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Publication date
1997

Published in
EMBO Journal

The EMBO Journal Vol.16 No.15 pp.4617–4627, 1997

BglF, the sensor of the *E.coli bgl* system, uses the same site to phosphorylate both a sugar and a regulatory protein

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The *Escherichia coli* BglF protein is a sugar permease that is a member of the phosphoenolpyruvate-dependent phosphotransferase system (PTS). It catalyses transport and phosphorylation of β-glucosides. In addition to its ability to phosphorylate its sugar substrate, BglF has the unusual ability to phosphorylate and dephosphorylate the transcriptional regulator BglG according to β-glucoside availability. By controlling the phosphorylation state of BglG, BglF controls the dimeric state of BglG and thus its ability to bind RNA and antiterminate transcription of the *bgl* operon. BglF has two phosphorylation sites. The first site accepts a phosphoryl group from the PTS protein HPr; the phosphoryl group is then transferred to the second phosphorylation site, which can deliver it to the sugar. We provide both *in vitro* and *in vivo* evidence that the same phosphorylation site on BglF, the second one, is in charge not only of sugar phosphorylation but also of BglG phosphorylation. Possible mechanisms that ensure correct phosphoryl delivery to the right entity, sugar or protein, depending on environmental conditions, are discussed.

Keywords: *bgl* system/β-glucosides/phosphorylation sites/protein phosphorylation/PTS

Introduction

The *Escherichia coli* BglF protein, which is a member of the phosphoenolpyruvate-dependent phosphotransferase system (PTS), catalyses concomitant transport and phosphorylation of β-glucosides (Fox and Wilson, 1968). In addition to its role in sugar transport, BglF functions as a negative regulator of *bgl* operon expression (Mahadevan *et al.*, 1987). This property of the enzyme is due to its unprecedented ability to phosphorylate not only its sugar substrate but also a protein essential for operon expression, BglG, a property not yet demonstrated for any other protein (Amster-Choder *et al.*, 1989). BglG is an RNA-binding protein which controls *bgl* operon expression by transcriptional antitermination (Mahadevan and Wright, 1987; Schnetz and Rak, 1988; Houman *et al.*, 1990). The ability of BglG to override termination of transcription depends on its phosphorylation state; BglF phosphorylates and dephosphorylates it depending on β-glucoside availability (Amster-Choder *et al.*, 1989; Amster-Choder and Wright, 1990; Schnetz and Rak, 1990). It was further shown that the reversible phosphorylation of BglG regulates its activity by modulating its dimeric state (Amster-Choder and Wright, 1992). Thus, in the absence of β-glucosides, BglF phosphorylates BglG; BglG~P is a monomer that cannot bind to its target RNA site and is inactive as a transcriptional antiterminator. In the presence of β-glucosides, BglF dephosphorylates BglG~P, allowing it to dimerize and function as a positive regulator of operon expression.

The phosphoryl flux in PTS starts with a phosphoryl group donated by phosphoenolpyruvate (PEP) which is passed down a phosphoryl transfer chain which consists of Enzyme I (EI) and HPr, which are not sugar specific, and Enzyme IIs (EIIs), which are specific for different sugars. The EIIs are composed of at least three well-recognized functional domains whose order is not conserved: IIA is a hydrophilic domain that possesses the first phosphorylation site, a conserved histidine which can be phosphorylated by P-HPr; IIB is a second hydrophilic domain that possesses the second phosphorylation site, usually a conserved cysteine that can be phosphorylated by P-IIA; and IIC is a hydrophobic domain which includes 6–8 transmembrane helices that presumably form the sugar translocation channel and at least part of its binding site (reviewed in Saier and Reizer, 1992; Postma *et al.*, 1993). BglF is the PTS EII for β-glucosides and is also designated II^{bgl}. Based on sequence comparisons with other PTS EIIs, several conserved residues in BglF were suggested to play a role in the transfer of the phosphoryl group from HPr to BglF and from BglF to the sugar. These residues were subjected to site-directed mutagenesis and the phosphorylation performance of the respective mutants was studied (Schnetz *et al.*, 1990). His547 (H547), located in the IIA domain, was identified as the first phosphorylation site (site 1), which is phosphorylated by HPr and transfers the phosphoryl group to the second phosphorylation site (site 2). Two residues were candidates for the second phosphorylation site, Cys24 (C24) and His306 (H306), both shown to be essential for the transfer of the phosphoryl group to the sugar. Based on the vast amount of information available on phosphorylation sites in related permeases (Postma *et al.*, 1993, and references therein), it was logical to assume that C24, which is located in the IIB domain, and not H306, located in the IIC domain, is the residue which accepts the phosphoryl group from H547 and transfers it on to the sugar, while H306, located in the IIC domain, is involved in transporting the sugar. *In vitro* phosphorylation experiments with distinct domains have shown unambiguously that the second phosphorylation site resides in the IIB domain of BglF and not in the IIC domain (Q.Chen and O.Amster-Choder, unpublished data).

Which site on BglF phosphorylates the transcriptional

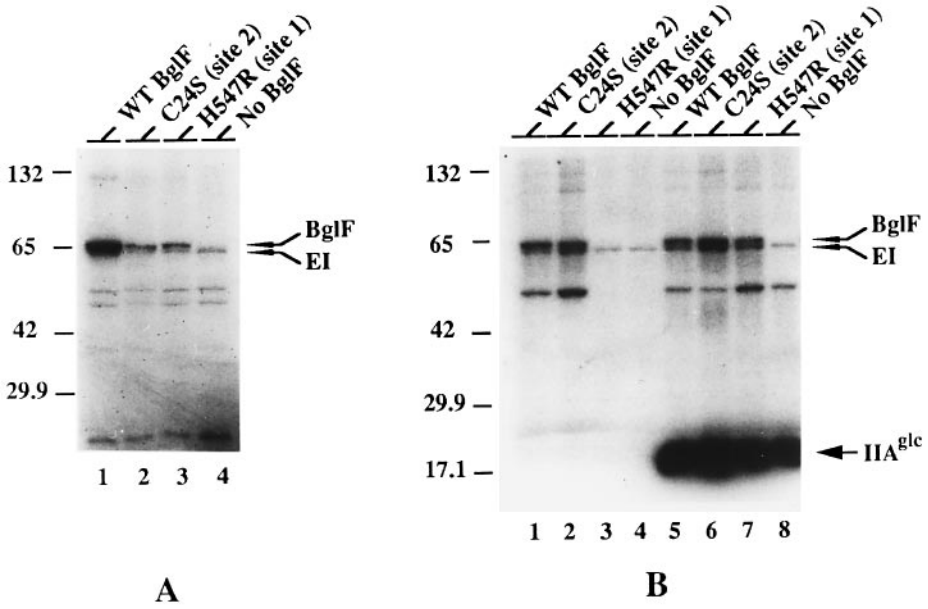

Fig. 1. Phosphorylation of BglF mutated in either one of its phosphorylation sites. (**A**) Membranes of cells that overproduce the various BglF derivatives were incubated with [^{32}P]PEP and a soluble protein extract prepared from the *Salmonella typhimurium* LJ144, which is enriched for EI, HPr and IIA^{glc} for 10 min (phosphorylation system A). (**B**) The various BglF derivatives were overproduced in LM1, a *crr* and *nagE E.coli* strain. Membranes were incubated with [^{32}P]PEP and purified EI and HPr (phosphorylation system B) for 10 min without (lane 1–4) or with (lanes 5–8) IIA^{glc}. H547R and C24S: mutations in the first and second phosphorylation sites of BglF ('site 1' and 'site 2') respectively. No BglF: membranes from cells which do not overproduce BglF, but are otherwise identical to the other membrane preparations used in each experiment, were included in the phosphorylation systems described above. Samples were analysed by SDS–PAGE followed by autoradiography. Molecular masses of protein standards are given in kilodaltons. Arrowheads indicate the positions of BglF, EI and IIA^{glc}.

regulator BglG? The basis for the ability of a protein to phosphorylate such different entities as a carbohydrate and a protein is unknown. Knowledge of whether a single phosphorylation site performs both transfer reactions or whether two different sites are involved, one for each reaction, is crucial for elucidating the relationship between recognition and phosphorylation. It was suggested previously that each of the two phosphorylation sites on BglF is in charge of a different phosphorylation function (Schnetz and Rak 1990), i.e. the site on IIA^{bgl} phosphorylates BglG and the site on IIB^{bgl} phosphorylates the sugar. These authors also suggested that IIA^{glc}, which is homologous to the IIA^{bgl} domain (Bramley and Kornberg, 1987) and was shown to complement BglF mutated in site 1 (Schnetz *et al.*, 1990), can transfer phosphoryl groups not only to site 2 of BglF but also to BglG. However, the observation that no [^{32}P]BglG was detected when non-phosphorylated BglG was incubated with [^{32}P]PEP and a soluble fraction of a *Salmonella typhimurium* strain overproducing EI, HPr and IIA^{glc} (Amster-Choder *et al.*, 1989) did not support transfer from IIA^{glc} to BglG. Here we provide both *in vivo* and *in vitro* evidence that the site on BglF which transfers a phosphoryl group to β-glucosides, site 2, is the same one that is used for transfer of a phosphoryl group to BglG. Thus, the phosphoryl group is transferred from site 1 to site 2 and then to either the sugar or to BglG. Therefore, not only is BglF unique in its ability to phosphorylate both a sugar and a regulatory protein, but, more interestingly, the phosphoryl group is donated to these totally different entities by the same site. Possible mechanisms that ensure correct phosphoryl delivery to the right entity, depending on environmental conditions, are discussed.

Results

To test which site(s) on BglF are involved in transfer of a phosphoryl group to β-glucosides and BglG, we mutated each of the two phosphorylation sites on BglF. His547 was mutated to an arginine (H547R), and Cys24 was mutated to a serine (C24S) (see Materials and methods). We then followed the ability of the mutant proteins to be phosphorylated and to donate the phosphoryl group to β-glucosides and to BglG *in vitro* on one hand, and to mediate β-glucoside utilization and to modulate BglG activity *in vivo* on the other hand.

Phosphorylation of wild-type and mutant BglF proteins

Membranes containing wild-type BglF, or BglF mutated in either one of its phosphorylation sites (C24S or H547R), were incubated in the *in vitro* phosphorylation system described previously (Amster-Choder *et al.*, 1989). The system, which will be referred to as system A, is crude and contains [^{32}P]PEP, a cytoplasmic extract prepared from the mutant strain of *S.typhimurium* LJ144 which expresses increased amounts of EI, HPr and IIA^{glc} (Saier and Feucht, 1975), and membranes prepared from *E.coli* K38 cells expressing the *bglF* alleles under the control of phage T7 promoter. All three BglF derivatives were detected by autoradiography following SDS–PAGE (Figure 1A, lanes 1–3). This polypeptide could not be detected when membranes of cells containing a similar plasmid which lacks the *bglF* gene were included in this *in vitro* system (Figure 1A, lane 4).

Subsequently, we have expressed the three *bglF* alleles (wild-type and the two mutants) in *E.coli* LM1, a strain

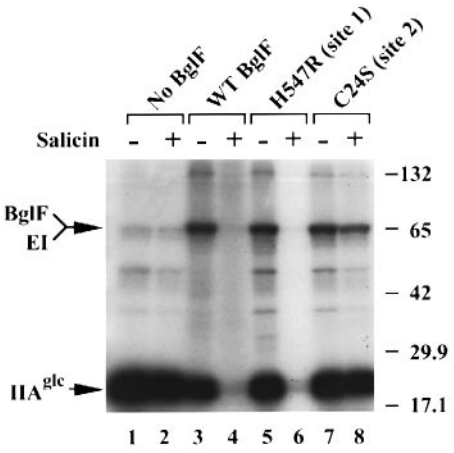

Fig. 2. BglF mutated in site 1, but not in site 2, is dephosphorylated by β-glucosides. The various BglF derivatives (wild-type, H547R and C24S) were overproduced in ZSC112ΔG, a *pstG* strain. Membranes were incubated with [^{32}P]PEP, purified EI, HPr and IIA^{glc}. The mixtures were incubated further with (+) or without (–) 0.2% salicin for 5 min. Samples were analysed by SDS–PAGE followed by autoradiography. Molecular masses of protein standards are given in kilodaltons. Arrowheads indicate the position of BglF, EI and IIA^{glc}.

deleted for the *crr* and *nagE* genes (and thus not expressing the IIA^{glc} and II^{nag} proteins which can substitute for IIA^{bgl}). The overproduction of the three BglF derivatives in this strain was demonstrated by metabolic labelling with [^{35}S]methionine (data not shown). Membranes prepared from LM1 producing the different BglF derivatives were incubated with [^{32}P]PEP and purified EI and HPr (referred to as system B). Phosphorylated proteins were detected by autoradiography following SDS–PAGE, and the results, presented in Figure 1B (lanes 1–3), demonstrate that the site 2 mutant (C24S) behaved like wild-type BglF, while a mutation in site 1 (H547R) abolished the ability of BglF to be phosphorylated by HPr. The control reaction contained membranes of LM1 bearing a similar plasmid lacking the *bglF* gene (Figure 1B, lane 4). Addition of purified IIA^{glc} restored site 1 mutant phosphorylation (compare lanes 3 and 7 in Figure 1B) while it did not affect phosphorylation of wild-type BglF and the site 2 mutant (Figure 1B, lanes 5 and 6 versus lanes 1 and 2). Thus BglF mutants are behaving as expected in the two *in vitro* systems utilized by us; BglF mutated in site 1 cannot accept a phosphoryl group from HPr, but this mutation can be complemented by IIA^{glc}, while a mutation in site 2 does not interfere with BglF phosphorylation by HPr. This is in agreement with the previously suggested heterologous phosphoryl transfer from IIA^{glc} to site 2 of BglF (Schnetz *et al.*, 1990).

Dephosphorylation of wild-type and mutant BglF proteins by β-glucosides

All of the published evidence to date suggests that the second phosphorylation site on BglF is the one involved in transferring the phosphoryl group to the sugar substrate. We have tested the ability of our mutant BglF proteins, pre-labelled by incubation with [^{32}P]PEP, and purified EI and HPr to donate a phosphoryl group to the β-glucoside salicin. As seen in Figure 2, the site 1 mutant protein (H547R), once phosphorylated by IIA^{glc}, behaves like wild-type BglF and is completely dephosphorylated upon addition of salicin (compare lane 3 with lane 4 and lane 5 with lane 6, for the wild-type and site 1 mutant, respectively). The phosphorylated site 2 mutant protein (C24S), on the other hand, is not chased by salicin (Figure 2, lanes 7 and 8).

IICB^{glc}, which is present in our membrane preparations in a significant amount (as demonstrated by Western blot analysis using monoclonal antibodies raised against this protein, data not shown), was reported to be phosphorylated by the IIA domain of BglF (Vogler *et al.*, 1988; Schnetz *et al.*, 1990). It can therefore lead to some dephosphorylation of BglF, which is independent of β-glucosides, due to the the presence of residual glucose contamination, detected occasionally in commercial salicin. To avoid this complication, the membranes containing the various BglF derivatives were prepared from strain ZSC112ΔG, which is mutated in the *ptsG* gene encoding IICB^{glc}. This strain expresses the *crr* gene at a relatively lower level (P.W.Postma, unpublished data). Therefore, to ensure phosphorylation of site 2 of the H547R mutant, IIA^{glc} was included in the phosphorylation reaction. Our conclusion from the results presented in this section is that our mutants behave as expected with regard to sugar phosphorylation (i.e. only the one that contains an intact second phosphorylation site can transfer the phosphoryl group to the sugar) and should thus serve as a reliable tool to study phosphorylation reactions catalysed by BglF.

Phosphorylation of BglG by wild-type and mutant BglF proteins

We have shown before that BglF, phosphorylated *in vitro*, can transfer a phosphoryl group to BglG (Amster-Choder *et al.*, 1989). The physiological significance of this result was demonstrated by the correlation between the behaviour of BglG mutants *in vivo* and their phosphorylation behaviour *in vitro*. BglF-dependent phosphorylation of BglG was also demonstrated *in vivo* (Amster-Choder and Wright, 1990). Thus the ability of BglG to be phosphorylated *in vitro* is an excellent indication for the *in vivo* situation.

We have tested the effect of the mutations in the phosphorylation sites of BglF on its ability to phosphorylate BglG. An extract of cells overproducing BglG (see Materials and methods) was added to mixtures containing the different BglF variants that had been pre-labelled for 10 min in system A. As was shown in Figure 1A, all three BglF variants examined (wild-type and mutants) are phosphorylated in this system (the site 1 mutant is labelled due to the presence of IIA^{glc} in this phosphorylation system, see above) and we could thus test their ability to transfer the phosphoryl group to BglG. The results, presented in Figure 3, demonstrate that a mutation in the first phosphorylation site of BglF does not prevent this protein from phosphorylating BglG. Some phosphorylation of BglG was detected 1 min after the addition of the BglG-containing extract, and the amount of phosphorylated BglG increased with time of incubation (Figure 3, lanes 9–12). The slight difference from the phosphorylation pattern of BglG by wild-type BglF (Figure 3, lanes 1–4) can be explained by the fact that H547R is labelled by IIA^{glc} and the phosphoryl flow is expected to be less efficient in this heterologous system than in the wild-type

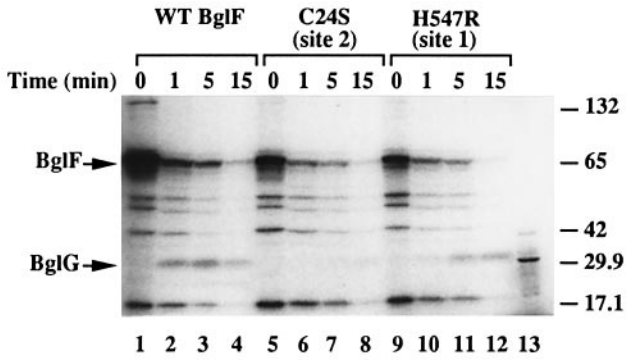

Fig. 3. BglF mutated in site 1, but not in site 2, phosphorylates BglG. Membranes containing the various BglF derivatives (wild-type, C24S and H547R) were labelled in phosphorylation system A, as described in Figure 1A. Extract of cells that overproduce BglG was added, and incubation was continued for the times indicated. Samples were analysed by SDS–PAGE followed by autoradiography. Lane 13 contains a ^{35}S-labelled sample of BglG. Molecular masses of protein standards are given in kilodaltons. Arrowheads indicate the positions of BglF and BglG.

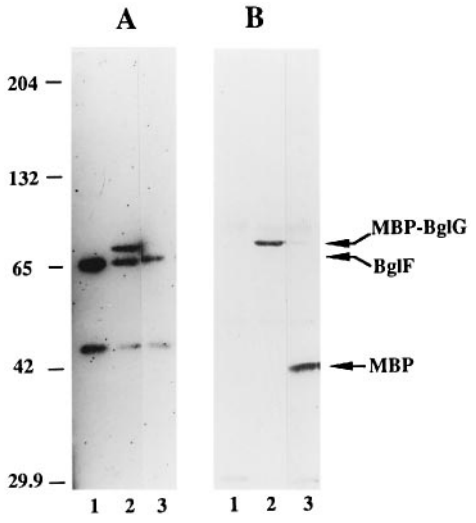

Fig. 4. BglF recognizes and phosphorylates BglG fused to MBP. BglF was labelled in phosphorylation system B (lanes 1), then further incubated for 15 min in the presence of either MBP–BglG (lanes 2) or MBP (lanes 3). Proteins were fractionated on a 5–12.5% SDS–polyacrylamide gradient gel and then blotted onto a nitrocellulose filter. The blot was probed with anti-MBP antibodies and analysed by autoradiography. (**A**) Autoradiography. (**B**) Western blot analysis. Molecular masses of protein standards are given in kilodaltons. Arrowheads indicate the positions of BglF, MBP–BglG and MBP. EI co-migrates with BglF in this gel system (see Figure 5).

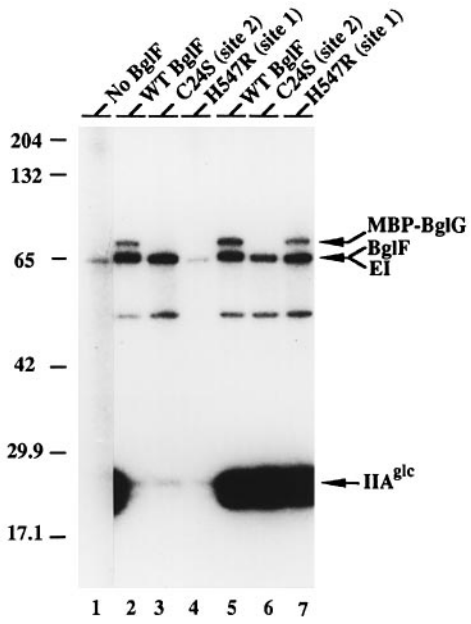

Fig. 5. BglF mutated in site 1, but not in site 2, phosphorylates MBP–BglG. The various BglF derivatives (wild-type, C24S and H547R) were labelled in phosphorylation system B in the absence (lanes 2–4) or presence (lanes 5–7) of IIA^{glc}. The mixtures were incubated further in the presence of MBP–BglG for 15 min. Proteins were fractionated on a 5–12.5% SDS–polyacrylamide gradient gel followed by autoradiography. Lane 1 contains a control with membranes from cells that do not overproduce BglF that were labelled in phosphorylation system B; it demonstrates that phosphorylated EI co-migrates with BglF in this gel system. Molecular masses of protein standards are given in kilodaltons. Arrowheads indicate the positions of MBP–BglG, BglF, EI and IIA^{glc}.

situation which involves intramolecular transfer of the phosphoryl group. In contrast to the behaviour of the site 1 mutant, no phosphorylation of BglG occurred with BglF mutated in site 2, even after incubating the labelled C24S with the BglG-containing extract for 15 min (Figure 3, lanes 5–8). Longer periods of incubation gave the same result (data not shown).

To assay for BglG phosphorylation by BglF in a purified system, and in light of the difficulty in purifying BglG due to its irreversible precipitation in inclusion bodies upon overproduction (A.Wright, unpublished data), we decided to measure phosphorylation of the fusion protein MBP–BglG (BglG fused to maltose-binding protein) which is soluble and can be purified on an amylose column (see Materials and methods). We first demonstrated that this fusion protein can be phosphorylated by wild-type BglF *in vitro* (Figure 4A, lane 2). To ensure that it is the BglG, and not the MBP moiety, that is phosphorylated by BglF, we incubated purified MBP with pre-labelled BglF and demonstrated that MBP, though present in the reaction in an amount which is equimolar to that of MBP–BglG (see Figure 4B for Western blot analysis), is not phosphorylated by BglF (Figure 4A, lane 3). We subsequently added purified MBP–BglG to the BglF variants that had been pre-labelled in system B. The results, presented in Figure 5, demonstrate that MBP–BglG can be phosphorylated by the site 1 mutant which was labelled in a reaction supplemented with IIA^{glc} (lane 7), but not by the site 2 mutant (lanes 3 and 6). No phosphorylation of MBP–BglG could be detected when it was added to membranes of cells that do not produce BglF, which were pre-labelled in phosphorylation system B (Figure 5, lane 1). Thus, phosphorylated EI and HPr cannot phosphorylate BglG.

Taken together, these results show conclusively that the second phosphorylation site in BglF (C24), and not the first, is in charge of delivering the phosphoryl group to BglG. These results also rule out the possibility raised before (Schnetz and Rak, 1990) that phosphorylated IIA^{glc} can deliver the phosphoryl group to BglG (see Figure 5, lane 6).

Table I. Plasmid-encoded BglF mutated in site 1, but not in site 2, can complement *bglF* strains and enable β-glucoside utilization

Plasmid	Plasmid-encoded BglF derivative	Complementation of *bglF* mutant strains[a]				
		MA231	AE304-1	AE304-2	AE304-4	PPA543 (IIA^{glc-}, II^{nag-})[b]
pBR322	−	−	−	−	−	−
pMN5	wild-type	+	+	+	+	+
pCQ-F	wild-type	+	+	+	+	+
pCQ-F1	H547R	+	+[c]	+[c]	+[c]	−
pCQ-F2	C24S	−	−	−	−	−

[a]Complementation was indicated by two alternative methods: (+) growth on minimal arbutin plates and red colonies on MacConkey arbutin plates; (−) no growth on minimal arbutin plates and white colonies on MacConkey arbutin plates. Complementation of strain MA231 was assayed only on MacConkey arbutin plates.
[b]The *crr* and *nag* genes of strain PPA543 were mutated (see Materials and methods). This strain is thus deficient for the IIA^{glc} and II^{nag} proteins. Strains PPA546 and PPA547 that also carry mutations in these genes behaved as PPA543 (not shown).
[c]The colour on MacConkey arbutin plates was pale red but the number of colonies on minimal arbutin plates was the same as for other plasmids.

β-glucoside phosphotransfer mediated by wild-type and mutant BglF proteins

Next we decided to substantiate our *in vitro* results regarding BglF-dependent BglG phosphorylation by *in vivo* studies. We first verified that our mutants behave as expected with regard to β-glucoside utilization.

To analyse the ability of the various BglF derivatives to transfer β-glucosides into the cell while phosphorylating them, we used strains defective in the *bglF* gene, and carried out complementation analyses with a series of plasmids encoding BglF derivatives: pMN5 and pCQ-F encode wild-type BglF; pCQ-F1 and pCQ-F2 encode BglF mutated in the first and second phosphorylation sites (H547R and C24S), respectively. Positive complementation of the chromosomal mutation in the *bglF* gene by the plasmid-encoded alleles was indicated both by growth on minimal medium containing arbutin as the sole carbon source and by the formation of red colonies on MacConkey arbutin plates. Utilization of the β-glucoside arbutin depends on the ability of the plasmid-encoded BglF derivatives to phosphorylate and transport this sugar which is then cleaved by the product of the unlinked locus *bglA*. Utilization of the β-glucoside salicin is prohibited in these strains due to the polarity of the mutation in the chromosomal *bglF* gene on the adjacent *bglB* gene, whose product preferentially cleaves phosphosalicin (Mahadevan *et al.*, 1987). We used several *bglF* strains which are wild-type for *crr* and *nagE* (Mahadevan *et al.*, 1987), and also isogenic strains defective in the *crr* and *nagE* genes which we have constructed (see Materials and methods). The results are presented in Table I. While the control wild-type *bglF* plasmids (pMN5 and pCQ-F) complemented all the *bglF* strains to Arb^+ (growth on minimal arbutin and red colonies on MacConkey arbutin), a mutation in site 2 abolished the ability of the plasmid-encoded BglF to complement any of these strains (no growth on minimal arbutin and white colonies on MacConkey arbutin in all strains containing pCQ-F2). The site 1 mutant (encoded by pCQ-F1) showed no complementation in *bglF* strains defective in the *crr* gene. However, *bglF* strains carrying the wild-type *crr* gene were complemented by the site 1 mutant and grew on minimal arbutin. They also led to the formation of red colonies on MacConkey arbutin, though paler in some cases than the same strains containing a plasmid which encodes wild-type BglF.

Thus, β-glucoside utilization can be restored in *bglF* strains by a plasmid-encoded BglF mutated in the first phosphorylation site, provided that the strain produces IIA^{glc}. The slight difference between the effect of the wild-type BglF and the site 1 mutant, observed with some strains (all originating from the same parental strain) in one of the complementation tests, i.e. colour on MacConkey arbutin, can be explained by the more efficient phosphoryl transfer from site 1 to site 2 when both sites are present on the same molecule than in the heterologous system (which necessitates phosphoryl flow from IIA^{glc} to site 2 of BglF). The other test, growth on minimal arbutin, is not sensitive to this difference. Also, strain MA231, which gives bright red colonies on MacConkey arbutin when transformed with pCQ-F1, might have a slighty higher level of IIA^{glc} which compensates for the intramolecular phosphoryl transfer.

Based on the results presented in this section, it can be concluded that our mutants behave as expected with regard to phosphotransfer of β-glucosides into the bacterial cell.

The effect of wild-type and mutant BglF proteins on BglG activity as a transcriptional antiterminator

BglF was shown before to exert its negative effect on operon expression by phosphorylating BglG, blocking its action as an antiterminator (Amster-Choder *et al.*, 1989). To establish which phosphorylation site on BglF is responsible for BglG negative regulation by phosphorylation, we tested the effect of the mutations in the two phosphorylation sites of BglF on the protein's ability to negatively regulate BglG. To address this question, we made use of strain MA200-1, whose chromosome carries a *bgl′–lacZ* fusion (a fusion of the *bgl* promoter and transcription terminator to *lacZ*) and a mutation in the *bglF* gene (Mahadevan *et al.*, 1987). Due to the mutation in the chromosomal *bglF* gene, BglG is not negatively regulated in this strain and therefore enables constitutive expression of the *lacZ* gene. Expression of plasmid-encoded wild-type BglF protein in MA200-1 renders *lacZ* expression inducible, i.e. β-galactosidase is produced only upon addition of β-glucosides to the growth medium. The β-galactosidase levels measured in MA200-1-containing plasmids which encode the various BglF derivatives, pCQ-F1 and pCQ-F2, in the absence and presence of

Table II. BglF mutated in site 1, but not in site 2, negatively regulates BglG transcription antitermination activity

Strain[a]	Plasmid	Plasmid-encoded BglF derivative	β-galactosidase activity (U)			
			βMG[b]		Salicin[c]	
			–	+	–	+
MA200-1	pMN5	Wild-type	6	50	6	128
	pCQ-F1	H547R	6	43	9	133
	pCQ-F2	C24S	30	36	30	59
PPA546 (*crr, nagE*)	pMN5	wild-type	5	100	3	324
	pCQ-F1	H547R	34	80	82	148
	pCQ-F2	C24S	68	152	73	133
PPA547 (*crr, nagE*)	pMN5	wild-type	4	82	5	283
	pCQ-F1	H547R	60	172	179	340
	pCQ-F2	C24S	85	180	70	138

[a]MA200-1 is Bgl$^+$ and it carries a *bgl–lacZ* transcriptional fusion. PPA546 and PPA547 are derivatives of MA200-1 but their *crr* and *nagE* genes were mutated.
[b]10 mM β-methylglucoside (βMG) were added to the growth medium when indicated.
[c]7 mM salicin were added to the growth medium when indicated.

β-glucosides, are given in Table II. BglF mutated in site 1 (H547R) behaved like wild-type BglF, allowing *lacZ* expression only upon addition of β-glucosides (two types of β-glucosides were used in this assay, salicin or β-methyl glucoside). Mutation in site 2 (C24S) abolished the ability of BglF to negatively regulate BglG and could not prevent constitutive expression of *lacZ*.

In order to study BglG regulation in a background deficient for IIA^{glc} and II^{nag}, which can substitute for IIA^{bgl} (and thus complement for mutations in this domain of BglF), we constructed two strains, PPA546 and PPA547, which are defective in their *crr* and *nagE* genes but are otherwise isogenic to MA200-1 (see Materials and methods). Introduction of pCQ-F1 and pCQ-F2 into these strains demonstrated that both BglF mutants were unable to regulate BglG and prevent constitutive expression of *lacZ* in this background. The control plasmid-encoded wild-type BglF allowed for *lacZ* expression only in the presence of β-glucosides, as in MA200-1 (Table II).

We can thus conclude unequivocally that the second phosphorylation site in BglF is in charge of BglG negative regulation *in vivo*. IIA^{glc} cannot complement or override the mutation in this site with respect to BglG regulation. However, a mutation in the first phosphorylation site of BglF, a site not involved in BglG regulation, can be complemented by IIA^{glc} as expected, and very likely by II^{nag} as well.

Discussion

It has been shown previously that BglF catalyses phosphorylation of either β-glucosides or a regulatory protein, BglG, depending on environmental conditions (Amster-Choder *et al.*, 1989; Amster-Choder and Wright, 1990; Schnetz and Rak, 1990). BglF, an EII of the PTS, has two phosphorylation sites. Similarly to other EIIs, the phosphoryl flows from the PTS protein HPr to the first phosphorylation site ('site 1') of BglF and then to its second site ('site 2'), which can deliver it to the sugar. How is BglG phosphorylation carried out by BglF? It has been suggested by Schnetz and Rak (1990) that each of the two phosphorylation sites on BglF is in charge of one phosphorylation function. According to their model, in the absence of sugar, the phosphoryl group in site 1 cannot be drained by site 2 to the sugar, leaving site 1 permanently phosphorylated; the phosphoryl group is then transferred from site 1 to BglG. They also suggested that IIA^{glc}, which is homologous to the IIA^{bgl} domain (Bramley and Kornberg, 1987) and can complement BglF mutated in site 1 (Schnetz *et al.*, 1990; this study), can transfer phosphoryl groups to site 2 of BglF or to BglG. However, the fact that no [^{32}P]BglG was detected when non-phosphorylated BglG was incubated with [^{32}P]PEP and a cellular fraction enriched for EI, HPr and IIA^{glc} (Amster-Choder *et al.*, 1989) did not support this model. As a matter of fact, despite the model presented by Schnetz and Rak, their results did not rule out the possibility that the same site, site 2, is in charge of both phosphorylation functions, i.e. sugar and BglG phosphorylation.

The results presented here demonstrate that the phosphoryl group is transferred from site 1 of BglF, His547, to its site 2, Cys24, and then either to the sugar or to BglG. This conclusion is based on *in vitro* and *in vivo* studies with BglF mutated either in site 1 (H547R) or in site 2 (C24S). Our *in vitro* studies prove unequivocally that the mutants behave the same with regard to their ability to transfer the phosphoryl group to β-glucosides and to BglG. A mutation in site 1 affects the ability of BglF to be phosphorylated by HPr but, once the mutant acquires a phosphoryl group on its second unchanged site (with the help of IIA^{glc} which can substitute for a defective IIA^{bgl}), it can transfer it both to the sugar and to BglG. A mutation in the second phosphorylation site abolishes both activities, sugar and BglG phosphorylation. It is thus obvious that the second site is the one that donates the phosphoryl group to both entities. To remove any doubt that these results have relevance for the *in vivo* situation, we have also shown that the effect of the mutations in BglF on its biological functions is consistent with the phosphoryl flux demonstrated *in vitro*. Since BglF inhibits BglG activity as a transcriptional antiterminator by phosphorylation, we expected a mutation in the site which is in charge of BglG phosphorylation to cancel the ability of BglF to inhibit BglG action. Indeed, BglF mutated in

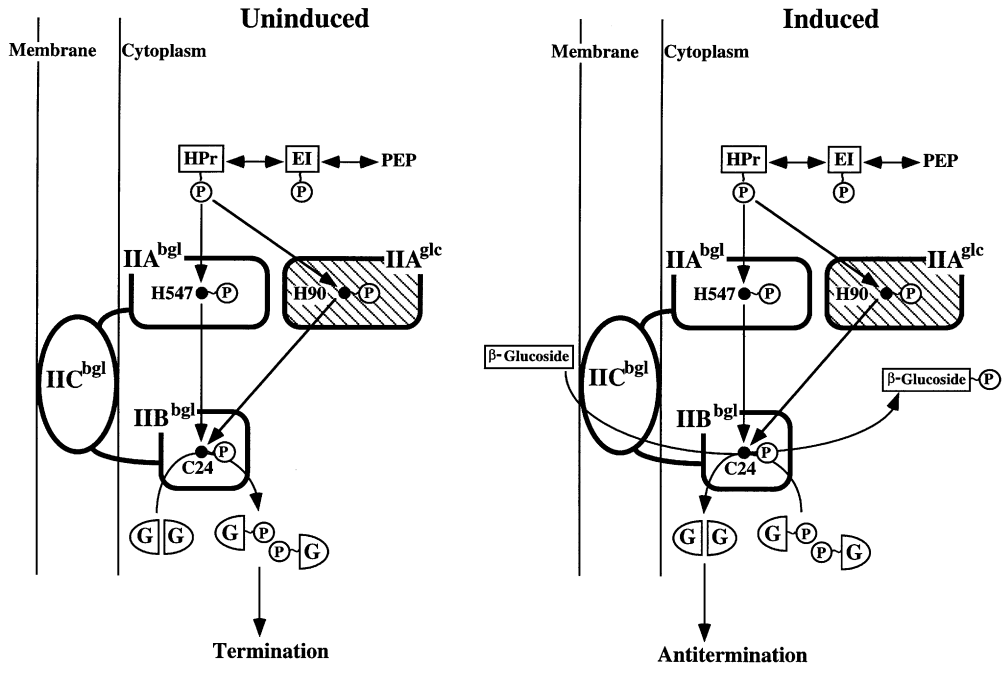

Fig. 6. Phosphoryl flow in the *bgl* system in the absence (Uninduced) and presence (Induced) of β-glucosides.

site 2, which prevents the bacterial cell from utilizing β-glucosides, cannot negatively regulate BglG activity. A mutation in site 1 affected both functions of BglF only in a strain deficient for the proteins IIA^{glc} and II^{nag}. In strains expressing these proteins, that can complement the mutation in site 1 of BglF and deliver the phosphoryl group from HPr to site 2 of BglF, the site 1 mutant carried out both functions.

A model for the phosphoryl flux from PEP to the components of the *bgl* system, which is consistent with our observations, is shown in Figure 6. A phosphoryl group is transferred from PEP through EI and HPr to the IIA domains of PTS proteins, among them IIA^{bgl} and IIA^{glc}, the latter being a key regulatory protein constitutively produced in the *E.coli* cell. These two IIAs, which are homologous to each other, are phosphorylated on a histidine residue, IIA^{bgl} on His547 and IIA^{glc} on His90. The phosphoryl group can be transferred from each of these histidines to Cys24 in the IIB^{bgl} domain. Under non-inducing conditions, Cys24 of BglF delivers phosphoryl groups to the BglG molecules present in the cell. Phosphorylated BglG cannot dimerize and thus cannot bind to the *bgl* transcript and antiterminate transcription. Transcription of the *bgl* operon is terminated prematurely. Addition of β-glucosides stimulates BglF to dephosphorylate BglG and to phosphorylate the β-glucosides (Amster-Choder *et al.*, 1989; Amster-Choder and Wright, 1990). Non-phosphorylated BglG dimerizes, binds its RNA target, antiterminates transcription of the *bgl* operon and leads to Bgl protein production. More β-glucosides can be phosphorylated and transported into the cell. Thus, under inducing conditions, the phosphoryl group is donated to the sugar by the phosphorylated Cys24, which is the same residue responsible for the phosphorylation of BglG under non-inducing conditions. The graphic illustration, showing the involvement of Cys24 in BglG dephosphorylation, represents a suggestion by which BglG can deliver the phosphoryl group back to Cys24 of BglF. Such phosphoryl flow from BglG back to BglF is the reverse reaction of BglG phosphorylation. The phosphorylation reactions between the different components of PTS were shown to be reversible in all cases when reversibility was tested. However, BglG is not a PTS member, according to the current definition of PTS proteins, since in no other case has a PTS EII been shown to phosphorylate a non-PTS protein, though it was suggested in several cases (see below). Further studies of the phosphorylation reaction of BglG by BglF, which we intend to pursue in the future, should provide an answer to whether this reaction is reversible.

Mechanisms that can possibly control phosphoryl flux in the bgl system

What are the possible mechanisms that allow the β-glucosides to divert the phosphoryl group away from BglG to sugar transport? The key is likely to lie in different recognition of the two entities, sugar and protein, by BglF. Different recognition can be achieved by different recognition sites for the sugar and for BglG, by alternative conformations that BglF can adopt under different conditions, and by a combination of both. Because the same active site on BglF delivers the phosphoryl group to BglG and to β-glucosides, recognition of the two entities is expected to be specified by sites other than the active site. If this is the case, it should be possible to engineer or select for BglF derivatives that can transport β-glucosides but cannot regulate BglG, or vice versa. We have preliminary evidence that such variants of BglF exist (Q.Chen and O.Amster-Choder, unpublished data).

The recognition sites are not necessarily expected to be specified by a consecutive sequence of amino acids. They might rather be created by sequences in different domains of BglF (which is composed of three distinct domains) that are brought together due to a certain way of folding

of the protein. An intriguing mechanism might be that the sugar induces a conformational change by binding to the BglF permease: a sugar-bound permease dephosphorylates BglG and phosphorylates the sugar; BglF, not bound to sugar, folds into a conformation that phosphorylates BglG. An example of such a conformational change is dimerization of BglF, which might be induced by substrate binding, similarly to ligand-induced dimerization of eukaryotic receptors. This possibility is currently under study. Moreover, BglF might alternate between a sugar-bound conformation and a BglG-bound conformation, the first being more favourable. Since BglG is a soluble protein present in catalytic amounts in the cell, a likely possibility that can ensure rapid and efficient response to environmental changes is recruitment of BglG molecules to the membrane. The physical attachment between BglF and BglG, if it exists, is expected to prevail as long as BglF is not bound to the sugar; induction of a conformational change in BglF due to binding of β-glucosides might very well lead not only to dephosphorylation of BglG, but also to its detachment from BglF due to lack of affinity between the sugar-bound conformation of BglF and BglG. Alternatively, the detachment might be the result of the conformational change that dephosphorylation induces in BglG, i.e. BglG dimerization. The latter option is less favourable since it requires dimerization of membrane-bound BglG rather than dimerization of free and soluble BglG. Such a process does not seem to be adequate for generating a quick response to the external stimulus, which is the presence of β-glucosides.

Does BglF represent a new class of EIIs of PTS?

The *bgl* system in *E.coli* is the first member of a new family of bacterial systems involved in signal transduction (Amster-Choder and Wright, 1993). Indeed the BglF is a PTS permease, and as such is responsible for the transfer of a sugar into the cell while phosphorylating it. However, BglF has novel capabilities, not yet demonstrated for any other PTS permease; in addition to phosphorylating carbohydrates, BglF phosphorylates and dephosphorylates the transcriptional regulator BglG (Amster-Choder *et al.*, 1989; Amster-Choder and Wright, 1990; Schnetz and Rak, 1990), which leads to transcription antitermination via a novel mechanism (Houman *et al.*, 1990). BglF, together with BglG, constitutes a system which transduces a signal from the cell surface to the transcription machinery and thus controls gene expression in response to an external stimulus. Although the *bgl* system is composed of two components, a sensor and a response-regulator, it is not a member of the family of two-component systems involved in processing sensory data and regulating gene expression (reviewed in Parkinson *et al.*, 1993; Russo and Silhavy, 1993). This is because the Bgl proteins do not share any homology with proteins of the two-component family which was studied intensively in bacteria and later discovered in eukaryotes (reviewed in Swanson and Simon, 1994).

Is BglF a unique EII of PTS, or do other PTS permeases stretch their activity beyond sugar phosphotransfer and control the activity of transcription regulatory proteins by phosphorylation? Although not directly proven yet, BglF-like PTS EIIs were suggested to exist in various organisms. Based on predicted amino acid sequence homology to the Bgl proteins and nucleotide sequence homology to the *cis* elements involved in *bgl* operon induction, several systems were suggested to affiliate to the *bgl* family of sensory systems. These systems seem to consist of BglG-like antiterminators negatively regulated by BglF-like EIIs. BglP and SacX from *Bacillus subtilis* are examples of proteins that were suggested to perform similarly to BglF. BglP, the *B.subtilis* β-glucoside phosphotransferase EII, negatively regulates the activity of LicT, a BglG-like transcriptional antiterminator (Kruger and Hecker, 1995; Le Coq *et al.*, 1995). SacX, which shows strong homology to sucrose-specific PTS permeases (Zukowski *et al.*, 1990), negatively regulates the activity of SacY (Aymerich and Steinmetz, 1987), another BglG homologue (Aymerich and Steinmetz, 1992). The similarity of these protein pairs to the *bgl* system led to the proposal, yet to be proven, that BglP and SacX play a similar role to BglF and inhibit the antitermination activity of LicT and SacY, respectively, by phosphorylation. Unlike BglF, SacX constitutes only part of the EII sucrose permease, and its counterpart has not been identified indisputably yet. Another putative *bgl*-like system in *B.subtilis* is composed of the four proteins suggested to form a PTS EII complex, designated *lev*-PTS, and the transcriptional regulator LevR, which has one domain homologous to BglG (Martin-Verstraete *et al.*, 1990; Debarbouille *et al.*, 1991). Interestingly, unlike BglG, LevR, as well as another BglG-like antiterminator from *B.subtilis*, SacT, were shown to be positively regulated and *in vitro* phosphorylated by the PTS general proteins, EI and HPr (Arnaud *et al.*, 1992, 1996; Stulke *et al.*, 1995). This does not rule out the possibility that these proteins are also negatively regulated by phosphorylation by their BglF-like partners. Another *bgl*-like system seems to exist in *Erwinia chrysanthemi*. Based on sequence homology between the *arb* genes in this organism and the *bgl* operon in *E.coli*, the *arbF* gene product was also suggested to resemble BglF and, in addition to β-glucoside phosphotransfer, to negatively regulate the *arbG* gene product, suggested to resemble BglG (El Hassouni *et al.*, 1992). Thus, indirect indications for the existence of BglF-like EIIs that regulate the activity of their cognate transcriptional antiterminators by reversible phosphorylation keep accumulating. They are based on resemblance to *bgl* and await direct proof, biochemical or otherwise. Nevertheless, it seems that the definition of PTS proteins might have to be extended in the future to include the PTS-dependent transcriptional antiterminators. It is too early to ask whether the BglF-like permeases use the same active site to phosphorylate their sugar substrate and cognate antiterminator protein. However, an intelligent guess is that they do, since the use of the same active site for the two phosphorylation reactions is probably not a coincidence. Rather, it seems to be an inherent feature of the mechanism underlying signal transduction, that reflects the competition of the two entities for the phosphoryl group, to prevent phosphorylation of both simultaneously and to ensure efficient response to the stimulus.

Materials and methods

Strains

The *E.coli* K12 strains used in this work are listed in Table III. LM1 contains mutations in the *nagE* and *crr* genes which code for II^{nag} and

Table III. Strains

Strain and/or plasmid	Relevant genotype	Source, derivation or reference
E.coli		
K38	*HfrC trpR thi* λ^+	C.Richardson
LM1	*crr-1 manA manI nagE thi-1 his-1 argG6 metB galT rpsL*	Lengeler *et al*. (1981)
MC1061	*hsdR mcrB araD139* Δ*(araABC-leu)7679* Δ*lacX74 galU galK rpsL thi*	Maniatis *et al*. (1989)
MA231	*F*$^-$ *recA56 bglR bglF31 trpB proC::Tn5 ilvO tna*$^+$ *lac*	Mahadevan *et al*. (1987)
AE304-1	*F*$^-$ *tna::Tn10 bglF1* Δ*lacX74 thi bglR11 (bglR::IS1) tsx (T6)*^{r}	Mahadevan *et al*. (1987)
AE304-2	As AE304-1 except *bglF2* instead of *bglF1*	Mahadevan *et al*. (1987)
AE304-4	As AE304-1 except *bglF3* instead of *bglF1*	Mahadevan *et al*. (1987)
MA200-1	*F*$^-$ *bglF201 srl::*Tn*10 recA56* Δ*lacX74 thi bglR11 (bglR::IS1)* λ*bglR7 bglG′ lacZ*$^+$ *lacY*$^+$ ϕ*(bgl-lac)*	Mahadevan *et al*. (1987)
CAG18468	*nupC510::*Tn*10*	Singer *et al.* (1989)
CAG12077	*zbe-280::*Tn*10*	Singer *et al.* (1989)
PPA237	as LM1 but *nupC510*::Tn*10*	P1(CAG18468)×LM1 to Tet^r
PPA531	as LM1 but *zbe-280*::Tn*10*	P1(CAG12077)×LM1 to Tet^r
PPA503	as AE304-4 but excision of Tn*10*	excision of Tn*10* (Tet^s)
PPA517	as PPA503 but *crr-1 nupC510*::Tn*10*	P1(PPA237)×PPA503 to Tet^r
PPA527	as PPA517 but excision of Tn*10*	excision of Tn*10* (Tet^s)
PPA543	as PPA527 but *nagE zbe-280*::Tn*10*	P1(PPA531)×PPA527 to Tet^r
PPA501	as MA200-1 but excision of Tn*10*	excision of Tn*10* (Tet^s)
PPA501/pGE82		Kan^r transformant
PPA515/pGE82	as PPA501/pGE82 but *crr-1 nupC510*::Tn*10*	P1(PPA237)×PPA501/pGE82 to Tet^r
PPA521/pGE82	as PPA515/pGE82 but excision of Tn*10*	excision of Tn10 (Tet^s)
PPA546/pGE82	as PPA521/pGE82 but *nagE zbe-280*::Tn*10*	P1(PPA531)×PPA521/pGE82 to Tet^r
PPA547/pGE82	as PPA521/pGE82 but *nagE zbe-280*::Tn10	as above but another transductant
ZSC112ΔG	Δ*ptsG::cat manZ glk-7 thi rpsL*	Buhr *et al*. (1994)
S.typhimurium		
LJ144	*cpd-401 cysA1150*/F′198 (*ptsI*$^+$ *ptsH*$^+$ *crr*$^+$)	Saier and Feucht (1975)

Abbreviation used: Tet^s, tetracycline sensitivity; Tet^r, tetracycline resistance; Kan^r, kanamycin resistance; P1, phage P1.

IIA^{glc} respectively. MA231, AE304-1, AE304-2 and AE304-4 carry a defective *bglF* gene. MA200-1 carries a *bgl′–lacZ* fusion on its chromosome and a defective *bglF* gene. PPA543 is a *crr* and *nagE* derivative of AE304-4; PPA546 and PPA547 are *crr* and *nagE* derivatives of MA200-1. Construction of these strains involved the construction of several intermediary strains, all listed in Table III.

P1 phage transductions were conducted as described by Arber (1958). Tn*10* excision was indicated by growth on fusaric acid plates which enable the selection for loss of tetracycline resistance (Maloy and Nunn, 1981). The mutations in the *crr* and *nagE* genes were confirmed by the inability of the mutants to grow on succinate and the ability to grow in the presence of streptozotocin (Lengeler, 1980), respectively. The *crr* genotype of PPA543, PPA546 and PPA547 was also confirmed by the lack of IIA^{glc} in rocket electrophoresis experiments using antibodies against IIA^{glc} (Scholte *et al.*, 1981). The *nagE* genotype is linked to streptozotocin resistance and was used to identify the *nagE* transductants (Lengeler, 1980).

The *S.typhimurium* strain LJ144 contains the *ptsHI-crr* genes on an *E.coli* plasmid, F′198, and thus produces increased levels of EI, HPr, and IIA^{glc} (Saier and Feucht, 1975).

Plasmids

Plasmids pT712 and pT713, containing the phage T7 late promoter, and plasmid pGP1-2, carrying the T7 RNA polymerase gene under control of the λCI857 repressor, were obtained from Bethesda Research Laboratories. Plasmid pT7FH-G carries the entire *bglG* gene cloned downstream of the T7 promoter in pT713; plasmid pT7OAC-F carries the entire *bglF* gene cloned downstream of the T7 promoter in pT712 (Amster-Choder *et al.*, 1989). Plasmids pT7CQ-F1 and pT7CQ-F2 are derivatives of pT7OAC-F that encode for BglF with either the His547 mutated to Arg (H547R) or the Cys24 mutated to Ser (C24S) respectively (the procedure for site-directed mutagenesis is described below). Plasmid pMN5 carries the entire *bglF* gene cloned in pBR322 (Mahadevan *et al.*, 1987). Plasmids pCQ-F1 and pCQ-F2 contain a 2099 bp *Hin*dIII–*Eco*RI fragment from pT7CQ-F1 (encoding the H547R mutant) or from pT7CQ-F2 (encoding the C24S mutant) ligated to the 4330 bp *Hin*dIII–*Eco*RI fragment of pBR322 respectively. Plasmid pMBP-BglG, obtained from A.Wright, carries a fusion between the *MalE* gene and the entire *bglG* gene cloned downstream of the Ptac promoter. Plasmid pLysS carries the T7 lysozyme gene cloned in pACYC184 (Studier *et al.*, 1990). Plasmid pGE82 which carries the *recA*$^+$ gene and confers kanamycin resistance was obtained from R.A.Bender and was used during strain construction (see Table III).

Media

Enriched media, M9 salts and M63 salts minimal media were prepared essentially as described by Miller (1972). The minimal medium used for [^{35}S]methionine labelling was the same as that used by Tabor and Richardson (1985) with 0.4% succinate as carbon source. Ampicillin (200 μg/ml), kanamycin (30 μg/ml), tetracycline (10 μg/ml) or chloramphenicol (30 μg/ml) were included in the media when growing strains which carry transposable elements or contain plasmids that confer resistance to either one of these antibiotics. Fusaric acid plates were prepared as described by Maloy and Nunn (1981). Plates containing streptozotocin were prepared as described by Lengeler (1980). MacConkey arbutin plates were prepared as described previously (Schaefler, 1967). MacConkey lactose plates were prepared from lactose MacConkey agar (Difco). Minimal arbutin plates were prepared from M9 salts minimal medium supplied with 0.4% arbutin.

Chemicals

[γ-^{32}P]ATP (3000 Ci/mmol) was obtained from Rotem Industries LTD (Israel). [^{35}S]methionine (1200 Ci/mmol) was obtained from Du Pont. PEP, pyruvic acid and pyruvate kinase were obtained from Sigma. Amylose resin, MBP, anti-MBP antiserum and maltose were obtained from New England Biolabs. [^{32}P]PEP was prepared and separated from [^{32}P]ATP as described before (Amster-Choder, *et al.*, 1989). EI, HPr and IIA^{glc} were obtained from J.Reizer. Monoclonal antibodies against IICB^{glc} were obtained from B.Erni.

Molecular cloning

All manipulations with recombinant DNA were carried out by standard procedures (Maniatis *et al.*, 1989). Restriction enzymes and other enzymes used in recombinant DNA experiments were purchased commercially and were used according to the specifications of the manufacturers.

Measurements of β-galactosidase activity

Assays for β-galactosidase activity were carried out as described by Miller (1972). Cells were grown in minimal medium which was

supplemented with 0.2% lactate as carbon source. Due to a *crr* mutation in two out of the three strains used, we supplemented the medium in all cases with 5 mM cAMP.

Preparation of cell extracts and membrane fractions

Cell extracts enriched for BglG and membrane fractions enriched for the various BglF derivatives (wild-type, H547R and C24S) were prepared as described before (Amster-Choder *et al.*, 1989). The proteins were expressed from their respective genes cloned under T7 promoter control in plasmids pT7FH-G, pT7OAC-F, pT7CQ-F1 and pT7CQ-F2. Expression of T7 RNA polymerase, specified by plasmid pGP1-2 which is compatible with the above plasmids, was induced thermally. For preparing extracts and membranes used in the *in vitro* phosphorylation system A (see below), the *E.coli* K38 strain was used as a host. The *E.coli* LM1 strain, containing mutations in the *crr* and *nagE* genes that code for IIA^{glc} and II^{nag} respectively, was used as a host when preparing cellular fractions used in the *in vitro* phosphorylation system B. Membranes of the *E.coli* strain ZSC112ΔG were used to study dephosphorylation of the various BglF derivatives in the presence of β-glucosides *in vitro*.

Membrane fractions lacking BglF were prepared either from strain K38/pGP1-2/pT712 or from strain LM1/pGP1-2/pT712 and were used in control experiments in phosphorylation systems A or B respectively.

A soluble fraction from *S.typhimurium* LJ144, which overproduces EI, HPr and IIA^{glc}, was prepared as described by Begley *et al.* (1982).

Purification of MBP–BglG

The expression and purification of MBP–BglG were carried out basically as recommended by New England Biolabs with some modifications. A culture of MC1061/pLysS/pMBP-BglG was grown with aeration to OD_{600} = 0.3 in L broth containing 0.1% glucose, 200 μg/ml ampicillin and 30 μg/ml chloramphenicol at 37°C. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.07 mM, to induce expression of pMBP-BglG, and growth was continued with aeration for an additional 2 h. The cells were then harvested by centrifugation at 4000 *g* for 20 min in the cold and the pelleted cells were frozen. Freezing and thawing the cells enhance lysis by the lysozyme expressed from pLysS. The pellet was resuspended in column buffer [20 mM Tris–HCl, pH 7.5, 200 mM NaCl, 1 mM EDTA, 20 mM phenylmethylsulfonyl fluoride (PMSF)] and sonicated. After removal of unbroken cells by centrifugation at 4000 *g* for 20 min, the supernatant was mixed gently with 1/10 volume of amylose resin overnight at 4°C. The resin then was packed in a column. The column was washed once with column buffer, once with column buffer containing 0.01% Triton X-100, and again with column buffer. The MBP–BglG was eluted with column buffer containing 10 mM maltose, and fractions were collected. The fractions were analysed by SDS–PAGE and those containing MBP–BglG were dialysed against column buffer to remove the maltose. The protein concentration was determined by the Bradford assay using a kit purchased from Bio-Rad.

In vitro phosphorylation systems

System A. Membranes enriched for the various BglF derivatives and cell extract enriched for BglG were prepared by overproducing these proteins in *E.coli* strain K38, which contains normal levels of IIA^{glc} and II^{nag} (see above). The *S.typhimurium* LJ144 soluble extract was used as the source of EI and HPr. The various phosphorylation reactions were carried out as described by Amster-Choder *et al.* (1989).

System B. To establish a phosphorylation system that lacks IIA^{glc} and II^{nag}, membranes enriched for the various BglF derivatives were prepared by overproducing these proteins in the *crr* and *nagE E.coli* strain LM1 or in the *ptsG E.coli* strain ZSC112ΔG (see above). Membrane fractions, at a final protein concentration of 0.9 mg/ml, were labelled by incubation at 30°C in a mixture containing 10 μg/ml EI, 40 μg/ml HPr, 10 μM [^{32}P]PEP and PLB buffer (50 mM Na_2HPO_4, pH 7.4, 0.5 mM $MgCl_2$, 1 mM NaF and 2 mM dithiothreitol). IIA^{glc} was added to a final concentration of 100 μg/ml when indicated. After incubation for 10 min, reactions were either terminated by addition of electrophoresis sample buffer or incubated further as described below. To study dephosphorylation by β-glucosides, salicin was added to a final concentration of 0.2% and incubation was continued at 30°C for 5 min. To study BglG phosphorylation, MBP–BglG or MBP (as a control), which were first adjusted to the PLB buffer concentration indicated above, were added to a final concentration of 10 μM, and incubation was continued at 30°C for 15 min.

Western blot analysis

Protein extracts were fractionated on a 5–12.5% gradient SDS–polyacrylamide gel and blotted onto a nitrocellulose filter (Schleicher & Schuell) using transfer buffer (25 mM Tris, 190 mM glycine, 20% methanol). The nitrocellulose membrane was then blocked by incubation in 1% fat milk for 1 h at room temperature. Incubation with anti-MBP antiserum, diluted 1:5000 in 1% fat milk, was carried out overnight at 4°C and was followed by three washes of 5 min in phosphate-buffered saline (PBS; 80 mM Na_2HPO_4, 20 mM NaH_2PO_4 and 100 mM NaCl). Alkaline phosphatase-conjugated goat anti-rabbit IgGs (Jackson ImmunoResearch Laboratories Inc.) were diluted 1:5000 in 1% fat milk and the blot was incubated in it for 2 h at room temperature. The blot was then washed three times in PBS for 5 min, once in AP buffer (100 mM Tris–HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) for 10 min and developed in a solution of 0.33 mg/ml NBT (nitro blue tetrazolium, Sigma) and 0.165 mg/ml BCIP (5-bromo-4-chloro-3-indolyl phosphate, Sigma) in AP buffer.

[^{35}S]methionine labelling of BglG

Cells containing the plasmids carrying the *bglF* or *bglG* genes under the control of the phage T7 promoter were induced and labelled with [^{35}S]methionine in the presence of rifampicin (Sigma) as described by Tabor and Richardson (1985).

Site-directed mutagenesis

Site-directed mutagenesis was carried out by overlap extension with PCR as described by Ho *et al.* (1989). The primers 5′-CTGATGCATAGCGCTACGCGA-3′ and its complementary oligo, or 5′-ATCCTGATACGCGTCGGTATC-3′ and its complementary oligo were used to mutate the *bglF* gene to its alleles that encode BglF derivatives with the Cys24 replaced by Ser or the His547 replaced by Arg, respectively. The mutations introduced new sites for restriction enzymes which were useful during the screening for the mutant plasmids. The mutations were confirmed by sequencing.

Electrophoresis and autoradiography

Proteins were incubated for 30 min at 30°C in electrophoresis sample buffer containing 62.5 mM Tris–HCl (pH 6.8), 2% SDS, 5% β-mercaptoethanol, 10% glycerol and 0.01% bromophenol blue. In most cases, electrophoresis of proteins was carried out on 10% SDS–polyacrylamide gels as described by Laemmli (1970). Gradient SDS–polyacrylamide gels (5–12.5%) were used where indicated. After electrophoresis, gels were dried and exposed to Kodak XAR-5 X-ray film at –70°C.

Acknowledgements

We are grateful to Dr Jonathan Reizer for the gift of the purified proteins, Enzyme I, HPr and IIA^{glc}, Dr Andrew Wright for the gift of plasmid pMBP-BglG and Dr Bernard Erni for the gift of anti-$IICB^{glc}$ monoclonal antibodies and strain ZSC112ΔG. We thank Dr A.Wright for critical reading of the manuscript. This research was supported by the Israel Science Foundation administered to O.A.-C. by the Israel Academy of Sciences and Humanities and The Scheuer Research Foundation. O.A.-C. is a recipient of an Alon Fellowship.

References

Amster-Choder,O. and Wright,A. (1990) Regulation of activity of a transcriptional antiterminator in *E.coli* by phosphorylation. *Science*, **249**, 540–542.

Amster-Choder,O. and Wright,A. (1992) Modulation of dimerization of a transcriptional antiterminator protein by phosphorylation. *Science*, **257**, 1395–1398.

Amster-Choder,O. and Wright,A. (1993) Transcriptional regulation of the *bgl* operon of *E.coli* involves phosphotransferase system-mediated phosphorylation of a transcriptional antiterminator. *J. Cell. Biochem.*, **51**, 83–90.

Amster-Choder,O., Houman,F. and Wright,A. (1989) Protein phosphorylation regulates transcription of the β-glucoside utilization operon in *E.coli*. *Cell*, **58**, 847–855.

Arber,W. (1958) Transduction of chromosomal genes and episomes in *Escherichia coli*. *Virology*, **1**, 273–288.

Arnaud,M., Vary,P., Zagorec,M., Klier,A., Debarbouille,M., Postma,P., and Rapoport,G. (1992) Regulation of the *sacPA* operon of *Bacillus subtilis*: identification of phosphotransferase system components involved in SacT activity. *J. Bacteriol.*, **174**, 3161–3170.

Arnaud,M., Debarbouille,M., Rapoport,G., Saier,M.H.,Jr and Reizer,J. (1996) *In vitro* reconstitution of transcriptional antitermination by the SacT and SacY proteins of *Bacillus subtilis*. *J. Biol. Chem.*, **51**, 18966–18972.

Aymerich,S. and Steinmetz,M. (1987) Cloning and preliminary characterization of the *sacS* locus from *Bacillus subtilis* which controls the regulation of the exoenzyme levansucrase. *Mol. Gen. Genet.*, **208**, 114–120.

Aymerich,S. and Steinmetz,M. (1992) Specificity determinants and structural features in the RNA target of the bacterial antiterminator proteins of the BglG/SacY family. *Proc. Natl Acad. Sci. USA*, **89**, 10410–10414.

Begley,G.S., Hansen,D.E., Jacobson,G.R. and Knowles,J.R. (1982) Stereochemical course of the reactions catalyzed by the bacterial phosphoenolpyruvate:glucose phosphotransferase system. *Biochemistry*, **21**, 5552–5556.

Bramley,H.F. and Kornberg,H. (1987) Sequence homologies between proteins of bacterial phosphoenolpyruvate-dependent sugar phosphotransferase system: identification of possible phosphate carrying histidine residues. *Proc. Natl Acad. Sci. USA*, **84**, 4777–4780.

Buhr,A., Flükiger,K. and Erni,B. (1994) The glucose transporter of *Escherichia coli*: overexpression, purification and characterization of functional domains. *J. Biol. Chem.*, **269**, 23437–23443.

Debarbouille,M., Martin-Verstraete,I., Klier,A. and Rapoport,G. (1991) The transcriptional regulator LevR of *Bacillus subtilis* has domains homologous to both σ^{54}- and phosphotransferase system-dependent regulators. *Proc. Natl Acad. Sci. USA*, **88**, 2212–2216.

El Hassouni,M., Henrissat,B., Chippaux,M. and Barras,F. (1992) Nucleotide sequences of the *arb* genes, which control β-glucoside utilization in *Erwinia chrysanthemi:* comparison with the *Escherichia coli bgl* operon and evidence for a new β-glycohydrolase family including enzymes from eubacteria, archaebacteria, and humans. *J. Bacteriol.*, **174**, 765–777.

Fox,C.F. and Wilson,G. (1968) The role of a phosphoenolpyruvate-dependent kinase system in β-glucoside catabolism in *E.coli*. *Proc. Natl Acad. Sci. USA*, **59**, 988–995.

Ho,S.N., Hunt,H.D., Horton,R.M., Pullen,J.K. and Pease,L.R. (1989) Site directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene*, **77**, 51–59.

Houman,F., Diaz-Torres,M.R. and Wright,A. (1990) Transcriptional antitermination in the *bgl* operon of *E.coli* is modulated by a specific RNA binding protein. *Cell*, **62**, 1153–1163.

Kruger,S. and Hecker,M. (1995) Regulation of the putative *bglPH* operon for aryl-β-glucoside utilization in *Bacillus subtilis*. *J. Bacteriol.*, **177**, 5590–5597.

Laemmli,U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685.

Le Coq,D., Lindner,C., Kruger,S., Steinmetz,M. and Stulke,J. (1995) New β-glucoside (*bgl*) genes in *Bacillus subtilis*: the *bglP* gene product has both transport and regulatory functions similar to those of BglF, its *Escherichia coli* homolog. *J. Bacteriol.*, **177**, 1527–1535.

Lengeler,J. (1980) Characterization of mutants of *Escherichia coli* K12, selected by resistance to streptozotocin. *Mol. Gen. Genet.*, **179**, 49–54.

Lengeler,J., Auburger,A.-M., Mayer,R. and Pecher,A. (1981) The phosphoenolpyruvate-dependent carbohydrate:phosphotransferase system enzymes II as chemoreceptors in chemotaxis of *Escherichia coli* K12. *Mol. Gen. Genet.*, **183**, 163–170.

Mahadevan,S. and Wright,A. (1987) A bacterial gene involved in transcription antitermination: regulation at a rho-independent terminator in the *bgl* operon of *E.coli*. *Cell*, **50**, 485–494.

Mahadevan,S., Reynolds,A.E. and Wright,A. (1987) Positive and negative regulation of the *bgl* operon in *E.coli*. *J. Bacteriol.*, **169**, 2570–2578.

Maloy,S.R. and Nunn,W.D. (1981) Selection for loss of tetracycline resistance by *Escherichia coli*. *J. Bacteriol.*, **145**, 1110–1112.

Maniatis,T., Fritsch,E.F. and Sambrook,J. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Martin-Verstraete,I., Debarbouille,M., Klier,A. and Rapoport,G. (1990) Levanase operon of *Bacillus subtilis* includes a fructose-specific phosphotransferase system regulating the expression of the operon. *J. Mol. Biol.*, **214**, 657–671.

Miller,J. (1972) *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Parkinson,J.S. (1993) Signal transduction schemes of bacteria. *Cell*, **73**, 857–871.

Postma,P.W., Lengeler,J.W. and Jacobson,G.R. (1993) Phosphoenolpyruvate:carbohydrate phosphotransferase systems of bacteria. *Microbiol. Rev.*, **57**, 543–594.

Russo,F.D. and Silhavy,T.J. (1993) The essential tension: opposed reactions in bacterial two-component regulatory systems. *Trends Microbiol.*, **1**, 307–310.

Saier,M.H.,Jr and Feucht,B.U. (1975) Coordinate regulation of adenylate cyclase and carbohydrate permeases by the phosphoenolpyruvate:sugar phosphotransferase system in *S.typhimurium*. *J. Biol. Chem.*, **250**, 7078–7080.

Saier,M.H.,Jr and Reizer,J. (1992) Proposed uniform nomenclature for the proteins and protein domains of the bacterial phosphoenolpyruvate:sugar phosphotransferase system. *J. Bacteriol.*, **174**, 1433–1438.

Schaefler,S. (1967) Inducible system for the utilization of β-glucosides in *Escherichia coli*. Active transport and utilization of β-glucosides. *J. Bacteriol.*, **93**, 254–263

Schnetz,K. and Rak,B. (1988) Regulation of the *bgl* operon of *E.coli* by transcriptional termination. *EMBO J.*, **7**, 3271–3277.

Schnetz,K. and Rak,B. (1990) β-Glucoside permease represses the *bgl* operon of *E.coli* by phosphorylation of the antiterminator protein and also interacts with glucose-specific enzyme II, the key element in catabolic control. *Proc. Natl Acad. Sci. USA*, **87**, 5074–5078.

Schnetz,K., Surtina,S.L., Saier,M.H. and Rak,B. (1990) Identification of catalytic residues in the β-glucoside permease of *E.coli* by site-directed mutagenesis and demonstration of interdomain cross-reactivity between the β-glucoside and glucose systems. *J. Biol. Chem.*, **265**, 13464–13471.

Scholte,B.J., Schuitema,A.R. and Postma,P.W. (1981) Isolation of III^{Glc} the phosphoenolpyruvate-dependent glucose phosphotransferase system in *Salmonella typhimurium*. *J. Bacteriol.*, **148**, 257–264.

Singer,M. *et al*. (1989) A collection of strains containing genetically linked alternating antibiotic resistance elements for genetic mapping of *Escherichia coli*. *Microbiol. Rev.*, **53**, 1–24.

Studier,F.W., Rosenberg,A.H., Dunn,J.H. and Dubendorff,J.W. (1990) Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol.*, **185**, 60–89.

Stulke,J., Martin-Verstraete,I., Charrier,V., Klier,A., Deutscher,J. and Rapoport,G. (1995) The HPr protein of the phosphotransferase system links induction and catabolite repression of the *Bacillus subtilis* levanase operon. *J. Bacteriol.*, **177**, 6928–6936.

Swanson,R.V. and Simon,M.I. (1994) Bringing the eukaryotes up to speed. *Curr. Biol.*, **4**, 234–237.

Tabor,S. and Richardson,C.C. (1985) A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. *Proc. Natl Acad. Sci. USA*, **82**, 1074–1078.

Vogler,A.P., Broekhuizen,C.P., Schuitema,J.W. and Postma,P.W. (1988) Suppression of III^{Glc}-defects by Enzymes II^{Nag} and II^{Bgl} of the PEP:carbohydrate phosphotransferase system. *Mol. Microbiol.*, **2**, 719–726.

Zukowski,M.M., Miller,L., Cosgwell,P., Chen,K., Aymerich,S. and Steinmetz,M. (1990) Nucleotide sequence of the *sacS* locus of *Bacillus subtilis* reveals the presence of two regulatory genes. *Gene*, **90**, 153–155.

Received on December 16, 1996; revised on April 28, 1997