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

Qing Chen, Jos C.Arents1, Rechien Bader1, Pieter W.Postma1 and Orna Amster-Choder2

Introduction

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 regulatory BglG protein 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

BglG, 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 protein 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
Q. Chen et al.

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 [32P]PEP and a soluble protein extract prepared from the Salmonella typhimurium LJ144, which is enriched for EI, HPr and IIA\(^{\text{agc}}\) 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 [32P]PEP and purified EI and HPr (phosphorylation system B) for 10 min without (lane 1–4) or with (lanes 5–8) IIA\(^{\text{agc}}\). 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\(^{\text{agc}}\).

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\(^{\text{agc}}\) phosphorylates BglG and the site on IIB\(^{\text{bgl}}\) phosphorylates the sugar. These authors also suggested that IIA\(^{\text{agc}}\), which is homologous to the IIA\(^{\text{bgl}}\) 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 [32P]BglG was detected when non-phosphorylated BglG was incubated with [32P]PEP and a soluble fraction of a Salmonella typhimurium strain overproducing EI, HPr and IIA\(^{\text{agc}}\) (Amster-Choder et al., 1989) did not support transfer from IIA\(^{\text{agc}}\) to BglG. Here we provide both in vivo and in vitro evidence that the site on BglF which transfers a phosphoryl group to \(\beta\)-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 \(\beta\)-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 \(\beta\)-glucosides and to BglG in vitro on one hand, and to mediate \(\beta\)-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 [32P]PEP, a cytoplasmic extract prepared from the mutant strain of S.typhimurium LJ144 which expresses increased amounts of EI, HPr and IIA\(^{\text{agc}}\) (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
deleted for the \(crr\) and \(nagE\) genes (and thus not expressing the \(IIA^{\text{gel}}\) and \(II^{\text{mag}}\) proteins which can substitute for \(IIA^{\text{bg}}\)). The overproduction of the three \(BglF\) derivatives in this strain was demonstrated by metabolic labelling with \(\text{[35S]methionine}\) (data not shown). Membranes prepared from \(LM1\) producing the different \(BglF\) derivatives were incubated with \(\text{[32P]PEP, purified EI, HPr and IIA^{\text{gel}}}\). 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 \(IIA^{\text{gel}}\).

**Fig. 2.** \(BglF\) mutated in site 1, but not in site 2, is dephosphorylated by \(\beta\)-glucosides. The various \(BglF\) derivatives (wild-type, H547R and C24S) were overproduced in \(ZSC112\Delta G\), a \(pstG\) strain. Membranes were incubated with \(\text{[32P]PEP, purified EI, HPr and IIA^{gel}}\). The mixtures were incubated further with (+) or without (−) 0.2% salicin for 5 min. Samples were analysed by SDS–PAGE followed by autoradiography.

Phosphorylation of \(BglF\) and \(BglG\) proteins by \(\beta\)-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 \(\text{[32P]PEP, purified EI and HPr to donate a phosphoryl group to the } \beta\text{-glucoside salicin. As seen in Figure 2, the site 1 mutant protein (H547R), once phosphorylated by } II^{\text{gel}}, \text{ behaves like wild-type } BglF \text{ 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).}

\(II^{\text{gel}}, \text{ which is present in our membrane preparations in a significant amount (as demonstrated by Western blot analysis using monoclonal antibodies raised against this protein, data not shown), was reported to be phosphorylated by the } II^{\text{gel}} \text{ domain of } BglF \text{ (Vogler et al., 1988; Schnetz et al., 1990). It can therefore lead to some dephosphorylation of } BglF, \text{ which is independent of } \beta\text{-glucosides, due to the } \text{the presence of residual glucose contamination, detected occasionally in commercial salicin. To avoid this complication, the membranes containing the various } BglF \text{ derivatives were prepared from strain } ZSC112\Delta G, \text{ which is mutated in the } ptsG \text{ gene encoding } II^{\text{gel}}. \text{ This strain expresses the } crr \text{ gene at a relatively lower level (P.W. Postma, unpublished data). Therefore, to ensure phosphorylation of site 2 of the } H547R \text{ mutant, } II^{\text{gel}} \text{ was included in the phosphorylation reaction. Our conclusion from the results presented in this section is that our mutants behave as expected with regard to sugar phosphorylation (i.e. only the one that contains an intact second phosphorylation site can transfer the phosphoryl group to the sugar) and should thus serve as a reliable tool to study phosphorylation reactions catalysed by } BglF.\)
Q. Chen et al.

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 35S-labelled sample of BglG. Molecular masses of protein standards are given in kilodaltons. Arrowheads indicate the positions of BglF and BglG.

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\textsubscript{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\textsuperscript{glc}.

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 \textit{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\textsuperscript{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\textsuperscript{glc} can deliver the phosphoryl group to BglG (see Figure 5, lane 6).

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

4620
**Table I.** Plasmid-encoded BglF mutated in site 1, but not in site 2, can complement bglF strains and enable β-glucoside utilization

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Plasmid-encoded BglF derivative</th>
<th>Complementation of bglF mutant strainsa</th>
<th>MA231</th>
<th>AE304-1</th>
<th>AE304-2</th>
<th>AE304-4</th>
<th>PPA543 (IIA^β-), II^βB^b</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBR322</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pMN5</td>
<td>wild-type</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pCQ-F</td>
<td>wild-type</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>pCQ-F1</td>
<td>H547R</td>
<td>+</td>
<td>+ +</td>
<td>+ +</td>
<td>+ +</td>
<td>+ +</td>
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</tr>
<tr>
<td>pCQ-F2</td>
<td>C24S</td>
<td></td>
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</tr>
</tbody>
</table>

aComplementation 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.

bThe crr and nag genes of strain PPA543 were mutated (see Materials and methods). This strain is thus deficient for the IIA^γc and II^B^β^B proteins. Strains PPA546 and PPA547 that also carry mutations in these genes behaved as PPA543 (not shown).

cThe 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^γc. 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^γc 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 slightly higher level of IIA^γc 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 bglF–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
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-methylglucoside). 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\textsubscript{bgl} and II\textsubscript{a\textsuperscript{ag}}, which can substitute for IIA\textsubscript{bgl} (and thus complement for mutations in this domain of BglF), we constructed two strains, PPA546 and PPA547, which are defective in their \textit{crr} and \textit{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 \textit{in vivo}. IIA\textsubscript{bgl} 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\textsubscript{bgl} as expected, and very likely by II\textsubscript{a\textsuperscript{ag}} 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\textsubscript{bgl}, which is homologous to the IIA\textsubscript{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 BglG or to BglG. However, a mutation in the first phosphorylation site of BglF, His547, which is complemented by IIA\textsubscript{bgl} as expected, and very likely by II\textsubscript{a\textsuperscript{ag}} as well.

In order to study BglG regulation in a background deficient for IIA\textsubscript{bgl} and II\textsubscript{a\textsuperscript{ag}}, which can substitute for IIA\textsubscript{bgl} (and thus complement for mutations in this domain of BglF), we constructed two strains, PPA546 and PPA547, which are defective in their \textit{crr} and \textit{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).

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

<table>
<thead>
<tr>
<th>Straina</th>
<th>Plasmid</th>
<th>Plasmid-encoded BglF derivative</th>
<th>β-galactosidase activity (U)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>βMG\textsuperscript{b}</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>–</td>
</tr>
<tr>
<td>MA200-1</td>
<td>pMN5</td>
<td>Wild-type</td>
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</tr>
<tr>
<td></td>
<td>pCQ-F1</td>
<td>H547R</td>
<td>6</td>
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<td></td>
<td>pCQ-F2</td>
<td>C24S</td>
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</tr>
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<td>wild-type</td>
<td>5</td>
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<tr>
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<td>pCQ-F1</td>
<td>H547R</td>
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</tr>
<tr>
<td></td>
<td>pCQ-F2</td>
<td>C24S</td>
<td>68</td>
</tr>
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<td>wild-type</td>
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<tr>
<td></td>
<td>pCQ-F2</td>
<td>C24S</td>
<td>85</td>
</tr>
</tbody>
</table>

\textsuperscript{a} MA200-1 is Bgl\textsuperscript{+} and it carries a \textit{bgl}–\textit{lacZ} transcriptional fusion. PPA546 and PPA547 are derivatives of MA200-1 but their \textit{crr} and \textit{nagE} genes were mutated.

\textsuperscript{b} 10 mM β-methylglucoside (βMG) were added to the growth medium when indicated.

\textsuperscript{c} 10 mM salicin were added to the growth medium when indicated.
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^Bgl^ and IIa^Glc^ 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 EIIIs 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 EIIIs were suggested to exist in various organisms. Based on predicted amino acid sequence homology to the Bgl proteins and nucleotide sequence homology to the *eis* 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 EIIIs. BglIP and SacX from *Bacillus subtilis* are examples of proteins that were suggested to perform similarly to BglF. BglIP, 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 EIIIs 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 *err* genes which code for HPr and
### Table III, Strains

<table>
<thead>
<tr>
<th>Strain and/or plasmid</th>
<th>Relevant genotype</th>
<th>Source, derivation or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K8</td>
<td>HisC trpR thi λ'</td>
<td></td>
</tr>
<tr>
<td>LM1</td>
<td>crr-1 man3 man1 nagE thi-1 his-1 argG6 metB gatT rpsL</td>
<td>C. Richardson (1981)</td>
</tr>
<tr>
<td>MC1061</td>
<td>hisd488 araR139 ΔarabACE-lee769 ΔlacX74 galU galK rpsL thi</td>
<td>Maniatis et al. (1989)</td>
</tr>
<tr>
<td>MA231</td>
<td>F' recA456 bglR bglF31 trpC::Tn5 lvO mota' lac</td>
<td>Mahadevan et al. (1987)</td>
</tr>
<tr>
<td>AE304-1</td>
<td>F' trpC::Tn10 bglF1 ΔlacX74 thi bglR11 (bglR::IS1) tss (T6) 10</td>
<td>Mahadevan et al. (1987)</td>
</tr>
<tr>
<td>AE304-2</td>
<td>As AE304-1 except bglF2 instead of bglF1</td>
<td>Mahadevan et al. (1987)</td>
</tr>
<tr>
<td>AE304-4</td>
<td>As AE304-1 except bglF3 instead of bglF1</td>
<td>Mahadevan et al. (1987)</td>
</tr>
<tr>
<td>MA200-1</td>
<td>F' bglF201 srl::Tn10 recA456 ΔlacX74 thi bglR11 (bglR::IS1) bglR7 bglG lacZ2 lacY7 bglF lacZ (bgl-lac)</td>
<td>Mahadevan et al. (1987)</td>
</tr>
<tr>
<td>CAG18468</td>
<td>smpC50::Tn10</td>
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<tr>
<td>CAG12077</td>
<td>zbe-280::Tn10</td>
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<td>PPA237</td>
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</tr>
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<tr>
<td>PPA501</td>
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</tr>
<tr>
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<tr>
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</tr>
<tr>
<td>ZSC112AG</td>
<td>ΔptsG::cat manZ gk-7 thi rpsL</td>
<td></td>
</tr>
<tr>
<td><strong>S. typhimurium</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L1J44</td>
<td>cplt-401 cysA1150F'198 (pots' ptsH' crr')</td>
<td>Saier and Feucht (1975)</td>
</tr>
</tbody>
</table>

**Abbreviation used**: Tet', tetracycline sensitivity; Tett', tetracycline resistance; Kan', kanamycin resistance; P1, phage P1.

**Plasmids**

Plasmids pT712 and pT713, containing the phase T7 late promoter, and plasmid pGPI-2, carrying the T7 RNA polymerase gene under control of the 2×C857 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 pT7CF-1 and pT7CF-F2 are derivatives of pT7OC-F that encode BglF with either the His547 mutated to Arg (His57R) or the Cys24 mutated to Ser (C24S) respectively (the procedure for site-directed mutagenesis is described below). Plasmid pMN5 carries the entire bglG gene cloned in PBR322 (Mahadevan et al., 1987). Plasmids pCQ-F1 and pCQ-F2 contain a 2099 bp HindIII- EcoRI fragment from pT7CF-1 (encoding the H547R mutant) or from pT7CF-F2 (encoding the C24S mutant) ligated to the 4330 bp HindIII- EcoRI fragment of pBR322 respectively. Plasmid pMBP-BglG, obtained from A. Wright, carries a fusion between the MadE gene and the entire bglG gene cloned downstream of the Pae 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 [35S]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 one or more 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 (Schafer, 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**

[5-35S]ATP (3000 Ci/mmol) was obtained from Rotem Industries LTD (Israel). [35S]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. [3P]PEP was prepared and separated from [3P]PAP as described before (Amster-Choder, et al., 1989). El, HPr and IIAβ were obtained from J.Reizer. Monoclonal antibodies against ICBβ were obtained from B.Ernst.

**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
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 pGPI-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^β^ and IIE^β^ respectively, was used as a host when preparing cellular fractions used in the *in vitro* phosphorylation system B. Membranes of the *E. coli* strain Z5C112A.G were used to study dephosphorylation of the various BglF derivatives in the presence of β-glucosides in vitro. Membrane fractions lacking BglG were prepared either from strain K38/pGPI-2/pT712 or from strain LM1/pGPI-2/pT712 and were used in control experiments in phosphorylation systems A or B respectively.

A soluble fraction from *S. typhimurium* L1414, which overproduces EI, HPr and IIA^β^, 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 MBP-BglG, and growth was continued with 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 amylene 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^β^ and IIE^β^ (see above). The *S. typhimurium* L1414 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^β^ and IIE^β^, 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 *psc* *E. coli* strain Z5C112A.G (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 [β-³²P]PEP and PLB buffer (50 mM Na₂HPO₄, pH 7.4, 0.5 mM MgCl₂, 1 mM NaF, and 2 mM dithiothreitol). IIA^β^ 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 Na₂HPO₄, 20 mM NaH₂PO₄ 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 MgCl₂) 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.

**[35S]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 [35S]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’-CTGATGCATA-GCCGCTACCGA-3’ and its complementary oligo, or 5’-ATCCGT-ATACGCCGTGATC-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^6^, 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 Z5C112A.G. 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**


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4627