Spatial and temporal organization of the *E. coli* PTS components

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The phosphotransferase system (PTS) controls preferential use of sugars in bacteria. It comprises of two general proteins, enzyme I (EI) and HPr, and various sugar-specific permeases. Using fluorescence microscopy, we show here that EI and HPr localize near the *Escherichia coli* cell poles. Polar localization of each protein occurs independently, but HPr is released from the poles in an EI- and sugar-dependent manner. Conversely, the β -glucoside-specific permease, BglF, localizes to the cell membrane. EI, HPr and BglF control the β -glucoside utilization (*bgl*) operon by modulating the activity of the BglG transcription factor; BglF inactivates BglG by membrane sequestration and phosphorylation, whereas EI and HPr activate it by an unknown mechanism in response to β -glucosides availability. Using biochemical, genetic and imaging methodologies, we show that EI and HPr interact with BglG and affect its subcellular localization in a phosphorylation-independent manner. Upon sugar stimulation, BglG migrates from the cell periphery to the cytoplasm through the poles. Hence, the PTS components appear to control *bgl* operon expression by ushering BglG between the cellular compartments. Our results reinforce the notion that signal transduction in bacteria involves dynamic localization of proteins.

The EMBO Journal (2010) 29, 3630–3645. doi:10.1038/emboj.2010.240; Published online 5 October 2010

Subject Categories: signal transduction; microbiology & pathogens

Keywords: bacterial polarity; bacterial sensory systems; cell poles; subcellular localization of proteins; transcription antitermination

Introduction

The phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS) controls preferential use of carbon sources in bacteria. It is involved in the uptake of a large number of energetically preferred sugars (PTS sugars), as well as in movement toward these carbon sources (chemotaxis), and in the regulation of many other metabolic

pathways (reviewed in Deutscher *et al.*, 2006). The basic composition of the PTS is similar in all species studied so far. It is comprised of the phosphocarrier proteins enzyme I (EI) and HPr, which are common to all PTS substrates (designated 'general PTS proteins'), as well as sugar-specific permeases, termed enzymes II (EIIs), which catalyse the transport of PTS sugars across the cytoplasmic membrane along with their simultaneous phosphorylation. The phosphate flux in PTS starts with a phosphoryl group, donated by PEP to EI, which then passes it via HPr to the various sugar-specific permeases. The PTS plays a crucial role in both global control mechanisms, such as carbon catabolite repression and inducer exclusion (Deutscher, 2008), as well as in specialized mechanisms that govern expression of sugar utilization genes in both gram-negative and gram-positive bacteria (reviewed in Lengeler and Jahreis, 2009).

Modulation of the various mechanisms by the PTS depends on the phosphorylation state of the PTS components, but does not necessarily involve phosphorylation of the components of these pathways. In fact, steric interactions between PTS components and auxiliary proteins have been demonstrated, including interactions of the soluble component of the glucose permease, IIA^{glc}, with adenylate cyclase, with permeases of non-PTS sugars and with glycerol kinase (Deutscher *et al.*, 2006). The diversity and complexity of the PTS activities raise the issue of the cellular distribution of the PTS components within the cell. On the basis of theoretical models for molecular crowding and *in vitro* measurements of the phosphate flux in PTS under conditions that presumably mimic intracellular conditions, it has been speculated that PTS enzymes form multiprotein complexes (Rohwer *et al.*, 1998). The structure of EI complexed with HPr and of HPr complexed with the soluble domain of EIIs, which accepts the phosphate from HPr, has been determined (Garrett *et al.*, 1999; Wang *et al.*, 2000), but the subcellular localization of these putative complexes remained unknown. EI and HPr have generally been regarded as freely diffusible cytoplasmic proteins, but there have been reports on the association of both proteins with the cytoplasmic membrane (Ghosh *et al.*, 1989; Ye and Saier, 1995; Dubreuil *et al.*, 1996). It has also been reported that EI is present as discrete foci whose subcellular localization depends on growth conditions (Patel *et al.*, 2004). The EII sugar permeases were always assumed to be localized around the cell periphery in association with the cytoplasmic membrane, an assumption that has been experimentally verified in the case of the mannitol permease (Maddock and Shapiro, 1993). These disparate observations concerning the localization of the PTS components led us to conduct a comprehensive study of their distribution within living *Escherichia coli* cells.

Among the specialized regulatory mechanisms that the PTS modulates, control of catabolic genes expression by a novel family of sensory systems, which are composed of PTS sugar permeases and transcriptional antiterminators, has been extensively studied (van Tilbeurgh and Declerck,

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Received: 13 March 2010; accepted: 6 September 2010; published online: 5 October 2010

2001; Amster-Choder, 2005). The paradigm for this family is the aromatic β -glucoside utilization system in *E. coli* K12, comprising a membrane-bound β -glucoside permease (BglF) and a transcription factor (BglG) (reviewed in Amster-Choder, 2005). Together, these two proteins control expression of the β -glucoside utilization operon (*bgl*) in the following way. In the absence of β -glucosides, BglF recruits BglG to the membrane, phosphorylates it and remains associated with it, maintaining it in an inactive state. In the presence of β -glucosides, BglG is dephosphorylated by BglF resulting in its release and subsequent dimerization. The non-phosphorylated BglG dimer binds to the emerging *bgl* transcript to prevent premature termination of transcription, thus leading to expression of the β -glucoside utilization genes. Notably, negative regulation of BglG by BglF involves, besides phosphorylation, their physical association near the membrane to form a pre-complex, apparently to control the timing of BglG activity (Lopian *et al*, 2003). We have recently shown that following the sugar-triggered dissociation of the BglF-BglG pre-complex, BglG requires the presence of both EI and HPr for activity. Activation of BglG by the general PTS proteins is not mediated by the BglF sugar permease and, unlike BglG homologues from gram-positive organisms, it does not require phosphorylation by HPr (Raveh *et al*, 2009), but the nature of the mechanism underlying BglG activation remained unknown. In gram-positive bacteria, HPr-mediated phosphorylation of BglG homologues drives dimer formation, thus modulating their RNA-binding activity (van Tilbeurgh and Declerck, 2001). We suggested that a similar mechanism operates in *E. coli*, that is, the general PTS proteins provoke a conformational change that stabilizes the active BglG dimer, albeit not via phosphorylation but perhaps by physical interaction (Raveh *et al*, 2009). Given the likelihood that EI and HPr occupy specific niches within the cell, we aimed at exploring also the nature and the subcellular site(s) of interaction between the PTS proteins and the BglG transcription factor.

In this paper, we show that both EI and HPr localize mainly near the cell poles. Polar localization of each protein is independent of the other and does not require their phosphorylation sites. Yet, localization of HPr is affected by EI, so that in the presence of PTS sugars, HPr is released to the cytoplasm, provided that EI or its N-terminal domain, which interacts with HPr, is present in the cell. IIA^{glc}, which accepts the phosphate from HPr and serves as a mediator between the general PTS proteins and a number of global regulatory pathways, is observed as spread evenly throughout the cell. Conversely, the β -glucoside PTS permease, BglF, is observed as a ring around the cell membrane. Both EI and HPr are shown to affect the subcellular localization of BglG by a mechanism that does not require the phosphorylation sites of EI, HPr or BglG. This effect on BglG subcellular localization is apparently implemented by physical interaction, as we also demonstrate that EI and HPr have the capacity to interact with BglG, both *in vitro* and *in vivo*, in a phosphorylation-independent manner. Finally, we show that following the addition of β -glucoside, BglG migrates from the membrane, where it was held by BglF prior to sugar stimulation, to the cell poles and is subsequently released to the cytoplasm concomitantly with HPr. Based on our results, we propose that the coordinated relocation of the BglG transcription factor from one cellular compart-

ment to another by the PTS components is a major feature in the mechanism underlying the control of BglG activity and, hence, in the expression of the sugar utilization genes.

Results

The general PTS proteins localize at the cell poles, whereas a PTS sugar permease localizes around the cell periphery

To study the subcellular localization of EI, the gene expressing the fluorescent mCherry protein was fused in-frame to the last codon of the *ptsI* gene in the chromosome of the *E. coli* strain MG1655. The chromosomally encoded EI-mCherry fusion protein was functional, as indicated by the ability of the engineered strain to grow on mannitol and to generate red colonies on MacConkey-mannitol plates. Fluorescence microscopy results indicated that EI-mCherry, expressed from a single-copy gene fusion at the *pts* locus on the chromosome, localizes as discrete foci near the cell poles regardless of growth phase (Figure 1A), growth temperature (not shown) or the growth medium used (rich or minimal medium, supplemented with PTS or non-PTS carbon sources; Supplementary Figure S1). The majority of cells contained a single focus near one pole, with a minority having a second weaker focus at the opposite pole (Supplementary Table SI).

Essentially, the same results were obtained when EI-mCherry was expressed from a multicopy plasmid under the control of the arabinose promoter (compare Figure 1B with A), except that a greater fraction of cells had two foci showing increased intensity, with a minority having a third weaker focus located at or near the site of cell division, destined to become a future pole (Supplementary Table SI). Apparently, the three-fold increase in EI level due to its expression from a plasmid (Supplementary Figure S2A) exposed additional minor foci of EI that could not be revealed at the chromosome-encoded level. Some plasmid-encoded EI was observed in the cytoplasm during stationary phase (Figure 1B, lower panel) or at higher overexpression levels (not shown), suggesting that the polar site might have limited occupancy. Formation and localization of EI-mCherry foci occurred even in the absence of other PTS components (Figure 2A, EI-mCherry expressed in Δ *pts* MG1655 cells). Importantly, when the mCherry protein itself was expressed in the same strain, it was evenly distributed throughout the cell (Figure 1C), indicating that EI directs the fluorescent protein to the poles rather than the mCherry component. Taken together, the results indicate that EI localizes at or near the poles consistently; expression of EI from a plasmid does not alter this localization pattern but allows for the detection of more subtle foci.

Based on theoretical models for molecular crowding and on *in vitro* studies, it has been speculated that the PTS enzymes form multiprotein complexes (Rohwer *et al*, 1998). As HPr is immediately downstream of EI in the phosphorylation cascade, we examined its subcellular localization using an HPr-mCherry fusion expressed in MG1655 Δ *pts* cells. The results presented in Figure 2B show that the pattern of HPr-mCherry localization resembles that of EI in that it forms foci primarily near the poles (a little more than half of the cells had one focus and the rest had two foci, with a minority having a third focus near the new forming pole, see Supplementary Table SI). As the MG1655 Δ *pts* cells produce no EI, recruitment of HPr to the poles is not mediated by EI.

Figure 1 The EI protein localizes to the *E. coli* cell poles. MG1655($\Delta ptsI$ -mCherry) cells, which express the EI protein fused to mCherry from the chromosome (A), MG1655 $\Delta ptsI$ cells containing a plasmid that encodes the EI-mCherry fusion (B), and MG1655 cells containing a plasmid that encodes just the mCherry protein (C) were grown in minimal medium supplemented with glycerol. (A, B) Pictures were taken when the cells reached the following optical density (O.D.₆₀₀): 0.1–0.2 (early log), 0.4–0.5 (mid log), 0.7–0.8 (late log) and 1–2 (stat., stationary phase). The mCherry proteins were observed by fluorescence microscopy (red, mCherry), and cells were observed with phase microscopy (grey, phase). An overlay of the signals from the fluorescence and phase microscopy is shown (grey and red, merge). Scale bar corresponds to 1 μm .

Of note, all mCherry-tagged EI and HPr proteins used in this study were functional, as evidenced by their ability to complement $\Delta ptsI$ MG1655 cells, when expressed with their partner protein, HPr and EI, respectively (see Supplementary

data). The relative levels of the tagged proteins were compared by immunoblot analysis (Supplementary Figure S2); the level of the plasmid-encoded mCherry-tagged EI was found to be approximately three times higher than when

Figure 2 EI and HPr localize to the cell poles independently, but the distribution of HPr in the cell is affected by EI. MG1655 Δpts (A, B and E, F), MG1655 (C, D) or MG1655 $\phi(ptsI-mCherry)$ (G) cells, containing plasmids that encode EI-mCherry (A, C), HPr-mCherry (B, E), EI-mCherry and HPr (E), HPr-mCherry and EI (F) and HPr-GFP (G), were grown in rich medium. The mCherry and GFP fusion proteins were observed by fluorescence microscopy (red, mCherry; green, GFP), and cells were observed with phase microscopy. Overlays of the signals from the fluorescence and phase microscopy (grey and red, merge1) or of the GFP and mCherry fluorescent signal (green and red, merge3) are shown. DNA was visualized with DAPI stain, and overlays of the signals from the mCherry and DAPI are shown (red and blue, merge2). Scale bar corresponds to 1 μm .

expressed from the chromosome (compare lanes 1 and 4 in Supplementary Figure S2A and see explanation in the figure legend), whereas the levels of the plasmid-encoded EI and HPr were comparable (Supplementary Figure S2B, lane 5), similar to the native situation.

To determine whether the general PTS proteins affect each other's localization, we compared the localization pattern of EI and HPr in cells expressing each protein individually to the pattern that is obtained when the two proteins are co-expressed. For this purpose, we expressed EI-mCherry or

HPr-mCherry in MG1655 Δpts cells (Figure 2A and B) and in the parental MG1655 strain, in which the endogenous PTS proteins are expressed from the chromosomal *pts* operon (Figure 2C and D). In addition, we expressed EI-mCherry or HPr-mCherry from a plasmid together with HPr or EI (not tagged with mCherry), respectively, in MG1655 Δpts cells (Figure 2E and F). The EI-mCherry was detected almost exclusively at the poles in all backgrounds, regardless of whether HPr was also expressed (Figure 2A, C and E). Hence, EI polar localization is not dependent on the *pts*

context of the cell. The results with HPr were somewhat different; HPr was observed near the poles in all three backgrounds, but, whereas in cells lacking EI, it was confined to the poles area (Figure 2B), in cells expressing EI either from the chromosome or from a plasmid, a fraction of HPr was distributed throughout the cell (Figure 2D and F, respectively; and Supplementary Figure S3A). The possibility that the partial dispersion of the fluorescent signal in the presence of EI stems from free mCherry liberated due to clipping of HPr-mCherry was ruled out by immunoblot analysis (Supplementary Figure S2A and B, compare lane 5 with lane 3). An overlay of the DNA stain (4,6-diamidino-2-phenylindole (DAPI)) and the fluorescent signals, shown in Figure 2, indicates that the location of the PTS proteins does not overlap with the DNA, that is, they are confined to the polar regions that are depleted of DNA. To further examine EI and HPr subcellular localization, we co-expressed a green fluorescent protein (GFP)-tagged HPr and mCherry-tagged EI, the latter being expressed either from the chromosome or from a plasmid. The results, shown in Figure 2G and Supplementary Figure S4, substantiate our finding that although the two proteins localize similarly near the poles, most probably to the same structure, they are recruited to the poles independently. This conclusion is not based on the incomplete overlap between the GFP-HPr and the mCherry-EI foci, which could be due to chromatic aberrations or slight movement of complexes between image takings, but, rather, on the observation that in some cells, the stronger GFP focus and the stronger mCherry focus localize to different poles (Supplementary Figure S4B and C). The differences between the GFP- and mCherry-tagged proteins were less noticeable when both are expressed from a plasmid (Supplementary Figure S4D), unless their level of expression was reduced (Supplementary Figure S4E). Additionally, the localization of GFP-HPr, like that of the mCherry-HPr, is affected by expression of EI in the cell, that is, when expressed in Δpts cells, HPr-GFP is mostly confined to the poles (Supplementary Figure S4A), but in cells expressing also EI, the HPr-GFP is also detected in the cytoplasm (Figure 2G; Supplementary Figure 4SB–E). The results presented thus far indicate that EI and HPr are recruited to the cell poles in the absence of each other. Nevertheless, the pattern of HPr distribution in the cell is affected by EI. Numerous controls corroborated these results, which were highly reproducible.

To extend our study of the subcellular localization of the PTS components, we aimed at looking at PTS sugar permeases. The subcellular localization of PTS permeases was directly observed only in one case (Maddock and Shapiro, 1993) and never in live cells. We chose to look at a typical sugar-specific PTS permease, BglF, which has an integral membrane domain that catalyses the concomitant transport and phosphorylation of β -glucosides. BglF was shown to purify with the membrane fraction (Amster-Choder *et al*, 1989), but its localization pattern was never directly examined and, hence, its confinement to the polar membrane regions could not have been ruled out. To address this issue, we fused the *bglF* gene, encoding the β -glucoside-specific permease, to GFP and expressed the fusion protein in MG1655 pts^+ cells together with either EI-mCherry or HPr-mCherry. The GFP-tagged BglF protein was functional, as evidenced by its ability to complement *bglF*[−] cells and enable them to use β -glucosides. Fluorescence microscopy of the

above strains (Figure 3, upper panels) showed that BglF-GFP was localized around the periphery of the cell, perhaps with slight preference for the poles. Notably, the level of BglF-GFP in the cells shown here was comparable to the level encoded by the chromosomal *bgl* operon after β -glucoside induction (Supplementary Figure S2C, compare lane 2 with lane 1; for quantifications see legend). We also examined the subcellular localization of IIA^{glc}, which is encoded by the third gene of the *pts* operon, *crr*, by fusing it to GFP. The IIA^{glc}-GFP was evenly spread in the cell (Supplementary Figure S5).

The picture that emerges from the results presented thus far on the subcellular localization of the PTS components is the following: EI, which starts the PTS phosphorylation cascade, clusters at the cell poles; HPr, which is phosphorylated by EI, is detected both at the poles and throughout the cell; IIA^{glc}, which is phosphorylated by HPr, is evenly spread in the cell; and the sugar-specific PTS permeases, also phosphorylated by HPr, localize with the cell membrane.

Recruitment of the EI and HPr to the poles is phosphorylation independent, but the stimulus-induced release of HPr from the poles requires HPr phosphorylation site

To explore the role of phosphorylation in the distribution of the general PTS proteins, we examined the effect of mutations in EI and HPr, which eliminate phosphorylation, on their distribution in the cell. For this purpose, we fused the genes encoding the HPr(H15A) and EI(H189A) mutant proteins to the mCherry gene and introduced the plasmids expressing these fusion proteins into MG1655. Fluorescence microscopy of these cells indicated that both mutant proteins localize to polar foci (Figure 4A and B). Hence, recruitment of EI and HPr to the poles is phosphorylation independent.

Next, we attempted to identify the domain of EI, which is responsible for its polar localization. EI is composed of two discrete domains that fold independently, the N-terminal domain (EI-N), which contains the phosphorylation site and interacts with HPr, and the C-terminal domain (EI-C), which binds to PEP and mediates dimerization (LiCalsi *et al*, 1991; Oberholzer *et al*, 2005; Teplyakov *et al*, 2006). We fused each of the EI domains separately to mCherry and observed their localization in the cell. As shown in Figure 4D, the C-terminal domain of EI localizes to the cell poles. In contrast, the N-terminal domain, which contains the phosphorylated histidine, does not localize to the poles and, instead, is spread out in the cytoplasm (Figure 4C). Thus, not only is EI's phosphorylatable residue unnecessary for EI polar localization, but the domain that directs EI to the poles lacks the phosphorylation site.

When both EI and HPr proteins were expressed in the cell, a portion of the HPr molecules was spread throughout the cell (see Figure 2D and F). A competition between EI and HPr for a common polar cue could account for such a distribution. However, the exclusive localization of HPr(H15A) mutant protein near the poles in pts^+ cells (Figure 4A) indicates that the two proteins do not compete for polar recruitment, as the mutant HPr protein is fully accommodated at the poles together with wild-type EI. Based on these results, we conclude that EI allows the release of some HPr from the poles, provided that HPr has its phosphorylation site. To further examine the EI-stimulated release of HPr from the poles, we expressed HPr-mCherry together with each of the EI domains

Figure 3 HPr is released from the cell poles upon addition of a PTS sugar. Fluorescence microscopy images of cells expressing BglF-GFP and HPr-mCherry (A) or BglF-GFP and EI-mCherry (B) (green, GFP; red, mCherry; green and red, merge). Pictures were taken before (t0) and 5, 15 and 30 min after the addition of 1% arbutin (t5, t15 and t30). The GFP fluorescent signals were two-dimensional deconvolved. Scale bar corresponds to 1 μ m.

in a Δ pts background. When HPr was expressed with EI-N, it was observed both at the poles and in the cytoplasm (Figure 4E), but when co-expressed with EI-C, it was detected only in polar foci (Figure 4F). Hence, the N-terminal domain of EI alone, which contains the phosphorylation site and interacts with HPr, can lead to the release of HPr from the poles, although at a somewhat decreased efficiency than intact EI (Supplementary Figure S3, compare A and B). Together with the requirement for HPr phosphorylation site, these results suggest that phosphorylation of HPr by EI underlies HPr release from the poles. Although EI-N does not bind to PEP and, hence, is not autophosphorylated, cross-phosphorylation with EI/HPr homologues is possible (see Discussion). Alternatively, an interaction between HPr and EI-N, which involves the HPr phosphorylation site, that is, H15, but not phosphorylation *per se*, triggers HPr release from the poles.

What are the signals that cause HPr to leave the cell poles? As the cascade of phosphorylation reactions in the PTS is induced in the presence of PTS sugars, we tested the cellular distribution of HPr, as well as of the other components in this cascade, following sugar stimulation. We grew MG1655 cells expressing HPr-mCherry together with the BglF-GFP, or EI-mCherry and BglF-GFP, in a minimal medium lacking PTS sugars. We then followed the localization of the fluorescent proteins at 5-min intervals, after the addition of a β -glucoside sugar (arbutin). The results are presented in Figure 3. In the absence of arbutin (t0), BglF was detected as a ring around

the periphery of the cell, EI was in its typical polar location and HPr was mainly at the poles (Figure 3A and B, upper panels). With time, the fraction of HPr that is distributed throughout the cell increased (t15) until most cells lacked polar HPr foci (t30) (Figure 3A). Dispersion of the fluorescent signal due to HPr-mCherry clipping in the presence of the sugar was ruled (Supplementary Figure S2A and B). In contrast, the localization of BglF and EI did not change following sugar stimulation (Figure 3B). The release of HPr from the poles was observed also after stimulating cells expressing HPr-Cherry with other PTS sugars, such as glucose (not shown). Similar results were obtained with cells expressing GFP-tagged HPr (Supplementary Figure S4, compare B and C). These results imply that upon addition of PTS sugars, HPr is released from the poles and starts to spread throughout the cell. Taken together, the release of HPr from the poles is induced by sugar stimulation and requires EI-N.

The PTS proteins affect the subcellular localization of the BglG transcription factor in a phosphorylation-independent manner

We have recently shown that both EI and HPr are essential for the activity of BglG, which positively regulates expression of the β -glucoside utilization operon by preventing premature termination of transcription (Raveh *et al*, 2009). Activation of BglG by the general PTS proteins does not require phosphorylation of BglG nor is it mediated by the BglF sugar permease. In the light of the present information on EI and HPr

Figure 4 Recruitment of the PTS proteins to the poles is phosphorylation independent, but the release of HPr from the poles requires its phosphorylation site. (A–D) Images of MG1655 cells expressing the following proteins fused to mCherry: HPr(H15A) (A), EI(H189A) (B), EI-N (C) and EI-C (D). (E, F) Images of MG1655Δ*pts* cells containing a plasmid, which encodes HPr-mCherry and either EI-N (E) or EI-C (F). The mCherry fusion proteins were observed by fluorescence microscopy (red, mCherry), and the cells were observed with phase microscopy (grey, phase). Also, an overlay of the signals from the fluorescence and phase microscopy is shown (grey and red, merge). Scale bar corresponds to 1 μm.

localization, we decided to examine the subcellular localization of BglG. When expressed in MG1655 Δ*pts* cells, BglG fused to GFP was evenly distributed in the cytoplasm

(Figure 5A). However, when co-expressed with either EI or HPr fused to mCherry, a significant amount of the BglG-GFP was detected at the poles together with the general PTS proteins (Figure 5B and C, respectively; see quantification in Supplementary Figure S6A). Similar results were obtained when BglG-GFP was co-expressed with EI or HPr that were not fused to mCherry (not shown). These results indicate that the localization of BglG is affected by each of the general PTS proteins, and suggests that BglG associates with the PTS proteins at the cell poles. When BglG was co-expressed with both EI and HPr, one of them fused to mCherry (Figure 5D and E), the general pattern of BglG cellular distribution was similar, although the polar fraction was slightly reduced (compare Figure 5D and E with B and C, respectively; see quantification in Supplementary Figure S6B). This pattern of BglG localization correlates with the normal distribution of the co-expressed EI (polar) and HPr (polar and spread). DAPI-stain images indicated again that the location of the polar foci does not overlap with the DNA (not shown). When expression of EI and HPr was not induced (no arabinose added), no PTS proteins were detected and BglG-GFP was evenly distributed in the cell (not shown). Taken together, each PTS protein by itself affects BglG localization.

To determine whether phosphorylation is required for the ability of the PTS proteins to control BglG localization, we examined the effect of mutant EI and HPr proteins, which lack phosphorylation sites, as well as of the individual EI domains on BglG localization. To this end, BglG-GFP was co-expressed in MG1655 Δ*pts* with one of the following proteins fused to mCherry: EI(H189A), HPr HPr(H15A), EIN or EIC. The observed co-localization of BglG with HPr(H15A) (Figure 5G) indicates that phosphorylation of BglG by HPr is not required for recruitment of BglG to the poles. Notably, BglG does not localize to the poles when co-expressed with EI(H189A) (Figure 5F). However, this is apparently not due to the requirement for phosphorylated EI to recruit BglG to the poles, as BglG co-localizes at the poles with the C-terminal domain of EI, which does not contain the phosphorylation site H189 (Figure 5I). In the presence of EI's N-terminal domain, which does not localize to the poles (see Figure 4C), BglG spreads out in the cytoplasm (Figure 5H). Hence, not only is EI phosphorylation domain dispensable for recruitment of BglG to the poles, but the domain in charge of BglG recruitment does not contain the phosphorylation site. Detection of a fraction of BglG-GFP at the poles of cells that express HPr and EIC or HPr and EIN (not shown) demonstrates that BglG does not co-localize with EIN, and that the latter does not prevent recruitment of BglG to the poles.

Next, we asked whether phosphorylation of BglG participates in the mechanism that underlies its recruitment to the poles by the general PTS proteins. For this purpose, we used BglG derivatives that are mutated in the conserved histidines at position 160 or 208, which are required for BglG phosphorylation (Amster-Choder *et al*, 1989; Chen *et al*, 1997b; van Tilbeurgh and Declerck, 2001). These mutations do not affect the intrinsic tendency of BglG to spread out in the cytoplasm, as indicated by the even distribution of the mutant BglG proteins, BglG(H160Y) and BglG(H208R), each fused to GFP, in the cytoplasm of MG1655 Δ*pts* cells (Supplementary Figure S7-I and S7-II, panel A, respectively). When BglG(H160Y)-GFP or BglG(H208R)-GFP was co-expressed with wild-type,

Figure 5 EI and HPr affect BglG subcellular localization in a phosphorylation-independent manner. (A) Fluorescence microscopy (green, left), FM4-64 staining (red, middle) and overlay (merge, right) images of MG1655 Δpts cells expressing BglG-GFP. (B–I) Fluorescence microscopy images of MG1655 Δpts cells containing a plasmid that encodes BglG-GFP and a second plasmid encoding EI-mCherry (B), HPr-mCherry (C), EI-mCherry and HPr (D), HPr-mCherry and EI (E), EI(H189A)-mCherry (F), HPr(H15A)-mCherry (G), EI-N-mCherry (H) and EI-C-mCherry (I) (green, GFP; red, mCherry; green and red, merge). Scale bar corresponds to 1 μ m.

mutant or domain derivatives of either HPr or EI, all fused to mCherry, in MG1655 Δpts , the localization patterns were the same as with wild-type BglG (compare Supplementary Figure S7-I and S7-II, panel B-I with Figure 3, panel B-I). Therefore, BglG phosphorylation is not a prerequisite for its co-localization with the general PTS proteins. Taken together, phosphorylation of neither the general PTS proteins nor BglG is required for recruitment of BglG to the cell poles.

EI and HPr interact with the BglG transcription factor *in vitro* and *in vivo*

Activation of BglG by the general PTS proteins in a phosphorylation-independent manner (Raveh *et al*, 2009) raised the possibility that the regulatory mechanism underlying the activation involves physical interaction between BglG and one of the PTS proteins or both. The fluorescence microscopy results, presented thus far, suggested that indeed BglG associates with EI and HPr in the cell. This hypothesis was supported by the co-purification of EI with BglG from cellular extracts. In brief, His-tagged BglG was expressed in *E. coli* cells at a low level, and BglG-bound proteins/complexes were

purified on a nickel column and analysed by SDS-PAGE alongside proteins that were purified from cells expressing a His-tagged irrelevant peptide, but no BglG (control) (see Supplementary data). Proteins detected only in BglG-expressing cells were isolated from the gel, and their identity was determined by mass spectrometry. EI repeatedly co-purified with BglG in this pull-down assay. Of note, the gel from which the proteins were isolated was unsuitable for detecting small proteins such as HPr. These observations encouraged us to investigate the nature of the association of BglG with the general PTS proteins.

First, we asked whether BglG has the capacity to directly bind to HPr and/or to EI *in vitro* by using the Far-western technique. Purified BglG fused to maltose-binding protein (MBP-BglG) was subjected to SDS-PAGE and blotted onto a nitrocellulose filter; the membrane was then incubated with purified His-tagged HPr or with His-tagged EI and then with antibodies against the His tag. A strong signal was detected with both His-HPr and His-EI (Figure 6A and B, lane 1, respectively). When MBP alone was probed with His-HPr or His-EI, no binding was observed (Figure 6A and B, lane 7,

Figure 6 Far-western analysis of the interaction between BglG and the general PTS proteins. The following purified proteins were fractionated by SDS-PAGE: BglG (lane 1), PRD1 (lane 2), PRD2 (lane 3), BglG(D100N) (lane 4), BglG(H160Y) (lane 5), BglG(H208R) (lane 6) and MBP (lane 7). The proteins were blotted onto a nitrocellulose filter and probed with the following His-tagged proteins: HPr (A), EI (B), HPr(H15A) (C), EI(H189A) (D), EI-N (E) or EI-C (F) and then with anti-His antibodies (A–F), or stained with Coomassie blue (G).

respectively), indicating that HPr and EI interact with BglG and not with the MBP moiety of MBP-BglG. We then asked to which domain of BglG would each one of the PTS proteins prefer to bind. BglG and its homologues consist of an RNA-binding domain followed by two homologous domains, PRD1 and PRD2 (PTS-regulation domains) (van Tilbeurgh and Declerck, 2001). Therefore, we probed filter-immobilized MBP-PRD1 and MBP-PRD2 with either His-HPr or His-EI and then with antibodies against the His tag. A strong interaction between EI and the PRD2 domain of BglG was observed (Figure 6B, lane 3), but the combination of HPr and either the PRD1 or PRD2 domain of BglG showed almost no interaction (Figure 6A, lanes 2 and 3). These results imply that BglG has the capacity to directly interact with each of the general PTS proteins. Although the interaction with EI is via the PRD2 domain of BglG, HPr recognizes and binds to intact BglG.

To examine whether the PTS proteins bind to BglG *in vivo*, we used the LexA-based bacterial two-hybrid system (Dmitrova *et al*, 1998). In this system, the proteins of interest are fused either to the wild-type LexA repressor DNA-binding domain (WT LexADBBD), or to LexADBBD with an altered specificity (mutant LexADBBD), and introduced into a strain that harbours a chromosomal copy of *lacZ* under the control of a LexA-hybrid operator. Transcriptional repression is achieved upon co-expression of both hybrid proteins, provided that they bind to each other. The hybrid proteins were expressed in a Δpts background, in which there is no expression of the general PTS proteins from the chromosome and the results are presented in Table I. The leucine zipper domains of Fos and Jun fused to the WT and mutant

LexADBBD, respectively, served as a positive control (99% repression), and the two LexADBBD served as a negative control (0% repression). The combination of EI or HPr fused to WT LexADBBD and BglG fused to mutant LexADBBD resulted in a heterodimer formation, as indicated by the 70% or 50% transcriptional repression, respectively. The combination of EI with the individual PRD domains showed that EI interacts only with the PRD2 domain (65% repression with PRD2, compared with 0% repression with PRD1). However, the combination of HPr with either one of the PRD domains showed no interaction (0% repression with PRD1 or PRD2). Taken together, the results obtained *in vivo*, which are in complete agreement with the *in vitro* results, indicate that BglG can interact directly with either one of the general PTS proteins; the BglG domain that interacts with EI was identified by both methods as PRD2, whereas HPr requires intact BglG for the interaction.

Interaction of BglG with the general PTS proteins does not require the conserved phosphorylation sites on the interacting partners

To examine whether the residues that are conserved among BglG-like antiterminators, which are required for their regulation by phosphorylation (Amster-Choder *et al*, 1989; Chen *et al*, 1997b; van Tilbeurgh and Declerck, 2001), are required for the interaction with the PTS proteins, we applied the two methodologies described above. Using the Far-western technique, we found that mutations in the three conserved residues, D100 and H160 in PRD1 and H208 in PRD2, did not preclude the interaction of BglG with either HPr or EI *in vitro* (Figure 6A and B, lanes 4–6, respectively). The effect of the above mutations on BglG–HPr and BglG–EI binding *in vivo* was examined using the LexA-based two-hybrid system. As shown in Table I, all three BglG mutant proteins repressed transcription, similar to WT BglG, when co-expressed with either PTS protein. Taken together, the conserved residues in BglG, which are required for its regulation by phosphorylation, are not required for the interaction of BglG with HPr or EI. These results are in accord with the lack of effect of replacement of these residues on co-localization of BglG with HPr and EI at the poles (see above).

To test whether the phosphorylation sites on HPr and EI are required for the interaction with BglG, we examined the interaction of BglG with the general PTS proteins mutated in their phosphorylation sites. To this end, we probed filter-immobilized purified BglG proteins (WT, PRD domains or mutants) with His-HPr(H15A) or His-EI(H189A) and anti-His antibodies. The pattern of interaction of HPr(H15A) with the BglG proteins was similar to that of wild-type HPr, that is, HPr(H15A) reacted with wild-type BglG, as well as with its three mutant derivatives, but not with the individual PRD domains (Figure 6C). In contrast to HPr, no signal was observed when EI(H189A) was probed with any of the BglG proteins (Figure 6D). This lack of interaction might suggest that EI phosphorylation site is required for the interaction with BglG. Alternatively, the conformation of this mutant EI protein has a strong effect on the interaction with BglG. To discern between these possibilities, we asked whether EI phosphorylation domain is crucial for the interaction with BglG. To address this question, filter-immobilized BglG proteins (WT, PRD domains or mutants) were probed either with the EIN domain or with EIC. When probing with EIC, the

Table I Analysis of the interaction of the general PTS proteins and their derivatives with BglG and its derivatives by the two-hybrid LexA-based system^a

BglG protein fused to mutant repressor ^c	PTS protein fused to WT repressor ^b Repression ^d of <i>PlacUV5^e-lacZ</i> (%)					
	HPr	EI	HPr(H15A)	EI(H189A)	EI(Nter)	EI(Cter)
WT	50	70	55	7	0	68
PRD1	0	0	16	0	1	7
PRD2	0	65	25	11	16	65
D100N	58	63	31	16	15	37
H160Y	49	65	52	6	7	56
H208R	45	60	55	2	10	64

^aThe experiment was performed with an *E. coli* SU202 Δ pts strain, which carries a hybrid LexA operator op408/op+::lacZ fusion on its chromosome (Dmitrova *et al*, 1998). The negative control, performed with pMS604 and pDP804 that express wild-type and mutant LexADBBD, respectively, yielded 0% repression of *P_{lacUV5}-lacZ*. The positive control, performed with Fos and Jun leucine zipper fused to wild-type and mutant LexADBBD, respectively, yielded 99% repression of *PlacUV5-lacZ*. All values represent the average of at least four independent measurements. The s.d. ranged from 2 to 5% repression.

^bCloned in pLL3, a derivative of pMS604, see Supplementary data.

^cCloned in pLL1, a derivative of pDP804, see Supplementary data.

^dPercent repression was calculated as 1-(β -galactosidase activity with repressor/ β -galactosidase activity without repressor).

^eA modified *PlacUV5* bearing a mutation, which decreases the level of expression in the presence of inducer IPTG (Dmitrova *et al*, 1998).

pattern of reaction was similar to the one observed with intact EI protein, that is, all BglG proteins, except for the PRD1 domain, reacted with both EI and its C-terminal domain (Figure 6F, compare with Figure 6A). In contrast, when EIN was incubated with the BglG derivatives, no binding was observed (Figure 6E). These results imply that the C-terminal domain of EI, which does not contain the phosphorylation site, mediates the interaction with BglG.

An interaction between HPr(H15A) and BglG, as well as its three mutants, was demonstrated also *in vivo* (Table I), implying once again that phosphorylation of BglG by HPr is not required for the interaction between the proteins. Again, the combination of HPr(H15A) with the BglG domains yielded low levels of interaction. There was a correlation between the intensity of the interaction of HPr(H15A) with the BglG mutants observed *in vitro* and the degree of interaction *in vivo*, that is, the interaction of HPr(H15A) with BglG(D100N) was somewhat reduced compared with the interaction with the other BglG mutants. Based on the *in vitro* and *in vivo* results, we can conclude that the phosphorylation site of HPr is not required for BglG–HPr interaction. Similar to the results obtained *in vitro*, the combination of EI(H189A) or EIN with the BglG proteins in the two-hybrid system showed no interaction (Table I). The interaction pattern of EIC in combination with all the BglG proteins was similar to that seen with wild-type EI (Table I). The finding that the C-terminal domain of EI, which lacks the phosphorylation site, is the domain that mediates the interaction with BglG and is sufficient for this interaction implies that, like HPr, the phosphorylation site of EI is not required for BglG–EI interaction.

Taken together, the results obtained with the three experimental approaches, Far-western, two-hybrid and fluorescence microscopy, are in complete agreement. The conclusions that emerge are that both EI and HPr interact with BglG and that the phosphorylation sites of the interacting partners are dispensable for this interaction.

Sugar stimulation triggers BglG migration from the cell membrane through the poles to the cytoplasm

The experiments described above, which demonstrated that BglG localizes with the general PTS proteins at the cell poles,

were performed using strain MG1655, in which the *bgl* operon is not expressed (wild-type *E. coli* strains are *bgl*⁰; Schaefer, 1967). Hence, BglF, the β -glucoside permease and BglG-negative regulator, is not expressed in these cells. Previously, we have shown that BglG is recruited to the membrane by BglF in a PTS-dependent manner and is released to the cytoplasm following the addition of β -glucosides (Lopian *et al*, 2003). To reconcile these findings and to elucidate the sequence of events, in terms of BglG subcellular localization, we co-expressed the two Bgl proteins (BglG-GFP and BglF) together with both general PTS proteins, only one tagged with mCherry (i.e. EI-mCherry and HPr or HPr-mCherry and EI) and followed BglG localization in the absence of the stimulating sugar or after the addition of the β -glucoside sugar (arbutin) at 5-min intervals. In the absence of arbutin, BglG was detected mainly as a ring around the periphery of the cell, whereas EI and HPr were in their typical polar location (Figure 7A and B, t0). Five minutes after arbutin addition, BglG started to accumulate at the poles (Figure 7A and B, t5). Larger fragments from the fields shown in Figure 7, before and 5 min after arbutin addition, are shown in Supplementary Figure S8. The enrichment in BglG polar fraction 5 min after arbutin addition is quantitatively shown in Supplementary Figure S9. Fifteen minutes after arbutin addition, most of the BglG-GFP and HPr-mCherry were released to the cytoplasm, whereas EI remained at the poles (Figure 7A and B, t15). The level of both Bgl proteins in this experiment (Supplementary Figure S2C, lane 4; Supplementary Figure S2D, lane 3) was significantly lower than the levels encoded by the chromosomal *bgl* operon after β -glucoside induction (Supplementary Figure S2C and E, lane 1; for quantifications see legend). Keeping the same low-expression level for BglG, but increasing BglF level significantly (Supplementary Figure S2C, lane 6), slowed BglG release from the membrane and allowed the detection of BglG-GFP foci at the membrane after sugar stimulation till its accumulation at the poles (Supplementary Figures S10 and S11), illustrating the putative route of BglG from the cell periphery, where in association with BglF, towards the polarly localized EI and HPr. These results imply that in the absence of the stimulating sugar, BglG is engaged in a pre-complex with BglF near the membrane;

Figure 7 BglG migrates from the membrane periphery through the cell poles to the cytoplasm after addition of the stimulating sugar. Fluorescence microscopy images of MG1655 cells expressing BglG-GFP, BglF, HPr-mCherry and EI (**A**) or BglG-GFP, BglF HPr and EI-mCherry (**B**) (green, GFP; red, mCherry; green and red, merge) and phase-contrast images of the same cells (grey, phase). Pictures were taken before (t0) and 5, 15 and 30 min after the addition of 1% arbutin (t5, t15 and t30). The fluorescent signals of the BglG-GFP at t0 were two-dimensional deconvolved to reveal the localization of most BglG molecules. Scale bar corresponds to 1 μ m.

shortly after the addition of β -glucoside, BglG migrates to the poles, where the PTS proteins are present, and is subsequently released to the cytoplasm concomitantly with HPr.

Discussion

Cellular organization of the PTS

A central issue in current biology is how cell metabolism functions as a system according to nutrient availability. The bacterial PTS can be considered as a 'metabolic nervous system'. It integrates signals that are exerted by various carbon sources to enable hierarchical uptake of sugars and to adjust cell metabolism accordingly. Integration of these signals involves cross-talk between the PTS and various global and specialized signalling systems (Deutscher *et al*, 2006), but the strategy that underlies coordination of these well-controlled cross-talks is not known. The results presented here suggest that spatial organization of the PTS and auxiliary systems is a key element in this strategy. Such a strategy seems suitable for the intracellular environment of exponentially growing *E. coli* cells, which are crowded with macromolecules, mostly proteins, at high concentrations (Zimmerman and Trach, 1991) that apparently limit diffusion rates and create a need for co-localization of metabolic pathways (Ovadi and Saks, 2004). Formation of multiprotein metabolon by the PTS enzymes has been speculated (Rohwer *et al*, 1998) and assumed to improve PTS function (Norris *et al*, 1999), but its localization to the poles has not been foreseen. In fact, the embedding of PTS sugar permeases in the inner membrane made this compartment seem compatible for the association of the putative PTS complex. Reports on the association of the soluble EI and HPr with the cytoplasmic membrane (Ghosh *et al*, 1989; Ye and Saier, 1995; Dubreuil *et al*, 1996), as well as the presumed membrane anchor of IIA^{glc} (Wang *et al*, 2003; Meadow *et al*, 2006) supported this speculation.

In the current work, we studied the subcellular localization of the PTS proteins and the components of a sensory system, which is regulated by the PTS. Our results reveal a meticulous organization of the studied systems, which involves gathering of the general PTS proteins near the poles, as opposed to the membrane localization of the PTS sugar permeases, stimulus-triggered release of the small phosphocarrier protein HPr from the poles, and dynamic relocation of a PTS-regulated transcription factor from one cellular compartment to another (membrane, poles and cytoplasm), depending on sugar availability. A major feature of the overall organization of the *E. coli* PTS is the localization of the system's 'control centre' at or near the cell poles. Polar localization is an intrinsic property of each of the general PTS proteins, as EI and HPr localize to the poles independent of each other and their foci do not fully overlap. However, whereas EI remains at this location, HPr leaves the poles upon stimulation. Bearing in mind the diverse targets of HPr, its relocation seems like a requirement. Clearly, HPr needs to phosphorylate the IIA domains of sugar permeases, which are soluble, but are required to be also near the membrane to phosphorylate the IIB domains, which phosphorylate the incoming sugars. Our fluorescence microscopy images, showing HPr discharging from the poles and spreading throughout the cell upon sugar stimulation (Figure 3), are compatible with the

presence of a fraction of HPr near the membrane. In addition, HPr activates transcription regulators, such as BglG (Raveh *et al*, 2009), shown here to be released from the poles concomitantly with HPr. Another major target of HPr is the multifunctional protein IIA^{glc}, which was observed by us as spread throughout the cell, not precluding the possibility that a fraction of this protein is present near the membrane as well. We suggest that the small HPr protein acts as a messenger that shuttles between the control centre of the PTS at the poles and other cellular compartments. This is reminiscent of the small CheY regulator, which is phosphorylated by the chemotaxis polar complex and then diffuses to the flagella that are randomly distributed around the cell (Kentner and Sourjik, 2006).

EI was previously observed in polar, punctuate or diffuse distributions, depending on the growth conditions (Patel *et al*, 2004). Apparently, the different localization patterns resulted either from overexpression of the fluorescent EI fusion protein and/or from visualizing colony cells that were spread on slides in some of the experiments. In our experiments, EI was observed mainly at the poles at all growth conditions documented by Patel *et al*, but when expressed from a plasmid, EI could also be detected in the cytoplasm, especially during stationary phase, suggesting saturation of the target docking sites upon overexpression. Hence, there seems to be a limited occupancy for EI at the polar site, but this occupancy is sufficient for the chromosomally encoded EI level.

The recruitment of both general PTS proteins to the poles is phosphorylation independent. In contrast, the release of HPr from the poles requires its phosphorylation site, as indicated by the exclusive localization of HPr(H15A) mutant to the poles. EI N-terminal domain is sufficient to enable the release of HPr from the poles. EI-N was shown to recognize and to bind to HPr (Garrett *et al*, 1999) and reversible phosphoryl transfer between the two proteins has been demonstrated (LiCalsi *et al*, 1991). Although EI-N does not bind to PEP, the *E. coli* chromosome encodes five EI homologues and six HPr homologues (Tchieu *et al*, 2001) that can potentially transfer the phosphoryl group to EI-N. At least for one homologous system, phosphate exchange with the general PTS proteins was demonstrated (Powell *et al*, 1995). On the other hand, the fact that such cross-phosphorylation was not sufficient to cause the release of HPr from the poles in the absence of EI might suggest that steric interaction between HPr and EI-N, which involves the phosphorylation site of HPr, rather than phosphorylation *per se*, might trigger HPr release from the poles. Importantly, when expression of the general PTS proteins was increased rigorously (see Materials and methods), only a fraction of HPr was released from the poles. Hence, EI is required for HPr release, but complete dispersion of all HPr occurs only upon addition of PTS sugars.

Spatial regulation of auxiliary proteins by the PTS

The interaction and spatial relationship between the general PTS proteins and an auxiliary factor regulated by the PTS were also explored in this study. We show that localization of the BglG transcription factor, which enables expression of a PTS sugar utilization operon, *bgl*, is determined by its consecutive interaction with several PTS components, that is, with the BglF permease and with the general PTS proteins. It has been known for quite some time that the activity of

BglG homologues depends on the general PTS proteins. In gram-positive bacteria, activation of these factors involves their phosphorylation by HPr (Deutscher *et al*, 2006), but in *E. coli* this activation is phosphorylation independent (Raveh *et al*, 2009). In accord with that, the results presented here suggest that activation of BglG is achieved through its physical interaction with both EI and HPr that does not involve the phosphorylation sites of the interacting partners. The lack of interaction between EI(H189A) and BglG is apparently not due to the mutation, as the C-terminal domain of EI, which lacks H189, mediates the interaction and is sufficient for it. Structural studies have shown that the activity of EI relies on swivelling between a compact and a relaxed conformation (Teplyakov *et al*, 2006). EI mutated in H189 was suggested to be 'stuck' in the compact conformation (Patel *et al*, 2006), which apparently precludes its interaction with BglG due to sequestration of the sequence in the C-terminal domain that mediates this interaction. Notably, HPr requires intact BglG for the interaction, whereas EI, like BglF (Lopian *et al*, 2003), interacts with the PRD2 domain of BglG, suggesting that the two interactions are dissimilar, probably having different roles in BglG relocation and activation. The finding that BglG interacts with each PTS protein independently of the other supports this hypothesis.

The interaction patterns between the different EI and HPr derivatives (wild-type, mutants and domains) and BglG, obtained with the Far-western and the two-hybrid system, correlated well with the ability of these proteins to activate BglG as an antiterminator (Raveh *et al*, 2009) and with the colocalization data obtained by fluorescence microscopy, providing a solid support for our model that physical interactions at specific cellular compartments underlie the activation mechanism. The recruitment of BglG to the PTS proteins at the poles seems an intermediate step between BglG localization around the cell periphery, where in complex with the sugar permease BglF, and its cytoplasmic distribution. What triggers the release of BglG from the poles? Phosphotransfer of PTS sugars drains the phosphate from the PTS cascade, thus changing the ability of proteins in this cascade to interact with auxiliary proteins (this is the basis for catabolite repression and inducer exclusion; Deutscher *et al*, 2006). Although, phosphorylation is not required for BglG recruitment, its release from both HPr and EI might very well be related to such sugar-triggered changes. Then again, interaction of the incoming sugar with BglG cannot be ruled out. How BglG gets to the membrane is an open question. Is it by diffusion and capture or perhaps by targeted protein synthesis? This subject is currently under investigation.

Another question concerns the purpose of recruiting BglG to the poles. One possibility is that EI and/or HPr act as chaperons that guarantee BglG dimer conformation. The cellular population of BglG homologues was suggested to be heterogeneous, with respect to their conformation. In gram-positive bacteria, HPr-mediated phosphorylation of BglG homologues was suggested to affect the relative amounts of the different conformers in favour of dimers, thereby modulating their nucleic acid-binding activity (van Tilbeurgh and Declerck, 2001). It is reasonable to assume that a similar mechanism operates in *E. coli*, that is, the general PTS proteins provoke a conformational change that stabilizes the BglG active dimer, albeit the interaction between the two proteins does not involve phosphorylation. The non-essenti-

ality of BglG phosphorylation sites for these interactions supports the hypothesis that EI and HPr assist in BglG folding into a dimer conformation, in which these residues are buried (van Tilbeurgh and Declerck, 2001). Another possible rationale for the recruitment of BglG to the polar PTS cluster might be the need for HPr to target BglG to its site of activity. The observation that a sizable fraction of HPr remains at the pole, whereas BglG is released completely upon β -glucoside stimulation does not disagree with such a scenario, as regulation of BglG is only one of HPr numerous functions. Moreover, BglG-GFP was expressed in our experiments at a lower amount than its native level (Supplementary Figure S2E), whereas HPr level was three-fold higher than the chromosome-encoded level (Supplementary Figure S2A). Nevertheless, the ability of the HPr(H15A) mutant protein, which is not, or hardly, released from the poles, to activate BglG (Raveh *et al*, 2009), is not in favour of this possibility. Apparently, the interactions with the general PTS proteins are more about stabilizing the active conformation of BglG and launching it to the transcription machinery. Our findings highlight the importance of steric interactions in signal transduction and in cross-talk between signalling pathways.

Polar organization of sensory systems

The idea that bacteria have a well thought-out internal organization, which affects many of their vital processes, has only recently been appreciated. In rod-shaped bacteria, the list of proteins that localize to the cell poles is growing rapidly. One example, which is relevant to our study, is the chemotaxis complex, composed of receptors and signalling proteins that cluster predominantly near the poles (Kentner and Sourjik, 2006). The PTS and chemotaxis systems obviously need to communicate and cooperate. Phosphoryl transfer between the two systems has not been demonstrated, but interaction between EI and CheA was suggested (Lux *et al*, 1995). Localization of both systems near the poles can clearly facilitate interactions between their components. It remains to be studied whether recruitment of the PTS and chemotaxis proteins to the poles is interdependent.

Finally, the distribution of the PTS proteins between the poles is asymmetric, similar to the chemotaxis and most other proteins that localize near the poles (Ebersbach and Jacobs-Wagner, 2007; Shapiro *et al*, 2009). This is explained by the inherent asymmetry of bacterial cells, that is, the 'new' poles generated at the cell division plane differ from old poles from the previous round of cell division (Shapiro *et al*, 2002). When EI was expressed from the chromosome, it was observed mainly near one pole, whereas expression of EI from a plasmid resulted in its detection in both poles and near the future cell division site, similar to lateral chemotaxis clusters that also localized to future cell division sites (Thiem *et al*, 2007). Apparently, signalling proteins localize preferentially near one pole, but they are also present, although often below the detection threshold, in both poles and in the poles to be.

Materials and methods

Bacterial strains and plasmids

All plasmids used in this study and the proteins they encode are listed in Table II. More details on the bacterial strains and plasmid construction are given in Supplementary data. Expression, stability and functionality assays of the engineered PTS and Bgl proteins are described in Supplementary data.

Table II Plasmids used in this study and the proteins they encode

Plasmid	Plasmid-encoded proteins	Source/ref. ^a
pBADLLmCherry	mCherry	
pBADLLHPr-mCherry	HPr-mCherry	
pBADLLEI-mCherry	EI-mCherry	
pBADLLHPr-mCherry&EI	HPr-mCherry and EI	
pBADLLEI-mCherry&HPr	EI-mCherry and HPr	
pBADANSHPr-GFP	HPr-GFP	
pBADANSHPr-GFP&EI	HPr-GFP and EI	
pBADLLHPr(H15A)-mCherry	HPr(H15A)-mCherry	
pBADLLEI(H189A)-mCherry	EI(H189A)-mCherry	
pBADLLEI-N-mCherry	EI-N-mCherry	
pBADLLEI-C-mCherry	EI-C-mCherry	
pBADLLHPr-mCherry&EI-N	HPr-mCherry and EI-N	
pBADLLHPr-mCherry&EI-C	HPr-mCherry and EI-C	
pBADANEI-mCherry&Kan	EI-mCherry and Kan ^R	
pJS185	BglG-GFP	A Wright
pLLK12F	BglF	
pZLF	BglF	
pLLK12F-GFP	BglF-GFP	
pJPK12IIA ^{glu} -GFP	IIA ^{glu} -GFP Kan ^R	
pJPA12IIA ^{glu} -GFP	IIA ^{glu} -GFP Amp ^R	
pET15b	His ₆ -T7 peptide	Novagen Corp
pLFHG	His ₆ -BglG	L Fux
pANSMG	MBP-BglG	Lopian <i>et al</i> (2003)
pANSMG(D100N)	MBP-BglG(D100N)	Lopian <i>et al</i> (2003)
pANSMG(H160Y)	MBP-BglG(H160Y)	Lopian <i>et al</i> (2003)
pANSMG(H208R)	MBP-BglG(H208R)	Lopian <i>et al</i> (2003)
pANSM-PRD1	MBP-BglG PRD1 (residues 65–172)	
pANSM-PRD2	MBP-BglG PRD2 (residues 175–278)	
pMAL-c2	MBP-LacZ'	NEB
pLF-HPr	His ₆ -HPr	Fux <i>et al</i> (2003a)
pLF-EI	His ₆ -EI	Fux <i>et al</i> (2003a)
pETLL-HPr(H15A)	His ₆ -HPr(H15A)	
pETLL-EI(H189A)	His ₆ -EI(H189A)	
pETLL-EI-N	His ₆ -EI-N	
pETLL-EI-C	His ₆ -EI-C	
pMS604	LexADBD(WT) ^b -Fos leu zipper	Dmitrova <i>et al</i> (1998)
pDP804	LexADBD(mutant) ^c -Jun leu zipper	Dmitrova <i>et al</i> (1998)
pLL1	LexADBD(mutant)	Lopian <i>et al</i> (2003)
pLL3	LexADBD(WT)	Lopian <i>et al</i> (2003)
pLL1-G	LexADBD(mutant)-BglG(WT)	Lopian <i>et al</i> (2003)
pLL1-G(H208R)	LexADBD(mutant)-BglG(H208R)	Lopian <i>et al</i> (2003)
pLL1-G(H160Y)	LexADBD(mutant)-BglG(H160Y)	Lopian <i>et al</i> (2003)
pLL1-G(D100N)	LexADBD(mutant)-BglG(D100N)	Lopian <i>et al</i> (2003)
pLF1-PRD1	LexADBD(mutant)-BglG PRD1(aa 65–172)	Fux <i>et al</i> (2003b)
pLF1-PRD2	LexADBD(mutant)-BglG PRD2(aa 175–278)	Fux <i>et al</i> (2003b)
pLL3-HPr	LexADBD(WT)-HPr(WT)	
pLL3-EI	LexADBD(WT)-EI(WT)	
pLL3-HPr(H15A)	LexADBD(WT)-HPr(H15A)	
pLL3-EI(H189A)	LexADBD(WT)-EI(H189A)	
pLL3-EI-N	LexADBD(WT)-EI-N	
pLL3-EI-C	LexADBD(WT)-EI-C	

^aPlasmids for which no reference has been cited were constructed as part of this work.

^bLexADBD(WT) is a fragment of wild-type LexA repressor (amino acids 1–87), which contains a domain that binds to WT LexA operator.

^cLexADBD(mutant) is a fragment of LexA 408 repressor (amino acids 1–87), which contains a domain that binds to an altered LexA operator (op 45).

Proteins

MBP was purchased from NEB. MBP-tagged BglG derivatives were purified as described previously (Chen *et al*, 1997a). His-tagged proteins were expressed in BL21(DE3) and purified as described before (Lopian *et al*, 2003).

Far-western analysis

Proteins were separated on 10% SDS–polyacrylamide gels. Gels were subjected to Far-western analysis as described previously (Nussbaum-Shochat and Amster-Choder, 1999) or stained with Coomassie blue.

Two-hybrid system analysis

The LexA-based two-hybrid system was used to monitor interaction between proteins as described previously (Lopian *et al*, 2003).

Fluorescence microscopy

MG1655 and its Δ pts derivative, transformed with the different plasmids, were grown to early log phase in either LB or M9 minimal medium, containing either 0.4% glycerol or 0.4% glucose or 40 mM lactose or 20 mM mannitol or 20 mM ribose as a carbon source and the appropriate antibiotics. Essentially, the same results were obtained with cells grown at 30 or 37°C, but most results presented here were obtained at 30°C. Expression of the *ptsI* and *ptsH* alleles was induced by adding 0.1% arabinose for 1 h in the results presented here, but essentially the same results were obtained between 0.02 and 2% arabinose. Expression of all other alleles was not induced, unless otherwise stated. When indicated, 1% arbutin or 0.5% glucose was added to the growing culture.

Fluorescence microscopy was carried out as described previously (Bejerano-Sagie *et al*, 2006). In brief, samples (0.5 ml) of culture were removed, gently centrifuged, and resuspended in 10 μ l of

phosphate-buffered saline. When indicated, samples were supplemented with the fluorescent membrane stain FM4-64 at 1 µg/ml or with the DNA stain DAPI at 2 µg/ml. For time-lapse microscopy observations, a chamber slide containing 1% agarose was used. We have verified that this procedure of sample preparation has no effect on cell morphology, both for wild-type and mutant cells. Cells were visualized and photographed using an Axioplan2 microscope (Zeiss) equipped with a CoolSnap HQ camera (Photometrics, Roper Scientific). System control, image processing and quantification of the fluorescent signals were performed using MetaMorph software (Molecular Devices).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Acknowledgements

We are in debt to Sigal Ben-Yehuda for her invaluable help with the fluorescence microscopy and for her important comments on the paper. We appreciate helpful discussions with Keren Di-Nur Galya Monderer-Rothkoff and Hadas Raveh. We thank Andrew Wright for critically reading our paper. This research was supported by the Israel Science Foundation founded by the Israel Academy of Sciences and Humanities.

Conflict of interest

The authors declare that they have no conflict of interest.

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