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Insight into Bacterial Phosphotransferase System-Mediated Signaling by Interspecies Transplantation of a Transcriptional Regulator▽†

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The bacterial sugar:phosphotransferase system (PTS) delivers phosphoryl groups via proteins EI and HPr to the EII sugar transporters. The antitermination protein LicT controls β-glucoside utilization in *Bacillus subtilis* and belongs to a family of bacterial transcriptional regulators that are antagonistically controlled by PTS-catalyzed phosphorylations at two homologous PTS regulation domains (PRDs). LicT is inhibited by phosphorylation of PRD1, which is mediated by the β-glucoside transporter EII^{Bgl}. Phosphorylation of PRD2 is catalyzed by HPr and stimulates LicT activity. Here, we report that LicT, when artificially expressed in the nonrelated bacterium *Escherichia coli*, is likewise phosphorylated at both PRDs, but the phosphoryl group donors differ. Surprisingly, *E. coli* HPr phosphorylates PRD1 rather than PRD2, while the stimulatory phosphorylation of PRD2 is carried out by the HPr homolog NPr. This demonstrates that subtle differences in the interaction surface of HPr can switch its affinities toward the PRDs. NPr transfers phosphoryl groups from EI^{Ntr} to $EIIA^{Ntr}$. Together these proteins form the paralogous PTS^{Ntr}, which controls the activity of K^+ transporters in response to unknown signals. This is achieved by binding of dephosphorylated $EIIA^{Ntr}$ to other proteins. We generated LicT mutants that were controlled either negatively by HPr or positively by NPr and were suitable bio-bricks, in order to monitor or to couple gene expression to the phosphorylation states of these two proteins. With the aid of these tools, we identified the stringent starvation protein SspA as a regulator of $EIIA^{Ntr}$ phosphorylation, indicating that PTS^{Ntr} represents a stress-related system in *E. coli*.

In many bacteria the phosphoenolpyruvate (PEP)-dependent sugar phosphotransferase system (transport PTS) represents the major carbohydrate uptake system (5, 48). The transport PTS is also a global signaling system that orchestrates carbohydrate utilization and coordinates it with other processes (15). It is composed of the two general phosphotransferases enzyme I (EI) and histidine protein (HPr), which deliver phosphoryl groups from phosphoenol-pyruvate to the EII enzymes (EIIs). The EIIs are carbohydrate-specific transport proteins and consist of three domains which may be fused or encoded separately as distinct proteins. The EIIC domain is membrane bound and forms the sugar translocation channel, while the EIIA and EIIB domains are cytoplasmic and sequentially transfer phosphoryl groups from HPr to the substrate during transport. The phosphorylation state of the PTS proteins is modulated in response to the available carbon source (8, 28). This state is sensed and used to regulate other protein activities, either through protein binding or by transfer of phosphoryl groups. Paradigmatic examples include the $EIIA^{Glc}$ protein of the glucose transporter, which controls carbon catabolite repression (CCR) and inducer exclusion in *Enterobacteriaceae*, and HPr, which carries out the analogous regulation in *Firmicutes* (for reviews, see references 14 and 24).

In addition to the transport PTS, many *Proteobacteria* possess the paralogous PTS^{Ntr} (for review, see reference 45). In analogy to the canonical PTS, the PTS^{Ntr} constitutes a phosphorylation cascade working in the direction $PEP \rightarrow EI^{Ntr} \rightarrow NPr \rightarrow EIIA^{Ntr}$ (43, 49, 50, 72). Associated EIIBC domains are lacking and, accordingly, PTS^{Ntr} is believed to exclusively serve regulatory functions. PTS^{Ntr} affects numerous processes, such as nitrogen and carbon assimilation and virulence (17, 49, 66), but mechanistic knowledge is often lacking. Recently, a direct role of PTS^{Ntr} in regulation of K^+ uptake was revealed in *Escherichia coli*. $EIIA^{Ntr}$ binds to the low-affinity K^+ transporter TrkA (35) and to the sensor kinase KdpD (41). Binding to TrkA inhibits K^+ uptake at high external K^+ concentrations, while binding to KdpD at low K^+ concentrations stimulates its kinase activity, thereby activating the response regulator KdpE, which in turn stimulates synthesis of the high-affinity K^+ transporter KdpFABC. Absence of $EIIA^{Ntr}$ increases K^+ uptake through TrkA, resulting in extraordinarily high intracellular K^+ concentrations, which have been suggested to globally redirect gene expression from σ^{70}- to σ^S-dependent transcription (34). The physiological meaning of this regulation is unknown. Only dephosphorylated $EIIA^{Ntr}$ is competent to bind to TrkA and KdpD (35, 41). Therefore, the identification of the signal(s) that controls phosphorylation of PTS^{Ntr} is expected to reveal the biological role of this regulation.

In addition to EI-, HPr-, and EIIA-like proteins, the EII transport proteins are also involved in regulation (47). Transcriptional antitermination proteins of the BglG/SacY family are regulatory proteins that are controlled by the activities of EIIs (for reviews, see references 15, 59, and 64). Common to these proteins are two iterative homologous PTS regulation domains (PRDs) and an N-terminal RNA-binding domain.

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When active, they bind a defined sequence in their target mRNAs and prevent formation of an overlapping transcriptional terminator that blocks synthesis of a specific EII and the associated catabolic functions. The activity of the antitermination proteins is in turn controlled by PTS-catalyzed phosphorylation of conserved histidines in the PRDs. One of the best-characterized members of this family is LicT of *Bacillus subtilis*, which regulates expression of the *bglPH* operon coding for the β-glucoside PTS transporter EII^{Bgl} (BglP) and the β-glucosidase BglH (33, 55). In the absence of β-glucosides, LicT is inactivated by BglP-mediated phosphorylation of histidines 100 and 159 located in PRD1. Although these sites are not phosphorylated in the presence of substrate, this is not sufficient for activity. In addition, LicT requires phosphorylation of histidines 207 and/or 269 in PRD2 for activity, which is catalyzed by HPr (37, 62). Evidence suggests that phosphorylation of either one of these residues is sufficient for activation of LicT (62). This second, antagonistically acting phosphorylation plays a role in CCR and downregulates LicT activity in the presence of preferred PTS substrates (38). Similar models have been proposed for the homologous proteins SacT and GlcT from *B. subtilis* and BglG from *E. coli* (2, 20, 22, 54). Structural studies suggest that LicT oscillates between several conformations involving switches in the regions linking the three protein domains that ultimately control dimerization (12, 25, 65). Phosphorylation of PRD2 leads to stabilization of the active LicT dimer, and this is accomplished through contacts at the PRD interfaces. In contrast, phosphorylation of PRD1 shifts the equilibrium toward the inactive monomeric form of LicT.

A still-open question concerning BglG/SacY-type antiterminators concerns the identity of the protein that catalyzes the phosphorylations in PRD1. It was shown that *in vitro* HPr phosphorylates not only PRD2 but also PRD1 of several antiterminators, including LicT, SacY, and GlcT from *B. subtilis.* Addition of the cognate EII stimulates this phosphorylation (37, 54, 61, 62). One explanation was that all phosphorylations are catalyzed by HPr and that the kinetics of phosphorylation of PRD1 is just modified by the EII (61). Alternatively, nonphysiological high HPr concentrations in the *in vitro* assays were considered to be responsible for the observed phosphorylation of PRD1, which therefore should be irrelevant for regulation *in vivo* (62). However, for GlcT, experimental evidence suggests a direct phosphoryl group transfer from the EII to the antitermination protein (54), leaving the role of the HPr in this reaction obscure.

We are interested in the regulatory functions of the PTS and in characterization of the underlying protein-protein interactions. For a better understanding of these interactions, we started to swap PTS components of the Gram-negative organism *E. coli* with their homologous counterparts from the Gram-positive bacterium *B. subtilis.* Application of this approach to HPr revealed a signature motif in its interacting surface that determines the specificity of HPr for either Gram-negative or -positive EIIs (52). In the present study, we extended this approach to the antitermination proteins and transplanted the *B. subtilis licT* gene to *E. coli*. Our data reveal that LicT becomes phosphorylated at both PRDs, even in *E. coli*. By using transposon mutagenesis, mutational analysis, and phosphorylation assays, the responsible phosphoryl group donors were identified. Surprisingly, HPr from *E. coli* phosphorylates PRD1 rather than PRD2 of LicT, leading to its inactivation. In contrast, the activating phosphorylation at PRD2 is catalyzed by the HPr homolog NPr of the PTS^{Ntr}. Hence, subtle differences in the interaction surface of HPr-like proteins determine whether PRD1 or PRD2 becomes phosphorylated. The data provide insight into the specificity determinants required for interaction with either PRD1 or PRD2 of LicT. Most importantly, we show that LicT is a useful tool for the identification of factors that alter the phosphorylation state of PTS^{Ntr} in *E. coli*. In making the first use of this device, we identified the stringent starvation protein SspA as a regulator of phosphorylation of $EIIA^{Ntr}$, suggesting that the PTS^{Ntr} has a role in the stress response.

TABLE 1. Plasmids used in this study

Plasmid name	Relevant structure[a]	Reference
pFDX2656	*tacOP*-SD_{licT}-*licT tet* ori p15A	55
pFDX2676	*lacI*^{q} P_{16}-*bglt*$_2$-*lacZ tet* ori ColEI	22
pFDX3194	*tacOP*-SD_{licT}-*licT* in MCS of pLDR10	This work
pFDX3219	*tacOP*-SD_{bglG}-*licT* in MCS of pLDR10	This work
pFDX3526	*lacI*^{q} P_{16}-*bglt*$_2$-*lacZ-lacY tet* ori ColEI	This work
pFDX4254	mTn*10*::*ery* λP_R::ats transposase, *cat*, *cis* elements of ori pSC101	This work
pFDX4260	Same as pFDX3219, but *licT* has H100A mutation	This work
pFDX4261	Same as pFDX3219, but *licT* has H100D mutation	This work
pFDX4262	Same as pFDX3219, but *licT* has H159A mutation	This work
pFDX4263	Same as pFDX3219, but *licT* has H159D mutation	This work
pFDX4275	Same as pFDX3219, but *licT* has H207A mutation	This work
pFDX4277	Same as pFDX3219, but *licT* has H269A mutation	This work
pFDX4278	Same as pFDX3219, but *licT* has H269D mutation	This work
pFDX4279	Same as pFDX3219, but *licT* has H207D mutation	This work
pFDX4291	Operatorless P_{tac}-SD_{sacB}-MCS *cat* ori pSC101	31
pFDX4292	*npr* under P_{tac} control in pFDX4291	31
pFDX4294	*ptsN* under P_{tac} control in pFDX4291	31
pFDX4296	*ptsN-yhbJ-npr* under P_{tac} control in pFDX4291	31
pFDX4298	*ptsP* under P_{tac} control in pFDX4291	This work
pFDX4334	*npr*(*H16A*) under P_{tac} control in pFDX4291	This work
pFDX4583	*lacI*^{q} P_{16}-*bglt*$_2$-*lacZ tet* ori p15A	This work
pFDY226	*lacI*^{q} P_{16}-*bglt*$_2$-*lacZ bla* ori ColEI	55
pLDR8	λ*int* under control of λP_R, λ*c*I857 *neo* ori pSC101-*rep*(Ts)	16
pLDR10	*bla* λ*attP neo* ori ColEI MCS	16

[a] SD, Shine-Dalgarno sequence; MCS, multiple cloning site.

MATERIALS AND METHODS

Growth conditions and plasmids. LB was used as standard medium. Where necessary, antibiotics were added to final concentrations of 50 μg/ml (ampicillin), 10 μg/ml (tetracycline), 20 μg/ml (chloramphenicol), 40 μg/ml (kanamycin), and 50 μg/ml (erythromycin). MacConkey-lactose plates were prepared from Difco MacConkey agar base supplemented with 0.5% (wt/vol) lactose. For induction of the *tacOP* promoter, isopropyl-β-D-thiogalactopyranoside (IPTG; 0.1 or 1 mM) was added as indicated. Plasmids were constructed following standard techniques. Their relevant structures are given in Table 1. When necessary, protruding DNA ends were filled in with Klenow polymerase. Sequences of oligonucleo-

TABLE 2. *E. coli* strains used in this study

E. coli strain	Genotype[a]	Reference or construction
IBPC903	*hfrH lacZ43* λ *relA1 spoT1 thi1* Δ*pcnB*::*kan*	30
JW0740	Δ(*araD-araB*)*567* Δ*lacZ4787*(::rrnB-3) λ$^-$ Δ*galK729*::*kan rph-1* Δ(*rhaD-rhaB*)*568 hsdR514*	4
JW1510	Δ(*araD-araB*)*567* Δ*lacZ4787*(::rrnB-3) λ$^-$ Δ*lsrF738*::*kan rph-1* Δ(*rhaD-rhaB*)*568 hsdR514*	4
JW1511	Δ(*araD-araB*)*567* Δ*lacZ4787*(::rrnB-3) λ$^-$ Δ*lsrG739*::*kan rph-1* Δ(*rhaD-rhaB*)*568 hsdR514*	4
JW3197	Δ(*araD-araB*)*567* Δ*lacZ4787*(::rrnB-3) λ$^-$ Δ*sspB756*::*kan rph-1* Δ(*rhaD-rhaB*)*568 hsdR514*	4
JW3198	Δ(*araD-araB*)*567* Δ*lacZ4787*(::rrnB-3) λ$^-$ Δ*sspA757*::*kan rph-1* Δ(*rhaD-rhaB*)*568 hsdR514*	4
LR2-175	F$^-$ *thi-1 argG6 metB1 hisG1 lacY1 galT6 xyl-7 rpsL104* Δ*phoA8 supE44 galP50 manI161 manA162 nagE167 ptsG168 fru-174*	57
R1279	CSH50 Δ(*pho-bgl*)*201* Δ(*lac-pro*) *ara thi*	22
R1653	Same as R1279, but Δ[*ptsH-ptsI-crr*]::*neo*	22
R1958	Same as R1279, but *attB*::*tacOP-bglG* (*bla*)	22
R2051	Same as R1279, but *attB*::*tacOP-bglG* (*bla*) Δ[*ptsH-ptsI-crr*]::*neo*	22
R2077	Same as R1279, but *attB*::*tacOP*-SD$_{licT}$-*licT* (*bla*)	pFDX3194/NotI→R1279, this work
R2079	Same as R1279, but *attB*::*tacOP*-SD$_{bglG}$-*licT* (*bla*)	pFDX3219/NotI→R1279, this work
R2105	Same as R1279, but *attB*::*tacOP-SD$_{licT}$-licT* (*bla*) Δ[*ptsH-ptsI-crr*]::*neo*	T4-GT7 (TP2811)→R2077, this work
R2107	Same as R1279, but *attB*::*tacOP*-SD$_{bglG}$-*licT* (*bla*) Δ[*ptsH-ptsI-crr*]::*neo*	T4-GT7 (TP2811)→R2079, this work
R2404	Same as R1279, but Δ*ptsP*	31
R2409	Same as R1279, but Δ*ptsP* Δ[*ptsH-ptsI-crr*]::*neo*	31
R2413	Same as R1279, but Δ[*ptsN-yhbJ-npr*]	31
R2415	Same as R1279, but Δ[*ptsN-yhbJ-npr*] Δ[*ptsH-ptsI-crr*]::*neo*	31
R2449	Same as R1279, but *attB*::*tacOP*-SD$_{licT}$-*licT* (*bla*) Δ*ptsP* Δ[*ptsH-ptsI-crr*]::*neo*	pFDX3194/NotI→R2409, this work
R2451	Same as R1279, but *attB*::*tacOP*-SD$_{licT}$-*licT* (*bla*) Δ[*ptsN-yhbJ-npr*] Δ[*ptsH-ptsI-crr*]::*neo*	pFDX3194/NotI→R2415, this work
R3555	Same as R1279, but *attB*::*tacOP*-SD$_{bglG}$-*licT* (*bla*) Δ[*ptsN-yhbJ-npr*]	pFDX3219/NotI→R2413, this work
R3557	Same as R1279, but *attB*::*tacOP*-SD$_{bglG}$-*licT* (*bla*) Δ[*ptsN-yhbJ-npr*] Δ[*ptsH-ptsI-crr*]::*neo*	pFDX3219/NotI→R2415, this work
TP2811	F$^-$ *xyl argH1* Δ*lacX74 aroB ilvA* Δ[*ptsH-ptsI-crr*]::*neo*	36
TP2862	F$^-$ *xyl argH1* Δ*lacX74 aroB ilvA* Δ*crr*::*neo*	36
Z267	Same as R1279, but *attB*::*kdpFA′-lacZ* (*aadA*)	41
Z269	Same as R1279, but Δ*ptsP attB*::*kdpFA′-lacZ* (*aadA*)	41
Z501	Same as R1279, but *ptsN*-3×FLAG::*kan attB*::*kdpFA′-lacZ* (*aadA*)	PCR BG741/742→Z267, this work
Z502	Same as R1279, but *ptsN*-3×FLAG::*kan* Δ*ptsP attB*::*kdpFA′-lacZ* (*aadA*)	T4-GT7 (Z501)→Z269, this work
Z504	Same as R1279, but *ptsN*-3×FLAG *attB*::*kdpFA′-lacZ* (*aadA*)	Z501 cured from *kan*, this work
Z505	Same as R1279, but *ptsN*-3×FLAG Δ*ptsP attB*::*kdpFA′-lacZ* (*aadA*)	Z502 cured from *kan*, this work
Z522	Same as R1279, but *sspB*::*kan ptsN*-3×FLAG *attB*::*kdpFA′-lacZ* (*aadA*)	T4-GT7 (JW3197)→Z504, this work
Z523	Same as R1279, but *sspA*::*kan ptsN*-3×FLAG *attB*::*kdpFA′-lacZ* (*aadA*)	T4-GT7 (JW3198)→Z504, this work
Z540	Same as R1279, but *lsrF*::*kan ptsN*-3×FLAG *attB*::*kdpFA′-lacZ* (*aadA*)	T4-GT7 (JW1510)→Z504, this work
Z541	Same as R1279, but *lsrG*::*kan ptsN*-3×FLAG *attB*::*kdpFA′-lacZ* (*aadA*)	T4-GT7 (JW1511)→Z504, this work
Z548	Same as R1279, but *galK*::*kan ptsN*-3×FLAG *attB*::*kdpFA′-lacZ* (*aadA*)	T4-GT7 (JW0740)→Z504, this work
Z549	Same as R1279, but *pcnB*::*kan ptsN*-3×FLAG *attB*::*kdpFA′-lacZ* (*aadA*)	T4-GT7 (IBPC903)→Z504, this work
Z571	Same as R1279, but *sspAB*::*cat ptsN*-3×FLAG *attB*::*kdpFA′-lacZ* (*aadA*)	PCR BG851/852→Z504, this work
ZSC103a	*ptsG manA glk-7 rpsL*	9
ZSC112L	*ptsM glk strA ptsG*::Cm	9

[a] SD, Shine-Dalgarno sequence.

tides used for mutagenesis and PCR cloning are given in Table S1 of the supplemental material. Details on plasmid construction are also provided in the supplemental material.

Strain constructions. The genotypes of relevant *E. coli* strains are given in Table 2. The *tacOP-licT* cassettes were integrated into the λ*attB* site on the chromosome by using the λ*att* integration system as described previously (16). Briefly, originless DNA fragments carrying the respective cassette, the λ*attP* site, and the *bla* selection marker were isolated by NotI digestion and circularized by self-ligation. Subsequently, they were introduced into target strains, which contained helper plasmid pLDR8 carrying the temperature-inducible λ integrase

gene and a temperature-sensitive origin of replication. Selection of transformants at 42°C on ampicillin-containing plates resulted in site-specific recombination of the circularized DNA fragments with the chromosomal *attB* site.

Strain Z501 carrying the *ptsN*-3×FLAG::*kan* allele was constructed using plasmid pSUB11 (63). Briefly, the *kan* cassette of plasmid pSUB11 was amplified by PCR using primers BG741/BG742, and the PCR fragment was introduced into strain Z267 to obtain strain Z501 upon recombination. The *ptsN*-3×FLAG::*kan* allele of Z501 was moved to strain Z269 by phage T4-GT7 transduction (70), which resulted in strain Z502. The *kan* cassettes of strains Z501 and Z502 were removed using plasmid pCP20 (10), which yielded strains Z504 and Z505. Subsequently, various deletion alleles tagged with a *kan* cassette were introduced into strain Z504 by transduction using phage T4-GT7, which yielded strains Z522 to Z549. The *sspAB* double mutation present in strain Z571 was constructed by introducing into strain Z504 a PCR fragment that was generated using primers BG851/BG852 and the template plasmid pKD46, as described previously (10). Strain constructions were verified by diagnostic PCR.

Determination of β-galactosidase activity. β-Galactosidase assays were carried out as described previously (42). Cells were grown in minimal medium (M9) containing proline (20 μg/ml), thiamine (1 μg/ml), Casamino Acids (0.66% [wt/vol]), the appropriate antibiotics, and 1% (wt/vol) glycerol as carbon source if not otherwise indicated. Activities were determined from exponentially growing cells. Presented values are the means of at least three measurements from independent cultures.

Radioactive labeling of proteins *in vivo*. *In vivo* protein phosphorylation using $H_3[^{32}P]O_4$, labeling with [^{35}S]methionine, and subsequent SDS-PAGE were performed as described previously (22, 23). Dried gels were autoradiographed by phosphorimaging, and signals were quantified using the Quantity One software (Bio-Rad).

Determination of phosphorylation state of EIIA^{Ntr}. Cultures of strains carrying the 3×FLAG epitope fused to the 3′ end of the gene *ptsN* were grown to an optical density at 600 nm (OD_{600}) of 0.5 to 0.8. Cells corresponding to 1 OD_{600} unit were harvested by centrifugation (4°C, 13,000 rpm, 10 min), and the pellets were resuspended in 200 μl sample buffer containing 10% glycerol and 0.05% bromophenol blue. Thirteen-microliter aliquots of these suspensions were directly loaded on nondenaturing gels prepared from 12% modified acrylamide ProSieve 50 (Lonza) in 375 mM Tris-HCl (pH 8.7). Exposure of the gels to a voltage of 100 V resulted in release of the proteins by dielectric breakdown, as reported previously for *Pseudomonas putida* (44). Gels were run at 4°C using 50 mM Tris-HCl (pH 8.7), 192 mM glycine as running buffer. After separation, the proteins were electroblotted for 1 h at 0.8 mA cm^{-2} to a polyvinyl difluoride membrane. EIIA^{Ntr}-3×FLAG was detected using anti-FLAG antibodies produced in rabbits (Sigma) and diluted 1:10,000. The antibodies were visualized using rabbit IgG antibodies conjugated to alkaline phosphatase and the CDP* detection system (Roche Diagnostics).

Transposon mutagenesis. The target strains R2079 and R2107 carrying the reporter plasmid pFDX3526 were grown in LB medium at 37°C to an OD_{600} of 0.5 to 1.0. Cells were transformed with the transposon delivery plasmid pFDX4254 by electroporation and subsequently stored at −80°C until their use. Aliquots were thawed and plated on LB plates containing erythromycin and tetracycline and incubated at 33°C. The obtained colonies were replica plated on MacConkey-lactose plates containing the same antibiotics and incubated overnight at 33°C. Subsequently, the colonies were screened for the phenotype of interest. To exclude that a second mutation elsewhere on the chromosome was responsible for the phenotype, the transposon insertions of mutants of interest were transduced to the respective parent strain by using phage T4-GT7 (70). The mini-Tn*10* (mTn*10*) insertion sites of positive clones were mapped by sequencing of chromosomal DNA by using primer 742.

RESULTS

The *E. coli* transport PTS phosphorylates and inhibits activity of LicT. In order to study the potential regulation of the *B. subtilis* antiterminator protein LicT in *E. coli*, the *licT* gene was placed under the control of the IPTG-inducible *tacOP* to allow for regulated expression. Since the Shine-Dalgarno (SD) translation initiation sequences preceding *B. subtilis* genes are known to yield exceptionally high translation initiation rates in *E. coli* (67), two different constructs were made. One carried the native SD sequence of *licT*, while in the other construct the SD sequence of the *E. coli bglG* gene was used to start translation of *licT*. The latter situation also allows for comparison with an isogenic construct that carries *bglG*, which was used in previous studies (22, 23). Thus, these constructs should yield comparable expression levels for *licT* and *bglG*. To determine whether LicT requires HPr for activity, as in its natural host, the *licT* alleles were integrated into the chromosome of *E. coli* strain R1279 (referred to in Fig. 1 as wild-type or *pts*$^+$, respectively) and an isogenic mutant carrying a deletion of the *ptsH-ptsI-crr* operon (coding for HPr, EI, and EIIA^{Glc}, respectively). The strains also carried a deletion of the *bgl* operon to avoid interference with the naturally encoded BglG protein and its negative regulator, BglF, which is a homolog of *B. subtilis* BglP. To assess antitermination activity, a reporter plasmid was used that carries the *bgl* terminator t_2 between a constitutive promoter (P_{16}) and the *lacZ* reporter gene, as has been described previously (22, 23, 52) (Fig. 1A). It has been shown that in the heterologous host *E. coli*, LicT efficiently alleviates termination at terminator t_2 of the *bgl* operon (55).

The various strains were grown in the absence or presence of IPTG, and β-galactosidase activities were determined. When synthesis of BglG was induced by the addition of IPTG, termination was alleviated in the wild-type strain, as previously shown, resulting in synthesis of 274 units of β-galactosidase activity (Fig. 1A). Without IPTG, only background levels of enzyme activity were obtained, indicating the known, weak leakiness of the terminator (22, 52). However, when *licT* was expressed to the same extent as *bglG* (Fig. 1A, *licT* with the SD of *bglG*), only background levels of enzyme activity were detectable in the wild-type strain, even in the presence of IPTG, while 112 units were obtained when LicT was under the control of its own, more efficient SD sequence. This picture changed drastically when the strains lacking the *ptsH-ptsI-crr* operon were employed. While the activity of BglG dropped to background levels as expected (22), the activity of LicT was enormously stimulated, resulting in 146 units of enzyme activity with the *bglG* SD sequence and even 1,703 units when under the control of its own SD sequence. It can thus be concluded that, in contrast to BglG, the activity of LicT does not require the presence of EI and HPr in *E. coli* but that it is, on the contrary, inhibited by the *E. coli* transport PTS.

Based on these unexpected results, we wanted to learn whether LicT is phosphorylated by the *E. coli* transport PTS, i.e., whether inactivity correlates with phosphorylation. We therefore performed metabolic $H_3[^{32}P]O_4$ labeling of strains that expressed *licT* from a plasmid. This technique allows the detection of *in vivo*-phosphorylated proteins. A phosphorylated protein was indeed detected on the gel at the position expected for LicT, which greatly increased in signal strength when synthesis of LicT was induced by IPTG (Fig. 1B, lanes 2 and 3). This signal was undetectable in the strains lacking *licT*, demonstrating that it is LicT (Fig. 1B, lanes 1 and 4). When assayed in the strain missing the *ptsH-ptsI-crr* operon, the intensity of the LicT phosphorylation signal drastically decreased (Fig. 1B, lanes 5 and 6). However, residual phosphorylation of LicT was detectable, which amounted to about 10% of that seen in the wild-type strain. Since a defect of the PTS has pleiotropic effects, we compared LicT protein levels in these strains by pulse-labeling with [^{35}S]methionine and SDS-PAGE (Fig. 1C). Synthesis of LicT was unaffected by the differences in strain backgrounds, confirming that the differences in

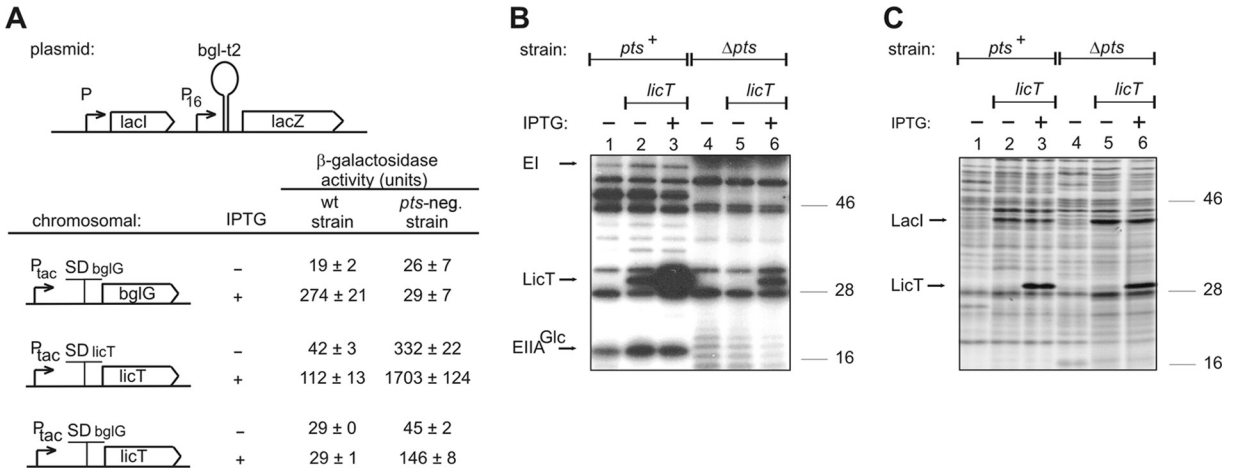
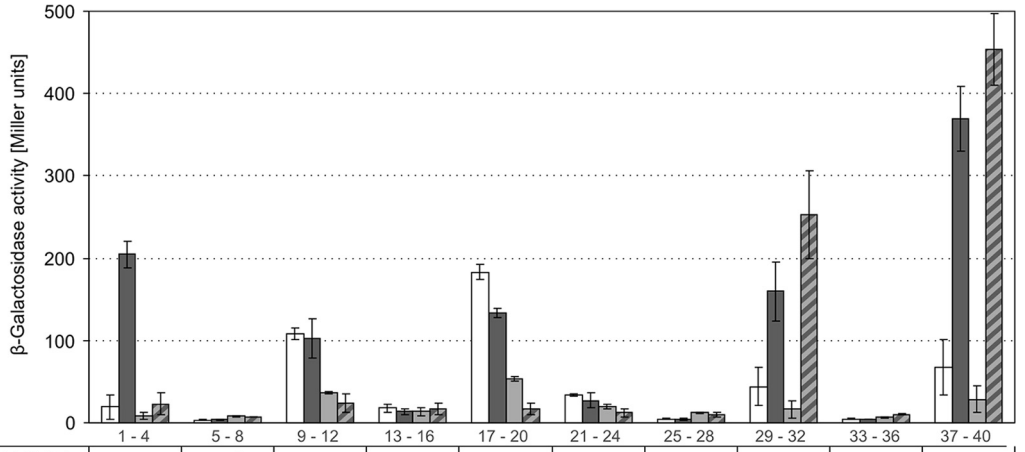


FIG. 1. Regulation of LicT activity by the transport PTS in *E. coli*. (A) LicT is inhibited rather than activated by the transport PTS. The genes *bglG* and *licT*, which are under the control of *tacOP* (P_{tac}) and thus inducible with IPTG, were introduced into the chromosomes (λ attachment site) of isogenic pairs of wild-type (wt) and *pts*-negative strains. The *licT* gene was integrated with its native translation initiation sequences (SD_{licT}) and, in addition, with those of *bglG* (SD_{bglG}). The resulting strains R1958, R2051, R2077, R2105, R2079, and R2107 were transformed with antitermination reporter plasmid pFDX2676, which carries the constitutive promoter P_{16} and the *lacZ* reporter gene separated by terminator $bglt_2$ and in addition the *lacI* gene, providing a repressor for the control of *tacOP* (top). β-Galactosidase activities indicate the efficiency of transcriptional antitermination at the terminator $bglt_2$. (B) LicT is phosphorylated *in vivo* by the *E. coli* transport PTS and by an additional activity. Strain R1279 (*pts*$^+$; lanes 1 to 3) and its isogenic Δ*pts* derivative R1653 (lanes 4 to 6) were doubly transformed with plasmid pFDX2656 carrying the *tacOP*-controlled *licT* gene and plasmid pFDY226, providing synthesis of the Lac repressor (lanes 2, 3, 5, and 6). Aliquots of cultures were labeled with $H_3[^{32}P]O_4$ in the presence or absence of 1 mM IPTG as inducer for LicT synthesis, the proteins were separated by SDS-PAGE, and bands were subsequently visualized by autoradiography. (C) Synthesis of LicT is not affected by the Δ[*ptsH-ptsI-crr*] mutation. The transformants from panel B were labeled with [^{35}S]methionine, and labeled proteins were analyzed by SDS-PAGE and autoradiography.

strengths of the phosphorylation signals are not based on differences in LicT protein levels. In order to unambiguously verify that the IPTG-inducible protein band is indeed LicT, we identified the protein present in this band by mass spectrometry. To this end, total protein extracts of the corresponding strains were separated by SDS-PAGE followed by Coomassie staining (see Fig. S1 in the supplemental material). Once more, a single IPTG-inducible protein band migrating at the position expected for LicT (molecular mass, 32.3 kDa) appeared exclusively in the transformants carrying the *tacOP*::*licT* allele on a plasmid and when IPTG was also present (see Fig. S1, lanes 4 and 8). The respective bands were isolated and analyzed by mass spectrometry. Exclusively, peptides matching LicT were identified, verifying that the IPTG-inducible protein band consists of LicT (for details, see the results of mass spectrometric analyses provided in the supplemental material).

Taken together, we can conclude that LicT is inhibited and at the same time strongly phosphorylated by the *E. coli* transport PTS, while it is active and still weakly phosphorylated by an activity of yet-unknown origin in the absence of a functional transport PTS.

Control of LicT activity by antagonistically acting phosphorylation activities of its PRDs is preserved in *E. coli*. In the genuine host *B. subtilis*, activity of LicT is regulated by multiple phosphorylations of the conserved histidines in the PRDs. Phosphorylation of PRD1 is mediated by BglP and inactivates LicT, while phosphorylation of PRD2 by HPr is a prerequisite for its activity. Our data suggested that in wild-type *E. coli* LicT is likewise inactivated by phosphorylation catalyzed by a yet-unknown protein of the transport PTS. Surprisingly, LicT was highly active in the absence of HPr. The residual phosphorylation of LicT in the Δ[*ptsH-ptsI-crr*] mutant suggested that another protein can take over the activating function of HPr. To assess whether LicT is subject to dual phosphorylation of its PRDs also in *E. coli*, we tested LicT mutants carrying individual amino acid exchanges of each of the four phosphorylated histidines. The effects of mutations in these sites have been extensively investigated in the authentic host, *B. subtilis* (62). It was shown that the nonphosphorylated and phosphorylated states of the individual histidines could be mimicked by alanine and aspartate substitutions, respectively. Therefore, *licT* mutants carrying Ala as well as Asp substitutions of each of the four histidines were placed on plasmids and introduced into the "wild-type" strain, R1279, and the isogenic Δ[*ptsH-ptsI-crr*] mutant, R1653. A compatible antitermination reporter plasmid allowed for the monitoring of LicT activity. Expression of wild-type *licT* resulted in 10-fold-higher antitermination activities in the Δ[*ptsH-ptsI-crr*] mutant than the wild-type strain (Fig. 2, columns 1 and 2), reflecting the results obtained before with the chromosomally encoded *licT* alleles (Fig. 1A). Examination of the various *licT* alleles yielded results that were generally very similar to those obtained previously with these mutants in *B. subtilis* (62). Substitution of the negative regulatory sites His100 and His159 with Ala residues resulted in high antitermination activities in both the wild-type strain and the Δ[*ptsH-ptsI-crr*] mutant (Fig. 2, columns 9, 10, 17, and 18). In contrast, the Asp substitutions of these sites led to low antitermination activities (Fig. 2, columns 13, 14, 21, and 22). This indicated that both histidines are also phosphorylated in the *E. coli* wild-type strain, causing inactivation of LicT, while they are not phosphorylated in the Δ[*ptsH-ptsI-crr*] mutant, leading to a high LicT activity. Substitution of the positive regulatory



Strain \ LicT allele	HHHH	empty vector	AHHH	DHHH	HAHH	HDHH	HHAH	HHDH	HHHA	HHHD
wt	19	3	108	18	183	33	5	44	4	67
Δ[*ptsH-ptsI-crr*]	205	4	102	14	133	27	5	160	4	369
Δ[*ptsN-npr*]	8	8	37	14	54	20	12	17	7	29
Δ[*ptsH-ptsI-crr*] Δ[*ptsN-npr*]	23	7	24	16	17	12	10	253	11	454

FIG. 2. Effects of mutations of the four histidine phosphorylation sites on LicT activity in wild-type *E. coli* and mutants lacking a functional transport PTS, PTS^{Ntr}, or both. Strains R1279 (wild type), R1653 (Δ[*ptsH-ptsI-crr*]), R2413 (Δ[*ptsN-npr*]), and R2415 (Δ[*ptsH-ptsI-crr*] Δ[*ptsN-npr*]) were transformed with antitermination reporter plasmid pFDX4583 and, in addition, with the following plasmids carrying various *licT* alleles under *tacOP* control: pFDX3219 (wild-type *licT*; columns 1 to 4), pLDR10 (empty plasmid; columns 5 to 8), pFDX4260 [*licT*(*H100A*); columns 9 to 12], pFDX4261 [*licT*(*H100D*); columns 13 to 16], pFDX4262 [*licT*(*H159A*); columns 17 to 20], pFDX4263 [*licT*(*H159D*); columns 21 to 24], pFDX4275 [*licT*(*H207A*); columns 25 to 28], pFDX4279 [*licT*(*H207D*); columns 29 to 32], pFDX4277 [*licT*(*H269A*); columns 33 to 36], pFDX4278 [*licT*(*H269D*); columns 37 to 40]. For the induction of LicT synthesis, 0.1 mM IPTG was added to the cultures. The β-galactosidase activities produced by the transformants are depicted. The values corresponding to the individual columns are shown below the graph.

site His207 or His269 with Ala resulted in completely inactive LicT variants in both strains (Fig. 2, columns 25, 26, 33, and 34), suggesting that LicT requires phosphorylation at these sites for activity even in *E. coli*. In contrast, substitution of these sites with Asp restored wild-type LicT behavior (Fig. 2, columns 29, 30, 37, and 38), demonstrating that these exchanges are able to mimic phosphorylation of these sites. Collectively, the data indicate that LicT is controlled by antagonistically acting phosphorylations even in *E. coli*. In the wild-type strain, LicT is phosphorylated at His100 and His159, leading to its inactivity. Although these inhibitory phosphorylations do not occur in the Δ[*ptsH-ptsI-crr*] mutant, this is not sufficient for LicT activity. In addition, LicT requires phosphorylation of His207 and/or His269 in PRD2 for its activity, as is the case in its genuine host.

In order to also provide biochemical evidence that LicT is phosphorylated at histidine residues when present in *E. coli*, we tested the stability of the phosphoryl group bonds in LicT toward treatment with hydroxylamine, NaOH, heat, and acid (see Fig. S2 and its legend in the supplemental material for details). By this approach, the phosphoryl groups on BglG were previously characterized as phosphoamidates, i.e., N-phosphates (1). Therefore, we used phospho-BglG as a control and tested it in parallel to phospho-LicT. Cells overproducing LicT or BglG were labeled with $H_3[^{32}P]O_4$, and the labeled proteins were separated by SDS-PAGE. Treatment of the gel with hydroxylamine resulted in the disappearance of phospho-BglG as well as phospho-LicT, while an alkali treatment of the gel had no effect (see Fig. S2A to C). Treatment of the samples with heat for increasing times prior to their being loaded onto the gels resulted in increased loss of the LicT phosphorylation signal, and this disappearance was drastically accelerated in the presence of HCl (see Fig. S2D). Phosphoester amino acids are stable in the presence of hydroxylamine, acid, and heat but sensitive to alkali treatment. Phospho-tyrosines and phospho-cysteines are stable under acidic as well as basic conditions, while acyl-phosphates are labile under both conditions (1, 3). In sum, the results are only compatible with the properties of phospho-amidates. Only one case of phosphorylation of a protein at an arginine is known in bacteria, while phosphorylation of lysines has not been reported (7, 19). Thus, these results provide further evidence that in *E. coli* exclusively histidine(s) is phosphorylated in LicT, as is the case in the cognate host.

The transposon mutagenesis screen for relief of inhibition of LicT activity in *E. coli* yielded mutations in the *ptsH-ptsI-crr* operon. Next, we were keen to identify the unknown phosphoryl group donors responsible for regulation of LicT activity in *E. coli*. Thereby, we hoped to learn more about the specificity determinants required for interactions of proteins with either PRD1 or PRD2 of LicT. To identify the negative regulator of LicT, a transposon mutagenesis screen was performed. For this purpose, we used a modified antitermination reporter plasmid that additionally carried the gene *lacY*, encoding lactose permease, transcriptionally fused to the upstream-located P_{16}-$bglt_2$-*lacZ* reporter cassette (Fig. 3B, top). Thus, only cells that contain active LicT express the *lacZY* genes and are able to utilize lactose. Accordingly, the Δ[*ptsH-ptsI-crr*] mutant R2107

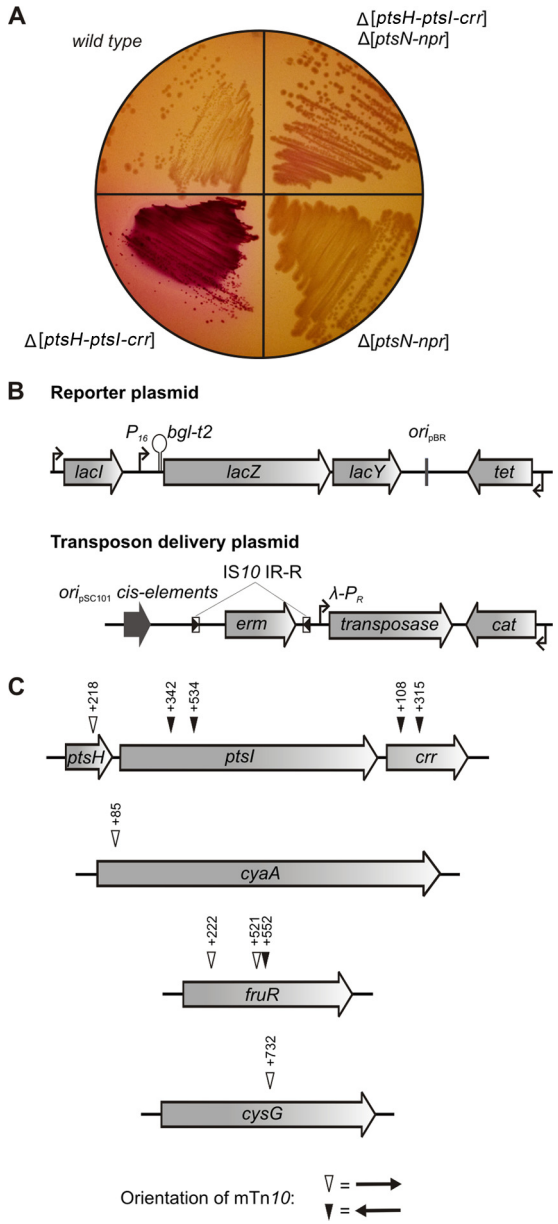


FIG. 3. Transposon mutagenesis screen for identification of the negative regulator of LicT activity in *E. coli*. (A) Phenotypes of strains R2079 (wild type), R2107 (Δ[*ptsH-ptsI-crr*]), R3555 (Δ[*ptsN-npr*]), and R3557 (Δ[*ptsH-ptsI-crr*] Δ[*ptsN-npr*]) on MacConkey-lactose plates. All strains carried reporter plasmid pFDX3526 (see panel B) and the *tacOP*::*licT* cassette on the chromosome. (B) Relevant structures of the reporter plasmid pFDX3526 (top) and the transposon delivery plasmid pFDX4254 (bottom) used in the mutagenesis screens. As a contrast with the reporter plasmids pFDX2676 and pFDX4583 (Fig. 1A and Fig. 2), plasmid pFDX3526 additionally carries the *lacY* gene, encoding lactose permease, in tandem with *lacZ*. Therefore, high LicT activity leads to expression of *lacZY*, which allows the bacterium to transport and utilize lactose as a carbon source. This permits the screening of LicT activity on MacConkey-lactose indicator plates. Transposon delivery plasmid pFDX4254 carries a transposable erythromycin resistance gene encompassed by copies of the right inverted repeat (IR-R) of IS*10*. The ats transposase, which is an IS*10*-derived transposase with relaxed target site recognition (6), is encoded outside the mTn*10* cassette and expressed constitutively from the λ-P_R promoter. The plasmid carries only the *cis* elements of plasmid pSC101 required for replication, while the *repA* gene is absent. Thus, pFDX4254 can only replicate in engineered host strains that provide the *repA* gene in *trans*. This minimizes the possibility of multiple transpositions, since the plasmid is rapidly lost by the target strains. (C) Transposon insertions obtained in the screen for loss of inhibition of LicT activity in wild-type strain R2079. Cells of strain R2079 carrying *licT* on the chromosome and reporter plasmid pFDX3526 were transformed with transposon delivery plasmid pFDX4254 by electroporation. Transposant colonies were selected on LB plates containing erythromycin and tetracycline and subsequently replica plated on MacConkey-lactose plates containing the same antibiotics. Colonies exhibiting a dark red phenotype, indicating lactose fermentation, were isolated, and the transposon insertion sites were identified by sequencing. The location and the orientation of the transposon insertions are indicated by open and closed triangles, respectively. The positions of the mTn*10* insertions are indicated relative to the first nucleotide of the respective gene.

carrying this reporter plasmid and the *tacOP*::*licT* cassette on the chromosome formed red colonies on MacConkey-lactose plates, indicating lactose utilization, while colorless colonies were obtained with the isogenic "wild-type" R2079 (Fig. 3A). This phenotypic difference appeared to be suitable for the screening of mutations that alter activity of LicT.

The "wild-type" strain R2079 carrying *licT* was subjected to transposon mutagenesis by using the mTn*10* transposon delivery plasmid pFDX4254 (Fig. 3B, bottom). Ten mutants were isolated that exhibited a Lac$^+$ phenotype. Mapping of the mTn*10* insertion sites revealed insertions in the *ptsH-ptsI-crr* operon in five mutants (Fig. 3C). One insertion disrupted *ptsH*, while *ptsI* and *crr* were hit two times each at different positions. The remaining insertions were located in *cyaA*, which encodes adenylate cyclase, in *fruR*, which encodes the pleiotropic transcriptional regulator Cra, and in *cysG*, which codes for a siroheme synthase (Fig. 3C). The genes *cyaA*, *fruR*, and *cysG* encode proteins that are not involved in phosphorylations, indicating that their disruption acted indirectly on LicT activity or on lactose utilization. The gene *cysG* is required for biosynthesis of siroheme, which is a prosthetic group present in sulfite and nitrite reductases (69). The inactivation of *cya* has pleiotropic effects on the expression of numerous genes due to the pivotal role of cyclic AMP (cAMP) in CCR. Notably, cAMP-CRP is also required for high expression of *ptsH* and *ptsI* (13, 46), raising the possibility that the *cyaA*::mTn*10* mutation acted through downregulation of *ptsH* and *ptsI* expression on LicT activity. FruR is a global transcriptional regulator that controls synthesis of enzymes of the Krebs cycle and gluconeogenesis (51). The mechanism underlying the Lac$^+$ phenotype of the *fruR* mutants remains to be determined.

In *E. coli*, the phosphorylation that inhibits LicT activity is apparently catalyzed by HPr and does not involve an enzyme II. The results from the transposon mutagenesis suggested that the negative regulator of LicT is encoded in the *ptsH-ptsI-crr* operon. Apart from *crr*, which encodes EIIA^{Glc}, a gene coding for an EII of the PTS could not be identified, suggesting that inactivation of LicT in *E. coli* does not involve a PTS transporter. Antitermination proteins of the BglG/SacY family are usually only active in the presence of specific substrate for their cognate EII. In order to further assess whether inactivation of LicT requires a specific EII in *E. coli*, we determined LicT activity in wild-type cells grown in minimal medium supple-

TABLE 3. Effect of carbon source on antitermination activity of LicT in *E. coli*[a]

Carbon source	β-Galactosidase activity (Miller units)	% phosphorylation of $EIIA^{Glc}$ ($EIIA^{Glc}$~P)
Glycerol	107 ± 9	47 ± 4
Trehalose	73 ± 6	ND[b]
Mannose	78 ± 4	48 ± 22
Sorbitol	82 ± 4	26 ± 10
Fructose	132 ± 2	24 ± 3
Mannitol	199 ± 3	14 ± 6
N-Acetyl-glucosamine	422 ± 23	17 ± 10
Glucose	645 ± 31	5 ± 2

[a] β-Galactosidase activities (means ± standard deviations) were determined from cultures of strain R2077-pFDX2676 grown in M9 minimal medium supplemented with the indicated carbon source in the presence of 1 mM IPTG as inducer for LicT expression. The relative amounts of phosphorylated $EIIA^{Glc}$ protein (according to reference 8) reflecting the phosphorylation status of EI and HPr are shown for comparison.
[b] ND, not determined.

mented with different carbon sources (Table 3). Interestingly, there was no substrate that specifically caused activation of LicT. In contrast, activity of LicT varied with the carbon source and was highest when cells utilized glucose, *N*-acetyl-glucosamine (NAG), or mannitol, which are preferred substrates for *E. coli*. Their utilization leads to preferential dephosphorylation of the general PTS proteins EI, HPr, and $EIIA^{Glc}$, while these proteins are preferentially phosphorylated when less-preferred carbon sources, such as mannose or glycerol, are utilized (8, 28). Thus, there is an inverse correlation of LicT activity and the degree of phosphorylation of the general PTS enzymes (Table 3). These results are incompatible with a mechanism that involves a specific EII controlling the activity of LicT. They are most easily explained by a drain of the phosphoryl groups from PRD1 of LicT via HPr to the incoming sugar.

How can the mTn*10* insertions in gene *crr* be explained? Several EIIs of the PTS depend on phosphorylation by $EIIA^{Glc}$. Although our experiments made phosphorylation of LicT by a specific EII unlikely, we wanted to ultimately rule out that $EIIA^{Glc}$ or an $EIIA^{Glc}$-dependent EII catalyzes this phosphorylation. Therefore, we tested phosphorylation of LicT in mutants lacking $EIIA^{Glc}$ or $EIICB^{Glc}$ (encoded by *ptsG*) by ^{32}P labeling. The absence of these proteins did not impair phosphorylation of LicT (see Fig. S3 in the supplemental material). Thus, a reasonable explanation for the Lac^+ phenotype of the *crr*::mTn*10* mutants might be an increased LacY activity in this mutant. The activity of LacY is limited by an autoregulatory loop that involves interaction with $EIIA^{Glc}$ (29). Alternatively, the mTn*10* insertions in *crr* could destabilize the *pts* operon transcripts, leading to poor synthesis of EI and HPr. Indeed, destabilization of the *ptsHI* mRNA by insertions has been observed in *Lactobacillus sake* (58). In sum, the data provide evidence that the phosphorylation inhibiting LicT activity in *E. coli* is directly catalyzed by HPr and does not involve a specific EII.

A transposon mutagenesis screen for mutations leading to loss of LicT activity in *E. coli* identified EI^{Ntr} and NPr. The next task was to identify the phosphoryl group donor responsible for activity of LicT in the Δ[*ptsH-ptsI-crr*] mutant. Therefore, once more a transposon mutagenesis was performed, but this time the Δ[*ptsH-ptsI-crr*] mutant carrying the *tacOP*::*licT* cassette on the chromosome (strain R2107) (Fig. 3A) was used. Twenty-one insertion mutants were isolated, which exhibited colorless colonies on MacConkey-lactose plates (Fig. 4). Nine of these mutants carried insertions in the gene *ptsP*, encoding EI^{Ntr}, and one mutant carried the mTn*10* in the gene *npr*, encoding NPr, which delivers phosphoryl groups from EI^{Ntr} to $EIIA^{Ntr}$. In five mutants, the insertions were located in the *gal* operon and likely impaired utilization of the galactose

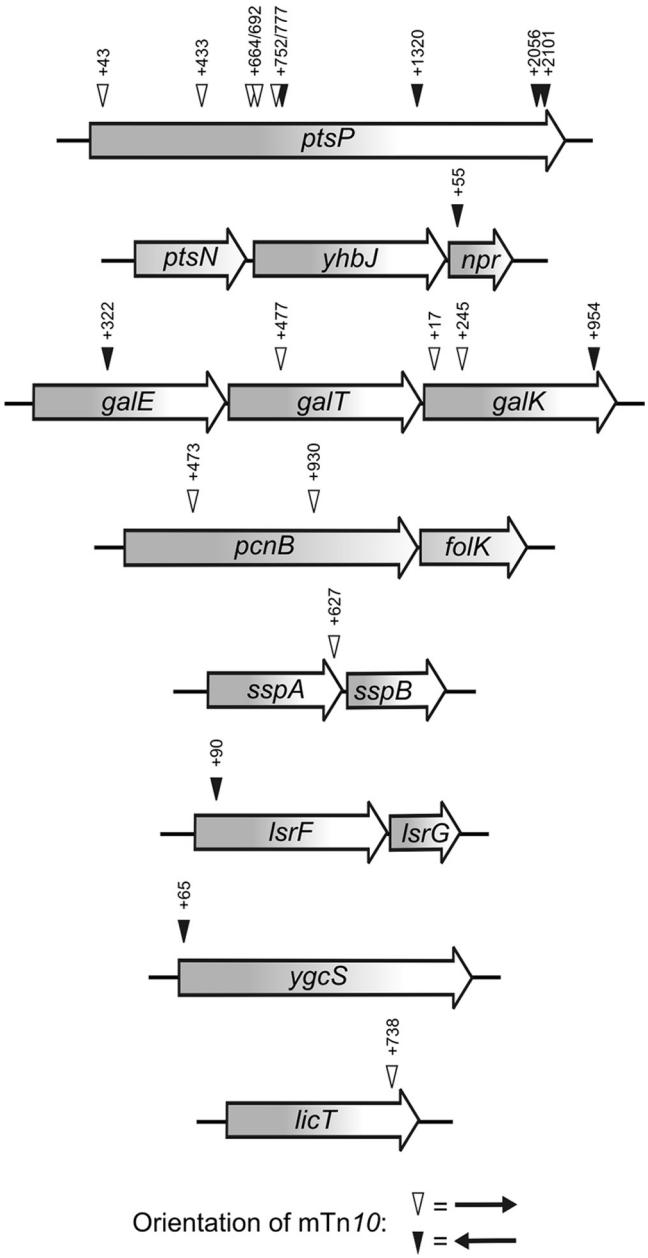


FIG. 4. Transposon mutagenesis screen for identification of genes required for LicT activity in *E. coli*. Transposon insertions obtained in the screen for loss of LicT activity in the Δ[*ptsH-ptsI-crr*] strain R2107 are shown. The reporter plasmid and the transposon delivery plasmid used in the screen are described in Fig. 3B. The screen was carried out as described in the legend to Fig. 3B except that mutants exhibiting colorless colonies were isolated and characterized.

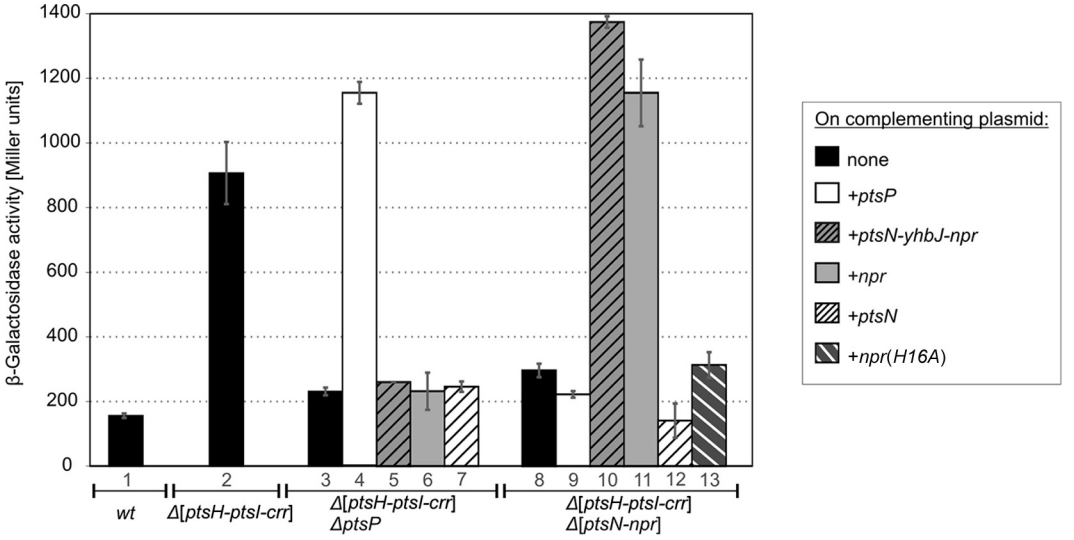


FIG. 5. In *E. coli*, activity of LicT requires EI^{Ntr} and NPr. Strains R2077 (wild type; column 1), R2105 (Δ[*ptsH-ptsI-crr*]; column 2), R2449 (Δ[*ptsH-ptsI-crr*] Δ*ptsP*; columns 3 to 7), and R2451 (Δ[*ptsH-ptsI-crr*] Δ[*ptsN-npr*]; columns 8 to 13) were used for complementation analysis. These strains carried the chromosomal *tacOP*::*licT* cassette, reporter plasmid pFDX3526, and, in addition, one of the following complementation plasmids: pFDX4291 (empty plasmid; columns 1, 2, 3, and 8), pFDX4298 (*ptsP*; columns 4 and 9), pFDX4296 (*ptsN-yhbJ-npr*; columns 5 and 10), pFDX4292 (*npr*; columns 6 and 11), pFDX4294 (*ptsN*; columns 7 and 12), and pFDX4334 [*npr*(*H16A*); column 13]. The β-galactosidase activities produced by these transformants in the presence of 0.1 mM IPTG are shown.

moiety of lactose, explaining the Lac$^-$ phenotype. In one case, the *licT* gene itself was disrupted. Two mutants carried disruptions of gene *pcnB*, which codes for poly(A) polymerase. Mutations in *pcnB* drastically reduce the copy number of ColEI-type plasmids (40), such as the reporter plasmid used in the screen, which may explain the Lac$^-$ phenotype. The remaining insertions were located in the genes *sspA*, encoding the stringent starvation protein A (26), *lsrF*, which plays a role in processing of the quorum-sensing signal AI-2 (71), and *ygcS*, which encodes a putative transport protein of so-far-unknown function (Fig. 4). None of the latter genes is known to encode a protein involved in phosphoryl group transfer reactions.

EI^{Ntr} and NPr are essential for activity of LicT in *E. coli*, whereas EIIA^{Ntr} is dispensable. The results of the mutagenesis screen indicated that the genes *ptsP* and *npr* are required for the activity of LicT in *E. coli*. To dissect the involvement of PTS^{Ntr} in activation of LicT, a complementation analysis was carried out. Double mutants were tested that carried a deletion of the *ptsH-ptsI-crr* operon and additionally lacked *ptsP* or the gene cluster *ptsN-yhbJ-npr* (*ptsN* encodes EIIA^{Ntr}). The strains carried the *tacOP*::*licT* cassette on the chromosome and a reporter plasmid for monitoring LicT activity. In both double mutants, expression of the *lacZ* reporter gene was much lower than in the Δ[*ptsH-ptsI-crr*] single mutant (Fig. 5, columns 1, 2, 3, and 8). Complementation of the Δ[*ptsH-ptsI-crr*] Δ*ptsP* mutant with a plasmid expressing *ptsP* restored high *lacZ* expression, while plasmids carrying either *npr* or *ptsN* or the complete *ptsN-yhbJ-npr* gene cluster had no effect (Fig. 5, columns 3 to 7). Similarly, the presence of plasmids that expressed *npr* restored high *lacZ* expression in the Δ[*ptsH-ptsI-crr*] Δ[*ptsN-yhbJ-npr*] mutant, while complementation with plasmids carrying *ptsP* or *ptsN* was without effect (Fig. 5, columns 8 to 12). These data show that both EI^{Ntr} and NPr are required for LicT activity in *E. coli*, whereas EIIA^{Ntr} is dispensable.

Evidence for phosphoryl group transfer from NPr to PRD2 of LicT. Phosphorylation of NPr requires the presence of EI^{Ntr}. Hence, the data suggested a transfer of phosphoryl groups from EI^{Ntr} via NPr to LicT. To test this possibility, the phosphorylation site His16 in NPr was exchanged for an Ala residue. Indeed, this mutation abolished the ability of NPr to stimulate *lacZ* expression and, hence, LicT activity in the Δ[*ptsH-ptsI-crr*] Δ[*ptsN-yhbJ-npr*] mutant (Fig. 5, columns 8, 11, and 13). Hence, NPr is most likely the protein donating phosphoryl groups to LicT.

Next, we wanted to confirm that NPr is responsible for phosphorylation of the histidines in PRD2 of LicT. To this end, we investigated the activities of the various LicT variants carrying mutations of the four histidine phosphorylation sites in the Δ[*ptsN-yhbJ-npr*] and Δ[*ptsH-ptsI-crr*] Δ[*ptsN-yhbJ-npr*] mutants (strains R2413 and R2415) (Fig. 2). None of the LicT variants exhibited high antitermination activity in the Δ[*ptsN-yhbJ-npr*] strain. Notably, even the mutants carrying the His100Ala and His159Ala exchanges exhibited only low activities, although they were active in the wild-type strain (Fig. 2, columns 9, 11, 17, and 19). This suggests that NPr transfers phosphoryl groups to LicT not only in the Δ[*ptsH-ptsI-crr*] mutant but also in the wild type. In the Δ[*ptsH-ptsI-crr*] Δ[*ptsN-yhbJ-npr*] double mutant, exclusively the His207Asp and His269Asp LicT mutants gained activity (Fig. 2, columns 32 and 40), demonstrating that the lack of activation by NPr can be compensated by Asp mutations mimicking phosphorylation of the His residues in PRD2 of LicT. In sum, the data indicate that NPr transfers phosphoryl groups to PRD2 of LicT, leading to its activation.

Mutations in *sspA* impair phosphorylation of EIIA^{Ntr}, the effector protein of the PTS^{Ntr}. According to our data, NPr activates LicT by its phosphorylation in *E. coli*. Normally, NPr transfers phosphoryl groups from EI^{Ntr} to EIIA^{Ntr}. When non-

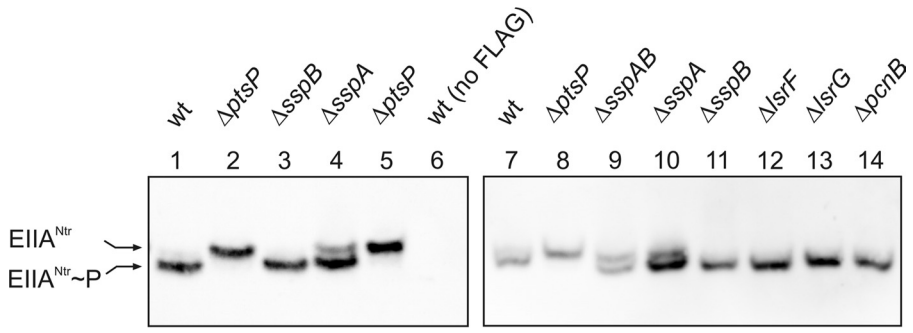


FIG. 6. Determination of the phosphorylation state of EIIA^{Ntr} in various mutants. Strains that carried a 3×FLAG-tagged *ptsN* gene in the chromosome and had the indicated genotypes were grown in minimal glucose medium. Crude extracts were separated by nondenaturing PAGE. EIIA^{Ntr}-3×FLAG was subsequently detected by Western blotting using anti-FLAG antiserum. The following strains were employed: Z504 (wild type; lanes 1 and 7), Z505 (Δ*ptsP*; lanes 2 and 5), Z522 (Δ*sspB*::*kan*; lanes 3 and 11), Z523 (Δ*sspA*::*kan*; lanes 4 and 10), Z571 (Δ*sspAB*::*kan*; lane 9), Z540 (Δ*lsrF*; lane 12), Z541 (Δ*lsrG*; lane 13), Z549 (Δ*pcnB*; lane 14). As a control for specificity of the antiserum, the wild-type strain lacking the 3×FLAG sequence was also employed (Z267; lane 6).

phosphorylated, EIIA^{Ntr} interacts with target proteins to regulate the activities of K^+ transporters. However, the signal(s) that triggers (de)phosphorylation of PTS^{Ntr} and thereby its regulatory output is essentially unknown. Therefore, we reasoned that LicT could be a useful tool for the identification of factors that influence the phosphorylation state of PTS^{Ntr}. Alongside the mutations in *ptsP* and *npr*, we had identified additional mutations in the screen for loss of LicT activity (Fig. 4). One possibility was that these additional mutations mechanistically acted through impaired phosphorylation of EI^{Ntr} and/or NPr, resulting in low LicT activity and, in parallel, in reduced phosphorylation of EIIA^{Ntr}. To address this possibility, we determined the phosphorylation state of EIIA^{Ntr}. To this end, we applied nondenaturing polyacrylamide gel electrophoresis, which enables the simultaneous detection of the phosphorylated as well as nonphosphorylated forms of a phosphoprotein. To allow for the detection of EIIA^{Ntr}, a sequence coding for the 3×FLAG epitope was fused to the 3′ end of gene *ptsN* at its natural chromosomal locus. Next, we separated extracts of the "wild-type" strain Z504 and the isogenic Δ*ptsP* mutant Z505 by nondenaturing PAGE and detected EIIA^{Ntr}-3×FLAG by subsequent Western blotting using anti-FLAG antiserum. In the wild-type strain, only a faster-migrating EIIA^{Ntr} band could be detected, while in the *ptsP* mutant only a slower-migrating band was detectable (Fig. 6, compare lanes 1 and 7 with lanes 2, 5, and 8). This result indicated that EIIA^{Ntr} is almost completely phosphorylated in the wild-type strain and nonphosphorylated in the absence of EI^{Ntr}, at least under the conditions employed. Next we tested isogenic mutants carrying deletions of genes that were identified in the screen for reduced LicT activity (Fig. 4). Deletion mutants of the genes *sspB* and *lsrG* were also employed to account for polar effects of the mTn*10* insertions in *sspA* and *lsrF*. Deletion of *sspB*, *lsrF*, *lsrG*, *pcnB*, or *galK* had any effect on the phosphorylation state of EIIA^{Ntr} (Fig. 6, lanes 3 and 11 to 14 and data not shown). In contrast, two bands corresponding to nonphosphorylated and phosphorylated EIIA^{Ntr} were detectable in the *sspA* mutant (Fig. 6, lanes 4 and 10). Since the isolated mTn*10* insertion in *sspA* (Fig. 4) supposedly also affected the expression of *sspB*, an *sspA sspB* double mutant was employed. However, the additional presence of the *sspB* mutation had no further effect on the ratio of the two forms of EIIA^{Ntr} compared to the *sspA* single mutant (Fig. 6, lanes 9 and 10). Thus, the *sspA* mutation appears to be sufficient for the inhibition of the phosphorylation of EIIA^{Ntr}, demonstrating that the SspA protein influences EIIA^{Ntr} phosphorylation in a positive way. Hence, LicT in general is a useful tool for the identification of modulators of the phosphorylation state of EIIA^{Ntr}, the regulatory output device of the PTS^{Ntr}.

DISCUSSION

Antitermination proteins of the BglG/SacY family are widespread and found in both Gram-positive and Gram-negative bacteria. In *E. coli*, the antitermination protein BglG regulates the *bgl* operon required for β-glucoside utilization (56). In *B. subtilis*, the analogous regulation is carried out by LicT, which shares 42% sequence identity with BglG. A phylogenetic study suggested that the *bgl* system was acquired by a horizontal gene transfer event, presumably from a Gram-positive bacterium such as *B. subtilis* (53). Not surprisingly, both antitermination proteins are similarly regulated in their hosts, involving EII^{Bgl}- and HPr-catalyzed phosphorylation of their PRDs (Fig. 7A) (20, 22, 37, 62). In the present study, we showed that despite these similarities, LicT is not regulated the same way when transferred to *E. coli*. Surprisingly, LicT gained activity only in the absence of the general PTS proteins EI and HPr. Our results indicate that in *E. coli* LicT is phosphorylated by HPr at PRD1 rather than PRD2, leading to its inactivity. In the absence of HPr, LicT is active in *E. coli*, but this activity requires the HPr homolog NPr, which apparently phosphorylates LicT at PRD2 (see the model in Fig. 7B). These observations not only provide further insight into the role of HPr for the control of antitermination proteins (see below) but also may be useful for the design of reporter systems suited for monitoring the phosphorylation states of HPr and NPr in living *E. coli* cells. The LicT variants carrying His→Asp exchanges in PRD2 lost the requirement for NPr and were exclusively subject to negative regulation by HPr~P (Fig. 2). Thus, activity of these mutants and, accordingly, reporter readout are directly coupled to the degree of HPr dephosphorylation. Using green fluorescent protein (GFP) as the reporter gene may even allow

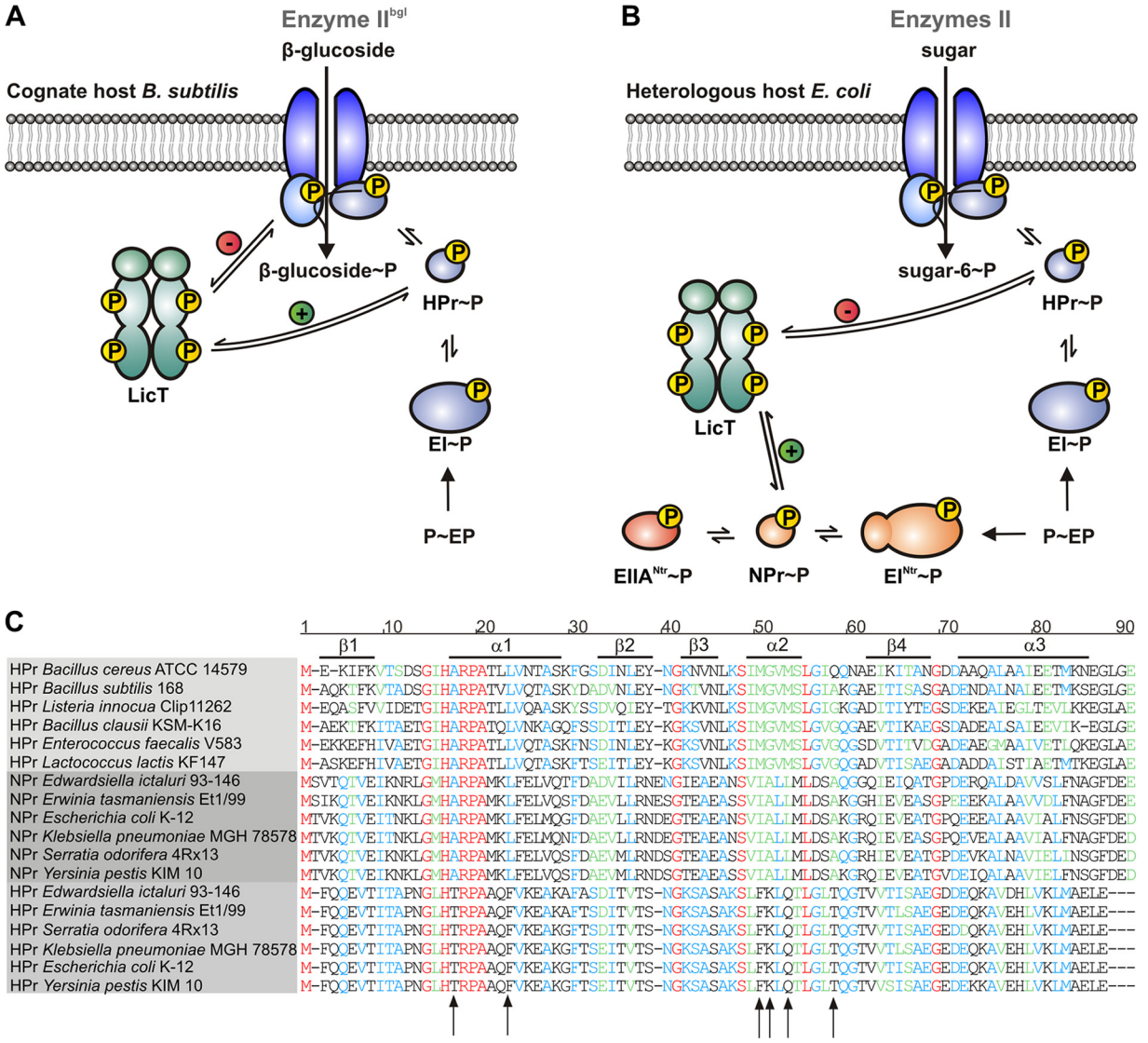


FIG. 7. Regulation of the *B. subtilis* antitermination protein LicT by proteins of the PTS in its cognate host (A) and in *E. coli* (B). (A) In *B. subtilis* the β-glucoside transporter EII^{Bgl} (BglP) inhibits LicT activity in the absence of substrate. This inhibition involves the transfer of phosphoryl groups to PRD1. Substrate availability results in the reversible process. In addition, LicT requires phosphorylation of PRD2 for activity, which is directly catalyzed by HPr. The model is based on previously published evidence (33, 37, 62). (B) In *E. coli*, activity of LicT is likewise controlled by antagonistically acting phosphorylations (present study). However, in this host the inhibitory-acting phosphorylation of PRD1 is catalyzed by HPr, while the stimulatory-acting phosphorylation of PRD2 is carried out by the HPr homolog NPr. NPr is a member of the regulatory PTS^{Ntr}. Thus, LicT is a tool suitable for the identification of conditions that alter the phosphorylation state of PTS^{Ntr} and thereby its regulatory output. (C) Amino acid sequence alignments of HPr proteins from *Firmicutes* and NPr and HPr proteins from *Enterobacteriaceae*. The positions of the α-helices and β-sheets are indicated at the top. Residues within the interaction surface that are identical or similar in the Gram-positive HPr and NPr proteins, but differ in the Gram-negative HPrs, are indicated with arrows. Identical residues are shown in red, conserved residues are shown in blue, and similar residues are shown in green.

monitoring of the phosphorylation state of HPr in single cells. So far, cAMP/CRP-dependent reporter systems, which reflect the PTS phosphorylation state only indirectly, have been used in system biology approaches addressing the PTS (8). Similarly, the LicT mutants carrying the His→Ala exchanges in PRD1 escape negative regulation by HPr but are still regulated by NPr, and they are therefore useful for monitoring its phosphorylation state *in vivo*. Indeed, by using LicT as a tool we identified the protein SspA as a modulator of the phosphorylation state of NPr and thus of PTS^{Ntr} (see below).

How can the unexpected rerouting of phosphoryl transfer reactions, as observed here for LicT in *E. coli*, be explained? The fold of HPr-like proteins consists of three α-helices on top of a four-stranded anti-parallel β-sheet. HPr and NPr interact with other proteins through the same narrow region, composed of helices α1 and α2 and the loops preceding α1 and following α2 (15, 68). However, several residues in the interaction surface are not conserved between Gram-positive and Gram-negative HPr proteins (Fig. 7C). These differences prevent efficient interaction of *B. subtilis* HPr with the EIIs from *E. coli*, as

shown previously (52), and supposedly they are also responsible for the inability of *E. coli* HPr to phosphorylate PRD2 of LicT, as observed here. Interestingly, several features of the interaction surface of the Gram-positive HPrs are shared with the NPr proteins, but not with the Gram-negative HPrs (Fig. 7C). These similarities include hydrophobic aliphatic residues, i.e., Ala16 and Leu22 in helix α1 and Met, Ile, or Leu at positions 48 and 51 in helix α2. Moreover, Ala or Gly residues are present at positions 49 and 56. In contrast, Gram-negative HPrs contain Phe at positions 22 and 48, Thr at positions 16 and 56, Lys at position 49, and Gln at position 51. In conclusion, hydrophobic aliphatic residues, in particular in α-helix 2, are the likely features that allow NPr to phosphorylate PRD2 of LicT. The aromatic and polar residues present at the corresponding positions in HPr of *E. coli* may prevent interaction with PRD2, but at the same time they trigger efficient interaction with PRD1 of LicT. Thus, subtle differences in the interaction surface decide whether HPr preferentially phosphorylates PRD2 or the homologous PRD1, switching it from an activator to an inhibitor of LicT activity.

It has been speculated for several antitermination proteins from *B. subtilis* that phosphorylation of PRD1 is catalyzed by HPr and that the cognate EII modulates this reaction in response to substrate availability. This hypothesis is also supported by our observation that LicT becomes efficiently phosphorylated at PRD1 by HPr of *E. coli*. Thus, it is conceivable that the previously observed phosphorylation of PRD1 by the cognate HPr protein *in vitro* (see the introduction) reflects HPr's natural ability to also carry out this reaction *in vivo*. A plausible mechanism may involve binding of the antitermination protein by the phosphorylated form of the cognate EII and its presentation in a manner that increases the affinity of HPr for PRD1. Indeed, EII^{Bgl}-dependent membrane sequestration has been reported for BglG from *E. coli* (23, 39). Alternatively, phosphorylation of PRD1 by HPr could be an evolutionary relic reflecting the phylogeny of the antitermination proteins. It is conceivable that an ancestral antitermination protein was only negatively controlled by HPr-mediated phosphorylation of a single "PRD." Perhaps, this protein controlled synthesis of EI and HPr (as observed for GlcT in recent *Firmicutes*) and of a prototypical EII. This antitermination protein would only be active during sugar transport, which would cause withdrawal of the inhibitory phosphoryl groups from PRD1 via HPr to the EII, a behavior similar to that observed here for LicT in *E. coli* (Table 3). This simple feedback loop would have coupled synthesis of the PTS proteins to substrate availability. The acquisition of additional EIIs during evolution required an additional substrate-specific regulation mechanism to avoid unspecific activation by transport of any PTS substrate. The EIIB domains could have taken over the task to phosphorylate PRD1, and the cognate HPr proteins progressively lost this ability, while it was still retained by noncognate HPrs, as observed in the present study. Although not homologous, the interaction surfaces of HPr and the EIIB domains share similar topologies, as also reflected by their common ability to interact with EIIA domains. Thus, such a phylogeny appears plausible from a structural point of view as well.

The pivotal role of EI and HPr at the top of the PTS phosphorylation cascade, their important physiological roles, and their dissimilarities to eukaryotic proteins make these proteins attractive potential targets for antimicrobial chemotherapy. EI-deficient mutants of several human pathogens have been shown to be attenuated in mice (11, 32, 60). Previous studies had already shown that LicT is inactive in wild-type *E. coli* but active in mutants lacking HPr and/or EI (21, 27). It was proposed that this gain of function in response to the loss of EI/HPr activity makes LicT an excellent reporter for high-throughput screening of inhibitors of the general PTS enzymes EI and HPr in living *E. coli* cells (27). Although the principal design of this system is appealing, the findings here reveal a hitherto-hidden Achilles heel. The transport PTS and PTS^{Ntr} are highly homologous systems and can even exchange phosphoryl groups under certain conditions (49, 72). Thus, compounds that inhibit the EI autophosphorylation cycle or the subsequent phosphoryl group transfer to HPr may also inhibit the homologous protein of PTS^{Ntr}. As a consequence, reporter readout would be negative, and the respective compounds would escape their identification. However, using LicT mutants with the His→Asp exchanges in PRD2 rather than the wild-type LicT would circumvent this problem, because it would make PTS^{Ntr} activity irrelevant for reporter readout.

Most importantly, our results demonstrate that the LicT-based reporter system is a useful tool for the identification and investigation of regulators of the phosphorylation state of PTS^{Ntr} in *E. coli* and perhaps other *Proteobacteria*. Little is known about the phosphorylation state of PTS^{Ntr} in *E. coli* and how it might be controlled. Here, we show that $EIIA^{Ntr}$ is present almost completely in its phosphorylated form in wild-type cells grown under standard conditions in minimal glucose medium (Fig. 6). This suggests the existence of so-far-unknown conditions able to inhibit the PTS^{Ntr} phosphorylation cascade and to increase the concentration of dephosphorylated $EIIA^{Ntr}$, which finally regulates synthesis and activities of K^+ transporters and perhaps other functions. Interestingly, EI^{Ntr} carries an N-terminal GAF signaling domain, which could modulate activity of the full-length protein in response to binding of an unknown ligand, either a metabolite or a protein (45). By using LicT as a tool, we identified the protein SspA as a regulator of the phosphorylation state of $EIIA^{Ntr}$. In *sspA* mutants, LicT was less active, and at the same time phosphorylation of $EIIA^{Ntr}$ was inhibited, at least partially. Thus, SspA is required for full EI^{Ntr} autophosphorylation activity and/or efficient phosphorylation of NPr. SspA is highly conserved in Gram-negative bacteria and was shown to play an important role in the acid stress response by activating expression of the transcription factor GadE, which is crucial for acid resistance (26). On the other hand, K^+ plays a pivotal role in regulation of intracellular pH (18). Thus, one possibility is that PTS^{Ntr} regulates K^+ uptake in response to acid stress, which could involve its interaction with SspA.

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