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Regulation of expression of general components of the phosphoenolpyruvate: carbohydrate phosphotransferase system (PTS) by the global regulator SugR in *Corynebacterium glutamicum*

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Abstract The phosphoenolpyruvate: carbohydrate phosphotransferase system (PTS) catalyzes transport of carbohydrates by coupling carbohydrate translocation and phosphorylation. Enzyme I and HPr, encoded in *ptsI* and *ptsH*, respectively, are cytoplasmic proteins commonly used for transport of variety of PTS sugars. In this study, we investigated the role of SugR on the expression of the *ptsI* and *ptsH* which increases in the presence of PTS sugars in *Corynebacterium glutamicum*. Disruption of *sugR* resulted in the increased expression of *ptsI* and *ptsH* in the absence of PTS sugar. Introduction of a plasmid containing *sugR* gene complemented the effect of *sugR* disruption. SugR was purified and binding to the promoter regions of *ptsI* and *ptsH* was indicated by EMSA. DNase I footprinting analysis indicated the binding sites of SugR on the promoter region of divergently transcribed *ptsI* gene and *fructose-pts* operon. The binding sites contain a possible SugR binding motif which is conserved in the promoter regions of general and sugar-specific *pts* genes. Mutations in this motif resulted in the decrease of SugR binding to the *ptsI* promoter. These results suggest that SugR represses *ptsI* and *ptsH* in the absence of PTS sugar and derepression is the mechanism for the induction of the general components of PTS.

Keywords PTS · SugR · ptsI · Corynebacterium · Glutamicum

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Introduction

In many bacteria, the phosphoenolpyruvate (PEP): carbohydrate phosphotransferase system (PTS) catalyzes transport of carbohydrates by coupling carbohydrate translocation and phosphorylation (Postma et al. 1993; Kotrba et al. 2001a). The PTS consists of two cytoplasmic proteins, enzyme I (EI) and HPr, and membrane-bound sugar specific enzyme II complexes (EIIs). The phosphoryl group from PEP is sequentially transferred to EI, HPr, EIIs, and finally to the substrate as it is translocated across the membrane. In most cases, EI and HPr participate in the phosphorylation of all PTS carbohydrates and have been called the general PTS proteins (Postma et al. 1993). 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).

It is known that expression of general PTS proteins is increased in the presence of PTS sugar especially by glucose (Postma et al. 1993). The mechanism for the induction has been extensively studied in *Escherichia coli* and in *Bacillus subtilis*. In *E. coli*, *ptsH*, and *ptsI* are cotranscribed with *crr*, which encodes for the EIIA component of the glucose-EII. Global regulator Mlc is responsible for the glucose induction of the *pts* operon (Kim et al. 1999; Plumbridge, 1999; Tanaka et al. 1999). In the absence of glucose, Mlc represses the transcription of the *pts* operon. In the presence of glucose, transport of glucose results in the dephosphorylation of $EIICB^{Glc}$ and this dephosphorylated $EIICB^{Glc}$ interacts and inhibits the activity of Mlc, resulting in the induction of the *pts* operon (Lee et al. 2000; Nam et al. 2001; Tanaka et al. 2000). In

B. subtilis, the *ptsGHI* operon expression is controlled by the GlcT antiterminator (Stülke et al. 1997). GlcT is active in its dephosphorylated form which is generated by the transport of glucose. Activated GlcT inhibits the rho-independent terminator preceding the *ptsGHI* operon and transcription proceeds to the 3′ end of *ptsGHI*.

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

In *C. glutamicum*, there are general components of PTS encoded in *ptsI* and *ptsH*, and fructose-, glucose-, and sucrose-specific EIIs which are encoded in *ptsF*, *ptsG*, and *ptsS*, respectively (Mori and Shiio, 1987; Dominguez and Lindley, 1996; Parche et al. 2001; Moon et al. 2005; Yukawa et al. 2007). Recently, we found that *ptsH* is cotranscribed with *fructose-pts*, although major transcripts are generated from its own promoter in *C. glutamicum* R (Tanaka et al. 2008). The gene encoding *ptsI* is divergently transcribed from the *fructose-pts* operon (Fig. 1a). Expression of these general components of PTS increases in the presence of glucose, fructose and sucrose. It is interesting to note that fructose is the most effective-inducing sugar for general components of PTS, which is different from the cases in other bacteria (Tanaka et al. 2008).

There are two DeoR-type transcriptional regulators which are located close to *ptsI* and *ptsH*. We recently found that one of the DeoR-type regulators, FruR, is involved in the regulation of the expression of *ptsI* and *ptsH* as well as *ptsF*. FruR reduces the induction level of these *pts* genes in the presence of fructose. However, FruR is not responsible for the induction of general component of PTS by PTS sugar, since the induction of *pts* genes by PTS sugar was still observed in the *fruR* disrupted cells. Recently, it was reported that SugR, the other DeoR-type regulator, regulates the expression of *ptsG*, *ptsF*, and *ptsS* (Engels and Wendisch, 2007). We also found that SugR, in addition to FruR, bound to the *ptsI-fruR* intergenic region containing the promoters of the *ptsI* and *fructose-pts* operon (Tanaka et al. 2008). However, it was not reported whether expression of general PTS was regulated by SugR. In this study, we examined the involvement of SugR in the regulation of expression of *ptsH* and *ptsI*. We also investigated SugR binding on the *ptsI-fruR* promoter region.

Materials and methods

Media and growth conditions *C. glutamicum* R (Yukawa et al. 2007) was grown aerobically at 33°C in BT minimal medium with casamino acids (Kotrba et al. 2001b) supplemented with 1% (*w*/*v*) acetate and indicated sugars added at 1% (*w*/*v*) as carbon sources. Antibiotics were added at the following final concentrations: kanamycin 50 μg ml^{-1} and chloramphenicol 5 μg ml^{-1}. Bacterial growth was monitored by determining the optical density at 610 nm.

Bacterial strains and plasmids *C. glutamicum* R (Yukawa et al. 2007) was used as a wild-type strain. The *sugR* gene disrupted strain was constructed by the transposon-mediated mutagenesis method as described previously (Suzuki et al. 2006). *sugR* gene disruption was confirmed by DNA sequencing. Transposon was inserted at 34 bases downstream from the 5′ end of the *sugR* ORF. Construction of recombinant plasmid pCRC803 containing *sugR* gene was carried out as follows. *sugR* gene was amplified from *C. glutamicum* R genomic DNA by PCR using primers EcoRI-sugR-23F and SphI-sugR-863R (Table 1). The amplified DNA fragment was digested with *Eco*RI and *Sph*I, and cloned into the corresponding site on pCRB1 (Nakata et al. 2003) to construct pCRC803. Construction of SugR overexpression plasmid pCRC805 was carried out as follows. *sugR* gene was amplified from *C. glutamicum* R genomic DNA by PCR using primers NdeI-sugR-1F and HindIII-sugR-863R (Table 1). The amplified DNA fragment was digested with *Nde*I and *Hind*III, and cloned into the corresponding site on pColdI (Takara) to construct pCRC805.

Real-time reverse transcriptase-polymerase chain reaction - Total RNA was isolated from exponentially growing cells (OD_{610} of 1.2) in BT medium supplemented with 1.0% (*w*/*v*) carbon source using the RNeasy Kit (QIAGEN). Total RNA of 20 ng 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 reverse transcriptase-polymerase chain reaction (RT-PCR) mixture (20 μl) contained 500 nM of each primer, 10 μl of Power SYBR Green PCR Master Mix, 8 U of RNase Inhibitor and 5 U of MuLV reverse transcriptase (Applied Biosystems). The primers used in these reactions are listed in Table 1. Reactions were performed using the ABI 7500 Fast Real-Time PCR System (Applied Biosystems) with the following cycle parameters: 1 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 result of 16S rRNA was used as an internal control.

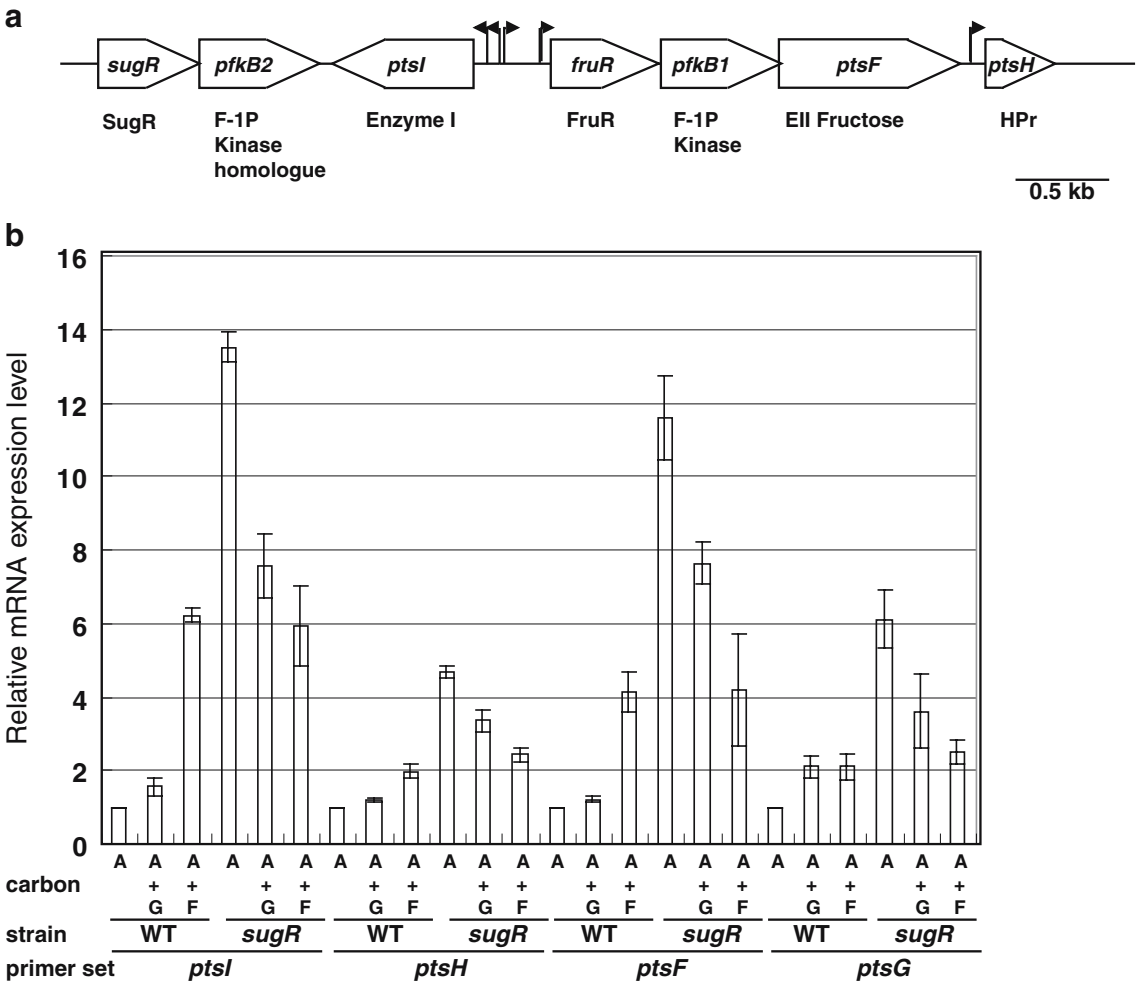

Fig. 1 Effect of *sugR* disruption on the expression of *pts* genes. **a** Genetic organization of the genes around the *fructose-pts* operon. *Open arrows* represent the coding regions. Deduced functions of each gene are indicated below. The promoters are indicated by *arrows*. **b** Real-time RT-PCR analysis of *pts* expression. Total RNAs prepared from wild-type (WT) or *sugR* disrupted 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, were subjected to real-time RT-PCR analysis using primers specific for the *ptsI*, *ptsH*, *ptsF*, *ptsG*, and *ptsS*. The ratio against wild type cells grown in the presence of acetate is indicated. The values are the means of three independent experiments and standard deviations are indicated on the bar tops. *A* Acetate, *G* glucose, and *F* fructose

Purification of SugR Overexpression of His_6-SugR was achieved by using a Cold-shock expressing vector pCold I (Takara). *E. coli* cells harboring pCRC805 were grown at 37°C in 100 ml of LB medium to OD_{610} of 0.5. Cultures were incubated 30 min at 15°C and then IPTG was added at a final concentration of 0.1 mM and shaken for 24 h at 15°C. Cells were harvested by centrifugation and suspended in 900 μl of His binding buffer (0.5 M NaCl, 20 mM Tris–HCl, 5 mM imidazole pH 7.9) (Novagen), 100 μl of Fast Break Cell Lysis Reagent (Promega), and 0.2 mg of lysozyme. The mixture was incubated for 15 min at room temperature and centrifuged for 5 min at 12,000 ×*g* and the supernatant was pooled. His_6-SugR was purified using His Bind Resin and Buffer Kit (Novagen) according to the procedure specified by the manufacturer.

Electrophoretic mobility-shift assay Electrophoretic mobility-shift assay (EMSA) was carried out in a total volume of 20 μl of binding buffer (20 mM Tris–HCl, pH 7.9, 100 mM NaCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM DTT, 5% (*w*/*v*) glycerol, and 50 μg/ml bovine serum albumin). The *ptsI-fruR* promote*, ptsH* promoter and *ptsI* ORF DNA fragments were amplified by PCR using primers listed in Table 1. The DNA fragments containing nucleotide substitutions at putative SugR binding sites were prepared as follows. First, *ptsI* promoter DNA fragments listed in Table 1 (ptsIwtF and ptsIwtR, ptsImut1F and ptsImut1R, ptsImut2F and ptsImut2R, and ptsImut3F and ptsImut3R) were annealed and digested by *Eco*RI and *Hind*III and cloned into the corresponding site of pCRA1. The DNA fragments containing *ptsI* promoter region were amplified by PCR using LSVRI and LSVH3 (Table 1).

Table 1 Oligonucleotides used as primers

Primer	Sequence (5′-3′)
Primers used for cloning	
EcoRI-sugR-23F	GCGGAATTCGGCTTTTTGCTTTAAGGAGTGACATG
SphI-sugR-863R	CCGGCATGCGATTTTGTTTGCTTCGCCAATTTCG
NdeI-sugR-1F	CCGCATATGTACGCAGAGGAGCGCCGTC
HindIII-sugR-863R	GCGAAGCTTCATTCTGCAATCACAACTTCTACATCG
Primers used for real time RT-PCR	
ptsIF	GGCTTCGAACATGGAGATGAAC
ptsIR	TGGCGTAAGGCTGTCATCAAG
ptsHF	GCTCCGATGATGACGAAGAGA
ptsHR	CGATAAGCGCAGCGATTTTC
ptsFF	CAGGACTGGCTATCGTCAATGTC
ptsFR	CGAGGTCGAAACACATCATGAG
ptsGF	CTGATCACTTTTGACGCTGACTTC
ptsGR	CGAATTTTGCGGCGTTAGAC
ptsSF	TGCGGCGAACCAAGTAAAG
ptsSR	GGTGTGTGCAGTTGTTACCTTCTT
16SF	TCGATGCAACGCGAAGAAC
16SR	GAACCGACCACAAGGGAAAAC
Primers used for EMSA and DNase I foot printing	
ptsI-P	CGGCTAGCCACCCATCCTCACAATC
fruR	GCGAAGCTTAACCTCGCTGATGTTGGAG
ptsH-1	GCGCCCGGGCTGACCCATTCCGTGTGATC
ptsH-2	GCGCCCGGGCTTGGAAGCCATGGAAAGTGTCC
ptsORF-1	GATCATGCCTCACCTGGACTTTG
ptsIORF-2	CGCGCTAGCGACTGCTGCGTCGATCACTGC
ptsIwtF	GCGGAATTCACCACAAAATCAACAGTTGTGCAACTATT CAAACATTTGGATATTGACAAGCTTCGC
ptsIwtR	GCGAAGCTTGTCAATATCCAAATGTTTGAATAGTTGCA CAACTGTTGATTTTGTGGTGAATTCCGC
ptsImut1F	GCGGAATTCACCACAAAATCAACAGTCCATGGTAGATT CAAACATTTGGATATTGACAAGCTTCGC
ptsImut1R	GCGAAGCTTGTCAATATCCAAATGTTTGAATCTACCATG GACTGTTGATTTTGTGGTGAATTCCGC
ptsImut2F	GCGGAATTCACCACAAAATGTCACGTTGTGCAACTATTA GCTACTTTGGATATTGACAAGCTTCGC
ptsImut2R	GCGAAGCTTGTCAATATCCAAAGTAGCTAATAGTTGCAC AACGTGACATTTTGTGGTGAATTCCGC
ptsImut3F	GCGGAATTCACCACAAAATGTCACGTCCATGGTAGATTA GCTACTTTGGATATTGACAAGCTTCGC
ptsImut3R	GCGGAATTCACCACAAAATGTCACGTCCATGGTAGATTA GCTACTTTGGATATTGACAAGCTTCGC
LSVRI	GTATGTTGTGTGGAATTGTGAGC
LSVH3	AAGGCGATTAAGTTGGGTAACG
Primers used for DNase I footprinting	
ptsI[a]	AACGGACTCCACCGACAACG
fruR[a]	TGTCTTTCCGTTTGGCTGACCATG

[a] Indicates an IRD700 fluorescent label at 5′ end.

Two nanomolar of DNA fragments were incubated with the indicated concentration of the purified SugR for 10 min at 33°C. The mixture was fractionated by electrophoresis on a native 5% (*w*/*v*) polyacrylamide gel containing 5% (*w*/*v*) glycerol in 1/2 TBE (45 mM Tris-borate, pH 8.3, 1 mM EDTA) at room temperature. The DNA bands were stained with ethidium bromide and visualized by UV irradiation.

DNase I footprinting The *ptsI-fruR* promoter DNA fragment was amplified by using primer sets listed in Table 1

(fruR and ptsI* and ptsI-P and fruR*). IRD-700 Labeled DNA fragments (2 nM) were mixed with purified SugR in a total volume of 100 μl of a binding buffer (20 mM Tris–HCl, pH 7.9, 100 mM NaCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM DTT, 5% (*w*/*v*) glycerol, and 50 μg/ml bovine serum albumin) containing 5 mM $CaCl_2$. The mixtures were incubated for 5 min at 33°C and then placed at 25°C for 5 min. DNase I (Takara) was added at 0.025 U and incubation was continued for 1 min at 25°C. The mixture was treated with phenol and precipitated with ethanol and loaded onto a 5.5% (*w*/*v*) polyacrylamide sequence gel next to the sequencing ladder and run in the LI-COR DNA sequencer model 4000 (Aloka).

Results

Effect of sugR disruption on the expression of the general components of PTS The gene encoding the DeoR-type transcriptional regulator SugR is present in the vicinity of the *ptsI* gene (Fig. 1a). We hypothesized that SugR is involved in the induction of general *pts* genes in the presence of PTS sugar. Accordingly, we analyzed the effect of *sugR* (*cgR1761*) disruption on the expression of general components of the PTS in the presence or absence of PTS sugars. *C. glutamicum* R wild-type cells or *sugR* disrupted cells were grown in minimal medium supplemented with acetate in the presence of or absence of glucose or fructose. Total RNAs were prepared and analyzed by real-time RT-PCR using primers specific for *ptsI* and *ptsH*. As we noted previously (Tanaka et al. 2008), the level of *ptsI* mRNA increased in the presence of PTS sugar in wild type cell (Fig. 1b). Fructose was a more effective inducer than glucose. Then, we examined the *ptsI* mRNA level in sugR disrupted cells. The levels of *ptsI* mRNA was higher in *sugR* disrupted cells than wild type cells in the absence of PTS sugar. Addition of glucose to the culture of *sugR* disrupted cells slightly reduced the induction of *ptsI*, but there was still higher expression than in wild type cells grown in the presence of glucose. Additions of fructose further reduced the expression level of *ptsI* in *sugR* cells to almost the same level of induction in wild type cells grown in the presence of fructose. Similar results were observed for expression of *ptsH*, although the effects of PTS sugar and *sugR* disruption were smaller than those on *ptsI* expression. In addition, we also analyzed expression of *ptsF* and *ptsG* encoding EIIs specific for fructose and glucose, respectively. Similar to *ptsI* and *ptsH*, the expressions of *ptsF* and *ptsG* showed high levels of expressions in the *sugR* disrupted cells in the absence of PTS sugar, and the addition of PTS sugar reduced the expressions of *ptsF* and *ptsG*.

To confirm that the effect of *sugR* disruption is caused by inactivation of SugR and not by other gene products, we introduced the plasmid pCRC803, which had the *sugR* gene, to the *sugR* disrupted cells and investigated *pts* gene expression (Fig. 2). The level of *ptsI* mRNA was higher in the *sugR* disrupted cells having vector plasmid pCRB1 than wild type cells containing the same plasmid in the absence of fructose, as expected from the results in Fig. 1. In the *sugR* cells containing pCRC803, expression of *ptsI* was decreased more than with the *sugR* cells having vector plasmid in the absence of fructose, and addition of fructose increased the expression to a pattern similar to the wild type cells containing the vector plasmid (Fig. 2). These results indicated that the *sugR* disruption effect was complemented. Similar results were observed for the expression of *ptsH* for general PTS, and sugar-specific *pts* genes, *ptsF*, and *ptsG*. We conclude that SugR inhibits the expression of *ptsI* and *ptsH* in the absence of PTS sugar.

Binding of SugR to the promoter regions of the ptsH and ptsI promoter In *C. glutamicum*, *ptsI* is located adjacent to the *fruR-ptsF-pfkB-ptsH* operon, and is transcribed divergently. Between *ptsI* and *fruR*, there are two promoters for *ptsI* (*ptsI*-P1 and *ptsI*-P2) and two promoters for *fruR* (*fruR*-P1 and *fruR*-P2) (Tanaka et al. 2008). *ptsH* has its own promoter and the majority of the *ptsH* transcripts are produced from the *ptsH* promoter (Tanaka et al. 2008). Previously, we observed the binding of SugR to *fruR-ptsI* promoter DNA by DNA affinity purification analysis (Tanaka et al. 2008). To test whether SugR binds to *ptsI* and *ptsH* promoter region, we conducted EMSA using *ptsI* or *ptsH* promoter DNA. Hexa-histidine-tagged SugR was overexpressed and purified from *E. coli*. Then, SugR was mixed with *ptsI* DNA promoter fragments covering the *ptsI-fruR* promoter region and the *ptsH* promoter fragment. We also incubated SugR with the coding region of *ptsI* which is assumed to not have a SugR binding site. Using *ptsI-fruR* promoter fragment, addition of SugR resulted in the appearance of three mobility retarded bands, two of which appeared at 7.5-fold molar excess SugR, and slowest mobility band appeared at 15-fold molar excess of SugR (Fig. 3, lanes 1–4).

By using the *ptsH* promoter fragment, we observed two shifted bands, one band appeared at 7.5-fold molar excess of SugR and another band appeared at 15-fold molar excess of SugR (Fig. 3, lanes 5–8). DNA fragment of the *ptsI* coding region showed no shifted bands (Fig. 3, lanes 9–12). We conclude that SugR binds to the *ptsI-fruR* promoter region and the *ptsH* promoter region, which suggest that the effect of SugR on the expression of *ptsI* and *ptsH* is a direct one. Engels and Wendisch (2007) also showed that purified SugR binds to the *fruR-ptsI* promoter region by EMSA,

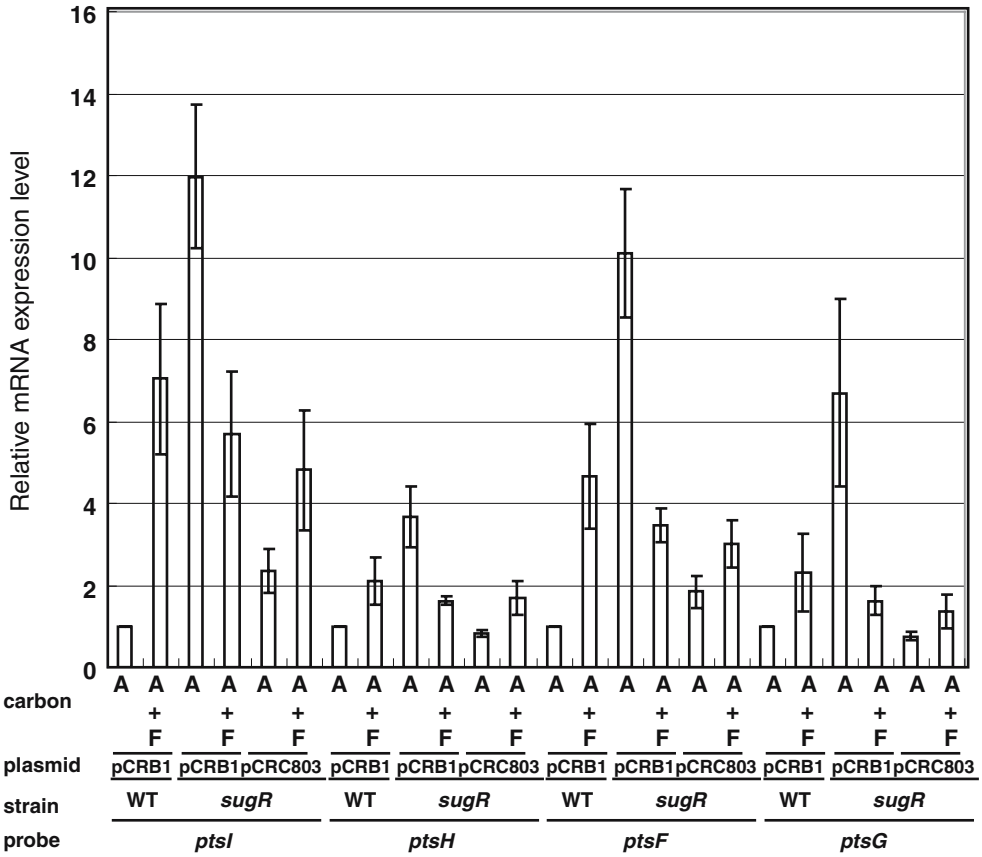

Fig. 2 Complementation of *sugR* disruption. Total RNAs, prepared from wild type (*WT*) or *sugR* disrupted cells with either pCRB1 or pCRA803 grown in BT minimal medium supplemented with casaminoacids and 1.0% (*w*/*v*) acetate with or without 1.0% (*w*/*v*) fructose, were subjected to real-time RT-PCR analysis using primers specific for the *ptsI*, *ptsH*, *ptsF*, and *ptsG*. The values are the means of three independent experiments and standard deviations are indicated on the bar tops

which corresponds to our *ptsI* results. The result of mobility shift assay also suggests that each promoter region contains multiple SugR binding sites.

Determination of the SugR binding sites on ptsI-fruR promoter region The result of EMSA suggested that several SugR binding sites are present in *ptsI-fruR* promoter region. To determine the binding sites of SugR, we conducted DNase I footprint analysis on *ptsI-fruR* promoter region. There are two promoters for *ptsI*. The initiation site is located at 103 nucleotide and 105 nucleotide upstream of the GTG start codon (*ptsI*-P1 promoter), and 37 nucleotide upstream of the start codon (*ptsI*-P2 promoter) (Tanaka et al. 2008). There are also two promoters for *fruR*. The initiation site is located 294 nucleotide upstream of the ATG start codon (*fruR*-P1 promoter), and 123 and 126 nucleotide upstream of the ATG start codon of *fruR* (*fruR*-P2 promoter). −10 regions of *ptsI*-P1 and *fruR*-P1 are overlapping (Tanaka et al. 2008).

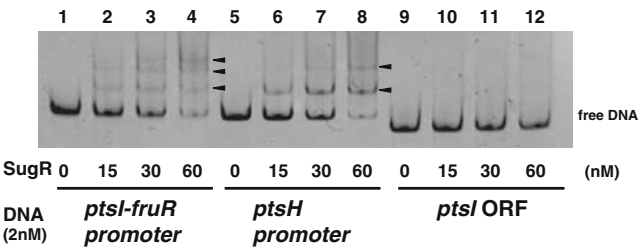

Fig. 3 Electro mobility shift assay of binding of SugR to the *ptsI-fruR* promoter and *ptsH* promoter. The 432 bp fragment (2 nM) containing *ptsI-fruR* promoter region, 462 bp fragment (2 nM) containing *ptsH* promoter, and 415 bp fragment (2 nM) containing *ptsI* ORF were incubated with indicated amount of SugR. The primers used to generate each DNA fragment are listed in Table 1

The *ptsI-fruR* promoter DNA fragment was fluorescently labeled at either the *ptsI* proximal end or the *fruR* proximal end. These end-labeled DNA fragments were incubated with SugR and challenged by DNase I digestion. Two regions were protected from the nuclease attack by DNase I. One protected region located from *ptsI*-P2 promoter to *ptsI*-P1 (*fruR*-P1) promoter. Another protected region was located downstream of *fruR*-P2 promoter (Fig. 4). The DNA sequence of *ptsI-fruR* promoter regions is shown in Fig. 5a. The protected region proximal to *ptsI* covers the −35 region of *ptsI*-P2, the −10 and the transcription initiation site of *ptsI*-P1 and the −10 region of *fruR*-P1. The length of protected region proximal to *ptsI* is 67 bp,

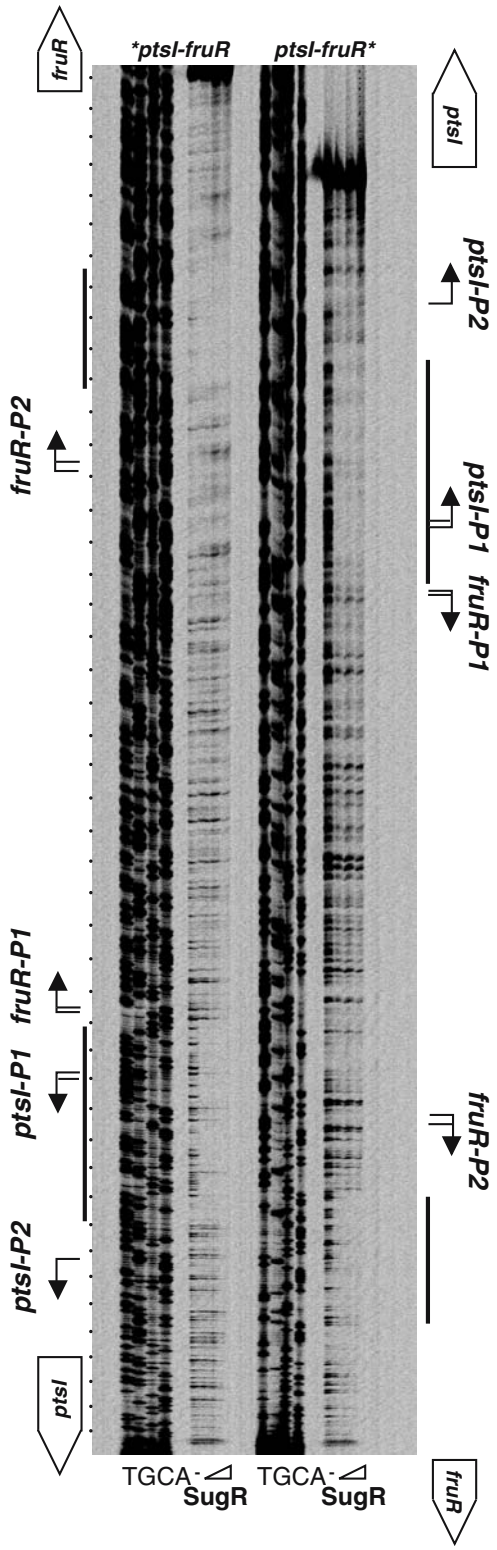

Fig. 4 DNase I footprinting analysis of the SugR binding to the *ptsI-fruR* promoter. The 487 bp *ptsI-fruR* promoter fragment fluorescently labeled at *ptsI* terminal or at *fruR* terminal was incubated with SugR (0, 20, 40, 80, and 160 nM). The mixture was subjected to a partial digestion by DNase I. The products were loaded next to the sequencing product of *ptsI-fruR*. The protected regions by SugR are shown by *vertical lines*. Transcriptional start sites of *fruR* and *ptsI* are shown by *arrows*

while the *fruR* proximal region is 38 bp long. This and the result of EMSA using *fruR-ptsI* DNA fragment suggest that the DNA region has three SugR binding sites, two of which are located in the protected region proximal to *ptsI*, and one of which is located downstream of *fruR*-P2. The putative binding sites have repeats of TG (T) $_{2–5}$ G (Fig. 5b) which we previously pointed out is present in all the promoter regions of PTS-sugar inducible *pts* genes, i.e., *ptsH*, *ptsI*, *ptsG*, *ptsF* (*fruR-ptsF* operon), and *ptsS* (Tanaka et al. 2008). The protected region proximal to *ptsI* also contains the GTTGCACA sequence which was found to be a motif partly conserved in the SugR binding upstream of *ptsG*, *ptsS*, and *fruR* (Engels and Wendisch 2007). We additionally found that the GTTGCACA motif is also present in the *ptsH* promoter (Fig. 5b). However, there seems to be no such sequence at the protected region downstream of the *fruR*-P2 promoter (Fig. 5a).

Effect of nucleotide change at ptsI promoter on SugR binding The result of DNaseI footprinting suggests that SugR recognizes either or both TG (T) $_{2–5}$ G and GTTGCACA sequences. To determine the role of these sequences on SugR binding, we constructed various mutated *ptsI* promoter DNA fragments that changed the TG (T) $_{2–5}$ G or GTTGCACA to random sequences (Fig. 6a). The DNA fragment that covers the −35 region of *ptsI*-P2 promoter was prepared. Mut1 changed the TG (T) $_{2–5}$ G motif, Mut2 changed the GTTGCACA motif and Mut3 changed both motifs. SugR bound to wild type *ptsI* promoter fragment as expected (Fig. 6b, lanes 1–3). Mutation of the TG (T) $_{2–5}$ G motif reduced the binding of SugR to this fragment (Fig. 6b, lanes 4–6). Mutation of the GTTGCACA motif also modestly reduced the SugR binding (Fig. 6b, lanes 7–9). The double mutation (Mut3) showed the most inhibitory effect on SugR binding (Fig. 6b, lanes 10–12). These results suggest that both GTTGCACA and TG (T) $_{2–5}$ G sequences are recognized by SugR, and the presence of both motifs enhances the SugR binding to target DNA.

Discussion

In our previous paper (Tanaka et al. 2008), we reported that the expression of general components of the PTS (*ptsI* and *ptsH*) as well as sugar-specific PTS components (*ptsF*, *ptsG*, and *ptsS*) was increased in the presence of PTS sugar in *C. glutamicum*. In this study, we revealed that disruption of *sugR*, which was reported to repress the expression of *ptsG*, *F*, and *S* (Engels and Wendisch 2007) induced the expression of *ptsH* and *ptsI* in the absence of PTS sugar. Introduction of *sugR* in *trans* resulted in the

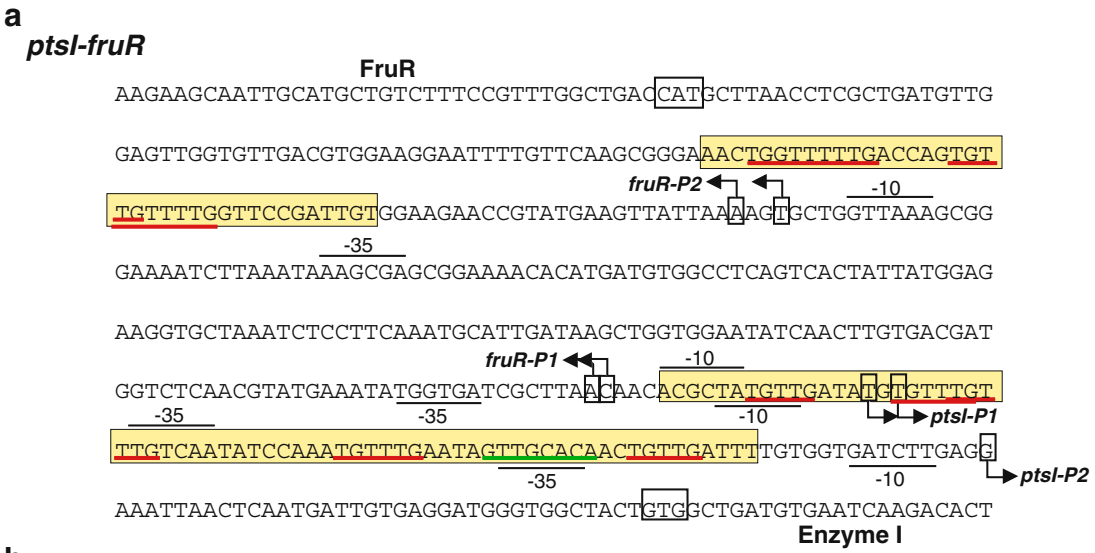

Fig. 5 SugR binding regions of *pts* promoters. **a** The nucleotide sequence between divergently transcribed *ptsI* and *fruR*. *Yellow box* represents the regions protected by SugR from DNase I digestion. Promoters of *ptsI* and *fruR* are depicted as *arrows*. Possible SugR binding motif (GTTGCACA) suggested by Engels and Wendisch (2007) is *underlined by green color*. The motif previously found in promoters of *pts* genes, TG (T) $_{2–5}$ G (Tanaka et al. 2008), is *underlined by red color*. The −10 and the −35 hexamers are underlined by *black color*. **b** Comparison of the promoter regions of *pts* genes. *Yellow box* represents the regions protected by SugR from DNase I digestion. Possible SugR binding motif suggested by Engels and Wendisch (2007) are *boxed by green*. TG (T) $_{2–5}$ G motifs are *boxed by red*. Numbers from the transcription initiation sites from *ptsI*-P1, *fruR*-P2, *ptsG*, *ptsH*-P1, and *ptsS* are indicated

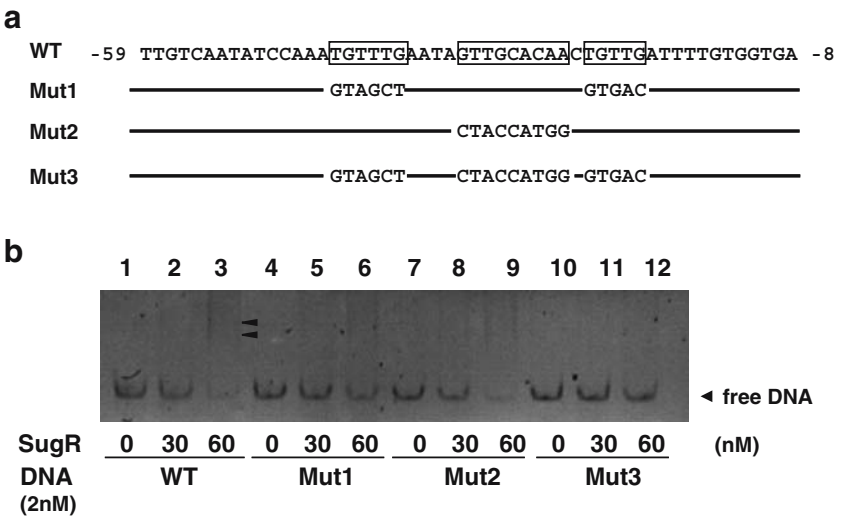

Fig. 6 EMSA of binding of SugR to the mutated *pts* promoter. **a** DNA fragments used in this study. The *ptsI* promoter regions are shown. Either or both putative SugR binding motifs (GTTGCACA and TG (T) $_{2–5}$ G) were substituted randomly. Putative SugR binding motifs are *boxed*. Only changed nucleotide sequences are depicted for mutated fragments. **b** EMSA of SugR binding to *ptsI* promoter. The *ptsI*-P2 promoter DNA fragments depicted above (2 nM) were incubated with indicated amount of SugR

repression of *ptsH* and *ptsI* expression in the absence of PTS sugar, supporting the notion that SugR represses the *ptsH* and *ptsI* promoter in the absence of PTS sugar. Therefore, expressions of both cytoplasmic components and transporter complexes of PTS seem to be controlled globally by SugR.

Engels and Wendisch (2007) reported that SugR binds to the *ptsG* promoter region, and this binding is inhibited by fructose-6-phosphate. They suggested that expression of *ptsG* is repressed by SugR in the absence of PTS sugar and inhibition of SugR activity by fructose-6-phosphate results in the induction of *ptsG*. The regulation of general PTS expression may be also controlled by such a mechanism. However, we observed that expressions of *ptsH*, *ptsI*, *ptsF*, and *ptsS* are more induced in the presence of fructose than in the presence of glucose. Since it was reported that intracellular concentrations of fructose-6-phosphate during growth on glucose is higher than during growth on fructose (Georgi et al. 2005), it is difficult to explain the expression pattern of *pts* genes simply by the action of SugR and fructose-6-phosphate.

It is interesting to note that addition of PTS sugar reduced the expressions of *ptsH*, *ptsI*, *ptsG*, and *ptsF* genes in *sugR* disrupted cells. These results suggest the existence of another transcriptional regulator(s) that decreases *pts* gene expression in the presence of PTS sugar. One candidate is FruR which is located upstream of *ptsF* gene (Fig. 1a). Previously, we have demonstrated that FruR reduces the induction of *ptsF-operon*, *ptsI*, and *ptsH* induction in the presence of fructose (Tanaka et al. 2008). However, other regulator(s) should also exist, since FruR does not regulate *ptsG* expression. Expression of *C. glutamicum pts* genes may be controlled in a complicated manner, as in the case of other bacteria. In *E. coli*, expression of *ptsG* is negatively regulated by Mlc and positively regulated by CRP-cAMP. In the presence of glucose, activity of Mlc is inhibited and results in the induction of the *ptsG*, while the activity of CRP-cAMP is reduced. Hence, full induction of *ptsG* is not observed in wild-type cell grown in glucose. The transcriptional regulators and mechanisms involved in the regulation of *C. glutamicum* PTS have many differences from that of other bacteria. Further investigation is required to clear the mechanism of the regulation of *pts* gene expression.

By EMSA and DNase I footprinting analysis, we investigated SugR binding to the *ptsI* promoter region. Between *ptsI* and *fruR*, there are two promoters for *ptsI* (*ptsI*-P1 and *ptsI*-P2) and two promoters for *fruR* (*fruR*-P1 and *fruR*-P2) (Fig. 5a). The −10 regions of *ptsI*-P1 and *fruR*-P1 are overlapping. There are several TG (T) $_{2–5}$ G sequences which are found in the promoters of *pts* genes upregulated in the presence of PTS sugar (Fig. 5b). Two regions were protected from the nuclease attack by DNase I. One protected region was located between the *ptsI*-P2 promoter to the *ptsI*-P1 *fruR*-P1 promoter. Another protected region was located downstream of the *fruR*-P2 promoter (Fig. 5a). We found that the TG (T) $_{2–5}$ G sequence is present in these regions. Engels and Wendisch examined the SugR binding site by EMSA using several deletion sets of the *ptsG* promoter. They suggested that the GTCGGACA or GTTGCACA motif is present at the upstream regions of *ptsG*, *ptsS*, and the *fruR-ptsF* operon. We found that this motif is also present at the *ptsH* promoter region, and showed the binding of SugR to the region by EMSA. However, the GTCGGACA or GTTGCACA sequence is not present in the *fruR*-P2 promoter region that was protected by SugR against DNase I attack, although GTTGCACA is present at the *pts*-P1 region that was protected by SugR. These results suggest that the TG (T) $_{2–5}$ G sequence in addition to GTTGCACA is involved in SugR binding to DNA. We tested this hypothesis by examining the effect of mutation at these putative SugR binding motifs. We found that both sequences contribute to the binding of SugR to target DNA. These motifs are present in all the *pts* upstream regions. However, the locations of these motifs are different for each *pts* promoter. Especially, there is no GTCGGACA or GTTGCACA in the *fruR*-P2 promoter region. It is interesting how these differences affect the action of SugR.

In summary, we demonstrated that SugR controls *ptsH* and *ptsI* expression that means SugR regulates expressions of both general cytoplasmic components and transporter complexes of PTS. We also found that the TG (T) $_{2–5}$ G sequence was involved in the SugR binding in addition to the GTTGCACA sequence.

During the submission of this manuscript, Gaigalat et al (2007) also reported that SugR regulates the expression of *ptsH* and *ptsI*. They showed that *sugR* gene disruption resulted in the increased expression of *ptsH* and *ptsI* mRNA in the absence of PTS sugar. They determined a 21 bp sequence in the *ptsI-fruR* promoter region as the SugR binding site containing the TG (T) $_{2–5}$ G motif determined in this study. They also showed that fructose-1-phosphate, fructose-1,6-bis-phosphate and glucose-6-phosphate can act as negative effectors of the SugR repressor. The highly efficient effector, fructose 1-phosphate, which is generated during the transport of fructose into the cell by fructose-PTS, may cause higher expression of *pts* genes in cells grown on fructose than in those grown on glucose.

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