Phosphotransferase protein EIIA\textsuperscript{Ntr} interacts with SpoT, a key enzyme of the stringent response, in \textit{Ralstonia eutropha} H16

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EIIA\textsuperscript{Ntr} is a member of a truncated phosphotransferase (PTS) system that serves regulatory functions and exists in many \textit{Proteobacteria} in addition to the sugar transport PTS. In \textit{Escherichia coli}, EIIA\textsuperscript{Ntr} regulates K\textsuperscript{+} homeostasis through interaction with the K\textsuperscript{+} transporter TrkA and sensor kinase KdpD. In the \textit{β-Proteobacterium Ralstonia eutropha} H16, EIIA\textsuperscript{Ntr} influences formation of the industrially important bioplastic poly(3-hydroxybutyrate) (PHB). PHB accumulation is controlled by the stringent response and induced under conditions of nitrogen deprivation. Knockout of EIIA\textsuperscript{Ntr} increases the PHB content. In contrast, absence of enzyme I or HPr, which deliver phosphoryl groups to EIIA\textsuperscript{Ntr}, has the opposite effect. To clarify the role of EIIA\textsuperscript{Ntr} in PHB formation, we screened for interacting proteins that co-purify with Strep-tagged EIIA\textsuperscript{Ntr} from \textit{R. eutropha} cells. This approach identified the bifunctional ppGpp synthase/hydrolase SpoT1, a key enzyme of the stringent response. Two-hybrid and far-Western analyses confirmed the interaction and indicated that only non-phosphorylated EIIANtr interacts with SpoT1. Interestingly, this interaction does not occur between the corresponding proteins of \textit{E. coli}. Vice versa, interaction of EIIA\textsuperscript{Ntr} with KdpD appears to be absent in \textit{R. eutropha}, although \textit{R. eutropha} EIIA\textsuperscript{Ntr} can perfectly substitute its homologue in \textit{E. coli} in regulation of KdpD activity. Thus, interaction with KdpD might be an evolutionary ‘ancient’ task of EIIA\textsuperscript{Ntr} that was subsequently replaced by interaction with SpoT1 in \textit{R. eutropha}. In conclusion, EIIA\textsuperscript{Ntr} might integrate information about nutritional status, as reflected by its phosphorylation state, into the stringent response, thereby controlling cellular PHB content in \textit{R. eutropha}.

INTRODUCTION

Differential protein phosphorylation plays a crucial role in signal transduction in all living organisms. In bacteria, the phosphoenolpyruvate (PEP)-dependent carbohydrate phosphotransferase system (PTS) has a dual role in carbohydrate uptake and signalling (Deutscher et al., 2006). Basically, PTS provides a phosphorylation cascade that couples transport of carbohydrates with their phosphorylation. First, enzyme I (EI) transfers phosphoryl groups from PEP to histidine protein (HPr). Subsequently, HPr phosphorylates the A domains of the enzyme II (EII) transporters. The phosphoryl groups are then transferred to the B domains and finally to the substrates during their uptake through the C domains. The three domains of EII transporters may be fused or exist as independent proteins. The phosphorylation state of the PTS is sensed to coordinate various processes such as carbon catabolite repression, chemotaxis, biofilm formation or the activities of transcriptional regulators with carbohydrate availability (Deutscher, 2008; Görke & Stülke, 2008; Joyet et al., 2013; Neumann et al., 2012; Ymele-Leki et al., 2013). Most \textit{Proteobacteria} possess a parallel PTS, PTS\textsuperscript{Ntr}, which exclusively functions in regulation (Pflüger-Grau & Görke, 2014)
of EIIA Nag and dedicated EI and HPr domains. Similar phosphorylate EIIANtr and EIIAMan, which is another EIIA-like contrast, canonical HPr and EI proteins serve to phos-
dulation of HPr by EI, which takes place at His15 (Deutscher et al., 2006). In Gram-positive bacteria, HPr(Ser)-P triggers a phosphorylation chain working in the order EI Ntr and thereby the phosphorylation state of PTS^Ntr (Lee et al., 2013).

To date, little is known about the role of EIIA^Ntr in β-Proteobacteria, for which Ralstonia eutropha H16 has become a model organism. R. eutropha is a facultative chemolithooautotrophic bacterium that has attracted much interest, because it produces the carbon storage compound poly(3-hydroxybutyrate) (PHB), which is a biodegradable thermoplastic with manifold industrial applications (Reinecke & Steinbüchel, 2009). PHB accumulation is controlled by the stringent response, and occurs when cells are exposed to conditions of nitrogen starvation and carbon excess (Brigham et al., 2012). R. eutropha possesses a single EII (Fig. 1), which catalyses uptake of N-acetylglucosamine and is encoded by nagE and nagF (Kaddor & Steinbüchel, 2011). NagF is a fusion protein composed of EIIA^Nag and dedicated EI and HPr domains. Similar to other β-Proteobacteria, R. eutropha contains EIIA^Ntr but lacks EI^Ntr and NP^r (Pflüger-Grau & Görike, 2010). In contrast, canonical HPr and EI proteins serve to phosphorylate EIIA^Ntr and EIIA^Man, which is another EIIA-like protein (Kim et al., 2013; Krause et al., 2009). R. eutropha also possesses HPr kinase/phosphorylase (HPrK/P), which phosphorylates HPr at Ser46 (Fig. 1; Krause et al., 2009). Generally, this phosphorylation inhibits phosphorylation of HPr by EI, which takes place at His15 (Deutscher et al., 2006). In Gram-positive bacteria, HPr(Ser)-P triggers carbon catabolite repression (Deutscher, 2008; Görike & Stülke, 2008). HPrK/P is essential in R. eutropha, and the deleterious effect of its absence was attributed to hyperphosphorylation of HPr by EI (Krause et al., 2009). Thus, HPr may fulfil an important role in R. eutropha, but it is unknown whether HPr itself or its phosphoryl-group acceptors EIIA^Ntr and/or EIIA^Man carry out this function.

Interestingly, EI, HPr and EIIA^Ntr affect PHB accumulation in R. eutropha. Absence of EI and/or HPr decreases the cellular PHB content, which was attributed to a faster mobilization of PHB after exhaustion of the extracellular carbon source (Kaddor & Steinbüchel, 2011; Pries et al., 1991). In contrast, knockout of EIIA^Ntr has the opposite effect, consistent with an inhibitory role of non-phosphorylated EIIA^Ntr on PHB accumulation. Similar effects were observed in other PHB producers, such as Azotobacter vinelandii and P. putida (Segura & Espin, 1998; Velázquez et al., 2007). Furthermore, knockout of EI and HPr has serious effects on the global R. eutropha proteome, namely proteins involved in amino acid metabolism, translation and gluconeogenesis are misregulated in the mutant (Kaddor et al., 2012). However, the molecular basis for these effects remains to be clarified.

To gain insight into the role of EIIA^Ntr in R. eutropha, we searched for protein interaction partners using a ligand-fishing approach. To this end, Strep-tagged EIIA^Ntr was overexpressed in R. eutropha and subjected to Strep-Tactin affinity chromatography. A co-purifying protein was isolated and identified as bifunctional ppGpp synthase/hydrolase SpoT1, a key enzyme of the stringent response. Two-hybrid and far-Western analyses validated the interaction of EIIA^Ntr and SpoT1. As observed in other bacteria, exclusively non-phosphorylated EIIA^Ntr is involved in interaction. Interestingly, there is no interaction between the corresponding homologous proteins of E. coli. Vice versa, interaction of EIIA^Ntr with the sensor kinase KdpD does not occur in R. eutropha, suggesting that EIIA^Ntr carries out diverse functions in different bacteria. Most importantly, our results suggest that R. eutropha EIIA^Ntr regulates the stringent response through interaction with SpoT1. This could explain the previously observed impact of EI, HPr and EIIA^Ntr on cellular PHB content and the global proteome.

METHODS

Strains, plasmids and growth conditions. The bacterial strains, plasmids and primers used in this study are listed in Tables 1 and 2. E. coli was routinely grown in Luria–Bertani (LB) broth at 37 or 30 °C. R. eutropha H16 was cultivated in nutrient broth or mineral medium supplemented with 0.2% (w/v) Na^+ -succinate as the carbon source (Windholz & Bowien, 1990) at 30 °C. If required, the medium contained ampicillin (100 µg ml^-1), kanamycin (60 µg ml^-1) or tetracycline (15 µg ml^-1 for E. coli and 20 µg ml^-1 for R. eutropha) as selective antibiotics. The ligand-fishing vectors pGP1655 and pGP1656 were transferred from E. coli strain S17-1 to R. eutropha by biparental mating (Srivastava et al., 1982).

E. coli strains XL-1-blue and DH5α were used for DNA cloning. The derivatives pGP1655 and pGP1656 of plasmid pBBR1MCS-3 were assembled in several steps. First, the cbb-2-Pt-349 promoter was amplified by PCR using primers DK15/DK16 and plasmid pBKM9 (Jefke et al., 1999) as the template. This DNA fragment was used to replace the XbaI–KpnI fragment of plasmid pGP798 K, resulting in
plasmid pGP1651. Subsequently, a fragment encoding the Strep-tag was generated by hybridization of oligonucleotides KK1/KK2 and inserted between the NdeI/KpnI sites on plasmid pGP1651, resulting in plasmid pGP1652. Next, ptsN was amplified from the R. eutropha genome using primers KK5/KK6 and inserted between the NheI and KpnI sites on plasmid pGP1652, generating plasmid pGP1653. Finally, the expression cassettes of plasmids pGP1652 and pGP1653, carrying the strep-tag and the strep-ptsN fusion gene, respectively, under cbbc-PL-M9 promoter control, were used to replace the XbaI–SacI fragment of plasmid pGP792.

The various plasmids used in the bacterial two-hybrid (BACTH) assays were derivatives of previously described plasmids pKT25, pUT18 and pUT18C (Karimova et al., 1998). Plasmids pGP1659 and pGP1675 were obtained by amplification of genes kdpD1 and kdpD2 from the R. eutropha genome using the primer combinations KK13/KK21 and KK15/KK20, respectively. The PCR fragments were subsequently inserted between the XbaI/EcoRI sites on plasmid pUT18. Plasmid pGP1660 was constructed by amplification of the R. eutropha ptsN gene using primers KK9/KK25 and subsequent ligation of the PCR fragment to vector pKT25, which was cleaved with XbaI/KpnI. Plasmids pGP1677 and pGP1685 were obtained by insertion of PCR fragments between the XbaI/KpnI sites on plasmid pUT18C. The PCR fragments were amplified using the primer combinations KK29/KK30 (R. eutropha spoT1) and KK39/KK40 (E. coli spoT). Plasmid pGP1687 was constructed by insertion of the PCR fragment obtained with primers KK42/KK43 (R. eutropha spoT2) between the XbaI/EcoRI sites on plasmid pUT18C.

Plasmids pGP2500, pGP2497 and pGP2501 allow for overexpression and purification of R. eutropha EIIANtr and its mutant variants, using E. coli as host. The corresponding alleles were amplified by PCR using primers KK5/CZ92 and the corresponding pKT25 derivatives as templates. The DNA fragments were cloned between the NheI and PstI sites on plasmid pBGG237. Plasmid pGP2494 allows for overexpression and purification of R. eutropha SpoT1 with a C-terminal 10×His tag from E. coli. For its construction, the spoT1 gene was amplified with primers CZ85/CZ86 and inserted between the NdeI/XbaI sites on plasmid pKES170.

Site-directed mutagenesis. Mutation of the phosphorylation site of EIIANtr was carried out by combined chain reaction (Bi & Stambrook, 1998). PCRs were performed that contained the 5′-phosphorylated mutagenesis primers BG671 or CZ91, in addition to

Fig. 1. The repertoire of PTS proteins in R. eutropha H16 and a role for EIIANtr. R. eutropha possesses a single EI for uptake of N-acetylglucosamine (GlcNAc). The triphosphoryl transfer protein NagF delivers phosphoryl groups from PEP to the NagE transporter via fused EI, HPr and EIIA domains. Separately encoded EI (gene locus H16_A0326) and HPr (gene locus H16_A0325) are present, which serve to phosphorylate EIIA[Ntr] (PtsN; gene locus H16_A0384) and EIIA[Man] (PtsM; gene locus H16_A0324). HPr can also be phosphorylated by HPrK/P (locus H16_A0383) at Ser46, which might limit its phosphorylation by EI at His15, and thereby also determine the phosphorylation state of EIIA[Ntr]. Non-phosphorylated EIIA[Ntr] forms a complex with SpoT1, a key enzyme of the stringent response. This could alter the ppGpp level and thereby affect PHB formation and global gene expression.
Table 1. Strains and plasmids used in this study

<table>
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<th>Strain/plasmid</th>
<th>Genotype or relevant structure</th>
<th>Reference</th>
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<td><strong>Strains</strong></td>
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<td>H16</td>
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<td>S17-1</td>
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<td>XL1-blue</td>
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<td>Lüttmann et al. (2009)</td>
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<td><strong>Plasmids</strong></td>
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<td>pUT18C-zip</td>
<td>Leucine zipper of GCN4 fused to T18 in pUT18C</td>
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E.c., *E. coli*; MCS, multiple cloning site; R.e., *R. eutropha* H16.
Table 2. Oligonucleotides used in this study

Restriction sites are underlined, positions deviating from the wild-type sequence are in bold and [P] indicates 5′-phosphorylation of the oligonucleotide. E.c., E. coli; fwd, forward; R.e., R. eutropha H16; rev, reverse.

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Purification of Strep-tagged EIIA<sup>Ntr</sup> from <i>R. eutropha</i> for ligand fishing. The <i>R. eutropha</i> H16 transconjugants carrying plasmids pGP1655 and pGP1656 were grown in mineral medium at 30 °C. The cells were harvested at OD<sub>600</sub> 0.8, washed with ZAP buffer (50 mM Tris/HCl pH 7.5, 200 mM NaCl) and crude lysates were prepared by passage through a One Shot cell disrupter (Constant Systems) at 2.50 kbar. The lysates were cleared by low-speed (8000 r.p.m., 20 min) and high-speed centrifugations (35 000 r.p.m., 1 h, 4 °C, FiberLite rotor F50L-8× 39; Thermo Scientific). Subsequently, the standard protocol for purification of Strep-tagged proteins was carried out (Landmann et al., 2011). Aliquots of the purification steps and the eluates were separated by 12.5% SDS-PAGE (Lüttermann et al., 2012) and gels were subsequently stained with Coomassie brilliant blue or with silver (Blum et al., 1987) for the detection of co-purifying proteins.

**MS.** The three protein bands indicated in Fig. 3 were excised from the gel and subjected to MS. The protease digestes and MS were performed by the Center for Molecular Medicine of the University of Cologne, Cologne, Germany (http://zmmk-bioanalytik.uni-koeln.de/). In band I, 17 unique peptides were detected, which covered the sequence of the MetE protein (UniProt Q0K0V6; gene locus H16_B1581) to an extent of 24 % with a score of 85. MetE was also detected in band II by 24 unique peptides with sequence coverage of 34 % and a score of 131. Analysis of band III detected 35 unique peptides matching StpT1 (UniProt Q0KD21; gene locus H16_A0955) with a sequence coverage of 35 % and a score of 181. No additional proteins of <i>R. eutropha</i> were detectable in the three bands.

**BACTH assay and determination of β-galactosidase activities.** The BACTH system measures reconstitution of adenylate cyclase CyaA in <i>E. coli</i> strain BTH101, which lacks endogenous adenylate cyclase activity (Karimova et al., 1998). In this system, the Bordetella pertussis CyaA protein is split into two fragments, T18 and T25, which are incapable of interacting. However, when fused to proteins that interact with each other, adenylate cyclase activity is restored, resulting in CAMP synthesis. The CAMP level is monitored using the cAMP-CRP-dependent β-galactosidase gene lacZ as a reporter. For interaction analysis, strain BTH101 was co-transformed with plasmids encoding the respective T18- and T25-fusion proteins. For visual monitoring of putative interactions, the co-transformants were spotted onto LB plates supplemented with the appropriate antibiotics, 1 mM IPTG and 40 µg X-Gal ml<sup>−1</sup>. Plates were incubated for 36 h at 30 °C. To evaluate the strength of interaction, quantitative β-galactosidase assays were performed. The co-transformants were grown in LB containing 0.5 mM IPTG at 30 °C with shaking (200 r.p.m.). To evaluate the strength of interaction, quantitative β-galactosidase assays were performed. The co-transformants were grown in LB containing 0.5 mM IPTG at 30 °C with shaking (200 r.p.m.).

**Purification of recombinant proteins from <i>E. coli</i> and far-Western dot blot analysis.** <i>E. coli</i> strain BL21(DE3) carrying plasmids pGP2500, pGP2497 and pGP2501 was used for overproduction of the Strep-tagged <i>R. eutropha</i> EIIA<sup>Ntr</sup> variants. Plasmid pBG217 was used for overproduction of <i>E. coli</i> Strep-EIIA<sup>Ntr</sup> and plasmid pGP2494 allowed for overexpression of <i>R. eutropha</i> SpT1-His<sub>10</sub> protein. The recombinant proteins were purified by
Strep-Tactin and Ni²⁺-NTA affinity chromatography, respectively, and subsequently used for far-Western dot blot analysis as described previously (Lüttrmann et al., 2012).

RESULTS

Broad-host-range vector for protein ligand fishing using Strep-Tactin affinity chromatography

Strep-Tactin affinity chromatography has emerged as a useful tool for the isolation of interaction partners by their co-purification with Strep-tagged bait proteins (Göpel et al., 2013; Herzberg et al., 2007). Therefore, we adopted this approach for the identification of protein interaction partners in R. eutropha H16. To this end, the generalized ligand-fishing vector pGP1655 was constructed, which mediates overexpression of N-terminally Strep-tagged proteins in R. eutropha (Fig. 2a). This vector is able to replicate in various Proteobacteria (Kovach et al., 1995) and might be useful in a wide range of species. The plasmid carries a mob site that allows for its conjugational transfer from E. coli strains carrying the tra genes to other bacteria. Finally, it encodes the Strep epitope downstream of the artificial cbb-P₄-M9 promoter (Fig. 2b), which directs high constitutive expression levels in R. eutropha (Jelfke et al., 1999). Restriction sites downstream of the strep-tag sequence facilitate the construction of fusion genes (Fig. 2b). To fish for interaction partners of EIIAᵦⁿʳ, we constructed the derivative plasmid pGP1656, which triggers synthesis of Strep-tagged R. eutropha EIIAᵦⁿʳ. The corresponding R. eutropha transconjugant was subjected to the Strep-Tactin affinity chromatography protocol. Analysis of the various purification steps by SDS-PAGE revealed the presence of R. eutropha Strep-EIIAᵦⁿʳ protein (molecular mass 19.28 kDa) in elution fractions I and II (Fig. 2c). Thus, plasmid pGP1655 is a suitable tool for overproduction and purification of Strep-tagged proteins using R. eutropha as host.

Ligand fishing in R. eutropha identifies SpoT1 as a potential interaction partner of EIIAᵦⁿʳ

The elution fractions derived from purification of Strep-EIIAᵦⁿʳ were separated by SDS-PAGE, and gels were subsequently silver stained to detect co-purifying proteins (Fig. 3, lanes 4–9). To identify proteins binding non-specifically to the Strep-Tactin matrix or the Strep-tag, the co-purification protocol was also carried out with a transconjugant containing the empty ligand-fishing vector pGP1655 as a negative control (Fig. 3, lanes 1–3). Three faint bands specifically co-purifying with Strep-EIIAᵦⁿʳ could be detected (Fig. 3, numbered bands in lanes 4 and 5). The protein bands were isolated and subjected to MS for their identification. This analysis identified 5-methyltetrahydropteroylglutamate-homocysteine methyltransferase (molecular mass 88 039 Da) encoded by gene metE in bands I, as well as band II. MetE is an abundant protein that has been observed as a contaminant in previous ligand-fishing experiments (F. Meyer and J. Stülke, unpublished data), and therefore it was not further analysed. Analysis of band III identified the GTP pyrophosphokinase SpoT1 (molecular mass 85 295 Da). SpoT1 is the R. eutropha orthologue of the SpoT protein (Brigham et al., 2012), which is a key enzyme of the stringent response elicited by ppGpp (Boutte & Crosson, 2013).

Two-hybrid analysis validates that EIIAᵦⁿʳ interacts with SpoT1 but not with its paralogue SpoT2

To verify the interaction of EIIAᵦⁿʳ with SpoT1, we made use of the BACTH system that is based on heterodimerization of the T18 and T25 fragments of split B. pertussis adenylate cyclase (CyaA). Reconstitution of CyaA activity can be monitored in E. coli by using lacZ expression as a reporter readout. Indeed, blue colonies on LB X-Gal indicator plates, indicating high β-galactosidase activities and therefore interaction, were observed when the R. eutropha EIIAᵦⁿʳ and SpoT1 fusion proteins were tested (Fig. 4a). In contrast, the colonies remained colourless, indicating low β-galactosidase activities, when interaction of EIIAᵦⁿʳ with the leucine zipper fragment of the yeast transcription factor GCN4 was employed as a negative control (Fig. 4a). Homo-dimerization of the GCN4 leucine zipper serves as a positive control in this two-hybrid system (Fig. 4a). To confirm these data, quantitative β-galactosidase measurements were carried out. The transformant containing the T25–EIIAᵦⁿʳ and T18–SpoT1 fusion proteins produced high β-galactosidase activities that were comparable to those produced by the positive control (Fig. 4b, compare 1 and 10), which is in perfect agreement with the phenotypes observed on the X-Gal plates. Essentially the same result was obtained when the interaction was tested in the opposite orientation, i.e. when EIIAᵦⁿʳ and SpoT1 were fused to the T18 and T25 domains, respectively (data not shown).

Like many bacteria, R. eutropha H16 harbours a paralogue of SpoT1, which is encoded by gene spoT2 (gene locus H16_A1337). SpoT2 is the orthologue of RelA, which synthesizes ppGpp in response to amino acid starvation (Brigham et al., 2012). Given the similarity of both proteins, which share 32 % sequence identity, we wondered whether EIIAᵦⁿʳ might also interact with SpoT2. However, only low β-galactosidase activities were detectable when the EIIAᵦⁿʳ and the SpoT2 fusion proteins were co-expressed (Fig. 4a, b – bar 2). These data suggest that EIIAᵦⁿʳ specifically interacts with SpoT1, but not with its paralogue SpoT2.

Evidence that phosphorylation of EIIAᵦⁿʳ impedes interaction with SpoT1

The ability of EIIAᵦⁿʳ to interact with its partner proteins is regulated by its phosphorylation state. As far as is known, non-phosphorylated EIIAᵦⁿʳ is involved in protein–protein
interaction. To learn whether this also holds true for the interaction of \textit{R. eutropha} EIIA\textsuperscript{Ntr} with SpoT1, we exchanged the phosphorylation site His67 in \textit{R. eutropha} EIIA\textsuperscript{Ntr} with an alanine or a glutamate residue. Often, the non-phosphorylated and phosphorylated states of protein phosphorylation sites can be mimicked by Ala and Glu substitutions, respectively (Dissmeyer & Schnittger, 2011). First, we tested the impact of these mutations in the context of the BACTH system. Both, the qualitative and quantitative \(\beta\)-galactosidase assays indicated that the EIIA\textsuperscript{Ntr}-H67A mutant fully retains the ability to interact with SpoT1, whereas the H67E substitution abolished interaction (Fig. 4a, b – bars 4 and 7). None of the EIIA\textsuperscript{Ntr} variants was able to interact with SpoT2, confirming the results obtained with the wild-type protein (Fig. 4a, b – bars 5 and 8).

Next, we confirmed these results by a different method, i.e. far-Western analysis. The various \textit{R. eutropha} Strep-EIIA\textsuperscript{Ntr} variants were purified from \textit{E. coli} and spotted in serial dilutions on a nylon membrane. The membrane was subsequently incubated in a solution containing purified \textit{R. eutropha} SpoT1-His10 protein. Finally, potential protein complexes were detected using anti-His-tag antiserum. This procedure yielded strong signals for wild-type EIIA\textsuperscript{Ntr} and the EIIA\textsuperscript{Ntr}-H67A mutant, indicating complex formation with SpoT1, whereas much weaker signals were obtained for the EIIA\textsuperscript{Ntr}-H67E mutant (Fig. 5). Thus, the far-Western data are in perfect agreement with the results obtained in the BACTH assay. They consistently show that \textit{R. eutropha} EIIA\textsuperscript{Ntr} interacts with SpoT1 in \textit{vivo} and \textit{in vitro}. Moreover, phosphorylation of EIIA\textsuperscript{Ntr} as mimicked by a His\textrightarrow{Glu} exchange inhibits this interaction.

### Interactions of EIIA\textsuperscript{Ntr} with SpoT1 and KdpD are not conserved

We wanted to determine whether the interaction of EIIA\textsuperscript{Ntr} with SpoT1 also takes place in \textit{E. coli}, i.e. whether the homologous proteins of \textit{E. coli} also interact. To this end, we repeated the BACTH assay with the corresponding \textit{E. coli} proteins, which were fused to the T25 and T18 fragments of CyaA. No interaction was detectable between these proteins (Fig. 4a). Moreover, no cross-species interaction could be observed, i.e. EIIA\textsuperscript{Ntr} of \textit{R. eutropha} was also unable to interact with the \textit{E. coli} SpoT protein (Fig. 4a, b – bars 3, 6, 9). Vice versa, EIIA\textsuperscript{Ntr} of \textit{E. coli} did not interact with the \textit{R. eutropha} SpoT1 protein (Fig. 4a). To confirm this result, we included the \textit{E. coli} EIIA\textsuperscript{Ntr} protein in the dot blot far-Western analysis. In agreement, complexes between \textit{E. coli}
EIAr and SpoT1 of R. eutropha were not detectable in the far-Western blot (Fig. 5). In conclusion, the EIAr–SpoT interaction observed for the R. eutropha proteins does not hold for the homologous E. coli proteins. Moreover, E. coli EIAr is unable to cross-interact with SpoT1 of R. eutropha.

In E. coli, EIAr stimulates the kinase activity of KdpD through interaction, leading to higher expression levels of the kdpFABC operon (Lüttmann et al., 2009). R. etutropha H16 encodes two distinct proteins with significant homology to E. coli KdpD (Pohlnann et al., 2006). The first, KdpD1 (gene locus H16_A2600), shares 47 % sequence identity with KdpD from E. coli. The second, KdpD2 (gene locus H16_B0895), is present in a gene cluster reminiscent of the E. coli kdpFABCDE operon and therefore it might represent the genuine orthologue of KdpD in E. coli. KdpD2 is 46 % identical with E. coli KdpD. We wanted to determine whether either one of these KdpD homologues interacts with the R. etutropha EIAr protein, i.e. whether the EIAr–KdpD interaction also takes place in R. etutropha. Therefore, corresponding two-hybrid plasmids encoding fusions of the R. etutropha KdpD proteins to the N-terminus of CyaA-T18 were constructed and tested in the BACTH assay. For comparison, a construct encoding a fusion of E. coli KdpD to the T18 fragment was included in this analysis. None of the EIAr proteins, regardless of whether derived from R. etutropha or E. coli, interacted with KdpD1 or KdpD2 of R. etutropha in the BACTH assay (Fig. 4a).

A control experiment revealed self-interaction of both KdpD proteins in the BACTH assay, confirming functionality of the fusion constructs (data not shown). In contrast, interaction of the E. coli EIAr variants with E. coli KdpD was readily detectable (Fig. 4a), as expected from previous results (Lüttmann et al., 2009). To confirm these results further, the various interactions were additionally tested in the opposite direction. In this case, the KdpD proteins were fused to the N-terminus of the T25 domain, whereas the EIAr variants were fused to the C-terminus of the T18 domain. Once again, the BACTH assay detected interaction of EIIAr with E. coli KdpD, but not with the KdpD1 and KdpD2 proteins of R. etutropha (Fig. S1, available in the online Supplementary Material). In agreement, interaction of R. etutropha KdpD2 with EIAr was also not detectable when KdpD2 was fused to the C-terminus of CyaA-T18 (Fig. S2). In conclusion, the interaction of EIAr with KdpD as present in E. coli is not detectable for the corresponding R. etutropha proteins.

**E. coli EIAr protein can be functionally replaced by its homologue from R. etutropha in the control of kinase KdpD activity**

Our results indicated that interaction of EIAr with SpoT1 and KdpD is confined to the respective cognate organisms R. etutropha and E. coli, and does not occur in other species. In this respect, it was surprising to learn from our BACTH analysis that R. etutropha EIAr interacts with the KdpD protein of E. coli (Figs 4a and S1). This crossexpression was maintained when the phosphorylation site His67 in R. etutropha EIAr was exchanged with an Ala residue, but appeared weaker when the protein carried the His67Glu exchange. This reflects the properties of the E. coli EIAr protein, which interacts with KdpD when non-phosphorylated (Lüttmann et al., 2009). We wanted to know whether the interaction also results in productive regulation, i.e. whether R. etutropha EIAr stimulates expression of the kdpFABC operon when present in E. coli. To this end, we complemented an E. coli ptsN mutant carrying a kdpFA’–lacZ reporter fusion with plasmids encoding EIAr either from R. etutropha or from E. coli. The transformants were grown in LB and the β-galactosidase activities were determined. LB contains ≥ 5 mM K+. At this K+ concentration, kdpFA’–lacZ fusion is completely repressed (Fig. 6). Complementation with plasmids overexpressing EIAr of R. etutropha or E. coli markedly stimulated expression of the fusion, whereas the empty expression vector had no effect (Fig. 6). These data show that R. etutropha EIAr can substitute its E. coli homologue in regulation of KdpD kinase activity.
DISCUSSION

The truncated PTS with EIIA^Ntr as the regulatory output domain is a signal transduction device controlling import-ant activities in various bacteria (Pfülger-Grau & Görke, 2010). In this study, we investigated the putative function of EIIA^Ntr in R. eutropha H16, a model organism of β-Proteobacteria. Previous work suggested a role of the phosphorylation cascade EI^A-HPr-A-EIIANtr in controlling PHB accumulation (Kaddor & Steinbüchel, 2011; Pries et al., 1991). Here, we identify the bifunctional ppGpp synthase/hydrolase SpoT1 as the interaction partner of EIIA^Ntr in R. eutropha. This was achieved by a ligand-fishing approach (Fig. 3), for which a broad-host-range bait vector was established (Fig. 2). This vector might also be useful for the identification of protein–protein interactions in other bacterial species. BACTH assays and far-Western analysis indicated that non-phosphorylated EIIANtr interacts with SpoT1 (Figs 4 and 5), recapitulating what has been observed for interactions performed by EIIANtr in other bacteria (Pfülger-Grau & Görke, 2010). Recently, the stringent control was shown to be essential for PHB accumulation in R. eutropha (Brigham et al., 2012). Hence, our finding offers the possibility that EIIA^Ntr affects the cellular PHB content by modulating the stringent response through interaction with SpoT1 (Fig. 1). This mechanism could also explain the interaction of EIIANtr with SpoT1 in R. eutropha.
pleiotropic effects caused by knockouts of EI, HPr and EIIANtr (Kaddor et al., 2012).

The stringent control is a widely conserved stress response that allows bacteria to adapt to nutrient deprivation and other stresses by globally reprogramming gene expression (Boutte & Crosson, 2013; Potrykus & Cashel, 2008). This is achieved through synthesis of the nucleotide ppGpp, which downregulates functions involved in translation and replication, and activates stress responses. In E. coli, the ppGpp level is determined by the activities of two paralogous proteins, RelA and SpoT. RelA synthesizes ppGpp in response to amino acid starvation, while SpoT hydrolyses this nucleotide. However, under specific stress conditions, such as carbon or phosphate starvation, SpoT can also gain ppGpp synthase activity (Boutte & Crosson, 2013). Similar to E. coli, R. eutropha contains two proteins involved in ppGpp metabolism, SpoT1 and SpoT2, which are the orthologues of SpoT and RelA, respectively (Brigham et al., 2012). A recent study showed that spoT2 mutants are unable to produce PHB and that chemical induction of the stringent response increases PHB accumulation (Brigham et al., 2012). It was concluded that nitrogen starvation induces ppGpp synthesis by SpoT1 and consequently PHB production. Moreover, a second ppGpp spike coinciding with the beginning of PHB degradation was observed in other PHB-producing bacteria (Ruiz et al., 2001). Taking this into account, an integral regulatory role of ppGpp throughout the PHB cycle was proposed by Brigham et al. (2012). When nitrogen becomes newly available during PHB production, SpoT2 activity slows down. Instead, the ppGpp synthase activity of SpoT1 becomes activated triggering intermediate levels of ppGpp, which stimulate PHB degradation while still repressing functions for translation and amino acid metabolism (Brigham et al., 2012). Thus, one possibility is that EIIANtr accumulates in its non-phosphorylated form during the PHB synthesis cycle and subsequently stimulates PHB degradation through transient activation of the ppGpp synthase activity of SpoT1. Alternatively, EIIANtr could inhibit the ppGpp hydrolase activity of SpoT1 at the onset of PHB mobilization. This could explain the previously observed antagonistic effects of knockouts of EI/HPr and EIIANtr on cellular PHB contents (Kaddor & Steinbüchel, 2011). Further, it would provide an explanation for the aberrant levels of proteins involved in translation and amino acid metabolism in a mutant lacking HPr and EI (Kaddor et al., 2012). Finally, a disturbed ppGpp metabolism that may result from hyperphosphorylation of HPr by EI could also account for the deleterious effect of an HPrK/P knockout (Krauße et al., 2009).

It is worth mentioning that the protein chain leading to phosphorylation of EIIANtr provides multiple entry points for integrating information about the carbon and nitrogen status. In E. coli, α-ketoglutarate, which accumulates during nitrogen limitation, inhibits autophosphorylation of EI (Doucette et al., 2011). Moreover, the kinase activity of HPrK/P is activated by high fructose-1,6-bisphosphate concentrations, reflecting carbon overflow conditions (Jault et al., 2000; Singh et al., 2008). Thus, high α-ketoglutarate and fructose-1,6-bisphosphate concentrations may accompany PHB synthesis, and could potentially inhibit phosphorylation of HPr by EI, and thereby trigger accumulation of non-phosphorylated EIIANtr during the PHB cycle.

The interaction of SpoT with EIIANtr, as present in R. eutropha, appears to be absent in E. coli (Fig. 4). Moreover, EIIANtr of E. coli is not able to interact with the R. eutropha SpoT1 protein (Figs 4a and 5), and EIIANtr of R. eutropha does not interact with SpoT of E. coli (Fig. 4a, b). Obviously, E. coli EIIANtr lacks features that allow R. eutropha EIIANtr to form a complex with SpoT1. This might also be reflected by the less pronounced homology of both proteins, which share 29% sequence identity (Kaddor & Steinbüchel, 2011). E. coli does not accumulate biodegradable polyesters for carbon storage and therefore regulation of SpoT activity by EIIANtr might not be required. To discover whether both processes indeed coincide, it will be interesting to determine whether interaction of EIIANtr with SpoT is conserved in other Proteobacteria that produce polyesters for carbon storage, such as Pseudomonas species (Velázquez et al., 2007). Interestingly, R. eutropha EIIANtr can replace its homologue in E. coli in binding and regulation of KdpD, although this interaction does not take place in R. eutropha itself (Figs 4a and 6). This surprising observation may indicate that regulation of KdpD by EIIANtr is an evolutionary ‘ancient’ process, which already existed in the last
common ancestor of both bacteria. In agreement, interaction of EIIA<sup>Ntr</sup> with KdpD has also been observed in<br>Rhizobium leguminosarum (Prell et al., 2012), which is only distantly related to E. coli. In <i>R. eutropha</i> this interaction might have been lost during evolution, putatively by divergence of the KdpD protein(s). In lieu thereof, interaction of EIIA<sup>Ntr</sup> with SpoT has been evolved. In conclusion, EIIA<sup>Ntr</sup> might fulfil different functions depending on the species under investigation. Thus, PTS<sup>Ntr</sup> is a versatile system that can be recruited to establish various regulatory circuits in the bacterial cell.

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**REFERENCES**


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