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Mutations lowering the phosphatase activity of HPr kinase/phosphatase switch off carbon metabolism

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The oligomeric bifunctional HPr kinase/P-Ser-HPr phosphatase (HprK/P) regulates many metabolic functions in Gram-positive bacteria by phosphorylating the phosphocarrier protein HPr at Ser46. We isolated *Lactobacillus casei hprK* alleles encoding mutant HprK/Ps exhibiting strongly reduced phosphatase, but almost normal kinase activity. Two mutations affected the Walker motif A of HprK/P and four a conserved C-terminal region in contact with the ATP-binding site of an adjacent subunit in the hexamer. Kinase and phosphatase activity appeared to be closely associated and linked to the Walker motif A, but dephosphorylation of seryl-phosphorylated HPr (P-Ser-HPr) is not simply a reversal of the kinase reaction. When the *hprK*V267F allele was expressed in *Bacillus subtilis*, the strongly reduced phosphatase activity of the mutant enzyme led to increased amounts of P-Ser-HPr. The *hprK*V267F mutant was unable to grow on carbohydrates transported by the phosphoenolpyruvate:glycose phosphotransferase system (PTS) and on most non-PTS carbohydrates. Disrupting *ccpA* relieved the growth defect only on non-PTS sugars, whereas replacing Ser46 in HPr with alanine also restored growth on PTS substrates.

Keywords: bifunctional enzymes/carbohydrate metabolism/HPr/HPr kinase:P-Ser-HPr phosphatase/PEP:glycose phosphotransferase system

Introduction

Protein phosphorylation is important for the regulation of most cellular functions in eukaryotes and prokaryotes. One of the best-studied bacterial protein kinases is the enzyme catalyzing the ATP-dependent, metabolite-activated phosphorylation of Ser46 in HPr (Deutscher and Saier, 1983; Deutscher *et al*., 1986), a phosphocarrier protein of the phosphoenolpyruvate (PEP):glycose phosphotransferase system (PTS) (Postma *et al*., 1993) (Figure 1). In *Bacillus subtilis*, seryl-phosphorylated HPr (P-Ser-HPr) controls the expression of ~10% of the genome (Moreno *et al*., 2001). The P-Ser-HPr-regulated genes are implicated in many cellular functions such as nitrogen metabolism, carbon catabolite repression (CCR) or activation (Deutscher *et al*., 1994), stress response, cytochrome *c* synthesis and regulation of central metabolic pathways such as glycolysis or the tricarboxylic acid (TCA) cycle (Stülke and Hillen, 2000; Deutscher *et al*., 2001). To carry out these functions, P-Ser-HPr interacts with the catabolite control protein A (CcpA) (Deutscher *et al*., 1995; Jones *et al*., 1997), a member of the LacI/GalR repressor family (Henkin *et al*., 1991). P-Ser-HPr functions as catabolite co-repressor by allowing CcpA to bind to catabolite response elements (*cre*) (Fujita *et al*., 1995), operator sites located in front of P-Ser-HPr/CcpA-controlled genes and operons (Weickert and Chambliss, 1990; for reviews see Stülke and Hillen, 2000; Deutscher *et al*., 2001). P-Ser-HPr has been suggested also to regulate carbohydrate uptake via the PTS, since it is a poor substrate for the PEP-dependent protein kinase enzyme I (EI) (Deutscher *et al*., 1984, 1994; Ye and Saier, 1996). EI phosphorylates HPr at His15 (Figure 1), which represents the first step of the PTS phosphorylation cascade leading to the phosphorylation of carbohydrates during their transport (Postma *et al*., 1993). In addition, P-Ser-HPr has been reported to inhibit the activity of several non-PTS permeases (Ye and Saier, 1995; Gauthier *et al*., 1997; Dossonnet *et al*., 2000; Viana *et al*., 2000), thereby preventing the entry of the inducer for the corresponding catabolic operon. This phenomenon was therefore called inducer exclusion in analogy to a similar regulatory function carried out by unphosphorylated $EIIA^{Glc}$ in Gram-negative bacteria (Postma *et al*., 1993).

Interestingly, HPr kinase and the first discovered bacterial protein kinase, isocitrate dehydrogenase kinase from *Escherichia coli*, are bifunctional enzymes also catalyzing the dephosphorylation of their substrates (LaPorte and Koshland, 1982; Kravanja *et al*., 1999; Dossonnet *et al*., 2000; Huynh *et al*., 2000). These two enzymes exhibit no similarity to eukaryotic protein kinases or P-protein phosphatases nor do they exhibit similarity to each other. HPr kinase/P-Ser-HPr phosphatase (HprK/P) contains a Walker motif A similar to nucleotide-binding proteins (Galinier *et al*., 1998). Experiments aimed at localizing domains containing the Idh kinase or P-Idh phosphatase activity provided no clearcut results, since mutations leading to normal kinase but reduced phosphatase activity affected amino acids in all parts of the protein (Ikeda and La Porte, 1991; Miller *et al*., 2000). We tried to address the question of how the opposing activities of bifunctional enzymes are organized and controlled by carrying out random mutagenesis with *Lactobacillus casei hprK*. We were able to isolate *hprK* alleles encoding enzymes exhibiting almost normal kinase, but strongly reduced phosphatase activity. The

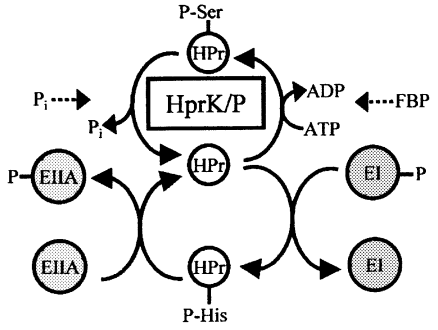

Fig. 1. Schematic presentation of ATP- and PEP-dependent HPr phosphorylation. The ATP-dependent phosphorylation of HPr by HprK/P at Ser46 is stimulated by FBP, whereas dephosphorylation of P-Ser-HPr is activated by P_i. Within the PTS phosphorylation cascade, HPr is phosphorylated intermediately by EI at His15 and transfers its phosphoryl group to various EIIAs.

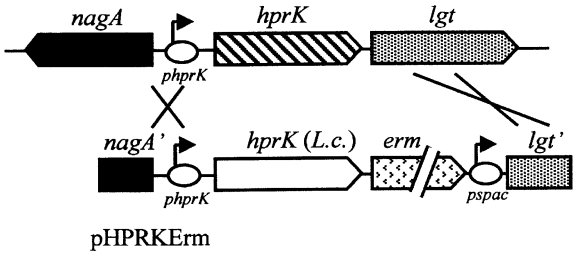

Fig. 2. Replacement of *B.subtilis hprK* with the *L.casei hprK* alleles. The integrative plasmid pHPRKErm carries the 3′ part of *nagA*, the *B.subtilis hprK* promoter, the *L.casei hprK* gene, an erythromycin resistance cassette, the *spac* promoter and the 5′ part of *lgt*. Double crossover allows replacement of the chromosomal *B.subtilis hprK* with the *L.casei hprK* and expression of *lgt* and the downstream genes from the *spac* promoter.

mutations affected either the nucleotide-binding site or a C-terminal conserved region, which is in close contact with the Walker motif A of a neighboring subunit in the HprK/P hexamer, and completely disturbed carbon metabolism when introduced into *B.subtilis*.

Results

hprK mutations leading to reduced expression of a CCR-sensitive reporter gene fusion

Random mutagenesis with the *hprK* gene of *L.casei* was carried out as described in Materials and methods. The resulting *hprK* alleles were inserted in the integrative plasmid pHPRKErm and subsequently used to replace the *hprK* gene in the *B.subtilis* strain QB7144, which carries a xylose-inducible catabolite repression-sensitive *ynaJ′–lacZ* fusion (Galinier *et al*., 1999). In the integrants, the *L.casei hprK* alleles are under control of the *B.subtilis hprK* promoter (Figure 2). *L.casei hprk* was used, since it differs sufficiently from *B.subtilis hprK* to prevent random recombination. Integration of the *L.casei* wild-type *hprK* led to the formation of blue colonies on solid CSK medium containing xylose and 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal), whereas addition of glucose repressed *ynaJ′–lacZ* expression (white colonies). Transformants synthesizing HprK/P with unbalanced enzyme activities leading to increased amounts of P-Ser-HPr were expected to form white colonies even in the absence of glucose. Out of 4000 clones tested, seven formed pale blue (TG102 and TG103) or white colonies on xylose- and X-Gal-containing medium. Pale blue or white clones were also obtained when QB7144 was transformed with chromosomal DNA isolated from the seven strains. β-galactosidase assays confirmed that the xylose-induced expression of the *ynaJ′–lacZ* fusion in these strains was 2- to 100-fold lower compared with QB7144 (Table I).

Table I. Expression of the *ynaJ′–lacZ* fusion in QB7144 and its derivatives carrying various *hprK* alleles

Strain	*hprK* allele	β-galactosidase activity[a]
QB7144	*hprK*+ *B.subtilis*	568
TG100	*hprK*+ *L.casei*	562
M181A	G58S G270R	7
TG101	V267F	5
TG102	D34V L152M H223Y	247
TG103	N272I	253
TG104	G160S	106
TG200	G270E	6
TG201	E163K	17
TG203	V267F and *ccpA*	582
TG113	*hprK*+ *B.subtilis*	563
TG114	V265F *B.subtilis*	35

[a]β-galactosidase activity was measured in cells grown in CSK medium containing 0.2% xylose. Presented are the mean values of three independent experiments, which exhibited standard deviations of less than ±10%.

The hprK mutations are located in two conserved regions

To identify the mutations responsible for the reduced expression from the *ynaJ* promoter, the *L.casei hprK* alleles of the integrants exhibiting reduced β-galactosidase activity were amplified by PCR and entirely sequenced. In all cases, at least one mutation could be detected in the *hprK* genes. These mutations are listed in Table II. The *hprK* alleles integrated in strains TG102 and M181A contained three and two mutations, respectively, and were not analyzed further. In M181A, the G270R mutation is probably responsible for the reduced *ynaJ′–lacZ* expression, since G270 was also affected in strain TG200 (G270E mutation). In summary, the *hprK* mutations leading to reduced expression from the *ynaJ* promoter are located in two defined regions. One is the presumed ATP-binding site (Walker motif A) extending from position 151 to 164 in the amino acid sequence of *L.casei* HprK/P, and the other is a C-terminal region (position 265–275), which, similarly to the ATP-binding site, is well conserved in all HprK/Ps (Figure 3).

The V267FLc hprK allele prevents growth on PTS and non-PTS sugars

The V267F replacement in *L.casei* HprK/P was found to exert the strongest repressive effect on the expression of the *ynaJ′–lacZ* fusion (Table I). The V267F *hprK*-containing integrant TG101 was therefore chosen to test in a more general way the effect of this mutation on sugar metabolism (Table III). In contrast to a strain carrying wild-type *L.casei hprK*, the V267FLc *hprK* mutant was

unable to grow on minimal medium supplemented with the PTS substrates glucose, fructose, mannitol or maltose (Reizer *et al*., 1999) or with the non-PTS substrates gluconate, ribose or glucitol. Among the carbon sources tested, glycerol was the only one that could be utilized by the V267FLc *hprK* mutant. We also measured the transport activity of the V267FLc *hprK* mutant with the PTS substrates glucose and mannitol. The strain carrying wild-type *L.casei hprK* (TG100) exhibited normal uptake of these two carbohydrates, whereas transport of these PTS substrates was almost completely abolished in the V267FLc *hprK* mutant TG101 (Figure 4). CCR was not responsible for the reduced PTS activity, since introducing a *ccpA* mutation into strain TG101 providing the double mutant TG203 had little effect on mannitol transport, and glucose uptake was 3- to 4-fold lower than in the *hprK*$^+$ strain (Figure 4).

To test whether expression of *ptsG*, which codes for the glucose-specific EIICBA, was lowered in strain TG101, the *L.casei* wild-type and the V267F *hprK* alleles were introduced into strain QB7035, which carries a *ptsG′–′lacZ* fusion (Stülke *et al*., 1997), providing strains TG109 and TG110, respectively. β-galactosidase activity measured in both strains after growth in the presence and

Table II. Mutations in *hprK* of *L.casei* leading to the observed reduced *ynaJ′–lacZ* expression

Mutant	Codon changes	Amino acid replacements
M181A	GGT→AGT; GGG→AGG	G58S; G270R
TG101	GTT→TTT	V267F
TG102	GAT→GTT; CTG→ATG; CAT→TAT	D34V; L152M; H233Y
TG103	AAC→ATC	N272I
TG104	GGC→AGC	G160S
TG200	GGG→GAG	G270E
TG201	GAA→AAA	E163K

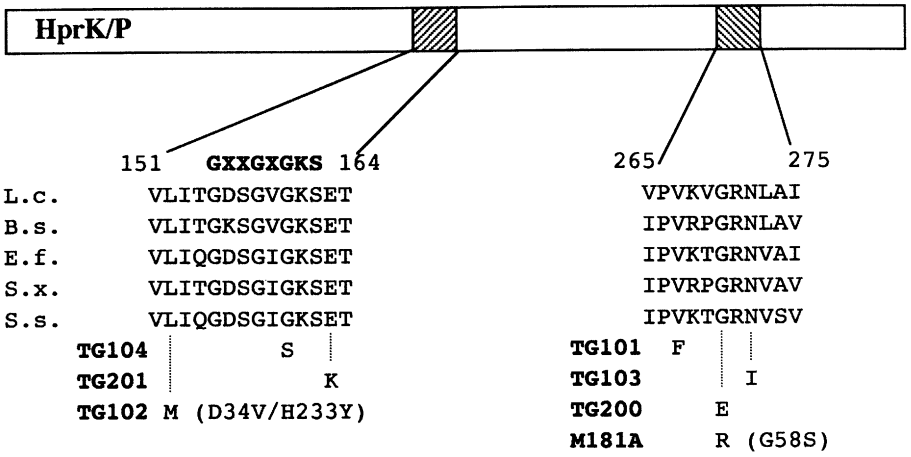

Fig. 3. Location of the mutations leading to reduced P-Ser-HPr phosphatase activity within two conserved regions of HprK/P. Aligned are the corresponding conserved regions of HprK/P from *L.casei* (L.c.), *B.subtilis* (B.s.), *Enterococcus faecalis* (E.f.), *Staphylococcus xylosus* (S.x.) and *Streptococcus salivarius* (S.s.). The consensus sequence of the Walker motif A is presented above the aligned sequences. Numbering refers to *L.casei* HprK/P.

Table III. Growth of various *B.subtilis* strains in C mineral medium supplemented with different carbon sources

Strain and relevant genotype[a]	PTS sugars				Non-PTS sugars			
	Glucose	Fructose	Mannitol	Maltose	Glycerol	Gluconate	Ribose	Glucitol
TG100 *hprK*$^+$	+	+	+	+	+	+	+	+
TG101 *hprK*V267F	–	–	–	–	+	–	–	–
TG202 *ccpA*	+	+	+	+	+	+	+	+
TG203 *ccpA hprK*V267F	+/–	–	–	+/–	+	+	+	+
TG107 *ptsH1*	+	+	+	+	+	+	+	+
TG108 *ptsH1 hprK*V267F	+	+	+	+	+	+	–	+
TG122 *ptsH1 crh hprK*V267F	+	+	+	+	+	–	+	+

[a]Strains were grown on solid C minimal medium supplemented with the indicated carbon sources (0.2% w/v) at 37°C and growth was checked after 2 days. (+), growth with a colony size >3 mm; (–), no growth at all or formation of tiny translucent colonies <0.5 mm in diameter; (+/–), intermediate growth with a colony size between 1 and 2 mm. Glutamate (0.25 mM) was added to the minimal medium to allow growth of *ccpA* mutants (Faires *et al*., 1999).

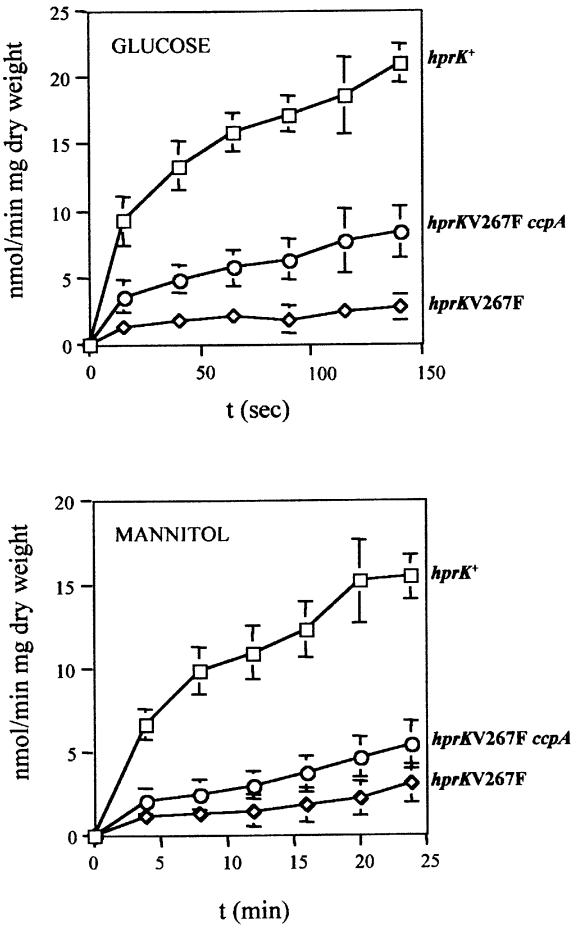

Fig. 4. Glucose and mannitol transport studies were carried out with *B.subtilis* strains containing the *L.casei* wild-type (squares) or V267F *hprK* allele (diamonds), or with the V267FLc *hprK ccpA* double mutant (circles).

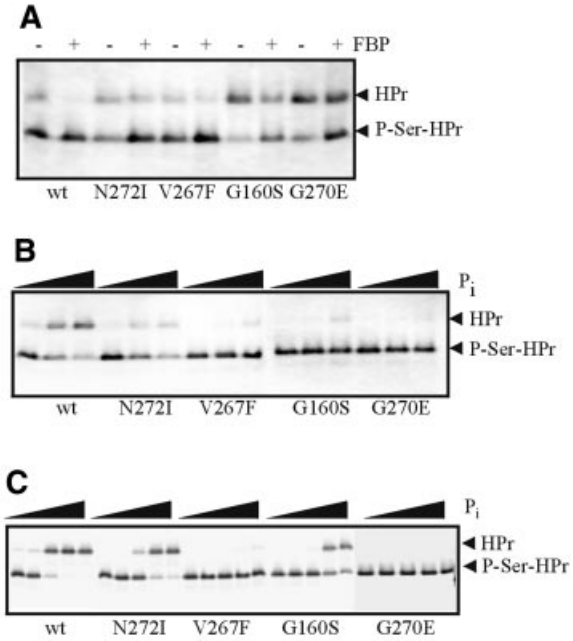

Fig. 5. HPr kinase and P-Ser-HPr phosphatase assays with *L.casei* wild-type and N272I, V267F, G160S and G270E mutant HprK/Ps. (**A**) HPr kinase assays with 20 ng of HprK/P in the absence (–) and presence (+) of 10 mM FBP. Samples were incubated for 150 s at 37°C. (**B**) Phosphatase assays with 40 ng of HprK/P and increasing concentrations of P_i (0, 0.2 and 2 mM). Samples were incubated for 5 min at 37°C. (**C**) Inhibition of P-Ser-HPr formation in the presence of 200 ng of HprK/P and 10 mM FBP by increasing concentrations of P_i (0, 1, 5, 20 and 40 mM). Samples were incubated for 10 min at 37°C.

absence of glucose was stimulated similarly by the presence of glucose: from 97 to 1171 U for strain TG109 and from 119 to 1018 U for the V267FLc *hprK* mutant TG110. These results established that the V267F *hprK* allele does not affect the synthesis of the glucose-specific EII but rather its transport activity.

To exclude the possibility that the effects caused by the *L.casei* V267F *hprK* allele on *B.subtilis* carbohydrate utilization were due to the heterologous system used in the above experiments, a corresponding *B.subtilis hprK* mutant was constructed by replacing the wild-type gene in strain QB7144 with the *B.subtilis* V265F *hprK* allele. Xylose-induced expression of the *ynaJ′–lacZ* fusion in the *B.subtilis* V265F *hprK* mutant TG114 was strongly repressed compared with the isogenic *hprK*$^+$ strain TG113 (Table I). In addition, the V265F *hprK* mutant exhibited growth characteristics on PTS and non-PTS sugars identical to those observed with the V267FLc *hprK* mutant (data not shown).

The hprK mutations primarily lower the phosphatase activity of HprK/P

To test whether the *hprK* mutations would unbalance the two opposing activities of the bifunctional HprK/P, which might be responsible for the observed changes in *ynaJ′–lacZ* expression and carbohydrate metabolism, four *L.casei hprK* alleles were inserted into His tag expression vectors and the encoded HprK/Ps were purified and tested for HPr kinase and phosphatase activity. In the V267F, G270E and N272I mutant HprK/Ps, the HPr kinase activity was only slightly reduced, whereas G160S mutant HprK/P exhibited low HPr kinase activity (Figure 5A). However, in all cases, the HPr kinase activity was still stimulated by the presence of 10 mM fructose-1,6-bisphosphate (FBP), as previously observed for wild-type HprK/P (Dossonnet *et al*., 2000). The phosphatase activity, which is stimulated by inorganic phosphate (P_i) (Dossonnet *et al*., 2000), was much more strongly affected in the four mutant enzymes. Even in the presence of 2 mM P_i, no phosphatase activity could be detected with V267F and G270E mutant HprK/Ps, and only low activity was observed with the other two mutant enzymes (Figure 5B). P_i has been shown to inhibit the FBP-stimulated formation of P-Ser-HPr by *L.casei* wild-type HprK/P, probably by stimulating the opposing P-Ser-HPr phosphatase activity (Dossonnet *et al*., 2000). However, even when the P_i concentration was increased up to 40 mM, no or only slight inhibition of P-Ser-HPr formation in the presence of 10 mM FBP was observed with V267F and G270E HprK/Ps (Figure 5C). The diminished inhibitory effect of P_i on HPr phosphorylation by the different mutant HprK/Ps correlated well with the reduced *ynaJ′–lacZ* expression observed in strains carrying the corresponding *hprK* alleles (Table I).

The effects of FBP and P_i on HPr phosphorylation were also determined for *B.subtilis* wild-type and V265F

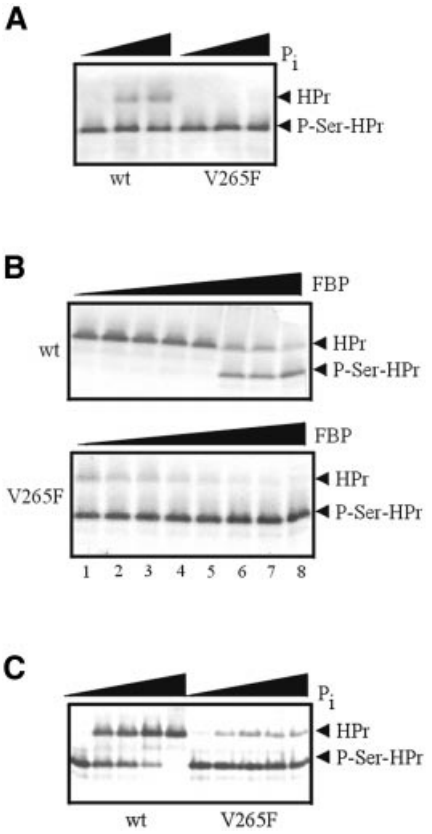

Fig. 6. HPr kinase and P-Ser-HPr phosphatase assays with *B.subtilis* wild-type and V265F mutant HprK/P. (**A**) Phosphatase assays with 40 ng of HprK/P and increasing concentrations of P_i (0, 0.2 and 2 mM). Samples were incubated for 5 min at 37°C. (**B**) Stimulation of the HPr kinase activity (200 ng of HprK/P) by increasing amounts of FBP (0, 0.2, 0.5, 1, 2, 5, 10 and 20 mM) in the presence of 5 mM P_i. Samples were incubated for 10 min at 37°C. (**C**) Inhibition of P-Ser-HPr formation in the presence of 200 ng of HprK/P and 10 mM FBP by increasing concentrations of P_i (0, 5, 10, 20 and 40 mM). Samples were incubated for 10 min at 37°C.

HprK/P. As observed with the corresponding *L.casei* mutant enzyme, V265F HprK/P exhibited no detectable phosphatase activity (Figure 6A). As a consequence, in the presence of 5 mM P_i and 2 mM FBP, HPr was almost completely phosphorylated by the mutant enzyme, whereas HPr was completely dephosphorylated when wild-type HprK/P was used under identical conditions (compare lanes 5 of Figure 6B). Even when the concentration of P_i was increased to 40 mM, phosphorylation of HPr by V265F HprK/P was barely diminished (Figure 6C). The chosen concentrations of FBP and Pi, which can vary over a wide range depending on whether the organisms grow on a rapidly metabolizable carbon source or not, lie within the range reported for Gram-positive bacteria (Thompson and Torchia, 1984).

Is P-Ser-HPr dephosphorylation a reversal of the kinase reaction?

The finding that the mutations leading to low phosphatase activity of HprK/P were located either directly in the Walker motif A or in a conserved C-terminal region in contact with the ATP-binding site of an adjacent subunit

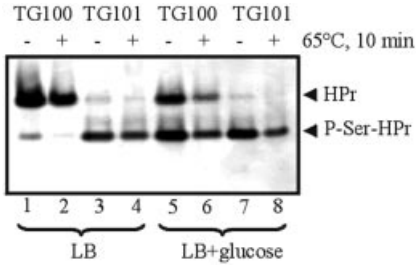

Fig. 7. Western blot with crude extracts prepared from LB- and LB plus glucose-grown TG100 and TG101 cells. Aliquots of the crude extracts were either loaded directly on a non-denaturing polyacrylamide gel or kept at 65°C for 10 min, which causes complete hydrolysis of the P-His bond, but leaves P-Ser-HPr intact. After electrophoresis, which allows separation of the various HPr forms (HPr, P-HPr and doubly phosphorylated HPr), HPr bands were detected with rabbit polyclonal antibodies directed against *B.subtilis* HPr.

(Fieulaine *et al.*, 2001) suggested that kinase and phosphatase activity are closely associated and that the Walker motif A might be the active site for both reactions. One possible explanation was that P-Ser-HPr dephosphorylation was a reversal of the kinase reaction. However, the presence of ADP at up to 5 mM had no stimulatory effect on the phosphatase activity of HprK/P (data not shown). Nevertheless, in the presence of 5 mM ADP, ~15% of the radioactive products of the phosphohydrolase reaction with [^{32}P]P-Ser-HPr and HprK/P were found to co-migrate with [γ-^{32}P]ATP during thin-layer chromatography (TLC), whereas the major radioactive spot co-migrated with labeled P_i. When 5 mM P_i, which stimulates the phosphatase activity, was also present during [^{32}P]P-Ser-HPr dephosphorylation, no product co-migrating with [γ-^{32}P]ATP could be detected (data not shown).

The V267FLc hprK mutation increases the intracellular amount of P-Ser-HPr

The enhanced formation of P-Ser-HPr observed *in vitro* with the mutant HprK/Ps suggested that expression of the mutant *hprK* alleles in *B.subtilis* might also lead to increased amounts of P-Ser-HPr *in vivo*. To test this assumption, we carried out western blots with crude extracts prepared from the *hprK*$^+$ strain TG100 or the V267FLc *hprK* mutant TG101 grown in Luria Bertani (LB) medium or in glucose-containing LB medium as described in Materials and methods. LB-grown TG100 cells contained ~85% unphosphorylated HPr (Figure 7, lane 1) and 15% P-His-HPr, which was converted to unphosphorylated HPr when kept for 10 min at 65°C (Figure 7, lane 2) (Waygood *et al.*, 1985). There was almost no P-Ser-HPr present in LB-grown TG100 cells. Growth on LB plus glucose increased the amount of P-Ser-HPr in TG100 to ~50%. The residual HPr was present in unphosphorylated form and there was little or no P-His-HPr (Figure 7, lanes 5 and 6). In contrast, in LB- and LB plus glucose-grown V267FLc *hprK* mutant cells, almost all HPr was present as P-Ser-HPr (Figure 7, lanes 3, 4, 7 and 8). No doubly phosphorylated HPr could be detected under the experimental conditions employed, either in TG100 or in the V267FLc *hprK* mutant strain.

The ptsH1 mutation restores growth on PTS and non-PTS carbohydrates

If the increased amount of P-Ser-HPr in the QB7144 derivatives expressing the *L.casei hprK* alleles was responsible for the observed permanent CCR and for their failure to grow on PTS and non-PTS carbohydrates, introduction of the *ptsH1* mutation (Eisermann *et al*., 1988), which prevents phosphorylation of HPr at Ser46, was expected to relieve the growth defects and the permanent CCR. Chromosomal DNA of strains TG100 and TG101 was therefore used to transform the *ptsH1* strain GM1222 and the isogenic *ptsH*$^+$ strain GM1221 as described in Materials and methods. In contrast to the V267FLc *hprK* mutant TG101, the V267FLc *hprK ptsH1* double mutant TG108 obtained was capable of growing on the PTS carbohydrates glucose, fructose, mannitol and maltose and on the non-PTS carbohydrates glycerol, gluconate and glucitol, similarly to *ptsH*$^+$ (TG105) and *ptsH1* (TG107) strains carrying *L.casei* wild-type *hprK* (Table III). However, growth on the non-PTS carbohydrate ribose could not be restored by the *ptsH1* mutation. We therefore inactivated the *crh* gene, which codes for an HPr-like protein also phosphorylated by HprK/P, and obtained the *ptsH1 crh* double mutant TG122. Similarly to P-Ser-HPr, P-Ser-Crh can function as catabolite co-repressor/co-activator for certain genes and operons in *B.subtilis* (Galinier *et al*., 1997, 1999; Martin-Verstraete *et al*., 1999; Presecan-Siedel *et al*., 1999; Zalieckas *et al*., 1999). The ribose operon seems to be one of these P-Ser-Crh-controlled transcription units, since, in contrast to the *ptsH1* mutant TG108, the *ptsH1 crh* double mutant TG122 was capable of growing on ribose. Strain TG122 had lost the capacity to grow on gluconate as the sole carbon source. Poor growth on gluconate has been observed previously with *ptsH1 crh* double mutants and is not related to the V267F *hprK* mutation.

Inactivation of ccpA restores growth only on non-PTS carbohydrates

The finding that compared with an *hprK*$^+$ strain expression of the *ptsG′–′lacZ* fusion was not altered in the V267FLc *hprK* mutant TG110 suggested that permanent CCR might not be the only cause of the impaired growth of the *hprK* mutants on PTS substrates such as glucose. Additional effects of P-Ser-HPr on PTS transport activity were thought to become detectable in strain TG203 carrying the V267F *hprK* mutation and a *ccpA* disruption, which was expected to cause a relief from CCR without altering the elevated amount of P-Ser-HPr. In TG203, β-galactosidase activity was indeed inducible with xylose, similarly to the *hprK*$^+$ strain TG100 (Table I), confirming that the *ccpA* mutation caused a relief from permanent CCR. In addition, TG203 had regained the capacity to grow on the non-PTS substrates glucitol, gluconate and ribose. However, it was not able to grow on the PTS substrates fructose and mannitol, and barely grew on the PTS sugars glucose and maltose, suggesting that PTS transporters are also regulated by P-Ser-HPr at the activity level in a CCR-independent manner.

Discussion

The phosphocarrier protein HPr, which participates in PTS-catalyzed sugar transport, is the target of two phosphorylation reactions (Figure 2) and represents the major regulator of carbon metabolism in Gram-positive bacteria. PEP-dependent phosphorylation via EI and P-His-HPr controls the activity of catabolic enzymes, antiterminators and transcriptional activators, whereas P-Ser-HPr plays a role in inducer exclusion and CCR (Stülke and Hillen, 2000; Deutscher *et al*., 2001). Formation of P-Ser-HPr is regulated by the bifunctional enzyme HprK/P, which phosphorylates HPr during high throughput through glycolysis, whereas it dephosphorylates P-Ser-HPr when the concentration of glycolytic intermediates drops.

Bifunctional enzymes carrying opposing enzyme activities have been detected in eukaryotic cells, including human (Heine-Suner *et al*., 1998), animal (Hasemann *et al*., 1996) and plant cells (Draborg *et al*., 1999), and prokaryotic cells (Johansson and Gest, 1977; LaPorte and Koshland, 1982; Garcia and Rhee, 1983; Zhu *et al*., 2000). Many of these enzymes catalyze the modification/demodification (phosphorylation, adenylylation, uridylylation) of proteins implicated in the regulation of essential cellular functions. In most cases, effector molecules bind to these bifunctional enzymes and specifically stimulate or inhibit one of the two opposing activities. Although the structure of some bifunctional enzymes has been determined (Hasemann *et al*., 1996; Thompson *et al*., 1998) and in a few cases domains could be identified harboring one of the two opposing activities (Jaggi *et al*., 1997; Lee *et al*., 1997), knowledge about the regulation of the enzymatic activities of bifunctional enzymes and why there is an advantage in having two opposing activities organized on a single polypeptide chain remains very limited.

We report here the isolation and characterization of mutant HprK/Ps, in which the balance between kinase and phosphatase activities was changed in favor of the kinase activity. Expression of the corresponding *hprK* alleles led to enhanced conversion of HPr to P-Ser-HPr and to CCR even in the absence of a repressing sugar. Surprisingly, the *hprK* mutants were unable to grow on most PTS and non-PTS carbohydrates. This inability was clearly related to the increased amounts of P-Ser-HPr or P-Ser-Crh, since introducing *ptsH1* or *ptsH1 crh* mutations restored growth on all carbohydrates. Only glycerol was metabolized normally by the *hprK* mutants. Although the *glpFK* operon coding for the glycerol facilitator and glycerol kinase is preceded by a potential *cre* (Miwa *et al*., 2000), CcpA-dependent CCR probably plays no or only a minor role for the *glpFK* operon, and other CcpA-independent CCR mechanisms seem to exist (Deutscher *et al*., 1994).

Disruption of the *ccpA* gene, which prevents CCR probably without diminishing the elevated amounts of P-Ser-HPr in the *hprK* mutants, restored full growth only on non-PTS, but not on PTS carbohydrates. P-Ser-HPr is a poor substrate for the PEP-dependent phosphorylation by EI. If almost all HPr is converted to P-Ser-HPr, as was observed with the V267FLc *hprK* mutant, PTS substrates are transported so slowly that they no longer support growth. In wild-type cells, formation of P-Ser-HPr in response to increasing amounts of FBP and other

glycolytic intermediates is probably used as a feedback mechanism to slow the PEP-dependent phosphorylation of HPr and consequently PTS transport. The slow growth of the V267FLc *hprK ccpA* double mutant on glucose and maltose (Table III) and the elevated glucose transport activity observed with this strain compared with the V267FLc *hprK* mutant (Figure 4) are probably due to the synthesis of glucose- or maltose-specific non-PTS transporters such as GlcP, GlcU or YvdHI (Deutscher *et al*., 2001), which might be subject to CcpA-dependent CCR.

Although the high amounts of P-Ser-HPr almost completely prevented PTS carbohydrate transport in the V267FLc *hprK* mutant, the remaining low PTS phosphocarrier activity seemed to be sufficient to allow phosphorylation of PTS-controlled transcriptional regulators. Synthesis of EIICBA^{Glc}, which is encoded by *ptsG*, is regulated by the antiterminator GlcT (Stülke *et al*., 1997). GlcT was proposed to be inactive when it becomes phosphorylated by P-EIICBA^{Glc}. The finding that in a V267FLc *hprK* mutant expression of a *ptsG′–′lacZ* fusion was similarly low as in the isogenic *hprK*$^+$ strain suggested that GlcT was inactivated normally by P-EIICBA^{Glc}. Although glucose was barely transported by the V267FLc *hprK* mutant due to the interruption of the PTS phosphorylation cascade at the P-His-HPr level, the presence of glucose supposedly led to rapid dephosphorylation of P-EIICBA^{Glc} and therefore to GlcT activation and the observed *ptsG′–′lacZ* induction.

The mutations that allowed dissection of the kinase and phosphatase activities of HprK/P affected two conserved regions: the Walker motif A and a C-terminal region extending from position 265 to 275 (Figure 3), which, according to the structure of *L.casei* HprK/P, is in contact with the Walker motif A of a neighboring subunit in the hexamer (Fieulaine *et al*., 2001). All mutations strongly diminished the P-Ser-HPr phosphatase activity. The HPr kinase activity was much less affected, even when the mutations were located in the ATP-binding site. These results suggest that the opposing activities of HprK/P are tightly associated and that the Walker motif A is implicated in both reactions. This assumption was supported further by the finding that P_i, the activator of the phosphatase reaction, binds to the Walker motif A (Fieulaine *et al*., 2001). However, P-Ser-HPr dephosphorylation is not simply a reversion of the kinase reaction, since the presence of 5 mM ADP had no stimulatory effect on the phosphatase activity. In addition, the major radioactive product obtained when [^{32}P]P-Ser-HPr dephosphorylation was carried out in the presence of 5 mM ADP co-migrated with P_i during TLC and only a small amount of ATP was formed. If 5 mM P_i was also present, it completely prevented the formation of ATP. Since the intracellular concentration of P_i is in the range of 3–50 mM depending on the metabolic state of the bacteria (Thompson and Torchia, 1984), formation of ATP from ADP and P-Ser-HPr is probably not of physiological significance. P_i binds to the position in the Walker motif A of HprK/P normally occupied by the β-phosphate of the nucleotide (Fieulaine *et al*., 2001). Competition between P_i and ATP for the common binding site is probably responsible for the inhibition of the kinase activity of HprK/P by P_i. The most striking characteristic of the mutant HprK/Ps was the decrease or even loss of the inhibitory effect of P_i (Figures 5C and 6C). These results suggested that some of the *hprK* mutations described in this study cause structural changes, reducing the affinity for P_i without lowering the affinity for ATP too much. Interestingly, amino acids G160 and E163, which are affected in the *hprK* mutants TG104 and TG201, are part of the P_i-binding site (Fieulaine *et al*., 2001). Binding of FBP might lead to structural changes similar to those caused by the *hprK* mutations, since, in the presence of FBP, higher amounts of P_i are necessary to inhibit P-Ser-HPr formation. The question therefore arises of whether the conserved C-terminal region affected by four mutations might be part of the FBP-binding site (Jault *et al*., 2000).

With carbohydrates being the major source of carbon and energy, the almost complete inability of the *hprK* mutants to utilize carbohydrates drastically limits their growth capacities in their natural environment. Similarly to the V267F *hprK* mutation leading to the accumulation of P-Ser-HPr, the specific inactivation of the P-Idh phosphatase activity of the second well-characterized bacterial bifunctional protein kinase/P-protein phosphatase, IdhK/P of *E.coli* (LaPorte and Koshland, 1982), led to the accumulation of phosphorylated, barely active Idh and to growth arrest probably due to the loss of TCA cycle activity when >85% of Idh was phosphorylated (Ikeda and La Porte, 1991). The severe growth defects observed with mutants, in which the target proteins of these two bifunctional enzymes were present primarily in their modified form, might be one reason why organisms developed proteins in which modifying/demodifying enzyme activities are organized within a single polypeptide chain. If these enzyme activities were carried by two proteins, frameshift or any other mutations inactivating the demodifying enzyme would lead to the accumulation of the modified target protein and hence to severe growth defects. However, if the two opposing activities are tightly associated on one polypeptide chain, as observed with HprK/P, then most mutations will inactivate both enzyme activities. In agreement with this concept, mutants carrying a disrupted *hprK* gene had lost both activities and were able to grow on most carbohydrates (Galinier *et al*., 1998; Reizer *et al*., 1998; Dossonnet *et al*., 2000). To knock out only one of the two enzyme activities will require very specific mutations, such as those isolated during this study, which will occur with low frequency. Although this concept needs further experimental support, lowering the probability of lethal or growth-inhibiting mutations might be one advantage of organizing opposing enzyme activities on a single polypeptide chain.

Materials and methods

Bacterial strains and growth conditions

The *B.subtilis* strains used in this study are listed in Table IV. Cells were grown at 37°C under agitation in LB medium, C mineral medium or CSK medium (Martin *et al*., 1989) supplemented with different carbon sources at 0.2% (w/v). To allow a better growth of the *ccpA* mutant strains, 0.25 mM glutamate was added to the minimal medium (Faires *et al*., 1999). When required, erythromycin and chloramphenicol were used at concentrations of 5 μg/ml. Spectinomycin was used at 100 μg/ml and neomycin at 10 μg/ml. X-Gal was added at a final concentration of 20 μg/ml. β-galactosidase activity was determined in exponentially growing cells as previously described (Miller, 1972).

Table IV. *Bacillus subtilis* strains used in this study

Strain	Genotype	Reference or construction
GM1090	*trpC2 pheA1 ccpA*::*spc*	M.Steinmetz
GM1221	*trpC2 pheA1 ΔlacA amyE*::(*gntRK′-lacZ*) *ptsH*$^+$ *cat*	Deutscher *et al*. (1994)
GM1222	*trpC2 pheA1 ΔlacA amyE*::(*gntRK′-lacZ*) *ptsH1 cat*	Deutscher *et al*. (1994)
M181A	*trpC2 amyE*::(*ynaJ′-lacZ cat*) *hprK*::(*hprK L.c.* G58S G270R-*erm-pspac*)[a]	pHPRErm(*L.c.* G58S G270R)[b]→QB7144
QB7035	*trpC2 amyE*::(*ptsG′-′lacZ cat*)	Stülke *et al*. (1997)
QB7096	*trpC2 crh*::*aphA3*	Presecan-Siedel *et al*. (1999)
QB7144	*trpC2 amyE*::(*ynaJ′-lacZ cat*)	Galinier *et al*. (1999)
TG100	*trpC2 amyE*::(*ynaJ′-lacZ cat*) *hprK*::(*hprK*$^+$ *L.c.-erm-pspac*)	pHPRKErm(*L.c.*wt)→QB7144
TG101	*trpC2 amyE*::(*ynaJ′-lacZ cat*) *hprK*::(*hprK L.c.* V267F-*erm-pspac*)	pHPRErm(*L.c.*V267F)→QB7144
TG102	*trpC2 amyE*::(*ynaJ′-lacZ cat*) *hprK*::(*hprK L.c.*D34V L152M H233Y-*erm-psapc*)	pHPRKErm(*L.c.* D34V L152M H233Y)→QB7144
TG103	*trpC2 amyE*::(*ynaJ′-lacZ cat*) *hprK*::(*hprK L.c.* N272I-*erm-pspac*)	pHPRKErm(*L.c.*N272I)→QB7144
TG104	*trpC2 amyE*::(*ynaJ′-lacZ cat*) *hprK*::(*hprK L.c.* G160S-*erm-pspac*)	pHPRKErm(*L.c.*G160S)→QB7144
TG105	*trpC2 pheA1ΔlacA amyE*::(*gntRK′-lacZ*) *hprK*::(*hprK*$^+$ *L.c.-erm-pspac*)	TG100→GM1221
TG106	*trpC2 pheA1ΔlacA amyE*::(*gntRK′-lacZ*) *hprK*::(*hprK L.c.*V267F-*erm-pspac*)	TG101→GM1221
TG107	*trpC2 pheA1ΔlacA amyE*::(*gntRK′-lacZ*) *ptsH1 hprK*::(*hprK*$^+$ *L.c.-erm-pspac*)	TG100→GM1222
TG108	*trpC2 pheA1ΔlacA amyE*::(*gntRK′-lacZ*) *ptsH1 hprK*::(*hprK L.c.*V267F-*erm-pspac*)	TG101→GM1222
TG109	*trpC2 amyE*::(*ptsG′-′lacZ cat*) *hprK*::(*hprK*$^+$ *L.c.-erm-pspac*)	TG100→QB7035
TG110	*trpC2 amyE*::(*ptsG′-′lacZ cat*) *hprK*::(*hprK L.c.*V267F-*erm-pspac*)	TG101→QB7035
TG113	*trpC2 amyE*::(*ynaJ′-lacZ cat*) *hprK*::(*hprK*$^+$ *B.s.-erm-pspac*)	pHPRKErm(*B.s.* wt)→QB7144
TG114	*trpC2 amyE*::(*ynaJ′-lacZ cat*) *hprK*::(*hprK B.s.*V265F-*erm-pspac*)	pHPRKErm(*B.s.*V265F)→QB7144
TG121	*trpC2 pheA1ΔlacA amyE*::(*gntRK′-lacZ*) *ptsH1 crh*::*aphA3*	QB7096→GM1222
TG122	*trpC2 pheA1ΔlacA amyE*::(*gntRK′-lacZ*) *ptsH1 crh*::*aphA3 hprK*::(*hprK L.c.*V267F-*erm-pspac*)	TG101→TG121
TG200	*trpC2 amyE*::(*ynaJ′-lacZ cat*) *hprK*::(*hprK L.c.* G270E-*erm-pspac*)	PHPRKErm(*L.c.*G270E)→QB7144
TG201	*trpC2 amyE*::(*ynaJ′-lacZ cat*) *hprK*::(*hprK L.c.* E163K-*erm-pspac*)	PHPRKErm(*L.c.*E163K)→QB7144
TG202	*trpC2 amyE*::(*ynaJ′-lacZ cat*) *hprK*::(*hprK*$^+$ *L.c.-erm-pspac*) *ccpA*::*spc*	GM1090→TG100
TG203	*TrpC2 amyE*::(*ynaJ′-lacZ cat*) *hprK*::(*hprK L.c.* V267F-*erm-pspac*) *ccpA*::*spc*	GM1090→TG101

[a]*L.c.* refers to *hprK* from *L.casei*, and *B.s.* to *hprK* from *B.subtilis*.
[b]Arrows indicate transformation by plasmid or chromosomal DNA.

Plasmid construction

The *hprK* gene from *L.casei* was amplified by PCR using genomic DNA and oligo1 and 2 (Table V). The 5′ end of the *B.subtilis nagA* gene, which is located upstream of *hprK*, was amplified by PCR using oligo3 and 4 as primers and *B.subtilis* 168 chromosomal DNA as template. The products were digested with *Kpn*I–*Nco*I and *Sac*I–*Nco*I, respectively, and cloned into pUC19 digested with *Kpn*I and *Sac*I to provide pHPRK. The *erm* gene and the *spac* promoter from plasmid pMUTIN (Vagner *et al*., 1998) were amplified by PCR using oligo5 and 6 (Table V). The resulting 1.6 kb fragment was digested with *Kpn*I–*Eco*RI and ligated in one step to pUC19 digested with *Kpn*I–*Hin*dIII and to the *Hin*dIII–*Eco*RI-digested 400 bp PCR fragment (amplified with oligo7 and 8) containing the 5′ part of the *B.subtilis lgt* gene providing plasmid pErm. The 1.4 kb *Sac*I–*Kpn*I fragment of pHPRK was ligated into *Sac*I–*Kpn*I-digested pErm. The resulting pHPRKErm contained the *hprK* gene of *L.casei*, which is under the control of the *B.subtilis hprK* transcription and translation initiation signals, an erythromycin resistance marker and the 5′ end of the *lgt* gene under control of the *spac* promoter (Figure 2). *Pfu* DNA polymerase (Promega) was used for all PCRs, and the correct sequence of all PCR products was confirmed by DNA sequencing. *Escherichia coli* NM522 (Stratagene) was used as a host for all plasmid constructions.

Table V. Oligonucleotides used in this study

oligo1 ACT*CCATGG*CAGACAGCGTG	Construction of pHPRKErm
oligo2 TAC*GGTACC*AATGAACTTCCAG	
oligo3 GTC*GAGCTC*GGAAAAGCTAGC	
oligo4 TTG*CCATGG*TATGTTCCTCC	
oligo5 GAAT*GGTACC*TCTAGCACAAA	
oligo6 CGG*GAATTC*AAGCTTAATTG	
oligo7 GCG*GAATTC*ATTGAAGACGG	
oligo8 CGC*AAGCTT*CCAGAACGAAA	
HPrKBam GTG*GGATCC*ATGGCAGACAGC	Cloning of *L.casei hprK* in pQE30
HPrKBs1 CATA*CCATGG*CAAAGGTTCG	Cloning of *B.subtilis hprK* in pHPRKErm
HPrKBs2 GAA*GGATCC*ATGGCAAAGGTTCG	Cloning of *B.subtilis* hprK in pQE30
HPrKBs3 AAC*GGTACC*TCCTATTCTTCTTG	
V265F2 GACGAT<u>T</u>CCCTTCCGCCCAGGCC	Mutagenesis of *B.subtilis hprK*

Random mutagenesis of L.casei hprK

PCR-based random mutagenesis was used to introduce mutations into *L.casei hprK*. The *hprK* gene was amplified by PCR in two separate reactions using oligo1 and 2. The first reaction was performed with 200 μM dGTP, dTTP, dCTP and 20 μM dATP, and the second with 200 μM dATP, dTTP, dCTP and 20 μM dGTP. For both reactions, 30 cycles comprised of the following steps were carried out: 30 s at 94°C, 30 s at 50°C and 1.5 min of extension at 72°C. The reactions were performed in a total volume of 50 μl containing 2.5 U of *Taq* DNA polymerase and 20 pmol of each oligonucleotide. The 1 kb PCR products from both reactions were isolated, mixed, digested with *Nco*I and *Kpn*I and used to replace the wild-type *hprK* gene with the different mutant alleles in pHPRKErm cut with the same enzymes. Strain NM522 was transformed with the pHPRKErm derivatives carrying the *L.casei hprK* alleles, and ~10 000 *E.coli* clones were scraped off from the Petri dishes, pooled and grown for 2 h at 37°C in 0.5 l of LB medium containing 100 μg/ml ampicillin. Plasmid DNA was isolated from these cells and used to transform *B.subtilis* strain QB7144, which carries a xylose-inducible *ynaJ′–lacZ* fusion and therefore forms blue colonies when grown on solid LB medium containing xylose and X-Gal. The *ynaJ* gene is part of the *xyn* operon and supposedly encodes a β-xyloside-specific permease (Galinier *et al*., 1999). The pHPRKErm derivatives were integrated in the *B.subtilis* chromosome, thereby replacing *B.subtilis hprK* with the *L.casei hprK* alleles. In the integrants, *L.casei hprK* is expressed from the *B.subtilis hprK* promoter and *lgt* from the *spac* promoter (Figure 2). Integrants carrying wild-type *L.casei hprK* formed blue colonies when grown on solid LB medium containing xylose and X-Gal.

Construction of ccpA, ptsH1, crh and hprK mutants

Strains carrying the *L.casei* wild-type or V267FLc *hprK* allele were used to construct *ccpA* and *ptsH1* mutants and a *ptsH1 crh* double mutant. To construct *ccpA* mutants, chromosomal DNA was isolated from strain GM1090 carrying a spectinomycin-disrupted *ccpA* gene. The isolated

DNA was used to transform strains TG100 and TG101, and spectinomycin-resistant clones were selected, providing strains TG202 and TG203.

For the construction of the *ptsH1* mutants carrying the *L.casei* wild-type or V267F *hprK* allele, chromosomal DNA was isolated from strains TG100 and TG101 and used to transform the *ptsH1* strain GM1222 and its isogenic *ptsH*$^+$ strain GM1221 (Deutscher *et al*., 1994). Erythromycin-resistant transformants were isolated, providing strains TG105–108. The *ptsH1 crh* double mutant TG121 was obtained by transforming GM1222 with chromosomal DNA of QB7096, in which the *crh* gene is disrupted with a kanamycin resistance cassette (Presecan-Siedel *et al*., 1999). DNA of strain TG101 was used to introduce the V267FLc *hprK* allele into TG121, providing strain TG122.

To obtain the *B.subtilis hprK* allele expressing V265F mutant HprK/P, the 3′ part of *hprK* was amplified by using oligonucleotides V265F2 (mutagenic) and HPrKBs3 (Table V). The resulting 160 bp DNA fragment was used as primer for PCR amplification in combination with the oligonucleotide HPrKBs1. The obtained 1 kb PCR product was digested with *Nco*I–*Kpn*I and cloned in *Nco*I–*Kpn*I-digested pHPRKErm, allowing the replacement of *L.casei hprK* with *hprK*V265FBs. *Bacillus subtilis* wild-type *hprK* was amplified by using oligonucleotides HPrKBs1 and HPrKBs3, and the PCR product was used to replace the *L.casei hprK* gene in pHPRKErm with *B.subtilis hprK* by following the procedure described for the V265FBs *hprK* allele. The *B.subtilis hprK* alleles were subsequently integrated in the chromosome of QB7144 by double recombination, providing strains TG113 (*hprK*$^+$) and TG114 (V265F *hprK*).

Protein purification, and HPr kinase and P-Ser-HPr phosphatase assays

The different *L.casei hprK* alleles were amplified by PCR using oligo2 and HPrKBam as primers (Table V) (Dossonnet *et al*., 2000), cut with *Bam*HI and *Kpn*I and cloned into the expression vector pQE30 cut with the same enzymes. The *B.subtilis* V265F mutant *hprK* was amplified using oligo3 and HPrKBS2 as primers and also inserted into pQE30 cut with *Bam*HI and *Kpn*I. The *E.coli* M15[pREP4] strains transformed with the expression vector carrying the different *hprK* alleles were cultivated in 1 l of LB containing 100 mg of ampicillin and 25 mg of kanamycin. Expression of the different *L.casei hprK* alleles was induced with isopropyl-β-D-galactopyranoside (IPTG), and purification of the various mutant HprK/Ps on Ni-NTA columns (Qiagen) was carried out as previously described for the wild-type protein (Dossonnet *et al*., 2000). HprK/P-containing fractions were pooled, dialyzed at 4°C against 50 mM Tris–HCl pH 7, containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and stored in aliquots at –80°C. *Bacillus subtilis* HPr(His$_6$) was purified as previously described (Galinier *et al*., 1997). P-Ser-HPr(His$_6$) was prepared by ATP-dependent phosphorylation with purified *B.subtilis* HprK/P (Galinier *et al*., 1998) and was separated from unphosphorylated HPr (Deutscher *et al*., 1986).

The HPr(Ser) kinase and P-Ser-HPr phosphatase assays were performed in 20 µl of 50 mM Tris–HCl pH 7.4, containing 5 mM $MgCl_2$, 1 mM ATP (only for the kinase reaction), 20–200 ng of HprK/P(His$_6$) and varying amounts of FBP or potassium phosphate. The reactions were started by adding 2.5 µg of HPr(His$_6$) (kinase assay) or P-Ser-HPr(His$_6$) (phosphatase assay) (Dossonnet *et al*., 2000). After incubation at 37°C, the reactions were stopped by heating the assay mixtures for 5 min at 75°C. The different forms of HPr were separated by electrophoresis on non-denaturing 12.5% polyacrylamide gels that were stained with Coomassie Blue.

[^{32}P]P-Ser-HPr dephosphorylation

Bacillus subtilis [^{32}P]P-Ser-HPr(His$_6$) was synthesized with [γ-^{32}P]ATP and HprK/P (Galinier *et al*., 1998). The reaction products were loaded onto an Ni-NTA column and [^{32}P]P-Ser-HPr(His$_6$) was eluted with 300 mM imidazole and subsequently desalted on a PD10 column (Pharmacia) run with 20 mM ammonium bicarbonate. After lyophilizing, aliquots of the [^{32}P]P-Ser-HPr(His$_6$) preparation were used for dephosphorylation assays carried out for 30 min at 37°C in 20 µl of 50 mM Tris–HCl pH 7.4, containing 0.2 µg of HprK/P and either 5 mM ADP, 5 mM P_i or both compounds. The reaction was stopped by heating the samples for 5 min at 70°C. TLC was performed with 4 µl aliquots on Polygram CEL 300 PEI sheets (Macherey-Nagel) using 0.3 M KH_2PO_4 or K_2HPO_4 as solvent. [γ-^{32}P]ATP and [^{33}P]P_i were used as standards. Electrophoresis of 5 µl aliquots of the phosphatase assay mixtures on 15% polyacrylamide–1% SDS gels was used to determine to what extent [^{32}P]P-Ser-HPr(His$_6$) was dephosphorylated, which was always >95%, except in the control experiment where no HprK/P was added.

Western blots

Western blots were carried out with crude extracts prepared from strains TG100 and the V267FLc *hprK* mutant TG101 by following the method described by Gauthier *et al*. (1997). Cells were grown in LB medium or LB medium containing 0.5% glucose to an OD_{600} of between 0.25 and 0.35 before preparing crude extracts, which were separated on non-denaturing 10% polyacrylamide gels. On non-denaturing gels, HPr and phosphorylated HPr migrated to different positions and could be detected subsequently with polyclonal antibodies directed against His-tagged *B.subtilis* HPr.

Sugar transport studies

Bacillus subtilis strains were grown to an OD_{600} of between 0.6 and 0.7 in 25 ml of LB medium containing 0.2% of either glucose or mannitol to induce the corresponding carbohydrate transport system. Cells were collected by centrifugation, washed twice with 50 mM sodium phosphate buffer pH 7, containing 10 mM $MgCl_2$ and resuspended in 500 µl of 50 mM Tris-maleate buffer pH 7.4, containing 5 mM $MgCl_2$. Transport assays were performed at 37°C with 1 ml of cell suspensions (OD_{600} = 4) prepared with the latter buffer. At time 0, ^{14}C-labeled sugars (0.5 mCi/mmol) were added to a final concentration of 0.5 mM. Aliquots of 100 µl were withdrawn at different time intervals, rapidly filtered through nitrocellulose filters (0.45 µm pore size) and washed twice with 5 ml of ice-cold transport buffer. Radioactivity retained in the filters was determined by liquid scintillation counting.

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