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 bgfF. Here we report that C. glutamicum R contains an additional β-glucoside PTS gene, bgfF2, organized in a cluster with a putative phospho-β-glucosidase gene, bgfA2, and a putative antiterminator,.bgfG2. While single gene disruption strains of either bgfF or bgfF2 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 bgfF and bgfF2 was induced in the presence of either salicin or arbutin, although disruption of bgfG2 affected only bgfF2 expression. Moreover, in the presence of either salicin or arbutin, glucose completely repressed the expression of bgfF but only slightly repressed that of bgfF2. We conclude that BgfF and BgfF2 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 bgfF and bgfF2 requires the general PTS.

INTRODUCTION

The phosphoenolpyruvate : carbohydrate phosphotransferase system (PTS) catalyses carbohydrate transport 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 EIIAGlc regulates the level of cyclic AMP, which binds to CRP protein, a transcriptional regulator of many catabolic genes. In addition, unphosphorylated EIIAGlc 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 (Kinosita et al., 1957; Kelle et al., 2005). *C. glutamicum* R can provide high yields of lactate and pyruvate (Kotrba et al., 1987; Dominguez & Lindley, 1996; Kotrba & Shiio, 1987). It has been reported that *C. glutamicum* strains ATCC 13032 and ATCC 13869, it can preferentially utilize glucose has been reported in only a few cases (Kotrba et al., 1997; Wendisch et al., 2000). In this study, we describe identification of a new antiterminator, which is the underling 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.

*C. glutamicum* R comprises three genes, *bglA2*, *bglG2*, and *bglF2*, which encode β-glucosidase and antiterminators, respectively. Efficient transcriptional regulation of the PTS in *C. glutamicum* R strain with a deleted *bglF* gene still contains β-glucosidase 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 (EIIBCAβββ), phospho-β-glucosidase and positive transcription regulator, respectively. Efficient expression of the β-glucoside PTS requires the *bglG* gene. BglG shows 53% similarity to LtcT 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 *bglF* gene, suggesting that *C. glutamicum* R *bglF* gene 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 EIIBCAβββ, 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*, *ptsD* 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 (P*bglF-lacZ* or P*bglF2-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 Dral 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 (P*bglFARAT-lacZ* or P*bglF2ARAT-lacZ*) was conducted as follows. The plasmids containing *bglF*- or *bglF2-lacZ* were used as a template for inverse PCR using primer sets RtgF1–120–R and RtgF1–60–F for the *bglF* promoter and RtgF2–150–R and RtgF2–60–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 LlacZL7-4354F and Ind7Insert-checkR, or LlacZL7-6425R 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 refractometer index detector.

**Real-time RT-PCR.** Total RNA was isolated from exponentially growing cells (OD610 1.2) using the RNaseasy 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 μl) contained 500 nM of each primer, 10 μl 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 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. The level 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 EIIA domain (His-575 residue of BglF2) are highly conserved (Fig. 1b). We previously reported that point mutation on the EIIC domain

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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 EIIAs are indicated by dots, and conserved residues in all three EIIAs 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 PbglF2ARAT-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 EIIBCA<sub>bgl</sub> (V317A or V317M) allowed the cells to transport cellobiose, which is not a substrate for wild-type EIIBCA<sub>bgl</sub>, 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 <i>bglF2</i> on a β-glucoside (salicin) as the sole carbon source (Fig. 2a). A disruptant lacking either <i>bglF</i> or <i>bglF2</i> grew on minimal medium with salicin as well as did the wild-type. Simultaneous disruption of both <i>bglF</i> and <i>bglF2</i> resulted in severe defects in growth on salicin. We also tested the growth on plates supplemented with several β-glucosides (Table 1). Wild-type <i>C. glutamicum</i> R grows on arbutin, methyl β-D-glucoside and salicin, but not on cellobiose (Kotra <i>et al.</i>, 2003). A disruptant lacking a general component of the PTS (<i>ptsH</i> or <i>ptsI</i>) 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 <i>bglF</i> or <i>bglF2</i> did not affect the utilization of any of the carbon sources tested. Simultaneous disruption of <i>bglF</i> and <i>bglF2</i> 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 <i>bglF2</i> is involved in β-glucoside utilization, sugar concentration in the medium was measured in aerobic growth conditions (Fig. 2b, c). Because the <i>bglF</i> <i>bglF2</i> 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, <i>bglF</i> disruptant, and <i>bglF2</i> disruptant strains showed similar growth while the <i>bglF bglF2</i> 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, <i>bglF</i> mutant and <i>bglF2</i> mutant strains, and the β-glucosides were completely utilized in 8 h cultivation. The <i>bglF bglF2</i> 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 <i>bglF</i> and <i>bglF2</i> genes was investigated by RT-PCR analysis (Fig. 3). Expression of many <i>bgl</i> genes in other bacteria is subject to glucose repression. Therefore, 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
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

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<th>Table 1. Utilization of β-glucosides by bgl gene deletion strains</th>
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<td>Minimal BT medium plates were supplemented with 1% (w/v) carbon sources as indicated. Growth on plates visible after 24 h is indicated as +.</td>
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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 genes 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 PbglFARAT-lacZ and PbglF2ARAT-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, pts 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 pts or ptsH mutant strains (Fig. 5a, b). These results indicate that the PTS is
required for the induction of \( bglF \) and \( bglF2 \) expression in response to salicin.

**DISCUSSION**

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

Unlike \( C. \ glutamicum \) R, \( C. \ glutamicum \) ATCC 13032 does not grow on \( \beta \)-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.

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**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 represents the ORFs. Deduced functions of the proteins ended 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-glucose 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 PTSSs 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 PTSSs 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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REFERENCES


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