Dynamic Localization of a Transcription Factor in *Bacillus subtilis*: the LicT Antiterminator Relocalizes in Response to Inducer Availability

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*Bacillus subtilis* transports β-glucosides such as salicin by a dedicated phosphotransferase system (PTS). The expression of the β-glucoside permease BglP is induced in the presence of the substrate salicin, and this induction requires the binding of the antiterminator protein LicT to a specific RNA target in the 5′ region of the *bglP* mRNA to prevent the formation of a transcription terminator. LicT is composed of an N-terminal RNA-binding domain and two consecutive PTS regulation domains, PRD1 and PRD2. In the absence of salicin, LicT is phosphorylated on PRD1 by BglP and thereby inactivated. In the presence of the inducer, the phosphate group from PRD1 is transferred back to BglP and consequently to the incoming substrate, resulting in the activation of LicT. In this study, we have investigated the intracellular localization of LicT. While the protein was evenly distributed in the cell in the absence of the inducer, we observed a subpolar localization of LicT if salicin was present in the medium. Upon addition or removal of the inducer, LicT rapidly relocalized in the cells. This dynamic relocalization did not depend on the binding of LicT to its RNA target sites, since the localization pattern was not affected by deletion of all LicT binding sites. In contrast, experiments with mutants affected in the PTS components as well as mutations of the LicT phosphorylation sites revealed that phosphorylation of LicT by the PTS components plays a major role in the control of the subcellular localization of this RNA-binding transcription factor.

The heterotrophic soil bacterium *Bacillus subtilis* lives in an environment that strongly fluctuates with respect to nutrient availability. To cope with this challenge, *B. subtilis* is able to select from a large variety of potential substrates the carbon source that is most advantageous for growth and propagation. In *B. subtilis* and many other bacteria, the phosphoenolpyruvate (PEP)-dependent sugar phosphotransferase system (PTS) is the key player in the regulated transport and catabolism of carbohydrates (1, 2). The PTS comprises a family of carbohydrate-specific transport proteins collectively called enzymes II (EIIs). The driving force for transport is provided by phosphorylation of the substrate during its uptake. The phosphoryl groups are derived from PEP and are delivered to the EIIs via the sequential (de)phosphorylation of the two general PTS proteins enzyme I (EI) and histidine protein (HPr) at histidine residues. The EIIs are composed of the two cytoplasmic EIIA and EIIB domains and of the EIIC domain, which forms the membrane channel. HPr donates the phosphoryl groups to a histidine residue in the IIA domain, from which it is transferred to a cysteine or histidine residue in the EIIB domain and subsequently to the translocated sugar.

The expression of the functions required for transport and utilization of a particular PTS sugar is usually strictly controlled by dedicated regulatory proteins. The regulation of PTS operons may take place at the level of transcription initiation by repressors or activators or by controlling transcript elongation by RNA-binding antitermination proteins that modulate RNA structures (1, 3). BglG-type transcriptional antitermination proteins represent a widespread family of regulators that control and are controlled by PTS proteins (1, 4, 5). The antitermination proteins bind a conserved RNA antiterminator sequence (RAT) in their target RNAs and thereby prevent the formation of a transcriptional terminator leading to expression of the target genes (6, 7). The antitermination proteins are composed of an N-terminal RNA-binding domain followed by two iterative PTS regulatory domains (PRDs), which receive information about carbohydrate availability from the PTS proteins (4, 8). *B. subtilis* contains four antitermination proteins of the BglG family, including the well-characterized LicT protein. LicT regulates expression of the *bglPH* operon, encoding the β-glucoside-specific EII transporter BglP and the phospho-β-glucosidase BglH, and of the *bglS* gene, which codes for a β-D-glucanase (9, 10, 11). In the absence of β-glucosides such as salicin, BglP inactivates LicT by its phosphorylation on histidine residues 100 and 159, located in the first PRD (PRD1). The phosphoryl groups are transferred back to BglP, if a substrate becomes available. However, to gain antitermination activity, LicT additionally requires phosphorylation by HPr, which takes place at histidine residues 207 and 269, located in the second PRD (PRD2) (12, 13). The latter phosphorylation/dephosphorylation is involved in carbon catabolite regulation downregulating LicT when preferred PTS sugars become available. Structural analysis suggests that phosphorylation of PRD2 stabilizes the active LicT dimer through contacts at the PRD interfaces (14, 15). In contrast, phosphorylation of PRD1 leads to inactivation of LicT, presumably due to its monomerization (1, 8). Comparable regulatory mechanisms, involving antagonistically acting phosphorylations catalyzed by a cognate EII and HPr, were also proposed for the homologous proteins SacT and GlcT from *B. subtilis* and BglG from *Escherichia coli* (16, 17, 18).

In recent years it has been learned that bacterial cells exhibit a highly complex spatial organization which is often dynamic (19).
Enzymatic activities of the PTS were determined colorimetrically by the method described by Haring and van Merdon (26). E. coli DH5α and the B. subtilis strain MZ303 (these plasmids were prepared by the addition of 15 μg l⁻¹ Bacto agar (Difco) to sporulation medium (8 g of nutrient broth per liter, 1 mM MgSO₄, and 13 mM KCl, supplemented after sterilization with 2.5 μM FeSO₄, 500 μM CaCl₂, and 10 μM MnCl₂). Enzymatic activities of the PTS were determined colorimetrically by the method described by Haring and van Merdon (26). E. coli DH5α and the B. subtilis strain MZ303 (these plasmids were prepared by the addition of 15 μg l⁻¹ Bacto agar (Difco) to sporulation medium (8 g of nutrient broth per liter, 1 mM MgSO₄, and 13 mM KCl, supplemented after sterilization with 2.5 μM FeSO₄, 500 μM CaCl₂, and 10 μM MnCl₂).

Transformation and enzyme assays. Chromosomal DNA of B. subtilis was isolated using the DNAeasy tissue kit (Qiagen) according to the supplier’s protocol. B. subtilis was transformed with plasmids and chromosomal DNA according to the two-step protocol (30). Transformants were selected on SP plates containing antibiotics as described above. For enzyme assays, cells were harvested in the exponential growth phase at an optical density of 0.6 nmol of 50 μM FeSO₄, 500 μM CaCl₂, and 10 μM MnCl₂). Enzymatic activities of the PTS were determined colorimetrically by the method described by Haring and van Merdon (26). E. coli DH5α and the B. subtilis strain MZ303 (these plasmids were prepared by the addition of 15 μg l⁻¹ Bacto agar (Difco) to sporulation medium (8 g of nutrient broth per liter, 1 mM MgSO₄, and 13 mM KCl, supplemented after sterilization with 2.5 μM FeSO₄, 500 μM CaCl₂, and 10 μM MnCl₂).

Plasmid constructions. To facilitate the fusion of B. subtilis proteins to the green and yellow fluorescent proteins (GFP and YFP, respectively), we constructed the plasmids pGP1870 and pGP1871, respectively. For this purpose, we amplified the gfp and yfp gene fragments using the primer pairs ML200/ML221 and ML222/ML223 and plasmids pSG1511 (31) and pYFP (32) as the templates, respectively. The amplicons were digested with HindIII and cloned into the vector pUS19 (33). The vectors pGP1870 and pGP1871 allow the construction of GFP/YFP fusions and integration of the plasmid into the chromosome via Campbell-type recombination between the cloned gene fragment and the chromosomal copy of the gene (see http://www.subtiwiki.uni-goettingen.de/wiki/index.php/PGP1870 and http://www.subtiwiki.uni-goettingen.de/wiki/index.php/PGP1871).

To fuse the LiCT protein to the green and yellow fluorescent proteins, we amplified the 3’ 600 bp of the iceT gene lacking a stop codon using the primer pair FR114/FR115 and chromosomal DNA of B. subtilis 168 as the template and cloned the ampiclon between the BamH1 and Sall sites of pGP1870 and pGP1871, respectively. The resulting plasmids were pGP1292 (licT-gfp) and pGP1296 (licT-yfp). Integration of these plasmids

Table 1. B. subtilis strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source or reference</th>
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<tr>
<td>168</td>
<td>trpC2</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>GM1112</td>
<td>saclA-D3 sacB23 sacT14 bglP::Tn10 (cat'zery) amyE(sacB::lacZ Phb)</td>
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<tr>
<td>GP427</td>
<td>trpC2 ΔlicT::ermC</td>
<td>7</td>
</tr>
<tr>
<td>GP475</td>
<td>trpC2 ΔbglP::ermC</td>
<td>GM1112 → 168</td>
</tr>
<tr>
<td>GP1225</td>
<td>trpC2 licT-gfp spc</td>
<td>pGP1292 → 168</td>
</tr>
<tr>
<td>GP1226</td>
<td>trpC2 licT-yfp spc</td>
<td>pGP1296 → 168</td>
</tr>
<tr>
<td>GP136</td>
<td>trpC2 ΔbglP::erm licT-gfp spc</td>
<td>pGP475 → GP1225</td>
</tr>
<tr>
<td>GP142</td>
<td>trpC2 amyEc::p(salB::lacZ phi) licT-gfp spc</td>
<td>pGP1225 → QB5335</td>
</tr>
<tr>
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</tr>
<tr>
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<td>trpC2 amyEc::p(salB::lacZ phi) LisC::ermC</td>
<td>pGP472 → QB5335</td>
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<td>GP157</td>
<td>ΔptsH::cat licT-gfp spc</td>
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<td>pGP1306 → 168</td>
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<td>pGP1308 → 168</td>
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<td>This work</td>
</tr>
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<td>GP162</td>
<td>trpC2 ΔRAT::gcn4::kan</td>
<td>GP1262 → GP1261</td>
</tr>
<tr>
<td>GP163</td>
<td>trpC2 ΔRAT::gcn4::kan</td>
<td>GP1262 → GP1265</td>
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<tr>
<td>GP164</td>
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<td>pGP1292 → GP1265</td>
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<tr>
<td>GP166</td>
<td>trpC2 bglP::cfp ermC</td>
<td>This work</td>
</tr>
<tr>
<td>GP167</td>
<td>trpC2 pch::cfp ermC</td>
<td>This work</td>
</tr>
<tr>
<td>GP168</td>
<td>trpC2 pch::cfp ermC</td>
<td>This work</td>
</tr>
<tr>
<td>MZ303</td>
<td>ΔptsH::cat</td>
<td>16</td>
</tr>
<tr>
<td>QB535</td>
<td>trpC2 amyEc::p(salB::lacZ phi)</td>
<td>36</td>
</tr>
</tbody>
</table>

* Arrows indicate construction by transformation.

B. subtilis was grown in C minimal medium supplemented with auxotrophic requirements (at 50 mg l⁻¹) as indicated below (29), and carbon sources were used at a concentration of 0.5% (wt/vol) unless stated otherwise. The basal medium used in this study, CSE, is C minimal medium supplemented with succinate and glutamate (29). Sporulation (SP) plates were prepared by the addition of 15 g l⁻¹ Bacto agar (Difco) to sporulation medium (8 g of nutrient broth per liter, 1 mM MgSO₄, and 13 mM KCl, supplemented after sterilization with 2.5 μM FeSO₄, 500 μM CaCl₂, and 10 μM MnCl₂).

Materials and Methods

Bacterial strains and growth conditions. The B. subtilis strains used in this study are listed in Table 1. E. coli DH5α (28) was used for plasmid constructions and transformation using standard techniques (28).

Luria-Bertani (LB) broth was used to grow E. coli and B. subtilis. When required, media were supplemented with antibiotics at the following concentrations: ampicillin, 100 μg ml⁻¹ (for E. coli); spectinomycin, 150 μg ml⁻¹; kanamycin, 7.5 μg ml⁻¹; pheolmycin, 6 μg ml⁻¹; chloramphenicol, 5 μg ml⁻¹; and erythromycin plus lincomycin, 2 and 25 μg ml⁻¹, respectively (for B. subtilis).
into the chromosome of *B. subtilis* leads to the in-frame fusion of the gfp or yfp alleles to the entire licT gene lacking its stop codon. To fuse mutant variants of LicT to GFP, PCR-based mutagenesis (34) was performed to obtain the alleles coding for LicT(H100A), LicT(H207A), and LicT(100A 207A). For this purpose, the licT gene was amplified using the outer primer pair FR162/FR115 and the mutagenesis primers FR164 ([for LicT(H100A)] and FR163 [for LicT(H207A)]. The mutated alleles were cloned between the BamHI and SalI sites of pGP1870, giving pGP1306 and pGP1307. To obtain the double mutant allele, pGP1306 was used as the template in the mutagenesis PCR and FR163 as the mutagenesis primer. After cloning of the fragment into pGP1870, the resulting plasmid was pGP1308.

**Strain construction by long flanking homology PCR mutagenesis.**

To replace the RNA antiterminator (RAT) sequences of the primer. After cloning of the fragment into pGP1870, the resulting plasmid the template in the mutagenesis PCR and FR163 as the mutagenesis and pGP1307. To obtain the double mutant allele, pGP1306 was used as the template in the mutagenesis PCR and FR163 as the mutagenesis primer. After cloning of the fragment into pGP1870, the resulting plasmid was pGP1308.

To fuse the PTS components to the cyan fluorescent protein (CFP), we performed a fusion PCR with amplicons of the *B. subtilis* chromosomal bglP gene, of the cfp ermC cassette amplified from plasmid pBP20 (K. Gunka and F. M. Commichau, unpublished data), and of the downstream bglH gene. In a similar way, we obtained a fusion PCR product that allowed the construction of the psh-cfp strain. To avoid interference with the expression of the downstream bglH and pts genes, respectively, the terminator downstream of the ermC gene was omitted in the PCR. For the generation of a psh-cfp strain, we used the same procedure, but in this case the ermC terminator was included.

**Western blotting.** For Western blot analysis, proteins were separated by 12% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad) by electroblotting. Rabbit anti-CFP polyclonal antibodies (Biozol, Eching, Germany; 1:10,000) served as primary antibodies. The antibodies were visualized by using anti-rabbit immunoglobulin alkaline phosphatase secondary antibodies (Promega) and the CDP-Star detection system (Roche Diagnostics), as described previously (29).

**Microscopy.** For fluorescence microscopy, cells were grown in CSE, CSE-salicylic acid (0.1% [wt/vol]), or CSE-sorbitol (0.5% [wt/vol]) medium to an OD<sub>600</sub> of 0.3 to 0.4, harvested, and resuspended in phosphate-buffered saline (pH 7.5; 50 mM). Fluorescence images were obtained with an Axioplan 40 FL fluorescence microscope, equipped with digital camera Axiocam MRm and AxioVision Rel 4.8 software for image processing (Carl Zeiss, Göttingen, Germany) and Neofluar series objective at a primary magnification of ×100. The applied filter sets were the YFP HC-Filterset (BP, 500/24; FT; 520; LP, 542/27; AHF Analysentechnik, Tubingen, Germany) for YFP detection, the eGFP HC-Filterset (BP, 472/30; FT, 495; LP, 520/35; AHF Analysentechnik) for eGFP detection, filter set 47 (BP, 436/20; FT, 455; LP, 480/40; Carl Zeiss) for CFP visualization, and filter set 49 (G, 365; FT, 395; LP, 445/50; Carl Zeiss) for 4',6-diamidino-2-phenylindole (DAPI) visualization. The overlays of fluorescent and phase-contrast images were prepared for presentation with Adobe Photoshop Elements 8.0 (Adobe Systems, San Jose, CA).

**RESULTS**

**Localization of PTS proteins involved in salicin uptake.** Salicin is taken up by a phosphotransferase system composed of the general components enzyme I and HPr and the β-glucoside-specific permease BglP. To investigate the localization of these proteins, we constructed a set of strains expressing these PTS components fused to the cyan fluorescent protein at their C termini (Table 1). These fusion proteins were encoded at the authentic loci of the PTS components. Prior to the investigation of protein localization, we tested whether the fusion proteins were biologically active. For this purpose, we compared the growth of these strains on minimal medium containing glucitol, glucose, or salicin to that of wild-type *B. subtilis* 168. All strains grew well with the non-PTS carbon source glucitol. In contrast, the strain expressing the HPr-CFP protein (GP1267) did not grow on glucose and salicin, whereas the bacteria with enzyme I-CFP (GP1268) or BglP-CFP (GP1266) grew well in the presence of these carbon sources (data not shown). These results indicate that the tagged enzyme I and BglP proteins were active in the transport of glucose (enzyme I) and salicin (enzyme I and BglP), whereas the tagged HPr protein was unable to participate in PTS-dependent sugar uptake. This suggests that the HPr-CFP protein was inactive, and therefore, the following experiments were performed only with the strains expressing active fusion proteins.

To localize enzyme I and BglP, the strains GP1268 and GP1266 were grown in CSE minimal medium with salicin or glucitol as the carbon source. For enzyme I-CFP, a bright fluorescence that was evenly distributed in the cell was observed under both conditions (Fig. 1A). This result is in good agreement with the established constitutive expression of the ptsHI operon (36) and with the absence of a membrane anchor in enzyme I. For BglP, the result was different. This protein gave bright signals in the membrane region of the cell when the bacteria were grown in the presence of the BglP substrate, salicin. In contrast, only background signals were detected during growth with glucitol (Fig. 1B). These observations precisely matched our expectations since BglP is known to be the membrane-bound permease for salicin uptake, and its expression is strongly dependent on presence of the inducer salicin (9).
The medium. This induction is controlled by the transcriptional antiterminator antiterminator LicT, which in the presence of salicin binds the RAT sequence in the \textit{bglP} leader mRNA in order to allow transcript elongation beyond the intrinsic terminator located in the leader region (9, 11). This prompted us to test the localization of the antiterminator protein LicT as well. For this purpose, we constructed strains with LicT fused to either the green or the yellow fluorescent protein (GP1225 and GP1229, respectively). To assess the activity of the LicT fusion proteins, we constructed a set of strains carrying a fusion of the LicT-regulated \textit{bglP} promoter to a promoterless \textit{lacZ} gene encoding \(\beta\)-galactosidase. The resulting strains were cultivated in minimal medium without an added sugar or in the presence of salicin or salicin and glucose. As shown in Table 2, no \(\beta\)-galactosidase activity was detectable with the wild-type strain in minimal medium without salicin. In the presence of salicin, the expression was induced to about 1,840 U per mg of protein. If both glucose and salicin were available, only 113 U of activity was detected. In the absence of a functional licT gene (GP1245), no \textit{bglP} expression was observed under all conditions tested. These results are in excellent agreement with previous observations (37). They show the salicin-dependent induction as well as glucose-mediated carbon catabolite repression of LicT activity. Moreover, it is obvious that a functional licT gene is essential for the expression of \textit{bglP}. The strains expressing the LicT fusion proteins exhibited an expression pattern of \textit{bglP} that was very similar to that of the strain carrying the nontagged wild-type LicT protein (Table 2). Therefore, the fluorescently labeled LicT proteins have retained full activity in antitermination as well as the physiological control of their activity by PTS components.

To study the localization of LicT, we grew strain GP1225 expressing LicT-GFP in CSE minimal medium without an added sugar or in the presence of glucitol, salicin, or glucose. The results are shown in Fig. 2. Generally, the fluorescence was much weaker than observed for the other fusions used in this study. This is in good agreement with the rather weak level of \textit{licT} transcription (38). In the absence of any sugar as well as in the presence of the noninducing carbon sources glucitol and glucose, LicT was evenly distributed throughout the cell. In contrast, we observed an accumulation of the protein in the subpolar regions when the bacteria were grown in the presence of the inducer salicin, i.e., when LicT was active (Fig. 2C). Similar results were obtained with LicT fused to YFP (data not shown). These results suggest a correlation between LicT activity and its intracellular localization.

### Dynamic relocalization of LicT upon a nutrient shift

As shown above, the localization of LicT is controlled by the availability of the inducer salicin and is thus correlated to its activity in antitermination. If, in turn, the localization would reflect the activity of the protein, one would expect a rapid relocalization when the nutrient supply changes. To address this issue, we cultivated \textit{B. subtilis} GP1225 expressing LicT-GFP in CSE minimal medium to the mid-logarithmic phase and transferred the bacteria to fresh medium containing the inducer salicin. The localization of LicT-GFP was analyzed prior to the shift and 5, 15, and 30 min after the addition of salicin. As shown in Fig. 3A, LicT was evenly distributed throughout the cell in the CSE medium. This result is in good agreement with the results described above (Fig. 2A). Five minutes after the nutrient shift, LicT started to concentrate in the subpolar regions of the cells. This process continued, and after 15 min the great majority of cells had LicT in the subpolar regions. The same localization was also observed 30 min after the nutrient shift.

Next, we tested whether the LicT localization would also alter when cells are shifted from a medium with the inducer salicin to a medium without salicin. For this purpose, GP1225 was grown to the mid-logarithmic phase in CSE medium containing salicin. Then, the cells were washed and resuspended in fresh CSE medium containing glucitol. Again, the LicT localization was observed before and after the shift. As shown in Fig. 3B, LicT exhibited the subpolar localization typical for the active protein during growth with salicin. After the shift to glucitol-containing medium, the localization changed and eventually the protein was again homogeneously distributed in the cells as observed before for inactive LicT irrespective of the noninducing carbon source available. However, this delocalization of LicT was slower; only after 15 min did most cells exhibit the homogeneous distribution of LicT. The process was finished in the last sample analyzed 30 min after the medium shift.

Taken together, our results demonstrate that LicT changes its intracellular localization in response to the presence of the inducer salicin and the accompanying change in LicT activity.

### Role of the LicT RNA targets for the localization of LicT

As reported above, active LicT concentrates in the subpolar regions

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**TABLE 2 Activities of LicT fusion proteins**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>(\beta)-Galactosidase activity (U/mg of protein)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>CSE-Sal</td>
</tr>
<tr>
<td>QB3335</td>
<td>Wild type</td>
<td>0.5 ± 0.5</td>
</tr>
<tr>
<td>GP1245</td>
<td>LicT</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>GP1242</td>
<td>licT-gfp</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>GP1243</td>
<td>licT-yfp</td>
<td>2.5 ± 0.5</td>
</tr>
</tbody>
</table>

\*All measurements were performed at least in triplicate. Values are means ± standard deviations. Sal, salicin; Glc, glucose.

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**FIG 2** The cellular localization of LicT correlates with its activity. Shown are fluorescence micrographs of GP1225 cells expressing a LicT-GFP fusion in the mid-logarithmic phase grown in CSE minimal medium without carbon sources (A) or with glucitol (gluc) (B), salicin (sal) (C), or glucose (glc) (D). Scale bar, 2 \(\mu\)m.
observed a subpolar localization of LicT in both media (Fig. 5A).

For the constitutively active LicT-H100A protein (GP1258), the localization of LicT was similar to that observed in the bglP mutant; i.e., LicT accumulated in the subpolar regions of the cells (Fig. 5B).

The results obtained with the PTS mutants suggest that it is not the activity of LicT that is important for the intracellular localization, since LicT is constitutively active in bglP mutants but completely inactive in ptsH mutants. Thus, specific phosphorylation events seem to control the localization of LicT.

**Impact of the different phosphorylation sites on LicT localization.** The conserved histidine residues in PRD1 and PRD2 are phosphorylated by BglP and HPr, respectively. To study the impact of these phosphorylation events in more detail, we constructed a set of strains that express LicT variants with mutations in His-100 (PRD1, H100A), His-207 (PRD2, H207A), or both residues. To exclude the possibility that the mutations affected the stability and thus the intracellular accumulation of the LicT-GFP fusion proteins, we cultivated the different strains in CSE minimal medium without an added carbohydrate or with salicin or glucitol. The amounts of LicT-GFP were analyzed by a Western blot using antibodies that recognize GFP. As shown in Fig. 6, the different LicT variants were expressed in each medium. Therefore, the mutations did not affect the stability of the mutant LicT-GFP fusion proteins.

For the determination of LicT localization, the bacteria were grown in CSE minimal medium supplemented with salicin or glucitol. For the constitutively active LicT-H100A protein (GP1258), we observed a subpolar localization pattern of LicT under both conditions (Fig. 7A). This result is in excellent agreement with the LicT localization in the bglP mutant, in which H-100 cannot be phosphorylated. The inactive Lic-H207A protein was found to be...
evenly distributed under both conditions (Fig. 7B). Similarly, the inactive LicT-H100A/H207A double mutant protein was evenly distributed both in the presence and absence of salicin (Fig. 7C).

Taken together, our data support the idea that the phosphorylation of LicT is crucial for its localization: all mutant proteins that are no longer responsive to the presence of salicin in their activity do also exhibit an unregulated localization. Similarly, in the PTS mutants that result either in constitutive LicT activity or in its complete inactivity, no response of LicT localization to the nutrient supply was observed. However, while the inactive LicT mutant proteins were always found throughout the cytoplasm, a subpolar localization was observed for the LicT protein that was inactive due to the absence of HPr. This difference suggests not only that the phosphorylation events are required for the correct intracellular recruitment of LicT but also that the integrity of the protein is important (see Discussion).

DISCUSSION

In this work, we provide the first analysis of the intracellular localization of PTS components and of a PTS-controlled transcription factor in B. subtilis.

Our results demonstrate that the general PTS protein enzyme I is evenly distributed in the cytoplasm. This is in good agreement with the annotation of this protein as a soluble PTS protein. However, contradictory results have been reported for the localization of enzyme I in E. coli. Similar to our results, E. coli enzyme I was found to be distributed in the cytoplasm by Neumann et al. (22), whereas a spotty distribution was reported by Patel et al. (21). Finally, polar localization of enzyme I was observed by Lopian et al. (20). It is difficult to judge the reason for these obvious differences. One factor may be the overexpression of enzyme I and the use of different fluorescence tags. In this work, we expressed the fluorescence-tagged PTS proteins from their own promoter, thus avoiding any effect of artificial expression.

A study on the subcellular localization of the β-glucoside-specific antitermination protein BglG indicated that this protein is recruited to the membrane by its cognate permease and sensor, BglF, and that BglG is released into the cytoplasm if the inducer and substrate salicin becomes available (23). Our results suggest that the localization of LicT in B. subtilis does also depend on the functional β-glucoside permease BglP: when no substrate is available, and BglP phosphorylates LicT on the PRD1 and thereby inactivates the protein, LicT is homogeneously distributed throughout the cytoplasm. In contrast, lack of phosphorylation of PRD1 when salicin is available (phosphate flux from BglP to the substrate) or the bglP gene is deleted results in subpolar localization of LicT. It should be noted that no membrane association of LicT was detected under all conditions tested in this work. Thus, the localization pattern of LicT differs from that of its E. coli homolog BglG. However, both proteins relocalize upon addition of the in-
ducer, and in both cases this relocalization is achieved after about 15 min (Fig. 3) (20). It is interesting that a very recent study on the B. subtilis PRD-type transcription factor MtlR revealed that this protein is recruited to the membrane by its cognate mannitol-specific EIIB domain of the mannitol permease MtlA. Moreover, this membrane association was found to be important for the activity of MtlR (27). Thus, PRD-type transcription factors seem generally to require specific localization patterns for their activity; however, the precise localization differs from one protein to the other.

The determinants of intracellular protein localization are only poorly understood. While transmembrane domains can easily be identified in a protein, it is not known which factors drive the differential localization of LicT in the presence or absence of the inducer salicin. For example, the peripheral membrane protein DivlVA binds primarily to sites that exhibit a negative membrane curvature (39, 40). In this case, it was suggested that DivlVA multimers bridge the curvature (40). Another factor that often determines the localization of proteins is recruitment by other proteins or macromolecules. In B. subtilis, this is best studied for the assembly of the spore coat, which depends on the initial ATP-driven polymerization of SpoIVA on the spore surface (41, 42). Similarly, protein-dependent recruitment was proposed for the inactive BglG and the active MtlR transcription factors. In the case of LicT, the localization determinants are unknown. Interestingly, LicT active in transcriptional antitermination localizes to the same regions of the cell as the ribosomes (43, 44). Given the coupling between transcription and translation in bacteria, it is tempting to speculate that the RNA molecules to which LicT binds recruit the protein to the subpolar regions of the cell. Surprisingly, our results indicate that differential LicT localization is observed even in a strain devoid of the bglPH and bglS RAT RNA regions (Fig. 4B). One might argue that LicT may bind other RAT sequences in the absence of its cognate targets. However, as shown previously, LicT is unable to bind any of the other RAT sequences present in B. subtilis (45). Thus, phosphorylation is likely to be the driving force for LicT (re)localization.

Our analyses with PTS mutants and LicT phosphorylation site mutants support the idea that the PTS-dependent phosphorylation of LicT controls its subcellular localization. Indeed, mutations affecting enzyme I or HPr resulted in the absence of phosphorylation of the PRD1 of LicT, and both mutations did also result in the prevention of the homogeneous distribution of LicT that is normally observed when LicT is phosphorylated on PRD1 in the absence of the inducer salicin. The loss of phosphorylation of both PRD1 and PRD2 gave contradicting results: while subpolar LicT localization was observed in the ptsH mutant lacking HPr, the nonphosphorylatable LicT-H100A/H207A protein was homogeneously distributed in the cytoplasm. Though counterintuitive, these observations are not unprecedented: phosphorylation-dependent protein localization or protein-protein interactions sometimes require not only the presence or absence of the phosphoryl group; the requirements for correct localization/interaction may extend to the correct amino acid sequence of the proteins. This was reported for the localization of E. coli HPr, which depends on the presence of the phosphorylation site (His-15) for proper polar localization (20). Similarly, the interaction between the B. subtilis HPr protein and the CcpA transcription factor depends on the integrity of the His-15 phosphorylation site (46, 47). Finally, the presence or absence of the phosphorylation site His-15...
in HPr and its regulatory paralog Crh is a major determinant for the specificity of the regulatory interaction between Crh and the methylglyoxal synthase MgsA (48).

While the field of intracellular protein localization in bacteria is still in its infancy, it is becoming more and more obvious that proteins are very dynamic and that they relocalize depending on the growth conditions. The analysis of the driving forces and the molecular mechanisms behind this movement of proteins will be a major challenge for future research.

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