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Identification of a Novel Genetic Locus Affecting *ptsG* Expression in *Escherichia coli*

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Abstract The phosphoenolpyruvate-dependent carbohydrate phosphotransferase system (PTS) is responsible for the simultaneous transfer and phosphorylation of various carbon sources in *Escherichia coli*. The *ptsG* gene encoding the enzyme $IICB^{Glc}$, the membrane component of the glucose-specific PTS, is repressed by Mlc and activated by the CRP•cAMP complex; various other factors, such as Fis, FruR, and ArcA, are also known to be involved in *ptsG* regulation. Thus, in an attempt to discover a novel gene affecting the regulation of *ptsG*, a mutant with a decreased *ptsG* transcription in the presence of glucose compared with the wild-type strain was screened using transposon random mutagenesis. The mutant was found to have a transposon insertion in *yhjV*, a putative gene encoding a transporter protein whose function is yet unknown.

Key words: *Escherichia coli*, PTS, *ptsG*, random mutagenesis, *yhjV*

The PEP:carbohydrate phosphotransferase system (PTS) in bacteria catalyzes the concomitant uptake and phosphorylation of sugars [10, 13, 15, 16]. The PTS consists of common cytoplasmic proteins, such as enzyme I (EI) and the histidine phosphocarrier protein (HPr), and carbohydrate-specific enzyme II complexes (EIIs). In *Escherichia coli*, the glucose-specific EIIs are composed of two components, cytoplasmic enzyme IIA^{Glc} ($EIIA^{Glc}$) and transmembrane enzyme $IICB^{Glc}$ ($EIICB^{Glc}$). In the process of transporting glucose, the phosphate originating from PEP is sequentially transferred to EI, HPr, $EIIA^{Glc}$, $EIICB^{Glc}$, and finally to glucose, converting it into glucose-6-phosphate [15].

$EIICB^{Glc}$ is encoded by *ptsG* located at 25 min of the chromosomal locus. *ptsG* transcription is regulated through two global systems: positively by CRP·cAMP and negatively by Mlc [4, 11, 14], as observed in all Mlc-regulated genes [1, 3, 14, 15, 22]. A novel induction mechanism for the Mlc regulon by glucose has also been identified, where the unphosphorylated $EIICB^{Glc}$ formed upon glucose uptake can relieve the repression of the Mlc regulon by sequestering Mlc through a direct protein-protein interaction [5, 10, 23]. Apart from these two major regulators, several other effector molecules, including heat-shock RNA polymerase [20], FruR [19], Fis [21], and ArcA [2], are known to influence *ptsG* expression. In addition to the regulations at the transcriptional level, a posttranscriptional control of *ptsG* expression through the modulation of mRNA degradation in response to the glycolytic flux has also been reported [4]. Therefore, these observations suggest that *ptsG* expression is regulated in a highly sophisticated manner in response to various environmental changes in order to maintain an optimum expression level, meaning the possibility of additional unknown factor(s) being involved in the regulation of *ptsG* expression is quite high. Accordingly, to find a novel genetic locus influencing *ptsG* expression, the present study screened a mutant that showed a lower *ptsG* expression level than the wild-type in the presence of glucose. *E. coli* MC4100 [F-*ara*D139 (*arg*F-*lac*) U169 *rps*L150 *thi*A1 *rel*A1 *flb*5301 *deo*C1 *pts*F25 *rbs*R] was used as the wild-type, and strain OH194 harboring a *lacZ* transcriptional fusion to the *ptsG* promoter was used for the random mutagenesis [12]. The cells were grown at 37°C in an M9 Minimal Medium containing X-gal, glucose, and kanamycin or a TB medium (tryptone 1%, NaCl 0.8%) under aerobic conditions. The TB medium was supplemented with 0.2% glucose when necessary. Bacterial growth was determined by measuring the optical density at 600 nm.

Isolation of Mutants Showing Lower *ptsG* Expression than Wild-type

Considering the regulation mechanism of *ptsG* by Mlc, it was expected that OH194 would form a white colony in

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the absence of glucose, whereas forming a blue colony in the presence of glucose on a medium containing X-gal. As such, the anticipated colony color change was observed on a minimal media plate containing X-gal. The OH194 was randomly mutagenized by EZ-Tn5 (Epicenter) using a kanamycin-resistant gene as the marker. A random mutant pool was constructed, and white or pale blue colonies selected on the M9 supplemented with kanamycin, glucose, and X-gal. Whereas some of the mutants had insertions in the *lacZ* gene, one had an insertion elsewhere in the chromosome, and therefore, this mutation was transferred to MC4100 by P1 transduction and given the name DW5.

Identification of Transposon Insertion Site

To examine where the transposon was inserted in the chromosome, the whole genomic DNA from the mutant was subjected to PCR-based sequencing using universal primers annealing to the chromosomal DNA adjacent to the transposon and transposon-specific primers. The chromosomal DNA was prepared from DW5 using DNAzol (Life Technologies, Inc.) and 1 ml of the harvested culture. The pellet was suspended in 100 μl of a TES/sucrose/lysozyme buffer [20 mM Tris-Cl (pH 8.0), 1 mM EDTA, 50 mM NaCl, 8% sucrose, and 1 mg/ml lysozyme] and placed at room temperature for 15 min. After adding 1 ml of DNAzol, the mixture was centrifuged at 15,000 ×*g* for 10 min at 4°C to remove any cell debris. The chromosomal DNA in the supernatant was then precipitated, washed with ethanol, dried at 37°C, and suspended in 100 μl of 8 mM NaOH. Using 50–100 ng of the chromosomal DNA as the template, a DNA Walking *SpeedUp*™ Kit (Seegene, Korea) was applied to sequence the genetic locus flanking transposon according to the manufacturer's instructions. After three sequential PCR amplifications including two nested PCRs, the final PCR product was directly sequenced. As a result of a blast search, the transposon was inserted into *yhjV*, whose function is yet not known, except that it encodes a putative transporter protein.

Characterization of Mutant

The expression of *ptsG* in the mutant strain was compared with that in the wild-type using a β-galactosidase assay [9] with certain modifications. The *E. coli* was incubated at 37°C in the TB medium with or without glucose and 1 ml of the culture collected by centrifugation at 5,000 ×*g*. The cell pellet was then suspended in 1 ml of a Z-buffer (60 mM $Na_2HPO_4 \cdot 7H_2O$, 40 mM $NaH_2PO_4 \cdot H_2O$, 10 mM KCl, 1 mM $MgSO_4 \cdot 7H_2O$, 50 mM mercaptoethanol, pH 7.0) and placed on ice for 20 min. The optical density at 600 nm was measured to determine the cell growth. The sample was then diluted with the Z-buffer to 1 ml at different proportions depending on the β-galactosidase activity. To permeabilize the diluted cells, 15 μl of 0.1% SDS and 30 μl of chloroform were added. After vortexing the mixture thoroughly, 0.2 ml of 4 mg/ml *o*-nitrophenyl-β-D-galactoside (ONPG) was added to start the reaction. The sample was placed at room temperature until it turned sufficiently yellow (OD_{420}= 0.6–0.9). The reaction was stopped by adding 0.5 ml of 1 M Na_2CO_3 and the reaction time recorded. One ml of the final mixture was transferred and centrifuged to remove any debris and chloroform, and the optical density measured at 420 nm. The following equation was used to calculate the units of β-galactosidase activity:

$$\text{Miller Units}=1{,}000\times[(OD_{420}-1.75\times OD_{550})]/(T\times V\times OD_{600})$$

where T=reaction time (min); V=volume of culture used in assay (ml).

When there was no external glucose, the β-galactosidase activity of the mutant strain was not significantly different to that of the wild-type strain (data not shown). Conversely, in the presence of glucose, the *ptsG-lacZ* expression was about 50% lower in the mutant compared with the wild-type cells, as shown in Fig. 1A.

The *ptsG* transcription level was also examined using a primer extension assay of the bacterial cells grown in the

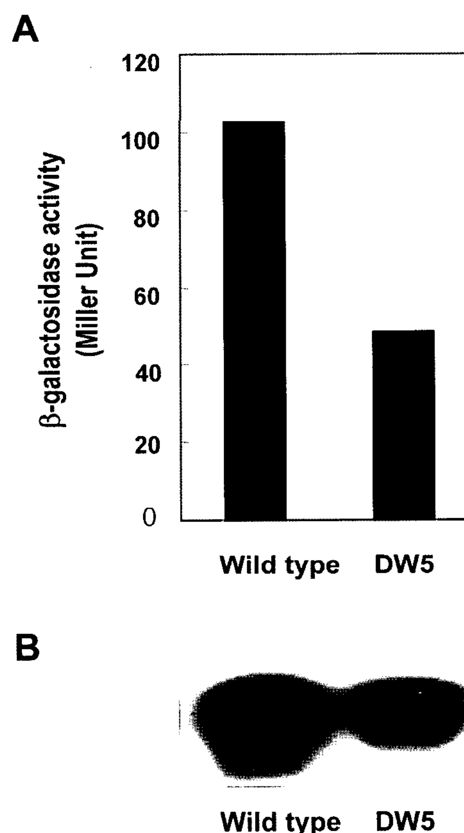

Fig. 1. Selected mutant showing decreased level of *ptsG* expression in presence of glucose.
The wild-type and mutant (DW5) strains were grown in TB containing 0.2% glucose. Aliquots of the cultures in the exponential growth phase were used for a β-galactosidase assay (**A**) and primer extension assay (**B**).

presence of glucose. The *E. coli* was grown in TB in the presence or absence of glucose, and then the cells were harvested, suspended in 1 ml of a Trizol Reagent (Life Technologies, Inc.), and the total RNA prepared according to the manufacturer's instructions. To obtain a labeled primer, 200 ng of a primer corresponding to the *ptsG* mRNA, PG1 (5'-AATTGAGAGTGCTCCTGAGTATGGGTGC-3'), was incubated at 37°C for 30 min with 10 units of T4 polynucleotide kinase (Life Technologies, Inc.) and 40 μCi of [γ-^{32}P]-dATP (Amersham Pharmacia Biotech). The T4 polynucleotide kinase was then inactivated by heating at 70°C for 10 min, and the labeled primer purified using MicroSpin™ G-25 columns (Amersham Pharmacia Biotech). Thirty mg of the total RNA was mixed with 2 μl of the labeled primer to make 20 μl, and then incubated at 60°C for 3 min. After 1 h at room temperature, a reverse transcription reaction was carried out with 50 μl of the reaction mixture [10 mM $MgCl_2$, 5 mM dithiothreitol, 100 ml/ml actinomycin D, 770 μM dNTPs, 20 mM Tris buffer (pH 8.3), 100 units Superscript II (Invitrogen)] at 40°C for 70 min. Two μl of 0.5 M EDTA and 1 μl of 170 units of RNase T1 (Epicenter) were added to stop the reaction. The mixture was then incubated at 37°C for 30 min, ethanol precipitated, and suspended in 3.75 μl of a Tris-EDTA buffer and 5 μl of a sequencing dye [80% distilled deionized formanide, 10 mM EDTA (pH 8.0), 1 mg/ml xylene cyanol FF, and 1 mg/ml bromophenol blue]. The mixture was denatured at 90°C for 3 min and electrophoresed on a 6% acrylamide-8 M urea gel, which was then dried and the signals analyzed quantitatively using a BAS2500 (Fuji, Japan) [7, 8, 17, 18]. Figure 1B shows that the expression of *ptsG* mRNA by the mutant was about 2.5-fold repressed compared with that by the wild-type, MC4100, suggesting that the mutation affected the *ptsG* expression at the transcriptional level. The mutation effects on the *ptsG* transcription may have been indirect, as *yhjV* encodes a putative transporter protein.

It is interesting to note that the mutant only exhibited a significant difference in the growth rate from the wild-type when it was grown in the presence of glucose. As such, while maintaining a similar growth pattern to the wild-type strain in the absence of glucose, the growth of the mutant was repressed up to 2-fold in the presence of glucose (Fig. 2), probably because of a slow glucose uptake due to the reduced *ptsG* expression. In *E. coli*, the favorite carbon source, glucose, is transported via glucose-specific PTS, and the accompanying phosphor relay through PTS triggers many important metabolic activities, as well as glycolysis [6, 11, 15]. Thus, although the significance of the role of the *yhjV* gene in PTS is still unclear, the present observations would seem to indicate that the *yhjV* gene affects one of the many control mechanisms involved in the highly complicated regulation of PTS. However, the mechanism by which the putative transporter protein encoded by *yhjV* affects *ptsG* transcription remains to be studied.

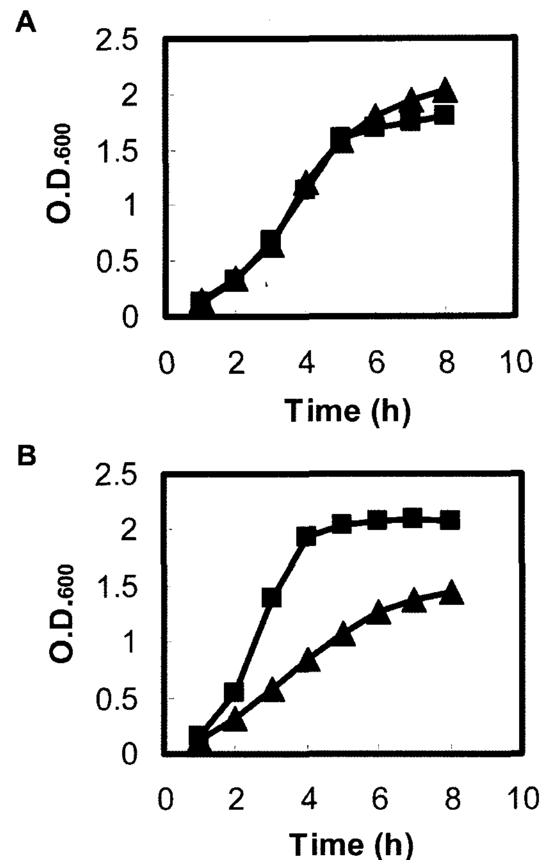

Fig. 2. Difference in growth rate between wild-type and mutant strains in the presence of glucose.
The *E. coli* MC4100 strain (square) and mutant DW5 (triangle) were grown in TB in the absence (**A**) and presence (**B**) of 0.2% glucose. The growth was measured by the optical density at 600 nm.

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