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Hierarchical Control *versus* Autoregulation of Carbohydrate Utilization in Bacteria

M. G. W. Gunnewijk[1], P. T. C. van den Bogaard[2], L.M. Veenhoff[1], E.H. Heuberger[1], W. M. de Vos[2], M. Kleerebezem[2], O. P. Kuipers[2,3] and B. Poolman[1]*

[1]Department of Biochemistry, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands
[2]Wageningen Centre for Food Sciences, NIZO food research, Department of Flavour and Natural Ingredients, P.O. Box 20, 6710 BA Ede, The Netherlands
[3]Present address: Department of Genetics, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands

Abstract

The involvement of phospho*enol*pyruvate:sugar phosphotransferase (PTS) proteins, like HPr and IIA^{Glc}, in the regulation of carbohydrate utilization has been well established in Gram-negative and Gram-positive bacteria. The majority of the studies of PTS-mediated regulation have been concerned with the hierarchical control of carbohydrate utilization, which results in the preferential utilization of a particular carbohydrate from a mixture of substrates. The underlying mechanisms of PTS-mediated hierarchical control involve the inhibition of expression of other catabolic enzymes and transporters and/or the allosteric regulation of their activity, which prevents the transcriptional inducer to be formed or taken up into the cell. More recently, it has become clear that PTS components allow also the cell to tune the uptake rate(s) to the carbohydrate availability in the medium and the metabolic capacity of the cell. The different phosphorylated species of HPr play a central role in this autoregulatory control circuit, both at the gene and at the protein level. Our knowledge of hierarchical control and autoregulation of carbohydrate utilization in bacteria is discussed.

Introduction

The first step in the metabolism of almost any carbohydrate is the transport of the molecule into the cell. In bacteria carbohydrates are taken up by *primary* or *secondary transport* systems or *group translocation* systems. Primary transport of sugars is driven by ATP (or related energy-rich compounds), whereas secondary transport is driven by the electrochemical gradient(s) of the translocated solute(s) across the membrane (Poolman and Konings, 1993). Among the secondary transport systems one can distinguish *symporters* (cotransport of two or more solutes), *uniporter*s (transport of one molecule) and *antiporters* (countertransport of two or more solutes). Sugar symporters usually couple the uphill movement of the sugar to the downhill movement of a proton (or sodium ion), *i.e.*, the electrochemical proton (or sodium ion) gradient drives the accumulation of sugar. Sugar uptake by group translocation is unique for bacteria and involves phospho*enol*pyruvate:sugar phosphotransferase systems (PTSs) (Postma *et al.,* 1993). The PTS catalyzes the uptake of sugar concomitant with its phosphorylation. The phosphoryl group is transferred from phospho*enol*pyruvate (PEP) via the general energy coupling proteins Enzyme I and HPr, and the sugar-specific phosphoryl transfer proteins/domains IIA and IIB. IIB~P transfers the phosphoryl group to the sugar that is translocated via the sugar-specific IIC protein/domain (Figure 1). IIA, IIB and IIC can be separate proteins, domains in a single polypeptide or linked as pairs in any possible combination (Robillard and Lolkema, 1988; Saier and Reizer, 1992; Lengeler *et al.*, 1994).

Most bacterial cells have the capacity to utilize several carbohydrates as carbon and energy source and possess various transport proteins and catabolic enzymes for the metabolism of the different carbohydrates. In addition, different mechanisms that control the transport and the first steps of metabolism of a particular carbohydrate have evolved. These mechanisms generally result in a sequential uptake and metabolism of the available carbohydrates in time and/or a tuning of the metabolic rate to the needs of the cell. These two regulatory phenomena, hereafter referred to as hierarchical control and autoregulation, are universal and have been reported for many bacteria. Hierarchical control of carbohydrates has been explained by (i) inhibition of expression of genes encoding enzymes that are involved in transport and metabolism of less preferred carbohydrates (Cohn and Horibata, 1959); (ii) inhibition of activity of enzymes that effect the uptake or production of the transcriptional inducer (hereafter referred to as inducer exclusion; Mcginnis and Paigen, 1969; Dills *et al.*, 1980); and (iii) stimulation of efflux of intracellular inducer, that is, the carbohydrate or the phosphorylated derivative (this phenomenon is referred to as inducer expulsion, Reizer and Panos, 1980; Thompson and Saier, 1981; Romano *et al.*, 1987). The inducer exclusion and expulsion mechanisms result in a lowering of the intracellular inducer concentration, and, thereby, indirectly affect gene expression. Autoregulatory control of carbohydrate utilization, on the other hand, occurs via adjustment of the rate of transport of a particular carbohydrate to the rate of its metabolism and the availability of the substrate, hereby providing a feedback or feedforward control to the pathway. Like with hierarchical control, autoregulation involves both control of gene

*For correspondence. Email B.Poolman@chem.rug.nl; Tel. (+3150) 3634190; Fax. (+3150) 3634165.

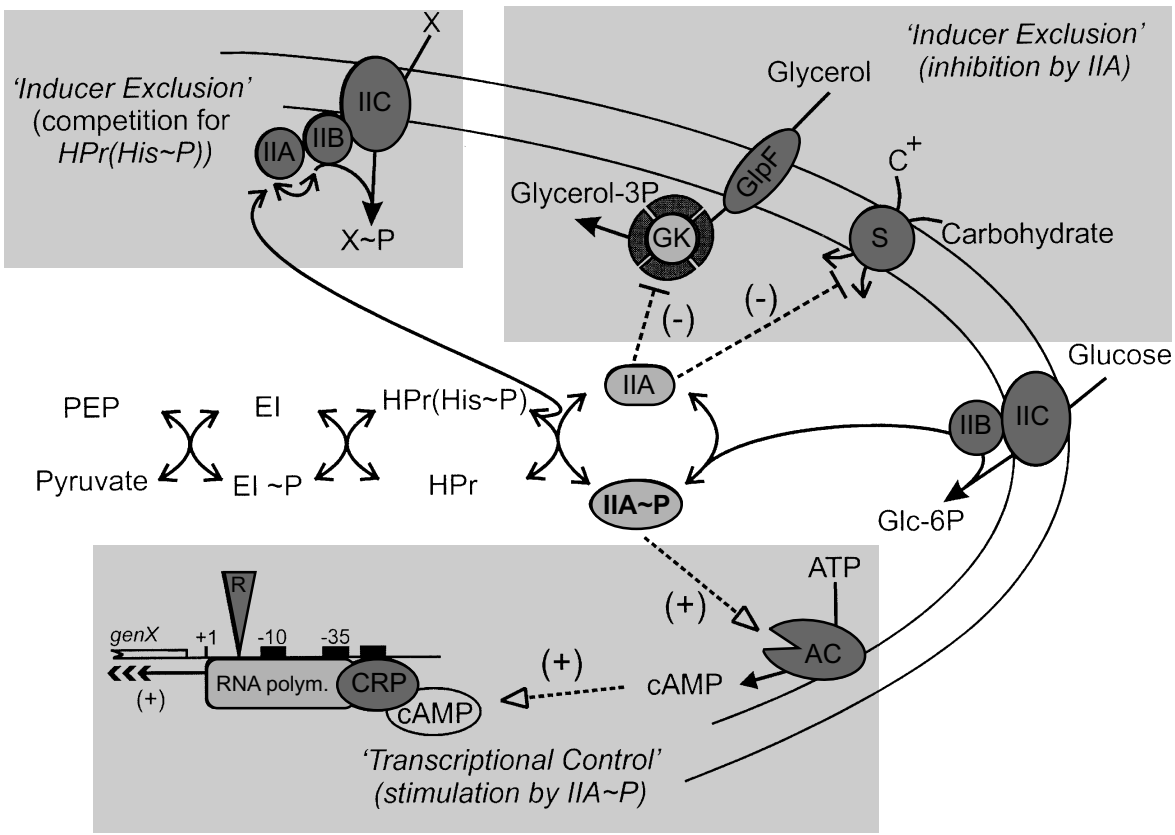

Figure 1. Schematic representation of the phospho*enol*pyruvate:glucose phosphotransferase system of Gram-negative enteric bacteria. The central role of IIA^{Glc}(~P) in controlling transcription and inducer exclusion is illustrated. EI, Enzyme I; Glc-6P, glucose-6P; CRP, cAMP receptor protein; RNA polym., RNA polymerase; R, repressor; C^+, cation; S, secondary transport protein; GK, glycerol kinase; and GlpF, glycerol facilitator.

transcription and control of enzyme/transporter activity. The two regulatory mechanisms differ in the sense that autoregulation of carbohydrate utilization controls the catabolic activities within a specific metabolic pathway, whereas hierarchical control involves the metabolic pathway of the preferred carbohydrate as well as that of the less preferred carbohydrate.

The mechanisms that result in the regulation of the initial steps of carbohydrate metabolism have been studied in two groups of bacteria: Gram-negative, enteric bacteria, such as *Escherichia coli* and *Salmonella typhimurium*; and Gram-positive, low GC bacteria, such as *Bacillus subtilis* and several streptococcal and lactobacilli species. For these groups of bacteria it has been well-established that the PTS system plays a crucial role in the mechanisms underlying hierarchical control of carbohydrate utilization. More recent experiments have indicated that autoregulation of transport and metabolism of carbohydrates is also controlled by the PTS.

How does the PTS exert all these regulatory functions? The PTS is able to sense the availability of carbohydrates and the metabolic capacity of the cell to metabolize these carbohydrates by adjusting the phosphorylation state of the PTS components; key players are the IIA^{Glc} and HPr proteins. As the phosphoryltransfer steps in the PTS system are reversible, the addition of a PTS substrate to the cell induces a dephosphorylating signal that is transmitted either directly, via $IICB^{Glc}$ in the case of glucose, to the central regulatory proteins IIA^{Glc} and HPr or indirectly by rerouting the phosphoryl transfer to other substrate-specific EII complexes, resulting in reduced ratios of HPr(His~P)/HPr and IIA^{Glc}~P/IIA^{Glc} (Figure 1 and 2; Postma *et al.*, 1993). On the contrary, in the absence of a PTS substrate, a high [PEP]/[pyruvate] ratio will favor the histidine-phosphorylated state of HPr and IIA^{Glc} (Weigel *et al.*, 1982a; Hogema *et al.*, 1998b). Besides being phosphorylated at His-15, the HPr protein of several Gram-positive bacteria can also be phosphorylated at Ser-46 in an ATP-dependent protein kinase catalyzed reaction (Deutscher and Saier, 1983; Reizer *et al.*, 1984). The reverse reaction, the hydrolysis of HPr(Ser-P), is catalyzed by a cytosolic HPr(Ser-P)phosphatase, which is stimulated by high concentrations of phosphate (Deutscher *et al.*, 1985). HPr(Ser)kinases of several Gram-positive bacteria, including *B. subtilis*, *Streptococcus pyogenes*, *Lactobacillus brevis* and *Lactococcus casei,* are stimulated by early glycolytic intermediates, in particular fructose 1,6-bisphosphate (FDP) (Deutscher and Engelman 1984; Reizer *et al.*, 1984; 1988; 1998; Galinier *et al.*, 1998 and Dossonnet *et al.*, 2000). The HPr kinases from *S. salivarius*, *S. mutans* Ingbritt and *E. faecalis* do not seem to be stimulated by FDP or other glycolytic intermediates, but instead, these enzymes are controlled by ATP and Pi levels in the cell (Thevenot *et al.*, 1995; Brochu *et al.*, 1999, Kravanja *et al.*, 1999). Recent experiments on the HPr(Ser)kinase of *B. subtilis* showed that stimulation by FDP essentially occurred at low ATP and enzyme concentrations, and that positive cooperativity for FDP

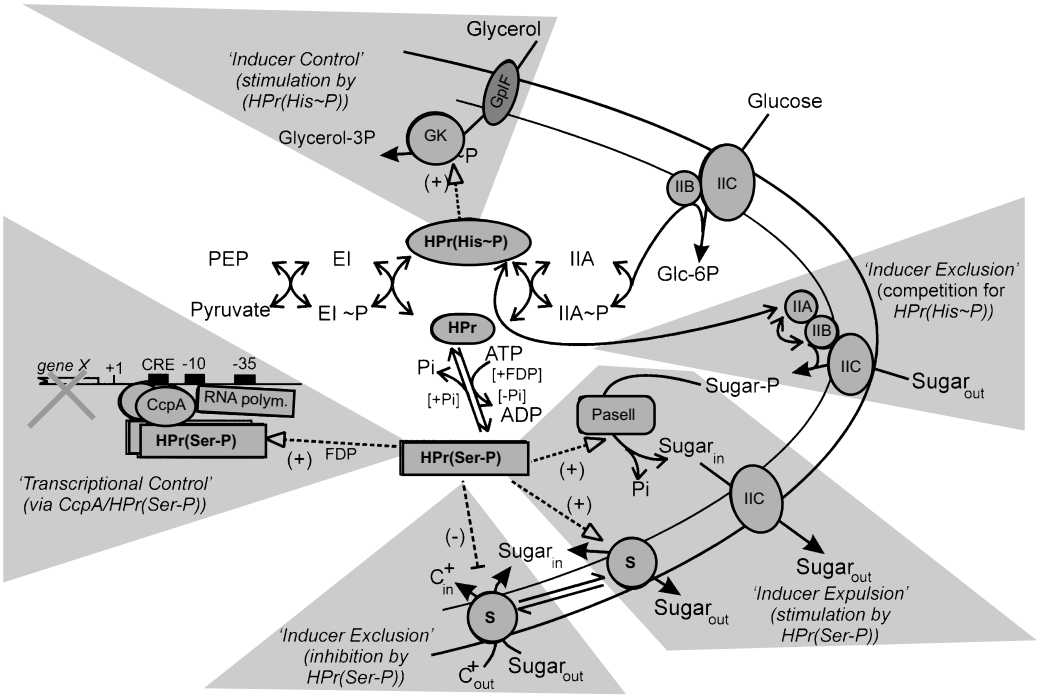

Figure 2. Schematic representation of the phospho*enol*pyruvate:glucose phosphotransferase system of Gram-positive bacteria. The central role of the HPr species in controlling transcription, inducer exclusion, inducer expulsion and inducer control is illustrated. CRE, Catabolite Responsive Element; FDP, fructose-1,6-bisphosphate; and Pi, free phosphate. Other symbols as in the legends of Figure 1.

binding is related to oligomerization of the enzyme (Jault *et al*., 2000). Overall, the formation of HPr(Ser-P) is proposed to be governed by the relative cellular concentrations of ATP, Pi and/or FDP, which are indicators of the energy status of the cells (Mason *et al*., 1981; Thompson and Torchia, 1984). This is supported by direct observations on the relative levels of HPr, HPr(Ser-P), HPr(His~P) and the doubly phosphorylated species HPr(Ser-P/His~P) in the cell. It has been shown that, HPr(Ser-P) is the dominant phosphorylated form of HPr in rapidly growing streptococcal cells, whereas free HPr and HPr(His~P) are the major species in slowly growing cells (Vadeboncoeur *et al*., 1991; Thevenot *et al*., 1995; Gunnewijk and Poolman, 2000a). HPr(Ser-P/His~P) is always a minor species (ranging from 5 – 30 % of total HPr present in different streptococci) and only present in rapidly growing cells; its physiological function has not been studied in detail.

This paper describes how the phosphorylated states of the PTS proteins control the transport and metabolism of carbohydrates. Specifically, their role in controlling hierarchical utilization is discussed as well as the recently described role of the PTS in the autoregulation of carbohydrate utilization. The former focuses mainly on the different mechanisms of hierarchical control that have evolved in Gram-negative enteric and low GC Gram-positive bacteria. It becomes clear that in some species more than one PTS-mediated regulatory mechanism is operative. The autoregulatory mechanism follows primarily from recent observations made for lactose transport and metabolism in *Streptococcus thermophilus*. This autoregulatory mechanism is unique for its involvement of a IIA-like protein, which is unusual for PTS-mediated regulation in Gram-positive bacteria.

Hierarchical Control of Carbohydrate Utilization

Hierarchical control of carbohydrate utilization was first described by Monod in 1942. This study demonstrated that when *E. coli* was grown on a mixture of carbohydrates, its growth curve was biphasic as a result of the sequential use of carbohydrates. For instance, glucose is used first when present in combination with lactose, melibiose, maltose and/or raffinose. The molecular basis for hierarchical carbohydrate utilization is well understood, in particular in enteric bacteria, and is generally referred to as catabolite repression. Catabolite repression is defined as the inhibitory effect of a preferred carbohydrate on the expression of other (catabolic) genes, and in fact, also includes inducer exclusion and inducer expulsion. These latter two regulatory phenomena have in common that the intracellular inducer concentration will be reduced and thereby, indirectly, affect gene expression. Most often the activity of the transport protein is modified such that the uptake of the inducer is prevented or the accumulated inducer is expelled from the cell. In some cases the first step(s) of the metabolism that produce(s) the transcriptional inducer is inhibited. The different mechanisms of inducer exclusion and inducer expulsion will be discussed in detail below. In the control of gene expression not only "inducer-specific" transcription factors, but also "general" ones are involved. In enteric bacteria, general transcriptional control is mediated by CRP (cAMP receptor protein), which requires cAMP as cofactor. The synthesis of cAMP is catalyzed by adenylate cyclase, which is under PTS control as it can be activated by IIA^{Glc}~P. In Gram-positive bacteria cAMP is not present and CcpA, the equivalent of CRP, is regulated by HPr(Ser-P). In addition to these strategies for controlled gene expression, Gram-negative and Gram-

positive bacteria have also developed transcriptional regulators that contain PTS regulation domains (PRD-containing proteins). In the next four sections the above mentioned PTS-mediated mechanisms underlying catabolite repression are discussed.

Catabolite Repression by Inducer Exclusion or Inducer Expulsion

Inducer exclusion is established by different mechanisms in Gram-negative and Gram-positive bacteria and involves different PTS proteins. In Gram-negative enteric bacteria inducer exclusion is determined by the phosphorylation state of IIA^{Glc} (Figure 1). In the presence of glucose, the activities of non-PTS carbohydrate transport proteins specific for lactose, maltose, melibiose or raffinose are inhibited by allosteric interaction of the unphosphorylated form of IIA^{Glc} (Nelson *et al.*, 1983; Postma *et al.*, 1984; Misko *et al.*, 1987; Dean *et al.*, 1990; Titgemeyer *et al.*, 1994; Hoischen *et al.*, 1996). Not only transport proteins, but also catabolic enzymes can be inhibited via allosteric interaction with IIA^{Glc}, as observed for the glycerol kinase (Postma *et al.*, 1984; Novotny *et al.*, 1985). Inhibition of glycerol kinase prevents the formation of L-glycerol-3-phosphate, the inducer for transcription of the *glp* operon, and thus prevents the expression of the catabolic enzymes specific for glycerol metabolism. This example of inducer exclusion is well-understood at a molecular level as the complex of glycerol kinase and IIA^{Glc} has been crystallized and the structure has been elucidated at 2.6 Å resolution (Hurley *et al.*, 1993). These studies have revealed that *E. coli* IIA^{Glc} binds to *E. coli* glycerol kinase at a region that is distant from the catalytic site of glycerol kinase, suggesting that long-range conformational changes mediate the inhibition of glycerol kinase by IIA^{Glc}. The binding of glycerol kinase to IIA^{Glc} involves mainly hydrophobic and electrostatic interactions, but also one hydrogen bond, involving an uncharged aspartate and a Zn(II) binding site (Feese *et al.*,1994). The Zn(II) binding site is made up of the two active-site histidines of IIA^{Glc} (His75 and His90), Glu478 of glycerol kinase and a H_2O molecule. Phosphorylation of IIA^{Glc} destroys the intermolecular Zn(II) binding site and disrupts the interactions between IIA^{Glc} and glycerol kinase.

In Gram-positive bacteria, inducer exclusion does not involve regulation by IIA^{Glc}, but rather allosteric control by HPr(Ser-P) or control via HPr(His~P)-dependent phosphorylation (Figure 2). In *L. brevis*, HPr(Ser-P) has been implicated in the control of uptake of non-PTS carbohydrates such as glucose, lactose and ribose (Ye *et al.*, 1994 a,b). Recent studies in *ptsH* and *hprK* mutants of *L. casei* showed that HPr(Ser-P) can also act in inducer exclusion by inhibiting the uptake of the non-PTS carbohydrate maltose (Viana *et al.*, 2000; Dossonet *et al.*, 2000). In some Gram-positive bacteria, like *E. faecalis, E. casseliflavus* and *B. subtilis*, the concentration of the transcriptional inducer for the *glp*-operon is controlled via the activity of glycerol kinase. The net result of this regulation is equivalent to the allosteric control of glycerol kinase from enteric bacteria by IIA^{Glc}, but the underlying mechanisms are different. In the Gram-positive bacteria, glycerol kinase is stimulated via HPr(His~P)-dependent phosphorylation (Deutscher and Sauerwald 1986; Wehtje *et al.*, 1995; Charrier *et al.*, 1997). The phosphorylation of glycerol kinase by HPr is reversible, and the dephosphorylated, less active form of glycerol kinase is dominant when a PTS-substrate is present in the medium (Deutscher *et al.*, 1993). HPr also affects the hierarchical utilization of PTS carbohydrates via competition for HPr(His~P), a general mechanism that is operative in both Gram-negative and Gram-positive bacteria. As the affinity of HPr(His~P) varies for the carbohydrate-specific IIA proteins/domains, competition for HPr(His~P) leads to hierarchical uptake of PTS carbohydrates.

For some low-GC Gram-positive bacteria catabolite repression is achieved by a mechanism in which the inducer is expelled from the cell. Two types of inducer expulsion mechanisms have been demonstrated (Figure 2). In homofermentative lactic acid bacteria like *E. faecalis*, *S. pyogenes, S. bovis* and *L. lactis*, which transport most sugars via PTS systems, lactose and glucose accumulate in the cytoplasm in their phosphorylated forms (Reizer and Panos, 1980; Thompson and Saier, 1981, Ye *et al.*, 1996). Addition of a rapidly metabolizable sugar results in dephosphorylation of the accumulated sugar-P, which is followed by a rapid efflux of the free sugar from the cell (Reizer *et al.*, 1983). A sugar-P phosphatase (Pase II) has been identified in all these four species and this enzyme seems to be absent in *S. aureus*, *S. mutans*, *S. salivarius* or *B. subtilis*, organisms that do not exhibit the sugar-P hydrolysis dependent expulsion phenomenon (Cook *et al.*, 1995 a, b; Ye *et al.*, 1996). Based on *in vitro* studies with toluenized vesicles or purified Pase II, it has suggested that HPr(Ser-P) stimulates PaseII (Ye *et al.*, 1994 c, d; Ye and Saier, 1995a). A second type of inducer expulsion was observed in heterofermentative lactobacilli such as *L. brevis*. These bacteria transport lactose and glucose via proton symport mechanisms and accumulate these substrates as free (non-phosphorylated) cytoplasmic sugars. Binding of HPr(Ser-P) to the glucose/H^+ and lactose/H^+ symporters is thought to alter their energy coupling mechanism, resulting in a conversion of the systems from carbohydrate-proton symport into carbohydrate uniport (Ye and Saier, 1995 a,b). Consequently, accumulated substrates are expelled from the cell, down the concentration gradient, and thereby the inducer levels are lowered (Romano *et al.*, 1987; Ye *et al.*, 1994 a, b).

Catabolite Repression by Regulation of cAMP Synthesis

Catabolite repression in enteric bacteria involves inhibition of the uptake of the inducer (inducer exclusion [Inada *et al.*, 1996a; Kimata *et al.*, 1997]) as well as a lowering of the cAMP levels via a diminished adenylate cyclase activity (Figure 1). In the absence of a PTS-carbohydrate, the phosphorylated form of IIA^{Glc} activates adenylate cyclase and stimulates cAMP production. Binding of cAMP to the cAMP receptor protein (CRP) enables the complex to bind to specific sites in several promoter regions and allows transcriptional regulation (either positively or negatively) of at least 45 genes and operons in *E. coli* and *S. typhimurium.* This subject has been the topic of many reviews and is not described further here (Botsford and Harman, 1992; Kolb *et al.*, 1993; Postma *et al.*, 1993).

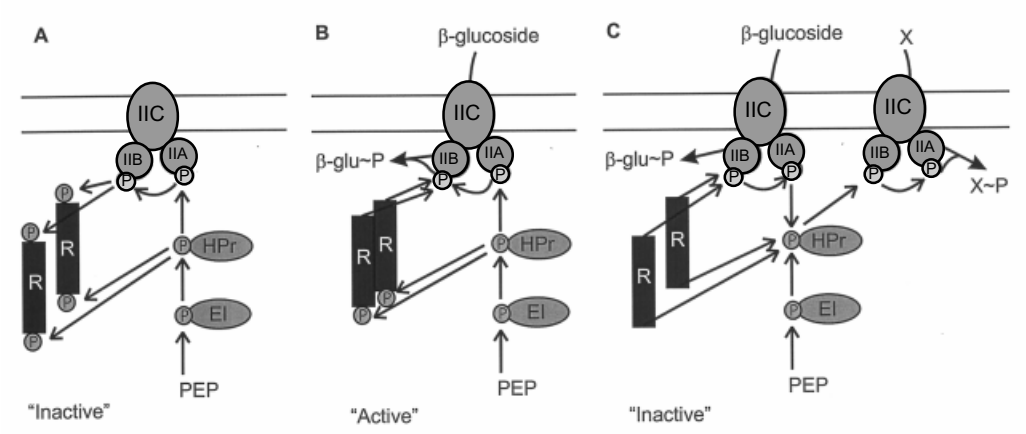

Figure 3. Model for activity control of PRD-containing transcriptional regulators as suggested for BglG by Görke and Rak, 1999. (A) In the absence of any PTS sugar the phosphorylated form of IIB phosphorylates the transcriptional regulator (R) leading to its monomer formation, the inactive form. In addition, HPr may phosphorylate R at a second site, which is without effect when R is phosphorylated by IIB. (B) In the presence of ß-glucosides (but absence of other PTS substrates), IIB dephosphorylates R, which is necessary but not sufficient for its activation. HPr directly transfers phosphoryl groups to a distinct site within R, which allows the protein to dimerize to the active form and stimulate transcription of the specific operon. (C) Appearance of any other PTS sugar leads to additional competition for phosphoryl groups and the transport of other PTS sugars reduces the level of HPr(His~P). As a consequence, HPr is underphosphorylated and therefore unable to activate R by phosphorylation.

CcpA-Mediated Catabolite Repression

In many Gram-positive bacteria the general transcription factor, CcpA, mediates the repression of several catabolic genes (Figure 2) (Hueck and Hillen, 1995). HPr(Ser-P) has been shown to interact with CcpA, allowing the latter protein to bind specifically to a *cis*-acting sequence. This sequence, named catabolite-responsive element (*cre*), is present in or near the promoter regions of many catabolite repression sensitive operons (Weickert and Chambliss, 1990; Fujita *et al*., 1995; Deutscher *et al*., 1995; Henkin, 1996; Gösseringer *et al*., 1997; Kim *et al*., 1995). The HPr(Ser-P)/CcpA complex forms a ternary complex with *cre*, consisting of two molecules of HPr(Ser-P), the CcpA dimer and the *cre* sequence (Jones *et al*., 1997). Both the formation of the HPr(Ser-P)/CcpA-complex and its binding to the *cre* sequences is stimulated by FDP (Deutscher *et al*., 1995; Kim *et al*., 1998). The histidine residue at position 15 in the HPr protein, the active site for PEP-dependent Enzyme I phosphorylation, is important for CcpA-mediated repression, as mutation or phosphorylation of His-15 blocks the interaction of HPr(Ser-P) with CcpA. This suggests a link between catabolite repression and PTS-mediated carbohydrate transport (Deutscher *et al*., 1995; Reizer *et al*., 1996). Indeed, the uptake of glucose or other rapidly metabolizable PTS carbohydrates leads to dephosphorylation of the PTS proteins and to an increase of the concentrations of glycolytic intermediates that activate the HPr(Ser)kinase. As a result the levels of HPr(Ser-P) rise and strong CcpA-dependent repression is observed under these conditions, leading to hierarchical carbohydrate utilization.

Catabolite control by CcpA not only involves repression but also activation of genes and operons. In *B. subtilis*, transcription of the *alsS* and *ackA* genes, encoding α-acetolactate synthase and acetate kinase, respectively, is activated by CcpA when glucose is present in the medium (Grundy *et al*., 1993; Renna *et al*., 1993). More direct evidence for a link between catabolite control and glycolytic activity has been reported for *Lactococcus lactis*. CcpA was found to be a transcriptional activator of the *las* operon, thereby controlling the production of the three key glycolytic enzymes, that are phosphofructokinase, pyruvate kinase and lactate dehydrogenase (Luesink *et al*., 1998).

Catabolite Repression Involving PRD-Containing Proteins

The expression of genes encoding enzymes of several PTS-dependent metabolic pathways, such as the β-glucoside operons in *E. coli* and *B. subtilis*, the sucrose regulon and the levanase operon in *B. subtilis,* and the mannitol operon in *Bacillus stearothermophilus,* are controlled by the PTS (Mahadevan and Wright, 1987; Crutz *et al*., 1990; Martin-Verstraete *et al*., 1990; Arnaud *et al*., 1992; Le Coq *et al*., 1995; Tobisch *et al*., 1999a; Stulke *et al*., 1997; Henstra *et al*., 1999). The transcription of these operons is initiated by specific activators, like LevR and MtlR, or controlled by specific antiterminators, like BglG, LicT and SacT. The activities of these transcriptional regulators are controlled via the phosphorylation states of their PRD-domains (PTS Regulation Domain; reviewed by Stulke *et al*., 1998). In the absence of PTS substrates, the phosphorylated IIB component of the carbohydrate-specific permease phosphorylates a PRD, thereby inactivating the regulator and thus preventing the transcription of the respective operons (Figure 3A). In the presence of the PTS substrate, the phosphate group is transferred to the PTS substrate, leaving the regulator unphosphorylated and active for induction of the operon (Figure 3B). In this way transcription is controlled in response to the availability of the inducer. In addition, some of these PRD-containing transcriptional regulators also require HPr(His~P)-dependent phosphorylation for activity (Stulke *et al*., 1995; Tobisch *et al*., 1999a; Görke and Rak, 1999; Henstra *et al*., 1999). In MtlR of *B. stearothermophilus* a IIA-like domain is present in addition to two PRDs, which results in an intricate interplay of multiple (de)phosphorylation reations, that is HPr- and IIB-mediated as well as interdomain phosphoryl transfer (Henstra *et al*., 2000).

Regulation of these transcription factors by the carbohydrate-specific IIB and the general PTS protein HPr

is an example of an elegant dual control mechanism that senses both the presence of a specific PTS substrate and the need of the cell to transport and metabolize this substrate. Since the level of HPr(His~P) is, amongst others, determined by the rate of uptake of the PTS carbohydrate itself, transcription is tuned (autoregulated) to the availability of the PTS substrate. The level of HPr(His~P) decreases more strongly with the presence of a more preferred PTS carbohydrates, due to a more rapid uptake or competition of different IIA components for HPr(His~P), which leads to hierarchical carbohydrate utilization both at the level of PTS uptake and transcription. Besides this competition for HPr(His~P), the rapid metabolism of carbohydrates will lead to HPr(Ser-P) formation. This results in an additional decrease in the formation of HPr(His~P) as free HPr will be no longer available for PEP-dependent phosphorylation by Enzyme I (Saier, 1989). Therefore, it has been proposed that HPr(His~P)-dependent stimulation of transcriptional regulators is another strategy by which catabolite repression can be manifested in Gram-positive bacteria. Indeed, CcpA-independent catabolite repression is observed for the *bgl, lic* and *lev* operons in *ccpA* mutants of *B. subtilis* (Martin-Verstraete *et al*., 1995; Kruger *et al*., 1996; Tobisch *et al*., 1999b). This catabolite repression is dependent on the presence of a specific transcriptional regulator (LicT, LicR or LevR).

Autoregulation of Carbohydrate Utilization

Besides hierarchical control, the PTS also mediates autoregulation of carbohydrate utilization. The mechanistic concepts of the autoregulatory control circuits are emerging and, in a few cases, it has been shown that the rate of carbohydrate uptake is tuned to the metabolic capacity of the cell and the carbohydrate availability in the medium. In Gram-positive bacteria, HPr(Ser-P), which is formed at high rates of metabolism, is thought to autoregulate the uptake rate of PTS carbohydrates. *In vitro* experiments with membrane vesicles of *L. lactis* demonstrated that HPr(Ser-P) inhibited PTS-mediated uptake of 2-deoxyglucose (2DG) and thiomethyl-β-galactoside (TMG) (Ye *et al*., 1994 c, d). *In vivo* studies with *ptsH* mutants of *B. subtilis* and *S. aureus* showed that HPr(Ser) phosphorylation reduced the fermentation response of several PTS carbohydrates (Reizer *et al*., 1989a; Ye and Saier, 1996). Based on kinetic studies, it was suggested that HPr(Ser-P) inhibits PTS uptake by reducing the phosphoryltransfer rates from Enzyme I via HPr to the IIA proteins (Reizer *et al*., 1989a, 1992). When HPr was phosphorylated on Ser-46, the affinity of Enzyme I and the affinities of several IIA proteins for HPr are reduced, and also the maximal velocities of these phosphoryl transfer reactions are reduced. It is worth noting that this HPr(Ser-P)-mediated inhibition of PTS carbohydrate uptake does not create a hierarchy in carbohydrate utilization as the inhibition was general in all PTS systems tested.

In contrast to what might be expected, also the metabolism of non-PTS carbohydrates can trigger PTS-mediated mechanisms to autoregulate the rate of metabolism. In Gram-negative bacteria, non-PTS carbohydrates like glucose-6-P, gluconate or lactose induce catabolite repression by decreasing the concentration of both cAMP and CRP (Perlman *et al*., 1969; Okinaka and Dobrogosz, 1966; Epstein *et al*., 1975; Inada *et al*., 1996b; Hogema *et al*., 1997). Moreover, there is evidence that the metabolism of non-PTS carbohydrates influences the intracellular [PEP]/[pyruvate] ratio (Hogema *et al*., 1998b). Since this ratio is the driving force for the phosphorylation of the PTS proteins, non-PTS carbohydrates can influence the phosphorylation state of IIA^{Glc} and thereby regulate IIA^{Glc}-mediated inducer exclusion (as described earlier). In agreement are the characteristics of *E. coli lacY* mutants that express a lactose carrier protein, which is insensitive for IIA^{Glc}-mediated inducer exclusion. In this mutant lactose is consumed much faster than in the wild type, larger amounts of glucose are excreted, and stronger dephosphorylation of IIA^{Glc} is observed (Hogema *et al*., 1998b; 1999). The growth rate is retarded in strains in which the mutations leading to insensitivity to inducer exclusion are combined with an increased *lac* expression. On the basis of these observations a model has been proposed, in which the metabolism of non-PTS carbohydrates determines the phosphorylation state of IIA^{Glc}, and, thereby controls inducer exclusion and transcriptional regulation via control of adenylate cyclase activity (Hogema *et al*. 1998b, 1999). This autoregulatory circuit is an important mechanism for *E. coli* cells to adjust the uptake rate to its metabolic capacity.

Recently, in the Gram-positive organism *S. thermophilus* evidence was obtained for autoregulation of the transport of the non-PTS carbohydrate lactose. This involved the tuning of the uptake to the rate of sugar metabolism. In the sections below we will discuss how transport and metabolism of lactose is regulated at the level of gene transcription and protein activity. The underlying mechanisms involve the HPr(Ser-P)/CcpA complex and HPr(His~P)-mediated phosphorylation of the IIA-like domain of the non-PTS transport protein, LacS.

Enzymatic and Structural Features of the LacS Protein

In *S. thermophilus*, lactose is taken up via the secondary transport protein LacS (Poolman *et al*., 1996). LacS catalyzes two modes of transport, solute-H^+ symport, driven by the proton motive force (Δp) and lactose/galactose exchange, which is driven by the concentration gradients of lactose and galactose across the membrane (Foucaud and Poolman, 1992). The lactose/galactose exchange reaction via LacS is the most relevant transport mode *in vivo* as it is much faster than the lactose/H^+ symport reaction (Knol *et al*., 1996). In addition, the galactose moiety of lactose cannot be metabolized in most *S. thermophilus* strains, and therefore galactose has to be expelled from the cell. Kinetic studies have revealed that the affinity of LacS for galactose and lactose at the cytoplasmic binding site is 20-fold higher than at the extracellular binding site, and that in this conformation galactose is preferred over lactose (Veenhoff and Poolman, 1999). These observations are consistent with the view that LacS is designed to catalyze an efficient lactose/galactose exchange rather than a unidirectional lactose influx.

Although, the LacS protein is not a PTS transport system, the protein has a carboxyl-terminal hydrophilic domain of about 160 amino acids, which is homologous to IIA proteins/domains of various PTS systems. Interestingly,

the LacS protein is, amongst others, homologous to the melibiose transport proteins of *S. typhimurium* and *E. coli,* which lack a IIA-like domain, but are regulated by IIA^{Glc} as discussed elsewhere (Poolman *et al*., 1996). The hydrophilic domain of LacS, called IIA^{LacS}, has 34 % residue identity with IIA^{Glc} of *E. coli* and contains several residues that are conserved in the active site of PTS IIA proteins/domains (Poolman *et al*., 1989). These conserved residues include His-552 and His-537, corresponding to His-90 and His-75 of IIA^{Glc} from *E. coli*, respectively. The former histidine residue has been shown to be the phosphoryl-accepting site for HPr(His~P) phosphorylation (Dörschug *et al*., 1984), whereas His-75 is involved in stabilization of the phosphoryl group, when bound to His-90, and in the transfer of the phosphoryl group to the membrane protein $IICB^{Glc}$ (Presper *et al*., 1989; Meadow and Roseman, 1996).

On the basis of amino acid alignments, we anticipated that IIA^{LacS} would be able to carry out one or more functions associated with IIA^{Glc}, *i.e.*, phosphorylation by HPr(His~P), phosphoryl transfer to the glucose specific IIB domain and/or affecting other catabolic functions such as inducer exclusion of non-PTS substrates. To study the properties of the IIA^{LacS} domain, a IIA^{LacS} protein comprising the carboxyl-terminal 173 residues of LacS was produced in *E. coli* (Gunnewijk *et al*., 1999). Biochemical characterization of purified wild type and mutant IIA^{LacS} protein showed that (i) HPr(His~P) can phosphorylate IIA^{LacS}; (ii) phosphorylation of IIA^{LacS} by HPr(His~P) from *S. thermophilus* is 5 orders of magnitude slower than phosphorylation of IIA^{Glc} by HPr(His~P) from *E. coli;* (iii) the phosphorylation-site corresponds most probably to His-552; and (iv) IIA^{LacS} is unable to transfer the phosphoryl group to *E. coli* IIB^{Glc}. These results suggest that IIA^{LacS} has evolved into a protein (domain), whose main function is not to transfer phosphoryl groups rapidly. Instead, the unphosphorylated form of IIA^{LacS} was able to inhibit the activity of glycerol kinase (inducer exclusion). The extent of inhibition of glycerol uptake was dependent on the actual uptake rate of glycerol [amount of glycerol kinase], suggesting that IIA^{LacS} inhibits glycerol kinase by forming a (stoichiometric) complex with the enzyme. In relation to these regulatory properties of IIA^{LacS}, carbohydrate fermentation studies in *S. thermophilus* showed that the IIA^{LacS} is involved in controlling the lactose uptake rate as deletion of the IIA^{LacS} domain increases the rate of lactose utilization relative to that of the wild type (Gunnewijk *et al*., 1997).

Regulation of Carbohydrate Utilization in *Streptococcus thermophilus*

S. thermophilus has a very limited capacity to utilize carbohydrates. Lactose and sucrose are fermented most rapidly, glucose is used very slowly by most strains, and only one or few other carbohydrates can be used by most strains. *S. thermophilus* co-metabolizes sucrose and lactose, a PTS and a non-PTS substrate, respectively, indicating that the utilization of these carbohydrates is not (strongly) hierarchically controlled. Instead, it has been proposed that HPr(His~P)-mediated regulation of the lactose uptake rate serves to control the flux of glycolysis. This mechanism is based on studies of the kinetic properties of phosphorylated and unphosphorylated LacS, and on the LacS levels of the cell as a function of the phosphorylation state of HPr. The various species of HPr present in lactose-growing *S. thermophilus* cells have been quantified at different stages of growth (Gunnewijk and Poolman, 2000). HPr(Ser-P) appears to be the dominant phosphorylated species in the exponentional phase of growth, whereas HPr(His~P) dominates in the stationary phase. Similar results were obtained when *S. thermophilus* cells were grown on sucrose (Gunnewijk and Poolman, unpublished results). The fact that the levels of HPr(Ser-P), HPr(His~P) and HPr are similar in sucrose- and lactose-growing cells suggest that the rate of glycolysis of both carbohydrates is sufficiently high to keep Ser-46 phosphorylated and that the drain of phosphoryl transfer to sucrose is minor compared to the phosphorylation activity of Enzyme I. The similar HPr(His~P)/HPr ratios also suggest that the PEP/pyruvate ratios are comparable in sucrose and lactose growing cells. Although PEP levels have not been measured in *S. thermophilus*, it has been firmly established for other lactic acid bacteria that concentrations of PEP are relatively low in rapidly metabolizing cells, whereas PEP concentrations increase under conditions of carbohydrate limitation (Mason *et al*., 1981; Thompson and Torchia, 1984; Konings *et al*., 1989). The increase in HPr(His~P) in *S. thermophilus* at later stages of growth would thus correlate with the increased PEP levels and a decreased metabolic activity.

Regulation of the Lactose Transport System

The effect of HPr(His~P)-mediated phosphorylation on lactose transport has been studied *in vitro* using a membrane system, in which purified LacS protein was incorporated into liposomes with the IIA^{LacS} domain facing outwards (Gunnewijk and Poolman, 2000b). This system allowed phosphorylation and manipulation of activity of LacS by adding PEP, Enzyme I and/or HPr to the outside medium. Upon phosphorylation of LacS the maximal rate of lactose exchange transport was increased, whereas the rate of Δp-driven lactose uptake at low lactose concentrations was somewhat decreased. This apparent inhibition is in agreement with earlier results obtained with hybrid membrane vesicles, and reflects a somewhat lower affinity of LacS for lactose when the protein is in the phosphorylated state. This inhibition is not observed when the activity of the protein is analyzed under saturating lactose concentrations. Moreover, the inhibition of Δp-driven lactose uptake is not observed with LacS mutants that lack IIA^{LacS} [LacS(Δ160)] or the active-site histidine residue [LacS(H552R)] (Poolman *et al*., 1995a). These results indicate that HPr(His~P)-mediated phosphorylation of IIA^{LacS} at the conserved His-552 residue modulates lactose transport activity of the LacS protein. In line with a range of kinetic studies (Foucaud and Poolman, 1992; Poolman *et al*., 1995b), it has been proposed that phosphorylation affects the rate constants for the reorientation of the ternary complex (LacS with bound lactose plus proton), which is rate-determining for exchange transport but not for Δp-driven uptake. Since the lactose/galactose exchange reaction and not the Δp-driven uptake is most relevant in lactose (glycolysing)-metabolizing cells of *S. thermophilus,* HPr(His~P)-mediated phosphorylation of LacS evokes

maximal activity of the lactose transport protein *in vivo* by increasing the V_{max} of the lactose/galactose exchange reaction. This condition is met in cells at the late-exponentional and stationary phase of growth, when HPr(His~P) is the dominant species of HPr (Gunnewijk and Poolman, 2000a).

The transition from HPr(Ser-P) to HPr(His~P) at the late-exponentional phase of growth parallels an increase in the extent of LacS phosphorylation, a decrease in lactose and an increase in galactose concentration in the growth medium. Since the K_m^{out} for lactose is higher than that for galactose (Veenhoff and Poolman, 1999), the decrease in lactose/galactose ratio in the medium will reduce the lactose transport capacity as growth proceeds. This will at some point during growth be reflected in a reduced glycolytic activity, to which the HPr(Ser-P)/HPr(His~P) ratio is very sensitive (Reizer *et al.*, 1984; 1989b; Deutscher *et al.*, 1985; Deutscher and Engelman, 1984). By increasing the specific activity upon HPr(His~P)-mediated phosphorylation, *S. thermophilus* is able to compensate for a decrease in lactose concentration (and galactose accumulation in the medium) by adjusting the lactose uptake rate rapidly. Another, but slower, response involves adjustment of the LacS expression levels, which is described in the next section.

Transcriptional Control of the *lac* Operon in *S. thermophilus*

The observed transition from HPr(Ser-P) to HPr(His~P) at the late-exponential phase of growth parallels an increase in LacS level (Gunnewijk and Poolman, 2000). At stationary phase, the expression level is about 10 times higher than the basal LacS level at early-exponential phase of growth, indicating that HPr(Ser-P) plays a role in repression of the *lac* operon. Consistent with a higher basal level of LacS in *S. thermophilus* ST11ΔlacS/pGKhis, the increase in expression was less than two-fold when early-exponential and late-exponential or stationary phase were compared. Importantly, the time point of transition from HPr(Ser-P) to HPr(His~P) is dependent on the basal level of LacS expression, as the transition occurred at later steps of growth in *S. thermophilus* ST11ΔlacS/pGKhis when compared to the wild-type ST11 strain. These results suggest that the formation of HPr(Ser-P) is determined by the rate of lactose transport and/or metabolism.

Direct evidence for HPr(Ser-P)/CcpA-mediated regulation of the *lac* operon comes from studies with *ccpA* disruption strains of *S. thermophilus*. Disruption of the *ccpA* gene impairs the growth of *S. thermophilus* on several sugars as has been observed for other Gram-positive bacteria (Hueck *et al.*, 1995; Egeter and Brückner, 1996; Monedero *et al.*, 1997). The *lacS* promoter contains a *cre* site, overlapping the –10 box and the transcriptional start site (Poolman, 1993), suggesting that the *lacSZ* expression is repressed by CcpA (Henkin, 1996). In accordance, disruption of the *ccpA* gene *in S. thermophilus* CNRZ302 results in derepression of *lacSZ* transcription during exponential growth on lactose (van den Bogaard *et al.*, 2000). Moreover, the rates of lactose uptake and galactose excretion are at least 4-fold increased in the *ccpA* disruption strain relative to wild-type cells. The increased lactose uptake and hydrolysis does not result in an increased growth rate on lactose, but leads to massive expulsion of glucose into the fermentation medium. Apparently, loss of a functional CcpA in *S. thermophilus* uncouples the control of metabolism over transport and *vice versa*, as glycolysis can no longer keep up with the massive lactose intake. Thus, the *S. thermophilus ccpA* disruption mutant has a lactose transport capacity that exceeds the maximal glycolytic rate. The data indicate that a concerted activity of HPr(Ser-P) and CcpA results in fine-tuning of lactose transport and hydrolysis capacity in order to accommodate maximal glycolytic flux.

Although most *S. thermophilus* strains cannot use galactose as a carbon source, galactose-fermenting mutants of strain CNRZ302 are readily obtained (Hutkins *et al.*, 1985; Vaughan *et al.*, 2001). Galactose is taken up by the LacS protein (Poolman, 1993), and fermented via the Leloir pathway (Vaughan *et al.*, 2001), but growth is slower than with lactose. When *ccpA* is disrupted in the galactose-fermenting strain, derepression of *lacSZ* transcription is not observed during growth on galactose (van den Bogaard *et al.*, 2000). This suggests that the glucose moiety derived from lactose induces repression of the *lacS* promoter. The repression is not observed when glucose is present in the growth medium, which is due to the low rate of uptake of glucose. The LacS transport protein of *S. thermophilus*, on the other hand, constitutes a very fast and efficient system for lactose uptake, leading to high intake of glucose into glycolysis. This results in relatively high intracellular HPr(Ser-P) concentrations (Deutscher *et al.*, 1995) and, consequently, repression of the *lacS* promoter. Repression of the *lac* operon in *S. thermophilus* is not carbon-source dependent but is determined by the rate of glycolysis relative to sugar uptake. Current studies aim at relating glycolytic activity in *S. thermophilus* to catabolite control (van den Bogaard *et al.*, unpublished results). Analysis of the steady state pool of glycolytic intermediates isolated from wild-type and *ccpA*-disruption strains grown on different carbon sources should elucidate the key-glycolytic steps controlled by HPr(Ser-P) and CcpA. Probably, FDP, PEP and/or ATP function as the intracellular indicators of this glycolytic flux, as has been suggested for other Gram-positive bacteria (Deutscher and Engelman 1984; Reizer *et al.*, 1984; 1989b; 1998; Galinier *et al.*, 1998; Kravanja *et al.*, 1999 and Dossonnet *et al.*, 2000; Jault *et al.*, 2000).

Recently, the *S. thermophilus ptsHI* operon, encoding HPr and Enzyme I, has been cloned and several HPr mutants have been constructed that abolish or mimic phosphorylation at residues His15 or Ser46 (van den Bogaard *et al.*, unpublished results). The expression of these mutants in a *ptsH* knock-out strain will give more insight in the physiological role of the different phosphorylated forms of HPr in the regulation of lactose metabolism (Deutscher *et al.*, 1994; Ye *et al.*, 1994 a, b; Luesink *et al.*, 1998). Furthermore, expression of HPr mutants that are affected in their affinity for IIA^{LacS} or their phosphorylation capacity could yield further evidence for the role of HPr in the modulation of the LacS exchange activity as indicated by the *in vitro* experiments.

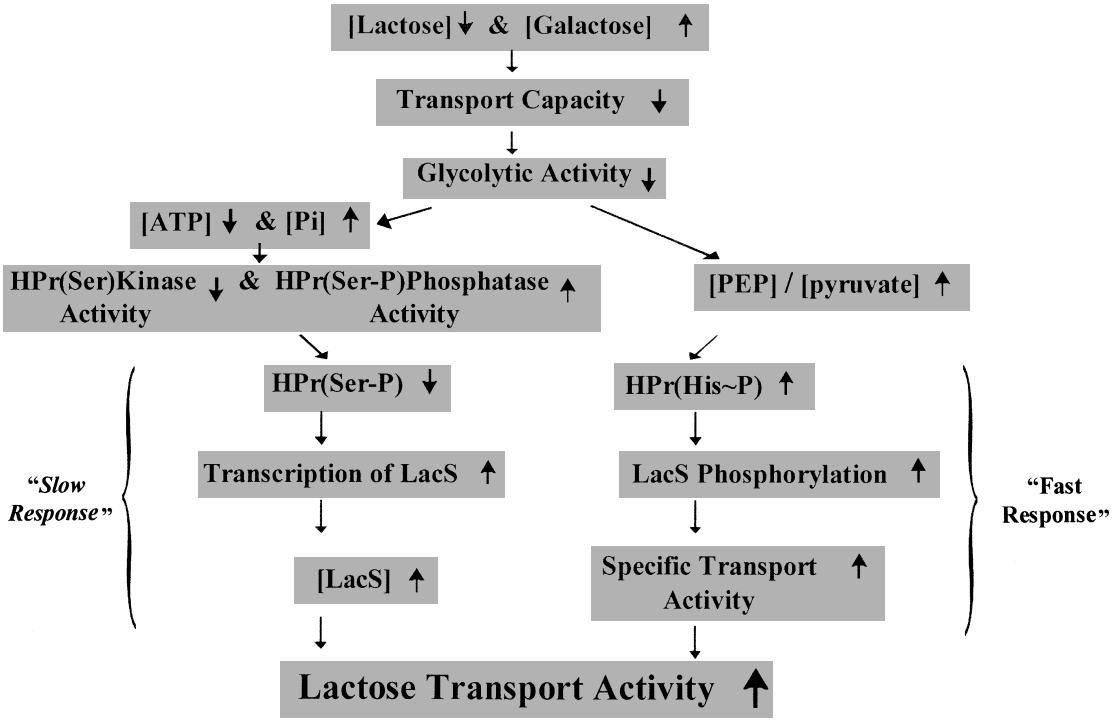

Figure 4. Schematic representation of the regulation of lactose transport capacity in *S. thermophilus*. The arrows in the boxes represent a decrease or increase of the concentration or activity of the indicated component.

Model for Autoregulation of Lactose Transport and Metabolism in *S. thermophilus*

On the basis of knowledge described in the previous sections a model has been proposed for the control of lactose transport and metabolism in *S. thermophilus* (Figure 4). The rate of lactose transport via LacS is very susceptible to the lactose/galactose ratio in the growth-medium as the transporter has a higher affinity for galactose (end-product of the fermentation) than for the substrate lactose. This implies that the transport capacity will decrease when galactose accumulates in the medium even with millimolar concentrations of lactose available. At some point during growth this will be reflected in a reduced glycolytic activity, which affects the concentrations of different glycolytic intermediates to which the HPr(Ser-P)/HPr(His~P) ratio is very sensitive. ATP is an effector of HPr(Ser) kinase, whereas Pi is an inhibitor. Besides, controlling HPr(Ser)kinase, the HPr(Ser-P)phosphatase is stimulated by Pi and inhibited by ATP (Deutscher and Saier, 1983; Reizer *et al.*, 1984; 1989b; Deutscher *et al.*, 1985). The intracellular concentrations of ATP, PEP and Pi vary in response to the carbohydrate availability as has been firmly established for other lactic acid bacteria. ATP levels are relatively high in rapidly metabolizing cells, whereas Pi and PEP are low under these conditions. These latter compounds become high at the end of the exponentional phase of growth and remain high in the stationary phase (Mason *et al.*, 1981; Thompson and Torchia, 1984; Konings *et al.*, 1989). These physiological parameters form the basis for the proposed changes in enzyme activity when the glycolytic activity decreases. Increasing PEP concentrations will induce a rise in the HPr(His~P) concentration due to PEP-dependent Enzyme I phosphorylation of HPr. Consequently, the LacS protein becomes phosphorylated, which in turn increases the maximal transport rate of the LacS protein. At the same time, the modified activities of HPr(Ser)kinase and HPr(Ser-P)phosphatase will decrease the HPr(Ser-P) concentration. Accordingly, a relief of the HPr(Ser-P)/CcpA-mediated repression of the *lac*S promoter will result in the synthesis of more LacS and β-galactosidase, which in turn will provide more glucose for glycolysis. In this way the uptake of lactose is tuned to the lactose/galactose ratio in the medium as well as the glycolytic capacity of the cell. This autoregulatory mechanism allows *S. thermophilus* to co-metabolize lactose and sucrose.

Concluding Remarks

Hierarchical control of carbohydrate transport and metabolism results in the preferential utilization of one carbohydrate over another. Autoregulatory control, on the other hand, adjusts the activity of the first step(s) of metabolism, most often the transport activity, to the metabolic capacity of the cell and to the availability of a particular substrate. In both cases, regulation of the activity of existing transporters/enzymes as well as gene regulation plays a role. For both regulatory processes, components of the PTS form the underlying signaling pathway that senses the availability of carbon sources and the metabolic needs of the cell to respond to these signals. It does so via modulation of the phosphorylation state of the PTS components in a PEP/Enzyme I or ATP/HPr(Ser)kinase dependent manner. Specific PTS components are the effectors that control transport and metabolism of several carbohydrates. In Gram-negative enteric bacteria IIA^{Glc} is

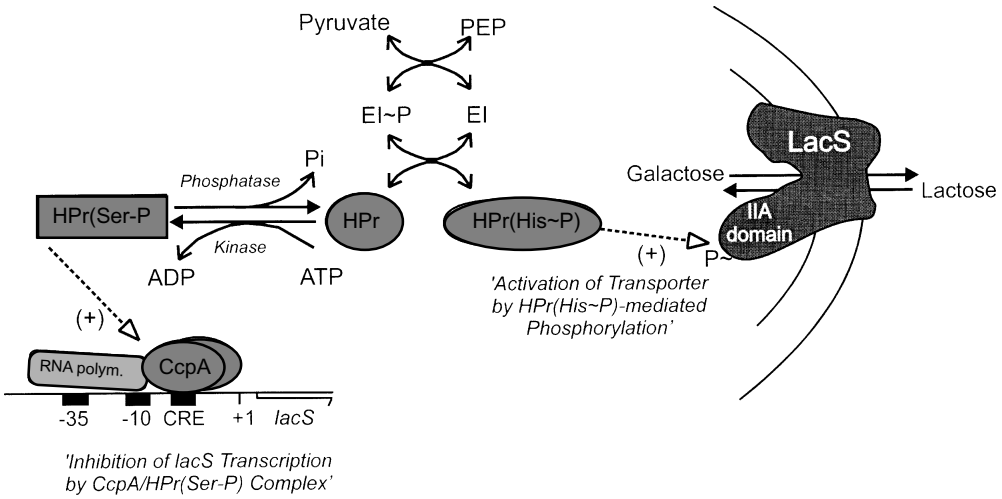

Figure 5. Regulation of lactose transport in *S. thermophilus*. Schematic representation of HPr(His~P)-mediated phosphorylation of LacS (stimulation of lactose transport activity), and HPr(Ser-P)/CcpA-mediated regulation of *lac*S transcription. The depicted symbols are described in the legends of Figure 2.

the critical effector molecule (Figure 1), whereas in Gram-positive bacteria HPr assumes this role (Figure 2). The regulation of transport and metabolism of the non-PTS sugar lactose in *S. thermophilus* is unprecedented and shows how metabolism is fine-tuned to the carbohydrate availability and metabolic capacity of the cell (Figure 5). We can conclude that evolution has generated a wide range of regulatory mechanisms for the control of carbohydrate utilization, but in most, if not all, of these mechanisms, the components of the PTS system form the intricate pathway to sense the changes and convert the signal(s) into an adequate response.

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