The role of protein phosphorylation in regulation of carbon catabolite repression in *Bacillus subtilis*

PhD Thesis

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I hereby declare that the PhD thesis entitled, “The role of protein phosphorylation in regulation of carbon catabolite repression in *Bacillus subtilis*” has been written independently and with no other sources and aids than quoted.

Kalpana Singh

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List of Abbreviations

ABC	ATP binding cassette
ATP	adenosine triphosphate
bp	base pairs
CCR	carbon catabolite repression
DNA	deoxyribonucleic acid
DTT	Dithiothreitol
ermC	Erythromycin
E I	enzyme I
EII	enzyme II
FBP	fructose 1,6-bisphosphate
glu	Glucose
G6P	glucose-6-phosphate
His	Histidine
IPTG	Isopropyl-β-D-thiogalactopyranoside
KDa	Kilodaltons
LB	Luria Bertani
LFH	long flanking homology
MIN	Minutes
mM	Millimolar
MW	molecular weight
NTA	nitrilotriacetic acid
OD	optical density
ONPG	2-nitrophenyl-β-D-galactosidase
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEP	Phosphoenolpyruvate
pH	power of hydrogen
PNPP	p-nitro phenyl phosphate
PNPX	p-nitro phenyl xyloside
PP2C	Protein phosphatase 2C
PPP	pentose phosphate pathway
PPP	protein phosphatase P
p.s.i	per square inch
PTS	phosphotransferase system
PVDF	polyvinylidene difluoride membrane
rpm	rotation per minute
Ser	Serine
Spc	Spectinomycin
RNA	ribonucleic acid
TCA	tricarboxylic acid
Tris	Tris(hydroxymethyl)aminomethane
Wt/vol	weight per volume
WT	Wild type

Abstract

Bacteria have developed sophisticated mechanisms to adapt to a variety of environmental conditions, including the availability and supply of different carbon sources. If provided with a mixture of nutrients, many bacteria can selectively utilize the carbon source that allows the most rapid growth. Simultaneously, the cells repress the functions involved in the utilization of secondary carbon sources. The underlying phenomenon is called carbon catabolite repression (CCR). CCR is a paradigm of signal transduction and is achieved by different molecular mechanisms in Gram-negative and Gram-positive bacteria. In *Bacillus subtilis*, the transcriptional regulator CcpA is the master regulator of the global CCR. In the presence of a preferred carbon source, CcpA is activated and binds to its DNA operator sites, the catabolite responsive elements (*cre*). This leads to the repression of numerous genes involved in metabolism and utilization of secondary carbon sources. The DNA binding activity of CcpA is stimulated by interaction with its phosphorylated cofactors, HPr(Ser-P) and Crh(Ser-P). HPr and Crh are phosphorylated by the ATP dependent bifunctional enzyme, the HPrK/P at Ser-46 residue. HPrK/P is an allosteric enzyme and possesses both kinase and phosphorylase activity, thereby catalysing the (de)phosphorylation of HPr and Crh. The antagonistic activities of HPrK/P are triggered by metabolites such as fructose 1,6-bisphoaphate (FBP), ATP and inorganic phosphate (P_i). HPr is also a part of the phosphoenol pyruvate dependent phosphotransferase system (PEP:PTS). The PTS is involved in carbohydrate uptake in many bacteria. When participating in sugar transport, HPr is phosphorylated at histidine-15 (His-P) residue. The two phosphorylation events (Ser-P and His-P) are considered to be mutually exclusive and only HPr(Ser-P) can participate in CCR. According to the present model of CCR in *B. subtilis*, the metabolism of a preferred carbon source increases the intracellular level of FBP. A high FBP concentration stimulates the HPrK/P kinase activity, leading to high amounts of intracellular HPr(Ser-P), which causes CCR.

Glucose is the most preferred carbon source for many organisms and the current model of CCR in *B. subtilis* is based on studies, which focussed on glucose as the repressing carbon source. In this study, CCR was studied in the presence of various carbon sources, which are catabolised and taken up by different metabolic pathways. The different carbon sources formed a hierarchy in their ability to cause CCR. As a reporter system for CCR expression of the β-xylosidase (XynB) was analysed. Most of the strong repressing carbon sources were found to be PTS substrates. It could be demonstrated that repression by each

carbon source is mediated via the CcpA dependent pathway, except for sorbitol. This highlights the existence of a CcpA independent mechanism of CCR of *xynB* expression.

For the CcpA dependent pathway of CCR, HPr was demonstrated to be the relevant corepressor *in vivo*. A strain lacking the second cofactor of CcpA, Crh exhibited similar repression of the reporter system as the wild type strain. Western blot analysis revealed that the amounts of HPr, HPrK/P and CcpA do not vary with the carbon source. This ruled out the possibility that the differential CCR exerted by the different carbon sources is caused by different intracellular amounts of these proteins. Therefore, the phosphorylation state of HPr *in vivo* was analysed. The presence of strong repressing carbon sources generated high intracellular HPr(Ser-P) levels as compared to the weaker repressing carbon sources. Moreover, weaker repressing carbon sources allowed formation of large amounts of HPr(His-P). Thus, it could be well established that the different repressing potential of various carbon sources are derived from the ability to generate differential intracellular levels of HPr(Ser-P).

I then addressed the question, how *B. subtilis* cells may judge the quality of a given carbon source, in terms of being a stronger or weaker repressing sugar. Since PTS carbon sources generally exerted a strong CCR, I analysed if an active PTS may affect the intracellular generation of HPr(Ser-P). Preventing the phosphorylation at histidine residue of HPr, resulted in a complete relief from CCR by PTS substrates. This was expected because these substrates can not be taken up in this mutant. However, the repression by non-PTS substrates did not change as compared to a wild type strain. This interesting observation clearly indicated that even if HPr does not participate in PTS, its participation in CCR can not be increased. Hence, there is no competition between His- and Ser- phosphorylated froms of HPr. Thus, differences in HPr(Ser-P) amounts must originate from the differential kinase activity of HPrK/P. Since the activity of the HPr kinase is believed to be triggered by FBP, we determined the intracellular FBP concentrations in cells grown on the different carbon sources. It turned out that on most sugars the intracellular FBP level is high enough to achieve full activation of the HPrK/P kinase activity. Thus, in contrast to the existing model, there must be further factors or metabolites, which affect the HPrK/P activity *in vivo*.

A hint regarding this possible additional factor, which may modulate the kinase activity of HPrK/P could also be gathered in this work. The relieved repression in a strain carrying a *ΔhprK* polar mutation could not be restored by the ectopic expression of *hprK*. This suggested a possible role of the genes downstream of *hprK* in CCR.

HPr(Ser-P) is also known to be a target of the Ser/Thr phosphatase, PrpC in *Mycoplasma pneumoniae.* Thus, the possible role of this phosphatase in CCR was also

analysed in *B. subtilis*. It could be established that *B. subtilis* PrpC can indeed dephosphorylate HPr(Ser-P) *in vitro. In vivo,* a *prpC* mutation had a minor effect on the phosphorylation state of HPr and none on CCR.

In summary, the global CCR mechanism in *B. subtilis* is governed solely by HPrK/P activity. Depending on the available carbon source, a concerted action of known and unknown factors/metabolites leads to the modulation of HPrK/P activity, which ultimately determines the strength of CCR.

1. Introduction

1.1 The prokaryotic way of life

For prokaryotes, adaptation is the key to survival. They rarely live in a constant environment and face challenges like changes in the physical and chemical composition of their habitats, limited nutrient supply etc. Moreover, in their natural habitat, bacteria have to compete with other microorganisms for the available resources.

Bacillus subtilis is a low G+C content, heterotrophic, Gram-positive soil bacterium and has developed sophisticated mechanisms to thrive well in the rough conditions of its natural habitat. This is achieved primarily by the combination of an economical use of the limited nutrient sources and intricate gene/cell regulation mechanisms (Stülke and Hillen, 2000).

Likewise, the ability to sense and migrate towards preferred carbon and nitrogen sources allows the bacterium to make use of new resources and to escape from unfavourable conditions. Upon conditions of prolonged stress and nutrient deprivation, *B. subtilis* is able to initiate the process of sporulation. During sporulation, dormant, heat and stress resistant endospores are formed that allow *B. subtilis* to survive unfavourable conditions. The process of spore formation made *B. subtilis* a model organism for the study of this simple developmental process (Errington, 1993).

1.2 Signal transduction and regulation in bacteria

Signal transduction in any organism serves the purpose of mediating response to a chemical or physical signal from its environment. The resulting intracellular effects of this signal sensing can be a change in gene expression, alterations in metabolism, the continued proliferation and death of the cell or the stimulation or suppression of locomotion. Protein modification is one of the principal mechanisms for the regulation of cellular functions in response to extracellular stimuli in both eukaryotes and prokaryotes. In this respect, protein phosphorylation and dephosphorylation provides the cell with signals that reflect rapid changes in the cell physiology. The prominence of phosphorylation derives from various attributes. Primarily, the high charge density, the ability to form strong salt bridges and the propensity to form multiple hydrogen bonds renders phosphate a potent agent for altering the chemical structure of a protein (Johnson and Barford, 1993). In addition, phosphorylation events are reversible and thus, unlike other covalent modifications, proteins can be readily

converted back to their native state *in vivo*. Thus, depending on the stimulus perceived, a protein can exist in physically and functionally distinguishable states.

A number of amino acid residues can accept phosphate. Commonly phosphorylated residues include histidine, tyrosine, serine, threonine and aspartate. Chemical properties and the intrinsic free energy of phosphorylation of the amino acids as compared to the same residue when they are part of a protein, also determines the cellular function of different types of phosphorylation. For instance, the free energy of hydrolysis of phosphoserines within proteins is higher than that of free phosphoserine, thus this mode of phosphorylation is usually involved in induction of energetically unfavourable conformational changes (Sprang *et al.*, 1988). On the other hand, the free energy of phosphohistidines within proteins is similar to that of free phosphohistidines, suggesting that this phosphorylation is independent of the protein of which it is a part (Stock *et al.*, 1990).

A number of cellular processes in prokaryotes including control of metabolism, carbohydrate transport, gene transcription, cellular differentiation, infective mechanisms of pathogens, osmoregulation, chemotaxis and phototaxis are subjected to phosphorylation dependent regulation (Alex and Simon, 1994; Cozzone, 1993; Mann, 1994; Stock *et al.*, 1992).

1.2.1 Modes of phosphorylation dependent signal transduction in Bacteria

In bacteria two main modes of signal transduction prevail. Firstly, the extracellular signal can be perceived or sensed by a transmembrane protein that generates an intracellular response (Hellingwerf *et al.*, 1998). Alternatively, the signalling molecule is transported intracellularly and its presence is then sensed by the cell, for e.g. lactose in the regulation of *lac* operon in *Escherichia coli* (see section 1.4 for details). The *lac* operon is induced in the presence of lactose in the media. Lactose is converted intracellularly into allolactose, which acts as an inducer of the *lac* operon (Pardee *et al*., 1959). Many of these regulatory mechanisms in prokaryotes are controlled by phosphorylation systems (Cozzone, 1988; Saier, Jr., 1993). They include:

a) ATP dependent protein kinases/phosphatases: This classical mode of phosphorylation includes enzymes that utilize alcoholic (serine and threonine) or phenolic groups (tyrosine) as acceptors to generate phosphate esters. In addition, basic amino acids (histidine, lysine and arginine) can be phosphorylated producing phosphoramidates. Furthermore, acyl groups can serve as acceptors (aspartic and glutamic acid) to generate mixed phosphate-carboxylate acid anhydrides (Cozzone, 1993). Interestingly,

most of the bacterial kinases that belong to this class are known to be activated by intracellular metabolites (Saier, Jr., 1993). Various types of protein kinases and phosphatases commonly encountered in prokaryotes are listed in Table. 1. For any particular system, the protein kinase and protein phosphatase may be present either as two separate proteins, or unified in a single bifunctional protein (Saier, Jr., 1993). The best examples of bifunctional kinase/phosphatase are the isocitrate dehydrogenase kinase/phosphatase (AceK) from *E. coli* (Garnak and Reeves, 1979; LaPorte, 1993) and HPr(Ser-P) kinase/phosphorylase from Firmicutes (Galinier *et al.*, 1998; Poncet *et al.*, 2004; Reizer *et al.*, 1998). An example of kinase and phosphatase activities residing in separate proteins is the serine threonine kinase RbsW in *B. subtilis*, which acts in conjugation with two phosphatases RbsU and RbsP to modulate the phosphorylation state of the anti-sigma factor RbsV (Yang *et al.*, 1996).

b) Two-component system: This type of phosphorylation system responds to an external stimulus by mediating an ATP dependent autophosphorylation at a histidyl residue of a sensor kinase. The phosphate is subsequently transferred to an aspartate residue of a second protein called the response regulator, whose activity is modulated in a phosphorylation dependent manner. Histidine protein kinases and response regulators have been identified in a wide range of Gram-positive and Gram-negative species (Fabret *et al.*, 1999; Mascher *et al.*, 2006). The paradigm for the two-component system is the EnvZ/OmpR in *E. coli.* EnvZ and OmpR are the transmembrane sensor and its cognate response regulator, respectively, that regulate the transcription of porin genes in response to medium osmolarity in *E. coli* (Cai and Inouye, 2002; Yoshida *et al.*, 2002).

c) Phosphotransferases: In this system, a phosphoryl group is translocated via a set of relay proteins to the final phosphate acceptor. It involves the well characterised PEP: PTS (phosphoenolpyruvate phosphotransferase system) (Postma *et al.*, 1993). This system is involved in the concomitant uptake and phosphorylation of a number of carbon sources. The different relay proteins receive the phosphate group at the imidazole ring of a histidine residue on either N-1 or the N-3 position. In addition, cystiene residues are phosphorylated in the PTS transport proteins (see below). This phosphorelay gives rise to high-energy bonds, providing the energy for the translocation and phosphorylation of the incoming carbon source. The PTS is regarded as a bifunctional system as besides being involved in sugar uptake, it also performs regulatory functions in the cell (discussed below in detail).

Table. 1. Protein kinases and phosphatases in prokaryotes

Kinases family	Example/Organism	Reference
Eukaryotic kinases homologues	AfsK from *Streptomyces coelicolor*A(3)2	Matsumoto *et al.*, 1994
Ser/Thr/Tyr specific histidine kinase homologues	SpoIIAB from *B. subtilis*	Min *et al.*, 1993
Acek (Isocitrate dehydrogenase kinase/phosphatase)	*E. coli*	LaPorte and Koshland, Jr., 1982
HPrK/P	Firmicutes, Gram-negative bacteria	Boël *et al.*, 2003; Reizer *et al.*, 1998
Phosphatases family[a]		
PPP	PrpA and PrpB from *E. coli*	Missiakas and Raina, 1997
PPM	PrpC from *B. subtilis*	Obuchowski *et al.*, 2000
Low MW PTP/Conv. PTP	YopH from *Yersinia pseudotuberculosis*	Guan and Dixon, 1990

[a] PPP (Protein phosphatase P) and PPM (Protein phosphatase M) characterized by their dependency on metal ions, inhibitor sensitivity; PTP-Protein tyrosine phosphatases.

1.3 Sugar utilization and metabolism in *B. subtilis*

1.3.1 Carbon sources utilized by *B. subtilis*

B. subtilis, like many other prokaryotes, can utilize a multitude of carbon sources including various complex carbohydrates such as polysaccharides composed of glucose (glycogen and starch), hemicellulosic substrates which are composed of xylose, mannose, galactose and arabinose monomers and polymers like xylan and arabinoxylans. Uptake of a little less than 20 carbon sources has already been reported for *B. subtilis*. These complex carbohydrates represent a major content of the plant biomass. *B. subtilis* secretes a large number of polysaccharide degrading enzymes, such as α-amylase, pullulanase, endo-β-1,4-mannanase, levanase, endoglucanase and xylanase, which allow the organism to break down polysaccharides into smaller entities (Deutscher *et al*., 2002). Glucose in general is the most

preferred carbon source. The various carbohydrates are subsequently taken up by different transporters that catalyze uptake of the substrates using different mechanisms.

B. subtilis employs primary and secondary active transporters, facilitators or multiprotein complexes like ATP-binding cassette (ABC) transporters and the PEP:PTS for the uptake of carbon sources (Saier *et al*., 2002). Facilitators mediate the energy independent uptake of substrates. Secondary active transporters utilize the energy derived from the electrochemical potential gradient of a substance across the membrane to drive transport. The symport or antiport of usually H^+ or Na^+ ions provides the energy in these cases. Primary active transporters use the hydrolysis of ATP or PEP to mediate the uptake of substrates, for e.g ABC transporter and PTS system. The PTS systems have not yet been identified in eukaryotes and archea. Uptake via PTS system introduces a phosphate in the incoming sugar, thus making the carbon source directly available for metabolism (Fig. 1 and Fig. 2).

1.3.2 The PTS: an economical way of sugar uptake

The PTS system is a complex multienzyme uptake system, dedicated for the concomitant phosphorylation and transport of carbohydrates across the cell membrane (Postma *et al.*, 1993). Phosphorylation incorporates a negative charge on the sugar molecule preventing its escape from the cell.

The PTS system also provides the cell with a very economical way of sugar uptake. The phosphate is derived from PEP which has about -14.7 kcal/mol of free energy of hydrolysis and is equivalent to one ATP in terms of energy. This allows both uptake and phosphorylation of the sugar molecule by the expenditure of only one ATP equivalent (Postma *et al.*, 1993). In contrast, in the case of ATP transporters, 2 ATP equivalents are required for the uptake and the subsequent phosphorylation of the sugar. For this reason, many bacteria, which rely on substrate level phosphorylation for the generation of ATP (e.g. facultative anaerobic bacteria), use the PTS as the main sugar uptake system.

The PTS in general is composed of three components, namely energy coupling proteins Enzyme I (EI) (*ptsI*), the Histidine-containing protein (HPr) (*ptsH*) and several sugar specific Enzyme IIs (EII). EI and HPr are cytoplasmic proteins involved in the uptake of all PTS carbohydrates in *B. subtilis*. EIIs are multidomain proteins. The domains can be organised as several individual polypeptides or as a single fused protein (Meadow *et al.*, 1990; Postma *et al.*, 1993). The phosphoryl transfer is mediated by the EIIA and EIIB domains and the membrane bound EIIC (and EIID, if present) domain forms the sugar translocation channel. *B. subtilis* genome encodes 16 sugar specific EII complexes, however

only one EI-like protein and two HPr-like proteins (HPr and Crh) are present (Reizer *et al.*, 1999). Transport of sugars by PTS occurs with their concomitant phosphorylation (Meadow *et al.*, 1990; Postma *et al.*, 1993).

The overall reaction catalysed by the PTS is schematically depicted in Fig. 1. EI is autophosphorylated at histidine 189 residue using PEP as a substrate, resulting in the formation of pyruvate. Once phosphorylated, EI forms homodimers and subsequently transfers the phosphate to HPr (Dimitrova *et al.*, 2003). The phosphate from EI is transferred to histidine 15 (His-P) residue of HPr. HPr is a monomeric protein, which in turn phosphorylates a histidyl residue of various EIIAs. EIIB domain then receives the phosphate on a cysteyl (or histidyl) residue from EIIA and phosphorylates the sugar molecule during its translocation through the membrane spanning EIIC domain (or EIIC and EIID). The phosphorylated carbohydrate is ultimately released into the cytoplasm.

Of the two HPr like proteins, exclusively HPr participates in sugar transport. Crh (catabolite repression HPr) does not participate in PTS. It possesses a glutamine at position 15 instead of a histidine residue, and thus cannot mediate the phosphotransfer reaction required for PTS activity (Galinier *et al.*, 1997).

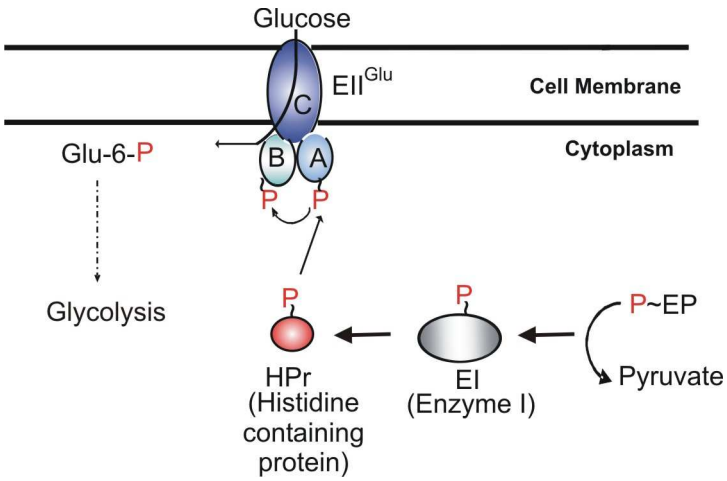

Figure. 1. The glucose specific Phosphoenolpyruvate:Phosphotranferase system (PEP:PTS) in *B. subtilis*. PEP serves as the phosphoryl donor for the phosphorylation cascade formed by the proteins EI, HPr, EIIA, and EIIB. The sugar transport step is catalysed by the membrane spanning EIIC (and EIID, if present) domain and the required energy is derived from the hydrolysis of PEP. The PTS system dedicated for the uptake of glucose in *B. subtilis* is depicted, in which all domains of glucose permease are fused to form a single polypeptide, leading to the arrangement, EIICBA (Postma *et al.*, 1993; Stülke and Hillen, 2000).

1.3.3 General pathways for the catabolism of carbohydrates

Bacteria convert the carbon sources utilized for growth into building blocks, reducing power and energy for maintenance and anabolism of molecules through a series and network of central metabolic reactions. The core of the carbon metabolic network is composed of three main metabolic pathways: the Embden-Meyerhoff-Parnass (EMP) pathway (or glycolysis), the pentose phosphate pathway (PPP) and the tricarboxylic acid cycle (TCA) or Krebs cycle (Fig. 2). This central metabolic core is highly conserved in most bacterial species. Thus, many steps in the metabolism of sugars are not specific to *B. subtilis*, but shared by a diversity of prokaryotes (Steinmetz, 1993).

B. subtilis harbours all glycolytic enzymes required for the conversion of sugar molecules starting from glucose-6-P (G6P) to pyruvate (Ludwig *et al.*, 2001). G6P is generated during PTS mediated uptake of glucose and by the metabolism of certain disaccharides (Skarlatos and Dahl, 1998). In the next step, G6P is converted to fructose-6-phosphate (F6P). F6P is then converted into fructose 1,6-bisphosphate (FBP) which is subsequently split into two C-3 molecules, dihydroxyacteone phosphate (DHAP) and glyceraldehyde-3-phosphate (see Fig. 2). Glyceraldehyde-3-P is converted to PEP by a series of enzymes. In the final step, pyruvate is generated from PEP leading to a net gain of two ATP and two NADH molecules. Pyruvate then enters the Krebs cycle.

Sugars like D-fructose, D-mannitol, sorbitol, myo-inositol, glucosamine and glycerol are catalytically converted to various intermediates of glycolysis. Pentoses including arabinose, ribose and xylose are metabolised via the pentose phosphate (PPP) pathway. Gluconate is also metabolised via the PPP as *B. subtilis* lacks the Entner Doudoroff pathway, which is utilized by various bacteria like *Enterococcus faecalis* to catabolize gluconate. Metabolic routes required for the assimilation of various sugars and their uptake systems are depicted in the figure 2. Depending on the carbon source used, the flux through this central metabolic network is differentially distributed. For example, when glucose or glycerol is utilized as the sole carbon source, the flux through the initial steps of glycolysis is not similar. When a carbon source that feeds into the Krebs cycle is used as the sole carbon source, the flux through the glycolytic steps is in the reverse order to generate metabolites like F6P and G6P, the building blocks for anabolism (gluconeogenesis).

The various reactions in the central metabolism of *B. subtilis* are highly capable of adapting to the availability of carbon sources and to their different concentrations. Diverse mechanisms including allosteric control of enzymes and transcriptional regulation are involved in achieving these regulations (Stülke and Hillen, 2000).

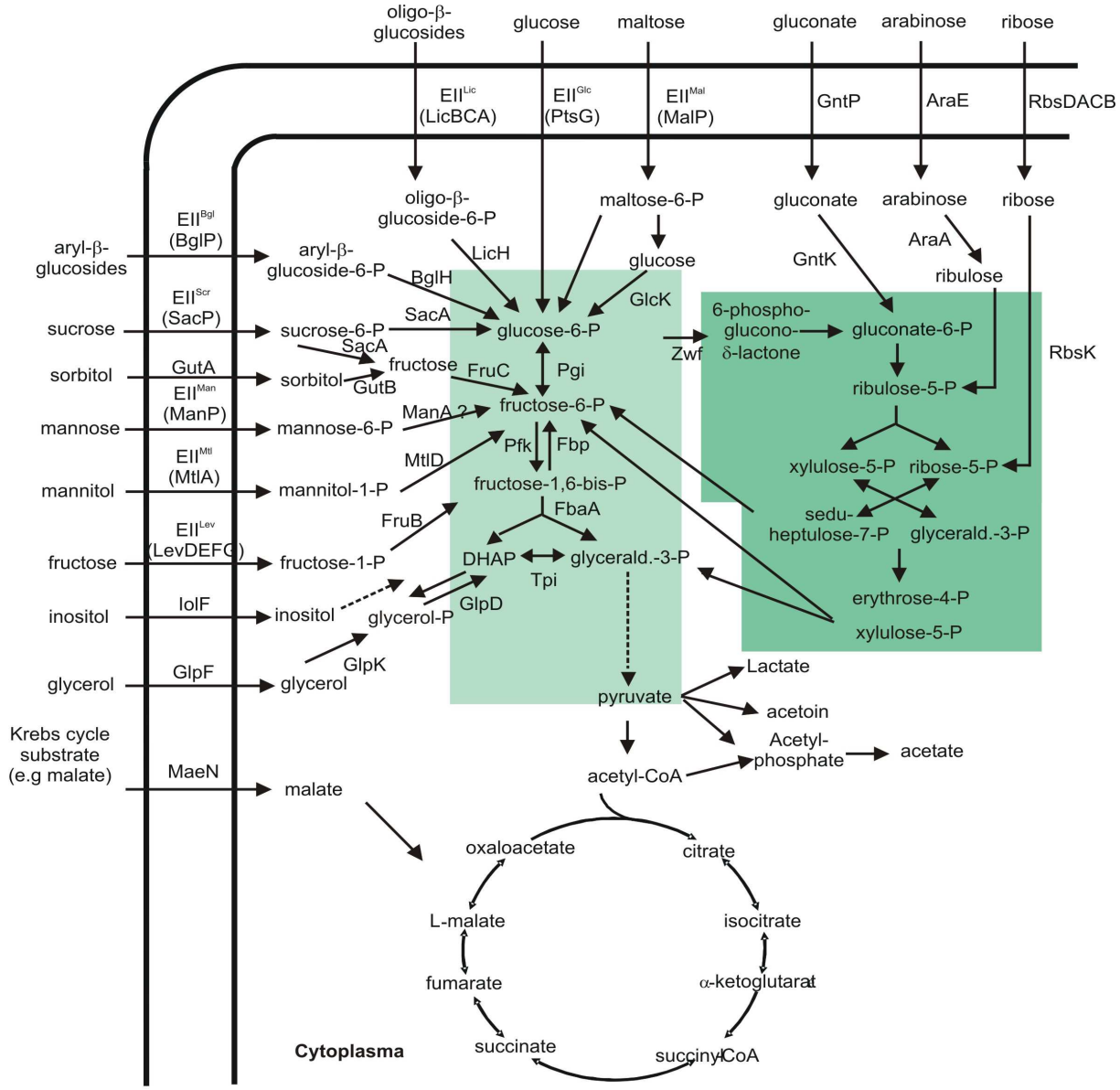

Figure. 2. Schematic representation of the transport systems and the metabolic routes required for the uptake and assimilation of various carbon sources which can be utilized by *B. subtilis*. Both PTS and non-PTS transport systems are represented. For sugars that are taken up by the PTS system, respective EIIs are depicted. Glycolytic pathway is indicated in light green and pentose phosphate pathway in dark green. The various enzymes involved in metabolic pathways connecting the assimilated carbon source and its subsequent entry into the central metabolic core are also shown. LicH, 6-phospho-β-glucosidase; BglH, 6-phospho-β-glucosidase; SacA, sucrose-6-P-hydrolase; FruC, fructokinase; GutB, glucitol dehydrogenase; ManA, mannose-6-P-isomerase; MtlD, mannitol-1-P-dehydrogenase; FruB, 1-phosphofructokinase; GlpK, glycerol kinase; GlpD, glycerol dehydrogenase, Pgi, glucose-6-P-isomerase; Pfk, 6-phosphofructokinase; Fbp, fructose-1,6-bisphosphatase; Fba, fructose-1,6-bisphosphate aldolase; Tpi, triose-phosphate-isomerase; Zwf, gluconate-6-P-dehydrogenase; GlcK, glucose kinase; GntK, gluconate kinase; Ara, L-arabinose isomerase; RbsK , ribokinase (adapted and modified from Görke and Deutscher, 2007).

1.4 Regulation of carbon metabolism

Usually bacteria differentially regulate gene expression, such that only the genes involved in the utilization of the available carbon sources are switched on. In the absence of a substrate, the corresponding subsets of genes are repressed. This mechanism is defined as induction and in *B. subtilis* it is achieved by various regulatory mechanisms mainly at the level of transcription of various catabolic operons (Steinmetz, 1993). Different operons are employ different mechanisms to be induced in the presence of their respective substrates. The regulation of *lac* operon in *E. coli* is a classical example of inducible gene expression. The *lac* operon is involved in the transport and metabolism of lactose in *E. coli* and consists of three structural genes *lacZ*, *lacY* and *lacA* encoding for β-galactosidase, β-galactosidase permease and β-galactosidase transacetylase, respectively. The operon is under the negative control of the lactose repressor encoded by the *lacI* gene. In the absence of the inducer lactose, LacI binds to the operator sequence preceding the operon genes and represses the expression of the operon. When cells are grown in the presence of lactose, a lactose metabolite called allolactose is generated intracellularly, which when bound to LacI, prevents the repression of the operon (Pardee *et al*., 1959). Similarly, the expression of the *xyn* and *xyl* operons, which are involved in the metabolism and uptake of xylose, in *B. subtilis* are induced in the presence of the substrate xylose (see chapter 3 and 5).

When exposed to a multitude of alternative nutrients, cells respond in a more complex manner. They selectively take up the carbon source that provides them with the most energy and growth advantage. Simultaneously, they repress the various functions involved in the catabolism and uptake of less preferred carbon sources. This phenomenon is commonly referred as carbon catabolite repression (CCR) (Deutscher, 2008; Görke and Stülke, 2008). CCR was first described in *B. subtilis* by Jacques Monod in 1942. Monod observed a hierarchical utilization of various carbon sources, glucose being the most preferred carbon source (Monod, 1942). He classified the carbon sources utilized by *B. subtilis* in two groups, A and B. When present in a mixture, the bacteria utilize preferentially the substrates of group A, which include glucose, fructose and mannitol. After a delay of growth, the cells make use of the group B carbon sources such as sorbitol, arabinose and maltose. This biphasic growth was termed as diauxie (Monod, 1942). Extended studies on this phenomenon established glucose as the preferred carbon source for many organisms.

Repression of utilization of secondary carbon sources can be mediated via several mechanisms. These mechanisms have been studied in detail in both, Firmicutes such as *B. subtilis* and Enterobacteriaceae such as *E. coli* (Deutscher, 2008; Görke and Stülke, 2008).

Even though the main regulatory mechanisms operative in these bacteria are quite different, they mostly employ components of the PEP:PTS. In general, repression of utilization of a secondary carbon source can be achieved by the following mechanisms (Görke and Stülke, 2008; Stülke and Hillen, 2000):

a) The intracellular formation of the specific inducer of catabolic operons can be prevented (inducer exclusion) in the presence of a preferred carbon source.

b) The activity of the operon specific regulatory proteins can be altered, leading to an anti induction effect (induction prevention).

c) A number of catabolic operons can be regulated/repressed by employing a global regulator in response to the availability of the preferred carbon source.

1.4.1 The regulatory role of PTS in CCR: linking sugar uptake and metabolism.

1.4.1.1 CCR in *E. coli*

All phospho transfer events during PTS transport activity are reversible, except for the transfer of the phosphoryl group to the sugar molecule. Thus, at any given time the phosphorylation state of PTS proteins depends on the presence of a PTS substrate and the metabolic state of the cell i.e. the PEP:pyruvate ratio (Görke and Stülke, 2008; Hogema *et al.*, 1997; Hogema *et al.*, 1998). The varying phosphorylation state of the PTS proteins provides the cell with a sensory mechanism to sense the availability of preferred carbon sources in the medium.

In *E. coli*, $EIIA^{glu}$ is the master regulator of carbon catabolite repression. In the presence of glucose or other rapidly metabolizable substrate, $EIIA^{glu}$ is mainly present in the dephosphorylated form since the phosphate is directed towards the incoming sugar. Unphosphorylated $EIIA^{glu}$ can interact with transport systems of several non-PTS sugars for e.g. lactose, maltose, mellibiose and inhibits their activity, excluding the inducer of the respective catabolic operon from the cell (Görke and Stülke, 2008; Saier, Jr. and Roseman, 1976). Inducer exclusion is one of the two main mechanisms for catabolite repression in *E. coli*. Various non PTS substrates like G6P or lactose can also exert CCR by modulating the PEP:pyruvate ratio by regulating the flux through glycolysis (Hogema *et al.*, 1997; Hogema *et al.*, 1998). In the presence of a rapidly metabolizable sugar, PEP:pyruvate ratio decreases in the cell. The decrease in the intracellular concentration of PEP, the phospho donor of PTS, eventually leads to unphosphorylated $EIIA^{glu}$.

On a global level, an additional mechanism exists in *E. coli.* In the absence of glucose, $EIIA^{glu}$ is phosphorylated and activates adenylate cyclase (Cya) by binding to it. This leads to

an increase in intracellular cyclic AMP (cAMP) levels. The transcriptional activator Crp (cAMP receptor protein) is the global regulator of CCR in *E. coli* and requires cAMP to be functionally active. Thus in the presence of glucose, Crp is inactivated and various catabolic operons under the control of Crp/cAMP can not be expressed (Fig. 3) (Görke and Stülke, 2008; Gosset *et al.*, 2004; Martinez-Antonio and Collado-Vides, 2003).

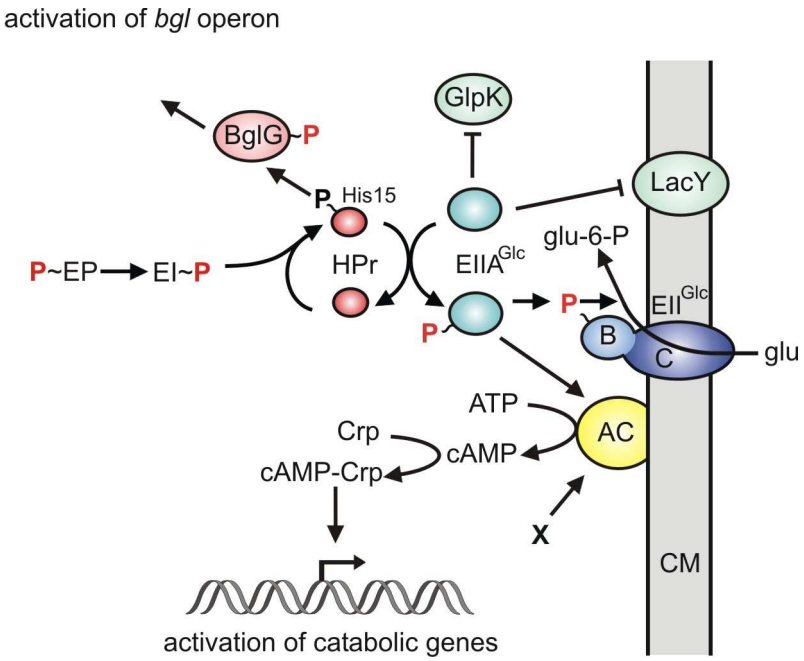

Figure. 3. CCR in *E. coli*. In the presence of glucose, $EIIA^{glu}$ is unphosphorylated and in this form it interacts with transporters of secondary carbon sources and metabolic enzymes and inactivates them. In the absence of glucose, $EIIA^{glu}$ retains its phosphate and in this state it binds and activates adenylate cyclase (AC), which leads to cyclic AMP (cAMP) synthesis. An unknown 'factor x' is also required for the activation of AC. High cAMP concentrations trigger the formation of cAMP–CRP complexes, which bind and activate the promoters of catabolic genes (adapted from Görke and Stülke, 2008).

1.4.1.2 CCR in *B. subtilis*

In Firmicutes like *B. subtilis*, the cAMP dependent pathway of catabolite repression does not exist. In contrast, the PTS phosphocarrier protein HPr is the key regulator of CCR in these organisms. Unlike in Enterobacteriaceae, HPr in Firmicutes can also be phosphorylated at the Serine 46 residue in an ATP dependent reaction catalysed by a bifunctional enzyme called HPrK/P (Deutscher and Saier, 1983; Reizer *et al.*, 1998). However, both forms of HPr, HPr(His-P) and HPr(Ser-P), are involved in CCR.

Inducer exclusion also exists in Firmicutes. Repression of glycerol metabolism in the presence of a rapidly metabolizable carbon source is a well investigated example of inducer exclusion regulated by HPr(His-P) in *B. subtilis*. Glycerol is taken up by the facilitator protein, GlpF and converted to glycerol-3-phosphate (G3P) in an ATP dependent manner by

the glycerol kinase, GlpK (Pettigrew *et al.*, 1988). G3P serves as the inducer for the *glpFK* operon and functions by activating the operon specific antiterminator protein in *B. subtilis* called, GlpP (Darbon *et al.*, 2002; Rutberg, 1997). The glycerol kinase is activated by HPr(His-P) dependent phosphorylation. Thus, in the presence of a PTS sugar, HPr(His-P) donates the phosphate to an incoming sugar rather than activating the glycerol kinase. This prevents the intracellular generation of the inducer G3P and ultimately the repression of the *glpFK* operon (Darbon *et al.*, 2002). Due to this reason, mutations in either of the general PTS proteins abolish growth of the bacterium on glycerol as the sole carbon source (Beijer and Rutberg, 1992; Reizer *et al.*, 1984). Inducer exclusion mediated by HPr(Ser-P) has also been reported in some organisms. In *Lactobacillus brevis,* HPr(Ser-P) has been suggested to bind the galactose/H^+ symporter, GalP and inhibit galactose uptake in the presence of glucose (Ye and Saier, Jr., 1995). Similarly in *L. casei* and *L. lactis*, presence of glucose inhibits maltose uptake at the transport step itself, thus repression being achieved by HPr(Ser-P) (Dossonnet *et al.*, 2000; Viana *et al.*, 2000).

Another interesting mode of catabolite repression is by antagonising the positive activity of various operon specific regulators. This mechanism called induction prevention exists in a variety of bacteria, e.g. in *E. coli* and *B. subtilis.* One of the interesting examples of this mode of repression is one which is mediated by PTS. Various catabolic genes require transcriptional regulators which contain conserved regulatory domains that can be phosphorylated by PTS proteins (PTS regulatory domains, PRDs) (Stülke *et al.*, 1998) (Fig. 4). These regulators can act as transcription antiterminators or transcription activators and are active only in the presence of specific inducers. PRDs usually contain two histidyl residues which are phosphorylated by either HPr(His-P) or EIIB(P) (Deutscher *et al.*, 2006; Görke and Deutscher, 2007). Most PRD containing proteins have two PRDs. In *B. subtilis*, there are four PTS controlled antiterminator proteins regulating the transcription of several genes. GlcT and SacY control the expression of the *ptsG(HI)* and *sacB* operon, respectively (Crutz *et al.*, 1990; Görke and Deutscher, 2007; Schmalisch *et al.*, 2003; Stülke *et al.*, 1997). SacT and LicT control the expression of *sacPA* and *bglPH* operon, respectively (Aymerich and Steinmetz, 1992; Krüger and Hecker, 1995). These antiterminator proteins have the similar domain organisation containing PRD-1 and PRD-II. The PRD-I can be negatively phosphorylated by their specific EIIs in the absence of the cognate substrate and rendered inactive. The PRD-II can be positively phosphorylated by HPr(His-15). In the presence of glucose or any other rapidly metabolizable carbon source, such antiterminators compete for the limited pool of HPr(His-P) with EIIAs. Thus the activating phosphorylation by

HPr(His-P) is limited. *In vitro* experiments suggest that the rate of transfer of phosphate is faster towards EIIAs and thus antiterminators are rendered functionally inactive (Lindner *et al.*, 1999). The antiterminator BglG from *E. coli* is also regulated by the antagonistic phosphorylation on PRD-I and PRD-II. BglG modulates the transcriptional antitermination of the *bgl*-operon and is negatively regulated by phosphorylation at PRD1 by its cognate EII and activated by phosphorylation by HPr(His-P) (Görke and Rak, 1999) (Fig. 3).

Interestingly, the PRD-II of GlcT and SacY can also be phosphorylated by HPr, but the activity of GlcT and SacY is not severly influenced by the phosphorylation state of PRD-II (Bachem and Stülke, 1998; Schmalisch *et al.*, 2003; Tortosa *et al*., 1997).

1.4.2 Global mechanism of carbon catabolite repression in *B. subtilis*

1.4.2.1 Role of HPr(Ser-P) and CcpA

As mentioned before, HPr is the key regulator of catabolite repression in Firmicutes. In these organisms HPr can be phosphorylated at serine 46 residue. The regulatory role of HPr(Ser-P) in catabolite repression in Firmicutes was initially identified in *Streptococcus pyogenes*. In this organism HPr(Ser-P) is involved in inducer exclusion of several secondary carbon sources when the cells grow in the presence of glucose (Deutscher and Saier, Jr., 1983). In 1994, the first report that identified HPr(Ser-P) as a global regulator in catabolite repression was published. Deutscher and co-workers demonstrated that in a *B. subtilis ptsH1* (serine 46 to alanine replacement) mutant, a number of catabolic enzymes are only partially or not at all repressed by glucose (Deutscher *et al.*, 1994).

Later on, a pioneering work on CCR of α-amylase synthesis in *B. subtilis* led to the identification of another important player in CCR mechanism: the DNA binding protein called CcpA (carbon catabolite protein A) (Henkin *et al.*, 1991). Global expression studies using proteomics and transcriptomics approaches demonstrated that the repression of a number of a similar set of genes is relieved in the presence of glucose in a *ccpA* as well as in a *ptsH1* mutant, implying that they both contribute to the same repression mechanism (Lorca *et al.*, 2005).

The CcpA protein is a member of the LacI/GalR family of transcriptional regulators. Independent approaches identified a specific interaction between CcpA and HPr(Ser-P). Both retardation elution experiments and nuclear magnetic resonance spectroscopy demonstrates that HPr(Ser-P) specifically forms a complex with CcpA (Jones *et al.*, 1997). Interestingly, only the serine phosphorylated form of HPr can interact with CcpA. This phosphorylation increases the binding affinity of CcpA for HPr over 50 folds, indicating the importance of

serine phosphorylation for the specificity and affinity of this interaction (Jones *et al.*, 1997). The inability of HPr(His-P) to interact with CcpA is explained by the crystal structure available for the ternary complex of CcpA/HPr(Ser-P)/*cre*. Histidine 15 of HPr is engaged in hydrogen bonding with an aspartate residue of CcpA and thus introduction of phosphate at this residue causes steric hindrances for its interaction with CcpA (Schumacher *et al.*, 2004).

The CcpA protein forms a dimer of two identical subunits and consists of an N-terminal DNA binding domain and a large C-terminal oligomerization and effector binding domain (Jones *et al.*, 1997; Schumacher *et al.*, 2004).

The interaction of HPr(Ser-P) with CcpA stimulates its DNA binding ability and the complex binds to a conserved 14 nucleotide long imperfect-palindromic DNA sequence called catabolite repressive element (Aung-Hilbrich *et al.*, 2002; Seidel *et al.*, 2005; Weickert and Chambliss, 1990). These regulatory *cre* sites occur in single or multiple copies in different catabolic operons at different positions with respect to the promoter. Repression by CcpA usually requires *cre* sequences to be located downstream of the promoter or overlapping with the promoter in front of the structural genes (Miwa *et al.*, 1997). Roughly 10% of the genome is under the control of CcpA mediated regulation and the majority of these genes are repressed by CcpA. Most of the genes regulated by CcpA encode proteins involved in utilization of secondary carbon sources (Blencke *et al.*, 2003; Moreno *et al.*, 2001; Yoshida *et al.*, 2001).

In certain cases, *cre* sites are present upstream of the promoter region. In such cases the transcription units are subjected to activation by CcpA. The genes involved in overflow metabolism (*pta, ackA, alsSD*) are induced in the presence of glucose in a CcpA/*cre* dependent manner (Lorca *et al.*, 2005). Interestingly, genes lacking a functional *cre* site can still be regulated by CcpA, for example, the expression of the *gapA* operon. The *gapA* operon includes the glycolytic genes *gapA*, *pgk*, *tpi*, *pgm* and *eno* encoding for glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, triose phosphate isomerase, phosphoglycerate mutase and enolase, repectively (Kunst *et al.*, 1997; Leyva-Vazquez and Setlow, 1994). This mode of regulation of *gapA* operon is achieved by a novel mechanism. For unknown reasons, a *ccpA* mutant contains larger amounts of HPr(Ser-P) than the wild type in *B. subtilis* and *E. faecalis* (Leboeuf *et al.*, 2000; Ludwig *et al.*, 2002). This increased percentage of HPr(Ser-P) slows down the glucose uptake, as the substrate for PTS transport, HPr(His-P), is not available in the required amounts. In the absence of glycolytic substrates like glucose, expression of the *gapA* operon is repressed by a transcriptional repressor called CggR. On the other hand, CggR is inactivated by elevated amounts of FBP, which explains

why the activation of *gapA* operon is not functional in a *ccpA* mutant strain (Doan and Aymerich, 2003; Zorrilla *et al.*, 2007).

1.4.2.2 Role of Crh in CCR in *B. subtilis*

Although most catabolite repressed genes are relieved from catabolite repression in *ccpA* and *ptsH1* mutant, the degree of relief is found to be quite different for some genes and operons for the two mutants. For example the *amyE, hut, cta, xyn* and *iol* operons are only partially relieved from glucose exerted CCR in a *ptsH1* mutant, whereas it is completely relieved in *ccpA* mutant (Deutscher *et al.*, 1994; Galinier *et al.*, 1997; Voskuil and Chambliss, 1996; Zalieckas *et al.*, 1999). Thus, a second co-repressor for CcpA should account for these differences. Crh, a protein which exhibits 45 % sequence identity to HPr was found to be this co-repressor. Similar to HPr, Crh can be phosphorylated at serine 46 residue by HPrK/P and this phosphorylated form interacts with CcpA. However, biochemical studies indicate that Crh(Ser-P) binds CcpA with an up to 10 fold reduced affinity as compared to HPr(Ser-P). The reason for this low affinity has been attributed to weaker interaction of one of its contact region to CcpA (Schumacher *et al.*, 2006).

The genes, which are partially relieved in a *ptsH1* mutant, are completely relieved in a *ptsH1 crh* double mutant. Although these *in vivo* and *in vitro* results unequivocally establish that Crh(Ser-P) can contribute to CCR in *B. subtilis*, some observations question this role of Crh. For example, if only *crh* gene is disrupted, no effect on CCR is observed (Galinier *et al.*, 1997).

1.4.2.3 HPrK/P: The central player of CCR

HPrK/P plays a key role in CCR in the Firmicutes. It catalyses the first step of the global carbon catabolite repression response i.e the phosphorylation of HPr and Crh at their serine 46 residues. HPrK/P exhibits no similarity to eukaryotic Ser or Thr protein kinases. In contrast, it possesses a nucleotide binding motif called P-loop (or Walker A motif) present in many ATPase or GTPase activity exhibiting proteins, nucleotide binding proteins, ABC transporters, proteases and chaperons. The crystal structure of HPrK/P from three organisms *Staphylococcus xylosus*, *Mycoplasma pneumoniae* and a truncated version from *Lactobacillus casei* reveals that HPrK/P is a hexamer in the cell with subunits folding into two distinct structural domains. The C-terminal domain carries both kinase and phosphorylase activity, whereas the N-terminal domain has no defined function, yet. The C-terminal domain contains the active site, the Walker A motif forming the phosphate binding loop (P-loop) of the nucleotide binding site is composed of an N-terminal domain, followed by an active site Walker A (Allen *et al.*, 2003; Fieulaine *et al.*, 2002; Marquez *et al.*, 2002). One hexamer

binds six HPr molecules on its surface. The crystal structure of the complex of the truncated version of *L. casie*, revealed that binding of one HPr molecule occurs via two interface regions formed by one HPr molecule and two adjacent HPrK/P subunits located within one trimer of the hexamer (Poncet *et al.*, 2004).

HPrK/P has been designated as a bifunctional enzyme as it can carry out both the phosphorylation and dephosphorylation of HPr(Ser-P) and Crh(Ser-P) *in vitro.* It was found that the presence of inorganic phosphate (P_i) stimulates dephosphorylation of these substrates. Indeed, the structural analysis suggests that the P-loop motif of HPrK/P can bind not only ATP but also P_i at the same position as the β-phosphate of the nucleotide (Fieulaine *et al.*, 2001). Thus, a dephosphorylation reaction mechanism in which inorganic phosphate carries out a nucleophilic attack on the phosphoryl group in HPr(Ser-P) has been proposed. The result of this dephosphorylation reaction is the generation of pyrophosphate (PP_i). This phosphorylase reaction is reversible and HPrK/P can use PP_i to phosphorylate HPr *in vitro*. Thus, HPrK/P can theoretically use both ATP and PP_i as a phospho donor (Mijakovic *et al.*, 2002).

1.4.2.4 Interplay of protein factors (HPr, Crh, HPrK/P and CcpA) and small molecule effectors: a model to describe CCR in *B. subtilis*

One of the consequences of carbon source utilization and assimilation is a change in the metabolic state of the cell where the energy charge (ATP/ADP ratio) and the intracellular concentrations of various metabolites changes. Most of the metabolic enzymes and transcriptional regulators involved in carbon metabolism are allosterically regulated and their activity is modulated by binding of small effector molecules (Saier, Jr., 1993). CCR in Firmicutes and in Enterobacteriacea is also affected by various metabolic intermediates besides the protein factors described above.

So far catabolite repression has always been described with respect to glucose as the repressing sugar. Glucose is known to generate high amounts of intracellular HPr(Ser-P). Approximately 60 % of the total HPr in *B. subtilis* is phosphorylated at its serine residue and a very little fraction is observed to be histidine phosphorylated (Ludwig *et al.*, 2002; Monedero *et al.*, 2001).

It has been reported that both the kinase and the phosphorylase activity of *B. subtilis* HPrK/P can be allosterically regulated. A number of metabolic intermediates were screened

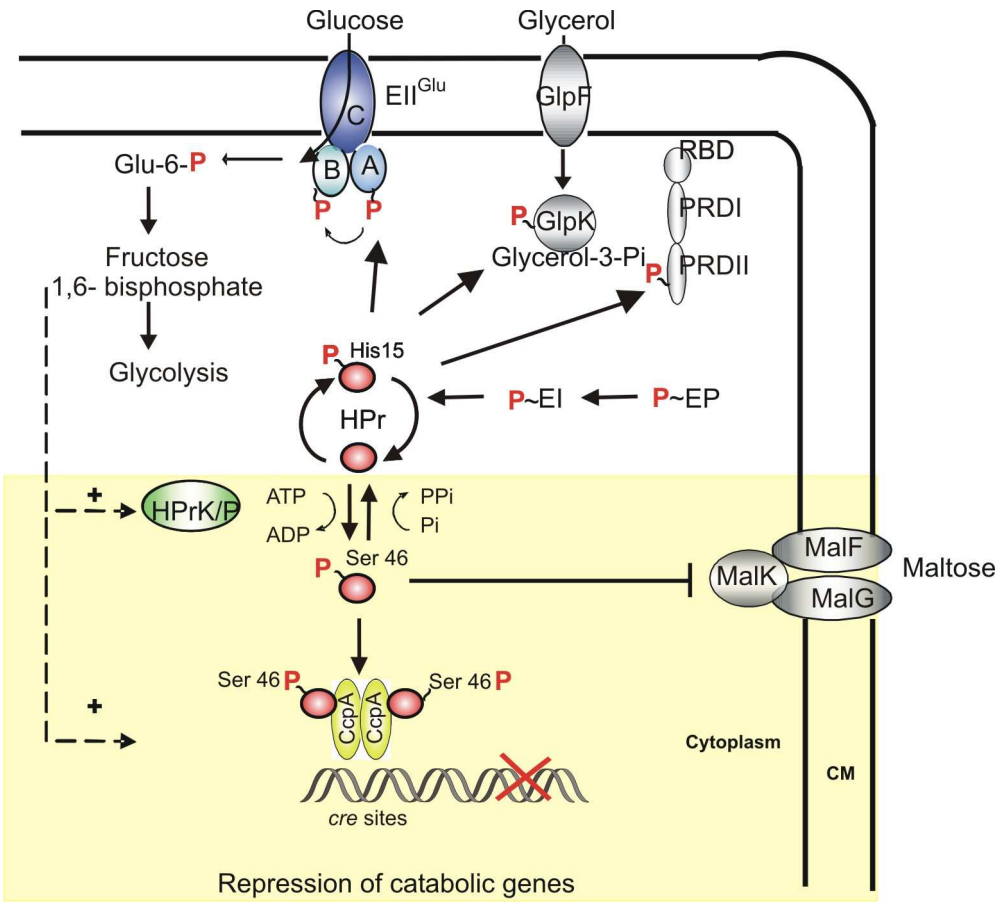

Figure. 4. Modes of catabolite repression in low G+C content Gram-positive bacteria (Görke and Stülke, 2008). In the presence of a rapidly metabolizable carbon source like glucose, the intracellular concentration of metabolites like FBP increases, leading to an allosteric activation of kinase activity of HPrK/P and ultimately to the generation of HPr(Ser-P). Serine phosphorylated HPr interacts with CcpA dimer (two HPr molecules). The protein complex binds to the operator sequences, *cre* sites and represses the transcription units bearing *cre* sites overlapping with or downstream of their promoter region. HPr(Ser-P) can also interact with transporters, inhibiting the intracellular accumulation of secondary carbon sources (illustrated in the part of the figure highlighted in yellow colour). The presence of glucose leads to a net dephosphorylation of PTS proteins including HPr(His-P). This low level of HPr(His-P) does not allow sufficient phosphorylation dependent activation of GlpK and PRD bearing proteins like LicT in *B. subtilis*. In some Gram-positive bacteria, HPr(Ser-P) also mediates inducer exclusion of secondary substrates by interacting with their transporter proteins. For e.g. in *L. casie* and *L. lactis*, HPr(Ser-P) is suggested to bind to MalK (adapted and modified from Görke and Stülke, 2008).

and it was shown *in vitro* that FBP can stimulate the ATP dependent kinase activity of HPrK/P (Jault *et al.*, 2000). In this context, the intracellular concentration of FBP, ATP and P_i vary largely depending on whether the cell utilized a favourable carbon source or not. *B. subtilis* cells grown in the presence of glucose contain 10 fold more FBP (from 1.4 mM to 14 mM) and PP_i (1.2 mM to 6 mM) than cells grown on succinate (Mijakovic *et al.*, 2002). On the other hand when cells are starved, concentration of inorganic phosphate increases drastically as observed in the case of *Streptococcus bovis* (upto 30 mM) (Asanuma and Hino, 2003).

Interaction of HPr(Ser-P) and CcpA has also been shown to be affected by various metabolites. Structural studies using *Bacillus megaterium* CcpA and HPr(Ser-P) demonstrated that the presence of FBP and G6P bolster cross interaction between arginine 17 in HPr(Ser-P) and the aspartate residues 69 and 99 of the CcpA subunit. This further stabilizes the closed DNA binding conformation of CcpA and enhances the interaction of the CcpA-HPr(Ser-P) complex with its cognate target DNA (Schumacher *et al.,* 2007).

For *B. subtilis gnt cre* site and *B. megaterium xyl cre* site, G6P has also been shown to allow CcpA binding to *cre* independent of HPr(Ser-P). However, these effects were observed only under non physiological conditions such as very high concentrations of G6P for *gnt cre* and low pH for *xyl cre* (Miwa *et al.,* 1997).

Based on the above observations, a general CCR mechanism has been proposed for *B. subtilis* and other Firmicutes. In the presence of a rapidly metabolizable carbon source such as glucose, an increases in FBP in cells stimulates the HPrK/P catalyzed formation of HPr(Ser-P), resulting in the formation of a complex between HPr(Ser-P) and CcpA and this repressor complex then binds to the *cre* sites (Fig. 4) (Görke and Stülke, 2008).

1.5 Aim of this work

CCR can be defined as the repression of functions required for the utilization of secondary carbon sources, in the presence of a preferred and rapidly metabolized carbon source. Glucose is often the preferred carbon source for many organisms. Catabolite repression mediated by glucose has been extensively studied in the model organisms *E. coli* and *B. subtilis*. In *E. coli,* the repressing potential of various other carbon sources besides glucose has also been studied and it was shown that a large number of carbohydrates in addition to glucose exert CCR. In *B. subtilis* most of the available knowledge regarding CCR is derived from experiments using glucose as the repressing carbon source. One of the aims of this work was to systematically study the repressing potential of various carbon sources besides glucose. It was also required to understand if the ability of a carbon source to cause CCR is related to its uptake mechanism and/or the catabolic pathway of a given substrate. To analyse this, carbon sources which differ from glucose in their chemical nature and their uptake mechanisms, were used to study CCR in *B. subtilis*. During this work, the factors contributing to CCR and the phosphorylation state of HPr were analysed.

A further aim of this work was to analyse the role of PrpC in CCR. PrpC is a Ser/Thr phosphatase and dephosphorylates HPr(Ser-P) in *M. pneumoniae*.

List of publications

Singh,K.D., Halbedel,S., Görke,B., and Stülke,J. (2007) Control of the phosphorylation state of the HPr protein of the phosphotransferase system in *Bacillus subtilis*: implication of the protein phosphatase PrpC. *J Mol Microbiol Biotechnol* **13**: 165-171.

Singh,K.D., Schmalisch,M.H., Stülke,J., and Görke,B. (2008) Carbon Catabolite Repression in *Bacillus subtilis*: A Quantitative Analysis of Repression Exerted by Different Carbon Sources. *J Bacteriol*. DOI: 10.1128/JB.00848-08

2. Control of the Phosphorylation State of the HPr Protein of the Phosphotransferase System in *Bacillus subtilis*: implication of the Protein Phosphatase PrpC

The results described in this chapter were published in:

Singh,K.D., Halbedel,S., Görke,B., and Stülke,J. (2007) Control of the phosphorylation state of the HPr protein of the phosphotransferase system in *Bacillus subtilis*: implication of the protein phosphatase PrpC. *J Mol Microbiol Biotechnol* **13**: 165-171.

Author contribution:

This study was designed and interpreted by Kalpana Singh, Boris Görke and Jörg Stülke. All experiments were performed by Kalpana Singh. Sven Halbedel contributed in suggesting the role of PrpC in *M. pneumoniae*. This paper was written by Jörg Stülke and Kalpana Singh.

Abstract

In the Gram-positive bacterium *Bacillus subtilis* as well as in other Firmicutes, the HPr protein of the phosphotransferase system (PTS) has two distinct phosphorylation sites, His-15 and Ser-46. These sites are phosphorylated by the Enzyme I of the PTS and by the ATP-dependent HPr kinase/ phosphorylase, respectively. As a result, the phosphorylation state of HPr reflects the nutrient supply of the cell and is in turn involved in several responses at the levels of transport activity and expression of catabolic genes. Most important, HPr(Ser-P) serves as a cofactor for the pleiotropic transcription regulator CcpA. In addition to the proteins that phosphorylate HPr, those that are involved in the dephosphorylation are important in controlling the overall HPr phosphorylation state and the resulting regulatory and physiological outputs. In this study, we found that in addition to the phosphorylase activity of the HPr kinase/ phosphorylase the serine/ threonine protein phosphatase PrpC uses HPr(Ser-P) as a target.

Introduction

In order to respond to changes in the environmental conditions or in the supply of nutrients, all living cells are capable of sensing such changes and of transducing the corresponding signals in a way that allows an adaptation of the cell. The signal transduction processes often involve covalent modifications of proteins that result either in changes of enzymatic activity or affect the cellular gene expression programme. Among these modifications protein phosphorylation is the most prominent one since it can affect the activity of a protein drastically due to the large size of the phosphate group and its strong negative charge (Huffine and Scholtz, 1996; Johnson and Barford, 1993; Kennelly and Potts, 1996).

In bacteria, three major types of signal transduction systems are controlled by reversible phosphorylation. These include the two-component systems, the stressosome regulatory systems involving sigma factors and anti-sigma factors, and the phosphoenolpyruvate:sugar phosphotransferase system-derived regulatory systems (PTS) (Pané-Farré *et al.*, 2005; Postma *et al.*, 1993; Stock, 2000; Stülke *et al.*, 1998). In each case, phosphorylation results in the reversal of the biological activity of the phosphorylated protein and thus in a signalling process.

The PTS was discovered as a sugar transport system that couples the transport of a substrate to its concomitant phosphorylation (Postma *et al.*, 1993). The phosphate group is

derived from phosphoenolpyruvate and is transferred via the two general energy-coupling proteins Enzyme I and HPr and the A and B domains of the sugar permease (IIA and IIB) to the incoming sugar. In *Escherichia coli*, the phosphorylation state of the glucose-specific IIA controls the synthesis of the signalling molecule cAMP and the activity of a set of sugar transporters (see Postma *et al*., 1993 for review). In many other bacteria, HPr rather than the IIA protein is crucial for signal transduction. In the low GC-branch of the Gram-positive bacteria (i.e., the Firmicutes) and in many Gram-negative bacteria (with the notable exception of the enteric bacteria), HPr is not only phosphorylated by Enzyme I of the PTS at a histidine residue at position 15 (His-15) but also by a metabolite-controlled kinase called HPr kinase/phosphorylase (HPrK/P) on Ser-46 (Boël *et al.*, 2003; Poncet *et al.*, 2004).

In *Bacillus subtilis*, HPr(His-P) is present in cells grown in the absence of preferred carbon sources such as glucose (Ludwig *et al.*, 2002; Monedero *et al.*, 2001). This form of HPr is involved in sugar transport by the PTS and can transfer its phosphate to glycerol kinase and several transcriptional regulators to stimulate their activity (Darbon *et al.*, 2002; Stülke *et al.*, 1998). If glucose is present in the medium, the HPrK/P is activated by high intracellular concentrations of ATP and fructose 1, 6-bisphosphate, and a large portion of HPr is present as HPr(Ser-P) (Hanson *et al.*, 2002; Jault *et al.*, 2000; Ludwig *et al.*, 2002; Monedero *et al.*, 2001). This form of HPr forms a complex with the transcription factor CcpA, and the complex binds target sites (catabolite reponsive elements) in the promoter regions of genes that are subject to catabolite regulation. Binding of the CcpA-HPr(Ser-P) complex results in transcription repression or activation of large sets of genes and operons (Blencke *et al.*, 2003; Moreno *et al.*, 2001). If the preferred carbon sources become limiting, the activity of HPrK/P switches and the protein acts as a phosphorylase to dephosphorylate HPr(Ser-P) (Kravanja *et al.*, 1999; Mijakovic *et al.*, 2002). This results in the dissociation of the CcpA-HPr(Ser-P) complex and thus in relief of carbon catabolite repression by CcpA.

In addition to the HPrK/P, *B. subtilis* encodes several other serine/threonine protein kinases and phosphatases, and many proteins are phosphorylated on serine or threonine residues *in vivo* (Leviné *et al*., 2006; Obuchowski, 2005). Three of the kinases and four of the phosphatases are involved in the control of sigma factor activities (Hughes and Mathee, 1998). For three other suspected serine/ threonine kinases it is not even clear whether they have a biological activity as kinases. Finally, the PrkC serine kinase phosphorylates the translation factor EF-G (Gaidenko *et al.*, 2002). The largest group of the serine phosphatases found in *B. subtilis* belongs to the metallophosphatases of the PP2C family (Obuchowski, 2005). Among the PP2C phosphatases is the PrpC phosphatase which is encoded in an operon

with the PrkC kinase and which was shown to dephosphorylate PrkC (if autophosphorylated on a threonine residue) as well as EF-G that had been phophorylated by PrkC (Gaidenko *et al.*, 2002; Obuchowski *et al.*, 2000). The *prpC-prkC* gene couple encoding the phosphatase and kinase is present in many bacteria among them the mollicutes such as *Mycoplasma genitalium* and *Mycoplasma pneumoniae* (Halbedel *et al.*, 2006; Obuchowski *et al.*, 2000). Recently, we provided evidence that the PrpC phosphatase of *M. pneumoniae* is capable of dephosphorylating HPr(Ser-P) and that this enzyme is involved in controlling the intracellular phosphorylation state of HPr (Halbedel *et al.*, 2006).

In this work, we studied the biochemical activity of *B. subtilis* PrpC with HPr(Ser-P) as the substrate. We provide evidence that PrpC is involved in preventing the accumulation of HPr(Ser-P) in cells grown in the absence of glucose.

Experimental procedures

Bacterial strains and growth conditions

B. subtilis strain GP278 (*trpC2* Δ*xylR*::*ermC amyE*::*xynP-lacZ*) was used as wild type reference. This strain was obtained by transformation of *B. subtilis* QB7144 (Galinier *et al.*, 1999) with plasmid pIW11xylR (Kraus *et al.*, 1994). The *prpC* deletion strain GP281 was constructed by transformation of GP278 with chromosomal DNA of strain OMG401 (Δ*prpC*::*aphA3*) (Obuchowski *et al.*, 2000). The *E. coli* strains BL21 (DE3) (Sambrook *et al.*, 1989), NM522 (Gough and Murray, 1983) and DH5α (Sambrook *et al.*, 1989) were used as hosts for the overproduction of PrpC, HPr and HPrK/P, respectively. *E. coli* was grown in Luria-Bertani broth supplemented with 100 μg/ml ampicillin when necessary. For western blotting experiments *B. subtilis* strains were grown in C minimal medium (Faires *et al.*, 1999) supplemented with L-tryptophan (100 μg/ml), potassium glutamate (0.8 %, g/l) and sodium succinate (0.6 %, g/l) as single carbon source.

Protein purification

Hexa-histidine tagged recombinant PrpC, HPr and HPrK/P proteins were purified as described previously using the expression vectors pE5635, pAG2, and pGP205, respectively (Gaidenko *et al.*, 2002; Galinier *et al.*, 1997; Hanson *et al.*, 2002). Briefly, the *E. coli* transformants were grown to an OD_{600}= 0.8 and protein expression was induced by the addition of 1 mM IPTG. Growth was continued 3 hours before the cultures were harvested and passed through a French pressure cell at 1000 p.s.i. The lysates were centrifuged at 45000

rpm for 45 min (Sorvall ultra pro, Dupont) and the supernatants were loaded on Ni^{+2}-NTA superflow columns (Qiagen). Proteins were eluted with an imidazole gradient and fractions containing the pure proteins were pooled.

Preparation of serine-phosphorylated HPr

$(His)_6$-HPr(Ser-P) was prepared as described (Hanson *et al.*, 2002). Briefly, purified $(His)_6$-HPr (20 µM) was mixed with 350 nM HPrK/P protein and incubated at 37°C for 30 min in an assay buffer containing 25 mM Tris/HCl pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 10 mM ATP and 20 mM fructose-1,6-bisphosphate. The reaction was stopped by thermal inactivation of HPrK/P at 95°C for 5 min and $(His)_6$-HPr(Ser-P) was purified by passing the reaction mixture through ultrafiltration spin columns (5 KDa exclusion size) according to the manufacturers protocol (Sartorius).

Dephosphorylation of HPr(Ser-P) and of para-nitrophenyl phosphate (PNPP) by PrpC *in vitro*

For the dephosphorylation of $(His)_6$-HPr(Ser-P) by PrpC, activity assays were carried out with varying amounts of purified PrpC in 50 µl dephosphorylation assay buffer containing 50 mM Tris/HCl pH 7.5, 2 mM $MnCl_2$, 1 mM dithiothreitol (DTT) and 8 µg $(His)_6$-HPr(Ser-P) at 37°C for 40 min. The reactions were stopped by placing the samples on ice before they were loaded on 10% native polyacrylamide gels (Ludwig *et al.*, 2002) followed by coomassie blue staining. Dephosphorylation assays with PNPP as a substrate were performed by adding 0.9 µM PrpC to 1 ml dephosphorylation assay buffer containing 25 mM PNPP instead of $(His)_6$-HPr(Ser-P). The reactions were incubated at 30°C for 25 min and stopped by the addition of 100 µl of 0.5 M EDTA pH 8.0. The end product *p*-nitrophenol was photometrically quantified by measuring the absorbance at 420 nm.

Western blotting

The phosphorylation state of HPr *in vivo* was assayed by western blot analysis as follows: *B. subtilis* strains were grown to an OD_{600} of 0.6 and then HCl was added directly to the culture in order to abolish phosphatase activity of HPrK/P by decreasing the pH to 4.5. Crude cell extracts were prepared as described (Gauthier *et al.*, 1997) and 1 µg of total cell protein was loaded on 10% native polyacrylamide gels, allowing the separation of phosphorylated and non-phosphorylated HPr. To distinguish HPr(His-P) from HPr(Ser-P), a second aliquot of each crude extract was incubated at 70°C for 10 min before gel electrophoresis. Proteins were

blotted to a PVDF membrane and HPr species were detected using polyclonal rabbit antibodies directed against *B. subtilis* HPr (Monedero *et al.*, 2001).

Results and Discussion

HPr(Ser-P) is a target for PrpC

In a previous work, we have shown that the PrpC phosphatase of *M. pneumoniae* can dephosphorylate HPr(Ser-P) (Halbedel *et al.*, 2006). We considered therefore the possibility that the *B. subtilis* phosphatase might also be capable of using HPr(Ser-P) as a substrate. To test this idea, we purified the His-tagged *B. subtilis* HPr and HPrK/P and phosphorylated HPr at Ser-46 on a preparative scale. Moreover, the *B. subtilis* PrpC phosphatase was purified. The purified HPr(Ser-P) was used in a dephosphorylation assay to detect PrpC phosphatase activity. As shown in Fig. 5, HPr(Ser-P) migrates much faster than non-phosphorylated HPr on a native polyacrylamide gel. The addition of PrpC resulted in a disappearance of the phosphorylated form of HPr whereas we observed an accumulation of non-phosphorylated HPr. As can be seen in Fig. 5, small amounts of PrpC were sufficient to cause complete dephosphorylation. These findings indicate that PrpC is capable of using HPr(Ser-P) as a substrate *in vitro*.

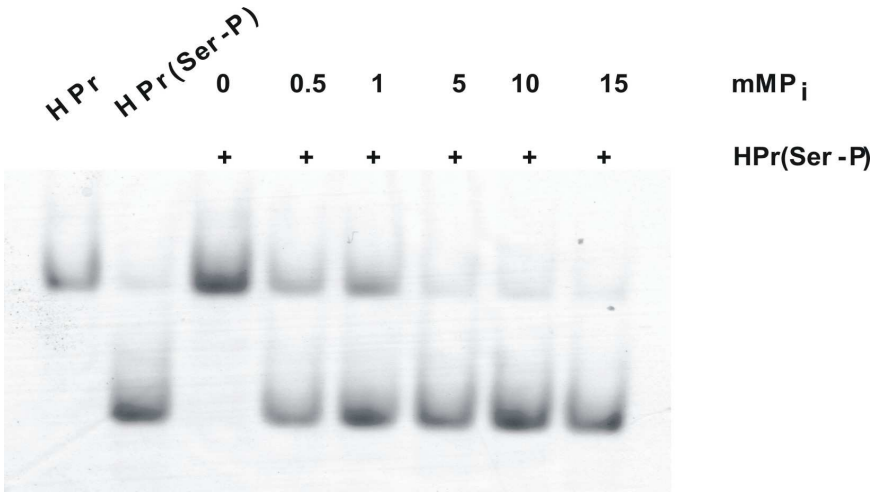

Figure. 5. Dephosphorylation of HPr(Ser-P) by PrpC *in vitro*. Increasing amounts of purified PrpC were incubated with 16 µM of HPr(Ser-P in dephosphorylation assay buffer at 37°C for 40 min and phosphorylated and non-phosphorylated forms of HPr were separated on a 10% native polyacrylamide gel (lanes 3-8). 8 µg of purified HPr and HPr(Ser-P) were loaded in lane 1 and lane 2 respectively as controls.

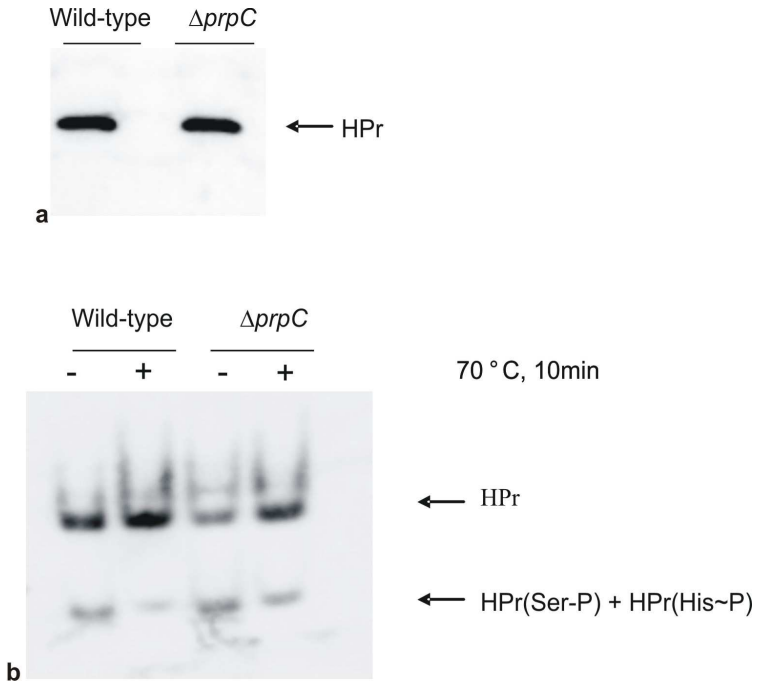

Figure. 6. Deletion of *prpC* increases the amount of HPr(Ser-P) *in vivo*. The wild type strain GP278 and the *prpC* mutant GP281 were grown in minimal medium supplemented with succinate as a single carbon source. a Comparison of the amounts of cellular HPr in the wild type strain and the *prpC* mutant. Total cell protein (5 µg) of the wild type strain GP278 and the *prpC* mutant GP281 was loaded onto 15% denaturing SDS polyacrylamide gel followed by elctroblotting onto PVDF membrane. HPr was then detected using polyclonal rabbit antiserum against *B. subtilis* HPr. b Determination of the HPr phosphorylation pattern. Protein extracts were separated on a 10% native polyacrylamide gel and HPr was detected by western blotting. Prior to loading, an aliquot of each cell extract was incubated at 70°C for 10 min to cause the loss of the histidine-bonded phosphoryl-group leaving HPr(Ser-P) as single phosphorylated form (lanes 2 and 4).

PrpC affects the HPr phosphorylation state *in vivo*

In *B. subtilis* growing in glucose, a large part of HPr is phosphorylated on Ser-46 by HPrK/P. In contrast, only a small fraction of HPr(Ser-P) is present in minimal medium without glucose, due to the low kinase and predominant phosphorylase activities of HPrK/P under these conditions (Ludwig *et al.*, 2002; Monedero *et al.*, 2001). Since PrpC is capable of dephosphorylating HPr(Ser-P) *in vitro* we asked whether it might also be involved in controlling the HPr phosphorylation state *in vivo* and thus contribute to the low amount of HPr(Ser-P) observed in glucose-free minimal medium. To address this question, we grew the *B. subtilis* wild type strain GP278 and its isogenic *prpC* mutant GP281 in CSE minimal medium and prepared protein extracts for the analysis of the HPr amounts and the *in vivo* HPr phosphorylation pattern. The results of this experiment are shown in Fig. 6. In both strains, comparable amounts of HPr were present (Fig. 6a). Both phosphorylated and non-phosphorylated HPr were detected in the wild type strain (Fig. 6b).

The molecular nature of the phosphorylation can easily be revealed by heating the cell extracts: histidyl-phosphate is heat-labile, whereas seryl-phosphate is heat-stable. We observed that the phosphorylation of HPr disappeared upon heating of an aliquot of the cell extract. This indicates that HPr in the wild type strain had been phosphorylated predominantly on His-15. This finding is in good agreement with previous studies (Ludwig *et al.*, 2002; Monedero *et al.*, 2001). A different result was obtained with the isogenic *prpC* mutant GP281. In this strain, a larger portion of HPr was present in the phosphorylated form (Fig. 6b). Upon heating, a small but significant fraction of heat-stable phosphorylation was detected demonstrating that HPr(Ser-P) is present in this mutant even in glucose-free CSE medium. A quantitative analysis revealed that about 10% of HPr are phosphorylated on Ser-46 in the wild type strain whereas 30% of HPr(Ser-P) were detected in the *prpC* mutant GP281. Thus, PrpC is involved in the control of the *in vivo* HPr phosphorylation state and is required for complete dephosphorylation of HPr in cells grown in the absence of glucose.

Inhibition of PrpC activity by inorganic phosphate

Previously, the *B. subtilis* PrpC activity was shown to be inhibited by β-glycerolphosphate and some divalent cations such as Ca^{2+} and Zn^{2+} (Obuchowski *et al.*, 2000). In *M. pneumoniae*, PrpC is also subject to inhibition by inorganic phosphate (Halbedel *et al.*, 2006). We tested therefore the response of *B. subtilis* PrpC activity to the presence of increasing concentrations of inorganic phosphate (P_i). As shown in Fig. 8, complete dephosphorylation of HPr(Ser-P) was observed in the absence of any phosphate. In contrast, the presence of 0.5 mM P_i was sufficient to cause a partial inhibition of PrpC activity, and this inhibition was even more pronounced at higher P_i concentrations.

To get a quantitative impression of the inhibition of PrpC by P_i, we studied the activity of PrpC using the synthetic substrate *p*-nitrophenyl phosphate (PNPP). The K_M and v_{max} values have been reported previously (Obuchowski *et al.*, 2000). In the presence of P_i, the PrpC activity was strongly inhibited in a competitive manner (K_i 110 ± 10 µM) (Fig. 7). As found for the other kinetic parameters (Halbedel *et al.*, 2006; Obuchowski *et al.*, 2000), the enzymes from *M. pneumoniae* and *B. subtilis* are similar also with respect to inhibition by phosphate.

It is interesting to note that the intracellular concentration of P_i is high under the conditions that were used to observe PrpC-dependent HPr(Ser-P) dephosphorylation (Mijakovic *et al.*, 2002). The results presented in Fig. 6b suggest that the inhibitory effect of P_i observed *in vitro* is counteracted *in vivo*, possibly by other metabolites. This idea is in good

agreement with our observation that PrpC is only a minor player in the control of the HPr phosphorylation state (see Fig. 6b).

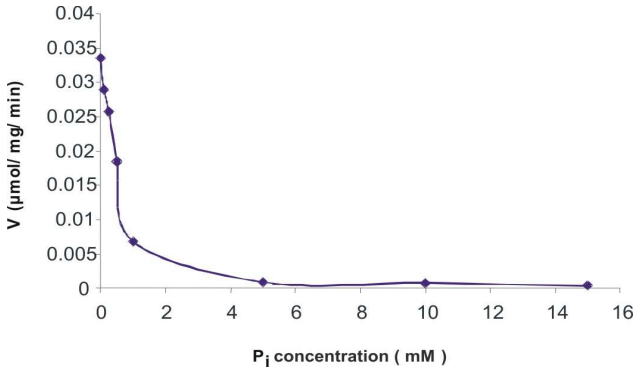

Figure. 7. Inhibition of PrpC phosphatase activity towards PNPP by inorganic phosphate. Dephosphorylation of para-nitrophenyl phosphate (PNPP) by PrpC (0.9 µM) in the presence of increasing concentrations of inorganic phosphate [0.5-15 mM] was investigated. The rate of para-nitrophenol formation is plotted against the P_i concentration.

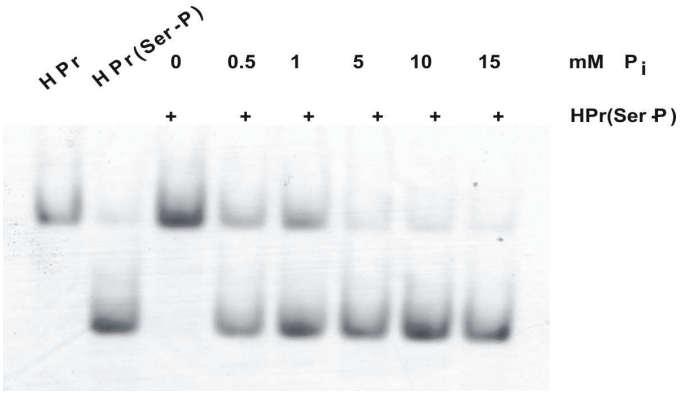

Figure. 8. Inhibition of PrpC phosphatase activity towards its natural target HPr(Ser-P) by inorganic phosphate. Dephosphorylation of HPr(Ser-P) by PrpC (0.9 µM) was studied in the presence of different P_i concentrations (lanes 3-8). Subsequently, phosphorylated and non-phosphorylated forms of HPr were separated on a 10% native polyacrylamide gel. Purified HPr and HPr(Ser-P) (5 µg) were loaded as controls in lanes 1 and 2, respectively.

PrpC-catalyzed HPr(Ser-P) dephosphorylation in search of a function

As shown in this work, PrpC contributes in keeping the cellular level of HPr(Ser-P) low in a glucose-free medium. This might ensure that no HPr(Ser-P) is available for CcpA-dependent gene regulation in the absence of glucose. If this would be true, one would expect to detect some "constitutive repression" of genes subject to carbon catabolite repression in a glucose-free medium in a *prpC* mutant strain. To test this assumption we used the promoter of the *xynPB* operon encoding β-xyloside permease and β-xylosidase, which is about hundred-fold repressed in the presence of glucose (Galinier *et al.*, 1999). However, the expression levels in

glucose-free medium were similar for the wild type and the *prpC* mutant (data not shown) suggesting that PrpC is not involved in carbon catabolite repression. This seems to be surprising, but one has to keep in mind that only low amounts of HPr(Ser-P) are present in the absence of PrpC. This concentration may not be sufficient to form a stable complex with CcpA. Moreover, the formation of the HPr(Ser-P)-CcpA complex and binding of the complex to the DNA target sites is simulated by fructose-1.6-bisphosphate (Seidel *et al.*, 2005). However, the intracellular concentration of this metabolite is low in cells grown in the absence of glucose (Mijakovic *et al.*, 2002). Thus, no CcpA-dependent catabolite repression might be possible even though low amounts of HPr(Ser-P) are detectable in the *prpC* mutant.

The formation of HPr(Ser-P) has a second effect which is not directly related to its activity as a cofactor in carbon catabolite repression: HPr(Ser-P) is a very poor target for Enzyme I of the PTS and is therefore not available for sugar transport (Deutscher *et al*., 1984). This results in impaired PTS sugar transport if a large portion of HPr is phosphorylated on Ser-46 (Ludwig *et al.*, 2002). Thus, a complete dephosphorylation of HPr jointly achieved by the activities of HPrK/P and PrpC may ensure the availability of free HPr for PTS phosphate transfer to all sugar permeases and may thus be a precaution allowing a rapid adaptation to an improving nutrient supply.

Conclusion

Our data suggest that PrpC is capable of dephosphorylating HPr(Ser-P). However, this activity seems to be of limited relevance in living cells of *B. subtilis*, and HPr(Ser-P) may be just a minor substrate for PrpC *in vivo*. While the physiological role of PrpC-dependent HPr(Ser-P) dephosphorylation in *B. subtilis* remains to be uncovered, it may be important in organisms in which the HPrK/P does not exhibit phosphorylase activity, as shown for *M. pneumoniae* (Halbedel *et al*., 2006). With the discovery of HPrK/P in many bacteria, among them proteobacteria and spirochaetes, PrpC is an attractive candidate to study the control of the HPr phosphorylation state in those bacteria.

3. Carbon catabolite repression in *Bacillus subtilis*: A quantitative analysis of repression exerted by different carbon sources

The results described in this chapter were published in:

Singh,K.D., Schmalisch,M.H., Stülke,J., and Görke,B. (2008) Carbon Catabolite Repression in Bacillus subtilis: A Quantitative Analysis of Repression Exerted by Different Carbon Sources. *J Bacteriol.* DOI: 10.1128/JB.00848-08

Author contributions:

This study was designed by Kalpana Singh, Jörg Stülke and Boris Görke. All experiments were performed by Kalpana Singh. Matthias Schmalisch performed the experiment for the estimation of intracellular FBP levels and started this project during his Ph.D. work. This paper was written by Boris Görke.

Abstract

In many bacteria glucose is the preferred carbon source and represses the utilization of secondary substrates. In *Bacillus subtilis*, this carbon catabolite repression (CCR) is achieved by the global transcription regulator CcpA, whose activity is triggered by the availability of its phosphorylated cofactors HPr(Ser46-P) and Crh(Ser46-P). Phosphorylation of these proteins is catalyzed by the metabolite-controlled HPr kinase HPrK/P. Recent studies focused on glucose as repressing substrate. Here, we show that many carbohydrates cause CCR. The substrates form a hierarchy in their ability to exert repression via the CcpA-mediated CCR pathway. Of the two co-factors, HPr is sufficient for complete CCR. In contrast, Crh cannot substitute for HPr on substrates that cause a strong repression. Determination of the phosphorylation state of HPr *in vivo* revealed a correlation between the strength of repression and the degree of phosphorylation of HPr at Ser-46. Sugars transported by the phosphotransferase system (PTS) cause the strongest repression. However, the phosphorylation state of HPr at its His-15 residue and PTS transport activity have no impact on the global CCR mechanism, which is a major difference to the mechanism operative in *Escherichia coli*. Our data suggest that the hierarchy in CCR exerted by the different substrates is exclusively determined by the activity of HPrK/P.

Introduction

As for any organism, nutrient supply is of prime importance for bacteria. In their natural habitats, bacteria often encounter a mixture of different carbon sources that can potentially be used. Therefore, mechanisms have evolved in many bacteria that enable the selective uptake and metabolism of those carbon sources that allow the most rapid growth and that promise the best success in the competition with other bacteria or fungi. For many heterotrophic bacteria, glucose is the preferred source of carbon. In the presence of glucose, the genes required for the utilization of secondary carbon sources are not expressed and pre-existing enzymes are often inactivated to prevent the waste of resources. This phenomenon is referred to as carbon catabolite repression (CCR, for reviews see (Deutscher, 2008; Görke and Stülke, 2008).

CCR has been extensively studied in the model bacteria *Bacillus subtilis* and *Escherichia coli*. Although the physiological outcome is very similar, the global mechanisms underlying CCR are completely different in these bacteria. In *E. coli* and other enteric bacteria, the $EIIA^{Glu}$ domain of the glucose transporter is the central processing unit in CCR.

This protein is part of the phosphoenolpyruvate (PEP):carbohydrate phosphotransferase system (PTS), which is responsible for the uptake and concomitant phosphorylation of numerous carbohydrates in many bacteria (Deutscher *et al.*, 2006). In this system, the two general phosphotransferases, Enzyme I (EI) and histidine protein (HPr), transfer phosphoryl-groups from PEP to the various sugar transporters, named Enzymes II (EIIs). In the absence of glucose, the $EIIA^{Glu}$ domain is preferentially phosphorylated. In this form, $EIIA^{Glc}$ activates the adenylate cyclase, which leads to an increase in the intracellular cAMP concentration. Binding of cAMP activates the transcription activator CRP (cAMP receptor protein), which is in turn required for the expression of numerous secondary catabolic genes. In the presence of glucose, $EIIA^{Glu}$ is predominantly un-phosphorylated and therefore unable to activate adenylate cyclase. In addition, un-phosphorylated $EIIA^{Glu}$ inhibits transporters of secondary carbon sources by direct interaction. This operon-specific mechanism, which has been termed inducer exclusion, contributes to the repression of catabolic genes in the presence of glucose. In some cases, e.g. the *E. coli lac* operon, inducer exclusion might even be the decisive mechanism for CCR (Görke and Stülke, 2008).

A different mechanism of CCR is operative in the Gram-positive soil bacterium *B. subtilis* and other Firmicutes. Here, the global mechanism of CCR is mediated by the pleiotropic transcription factor CcpA (for reviews see (Fujita *et al.*, 2007; Warner and Lolkema, 2003). In the presence of glucose, CcpA represses several hundred catabolic genes and activates the transcription of some genes of overflow metabolism (Blencke *et al.*, 2003; Lulko *et al.*, 2007; Moreno *et al.*, 2001; Yoshida *et al.*, 2001). The ability of CcpA to bind its target sites, the catabolite responsive elements (*cre*), is in turn controlled by the presence of its cofactors, HPr(Ser-P) and Crh(Ser-P) (Galinier *et al.*, 1999; Schumacher *et al.*, 2004; Schumacher *et al.*, 2006). Unlike HPr in *E. coli*, the *B. subtilis* homolog contains a regulatory phosphorylation site, Ser-46, in addition to His-15, which is phosphorylated during phosphate transfer to the transported sugar. Crh is homologous to HPr, but it lacks His-15 and is therefore unable to participate in sugar transport (Galinier *et al.*, 1997). In the presence of glucose, HPr, and presumably also Crh, are phosphorylated by the bi-functional HPr kinase/ phosphorylase (HPrK/P) on Ser-46, whereas this site is less phosphorylated in cells growing in the absence of sugars (Ludwig *et al.*, 2002; Monedero *et al.*, 2001). Dephosphorylation of HPr(Ser-P) is catalyzed by the phosphorylase activity of HPrK/P (Mijakovic *et al.*, 2002) and, to some extent, by the protein phosphatase PrpC (Singh *et al.*, 2007). *In vitro* experiments suggested that the two antagonistic activities of HPrK/P are regulated by metabolites. High fructose 1,6-bisphosphate (FBP) and ATP or PP_i concentrations stimulate the kinase activity,

whereas the phosphorylase activity prevails when the P_i concentration is high (Jault *et al.*, 2000; Ramström *et al.*, 2003; Reizer *et al.*, 1998). Only HPr(Ser-P), but none of the other HPr forms, is able to productively interact with CcpA and to exert CCR (Reizer *et al.*, 1996; Schumacher *et al.*, 2004). *In vitro* experiments suggested that in addition to its role in the activation of HPrK kinase, FBP enhances DNA binding of the CcpA/HPr(Ser-P) complex by directly binding to CcpA (Schumacher *et al.*, 2007; Seidel *et al.*, 2005). The role of Crh in CCR is still unclear.

Traditionally, studies dealing with CCR have been focused on the repression exerted by glucose. Therefore, the terms carbon catabolite repression and glucose repression are often used synonymously. However, for the Gram-negative bacterium *E. coli* it was shown that a large number of carbohydrates in addition to glucose exert CCR (Bettenbrock *et al.*, 2007; Hogema *et al.*, 1998). Much less is known in *B. subtilis*. So far, CCR exerted by carbon sources different from glucose has not been systematically studied. In this work, we analyzed CcpA-mediated CCR exerted by a variety of carbon sources in *B. subtilis*. As a model for this study, we chose the *xynPB* operon, which encodes a β-xyloside transporter and β-xylosidase. These functions allow the uptake and degradation of β-xylosides to xylose (Fig. 9), (Galinier *et al.*, 1999; Lindner *et al.*, 1994). Xylose is subsequently converted to xylulose-5-P by enzymes encoded in the *xylAB* operon located downstream of the *xynPB* operon (Fig. 9). Both operons are repressed by the XylR protein, which binds to operator sites present in the respective promoter regions and prevents transcription initiation (Dahl *et al.*, 1994; Galinier *et al.*, 1999). Binding of xylose releases XylR from its operator sites. *B. subtilis* is not able to grow on xylose due to the lack of a xylose-specific permease (Lindner *et al.*, 1994; Schmiedel and Hillen, 1996a; Schmiedel and Hillen, 1996b). However, xylose can be slowly taken up by the AraE protein, which is sufficient for induction of *xynPB* and *xylAB* expression (Krispin and Allmansberger, 1998). In the presence of glucose, both operons are strongly repressed. Downstream of their promoters, *cre* sites are present, which are bound by the HPr-Ser46-P/CcpA complex thereby preventing transcription initiation (Galinier *et al.*, 1999; Kraus *et al.*, 1994).

In this study, we show that a variety of carbohydrates represses *xynPB* transcription via the CcpA-mediated CCR pathway, but each to a different degree. In general, most substrates transported by the PTS caused a strong repression, whereas CCR by non-PTS substrates was weaker. The Crh protein was completely dispensable for repression exerted by all of these substrates, whereas HPr was essential for the strong CCR caused by sugars like glucose, fructose and mannitol. Analysis of the HPr phosphorylation state *in vivo* revealed

that the strength of repression exerted by a particular substrate correlates well with the amount of HPr(Ser-P) in the cell. However, in contrast to *E. coli*, transport activity of the PTS has no direct role for the global CCR mechanism. Our data suggest that in *B. subtilis* the strength of CcpA-mediated CCR is determined exclusively by the metabolite-controlled activity of HPrK/P.

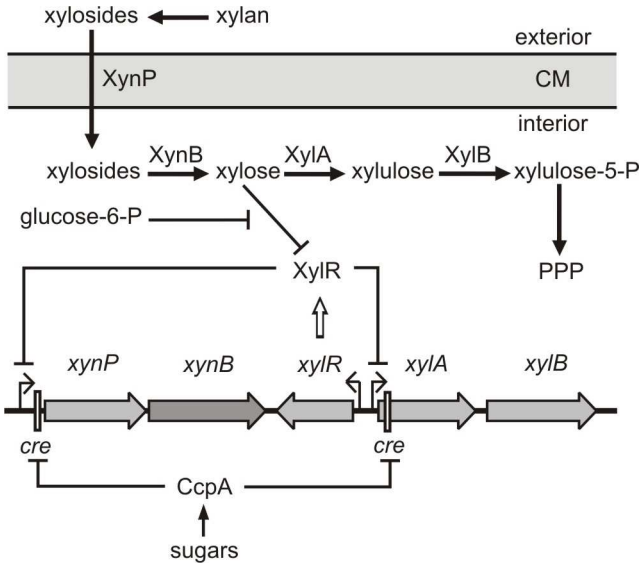

Figure. 9. Utilization of β-xylan in *B. subtilis*. Xylan can be degraded by an extracellular xylanase to β-xylosides, which are subsequently taken up by the β-xyloside transporter XynP encoded in the bi-cistronic *xynPB* operon. The β-xylosidase, XynB converts β-xylosides to xylose, which is further converted to xylulose-5-P by the functions encoded in the adjacent *xylAB* operon. Xylulose-5-P finally enters the pentose phosphate pathway (PPP). The *xynPB* and *xylAB* operons are repressed by binding of the Xyl repressor (XylR) to operator sites in the absence of the inducer xylose. Both operons are subject to global CCR, which is mediated by binding of CcpA to *cre* sites downstream of the promoters. In addition, glucose-6-phosphate contributes to CCR by acting as an anti-inducer for XylR.

Materials and Methods

Bacterial strains and growth conditions

The *B. subtilis* strains used in this study are listed in Table 2. The presence of the *ptsH1* mutation was verified by sequencing of chromosomal DNA of the relevant strains. *E. coli* DH5α (Sambrook and Russell, 2001) was used for plasmid propagation. *B. subtilis* was grown in CSE minimal medium supplemented with auxotrophic requirements (at 50 mg l^{-1}) (Martin-Verstraete *et al.*, 1995). If necessary, xylose was added at a concentration of 0.2% (w/v). Potentially repressing carbon sources were used at a concentration of 0.5% (w/v). *E. coli* was grown in LB medium and transformants were selected on plates containing ampicillin (100 µg ml^{-1}).

LB and SP plates were prepared by the addition of 17 g l^{-1} Bacto agar (Difco) to the medium. For the determination of growth rates, pre-cultures were grown overnight in CE minimal medium supplemented with auxotrophic requirements (at 50 mg l^{-1}) and a single carbon source at a concentration of 0.5 % (w/v). The cells were inoculated in the same medium to an OD_{600} of 0.1 and the bacteria were incubated at 37°C at 200 r.p.m. and the turbidity at 600 nm was recorded periodically. The growth rates (μ) were determined from the exponential phases of the growth curves using the formula $\mu = \log b - \log B / (\log 2) \times (\Delta t)$, where Δt is the time interval in min, b the OD_{600} of the culture at the end, and B the OD_{600} at the beginning of this time interval.

DNA manipulation

Transformation of *E. coli* and plasmid DNA extraction was performed using standard procedures (Sambrook and Russell, 2001). Restriction enzymes and DNA polymerases were used as recommended by the manufacturers. Chromosomal DNA of *B. subtilis* was isolated using the DNeasy Tissue Kit (Quiagen) according to the supplier's protocol. Plasmid pIW11xylR used for inactivation of the *xylR* gene has been described previously (Kraus *et al.*, 1994). Plasmid pGP811 used for the disruption of *ptsI* is a derivative of plasmid pHT181 (Lereclus and Arantes, 1992) carrying the 591 bp EcoRI fragment of *ptsI* inserted in its unique EcoRI site. Plasmid pGP650 carries the *hprK*-G158A allele under control of the constitutive *degQ36* promoter. The *hprK* allele was amplified using primers SK11 (GGCGGATCCGTGGCAAAGGTTCGCACAAAAGA) and KS12 (AAAAAGCTTGGTTCTATCGCTTCATTCATTTAACGC) and plasmid pGP407 (Hanson *et al.*, 2002) as template. Subsequently, the PCR fragment was digested with *BamH*I and *Hind*III and inserted between the same sites of plasmid pGP380 (Herzberg *et al.*, 2007).

Transformation and enzyme assays

B. subtilis was transformed with plasmids and chromosomal DNA according to the two-step protocol (Kunst and Rapoport, 1995). Transformants were selected on SP plates containing spectinomycin (100 μg ml^{-1}), kanamycin (5 μg ml^{-1}), or erythromycin plus lincomycin (2 and 25 μg ml^{-1}, respectively). For enzyme assays cells were harvested in exponential growth phase at an OD_{600} of 0.6 - 0.8. β-Galactosidase and β-xylosidase activities were determined in cell extracts using *o*-nitrophenyl galactopyranoside and *p*-nitrophenyl xyloside as substrates, respectively (Lindner *et al.*, 1994; Sambrook and Russell, 2001).

Western blot analysis

For Western blot analyses, crude cell extracts were separated by SDS PAGE and transferred to a polyvinylidene difluoride membrane (PVDF, BioRad) by electroblotting. The proteins were detected with rabbit polyclonal antisera raised against CcpA, HPr, or HPrK/P of *Bacillus megaterium* or *B. subtilis* (Hanson *et al.*, 2002; Küster *et al.*, 1996; Monedero *et al.*, 2001). The antibodies were visualized by using anti-rabbit IgG-AP secondary antibodies (Chemikon International, Temecula, U.S.A.) and the CDP* detection system (Roche Diagnostics).

Analysis of the phosphorylation state of HPr *in vivo*

HPr phosphorylation was assayed *in vivo* by Western blot analysis as follows. Bacteria were grown in CSE in the presence of the indicated carbon sources to an OD_{600} of 0.6. Cells were disrupted using a French press, and crude extracts were prepared as described previously (Ludwig *et al.*, 2002). Proteins (2 µg, respectively) were separated on non-denaturing 12% polyacrylamide gels. On these gels, phosphorylated HPr migrates faster than the non-phosphorylated protein. HPr(His-P) was dephosphorylated by incubation of the crude extract for 10 min at 70°C. After electrophoresis, the proteins were blotted to a PVDF membrane. The different forms of HPr were detected using antibodies directed against *B. subtilis* HPr (Monedero *et al.*, 2001).

Determination of fructose 1,6-bisphosphate concentrations

Protein-free cell extracts for the determination of FBP concentrations in *B. subtilis* were prepared as described previously with few modifications (Mijakovic *et al.*, 2002). Briefly, cells of the *B. subtilis* wild type strain 168 were grown in 50 ml CSE medium in the presence of the indicated carbon sources. For each growth condition at least three independent experiments were carried out. Cultures were harvested by centrifugation at room temperature for 5 min at 10,000g and pellets were subsequently frozen in liquid nitrogen. The pellets were resuspended in 0.6 M of cold perchloric acid and subsequently incubated on ice for 20 min. The precipitated proteins and cell debris were removed by centrifugation (4°C, 5 min, 13,000 rpm). The pH in the supernatant was adjusted to 7.4 with a solution of cold 0.6 M KOH in 100 mM Tris-HCl (pH 7.4). The precipitated $KClO_4$ was removed by centrifugation. The FBP concentrations were determined in the supernatants as described previously (Mijakovic *et al.*, 2002).

Table. 2. *B. subtilis* strains used in this study.

Strain	Genotype	Source[a]
168	*trpC2*	Laboratory stock
GP270	*trpC2 xylR*::*ermC*	pIW11xylR → 168
GP278	*trpC2 xylR*::*ermC amyE::(xynP-lacZ cat)*	Singh *et al.*, 2007
GP279	*trpC2 xylR*::*ermC crh*::*spc amyE::(xynP-lacZ cat)*	QB7097 → GP278
GP284	*trpC2 xylR*::*ermC ptsH1*	pIW11xylR → QB5223
GP287	*trpC2 xylR*::*ermC ptsH1 crh*::*spc*	pIW11xylR → QB7101
GP289	*trpC2 xylR*::*ermC hprK*::*aphA3*	QB7160 → GP270
GP297	*trpC2 xylR*::*ermC crh*::*spc*	QB7097 → GP270
GP853	*trpC2 xylR*::*ermC ccpA*::*spc*	QB5407 → GP270
GP858	*trpC2 ΔhprK*::*aphA3*	QB7160 → 168
GP864	*trpC2 ΔptsI::ermC*	pGP811 → 168
QB5223	*trpC2 ptsH1*	Martin-Verstraete *et al.*, 1995
QB5407	*trpC2 ccpA*::*spc*	Faires *et al.*, 1999
QB7097	*trpC2 crh*::*spc*	I. Martin-Verstraete
QB7101	*trpC2 ptsH1 crh*::*spc*	I. Martin-Verstraete
QB7144	*trpC2 amyE::(xynP-lacZ cat)*	Galinier *et al.*, 1999
QB7160	*trpC2 amyE*::*(levD-lacZ cat) hprK*::*aphA3*	Martin-Verstraete *et al.*, 1999

[a] Arrows indicate construction by transformation.

Results

CCR of β-xylosidase exerted by different carbon sources.

CCR has mainly been studied using glucose as repressing carbon source. To explore whether other carbon sources also exert CCR, we chose the *xynPB* operon as a model system. For this purpose, we measured the activity of the β-xylosidase XynB as a convenient reporter for regulatory events taking place at the *xynPB* promoter (see below). First, we tested the wild-type strain 168 in CSE minimal medium containing succinate as carbon source. In the absence

of xylose, only 14 units of β-xylosidase activity were detectable, whereas in the presence of xylose a high β-xylosidase activity of 945 units was measured (Table 3, column 1), reflecting the repression of the *xyn* operon by the XylR protein. Next, we added other carbon sources in addition to succinate and repeated the measurements. Xylose as inducer for XylR was also included. In all cases, the XynB activities were reduced in comparison to the culture grown on succinate (Table 3, column 1). Succinate is known to cause no CCR in *B. subtilis* (Blencke *et al.*, 2003; Galinier *et al.*, 1999). Glucose caused the strongest reduction of XynB activity (135-fold), which is in agreement with previous data (Galinier *et al.*, 1999). The sugars salicin, glycerol, mannitol and fructose also caused repression, but to a weaker extent (12- to 18-fold). Gluconate, sucrose and sorbitol repressed XynB activity eight-fold. Ribose, arabinose and maltose had only a two-fold effect. In conclusion, the substrates formed a hierarchy in their ability to exert CCR.

Table. 3. Catabolite repression of β-xylosidase by different carbon sources in various mutants. The values are the average of at least three independent experiments. Standard deviations are shown in parentheses.

	Enzyme activity in units/mg of protein						
Carbon source[a]	168[b] *wild-type*	GP270 *ΔxylR*	GP297 *ΔxylR, Δcrh*	GP284 *ΔxylR, ptsH1*	GP287 *ΔxylR, Δcrh, ptsH1*	GP289 *ΔxylR, ΔhprK*	GP853 *ΔxylR, ΔccpA*
- (CSE)	945 (281)	1585 (560)	2748 (540)	2590 (304)	2397 (214)	2287 (218)	2142 (384)
Ribose	497 (138)	939 (164)	1360 (169)	971 (263)	1346 (151)	n. g.[c]	1780 (454)
Arabinose	414 (136)	600 (153)	876 (35)	713 (100)	1498 (323)	1257 (70)	1437 (228)
Maltose	437 (127)	489 (32)	488 (105)	710 (81)	2226 (206)	2023 (408)	2078 (632)
Gluconate	116 (11)	201 (31)	378 (64)	244 (46)	1220 (179)	1163 (170)	1628 (554)
Sucrose	126 (20)	205 (12)	203 (13)	309 (88)	2770 (136)	2271 (292)	2858 (123)
Salicin	54 (6)	175 (19)	167 (4)	202 (37)	2850 (180)	2743 (667)	2049 (83)
Sorbitol	114 (20)	121 (29)	130 (52)	113 (19)	732 (66)	748 (61)	734 (51)
Glycerol	82 (15)	96 (20)	87 (6)	135 (17)	2164 (110)	1273 (160)	1138 (127)
Mannitol	72 (13)	79 (21)	78 (18)	606 (190)	2080 (395)	1689 (269)	1265 (454)
Fructose	60 (9)	63 (5)	63 (7)	231 (75)	2045 (206)	1638 (32)	1889 (147)
Glucose	7 (3)	44 (10)	60 (21)	173 (56)	2077 (500)	1679 (421)	1570 (153)

[a]added to CSE medium. [b]Xylose was added to induce *xynPB* expression. [c] = no growth

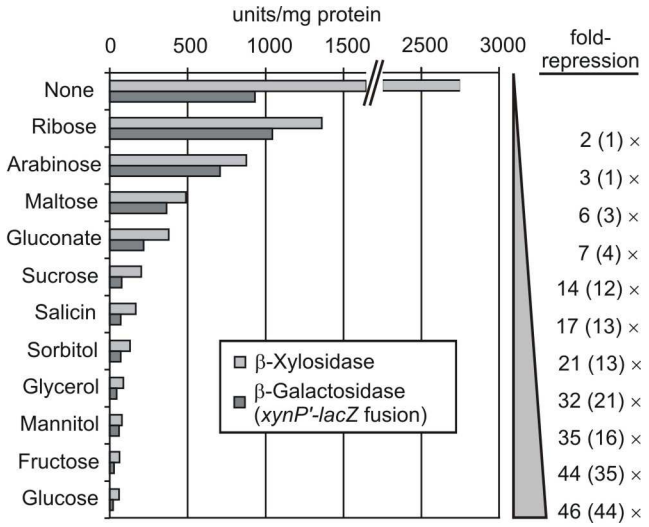

Figure. 10. Various carbohydrates repress the *xynPB* operon via the global CCR pathway. Shown are the β-xylosidase activities in the *ΔxylR Δcrh* mutant GP297 (light grey bars), in which CCR exclusively relies on the activities of HPrK/P, CcpA and HPr. To demonstrate that the β-xylosidase activities reflect transcription from the *xynPB* promoter, the isogenic strain GP279 was used, which carries in addition a *xynP'-lacZ* fusion on the chromosome. The β-galactosidase activities produced in this strain are shown for comparison (dark grey bars). The repression factors relative to the activities determined in pure CSE medium („none“) are depicted at the right.

To rule out that enzyme activity rather than synthesis of XynB was affected by the various carbon sources in these and the following experiments, we also performed similar experiments with isogenic strains carrying a transcriptional *xynP'-lacZ* fusion on the chromosome. The β-galactosidase activities in these strains correlated well with the corresponding β-xylosidase activities (Fig. 10 and data not shown). Somewhat lower repression factors were obtained in the LacZ assays in comparison to the XynB measurements, making the latter the more sensitive tool to measure CCR. In conclusion, the observed differences of XynB activities in this study reflect regulatory events taking place at the *xynPB* promoter.

The XylR repressor protein contributes to CCR of *xynPB* expression exclusively in the presence of glucose

As for many other systems, the *xynPB* operon underlies an operon-specific mechanism of CCR in addition to the general CCR pathway via CcpA. The XylR repressor also contributes to glucose repression of its target genes (Dahl *et al.*, 1995; Kraus *et al.*, 1994). Uptake of glucose generates glucose-6-phosphate, which binds to XylR and counteracts binding of the inducer xylose (Fig. 9). In principle, the repression of XynB activity by the other carbohydrates may also result from the combined activities of both mechanisms of CCR. To

analyze this possible interference, we repeated our experiments with a Δ*xylR* mutant. As expected, the *xylR* mutation caused complete de-repression of *xynPB* expression, i.e. high XynB activities were already detectable in the absence of the inducer xylose (Table 3, column 2). With respect to the available carbon sources, the enzyme activities followed a similar order as in the xylose-induced wild-type strain (Table 3, compare columns 1 and 2). In most cases, the activities were slightly higher in the *ΔxylR* mutant, which can be explained by incomplete de-repression in the wild-type strain. In contrast, there was a 6-fold relief from repression when the cells grew on glucose, which reflects the extra-repression exerted by glucose-6-P. In conclusion, XylR only contributes to CCR of *xynPB* expression when glucose is utilized and is not relevant for CCR elicited by the other carbon sources.

In the Δ*xylR* mutant, CCR of β-xylosidase completely depends on the general pathway of CCR

In the Δ*xylR* mutant, the substrates still formed a hierarchical order in their ability to cause repression. We wanted to determine whether this order is caused exclusively by the global CCR mechanism or whether additional mechanisms are still involved. If only global CCR is involved, an interruption of the general CCR pathway is expected to completely relieve repression. To test this possibility, we used *ΔxylR* strains, which additionally lacked HprK/P or CcpA or its cofactors Crh and HPr. In the latter case, a *Δcrh ptsH1* mutant was used, which encodes an HPr mutant with a non-phosphorylatable alanine rather than a serine at position 46. The HPr-Ser46Ala protein is unable to participate in CcpA-mediated CCR but retains its function in PTS sugar uptake (Deutscher *et al.*, 1994). All strains produced similar high β-xylosidase activities regardless of the carbon source, with the notable exception of sorbitol (Table 3, columns 5-7). On sorbitol a 3-fold repression was still detectable. On all other carbon sources, the elimination of the global regulators of CCR caused a complete relief from CCR. Hence, global CCR is responsible for the hierarchical order of repression caused by the different substrates in the *ΔxylR* mutant.

The amounts of HPrK, CcpA and HPr are not affected by the carbon source

It is possible that the differences in repression exerted by the different substrates are caused by varying amounts of HPrK or CcpA or its co-factors in the cell. To address this possibility, we determined the amounts of these proteins in cells grown on the various carbon sources. Protein extracts were separated by SDS polyacrylamide gel electrophoresis and subjected to Western blot analyses using specific antisera. Only small differences in the amounts of the

three proteins were detectable (Fig. 11). We also attempted to detect the Crh protein. However, although our antiserum readily detected purified Crh protein, no signals were obtained in cell extracts (data not shown). This observation is in agreement with our previous data suggesting that Crh is present in the cell in much lower amounts than HPr (Görke *et al.*, 2004). In conclusion, the cellular amounts of the global CCR regulators HPrK/P, CcpA and HPr are not affected by the carbon source.

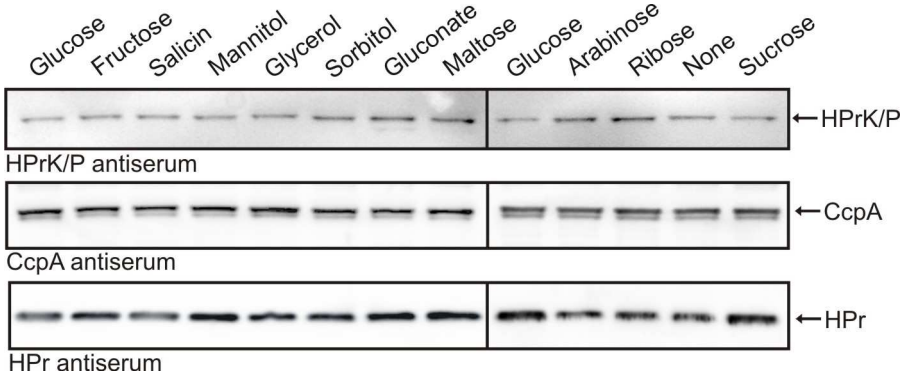

Figure. 11. The amounts of HPrK/P, CcpA and HPr are not affected by the utilized carbon source. Wild-type strain 168 was grown to exponential phase in CSE medium containing the indicated carbon sources and crude extracts were prepared. Of these protein extracts 10µg, 5µg and 2µg were loaded on 12.5% SDS-PAA gels for the detection of HPrK/P, CcpA and HPr, respectively. After gel electrophoresis proteins were blotted to a PVDF membrane and the proteins of interest were detected using specific antisera, respectively.

HPr and Crh are not interchangeable in CcpA-mediated CCR

In the Δ*xylR* mutant, only global CCR is operative in *xynPB* repression. Hence, the degree of repression exerted by the individual substrates should be reflected by the total number of HPr(Ser-P)/CcpA and Crh(Ser-P)/CcpA complexes competent in DNA-binding. So far, it is unclear to which extent each of these complexes actually contributes to CCR via CcpA under a certain condition. Hence, it is possible that the cell selectively prefers or relies on one of these complexes depending on the carbon source. To address this question, we compared Δ*xylR* Δ*crh*- and Δ*xylR ptsH1*-mutants in which either one of the co-factors for CcpA is missing. The XynB activities in the *ΔxylR Δcrh* mutant were very similar to those detected in the Δ*xylR* mutant (Table 3, compare columns 2 and 3; Fig. 10). Therefore, Crh is dispensable for CCR exerted by the different carbon sources. Obviously, HPr can completely compensate for the loss of Crh. A different result was obtained using the *ΔxylR ptsH1* mutant. In this case, a 3- to 4-fold relief from repression was observed when the cells grew on glucose or fructose. On mannitol, the β-xylosidase activities were even 8-fold higher (Table 3, compare column 4 with columns 2 and 3). These sugars exert the strongest repression in the wild type and in the

ΔxylR mutant. In contrast, repression by all other substrates was not significantly affected by the *ptsH1* mutation. These data indicate that Crh is unable to substitute for HPr in the presence of substrates that exert a strong CCR. Hence, HPr is essential for CCR.

The extent to which a substrate causes repression correlates with the amount of HPr(Ser-P) in the cell

The phosphorylation of HPr by the HPr kinase is a key step in the signaling pathway of CCR in *B. subtilis*. It is well established that significant amounts of HPr(Ser-P) are present during growth on glucose, whereas little HPr(Ser-P) is detectable in the absence of a sugar, i.e. in CSE minimal medium or LB (Ludwig *et al.*, 2002; Monedero *et al.*, 2001). However, the phosphorylation state of HPr has so far not been determined during growth in the presence of sugars other than glucose. In order to explore whether the strength of CCR exerted by a given substrate correlates with the amount of HPr(Ser-P) in the cell, we determined the HPr phosphorylation state *in vivo*. To this end, we prepared extracts from cells grown on the various carbohydrates and subjected them to non-denaturing polyacrylamide gel electrophoresis, which allows the separation of the non-phosphorylated and phosphorylated forms of HPr. HPr was subsequently detected by Western blot analysis (Fig. 12).

As reported previously (Ludwig *et al.*, 2002), HPr was present in three different forms when the cells grew in CSE medium: non-phosphorylated, single phosphorylated and doubly phosphorylated (Fig. 12, lane 1). HPr can be phosphorylated by the EI of the PTS on His-15 and by HPrK/P on Ser-46. To discriminate between both forms, aliquots of the extracts were incubated at 70°C before loading on the gel. Heating causes loss of phospho-histidine but not of phospho-serine bonds. As a result, the doubly phosphorylated HPr is converted to HPr(Ser-P) and HPr(His-P) is converted to non-phosphorylated HPr (Fig. 12, even numbered lanes). From the quantification of the band intensities in both lanes and their comparison, it was possible to calculate the relative amount of each form of HPr (Table 4). In CSE medium, only 13% of total HPr were phosphorylated at Ser-46 and 5% were doubly phosphorylated. The majority of HPr molecules were non-phosphorylated or phosphorylated at His-15. Very similar results were obtained when the cells grew on the weakly repressing sugars ribose, gluconate, arabinose or maltose. The level of HPr(Ser-P) slightly increased up to 32% on gluconate, which is the most strongly repressing substrate among these carbohydrates (Fig. 12, top panel; Table 4). In all cases, HPr(His-P) and doubly phosphorylated HPr were the predominant forms.

A very different result was obtained, when the cells grew on sugars that cause a stronger CCR, i.e. sorbitol, sucrose, salicin, mannitol, fructose and glucose. In these cases, almost no doubly phosphorylated HPr was detectable and heating of the extracts had almost no effect on the phosphorylation pattern (Fig. 12, lanes 11, 12 and 15-24). Hence, there was no detectable HPr(His-P) present in the cells. In contrast, 50-70% of all HPr molecules were phosphorylated at Ser-46 (Fig. 12, bottom panel; Table 4, rows 6-12). The only other detectable form was non-phosphorylated HPr. As an exception, glycerol behaved different from all other substrates: In contrast to the other strongly repressing substrates, it generated a somewhat lower level of HPr(Ser-P), i.e. 46%. Moreover, no un-phosphorylated HPr could be detected. In contrast, doubly phosphorylated HPr was a predominant form in this case (Fig. 12, lanes 13, 14).

In conclusion, sugars that exert strong (i.e. at least 10-fold, Fig. 10) repression of β-xylosidase activity also generate a high level of HPr(Ser-P) in the cell, whereas in the presence of weakly repressing substrates the amount of HPr(Ser-P) is significantly lower. In addition, the data suggest that the absence of histidine-phosphorylated HPr is a common feature of substrates that generate a strong CCR.

His-15-dependent phosphorylation of HPr has no impact on CcpA-mediated CCR. Interestingly, most of the carbohydrates that generated a strong CCR are substrates of the PTS. Glucose, fructose, mannitol, salicin and sucrose are all taken up by specific EIIs, which rely on HPr(His-P)-dependent phosphorylation for this function. Hence, utilization of all these substrates drains away the phosphoryl-groups bound to His-15 of HPr. Accordingly, no HPr(His-P) can be detected when the cells grow on these substrates (Fig. 12 and Table 4). On the weakly repressing substrates ribose, arabinose and gluconate, which are non-PTS substrates, a considerable fraction of HPr is phosphorylated at its His-15 residue by Enzyme I (Table 4). Hence, it is conceivable that in these cases EI and HPrK compete for the phosphorylation of HPr and that CCR is weak, because EI-dependent phosphorylation limits the HPr(Ser-P) amount in the cell and thereby CCR.

To test this possibility, we used a *ΔptsI* mutant in which HPr cannot get phosphorylated at His-15. If histidine phosphorylation of HPr limits CCR, one would expect an increased CCR by non-PTS substrates in this mutant. However, there was virtually no difference in CCR by non-PTS substrates between the *ΔptsI* mutant and the wild-type strain (Fig. 13). In contrast, on PTS-sugars (and glycerol) an almost complete relief from CCR was observed in the *ΔptsI* mutant. This was expected, because uptake and metabolism of these

substrates are not possible in the *ΔptsI* mutant. Hence, the cells actually use succinate for growth, which exerts no CCR. To support these data, we also investigated a mutant strain coding for an HPr-His15Ala variant. This strain exhibited β-xylosidase activities almost identical with those determined in the Δ*ptsI* mutant (our unpublished data). In conclusion, the phosphorylation state of HPr at its histidine residue has no direct impact on CcpA-mediated CCR. Therefore, solely different HPrK activities appear to account for the different CCR levels exerted by the various substrates.

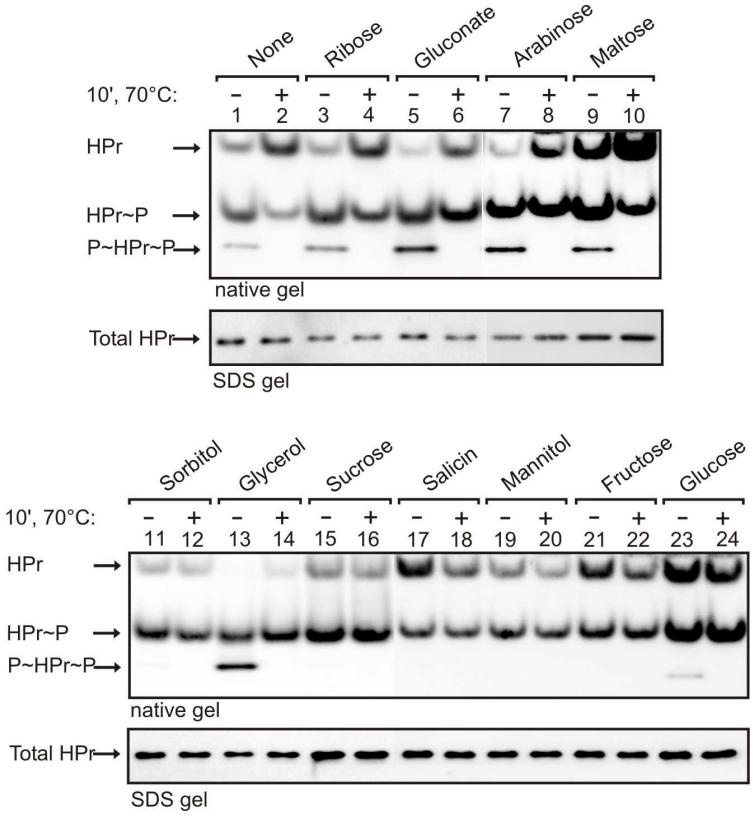

Figure. 12. Determination of the phosphorylation state of HPr in the presence of different carbon sources. Wild-type strain 168 was grown on CSE medium supplemented with the indicated carbohydrates. Protein extracts were prepared and separated on native 12% PAA gels (top panels). HPr was subsequently detected by Western blotting (odd-numbered lanes). To discriminate between HPr(Ser-P) and HPr(His-P) an aliquot of each cell extract was heated (70°C, 10 min) prior to loading (even-numbered lanes). This causes loss of the phospho-histidine bonds. The lysis buffer used affected the reliability of the Bradford assay for determination of protein concentrations. To account for the differences in the protein estimation and sample loading, 2 µg of each protein extract (according to Bradford-assay) were separated in parallel by SDS-PAGE and total HPr was detected by Western blot analysis (bottom panels).

Table. 4. Comparison of catabolite repression by different substrates, the relative amounts of the different HPr forms and the intracellular FBP concentration. Standard deviations are shown in parentheses.

Carbon source[a]	Fold-repression[b]	Relative amount of HPr form [%][c]				Fructose-1,6-bis-P [mM]	Growth rate μ [h^{-1}]
		HPr (Ser-P)	HPr (His-P)	HPr	HPr (Ser-P)(His-P)		
- (CSE)	-	13 (3)	40 (14)	42 (19)	5 (2)	1.8 (0.5)	0.29 (0.03)
Ribose	2	27 (1)	37 (6)	25 (9)	11 (4)	6.5 (0.1)	0.76 (0.07)
Arabinose	3	21 (1)	29 (9)	13 (10)	37 (1)	8.1 (0.5)	0.98 (0.06)
Maltose	6	13 (1)	47 (4)	25 (6)	15 (1)	10.7 (0.8)	0.85 (0.01)
Gluconate	7	32 (10)	29 (4)	9 (2)	30 (9)	12.3 (3.6)	0.94 (0.09)
Sucrose	14	66	0	33	1	11.5 (1.9)	0.85 (0.02)
Salicin	17	50 (9)	0	50 (9)	0	9.4 (2.1)	0.91 (0.04)
Sorbitol	21	66 (6)	0	32 (7)	2 (1)	4.4 (2.1)	0.83 (0.03)
Glycerol	32	46 (3)	12 (6)	0	42 (4)	4.3 (1.6)	0.94 (0.11)
Mannitol	35	70 (6)	0	30 (6)	0	4.4 (0.6)	0.86 (0.03)
Fructose	44	60 (2)	0	40 (3)	0	13.3 (3.0)	0.94 (0.07)
Glucose	46	58 (6)	0	35 (10)	7 (7)	14.1 (1.3)	0.95 (0.07)

[a]added to CSE medium; for growth rate determinations CE-medium plus a single carbon source was used. [b]in the *ΔxylR Δcrh* mutant. [c]mean values of at least two independent experiments are presented except for sucrose, which was performed once.

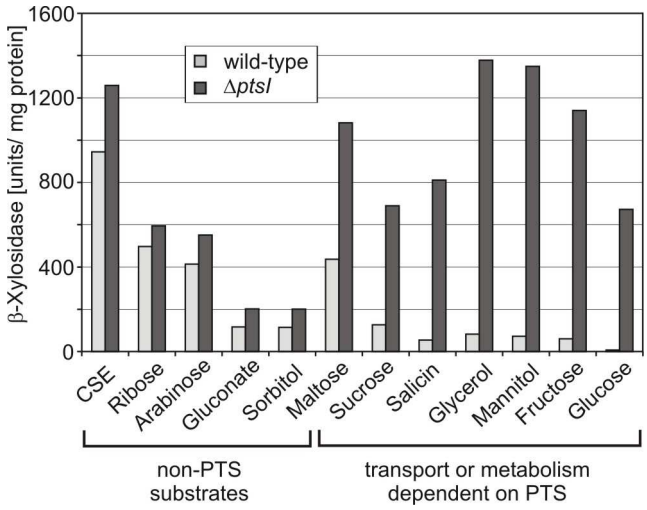

Figure. 13. Catabolite repression of β-xylosidase in a mutant lacking EI of the PTS. Strain GP864 (*ΔptsI*) was grown in CSE medium supplemented with the indicated carbohydrates and the β-xylosidase activities were determined (dark grey columns). The corresponding activities in the wild-type strain 168 are shown for comparison (light grey columns).

Activity of HPrK/P determines the level of CcpA-exerted CCR in *Bacillus subtilis*

Finally, we wanted to confirm that a low HPr kinase activity is responsible for the weak CCR exerted by substrates like ribose, arabinose, gluconate or maltose. To this end, we used a strain, which expresses the mutant *hprK*-G158A allele rather than wild-type *hprK*. The G158A exchange is located in the nucleotide binding Walker A motif of HPrK and abolishes phosphorylase activity *in vitro* (Hanson *et al.*, 2002). As a result, HPrK-G158A behaves as a constitutive kinase, triggering the slow phosphorylation of HPr. In order to see whether this is indeed the case *in vivo*, we introduced a plasmid carrying *hprK* G158A into a mutant strain lacking the wild-type gene. A transformant carrying the empty expression plasmid served as a control. These transformants as well as the wild type strain were grown in CSE and CSE + glucose and the phosphorylation state of HPr was determined (Fig. 14). As expected, no HPr(Ser-P) was detectable in the *ΔhprK* mutant (Fig. 14, lanes 3, 4 and 9, 10). In the strain expressing the *hprK* G158A allele, the fraction of HPr(Ser-P) increased to 38% when the cells grew in CSE, whereas only 13% HPr(Ser-P) were detectable in the wild-type (Fig. 14, compare lanes 1, 2 and 5, 6). In the presence of glucose 43% HPr(Ser-P) were detectable in the mutant expressing the *hprK* G158A allele (Fig. 14, lanes 7, 8 and 11, 12). The wild-type strain produced 58% HPr(Ser-P) under these conditions (Fig. 14, lanes 7 and 8). In conclusion, the HprK G158A mutant triggers the phosphorylation of HPr at its Ser residue even in the absence of a repressing sugar. To see whether this increased fraction of HPr(Ser-P) also correlates with a stronger CCR, we determined the β-xylosidase activities in these

strains (Table 5). In the *ΔhprK* mutant carrying the empty expression plasmid, very high β-xylosidase activities were detectable, i.e. *xynPB* expression was relieved from CCR. In contrast, the strain expressing the *hprK*-G158A allele produced only low activities in the range of 60 to 170 units. As an exception repression by glucose was somewhat stronger (21 units), which can be ascribed to the extra-repression by glucose-6-P via XylR. In conclusion, the data show that the HPrK-G158A variant phosphorylates HPr under all conditions and thereby triggers strong CCR on all substrates. This confirms that the different levels of CCR exerted by the various carbon sources (Fig. 10) solely result from different activities of HPrK/P.

Table. 5. Catabolite repression by the mutant HPrK/P-G158A protein lacking phosphorylase activity. The values are the average of at least two independent experiments.

Enzyme activity in units/mg of protein			
Carbon source[a]	168[b] *wild-type*	GP858+pGP380 *ΔhprK*	GP858+pGP650 *ΔhprK* + *hprK*-G158A
-	945 (281)	2077 (323)	178 (10)
Ribose	497 (138)	n. g. [c]	115 (30)
Arabinose	414 (136)	1175 (50)	138 (27)
Maltose	437 (127)	1265 (82)	142 (17)
Sucrose	126 (20)	1232 (145)	124 (66)
Gluconate	116 (11)	822 (97)	63 (6)
Sorbitol	114 (20)	990 (52)	90 (22)
Glycerol	82 (15)	n. g. [c]	70 (15)
Mannitol	72 (13)	1592 (460)	60 (8)
Fructose	60 (9)	1364 (355)	64 (8)
Salicin	54 (6)	981 (168)	46 (27)
Glucose	7 (3)	870 (11)	21 (4)

[a]added to CSE medium. [b]xylose was added to induce *xynPB* expression. [c] = no growth

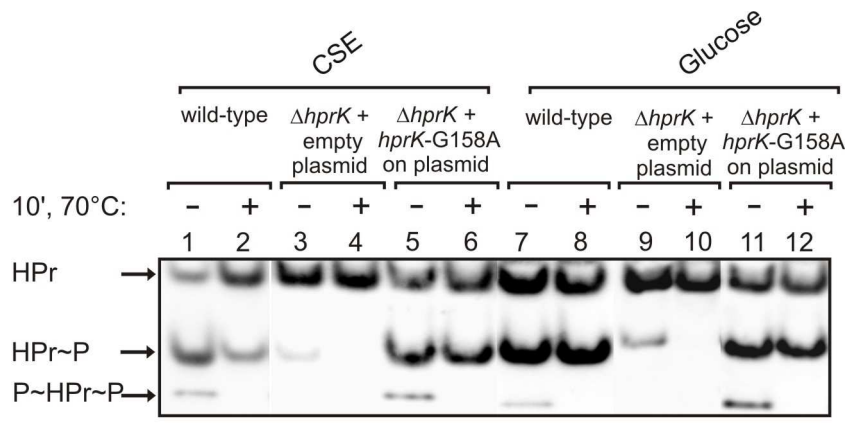

Figure. 14. The mutant HPrK/P-G158A protein phosphorylates HPr at Ser-46 even in the absence of a sugar. Strain GP858 (*ΔhprK*) carrying either plasmid pGP650 encoding the *hprK*-G158A allele (lanes 5, 6, 11, 12) or the empty plasmid (lanes 3, 4, 9, 10) was grown in CSE or CSE + glucose. Crude extracts of these strains were subjected to non-denaturating PAGE and the phosphorylation state of HPr was determined as described for Fig. 12. For comparison, the wild-type strain 168 is also shown (lanes 1, 2, 7, 8).

The intracellular FBP concentration and the strength of CCR exerted by a given carbohydrate do not strictly correlate

FBP has been identified as a key metabolite modulating the activity of HPrK. To see, if there is a correlation between the intracellular FBP concentration and the level of CCR exerted by a given substrate, we determined the FBP concentrations (Table 4). In cells grown in pure CSE medium, only 1.8 mM FBP was detectable, whereas the FBP concentration increased to 14.1 mM in the presence of glucose. These results are in perfect agreement with a previous study (Mijakovic *et al.*, 2002). High FBP concentrations in the range of 9.4 – 13.3 mM FBP were also detectable in cells grown on the strongly repressing sugars fructose, salicin and sucrose. However, there was no strict correlation for the remaining substrates, e.g. the utilization of ribose, arabinose and maltose generated rather high FBP concentrations (6.5 – 10.7 mM FBP), whereas CCR exerted by these substrates was weak. In contrast, the FBP concentrations were lower (4.3 - 4.4 mM) on mannitol, glycerol and sorbitol, which all exert a strong CCR. In conclusion, the different levels of repression exerted by the different carbohydrates cannot be explained by the different intracellular FBP levels alone.

Discussion

In this work, we show that in addition to glucose many other carbohydrates cause carbon catabolite repression in *B. subtilis*. These substrates form a hierarchical order in their capacity to exert repression, suggesting that they trigger the formation of active CcpA complexes to

different degrees (Table 3, Fig. 10). Our data show that the different carbon sources modulate the activities rather than the amounts of the proteins responsible for CCR. In fact, the data suggest a correlation between the ability of a sugar to cause repression and the phosphorylation state of HPr at its Ser-46 residue (Fig. 12). We could not observe any interference of the phosphorylation state of HPr at its His-15 residue with CcpA-mediated CCR of the *xynPB* operon, which served as a model system in this study. Our data suggest that at least in this case HPrK/P activity is the sole factor that accounts for the differences in repression by the various substrates. However, it should be emphasized that in other gene systems additional CcpA-independent mechanisms of CCR exist, which rely on HPr(His-P)-dependent phosphorylation (for a review see: (Görke and Deutscher, 2007)).

The substrates form a hierarchy in their capacity to exert repression. The general CCR pathway determines this hierarchy, since the absence of either HprK/P or CcpA or its cofactors resulted in complete de-repression on all substrates. Intriguingly, there is a good correlation between the hierarchy in repression (this work) and the hierarchy of carbon source utilization reported by Monod based on diauxic growth experiments (Monod, 1942). Monod classified the carbon sources utilized by *B. subtilis* in two groups, A and B: When present in a mixture, the bacteria first utilize the substrates of group A, which include glucose, fructose, mannitol and sucrose. Subsequently the cells make use of the group B carbohydrates e.g. sorbitol, arabinose or maltose. Based on our observation that group A sugars exert strong CCR, whereas repression by group B sugars is weak, it appears reasonable that the diauxic growth behavior observed by Monod is caused by CcpA-mediated CCR. However, as an inconsistency we observed a strong repression of *xynPB* by sorbitol, which was assigned by Monod to group B. Interestingly, disruption of the CcpA-mediated CCR did not completely relieve *xynPB* from repression when the cells grew on sorbitol (Table 3). In contrast, other CcpA-controlled catabolic genes like *rocG* were completely de-repressed in *ccpA* and *hprK* mutants grown on sorbitol (data not shown). Therefore, strong repression by sorbitol appears to be specific for the *xynPB* operon, which might explain the discrepancy to Monod's observations.

The Crh protein was completely dispensable for CCR exerted by the different substrates. A different result was obtained for a *ptsH1* mutant, in which the Crh protein is the only potential effector for CcpA. In this case, there was a significant relief from CCR exerted by glucose, fructose and mannitol, but not from CCR exerted by the other sugars. In a previous study it was shown that in a *ptsH1* mutant synthesis of gluconate kinase and glucitol dehydrogenase is relieved from repression by glucose and mannitol but not by glycerol

(Deutscher *et al.*, 1994). Collectively, these observations suggest that Crh cannot substitute for HPr when the cells grow on substrates that cause a very strong CCR via CcpA. This might be reflected by the up to 100-fold lower synthesis rate of Crh and its 10-fold lower affinity for CcpA, when compared with HPr (Görke *et al.*, 2004). Alternatively, Crh might play a more specific role in CCR, when the cells use weaker repressing substrates. Recent work suggested that Crh might be more important for CCR during the transition to stationary phase (Inacio and de Sa-Nogueira, 2007). In conclusion, HPr rather than Crh is the relevant effector for CcpA *in vivo*, at least during the exponential growth phase.

According to the current model of the global CCR mechanism in *B. subtilis*, repression is brought about by the HPrK-catalyzed phosphorylation of HPr at its Ser-46 residue. Indeed, during growth on weakly repressing carbon sources only a minor fraction of HPr was phosphorylated at Ser-46, whereas the majority of HPr molecules were phosphorylated at this site on strongly repressing substrates. This clearly shows that the cell modulates the strength of CCR by dynamically triggering the HPrK/P-dependent (de)phosphorylation of HPr. Interestingly, large amounts of HPr(His-P) and also doubly phosphorylated HPr were formed, when the cells grew on weakly repressing substrates. The formation of significant amounts of doubly phosphorylated HPr *in vivo* is surprising. Previous *in vitro* studies suggested that HPr(His-P) is a poor substrate for HPr kinase (Reizer *et al.*, 1998). Moreover, a Ser46Asp exchange in HPr, which mimics phosphorylation at this site, was shown to block EI-dependent phosphorylation *in vitro* (Reizer *et al.*, 1989). These effects were explained by a diminished affinity for the second phosphoryl group delivering protein. Obviously, the *in vitro* data do not adequately reflect the situation *in vivo*.

Most of the strongly repressing substrates are transported by the PTS (glucose, fructose, mannitol, salicin and sucrose) and are therefore expected to dephosphorylate HPr at its His-15 residue, which is in agreement with our finding that no HPr(His-P) was detectable in these cases. On the other hand, these observations raised the possibility that CCR is strong because dephosphorylation of HPr at His-15 makes the protein susceptible for HPr kinase–catalyzed phosphorylation, i.e. that EI and HPrK compete for phosphorylation of HPr. However, our experiments using mutants lacking EI were not in favor of this scenario (Fig. 13). Hence, the phosphorylation state of HPr at its His-15 residue is irrelevant for CcpA-mediated CCR in *B. subtilis*. This is very different to *E. coli*, where the absence of EI leads to permanent repression of secondary catabolic genes (Postma *et al.*, 1993). In *E. coli*, many substrates, which are taken up by the PTS cause CCR, because their transport not only de-phosphorylates the general PTS proteins EI and HPr, but also the $EIIA^{Glc}$ protein (Postma *et*

al., 1993). In contrast, PTS transport activity has no direct effect on CCR in *B. subtilis*. Our data using the constitutive HPrK-G158A allele (Fig. 14 and Table 5) demonstrate that low HPr kinase activity limits CCR by weakly repressing carbon sources. Hence, different HPrK/P activities account for the different repression levels exerted by the various substrates.
What are the molecular mechanisms that adjust the activity of HPrK/P to the available carbon source? The cells exhibited comparable growth rates on the different carbon sources except for succinate and ribose, on which growth was significantly slower (Table 4, last column). Therefore, it appears unlikely that the growth rate has a direct effect on HprK/P activity and therefore CCR. It is well known that the two antagonistic activities of HPrK/P are regulated by the concentrations of FBP, ATP and Pi. The central role of FBP for activity of the HPr kinase has been unequivocally proven *in vivo* and *in vitro*. *In vivo*, any mutation that prevents the formation of FBP results in a complete relief from CCR via CcpA, e.g. there is no CCR by glucose in mutants lacking the glycolytic enzymes glucose-6-phosphate isomerase or phosphofructokinase (Nihashi and Fujita, 1984). *In vitro*, HPr kinase activity is barely detectable below 1 mM FBP. With higher FBP concentrations HPr kinase activity sharply increases and reaches a plateau at about 5 mM FBP (Jault *et al.*, 2000; Reizer *et al.*, 1998). In our experiments we detected a low FBP concentration of 1.8 mM in the absence of a sugar (i.e. in CSE-medium), whereas in the presence of the various sugars, FBP concentrations in the range of 4.3 – 14.1 mM were detected. Therefore, all sugars generated FBP levels, which are theoretically sufficient for a high HPr kinase activity. This suggests that the different levels of repression exerted by the different carbohydrates cannot be explained by the different intracellular FBP concentrations alone. Therefore, in addition to FBP other metabolites might account for the substrate-dependent differences of HPrK/P activity. Indeed, for *Streptococcus bovis*, an inverse correlation between HPr(Ser-P) formation and the Pi concentration was observed *in vivo* (Asanuma and Hino, 2003). In addition, other metabolites like ATP, acetyl-phosphate and glyceraldehyde 3-phosphate were shown to modulate the activity of *B. subtilis* HPrK/P *in vitro* (Ramström *et al.*, 2003).

Hierarchical regulation is a wide-spread phenomenon if bacteria have the choice between different substrates. If only few substrates can be used, sophisticated regulatory networks with multiple transcription factors allow the consecutive expression of the respective enzymes. This was observed for the choice of the electron acceptors for *E. coli* respiration (Unden and Bongaerts, 1997). However, individual regulators for the repression of genes that are lower in the hierarchy cannot be employed if the bacteria have to choose between a plethora of substrates. Accordingly, in *E. coli* and *B. subtilis*, the hierarchy of

carbon sources in catabolite repression is established by a single signal, i.e. the control of the phosphorylation state of $EIIA^{Glc}$ and HPr, respectively ((Hogema *et al.*, 1998), this work).

4. Analysis of the *hprK* operon in *Bacillus subtilis*: Evidence for a role of a gene encoded downstream of *hprK* in carbon catabolite repression.

Author contributions:

This study was designed and interpreted by Kalpana Singh, Boris Görke and Jörg Stülke. All experiments were performed by Kalpana Singh.

Abstract

HPr Kinase/phosphorylase (HPrK/P) is an ATP dependent Ser/ Thr kinase and controls the phosphorylation state of the phospho-carrier protein HPr at its Ser-46 residue in *Bacillus subtilis* and other Gram-positive bacteria. Under conditions of good carbon supply HPr(Ser-P) is formed and activates the global repressor protein CcpA by direct interaction. The CcpA/HPr(Ser-P) complex represses numerous catabolic genes, a regulatory mechanism known as catabolite repression. In addition to its role in CCR, HPr is also part of the phosphotransferase system, which catalyzes the uptake of a variety of sugars. HPrK/P is encoded by the first gene of a penta-cistronic operon. Here, we analysed the putative functions of the genes of the *hprK* operon. Initially, we observed that a polar *hprK* mutation can not be complemented by the ectopic expression of *hprK*. In this mutant, CCR was drastically relieved, but exclusively on PTS substrates. In agreement, there was also a lower amount of HPr(Ser-P) present in the cell. On other carbohydrates, CCR was indistinguishable from the wild-type. Deletion analysis revealed that absence of *lgt*, the second gene of the operon, resulted in a slight relief from CCR regardless of the carbon source. Absence of the other genes downstream of *hprK* had no effect on HPrK/P activity. Since the *lgt* mutant and the mutant ectopically expressing *hprK* exhibited different CCR phenotypes, we conclude that over-expression of one of the genes downstream of *hprK* interferes with HPrK/P activity.

Introduction

Phosphorylation plays an important role in mediating signal transduction and regulation in bacteria (Hunter, 2000). A large number of physiological processes in bacteria are regulated by Ser/Thr kinases (Deutscher and Saier, Jr., 2005).The metabolite-controlled bifunctional HPr kinase/phosphorylase (HPrK/P) is one of the best studied Ser/Thr kinases in bacteria (Galinier *et al.*, 1998; Poncet *et al.*, 2004). HPrK/P triggers carbon catabolite repression (CCR) in *Bacillus subtilis* and other Firmicutes. CCR is a regulatory mechanism employed by bacteria in order to utilize the available nutrients in an economical way. In its natural habitat, *B. subtilis* often encounters a mixture of different carbon sources that can potentially be used. In this case, *B. subtilis* selectively utilizes the carbon source that permits the most rapid growth. For *B. subtilis* glucose is the preferred carbon source (Monod, 1942). During utilization of glucose, the genes required for the utilization of secondary substrates are not expressed. This phenomenon is referred to as CCR (Görke and Stülke, 2008).

HPrK/P catalyses both, the phosphorylation and dephosphorylation of the histidine-containing protein (HPr) and of its homologue Crh (catabolite repression HPr) at a serine residue (Ser-46) (Galinier *et al.*, 1998; Kravanja *et al.*, 1999). This phosphorylation triggers the interaction of HPr and Crh with the global transcriptional regulator protein CcpA (catabolite control protein A) (Schumacher *et al.*, 2004; Schumacher *et al.*, 2006; Seidel *et al.*, 2005). The resulting HPr(Ser-P)/CcpA and Crh(Ser-P)/CcpA complexes bind to operator sites on the DNA called *cre* (catabolite repression elements) (Weickert and Chambliss, 1990). In most cases, the *cre* sites are located in promoter regions. Binding of the CcpA complex usually abolishes promoter activity and thereby represses gene expression. *In vivo,* HPr is the relevant co-factor for CcpA, whereas Crh appears to be dispensable (Singh *et al.*, 2008).

In vitro experiments demonstrated that the activities of HPrK/P are allosterically controlled by metabolites. The kinase activity of *B. subtilis* HPrK/P is stimulated by fructose 1,6-bisphosphate (FBP), whereas the phosphorylase activity prevails in the presence of high inorganic phosphate (Pi) concentrations (Jault *et al.*, 2000). From *in vitro* experiments using *Enterococcus faecalis* HPrK/P, it was concluded that under *in vivo* conditions kinase activity might dominate when the concentrations of ATP and FBP are high in the cell, whereas the dephosphorylation should be prevalent when the concentration of Pi increases (Kravanja *et al.*, 1999)

HPr, the substrate of HPrK/P, is also a part of the phosphoenolpyruvate (PEP): carbohydrate phosphotransferase system (PTS). The PTS is an important carbohydrate uptake system in many bacteria. In this system, the general phosphotransferases EI and HPr transfer phosphoryl groups derived from PEP to the various sugar-specific Enzyme II (EII) transporters, which phosphorylate their substrates during the uptake step. In this case, HPr is phosphorylated at histidine-15 (His-15). The HPr homolog Crh lacks His-15 and is therefore unable to participate in sugar transport (Galinier *et al*., 1997). A recent analysis demonstrated that PTS-sugar transport, i.e. (de)phosphorylation of HPr at His-15 does not interfer *in vivo* with the CcpA-mediated pathway of CCR, i.e with phosphorylation of HPr at Ser-46 (Singh *et al.*, 2008).

In *B. subtilis,* gene *hprK* encoding the HPr kinase/phosphorylase, is the first gene of a penta-cistronic operon, which is conserved in many Firmicutes (Boël *et al.*, 2003). Downstream of *hprK*, the genes *lgt, yvoD, yvoE* and *yvoF* are present (Fig. 15) (Reizer *et al*., 1998; Deutscher, 2006). The second gene of the operon, *lgt,* codes for the prolipoprotein diacylglyceryl transferase, which catalyzes the first reaction of lipomodification of bacterial lipoproteins (Leskelä *et al.*, 1999). The lipid on lipoproteins is covalently bound to a cysteyl

residue and anchors the protein molecules to the outer surface of the cell membrane. The genes *yvoD* and *yvoE* code for an uncharacterized hydrophobic protein and a pyrophosphate, respectively. Sequence comparison indicates that *yvoE* encodes a homologue of the phosphoglycolate phosphatase of *E. coli*. Phosphoglycolate phosphatase in *E. coli* is involved in the dissimilation of the intracellular 2-phosphoglycolate formed in the DNA repair of 3'-phosphoglycolate ends (Teresa *et al.*, 2003). During the initial characterization of the protein encoded by *yvoE*, it was mistakenly assumed to be the HPr(Ser-P) phosphatase (Galinier *et al.*, 1998). Later it was established that dephosphorylation of HPr(Ser-P) is also catalyzed by HPrK/P, demonstrating that it is a bifunctional enzyme (Kravanja *et al.*, 1999). However, recent work suggested that YvoE might play yet another role in regulating the HPr(Ser-P) level in the cell. It was demonstrated *in vitro* that the dephosphorylation of HPr(Ser-P) by HPrK/P requires Pi as a substrate and generates pyrophosphate (PPi) as product (Mijakovic *et al.*, 2002). PPi can also act as phosphate donor for the phosphorylation of HPr by HPrK/P, at least *in vitro*. Therefore, it was suggested that *in vivo* the intracellular PPi level could affect the phosphorylation of HPr at Ser-46. However, it was demonstrated that YvoE has pyrophosphatase activity suggesting that this protein removes the pyrophosphate generated by the phosphorylase activity of HPrK/P (Mijakovic *et al.*, 2002). YvoE is not present in all Firmicutes, e.g. *Staphylococcus aureus* and *S. epidermis* lack the corresponding gene. However, these bacteria encode several YvoE homologues, which potentally could substitute for YvoE. The last gene of the *hprK* operon in *B. subtilis* is *yvoF*. The C-terminal half of the corresponding protein shares homology with acetyl transferases, including chloramphenicol acetyltransferase, serine acetyltranferase and thiogalactoside acetyltransferase (Reizer *et al.*, 1998). The function of this gene is unknown.

In this work, we analysed the putative roles of the genes encoded downstream of *hprK* for the activity and function of HPrK/P. Initially, we observed that a polar *hprK* mutation cannot be complemented by the expression of *hprK* from an ectopic locus. In this case, CCR triggered by a subset of carbohydrates, i.e. salicin, mannitol, fructose and glucose could not be restored. In contrast, other carbohydrates, e.g. gluconate, glycerol or sorbitol exerted CCR in this strain indistinguishable from the wild-type. A detailed deletion analysis revealed that mutation of *lgt* affects CCR, i.e. absence of this gene resulted in a slight relief from CCR regardless of the carbon source. Absence of the other genes downstream of *hprK* had no effect on HPrK/P activity. Since the *lgt* mutant and the mutant expressing *hprK* ectopically exhibited different CCR phenotypes, we conclude that over-expression of one of the genes downstream of *hprK* interferes with HPrK/P activity.

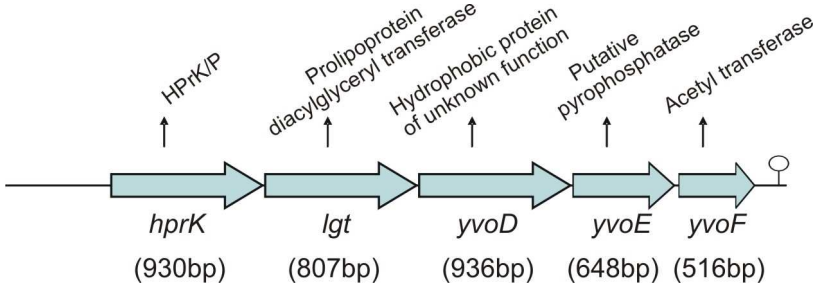

Figure. 15. Schematic representation of the *hprK* operon of *B. subtilis.* The protein product of each gene of the *hprK* operon is indicated by an arrow. The putative terminator is depicted by a lollipop (Reizer *et al.*, 1998). Numbers in parentheses indicate the length of each open reading frame in base pairs (ORF). The operon is drawn to scale.

Materials and Methods

Bacterial strains and growth conditions

The *B. subtilis* strains used in this study are listed in Table. 6. *E. coli* DH5α was used for plasmid propagation (Sambrook and Russel, 2001). *E. coli* was grown in Luria-Bertani medium and transformants were selected on plates containing ampicillin (100 μg ml^{-1}). *B. subtilis* was grown in CSE medium which is C minimal medium supplemented with 6 g/l potassium succinate and 8 g/l potassium glutamate (Martin-Verstraete *et al.*, 1995) and auxotrophic requirements (at 50 mg l^{-1}). Carbon sources were used at a concentration of 0.5% (w/v). LB and SP plates were prepared by addition of 17 g Bacto agar/l (Difco).

DNA manipulation

Transformation of *E. coli* and plasmid extraction was performed using standard procedures (Sambrook and Russel, 2001). Restriction enzymes and DNA polymerases were used as recommended by the manufacturers. Plasmid pGP634 was constructed by inserting a DNA fragment encompassing *hprK* and 700 bp of its upstream region,into the unique *Eco*RI- and *Sac*I- sites of plasmid pAC6 (Stülke *et al.*, 1997). The insert was amplified using primer SK3 and SK4. See table 7 for the list of oligonucleotides used in this study.

Construction of strains and characterization of phenotypes

Strains carrying deletion of the various genes of the *hprK* operon were generated using the Long Flanking homology PCR protocol as described previously (Wach, 1996). Briefly, cassettes carrying the kanamycin resistance gene were amplified from plasmid pDG780 using primers kan cassette fwd and kan cassette rev (Guerout-Fleury *et al.*, 1995). Next DNA

fragments of ~1000 bp were amplified carrying the sequences upstream and downstream of the region to be deleted, respectively. See Table. 7 for the oligonucleotides used for this purpose. The joining of the two fragments to the resistance cassette was performed in a second PCR as described previously (Jordan *et al.*, 2006). The resulting PCR products were used to transform *B. subtilis* strains GP270 (*ΔxylR)*. The integrity of the regions flanking the integrated resistance cassettes was verified by sequencing PCR products of ~1000 bp, which were obtained by PCR using chromosomal DNA of the respective mutants. The resulting strains were GP851 *(ΔxylR,* [*Δlgt, ΔyvoD, ΔyvoE, ΔyvoF*]), GP852 (*ΔxylR, ΔyvoE*), GP859 (*ΔxylR,* [*ΔyvoE, ΔyvoF*]) and GP861 (*ΔxylR,* [*Δlgt, ΔyvoD, ΔyvoE, yvoF*]).

Strain GP290 carrying the *hprK* gene ectopically integrated in the *amyE* locus was constructed by transformation of strain GP270 (*ΔxylR*) with plasmid pGP634, which was linearized by *Sac*I digestion. Plasmid pGP634 allows integration of the *hprK* gene into the *amyE* locus by a double crossing over. On this plasmid the cloned insert is flanked by sequences corresponding to the *amyE*-5' and *amyE*-3' regions. The *amyE* locus codes for non-essential α-amylase and can be used for integration of foreign DNA. A successful recombination disrupts the *amyE* gene, which was confirmed by an iodine/starch test for amylase activity. For this purpose strain GP290 was grown on SP medium supplemented with hydrolyzed starch (10 g/ l). Starch degradation was detected by sublimating iodine onto the plates.

Table. 6. *B. subtilis* strains used in this study

Strain	Genotype	Source[a]
168	*trpC2*	Laboratory stock
GP270	*trpC2 xylR::ermC*	Singh *et al.*, 2008
GP289	*trpC2 xylR::ermC ΔhprK::aphA3*	Singh *et al.*, 2008
GP290	*trpC2 xylR::ermC ΔhprK::aphA3 amyE:: hprK cat*	pGP634 → GP289
GP851	*trpC2 xylR::ermC Δ lgt-yvoF::aphA3*	LFH PCR product → GP270
GP852	*trpC2 xylR::ermC Δ yvoE::aphA3*	LFH PCR product → GP270
GP859	*trpC2 xylR::ermC ΔyvoE-yvoF::aphA3*	LFH PCR product → GP270
GP861	*trpC2 xylR::ermC ΔyvoD-yvoF::aphA3*	LFH PCR product → GP270

[a] Arrows indicate construction by transformation

Table. 7. List of oligonucleotides used in this study

Oligonucleotides[a]	**Sequence(5'-3')**[b]
SK3	5'GGCGAATT**C**GTGTAACAATTTTGATCAGTCCG
SK4	5'GGCGAGCTCTATTCTTCTTGTTCACCGTCTT
RNA probe	
SK13	5'ACGCTTATTAATACGAATGAATTA
SK14	*CTAATACGACTCACTATAGGGAGA*CTCATTTTCTCAAGCATAAA
LFH Oligonucleotides	
kan cassette fwd	CAGCGAACCATTTGAGGTGATAGG
kan cassette rev	CGATACAAATTCCTCGTAGGCGCTCGG
kan check fwd	CATCCGCAACTGTCCATACTCTG
kan check rev	CTGCCTCCTCATCCTCTTCATCC
Deletion of *lgt-yvoF*	
SK19; up fwd	5'GTGGCAAAGGTTCGCACAAAAGACGT
SK20; up rev	**CCTATCACCTCAAATGGTTCGCTG**AACGCCAACTCCTATTCTTCTTG
SK21;down rev	CTGAGCAAATACGACAATATTCATGCTTTT
SK22; down fwd	**CGAGCGCCTACGAGGAATTTGTATCG** CATCAGCGGACTTTTTTTGTTAAAATT
Deletion of *yvoE*	
SK23; up fwd	ATGAAGAAGATCTTTCTGGCCGGTC
SK24; up rev	**CCTATCACCTCAAATGGTTCGCTG**TCATATTGCTTCCTTTCCAACCGC
SK25; down fwd	**CGAGCGCCTACGAGGAATTTGTATCG** GTGAGAAAAACAGATCGTCATCCGGTCTCG
SK26; down rev	ATGGGAAGATGTTGGTGAGGATGCG
Deletion of *yvoD-yvoF*	
SK38; up fwd	ATGAATGAAGCGATAGAACCACTCAATCCGATAGCAT
SK39; up rev	**CCTATCACCTCAAATGGTTCGCTG**GTCTACTCCGCGTACCGCTCCTTCG AGTATC

[a]up rev= Reverse primer for the amplification of the region upstream of the gene to be deleted. Same applies for down fwd and down rev, the fragment amplified is downstream of the gene to be deleted.

[b] Restriction sites are underlined; Sequences complementary to the resistance cassette for LFH PCR are marked in bold; sequence in italics indicates the T7 promoter region.

Transformation and enzyme assays

B. subtilis was transformed using the two step protocol (Kunst and Rapoport, 1995). Transformants were selected on SP plates containing kanamycin (5 μg ml^{-1}) or erythromycin plus lincomycin (2 μg ml^{-1} and 25 μg ml^{-1} respectively). Cells were harvested in exponential

growth phase at an OD_{600} of 0.6-0.8. β-xylosidase activities were measured in cell extracts using p-nitrophenyl xyloside as substrate (Lindner *et al.*, 1994).

Western blot analysis

For western blot analysis crude cell extracts were prepared and were separated by either SDS or native PAGE, depending on the experimental requirements and transferred to a polyvinylidene difluoride membrane (PVDF, BioRad). The proteins were detected with rabbit polyclonal antisera against HPr, RocG, HPrK of *B. subtilis* or *B. megaterium* respectively (Commichau *et al.*, 2008; Hanson *et al.*, 2002; Monedero *et al.*, 2001). The purified strep-tagged RocG was kindly provided by Fabian Commichau. The antibodies were visualized by using anti-rabbit IgG-AP secondary antibodies (Promega, Madison, and U.S.A) and the CDP star detection system (Roche Diagnostics).

Phosphorylation state of HPr *in vivo*

HPr phosphorylation was assayed by Western blot analysis as follows**.** Cells were grown in CSE minimal medium supplemented with 0.5% glucose to an O.D of 0.6 followed by an addition of 12M HCl to adjust the pH of culture to 4.5. Cells were disrupted using a French press, and crude extracts were prepared as described before (Ludwig *et al.*, 2002). Proteins were loaded on a 10% native polyacrylamide (PAA) gel, allowing the separation of phosphorylated forms of protein from the non-phosphorylated ones. To distinguish the HPr(His-P) from HPr(Ser-P), we took advantage of the heat instability of the phospho-amide bond. An aliquot of each crude extract was incubated at 70°C for 10 min before separation on PAA gel. After electrophoresis, the proteins were blotted to a PVDF membrane. The different forms of HPr were detected using antibodies directed against *B. subtilis* HPr (Monedero *et al.*, 2001).

Northern analysis

RNA was prepared by the modified 'mechanical disruption protocol' described previously (Ludwig *et al.*, 2001). Briefly, 20 ml of cells were harvested at the exponential phase. After mechanical cell disruption, the frozen powder was immediately re-suspended in 3 ml lysis buffer [4 M guanidine isothiocyanate, 0.025 M sodium acetate, pH 5.3, 0.5% *N*-laurylsarcosine (wt/vol)]. Subsequently, total RNA was extracted using the RNeasy Mini kit according to the manufacturers protocol (Qiagen, Germany). Digoxigenin-labelled RNA probes directed against the *yvoE* transcript were obtained by *in vitro* transcription using T7

RNA polymerase (Roche Diagnostics) and a DNA-fragment as template, which was obtained by PCR using primers SK13 and SK14. The reverse primer SK14 contained a T7 RNA polymerase recognition sequence. *In vitro* RNA labelling, hybridization and signal detection were carried out according to the instructions of the manufacturer (DIG RNA labelling kit and detection chemicals; Roche Diagnostics). Total RNA was separated on a 0.8% agarose gel followed by transfer to a nylon membrane. The desired signals were detected after hybridisation with the RNA probe directed against *yvoE*.

Results

Construction of a strain, which expresses *hprK* ectopically from the *amyE* locus

We wanted to determine whether a deletion of the *hprK* gene can be complemented by the ectopic expression of *hprK* from a locus *in trans*. For this purpose, we constructed a mutant in which *hprK* was expressed from an ectopic location under the control of its native promoter while the native copy of the gene was absent. To achieve this, the *hprK* gene including 700 base pairs upstream was inserted into the *amyE* locus on the chromosome. At the same time, 209 codons of the endogenous *hprK* gene were deleted and replaced by a kanamycin cassette. To confirm, that *hprK* was indeed expressed from its ectopic location in this mutant (strain GP290), we performed a Western blot analysis using antiserum directed against HPrK/P. A set of three independent experiments suggested that similar amounts of HPrK/P is present in GP290, which expresses the *hprK* from the ectopic locus and the corresponding wild type

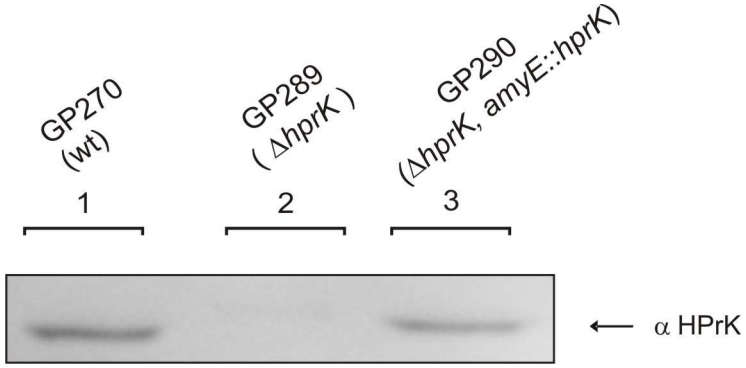

Figure. 16. Western blot to confirm the expression of the ectopic *hprK* gene in strain GP290. The strains carrying the mutations as indicated at top were grown in CSE minimal medium supplemented with 0.5% glucose and were harvested in the logarithmic phase (OD_{600} 0.6-0.8). In each case, 15 µg of total cell protein were separated on a 10% SDS-PAA-gel and blotted onto PVDF membrane. HPrK/P was detected using antibodies raised against HPrK/P. No signal was detected in strain GP289 (lane2), confirming the absence of HPrK/P.

strain GP270 (Fig. 16., compare lanes 1 and 3). No HPrK/P was detectable in the un-complemented *ΔhprK* mutant GP289 (Fig. 16, lane 2). In conclusion, the ectopic *hprK* gene is properly expressed.

The ectopic expression of *hprK* does not restore CCR in the *hprK::aphA3* mutant

In strains lacking a functional HPrK/P, gene expression is completely relieved from CCR, i.e. the repressive effect of preferred carbon sources on the expression of secondary catabolic genes is abolished (Galinier *et al.*, 1998; Hanson *et al.*, 2002; Martin-Verstraete *et al.*, 1999; Reizer *et al.*, 1998; Singh *et al.*, 2008). To check if the ectopically expressed HPrK/P is functionally active, we determined the carbon catabolite repression β-xylosidase (XynB) activity by different carbon sources. We have previously shown that activity of XynB is a perfect reporter for the CcpA-mediated pathway of CCR (Singh *et al.*, 2008). However, this requires deletion of the *xylR* repressor gene, which encodes the dedicated substrate dependent regulator of *xynB* expression. In *xylR* mutants, the expression of *xynB* exclusively relies on the activity of CcpA. Therefore, the *xylR* gene was deleted in all strains used throughout this study. The *ΔxylR* single mutant served as the "wild-type" control in these experiments.

As can be seen from the data presented in Table. 8, the wild-type strain produced high XynB activities in CSE-medium, i.e. when succinate, which exerts no CCR, was the single carbon source. The presence of an additional carbohydrate reduced the XynB activity (Table 8, column 1). Glucose, fructose and mannitol had the strongest repressing effect, whereas repression by other carbon sources was weaker, as reported recently (Singh *et al*., 2008). In the *ΔhprK* mutant high XynB activities were detected in all cases, reflecting the release from CCR (Table 8, column 2; Singh *et al*., 2008).

In the [*ΔhprK amyE::hprK*] mutant, which expresses *hprK* ectopically, CCR was perfectly restored when the cells grew on arabinose, maltose, gluconate, glycerol or sorbitol (Table 8, compare columns 1 and 3). Surprisingly, CCR was not restored to wild-type levels in the presence of salicin, mannitol, fructose or glucose. In these cases, a 5- to 14-fold relief from CCR was still detectable. In conclusion CCR of XynB activity was not restored on those substrates, which exert the strongest repression in the wild-type strain.

It could be possible that reduced CCR observed in the [*ΔhprK amyE::hprK*] mutant is confined to the *xyn* operon only. To address this possibility, we investigated CCR of *rocG* expression. The *rocG* gene codes for the enzyme glutamate dehydrogenase, RocG (Belitsky and Sonenshein, 1998). The expression of *rocG* is induced by arginine and strongly repressed.

Table. 8. Catabolite repression of β-xylosidase by different carbon sources in various mutants. Standard deviations are shown in parentheses except for the measurements done once.

β-xylosidase activity in units/mg of protein			
Carbon source[a]	GP270 wild-type	GP289 *ΔhprK*	GP290 *ΔhprK, amyE: hprK*
-	1585(560)	2287 (218)	2317(112)
Arabinose	600(153)	1257(70)	680(96)
Maltose	489(32)	2023(408)	665(77)
Gluconate	201(31)	1163(170)	286(35)
Glycerol	96(14)	1273(160)	98(4)
Sorbitol	121(29)	748(61)	186(2)
Salicin	175(14)	2743(667)	838
Mannitol	83(17)	1689(269)	958
Fructose	66(6)	1638(32)	340(44)
Glucose	44(10)	1679(421)	610(270)

[a]added to CSE medium (0.5%).

in the presence of preferred carbon sources such as glucose. This glucose-mediated CCR depends on CcpA (Belitsky *et al.*, 2004). To study CCR of the *rocG* gene, the various mutant strains were grown in arginine-containing CSE-medium in the absence or presence of glucose. The cells were harvested and after separation of the protein extracts by SDS-PAGE, a Western blot analysis using antibodies directed against RocG was performed. As expected from previous data (Commichau *et al.*, 2007), RocG was readily detectable in the wild-type strain, but its synthesis was strongly repressed upon addition of glucose (Fig. 17, compare lanes 1 and 2). The repression by glucose was abolished in the *ΔhprK* mutant (Fig 17, compare lanes 3 and 4). The same result was obtained for the [*ΔhprK amyE: hprK*] mutant, which expresses *hprK* from the *amyE* locus. This demonstrates that the weak CCR by glucose

observed in this mutant is not restricted to *xynB*, but does also apply to other genes such as *rocG*.

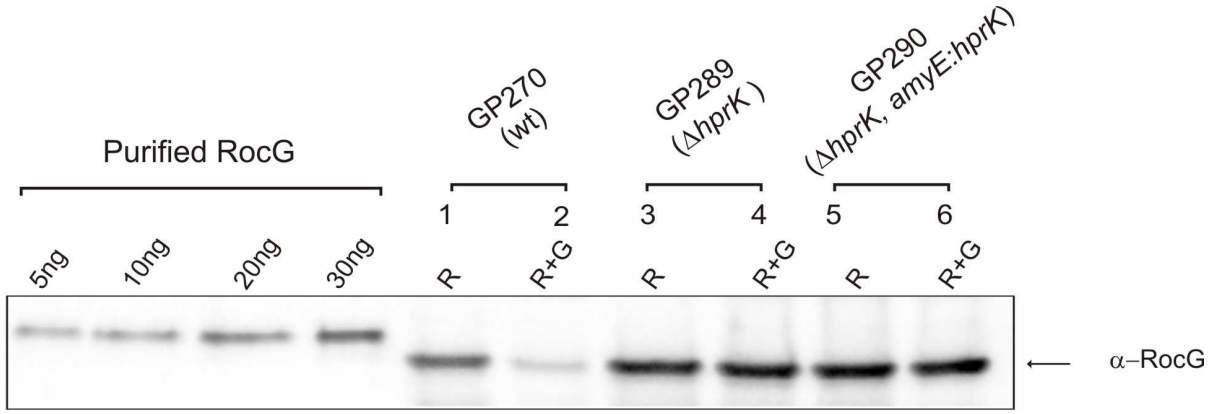

Figure. 17. Western blot analysis to determine CCR of RocG synthesis in various mutants. 15 µg total cell protein were separated on a 10% SDS-PAA-gel and blotted onto a PVDF membrane. The specific signal was detected using antibodies raised against purified *B. subtilis* RocG (Commichau *et al.*, 2008). Strains were grown in CSE minimal medium supplemented with 0.5% arginine (R) in the absence or presence of 0.5% w/v glucose (G). Purified Strep tagged RocG loaded in various amounts (5- 30 ng) served as control.

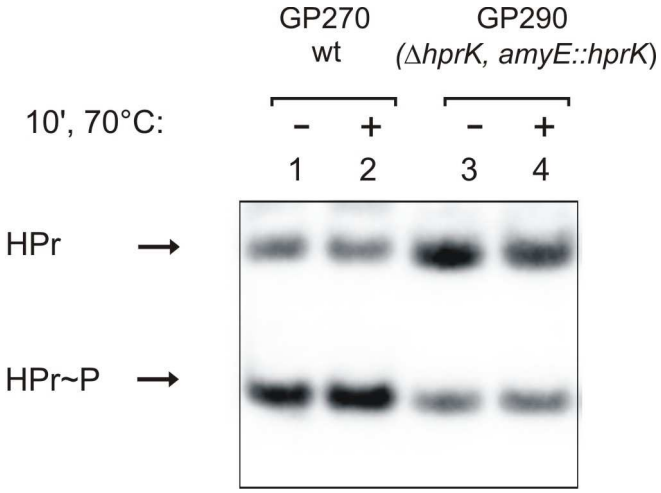

Figure. 18. Determination of the phosphorylation state of HPr in strains GP270 and GP290. Cells were grown in CSE + glucose and harvested at logarithmic phase as described before (Singh *et al.*, 2008). 1µg of total cell protein, each was separated on a 10% native PAA gel and HPr was detected by immuno-blotting in a western blot analysis. To discriminate between HPr(Ser-P) and HPr(His-P), an aliquot of each cell extract was heated at 70 °C prior to loading (even numbered lanes). Histidine phosphorylation is heat labile and thus the two singly phosphorylated forms of HPr can be easily distinguished.

Formation of HPr(Ser-P) is impaired in the mutant expressing *hprK* from an ectopic site

HPrK/P triggers CCR by phosphorylating the corepressors of CcpA (HPr and Crh). One possible explanation for the weak CCR by certain carbohydrates in the [*ΔhprK amyE: hprK*] mutant could be that the ectopically expressed HPrK/P is less active as a kinase as compared to its counterpart in the wild-type strain. In order to check this possibility, the phosphorylation state of HPr was assayed. For this purpose, cells were grown in CSE + glucose and protein extracts were separated on a native PAA-gel followed by Western blotting using antiserum directed against HPr (See Materials and Methods and Singh *et al*., 2008 for details on the procedure). In agreement with a recent publication (Singh *et al*., 2008), 56 % of the HPr molecules were phosphorylated at Ser-46 (Fig. 18, compare lane 1 and 2). In contrast, the [*ΔhprK amyE: hprK*] mutant, which expresses *hprK* ectopically, produced only 34 % HPr(Ser-P) (Fig. 18, compare lanes 3, 4 with lanes 1, 2). This observation clearly establishes that a lower amount of HPr(Ser-P) is produced in the [*ΔhprK amyE: hprK*] mutant in comparison to the wild-type, explaining the weaker CCR.

Is there a polar effect of the *hprK::aphA3* mutation on genes located downstream in the *hprK* operon?

Strain GP290 harbours the *hprK* gene in the *amyE* locus while most of the native *hprK* gene is deleted and replaced by the *aphA3*-kanamycin cassette (Fig. 19b) (Martin-Verstraete *et al.*, 1999). The DNA fragment used to replace the *hprK* gene contains the *aphA3* gene under control of its own promoter, which reads into the same direction as the authentic promoter of the *hprK* operon. In principle, it is conceivable that this arrangement generates polar effects on the expression of the genes located downstream in the *hprK* operon. This could account for the observed relief of CCR in strain GP290, provided that one of the genes downstream of *hprK* has a role in CCR. In principle, two different scenarios can be imagined:

1. The presence of the *aphA3*-promoter might generate a second transcript in addition to the transcript started at the authentic *hprK* promoter (Fig.19b). This could elevate the expression level of the genes encoded downstream of *hprK*.
2. The insertion of the *aphA3* cassette yields naked stretches of mRNA, which are not covered by ribosomes, e.g. the 3' sequence of the *hprK* gene following the *aphA3* resistance gene is not translatable. Non-translated RNAs are often rapidly degraded by RNases or may be subject to the formation of secondary structures that may block the translation of genes. Hence, this scenario would reduce expression of the downstream genes.

In order to explore whether the presence of the *aphA3* cassette affected the transcription of the genes encoded downstream of *hprK*, we performed a Northern analysis. The various strains were grown in CSE-medium supplemented with glucose and total RNA was isolated from cells grown to exponential phase and subsequently separated on a denaturating agarose gel and blotted onto a nylon membrane. Transcripts of the *hprK* operon were detected using an RNA probe directed against *yvoE* RNA. As expected, a single prominent signal of a size of ~4kb was detectable in the wild-type strain GP270 (Fig. 20, lane 1). This confirms, that the genes of the *hprK* operon are co-transcribed as a penta-cistronic mRNA from a single promoter located upstream of *hprK* (Fig. 19a). A transcript of similar length was also detectable in strains GP289 and GP290 carrying the *hprK::aphA3* cassette (Fig.20, lanes 2 and 3). However, in these strains an additional transcript of ~4.8 kb was also detectable. These results are in agreement with the scenario described in Fig. 19b, i.e. the *aphA3* promoter as well as the authentic *hprK* promoter contributes both to transcription of the genes downstream of the *aphA3* cassette. These results demonstrate that the genes *lgt-yvoF* are properly transcribed in the *hprK::aphA3* mutant, although the overall transcript level appears to be somewhat higher in comparison to the wild-type strain GP270. In conclusion, the results are in favour with a stronger transcription of genes *lgt-yvoF* in the strains carrying the *hprK::aphA3* cassette, although an aberrant translation of these genes cannot be excluded.

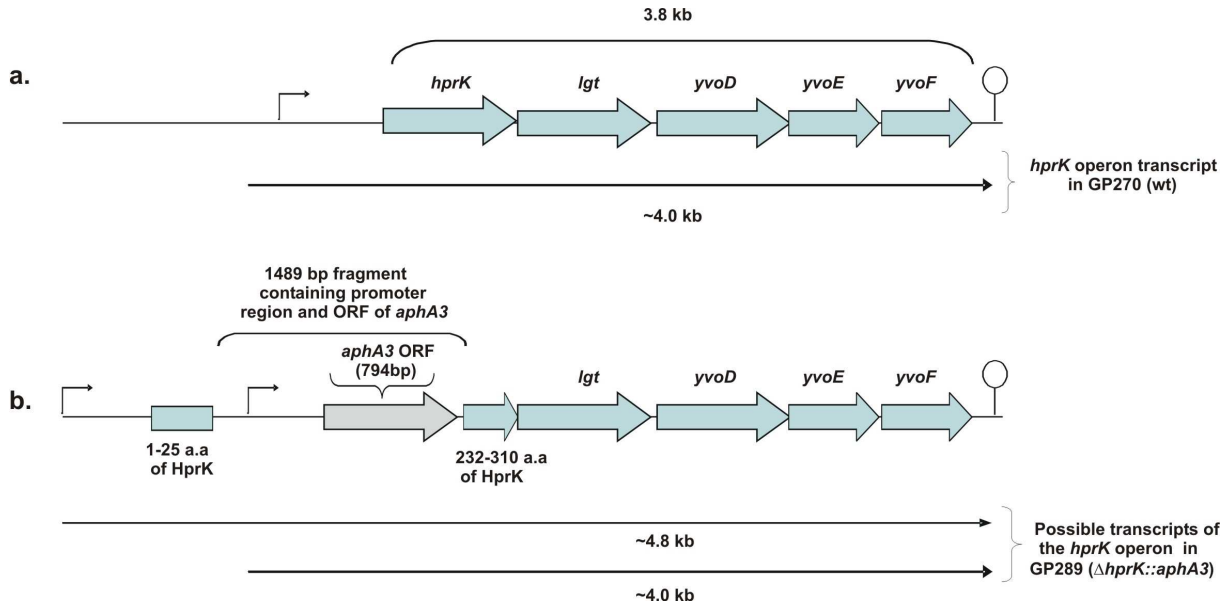

Figure. 19. Possible effects of the *hprK::aphA3* allele on the expression of the genes downstream of *hprK*. (a) The length of the expected transcript in wild-type strain GP270 originating from the native promoter. (b) Due to the insertion of the *aphA3* cassette, the length of the transcript originating from native promoter would increase by approximately 800 bp in strain GP290. An additional transcript is expected in strain GP290, originating from the promoter of the *aphA3* cassette.

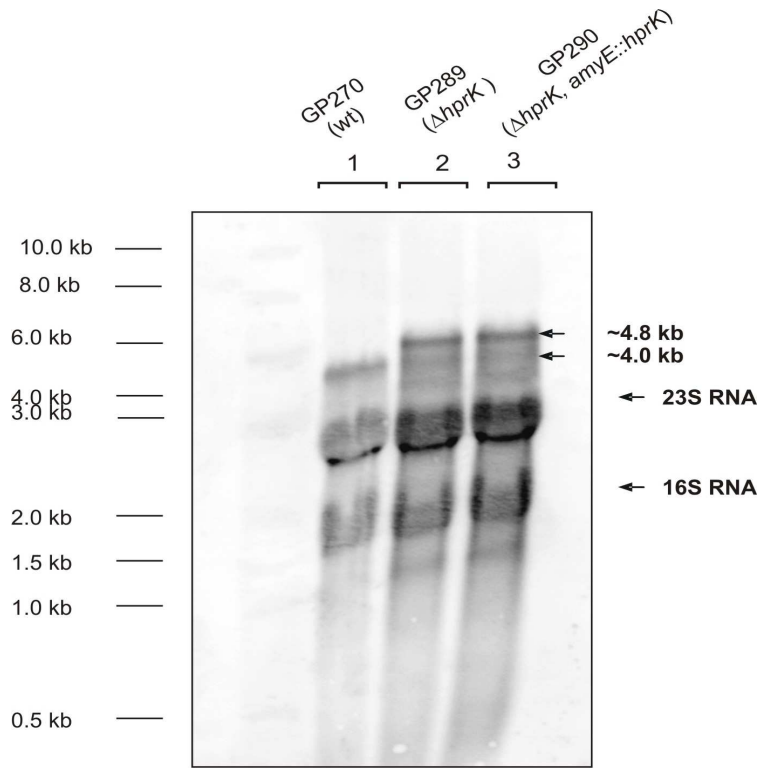

Figure. 20. Northern blot analysis of the *hprK* operon. Cells were grown in CSE minimal medium supplemented by 0.5 % of glucose. Total RNA was separated by electrophoresis on a 0.8% agarose gel and then blotted onto nylon membrane followed by hybridization with a riboprobe specific for *yvoE* mRNA. The specific signals detected by the *yvoE* riboprobe are marked by an arrow. 5µg of total RNA were loaded in each lane.

Deletion analysis of the *hprK* operon reveals that gene *lgt* is required for an undisturbed CCR

Our data raised the possibility that the disturbed CCR observed in the [*ΔhprK amyE: hprK*] strain, which expresses *hprK* ectopically, results from an altered expression level of the gene(s) downstream of the *hprK::aphA3* cassette. This suggests, that one or more of these genes i.e. *lgt*, *yvoD*, *yvoE* or *yvoF* might have a role in CCR. In order to identify these gene(s), we constructed several deletion mutants lacking one or more of these genes. In these mutants, the corresponding genes were replaced by the *aphA3* (kanamycin) resistance cassette, whereas the authentic *hprK* gene remained intact. These constructions resulted in strains GP851 (Δ*lgt*, Δ*yvoD*, Δ*yvoE*, Δ*yvoF*), GP859 (Δ*yvoE*, *yvoF*) and GP861 (Δ*yvoD*, Δ*yvoE*, Δ*yvoF*). Since a previous study suggested a role of YvoE in CCR (Mijakovic *et al.*, 2002), we also constructed a *ΔyvoE* single mutant (strain GP852). Subsequently, the strains were grown on different carbon sources and the β-xylosidase (XynB) activities were determined (Table. 9). The strains GP852, GP859 and GP861 produced XynB activities very similar to the wild-type (Table. 9, compare column 1 with columns 3-5). Therefore, the

absence of genes Δ*yvoD*, Δ*yvoE and* Δ*yvoF* has no effect on CCR of XynB activity. In contrast, in strain GP851 lacking all four genes *lgt*, *yvoD*, *yvoE* and *yvoF*, a two- to three-fold relief from CCR was detectable, regardless of the carbon source included in the growth medium (Table 9, compare columns 1 and 2). The only difference between strains GP851 and GP861 is the additional lack of gene *lgt* in GP851. Hence, gene *lgt* is required for an undisturbed CCR, at least in a situation in which the three genes following *lgt* are also absent. Moreover, it can be concluded that the absence of the pyro-phosphatase YvoE has no impact on CCR, at least under the experimental conditions used in his work.

Table. 9. Catabolite repression of β-xylosidase by different carbon sources in various mutants. Standard deviations are shown in parentheses except for the measurements done once.

	β-xylosidase activity in units/mg of protein				
Carbon source[a]	GP270 wild-type	GP851 Δ*lgt-yvoF*	GP852 Δ*yvoE*	GP859 Δ*yvoE-yvoF*	GP861 Δ*yvoD-yvoF*
Arabinose	600(153)	1292(13)	598(20)	660	684
Maltose	489(32)	1153(669)	570	n.d	n.d
Gluconate	243(76)	454(128)	324(70)	n.d	n.d
Sorbitol	121(29)	238(40)	126	n.d	n.d
Glycerol	96(14)	104(10)	81(3)	n.d	n.d
Mannitol	83(17)	523	93	94	90
Fructose	66(6)	75(8)	74(22)	n.d	n.d
Glucose	44(10)	140(70)	65(22)	40(13)	48(26)

[a]added to CSE medium (0.5%), n.d = Not determined

Discussion

HPrK/P plays a key role for CCR in *B. subtilis*: It triggers the phosphorylation of HPr at Ser-46 in response to the metabolic state in the cell. HPr(Ser-P) serves as co-factor for the global transcriptional regulator CcpA, which represses secondary catabolic genes in the presence of preferred substrates. HPrK/P is encoded by the first gene of a penta-cistronic operon. Little is known about the functions of the four genes co-transcribed with *hprK*. In this study, we provide evidence that at least one of these genes might also play a direct or indirect role in CCR.

Initially, we observed that a deletion of the authentic *hprK* gene could not be complemented by *hprK* expression from an ectopic locus. The respective mutant exhibited a partial relief from CCR, when grown on glucose, fructose, mannitol or salicin. Interestingly, CCR by other carbon sources was not affected. Since the native *hprK* gene was replaced by an *aphA3* antibiotic resistance cassette reading into the genes downstream, we speculated that a polar effect was responsible for the aberrant CCR in this strain. Indeed, Northern analysis demonstrated the presence of two transcripts: One started from the authentic *hprK* promoter and another one initiated from the foreign promoter preceding the antibiotic resistance cassette. As a result, the amounts of the *lgt-yvoF* transcript present in this mutant are higher than in the wild-type strain. These observations suggest that a higher expression level of one or more of the genes downstream of the resistance cassette was responsible for the partial loss of CCR.

To learn more about the putative roles of the proteins encoded in the *hprK* operon, we constructed mutants lacking one or more of the genes present downstream of *hprK* in the same operon. These experiments yielded several insights:

First, it turned out that the absence of *yvoE* had no effect on CCR. Gene *yvoE* encodes a protein exhibiting pyro-phosphatase activity *in vitro* (Mijakovic *et al*., 2002). HPrK/P uses P_i as substrate for the dephosphorylation of HPr(Ser-P) and thereby produces PP_i as product. It was proposed that the so-generated pyro-phosphate is hydrolyzed by YvoE. Furthermore it was shown that *in vitro* PP_i can substitute for ATP as substrate for the HPrK catalyzed phosphorylation of HPr. In addition, P_i was reported to inhibit the kinase activity and to stimulate the phosphorylase activity of HPrK/P *in vitro* (Fieulaine *et al*., 2002; Mijakovic *et al*., 2002). In this respect, a *yvoE* mutation should generate, if there is any effect at all, a higher cellular level of PP_i and perhaps less P_i. These conditions would stimulate the kinase activity of HPrK/P and thereby generate a stronger CCR. The absence of any effect of an *yvoE* mutation on CCR, as shown in this work, is may be due hydrolysis of PPi by other possible phosphatases, which can also utilize PPi, like YvoE.

Second, our results let us conclude that *yvoD* and *yvoF* have no essential function for CCR. The deletion of these genes had no detectable effect on CCR (Table 9). The putative functions of these genes remain obscure.

Finally, the data suggested that a functional *lgt* gene might be necessary for an undisturbed CCR: No effect on CCR could be detected in strains lacking the last three genes of the *hprK* operon. However, when *lgt* was additionally deleted, a partial relief from CCR could be detected. We cannot rule out yet, that in addition to *lgt* one of the three downstream

genes must be also absent to yield the observed phenotype. Future studies using a *lgt* single mutant should address this point and clarify whether absence of *lgt* is sufficient for the observed relief from CCR. In any case, the data demonstrate that presence of gene *lgt* is required for CCR, at least in the observed mutant background. Gene *lgt* encodes the prolipoprotein diacylglycerol transferase that catalyzes the first step in the lipomodification of bacterial lipoproteins (Leskelä *et al*., 1999). This modification anchors peripheral membrane proteins to the outer surface of the cytoplasmic membrane. There are three groups of lipoproteins known in Gram-positive bacteria. These include the substrate-binding domains of ABC transporters, proteins necessary for adhesion to various surfaces and proteins with enzymatic activity e.g. β-lactamases (Leskelä *et al*., 1999 and references therein). In *E. coli lgt* is an essential gene. In *B. subtilis* an *lgt* mutant is viable but disturbed in protein secretion and spore germination (Igarashi *et al.*, 2004; Leskelä *et al.*, 1999).In respect of all these findings, it is difficult to imagine how *lgt* could affect CCR. None of the transporters involved in the uptake of the carbohydrates tested in this study, is known to be a lipoprotein. Most likely, the *lgt* mutation affects CCR indirectly by the modification of the membrane composition and cell surface.

The phenotypes of the *Δlgt-yvoF* mutant and the mutant expressing the ectopic *hprK* copy were dissimilar. The *Δlgt-yvoF* mutant exhibited a partial relief from CCR (~2- to 4-fold) on all carbon sources. In contrast, in the ectopically complemented *hprK* mutant there was a much stronger relief from CCR, i.e. 5-14-fold, but only on a certain subset on carbohydrates: All these substrates, i.e. glucose, fructose, mannitol and salicin are transported by the PTS. On Non-PTS-substrates, CCR was unaffected. These different phenotypes make it rather unlikely that a diminished synthesis of genes *lgt-yvoF* is responsible for this selective relief from CCR. In contrast and in agreement with the Northern blot data, it appears conclusive that this phenotype is caused by the increased expression of genes *lgt-yvoF*, present downstream of the *hprK::aphA3* cassette.

How can the selective relief from CCR in the ectopically complemented *hprK* mutant be explained? We made the observation that the level of HPr(Ser-P), the active co-factor for CcpA, is significantly lower in this mutant in comparison to the wild-type. Hence, it is obvious, that the (de)phosphorylation step of HPr by HPrK/P is affected. Theoretically, it is conceivable that the over-expression of YvoE might affect CCR. YvoE removes pyrophosphate, which is also a substrate of HPrK kinase. This could lower HPrK kinase activity in the cell. However, such a mechanism should be operative independent of the carbon source utilized by the bacteria, which makes this explanation unlikely. An alternative

explanation might take into account that all carbohydrates, on which CCR was impaired, are substrates of the PTS. Hence, it is conceivable that transport of these substrates is negatively affected, which would result in lower amounts of FBP, which is required for the allosteric activation of HPr kinase. Indeed, the ectopically complemented *hprK* mutant grows significantly slower on these PTS substrates in comparison to the wild-type, which is in support with this idea (our unpublished observations). Future transport studies may clarify this point.

5. Discussion

5.1 Role of PrpC in regulating the phosphorylation status of HPr

So far, HPrK/P and EI were the only enzymes known to be involved in the (de)phosphorylation of HPr in Gram-positive bacteria. However, certain arguments suggest that there might be a need in *B. subtilis* for an additional protein that acts antagonistically to the kinase activity of HPrK/P. *In vitro* studies suggest that the ATP dependent kinase activity of *B. subtilis* HPrK/P is allosterically activated by FBP. In the presence of 10 mM FBP and 5 mM ATP, it can completely phosphorylate HPr in an 85 molar excess (Jault *et al.*, 2000). *B. subtilis* cells grown in the presence of glucose contain 14 mM FBP. However, 30-40% of the total HPr remains unphosphorylated. The majority is phosphorylated at Ser-46 (Monedero *et al.*, 2001; Singh *et al.*, 2008). The other substrate for HPrK/P, ATP, has also been found, *in vivo*, to be within the range of *in vitro* experiments performed. During the uptake of glucose by *L. lactis*, the concentration of ATP is about 8 mM (Neves *et al.*, 1999). Hence, there is a contradiction between the *in vivo* and the *in vitro* data. Having HPr completely phosphorylated at serine residue is not advantageous for the cell. It would inhibit further PTS-mediated uptake of glucose because no histidine phosphorylated HPr is available for phospho transfer (Monedero *et al.*, 2001). Thus, there should be a mechanism to achieve limit phosphorylation of HPr at its serine residue even in the presence of the most preferred carbon source which is glucose.

There are several possibilities that could explain the *in vivo* pattern of HPr(Ser-P) in the presence of glucose: a) The intracellular ratio of HPrK/P to the HPr molecules is much lower in comparison to the protein concentrations used in *in vitro* experiments. Therefore, only 60-70% of HPr can be phosphorylated at Ser-46. This possibility appears to be less likely because artificially increased intracellular amounts of HPrK/P protein did not alter the phosphorylation pattern of HPr, when the cells grew on glucose (see supplementary material, Fig. 23) b) A so far unidentified phosphatase may counteract the effect of phosphorylation achieved by HPrK/P.

Recently, Halbedel, *et al* demonstrated that in *M. pneumoniae* PrpC dephosphorylates Ser-46 phosphorylated HPr. *M. pneumoniae* is classified as a Gram-positive bacterium belonging to the genus *Mollicutes*, which possesses the smallest genome among bacteria. Interestingly, HPrK/P and PrpC are among the few regulatory proteins which *M. pneumoniae* retained (Himmelreich *et al.*, 1996). This prompted me to analyse the role of PrpC in regulating the phosphorylation state of HPr in *B. subtilis*. Indeed, in an *in vitro*

dephosphorylation reaction, PrpC could dephosphorylate Ser-46 phosphorylated HPr (Singh *et al.*, 2007).

PrpC of *B. subtilis* belongs to the PPM family of phosphatases and is a Mn^{+2} ion dependent Ser/Thr phosphatase (Obuchowski *et al.*, 2000). The members of PPM family are widely distributed and are represented by protein phosphatase 2C from humans, *Arabidopsis thaliana* and bovine pyruvate dehyrogenase phosphatase in eukaryotes (Das *et al.*, 1996; Meyer *et al.*, 1994; Vassylyev and Symersky, 2007). In prokaryotes, most of the available knowledge regarding the distribution of PPM phosphatases comes from protein sequence and conserved catalytic domain similarity searches. Only a subset of these phosphatases have been investigated experimentally (Shi *et al.*, 1998). In bacteria, PPMs have been most intensively studied in *B. subtilis*. They include SpoIIE, RsbU, RsbX and PrpC. All these proteins exhibit divalent metal ion stimulated protein phosphatase activity *in vitro* (Adler *et al.*, 1997; Duncan *et al.*, 1995; Yang *et al.*, 1996). SpoIIE is involved in a signal transduction cascade that targets a sigma factor, σ^Finvolved in activating the transcription of stress response proteins (Duncan *et al.*, 1995). RsbX and RsbU, constitute portions of a pathway that regulates the activity of sigma B. PrpC was shown to dephosphorylate elongation factor-G and PrkC, a membrane linked protein kinase, on their threonine residue in *B. subtilis* (Gaidenko *et al.*, 2002; Obuchowski *et al.*, 2000). PPM phosphatases can use a broad spectrum of phosphorylated substrates including the artificial substrate *p*-nitro phenyl phosphate (pNPP).

The biochemical properties of PrpC made it a suitable candidate for testing the possibility of being the phosphatase, acting against the HPrK/P kinase. Dephosphorylation of HPr(Ser-P) *in vitro* is indeed catalysed by PrpC as shown for the proteins from *B. subtilis* and *M. pneumoniae.* This reaction was inhibited by P_i (Halbedel *et al.*, 2006; Singh *et al.*, 2007). Dephosphorylation of HPr(Ser-P) by *B. subtilis* PrpC could be completely inhibited by 10 mM phosphate (Singh *et al.*, 2007). In the presence of a rapidly metabolizable carbon source like glucose, intracellular phosphate levels are much lower compared to the cells grown on a poor carbon source (Mason *et al.*, 1981; Thompson and Torchia, 1984). During the uptake of a rapidly metabolizable carbon source such as glucose by *L. lactis*, the concentration of P_i are about 5 mM. In contrast, in the absence of a rapidly metabolizable carbon source, the concentration of P_i increases to about 50 mM (Neves *et al.*, 1999). However, no difference was observed in the phosphorylation pattern of HPr in a *ΔprpC* mutant as compared to wild type cells, grown in the presence of glucose (see supplementary material, Fig. 24). Thus it appears unlikely, that PrpC affect the *in vivo* phosphorylation state of the HPr, at least in the presence of good carbon source like glucose. This observation is very different from the situation in *M.*

pneumoniae, where a *prpC* mutation drastically increases the intracellular HPr(Ser-P), in the presence of glucose and glycerol. In *M. pneumoniae* wild type cells, HPr(Ser-P) is present in considerable amounts in the presence of glycerol and glucose (Halbedel *et al.*, 2006).

Interestingly, when the cells were grown in the presence of succinate (CSE medium) as the sole carbon source, the *ΔprpC* strain exhibited an increased phosphorylation of HPr as compared to the wild type strain (30 % increase in the signal intensity). Succinate is a poor carbon source, and generates only 13% of HPr(Ser-P) in the wild type cells (Singh *et al.*, 2008). Succinate does not allow exertion of CCR of the reporter system used in this study (*xynB*). In a Δ*prpC* mutant the HPr(Ser-P) levels increased to 16% of the total HPr. However, these increased HPr(Ser-P) levels did not lead to stronger repression of *xynB*. Probably a minor increase in HPr(Ser-P) in *prpC* mutant does not lead to formation of the complex with CcpA, which is sufficient to repress the expression of the reporter system used here. This is in agreement with our finding that atleast 20% of intracellular HPr(Ser-P) is required to exert 3 fold repression (Singh *et al*., 2008). In this respect, it would be interesting to analyze the CCR of the catabolic operons that are repressed in the presence of succinate. For e.g, the expression of the *citM*, which encodes the Mg^{+}-citrate transporter, is known to be repressed in CSE medium (Warner *et al.*, 2000). In previous studies, PrpC has been implicated in controlling a number of stationary phase processes (Gaidenko *et al.*, 2002). In terms of the metabolic state of the cell, stationary phase is associated with nutrient limitation and an increasing intracellular phosphate concentration. This is in good agreement with the idea that PrpC functions under conditions poor carbon supply.

Crh, an HPr paralog in *B. subtilis* has been implicated in catabolite repression of some catabolic operons, when cells are grown in succinate or when cells enter stationary phase (Inacio and de Sa-Nogueira, 2007; Warner *et al.*, 2003). Crh can be phosphorylated at Ser-46 residue and thus it is tempting to speculate that PrpC may be involved in the (de)phosphorylation of Crh, especially when Crh-specific functions are required.

Generically, HPrK/P from *M. pneumoniae* has the kinase activity as its preferential mode of action. In comparison, HprK/P from *B. subtilis* requires 300-fold molar excess of ATP and stimulation by FBP to act as a kinase under otherwise similar conditions. Therefore, the requirement for an alternate phosphatase could be a property of the organisms bearing HPrK/Ps with low HPr(Ser-P) dephosphorylation activity. Thus, PrpC in *M. pneumoniae*, may be more important in effecting the phosphorylation state of HPr(Ser-P) and regulating the carbon metabolism, in contrast to the PrpC in *B. subtilis*. Interestingly, the residues involved in phosphate binding, as suggested by the crystal structure of human PP2C proteins, are

perfectly conserved in *M. pneumoniae* and *B. subtilis* PrpC proteins (Halbedel *et al.*, 2006, Obuchowski *et al*., 2000). Thus, the differences in acting as a phosphatase on HPr(Ser-P) *in vivo*, from these two organisms might reside in the subtle differences in the structure of HPr proteins.

Moreover, as evident from the findings from this work, PrpC can not be the answer to the presence of unphosphorylated HPr in *B. subtilis* under conditions of good carbon supply.

5.2 Hierarchical nature of catabolite repression exerted by various carbon sources

Much effort has been devoted to understand the mechanisms underlying catabolite repression in *B. subtilis*. Various regulatory mechanisms have been elucidated, among which global repression achieved by CcpA is the most important one (see introduction). The rearrangements in CcpA secondary structure, induced by HPr(Ser-P) and Crh(Ser-P) binding, are a prerequisite for adapting the DNA binding conformation and consequently for transcription regulation. Intensive mutagenesis studies, and in particular, the determination of the structure of the CcpA/HPr(Ser-P)-*cre* ternary complex, have contributed considerably to the understanding of CcpA function in CCR in Gram-positive bacteria (Kraus *et al.*, 1998; Kraus and Hillen, 1997; Küster-Schock *et al.*, 1999; Schumacher *et al.*, 2004). In this work, a systematic analysis of the global catabolite repression pathway is presented in which a variety of different of carbon sources were compared. Use of chemically different sugars provides the opportunity to evaluate every step involved in mediating catabolite repression, including uptake and metabolism of different carbon sources. In *E. coli*, the transport of a carbohydrate via the PTS is sufficient to elicit CCR (Deutscher, 2006). In contrast, in *B. subtilis*, metabolism of a repressing sugar has been found to be required for CCR (Nihashi and Fujita, 1984).

Repression of the expression of the *xynPB* operon provides an efficient reporter system for this study. The *xynPB* operon encodes for the xyloside transporter (XynP) and β-xylosidase (XynB). The metabolism of xylosides generates xylose, which is the inducer for the expression of the *xynPB* operon (Lindner *et al.*, 1994; Galinier *et al*., 1997). β-xylosidase activity can be measured in a colorimetric enzymatic assay. The promoter region of this operon bears two cis-acting elements: an operator site recognised by an operon specific repressor, XylR and a *cre* sequence, separated by ~ 200bp (see Fig. 21). Xylose, as an inducer for this system, releases XylR from its operator site. XylR is also known to act as an anti inducer in the presence of high intracellular concentration of G6P (Dahl *et al.*, 1995). Thus, in

a mutant strain lacking a functional XylR, only catabolite repression via CcpA/*cre* is operative (Kraus *et al.*, 1994). Moreover, use of a XylR mutant avoids the addition of xylose in the media. The presence of only a single *cre* site, further simplifies the situation. The Regulation of *xynPB* operon *via* CcpA is strongly regulated and the expression can be repressed by as much as 36 folds in the presence of glucose (Singh *et al.*, 2008). Additionally, as demonstrated in this work, a number of additional carbon sources also repressed the *xynPB* expression, making it an optimal reporter system. Catabolic operons like *iol* and *gnt* are not repressed in the presence of carbon sources like ribose and arabinose, thus limiting the use of sugars, categorized here as poor carbon sources, to be studied (Nihashi and Fujita, 1984).

The carbon sources used in this study include hexoses (glucose and fructose), pentoses (arabinose and ribose), sugar alcohols (glycerol and sorbitol), oligo-β-glucosides (salicin) and disaccharides (sucrose and maltose) and sugar acids (gluconate). They comprise both, PTS and non PTS carbon sources (see Fig. 2). The various carbon sources repressed *xynB* expression, to different degrees. In general, glucose and fructose (36- and 25-folds respectively) are the strongest repressing carbon sources, followed by mannitol, sorbitol, glycerol, salicin, sucrose and gluconate (20- to 7-folds). The weakest repression was exerted by maltose, arabinose and ribose (2- to 3-folds). No repression occurs when only succinate is included in the media (CSE medium). Additionally, the absence of CcpA or of its known co-repressor, HPr and Crh, completely abolished the repressing potential of almost every carbon source. This demonstrated that repression by various carbon sources is solely dependent on the CcpA mediated CCR pathway. Interestingly, the presence of sorbitol elicited a residual 3-4 fold repression in the Δ*ccpA* background. A deviating behavior for sorbitol, has been observed before. The expression of the *gltAB* operon in *B. subtilis*, encoding for the enzyme glutamate synthase, is induced in the presence of sorbitol. This induction is independent of CcpA and is impeded in an EI mutant, which can not accept phosphate from PEP (*ptsI* H189A). In *B. subtilis*, sorbitol uptake is mediated by a Sorbitol/H^+ symporter (GutA) and is independent of the PTS activity (Chalumeau *et al.*, 1978). A possible phospho transfer event from EI to a potential activator of *gltAB* operon was suggested to explain this observation (Schmalisch, 2004). However, in an EI mutant strain, XynB activity in the presence of sorbitol is not altered. Obviously, no mechanism parallel to that observed for the induction of *gltAB* operon by sorbitol prevails for *xynPB* operon. Indeed, this sorbitol induced, CcpA independent repression mechanism seems to be specific for the *xynPB* operon as the repression of the RocG was completely relieved in a *ccpA* mutant (Singh *et al.*, 2008). It could

also be established in this work that sorbitol exerts this residual repression at the level of the transcription of the operon.

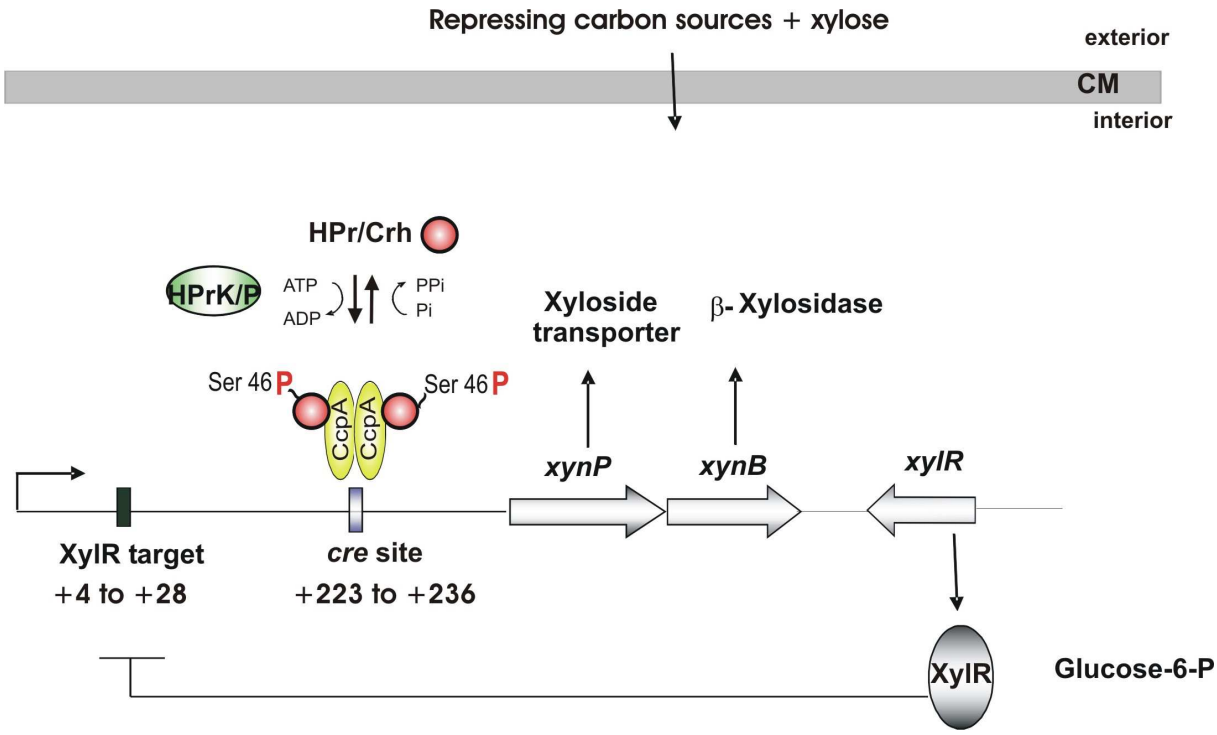

Figure. 21. Organisation and regulation of the *xynPB* operon in *B. subtilis*. The operon is under control of dual regulation. In the absence of the inducer xylose, the operon specific repressor XylR binds to the XylR target site, present 3bp downstream of the transcription start site (+1). A 14bp *cre* site recognised by CcpA is present downstream of the XylR target site. In the presence of a repressing carbon source, the CcpA/co-repressor complex is formed and represses the expression of the operon by binding to the *cre* site. In addition, G6P acts as an anti-inducer by binding to XylR and thereby opposing the inducing effect of xylose. This leads to an additional repression in the presence of glucose.

5.3 HPr(Ser-P) levels in the cell govern the strength of CcpA-medited catabolite repression by various carbon sources

The hierarchical nature of repression observed in the presence of different carbon sources suggested that the repressor complex, CcpA/co-repressor is formed to different extents. Since the intracellular levels of CcpA as well as of its cofactors remain constant, irrespective of the carbon source used (this work), it is conceivable that the extent of repressor complexes formed inside the cell is a function of phosphorylation of HPr and Crh. Previous investigations established that HPr(Ser-P) and Crh(Ser-P) do not act synergistically in allowing the binding of CcpA to the *xynPB* cre site (Galinier *et al.*, 1999). Infact, *in vivo*, HPr

seems to efficiently take over the function of being a co-repressor, in a *crh* mutant strain. Similar observations have been made for other catabolic genes subjected to CcpA regulated repression (Inacio *et al.*, 2003; Martin-Verstraete *et al.*, 1999). This clearly indicates that the CcpA mediated repression is not an additive function of the two co-repressors.

On the other hand, in a strain which expresses a *ptsH1* allele, coding for an HPr variant with non phosphorylatable alanine rather than a serine, a clearly different picture was observed. In such a mutant, the repression was relieved 3-8 fold in the presence of glucose, fructose and mannitol, but remained similar to a wild type strain in the presence of other carbon sources. These carbon sources exert the strongest repression. The HPrS46A variant is competent in PTS mediated sugar uptake (Deutscher *et al.*, 1994; Reizer *et al.*, 1989; Ye and Saier, Jr., 1996). Thus, impaired uptake of glucose, fructose and mannitol can not explain the weaker repression in this strain. Similarly, salicin and sucrose, which are also PTS substrates, when present in the media could elicit repression similar to the wild type strain (this work).

An alternative explanation can be envisioned as follows. In the presence of sorbitol and glycerol, Crh is capable of exerting 20 fold repression. It has been shown that as compared to HPr, Crh is synthesized in 100-fold lower amounts in the presence of glucose (Görke *et al*., 2004). Apparently, this low concentration of a CcpA co-repressor is sufficient to carry out a 20-fold repression of the *xynPB* operon. For achieving a stronger repression a higher concentration of co-repressor would be required. Furthermore, it was shown for the *B. subtilis* CcpA/Crh(Ser-P) complex that its binding to the *cre* site *in vitro* is not stimulated by metabolites like FBP and G6P (Schumacher *et al.*, 2007; Seidel *et al.*, 2005). Thus the repression observed in a *ptsH1* mutant strain is independent of the allosteric stimulation of the CcpA/Crh(Ser-P) complex by metabolites. Taken together, these findings suggest that a higher concentration of co-repressor and the allosteric activation of the CcpA complex by metabolites are required to achieve very strong repression.

Collectively, these findings clearly establish that the role of Crh in CCR only becomes prevalent in the absence of HPr. In addition, numerous *B. subtilis* operons are completely relieved from CCR in a *ptsH1* mutant, suggesting that their *cre* site might not be recognised by CcpA/Crh(Ser-P) (Deutscher *et al.*, 1994). In summary, HPr is the more relevant co-repressor of CcpA. Gene, *crh* has been only detected in *Bacilli*. The genomes of all other Gram-positive bacteria lack a *crh* gene. Thus, HPr(Ser-P) seems to be sufficient to mediate CCR in Gram-positive organisms, independently of whether they possess Crh or not (Deutscher *et al.*, 2006). Although, *crh* can partially complement the function of HPr, the primary function might be unrelated to CCR. For instance, it was demonstrated that

unphosphorylated Crh can inhibit the enzymatic activity of glyceraldehyde-3-phosphate dehydrogenase (GapA) (Pompeo *et al.*, 2007).

Most of the stronger repressing carbon sources were found to be PTS substrates. Presence of a PTS substrate stimulates the transcription of HPr, leading to a 3 fold increased amount in the cell as judged from the Lac-Z fusion data (Görke *et al*., 2004; Stülke *et al*., 1997). Moreover, western blot analysis suggested similar amounts of HPr regardless of the carbon source used (Singh *et al.*, 2008). Therefore, the differences in repression are not caused by different amounts of HPr present in the cell. Since, HPr can not bind CcpA unless its serine 46 residue is phosphorylated (Jones *et al.*, 1997), the intracellular HPr(Ser-P) rather than total HPr should decide the extent of repression. Indeed, large amounts of HPr(Ser-P) are formed in the presence of strongly repressing carbon sources such as glucose. In contrast, negligible amounts of HPr(Ser-P) are present when cells grew on CSE medium with succinate (Ludwig *et al.*, 2002; Monedero *et al.*, 2001). High amounts of intracellular HPr(Ser-P) could also be observed in the presence of other strongly repressing carbon sources such as fructose, sorbitol, glycerol, salicin, mannitol and sucrose. In the presence of these carbon sources, 50-70% of all HPr molecules are phosphorylated at serine residue. When the cells were grown on sugars that cause weaker repression, the HPr(Ser-P) amounts varied from 13- 32%, being lowest in the presence of succinate (CSE medium) and highest in the presence of gluconate (see Fig. 22). An analogous situation exists in *E. coli*, for which several carbon sources are suggested to cause CCR. This has been attributed to the ability of these carbon sources to affect the phosphorylation state of the master regulator of CCR, $EIIA^{glu}$ (Bettenbrock *et al.*, 2007; Hogema *et al.*, 1998). In *E. coli*, a correlation between the growth rate, the PEP/pyruvate ratio and the amount of dephosphorylated $EIIA^{glu}$ was demonstrated. Carbon sources, which support fast growth, regardless of being a substrate of the PTS, exert CCR. The fast growth on carbon sources reflects a high flux through glycolysis which is accompanied by a low PEP/pyruvate ratio in the cell. This can cause $EIIA^{glu}$ to be present in the unphosphorylated state, because the concentration of phosphate donor of the PTS, decreases in the cell (Hogema *et al.*, 1997; Hogema *et al.*, 1998). Owing to this, even pentoses such as arabinose can exert CCR in *E. coli* (Bettenbrock *et al.*, 2007). On the other hand, arabinose also supports fast growth of *B. subtilis* cells, (μ=0.98) but, exerts only 2 fold repression of the XynB activity. In general, in *B. subtilis* there is no strict correlation between the growth and the strength of repression exerted, by a given carbon source. Except for ribose and succinate on which growth was significantly slower, all other carbon sources allowed fast

growth of *B. subtilis* (specific growth rate (μ) of 0.83-0.98). In conclusion, HPr(Ser-P) levels in the cell can determine the hierarchy of the repression exerted by various carbon sources.

5.4 Modulation of the phosphorylation state of HPr by the nature of the carbon source

In this work, the repressing potential of various carbon sources is evaluated by using the *xynPB* operon as the reporter system. Using this system a correlation between the extent of the CcpA-mediated repression of the *xynPB* operon and the intracellular HPr(Ser-P) is demonstrated (see Table. 4, chapter 3). Interestingly, not only the HPr(Ser-P) levels varied depending on the carbon source, but also the HPr(His-P) levels showed variation. Most of the stronger repressing carbon sources are PTS substrates. These substrates generated 50-70 % of intracellular HPr(Ser-P). Moreover, HPr(His-P) is undetectable on these substrates and 40-50% of total HPr *in vivo* is present in the unphosphorylated form. It has been reported that glucose grown *S. salivarius* cells also contain only a small amount of HPr(His-P) (Thevenot *et al*., 1995). Presence of a PTS substrate drains away the P_i from the general PTS proteins, partly explaining the detection of unphosphorylated form of HPr and low amounts of HPr(His-P). This low amount of HPr(His-P) seems to be sufficient to allow the continued growth on a given PTS substrate.

Another interesting role of HPr(Ser-P) is in autoregulation of various catabolic operons. In *B. subtilis*, *glpFK* operon is repressed in the presence of glucose via two independent mechanisms: an antiterminator-dependent regulation and a CcpA/*cre* mediated repression. In the presence of constitutively active glycerol kinase, the expression of *glpFK* operon is always high, irrespective of the presence of the substrate glycerol in the media (Darbon *et al.,* 2002). Presence of glucose in such a situation mediates 3- to 4-fold CcpA dependent repression. Surprisingly, a similar level of repression could also be observed when glycerol was included in media (Darbon *et al.,* 2002). The repression was lost in a *ccpA* mutant, suggesting that glycerol can to some extent regulates its own uptake, via HPr(Ser-P). Similarly, *gutAB* operon encoding for glucitol permease and glucitol dehydrogenase, is induced in the presence of sorbitol (Chalumeau *et al.*, 1978). Deleting the negative regulatory *cre* site increased the induced levels of sorbitol dehydrogenase (Ye and Wong, 1994). These observations point to an autoregulatory mechanism of carbohydrate utilization in which the rate of carbohydrate uptake and metabolism is adapted to the metabolic state of the cell, i.e the intracellular concentration of glycolytic intermediates.

Table. 10. The *cre* sequences present in the regulatory region of various catabolic operons dedicated for the metabolism of the carbon sources used in this study.

Gene/operon	Function	*cre* sequence§	Position#	Reference*
Consensus *cre*		Half site ↓ Half site TGWAARCGYTWNCA 123456 7 891011121314		1, 2, 3
xynPB cre	Xylose metabolism	TGAAAGCGCTTTTA	D	4
rbs cre	Ribose metabolism	TGTAAACGGTTACA	O	5
araA cre	Arabinose metabolism	TGGAAACGGTTACA	D	6
araE cre	Arabinose metabolism	TGAAAGCGTTTTAT	D	6
gntRup cre	Gluconate metabolism	TGAAAGTGTTGCAT	O	7
gntRdown cre	Gluconate metabolism	TGAAAGCGGTACCA	D	7
bglPH cre	Aryl-β–glucoside metabolism	TGAAAGCGTTGACA	O	8
gutB cre	Sorbitol metabolism	TGTATGCACTTACA	D	9
glpFK cre	Glycerol metabolism	TGACACCGCTTTCA	O	10
mtlA cre	Mannitol metabolism	TGTAAGCGTTTTAA	O	11
mtlD cre	Mannitol metabolism	TGTGAACGAAACGA	D	11
lev cre	Fructose metabolism	TGAAAACGCTT$_{aA}$CA	U	12

. § - W=A or T, R=A or G, Y=C or T, N=any base

- Position indicate the relative location of *cre* site with respect to the transcription start site or the promoter. D, O, U indicates localization the *cre* downstream, overlapping, and upstream of the promoter/transcription start site. Repression via *cre* site requires *cre* site to be present downstream or overlapping with the promoter region. This allows repression of transcription by blocking the initiation of transcription or preventing the transcription elongation by a roadblock mechanism. For levanase operon, the *cre* site is localised upstream of the promoter. However in this case, it overlaps with the binding site for the activator of the operon, LevR, thus mediating repression.

*- 1 (Weickert and Chambliss, 1990), 2 (Kim and Chambliss, 1997), 3 (Martin-Verstraete *et al.*, 1995), 4 (Galinier *et al.*, 1999) , 5 (Strauch, 1995) , 6 (Inacio *et al.*, 2003), 7 (Miwa *et al.*, 1997), 8 (Krüger *et al.*, 1996) , 9 (Ye and Wong, 1994), 10 (Darbon *et al.*, 2002), 11 (Watanabe *et al.*, 2003), 12 (Galinier *et al.*, 1999).

- The dyad is indicated by an arrow.

However, any catabolic operon must resist a complete self repression, so that the respective catabolic genes are turned on when required. In *B. subtilis*, CcpA mediated repression can be avoided by lacking a potential *cre* site. This is the case for the *ptsGHI* operon which encodes for EII^{glu} and the general PTS components HPr and EI (Stülke *et al.*, 1997). This explains the exceptional position of glucose as a repressing sugar. It is plausible that autoregulation of glucose uptake is mediated by lowering the HPr(His-P) amounts in the cell to the extent that is sufficient to support rapid growth, but prevents deleterious

accumulation and uptake. This is evident by an earlier report where the uptake rate for glucose was diminished in wt *B. subtilis* cells in comparison to the strain bearing the *ptsH1* mutation, at high concentration of substrate (Ye and Saier, Jr., 1996).

However, all of the catabolic operons directly subjected to CcpA dependent catabolite repression, harbours a potential *cre* site. The CcpA dimer interacts with 7 bp of the 14 bp *cre* site (as shown in Table. 10) and makes 25 phosphate contacts (Schumacher *et al.*, 2004). As shown in Table. 10, *cre* sites not only show sequence deviations from the consensus sequence but also degeneracy in their location. The precise docking of the Helix turn Helix (HTH) motif of CcpA can differ, depending on the *cre* sequence. However, despite of this different docking, key base and phosphate contacts can be preserved (Schumacher *et al.*, 2004). Therefore, CcpA is flexible enough to bind half sites with altered sequences. Considering this, it is possible that the *cre* site of a particular catabolic operon responds strongly to the CcpA/HPr(Ser-P) complex formed in the presence of a stronger repressing carbon source. However, it is not strongly repressed by the HPr(Ser-P) amounts generated by the presence of the cognate substrate of the operon. This idea discussed above may explain how the self repression is avoided but hierarachy of repression is maintained by different carbon sources.

5.5 Formation of doubly phosphorylated HPr in *B. subtilis*

In the presence of weakly repressing carbon sources like succinate, ribose, maltose, arabinose, gluconate and glycerol, a considerable amount of HPr(Ser-P)(His-P) was detectable. The peculiarity of this observation resides in the following facts: a) In *B. subtilis* and other Gram-positive bacteria both phosphorylation events are considered to be mutually exclusive b) The presence of HPr(Ser-P)(His-P), is almost always accompanied by the presence of considerable amounts of histidine phosphorylated HPr except for glycerol (see Fig. 22).

Doubly phosphorylated HPr can be a result of either phosphorylation of HPr(Ser-P) by EI or phosphorylation of HPr(His-P) by HPrK/P. For *B. subtilis* HPr, a negative charge at serine 46, renders it a poor substrate for EI catalysed phosphorylation *in vitro* (K_M= 8-10 fold higher than that of wild type HPr) (Reizer *et al.*, 1992). Serine 46 of HPr faces the glutamate-84 side chain of EI, as observed in the structure of the *E. coli* HPr-EI complex (Garrett *et al.*, 1999). Thus, phosphorylation at Ser-46 is believed to result in a repulsion between the negatively charged carboxylate of Glu-84 and phosphate group of Ser-P causing a disturbance in the interaction of the two proteins (Garrett *et al.*, 1999). This glutamate is also present in EI of *B. subtilis* (Kunst *et al*., 1997).

Doubly phosphorylated HPr is also present in *M. pneumoniae* and oral *Streptococci* (Halbedel and Stülke, 2005; Thevenot *et al.*, 1995). However, in *M. pneumoniae* EI, Glu-84 is replaced with a threonine, explaining the formation of HPr(Ser-P)(His-P) in this organism (Halbedel, 2006). Similarly, in *S. salivarius*, EI requires an acidic pH to accept HPr(Ser-P) as a substrate *in vitro*. At acidic conditions, the negative charge of Glu-84 is neutralized to overcome the electrostatic repulsion of the serine phosphorylated HPr (Casabon *et al.*, 2006). Interestingly, *B. subtilis* EI was also shown to utilize HPr(Ser-P) as substrate, at slightly alkaline pH (Casabon *et al.*, 2006). However, *Bacilli* unlike *Streptococci* maintain an intracellular pH near neutrality (Breeuwer *et al.*, 1996). Thus, it is reasonable to assume that in *B. subtilis* HPr(Ser-P)(His-P) is not a result of EI activity on HPr(Ser-P). This assumption is further supported by the fact that in *B. subtilis* only a low level of intracellular HPr(Ser-P) was present, when the doubly phosphorylated form was also detected. Theoretically, doubly phosphorylated HPr can be considered a result of EI dependent phosphorylation of HPr(Ser-P) when HPr(Ser-P)(His-P) and HPr(Ser-P) forms are present together in the cell. For instance, in *S. mutans*, the concentration of doubly phosphorylated HPr concomitantly increases with the HPr(Ser-P) concentration.

The alternative possibility that HPr(Ser-P)(His-P) is formed *in vivo* as a result of HPrK/P activity on HPr(His-P), is also not in agreement with the available *in vitro* data. The phosphorylation of *B. subtilis* HPr by HPrK/P is shown to be inhibited when HPr was preincubated with PEP and EI. Additionally, HPrK/P exhibited a 10 times higher K_M for HPr allele with a glutamate at position 15 rather than a histidine (Reizer *et al.*, 1998). Since, the occurrence of HPr(Ser-P)(His-P) parallels the presence of HPr(His-P) in the cell, it appears conceivable that HPrK/P phosphorylates HPr(His-P). In contrast to the *in vitro* observations, it seems that there are conditions *in vivo* which stimulate the formation of doubly phosphorylated HPr by HPrK/P. As mentioned before, double phosphorylation of HPr was prevalent in the presence of histidine phosphorylated HPr, irrespective of the presence of unphosphorylated HPr. This suggests that HPrK/P can specifically utilize HPr(His-P) as a substrate. Thus, it can be proposed that in the presence of large amounts of intracellular HPr(His-P), HPrK/P can display an altered specificity towards its substrate. This seems to be an interesting mode of regulation to limit the HPr(Ser-P) amounts in the cell. In agreement with this idea, when *B. subtilis* cells grows in the presence of 0.1% glucose (as compared to 0.5%, which was used in this work), doubly phosphorylated HPr start appearing.

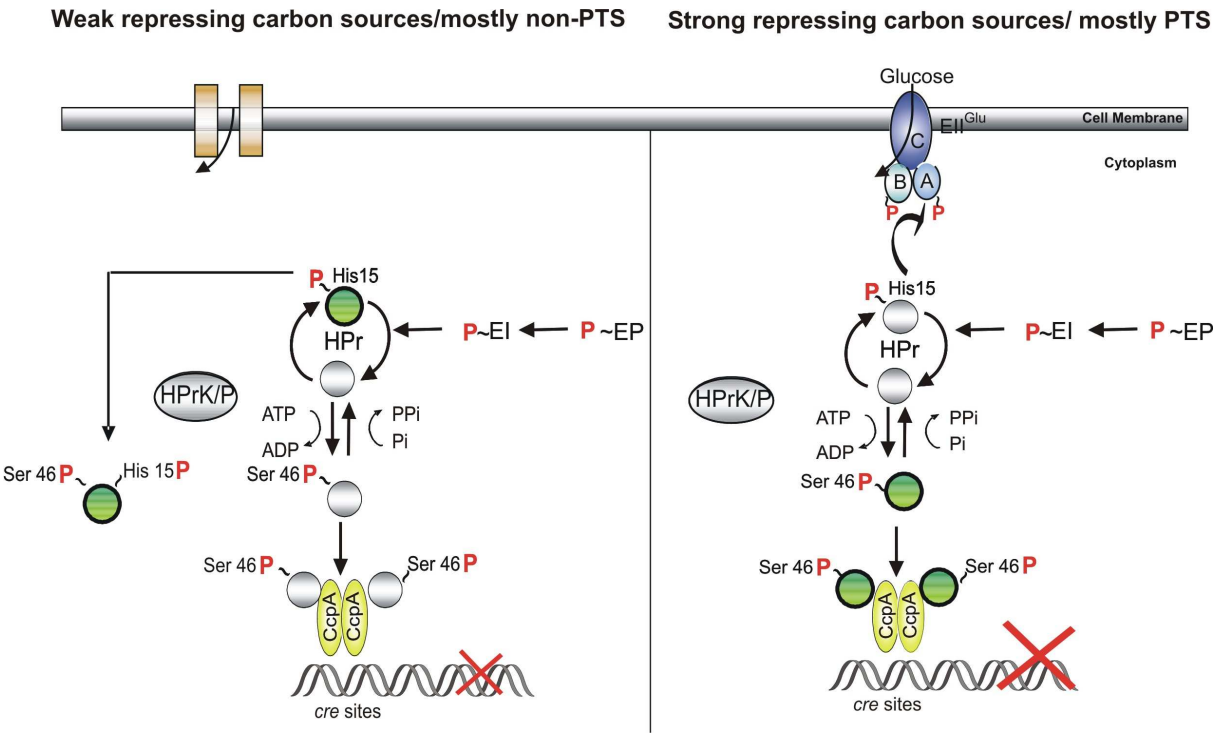

Figure. 22. The phosphorylation state of HPr in the presence of different carbon sources. The predominant forms of HPr are highlighted by a green colour. Most of the weakly repressing carbon sources are non-PTS carbon sources, except for maltose. In the presence of such carbon sources, the phosphate received by HPr via EI and PEP, is retained on HPr, resulting into high intracellular HPr(His-P). Low HPrK/P activity in the presence of these carbon sources, results in lower HPr(Ser-P) in the cells. Moreover, HPrK/P probably exhibits an altered substrate specificity in the presence of high intracellular HPr(His-P) and can generate doubly phosphorylated form. In the presence of strongly repressing carbon sources, HPr(Ser-P) is the predominant form. The remaining HPr is present in unphosphorylated form. Since strongly repressing carbon sources are mostly PTS substrates, the phosphate from the HPr(His-P) is directed towards the incoming carbon source. Glucose is shown as an example for strongly repressing carbon source.

On the other hand, the amount of unphosphorylated HPr remained similar (Schmalisch, 2004). Obviously, the presence of a lower concentration in the medium increases the phosphorylation of HPr at its histidine residue.

In *M. pneumoniae*, and oral *Streptococci*, doubly phosphorylated HPr is detected in the presence of a rapidly metabolized PTS sugar for e.g glucose. It is possible that doubly phosphorylated HPr transfers phosphate towards the EIIs (Halbedel and Stülke, 2005; Thevenot *et al.*, 1995). For instance, in *S. thermophilus*, lactose is taken up by the Lactose/H^+ symporter LacS. The rate of Lactose uptake is inhibited three-fold when IIA-like domain of LacS is phosphorylated by HPr(His-P) at a regulatory histidine residue. The rate of reversible phosphorylation of LacS was found to be the same regardless whether HPr(His-P) or HPr(Ser-P)(His-P) is used as phosphate donor (Cochu *et al.*, 2005). However, in *B. subtilis*, role of HPr(Ser-P)(His-P) in participating in PTS transport functions appears less likely. The HPr S46D variant from *B. subtilis*, which mimics the negative charge of phosphorylation at serine

46 (S46D), shows a markedly reduced rates of PTS sugar uptake when expressed in a heterologous system (*S. aureus*) (Reizer *et al.*, 1989). Moreover, the doubly phosphorylated form was only detected in the presence of non–PTS sugars in this work except for maltose.

5.6 Activity of HPrK/P determines CCR in *B. subtilis*

It was suggested that EI can compete with the kinase activity of HPrK/P leading to a reduced level of HPr(Ser-P). This was explained by the common interacting surface employed by HPr to bind HPrK/P and EI (Fieulaine *et al.*, 2002; Reizer *et al.*, 1989). The presence of weakly repressing carbon sources generated low levels of intracellular HPr(Ser-P) in the cell and considerable amount of HPr(His-P). This suggested that the PEP dependent phosphorylation of HPr limits the HPr(Ser-P) amounts in the cell in these carbon sources. An EI mutant however, did not show any increased CCR. In the absence of EI, neither HPr is phosphorylated at His-15 nor is it occupied by EI, thus avoiding any competition for HPrK/P. This leads to the conclusion that the phosphorylation at His-15 of HPr does not negatively regulate the phosphorylation at Ser-46 achieved by HPrK/P. Thus, the HPr(Ser-P) levels in the cell are exclusively determined by HPrK/P kinase activity. Moreover, results from this work and previously published reports support this idea. In the presence of HPrK/P variants, which lost their phosphorylase activity, and acts a constitutive kinase, a permanent state of repression was observed (Monedero *et al.*, 2001; Singh *et al.*, 2008). The constitutive kinase HPrK/P generates high amounts of intracellular HPr(Ser-P) even in the absence of a repressing carbon source (Monedero *et al.*, 2001; Singh *et al.*, 2008).

Most of the strongly repressing carbon sources are catabolized via glycolysis (see Fig. 2). It was also demonstrated that glucose drastically lost its repressing potential on the *iol* operon, when the flux of glucose utilization is directed towards PPP, rather than glycolysis (Nihashi and Fujita, 1984). Interestingly, the uptake of maltose, which is also a glycolytic sugar, did not lead to very strong repression. Catabolite repression studies using maltose as a potential sugar, considered it a weakly metabolized carbon source (Nihashi and Fujita, 1984). This is also supported by observations in this work where large amounts of HPr(His-P) and less HPr(Ser-P) were observed in the presence of maltose. Maltose is a PTS sugar and probably a low flux of PTS towards the maltose specific permease, limits uptake of maltose. This may lead to lower levels of HPr(Ser-P) because only low amounts of glycolytic intermediates including FBP are formed.

In vitro, HPrK/P exhibits kinase activity above 1 mM FBP and maximum activity at 5 mM FBP concentrations (Jault *et al.*, 2000). Here, we showed that 1.8 mM FBP is present intracellulary in the absence of a repressing sugar. In the presence of various other sugars, FBP concentrations in the range of 4.3-14.1 mM were detected. Therefore all sugars generated FBP levels, which are theoretically sufficient for a high kinase activity. The question that still holds is how the kinase activity of HPrK/P is modulated by the different carbon sources. Various possibilities could explain the differential kinase activities of HPrK/P. For instance, there might be an alternative allosteric activator of kinase activity. The role of FBP as a regulator of CCR has been demonstrated very coherently in *B. subtilis*. Any mutation that prevents the formation of FBP *in vivo* results in a complete relief of CCR (Nihashi and Fujita, 1984). Moreover, in *vitro* studies using several other intermediates of glycolysis for e.g G6P, fructose-6-P, 2-P-glycerate and of PPP such as gluconate-6-P, excluded the role of these metabolite in stimulating the kinase activity of HPrK/P (Reizer *et al*., 1998). The role of G6P in CCR has been implicated previously. Besides FBP, G6P has been suggested to modulate the CcpA/HPr(Ser-P) complex (Siedel *et al*., 2005; Schumacher *et al*., 2007). In this respect the intracellular amounts of G6P were estimated in wt cells in the presence of various carbon sources. Glucose generated highest levels of intracellular G6P, however there was no strict correlation between the strength of repression and G6P levels, in the presence of other carbon sources (see table 17 in supplementary material).

As an activator of HPrK/P kinase activity, FBP seems to play the most important role in *B. subtilis*. It is also possible that the effect of FBP on HPrK/P is similar under all conditions, but unknown signal antagonises the effect of FBP. As shown for the *L. casei* HPrK/P, the phosphate from the ATP binds at the P-loop and the base moiety of ATP interacts with Trp-237 of HPrK/P. This interaction stabilizes the kinase conformation (Chaptal *et al.*, 2007) Inorganic Pi, the substrate for the HPrK/P mediated dephosphorylation, occupies the position of the β- and γ- phosphate of ATP in the P-loop of *L. casei* HPrK/P (Fieulaine *et al*., 2001). This explains why P_i is an inhibitor os the kinase reaction. In contrast to most bifunctional enzymes, such as aspartokinaseI/ homoserine dehydrogenase I (Jullien *et al.*, 1988) or 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (Yang *et al.*, 2001), which possess two distinct active sites, the activities of HPrK/P are catalysed by the same site. Thus, the two conformations are probably in an ATP/P_i regulated equilibrium. This concept of equilibrium shift activation of antagonistic activities has been also observed for *E. coli* CheY (Dyer and Dahlquist, 2006). *In vitro*, the FBP dependent stimulation of the ATP dependent kinase activity is more obvious at ATP concentrations lower than 0.4 mM. As mentioned

before, the maximum kinase activity can be achieved already at 5 mM FBP concentrations. Interestingly, the phosphorylation achieved by HPrK/P in the presence of 0.5 mM ATP and 5 mM FBP can be totally inhibited by the presence of 0.5 mM P_i (Lavergne *et al.*, 2002). Thus, it is conceivable that relative changes in the intracellular ratio of ATP and P_i may affect the kinase conformation. Similarly, for *S. bovis,* an inverse relationship between the HPr(Ser-P) formation and the Pi concentration was observed (Asanuma and Hino, 2003).

An alternate mechanism, which could explain the low HPrK/P kinase activity in the presence of weakly repressing carbon sources, might be the binding of HPrK/P by an unknown inhibitor in a non competitive manner. In support of this, a relief from CCR was observed, when HPrK/P was expressed ectopically. Therefore, one possibility is that a protein encoded downstream of *hprK* in the same operon, opposes HPrK/P activity.

It was suggested that the N-terminal domain of HPrK/P, whose function is unknown can interact with phosphorylated metabolites. However, the truncated *L. casie* HPrK/P missing the first 127 amino acids exhibited both enzymatic activities and responded to all known effectors in the same way as the wild type enzyme (Fieulaine *et al*., 2001). Thus, FBP, ATP and Pi binding sites reside in the C-terminal domain. The N-terminal domain, which protrudes from the core hexamer exhibits similarity to UDP-*N*-acetylmuramoyl-$_L$-alanyl-$_D$-glutamate: *meso*-diaminopimelate ligase (MurE) of *E. coli* (Poncet *et al.*, 2004). MurE is an amide bond ligase involved in cell wall synthesis (Smith, 2006). This resemblance between the N-terminal domain of HPrK/P and the UDP interacting site of MurE, suggested that the N-terminal part of HPrK/P may interact with phosphorylated metabolites. Moreover, it has been demonstrated *in vitro* that a number of metabolic intermediates can inhibit the HPrK/P kinase activity including acetyl phosphate and glyceraldehyde phosphate (Ramström *et al.*, 2003). Acetyl phosphate has been postulated to be an indicator of glucose starvation. Synthesis of acetyl phosphate was found to be necessary for glucose starved cells of *E. coli* to survive glucose starvation (Nystrom, 1994). In this respect, it would be very interesting for the future studies to test the effect of an N-terminally truncated version of HPrK/P on CCR and on the phosphorylation pattern of HPr *in vivo*.

Summary and Conclusions

CCR is one of the most thoroughly investigated signal transduction system in bacteria. It allows the bacteria to adapt to changes in the availability and supply of different carbon sources. In general, CCR is defined as the selective utilization of a preferred carbon source which represses the functions for the utilization of secondary carbon sources. At the molecular level, CCR is achieved by the global transcriptional regulator CcpA, in *B. subtilis*. CcpA forms a repressor complex with serine phosphorylated HPr and Crh proteins in the presence of a preferred carbon source like glucose. This repressor complex binds to the operator sites on the DNA called *cre* sites and thereby repressing a number of catabolic genes and operons involved in utilization of secondary carbon sources. The regulatory phosphorylation of HPr and Crh is achieved by a bifunctional enzyme HPrK/P.

In this work, the repressing potential of various carbon sources besides glucose was analysed. A number of carbon sources could exert CcpA-mediated catabolite repression of the reporter system. Moreover, the substrates formed a hierarchy in their ability to exert repression. The different levels of repression by various carbon sources indicated the formation of the CcpA/co-repressor complex to different extents. CcpA and HPr levels were found to be similar in the cell, irrespective of the nature of the carbon source used. Thus, the phosphorylation pattern of the co-repressor was analysed. As a prerequisite for this experiment, it could be established that HPr and not Crh is the relevant co-repressor of CcpA. Thus, the focus was on analysing the level of HPr(Ser-P) in the cell. The presence of strong repressing carbon sources generated high intracellular HPr(Ser-P) as compared to the poor repressing carbon sources. Thus, it could be well established that the different repressing potential of various carbon sources is derived from the ability to generate different intracellular levels of HPr(Ser-P).

In the presence of poor repressing carbon sources, besides low intracellular HPr(Ser-P), considerable amounts of histidine phosphorylated HPr were also present. HPr is also a part of the sugar PTS. The PTS is involved in the concomitant uptake and phosphorylation of various carbon sources. As a part of PTS, HPr is phosphorylated at its His-15 residue and receives phosphate from PEP via EI. Phosphorylation at histidine residue might decrease the available HPr as a substrate for HPrK/P. Therefore, the possibility that the presence of HPr(His-P) negatively regulates the phosphorylation of HPr by HPrK/P was addressed. In an EI mutant, HPr(His-P) is not formed. However, the repression potential of the non-PTS carbon sources remained unchanged in the EI mutant. This clearly established that the

phosphorylation of HPr at Ser-46 is exclusively determined by HPrK/P activity and not by the PTS. Thus, in the presence of weaker repressing carbon sources, low HPr(Ser-P) levels are generated owing to the low kinase activity of HPrK/P. This hypothesis was confirmed by using an HPrK/P variant, which lost its phosphorylase activity. Such a variant exhibited a low but constitutive kinase activity and allowed repression even in the absence of a repressing carbon source.

Modulation of the HPrK/P activity is suggested to be achieved by an allosteric regulation by metabolites like FBP and P_i. Therefore, we determined the FBP concentration *in vivo*. It turned out that on most sugars the intracellular FBP level is high enough to achieve theoretically a complete activation of the HPrK/P kinase activity. Therefore, further factors should exist that regulate HPrK/P activity *in vivo*.

To probe into the possibility that HPr(Ser-P) levels can be affected by an enzyme other than HPrK/P, the role of PrpC was analysed. PrpC, a Ser/Thr phosphatase, has shown to dephosphorylate HPr(Ser-P) in *M. pneumoniae*. *B. subtilis* PrpC could dephosphorylate HPr(Ser-P) *in vitro*, but its absence or presence had no effect on CCR *in vivo*.

Taken together, this work coherently demonstrates the central role of HPrK/P in CCR in *B. subtilis*. Moreover, the hierarchy of repression exerted by various carbon sources could be well explained with the levels of HPr(Ser-P) generated in the cell. This work further highlights the fundamental differences in the mechanism of catabolite repression in the two model organisms *E. coli* and *B. subtilis*. PTS transport activity is the decisive factor of both global and operon-specific CCR in *E. coli*. In the absence of HPr(His-P) in *E. coli*, $EIIA^{glu}$ is rendered unphosphorylated and exerts strong catabolite repression. In contrast, the global CCR mechanism in *B. subtilis* is not directly affected by the PTS activity.

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Supplementary material

Oligonucleotides

Table. 11. Oligonucleotides

Name	Sequence 5' → 3' [#]	Description
SK1	CGTCAGGCCTATGGTTCAACAGAAAGTG GAAGT	Cloning of *B. subtilis crh* in pDG148 (fwd) (*Stu*I)
SK2	CGTCAGGCCTCTAAACTTCTTCTTGAACG TAAG	Cloning of *B. subtilis crh* in pDG148 (rev) (*Stu*I)
SK3	GGCGAATT**C**GTGTAACAATTTTGATCAG TCCG	Cloning of *B. subtilis hprK* with 700 bp upstream of the start codon (to include the putative promoter region) (fwd) (*EcoR*I)
SK4	GGCGAGCTCTATTCTTCTTGTTCACCGTC TT	Cloning of *B. subtilis hprK* (rev) (*Sac*I)
SK5	GTAAACTACGTGCCTGAACG	Amplification of *B. subtilis prpC*
SK6	CCGCCGCCTATGACGCG	Amplification of *B. subtilis prpC*
SK7	TGTTTTGGACTGAGACACTG	Amplification of regulatory region upstream of *B. subtilis xynP*
SK8	CTTGTGGACAAAGCCAATCAGA	Amplification of *B. subtilis prkC*
SK9	ATTCATCTTTCGGATACTCAATG	Amplification of *B. subtilis prkC*
SK10	ACGTAATCACCGACAAGAGGGGTA	Amplification of *B. subtilis prkC*
SK11	GGCGGATCCGTGGCAAAGGTTCGCACAA AAGA	Cloning of *B. subtilis hprK* in pGP380 (fwd) (*BamH*I)
SK12	CGTCCCCGGGCTATTCTTCTTGTTCACCG TCTT	Cloning of *B. subtilis hprK* (rev) (*Xma*I)
SK13	ACGCTTATTAATACGAATGAATTA	Amplification of *B. subtilis yvoE* (fwd) for RNA probe
SK14	*CTAATACGACTCACTATAGGGAGA*CTCATT TTCTCAAGCATAAA	Amplification of *B. subtilis yvoE* for RNA probe
SK15	GGCGGATCCATGAGTGACAAACAAGTA ACGACG	Cloning of *B. subtilis yvoE* in pHCMC05 (fwd) (*BamH*I)
SK16	CGTCCCCGGGTTACTTCACTCCAACGATT TGTAA	Cloning of *B. subtilis yvoE* in pHCMC05 (rev) (*Xma*I)
SK17	GGCGGATCCTGAATGAAGCGATAGAACC ACTC	Cloning of *B. subtilis lgt-yvoF* in pHCMC05 (fwd) (*BamH*I)
SK18	CGTCTCTAGATTATTCAGCGGACTTTTTC AATC	Cloning of *B. subtilis lgt-yvoF* in pHCMC05 (rev) (*Xba*I)
SK19	GTGGCAAAGGTTCGCACAAAAGACGT	Knockout of *B. subtilis lgt-yvoF* with LFH-PCR[*](up fwd). Complementary to *hprK*

Name	Sequence 5' → 3' [#]	Description
SK20	**CCTATCACCTCAAATGGTTCGCTG**AAC GCCAACTCCTATTCTTCTTG	Knockout of *B. subtilis lgt-yvoF* with LFH-PCR[*] (up rev). Sequence in bold is complementary to the forward primer of kanamycin cassette
SK21	CTGAGCAAATACGACAATATTCATGCTT TT	Knockout of *B. subtilis lgt-yvoF* with LFH-PCR[*] (down rev)
SK22	**CGAGCGCCTACGAGGAATTTGTATCG** CATCAGCGGACTTTTTTTGTTAAAATT	Knockout of *B. subtilis lgt-yvoF* with LFH-PCR[*] (down fwd) Sequence in bold is complementary to the reverse primer of kanamycin cassette
SK23	ATGAAGAAGATCTTTCTGGCCGGTC	Knockout of *B. subtilis yvoE* with LFH-PCR[*] (upfwd). Complementary to *yvoD*
SK24	**CCTATCACCTCAAATGGTTCGCTG**TCA TATTGCTTCCTTTCCAACCGC	Knockout of *B. subtilis yvoE* with LFH-PCR[*] (up rev). Complementary to *yvoE.* Sequence in bold is complementary to the forward primer of kanamycin cassette
SK25	**CGAGCGCCTACGAGGAATTTGTATCG** GTGAGAAAAACAGATCGTCATCCGGTCT CG	Knockout of *B. subtilis yvoE* with LFH-PCR[*] (down fwd). Sequence in bold is complementary to the reverse primer of kanamycin cassette
SK26	ATGGGAAGATGTTGGTGAGGATGCG	Knockout of *B. subtilis yvoE* with LFH-PCR[*] (down rev)
SK27	GGCCTCGAGGTTCTTTCAATTCATGACC GGGCTTGCC	Cloning of the region (-279 to +86, transcription start site as +1) upstream of *xynP* gene in pDG148 (*Ava*I)
SK28	GGCGGATCCCTCTTATCCCCCTTCCATCC ATATT	Cloning of the region (-279-+86, transcription start site as +1) upstream of *xynP* gene in pDG148 (*BamH*I)
SK29	GGCGGATCCCAAGCAAAATCTCCAGACG CATATCCAACT	Cloning of the region (-279 to+380, transcription start site as +1) upstream of *xynP* gene in pDG148 (*BamH*I)
SK30	CGTCAGGCCTATGAGCAATATTACGATC TACGATGTAGCG	Cloning of *B. subtilis ccpA* in pDG148 (fwd) (*Stu*I)
SK31	CGTCAGGCCTTTTCTTATGACTTGGTTGA CTTTCTAAGC	Cloning of *B. subtilis ccpA* in pDG148 (rev) (*Stu*I)
SK32	GGCGGATCCATGGTTCAACAGAAAGTGG AAGTTCG	Cloning of *B. subtilis crh* in pGP380 (fwd) (*BamH*I)
SK33	GGCCTGCAGCTAAACTTCTTCTTGAACG TAAGCAGCC	Cloning of *B. subtilis crh* in pGP380 (rev) (*Pst*I)
SK34	GGCGGATCCATGAGCAATATTACGATCT ACGATGTA	Cloning of *B. subtilis ccpA* in pGP380 (fwd) (*BamH*I)

Name	Sequence 5' → 3' [#]	Description
SK35	GGCCGTCGACTTTTCTTATGACTTGGTTGACTTTCTAAGC	Cloning of *B. subtilis ccpA* in pGP380 (rev) (*Sal*I)
SK36	GCCTCGTATGTTTCAACCATTTGTTCCAGG	Sequencing of inserts cloned in pHCMC05
SK37	GGCCTGCAGAACTTCTTCTTGAACGTAAGCAGCC	Cloning of *B. subtilis crh* in pGP382 (rev) (*Pst*I)
SK38	ATGAATGAAGCGATAGAACCACTCAATCCGATAGCAT	Knockout of *B. subtilis yvoD-yvoF* with LFH-PCR[*] (up fwd)
SK39	**CCTATCACCTCAAATGGTTCGCTG**GTCTACTCCGCGTACCGCTCCTTCGAGTATC	Knockout of *B. subtilis yvoD-yvoF* with LFH-PCR[*]. Sequence in bold is complementary to the forward primer of kanamycin cassette (up rev)
SK40	TCTCAATGATCCGTATGGCTATAAAAACAG	Sequencing of *B. subtilis yvoF*
SK41	AATCGTATTGATTGTTTTGGCTGTGGCAGC	Sequencing of *B. subtilis yvoD*
SK42	CGTCAGGCCTGTGGCAAAGGTTCGCACAAAAGACGTA	Cloning of *B. subtilis hprK* in pDG148 (fwd) (*Stu*I)
SK43	CGTCAGGCCTCTATTCTTCTTGTTCACCGTCTTCAATGAC	Cloning of *B. subtilis hprK* in pDG148 (rev) (*Stu*I)
SK44	GTTCTACATCCAGAACAACCTCTG	Sequencing of inserts cloned in pDG148
SK45	GCAACCGTTTTTTCGGAAGGAAATG	Sequencing of inserts cloned in pDG148
SK46	GCCAAGCTTGTTA**AAAGGAG**AATGATAAAAATGGCACAA	Cloning of *B. subtilis ptsH* in pdr79. Sequence in bold is the native ribosome binding site of *ptsH* (fwd) (*Hind*III)
SK47	GGCGTCGAC TTACTCGCCGAGTCCTTCGCTTTT.	Cloning of *B. subtilis ptsH* in pdr79 (rev) (*Sal*I)
SK48	CCGGGATCCAGATGAGTGTTAGTGAATCACATGATATTCA	Cloning of *B. subtilis yvcJ* in pSG1729 (fwd) (*BamH*I)
SK49	CCGGAATTCTTATTTCCGGCTTCTCTTTTCAATGTC	Cloning of *B. subtilis yvcJ* in pSG1729 (rev) (*EcoR*I)
SK50	CCGGGATCCAGATGGGACAAAAGCCGAAAATCGCAATCTTT	Cloning of *B. subtilis yvcK* in pSG1729 (fwd) (*BamH*I)
SK51	CCGGAATTCTCATTCTTTCAGTAAATCAACAAGAAGAGAGGCC	Cloning of *B. subtilis yvcK* in pSG1729 (rev) (*EcoR*I)
SK52	CCGGGTACCATGGTTCAACAGAAAGTGGAAGTTC	Cloning of *B. subtilis crh* in pMUTIN-cMyc (fwd) (*Kpn*I)
SK53	CCGATCGAT**AAGATCTTCTTCGCTAATAAGTTTTTGTTC**GCCAACTTCTTCTTGAACGTAAGCAGCC	Cloning of *B. subtilis crh* in pMUTIN-cMyc: Sequence in bold is c-Myc tag. Introduces myc tag C-terminally (rev)

Name	Sequence 5' → 3' [#]	Description
SK54	ATTTTATTCCTGGACGCGAA	Sequencing of *B. subtilis yvcJ*
SK55	GCTGAATGTAAGAGGAAAGGTT	Sequencing of *B. subtilis yvcK*
SK56	AACCTTTCCTCTTACATTCAGCAC	Sequencing of *B. subtilis yvcK*
SK57	GCCAAGCTTTAAAGA**AAAGGGG**AGATC TTATGGTTCAAC	Cloning of *B. subtilis crh* in pdr79. Sequence in bold ist the native ribosome binding site of *crh* (fwd) (*Hind*III)
SK58	GGCGTCGAC CTAAACTTCTTCTTGAACGTAAGC	Cloning of *B. subtilis crh* in pdr79 (rev) (*Sal*I)
SK59	GGCGAGCTCAATGAAAATTGCTTTGATC GCGCATG	Cloning of *B. subtilis mgsA* in pGP172 (fwd) (*Sac*I)
SK60	GGCGGATCCTTATACATTCGGCTCTTCTC CCCGAAGAA	Cloning of *B. subtilis mgsA* in pGP172 (rev) (*BamH*I)
SK61	GGCGTCGACTTATTCAGCGGACTTTTTCA ATCTTTC	Cloning of *B. subtilis hprK* operon in pGP380 (rev) (*Sal*I)
SK62	CAGGAATTCTCATGTTTGACAGCTTATC ATCGGCA	Amplification of cat cassette from pBGM35 (*EcoR*I)
SK63	GGCGAATTCTTATAAAAGCCAGTCATTA GGCCTA	Amplification of cat cassette from pBGM35 (*EcoR*I)
KS12	AAAAAGCTTGGTTCTATCGCTTCATTCAT TTAACGC	Cloning of *B. subtilis hprK* in pGP380 (*Hind*III)
pUC fwd	GTAAAACGACGGCCAGTG	Sequencing of inserts cloned in pGP380
pUC rev	GGAAACAGCTATGACCATG	Sequencing of inserts cloned in pGP380
NR1	CCTTTGTCAAAAAAGTAAATCAAAAG	Amplification of *B. subtilis xylR*
BG94	CTACAAGGTGTGGCATAATG	Sequencing of inserts cloned in pDG148
Kan fwd	CAGCGAACCATTTGAGGTGATAGG	Amplification of kan cassette from pDG780
Kan rev	CGATACAAATTCCTCGTAGGCGCTCGG	Amplification of kan cassette from pDG780
Kan checkfwd	CATCCGCAACTGTCCATACTCTG	Sequencing of kan cassette amplified from pDG780
Kan check rev	CTGCCTCCTCATCCTCTTCATCC	Sequencing of kan cassette amplified from pDG780

[#] restriction sites are underlined, introduced mutations or special sequences are bold, promoters are italic

[*] LFH-PCR: long flanking homology PCR

Plasmids

Table. 12. Plasmids used in this work

Plasmid	Resistance	Description	Reference
pAC6	Ampicillin/ Chloramphenicol	Transcriptional *lacZ* fusion	Stülke *et al.*, 1997
pAC7	Ampicillin/ Kanamycin	Translational *lacZ* fusion	Weinrauch *et al.*, 1991
pAG2	Ampicillin	Overproduction of His-tagged HPr	Galinier *et al.*, 1997
pDG148	Ampicillin/ Kanamycin	Overexpression under the control of *Pspac* promoter	Joseph *et al.*, 2001
pDG780	Amplicillin/ Kanamycin	Amplification of kanamycin cassette	Guerout-Fleury *et al.*, 1995
pdr79	Ampicillin/ Spectinomycin	Integration of a target protein in *amyE* locus under the control of *Pspac* promoter	Pan *et al.*, 2001
pdr90	Ampicillin/ Spectinomycin	Integration of a target protein in *amyE* locus under the control of *Phyper-spac* promoter	Srivatsan *et al.*, 2008
pdr110	Ampicillin/ Spectinomycin	Integration of a target protein in *amyE* locus under the control of *Pspank* promoter	Rokop *et al.*, 2004
pdr111	Ampicillin/ Spectinomycin	Integration of a target protein in *amyE* locus under the control of *Phyper-spank* promoter	Rokop *et al.*, 2004
pE5635	Ampicillin	Overproduction of His-tagged PrpC	Gaidenko *et al.*, 2002
pGP205	Ampicillin	Overproduction of His-tagged HPrK/P	Hanson *et al.*, 2002
pGP211	Ampicillin	Disruption of *hprK*	Hanson *et al.*, 2002
pGP380	Ampicillin/ Erythromycin	Overproduction of N-terminal Strep tagged target protein	Herzberg *et al.*, 2007
pGP811	Ampicillin/ Erythromycin	Disruption of *ptsI*	Schmalisch, 2004
pHCMC05	Ampicillin/ Chloramphenicol	Overexpression under the control of *Pspac* promoter	Nguyen *et al.*, 2005
pIW11xylR	Ampicillin/ Ermthromycin	Inactivation of *xylR* gene	Kraus *et al.*, 1994
pMUTIN-cMyc	Ampicillin/ Erythromycin	Construction of C-terminally c-Myc tagged target protein.	Kaltwasser *et al.*, 2002
pMUTIN-HA	Ampicillin/ Erythromycin	Construction of C-terminally HA tagged target protein	Kaltwasser *et al.*, 2002
pMUTIN-FLAG	Ampicillin/ Erythromycin	Construction of C-terminally FLAG tagged target protein	Kaltwasser *et al.*, 2002
pSG1729	Ampicillin/ Spectinomycin	Production of N-terminal *gfpmut1* fusion with the target protein under control of *Pxyl*. Integrates in *amyE* locus	Lewis and Marston, 1999

Plasmid	Resistance	Description	Reference
pSG1151	Ampicillin/ Chloramphenicol	Production of C terminal *gfpmut1* fusion with the target protein. Allows integration in the native locus	Lewis and Marston, 1999
pSG1187	Ampicillin/ Chloramphenicol	Production of C-terminal *yfp* fusion with the target protein under control. Allows integration in the native locus and the expression of the fusion protein driven by natural promoter	Feucht and Lewis, 2001
pSG1164	Ampicillin/ Chloramphenicol	Production of C terminal *gfpmut1* fusion with the target protein. Allows integration in the native locus and the expression of the downstream genes under the control of *Pxyl*.	Lewis and Marston, 1999
pSG1170	Ampicillin/ Chloramphenicol	Production of C terminal *gfpmut1* fusion with the target protein. Allows integration in the native locus and the expression of the downstream genes under the control of *Pspac* promoter	Lewis and Marston, 1999
pSG1154	Ampicillin/ Spectinomycin	Production of C-terminal *gfpmut1* fusion with the target protein under control of *Pxyl*. Integrates in *amyE* locus	Lewis and Marston, 1999

Table. 13. Plasmids constructed in this work

Plasmid	Construction	Description	Reference
pGP633	pDG148/*Stu*I	PCR-Prod *crh* orf, SK1+SK2/*Stu*I	
pGP634	pAC6/*EcoR*I + *Sac*I	PCR-Prod *hprK* orf + 700bp upstream of the orf, SK3+SK4/*EcoR*I+*Sac*I.	
pGP636	pHCMC05/ *BamH*I+*Xma*I	PCR-Prod *yvoE* orf, SK15+SK16/ *BamH*I+*Xma*I	
pGP637	pHCMC05/ *BamH*I+*Xma*I	PCR-Prod *lgt-yvoF* genes, SK17+SK18/ *BamH*I+*Xba*I	
pGP638	pDG148/ *Ava*I+*BamH*I	PCR-Prod *xynP* (-279 to +86), SK27+SK28 / *Ava*I+*BamH*I	
pGP639	pDG148/ *Ava*I+*BamH*I	PCR-Prod *xynP* (-279 to +380), SK27+SK29/*Ava*I+*BamH*I	
pGP640	pDG148/StuI	PCR-Prod *ccpA* orf, SK30+SK31/*Stu*I	
pGP641	pGP380/ *BamH*I+*Pst*I	PCR-Prod *crh* orf, SK32+SK33/ *BamH*I+*Pst*I	
pGP642	pGP380/ *BamH*I+*Hind*III	PCR-Prod *hprK* orf, SK11+KS12/ *BamH*I+*Hind*III	

Plasmid	Construction	Description	Reference
pGP643	pGP380/ *BamH*I+*Sal*I	PCR-Prod *ccpA* orf, SK34+SK35/ *BamH*I+*Sal*I	
pGP644	pDG148/*Stu*I	PCR-Prod *hprK* orf, SK42+SK43/ *Stu*I	
pGP645	pDG148/*Stu*I	PCR-Prod *hprK* G158A, SK42+SK43/ *Stu*I, pGP407 as a template to amplify the PCR product	
pGP646	pdr79/ *Hind*III+*Sal*I	PCR-Prod *ptsH* orf, SK46+SK47/ *Hind*III+*Sal*I, linearization of the plasmid via *Sca*I	
pGP647	pdr79/ *Hind*III+*Sal*I	PCR-Prod *crh* orf, SK57+SK58/ *Hind*III+*Sal*I, linearization of the plasmid via *Sca*I	
pGP648	pSG1729/ *BamH*I+*EcoR*I	PCR-Prod *yvcJ* orf, SK48+SK49/ *BamH*I+EcoRI, linearization of the plasmid via *Sca*I	
pGP649	pSG1729/ *BamH*I+*EcoR*I	PCR-Prod *yvcK* orf, SK50+SK51/ *BamH*I+EcoRI, linearization of the plasmid via *Sca*I	
pGP650	pGP380/ *BamH*I+*Hind*III	PCR-Prod *hprK*G158A, SK11+KS12/ *BamH*I+*Hind*III, pGP407 used as a template for the amplification of the insert	Singh *et al.*, 2008

Strains

Table. 14. Strains used in this work

Strain	Genotype	Reference
Escherichia coli		
BL21 (DE3)	F- *lon ompT rBmB hsdS gal* (cIts857*ind*1 Sam7 *nin*5 *lac*UV5- T7 gene1)	Sambrook *et al.*, 1989
DH5α	*recA1 endA1 gyrA96 thi hsdR17*rK-mK+*relA1 supE44* Φ80Δ*lac*ZΔ*M15* Δ(*lacZYA-argF*)U169	Sambrook *et al.*, 1989
NM522	*supE thi-1* Δ(*lac-proAB*) Δ(*mcrB-hsdSM*)5 (r_K-m_K) [F‘ *proAB lacI*^{q}ZΔM15]	Gough and Murray, 1983
Bacillus subtilis		
168	*trpC2*	Laboratory collection
GP202	*trpC2 hprK*::*spc*	Hanson *et al.*, 2002
GP505	*trpC2 ptsH*-H15A *amyE*::*(lacZ cat)*	Schmalisch, 2004
GS4	*trpC2 yvcJ (+369)*::*pX2 (cat)*	Boris Görke; unpublished
GS5	*trpC2 yvcK (+367)*::*pX2 (cat)*	Boris Görke; unpublished
MZ303	*trpC2* Δ*ptsH*::*cat*	Arnaud *et al.*, 1996

Strain	Genotype	Reference
OMG401	*trpC2* Δ*prpC*::*aphA3*	Madec *et al.*, 2002
OMG302	*trpC2* Δ*prkC*::*aphA3*	Madec *et al.*, 2002
PB703	*trpC2* Δ*prpC amyE*::*(ctc-lacZ cat)*	Gaidenko *et al.*, 2002
QB5223	*trpC2 ptsH1*	Martin-Verstraete *et al.*, 1995
QB5407	*trpC2 ccpA*::Tn*917 spc*	Faires *et al.*, 1999
QB7096	*trpC2 crh*::*aphA3*	Presecan-Siedel *et al.*, 1999
QB7097	*trpC2 crh*::*spc*	Martin-Verstraete; unpublished
QB7101	*trpC2 ptsH1 crh*::*spc*	Martin-Verstraete; unpublished
QB7102	*trpC2 ptsH1 crh*::*aphA3*	Presecan-Siedel *et al.*, 1999
QB7108	*trpC2 ptsH1 crh*::*aphA3, amyE:(Pspac crh cat)*	Galinier *et al.*, 1997
QB7144	*trpC2 amyE*::*xynP-lacZ*	Galinier *et al.*, 1999
QB7160	*trpC2* Δ*hprK*::*aphA3 amyE*::(PΔB *levD-lacZ* Cm^S)	Martin-Verstraete *et al.*, 1999

Table. 15. Strains constructed in this work

Strain	Genotype	Construction [a]	Reference
GP270	*trpC2 xylR*::*ermC*	pIW11*xylR* → WT168	Singh *et al.*, 2008
GP271	*trpC2 xylR*::*ermC crh*::*aphA3*	QB7096 → GP270	
GP272	*trpC2* Δ*prpC amyE*::*(ctc-lacZ cat) hprK*::*spc*	pGP211 → PB703	
GP273	*trpC2* Δ*prpC amyE*::*(pAC7 aphA3)*	pAC7 → PB703	
GP274	*trpC2* Δ*prpC amyE*::*(xynP-lacZ cat)*	QB7144 → GP273	
GP275	*trpC2* Δ*prpC amyE*::*(xynP-lacZ cat) xylR*::*ermC*	pIW11*xylR* → GP274	
GP276	*trpC2* Δ*prpC amyE*::*(xynP-lacZ cat) xylR*::*ermC crh*::*aphA3*	QB7096 → GP275	
GP277	*trpC2* Δ*prpC amyE*::*(xynP-lacZ cat) xylR*::*ermC hprK*::*spc*	pGP211 →GP275	
GP278	*trpC2 amyE*::*(xynP-lacZ cat) xylR*::*ermC*	pIW11*xylR* → QB7144	Singh *et al.*, 2007
GP279	*trpC2 amyE*::*(xynP-lacZ cat) xylR*::*ermC crh*::*aphA3*	QB7096 → GP278	Singh *et al.*, 2008

Strain	Genotype	Construction [a]	Reference
GP280	*trpC2 amyE*::*(xynP-lacZ cat) xylR*::*ermC hprK*::*spc*	pGP211 → GP278	
GP281	*trpC2 amyE*::*(xynP-lacZ cat) xylR*::*ermC* Δ*prpC*::*aphA3*	OMG 401 → GP278	Singh *et al.*, 2007
GP282	*trpC2 amyE*::*(xynP-lacZ cat) xylR*::*ermC* Δ*prkC*::*aphA3*	OMG 302 → GP278	
GP283	*trpC2 hprK*::*spc amyE*::*(hprK cat)*	pGP634 → GP202	
GP284	*trpC2 ptsH1 xylR*::*ermC*	pIW11*xylR* → QB5223	Singh *et al.*, 2008
GP285	*trpC2 amyE*::*(xynP-lacZ cat) xylR*::*ermC* Δ*prpC*::*aphA3 hprK*::*spc*	pGP211 → GP281	
GP286	*trpC2 amyE*::*(xynP-lacZ cat) xylR*::*ermC* Δ*prkC*::*aphA3 hprK*::*spc*	pGP211 → GP282	
GP287	*trpC2 ptsH1 crh*::*spc xylR*::*ermC*	pIW11*xylR* → GP7101	Singh *et al.*, 2008
GP288	*trpC2 hprK*::*spc amyE*::*(PhprK hprK cat) xylR*::*ermC*	pIW11*xylR* → GP283	
GP289	*trpC2 xylR*::*ermC* Δ*hprK*::*aphA3*	QB7160 → GP270	Singh *et al.*, 2008
GP290	*trpC2 xylR*::*ermC* Δ*hprK*::*aphA3 amyE*::*(PhprK hprK cat)*	pGP634 → GP289	
GP291	*trpC2 ptsH1 crh*::*aphA3 amyE*::*(Pspac-ptsH spc)*	pGP646 → GP293	
GP292	*trpC xylR*::*ermC amyE*::*(xynP-lacZ cat) crh*::*spc*	QB7097 → GP278	
GP293	*trpC2 ptsH1 crh*::*aphA3, xylR*::*ermC*	pIW11*xylR* → QB7102	
GP294	*trpC2 xylR*::*ermC ptsH1 crh*::*phA3 amyE*::*(Pspac -crh spc)*	pGP647 → GP293	
GP295	*trpC2 ptsH1 crh*::*aphA3 amyE*::*(Pspac-crh spc)*	pGP647 → QB7102	
GP296	*trpC2* Δ*ptsH*::*cat amyE*::*(Pspac -ptsH spc)*	pGP646 → MZ303	
GP297	*trpC2 xylR*::*ermC crh*::*spc*	QB7097 → GP270	Singh *et al.*, 2008
GP298	*trpC2 xylR*::*ermC amyE*::*(lacZ cat)*	pAC6 → GP270	
GP299	*trpC2 xylR*::*ermC ptsH-H15A amyE*::*(lacZ cat)*	pIWIIxylR → GP505	
GP851	*trpC2 xylR*::*ermC* Δ*lgt-yvoF*::*aphA3*	LFH PCR product → GP270	

Strain	Genotype	Construction [a]	Reference
GP852	*trpC2 xylR*::*ermC* Δ*yvoE*::*aphA3*	LFH PCR product → GP270	
GP853	*trpC2 xylR*::*ermC ccpA*::Tn*917 spc*	QB5407 → GP270	Singh *et al.*, 2008
GP854	*trpC2 yvcK (+367)*::*pX2 (cat) amyE*::*(Pxyl-GFP spc)*	pSG1729 → GS5	
GP855	*trpC2 hprK*::*spc amyE*::*(hrpK cat) xylR*::*ermC* Δ*lgt-yvoF*::*aphA3*	GP851→ GP 288	
GP856	*trpC2 hprK*::*spc xylR*::*ermC*	pGP211 → GP270	
GP857	*trpC2 hprK*::*spc xylR*::*ermC* Δ*lgt-yvoF*::*aphA3*	GP 851 → GP 856	
GP858	*trpC2* Δ*hprK*::*aphA3*	QB7160 → Wt168	Singh *et al.*, 2008
GP859	*trpC2 xylR*::*ermC* Δ*yvoE-yvoF*::*aphA3*	LFH PCR product → GP270	
GP860	*trpC2 crh*::*aphA3*	QB7096 → Wt168 "Genotype identical to QB7096"	
GP861	*trpC2 xylR*::*ermC* Δ*yvoD-yvoF*::*aphA3*	LFH PCR product → GP270	
GP862	*trpC2 amyE*::*(Pspac-ptsH spc)*	pGP646 → Wt 168	
GP863	*trpC2 xylR*::*ermC amyE*::*(Pspac-ptsH spc)*	pGP646 → GP270	
GP864	*trpC2* Δ*ptsI*::*ermC*	pGP811 → Wt168	Singh *et al.*, 2008
GP865	*trpC2 crh*::*aphA3* Δ*ptsI*::*ermC*	pGP811 → QB7096	
GP866	*trpC2, yvcJ (+369)*::*pX2 (cat) amyE*::*(Pxyl-yvcJ-GFP spc)*	pGP648 → GS4	
GP867	*trpC2 yvcK (+367)*::*pX2 (cat) amyE*::*(Pxyl.-yvcK-GFP spc)*	pGP649 → GS5	

[a] Arrows indicate construction by transformation.

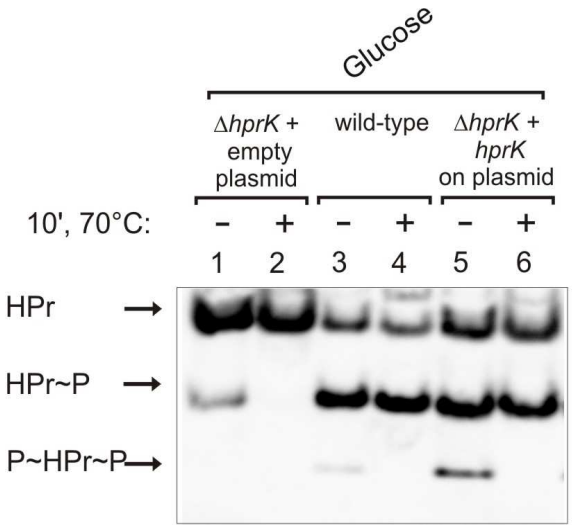

Figure. 23. The level of unphosphorylated HPr and HPr(Ser-P) in a strain expressing *hprk* from a multicopy vector (pGP642) is similar to the wild type strain. Cells were grown in CSE + glucose and harvested at logarithmic phase as described before (Singh *et al.*, 2007). 2µg of total cell protein, each was separated on a 12% native PAA gel and HPr was detected by immuno-blotting in a western blot analysis. To discriminate between HPr(Ser-P) and HPr(His-P), an aliquot of each cell extract was heated at 70 °C prior to loading (even numbered lanes). Histidine phosphorylation is heat labile and thus the two singly phosphorylated forms of HPr can be easily distinguished. In *ΔhprK* strain with empty plasmid (pGP380), no HPr(Ser-P) is detected (lane 1 and 2). Introducing the *hprK* on a multicopy plasmid (pGP642) restored the "wild type" phosphorylation pattern of HPr (compare lane3, 4 and lane 5, 6).

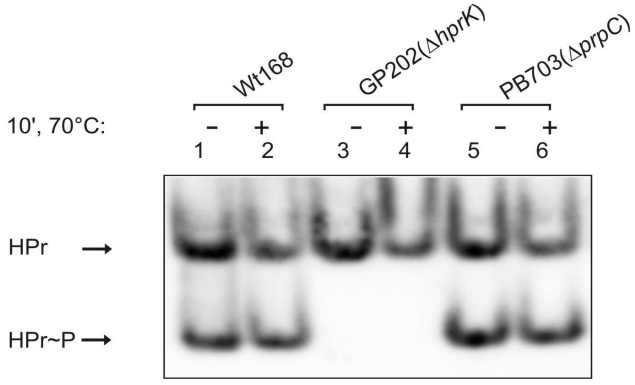

Figure. 24. Determination of the phosphorylation state of HPr in *ΔprpC* and *ΔhprK* mutants. Cells were grown in CSE + glucose and harvested at logarithmic phase as described before (Singh *et al.*, 2007). 1µg of total cell protein, each was separated on a 10% native PAA gel and HPr was detected by immuno-blotting in a western blot analysis. In *ΔhprK* strain, none of the phosphorylated form of HPr (Ser-P or His-15) was detected. In wt strain, 48% of the HPr was phosphorylated at serine residue, rest being the unphosphorylated form. Similar phosphorylation pattern of HPr was detected in PB703 (*ΔprpC*) strain, where 52% of the HPr(Ser-P) was detected.

Table. 16. Catabolite repression of *xynP-lacZ* fusion in the presence of glucose in *ΔprpC* mutant. The values are the average of at least two independent experiments. Standard deviations are shown in parentheses.

β-galactosidase activity in units/mg of protein			
Carbon source[a]	GP278 Δ*xylR*, *amyE::xynP-lacZ*	GP280 Δ*xylR*, Δ*hprK*, *amyE::xynP-lacZ*	GP275 Δ*xylR*, *ΔprpC*, *amyE::xynP-lacZ*
-	740(41)	1110(282)	498(175)
Glucose	31(3)	1941(674)	20(2)

[a]added to CSE medium (0.5%)

Intracellular Glucose-6-P (G6P) concentration

Protein-free cell extracts for the G6P concentrations in *B. subtilis* were prepared as described earlier with few modifications (Mijakovic *et al.*, 2002). Briefly, cells of the *B. subtilis* wild type strain 168 were grown in 50 ml of CSE medium in the presence of the indicated carbon sources (0.5%, w/v). For each growth condition, at least three independent experiments were carried out. Cultures were harvested by centrifugation at room temperature for 5 minutes at 10,000 g followed by freezing the pellet in liquid nitrogen. Pellets were resuspended in 0.6 M of cold perchloric acid and subsequently kept on ice for 20 minutes. The precipitated proteins and cell debris were removed by centrifugation (4°C, 5 minutes, 13,000 rpm). The pH in the supernatant was subsequently adjusted to 7.4 with a solution of cold 0.6 M KOH in 0.5 M glycyl glycine buffer (pH 7.4). The precipitated $KClO_4$ was removed by centrifugation. Aliquots of the final supernatants were used to measure the amounts of G6P in a direct photometric assay. For intracellular G6P estimation, reaction mixtures of 1ml were prepared, which contained 600 µl of cell extract and 100 mM of glycyl-glycine buffer (pH 7.4), 0.3 mM of NADP, 5 mM of $MgCl_2$ and 3.3 units of Glucose-6-phosphate dehydrogenase. The increase in absorbance at 340 nm was measured as indicator of the NADPH formation.

Table. 17. Intracellular amounts of G6P in wild type 168 grown in the presence of different carbon sources. The G6P concentrations were calculated by correlating the obtained values to a standard calibration curve (data not shown).

Carbon source[a]	G6P (mM) *
Glucose	0.88 (0.09)
Salicin	0.34 (0.01)
Mannitol	–
Fructose	0.43 (0.10)
Sorbitol	–
Glycerol	0.27 (0.10)
Gluconate	0.21 (0.01)
Arabinose	0.36 (0.13)
Maltose	0.65 (0.06)
Ribose	–
CSE	–

[a]added to CSE medium (0.5%), – = Below detection limit, * = The standard deviations are given in parentheses.

Curriculum vitae

Name	Kalpana Singh
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Education

1988-1998	High School, D.A.V. Public School, Haryana, India
1998-2000	Senior Secondary School, M.V.N. Public School, Haryana, India
2000-2004	Bachelor of Biotechnology, Guru Gobind Singh Indraprastha University, Delhi, India Thesis: Characterization of the subunit 4 of the RNA polymerase in *Saccharomyces cerevisiae*.
2004-Present	Program in Molecular Biology, International Max-Planck Research School, Göttingen, Germany PhD thesis at the Department of General Microbiology, Institute for Microbiology and Genetics (Supervised by Prof. Jörg Stülke and Dr. Boris Görke) Title: The role of protein phosphorylation in regulation of carbon catabolite repression in *Bacillus subtilis*