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Catabolite control of *Escherichia coli* regulatory protein BglG activity by antagonistically acting phosphorylations

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In bacteria various sugars are taken up and concomitantly phosphorylated by sugar-specific enzymes II (EII) of the phosphoenolpyruvate:sugar phosphotransferase system (PTS). The phosphoryl groups are donated by the phosphocarrier protein HPr. BglG, the positively acting regulatory protein of the *Escherichia coli bgl* (β-glucoside utilization) operon, is known to be negatively regulated by reversible phosphorylation catalyzed by the membrane spanning β-glucoside-specific EII^{Bgl}. Here we present evidence that in addition BglG must be phosphorylated by HPr at a distinct site to gain activity. Our data suggest that this second, shortcut route of phosphorylation is used to monitor the state of the various PTS sugar availabilities in order to hierarchically tune expression of the *bgl* operon in a physiologically meaningful way. Thus, the PTS may represent a highly integrated signal transduction network in carbon catabolite control.

Keywords: carbon catabolite control/gene regulation/ phosphoenolpyruvate:sugar phosphotransferase system/ protein phosphorylation/signal transduction

Introduction

Although it was initially thought that different mechanisms of signal transfer may exist for the three kingdoms of life, it has recently been discovered that eukaryotic-type protein kinases are also found in prokaryotes (for a review see Zhang, 1996), whereas two-component systems, which were thought to be restricted to bacteria, were found to be involved in eukaryotic signaling (for reviews see Loomis *et al*., 1997; Wurgler-Murphy and Saito, 1997). In contrast, the phosphoenolpyruvate:sugar phosphotransferase system (PTS), which is ubiquitous in bacteria, seems to be bacteria specific. The PTS is not only central in uptake and phosphorylation of various sugars, but also mediates chemotaxis towards these sugars, as well as transcriptional regulation of numerous genes (for reviews see Postma *et al*., 1993; Saier and Reizer, 1994).

The PTS catalyzes phosphotransfer reactions from phosphoenolpyruvate to various sugars, a process which involves at least three proteins. First, enzyme I (EI) autophosphorylates with phosphoenolpyruvate, and then donates the phosphoryl group to histidine protein (HPr), which in turn phosphorylates the respective sugar-specific transport proteins enzymes II (EII) from which phosphate is transferred to the incoming sugar concomitantly with its transport. EIIs consist of at least three functionally different domains, IIA, IIB and IIC, which may be present on the same polypeptide chain or may be encoded by different genes. The phosphocarrier protein HPr phosphorylates the respective EIIs at a histidine residue in the IIA domain, from which phosphate is transferred to a residue of the IIB domain, in most cases a cysteine, and then to the sugar. Membrane bound domain, IIC, forms the translocation channel and the sugar-specific substrate-binding site.

The PTS is also central in carbon catabolite repression, exerted in Gram-negative bacteria by the glucose-specific IIA^{Glc} (formerly $EIII^{Glc}$). Phosphorylated IIA^{Glc} is thought to activate adenylate cyclase, whereas non-phosphorylated IIA^{Glc} binds and inhibits various proteins concerned with uptake and metabolism of non-PTS sugars. This mechanism may, therefore, explain preferred utilization of some PTS carbohydrates over non-PTS carbohydrates. Preferential utilization of one PTS sugar over another has also been observed several times, but the cause of this hierarchical utilization remained unclear (Postma *et al*., 1993).

The transcriptional regulator BglG from *Escherichia coli* regulates expression of the *bgl* operon, encoding proteins concerned with regulated uptake and utilization of β-glucosidic sugars (Prasad and Schaefler, 1974; Schnetz *et al*., 1987). BglG was the first identified member of a fast-growing family of related proteins shown to be directly regulated by the PTS in a substrate-dependent manner (Rutberg, 1997; Tortosa *et al*., 1997; Stülke *et al*., 1998). BglG is the product of the first gene of the operon and acts as a transcriptional antiterminator at two ρ-independent terminators within the operon (Mahadevan and Wright, 1987; Schnetz and Rak, 1988). The second gene of the operon, *bglF*, encodes the β-glucoside-specific EII^{Bgl}, which is a member of the PTS-family of sugar transport proteins. EII^{Bgl} has also been shown to be a negative regulator of BglG (Mahadevan *et al*., 1987). In the absence of substrate, when phosphate cannot be transferred to the sugar, EII^{Bgl} phosphorylates and concomitantly inactivates BglG, thereby preventing transcription of the operon. When β-glucosides become available, EII^{Bgl} dephosphorylates and thereby reactivates BglG (Amster-Choder *et al*., 1989; Amster-Choder and Wright, 1990; Schnetz and Rak, 1990).

In this report we re-investigated regulation of BglG activity. Data presented show that to be active, BglG requires the presence of the general PTS proteins, EI and HPr. Complementation analysis revealed that phospho-HPr is necessary for activation and that the diphosphoryl transfer protein (DTP), which shares an HPr-like domain, can substitute for HPr in this process. Furthermore, we show that BglG is phosphorylated *in vivo* in a PTS-dependent reaction also in the absence of EII^{Bgl}, suggesting that HPr activates BglG by phosphorylation and that

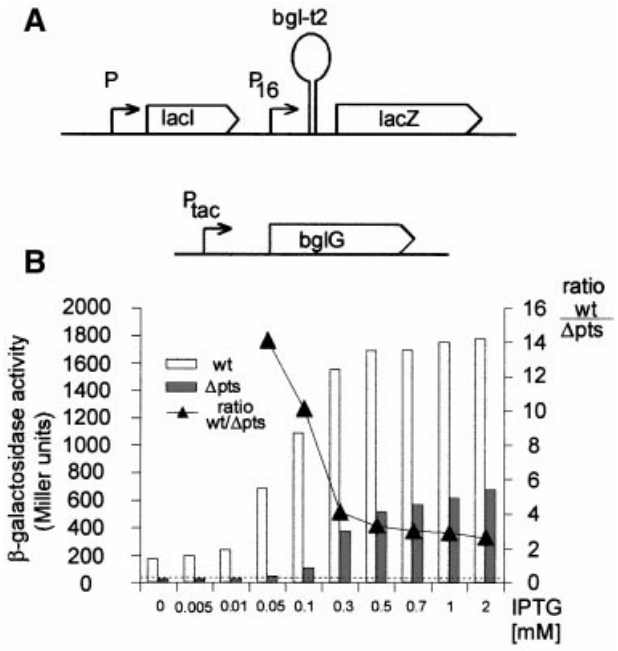

Fig. 1. BglG requires an intact PTS to be active, but its overproduction can partially overcome this requirement. Antitermination reporter plasmid pFDY226 (**A**) was cotransformed with *bglG* expression plasmid pFDX2469 (**B**) into *E.coli* strain R1279 (*pts*$^+$; white histograms) and its isogenic *Δpts* derivative R1653 (shaded histograms). Increasing levels of BglG synthesis were driven by increasing concentrations of inducer (IPTG) and β-galactosidase activity was determined as a measure for antitermination activity. The ratio of enzyme activity synthesized by the two strains is given as filled triangles. For IPTG concentrations below 50 μM enzyme activity remained at background levels with the *Δpts* strain which was 37 units for both strains when transformed with the antitermination test plasmid alone (dotted line).

two different and antagonistically acting phosphorylation reactions control BglG activity. When we investigated the possible role of this positively acting HPr-mediated phosphorylation, we found that this mechanism may function to downregulate BglG activity and thus *bgl* operon expression when other, more favorable PTS sugars become available, by draining the activating phosphoryl groups away from BglG. This control, which makes use of the reversible flow and limited pool of transferable phosphoryl groups within the PTS, represents an unprecedented level of carbon catabolite control in *E.coli*.

Results

BglG requires the PTS for its activity

To determine antitermination activity of BglG, we made use of our antitermination reporter plasmid described previously (Schnetz *et al*., 1996). In this plasmid (Figure 1A) the *lacZ* reporter gene is preceded by terminator *t2* of the *bgl* operon (bgl-t2) which in the absence of the active BglG protein blocks elongation of transcription initiated at constitutive promoter *P16*. Also present on the plasmid is the *lacI*^{q} gene providing the *lac* repressor for the controlled expression of *bglG* which is under *tacOP* control on a second plasmid (Figure 1B). In the absence of the *bglG*-containing plasmid, 37 units of β-galactosidase activity were synthesized in a wild type strain background as well as in an isogenic background carrying a deletion (*Δpts*) which encompasses genes *ptsH*, *ptsI* and *crr* encoding HPr, EI and IIA^{Glc}, respectively. This low activity reflects the leakiness of terminator *t2* (Schnetz and Rak, 1988; dotted line in Figure 1B). In the presence of the *bglG* expression plasmid, and with increasing concentrations of isopropyl-β-D-thiogalactopyranoside (IPTG) as inducer for BglG synthesis, enzyme activity increased in the wild type background from 177 units (no IPTG) to 1776 units (2 mM IPTG). In the *pts* deletion strain, enzyme activity did not start to rise above background at concentrations below 0.05 mM IPTG (49 units) and only reached 676 units at saturating concentrations (2 mM). Moreover, the ratio of enzyme activities (wt/Δpts) decreased with increasing IPTG concentrations from 14.1 (0.05 mM) to 2.6 (2 mM), indicating that BglG requires an intact PTS for full activity and that this requirement can be partially overcome by overproduction of BglG. To support this conclusion we verified that deletion of the *pts* operon does not negatively influence gene expression within our detection system. To this end we employed transformants carrying a plasmid (pFDY225), which is identical to the antiterminator test plasmid (Figure 1A) but lacks the *bgl* terminator. In addition we employed a plasmid (pFDX3549), which is isogenic to the BglG expression plasmid (Figure 1B) but carries the *lacZ* gene instead of *bglG*. Indeed, enzyme activities directed by these plasmids in the wild type strain were not significantly different from those synthesized in the isogenic *pts* deletion derivative (data not shown).

Activation of BglG requires phospho-HPr or phospho-DTP

Next we wanted to define in more detail the requirements for activation of BglG by the PTS. To avoid the problems imposed by overproduction of BglG due to high gene dosage with multi-copy plasmids, we inserted the *tacOP*-driven *bglG* expression cassette either with (Figure 2B) or without the *bglF* gene (encoding EII^{Bgl}) (Figure 2C) into the chromosome. The different strain backgrounds used together with complementing plasmids are given in Figure 2A. As can be seen from the data, β-galactosidase activity was low in all cases when IPTG was omitted. As expected, it remained low in the presence of IPTG when *bglF* was coexpressed together with *bglG* in the wild type strain and increased to 325 units when salicin was added as substrate for *bglF*-encoded EII^{Bgl} (Figure 2B, column 1) reflecting the relief from negative control exerted by the phosphorylated EII^{Bgl} on BglG activity (see Introduction). Likewise, BglG activity was constitutive and high in the absence of *bglF* (Figure 2C, column 1). In the *Δpts* background (columns 2), β-galactosidase activities were reduced to background levels verifying the PTS dependence of BglG activity demonstrated above. Activation of BglG as well as its negative regulation were fully complemented when HPr together with EI were expressed from a plasmid (columns 3). Expression of HPr alone did not result in complementation (column 4), whereas expression of EI alone led to partial complementation (columns 5). HPr thus appears not to be absolutely required for activation and negative control of BglG. However, it has been reported that a defect in HPr can be complemented for PTS sugar transport in mutants defective for *fruR* (Saier and Ramseier, 1996). The repressor FruR negatively

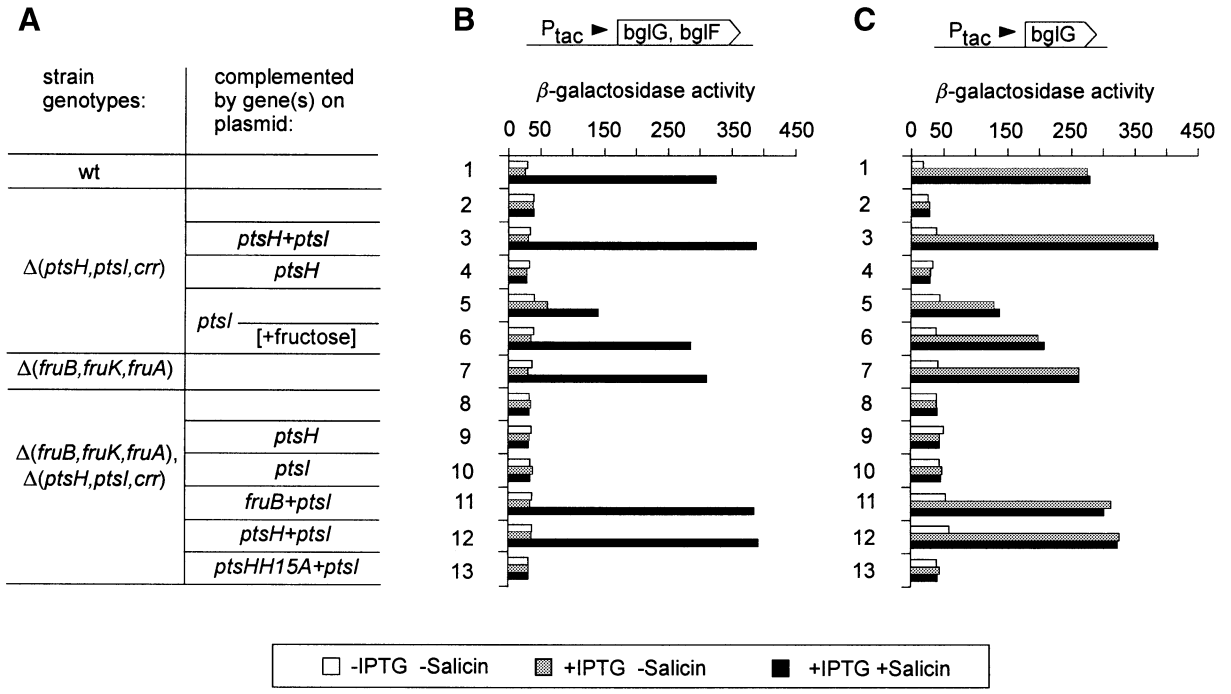

Fig. 2. BglG requires phospho-HPr for activation which can be substituted by phospho-DTP. Genes *bglG* and *bglF* (**B**) or *bglG* alone (**C**) under *tacOP* control were integrated into the chromosome by site-specific recombination to obtain single-copy situations and subsequently crossed into various strain backgrounds harboring different expression plasmids (**A**) also driven by *tacOP* and thus inducible by IPTG. In addition, antitermination reporter plasmids pFDX2676 or pFDX3158 were present. These plasmids are identical to plasmid pFDY226 (Figure 1A) but carry, for compatibility reasons, different antibiotic resistance markers. Inducers were added as indicated in the figure and β-galactosidase activities were determined as described in Materials and methods with the exception of lane 6. In these experiments, cells were pregrown in M9-fructose medium which was substituted by M9-glycerol at the time point when inducers were added. This was necessary to avoid interference of BglG activity due to transport of fructose (a PTS-sugar) (see data below and Discussion). The following strains were employed: in (B) lane 1, R1752; lanes 2–6, R2013; lane 7, R1971; lanes 8–13, R1977. In (C) lane 1, R1958; lanes 2–6, R2051; lane 7, R1974; lanes 8–13, R1979. These strains harbored the following plasmids: (B and C) lanes 1 and 2 and 7 and 8, pFDX2676; lane 3, pFDX2676/pFDX3155; lanes 4 and 9, pFDX3158/pFDX3160; lanes 5, 6 and 10, pFDX3158/pFDX3161; lane 11, pFDX3158/pFDX3221; lane 12, pFDX2676/pFDX3155; lane 13, pFDX3158/pFDX3223.

controls the fructose inducible *fru* operon which codes for fructose-specific PTS functions and a fructose-1-phosphate kinase. Responsible for the complementation is the product of the *fruB* gene, DTP (diphosphoryl transfer protein), which carries a domain homologous to HPr. DTP thus was a good candidate which possibly could substitute for HPr in BglG control. We therefore repeated the experiment shown in columns 5 with a culture pregrown in fructose as inducer of the *fru* operon. This pretreatment indeed resulted in better complementation of activation as well as negative control of BglG activity (columns 6). Consequently, we analyzed the involvement of *fru* operon functions. Deletion of the operon in the wild type strain had no appreciable effect (compare columns 1 and 7). We next crossed in the *pts* deletion resulting in the double-deletion strain *Δpts,Δfru*. As expected, enzyme activities were equally low in this strain as in the strain carrying the *pts* deletion (compare columns 2 and 8). They remained low when the defects were complemented by a *ptsH* (HPr) or *ptsI* (EI) expression plasmid (see columns 9 and 10). We can thus conclude that restoration of BglG activity and its negative control in the *pts* deletion strain expressing EI (columns 5 and 6) is due to a function encoded by the *fru* operon. Since the likely candidate is DTP, we complemented the double-deletion strain with an artificial operon encompassing *fruB* (DTP) and *ptsI*(EI). As can be seen from the data in column 11, the activity of BglG as well as its negative control were fully restored. It was likewise restored when the strain expressed *ptsH* and *ptsI* (columns 12), but not when the *ptsH* gene was replaced by an allele carrying a point mutation within the phosphorylation site (columns 13). Together, the data indicate that BglG is activated by the PTS and that this activation requires phosphorylated HPr, which can be substituted by the phosphorylated form of DTP.

BglG is phosphorylated by the PTS even in the absence of EII^{Bgl}

The requirement for phosphorylated HPr suggested that activation of BglG might involve HPr-catalyzed phosphorylation of BglG. This possibility was studied by *in vivo* phosphorylation of BglG. Strains carrying various plasmids with the relevant genes cloned downstream of *tacOP* were pulse labeled with $H_3[^{32}P]O_4$ in the presence or absence of IPTG as inducer, and the proteins were separated on SDS–PAGE. The resulting autoradiographs are shown in Figure 3. First we reproduced the EII^{Bgl}-dependent BglG phosphorylation demonstrated previously (Amster-Choder *et al*., 1989; Amster-Choder and Wright, 1990; Schnetz and Rak, 1990). When compared with the untransformed strain (Figure 3A, lane 1), a signal corresponding to the BglG protein can be observed without IPTG (Figure 3A, lane 2), and this signal increased strongly when IPTG was added (Figure 3A, lane 3). As expected from the previous data, the presence of salicin as substrate for EII^{Bgl} transport completely inhibited

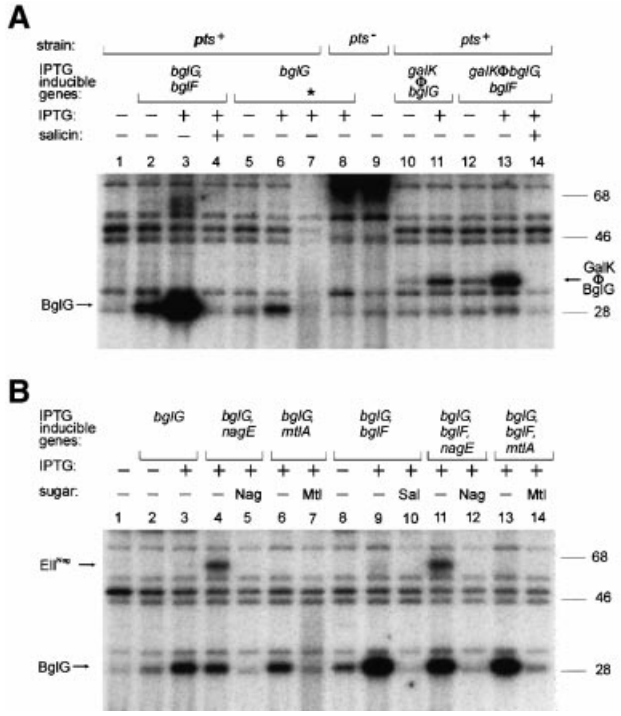

Fig. 3. Phosphorylation of BglG *in vivo*. (**A**) PTS-dependent phosphorylation of BglG also occurs in the absence of EII^{Bgl}. Transformants of strains R1279 (*pts*$^+$) and R1653 (*Δpts*) were grown with or without IPTG (0.1 mM) as inducer for *tacOP* driven expression of plasmid-encoded genes. Where indicated, salicin as substrate for EII^{Bgl} was added. Lac repressor was provided from a second plasmid (pFDY226) with the exception of lane 7 (marked with an asterisk), where the *lacI* gene was present on the *bglG*-containing plasmid. In this case the plasmid had an increased copy number and 1 mM IPTG was added as inducer. Aliquots of cultures were labeled with $H_3[^{32}P]O_4$, proteins separated by SDS–PAGE and gels subsequently exposed to X-ray films. Lane 1, R1279 (empty strain); lanes 2–4, R1279/pFDX3102 (*bglG*, *bglF*); lanes 5 and 6, R1279/ pFDX2942 (*bglG*); lane 7, R1279/pFDY45 (*bglG*); lane 8, R1653/ pFDX2942 (*bglG*); lane 9, R1653 (empty strain); lanes 10 and 11, R1279/pFDX3225 (*galKΦbglG*); lanes 12–14, R1279/pFDX3226 (*galKΦbglG*, *bglF*). (**B**) Massive PTS-sugar transport completely prevents phosphorylation of BglG in the absence as well as in the presence of EII^{Bgl}. Transformants of strain R1279 were labeled as described in (A). In contrast to (A), gene *bglF* controlled by *tacOP* was provided by a *lacI*-containing plasmid (pFDX3283) *in trans*. IPTG as inducer for gene expression and sugars salicin, *N*-acetyl-D-glucosamine or mannitol as substrates for their respective EIIs were added where indicated. Lane 1, R1279 (empty strain); lanes 2 and 3, R1279/pFDX2942 (*bglG*)/pFDY226; lanes 4 and 5, R1279/pFDX3293 (*bglG*, *nagE*)/pFDY226; lanes 6 and 7, R1279/pFDX3295 (*bglG*, *mtlA*)/pFDY226; lanes 8–10, R1279/pFDX2942 (*bglG*)/pFDX3283 (*bglF*); lanes 11 and 12, R1279/ pFDX3293 (*bglG*, *nagE*)/pFDX3283 (*bglF*); lanes 13 and 14, R1279/pFDX3295 (*bglG*, *mtlA*)/pFDX3283 (*bglF*).

phosphorylation of BglG (Figure 3A, lane 4). However, when *bglG* was expressed alone, an IPTG-dependent signal appeared at the position of BglG (Figure 3A, lanes 5 and 6). It disappeared when the same experiment was carried out with the *pts* deletion strain (Figure 3A, lanes 8 and 9). To unambiguously verify that the signal detected in the absence of EII^{Bgl} is due to the phosphorylated BglG protein, we repeated the experiments in the wild type strain with a *galK–bglG* fusion which expresses a protein with an increased molecular weight and for which EII^{Bgl}-dependent phosphorylation has previously been shown (Schnetz and Rak, 1990). Phosphorylated species were indeed observed at the expected positions and in an IPTG-dependent manner, not only in the presence of EII^{Bgl} (Figure 3A, lanes 12 and 13) but also in its absence (Figure 3A, lanes 10 and 11). Again, no phosphorylation was observed in the presence of EII^{Bgl} when salicin was present (Figure 3A, lane 14). As a control for BglG expression levels we performed [^{35}S]methionine pulse–chase labeling and SDS–PAGE with the identical transformants used in Figure 3A and in the presence or absence of IPTG and salicin as given in the figure. The resulting autoradiograph revealed that neither coexpression of EII^{Bgl} together with BglG nor the strain employed nor the presence of salicin had any appreciable effect on the amount or stability of BglG produced (data not shown), thus indicating that differences in ^{32}P-signal strengths of BglG at identical IPTG concentrations reflect differences in the efficiency of BglG phosphorylation rather than differences in the amount of protein present. We also learned from this series of experiments that care had to be taken in adjusting the expression level of BglG. A 5-fold higher expression (as revealed from the [^{35}S]methionine labeling; data not shown) resulted in an almost complete loss of the HPr-dependent phosphorylation signal of BglG (Figure 3A, lane 7). This effect could be attributed to the formation of insoluble inclusion bodies (Krieg, 1993; Chen *et al*., 1997a) which may sequester most of the available BglG protein. It may also explain why EII^{Bgl}-independent phosphorylation previously remained undetected (Schnetz and Rak, 1990).

Underphosphorylation of the PTS leads to dephosphorylation and concomitant inactivation of BglG

According to previous models (see Introduction) EII^{Bgl}, in the absence of β-glucosidic substrates, inhibits BglG by phosphorylation and reactivates it by dephosphorylation when substrates become available (negative-feedback control). The data presented above extend this model and demonstrate that activity of BglG is additionally under positive control of the PTS, dependent upon HPr-catalyzed phosphorylation, which should occur at a different site than that catalyzed by EII^{Bgl}. However, the observation that the presence of salicin as sugar substrate completely prevents phosphorylation of BglG (Figure 3A, lanes 4 and 14) at first seemed to contradict this conclusion. One has to bear in mind, however, that analysis of BglG activity (Figure 2) required single-copy situations, while the salicin-dependent dephosphorylation of BglG, which was performed under *in vivo* conditions (Figure 3A), could only be demonstrated when *bglG* and *bglF* were overexpressed from a plasmid. We therefore reasoned that the HPr-catalyzed phosphorylation of BglG was prevented due to massive salicin transport catalyzed by unphysiologically high concentrations of EII^{Bgl}. This might drain the entire phosphorylation capacity of the PTS towards the incoming sugar. To investigate this possibility, we performed two types of analysis. We analyzed phosphorylation of BglG expressed from a plasmid (Figure 3B) and in parallel studied the activity of BglG expressed from a single-copy gene, in both cases in the presence of overproduced EIIs other than EII^{Bgl} (Figure 4). To indicate the position of the phosphorylated form of BglG and IPTG dependence of its expression, the phosphorylation assays shown in

chromosomally encoded:	IPTG	salicin	other sugar	β-galactosidase activity	plasmid encoded: P_{tac} ► nagE	P_{tac} ► mtlA	P_{tac} ► bglF
P_{tac} ► bglG, bglF	–	–	–	30	27	25	26
	+	–	–	26	25	23	20
	+	+	–	325	331	395	31
	+	+	Nag	–	33	–	–
	+	+	Mtl	–	–	34	–
P_{tac} ► bglG	–	–	–	19	27	27	20
	+	–	–	274	269	296	21
	+	+	–	279	–	–	86
	+	–	Nag	–	32	–	–
	+	–	Mtl	–	–	34	–

Fig. 4. Massive PTS sugar transport drastically decreases BglG activity. Strains R1752 and R1958 carrying *tacOP*-controlled genes *bglG–bglF* and *bglG*, respectively, integrated into the chromosome were cotransformed with antitermination reporter plasmid pFDX2676 and one of the following plasmids for *tacOP*-controlled expression of different EIIs (where indicated): pFDX3297 (*nagE*; EII^{Nag}), pFDX3298 (*mtlA*; EII^{Mtl}), pFDX3299 (*bglF*; EII^{Bgl}). Given are β-galactosidase enzyme activities synthesized by these transformants in presence or absence of IPTG as inducer for gene expression and the various PTS sugar transport substrates. –, not determined.

Figure 3A, lanes 1, 5 and 6, were repeated in Figure 3B, lanes 1, 2 and 3, respectively. Neither overexpression of *nagE* (encoding EII^{Nag}) nor that of *mtlA* (encoding EII^{Mtl}) led to significant alterations in the state of phosphorylation of BglG (Figure 3B, lanes 4 and 6, respectively). The presence of the respective sugar substrates, *N*-acetyl-D-glucosamine (Nag) and D-mannitol (Mtl), however, completely prevented phosphorylation of BglG (Figure 3B, lanes 5 and 7). Lanes 8–10 correspond to lanes 2–4 in Figure 3A, with the difference that EII^{Bgl} was delivered from a second plasmid *in trans*. Again, the phosphorylated form of BglG appeared in an IPTG-dependent manner (Figure 3B, lanes 8 and 9) and disappeared when salicin was present (lane 10). As expected, this pattern did not alter, when in addition, *nagE* (lane 11) or *mtlA* (lane 13) was expressed as an operon fusion with *bglG*. However, the presence of the respective sugar substrates (Nag; Figure 3B, lane 12 or Mtl; lane 14) almost completely prevented phosphorylation of BglG. Again, we verified by [^{35}S]methionine pulse–chase labeling and SDS–PAGE with the identical transformants that the conditions employed had no unexpected effects on the expression level of BglG (data not shown). We can thus conclude that massive transport of a PTS substrate generally causes disappearance of the phosphorylated form of BglG, regardless of the presence of EII^{Bgl}.

To correlate the phosphorylation data with the activity of BglG, we performed the antitermination assay with the transformants used in Figure 2, columns 1. These cells were transformed with additional plasmids carrying *nagE*, *mtlA* or *bglF* under *tacOP* control (Figure 4). For comparison, the values without an additional plasmid (taken from Figure 2, column 1) are also included. It can be seen that the additional expression of *nagE* in the absence of *N*-actetyl-D-glucosamine (Nag) as substrate, or *mtlA* in the absence of D-mannitol (Mtl) did not influence the BglG activity originating from the *bglG* gene or the *bglG–bglF* operon. In contrast, the presence of the respective sugar substrates reduced activity to nearly background, not only when the *bglG* gene was expressed alone, but also when the *bglG–bglF* operon was expressed and salicin was included as inducer. These results support the notion derived from the phosphorylation data that phosphoryl groups within the PTS may be limiting for the activation of BglG. They support our conclusion that BglG must be phosphorylated in order to be active. Further support is derived from the data in the last column in Figure 4: overexpression of *bglF* from a plasmid in the strain containing *bglG* alone led to a drastic reduction of BglG activity even in the presence of salicin, and reduced it to almost background levels in the strain carrying the *bglG–bglF* operon.

Does the limitation of phosphoryl group availability play a role under physiological conditions?

We wanted to learn whether the inhibitory effect on BglG activation seen under conditions of overproduction of EIIs and in the presence of the respective sugar substrates points to a limited pool of phosphoryl groups under more physiological conditions. From this we hoped to learn more about its possible role in keeping the activity of BglG low under natural conditions when other, perhaps more favorable sugars are available. To this end we performed the antitermination assay as in Figure 2, columns 1 but replaced glycerol with other sugars. In the case of the *bglG–bglF* operon, salicin was in addition present as substrate for EII^{Bgl} and thus as inducer of BglG activity. The results are given in Figure 5. Glycerol is a non-PTS sugar, while the other sugars used are all substrates of the PTS. Compared with glycerol, all PTS sugars indeed led to a reduction of BglG activity to different extents.

Discussion

In view of the previous finding that the PTS negatively controls activity of BglG by EII^{Bgl}-catalyzed phosphorylation it might have been expected that any mutation

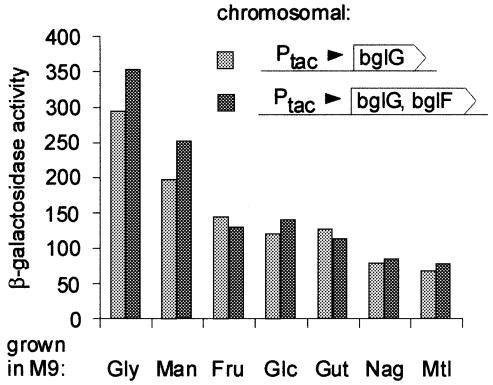

Fig. 5. Utilization of PTS sugars reduces BglG activity to different degrees. Strains R1958 (light gray histograms) and R1752 (dark gray histograms) carrying the chromosomally integrated *tacOP* driven *bglG* and *bglG–bglF* expression cassettes, respectively, were transformed with antitermination reporter plasmid pFDX2676. Cells were grown in M9-medium with the indicated sugar as carbon source. IPTG was added to induce synthesis of BglG and BglG together with EII^{Bgl}, respectively. In the case of R1752 salicin was additionally present to release BglG from negative control exerted by EII^{Bgl}.

preventing this phosphoryl group transfer would result in unrestrictedly high activity of BglG. Indeed, activity of BglG was high and constitutive in the absence of EII^{Bgl} (Figures 1 and 2) or when cysteine residue 24 within EII^{Bgl}, the phosphoryl group donor site for BglG phosphorylation, was replaced by a serine (Krieg, 1993; Chen *et al*., 1997a). However, contrary to expectation, BglG turned out to be inactive when the central energy-coupling proteins of the PTS, EI and HPr, were absent (Figure 2), suggesting a second level of BglG activity control requiring the PTS. Since evidence has been presented that the active form of BglG is a dimer (Amster-Choder and Wright, 1992), the positively acting signal may be necessary for dimer formation. This requirement can, however, be overcome, at least in part, by an increase in its concentration above the physiological level (Figure 1). Similar observations have been reported for LicT of the BglG family of antiterminators (Krüger and Hecker, 1995; Krüger *et al*., 1996) and the related transcriptional activator LevR (Stülke *et al*., 1995). Detailed genetic complementation analyses revealed that phospho-HPr is required for BglG activity, and it turned out that for this activation, HPr can be substituted by DTP, a component of the fructose-inducible PTS encoded by the *fru* operon. Suppression of HPr deficiency by DTP has been observed before for PTS transport functions, either in mutants lacking the *fru* repressor (Saier and Ramseier, 1996) or in cultures induced with fructose (Saier *et al*., 1970). The possible physiological role of the cross-talk observed here for BglG activation remains to be determined.

Since our results suggested that BglG might be directly phosphorylated by HPr, we performed *in vivo* phosphorylation analyses. Indeed, phosphorylated BglG species were detected in the absence of EII^{Bgl}, under conditions where BglG activity was constitutively high (Figure 3). On the other hand, BglG was inactive and not phosphorylated when expressed in a *Δpts* strain background. We therefore extend the previous model and conclude that activation of BglG might require both dephosphorylation by EII^{Bgl} and simultaneous phosphorylation by HPr (see model in Figure 6B). In the presence of EII^{Bgl} and in the absence of β-glucosides (and any other PTS sugars; see below) we observed a stronger signal for phospho-BglG (Figure 3A), suggesting that BglG might be doubly phosphorylated under these conditions (Figure 6A). Since this form is not active, inactivation of BglG by EII^{Bgl}-dependent phosphorylation may be dominant over that exerted by HPr.

The conclusion drawn in previous works, that the completely dephosphorylated form of BglG may be the active form, was derived from experiments demonstrating that BglG is completely dephosphorylated in the presence of EII^{Bgl} and β-glucosidic substrates under *in vivo* (Amster-Choder and Wright, 1990; Schnetz and Rak, 1990; this work) as well as *in vitro* conditions (Amster-Choder *et al*., 1989). This conclusion seems to contradict our model that BglG must be activated by HPr-mediated phosphorylation. According to the model proposed in Figure 6B, one might expect the addition of β-glucosides should lead to the appearance of a monophosphorylated form of BglG, since activation of BglG seems to require both dephosphorylation by EII^{Bgl} at one site and simultaneous phosphorylation by HPr at a distinct site. However, it has been proposed that only a limited pool of phosphoryl groups is available within the PTS due to slow autophosphorylation of EI (Meadow *et al*., 1990; Chauvin *et al*., 1994; Fomenkov *et al*., 1998). Furthermore, the phosphoryl groups appear to be exchangeable within the PTS due to the exceptionally high phospho-transfer potential of the PTS phosphoproteins, which is conserved until their transfer to the sugar substrate (Weigel *et al*., 1982). These characteristics are thought to form the basis for a wide variety of regulatory processes like the stringent regulation of sugar uptake and catabolite repression exerted in *E.coli* by the ratio [phospho-IIA^{Glc}]/[IIA^{Glc}] (Postma *et al*., 1993; Hogema *et al*., 1998) or chemotaxis towards PTS substrates, which is believed to monitor the phosphorylation state of EI (Lux *et al*., 1995). These characteristics also provide an explanation for the apparent contradiction: since in all *in vivo* BglG phosphorylation experiments carried out until now, EII^{Bgl} was provided in much higher concentrations than normally present inside the cell, it is conceivable that phosphoryl groups from HPr were all drained via EII^{Bgl} by the incoming β-glucoside. This would leave BglG in an unphosphorylated form and mask its HPr-catalyzed phosphorylation which occurs under normal conditions. To investigate whether this inhibition of HPr-catalyzed phosphorylation is generally caused by an overload of the PTS, we not only expressed EII^{Bgl} from a plasmid, but also EII^{Nag} and EII^{Mtl}, respectively, and studied BglG phosphorylation (Figure 3B) as well as its activity (Figure 4). Indeed, plasmid-dependent expression of all these EIIs in the presence of their respective sugar substrates completely abolished phosphorylation of BglG and simultaneously rendered the chromosomally expressed BglG inactive. The inhibitory effect of multiple copies of *bglF* (EII^{Bgl}) *in trans* to a single copy of *bglG* in the presence of salicin has been noticed before (Mahadevan *et al*., 1987), but an explanation could not be given at that time. The finding that transport of other PTS sugars not only prevents phosphoryl transfer from HPr to BglG but also from HPr to EII^{Bgl} and from there to the EII^{Bgl}-dependent phosphorylation site of BglG may not be surprising since the K_M of EII^{Bgl} for phospho-HPr with ~40 μM (Schnetz *et al*., 1990) is much higher than the

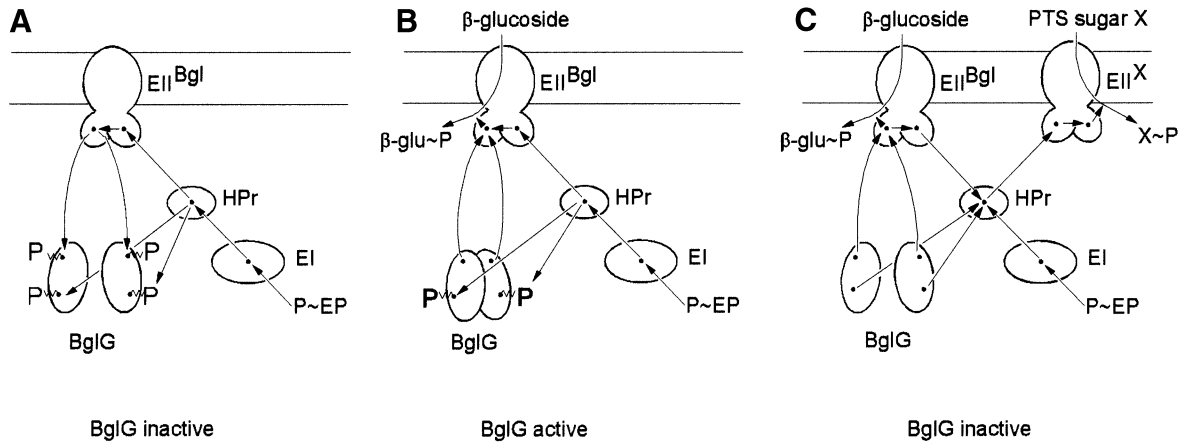

Fig. 6. Model for activity control of BglG as suggested by the data presented in this study. (**A**) In the absence of any PTS sugar, proteins of the PTS are all maintained in a phosphorylated state. Since phosphate cannot be transferred to β-glucosidic substrate, EII^{Bgl} phosphorylates BglG, which leads to its monomer formation, the inactive form. In addition HPr may phosphorylate BglG at a second site. (**B**) In the presence of β-glucosides (but absence of other PTS substrates), EII^{Bgl} dephosphorylates BglG, which is necessary but not sufficient for its activation. In addition, HPr directly transfers phosphoryl groups to a distinct site within BglG, which allows it to dimerize to the active form and alleviate transcription termination within the *bgl* operon. This model also includes a subtle mode of *bgl* operon autoregulation, since both phosphate flow to the sugar during transport and activation of BglG by HPr-catalyzed reversible phosphorylation have to share the limited pool of phosphoryl groups provided by the slowly autophosphorylating EI. (**C**) Appearance of any other PTS sugar leads to additional competition for phosphoryl groups. Since the K_M of EII^{Bgl} is higher than that reported for any other EII, it is conceivable, that phosphoryl groups are massively drained away from EII^{Bgl} in favor of the other sugar transport. This not only leads to severe reduction of β-glucoside transport but in addition inhibits the capacity of EII^{Bgl} to phosphorylate BglG. As a consequence, EII^{Bgl} is no longer able to repress BglG activity. It is therefore necessary to additionally couple control of activity of BglG to the general state of the PTS via the phosphorylation state of HPr. When phosphoryl groups become limited due to transport of other PTS sugars, HPr is underphosphorylated and therefore unable to activate BglG by phosphorylation. BglG is inactive.

reported K_M values of other EIIs (Postma *et al*., 1993). Thus, it is conceivable that if only negative control by EII^{Bgl} on BglG takes place, addition of another PTS sugar would result in dephosphorylation and activation of BglG even in the absence of β-glucosidic substrates. Therefore, additional control of BglG activity by HPr might be necessary to maintain BglG in an inactive state, when in the absence of β-glucosides, other PTS sugars are available (Figure 6C).

To learn whether cells utilize the limited pool of phosphoryl groups within the PTS for regulation of BglG activity under physiological conditions, we examined BglG activity in the presence of various sugars, which were taken up and utilized by the respective chromosomally encoded proteins (Figure 5). Indeed fermentation of all PTS sugars tested lowered BglG activity in the absence as well as in the presence of EII^{Bgl} and its substrate salicin, suggesting that part of the BglG population becomes dephosphorylated and inactivated under natural conditions (Figure 6C). Interestingly, PTS sugars differed in their ability to decrease the BglG activity which might reflect a hierarchical order of sugar preference. Hierarchical utilization of PTS carbohydrates has been observed previously (reviewed in Postma *et al*., 1993). Since the various EIIA domains of PTS permeases have different K_M values for phospho-HPr, it has been proposed that competition for phospho-HPr may be a primary mechanism for the observed hierarchy (Postma *et al*., 1993). In the case of control of BglG activity, competition of BglG with the various IIA domains of EIIs for phospho-HPr may determine the level of *bgl* operon expression and thus the β-glucoside transport capacity of the cell. This represents a novel type of control of gene expression and of carbon catabolite repression in *E.coli*.

BglG is the prototype of a family of related regulators all concerned with positive control of catabolic operons encoding proteins that function in uptake and utilization of PTS sugars. Most of these proteins act as antiterminators, but transcriptional activators are also found (for reviews see Rutberg, 1997; Stülke *et al*., 1998). They all share two conserved, homologous domains that function as the targets of PTS-mediated regulation and are thus called PTS regulation domains (PRD-1 and PRD-2; Tortosa *et al*., 1997; Stülke *et al*., 1998). Within the PRDs three highly conserved histidine residues are believed to be potential targets of PTS-mediated phosphorylation. Within BglG, these residues are H101 and H160, located in the PRD-1 domain, and H208 located in the PRD-2 domain (Tortosa *et al*., 1997; Stülke *et al*., 1998). Upon examination it has become clear that PRD-containing regulators are negatively regulated by their respective EIIs. For BglG as well as the homologous antiterminators, SacY, SacT and GlcT from *Bacillus subtilis*, mutants have been isolated which exhibit constitutive activity. Most of these mutations changed the first conserved histidine in PRD-1 or residues around that position (Mahadevan *et al*., 1987; Crutz *et al*., 1990; Debarbouille *et al*., 1990; Chen *et al*., 1997b; Bachem and Stülke, 1998). Thus, it has been proposed that in these antiterminators PRD-1 may be the target of negative regulation exerted by the respective sugar permeases (Stülke *et al*., 1998). In addition, it has been reported that activity of some of these PRD-containing regulators, i.e. antiterminators SacT and LicT and the activator LevR from *B.subtilis*, are dependent on the presence of EI and HPr (for an overview see Stülke *et al*., 1998). In fact, HPr-dependent phosphorylation of these proteins has been demonstrated *in vitro* (Stülke *et al*., 1995; Arnaud *et al*., 1996; Deutscher *et al*., 1997). The data presented here do not exclude the possibility that an unknown enzyme mediates the HPr-dependent,

Table I. Strains and plasmids used in this study

Strain/plasmid	Relevant genotype or structures	Source or reference
Strains		
TP2811	(= R1330) *F⁻ xyl argH1 ΔlacX74 aroB ilvA Δ(ptsH ptsI crr)::neo*	Levy *et al*. (1990)
W3110	(= R1128) *F⁻* λ⁻ IN(*rrnD-rrnE*)1	Bachmann (1972)
R1279	*Δ(pho-bgl)201 Δ(lac-pro) ara thi rpsL*	Schnetz *et al*. (1996)
R1653	as R1279 but *Δ(ptsH ptsI crr)::neo*	R1279×T4(TP2811); Krieg (1993)
R1752	as R1279 but *attB::bla Ptac bglG bglF*	R1279×pFDX3410; this work
R2013	as R1752 but *Δ(ptsH ptsI crr)::neo*	R1752×T4(TP2811); this work
R1958	as R1279 but *attB::bla Ptac bglG*	R1279×pFDX3157; this work
R2051	as R1958 but *Δ(ptsH ptsI crr)::neo*	R1958×T4(TP2811); this work
R1967	as R1279 but *Δ(fruB fruK fruA)*	R1279×pFDX3212; this work
R1971	as R1967 but *attB:: bla Ptac bglG bglF*	R1967×pFDX3410; this work
R1977	as R1971 but *Δ(ptsH ptsI crr)::neo*	R1971×T4(TP2811); this work
R1974	as R1967 but *attB::bla Ptac bglG*	R1967×pFDX3157; this work
R1979	as R1974 but *Δ(ptsH ptsI crr)::neo*	R1974×T4(TP2811); this work
Plasmids		
pFDY226	*lacI^q P_16 bglt2 lacZ* (*bla*; *oriR* pBR322)	Schnetz *et al*. (1996)
pFDY225	as pFDY226 but lacking the *bglt2*	Schnetz *et al*. (1996)
pFDX2676	as pFDY226 but *bla* replaced by *tet*	Krieg (1993)
pFDX3158	as pFDY226 but *bla* replaced by *cat*	this work
pFDX2469	*bglG* under control of *Ptac* (*tet*; *oriR* pACYC177)	this work
pFDX3549	as pFDX2469 but *bglG* replaced by *lacZ*	this work
pLDR8	*int* under control of λP_R, λcI_{857} (*neo*; *oriR* pSC101; rep^{TS})	Diederich *et al*. (1992)
pFDX3401	as pLDR8 but *neo* replaced by *cat*	this work
pLDR10	multiple cloning site; λ*attP* (*bla*; *cat*; *oriR* pBR322)	Diederich *et al*. (1992)
pFDX3410	pLDR10 carrying *Ptac bglG bglF* within multiple cloning site	this work
pFDX3157	pLDR10 carrying *Ptac bglG* within multiple cloning site	this work
pFDX3155	*ptsH ptsI* under control of *Ptac* (*cat*; *oriR* pACYC177)	this work
pFDX3160	*ptsH* under control of *Ptac* (*tet*; *oriR* pACYC177)	this work
pFDX3161	*ptsI* under control of *Ptac* (*tet*; *oriR* pACYC177)	this work
pFDX3212	flanking regions of *fru* chromosomal region (1kb resp.) (*tet*; *oriR* pSC101; rep^{TS})	this work
pFDX3223	*ptsHH15A ptsI* under control of *Ptac* (*tet*; *oriR* pACYC177)	this work
pFDX3221	*fruB ptsI* under control of *Ptac* (*tet*; *oriR* pACYC177)	this work
pFDX3102	*Ptac bglG bglF*; *SD* sequence of phage *T7gene10* preceding *bglG* (*tet*; *oriR* pACYC177)	this work
pFDX2942	as pFDX3102 but *bglF* removed	this work
pFDY45	*lacI^q Ptac bglG*; *SD T7gene10* preceding *bglG* (*bla*; *oriR* pBR322)	Schnetz and Rak (1990)
pFDX3225	as pFDX2942 but *galKΦbglG*	this work
pFDX3226	as pFDX3102 but *galKΦbglG*	this work
pFDX3293	as pFDX3102 but *bglF* replaced by *nagE*	this work
pFDX3295	as pFDX3102 but *bglF* replaced by *mtlA*	this work
pFDX3283	as pFDY226 but additionally carrying *bglF* under control of *Ptac*	this work
pFDX3297	*nagE* under control of *Ptac* (*neo*; *oriR* pACYC177)	this work
pFDX3298	*mtlA* under control of *Ptac* (*neo*; *oriR* pACYC177)	this work
pFDX3299	*bglF* under control of *Ptac* (*neo*; *oriR* pACYC177)	this work

EII^{Bgl}-independent phosphorylation described here, which in any case stimulates BglG activity. However, preliminary phosphorylation experiments with purified His-tagged BglG protein and either purified EI and HPr from *B.subtilis* or EI from *B.subtilis* and HPr from *E.coli* indicated that BglG can indeed be phosphorylated by HPr *in vitro* (C.Lindner, B.Görke, B.Rak and J.Deutscher, unpublished). It should be noted, however, that in another *in vitro* set up containing PEP, EI and HPr, and partly whole cell extracts, phosphorylation of BglG was only seen when membrane fractions containing EII^{Bgl} were also present (Chen *et al*., 1997a,b; Chen and Amster-Choder, 1998). The discrepancies between these results and our own remain to be clarified.

For antiterminators LicT and SacT, the conserved histidine residue in the PRD-2 domain (H208 in BglG) was proposed to be the HPr-dependent phosphorylation site (Stülke *et al*., 1998). Thus, these antiterminators might be negatively regulated by EII-catalyzed phosphorylation at PRD-1 and in addition positively controlled by HPr-dependent phosphorylation at PRD-2. In this report we provide evidence that BglG might be controlled by dual PTS-catalyzed phosphorylation in a similar way. Recently it was demonstrated that BglG can be phosphorylated at H208 *in vitro* and it has been suggested that this site may be the only one phosphorylated (Chen *et al*., 1997b). Since in this study, factor Xa cleavage sites were introduced into the PRD-1 domain of BglG, which possibly destroyed its function, it might not be surprising that phosphorylation of the PRD-1 was not detected. Furthermore, our own experiments showed that BglG-H208 mutant proteins were still phosphorylated *in vivo* (Krieg, 1993; B.Görke and B.Rak, unpublished), suggesting that there are at least two phosphorylation sites in BglG. Undoubtedly further studies will be necessary to identify the phosphorylation sites on BglG and to clarify the apparently conflicting findings.

Materials and methods

Plasmids and bacterial strains

The relevant structures of plasmids and the genotypes of strains are given in Table I. Plasmids were constructed using standard recombinant techniques (Sambrook *et al*., 1989). Details on their construction together with their completely compiled and annotated sequences can be obtained from the

Web under http://www-rak.biologie.uni-freiburg.de/supplements.htm. For PCR amplifications and subsequent cloning, chromosomal DNA of strain W3110 was used. The *fru* operon (*fruB*, *fruK*, *fruA*) was deleted according to Hamilton *et al*. (1989) as follows: strain R1279 was transformed with temperature-sensitive plasmid pFDX3212. Transformants were plated at non-permissive temperature (42°C) on LB plates containing tetracycline to select crossover events between the cloned *fru* border sequences in pFDX3212 and the corresponding chromosomal regions. Colonies were subsequently inoculated into LB broth at 30°C to allow resolution of the cointegrates by a second recombination event which could either result in deletion of the *fru* genes or in reversion. Deletion derivatives were obtained by screening of individual colonies for tetracycline sensitivity and a fructose-negative phenotype. Correct deletion in the resulting strain, R1967, was verified by PCR. Integration of the *tacOP–bglG* and *tacOP–bglG–bglF* expression cassettes into the chromosomes of strains R1279 and its isogenic *fru* operon deletion derivative R1967 was achieved by site-specific recombination into the bacteriophage λ attachment site, *attB*, using the integration system provided by Diederich *et al*. (1992). Vector moieties of plasmids pFDX3157 and pFDX3410 were removed by *Not*I digestion and the remaining DNA molecules were ligated, generating circular DNA molecules which carry the cassette to be integrated together with the *attP* sequence and the *bla* gene as a selection marker. The DNAs were used to transform strains R1279 and R1967 harboring the thermosensitive integrase expression plasmid pFDX3401. Subsequently, recombinants carrying the cassettes in the chromosome were selected at 42°C on ampicillin-containing plates (20 μg/ml) resulting in R1279-derived strains R1958 and R1752, and in R1967-derived strains R1974 and R1971, each carrying the *tacOP–bglG* and *tacOP–bglG–bglF* cassettes, respectively. Deletion of the *pts* operon was crossed into strains by T4GT7 transduction (Wilson *et al*., 1979) of the *neo* gene (kanamycin resistance) from strain TP2811.

Growth conditions

LB (Miller, 1972) was used as standard medium. Where necessary, antibiotics were added to final concentrations of 75 μg/ml (ampicillin), 12.5 μg/ml (tetracycline), 15 μg/ml (chloramphenicol) and 30 μg/ml (kanamycin) if not otherwise indicated.

Determination of β-galactosidase activity

β-galactosidase assays were carried out as described previously (Schnetz *et al*., 1996). Cells were grown in synthetic medium (M9) containing 1% (w/v) carbon source (glycerol, if not otherwise indicated), proline (20 μg/ml), thiamine (1 μg/ml), casamino acids [0.66% (w/v)] and the appropriate antibiotics according to the plasmids carried by the different transformants. In general, 1 mM IPTG was added as inducer for *tacOP* where indicated. Where appropriate, D-salicin (7 mM) as substrate for EII^{Bgl}, *N*-acetyl-D-glucosamine [0.5% (w/v)] as substrate for EII^{Nag}, and D-mannitol [0.5% (w/v)] as substrate for EII^{Mtl} were additionally added. Activities were derived from the average of three to five independent assays from at least two independent transformants each. Deviations were <20%.

In vivo protein phosphorylation

Cells were labeled with $H_3[^{32}P]O_4$ according to the procedure described previously (Schnetz and Rak, 1990) but with the following modifications. Overnight cultures grown in LB containing the appropriate antibiotics were diluted to an OD_{600} of 0.15 in the same medium and grown at 37°C to an OD_{600} of 0.5–0.8. After addition of IPTG (0.1 mM), D-salicin (7 mM), *N*-acetyl-D-glucosamine or D-mannitol [0.5% (w/v)] where indicated, growth was continued for 20 min. Cells were collected by centrifugation and resuspended in phosphate-free Tris medium containing 1% succinate, 1% glycerol, proline (20 μg/ml), thiamine (1 μg/ml), the appropriate antibiotics as well as the necessary inducers and sugars where indicated. After 1 h shaking at 37°C cells were collected by centrifugation and resuspended to an OD_{600} of 0.5 in the same medium. Of these suspensions 50 μl were labeled with $H_3[^{32}P]O_4$ as described before. SDS–PAGE and subsequent handling of the polyacrylamide gels were also as described before with the difference that proteins were separated on 13.5% gels.

In vivo labeling of proteins with [^{35}S]methionine

Overnight cultures grown in M9 supplemented with Difco 2× methionine assay [1% (v/v)], proline (20 μg/ml), thiamine (1 μg/ml), glycerol [1% (w/v)] and the appropriate antibiotics were diluted to an OD_{600} of 0.1 in the same medium and grown at 37°C to an OD_{600} of 0.5–0.8. Cells were harvested by centrifugation and resuspended to an OD_{600} of 0.5 in the same medium. To 100 μl of these suspensions 20 μl Difco 2× methionine assay, 60 μl M9 containing the necessary inducers and sugars [final concentrations: IPTG, 0.1 mM; D-salicin, 7 mM; *N*-acetyl-D-glucosamine or D-mannitol, 0.5% (w/v)] were added. After pre-incubation at 37°C for 5 min 20 μl M9 containing 20 μCi [^{35}S]methionine were added and further incubated for 2 min. Labeling was terminated by addition of unlabeled methionine (1 mg/ml). After a chase time of 2.5 min cells were collected by centrifugation and after lysis 0.01 OD_{600} cells were subjected to SDS–PAGE. Quantitation of radiolabeled bands was carried out by using the bio imaging analyzer FujiXBAS1000 (Fuji Photo Film) and the software PCBAS Tina 2.07c (Raytest).

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