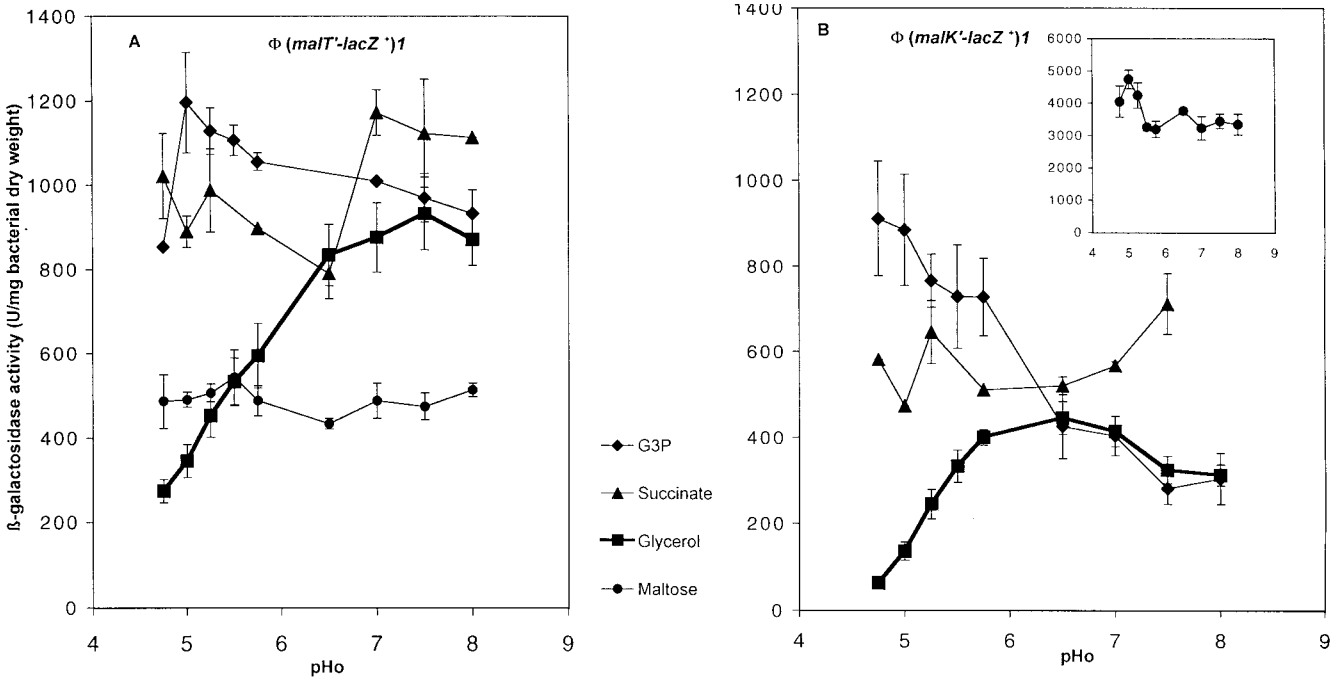

FIG. 2. pHo regulation of *malT* and *malK* during growth with different carbon sources. β-Galactosidase activity was assayed during growth at 30°C in MM adjusted to different pH values and supplemented with ampicillin and the indicated carbon source. (A) (*malT'-lacZ*$^+$*)1* operon fusion on pJEL250 (GPH8881); (B) (*malK'-lacZ*$^+$*)1* operon fusion on pJEL250 (GPH1768). (Inset) Induced *malK* expression with maltose as the carbon source. Error bars show standard deviations. The absence of error bars indicates that the deviation fell below the resolution limit of the graphing program.

pGPH11250 was digested with *Bst*EII and self-ligated to create pGPH9993, which contains *glpF* and part of *glpK*.

Nucleotide sequencing. Sequencing was carried out by Genome Express (Grenoble, France) using fluorescent dye terminator technology and then analyzed on a Applied Biosystems 373 automated sequencer.

In vivo genetic methods. Exponentially growing MC4100 cells were harvested by centrifugation, washed twice, and resuspended in 0.1 M sodium citrate (pH 5.5). *N*-Methyl-*N'*-nitrosoguanidine was added to a final concentration of 40 μg ml^{-1}. After 12 min at 37°C without shaking, cells were centrifuged and resuspended in 100 mM phosphate buffer (pH 7.0). This suspension was used to inoculate five independent expression cultures that were incubated at 37°C for at least 20 h. From each culture, electrocompetent cells were prepared and transformed with the pGPH1768 plasmid [(*malK'-lacZ*$^+$*)1* operon fusion]. After 1 h at 30°C, cells were centrifuged and resuspended in M63 medium. Appropriate dilutions were spread on MM-MES (pH 5.0) plates containing ampicillin, glycerol, and X-Gal. After 3 days at 30°C, blue colonies were picked and restreaked twice on the same medium.

Generalized transductions with P1*vir* were performed as described by Miller (18). Hfr mapping was performed using a set of Hfr Tn*10* strains (31) carrying the F plasmid at various sites scattered around the chromosome.

Curing strain from pGPH1768. An overnight GPH8840 subculture, grown in LB broth at 30°C, was used to inoculate fresh LB broth (10^3-fold dilution). These cultures were incubated at 30°C until the stationary phase was reached. Appropriate dilutions were spread on MacConkey medium with lactose added. Cultures were incubated at 30°C, and white colonies among the pink ones were picked and restreaked. The analysis showed that the white colonies did not harbor any plasmid. Strain GPH9244 was conserved for further study.

RESULTS

***malT* and *malK* pHo regulation without *glpFKX* induction.** The pHo regulation of the maltose regulon with glycerol as the carbon source was described (1). Because of the link between the *mal* and *glp* regulons, *malT* transcription and that of *malK*, according to the pHo, were compared with and without *glpFKX* expression. The *glpFKX* operon is not induced when succinate, pyruvate, lactate, ribose, or maltose is used as the carbon source, and glycerol and G3P are the carbon sources known to induce *glpFKX* expression.

When *glpFKX* was induced in the presence of glycerol (Fig. 2), *malT* and *malK* expressions were repressed at low pHo and reached higher levels as the pHo was increased. With G3P as the carbon source, *malT* expression was high regardless of the pHo (Fig. 2A), and *malK* expression was quite high at low pHo and decreased to the level observed with glycerol as the carbon source as the pHo became higher than 6.5 (Fig. 2B).

Without *glpFKX* induction, in the presence of succinate (Fig. 2), pyruvate, lactate, or ribose (data not shown), *malT* and *malK* expression was not repressed during growth in acidic medium. With maltose as the carbon source, *malT* expression was low regardless of the pHo. Thus, with these carbon sources, the maltose regulon was not pHo regulated. We hypothesize that the synthesis of GlpF, GlpK, or GlpX may induce the repression of the maltose regulon during growth in acidic medium.

The different results obtained with G3P and glycerol demonstrated that the repression of the maltose regulon by a Glp protein may be linked to the entry of glycerol via GlpF or to glycerol phosphorylation by GlpK. Further metabolism of glycerol, beyond G3P, would not be involved in maltose regulon repression during growth at low pHo.

Isolation and characterization of a mutant altered in the *glpFKX* promoter. In a mutant search aiming to identify genes whose products are involved in the putative pHo transduction pathway(s), we looked for mutants altered in the pHo regulation of the *malK* gene. MC4100 cells were treated with *N*-methyl-*N'*-nitrosoguanidine and then transformed with plas-

mid pGPH1768 carrying the (*malK'-lacZ*$^+$)*1* operon fusion. On buffered MM, adjusted to pH 5 and supplemented with X-Gal and glycerol as the carbon source, the basal (*malK'-lacZ*$^+$)*1* expression was too low for staining in blue bacterial colonies. Uninduced expression of the maltose regulon is used for expression in MM with glycerol as the carbon source. Mutants which displayed high expression of the fusion during growth at pHo 5 and in the absence of maltose were visualized as blue colonies. Seventeen independent mutants with an increased expression of *malK* in acidic medium (*iea*) were isolated. The *iea18* mutation is described in this paper.

In the parental background of strain MC4100, (*malK'-lacZ*$^+$)*1* expression (Fig. 3A) was very low at a pHo of 4.75 and increased as the pHo reached 5.5. In the *iea18* strain, uninduced (*malK'-lacZ*$^+$)*1* expression was high at low pHo and decreased to the level observed in the parental strain at high pHo (Fig. 3A). To study the effect of *iea18* on *malP* and *malT* transcription, strain GPH8840 was cured from pGPH1768 and transformed with pGPH9463 (strain GPH9473) or pGPH8881 (strain GPH9401), harboring, respectively, the (*malP'-lacZ*$^+$)*1* or the (*malT'-lacZ*$^+$)*1* operon fusion. Data from Fig. 3B show that *malP* promoter activity was high whatever the pHo in the *iea18* background and in the presence of glycerol plus maltose in the growth medium. The induced level of *malP* promoter activity was measured because its uninduced expression is too low to be detected. In the *iea18* background with glycerol as the carbon source, *malT* expression was as high, regardless of the pHo (Fig. 3C), as those observed in the presence of G3P as the carbon source (compare Fig. 3C and 2A).

The effect of the *iea18* mutation on the transcription of *ompF* and *ompC* porin genes was measured (Fig. 4). In the parental background, and with glycerol as the carbon source, *ompF* expression was higher in neutral than in acidic medium (Fig. 4A) whereas *ompC* expression was higher at low pHo than at pHo 7 (Fig. 4B). In the presence of the *iea18* mutation, *ompF* transcription was not further decreased in acidic medium, but there was no evidence of an effect on *ompC* expression.

The *iea18* mutation was associated with slower growth with glycerol as the carbon source. Hfr and P1 transduction mapping (31) showed that the mutation *iea18* was linked to a Tn*10* marker inserted close to *metF* (min 89).

To determine if the *iea18* mutation altered the *glpFKX* operon (min 88.7), chromosomal DNA from strains MC4100 and GPH8840 was isolated and used as a template in PCR amplifications with the primer pair PrGlpF-A10 (Fig. 1A) to amplify the *glpFKX* operon and its 5′ upstream region. Wild-type and mutant fragments of 4,374 bp were sequenced, and comparison of the sequences enabled us to identify a single mutation located 34 bp upstream of the transcription start site (Fig. 1B). The *iea18* mutation was renamed *glpFp18.* Such a mutation, located in the −35 region of the promoter and in the GlpR-3 binding site of GlpR and close to the CRP2 binding site of cAMP-CRP (Fig. 1B), could potentially yield an altered level of *glpFKX* operon transcription.

To test this hypothesis, transcriptional fusions of *lacZ* as the reporter gene and the wild-type *glpFp* or mutant *glpFp18* promoter were constructed. Fragments of 718 bp, from PrGlpF to the *Bam*HI site in *glpF* (Fig. 1A), were cloned in pJEL250 to yield a wild-type (*glpF'-lacZ*$^+$)*1* and a mutant (*glpFp18'-lacZ*$^+$)*1* operon fusion (pGPH11247 and pGPH11248, respectively). During growth in MM with glycerol as the carbon source, the *glpFp18* mutation reduced, regardless of the pHo, the β-galactosidase activity of the corresponding fusion by a factor of 10 to 12, compared to the wild-type promoter (Fig. 5).

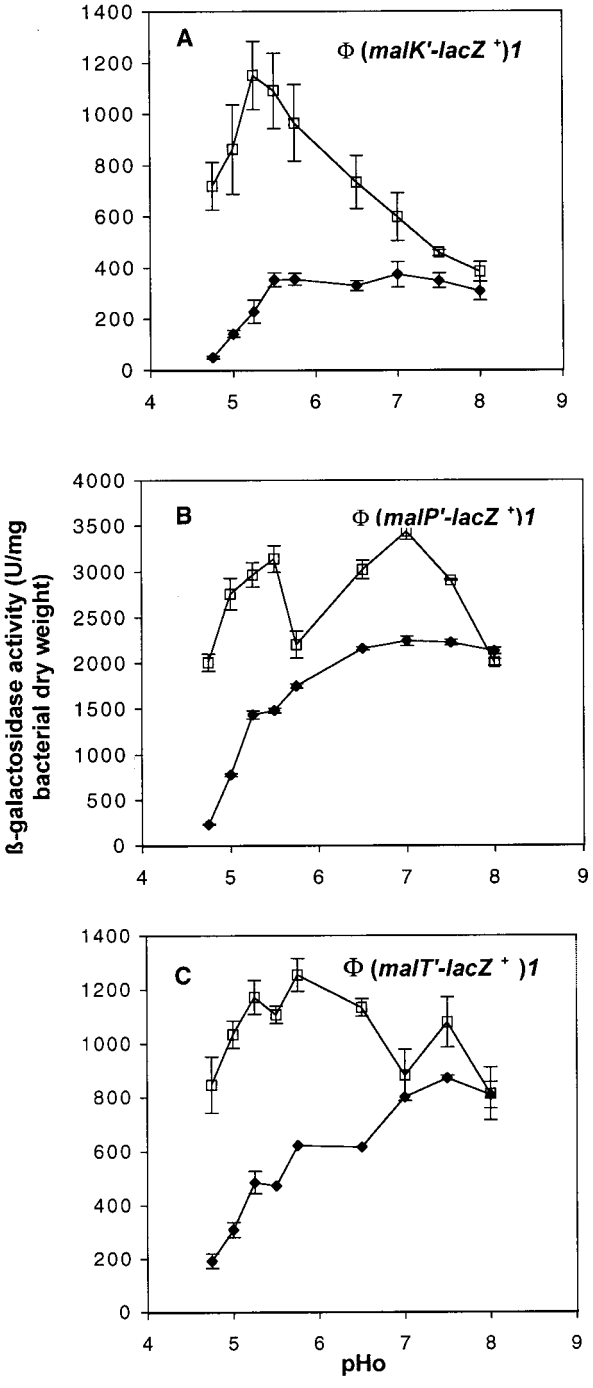

FIG. 3. Influence of the *iea18* mutation on *malK*, *malP*, and *malT* pHo regulation. β-Galactosidase activity was assayed during growth at 30°C in MM adjusted to different pHs and supplemented with ampicillin and glycerol (A and C) or glycerol and maltose (B). ◆, parental strain; □, *iea18* strain. (A) (*malK'-lacZ*$^+$)*1* operon fusion on pJEL250 (GPH1768 and GPH8840); (B) (*malP'-lacZ*$^+$)*1* operon fusion on pJEL250 (GPH9463 and GPH9473); (C) (*malT'-lacZ*$^+$)*1* operon fusion on pJEL250 (GPH8881 and GPH9401). Error bars are as in Fig. 2.

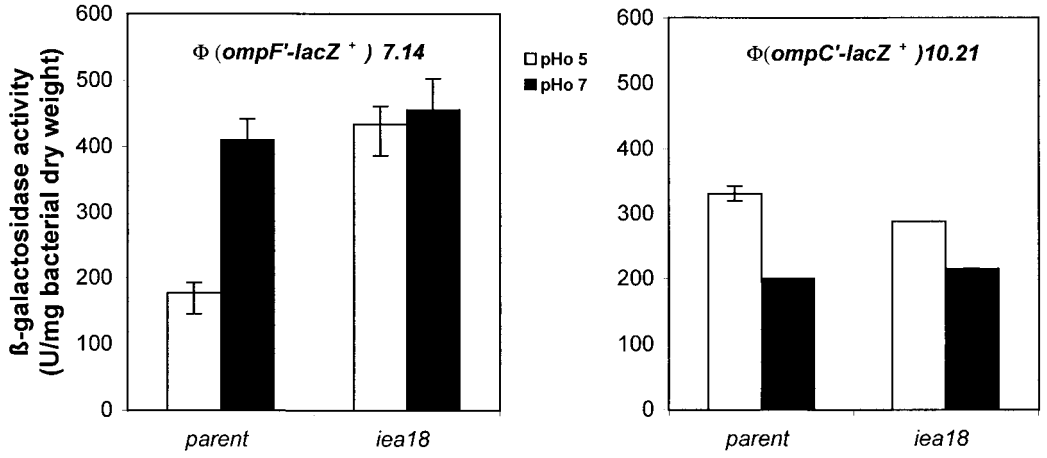

FIG. 4. *ompF* and *ompC* expression at pHo 5 and 7 in parental and *iea18* strains. Strains were grown at 30°C in MM adjusted to pH 5 or 7 and supplemented with glycerol as the carbon source. (A) GPH9604 parental strain and GPH9605 *iea18* strain; (B) GPH9910 parental strain and GPH9909 *iea18* strain. Error bars are as in Fig. 2.

glpFp18 is a promoter-down mutation of the *glpFKX* operon. Our results also indicate that growth at neutral or higher pHo, with glycerol as the carbon source, stimulated the transcription initiated at both *glpFp* (Fig. 5A) and *glpFp18* (Fig. 5B). Thus, the *glpFKX* operon belongs to the pH stimulon.

These results show that the *glpFp18* mutation leading to a decrease in *glpFKX* expression is responsible for an increase in *ompF*, *malT*, and MalT-dependent gene expression during growth in acidic medium. These results confirm that at least one of the proteins encoded by the *glpFKX* operon and expressed at the wild-type level could contribute to the repression, directly or indirectly, of *mal* and *ompF* gene expression during growth at low pHo.

The amplification of *glpK* in the cell decreases *mal* gene expression. To further analyze the involvement of proteins encoded by *glpFKX* in the repression of the maltose regulon, the *glpFKX* copy number was increased in the wild-type strain by introducing pGPH9925. The β-galactosidase activities

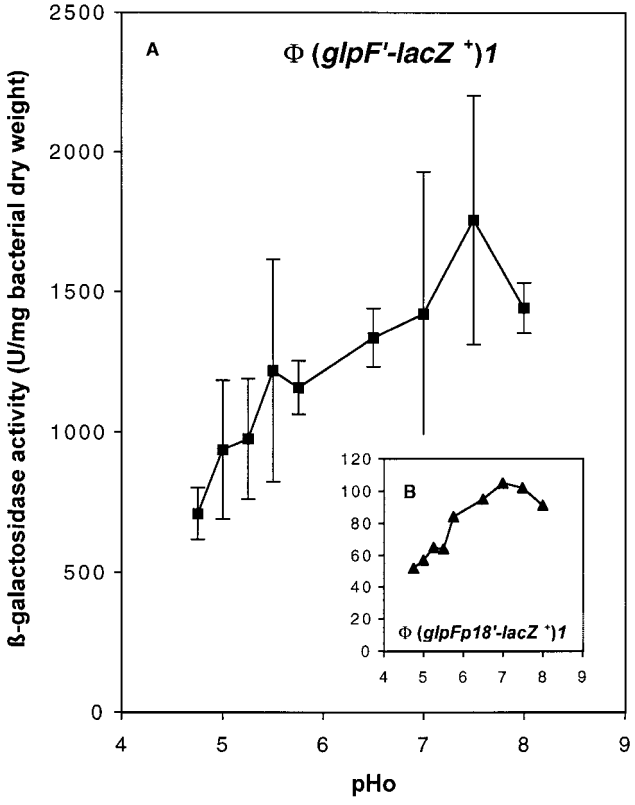

FIG. 5. Parental and mutant *glp* promoter activities according to the pHo. β-Galactosidase activity was assayed during growth at 30°C in MM adjusted to different pHs and supplemented with glycerol and ampicillin. (A) (*glpF'-lacZ*$^+$)*1* operon fusion on pJEL250 (pGPH11247 in MC4100); (B) (*glpFp18'-lacZ*$^+$)*1* operon fusion on pJEL250 (pGPH11248 in MC4100). Error bars are as in Fig. 2.

TABLE 2. Repression of the maltose regulon when *glp* genes are amplified

Fusion and introduced plasmid[a]	β-Galactosidase activity[b]		Repression by *glp*[c]	
	pHo 5	pHo 7	pHo 5	pHo 7
Φ(*malK'-lacZ*$^+$)*1* (pGPH1768)				
pACYC177	189 ± 11	339 ± 51		
pGPH9925 (*glpFKX*)	VL	VL	Full	Full
pGPH11250 (*glpFK*)	VL	VL	Full	Full
pGPH9993 (*glpF*)	136 ± 18	328 ± 29	1.4	1
pGPH11264 (*glpK*)	VL	90 ± 32	Full	3.8
pGPH11249 (*glpX*)	153 ± 40	295 ± 74	1.2	1.1
Φ(*malT'-lacZ*$^+$)*1* (pGPH8881)				
pACYC177	417 ± 86	593 ± 41		
pGPH9925 (*glpFKX*)	162 ± 35	194 ± 25	2.6	3.1
pGPH11250 (*glpFK*)	150 ± 21	244 ± 58	2.8	2.4
pGPH9993 (*glpF*)	328 ± 7	657 ± 53	1.3	1.1
pGPH11264 (*glpK*)	128 ± 37	232 ± 34	3.3	2.6
pGPH11249 (*glpX*)	395 ± 58	656 ± 93	1.1	0.9
Φ(*malP'-lacZ*$^+$)*1* (pGPH9463)				
pACYC177	1,261 ± 21	2,321 ± 163		
pGPH9925 (*glpFKX*)	ND	1,116 ± 26		2.1

[a] Fusions were in an MC4100 background in pJEL250.

[b] β-Galactosidase activity was assayed during growth at 30°C in MM adjusted to pH 5 or 7 and supplemented with glycerol [and maltose for Φ(*malP'-lacZ*$^+$)*1*], ampicillin, and kanamycin. ND, not done; VL, very low β-galactosidase activity (below the sensitivity threshold of the method used).

[c] Repression values correspond to the ratio between β-galactosidase activities of the strain with pACYC177 and the strain with amplified *glp* genes.

TABLE 3. Repression of *malK* when the glycerol regulon is controlled by an IPTG-inducible *tac* promoter

Carbon source	β-Galactosidase activity[a] with introduced plasmid		Repression by *glp*[b]
	pACT3	pGPH11542 (*glpFKX*)	
Glycerol	120 ± 13	24 ± 7	5
Maltose	2,870 ± 31	1,177 ± 231	2.4
G3P	92 ± 33	18 ± 7	5.1
Ribose	661 ± 47	36 ± 3	18.4
Succinate	177 ± 54	30 ± 7	5.9
Mannose	237 ± 18	112 ± 6	2.1
Glucose	27 ± 7	55 ± 10	0.5

[a] β-Galactosidase activity of a strain with Φ(*malK′-lacZ*$^+$)*1* in a GPH11296 background was assayed during growth at 30°C in MM adjusted to pH 7 and supplemented with the indicated carbon source (0.4%) and chloramphenicol. IPTG (5 or 10 μg/ml) was added to the medium.

[b] Repression values correspond to the ratio between β-galactosidase activities of the strain with pACT3 and the strain with pGPH11542.

of strains harboring either the (*malK′-lacZ*$^+$)*1*, the (*malT′-lacZ*$^+$)*1*, or the (*malP′-lacZ*$^+$)*1* operon fusion, in the presence of multiple copies of *glpFKX*, are shown in Table 2. At pHo 5 and 7, as GlpF, GlpK, and GlpX were overexpressed (pGHP9925), *malK* was fully repressed and *malT* and *malP* expression was reduced by factors of 3.1 and 2.1.

In order to determine which gene of the *glpFKX* operon has to be amplified to exert the repressional effect on the maltose regulon, internal deletions have been carried out in the *glpFKX* operon with the *glpFKX* promoter kept intact (Table 2). Whatever the pHo, *glpF* (pGPH9993) or *glpX* (pGPH11249) amplification did not reduce maltose gene expression. The repression of maltose genes was still established in the presence of GlpF and GlpK when the *glpX* gene was deleted. The amplification of *glpK* alone triggers *malK* and *malT* repression. Thus, GlpK is the protein playing the key role in the repression observed, even if the strongest repression was observed when the full *glpFKX* operon was amplified (full repression compared to 3.8-fold).

To investigate whether the repression was linked to GlpK itself or to its kinase activity on glycerol, a promoterless *glpFKX* operon was cloned under the IPTG (isopropyl-β-D-galactopyranoside)-inducible *tac* promoter of pACT3, resulting in pGPH11542. This construct allows *glpFKX* expression without the presence of glycerol in the growth medium. It was introduced into a strain with the (*malK′-lacZ*$^+$)*1* operon fusion on the chromosome at *att*λ. Table 3 shows β-galactosidase production by the strain carrying the (*malK′-lacZ*$^+$)*1* operon fusion with different carbon sources in the presence of IPTG. Without IPTG added to the growth medium, *malK* expression was not affected by the presence of pACT3 or pGPH11542 (data not shown). In the presence of IPTG, whatever the carbon source (with the exception of glucose), repression of *malK* was observed when *glpFKX* was present (5-fold with glycerol, 2.4-fold with maltose, 5.1-fold with G3P, 18.4-fold with ribose, 5.9-fold with succinate, and 2.1-fold with mannose). These data demonstrate that the maltose regulon repression by GlpK was not specific to growth with glycerol as the carbon source. Thus, neither phosphorylation of exogenous glycerol by GlpK nor glycerol metabolism would be required for repression.

Repression of the maltose regulon required the cAMP-CRP complex. The involvement of the cAMP-CRP complex in the repression of the maltose regulon by a high level of GlpK was characterized using strains carrying both *malTp1* and *malTp10* mutations in the control region of *malT*. These mutations render *malT* expression cAMP-CRP independent. Table 4 shows the effect of *glpFKX* amplification on a (*malTp1 malTp10′-lacZ*$^+$)*1* operon fusion and on a (*malK′-lacZ*$^+$)*1* operon fusion with the *malTp1 malTp10* mutations introduced into the control region of the chromosomal *malT* gene. The repression became residual on *malK* (1.7-fold instead of full repression) and was cancelled on *malT* (0.6- instead of 3-fold). The repression observed on *malK* would be a consequence of the repression exerted on *malT* expression. These data demonstrate that binding of cAMP-CRP to the *malT* promoter was required for *mal* genes to be repressed by *glpFKX* amplification.

The use of Δ*cya crp** derivatives allows the expression of cAMP-CRP-dependent genes independently of the amount of cAMP present in the cell. A *crp** mutation affects the coding sequence of *crp* and enables the Crp* protein to activate cAMP-CRP-dependent promoters, even in the absence of cAMP. In the Δ*cya crp** background, *malK* expression was 2-fold repressed instead of fully repressed and *malT* expression became 1.2-fold repressed by *glpFKX* instead of 3-fold (Table 4). The data show that the repression exerted by *glpFKX* amplification is cancelled when *malT* expression is no longer dependent on the amount of cAMP.

Is the repression exerted by *glpFKX* amplification restricted to *mal* genes? We were interested in establishing whether the repression observed with the amplification of *glpFKX* operates for all pHo-regulated genes. Table 5 shows that *glpFKX* amplification lowered *ompF* transcription twofold while *ompC* expression was not significantly modified. These results agree with an increased *ompF* expression in acidic medium in the presence of the *glpFp18* mutation (Fig. 4A). *ompA* expression, which was previously reported to be pHo independent, was not repressed by *glpK* amplification (Table 5). However, lactose

TABLE 4. Genetic analysis of *malT* and *malK* repression by *glpFKX* amplification

Fusion[a] tested and genetic background	Plasmid introduced	β-Galactosidase activity[b]	Repression by *glp*[c]
Φ(*malK′ -lacZ*$^+$)*1*			
MC4100	pACYC177	339 ± 51	
	pGPH9925 (*glpFKX*)	VL	Full
GPH8818 (*malTp1 malTp10*)	pACYC177	377 ± 77	
	pGPH9925 (*glpFKX*)	220 ± 57	1.7
GPH8549 (*cya crp**)	pACYC177	122 ± 10	
	pGPH9925 (*glpFKX*)	60 ± 11	2
Φ(*malT′-lacZ*$^+$)*1*			
MC4100	pACYC177	593 ± 41	
	pGPH9925 (*glpFKX*)	194 ± 25	3
GPH8549 (*cya crp**)	pACYC177	404 ± 95	
	pGPH9925 (*glpFKX*)	345 ± 71	1.2
Φ(*malTpl malTp10′-lacZ*$^+$)*1*, MC4100	pACYC177	720 ± 137	
	pGPH9925 (*glpFKX*)	1,145 ± 250	0.6

[a] The indicated operon fusion on pJEL250.

[b] β-Galactosidase activity was assayed during growth at 30°C in MM adjusted to pH 7 and supplemented with glycerol, ampicillin, and kanamycin. VL, very low β-galactosidase activity (below the sensitivity threshold of the method used).

[c] Repression values correspond to the ratio between β-galactosidase activities of the indicated fusion in the presence of pGPH9925 and in the presence of pACYC177.

TABLE 5. Effect of *glpFKX* amplification on *ompF, ompC, ompA*, and *lacZ* expression

Fusion or gene (in MC4100) tested	Plasmid introduced	β-Galactosidase activity[a]	Repression by *glp*[b]
Φ(*ompF′-lacZ*$^+$)*7.14*	pACYC177	372 ± 82	
	pGPH9925 (*glpFKX*)	191 ± 20	2
Φ(*ompC′-lacZ*$^+$)*10.21*	pACYC177	160 ± 42	
	pGPH9925 (*glpFKX*)	209 ± 30	0.8
Φ(*ompA′-lacZ*$^+$) (Hyb)	pACYC177	81 ± 4	
	pCJ102 (*glpK*)	76 ± 18	1
lacZ	pACYC177	4,520 ± 400[c]	
	pGPH9925 (*glpFKX*)	2,497 ± 71[c]	1.8

[a] β-Galactosidase activity was assayed during growth at 30°C in MM adjusted to pH 7 and supplemented with glycerol and appropriate antibiotics.
[b] Repression values correspond to the ratio of β-galactosidase activities produced by the indicated operon fusion without *glp* amplification to the activities produced with *glp* amplification.
[c] 1 mM IPTG was added to the medium.

operon expression, which is also known to be pHo independent, was repressed by a factor of 1.8 when *glpFKX* was amplified. Thus, the repression exerted by *glpFKX* amplification is neither specific to pHo-regulated genes (*lac* operon) nor effective on all pHo-regulated loci (*ompC* gene).

DISCUSSION

The results presented in this paper demonstrate that *mal* and *ompF* gene expression is correlated with *glpFKX* expression levels. A mutation that lowers *glpFKX* promoter activity has been isolated. This mutation allows an increased expression of *malT*, MalT-dependent genes, and *ompF* during growth in acidic medium. Conversely, *glpFKX* amplification repressed this set of genes. Our experiments show that GlpK is the protein which is involved, directly or indirectly, in this repression.

We attempted to discover whether glycerol phosphorylation was required for establishing this repression. The cloning of an IPTG-inducible promoter in front of a promoterless *glpFKX* operon allowed us to overexpress this operon without glycerol or G3P in the growth medium, and this demonstrated that repression of the maltose system occurred even without exogenous glycerol. This repression was strongest with ribose as the carbon source and no longer occurred with glucose as the carbon source. Thus, GlpK in excess exerted its effect independently of glycerol phosphorylation. Conversely, our experiments show that repression of *malT* transcription at low pHo was abolished with G3P as the carbon source, even when *glpK* expression was induced. These data indicate that repression at low pHo of *malT* transcription would require both glycerol and GlpK.

Eppler and Boos (10) previously analyzed the glycerol-dependent repression of *malT* transcription in rich medium. They demonstrated that glycerol needs to be phosphorylated to G3P but no further metabolism of G3P is required to establish repression. Repression is controlled by the level of IIA^{Glc} phosphorylation and, consequently, by the cAMP level. In this work, we show that the cAMP-CRP complex is also involved in the *glpFKX* amplification-dependent repression of *malT* transcription. The cAMP-CRP complex is linked to GlpK via the enzyme IIA^{Glc} of the PTS. Indeed, glycerol kinase together with glycerol formed a complex with the unphosphorylated form of enzyme IIA^{Glc} (25). As the phosphorylated form of IIA^{Glc} is thought to stimulate adenylate cyclase (21), we propose that *malT* repression by *glpFKX* amplification could be mediated by modification of the cAMP level. Overproduction of GlpK in the cell may titrate the unphosphorylated form of IIA^{Glc} and avoid its phosphorylation. A smaller amount of phosphorylated IIA^{Glc} would lead to weaker adenylate cyclase activation. In addition, the amount of cAMP would become too limited to fully activate a cAMP-dependent gene, such as *malT*. To test this hypothesis, we are currently investigating the phosphorylation state of IIA^{Glc} according to the amount of GlpK present and the effect of *glpFKX* amplification without IIA^{Glc} in the cell (*crr* background).

As the level of *mal* gene expression was shown to be linked to the amount of GlpK, we searched for a potential link between an increased amount of GlpK in acidic medium and maltose gene pHo regulation. We have shown that *glpK* expression is modulated by the pHo but with a lower *glpFKX* promoter activity at low pHo than at high pHo. Thus, if it exists, the link between the amount of GlpK and maltose gene pHo regulation would be indirect. In acidic medium, the weaker *glpFp18* promoter allowed the alleviation of *malT* repression that occurs in the parental background. This result indicates an interplay between *malT* pHo regulation and GlpK. This interplay could be at the level of cAMP synthesis. Thus, we formulated the hypothesis that *malT* transcription may follow the cAMP levels in the cell that would, in turn, change with the pHo. However, a decrease in *malT* expression driven by a smaller amount of cAMP-CRP could be demonstrated only if the amount of complex becomes too small to fully activate the promoter. As long as there is enough cAMP-CRP to bind to the low-affinity CRP-binding sites in front of *malT*, then *malTp* would be fully activated. In the wild-type strain, with glycerol in the growth medium, GlpK is induced and interacts with IIA^{Glc}, leading to low cAMP synthesis. The effect of pHo on the amount of cAMP would be observed as pHo regulation of *malT* expression. With the *glpFp18* mutation, small amounts of GlpK are produced and more IIA^{Glc} would be converted to its phosphorylated form, leading to enough cAMP, whatever the pHo, to fully activate *malT* transcription. With maltose as the carbon source, the level of *malT* transcription was low regardless of the pHo. This low expression level could be explained by the same mechanism. Indeed, induced MalK would be able to interact, as does GlpK, with IIA^{Glc}, limiting its conversion to the phosphorylated form. With maltose, no pHo regulation would be seen because the IIA^{Glc} affinity for MalK is higher than that for GlpK (30) and the pHo would have no effect on a weakly active adenylate cyclase.

In conclusion, GlpK is not directly responsible for pHo regulation. However, it may contribute in releasing the pHo effect on adenylate cyclase through its interaction with IIA^{Glc}, leading to a level of cAMP below that required for full *malT* activation. pHo could also influence the phosphorylation state of the enzyme IIA^{Glc} or the interactions of IIA^{Glc} with adenylate cyclase by unknown mechanisms. We are currently investigating these hypotheses.

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