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

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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.

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 (EIIIs). The phosphoryl group from phosphoenolpyruvate (PEP) is sequentially transferred to enzyme I, HPr, EIIIs 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 represses operon transcription. HPr phosphorylation at the Ser is catalysed by HPr (Ser) kinase, which is activated in positive bacteria, induction of the fructose-PTS gene, which is peculiar to *Corynebacterium* species in addition to these components of the PTS are located near the glucoside-specific PTS in addition to these components of the PTS are located near the glucoside-specific PTS in addition to these components of the PTS and the glucose-, fructose- and sucrose-specific EIIs of the PTS, has been demonstrated in several biochemical and genetic studies (Mori & Shio, 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 & Shio, 1987; Parche et al., 2001a). Expression of the *pts* genes of *C. glutamicum* has been examined in only a few laboratories (Gerstmeier 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 μg ml⁻¹; chloramphenicol, 5 μg ml⁻¹. Bacterial growth was monitored by determining OD₆₀₀.

#### 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′-GGCAGATTTACACATCAGGAGTGAAGCATG-3′) and HindIII-fruR-840R (5′-GGCAAGGCTTCGGACAGCGTTGAAATCAGAAC-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 *ptsF*-FLAG strain was constructed as follows. First, the 3′ region of the *ptsF* gene was amplified from *C. glutamicum* R genomic DNA by PCR using primers SacI-ptsF-300R (5′-GGCAAGGCTTCGGACAGCGTTGAAATCAGAAC-3′) and SacI-1762-25F (5′-GGCGTGCGATCTATGTTGCCACGAAATGCTGAC3′). The amplified DNA fragment was cloned into *SacI* and *SalI*, and cloned into the corresponding site on plasmid pCRA925 (Inui et al., 2004b). *SalI* and *NotI* restriction endonuclease sites were introduced at the 3′ terminus of the cloned *ptsF* by PCR using primers *SalI*-ptsF-1704F (5′-GGCAGTCTGACACCTTGATCTGATC3′) and *NorI*-ptsF-1707F (5′-GAGGCGGCCGCGCACTCTGATC3′) to construct pCRC801. DNA fragments coding for FLAG-epitope tag and chloramphenicol-resistance genes were amplified by PCR using primers *SalI*-FLAG-CmF (5′-GAGCAGTCTGACACCTTGATCTGATC3′) and *CmF*-NorI-FlagCmR (5′-GAGGCGGCCGCGCACTCTGATC3′) and cloned into the *SacI* and *SalI* sites of the corresponding site on pCRC801 to construct pCRC802. *C. glutamicum* R was transformed with pCRC802, and KmR and CmR colonies were selected. Integration of the *FLAG*-Clm fusion into the 3′ end of the *fruR* in *C. glutamicum* R genome by double cross-over was confirmed by colony PCR using primers mini-*Clm*-I (5′-GGCAGTCTGACACCTTGATCTGATC3′) and *ClmF*-NorI-I-ClmR (5′-GAGGCGGCCGCGCACTCTGATC3′) to construct pCRC801. 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 *SalI*-fruR (5′-GGCAGTCTGACACCTTGATCTGATC3′) and *CmF*-ptsF (5′-GGCAAGGCTTCGGACAGCGTTGAAATCAGAAC-3′). The amplified fragment was digested with *SalI* and cloned into the promoter–lacZ strain was constructed as follows. The promoter region of *fruR* was amplified by PCR using primers *SalI*-fruR (5′-GGCAGTCTGACACCTTGATCTGATC3′) and *CmF*-ptsF (5′-GGCAAGGCTTCGGACAGCGTTGAAATCAGAAC-3′). The amplified fragment was digested with *SalI* and cloned into the *SacI* site of the corresponding site on pBAC741 reporter plasmid, which has been described previously (Inui et al., 2007). These promoter–lacZ fusion plasmids were then digested with *KpnI* and *SalI*, and the DNA fragment containing the *fruR* promoter–lacZ gene was cloned into the corresponding site on pBAC741 (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 LaclZLR-4354F (5'-ATAACCCGGGACGGGTCTAG-3') and Ind7insert-checkR (5'-GGTACCGAACAACAGACGC-3'), or LaclZLR-6425R (5'-CGACCGCAAGTTCAAGC-3') and Ind7insert-checkF (5'-CGAGACTGGGAATAGGCTC-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–12 % (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 ºC for 20 min, and cDNA was synthesized at 42 ºC 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 µl RNase inhibitor and 5 µl MuLV reverse transcriptase (Applied Biosystems). The primers used in these reactions were: ptsI, ptsIF (5'-GGCTTCGAACTGGAGATGAAC-3') and ptsIR (5'-GGTGGTGAGCTTGCTCATCAAG-3'); and 16S rRNA, 16SF (5'-TGATGCAACGGGAAGAC-3') and 16SR (5'-GAACGACCAACAGGAAAAC-3'). Reactions were performed using the ABI 7500 Fast Real-Time PCR System (Applied Biosystems) with the following cycle parameters: one cycle of 50 ºC for 30 min and 95 ºC for 10 min, followed by 40 cycles of 95 ºC for 15 s and 60 ºC 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 (Na3HPO4/NaH2PO4, pH 7.0, 10 mM KCl, 1 mM MgSO4, 50 mM β-mercaptoethanol) with 2 % tolune 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 ptsf-fruR promoter DNA fragment was generated by PCR using primers promoter-ptsf (5'-GGCCGGCGGGCAGCATCGCCAGCTGAGGCACG-3') and bio-promoter-fruR (5'-AACGCAATGCATGGCCTTGCTTGTTGG-3'). The 5' terminal of the bio-promoter-fruR primer was labelled with biotin. PCR product (100 pmol) was coupled to 3 mg Dynabeads streptavidin (Dynal). 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 ptsf-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

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**Table 1. Oligonucleotides used as primers**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’–3’)</th>
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<tbody>
<tr>
<td>ptsI-1</td>
<td>ACATCAGGCGGACTAGGCA</td>
</tr>
<tr>
<td>ptsI-2</td>
<td>AAGCGACTCCACGGACAGC</td>
</tr>
<tr>
<td>ptsH-1</td>
<td>TTACGAGTCTGGAAAGCAGT</td>
</tr>
<tr>
<td>ptsH-2</td>
<td>AGCGATTGATGCTGGAC</td>
</tr>
<tr>
<td>ptsG-1</td>
<td>GTGTGCTGACGTGAGCA</td>
</tr>
<tr>
<td>ptsG-2</td>
<td>AGTACGATGAAATATGTTGCT</td>
</tr>
<tr>
<td>fruR-1</td>
<td>TGTCCGGCTGGTCGACATG</td>
</tr>
<tr>
<td>fruR-2</td>
<td>GGTGCGGATTAAAGAAAGCATTCG</td>
</tr>
<tr>
<td>ptsS-1</td>
<td>CTCGCGGGCCAAATTGCGCCAGGATG</td>
</tr>
<tr>
<td>ptsS-2</td>
<td>GCCGTAGACCGCAGTCAGTGTG</td>
</tr>
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</table>
of glucose did not increase the level of \textit{ptsF} mRNA (Fig. 1a, lane 2). In contrast, the addition of fructose increased the expression of \textit{ptsF} (Fig. 1a, lane 3), and sucrose also increased the \textit{ptsF} mRNA signal (Fig. 1a, lane 4). The expression pattern of \textit{ptsS} was similar to that of \textit{ptsF}, that is, the expression of \textit{ptsS} was induced by both fructose and sucrose (Fig. 1b). Glucose-specific \textit{pts} showed a different expression pattern. Significant \textit{ptsG} mRNA was detected in the absence of a PTS sugar (Fig. 1c, lane 1), and \textit{ptsG} expression increased in the presence of glucose (Fig. 1c, lane 2). Surprisingly, in the presence of fructose and sucrose, \textit{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 \textit{ptsH} was observed in the absence of PTS sugar (Fig. 1d, lane 1), and \textit{ptsH} expression increased in the presence of PTS sugar (Fig. 1d, lane 3). Both \textit{ptsH} and \textit{ptsI} expression increased in the presence of PTS sugar. Fructose was the most effective inducing sugar for \textit{ptsH} and \textit{ptsI} expression (Fig. 1d, e, lane 3). The results in Fig. 1(a, d) show that several transcriptional products are present for \textit{ptsF} and \textit{ptsH} genes. In summary, fructose and sucrose induced the expression of all \textit{pts} genes tested. We observed the induction of \textit{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 \textit{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 \textit{ptsI} gene (Fig. 1e), and suggests that the regulation of \textit{ptsI} expression by PTS sugars occurs mainly at the transcriptional level.

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**Fig. 1.** Effect of various PTS sugars on the expression of \textit{pts} genes. Total RNA (10 \(\mu\)g), 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 \textit{ptsF} probe; (b) hybridization with \textit{ptsS} probe; (c) hybridization with \textit{ptsG} probe; (d) hybridization with \textit{ptsH} probe; (e) hybridization with \textit{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.
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

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**Fig. 2.** Immunoblot analysis of cellular enzyme I level. Crude extract equivalent to OD$_{610}$ 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.

**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 \textit{ptsH} gene. This gene cluster is also conserved among \textit{C. glutamicum} ATCC 13032, \textit{Corynebacterium diptheriae} and \textit{Corynebacterium efficiens} (Parche et al., 2001b; Yokota & Lindley, 2005).

We analysed the operon structure of this region by Northern blotting using probes specific for \textit{fruR}, \textit{pfkB1}, \textit{ptsF} and \textit{ptsH} (Fig. 3b). A product of about 4.7 kb (product a) was detected by all four probes, indicating that \textit{fruR}, \textit{pfkB1}, \textit{ptsF} and \textit{ptsH} are transcribed as a polycistronic message. Using a \textit{ptsH} probe, an intense 0.5 kb transcript (product d) was detected. The \textit{ptsH} gene therefore seems to have its own promoter, as the expression pattern of \textit{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 \textit{ptsF}. This suggests therefore that these products are the result of the nuclease digestion.

**Determination of the transcriptional start sites of the \textit{pts} genes**

For further detailed analysis of the regulation of \textit{pts} gene expression, we examined the transcriptional initiation site of each \textit{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 \textit{pts} gene (Table 1). The position of the transcriptional start point was confirmed by experiments using two different primers for each \textit{pts} gene (Table 1). A major transcriptional start site of \textit{ptsH} (\textit{ptsH-P1} promoter) is the G residue located 102 nt upstream of the start codon of \textit{ptsH} (Fig. 4a), and we observed a minor signal corresponding to the T residue located 82 nt upstream of the start codon of \textit{ptsH} (\textit{ptsH-P2} promoter). Using a \textit{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 (\textit{ptsI-P1} promoter), and 37 nt upstream of the start codon (\textit{ptsI-P2} promoter). Using a \textit{ptsG}-specific primer, we detected a single band corresponding to a T residue, located 256 nt upstream of the start codon. The difference from our result may come from the slight difference in DNA sequence at the \textit{ptsG} promoter. The A residue located 262 nt upstream of the start codon in \textit{C. glutamicum} R is a G residue in the ATCC 13032 strain.

To determine the start site of the fructose-specific \textit{pts} operon, we used a \textit{fruR}-specific primer. We detected four bands corresponding to G and T (\textit{fruR-P1} promoter) and A and T residues (\textit{fruR-P2} promoter) located 294, 293, 123 and 126 nt, respectively, upstream of the ATG start codon of \textit{fruR}. A T residue located 62 nt upstream of the start codon was detected using a \textit{ptsS} primer (Fig. 4g). Nucleotide sequences of the \textit{pts} promoter regions are compared in Fig. 5. We found putative −10 and −35 hexamers. Comparison of each promoter showed that a TGTTT(TT)G sequence was conserved. The \textit{ptsI-P1} promoter region and the \textit{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 \textit{ptsG} mRNA expression, and an increase in \textit{ptsG} mRNA level was detected (Fig. 4d). These data correspond to the results of Northern blotting assays (Fig. 1).

**Effect of \textit{fruR} gene disruption on the expression of the \textit{pts} genes**

We were interested in how fructose regulates the expression of \textit{pts} genes, as fructose has the strongest inducing effect on \textit{pts} gene expression, and the general component of the PTS is located near the \textit{fructose-pts} operon, which differs from that of other bacteria. The \textit{fruR} gene encodes a DeoR-type regulator that is located upstream of \textit{ptsF} (Fig. 3a). This arrangement is conserved in many bacteria, and the regulator is responsible for the induction of the \textit{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 \textit{pts} genes.

We compared the expression of \textit{pts} genes in wild-type and \textit{fruR} cells. In cells grown in the presence of fructose, the mRNA levels of \textit{ptsI} and \textit{ptsH} were higher in the \textit{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 \textit{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 \textit{pts} mRNA level in the \textit{fruR} strain as in the wild-type, and that the induced level in response to fructose was increased by disruption of \textit{fruR}. Expression of \textit{ptsG} was hardly affected by the \textit{fruR} mutation with all PTS sugars (data not shown).

In the \textit{fruR} mutant we could not detect the \textit{fructose-pts} operon mRNA by Northern blotting analysis, probably because of the transposon insertion in the \textit{fruR} gene. In this regard, we observed that disruption of \textit{ptsF} completely eliminated the induction of \textit{pts} genes in response to fructose. We observed that disruption of \textit{pfkB1} completely inhibited growth in the presence of fructose. The phenotypes of the \textit{ptsF} and \textit{pfkB1} mutants were different from those of the \textit{fruR} mutant. These results suggest that \textit{ptsF} and \textit{pfkB1} are expressed in response to fructose in the \textit{fruR} cells, although we cannot rule out the possibility that...
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 $\beta$-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-
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 of PTS sugars (Fig. 2a) and the expression was induced by both fructose and sucrose (Fig. 2b). In addition, the expression of *ptsF* and *ptsS* was induced by fructose and sucrose (Fig. 3a, b). On the other hand, significant expression of *ptsG* was observed in the absence of PTS sugars (Fig. 3c).
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 tGtntg(TT)G 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 tG are well conserved among pts promoters, but the last three nucleotides are less well conserved. Among the pts promoters, the TGTTT(TT)G sequence is conserved, and may be an operator site for the pts operon (Gauriavud 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-pfkB1-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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