Identification of a second β -glucoside phosphoenolpyruvate : carbohydrate phosphotransferase system in *Corynebacterium glutamicum* R

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The phosphoenolpyruvate : carbohydrate phosphotransferase system (PTS) catalyses carbohydrate transport by coupling it to phosphorylation. Previously, we reported a *Corynebacterium glutamicum* R β -glucoside PTS encoded by *bglF*. Here we report that *C. glutamicum* R contains an additional β -glucoside PTS gene, *bglF2*, organized in a cluster with a putative phospho- β -glucosidase gene, *bglA2*, and a putative antiterminator, *bglG2*. While single gene disruption strains of either *bglF* or *bglF2* were able to utilize salicin or arbutin as sole carbon sources, a double disruption strain exhibited defects in utilization of both carbon sources. Expression of both *bglF* and *bglF2* was induced in the presence of either salicin or arbutin, although disruption of *bglG2* affected only *bglF2* expression. Moreover, in the presence of either salicin or arbutin, glucose completely repressed the expression of *bglF* but only slightly repressed that of *bglF2*. We conclude that BglF and BglF2 have a redundant role in β -glucoside transport even though the catabolite repression control of their encoding genes is different. We also show that expression of both *bglF* and *bglF2* requires the general PTS.

INTRODUCTION

The phosphoenolpyruvate : carbohydrate phosphotransferase system (PTS) catalyses transport of carbohydrates by coupling carbohydrate translocation to phosphorylation (Postma *et al.*, 1993; Kotrba *et al.*, 2001a). The PTS consists of two common cytoplasmic proteins, enzyme I (EI) and HPr, and an array of sugar-specific enzyme II complexes (EIIs). The phosphoryl group from phosphoenolpyruvate is sequentially transferred to EI, HPr, EIIs and finally to the substrate as it is translocated across the membrane.

The PTS also has a regulatory role in response to carbohydrate availability. In the presence of a rapidly metabolizable carbon source such as glucose, bacteria often preferentially utilize this carbon source by repressing expression of other catabolic genes. This phenomenon is called carbon catabolite repression. Phosphorylation states of PTS components have a critical role in carbon catabolite repression (Postma *et al.*, 1993; Deutscher *et al.*, 2006). In *Escherichia coli*, phosphorylation of EIIA^{Glc} regulates the level of cyclic AMP, which binds to CRP protein, a

transcriptional regulator of many catabolic genes. In addition, unphosphorylated EIIA^{Glc} inhibits many sugar transporters, resulting in the decrease of inducers in the cell (inducer exclusion). In *Bacillus subtilis* and other low-GC Gram-positive bacteria, HPr binds to CcpA protein to control the transcription of many catabolic genes. HPr is also involved in inducer exclusion in low-GC Gram-positive bacteria (Postma *et al.*, 1993; Deutscher *et al.*, 2006).

Expression of the β -glucoside PTS in many bacteria is upregulated in response to the substrate, and is often subject to strict glucose repression. In *E. coli*, *B. subtilis* and many other bacteria, expression of the β -glucoside PTS gene is controlled by an antitermination mechanism. In antitermination regulation, transcription terminates at a terminator present in the 5' UTR of mRNA in the absence of substrate. In the presence of substrate, BglG/SacY family transcriptional antiterminators recognize the ribonucleic antiterminator (RAT) sequence present in the 5' UTR of mRNA (Stülke & Hillen, 2000; Amster-Choder, 2005; Deutscher *et al.*, 2006). RAT partially overlaps the transcriptional terminator, and it is believed that binding of transcriptional antiterminator to RAT stabilizes the secondary structure of the RAT sequence and disrupts the terminator structure, which results in the elongation of transcripts to the end of the target gene. The BglG/SacY

Abbreviations: PRD, PTS regulation domain; PTS, phosphoenolpyruvate : carbohydrate phosphotransferase system; RAT, ribonucleic antiterminator.

A supplementary table of primers is available with the online version of this paper.

family antiterminator contains the PTS regulation domain (PRD). Phosphorylation of the PRD-1 site by EII of the PTS inhibits antiterminator activity, which is the underlying mechanism of upregulation of the β -glucoside PTS gene in response to the substrate. On the other hand, PRD-2 site phosphorylation by HPr is required for antitermination, which is related to glucose repression.

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; Kelle *et al.*, 2005). *C. glutamicum* R can provide high yields of lactate and succinate from sugar (Inui *et al.*, 2004a; Okino *et al.*, 2008a, b). In *C. glutamicum*, the general PTS (EI and HPr) as well as glucose-, fructose- and sucrose-specific PTS EIIs (Mori & Shiiro, 1987; Dominguez & Lindley, 1996; Kotrba *et al.*, 2001b; Parche *et al.*, 2001; Moon *et al.*, 2005), and additional *pts* genes (*cgR_2922* and *cgR_2923* of *C. glutamicum* R, *cg3365* and *cg3366* of *C. glutamicum* ATCC 13032) for unidentified substrates are present. However, a full understanding of the regulatory role of the PTS in *C. glutamicum* remains elusive. *C. glutamicum* can simultaneously utilize multiple carbon sources, and preferential utilization of glucose has been reported in only a few cases (Dominguez *et al.*, 1997; Wendisch *et al.*, 2000). The global catabolite repression mechanism involving the key roles of the PTS, as found in *E. coli* and *B. subtilis*, has not been identified in *C. glutamicum*.

A distinct feature of *C. glutamicum* R is that, unlike *C. glutamicum* strains ATCC 13032 and ATCC 13869, it can utilize the β -glucosides salicin and arbutin, which are transported via a β -glucoside-specific PTS (Kotrba *et al.*, 2003). The β -glucoside phosphotransferase and utilization system in *C. glutamicum* R comprises three genes, *bglF* (*cgR_2729*), *bglA* (*cgR_2728*) and *bglG* (*cgR_2727*), encoding β -glucoside PTS (EIIBCA^{bgl}), phospho- β -glucosidase and positive transcription regulator, respectively. Efficient expression of the β -glucoside PTS requires the *bglG* gene. BglG shows 53 % similarity to LicT from *B. subtilis* and BglG from *E. coli*, two transcriptional antiterminators belonging to the BglG/SacY family. A RAT-like sequence and a putative transcriptional terminator are present upstream of the *C. glutamicum* R *bglF* gene, suggesting that *C. glutamicum* R *bglF* is regulated via the antitermination mechanism.

A *C. glutamicum* R strain with a deleted *bglF* gene still consumes β -glucosides (Kotrba *et al.*, 2003), suggesting that this strain encodes another β -glucoside transporter. In this study, we describe identification of a new β -glucoside phosphotransferase and utilization system in *C. glutamicum* R. It comprises three genes, *bglF2* (*cgR_2610*), *bglA2* (*cgR_2609*) and *bglG2* (*cgR_2608*), putatively encoding EIIBCA^{bgl}, phospho- β -glucosidase and antiterminator, respectively. We also investigate involvement of antiterminators and general PTS components in the regulation of *bglF* and *bglF2* in response to the presence of β -glucoside and glucose.

METHODS

Media and growth conditions. *C. glutamicum* R was grown aerobically at 33 °C with shaking at 200 r.p.m. For the analysis of sugar concentration, *C. glutamicum* R was grown in 100 ml A medium (Inui *et al.*, 2007) supplemented with 1 % sugar, or in BT minimal medium (Inui *et al.*, 2007) supplemented with 0.2 % sugar. For the analysis of *bgl* expression, *C. glutamicum* R was grown in 10 ml A medium supplemented with 1 % (w/v) carbon sources. Bacterial growth was monitored by determining the OD₆₁₀.

Bacterial strains and plasmids. The strains used in this study are as follows. *C. glutamicum* R (Yukawa *et al.*, 2007) was used as a wild-type strain. The strains with various gene deletions (*bglF*, *bglF2*, *bglG*, *bglG2*, *ptsI* and *ptsH*) were constructed as described previously (Inui *et al.*, 2004b). A suicide vector, pCRA725, carrying the *sacB* gene was used for construction of markerless gene deletions. Oligonucleotide primers used for gene deletion are summarized in Supplementary Table S1, available with the online version of this paper. Briefly, DNA fragments encoding genes with an internal deletion were cloned into pCRA725. The resultant plasmids were introduced into *C. glutamicum* R and single-crossover cells were isolated by using kanamycin resistance. Isolated cells were cultivated on BT medium supplemented with 10 % sucrose and double-crossover cells were isolated. The gene deletions were confirmed by DNA sequencing of the PCR products around the deleted region.

The strains having *bglF*- or *bglF2-lacZ* fusion genes (*PbglF-lacZ* or *PbglF2-lacZ*) were constructed as described previously (Tanaka *et al.*, 2008a). The promoter region of *bglF* or *bglF2* was amplified by PCR using primers EcoRV-*bglF1*-400-F and EcoRV-*bglF1*-9-R for the *bglF* promoter and EcoRV-*bglF2*-400-F and EcoRV-*bglF2*-9-R for the *bglF2* promoter. The amplified fragment was digested with *EcoRV* and cloned into the *DraI* site of the pCRA741 reporter plasmid, which is described elsewhere (Inui *et al.*, 2007). Construction of mutants deleted for both RAT and the transcriptional terminator region of the *bglF*- or *bglF2-lacZ* fusion genes (*PbglFΔRAT-lacZ* or *PbglF2ΔRAT-lacZ*) was conducted as follows. The plasmids containing *bglF*- or *bglF2-lacZ* were used as a template for inverse PCR using primer sets RI-*bglF1*-120-R and RI-*bglF1*-60-F for the *bglF* promoter and RI-*bglF2*-150-R and RI-*bglF2*-60-F for the *bglF2* promoter. The amplified fragment was digested with *EcoRI* and self-ligated. These RAT-transcriptional terminator deletion plasmids were used to transform *C. glutamicum* R and recombinant cells were selected for kanamycin resistance. Insertion of the promoter-*lacZ* fusion gene between *cgR_0734* and *cgR_0735* was confirmed by PCR using primers LlacZLR-4354F and Ind7insert-checkR, or LlacZLR-6425R and Ind7insert-checkF.

Analysis of sugars. *C. glutamicum* cells were centrifuged (1000 g, 4 °C, 1 min), and supernatants were analysed for sugars. The concentration of salicin was measured by high-performance liquid chromatography (8020, Tosoh) with a 600 mm OA PAK-A TSK-GEL column (Tosoh). Sugars were detected with a refraction index detector.

Real-time RT-PCR. Total RNA was isolated from exponentially growing cells (OD₆₁₀ 1.2) using the RNeasy kit (Qiagen). A 20 ng sample of total RNA was used as template for analysis of the *bgl* genes and 0.4 ng was used for analysis of the 16S rRNA to generate cDNA and for the subsequent PCR. Each real-time RT-PCR mixture (20 μ l) contained 500 nM of each primer, 10 μ l of Power SYBR Green PCR Master Mix, eight units of RNase Inhibitor and five units of MuLV reverse transcriptase (Applied Biosystems). The primers used in these reactions are listed in Supplementary Table S1. 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 30 s. The result of 16S rRNA was used as an internal control.

RESULTS

Identification of a second set of β -glucoside-utilization genes

In a previous paper, we described the presence of a β -glucoside PTS encoded by *bglF* in *C. glutamicum* R (Kotrba *et al.*, 2003). However, disruption of the *bglF* gene did not prevent consumption of β -glucosides (Kotrba *et al.*, 2003). We inferred that there is another β -glucoside-utilizing system in *C. glutamicum* R. A search of the entire *C. glutamicum* R genome (Yukawa *et al.*, 2007) for candidate β -glucoside utilization genes revealed CgR_2610, CgR_2609 and CgR_2608, with 38 % amino acid sequence

identity and 57 % similarity to BglF, 40 % identity and 56 % similarity to BglA, and 34 % identity and 53 % similarity to BglG, respectively. We designated *cgR_2610*, *cgR_2609* and *cgR_2608* as *bglF2*, *bglA2* and *bglG2*, respectively (Fig. 1a). We compared the amino acid sequence of BglF2 with BglF (Fig. 1b). We also compared it with that of PtsG because BglF is a member of the PtsG family of EII (Postma *et al.*, 1993). Comparison of BglF2, BglF and PtsG revealed that the regions around the putative phosphorylation site on the EIIB domain (Cys-30 residue of BglF2) and the EIIC domain (His-575 residue of BglF2) are highly conserved (Fig. 1b). We previously reported that point mutation on the EIIC domain

Fig. 1. Two β -glucoside utilization systems in *C. glutamicum* R. (a) Organization of the two *bgl* gene clusters. Open arrows represent the coding regions for indicated genes. Deduced functions of each gene are indicated below. RAT-like sequences and putative transcriptional terminators are indicated as boxes and stem–loops, respectively. (b) Comparison of amino acid sequence between BglF, BglF2 and PtsG. The region around the phosphorylation site of the EIIB domain (amino acid residues 29–40 of BglF2), the substrate specificity domain of EIIC (residues 305–333 of BglF2) and the phosphorylation site of EIIC domain (residues 569–581 of BglF2) are shown. Conserved residues in two of three EIIs are indicated by dots, and conserved residues in all three EIIs are indicated by asterisks. The putative phosphorylation site in BglF2 (Cys-30 and His-575) and Val-325, in which mutation in BglF results in cellobiose recognition, are indicated. (c) Sequence of the upstream region of *bglF2*. The sequence of the region upstream of the translation initiation codon is shown. The region which has similarity with the RAT sequence is boxed. The palindromic sequence which partially overlaps with the RAT-like sequence is indicated by a pair of inverted arrows. The dashed line indicates the deleted region in the *PbgIF2* Δ *RAT-lacZ* strain. (d) Secondary structure of the RAT-like sequence of *bglF2*. The line to the right of the hairpin indicates the 5' region of the palindromic sequence. The secondary structure shown is based on comparison with the proposed secondary structure of other RAT sequences (Yang *et al.*, 2002).

(permease domain) of EIIBCAB^{agl} (V317A or V317M) allowed the cells to transport cellobiose, which is not a substrate for wild-type EIIBCAB^{agl}, suggesting that this amino acid is important for substrate recognition. Val-317 of BglF is conserved in BglF2 but not conserved in PtsG.

Involvement of BglF and BglF2 in β -glucoside utilization

We tested the growth of a strain with deleted *bglF2* on a β -glucoside (salicin) as the sole carbon source (Fig. 2a). A disruptant lacking either *bglF* or *bglF2* grew on minimal medium with salicin as well as did the wild-type. Simultaneous disruption of both *bglF* and *bglF2* resulted in severe defects in growth on salicin. We also tested the growth on plates supplemented with several β -glucosides (Table 1). Wild-type *C. glutamicum* R grows on arbutin, methyl β -D-glucoside and salicin, but not on cellobiose (Kotrba *et al.*, 2003). A disruptant lacking a general component of the PTS (*ptsH* or *ptsI*) did not grow on any of the tested β -glucosides, suggesting that these sugars are transported by the PTS or, alternatively, expression and/or activities of β -glucoside sugar utilization system are controlled by the PTS. Single disruption of either *bglF* or *bglF2* did not affect the utilization of any of the carbon sources tested. Simultaneous disruption of *bglF* and *bglF2* resulted in defective growth on arbutin and salicin, while colony formation was observed on methyl β -D-glucoside. These results indicate that both BglF and BglF2 transport arbutin and salicin. It is likely that another transport system is involved in methyl β -D-glucoside transport, although we did not rule out that BglF and/or BglF2 are also involved in the transport.

To test whether *bglF2* is involved in β -glucoside utilization, sugar concentration in the medium was measured in aerobic growth conditions (Fig. 2b, c). Because the *bglF bglF2* double disruptant does not grow on minimal medium with salicin, we used nutrient-rich medium (A medium) supplemented with 0.2 % salicin or arbutin (Fig. 2b, c). Wild-type, *bglF* disruptant, and *bglF2* disruptant strains showed similar growth while the *bglF bglF2* double disruptant strain showed slower growth and lower final cell density (data not shown). Salicin and arbutin concentrations decreased almost by equal measure during the growth of wild-type, *bglF* mutant and *bglF2* mutant strains, and the β -glucosides were completely utilized in 8 h cultivation. The *bglF bglF2* double disruptant showed very slow consumption of salicin or arbutin. Therefore, we concluded that both BglF and BglF2 are able to transport salicin and arbutin, and also postulate that both are major β -glucoside transporters.

Regulation of *bglF2* expression by β -glucosides

Expression of the *bglF* and *bglF2* genes was investigated by RT-PCR analysis (Fig. 3). Expression of many *bgl* genes in other bacteria is subject to glucose repression. Therefore,

Fig. 2. β -Glucoside utilization by *bgl* mutant strains. (a) Growth of wild-type and *bgl* mutants in minimal medium with 0.2 % salicin. (b, c) Concentration of salicin (b) and arbutin (c) in the culture medium of wild-type and *bgl* mutants grown in A medium with 0.2 % salicin or 0.2 % arbutin, respectively. $\circ$, Wild-type; $\square$, *bglF*; $\diamond$, *bglF2*; $\triangle$, *bglF bglF2*. The results shown are representative of at least two independent reproducible experiments.

we tested the effects of glucose added in the presence of salicin (Fig. 3a, b) or arbutin (Fig. 3c, d). We also tested the effect of fructose because expression of general as well as

Table 1. Utilization of β -glucosides by *bgl* gene deletion strains

Minimal BT medium plates were supplemented with 1% (w/v) carbon sources as indicated. Growth on plates visible after 24 h is indicated as +.

	WT	<i>ptsH</i>	<i>ptsI</i>	<i>bglF</i>	<i>bglF2</i>	<i>bglF bglF2</i>
Arbutin	+	—	—	+	+	—
Methyl β -D-glucoside	+	—	—	+	+	+
Salicin	+	—	—	+	+	—
Cellobiose	—	—	—	—	—	—

sugar-specific *pts* genes is strongly induced in the presence of fructose. The levels of *bglF* and *bglF2* mRNAs did not increase upon the addition of glucose or fructose. This indicates that the regulatory mechanism of *bglF* and *bglF2* is different from that of *pts* genes that are controlled by the transcriptional repressor protein SugR (Engels & Wendish, 2007; Gaigalat *et al.*, 2007; Tanaka *et al.*, 2008b). Indeed, we

found that *bglF* and *bglF2* mRNA levels did not increase upon the disruption of *sugR* gene (data not shown). As expected, *bglF* and *bglF2* expression was increased in the presence of salicin (Fig. 3a, b). The level of *bglF* mRNA was repressed to less than 10% by the addition of glucose (Fig. 3a, column 5). Addition of fructose partially repressed the expression of *bglF* (Fig. 3a, column 6). In contrast, expression of *bglF2* was decreased by only 20% by the addition of glucose (Fig. 3b, column 5) and by 50% by addition of fructose (Fig. 3b, column 6). Similar results were obtained with arbutin (Fig. 3c, d). These results indicate that *bglF* and *bglF2* are under different catabolite repression control by glucose.

Regulation of *bglF2* expression by BglG2 independent of BglG

We found that *bglG2*, coding for an antiterminator-like protein, is located near the *bglF2* gene on the *C. glutamicum* R chromosome. Moreover, there is a RAT-like sequence which overlaps a palindromic sequence (Fig. 1c, d) upstream of *bglF2*. These observations suggest that

Fig. 3. Expression of *bgl* genes in the presence of various PTS sugars. Total RNAs prepared from wild-type cells grown in A medium supplemented or not with 1.0% (w/v) PTS sugars were subjected to real-time RT-PCR analysis using primers specific for *bglF* (a, c) or *bglF2* (b, d). The relative mRNA level is indicated as the ratio compared to cells grown in the presence of salicin. The values are the means of three independent experiments and standard deviations are indicated. Abbreviations: Glc, glucose; Fru, fructose; Sal, salicin; Arb, arbutin.

bglF2 expression is controlled by an antitermination mechanism via BglG2. To confirm this, a strain with deleted *bglG2* was constructed and *bglF2* expression determined by RT-PCR analysis. As described previously (Kotrba *et al.*, 2003), *bglF* induction by salicin was not observed in the *bglG* mutant strain. The level of *bglF* mRNA was increased upon addition of salicin in the *bglG2* mutant strain, suggesting that BglG2 is not involved in the regulation of *bglF* expression (Fig. 4a). Expression of *bglF2* was not affected by *bglG* deletion (Fig. 4b). Deletion of *bglG2* resulted in a marked decrease in salicin induction of *bglF2* (Fig. 4b). The slight induction of *bglF2* by salicin in the *bglG2* disruption strain was completely abolished in a *bglG bglG2* double disruptant strain. These results indicate that BglG2 regulates the expression of *bglF2*, while there may be only minor cross-talk regulation by BglG of *bglF2* expression.

To further test whether or not antitermination is involved in the induction of *bglF* and *bglF2* genes, we examined effects of deletion of the putative RAT sequence and transcriptional terminator sequence on the promoter-*lacZ* gene fusions that integrated into the chromosome of *C. glutamicum* R. The levels of *lacZ* mRNA were determined by RT-PCR. As expected, the expression levels of the *bglF*- and *bglF2*-*lacZ* fusions were very low in the absence of

salicin, but markedly induced in its presence (the relative *lacZ* expression levels in the absence of salicin were 0.042 ± 0.023 and 0.070 ± 0.054 for *PbglF*- and *PbglF2*-*lacZ*, respectively, compared to those in the presence of salicin). Deletion of the putative *cis*-element for antitermination resulted in the expression of *lacZ* even in the absence of salicin, and expression was barely induced by salicin (the relative *lacZ* expression levels in the absence of salicin were 0.81 ± 0.08 and 1.48 ± 0.20 for *PbglF* Δ RAT-*lacZ* and *PbglF2* Δ RAT-*lacZ*, respectively, compared to those in the presence of salicin). These results indicate that the RAT-termination region is essential for the regulation of *bglF* and *bglF2*.

Effect of PTS disruption on *bgl* expression

The PTS is generally involved in the regulation of the activity of BglG/SacY-type transcriptional antiterminators. To test whether the PTS is involved in the regulation of the activity of BglG and/or BglG2, we investigated *bglF* and *bglF2* gene expression in *ptsI* (encoding EI) or *ptsH* (encoding HPr) mutants by RT-PCR analysis. In the absence of salicin, the levels of *bglF* and *bglF2* mRNAs were equally low in the wild-type, *ptsI* mutant and *ptsH* mutant strains (Fig. 5a, b). The marked induction of *bglF* and *bglF2* by supplementation with salicin observed in the wild-type strain was completely suppressed in the *ptsI* or *ptsH* mutant strains (Fig. 5a, b). These results indicate that the PTS is

Fig. 4. Effect of *bglG* or *bglG2* disruption on *bglF* and *bglF2* gene expression. Total RNAs prepared from cells grown in A medium supplemented or not with 1.0 % (w/v) PTS sugars were subjected to real-time RT-PCR analysis using primers specific for *bglF* (a) or *bglF2* (b). The relative mRNA level is indicated as the ratio compared to cells grown in the presence of salicin. The values are the means of three independent experiments and standard deviations are indicated. Abbreviations: Glc, glucose; Fru, fructose; Sal, salicin.

Fig. 5. Effect of disruption of the general PTS on *bglF* and *bglF2* expression. Total RNAs prepared from cells grown in A medium supplemented or not with 1.0 % (w/v) salicin (Sal) were subjected to real-time RT-PCR analysis using primers specific for *bglF* (a) or *bglF2* (b). The relative mRNA level is indicated as the ratio compared to cells grown in the presence of salicin. The values are the means of three independent experiments and standard deviations are indicated.

required for the induction of *bglF* and *bglF2* expression in response to salicin.

DISCUSSION

In this paper, we report a second β -glucoside PTS encoded by *bglF2*, which is organized on the *C. glutamicum* R chromosome in a cluster with a phospho- β -glucosidase gene, *bglA2*, and an antiterminator-like gene, *bglG2*. The gene organization is similar to that of the previously identified β -glucoside utilization system (*bglF*-*bglA*-*bglG*) of this strain. Although a *bglF2* mutant grew on β -glucoside as the sole carbon source, as does a *bglF* mutant, simultaneous disruption of *bglF* and *bglF2* abolished utilization of β -glucosides, and expression of these genes was similarly induced in the presence of β -glucoside. These results indicate that these two β -glucoside PTSs in *C. glutamicum* R are redundantly responsible for β -glucoside uptake.

Unlike *C. glutamicum* R, *C. glutamicum* ATCC 13032 does not grow on β -glucosides as sole carbon source. However, comparison of the genome sequence of *C. glutamicum* strains R (Yukawa *et al.*, 2007) and ATCC 13032 (Ikeda & Nakagawa, 2003; Kalinowski *et al.*, 2003) revealed that fragments of the genes corresponding to *bglA*-*bglG* and *bglF2* do exist in *C. glutamicum* ATCC 13032 (Fig. 6). Protein Cg2995 predicted from the *C. glutamicum* ATCC 13032 genome showed 96 % sequence identity to part of BglF2 of *C. glutamicum* R (amino acid residues 105–155), and Cg2996 also showed 94 % identity to another part of BglF2 (residues 157–226). Cg2994 showed 97 % amino acid

sequence identity to CgR_2607 of *C. glutamicum* R, and Cg2999 showed 99 % identity to CgR_2611, indicating that *bglF2* and the *cg2995*–*2996* region are present in the same position in the genome of the respective strains. BLAST searches revealed no region corresponding to *bglA2* or *bglG2* in the *C. glutamicum* ATCC 13032 genome, suggesting that these genes have been lost in this strain. Similarly, ORFs corresponding to fragments of *bglG* and *bglA* were found in the *C. glutamicum* ATCC 13032 genome (*cg3144*, *cg3146* and *cg3147*), but no sequence in this strain was found to correspond to *bglF* of *C. glutamicum* R. An ORF encoding a 175 aa product which overlaps *bglG* and *bglA* but inversely directed can be assigned in both these strains. We have not tested whether or not this gene is functional. These findings suggest that both the *bglF*-*bglA*-*bglG* and *bglF2*-*bglA2*-*bglG2* gene clusters were present in the common ancestor of *C. glutamicum* R and ATCC 13032. Then, both of these gene clusters were lost in *C. glutamicum* ATCC 13032 after the divergence into these strains. Differences in the environmental nutritional conditions that these strains have been subjected to may have led to the genetical alterations to the sugar utilization systems.

In this study, we demonstrated that expression of *bglF2* requires BglG2, which belongs to the BglG/SacY-type antiterminator family (Fig. 4a, b). The RAT-like sequence found upstream of *bglF2* is probably recognized by BglG2, leading to upregulation of *bglF2* expression by antitermination. Indeed, we found that in the absence of the RAT-transcriptional terminator region, *lacZ* reporter genes from *bglF* and *bglF2* were constitutively expressed. The

Fig. 6. Schematic representation of the organization of the genes around *bglF2* (a) and *bglF* (b) in *C. glutamicum* R and *C. glutamicum* ATCC 13032. Open arrows represent the ORFs. Deduced functions of the proteins encoded by the genes are as follows: CgR_2607, putative secreted or membrane protein; CgR_2611, putative ferredoxin reductase; CgR_2726, putative secreted protein; CgR_5055, hypothetical protein; CgR_2730, ferrienterobactin-binding periplasmic protein precursor FepB2; CgR_2731, ferric enterobactin transport protein; CgR_2732, putative iron-siderophore uptake system transmembrane protein; CgR_2733, ferric enterobactin transport system ATP-binding protein FepC). The dashed line indicates the deletion in *C. glutamicum* ATCC 13032.

BglG/SacY family antiterminator is known to be involved in PTS-dependent induction and carbon catabolite repression of catabolic genes in bacteria. As with *E. coli* and *B. subtilis* β -glucoside utilization genes, it is possible that *C. glutamicum* R β -glucoside PTS EII, BglF and/or BglF2 phosphorylate the PRD-1 site conserved in BglG/SacY-type antiterminators, BglG and/or BglG2, to inhibit the activity of the antiterminators, and expression of *bglF* and *bglF2* is induced by activation of the antiterminators dephosphorylated at the PRD-1 site along with uptake and phosphorylation of β -glucoside by the substrate-specific PTS EIIs. Our results revealed that there is only minor cross-talk regulation by BglG of *bglF2* and by BglG2 of *bglF*.

Interestingly, expression of *bglF2* was not repressed in the presence of glucose plus salicin, in contrast to *bglF* expression, which is subject to strict glucose repression. The difference in glucose repression may be due to distinct features of the antiterminators, BglG and BglG2. It is known that phosphorylation of several BglG/SacY-type antiterminators at the PRD-2 site by HPr is required for activation, and its dephosphorylation due to a decreased level of phosphorylated HPr in the presence of readily metabolizable carbon sources such as glucose could explain catabolite repression (Postma *et al.*, 1993; Deutscher *et al.*, 2006). It is noteworthy that activity of *B. subtilis* SacY and GlcT, belonging to the BglG/SacY-type antiterminators, whose target genes are not subject to glucose repression, does not depend on HPr. This suggests the possibility that *C. glutamicum* R BglG2 may not be under the control of the HPr-dependent PRD-2 phosphorylation. However, this possibility is ruled out by analysis of *bglF2* gene expression using mutants of a general PTS component, EI or HPr (Fig. 5). If antitermination is regulated by both PRD-1 and PRD-2 phosphorylation, *ptsI* or *ptsH* mutation would result in the inactivation of antiterminator, while if antitermination is regulated by PRD-1 and not PRD-2, *ptsI* or *ptsH* mutation would result in the activation of antiterminator. We revealed that in *ptsI* mutant and *ptsH* mutant cells, expression of *bglF* and *bglF2* was repressed in the presence and absence of salicin; this behaviour is different from that of *B. subtilis* SacY- and GlcT-regulated genes, whose expression is upregulated in *ptsH* mutant cells. It should be noted that addition of fructose partially repressed induction levels of both *bglF* and *bglF2* in the presence of salicin, which may be explained by decrease of PRD-2 phosphorylation of BglG and BglG2. To assess HPr-dependent regulation, it is important to determine the phosphorylation level of HPr in the presence of various PTS sugars.

The strong effect of glucose but not fructose on *bglF* expression in *C. glutamicum* R may be due to an unidentified catabolite repression mechanism other than antitermination. In *B. subtilis*, transcription initiation is also the target of CcpA-mediated catabolite repression of *bglPH* (Krüger & Hecker, 1995). One possible mechanism to explain the effect of glucose in *C. glutamicum* R is that a transcriptional regulator that responds to the presence of glucose is involved in the regulation of *bglF* expression but

not in the regulation of *bglF2* expression. Further studies are needed to elucidate the regulatory system underlying the different behaviour of *bglF* and *bglF2* in response to glucose but not fructose.

The presence of redundant β -glucoside PTSs is found in other bacteria, such as *E. coli*, *Streptococcus mutans* and *Pectobacterium carotovorum* (Parker & Hall, 1988; Cote *et al.*, 2000; Hong *et al.*, 2006). However, there are few studies on how the expression of these β -glucoside PTSs is coordinated. The two β -glucoside utilization systems in *C. glutamicum* R under different catabolite repression control shown in this study may serve to fine tune the expression level. It is possible that in the presence of β -glucoside and glucose, the requirement for β -glucoside transporter is not high, so only BglF2 is induced. In the absence of glucose on the other hand, the requirement for β -glucoside transporter increases and both BglF and BglF2 are induced.

We have developed *C. glutamicum* R mutants of the β -glucoside PTS that can utilize cellobiose in industrial applications (Kotrba *et al.*, 2003; Sasaki *et al.*, 2008). In addition, the expression pattern of the *bglF2* transcription system indicates that this promoter and regulator system may serve as a suitable inducible promoter, because its expression is efficiently repressed in the absence of inducer and strongly induced by the addition of inducer even in the presence of glucose.

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