Expression of *galP* and *glk* in a *Escherichia coli* PTS Mutant Restores Glucose Transport and Increases Glycolytic Flux to Fermentation Products

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Abstract: In *Escherichia coli,* the uptake and phosphorylation of glucose is carried out mainly by the phosphotransferase system (PTS). Despite the efficiency of glucose transport by PTS, the required consumption of 1 mol of phosphoenolpyruvate (PEP) for each mol of internalized glucose represents a drawback for some biotechnological applications where PEP is a precursor of the desired product. For this reason, there is considerable interest in the generation of strains that can transport glucose efficiently by a non-PTS mechanism. The purpose of this work was to study the effect of different gene expression levels, of galactose permease (GalP) and glucokinase (Glk), on glucose internalization and phosphorylation in a *E. coli* PTS$^-$ strain. The W3110 PTS$^-$, designated VH32, showed limited growth on glucose with a specific growth rate (μ) of 0.03 h^{-1}. A low copy plasmid family was constructed containing *E. coli galP* and *glk* genes, individually or combined, under the control of a *trc*-derived promoter set. This plasmid family was used to transform the VH32 strain, each plasmid having different levels of expression of *galP* and *glk*. Experiments in minimal medium with glucose showed that expression of only *galP* under the control of a wild-type *trc* promoter resulted in a μ of 0.55 h^{-1}, corresponding to 89% of the μ measured for W3110 (0.62 h^{-1}). In contrast, no increase in specific growth rate (μ) was observed in VH32 with a plasmid expressing only *glk* from the same promoter. Strains transformed with part of the plasmid family, containing both *galP* and *glk* genes, showed a μ value similar to that of W3110. Fermentor experiments with the VH32 strain harboring plasmids pv1Glk1GalP, pv4Glk5GalP, and pv5Glk5GalP showed that specific acetate productivity was twofold higher than in W3110. Introduction of plasmid pLOI1594, coding for pyruvate decarboxylase and alcohol dehydrogenase from *Zymomonas mobilis,* to strain VH32 carrying one of the plasmids with *galP* and *glk* caused a twofold increase in ethanol productivity over strain W3110, also containing pLOI1594. © 2003 Wiley Periodicals, Inc. *Biotechnol Bioeng* **83**: 687–694, 2003.

Keywords: glucose phosphotransferase transport system; phosphoenolpyruvate; ethanol; galactose permease; glucokinase

INTRODUCTION

Metabolic engineering has been defined as the purposeful modification of cellular activities with the aim of strain improvement. Enzymatic, transport, and regulatory activities are the potential targets for modification using the tools of genetic and protein engineering (Bailey, 1991). Presently, the majority of efforts for bacterial strain improvement by metabolic engineering have focused on modifying enzyme activities or their properties. It is noteworthy that, despite the important role that transport systems play in bacterial physiology, compared with enzyme function modifications, relatively few examples exist wherein transport functions have been modified directly in an attempt to improve the characteristics of a production strain. In the case of *Escherichia coli,* reported works have focused on modifying the sugar import capacity of the cell (Chatterjee et al., 2001; Chen et al., 1997; Chou et al., 1994; Flores et al., 1996; Hernández-Montalvo et al., 2001; Lindsay et al., 1995; Snoep et al., 1994). These efforts have relied, usually with other genetic modifications, on the elimination or modification of the functions of the glucose phosphotransferase system (PTS). This system belongs to the group translocator family of transporters, which are widespread in bacteria and absent in Archaea and eukaryotic organisms (Saier, 2000). PTS participates in transport and phosphoenolpyruvate (PEP)-dependent phosphorylation of several sugars. The system is composed of the soluble and non–

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sugar-specific protein components enzyme I and HPr. These proteins relay a phosphate group from PEP to sugar-specific enzymes IIA and IIB. The last component of this system, IIC, is an integral membrane permease that recognizes and transports the sugar molecules, which are phosphorylated by component IIB. The glucose-specific PTS proteins are soluble IIA^{Glc} and membrane-bound $IICB^{Glc}$ permease (see Fig. 1A). In addition to its role in sugar transport, PTS is also involved in the regulation of several cellular processes such as catabolite repression and chemotaxis (Postma et al., 1996). This regulation is mediated primarily by the IIA^{Glc} protein. Therefore, PTS forms part of a global regulatory network controlling the capability of cells to find, select, transport, and metabolize several types of carbon sources. It would then be expected that inactivation of PTS should have wide-ranging effects on bacterial physiology.

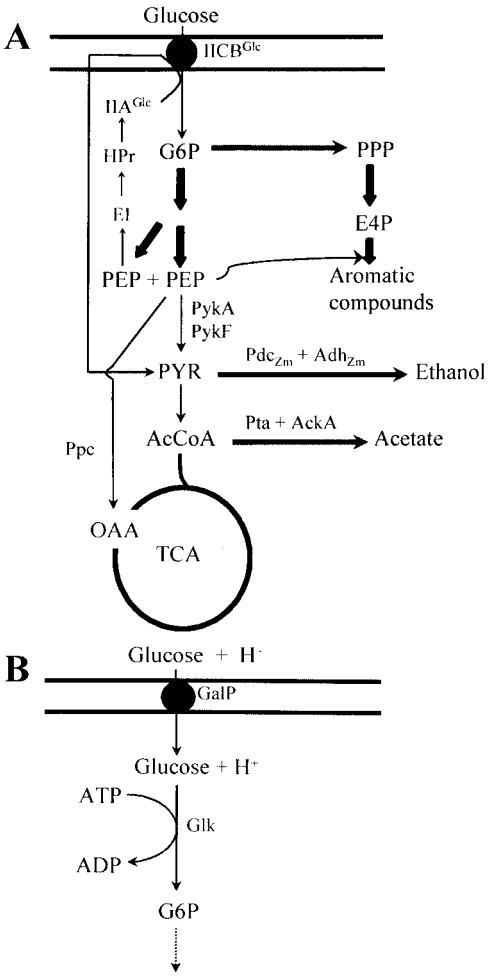

Figure 1. (A) Scheme of some of the metabolic pathways involved in glucose assimilation and acetate and ethanol formation. Solid arrows indicate multiple enzymatic reactions. G6P, glucose-6-phosphate; PEP, phosphoenolpyruvate; PPP, pentose phosphate pathway; E4P, eritrose-4-phosphate; PYR, pyruvate; AcCoA, acetyl-CoA; Ppc, phosphoenolpyruvate carboxylase; TCA, trycarboxylic acids cycle; PykA and PykF, the two pyruvate kinase isozymes present in *E. coli;* Pdc_{Zm}, pyruvate decarboxylase from *Zymomonas mobilis;* Adh_{Zm}, alcohol dehydrogenase from *Z. mobilis;* Pta, phosphotransacetylase; AckA, acetate kinase. (B) Glucose import and phosphorylation reactions in a PTS^- $glucose^+$ strain. GalP, galactose permease; EI, PTS enzyme I; HPr, PTS HPr enzyme; IIA^{Glc}, glucose specific PTS soluble component; $IICB^{Glc}$, glucose specific PTS membrane component.

With the purpose of determining the effect of PTS inactivation on the physiology of *E. coli,* we have generated mutants with an inactive PTS (PTS^- $glucose^-$ phenotype). These mutants were then subjected to a continuous culture selection method that allowed the isolation of revertants, with the capacity to obtain growth rates similar to that of a wild-type strain using glucose as carbon source (PTS^- $glucose^+$ phenotype) (Flores et al., 1996). It has been shown that, compared with the wild-type, these revertant mutants can achieve a higher metabolic flux and yield from glucose for the synthesis of aromatic compounds (Baez et al., 2001; Flores et al., 1996; Gosset et al., 1996). These results indicate that glucose internalization is not dependent on PEP consumption in PTS^- $glucose^+$ strains. It was also found that glucose catabolite repression of arabinose and xylose utilization is reduced in these strains. Therefore, these strains can assimilate sugar mixtures faster than a PTS^+ strain (Hernández-Montalvo et al., 2001).

Although the continuous culture selection method for isolating $glucose^+$ revertants has proved to be successful for some *E. coli* strains, for other strains it has not been possible to obtain PTS^- $glucose^+$ revertants using this process (unpublished results). Furthermore, it has been found that PTS^- $glucose^+$ revertants isolated from the continuous culture selection process can have very different metabolic characteristics (Flores et al., 2002). Finally, comparisons between several lines of PTS^- $glucose^+$ and isogenic wild-type strains have revealed that the former have a lower glucose transport capacity (Baez et al., 2001; Flores et al., 2002).

In this study, using a PTS^- $glucose^-$ strain, we explore the individual and combined roles of GalP and Glk in replacing glucose transport and phosphorylation. These two genes were expressed at different levels by using a set of *trc*-derived promoters of different strength. The second part of this work consists of the utilization of the promoter set to identify GalP and Glk levels that enabled a PTS^- strain to sustain high growth rates, and increase glycolytic flux to acetate or ethanol.

MATERIALS AND METHODS

E. coli Strains and Plasmids

The strains genotype and plasmids used are shown in Table I. TOP10 and XL1 blue strains were used for the propagation and amplification of the plasmids used in this work. The W3110 strain was used as host to evaluate the modulated expression of glucokinase. VH32, a PTS^- $LacI^-$ $LacZ^-$ W3110 derivative strain, was used to evaluate the roles of GalP and Glk in replacing glucose transport and phosphorylation previously provided by PTS. A VH32 derivative was also used to determine ethanol productivity in a PTS^- background. Cells were grown in Luria–Bertani

Table I. Strains and plasmids used and generated in this work.

Strain/plasmid	Characteristics	Reference
Strains		
TOP10	F⁻*mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*ZΔ M15 Δ*lac*X74*rec*A1*deo*R *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G	Invitrogen (Carlsbad, CA)
XL1 blue	*sup*E44, *hsd*R17, *rec*A1, *end*A1, *gyr*A46 (Nal^R), *thi, rel*A1, *lac*⁻ F′[*tra*D36 *pro*AB$^+$ *lac*I^q *lac*ZΔM15, Tn10 (Tet^R)]	Stratagene (La Jolla, CA)
NF9	PTS⁻ glucose$^+$ derivative of PB103, selected by a continuous culture method	Flores et al. (1996)
W3110	F⁻ λ⁻ INV(*rrn*D-*rrn*E)1	Jensen (1993)
VH30	W3110 Δ*ptsH, ptsI, crr*::km^R, glucose⁻	This work
VH31	VH30 Δ*lacI, lacZ*::Cat2	This work
VH32	VH30 Δ*lacI, lacZ*::*lox*P	This work
Plasmids		
pTrc99A	pKK233-2 derivative that has the *trc* promoter cloned before a multicloning site and the transcriptional terminator *rrn*B	Pharmacia Biotech
pCL1920	pSC101 derivative that confers spectinomycin resistance (50 μg/mL) in *Escherichia coli*	Lerner and Inouye (1990)
pvLac	R6K (*ori*γ) vector used to obtain the deletion of *LacI* and *LacZ* genes in the PTS-derivative	This work
pLOI1594	pUC19 derivative that harbors the *pdc* and *adhB* genes under the regulation region P_{zm4}	Martinez et al. (1999)
pTrc99A derivatives		
pTrcGlk	It harbors the fusion of the *glk* gene to *trc1*	This work
pTrcGalP	It harbors the fusion of the *galP* gene to *trc1*	This work
pCL1920 derivatives		
pCLv1Glk	It harbors the *glk* fusion to the promoter *trc1*	This work
pCLv2Glk	It harbors the *glk* fusion to the promoter *trc2*	This work
pCLv3Glk	It harbors the *glk* fusion to the promoter *trc3*	This work
pCLv4Glk	It harbors the *glk* fusion to the promoter *trc4*	This work
pCLv5Glk	It harbors the *glk* fusion to the promoter *trc5*	This work
pCLv1GalP	It harbors the *galP* fusion to the promoter *trc1*	This work
pCLv5GalP	It harbors the *galP* fusion to the promoter *trc5*	This work
pv1Glk1GalP	It harbors the fusion of *glk* to *trc1* and the *galP* fusion to *trc1*	This work
pv1Glk5GalP	Equal to pv1Glk1GalP, but the *galP* gene is fused to *trc5*	This work
pv4Glk5GalP	Equal to pv1Glk1GalP, but *glk* gene is fused to *trc4* and *galP* gene is fused to *trc5*	This work
pv5Glk1GalP	Equal to pv1Glk1GalP, but *glk* gene is fused to *trc5*	This work
pv5Glk5GalP	Equal to pv1Glk1GalP, but *glk* and *galP* genes are fused to *trc5*	This work

(LB) broth or LB agar plates (Maniatis et al., 1982) for all the recombinant DNA techniques. Antibiotics, when necessary, were used in the selection, propagation, and culture media at final concentrations of: carbenicillin, 200 μ/mL; spectomycin, 50 μ/mL; ampicillin, 200 μ/mL; and kanamycin, 33 μ/mL.

Construction of *pts*⁻ Strain VH30 and Its *lacI*⁻ *lacZ*⁻ Derivative VH32

A *ptsI*⁻ *ptsH*⁻ *crr*⁻ *lacI*⁻ *lacZ*⁻ derivative of W3110, was constructed in three steps. First, the *ptsI*⁻, *ptsH*⁻, *crr*⁻::Km deletion was transferred by P1 *vir* phage transduction using the NF9 strain as donor, according to the protocol described by Silhavy et al. (1984). In the next step, the *lacI*⁻ *lacZ*⁻ phenotype was constructed by transforming W3110 *pts*⁻ (VH30) with the suicide plasmid pvLac, which contains the oriγ from R6K harboring the upstream region of *lacI,* a DNA fragment containing Cm^R (Cat2 cassette, as described by Palmeros et al. [2000]) and the 3′ end of *lacZ.* This vector cannot replicate in a W3110 background, facilitating the selection of double recombinants Cm^R and *lacI*⁻ *lacZ*⁻ (strain VH31). To obtain strain VH32, antibiotic resistance was excised using the method described by Palmeros et al. (2000).

Construction of *trc*-Derived Promoter Set and the Vector Family Harboring *galP* and *glk* Fused Genes

The *trc* promoter variants were obtained using a quick-change site-directed mutagenesis kit (Stratagene, La Jolla, CA) and pTrcGlk and pTrcGalP vectors as templates. This promoter set was designed using data obtained with the P_{22} *ant* promoter by Moyle et al. (1991), who demonstrated the effect of point mutations in the −10 and −35 boxes of this promoter. Using the *trc* promoter (Brosius et al., 1985) as template (designated *trc1* in this work), four point mutations were generated in the −35 box to generate mutant promoters *trc2, trc3, trc4,* and *trc5* (see Fig. 2).

The *trc–glk* and *trc–galP* fusions were cloned in the pCL1920 vector; this plasmid family was named according to the presence of the *galP* or *glk* gene and the number of the promoter that each vector carries (Table I).

Inoculum Preparation

Plates made with M9 minimal media supplemented with 0.2% of glucose (Maniatis et al., 1982) were inoculated

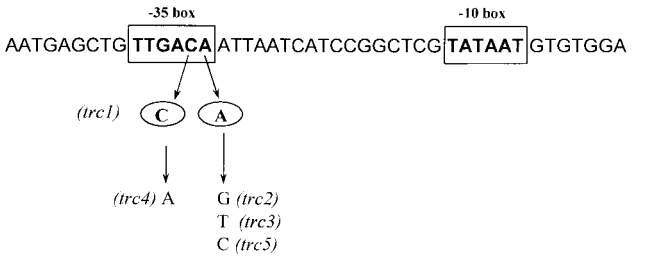

Figure 2. Construction of a *trc*-derived promoter family. The nucleotide sequences of the −35 and −10 boxes of the *trc* promoter shown in bold. Arrows show the changes in the nucleotide sequence made to the *trc* −35 box to generate the corresponding four mutant promoters *trc2, trc3, trc4,* and *trc5*. *Trc1* is the native −35 box.

directly from strain frozen vials and incubated overnight at 37°C. These plates were used to inoculate 250-mL flasks containing 50 mL of M9 (0.2% of glucose), which were incubated for 12 h at 37°C and 300 rpm in an orbital shaker (G25, New Brunswick Scientific, Inc., New Brunswick, NJ).

For ethanol production studies, Fermback flasks, containing 600 mL of LB media supplemented with 1.5% of glucose, were inoculated with cells from LB 2% glucose plates and incubated for 12 h at 30°C and 120 rpm.

Flask and Fermentor Cultures

Cells from flasks were used to inoculate fresh media at an initial OD_{600} of 0.05. Fermentor cultures were performed in 1 L of M9 minimal media supplemented with 0.2% glucose in a baffled stirred-tank fermentor equipped with a six-blade Rushton turbine and controlled by BLSL Biolafitte Maestro (Biolafitte, NJ). Working conditions were 600 rpm, 1 vvm (air volume × media volume^{-1} min^{-1}), 37°C, and pH 7 (controlled by automatic addition of 2.5% NH_3). Cultures were initiated at an OD_{600} of 0.05. Specific glucose consumption rate was calculated from the exponential growth data as the ratio between the specific growth rate and biomass yield.

Fermentor cultures for heterologous ethanol production were started at an initial OD_{600} of 1 using LB media supplemented with 1.5% of glucose at 600 rpm, 1 vvm, 30°C, and pH 6 (controlled by automatic additions of 2N KOH).

Analytical Methods

Protein concentration was determined using a commercial reagent Bradford (1976) (Pierce). Glucokinase assays were performed as described by Lessie et al. (1972). Biomass was measured as optical density at 600 nm using a spectophotometer (Lambda 11, Perkin Elmer, Pomona, CA) converted to dry cellular weight using a standard curve (1 OD_{600} = 0.37 g/L of dry cellular weight). Culture data represent the average of two fermentations. Glucose was determined with an enzymatic analyzer (Ektachem T60 II Multiple Analyzer, Kodak, NY). Acetate and ethanol concentration was determined by high-performance liquid chromatography (HPLC), using an Aminex HPX-87H column (300 × 7.8 mm; Biorad, Hercules, CA), and 5 m*M* H_2SO_4 as the mobile phase (0.5 mL/min) at 50°C. Initial rates of glucose uptake were measured according to Hernández-Montalvo et al. (2001) using [^{14}C]-glucose (1 m*M,* 5 mCi/mmol).

RESULTS

Generation and Characterization of a *trc* Promoter Set to Drive Different Expression Levels of *galP* and *glk* Genes

Genetic and biochemical characterization of PTS$^-$ glucose$^+$ strains obtained by a continuous culture selection method has revealed that the glucose$^+$ phenotype depends on the presence of functional *galP* and *glk* genes in the chromosome (Flores et al., 1996, 2002). These results suggest that, in these strains, glucose import depends on the activity of the GalP (galactose proton symporter), and glucose phosphorylation on the activity of glucokinase (see Fig. 1B). To determine the effect of increased GalP and Glk activities on the capacity of a PTS$^-$ strain to grow on glucose, we expressed the *galP* and *glk* genes alone or in combination from promoters with varying strength. All these promoters were characterized by fusing them with the *glk* gene harbored in the low copy plasmid pCL1920 (Lerner and Inouye, 1990), generating plasmids pCLv1Glk, pCLv2Glk, pCLv3Glk, pCLv4Glk, and pCLv5Glk. Glucokinase specific activity was determined from flask cultures of strain W3110 transformed with each of these plasmids and induced by the addition of isopropyl-β-thiogalactopyranoside (IPTG; 1 m*M*). The endogenous Glk specific activity present in strain W3110 under these culture conditions was 153 nmol min^{-1} $mg_{PROT}{}^{-1}$. These results show that the range of mutant promoter strength was modulated from 8% to 85% (16.7 ± 4.5 to 206.6 ± 55 nmol min^{-1} $mg_{PROT}{}^{-1}$) of that measured for the wild-type *trc1* promoter (Fig. 3).

Reconstitution of Glucose$^+$ Phenotype in a Strain Devoid of the Phosphotransferase System and Characterization of a New Group of PTS$^-$ Glucose$^+$ Strains

A mutant devoid of PTS cannot rapidly assimilate glucose and some other sugars; therefore, it displays an extremely low growth rate when using glucose as a carbon source (PTS$^-$ glucose$^-$ phenotype) (Flores et al., 1996; Postma et al., 1996). This reduced, but still existent, capacity to transport glucose depends on low-capacity, high-affinity systems like that composed of LamB (maltoporin) and Mgl (an ATP-driven ABC transporter) (Notley-McRobb and Ferenci, 1999a, 1999b). To determine the effect of increased GalP and Glk activities on growth capacity with glucose for a PTS$^-$ strain, the transformed strains, with plasmids carrying the *trc* promoter set controlling *galP* and *glk* expression, were grown in a shake flask with minimal media (M9)

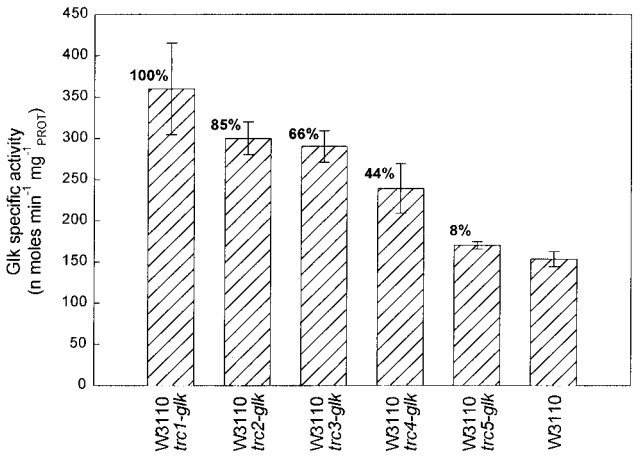

Figure 3. Characterization of the *trc*-derived promoter set. Glucokinase (Glk) specific activity was measured in strain W3110 that was transformed with plasmids containing the wild-type and four mutant *trc* promoters (for details see Fig. 2). Endogenous Glk activity present in the host strain (approximately 153 nmol min^{-1} $mg_{PROT}{}^{-1}$) was subtracted from total Glk activity, and relative levels were calculated using an additional glucokinase level, provided by pCLv1Glk as 100%. All assays were done at least four times, independently. The vectors used in these assays were pCLv1Glk, pCLv2Glk, pCLv3Glk, pCLv4Glk, and pCLv5Glk.

supplemented with 0.2% glucose as the carbon source. During the course of this work, it was found that IPTG inhibited growth of the PTS$^-$ strain VH30, even at low concentrations (unpublished results). Therefore, to avoid the use of IPTG as inducer of the *trc* promoters, the *lacI* and *lacZ* genes in VH30 were inactivated as described in "Materials and Methods," generating strain VH32. This strain was transformed with plasmid pCLv1GalP. As can be seen in Table II, expressing only *galP* from the *trc1* promoter enabled the PTS$^-$ glucose$^-$ VH32 strain to grow at a specific growth rate (μ) of 0.55 h^{-1}. This growth rate represents 89% of that measured for W3110 under the same culture conditions. In this work we define the glucose$^+$ phenotype for a PTS$^-$ strain as the capacity to sustain a growth rate of at least 80% of that observed in PTS$^+$ wild-type strain W3110. Therefore, strain VH32/pCLv1GalP acquired the glucose$^+$ phenotype as a consequence of the high-level expression of *galP*. To test if expression of *galP* from the weakest of the *trc* promoters still caused the reconstitution of the glucose$^+$ phenotype, VH32 transformed with plasmid pCLv5GalP was grown in minimal media displaying a μ of 0.51 h^{-1}, also showing the acquisition of the glucose$^+$ phenotype. On the other hand, expression of *glk* alone from *trc1* did not cause an increase in μ in strain VH32, maintaining the glucose$^-$ phenotype (Table II).

Table II. Specific growth rates of wild-type, PTS$^-$ glucose$^-$, and PTS$^-$ strains transformed with plasmids containing *galP* and *glk* genes driven by the *trc* promoter family.

Strain/plasmid	Specific growth rate (h^{-1})
W3110	0.62 (0.02)
VH32	0.03 (0.0)
VH32/pCL1920	0.03 (0.0)
VH32/pCLv1Glk	0.03 (0.0)
VH32/pCLv1GalP	0.55 (0.01)
VH32/pCLv5GalP	0.51 (0.01)
VH32/pv1Glk1GalP	0.59 (0.03)
VH32/pv1Glk5GalP	0.63 (0.02)
VH32/pv5Glk1GalP	0.64 (0.01)
VH32/pv5Glk5GalP	0.62 (0.02)
VH32/pv4Glk5GalP	0.64 (0.02)

All strains were grown in independent triplicate cultures using minimal medium with glucose as the carbon source. Values in parentheses indicate standard deviation.

galP and *glk* were fused to the five *trc*(*1–5*) promoters and cloned in the pCL1920 vector, generating the pvGlkGalP family of 25 vectors; this family provides combinations of different expression levels for both genes (data not shown). These vectors were used to transform strain VH32 to evaluate the combined effect of increased GalP and Glk activities on the capacity to grow with glucose. Strains carrying *glk* controlled by the strongest promoter, *trc1,* and the *galP* gene controlled by the strongest and weakest promoter (Table II) displayed μ values 16% and 24% higher than that obtained with strain VH32/pCLv1GalP, respectively. The μ of these strains was essentially the same as that of W3110. Similar experiments were conducted to determine the effect of the weakest expression level of *glk* by the *trc* promoter set—that is, those plasmids having *glk* controlled by *trc5,* whereas *galP* was controlled by the strongest and weakest promoter. These strains also showed μ values similar to that of W3110 (Table II). The μ values with other combinations from the pvGlkGalP family were similar to that obtained for the W3110 strain (data not shown). Furthermore, to avoid glucokinase activity limitation, we decided to characterize a vector with a slightly higher *glk* expression (pv4Glk5GalP), as shown in Table II; this combination also conferred a high μ to the VH32 strain.

Five strains were selected for further characterization, the control strain (W3110) and four VH32 derivatives. These derivative strains harbor plasmids pCLv1GalP (expressing GalP alone at the highest level), pv1Glk1GalP (expressing Glk and GalP, both at the highest level), pv5Glk5GalP (expressing Glk and GalP, both at the lowest level), and pv4Glk5GalP (expressing GalP at the lowest level and Glk at an intermediate level).

The glucokinase activity in the VH32 derivatives varied from 50% to 10% of the basal activity level found for the wild-type (Table III). In addition, for recombinants expressing both genes at the lower levels (strains transformed with vectors pv5Glk5GalP and pv4Glk5GalP), no significant difference (6%) was observed for glucokinase activity (Table III).

The rates of glucose transport and glucokinase activity were measured for these strains (Table III). In the glucose transport assays, it was found that the rate of glucose transport for strain VH32/pCLv1GalP was 40% higher than that in W3110. On the other hand, the glucose uptake rate in strain pv1Glk1GalP was only 22% of that measured for the

Table III. Determination of Glk specific activity, initial rate of glucose transport, specific glucose consumption rate (q_S), specific acetate productivity (q_{HAc}), and acetate yield from glucose ($Y_{HAc/Glc}$) for wild-type and PTS$^-$ strains in batch cultures.

Strain	Specific Glk activity (nmol min^{-1} mg^{-1} protein)	Glucose transport rate (nmol min^{-1} mg^{-1} protein)	q_S (g_{Glc} g_x^{-1} h^{-1})	q_{HAc} (g_{HAc} g_X^{-1} h^{-1})	$Y_{HAc/Glc}$ (g_{HAc}/g_{Glc})
W3110	153.3 (9.2)[a]	65.36 (2.8)	1.5 (0.01)	1.43 (0.5)	0.17 (0.05)
VH32/pCLv1GalP	127.6 (9.7)	93.89 (10.17)	1.32 (0.24)	2.74 (0.4)	0.37 (0.03)
VH32/pv1Glk1GalP	229.8 (14.7)	20.49 (1.08)	1.4 (0.15)	2.85 (0.5)	0.38 (0.03)
VH32/pv4Glk5GalP	181.5 (31.4)	34.93 (3.79)	1.53 (0.01)	3.4 (0.3)	0.43 (0.03)
VH32/pv5Glk5GalP	170.5 (9.9)	38.48 (7.54)	1.57 (0.2)	2.99 (0.2)	0.36 (0.02)

[a] Values in parentheses indicate standard deviation. All assays were done at least four times, independently. For kinetic parameters, all strains were grown in independent triplicates using minimal medium with glucose as the carbon source.

strain containing plasmid pCLv1GalP and 31% of that in W3110. Similar results were observed with VH32 transformed with pv5Glk5GalP or pv4Glk5GalP.

These results show that the presence of *glk* and *galP* in the same plasmid caused a reduction in expression level of the latter gene, when compared with expression from pCLv1GalP.

As part of the physiological characterization of these strains, specific rates of glucose consumption (q_s), acetate production (q_{HAc}), and acetate yield from glucose ($Y_{HAc/Glc}$) for five selected strains were determined in flask cultures (Table III). Remarkably, considering the different genotypes of these strains, q_s values were similar among them, but the q_{HAc} and $Y_{HAc/Glc}$ values for all the recombinant PTS$^-$ strains analyzed showed an approximately twofold increase compared with W3110.

Glycolytic Flux to Ethanol Is Increased in PTS$^-$ Strains Expressing *glk, galp, pdc,* and *adhII* Simultaneously

To determine whether the higher rate of acetate production, as observed in the VH32 glucose$^+$ strains, was the result of an increase in flux to pyruvate in glycolysis, we measured ethanol production from a strain engineered to convert pyruvate to ethanol. Strains VH32/pCLv1GalP, VH32/pv4Glk5GalP, and W3110 were transformed with plasmid pLOI1594 (Martinez et al., 1999) containing the *Zymomonas mobilis pdc* and *adhB* genes and encoding the pyruvate decarboxylase (Pdc_{Zm}) and alcohol dehydrogenase II ($AdhII_{Zm}$) enzymes, respectively (see Fig. 1A). The presence of these two genes generates a new pathway that increases the capacity of *E. coli* to produce ethanol. The resulting strains were cultured on rich medium (Luria broth) supplemented with 15 g/L of glucose under aerobic conditions (Fig. 4). During the first 3 h of the culture, strain VH32/pv4Glk5GalP/pLOI1594 grew with a μ of 0.54 h^{-1}, 38% faster than the wild-type W3110/pLOI1594 (0.39 h^{-1}). However, after 4 h of culture time, growth stopped for the strain carrying plasmid pv4Glk5GalP, possibly due to the metabolic burden provoked by overexpression of four genes (*galP, glk, pdc,* and *adhB*). Meanwhile, the other two strains continued growing until glucose depletion. The biomass value obtained with VH32/pv4Glk5GalP/pLOI1594 was approximately half of that obtained with the control strain and VH32 expressing GalP alone. Glucose consumption followed the same pattern for the control strain and for VH32/pCLv1GalP. However, the specific glucose consumption

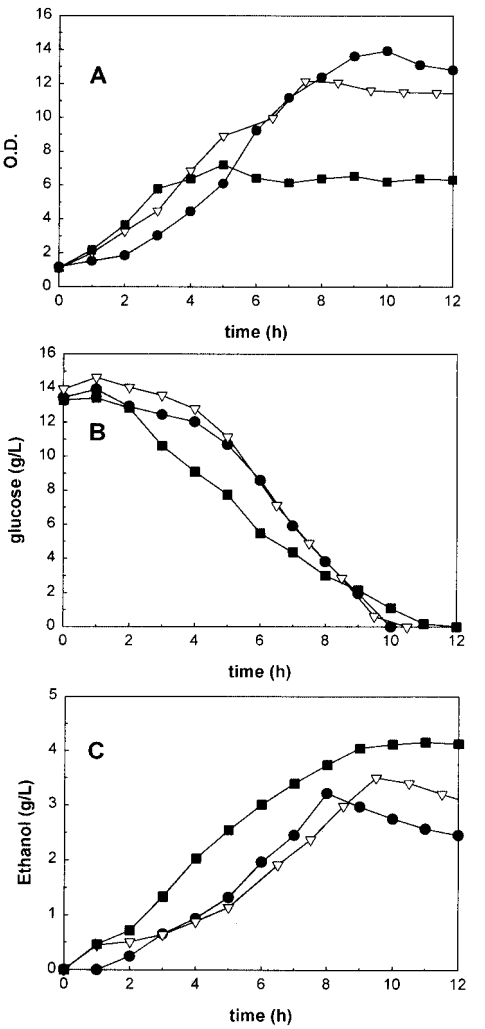

Figure 4. Growth kinetics of wild-type (W3110) and two PTS$^-$ glucose$^+$ strains performed for ethanol production. Strains were transformed with vector pLOI1594 (see Table I) to redirect pyruvate flux to ethanol production. (A) Growth; (B) glucose consumption; and (C) ethanol production. (●) W3110/pLOI1594; (▽) VH32/pCLv1GalP/pLOI1594; and (■) VH32/pv4Glk5GalP/pLOI1594.

rate was higher for the VH32 strain expressing both Glk and GalP. As a result of this behavior, the specific ethanol production rate was higher for VH32/pv4Glk5GalP/pLOI1594 during growth (0.2 $g_{EtOH}\ g_X^{-1}\ h^{-1}$) and nongrowth stages (0.15 $g_{EtOH}\ g_X^{-1}\ h^{-1}$) as compared with values obtained for the growth stage of W3110/pLOI1594 and VH32/pCLv1GalP/pLOI1594 (0.1 $g_{EtOH}\ g_X^{-1}\ h^{-1}$).

DISCUSSION

Reconstitution and Characterization of Glucose$^+$ Phenotype in *E. coli* Devoid of the Phosphotransferase System

Because the specific activities of GalP and Glk required to enable rapid glucose transport were not known a priori, we decided to use a strategy based on the generation of promoter strength diversity to control *galP* and *glk* gene expression, followed by characterization and selection of the strain(s) with the desired phenotype. By modifying the −35 box sequence of the *trc* promoter, it was possible to generate a set spanning an order of magnitude in promoter strength. This strategy, combined with the inactivation of *lacI* and *lacZ,* enabled the generation of strains with different and defined levels of expression for the genes used in this study. Although the same result probably could have been obtained by inactivating only *lacI,* simultaneous inactivation of *lacZ* avoids the potential metabolic burden of its derepressed expression.

It is important to point out that all strains used in this study have a functional *galP* gene in the chromosome. However, *galP* expression is repressed when *E. coli* is growing in the presence of glucose. The replacement of the native regulatory region of *galP* by the *trc* promoter family enables expression of this gene in the presence of glucose. Expression of *galP* from the *trc5* (weakest) promoter was sufficient to regenerate a glucose$^+$ phenotype in strain VH32. In contrast, expression of *glk* alone from *trc1* did not cause an increase in μ, and hence on glucose transport. These results show that, in PTS$^-$ glucose$^-$ strain VH32, glucose internalization and not phosphorylation was the limiting factor for rapid growth on glucose. GalP expression alone from *trc1* caused a significant increase in μ (89% of the value for the PTS$^-$ strain). Once inside the cell, glucose was phosphorylated by the chromosome-encoded Glk activity (see Fig. 1B). However, the strains expressing only *galP* did not reach the specific growth rate of the wild-type strain. This indicates that once GalP is not limiting glucose internalization, then Glk becomes the activity limiting glycolytic flux and, therefore, cell growth. This represents total recovery of μ in strains with plasmids containing both *galP* and *glk,* as compared with those having only *galP* (see Table II).

Determination of the strength of each mutant promoter relative to that of native *trc* makes this set useful for the expression of other genes in metabolic engineering studies. In addition, if lower promoter strengths are needed for a particular application, the sequences of the −35 or −10 boxes can be further modified to achieve the desired results. It should also be pointed out that placing the transcriptional unit in plasmids with different copynumbers could alter the expression level of a particular gene with each of these promoters.

Increased Flux to Pyruvate and Its Products Acetate and Ethanol in PTS$^-$ Glk$^+$ GalP$^+$ Strains

Acetate production under aerobic conditions in *E. coli* is the result of an imbalance between glycolytic and TCA fluxes (Han et al., 1992; Majewski and Domach, 1990). Therefore, comparison of the rates of production and yield of acetate from glucose is an indirect method for comparison of glycolytic fluxes at the level of the pyruvate node. As shown in Table III, both q_{HAc} and $Y_{HAc/Glc}$ values were about twofold higher when compared with W3110 values. Because q_s values were similar for all strains, these results indicate a redistribution of metabolic fluxes related to the synthesis and/or consumption of the acetate precursor, pyruvate (see Fig. 1A). Recent detailed flux analysis of two PTS$^-$ glucose$^+$ strains (PB12 and PB13, obtained by the continuous culture selection method), revealed that significant changes in flux distribution in central metabolic pathways occurred as a result of the elimination of PTS (Flores et al., 2002). In these strains it was found that flux through the glycolysis pathway was higher by an average of 19% over a parental PTS$^+$ strain.

Ethanol production experiments using pLOI1594 showed that, compared with a PTS$^+$ strain, more glucose-derived carbon flux was channeled to pyruvate when the *galP* and *glk* genes were expressed in a PTS$^-$ background. This behavior shows that the GalP- and Glk-dependent transport and phosphorylation of glucose, which is not regulated like PTS, causes an increase in carbon flux into glycolysis. It is important to note that a PTS$^-$ strain expressing only the *galP* gene was able to grow at a rate similar that obtained for the wild-type strain (Table III). However, in conditions under which growth and synthesis of a product such as ethanol is desired, *galP* overexpression is not sufficient, because *glk* expression is also required to increase productivity. Glucose transport and glucose consumption rates suggest that the increase in productivity can be a result of decreased glucose uptake, or a balanced activity between GalP and Glk, which results in no intracellular accumulation of glucose.

Metabolic Consequences of Phosphotransferase System Inactivation

In addition to its function in glucose transport, PTS plays an important and complex regulatory role in catabolic repression (Hogema et al., 1998; Postma et al., 1996). It is expected that a strain without PTS should have impaired catabolic repression. Results with strains in which IIBC^{GLC} has been inactivated show that glucose repression of hexose or pentose consumption is reduced or eliminated (Chatterjee et

al., 2001; Nichols et al., 2001). Characterization of PTS$^-$ glucose$^+$ strains with inactive enzyme I, HPr, and IIA^{GLC}, and obtained by the continuous culture selection method, has revealed that glucose catabolic repression is partially abolished. Hence, glucose and arabinose can be assimilated simultaneously (Hernández-Montalvo et al., 2001). This is a useful characteristic as the bacteria can consume sugar mixtures faster than a wild-type strain.

It has been shown previously that strains with the PTS$^-$ glucose$^+$ phenotype have characteristics useful for biotechnological applications (Flores et al., 1996; Gosset et al., 1996). By decoupling glucose transport from PEP consumption, the metabolic availability of this intermediate molecule is significantly increased when compared with a PTS$^+$ strain. This has been demonstrated using strains designed to direct carbon flow to the common aromatic pathway. In these reports, it was demonstrated that carbon flow to aromatics and yield from glucose is greatly increased as a result of the inactivation of PTS (Flores et al., 1996; Gosset et al., 1996). It should be expected that the production of other metabolites with PEP as precursor should also be enhanced in a PTS$^-$ glucose$^+$ strain. By applying strategies similar to the one described herein, it should now be possible to develop PTS$^-$ glucose$^+$ strains with defined levels of glucose transport and phosphorylation, from a low level to that of a wild-type strain.

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