A single V317A or V317M substitution in Enzyme II of a newly identified β-glucoside phosphotransferase and utilization system of Corynebacterium glutamicum R extends its specificity towards cellobiose

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A catabolic system involved in the utilization of β-glucosides in Corynebacterium glutamicum R and its spontaneous mutant variants allowing uptake of cellobiose were investigated. The system comprises a β-glucoside-specific Enzyme IIIC component (gene bglF) of the phosphotransferase system (PTS), a phospho-β-glucosidase (bglA) and an antiterminator protein (bglG) from the BglG/SacY family of transcription regulators. The results suggest that transcription antitermination is involved in control of induction and carbon catabolite repression of bgl genes, which presumably form an operon. Functional analysis of the bglF and bglA products revealed that they are simultaneously required for uptake, phosphorylation and breakdown of methyl β-glucoside, salicin and arbutin. Although cellobiose is not normally a substrate for BglF permease and is not utilized by C. glutamicum R, cellobiose-utilizing mutants can be obtained. The mutation responsible was mapped to the bgl locus and sequenced, and point mutations were found in codon 317 of bglF. These led to substitutions V317A and/or V317M in the putative PTS active-site H313 in the membrane-spanning IIC domain of BglF and allowed BglF to act on cellobiose. Such results strengthen the evidence that the IIC domains can be regarded as selectivity filters of the PTS.

INTRODUCTION

The phosphoenolpyruvate (PEP) carbohydrate phosphotransferase system (PTS) in many bacteria catalyses the import of several sugars and hexitols. The system consists of two general cytoplasmic proteins named Enzyme I and HPr that are common to the transport of most carbohydrates, and a range of carbohydrate-specific Enzymes II (Postma et al., 1993). The Enzymes II are modular proteins composed of cytoplasmic IIA and IIB, and a membrane-spanning IIC (sometimes also IID), which occur either as domains in a single polypeptide or as subunits of a complex. For carbohydrate transport, Enzyme I autophosphorylates from PEP and the phosphate is sequentially transferred to HPr, IIA, IIB and then to an incoming carbohydrate molecule during its translocation by IIC. The presence of general as well as glucose-, mannose-, fructose- and sucrose-specific components of the PTS has been demonstrated in the high-G+C Gram-positive soil bacterium Corynebacterium glutamicum in several biochemical and genetic studies (Mori & Shio, 1987; Malin & Bourd, 1991; Lee et al., 1994; Dominguez et al., 1998; Parche et al., 2001). Non-pathogenic corynebacteria, such as C. glutamicum strains ATCC 13032 or ATCC 13869, have a long history of commercial use as producers of various amino acids, notably lysine and glutamic acid (Kinoshita, 1985; Lessard et al., 1999). Another strain, C. glutamicum R, recently received our attention as it provided high yields of the plastics precursors lactate and succinate. By using a genetic approach employing inactivation of the ptsI gene encoding Enzyme I, we showed that for this bacterium, PTS represents the dominant sugar-uptake system (Kotrba et al., 2001). A distinct feature of C. glutamicum R is that, unlike strains ATCC 13032 or ATCC 13869, it is capable of PTS-dependent utilization of methyl β-glucoside and the natural aryl β-glucosides salicin and arbutin (Kotrba et al., 2001). Moreover, C. glutamicum R may adapt to growth on the 1,4-β-glucoside cellobiose, which could play an important role in the fermentation of hydrolysed cellulosic materials.

The β-glucoside utilization pathways that rely upon the PTS for carbohydrate uptake have been characterized in several...
bacteria. They include $\beta$-glucoside-specific Enzymes II from *Erwinia chrysanthemi* (el Hassouni et al., 1992), *abgGFA* from *Clostridium longisporum* (Brown et al., 1998), *bgIPT* from *Lactobacillus plantarum* (Marasco et al., 2000), *bgPH* from *Bacillus subtilis* (Krüger & Hecker, 1995), *casRAB* from *Klebsiella oxytoca* (Lai et al., 1997), *bvrABC* from *Listeria monocytogenes* (Brehm et al., 1999), and cryptic *bgI* from *Escherichia coli* (Schnetz et al., 1987). Expression of these operons is controlled by multi-domain antiterminators of the BglG/SacY family (Stülke et al., 1987). Expression of these operons is controlled by multi-domain antiterminators of the BglG/SacY family (Stülke et al., 1987). Expression of these operons is controlled by multi-domain antiterminators of the BglG/SacY family (Stülke et al., 1987). Expression of these operons is controlled by multi-domain antiterminators of the BglG/SacY family (Stülke et al., 1987). Expression of these operons is controlled by multi-domain antiterminators of the BglG/SacY family (Stülke et al., 1987). Expression of these operons is controlled by multi-domain antiterminators of the BglG/SacY family (Stülke et al., 1987).

In the present study we describe a $\beta$-glucoside phospho-transferase and utilization system from *C. glutamicum* R, which we believe represents the first example of such a system reported from Actinobacteria. Specifically, it comprises three genes, *bgIF*, *bgLA* and *bgIG*, encoding PTS permease, $\beta$-glucosidase and the positive transcription regulator, respectively. We demonstrate the potential of this system in the utilization of $\beta$-glucosides and its repression exerted by glucose. We further show that a single amino acid substitution is required for activity of BglF on cellobiose in adaptive cellobiose-utilizing mutants of *C. glutamicum* R.

**METHODS**

*C. glutamicum* strains and culture conditions. Strains used in this study are described in Table 1. *C. glutamicum* was routinely grown at 33°C in AR medium (Kurusu et al., 1990) with glucose or acetate added at 2% (w/v). Where appropriate, media contained 50 μg kanamycin (Km) ml⁻¹ or 5 μg chloramphenicol (Cm) ml⁻¹. AR medium without yeast extract (AA medium) and without both yeast extract and Casamino acids (minimal BT medium) were used for sugar utilization studies. To obtain cellobiose-utilizing (Cel⁺) mutants, $10^9–10^{10}$ viable cells were plated on BT medium supplemented with 0.5% (w/v) cellobiose. The cells from single colonies of 0.5–1.0 mm i.d., which typically appeared after 7–10 days of incubation at 33°C, were further cultivated in the same medium.

DNA techniques. Routine recombinant DNA manipulations and analyses were performed according to standard protocols (Sambrook et al., 1989). Electroporation was used to transform *C. glutamicum* with plasmid DNA (Vertès et al., 1993). Chromosomal DNA was isolated by the phenol extraction method described by Sambrook et al. (1989) and plasmid DNA was isolated by using the Qiagen Plasmid Midi Kit. *C. glutamicum* Bgl was incubated with 10 mg lysozyme ml⁻¹ at 37°C for 90 min prior to alkaline lysis. DNA fragments from chromosomal or plasmid DNA were amplified with ExTaq polymerase (Takara) and specific primers by PCR following appropriate protocols (Innis et al., 1990). The PCR products were purified by using the PCR Purification Kit from Qiagen, inserted into a suitable plasmid (see below) and sequenced.

The DNA sequences were determined on both strands of the linear template by the dideoxy chain-termination method using the BigDye Terminator Cycle Sequencing kit in the presence of 5% dimethyl sulfoxide and by use of the ABI Prism 377 DNA sequencer (Applied Biosystems–Perkin-Elmer). The DNA sequences were analysed by the

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**Table 1. *C. glutamicum* strains used in this study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant characteristic(s)*</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>R</td>
<td>Plasmidless wild-type strain</td>
<td>Kotrba et al. (2001)</td>
</tr>
<tr>
<td>R-CEL</td>
<td>Cel⁺ adaptive mutant of <em>C. glutamicum</em> R; <em>bgIF</em>(V317A)</td>
<td>Kotrba et al. (2001); this work</td>
</tr>
<tr>
<td>R-CEL2</td>
<td>Cel⁺ adaptive mutant of <em>C. glutamicum</em> R; <em>bgIF</em>(V317M)</td>
<td>This work</td>
</tr>
<tr>
<td>R-PDI5</td>
<td>Δpts; <em>C. glutamicum</em> R derivative, Km'</td>
<td>Kotrba et al. (2001)</td>
</tr>
<tr>
<td>R(bgl::npt)</td>
<td>ΔbgI, <em>C. glutamicum</em> R derivative, Km'</td>
<td>This work</td>
</tr>
<tr>
<td>R-CEL(bgl::npt)</td>
<td>ΔbgI, <em>C. glutamicum</em> R derivative, Km'</td>
<td>This work</td>
</tr>
<tr>
<td>ATCC 13869-C</td>
<td>Cured <em>C. glutamicum</em> ATCC 13869; Bgl⁺</td>
<td>Kurusu et al. (1990)</td>
</tr>
</tbody>
</table>

*Bgl⁻, $\beta$-glucoside-non-utilizing phenotype; Cel⁺, cellobiose-utilizing phenotype.
Genetyx version 4.0 program (Software Development, Japan), and database searches were performed using the BLAST server of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov).

Isolation of \textit{bgl} genes and construction of plasmids. The \textit{C. glutamicum} R \& FIX library (Kotrba et al., 2001) was screened by plaque hybridization using a probe obtained in PCR with degenerate primers. To do this, the primers 5'-ACIACAYTGCCACGCAATCGC-3' (PBG1, plus strand, [I, inosine; Y, T or C; M, A or G]) and 5'-GGTACCGGTAGATGCACACG-3' (PBG8, minus strand [R, A or G]) were designed for amino acid sequences of highly conserved regions of the \textit{bgl} genes. After being ligated into pUC18 digested with \textit{BglII}, the resulting fragments were cloned in \textit{E. coli} or \textit{Corynebacterium} spp. shuttle vectors from our laboratory. The resulting plasmid, pHSG-SB2, was used as a mixed hybridization probe. Detection was performed by using the Gene Images detection kit (Amersham Pharmacia Biotech) according to the recommended protocol. The signal was scanned in a luminescent image analyser model LAS-1000 (Fuji).

Analysis of sugar in culture media and \textit{in vitro} assay of PTS activity. For sugar uptake studies, the culture samples were removed at stated intervals and filtered through 0.2-μm filters (Millipore). Sugar concentrations were determined by HPLC using the model 8020 apparatus from TOSOH (Japan) with a 600 mm OA PAK-A column (TOSOH). The chromatography was performed at 40°C with 0.75 mM H$_2$SO$_4$ as a mobile phase (1.0 ml min$^{-1}$) and sugars were detected with a refraction index detector.

The combined \textit{bgl}-glucoside phosphorylation and phospho-\textit{bgl}-glucoside activities were determined essentially by the method of Kricke & Hall (1987). The cells from a culture that had reached the desired OD$_{600}$ were harvested by centrifugation (6000 g, 5 min, 4°C), washed in ice-cold 50 mM NaKHP0$_4$ buffer (pH 7.2) and resuspended in the same buffer to an OD$_{600}$ of 200. Cells were disrupted for 2 min with 0.1 mm Al$_2$O$_3$ beads in a Mini-Beadbeater device (BioSpec) set to the maximum speed. Crude cell-free extracts were assayed at 33°C in 50 mM NaKHP0$_4$ buffer (pH 7.2) containing 5 mM MgCl$_2$, 2 mM p-nitrophenyl \textit{β}-D-1,4-glucopyranoside (PNPG) and 2 mM PEP. The unit of activity was defined as the amount (pmol) of \textit{p}-nitrophenol released from PNPG per second per mg of total protein. Formation of \textit{p}-nitrophenol was monitored by measuring the \textit{A$_{410}$} at 30 s intervals in a period of 15 min. The protein content was determined by using the Bradford Reagent Kit (Bio-Rad).

RESULTS

Cloning of \textit{bgl} genes of \textit{C. glutamicum} R

Alignments of \textit{β}-glucoside-specific PTS permeases from different bacteria revealed several conserved regions (Fig. 2). Two of these conserved regions were used to design degenerate primers PBG1 and PBG8 (see Methods). These were used in a PCR with chromosomal DNA from...
C. glutamicum R as a template in a trial to amplify a portion of sequence encoding the respective permease. The reaction yielded a product of the expected size of 1.1 kb. Analysis of the polypeptide deduced from the nucleotide sequence suggested that it originated from a gene encoding β-glucoside-specific Enzyme II\textsuperscript{Bgl}. This PCR product was labelled and used to screen the C. glutamicum R λ FIX library by plaque hybridization.

Fig. 1. Organization of the 5.8 kb DNA region containing \textit{bgl} genes from \textit{C. glutamicum} R (GenBank accession number AF508972). (A) Physical map of cloned DNA fragments. The open reading frames designated \textit{bglF}, \textit{bglA} and \textit{bglG}, and encoded protein activities, are indicated. The positions of the putative promoter (\(P_{bgl}\)), transcription terminators (hairpin-like symbols) and RAT are shown. The \textit{bgl::npt} mutants were constructed by allelic exchange by using a pHSG396 derivative harbouring fr-FA in which an \textit{Apa}LI fragment was replaced with a Km\textsuperscript{r} cassette and homologous recombination. Abbreviations: S, \textit{Sal}I (\textit{Sal} originating from \(\lambda\) phage and used to clone fr-AG); P, \textit{Pst}I; B, \textit{Bam}HI; A, \textit{Apa}LI. (B) Nucleotide sequence of DNA upstream of \textit{bglF}. Potential\(-35\) and \(-10\) promoter consensus sequences and ribosome-binding site (RBS) are underlined. Possible transcription terminator sequences are marked with arrows. The putative RAT sequence is boxed. (C) Comparative analysis of RAT sequences. Multiple alignment and consensus in predicted secondary structures of the putative RAT from upstream of \textit{bglF} of \textit{C. glutamicum} R with RAT sequences situated upstream of \textit{B. subtilis} (\textit{Bs}u) \textit{licT} (Schnetz et al., 1996), \textit{bglF} (Krüger & Hecker, 1995) and \textit{sacB} (Crutz & Steinmetz, 1992), \textit{E. coli} (\textit{Eco}) \textit{bglG} and \textit{bglF} (Schnetz et al., 1987), and \textit{C. longisporum} (\textit{Clo}) \textit{abgF} (Brown & Thomson, 1998). Highly conserved nucleotides are boxed and a secondary-structure model of the RAT preceding \textit{bgl} is shown to the right.
A 4.1-kb *SalI* fragment (fr-FA) from a positive phage hybridized to the probe (data not shown) and analysis of its nucleotide sequence revealed the presence of one complete open reading frame, *orf2*, and partial *orf1* and *orf3* at the 5' and 3' ends, respectively. Based on the similarity of the deduced polypeptides with known proteins, the missing part from *orf3* was found on the 1.7-kb *SalI* fragment (fr-AG) from the same phage, where it was followed by *orf4* (Fig. 1A). Notable similarity of translated *orf2*, *orf3*, and *orf4* with proteins associated with transport and utilization of β-glucosides (see below) suggested their designation as *bgl* genes. The polypeptide predicted from *orf1* displayed 50% similarity to periplasmic binding protein involved in iron transport in *E. coli* (Elkins & Earhart, 1989).

**Identification of bgl genes by analysis of DNA sequence and homology search**

Analysis of the nucleotide sequence revealed that the coding sequences of all three *bgl* genes, named *bglF* (*orf2*), *bglA* (*orf3*) and *bglG* (*orf4*), were in the same translational reading frame. The DNA sequence spanning approximately 200 bp upstream of *bglF* contains several characteristic elements (Fig. 1B). The hexamers TTGCTT and CATAAT separated by 18 nucleotides corresponded well with consensus 235 and 210 promoter sequences, respectively. Downstream from the putative promoter was found a region of dyad symmetry with the potential to form a stable RNA stem-loop structure (228 to 9 kcal mol⁻¹, 2120 to 9 kcal mol⁻¹) which, followed by a T-rich stretch of nucleotides, resembled a factor-independent transcription terminator. Partially overlapping this region at the 5' end, a putative ribonucleic antiterminator (RAT) sequence was recognized. A comparison of this sequence with known RAT sequences (Fig. 1C), and its position relative to the putative terminator, suggested a strong relationship of this element to regulatory features characteristic of operons controlled by antiterminators of the BglG/SacY family (Stülke et al., 1998).

The choice of the translational start codons for putative *bgl* products was based on the position of a potential ribosome-binding site and on similarities to known proteins. The first

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Fig. 2. Comparative analysis of the predicted *bglF* product and its mutant variants from *C. glutamicum* R. (A) Diagrammatic representation of IICB *BglF* (*BglF*) of *C. glutamicum* R. The numbers at the extremes of the putative IIC domain represent the first and last amino acid residues of the putative transmembrane region. Residues corresponding by homology to those important for phosphoryl transfer (Schnetz et al., 1990) and the putative disaccharide-binding site (Lai et al., 1997) are indicated. The regions around conserved residues are shown together with consensus sequence derived from 10 β-glucoside-specific PTS permeases of the glucose–glucoside family. Residues conserved in at least nine permesases are shown in capital letters whilst those found in more than five permesases are shown in small letters. Indicated primers PBG1 and PBG8 were used to clone *bgl* genes. (B) Consensus around the site of *bglF* accumulating mutations. Highly conserved residues are boxed. An arrowhead points to the V317 of BglF that was a target for adaptive mutations, which resulted in V317A (*BglF A*) and V317M (*BglF M*).

http://mic.sgmjournals.org 1573
bgl gene, bglF, encoded a 64.8 kDa protein, 618 amino acids long. It was highly similar to other Enzymes IIbgl containing IIA, IIB and IIC domains fused in one polypeptide. The highest scores found were with BglF from E. coli (36% identity and 50% similarity: Schnetz et al., 1987), BglF from L. plantarum (35% identity and 52% similarity: Marasco et al., 2000) and CasA from K. oxytoca (33% identity and 51% similarity: Lai et al., 1997). The observed homologies and pattern of hydrophobicity (not shown) suggested a central membrane-spanning IIC domain flanked by hydrophilic IIB and IIA domains located in the N terminus and pattern of salicin consumption from minimal BT medium inducible bgl genes only in an adaptive
or methyl
that wild-type bglFA genes alone do not support cellobiose utilization.

C. glutamicum ATCC 13869-C harbouring individual bglF or bglA genes did not exhibit PTS/β-glucosidase activity and failed to grow on β-glucosides (Fig. 4A). It thus appeared that both BglF PTS permease and phospho-β-glucosidase were essential for utilization of β-glucosides in recombinant cells. To demonstrate their function in vitro, we combined

**Functional analysis of bgl genes in C. glutamicum ATCC 13869**

To further examine whether bgl genes confer the potential for utilization of β-glucosides, we subcloned promoterless bglFA (plasmid pCbglFA) and individual bglF and bglA genes (plasmids pCbglF and pCbglA, respectively) to provide their constitutive transcription from the lac promoter of the vector pCRA1 in C. glutamicum ATCC 13869-C (Fig. 4A). Unlike C. glutamicum R, C. glutamicum ATCC 13869 does not grow on methyl β-glucoside, salicin or arbutin. Southern hybridization further revealed the absence of the bglFA genes from the chromosome of this strain (data not shown). Phosphorylation and cleavage of β-glucoside analogue PNPG were also not detected under any conditions tested (Table 2). The PTS and β-glucosidase (PTS/β-glucosidase) activities were evaluated as combined activities in vitro. The transformation of strain ATCC 13869-C with pCbglFA resulted in constitutive, carbohydrate-independent expression of PTS/β-glucosidase activity (Table 2) and transformants grew on methyl β-glucoside and salicin (Fig. 4A) or arbutin (data not shown). Regardless of the high constitutive PTS/β-glucosidase activity in ATCC 13869-C harbouring pCbglFA, this strain failed to grow on cellobiose (Figs 4A and 5B), thereby suggesting that wild-type bglFA genes alone do not support cellobiose utilization.

**Induction of bgl genes and utilization of different sugars in parental strains and bgl disruptant mutants**

The induction of bglF and bglA was explored by using hybridization of total RNA from cells grown on various carbon sources with probes corresponding to these genes (Fig. 3A). The in vivo bgl transcripts were detected in C. glutamicum R grown on methyl β-glucoside, salicin and arbutin but not on acetate, glucose or cellobiose. Cellobiose induced transcription of bgl genes only in an adaptive Cel+ mutant of C. glutamicum R, named R-CEL (Table 1), which is capable of cellobiose utilization.

We further assessed the significance of the bglFA system in the β-glucoside PTS through its disruption by replacement recombination. For this purpose, the plasmid pDbglPF was constructed, which contained the Km+npt gene flanked by DNA regions located upstream and downstream from bglF (see Methods). The integration of npt from plasmid pDbglPF into the chromosomes of C. glutamicum R and R-CEL (Fig. 1A) via a double crossover event yielded strains R(bgl::npt) and R-CEL(bgl::npt), respectively. In spite of the apparent absence of salicin-inducible bglFA transcripts in R(bgl::npt) (Fig. 3A), the pattern of salicin consumption from minimal BT medium supplemented additionally with glucose remained unchanged (Fig. 3B). We were also unable to detect any change of phenotype in this strain grown on salicin, arbutin and methyl β-glucoside alone (data not shown). The R-CEL(bgl::npt) strain also retained its ability to grow on these β-glucosides. However, it failed to utilize cellobiose (Fig. 3C), which led us to conclude that the bgl genes play an indispensable role in utilization of this sugar in the R-CEL strain.

The presence of a single promoter preceding the bgl genes which encode closely related activities, and the gene spacing and putative regulatory features, which are similar to those of other PTSs, suggested that bglFAG represents the β-glucoside PTS operon. The inverted repeats with a putative stable RNA stem-loop structure (-189 kJ mol-1, 79·12 kJ mol-1) located immediately (9 bp) downstream from the bglG translation termination codon may allow factor-independent termination of the putative tricistronic transcript.

The deduced 52.7 kDa BglA of 469 amino acids has, throughout the full length of the protein, extensive homology to phospho-β-glucosidases, having 65%, 64% and 57% identity to BglA from Streptococcus mutans (35% identity and 52% similarity: Marasco et al., 2000) and CasA from K. oxytoca (33% identity and 51% similarity: Lai et al., 1997). The observed homologies and pattern of hydrophobicity (not shown) suggested a central membrane-spanning IIC domain flanked by hydrophilic IIB and IIA domains located in the N terminus and C terminus of BglF, respectively (Fig. 2A).

The translational start of the protein encoded by bglA is located 60 bp downstream from bglF (Fig. 1A). The deduced 52.7 kDa BglA of 469 amino acids has, throughout the full length of the protein, extensive homology to phospho-β-glucosidases, having 65%, 64% and 57% identity to BglA from Streptococcus mutans (35% identity and 52% similarity: Marasco et al., 2000) and CasA from K. oxytoca (33% identity and 51% similarity: Lai et al., 1997). The observed homologies and pattern of hydrophobicity (not shown) suggested a central membrane-spanning IIC domain flanked by hydrophilic IIB and IIA domains located in the N terminus and C terminus of BglF, respectively (Fig. 2A).

Assuming that the GTG codon positioned 39 bp downstream from bglA represents a translation initiation codon, a protein of 289 amino acids could be deduced from bglG. The predicted 31.7 kDa protein shares 53% similarity with LicT from B. subtilis (Schnetz et al., 1996) and BglG from E. coli (Schnetz et al., 1987), the transcriptional antiterminators belonging to the BglG/SacY family. The similarities were particularly significant for three distinct regions. The first 56 amino acid residues resembled the so-called co-antiterminator RNA binding domain, which was 75% similar to that of LicT from B. subtilis (Schnetz et al., 1996). Two other regions (amino acids 93–169 and 208–280) might, by similarity, represent duplicated PTS regulatory domains PRD-I and II (Stulke et al., 1998).

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crude extracts from strains transformed with pCbglF and pCbglA. As shown in Table 2, the PTS/β-glucosidase activity was restored to the level found in pCbglFA-transformed ATCC 13869-C. Interestingly, the phosphoβ-glucosidase (bglA) alone allowed pCbglA transformants to utilize methylβ-glucoside. This sugar seemingly entered the cell via another (glucose-specific?) PTS permease. It is noteworthy that methylβ-glucoside can also be a substrate for the glucose PTS in E. coli (Schaefler, 1967).

To examine whether bglG, encoding a putative transcriptional regulator, is required for induction of bgl genes, we compared growth and PTS/β-glucosidase activities in C. glutamicum ATCC 13869-C. Interestingly, the phosphoβ-glucosidase (bglA) alone allowed pCbglA transformants to utilize methylβ-glucoside. This sugar seemingly entered the cell via another (glucose-specific?) PTS permease. It is noteworthy that methylβ-glucoside can also be a substrate for the glucose PTS in E. coli (Schaefler, 1967).

Fig. 4. Functional analysis of bgl genes by complementing the β-glucoside-negative phenotype in C. glutamicum ATCC 13869-C. (A) Organization of plasmids used to constitutively express bgl genes and utilization of β-glucosides by the corresponding transformants. PCR-amplified genes were inserted into the multi-cloning site (MCS) of pCRA1 behind the lac promoter of the vector (the P<sub>lac</sub>-MCS-lacZ region originated from the pHSG298 part of the shuttle vector) as described in Methods. Sugar utilization was assayed on minimal BT medium supplemented with 0.5% β-glucoside. For PTS/β-glucosidase activities see Table 2. (B) Utilization of β-glucosides in transformants harbouring wild-type and mutant bgl genes. The wild-type bglFAG was restored from fr-FA and fr-AG (Fig. 1A) in pCRA1 as described in Methods. Plasmids pRbglFA and pRbglFAG are those which accumulated adaptive point mutations in codon 317 of bglF that resulted in V317A and V317M, respectively.

http://mic.sgmjournals.org 1575
-glucosidase activity in pRbglFAG-transformed ATCC 13869-C, but not in pRbglFA transformants, thereby suggesting that bglG product acts as a positive regulator.

Mutations that occur in Cel+ strains
A possible explanation for the occurrence of Cel+ strains was that a mutation within bglF and/or bglA was required to convert bglFAG into a cellobiose PTS. To test this hypothesis, we incubated C. glutamicum ATCC 13869-C transformed with pRbglFAG and pCbglFA on minimal BT medium supplemented with 0.5% cellobiose. Cel+ colonies appeared with both transformants at a frequency of 10−7. Sequence analysis of plasmids isolated from 15 independent Cel+ mutants showed that all contained one of two different single-nucleotide mutations in codon 317 of Table 2. Functional expression of bgl genes in C. glutamicum ATCC 13869-C
Cells were grown in AA medium supplemented with the carbon source indicated. Activities were measured in crude cell extracts from mid-exponential-phase cultures with PNPG as a substrate and expressed as pmol p-nitrophenol formed s−1 (mg total protein)−1. The 50% inhibition of the rate of p-nitrophenol formation was achieved at a PNPG-to-salicin ratio of 1:1. Three independent sets of experiments were carried out and representative results are shown. β-MG, methyl β-D-glucopyranoside; ND, not determined.

<table>
<thead>
<tr>
<th>Plasmid*</th>
<th>Combined PTS and phospho-β-glucosidase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0·4 % Acetate</td>
</tr>
<tr>
<td>pCRA1</td>
<td>0·0</td>
</tr>
<tr>
<td>pCbglFA</td>
<td>19·7</td>
</tr>
<tr>
<td>pCbglF</td>
<td>0·0</td>
</tr>
<tr>
<td>pCbglA</td>
<td>0·51</td>
</tr>
<tr>
<td>pRbglFAG</td>
<td>ND</td>
</tr>
<tr>
<td>pRbglFA</td>
<td>ND</td>
</tr>
</tbody>
</table>

*See Fig. 4(A) for the genetic organization of plasmids.
†0·4 % glucose was supplemented additionally (results in parentheses).
‡An equal amount of cell-free extract from pCbglA-transformed ATCC 13869-C (not included in calculation of specific activity) was additionally supplied to provide phospho-β-glucosidase (results in parentheses).

β-glucosidase activity in pRbglFAG-transformed ATCC 13869-C, but not in pRbglFA transformants, thereby suggesting that bglG product acts as a positive regulator.

![Fig. 5](image_url)

**Fig. 5.** Growth and cellobiose utilization in C. glutamicum strains. The cultures were grown in minimal BT medium supplemented with sugars as indicated below. Growth (as OD590, top of each panel) and sugar consumption (bottom) were monitored throughout growth. (A) Comparison of C. glutamicum R-CEL (open symbols) and R-CEL2 (solid symbols) in media containing either 0.5% glucose (growth, diamonds; sugar consumption not shown) or 0.5% cellobiose (growth, squares; sugar, triangles). (B) Growth and sugar consumption of C. glutamicum ATCC 13869-C transformed with pCbglFA (open symbols) or pCbglFA A (solid symbols) in medium supplemented with glucose (circles) and cellobiose (triangles). For genetic organization of pCbglFA see Fig. 4(A). Plasmid pCbglFA A is the same as pCbglFA but harbours a mutant bglF(V317A). It should be noted here that the PTS/β-glucosidase activities reported in Table 2 for ATCC 13869-C transformed with pCbglFA and values found in similar experiments with pCbglFA A-transformant (data not shown) were essentially identical.
Table 3. Influence of different carbon sources on activity of β-glucoside PTS

Cells were grown to OD₅₉₀ 2 ± 0.5 in AA medium supplemented with the carbon source indicated. The combined PTS and β-glucosidase activities were measured as described in the legend to Table 2 [1 U is 1 pmol p-nitrophenol formed from PNPG s⁻¹ (mg total protein)⁻¹]. Three independent sets of experiments were performed and representative results are shown. β-MG, methyl β-D-glucopyranoside; ND, not determined.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Combined PTS and phospho-β-glucosidase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0-4 % Acetate</td>
</tr>
<tr>
<td>R</td>
<td>bgfF</td>
<td>0-53</td>
</tr>
<tr>
<td>R-CEL</td>
<td>bgfF(V317A)</td>
<td>0-42</td>
</tr>
<tr>
<td>R-CEL2</td>
<td>bgfF(V317M)</td>
<td>0-56</td>
</tr>
<tr>
<td>R(bgl::npt)</td>
<td>ΔbgfG</td>
<td>0-33</td>
</tr>
<tr>
<td>R-CEL(bgl::npt)</td>
<td>ΔbgfG</td>
<td>0-45</td>
</tr>
</tbody>
</table>

*0-4 % glucose was supplemented additionally (results in parentheses).

bgfF, which caused an amino acid exchange. The point mutation GTG→GGC resulted in substitution V317A and GTG→ATG in V317M. bgfF genes carrying V317A (bgfF(V317A) and V317M (bgfF(V317M)) were the only mutated alleles identified and we did not find any mutations within bgfA or bgfG in the isolated plasmids. The respective mutant plasmids were named pRbgfF317A or pRbgfF317A and pRbgfF317M or pRbgfF317M. These plasmids were used to transform C. glutamicum R and ATCC 13869-C. All transformants harbouring the mutant plasmids grew on β-glucosides, including cellobiose (Fig. 4B), indicating that further mutations are not required for the Cel + phenotype. We also constitutively expressed bgfF(317A) and bgfF(317M) in the Enzyme I-deficient mutant of C. glutamicum R (strain R-PDI5; data not shown). The observation that pRbgfF317A and pRbgfF317M did not support growth of R-PDI5 on cellobiose was in good agreement with the idea that the transport of cellobiose is coupled to its phosphorylation.

Finally, to investigate whether a mutation in bgfF is also responsible for the Cel + phenotype in C. glutamicum R-CEL, we amplified the bgf locus from this strain in a PCR using Vent DNA polymerase and chromosomal DNA as a template. Sequencing of the products from three independent reactions showed that R-CEL carried the bgf(317A)AG genes. We were also able to isolate and, by sequencing of the bgf locus, identify a spontaneous Cel + mutant of C. glutamicum R, which harboured mutation V317M. This newly identified mutant was designated R-CEL2. Both R-CEL and R-CEL2 strains grew equally well in minimal BT medium with glucose (as well as methyl β-glucoside and salicin; data not shown) with a growth rate (μ) of about 0.65 h⁻¹, but evaluation of growth on 0.5 % cellobiose revealed significant differences between strains (Fig. 5A). The R-CEL strain both consumed cellobiose and grew (μ 0.23 h⁻¹) faster than R-CEL2 (μ 0.10 h⁻¹), indicating that mutations V317A and V317M do not provide an equally well-performing cellobiose PTS.

Effect of different carbon sources on PTS/β-glucosidase activity

To investigate the β-glucoside PTS and its regulation in more detail we measured the combined PTS and β-glucosidase activity in crude cell extracts from strains grown in AA medium supplemented with 0.4 % sodium acetate, 0.4 % glucose, 0.1 % methyl β-glucoside, 0.1 % salicin or 0.05 % cellobiose (Table 3). Compared to the control (acetate), the presence of methyl β-glucoside and salicin resulted in a 20- to 30-fold increase of PTS/β-glucosidase activity in C. glutamicum R, R-CEL and R-CEL2. This activity was induced by the same spectrum of β-glucosides in bgf-deficient mutants also, suggesting the presence of another PTS responsible for the observed Bgl + phenotype of bgf::npt strains. The levels of the PTS/β-glucosidase activity were actually similar in wild-type and R(bgf::npt) strains, suggesting that a second system may fully complement the bgf genes to provide sufficient activity for maximum (as compared to glucose) growth. Cellobiose induced the PTS/β-glucosidase activity only in R-CEL (28-fold) and R-CEL2 (9-fold).

We further explored the PTS/β-glucosidase activity in C. glutamicum R and its variants grown on mixtures of glucose and β-glucosides to test whether a favoured carbon source would repress the β-glucoside PTS. The PTS/β-glucosidase activities were then only about 10 % of those induced by β-glucosides alone (Table 3), thereby attesting that both β-glucoside utilization systems present in C. glutamicum R are subject to carbon catabolite repression and that β-glucosides should be considered alternative carbon sources in this bacterium. The finding that constitutive expression of bgfF(317A) resulted in simultaneous utilization of glucose and cellobiose (Fig. 5B) indicated that bgf-dependent utilization of β-glucosides is negatively regulated at the transcription level.
DISCUSSION

Analysis of sugar utilization in different strains of *C. glutamicum* revealed that *C. glutamicum* strain R can utilize several β-glucosides as sole carbon and energy sources (Kotrba et al., 2001). The evidence that transport of β-glucosides depends, in this strain, on the PTS prompted our efforts towards cloning of the respective PTS permease that ultimately led to isolation of the β-glucoside phosphotransferase and utilization system which is described here. The system isolated comprises three genes, *bglF*, *bglA* and *bglG*, which, considering their genetic organization, may constitute an operon. Independent transcription of *bglG* cannot be ruled out, however. The respective genes encode (i) PTS permease BglF, which is a β-glucoside-specific Enzyme II with all three functional domains synthesized as one IIBCA protein; (ii) phospho-β-glucosidase BglA; and (iii) putative transcription regulator BglG. The system isolated shares significant homology with β-glucoside PTS operons with permeases belonging to the glucose–cellobiose family of PTS transporters (Robillard & Broos, 1999) reported from Gram-positive and Gram-negative bacteria. The PTS permeases specific for cellobiose, which have been identified in, for example, *Bacillus stearothermophilus* (Lai & Ingram, 1993) or *B. subtilis* (Tobisch et al., 1997) are of the lactose–cellobiose family and do not have significant similarity to BglF.

Functional analysis of *bglF* and *bglA* performed in Bgl-- *C. glutamicum* ATCC 13869-C confirmed that BglF permease, along with the BglA hydrolase, supports transport and breakdown of methyl β-glucoside, salicin and arbutin *in vivo* and that the system isolated in this study is specific for these β-glucosides. The *bglFA* genes are absent from both *C. glutamicum* ATCC 13869 and ATCC 13032, which cannot grow at the expense of β-glucosides (Kotrba et al., 2001) and thus apparently also lack a functional homologue of the second, as yet unidentified, β-glucoside system of *C. glutamicum* R. Taking advantage of the available genome sequence of *C. glutamicum* ATCC 13032 (GenBank accession no. NC003450) and probing it with the *bglFAG* sequence in *silico*, we obtained the only positive hit in the DNA region which was identical with part of the *bglG* coding sequence corresponding to amino acids 96–289 of BglG (data not shown). It is noteworthy that in strain ATCC 13032 this sequence is located 3 kb upstream of an insertion element designated ISG2 (Quast et al., 1999). It is, therefore, tempting to speculate that the *bgl* homologue in strain ATCC 13032 was knocked out due to DNA rearrangements related to the transposition event.

In addition to β-glucosides transported by wild-type BglF, the permease that accumulated mutations V317A and V317M in strains R-CEL and R-CEL2, respectively, can also transport cellobiose, a disaccharide that is not normally utilized in *C. glutamicum* R. Considering that only phosphorylated β-glucosides can be hydrolysed by BglA, and given the fact that constitutive expression of mutant permeases along with phospho-β-glucosidase in the PTS-deficient *ptsI*::npt strain R-PD15 did not support growth on cellobiose, it appears that mutants BglF317A and BglF317M act on cellobiose as phosphorylating PTS Enzyme II. Our experiments did not fully exclude the possibility that phosphorylated cellobiose was hydrolysed by an as yet unidentified β-glucosidase in *C. glutamicum* strains. An examination of the amino acid sequence surrounding the V317 of BglF revealed that the site accumulating mutations is situated only three residues from H313. This histidine residue is highly conserved in all Enzymes II of the homologous BglF from *E. coli* was reported as being indispensable for phosphorylation of transported β-glucosides (Schnetz et al., 1990). From the close proximity of H313 and V317, it is tempting to speculate that substitution of V317 with a residue having either smaller (Ala) or larger (Met) side chains causes a local conformational change affecting the side chain of H313, such that it may participate in phosphorylation of incoming cellobiose. Intriguingly, residues corresponding to position 317 of BglF are also different from valine in the other three permeases (Fig. 2B) which recognize cellobiose as a substrate, namely BvB from *L. monocytogenes* (Brehm et al., 1999), CasA from *K. oxytoca* (Lai et al., 1997) and AscF from *E. coli* (Hall & Xu, 1992). Conversely, the valine residue is conserved in those Enzymes II of *C. glutamicum*, which do not transport cellobiose.

In view of the identification of the RAT/terminator overlap in the *bgl* sequence, and BglG, which may be regarded as a transcription antiterminator from the BglG/SacY family, and considering that *bglG* was confirmed as being indispensable for induction of the PTS/β-glucosidase activity in *C. glutamicum* ATCC 13969-C (Table 2), it appears that transcription of *bgl* genes is regulated via a transcription antitermination mechanism (Stülke et al., 1998; Görke & Rak, 1999). Accordingly, the RNA hybridization analysis demonstrated that the *bgl* genes are induced in *C. glutamicum* R and R-CEL by methyl β-glucoside, salicin and arbutin, and in the latter strain also by cellobiose. The availability of glucose further repressed PTS/β-glucosidase activity in *C. glutamicum* R and R-CEL (Table 3), and the β-glucosides salicin and cellobiose, respectively, were appreciably taken up from the medium only after all the glucose was consumed (Fig. 3B, C). It should be also noted that mutations V317A and V317M did not have equivalent phenotypes. Induction of *bgl*-borne PTS/β-glucosidase activity by cellobiose and the response to glucose availability was more pronounced in the R-CEL mutant. Accordingly, growth of R-CEL2 was slower on cellobiose than that of R-CEL, which was also reflected by the lower rate of cellobiose consumption. Our approach did not fully address the influence of V317A and V317M on transport of other β-glucosides, as these mutations are masked in *C. glutamicum* R-CEL and R-CEL2 by a complementary β-glucoside PTS. It is worth noting, however, that we did not observe any significant differences in growth and PTS/β-glucosidase activities in *C. glutamicum* ATCC
13869-C transformed with plasmids harbouring wild-type or mutant bglFAG when salicin or methyl β-glucoside was supplied as the only carbon and energy source (data not shown). This suggested that the reported substitutions at residue 317 only extended the substrate specificity towards cellobiose. The experimental data represented here encourage the concept that it is the IIC domain that is responsible for substrate specificity of Enzyme II and suggest that a specific PTS may adopt new substrates under unfavourable environmental conditions to support the growth and survival of bacteria.

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