In vitro Phosphorylation of Key Metabolic Enzymes from *Bacillus subtilis*: PrkC Phosphorylates Enzymes from Different Branches of Basic Metabolism

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Key Words

Protein phosphorylation • Phosphotransferase system • Glycolysis • Tricarboxylic acid cycle • Serine/threonine kinase

Abstract

Phosphorylation is an important mechanism of protein modification. In the Gram-positive soil bacterium *Bacillus subtilis*, about 5% of all proteins are subject to phosphorylation, and a significant portion of these proteins is phosphorylated on serine or threonine residues. We were interested in the regulation of the basic metabolism in *B. subtilis*. Many enzymes of the central metabolic pathways are phosphorylated in this organism. In an attempt to identify the responsible protein kinase(s), we identified four candidate kinases, among them the previously studied kinase PrkC. We observed that PrkC is indeed able to phosphorylate several metabolic enzymes in vitro. Determination of the phosphorylation sites revealed a remarkable preference of PrkC for threonine residues. Moreover, PrkC often used several phosphorylation sites in one protein. This feature of PrkC-dependent protein phosphorylation resembles the multiple phosphorylations often observed in eukaryotic proteins. The HPr

protein of the phosphotransferase system is one of the proteins phosphorylated by PrkC, and PrkC phosphorylates a site (Ser-12) that has recently been found to be phosphorylated in vivo. The agreement between in vivo and in vitro phosphorylation of HPr on Ser-12 suggests that our in vitro observations reflect the events that take place in the cell.

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Introduction

Adaptation to changing environmental conditions is essential for life. The Gram-positive soil bacterium *Bacillus subtilis* is permanently exposed to changing conditions, such as nutrient limitations or various physical stresses. In principle, there are three different mechanisms by which bacteria can adapt their metabolism to the environment: (1) alterations of gene expression, (2) changing the stabilities of proteins, and (3) modulation of enzymatic activities. Regulation of gene expression is by far the most intensively studied regulatory mechanism. There is a huge collection of studies concerning the response of *B. subtilis* to environmental changes at the proteome and/or transcriptome levels [Moreno et al., 2001;

Eymann et al., 2002; Blencke et al., 2003]. In the past few years, when transcriptome and proteome studies became very popular, most regulatory effects were attributed to changes in transcription. However, changes in transcription are not always accompanied by corresponding changes in metabolic fluxes and vice versa [Schilling et al., 2007]. Thus, posttranslational regulatory mechanisms require our attention to fully understand regulatory phenomena.

In the past few years, it turned out that extensive protein phosphorylation not only on histidine but also on serine, threonine and tyrosine residues occurs in bacteria [Kennelly and Potts, 1996; Deutscher and Saier, 2005]. In *B. subtilis*, phosphorylation on histidine residues occurs in the phosphotransferase system (PTS) as well as in PTS-controlled transcription regulators and glycerol kinase. The sensor kinases of the two-component regulatory systems constitute a second class of His-phosphorylated proteins [Fabret et al., 1999; Reizer et al., 1999]. In the PTS, some phosphotransfer domains are phosphorylated on cysteine residues from which the phosphate is transferred to the incoming sugar. In contrast, the response regulators of the two-component systems are phosphorylated on aspartate residues by their cognate sensor kinases [Fabret et al., 1999; Reizer et al., 1999]. Phosphorylation on serine residues has long been studied for the HPr protein of the PTS that is phosphorylated on Ser-46 by the metabolite-controlled HPr kinase/phosphorylase. HPr(Ser-P) then binds to the transcription factor CcpA and acts as a corepressor in carbon regulation [Görke and Stülke, 2008]. Regulatory phosphorylation of proteins on serine (and threonine) residues is also well established for the modulators of sigma factor activity in sporulation and stress response [Min et al., 1993; Yang et al., 1996]. Protein tyrosine phosphorylation is implicated in the control of the transcription repressor CtsR, of UDP-glucose dehydrogenases and of the single-stranded DNA-binding proteins [Grangeasse et al., 2007]. In total, about 200 proteins, representing 5% of all *B. subtilis* proteins, are now known to be phosphorylated [Lévine et al., 2006; Eymann et al., 2007; Macek et al., 2007].

With the large number of proteins phosphorylated on serine, threonine or tyrosine, the identification of the responsible kinase(s) is getting into the spotlight. So far, the HPr kinase and the three kinases involved in controlling sigma factor activities have been studied. All these kinases seem to phosphorylate clearly defined sites and are therefore not candidates for the phosphorylation of novel targets. In addition, the protein kinase PrkC can phosphorylate several proteins, including elongation factor G

and PrkC itself [Gaidenko et al., 2002; Madec et al., 2002]. PrkC is located in the cell membrane of vegetative cells and in the inner spore membrane of spores [Shah et al., 2008; Madec et al., 2002]. The *prkC* gene is part of the *prpC-prkC-cpgA* operon that is constitutively expressed throughout growth [Iwanicki et al., 2005]. Recently, stimulation of PrkC activity by muropeptides was reported. In spores, this activation of PrkC triggers germination, and *prkC* mutants are unable to germinate in response to muropeptides [Shah et al., 2008]. Homologues of PrkC are widespread in both Gram-positive and Gram-negative bacteria (fig. 1). This kinase is implicated in the phosphorylation of glycolytic enzymes and histone-like proteins as well as in pathogenicity in other Gram-positive bacteria [Jin and Pancholi, 2006; Kristich et al., 2007; Lomas-Lopez et al., 2007]. It is interesting to note that *prkC* is encoded in an operon with a corresponding protein phosphatase, PrpC [Madec et al., 2002]. This phosphatase is able to dephosphorylate HPr(Ser-P) in *B. subtilis* and *Mycoplasma pneumoniae* [Halbedel et al., 2006; Singh et al., 2007].

We were interested in the regulation of carbon and nitrogen metabolism in *B. subtilis*. In an analysis of the transcriptome and metabolic flux responses to organic acids, we noticed that there are some cases where the changes in transcription do not match those in the metabolic fluxes. This is exemplified by the expression of the *alsSD* operon encoding acetolactate synthase and acetolactate decarboxylase and the corresponding acetoin production. Therefore, we considered posttranscriptional events to be important for metabolic adaptation [Schilling et al., 2007]. At the same time, phosphoproteome analyses demonstrated that indeed many enzymes of basic metabolism are subject to phosphorylation in *B. subtilis* [Lévine et al., 2006; Eymann et al., 2007; Macek et al., 2007]. Thus, protein phosphorylation might indeed play an important role for the adaptation of *B. subtilis* metabolism to changing conditions. However, the kinases responsible for these phosphorylation events have so far not been identified.

Fig. 1. Phylogenetic tree of PrkC homologues. The tree is based on a multiple alignment generated with the CLUSTAL X v2.0.12. program [Larkin et al., 2007]. The guide tree has been calculated using the UPGMA algorithm. The final phylogenetic tree was obtained using the Tree View X v 0.5.0 program (<http://darwin.zoology.gla.ac.uk/~rpage/treeviewx/>).

Table 1. Plasmids used in this work

Plasmid	Gene	Protein	Affinity tag	Oligonucleotides	Vector	References
pAG2	<i>ptsH</i>	HPr	His-tag			Galinier et al. [1997]
pGP174	<i>glnA</i>	glutamine synthetase	Strep-tag			Heinrich et al. [2006]
pGP205	<i>hprK</i>	HPr kinase	His-tag			Hanson et al. [2002]
pGP371	<i>ptsH</i> *	HPr-S46A	His-tag	SH82/SH83	pWH844	
pGP563	<i>eno</i>	enolase	His-tag	eno forw1/eno rev	pWH844	
pGP819	<i>ywjH</i>	transaldolase	Strep-tag	NP11/NP12	pGP172	
pGP820	<i>tkt</i>	transketolase	Strep-tag	NP5/NP6	pGP172	
pGP821	<i>prkD</i>	protein kinase	Strep-tag	NP9/NP10	pGP172	
pGP822	<i>alsD</i>	α -acetolactate decarboxylase	Strep-tag	NP1/NP2	pGP172	
pGP823	<i>yabT</i>	protein kinase	Strep-tag	NP7/NP8	pGP172	
pGP824	<i>yxaL</i>	protein kinase	Strep-tag	NP13/NP14	pGP172	
pGP825	<i>prkC</i>	protein kinase	Strep-tag	NP3/NP4	pGP172	
pGP931	<i>icd</i>	isocitrate dehydrogenase	His-tag	FC17/FC18	pWH844	
pGP1100	<i>pyk</i>	pyruvate kinase	His-tag	pyk forw1/pyk rev	pWH844	

* Indicates mutant alleles.

In this study, we intended to identify the kinase(s) for some of the enzymes of central metabolism by in vitro analysis of protein phosphorylation. Our work demonstrates that PrkC is able to phosphorylate several enzymes of different metabolic pathways. Moreover, PrkC was also responsible for a novel modification of the HPr protein of the PTS on Ser-12, an event previously observed in vivo [Macek et al., 2007]. Thus, PrkC may play an important role in phosphorylating proteins with different functions in *B. subtilis*.

Results

Identification of Potential Serine/Threonine Protein Kinases in *B. subtilis*

The genome of *B. subtilis* encodes several proteins that are known or suspected to have serine/threonine kinase activity, among them the intensively studied HPr kinase/phosphorylase and the kinases involved in the control of sigma factor activities. In addition, the PrkC serine/threonine kinase has been studied before. Analysis of the annotated genome sequence revealed the presence of three additional potential kinases: PrkD that is similar to the kinase domain of PrkC [Madec et al., 2002], YxaL and YabT.

Purification of Selected Potential Protein Kinases and Phosphorylation Targets

The four genes encoding the selected proteins were cloned into the expression vector pGP172, fusing them to

an N-terminal Strep-tag. The proteins were expressed and purified using their affinity for Streptactin Sepharose. PrkC and PrkD were purified to apparent homogeneity. In contrast, we were unable to purify detectable amounts of YxaL. This may be due to the fact that YxaL is a secreted protein carrying a signal peptide [Tjalsma et al., 2004]. Similarly, only small amounts of YabT were obtained. Therefore, we concentrated our subsequent analyses on PrkC and PrkD.

Among the phosphoproteins that are involved in central metabolism, we selected glutamine synthetase (GlnA), enolase (Eno), transaldolase (YwjH), transketolase (Tkt), α -acetolactate decarboxylase (AlsD), isocitrate dehydrogenase (Icd), and pyruvate kinase (Pyk). These proteins were found to be phosphorylated in vivo on serine, threonine or tyrosine [Lévine et al., 2006; Eymann et al., 2007; Macek et al., 2007]. The corresponding genes were cloned into the expression vectors pWH844 and pGP172 to fuse the coding sequences to an N-terminal His- or Strep-tag, respectively (table 1). All these proteins were expressed in *Escherichia coli* and were purified to apparent homogeneity by affinity chromatography.

Autophosphorylation of PrkC and PrkD

First, we analyzed the autophosphorylation of the selected serine/threonine kinases, PrkC and PrkD. For this purpose, we incubated the two proteins in the presence of [γ - 32 P]ATP and analyzed them by SDS-PAGE. As shown in figure 2, both proteins exhibited autophosphorylation activity. This observation is in excellent agreement with

Fig. 2. Autophosphorylation of the serine/threonine kinases PrkC and PrkD. Two micrograms of each purified protein were incubated with 0.4 mM [γ - 32 P]ATP (480 Ci/mmol) at 37°C for 30 min in assay buffer. The samples were then heated for 10 min at 95°C and loaded on a 16% SDS-PAGE gel. The gel was dried and analyzed by using a Storm 860 Molecular Imager, Molecular Dynamics.

a previous report on the autophosphorylation of PrkC [Madec et al., 2003]. To characterize these autophosphorylation events in more detail, we determined the phosphorylation sites by mass spectrometry using an LTQ-Orbitrap mass spectrometer. For PrkC, we detected six phosphorylation sites: Ser-214, Thr-290, Thr-313, Thr-320 in the cytoplasmic kinase domain, and Thr-417 and Thr-498 in the extracytoplasmic domain. The phosphorylation sites in the kinase domain were also detected in a previous study that was exclusively devoted to the analysis of this domain [Madec et al., 2003]. Moreover, phosphorylation of Thr-290 was found to take place in vivo [Macek et al., 2007]. For PrkD, we found one phosphorylation site, Thr-174. It is interesting to note that the region surrounding this site is not conserved in PrkC. Similarly, the regions encompassing the phosphorylation sites in PrkC are not conserved in PrkD. Thus, both PrkD and

Fig. 3. Phosphorylation of potential target proteins by PrkC. Ten micrograms of the purified enzymes YwjH, GlnA, Icd and AlsD were incubated with 0.4 mM [γ - 32 P]ATP (480 Ci/mmol) in the absence and in the presence of 2 μ g PrkC at 37°C for 30 min in assay buffer. The samples were heated for 10 min at 95°C and loaded on a 16% SDS-PAGE gel. To obtain the autoradiogram, the gel was dried and analyzed using a Storm 860 Molecular Imager, Molecular Dynamics (a). In addition, the proteins were visualized by Coomassie-staining (b). co = Control (HPr phosphorylated by HPrK).

PrkC are mainly autophosphorylated on threonine residues, but the actual phosphorylation sites are not conserved even though 26 and 41% of identical and similar residues are conserved, respectively, for the N-terminal 200 amino acids of the two kinases.

Phosphorylation of the Potential Target Proteins

The principal aim of this study was the investigation of phosphorylation of central metabolic enzymes of *B. subtilis*. To address this question, we incubated the putative kinase substrates with [γ - 32 P]ATP and either of the two kinases. Of the seven selected proteins, four were phosphorylated by PrkC (fig. 3). These were the transaldolase YwjH, the glutamine synthetase GlnA, the isocitrate dehydrogenase Icd, and the α -acetolactate decar-

boxylase AlsD. In contrast, enolase, transketolase, and the pyruvate kinase PykA were not substrates for PrkC-dependent phosphorylation (data not shown). However, the phosphorylation efficiency differed for the PrkC substrates: the phosphorylation efficiency of YwjH, GlnA and Icd by PrkC was similar to that observed for HPrK-dependent phosphorylation of HPr (fig. 3). In contrast, only weak signals were observed with AlsD (fig. 3). This might be caused by differences in the number of phosphorylation sites or in different affinities of PrkC for its respective substrates. The kinase assays with PrkD revealed that only Icd and GlnA were phosphorylated by PrkD (data not shown). However, these phosphorylation signals were extremely faint, which questions their biological relevance. Thus, our analysis revealed selective phosphorylation of four enzymes of central metabolism by PrkC.

Determination of Phosphorylation Sites in the Target Proteins

In order to get new insights into PrkC substrates, we determined the phosphorylation sites of the proteins phosphorylated by PrkC. As observed for the autophosphorylation of PrkC, multiple phosphorylations with threonine as the major phosphoacceptor were observed. For the transaldolase YwjH, we observed six sites that were phosphorylated by PrkC. The phosphorylated amino acids were scattered throughout the protein. There was one phosphorylated serine residue, Ser-55, and five phosphothreonines, i.e. Thr-26, Thr-82, Thr-125, Thr-159, and Thr-184. Similar results were obtained with glutamine synthetase. This enzyme was phosphorylated on one serine residue, Ser-207, and three threonine residues, Thr-26, Thr-147, and Thr-286. It is interesting to note that Thr-26 is located in the N-terminal β -grasp domain of the enzyme that is thought to be involved in binding of small molecules [Burroughs et al., 2007], whereas the other phosphorylation events take place in the catalytic domain of glutamine synthetase. Isocitrate dehydrogenase is phosphorylated by PrkC on three threonine residues, Thr-138, Thr-147 and Thr-396. In contrast to the other PrkC substrates (including PrkC autophosphorylation), the α -acetolactate decarboxylase AlsD was phosphorylated by PrkC on a single serine residue, Ser-88.

Phosphorylation of HPr by PrkC

A recent analysis of the *B. subtilis* phosphoproteome revealed a novel phosphorylation of the HPr protein of the PTS on Ser-12 [Macek et al., 2007]. However, the source of this phosphorylation is not known so far. We

Fig. 4. Phosphorylation of wild-type HPr and HPr-S46A by PrkC and HPrK. Ten micrograms of the purified wild-type HPr (W) and HPr-S46A (M) were incubated with 0.4 mM [γ - 32 P]ATP (480 Ci/mmol) in the absence and in the presence of either 2 μ g PrkC or HPrK at 37°C for 30 min in assay buffer. The samples were heated for 10 min at 95°C and loaded on a 16% SDS-PAGE gel. The autoradiogram (a) and the Coomassie-stained gel (b) were prepared as described in figure 2.

considered the possibility that this phosphorylation might be caused by PrkC. Therefore, we assayed the phosphorylation of HPr in the presence of PrkC and [γ - 32 P]ATP. The HPr kinase that phosphorylates HPr on Ser-46 served as a control. As shown in figure 4, HPr was efficiently phosphorylated by the HPr kinase. Similarly,

Fig. 5. Identification of the site of PrkC-dependent phosphorylation in HPr. The fragment ion spectrum of the HPr T2 peptide (VTADSGIHAR) with a precursor ion mass 544.7 $[M+2H]^{2+}$ indicates a phosphate residue. The corresponding b- and y-ions

and the formation of the ion corresponding to the neutral loss of phosphoric acid from the doubly-charged precursor ion are highlighted and demonstrate the Ser-12 phosphorylation of HPr.

PrkC was capable of phosphorylating HPr. Both HPrK (with its phosphorylase activity) and PrpC, a protein phosphatase encoded in an operon with PrkC, are able to dephosphorylate HPr(Ser-P) [Mijakovic et al., 2002; Singh et al., 2007]. Therefore, we considered the possibility that Ser-46 of HPr was the phosphorylation site used by HPrK as well as by PrkC. To address this problem, we used a mutant variant of the HPr protein devoid of its HPrK-dependent phosphorylation site (HPr-S46A) and tested the phosphorylation of this protein by the two kinases. As expected, the mutant protein was no longer phosphorylated by HPr kinase, thus confirming the

conclusion that Ser-46 is the only phosphorylation site in HPr used by HPr kinase. In contrast, the mutant protein was still phosphorylated by PrkC, and the intensity of the phosphorylation signal was similar to that observed with the wild-type protein. These observations suggest that a site (or sites) distinct from Ser-46 are the primary target(s) of PrkC-dependent phosphorylation in HPr.

The use of a different phosphorylation site by PrkC may suggest that Ser-12, a site phosphorylated in vivo [Macek et al., 2007], is the target of PrkC. This was verified by mass-spectrometric determination of the PrkC-

dependent phosphorylation site (fig. 5). Indeed, the only phosphorylated site detected in this study was Ser-12. Thus, the in vitro activity of PrkC on HPr reflects the in vivo situation. This observation suggests that PrkC is the kinase that phosphorylates HPr in vivo on Ser-12.

Discussion

Protein phosphorylation is a very common event in all organisms [Ficarro et al., 2002; Levine et al., 2006; Eyermann et al., 2007; Macek et al., 2007; Macek et al., 2008]. As much as about 5% of all proteins can be phosphorylated in organisms so different as the sporulating bacterium *B. subtilis* and the genome minimalist *Mycoplasma genitalium* [Su et al., 2007]. Two major questions remain to be answered: (1) which kinases are responsible for these phosphorylation events, and (2) what is the effect of the phosphorylation on the activity of the target proteins?

In this work, we addressed the first of these questions. In *B. subtilis*, only five serine/threonine kinases have been studied so far. These are HPr kinase, RsbT kinase, RsbW kinase and SpoIIAB kinase, involved in the control of sigma factor activities and PrkC [Min et al., 1993; Reizer et al., 1998; Madec et al., 2002]. In this work, we focussed on PrkC and its relative, PrkD. Indeed, our in vitro experiments revealed that PrkC is able to phosphorylate several of the proteins that were found to exist as phosphoproteins in vivo.

PrkC is implicated in sporulation, biofilm formation and germination [Gaidenko et al., 2002; Madec et al., 2002; Shah et al., 2008], but only two substrates (EF-G and PrkC itself) have been identified. Our work confirms that PrkC might be of global importance as it phosphorylates enzymes from different pathways, such as transaldolase, glutamine synthetase, isocitrate dehydrogenase, α -acetolactate decarboxylase and HPr. However, among the eight phosphoproteins tested for phosphorylation by PrkC, three were not targets of this kinase. A global role for PrkC is supported by studies with other bacteria. In *Staphylococcus aureus*, PrkC phosphorylates glycolytic enzymes [Lomas-Lopez et al., 2007], whereas the α -subunit of RNA polymerase and the phosphoglucosamine mutase are substrates of the *Streptococcus pneumoniae* PrkC homolog, StkP [Nováková et al., 2005]. Moreover, PrkC homologs are important for virulence development and global gene expression in *S. pneumoniae*, *S. aureus*, *M. pneumoniae* and *Bacillus anthracis* [Sasková et al., 2007; Débarbouillé et al., 2009; Schmidl et al., 2010; Shaker et al., 2010]. Thus, PrkC can phosphorylate a wide

range of proteins involved in a variety of cellular functions.

Another remarkable feature of PrkC is its obvious preference for threonine. This study presents a significant increase of the known phosphorylation sites used by PrkC. Of the sites identified here, 16 are threonines and only 5 are serine residues. In a previous study on PrkC autophosphorylation, 7 phosphorylated threonine residues and 1 serine residue were reported [Madec et al., 2003]. Our results confirm the autophosphorylation of Ser-214 as well as of Thr-290, Thr-313 and Thr-320. We failed to detect the phosphorylation of a stretch of multiple threonine residues in the N-terminal part of PrkC, but instead we detected two phosphorylation sites in the extracellular domain of PrkC that was not included in the previous study [Madec et al., 2003]. In vivo phosphorylation of PrkC was observed on Thr-290 [Macek et al., 2007], thus providing a confirmation for our in vitro study. Studies on protein phosphorylation by PrkC proteins from *Streptococcus pyogenes* also revealed the use of threonine as the preferred phosphorylation target by PrkC [Jin and Pancholi, 2006]. The kinase domains of PrkC and PrkD are very similar to each other (about 30% identical residues). However, the sites of autophosphorylation were not conserved between the two proteins although the only phosphorylation site identified for PrkD was also a threonine. A comparison of the regions around the PrkC-dependent phosphorylation sites revealed little conservation of the primary sequences, suggesting that it is not the primary sequence context that serves for recognition by PrkC.

An interesting aspect of this work is the identification of HPr as a novel target for PrkC. HPr is a paradigm of protein phosphorylation with its His-15 and Ser-46 phosphorylated by enzyme I of the PTS and by the ATP-dependent HPr kinase, respectively [Reizer et al., 1998]. Analysis of the *B. subtilis* phosphoproteome revealed the phosphorylation of yet another site, Ser-12. We could attribute this phosphorylation event to the activity of PrkC. This finding suggests that our in vitro observations regarding PrkC-dependent protein phosphorylation reflect the in vivo activity of PrkC and the physiological interactions with the different target proteins. However, it is not known whether this phosphorylation affects the biological activity of HPr. The weak phosphorylation of Ser-12 by PrkC suggests that this phosphorylation does not affect the properties of the cellular HPr. Similar results have been obtained for the transcription antiterminator GlcT, which can be phosphorylated and inactivated to a minor degree by HPr under conditions in which its full activity

Table 2. Oligonucleotides used in this study

Oligonucleotide	Sequence (5' → 3')
CD13	AAACATATGGCTAGCTGGAGCCACCCGCAGTTC
eno forw1	AAGGATCCCCATACATTGTTGATGTTTATGCAC
eno rev	TTTCTGCAGTTACTTGTTTAAGTTGT AGAAAGAGTTG
FC17	AAAGGATCCGTGGCACAAGGTGAAAAAATTAC
FC18	TTTCTGCAGTTATTAGTCCATGTTTTTGAT
JS39	TCTATCAACAGGAGTCCAAGC
NP1	AAAGAGCTCCGATGAAACGAGAAAGCAACATTC
NP2	TTTGGATCCCTATTATTCAGGGCTTCCTTCAGT
NP3	AAAGAGCTCCGGTGCTAATCGGCAAGC
NP4	TTTAGATCTTCATTATTTCATCTTTTCGGATACTCAAT
NP5	AAAGAGCTCGATGGATACAATTGAAAAAGAAATCAG
NP6	TTTGGATCCCTCATTACTTATTGATTAATGCCTTAAC
NP7	AAAGAGCTCGATGATGAACGACGCTTTGAC
NP8	TTTAGATCTTCATTAGATTAAGAAAAAGATAATA
NP9	AAAGAGCTCGATGGCATTAAACTTCTAAAAAA
NP10	TTTGGATCCCTCATTATGTGACCGATTGAATGGC
NP11	AAAGAGCTCGATGTTATTCTTTGTTGATACAG
NP12	TTTGGATCCCTCATTATTTGTTCCAGTCTGCC
NP13	AAAGAGCTCGATGGTCAAGTCATTTTCG
NP14	TTTGGATCCCTCATTATTTCCCAAAAGCCATCAG
NP15	CCGGTGTTCACGGTGCGCCGCTTGG
NP16	CTCACCTGATTCTTTAACAGCTACAGCG
NP17	CTTGCTAAAGCTGCTGGCGAAAAGAAGCTG
NP18	ATCGTGAGCAAAACCCACGGCCAC
NP19	ATGTTACGCGATCAAACCGACGTC
NP20	GCAGCAGCCAACTCAGCTTCCTTTCCGGGC
pWH844 fw	TATGAGAGGATCGCATCACCAT
pyk forw1	AAGGATCCAGAAAAACTAAAATTG TTTGTACCATCGG
pyk rev	TTTCTGCAGTTAAAGAACGCTCGCA CGG
SH82	ATGGATCCATGGCACAACAAACATTTAAAG
SH83	ATAAAGCTTCTCGCCGAGTCCTTCG

is required. Again, this phosphorylation affects only a small fraction of the cellular GlcT pool and, therefore, does not influence GlcT-mediated regulation [Schmalisch et al., 2003].

Our work suggests that PrkC is a protein kinase which may have global importance in *B. subtilis*. However, important questions remain to be answered: which other proteins does PrkC phosphorylate? Phosphoproteome studies with *prkC* mutants might be helpful to address this question. Does PrkC-dependent phosphorylation serve for fine-tuning of the metabolism, does it tag the proteins for degradation, or does it serve completely different purposes? Finding the answers to these questions is challenging, but these answers will have a significant impact on our understanding of microbial physiology.

Experimental Procedures

Bacterial Strains, Oligonucleotides and Growth Conditions

B. subtilis 168 (wild type, laboratory collection) and QB5223 (*ptsHI*) [Martin-Verstraete et al., 1995] were used to isolate chromosomal DNA for the amplification of the desired genes. *E. coli* DH5 α and BL21 (DE3)/pLysS [Sambrook et al., 1989] were used for cloning experiments and protein expression, respectively. *B. subtilis* was grown in TY medium (1% w/v tryptone, 0.5% w/v yeast extract, and 1% w/v NaCl). *E. coli* was grown in Luria-Bertani medium (LB medium), and transformants were selected on plates containing ampicillin (100 μ g/ml). LB plates were prepared by the addition of 17 g Bacto agar (Difco) per liter of LB medium. The oligonucleotides used in this study are listed in table 2.

DNA Manipulation

Transformation of *E. coli* and plasmid DNA extraction were performed using standard procedures [Sambrook et al., 1989]. Restriction enzymes, T4 DNA ligase and DNA polymerases were used as recommended by the manufacturers. DNA fragments

were purified from agarose gels using the QIAquick PCR Purification kit (Qiagen, Germany). *Pfu* DNA polymerase was used for the polymerase chain reaction as recommended by the manufacturer. DNA sequences were determined using the dideoxy chain termination method [Sambrook et al., 1989]. All plasmid inserts derived from PCR products were verified by DNA sequencing. Chromosomal DNA of *B. subtilis* was isolated as described [Martin-Verstraete et al., 1995].

Plasmid Constructions

The plasmids used for the expression of tagged *B. subtilis* proteins in *E. coli* were constructed using the expression vectors pWH844 [Schirmer et al., 1997] or pGP172 [Merzbacher et al., 2004] that allow the fusion of the cloned proteins to an N-terminal His- or Strep-tag, respectively. These plasmids are listed in table 1.

For the expression of the S46A mutant variant of the HPr proteins, plasmid pGP371 was constructed by cloning of the *ptsHI* DNA fragment from *B. subtilis* QB5223 (table 1). *B. subtilis* HPr kinase and wild-type HPr were expressed using pGP205 and pAG2, respectively [Galinier et al., 1997; Hanson et al., 2002].

Overexpression and Purification of Recombinant Proteins

E. coli BL21(DE3)/pLysS was used as host for the overexpression of recombinant proteins. Expression was induced by the addition of IPTG (final concentration 1 mM) to exponentially growing cultures (OD₆₀₀ of 0.8). Cells were lysed using a French press (20,000 p.s.i., 138,000 kPa; Spectronic Instruments, UK). After lysis, the crude extracts were centrifuged at 15,000 g for 1 h. For purification of His-tagged proteins, the resulting supernatants were passed over an Ni²⁺ HiTrap chelating column (5 ml bed volume, Pharmacia) followed by elution with an imidazole gradient (from 0 to 500 mM imidazole in a buffer containing 10 mM Tris-HCl pH 7.5, 600 mM NaCl, 10 mM β -mercaptoethanol) over 30 ml at a flow rate of 0.5 ml/min. For proteins carrying an N-terminal Strep-tag, the crude extract was passed over a Streptactin column (IBA, Göttingen, Germany). The recombinant protein was eluted with desthiobiotin (Sigma, final concentration 2.5 mM).

After elution, the fractions were tested for the desired protein using 12.5% SDS-PAGE gels. The relevant fractions were combined and dialyzed overnight. Protein concentration was determined using the Bio-Rad dye-binding assay with bovine serum albumin serving as the standard.

Assays for Protein Phosphorylation

Activity assays of potential protein kinases were carried out with purified proteins in assay buffer (10 mM MgCl₂, 25 mM Tris-HCl pH 7.6, 1 mM dithiothreitol, 0.4 mM [γ -³²P]ATP (480 Ci/mmol)) using purified target proteins. For assays of HPr kinase activity, fructose 1,6-bisphosphate was added to a final concentration of 25 mM. The assays were carried out at 37°C for 30 min followed by thermal inactivation of the enzyme (10 min at 95°C). The assay mixtures were analyzed on 16% SDS-polyacrylamide gels. Proteins were visualized by Coomassie staining.

Protein Identification by Mass Spectrometry

Peptide preparation was carried out as recently described [Eymann et al., 2007]. Excised polyacrylamide gel pieces of stained protein bands were washed with 100 μ l of 200 mM NH₄HCO₃ in 50% CH₃CN for 20 min at 37°C and dried in a vacuum centrifuge

for 20 min. In-gel digestion with trypsin (Promega, Madison, Wisc., USA) was performed overnight at 37°C. After addition of 15 μ l formic acid (5%, v/v), samples were incubated in an ultrasonic bath for 20 min. The supernatant was transferred into microsample vials and stored for LC-MS/MS analysis at 4°C.

LC-MS/MS analysis was performed using either an LTQ-Orbitrap mass spectrometer (Thermo Electron, San Jose, Calif., USA) in conjunction with a nanoACQUITY UPLC™ system (Waters, Milford, Mass., USA) or a Q-Star Pulsar mass spectrometer (Applied Biosystems MDS Sciex) in conjunction with the Ettan MDLC system (GE Healthcare, Munich, Germany).

In the nanoACQUITY UPLC system, peptides were loaded onto a trapping column (nanoAcquity UPLC Column, Symmetry® C18 5 μ m, 180 μ m $\times$ 20 mm, Waters) and washed for 3 min with 99% buffer A (0.1% v/v formic acid) at a flow rate of 10 μ l/min and separated on an analytical column (BEH130 C18 1.7 μ m, 100 μ m $\times$ 100 mm, Waters) in a linear gradient from 99% buffer A to 60% buffer B (90% v/v acetonitrile, 0.1% v/v acetic acid) in 80 min. The flow rate during elution was set to 1 μ l/min. In the Ettan MDLC system, the peptides were loaded onto a trapping column (μ -Precolumn, PepMap™, C18, 300 μ m i.d. $\times$ 5 mm, LC Packings), washed for 15 min with buffer A and separated onto the analytical column (PepMap, C18, 75 μ m i.d. $\times$ 15 cm, LC Packings) by formation of a 70-min gradient of 99% buffer A to 60% buffer B at a flow rate of 200 nl/min.

The LTQ-Orbitrap was employed to acquire a full Orbitrap survey scan in the range *m/z* 300–2,000. Subsequently, MS/MS experiments of the three most abundant precursor ions were carried out in the LTQ. Meanwhile, the masses of the precursor ions were determined with high accuracy via single-ion mode scans in the Orbitrap cell of the mass spectrometer. The Qstar system was used to carry out a survey scan in the mass range of *m/z* 230–2,000 in the first step. In a second step, up to four precursor ions were selected for a fragmentation in MS/MS experiments. Product ions were detected in the range of *m/z* 70–2,000. The data were analyzed as described [Eymann et al., 2007].

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