Essential Role of the *hprK* Gene in *Ralstonia eutropha* H16

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Key Words

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Abstract

Ralstonia eutropha H16 possesses an incomplete phosphoenolpyruvate (PEP):sugar phosphotransferase system (PTS) composed of EI, HPr, EIIA^{Ntr} (PtsN) and EIIA^{Man} (PtsM). We could show that in vitro the incomplete PTS phosphorylation cascade is partially functional. HPr becomes phosphorylated by PEP and EI, and transfers the phosphoryl group to EIIA^{Ntr}, but only extremely slowly to EIIA^{Man}. Components of this system have previously been shown to regulate the metabolism of polyhydroxybutyrate. Downstream from *ptsN* this organism contains an *hprK* gene, which codes for a homologue of HPr kinase/phosphorylase. We show that this enzyme phosphorylates HPr using ATP as phosphoryl donor. Interestingly, *hprK* appeared to be essential in *R. eutropha* because this gene could not be deleted in the wild-type strain, but could be deleted in mutants lacking *ptsH* or *ptsI*. This suggests that an increase in the HPr and/or P~His-HPr concentrations might be responsible for the growth defect. To test this hypothesis, various *ptsH* alleles were introduced into the *ptsH hprK* double mutant. Complementation of this mutant was possible only with the *ptsH*(His15Ala) allele, but not with the wild-type or *ptsH*(Ser46Ala) alleles. We con-

clude that elevated amounts of His-15-phosphorylated HPr, formed in the *hprK* mutant, are responsible for its growth defect.

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Introduction

Ralstonia eutropha H16 is a facultatively chemoautotrophic β -proteobacterium capable of oxidizing hydrogen or formate as an energy source while assimilating carbon dioxide into cell material [Bowien and Kusian, 2002; Cramm, 2009]. For heterotrophic growth, this bacterium can utilize numerous carbon sources including the two sugars, fructose [Wilde, 1962] and *N*-acetyl-D-glucosamine [Jenni et al., 1988], both of which are exclusively metabolized via the Entner-Doudoroff pathway [Gottschalk et al., 1964; Pohlmann et al., 2006]. *N*-Acetyl-D-glucosamine is apparently transported and phosphorylated by a phosphoenolpyruvate (PEP):carbohydrate phosphotransferase system (PTS) composed of the two proteins, NagF, containing the three soluble domains enzyme IIA^{Nag} (EIIA^{Nag}) – HPr – enzyme I (EI), and NagE, consisting of the membrane-spanning EIIC^{Nag} and the cytosolic domain EIIB^{Nag} [Pohlmann et al., 2006].

The PTS proteins and domains form a phosphorylation cascade [Deutscher et al., 2006]. In the first step, EI uses

PEP to phosphorylate His-15 in HPr, which transfers the phosphoryl group to one of usually several EIAs present in PTS-containing bacteria. Finally, the corresponding EIIB accepts the phosphoryl group from EIIA and passes it on to the sugar bound to the EIIC. The phosphorylated sugar is subsequently released into the cytoplasm. In addition to sugar transport and phosphorylation, PTS components carry out numerous regulatory functions in carbon metabolism and other important cellular processes [Deutscher et al., 2006; Görke and Stülke, 2008]. The PTS components can modulate the activities of their target proteins by either phosphorylating them or by interacting with them in a phosphorylation-dependent manner.

In addition to the *N*-acetyl-D-glucosamine-specific PTS, *R. eutropha* H16 possesses an 'incomplete' PTS regulating the metabolism of the storage polyester poly- β -hydroxybutyrate [Pries et al., 1991; Reinecke and Steinbüchel, 2009]. This PTS is composed of the proteins EI, HPr, EIIA^{Ntr} and EIIA^{Man} encoded by *ptsI*, *ptsH* (also called *phbI* and *phbH*), *ptsN* and *ptsM*, respectively. The genes *ptsM*, *ptsH* and *ptsI* presumably form an operon on chromosome 1 of *R. eutropha* H16. The incomplete PTS lacks EIIB and EIIC components and therefore cannot transport and phosphorylate sugars. It was shown instead to carry out regulatory functions because *ptsI* and *ptsH* mutants of *R. eutropha* H16 were found to exhibit significantly reduced poly- β -hydroxybutyrate contents [Pries et al., 1991]. Interestingly, in the γ -proteobacterium *Azotobacter vinelandii* inactivation of *ptsI* and *ptsH* as well as replacement of the phosphorylatable His-68 in EIIA^{Ntr} diminished poly- β -hydroxybutyrate production, whereas inactivation of *ptsN* enhanced it [Noguez et al., 2008]. Similar observations were reported for polyhydroxyalkanoate production in *Pseudomonas putida*, another γ -proteobacterium [Velázquez et al., 2007]. These results suggest that EIIA^{Ntr} is the major player in the regulation of poly- β -hydroxybutyrate metabolism and that it becomes phosphorylated by PEP, EI and HPr. Phosphorylation of EIIA^{Ntr} seems to favor poly- β -hydroxybutyrate storage.

Like firmicutes, *R. eutropha* H16 contains an HPr kinase/phosphorylase (HprK/P) encoded by the *hprK* gene, which is located immediately downstream from *ptsN* on chromosome 1. HprK/P uses ATP to phosphorylate HPr at Ser-46. Generally, phosphorylation of HPr at one site drastically slows phosphorylation at the other site [Deutscher et al., 2006]. Only in the HPr of the incomplete *R. eutropha* PTS, but not in the HPr domain of NagF, the region flanking Ser-46 resembles that in HPr of firmicutes. It was therefore likely that only this HPr becomes phosphorylated by HprK/P.

We here report that the phosphorylation cascade formed by the incomplete PTS of *R. eutropha* H16 is indeed functional in vitro and that EI and HPr can phosphorylate EIIA^{Ntr}, but not EIIA^{Man}. In addition, HprK/P phosphorylates HPr in an ATP-dependent reaction. Surprisingly, it was not possible to delete the *hprK* gene in the *R. eutropha* wild-type strain. We provide evidence that the increase of the P~His-HPr concentration, caused by *hprK* inactivation, is responsible for the growth defect.

Results and Discussion

In vitro Phosphorylation Assays with *R. eutropha* PTS Proteins

To test whether the components of the truncated PTS are functional in vitro and whether HPr is indeed phosphorylated at its two phosphorylation sites, His-15 and Ser-46, the *R. eutropha* H16 *ptsH*, *ptsN*, *ptsM* and *hprK* genes were PCR-amplified, cloned in an expression vector and the corresponding His-tagged proteins were purified as described in Experimental Procedures. We did not succeed to purify *R. eutropha* EI and used the *Bacillus subtilis* homologue [Galinier et al., 1997] instead for in vitro phosphorylation experiments. To test for cross-reactivity of Ser-46 phosphorylation, *B. subtilis* HPr [Galinier et al., 1997] and HprK/P [Hanson et al., 2002] were also purified and used for the ATP-dependent HPr phosphorylation experiments. HPr of *R. eutropha* could be phosphorylated with *B. subtilis* EI and 0.01 mM [³²P]PEP (fig. 1a, lane 2). In addition, very strong phosphorylation of EIIA^{Ntr} occurred when it was incubated with EI, HPr and [³²P]PEP (fig. 1a, lane 6). No radioactive band was detectable when an identical experiment was carried out with EIIA^{Man} (fig. 1a, lane 4). When carrying out an additional phosphorylation experiment with EI, HPr, EIIA^{Man} and [³²P]PEP, a faint band migrating to the position of EIIA^{Man} became visible after prolonged exposure to a storage phosphor screen (data not shown). In any case, compared to EIIA^{Ntr}, EIIA^{Man} is only barely phosphorylated under the in vitro conditions employed. HPr of the incomplete *R. eutropha* PTS became also phosphorylated with HprK/P and 0.4 mM [γ -³²P]ATP (fig. 1b, lane 3). Purified HprK/P of *B. subtilis* also phosphorylated *R. eutropha* HPr and vice versa, HprK/P of *R. eutropha* modified the slightly slower migrating *B. subtilis* HPr (fig. 1b, lanes 4 and 5, respectively). The upper radioactive band in lane 4 migrates to the position of His-tagged *B. subtilis* HprK/P, which has previously been shown to autophosphorylate [Galinier et al., 1998].

Fig. 1. In vitro phosphorylation experiments with *R. eutropha* and *B. subtilis* PTS proteins. **a** PEP-dependent phosphorylation of EI (from *B. subtilis*), HPr, EIIA^{Ntr} and EIIA^{Man}. The 20- μ l reaction mixtures contained 100 mM Tris-HCl, pH 7.5; 20 mM MgCl₂; 1 mM DTT; 0.01 mM [32P]PEP (0.5 μ Ci, specific radioactivity 3 Ci/mmol) and the following proteins: lane 1, 2.4 μ g EI; lane 2, 2.4 μ g EI and 1.6 μ g HPr; lane 3, 1.6 μ g HPr; lane 4, 2.4 μ g EI, 1.6 μ g HPr and 4.0 μ g EIIA^{Man}; lane 5, 4.0 μ g EIIA^{Man}; lane 6, 2.4 μ g EI, 1.6 μ g HPr and 0.8 μ g EIIA^{Ntr}; lane 7, 0.8 μ g EIIA^{Ntr}. **b** ATP-dependent phosphorylation of HPr with HprK/P. The 7.5- μ l reaction mixtures contained 80 mM Tris-HCl, pH 7.4; 15 mM MgCl₂; 3 mM DTT; 0.4 mM [γ -³²P]ATP (1 μ Ci, specific radioactivity 3.75

Ci/mmol); 50 mM fructose-1,6-bisphosphate and the following proteins: lane 1, 1.3 μ g HPr of *R. eutropha*; lane 2, 1.4 μ g HprK/P of *R. eutropha*; lane 3, 1.3 μ g HPr and 1.4 μ g HprK/P of *R. eutropha*; lane 4, 1.3 μ g HPr of *R. eutropha* and 1.8 μ g HprK/P of *B. subtilis*; lane 5, 1.0 μ g HPr of *B. subtilis* and 1.4 μ g HprK/P of *R. eutropha*. For **a** and **b**, samples were incubated for 30 min at 37°C, and proteins were subsequently separated on 1% (w/v) SDS/15% (w/v) polyacrylamide gels, which were dried after electrophoresis. Radioactive bands were visualized using a PhosphorImager after 15 h exposure. EIIA indicates the migration position of EIIA^{Ntr} and EIIA^{Man}.

The hprK Gene Is Essential in the Wild-Type Strain, but Not in ptsH or ptsI Mutants

Surprisingly, despite extensive efforts we were not able to obtain *hprK* deletion mutants from the wild-type strain *R. eutropha* H16 when using plasmid pNHG1 Δ *hprK* in conjugation experiments with the *Escherichia coli* donor strain S17-1 (see Experimental Procedures). Conjugation experiments were carried out as previously described [Jeffke et al., 1999; Lenz et al., 1994] and both modes of single integration of pNHG1 Δ *hprK* into the *R. eutropha* chromosome, which leave an intact copy of *hprK*, were observed. However, in neither one of the two types of heterogenotes did the second recombination event occur. This would have led to excision of the vector and deletion of the *hprK* gene. In the more than 300 tested clones exhibiting the expected phenotype of homogenotes (growth on Nutrient Broth containing 20% sucrose, but not on

Nutrient Broth containing tetracycline) [Lenz et al., 1994] the wild-type *hprK* had been restored.

One possible explanation for the failure to obtain an *hprK* mutant was that the loss of P-Ser-HPr formation might lead to a growth defect. In this case, we expected that the construction of *ptsH* mutants should also be impossible. To test this hypothesis, we tried to delete an internal fragment of 63 bp from the *R. eutropha* *ptsH* gene, that includes the coding region for Ser-46 of HPr, but not for His-15. By using the same procedure applied for the construction of the *hprK* mutant, *ptsH* mutants could easily be isolated. It therefore seems that it is not the absence of P-Ser-HPr formation which prevents growth of the *hprK* mutant. We subsequently tested whether *hprK* deletion would be possible in the *ptsH* strain. Indeed, *hprK* deletion mutants were obtained with high frequency when using the *ptsH* mutant as recipient. Owing to the

Table 1. Number of *R. eutropha* $\Delta hprK$ $\Delta ptsH$ transconjugants obtained with either empty pBBR1MCS-3 or pBBR1MCS-3 carrying the indicated *ptsH* allele

Sample dilution ^a	Number of transconjugants			
	no insert	wild-type <i>ptsH</i>	<i>ptsH</i> (Ser46Ala)	<i>ptsH</i> (His15Ala)
10 ⁰	–	96	70	>2,000
10 ¹	–	3	3	>2,000
10 ²	>2,000	0	0	>1,000
10 ³	>2,000	0	0	217
10 ⁴	>1,000	0	0	17
10 ⁵	>500	0	0	0

^a Aliquots of the total conjugation sample were diluted as indicated before plating them on fructose minimal agar [Schlegel et al., 1961] containing tetracycline.

lack of phosphorylation of HPr at Ser-46, *hprK* mutants probably contain higher amounts of HPr and/or P~His-HPr than the wild-type strain. A possible and most plausible explanation for these findings was therefore that overproduction of one of these two HPr forms might lead to a growth defect, which of course would be prevented when *ptsH* was deleted. If P~His-HPr was the deleterious HPr form, inactivation of *hprK* should also be possible in a *ptsI* mutant lacking EI and therefore unable to form P~His-HPr. To test this assumption, nearly the entire *ptsI* gene (1662 bp) was deleted. Indeed, *ptsI* *hprK* double mutants were obtained at a frequency similar to that observed for the *ptsH* *hprK* double mutants. We therefore concluded that elevated amounts of P~His-HPr are probably responsible for the failure to obtain *hprK* deletion mutants from wild-type *R. eutropha* H16.

A ptsH hprK Double Mutant Can Be Complemented with the ptsH(His15Ala), but Not the Wild-Type and ptsH(Ser46Ala) Alleles

To further support the above hypothesis, we tried to introduce into the *ptsH* *hprK* double mutant plasmids expressing either wild-type *ptsH* or the *ptsH*His15Ala or *ptsH*Ser46Ala allele. The construction of the *ptsH* alleles, which contained their own ribosome-binding site, but were expressed from the *R. eutropha* *cbb* promoter, is described in Experimental Procedures. In the last step, the constructs were inserted into the broad-host-range vector pBBR1MCS-3. The resulting plasmids were used to transform *E. coli* S17-1, and conjugation experiments were carried out as previously described [Lenz et al., 1994]. The *cbb* promoter is fully induced during autotro-

phic growth and moderately active (about one fifth of its full activity) when *R. eutropha* is grown on fructose [Bowien and Kusian, 2002]. To avoid too strong expression of the *ptsH* alleles, aliquots of the conjugation experiments were plated on mineral medium containing 0.4% fructose and 20 µg/ml tetracycline. A large number of *R. eutropha* $\Delta ptsH$ $\Delta hprK$ transconjugants were obtained with the plasmid containing the *ptsH*(His15Ala) allele, which nevertheless was about 200-fold lower than the number of transconjugants obtained with empty pBBR1MCS-3 (table 1). However, compared to the *ptsH*His15Ala allele the number of transconjugants obtained with wild-type *ptsH* or the *ptsH*(Ser46Ala) allele was drastically reduced (about 2,000-fold lower). The few transconjugants obtained with the two latter *ptsH* alleles might result from mutations in the *cbb-ptsH* part of the plasmid or in the genome of the recipient strain. The entire experiment was repeated twice, and in each case similar numbers of transconjugants were obtained.

During the last decade, HprK/P has been intensively studied, and *hprK* deletions were carried out, not only in numerous firmicutes [for a review, see Deutscher et al., 2006] but also in *Neisseria meningitidis* [Boël et al., 2003], a β -proteobacterium like *R. eutropha* H16, and the α -proteobacterium *Sinorhizobium meliloti* [Pinedo and Gage, 2009]. It was therefore surprising that *hprK* is essential in *R. eutropha* H16. The failure to obtain *hprK* mutants of *R. eutropha* H16 turned out to presumably be due to an imbalance of the different HPr forms caused by the mutation. More specifically, the elevated amount of P~His-HPr appeared to be the major cause of the growth defect, because expression of *ptsH* alleles encoding HPr proteins with a phosphorylatable His-15 was incompatible with a concurrent *hprK* deletion. To the best of our knowledge, this is the first report that elevated production of P~His-HPr resulting from *hprK* inactivation can prevent growth. These findings suggest that in *R. eutropha* H16 the phosphorylation state of the proteins of the truncated PTS controls essential cellular functions. The molecular details of the mechanism responsible for the growth defect will therefore be the subject of future studies.

Experimental Procedures

Bacterial Strains, Plasmids and Growth Conditions

R. eutropha strain H16 and the derived *ptsH*, *ptsI* and *hprK* mutants were grown on mineral medium [Schlegel et al., 1961] supplemented with 0.4% pyruvate or 0.2% fructose and contained 20 µg/ml tetracycline when appropriate. To select for recombinants, the strains were also cultivated on Nutrient Broth medium

Table 2. PCR primers used in this study

Primer name	Primer sequence	Restriction site	Used for
DK3	AAAGGATCCATGCTGCAGAGGGACACCACCATCATC	<i>Bam</i> HI	<i>ptsH</i> synthesis
DK4	TTTAAGCTTCCTCACTCTCCCTCACCAAAGCGG	<i>Hind</i> III	<i>ptsH</i> synthesis
DK1	AAAGGATCCATGGCAGGCATTCTGATCATCGCGCAC	<i>Bam</i> HI	<i>ptsM</i> synthesis
DK2	TTTAAGCTTTTATTGATGATGGTGGTGTCCCTCTGCAG	<i>Hind</i> III	<i>ptsM</i> synthesis
DK5	AAAAGATCTATGAATCGTTTGGCCAAATTGCTGCCAC	<i>Bgl</i> II	<i>ptsN</i> synthesis
DK6	TTTAAGCTTTCAGGGATGCCATGCGATCAGCAAC	<i>Hind</i> III	<i>ptsN</i> synthesis
DK7	CCGCCCCACCAAGTCAGGATCCATGGAATCACC	<i>Bam</i> HI	<i>hprK</i> synthesis
DK8	TCAACGCCGTTGTAAGCTTATGAAAACGCCGG	<i>Hind</i> III	<i>hprK</i> synthesis
<i>ptsH</i> #1	GCTGGAATCCATCGACGTC		Verification of <i>ptsH</i> deletion
<i>ptsH</i> #2	TGGCGGTAAGCGAGGTGCGTCCGCCC		Verification of <i>ptsH</i> deletion
<i>ptsI</i> _Xf	GAGCATCATGGGCGTCTAGATGCTGG	<i>Xba</i> I	<i>ptsI</i> deletion
<i>ptsI</i> _Hr	GCGCGCACAAAGCTTGCGCC	<i>Hind</i> III	<i>ptsI</i> deletion
<i>ptsI</i> _Hf	CGATCCCGAGAAGCTTGCGGC	<i>Hind</i> III	<i>ptsI</i> deletion
<i>ptsI</i> _Br	CGTCTGGATCCGCAACCAGATCACC	<i>Bam</i> HI	<i>ptsI</i> deletion
<i>ptsI</i> Verify F	AAGATGTCCC GCAATGGTCGCC	–	Verification of <i>ptsI</i> deletion
<i>ptsI</i> Verify R	TTCGATGCCCAGGTGCGCAG	–	Verification of <i>ptsI</i> deletion
DK11	TCAGCGTCACCAGCAAGAAGCGTGTGTTCCG	–	<i>hprK</i> deletion
DK12	TATTGCAGGCCTTCATCTTGTCTCTGCTGGG	–	<i>hprK</i> deletion
DK13	TGTCCGAAATCGCGCAACTGCTTTCCGACC	–	<i>hprK</i> deletion
DK14	TTGCTCTCCTGGGTGTGGAAGAACTTCATGG	–	<i>hprK</i> deletion
DK9	ACTGGGCCTGGAAGTATCTCG	–	Verification of <i>hprK</i> deletion
DK10	ATTTTCATTGAAGGCCGCTGCCCCG	–	Verification of <i>hprK</i> deletion
DK15	AAATCTAGAGCAACTGGCGAAGGGTAAGG	<i>Xba</i> I	Construction of the <i>cbb</i> promoter
DK16	TTTGGTACCGTTTCATATGTGTCTCCTTGCGTGGTTGAG	<i>Kpn</i> I, <i>Nde</i> I	Construction of the <i>cbb</i> promoter
DK17	AAACATATGCTGCAGAGGGACACCACCATCATC	<i>Nde</i> I	Construction of <i>ptsH</i> and <i>ptsH</i> Ser46Ala
DK18	AAACATATGCTGCAGAGGGACACCACCATCATCAATA-AACTCGGCCTGGCTGCGCGCGCG	<i>Nde</i> I	Construction of <i>ptsH</i> His15Ala
DK20	TGCTCTTGGCGTCGACCTGGCGACC	<i>Sal</i> I	Construction of <i>ptsH</i> Ser46Ala
DK21	CCAGGTCGACGCGAAGGCCATCATGGGCGTCATGATGC	<i>Sal</i> I	Construction of <i>ptsH</i> Ser46Ala
DK22	TTTGGTACCCCTCACTCTCCCTCACCAAAGCGG	<i>Kpn</i> I	Construction of the all three <i>ptsH</i> alleles

containing 20% (w/v) sucrose. *E. coli* strain DH5 α was grown in LB medium containing either 20 μ g/ml tetracycline or 50 μ g/ml ampicillin.

Construction of *hprK*, *ptsH* and *ptsI* Deletion Mutants

To construct *R. eutropha* Δ *hprK* or Δ *ptsI* mutants, the flanking regions of the corresponding gene were PCR-amplified by using two primer pairs for each gene (table 2) and genomic DNA as template. The PCR products for each gene were cloned into the non-replicative *sacB*-containing suicide vector pNHG1 [Jeffke et al., 1999], providing pNHG1 Δ *hprK* and pNHG1 Δ *ptsI*, which were

subsequently used to transform the *E. coli* donor strain S17-1 [Simon et al., 1983]. Conjugation experiments were carried out as previously described [Jeffke et al., 1999; Lenz et al., 1994]. *R. eutropha* heterogenotes carrying the plasmid integrated in the genome by single recombination were identified by colony PCR. They were used to select for homogenotes resulting from a second recombination by growing them in Nutrient Broth medium at 30°C. After different time periods (7, 20 and 48 h), aliquots were plated on Nutrient Broth medium containing 20% sucrose (w/v). Under these growth conditions, the presence of the plasmid-encoded *sacB* gene is lethal due to SacB-catalyzed formation of tox-

ic amounts of levan from sucrose, and growth of strains having lost the plasmid by the second recombination is therefore favored. Colonies growing up under these conditions were tested for tetracycline-sensitivity. The second recombination can either restore the wild-type genotype or lead to the deletion of the corresponding gene. PCR with specific primers (table 2) was used to distinguish between these two possibilities. Deletion mutants for *ptsI* were obtained with a reasonable frequency. In contrast, for the deletion of the *hprK* gene from the wild-type strain more than 300 homogenotes were tested by PCR, but in none of them was the *hprK* gene deleted. However, when starting from the Δ *ptsH* (see below) or Δ *ptsI* strain, in each case, between two and four *hprK* deletion strains were detected among the first 30 homogenotes tested.

A mutant of *R. eutropha* H16 with a small in-frame deletion in the *ptsH* gene was obtained using a modified protocol for gene replacement mutagenesis. A 3.78 kb *HindIII*-*KpnI* fragment containing the *ptsH* and *ptsI* genes was transferred from pBH4550 (a kind gift from Alexander Steinbüchel) to LITMUS 28 [Pries et al., 1991]. Digestion of the resulting plasmid with *SalI* and *XhoI* led to a 63-bp in-frame deletion in the *ptsH* gene (Ala-44 to Glu-64), which was subsequently excised by digestion with *SnaBI* and *EcoRV* and inserted into the suicide vector pLO3 [Lenz and Friedrich, 1998], which had previously been cut with *PmeI*. The deleterious plasmid was introduced into *R. eutropha* H16 by conjugative transfer from the donor strain *E. coli* S17-1. Transconjugants were grown overnight in Nutrient Broth medium and plated on Nutrient Broth plates containing sucrose. Sucrose-resistant segregants were screened by PCR for the desired deletions using primers *ptsH*#1 and *ptsH*#2. One isolated *R. eutropha* H16 Δ *ptsH* mutant was used for further studies.

Protein Purification

In order to purify His-tagged proteins of *R. eutropha* H16, the *ptsH*, *ptsN*, *ptsM* and *hprK* genes were PCR-amplified and cloned into the His-tag expression vector pWH844 [Schirmer et al., 1997]. The resulting plasmids were used to transform *E. coli* DH5 α , and the corresponding proteins were purified as previously described [Schirmer et al., 1997]. Because we did not succeed to purify *R. eutropha* EI, we used the *B. subtilis* homologue [Galinier et al., 1997] for the in vitro phosphorylation assays. *B. subtilis* HPr [Galinier et al., 1997] and HprK/P [Hanson et al., 2002] were purified as previously described and used in the ATP-dependent phosphorylation experiments.

Protein Phosphorylation Experiments

Phosphorylation of PTS proteins with [³²P]PEP was carried out in 20- μ l reaction mixtures containing: 100 mM Tris-HCl, pH 7.5; 20 mM MgCl₂; 1 mM DTT; 0.01 mM [³²P]PEP (0.5 μ Ci, specific radioactivity 3 Ci/mmol) and one or several PTS proteins. The samples were incubated for 30 min at 37°C before the reaction was stopped by adding SDS sample buffer, and the proteins were separated by electrophoresis on a 1% SDS/15% polyacrylamide gel. After electrophoresis, the gels were dried and exposed to a phosphate storage screen for 15 h. Radioactive bands were visualized by using a PhosphorImager.

For the ATP-dependent phosphorylation of HPr with HprK/P, the 7.5- μ l reaction mixtures contained: 80 mM Tris-HCl, pH 7.4; 15 mM MgCl₂; 3 mM DTT; 0.4 mM [γ -³²P]ATP (1 μ Ci, specific radioactivity 3.75 Ci/mmol); 80 mM fructose-1,6-bisphosphate

and HPr and HprK/P either from *R. eutropha* or *B. subtilis*. The samples were incubated for 30 min at 37°C, and the reaction was stopped by heating the samples for 5 min to 95°C. Gel electrophoresis and detection of radioactive bands were carried out as described above for the PEP-dependent phosphorylation experiments.

Complementation of the *ptsH* *hprK* Double Mutant with Various Plasmid-Born *ptsH* Alleles

In order to construct the various *ptsH* alleles encoding HPr, in which either one of the two phosphorylatable residues His-15 or Ser-46 was replaced with Ala, we started with plasmid pUC19. For our cloning strategy, the intrinsic *NdeI* restriction site in pUC19 had first to be removed by cutting the vector with the restriction enzymes *EcoRI* and *DraII*, filling up the protruding ends and reclosing it. Subsequently, a 220-bp DNA fragment was amplified with appropriate primers (table 2), that carries in its center the *R. eutropha* *cbb* operon promoter flanked by an upstream *XbaI* and a downstream *KpnI* restriction site, with the latter being preceded by a *NdeI* site. This fragment was inserted into the modified pUC19 cut with the enzymes *XbaI* and *KpnI* providing plasmid pGP785. Wild-type *ptsH* and the *ptsH*His15Ala allele containing their own Shine-Dalgarno box were PCR-amplified with appropriate primers (table 2) using *R. eutropha* genomic DNA as template. For the construction of the *ptsH*Ser46Ala allele, genomic DNA, two inversely oriented overlapping mutagenic primers with identical restriction sites (*SalI*) and primers DK17 and DK22 (table 2) were used in a two-step PCR reaction. Subsequently, the amplified wild-type *ptsH* and the His15Ala and Ser46Ala alleles containing their authentic ribosome binding site and carrying *NdeI* and *KpnI* restriction sites at their 5' and 3' ends, respectively, were inserted into the *cbb* promoter-containing pUC19 derivative pGP785 cleaved with the same enzymes. This allowed expression of the *ptsH* alleles under the control of the *cbb* promoter. DNA sequencing, performed after each cloning step, confirmed the correct sequence of PCR-amplified DNA fragments. Cutting the pUC19-derived plasmids with *XbaI* and *KpnI* provided the three different *cbb*-*ptsH* fragments, which were inserted into the broad-host-range vector pBBR1MCS-3 [Kovach et al., 1995] cleaved with the same enzymes. The resulting plasmids were used to transform *E. coli* S17-1, and conjugation experiments were carried out as previously described [Lenz et al., 1994].

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