Regulation of the expression of phosphoenolpyruvate : carbohydrate phosphotransferase system (PTS) genes in *Corynebacterium glutamicum* R

Yuya Tanaka, Naoko Okai, Haruhiko Teramoto, Masayuki Inui and Hideaki Yukawa

Correspondence
Hideaki Yukawa
mmg-lab@rite.or.jp

Research Institute of Innovative Technology for the Earth (RITE), 9-2, Kizugawadai, Kizugawa, Kyoto 619-0292, Japan

The phosphoenolpyruvate : carbohydrate phosphotransferase system (PTS) catalyses the transport of carbohydrates by coupling carbohydrate translocation and phosphorylation. In *Corynebacterium glutamicum* R, the genes *ptsH* and *ptsI* encode general components of the PTS, and genes *ptsF*, *ptsS* and *ptsG* each encode fructose-, sucrose- and glucose-specific components of the PTS, respectively. In this study, we examined the mRNA levels of the *pts* genes in the presence or absence of PTS sugars. Glucose elevated the expression of *ptsG*, *ptsH* and *ptsI* genes, whereas fructose and sucrose induced the expression of all the *pts* genes examined, i.e. *ptsF*, *-S*, *-G*, *-H* and *-I*. We determined the transcriptional start sites of the *pts* genes and found that these promoters were activated in the presence of fructose. Disruption of *fruR*, which is a deoxyribonucleoside repressor (DeoR)-type transcriptional regulator co-transcribed with *ptsF*, resulted in enhanced induction of the *fructose-pts* operon, *ptsI*, and *ptsH* expression in response to fructose, indicating that FruR attenuates the induction of *ptsI*, *ptsH* and *fructose-pts* by fructose.

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INTRODUCTION

Corynebacterium glutamicum is a non-pathogenic, high-GC, Gram-positive soil bacterium which is widely used for the industrial production of amino acids, notably glutamic acid and lysine (Kinoshita *et al.*, 1957; Ikeda, 2003; Kelle *et al.*, 2005). We are investigating the *C. glutamicum* R strain, which can provide high yields of lactate and succinate from sugar (Inui *et al.*, 2004a; Okino *et al.*, 2005). To improve the production of organic acids, understanding the regulatory systems of sugar transport and metabolism is important.

In many bacteria, the phosphoenolpyruvate : carbohydrate phosphotransferase system (PTS) catalyses the transport of carbohydrates by coupling carbohydrate translocation and phosphorylation (Postma *et al.*, 1993; Kotrba *et al.*, 2001a). The PTS consists of two common cytoplasmic proteins, enzyme I and HPr, and an array of sugar-specific enzyme II complexes (EIIs). The phosphoryl group from

phosphoenolpyruvate (PEP) is sequentially transferred to enzyme I, HPr, EIIs and finally to the substrate as it is translocated across the membrane. In addition to carbohydrate uptake, the PTS regulates the expression of many catabolic genes, depending on its phosphorylation state (Postma *et al.*, 1993; Kotrba *et al.*, 2001a).

Expression of *pts* genes has been studied in many bacteria (Postma *et al.*, 1993; Vadeboncoeur *et al.*, 2000; Kotrba *et al.*, 2001b; Deutscher *et al.*, 2006). Generally, EII expression is induced in the presence of its substrate sugar. Expression of enzyme I and HPr, encoded by *ptsI* and *ptsH*, increases in the presence of different PTS sugars. In many bacteria, glucose is reported to be the most effective inducing sugar of *ptsI* and *ptsH* expression (De Reuse & Danchin, 1988; Stülke *et al.*, 1997; Viana *et al.*, 2000; Nothaft *et al.*, 2003). Regulation of *pts* gene expression is mainly controlled at the stage of transcription initiation or at transcription elongation. The mechanism for control of *pts* gene expression differs for the respective *pts* genes. For example, Mlc protein represses the expression of the *ptsHICrr* operon, which encodes general components of PTS, HPr and enzyme I, and the EIIA component of the glucose-PTS in *Escherichia coli* (Kim *et al.*, 1999; Plumbridge, 1999; Tanaka *et al.*, 1999). This repression is relieved by dephosphorylated glucose-PTS, which is

Abbreviations: DeoR, deoxyribonucleoside repressor; EII, enzyme II complex; PTS, phosphoenolpyruvate : carbohydrate phosphotransferase system.

A supplementary figure showing real-time RT-PCR analysis of *pts* gene expression is available with the online version of this paper.

generated by the transport of PTS sugar. In *Bacillus subtilis*, *ptsGHI* operon expression is controlled by the GlcT antiterminator. GlcT is active in its dephosphorylated form, which is generated by the transport of glucose. Activated GlcT inhibits the rho-independent terminator that precedes the *ptsG* ORF, and transcription proceeds to the 3' end of *ptsGHI* (Stülke *et al.*, 1997).

Expression of the *fructose-pts* operon is negatively regulated by Cra, which belongs to the GalR–LacI-type regulator family. Repression is relieved by fructose 1-phosphate in *E. coli*. In *B. subtilis*, expression of the *lev* operon, which is a fructose-PTS permease forming fructose 6-phosphate, is regulated by the antitermination mechanism mediated by LevR. This operon is also controlled by the CcpA–HPr complex. Ser-phosphorylated HPr binds to CcpA, which belongs to the GalR–LacI-type regulator family, and represses operon transcription. HPr phosphorylation at Ser is catalysed by HPr (Ser) kinase, which is activated in the presence of ATP and fructose 1,6-bisphosphate (Vadeboncoeur *et al.*, 2000). In other low-GC Gram-positive bacteria, induction of the *fructose-pts* operon is mediated by FruR, which is a deoxyribonucleoside repressor (DeoR)-type regulator encoded by the first gene of the *fructose-pts* operon. In *Spiroplasma citri*, FruR is an activator, whereas in *Streptococcus gordonii* and *Lactococcus lactis*, FruR is a repressor (Gaurivaud *et al.*, 2001; Loo *et al.*, 2003; Barrière *et al.*, 2005). It is proposed that fructose 1-phosphate modulates FruR activity in *L. lactis*.

In *C. glutamicum*, the presence of general components (enzyme I and HPr), as well as glucose-, fructose- and sucrose-specific EIIs of the PTS, has been demonstrated in several biochemical and genetic studies (Mori & Shiio, 1987; Dominguez & Lindley, 1996; Parche *et al.*, 2001a; Moon *et al.*, 2005), and there are additional *pts* genes for unknown substrates (NCgl2933 and NCgl2934 of *C. glutamicum* ATCC 13032). *C. glutamicum* R has a β -glucoside-specific PTS in addition to these *pts* genes (Kotrba *et al.*, 2001b). The genes that encode general components of the PTS are located near the *fructose-pts* gene, which is peculiar to *Corynebacterium* species (Fig. 3a).

PTS transport assays have revealed that the activity of the *C. glutamicum* fructose-PTS is induced when cells are cultured in the presence of fructose. In contrast, the activity of glucose-PTS is relatively constant (Mori & Shiio, 1987; Parche *et al.*, 2001a). Expression of the *pts* genes of *C. glutamicum* has been examined in only a few studies (Gerstmeir *et al.*, 2003; Kotrba *et al.*, 2003).

In this study, we investigated the expression of the general components of the PTS and the glucose-, fructose- and sucrose-specific EIIs of the PTS. We found that PTS sugars induced the expression of *pts* genes. In particular, fructose induced the expression of all the *pts* genes examined. We also investigated the role of FruR in the control of *pts* gene expression.

METHODS

Media and growth conditions. *C. glutamicum* R was grown aerobically at 33 °C in BT minimal medium with Casamino acids (Kotrba *et al.*, 2001b) supplemented with 1.0% (w/v) acetate, and various PTS sugars were added at 1.0% (w/v) as carbon sources. Antibiotics were added at the following final concentrations: kanamycin, 50 $\mu\text{g ml}^{-1}$; chloramphenicol, 5 $\mu\text{g ml}^{-1}$. Bacterial growth was monitored by determining OD₆₁₀.

Bacterial strains and plasmids. Construction of recombinant plasmid pCRC800 containing the *fruR* gene was carried out as follows. The *fruR* gene was amplified from *C. glutamicum* R genomic DNA by PCR using primers *EcoRI*-*fruR*-20F (5'-GCGGAATTCAACATCAGCGAGGTTAAGCATG-3') and *HindIII*-*fruR*-840R (5'-GCGAAGCTTGCGACAGCGTGAATCAATAC-3'). The amplified DNA fragment was digested with *EcoRI* and *HindIII*, and cloned into the corresponding sites on pCRB1 (Nakata *et al.*, 2003) to construct pCRC800.

C. glutamicum R (Yukawa *et al.*, 2007) was used as a wild-type strain. The *ptsI*-FLAG strain was constructed as follows. First, the 3' region of the *ptsI* gene was amplified from *C. glutamicum* R genomic DNA by PCR using primers *SacI*-*ptsI*-707F (5'-CGCGAGCTCGCGGACGAAGCTGAAGCAACCAAG-3') and *SalI*-1762-25F (5'-CGCGTCGACGTATCTGTTGAGCACCAATGAGCTTGAC-3'). The amplified DNA fragment was digested by *SacI* and *SalI*, and cloned into the corresponding site on plasmid pCRA925 (Inui *et al.*, 2004b). *SpeI* and *NotI* restriction endonuclease sites were introduced at the 3' terminus of the cloned *ptsI* by PCR using primers *SpeI*-*ptsI*-1704R (5'-CGGACTAGTGACTGCTGCGTCGATCACTGC-3') and *NotI*-*ptsI*-1707F (5'-GAGGCGGCCcACCACCTGTTGAGCTAAAAAGCCTC-3') to construct pCRC801. DNA fragments coding for FLAG-epitope tag and chloramphenicol-resistance genes were amplified by PCR using primers *SpeI*-FLAG-CmF (5'-CGGACTAGTGACAGCTACAAGGATGACGATGACAAGTAAGGCCCTTCCGGTTTTGGGGTAC-3') and Cm-*NotI*R1 (5'-CGCGCGGCCCGCGCTCTTCCTTTTGTAGAGTGCATTGATC-3'). The amplified DNA fragment was digested by *SpeI* and *NotI*, and cloned into the corresponding site on pCRC801 to construct pCRC802. *C. glutamicum* R was transformed with pCRC802, and Km^S and Cm^R colonies were selected. Integration of the FLAG-Cm fragment into the *C. glutamicum* R genome by double cross-over was confirmed by colony PCR using primers mini-CmL (5'-GCGAAGTGATCTTCCGTTTCG-3') and *ptsI*-1 F (5'-GTG-GCTACTGTGGCTGATGT-3'), or mini-CmR (5'-CACGACAGGTTTCCCGACTGG-3') and *EcoRI*-1761-23F (5'-GCGGAATTCGGCTTTTGCTTTAAGGAGTGACATG-3').

Disruption of the *fruR* gene was achieved by transposon-mediated mutagenesis, as described previously (Suzuki *et al.*, 2006). Disruption of the *fruR* gene was confirmed by DNA sequencing. The transposon was inserted at 463 bases downstream from the 5' end of the *fruR* ORF.

The *fruR* promoter-*lacZ* strain was constructed as follows. The promoter region of *fruR* was amplified by PCR using primers *SmaI*-*fruR* (5'-GCGCCCGGGAAGCAATTGCATGCTGTCTTTCCGTTTG-3') and *SmaI*-*ptsI* (5'-GCGCCCGGGCAGATCAGCCACAGTAGCCAC-3'). The amplified fragment was digested with *SmaI* and cloned into the *DraI* site of the pCRA741 reporter plasmid, which has been described previously (Inui *et al.*, 2007). These promoter-*lacZ* fusion plasmids were then digested with *KpnI* and *SmaI*, and the DNA fragment containing the *fruR* promoter-*lacZ* gene was cloned into the corresponding site on pHSG398 (Takara) to construct pCRC804. pCRC804 was used to transform wild-type *C. glutamicum* R or *fruR*-disruptant cells, and recombinant cells were selected for chloramphenicol resistance. Insertion of the promoter-*lacZ* fusion gene between CgR0734 and CgR0735 was confirmed by PCR using

primers LlacZLR-4354F (5'-ATAACCGGGCAGGGGTCTAG-3') and Ind7insert-checkR (5'-GCGTCACGAACAACAGACAGC-3'), or LlacZLR-6425R (5'-CGACGGCCAGTGCCAAGC-3') and Ind7insert-checkF (5'-CGAGACTGGAATTGAGGCTC-3').

Northern blotting analysis. Total RNA was isolated using the RNeasy Kit (Qiagen) from exponentially growing cells (OD₆₁₀ 1.2) in BT medium supplemented with 1.0% (w/v) carbon source. Ten micrograms of total RNA were resolved by 1.25% (w/v) agarose gel electrophoresis in the presence of formaldehyde and blotted onto Hybond-N+ membranes (GE Healthcare). The mRNAs were visualized using a non-radioactive nucleic acid labelling and detection system (GE Healthcare), according to the procedure specified by the manufacturer. The fluorescein-11-dUTP-labelled DNA probe was synthesized by a random-labelling kit (GE Healthcare). DNA fragments covering each *pts* gene were used as probes. The signal was scanned by a luminescent image analyser (Fuji model LAS-1000 CH).

Immunoblot analysis. A 10 ml aliquot of cell culture grown to OD₆₁₀ 1.0 was collected by centrifugation, and pellets were mixed with glass beads and 1.0 ml buffer (4% SDS, 5% 2-mercaptoethanol, 40 mM Tris/HCl, pH 6.8, 8 M urea, 0.1 mM EDTA). Cells were disrupted by vigorous vortexing and samples were centrifuged. Crude extract (10 µl) was loaded onto 0.1% (w/v) SDS-10% (w/v) polyacrylamide gels and electrophoresed. Western blotting was performed using anti-FLAG M2 mAb (Sigma) and horseradish peroxidase-conjugated anti-mouse antibody (GE Healthcare). Chemiluminescence reactions were done using the ECL Plus Western blotting detection system (GE Healthcare). The signal was scanned by a luminescent image analyser (Fuji model LAS-1000 CH).

Primer-extension analysis. Primer extension was carried out using appropriate gene-specific primers (Table 1) and total RNA (10–50 µg). Briefly, 1.5 pmol IRD700-labelled fluorescein primer was hybridized to RNA at 52 °C for 20 min, and cDNA was synthesized at 42 °C for 30 min using the AMV Reverse Transcriptase Primer Extension System (Promega) by the method described by the manufacturer. A 1.65 µl aliquot of sample was heat-denatured and loaded onto a denaturing 5.5% (w/v) polyacrylamide sequencing gel in parallel with the sequencing ladder and run in the LI-COR DNA sequencer (Aloka model 4000).

Real-time RT-PCR. A total of 20 ng RNA was used as template for analysis of the *pts* genes, and 0.4 ng was used for analysis of the 16S rRNA to generate cDNA and for the subsequent PCR reaction. Each real-time RT-PCR mixture (20 µl) contained 500 nM each primer, 12.5 µl Power SYBR Green PCR Master Mix, 8 U RNase inhibitor and 5 U MuLV reverse transcriptase (Applied Biosystems). The primers

used in these reactions were: *ptsI*, *ptsIF* (5'-GGCTTCGAACATG-GAGATGAAC-3') and *ptsIR* (5'-TGGCGTAAGGCTGTCAAG-3'); and 16S rRNA, 16SF (5'-TCGATGCAACGCGAAGAAC-3') and 16SR (5'-GAACCGACCACAAGGGAAAAC-3'). Reactions were performed using the ABI 7500 Fast Real-Time PCR System (Applied Biosystems) with the following cycle parameters: one cycle of 50 °C for 30 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. The 16S rRNA result was used as an internal control.

β-Galactosidase assay. *C. glutamicum* R was grown to OD₆₁₀ 1.2, and 1 ml culture sample was harvested and dissolved in 1 ml Z buffer (Na₂HPO₄/NaH₂PO₄, pH 7.0, 10 mM KCl, 1 mM MgSO₄, 50 mM β-mercaptoethanol) with 2% toluene to permeabilize the cells. β-Galactosidase activity was determined with permeabilized cells by the method described by Miller (1972).

Affinity purification of promoter binding protein. Biotin-labelled *ptsI*-*fruR* promoter DNA fragment was generated by PCR using primers promoter-*ptsI* (5'-GCGCCCGGGCAGATCAGCCACAGT-AGCCAC-3') and bio-promoter-*fruR* (5'-AAGCAATTGCATG-CTGTCTTTCCGTTT-3'). The 5' terminus of the bio-promoter-*fruR* primer was labelled with biotin. PCR product (100 pmol) was coupled to 3 mg Dynabeads streptavidin (Dyna). *C. glutamicum* R was grown in BT medium supplemented with 2.0% (w/v) fructose to OD₆₁₀ 3.0, harvested, washed with 50 ml cell wash buffer (50 mM Tris/HCl, pH 7.5, 50 mM NaCl) and suspended in 6 ml binding buffer (50 mM Tris/HCl, pH 7.5, 1 mM EDTA, 10% w/v, glycerol, 1 mM DTT, 100 mM NaCl, 0.05% (w/v) Triton X-100) with 1.5 g glass beads. Cells were disrupted by vigorous vortexing, and cell debris was removed by centrifugation at 12 000 g for 10 min and then at 50 000 g for 60 min. Supernatants were concentrated by using Amicon Ultra-15 centrifugal filter devices (Millipore) to 1.5 ml, and incubated with Dynabeads or *ptsI*-*fruR* promoter-coupled Dynabeads for 2 h at room temperature. Beads were washed six times with 300 µl binding buffer and twice with binding buffer containing 200 mM NaCl. Bound proteins were eluted with 50 µl binding buffer containing 1 M NaCl. Eluted fractions of 20 µl were loaded onto 0.1% (w/v) SDS-12% (w/v) polyacrylamide gels and electrophoresed. Proteins were transferred to Immobilon-P PVDF membranes (Millipore) and Coomassie Brilliant Blue-stained. The protein bands that appeared only for affinity-purified samples were excised, and the N-terminal protein sequence was determined by APRO Life Science Institute.

RESULTS

Effects of PTS sugars on *pts* mRNA expression

We first analysed the effects of various PTS sugars on *pts* mRNA levels. Total RNA prepared from *C. glutamicum* R wild-type cells grown in minimal medium with acetate in the presence or absence of PTS sugars (glucose, fructose and sucrose) was analysed by Northern blotting using probes specific for *ptsF*, *ptsS*, *ptsG*, *ptsH* and *ptsI*. Acetate was used as a base carbon source, as acetate gives relatively good growth among non-PTS carbon sources in *C. glutamicum* R, and previous research has resulted in the accumulation of much knowledge of acetate usage in *C. glutamicum*. We repeated Northern-blotting analysis more than twice and observed the same expression patterns of *pts* genes.

In the absence of PTS sugars, a very faint band was detected with the *ptsF* probe (Fig. 1a, lane 1), and addition

Table 1. Oligonucleotides used as primers

Primer	Sequence (5'–3')
<i>ptsI</i> -1	ACATCAGCCACAGTAGCCAC
<i>ptsI</i> -2	AACGGACTCCACCGACAACG
<i>ptsH</i> -1	TTACAGTCTTGAAGCCATG
<i>ptsH</i> -2	AGCGATGATGGATGCTGGAC
<i>ptsG</i> -1	GTCGTCAGTTTGGACGCCATGTC
<i>ptsG</i> -2	AGTCATCGAAGTAATATTGTCTG
<i>fruR</i> -1	TGTCTTTCCGTTTGGCTGACCATG
<i>fruR</i> -2	GGTCCAGTAAAGAAGCAATTGC
<i>ptsS</i> -1	CTTCGCCGCCAATGTCGCGCAGGATG
<i>ptsS</i> -2	GCGTAAACGCGTTGCACAGTGTG

Fig. 1. Effect of various PTS sugars on the expression of *pts* genes. Total RNA (10 µg), prepared from cells grown in BT minimal medium supplemented with Casamino acids and 1.0% (w/v) acetate with or without 1.0% (w/v) PTS sugars, was subjected to Northern blotting analysis. Abbreviations: A, acetate; G, glucose; F, fructose; S, sucrose. (a) Hybridization with *ptsF* probe; (b) hybridization with *ptsS* probe; (c) hybridization with *ptsG* probe; (d) hybridization with *ptsH* probe; (e) hybridization with *ptsI* probe. (f) Total RNAs used were stained with ethidium bromide and visualized by UV irradiation. The locations of the 23S rRNA and 16S rRNA are indicated.

of glucose did not increase the level of *ptsF* mRNA (Fig. 1a, lane 2). In contrast, the addition of fructose increased the expression of *ptsF* (Fig. 1a, lane 3), and sucrose also increased the *ptsF* mRNA signal (Fig. 1a, lane 4). The expression pattern of *ptsS* was similar to that of *ptsF*, that is, the expression of *ptsS* was induced by both fructose and sucrose (Fig. 1b). Glucose-specific *pts* showed a different expression pattern. Significant *ptsG* mRNA was detected in the absence of a PTS sugar (Fig. 1c, lane 1), and *ptsG* expression increased in the presence of glucose (Fig. 1c, lane 2). Surprisingly, in the presence of fructose and sucrose, *ptsG* expression increased to almost the same level as that seen with glucose induction (Fig. 1c, lanes 3 and 4). For general components of the PTS, expression of *ptsH* was observed in the absence of PTS sugar (Fig. 1d, lane 1), but very little expression was observed for *ptsI* mRNA (Fig. 1e, lane 1). Both *ptsH* and *ptsI* expression increased in the presence of PTS sugar. Fructose was the most effective inducing sugar for *ptsH* and *ptsI* expression (Fig. 1d, e, lane 3). The results in Fig. 1(a, d) show that several transcriptional products are present for *ptsF* and *ptsH* genes. In summary,

fructose and sucrose induced the expression of all *pts* genes tested. We observed the induction of *pts* genes by PTS sugars when cells were cultured in a rich medium with yeast extract and no added acetate (data not shown), indicating that a decrease in acetate usage is not the signal for the induction of *pts* gene expression by PTS sugars.

To analyse the effects of PTS sugars on the expression level of PTS proteins, we determined the relative levels of enzyme I by Western blotting. To do this, we introduced a FLAG-tag sequence to the C terminus of enzyme I, and determined the enzyme I level using an antibody against the FLAG-epitope tag. The enzyme I level increased when external glucose was present (Fig. 2, lanes 4 and 5). A further increase in enzyme I protein level was observed in the presence of fructose (Fig. 2, lane 6). No band corresponding to enzyme I was observed in wild-type cells (Fig. 2, lanes 1–3). This result corresponds to the results of the mRNA study of the *ptsI* gene (Fig. 1e), and suggests that the regulation of *ptsI* expression by PTS sugars occurs mainly at the transcriptional level.

Fig. 2. Immunoblot analysis of cellular enzyme I level. Crude extract equivalent to OD₆₁₀ 0.01, prepared from wild-type (WT; lanes 1–4) or *ptsI*-FLAG (lanes 5–8) cells grown in BT minimal medium supplemented with Casamino acids and 1.0% (w/v) acetate with or without 1.0% (w/v) glucose or 1.0% (w/v) fructose, was analysed by Western blotting using anti-FLAG M2 antibody.

Operon structure of the *fructose-pts*

According to the genome sequence of *C. glutamicum* R (Yukawa *et al.*, 2007), the *ptsF* and *ptsH* genes are adjacent to each other (Fig. 3a). The gene order and intergenic spacing suggest that *ptsF* and *ptsH* are transcribed as a polycistronic mRNA. *ptsI* is also located near *ptsF*. Between *ptsF* and *ptsI* there is the *fruR* gene, encoding a protein of 264 aa that shows homology to a member of the DeoR family of transcriptional regulatory proteins, and the *pfkB1* gene, encoding a protein of 330 aa that shows homology to fructose-1-phosphate kinase. The *fruR*, *pfkB1*, *ptsF* and *ptsH* genes are oriented in the same direction, while *ptsI* and the ORF that encodes a hypothetical protein located downstream of *ptsH* are oriented in the opposite direction (Fig. 3a). A rho-independent terminator structure is

Fig. 3. (a) Genetic organization of the *C. glutamicum* R *fructose-pts* operon. Open arrows represent the coding region for the *fruR*, *pfkB1*, *ptsF* and *ptsH* genes. Deduced functions of each gene are indicated below the respective gene. The transcription initiation sites identified in this study (Fig. 4) are indicated by arrows. A putative rho-independent terminator is indicated by a circle. (b) Northern blotting analysis of specific transcripts for *fruR*, *pfkB1*, *ptsF* and *ptsH* from *C. glutamicum* R. Total RNA was prepared from cells grown in BT minimal medium supplemented with Casamino acids and 1.0% (w/v) acetate, with or without 1.0% (w/v) fructose. Hybridization was done with probes specific for *fruR*, *pfkB1*, *ptsF* and *ptsH*. Major transcripts are indicated on the right of the panel; RNA standards (Invitrogen) are shown on the left. Asterisks on the left of the Northern blot indicate the position of 23S and 16S rRNAs. The right-hand panel is total RNA stained with ethidium bromide and visualized by UV irradiation. The locations of the 23S and 16S rRNA are indicated. M, RNA size marker.

present downstream of the *ptsH* gene. This gene cluster is also conserved among *C. glutamicum* ATCC 13032, *Corynebacterium diphtheriae* and *Corynebacterium efficiens* (Parche *et al.*, 2001b; Yokota & Lindley, 2005).

We analysed the operon structure of this region by Northern blotting using probes specific for *fruR*, *pfkB1*, *ptsF* and *ptsH* (Fig. 3b). A product of about 4.7 kb (product a) was detected by all four probes, indicating that *fruR*, *pfkB1*, *ptsF* and *ptsH* are transcribed as a polycistronic message. Using a *ptsH* probe, an intense 0.5 kb transcript (product d) was detected. The *ptsH* gene therefore seems to have its own promoter, as the expression pattern of *ptsH* mRNA was different from that of the other products. It is significant that product d was seen in the absence of fructose, and the amount of product d was more abundant than that of other mRNA products. For products b and c, the expression pattern was the same as that for product a, and we observed many bands by primer-extension analysis using a primer specific for *ptsF*. This suggests therefore that these products are the result of the nuclease digestion.

Determination of the transcriptional start sites of the *pts* genes

For further detailed analysis of the regulation of *pts* gene expression, we examined the transcriptional initiation site of each *pts* gene. Total RNA prepared from wild-type cells grown in a minimal medium in the presence or absence of PTS sugars (glucose or fructose) was analysed by primer-extension analysis using fluorescently labelled primers specific for each *pts* gene (Table 1). The position of the transcriptional start point was confirmed by experiments using two different primers for each *pts* gene (Table 1). A major transcriptional start site of *ptsH* (*ptsH*-P1 promoter) is the G residue located 102 nt upstream of the start codon of *ptsH* (Fig. 4a), and we observed a minor signal corresponding to the T residue located 82 nt upstream of the start codon of *ptsH* (*ptsH*-P2 promoter). Using a *ptsI*-specific primer, we detected three bands (Fig. 4b, c). The signals corresponded to T residues located at 103 and 105 nt upstream of the GTG start codon (*ptsI*-P1 promoter), and 37 nt upstream of the start codon (*ptsI*-P2 promoter). Using a *ptsG*-specific primer, we detected a single band corresponding to a T residue, located 256 nt upstream of the start codon (Fig. 4d). The transcriptional start site of *ptsG* has recently been reported in *C. glutamicum* ATCC 13032 (Engels & Wendisch, 2007). Those authors reported the start site at a G residue located 258 nt upstream of the start codon. The difference from our result may come from the slight difference in DNA sequence at the *ptsG* promoter. The A residue located 262 nt upstream of the start codon in *C. glutamicum* R is a G residue in the ATCC 13032 strain.

To determine the start site of the fructose-specific *pts* operon, we used a *fruR*-specific primer. We detected four bands corresponding to G and T (*fruR*-P1 promoter) and

A and T residues (*fruR*-P2 promoter) located 294, 293, 123 and 126 nt, respectively, upstream of the ATG start codon of *fruR*. A T residue located 62 nt upstream of the start codon was detected using a *ptsS* primer (Fig. 4g). Nucleotide sequences of the *pts* promoter regions are compared in Fig. 5. We found putative -10 and -35 hexamers. Comparison of each promoter showed that a TGT(TT)G sequence was conserved. The *ptsI*-P1 promoter region and the *fruR*-P1 promoter region were found to overlap.

The level of the primer-extension products increased in the presence of fructose (Fig. 4a–g). We also determined the effect of glucose on *ptsG* mRNA expression, and an increase in *ptsG* mRNA level was detected (Fig. 4d). These data correspond to the results of Northern blotting assays (Fig. 1).

Effect of *fruR* gene disruption on the expression of the *pts* genes

We were interested in how fructose regulates the expression of *pts* genes, as fructose has the strongest inducing effect on *pts* gene expression, and the general component of the PTS is located near the *fructose-pts* operon, which differs from that of other bacteria. The *fruR* gene encodes a DeoR-type regulator that is located upstream of *ptsF* (Fig. 3a). This arrangement is conserved in many bacteria, and the regulator is responsible for the induction of the *fructose-pts* operon (Gaurivaud *et al.*, 2001; Loo *et al.*, 2003; Barriere *et al.*, 2005). Thus, we examined the role of FruR on the expression of the *pts* genes.

We compared the expression of *pts* genes in wild-type and *fruR* cells. In cells grown in the presence of fructose, the mRNA levels of *ptsI* and *ptsH* were higher in the *fruR* mutant than in the wild-type (Fig. 6a, b). In the absence of PTS sugars, or in the presence of glucose, the bands for the mRNAs were faint in both the *fruR* mutant and the wild-type. The exposure time for detection of bands in the Northern blotting analysis was shorter in Fig. 6 than in Fig. 1 to avoid saturation of the band intensity. These results showed that fructose increased the *pts* mRNA level in the *fruR* strain as in the wild-type, and that the induced level in response to fructose was increased by disruption of *fruR*. Expression of *ptsG* was hardly affected by the *fruR* mutation with all PTS sugars (data not shown).

In the *fruR* mutant we could not detect the *fructose-pts* operon mRNA by Northern blotting analysis, probably because of the transposon insertion in the *fruR* gene. In this regard, we observed that disruption of *ptsF* completely eliminated the induction of *pts* genes in response to fructose. We observed that disruption of *pfkB1* completely inhibited growth in the presence of fructose. The phenotypes of the *ptsF* and *pfkB1* mutants were different from those of the *fruR* mutant. These results suggest that *ptsF* and *pfkB1* are expressed in response to fructose in the *fruR* cells, although we cannot rule out the possibility that

Fig. 4. Determination of the transcriptional initiation sites of the *pts* genes. Total RNA (10 or 50 µg) isolated from *C. glutamicum* R cultured in BT medium supplemented with Casamino acids and 1.0% (w/v) acetate, with or without 1.0% (w/v) fructose or glucose, was used for primer-extension analysis with the gene-specific primers listed in Table 1. The transcription initiation sites are indicated by arrows.

the negative effect of disruption of *fruR* on *ptsF* and *pfkB1* expression affects *pts* gene expression indirectly.

We then complemented the *fruR* mutant with pCRC800, which has the *fruR* gene expressed under the control of the *lac* promoter (Fig. 6d). We observed that induction of *ptsI* by fructose returned to the wild-type level in the strain containing pCRC800 (Fig. 6d, lane 6; Supplementary Fig. S1). These results suggested that FruR functions as a repressor of the *pts* gene.

To examine whether FruR controls the expression of the *fruR*-*pfkB1*-*ptsF* operon, we constructed a *fruR* promoter-*lacZ* fusion and then introduced this construct into the wild-type or the *fruR* mutant in a region of the genome known to be non-essential in *C. glutamicum* R (Yukawa *et al.*, 2007). We determined β -galactosidase activity in the presence or absence of fructose (Fig. 6f). In wild-type cells, a twofold increase in *fruR* promoter activity was observed

in the presence of fructose (Fig. 6f, columns 1 and 2). The induction by fructose seemed weaker than that observed by Northern analysis. This might have been a consequence of the fact that the *fruR*-*lacZ* promoter construct included only 135 bp of DNA upstream from the *fruR*-P1 promoter, which may be insufficient for full promoter activity. Alternatively, the difference might have been caused by post-transcriptional regulation. In *fruR*-deficient cells, *fruR* promoter activity increased to a level threefold higher than that in the wild-type (Fig. 6f, columns 3 and 4). In the absence of fructose, *fruR* promoter activity in *fruR*-deficient cells was the same as that in the wild-type (Fig. 6f, columns 1 and 3). These results suggested that FruR reduces the induction effect of fructose on the *fructose*-*pts* operon. By affinity-purification experiments using *ptsI*-*fruR* promoter DNA as a bait (Fig. 6g), we isolated two proteins of about 28 kDa that bound specifically to the promoter region. The determined N-

Fig. 5. Alignment of the promoter regions of *pts* genes. Promoter regions of the *fruR*, *ptsG*, *ptsH*, *ptsI* and *ptsS* genes are aligned. Transcription start (TS) sites (yellow), -10 regions and -35 regions are boxed. For each promoter, 101 nt are shown; 25 nt downstream of the -10 region and 70 nt upstream of the -10 region. A conserved TGT(TT)G sequence or its complement CAAACA sequence is underlined.

terminal amino acid sequence of one protein was VSQTE, which was identical to the N-terminal amino acid sequence of FruR lacking the first amino acid. This result demonstrated that FruR interacts directly with the promoter and suggested that FruR directly represses the expression of the *pts* gene.

In addition, the second protein we isolated that bound specifically to the promoter region was identified as SugR, which has recently been reported to bind the *fruR* promoter region (Engels & Wendisch, 2007).

DISCUSSION

Sugar transport by the PTS has been demonstrated before in *C. glutamicum* (Mori & Shiio, 1987; Dominguez &

Lindley, 1996; Parche *et al.*, 2001a; Moon *et al.*, 2005). The genes that encode the general PTS and the enzyme II specific for glucose, fructose and sucrose are known. By transport assay, it was demonstrated that glucose-PTS activity is relatively constitutive and that fructose-PTS activity is induced by fructose. However, the regulation of *pts* gene expression is largely unknown. In this paper we determined the expression patterns of *pts* genes in the presence of PTS sugars and determined the location of promoter regions, and finally we investigated the role of FruR on *pts* expression.

Expression of *ptsF* and *ptsS* showed similar patterns; low-level expression of these genes was detected in acetate or glucose cultures, and the expression was induced by both fructose and sucrose (Fig. 1a, b). On the other hand, significant expression of *ptsG* was observed in the absence

Fig. 6. Effect of *fruR* mutation on the expression of *pts* genes. Total RNA (10 μ g), prepared from wild-type (WT) or a *fruR* mutant grown in BT minimal medium supplemented with Casamino acids and 1.0 % (w/v) acetate with or without 1.0 % (w/v) PTS sugars, was subjected to Northern blotting analysis. Abbreviations: A, acetate; G, glucose; F, fructose. (a) Hybridization with *ptsI* probe; (b) hybridization with *ptsH* probe. (c) Total RNAs used in (a) and (b) were stained with ethidium bromide and visualized by UV irradiation; 23S and 16S rRNA are indicated. (d) Complementation of the *fruR* mutant. Total RNA was prepared from wild-type cells containing pCRA1 vector or the *fruR* mutant containing either pCRA1 or pCRC800 plasmid. Northern blotting analysis was performed with the *ptsI* probe. (e) Total RNAs used in (d) were stained with ethidium bromide and visualized by UV irradiation. (f) Effect of *fruR* mutation on the activity of the *fruR* promoter. *C. glutamicum* R cells having a *fruR* promoter–*lacZ* fusion gene with wild-type *fruR* or with a *fruR* gene disruption mutation were grown in BT medium with 1.0 % (w/v) acetate or 1.0 % (w/v) fructose. Culture samples were taken at OD₆₁₀ 1.2 and β -galactosidase activities were determined. Each value is the mean and SD of four independent experiments. (g) Affinity purification of FruR protein bound to the *ptsI*–*fruR* promoter DNA fragment. Cell extracts of *C. glutamicum* R grown in BT medium were incubated with magnetic beads–biotin-labelled *ptsI*–*fruR* promoter DNA fragment and bound proteins were eluted with 1 M NaCl. In lane 1, proteins were eluted from magnetic beads; in lane 2, proteins were eluted from the *ptsI*–*fruR* promoter fragment bound to magnetic beads. A molecular mass marker (BioRad) is shown to the left of the panel.

of PTS sugars, and *ptsG* expression was increased by glucose, fructose and sucrose (Fig. 1c). Glucose-PTS has the ability to transport fructose, although the contribution of glucose-PTS to fructose uptake is only minor (Dominguez *et al.*, 1998; Kiefer *et al.*, 2004). Thus, there might be some advantage for *C. glutamicum* R to increase *ptsG* expression in the presence of fructose. The expression of the general component of the PTS increased in the presence of PTS sugar (Fig. 1d, e). Interestingly, fructose is

the most effective inducing sugar, which is different from that which has been observed for other bacteria (De Reuse & Danchin, 1988; Stülke *et al.*, 1997; Viana *et al.*, 2000; Nothaft *et al.*, 2003). In other bacteria, expression of the general PTS is induced most significantly by glucose. It is also noteworthy that *ptsH* and *ptsI* are located near the *fructose-pts* operon in *C. glutamicum*, which also differs from the situation in *E. coli* and *B. subtilis*, in which *ptsH* and *ptsI* are co-transcribed with the *glucose-pts* genes. In *C.*

glutamicum, the uptake rate of fructose is faster than that of glucose (Kiefer *et al.*, 2004), which may be explained by the higher expression of the general component of PTS.

In *C. glutamicum*, sucrose metabolism produces fructose and glucose 6-phosphate, and fructose is exported to the outside of the cell. Fructose re-enters the cell via the fructose-PTS (Dominguez & Lindley, 1996; Pátek *et al.*, 2003; Moon *et al.*, 2005). This means that the effect of sucrose on the expression of *pts* genes may be caused by fructose generated inside the cell. By comparing the sucrose induction of *pts* genes for the wild-type and the *ptsF* mutant, it might be possible to determine whether sucrose induces *pts* gene expression directly or indirectly.

To investigate how *pts* gene expression is controlled, we determined the transcriptional start site of each *pts* gene. The nucleotide sequence that resembles the -10 hexamer is located upstream of each transcriptional start site. Alignment of *pts* promoters through their -10 regions is shown in Fig. 5. The sequence tgngnTA(c/t)aaTgg is proposed as an extended -10 consensus sequence for *C. glutamicum* (Pátek *et al.*, 2003). We compared this sequence with each *pts* promoter. Core hexamers are well conserved in all *pts* promoters, especially the first T and last T, while extended regions are poorly conserved. Only the G residue that is 2 nt upstream of the core hexamer and the G residue 1 nt downstream from the hexamer are conserved. We also found -35 -like sequences that are separated by 15–18 bp from the -10 regions. The hexamer ttGcca is proposed as a -35 consensus sequence (Pátek *et al.*, 2003). The first three nucleotides ttG are well conserved among *pts* promoters, but the last three nucleotides are less well conserved. Among the *pts* promoters, the TGT(TT)G sequence is conserved, and may be an operator site for the putative common transcriptional regulator for *pts*. This sequence is unlikely to be the binding site for FruR because FruR does not regulate the expression of *ptsG*, which has the sequence TGT(TT)G.

In many bacteria, the gene that encodes the transcriptional regulator located in front of the *fructose-pts* genes is responsible for the fructose-dependent induction of the *fructose-pts* operon (Gaurivaud *et al.*, 2001; Loo *et al.*, 2003; Barrière *et al.*, 2005). The regulatory mechanism is either derepression or activation of transcription in the presence of fructose. *C. glutamicum* also has a regulator FruR for the *fructose-pts* operon; hence, we determined the effect of *fruR* disruption on the expression of the *pts* genes. We found that the expression levels of *ptsI*, *ptsH* and the *fructose-pts* operon increased more in a *fruR* mutant than in wild-type cells in the presence of fructose, indicating that FruR decreases the induction effect of fructose. This is different from the role of FruR in other bacteria. These results also indicate that transcriptional regulator(s) other than FruR exist, as the increase in *pts* expression was still observed in the presence of PTS sugar in the *fruR* mutant. In general, the expression of a catabolic gene is controlled so as not to exceed the metabolic capacity. The role of FruR seems to be

to adjust the expression level of *pts* genes to prevent overflow of PTS sugars.

During the preparation of this manuscript, Engels & Wendisch (2007) reported that EII components of *pts* genes (*ptsG*, *fruR-pfkBI-ptsF* and *ptsS*) are regulated by a DeoR-type regulator SugR. SugR represses the expression of EII components of the PTS in the absence of PTS sugar, and addition of PTS sugar relieves this repression. Our results showed that the expression of cytoplasmic components of the PTS (*ptsH* and *ptsI*) is increased in the presence of PTS sugar. We also found that a conserved motif is found in *pts* promoters. One possibility is that SugR also controls the expression of *ptsH* and *ptsI*. We are now investigating whether SugR controls the expression of cytoplasmic components of the PTS.

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