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How Phosphotransferase System-Related Protein Phosphorylation Regulates Carbohydrate Metabolism in Bacteria†

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† Pieter W. Postma passed away during the preparation of this review. The loss that we experienced has been put into words by Gary R. Jacobson and Joseph W. Lengeler (368).

From a few peaks rising above the fog we try to imagine what the hidden landscape underneath might look like.

—Pieter W. Postma, during a hiking trip in the Beaujolais region in October 1997.

INTRODUCTION

Carbon Catabolite Repression

Given a certain extracellular environment, a bacterium needs only a subset of the enzymes encoded by the genome to propagate, and therefore, it regulates gene expression differentially. For instance, in case a particular substrate is absent, the genes encoding the enzymes required for uptake and subsequent metabolism of the substrate are often repressed. Availability of the substrate then leads to the relief of repression. The classical example of this mode of transcription regulation is the *lac* operon of *Escherichia coli* (367). More complex responses of cells can occur when they are exposed to multiple nutrients at the same time. More than a hundred years ago, it was observed that growth on glucose can lower the activity of certain enzymes in bacteria and yeast. This phenomenon became known as the glucose effect (see also reference 744). One of the first descriptions of the glucose effect stems from the work of Dienert and was published in 1900 in Paris, France. He observed that *Saccharomyces cerevisiae* cells, which had been adapted to galactose (i.e., they were able to utilize galactose), rapidly lost the adaptation when the cells were exposed to glucose or fructose (188). This observation was later confirmed, for example, by Söhngen and Coolhaas, in Wageningen, The Netherlands, who measured the influence of temperature on the glucose effect in yeast (820). One of the first quantitative analyses of the glucose effect was carried out by Stephenson and Gale in Cambridge, England, who measured the activity of *E. coli* galactozymase. Galactozymase was understood as being the entity of enzymes necessary for the degradation of galactose by a specific organism. Those authors found that glucose exerted a strong repressive effect on galactozymase activity (more than sevenfold), while the utilization of glucose was not affected by galactose (836). In the early 1940s, Jacques Monod observed that when cultivated in a synthetic medium containing sucrose and dextran, the gram-positive bacterium *Bacillus subtilis* first utilized sucrose and then stopped growing for a certain period (lag phase or phase of adaptation) before the cells resumed growth by utilizing dextran (572). Owing to the biphasic growth he had observed, Monod called this phenomenon diauxie. He extended his studies with *B. subtilis* and found that diauxie was a more general phenomenon. When this organism was provided with sucrose or glucose, for example, and a second carbohydrate such as maltose, mannitol, inositol, or sorbitol, it also showed diauxic growth; i.e., sucrose or glucose was utilized first before the bacterium started to transport and metabolize the "less favorable" carbon source. He finally extended his studies to the gram-negative bacterium *E. coli*, which exhibited diauxie when grown in the presence of glucose or fructose, for example, as the preferred carbon source and xylose, arabinose, rhamnose, maltose, lactose, or glucitol as the less favorable sugar (572). It appeared that in each organism, a specific hierarchy existed for the utilization of carbon sources, with glucose usually being on top of it. In subsequent years, it was found that preferred sugars such as glucose, fructose, or sucrose, as long as they are present in sufficient amounts in the growth medium, repress the synthesis of the enzymes necessary for the transport and metabolism of less favorable carbon sources. The phenomenon therefore became known as carbon catabolite repression (CCR) (137). When the preferred carbon source is exhausted, bacteria first need to synthesize the enzymes necessary for the transport and metabolism of the less favorable carbon source (lag phase) before they can resume growth. The glucose effect reported at the beginning of the last century can therefore be considered CCR.

The regulatory mechanisms underlying CCR have since been intensively studied, and it was found that the preferential use of one carbon source over the other involves either the prevention of transcription activation or the repression of transcription. Here, we define CCR, somewhat more broadly, as the inhibitory effect of a certain carbon source in the growth medium on gene expression and/or the activity of enzymes involved in the catabolism of other carbon sources. Gene expression and enzyme activity are regulated via protein-DNA, protein-protein, and protein-metabolite interactions, and these interactions are in turn modulated via protein modification. Interestingly, the bacterial phosphoenolpyruvate (PEP):carbohydrate phosphotransferase system (PTS), which catalyzes the uptake and concomitant phosphorylation of numerous carbohydrates, plays a major role in bacterial CCR. However, quite different mechanisms have evolved in gram-negative and gram-positive organisms, and different PTS proteins are implicated in the two types of bacteria (for earlier reviews on the subject, see references 90, 756, and 845). In some bacteria, CCR is governed by other preferences and is possibly mediated by additional mechanisms. For instance, α-proteobacteria of the genera *Sinorhizobium*, *Rhizobium*, and *Bradyrhizobium* (83, 889) and γ-proteobacteria of the *Pseudomonas* family (135) prefer acetate or tricarboxylic acid (TCA) cycle intermediates such as succinate over carbon sources such as glucose, fructose,

or lactose. The underlying mechanisms have not yet been unraveled, but in pseudomonads, they involve the catabolite repression control (Crc) protein (22, 331, 332, 573, 747, 982), and rhizobacteria, they involve inducer accumulation (83).

In this review, we will focus on mechanisms that control carbohydrate transport and metabolism in PTS-containing bacteria, and we will concentrate on the regulatory roles played by the components of the system. We start by discussing the individual components of the PTS and their properties. Subsequently, we will describe their role in catabolite repression and in other regulatory mechanisms governing carbon metabolism. We will pay particular attention to the mechanisms that have developed in the enteric bacteria *E. coli* and *Salmonella enterica* serovar Typhimurium (*γ-Proteobacteria*) and in low-G+C gram-positive bacteria (also known as *Firmicutes*) such as *B. subtilis* and *Lactobacillus casei*, but we will also refer to other proteobacteria and to gram-positive bacteria with high G+C content (also known as *Actinobacteria*). The two groups of gram-positive bacteria can be distinguished not only on the basis of the different G+C content of their DNA but also based on many other different characteristics. For example, actinobacteria have been shown to contain numerous proteins that have been detected only in members of this phylum so far (corynebacteria, streptomyces, mycobacteria, nocardiae, propionibacteria, bifidobacteria, leifsoniae, and rhodococci, to name just a few) (253). Important for this review is that most high-G+C gram-positive bacteria possess PTS components and transport sugars via this system but seem to be devoid of adenylate cyclase and HPr kinase/phosphorylase (41). Nevertheless, other PTS-related control mechanisms of carbon metabolism are operative.

The Phosphoenolpyruvate:Carbohydrate Phosphotransferase System

The PTS was discovered in *E. coli* by Kundig, Ghosh, and Roseman as a system that uses PEP to phosphorylate a number of hexoses, including *N*-acetylmannosamine, glucose, mannose, glucosamine, and *N*-acetylglucosamine (434). Subsequently, it was recognized that the PTS is in fact a transport system that catalyzes the uptake of numerous carbohydrates and their conversion into their respective phosphoesters during transport. After its discovery in *E. coli*, the PTS was found in many other bacterial species. The coupled transport and phosphorylation of carbohydrates were originally described as a two-step reaction catalyzed by two enzymes, enzyme I (EI) and enzyme II (EII), with the protein HPr as an intermediate phosphoryl donor:

$$\text{PEP} + \text{HPr} \overset{\text{EI}}{\Leftrightarrow} \text{pyruvate} + \text{phospho} \sim \text{HPr(P}\sim\text{HPr)}$$

$$\text{P}\sim\text{HPr} + \text{carbohydrate} \overset{\text{EII}}{\Leftrightarrow} \text{HPr} + \text{carbohydrate-P}$$

However, this representation is a bit misleading in the sense that EI, HPr, and EII behave in a functionally identical manner. They accept a phosphoryl group from a donor and donate it to an acceptor (i.e., they transfer a group), thus cycling between the phosphorylated and unphosphorylated states (depicted in Fig. 1). It is precisely this aspect of the mechanism that is exploited by the cell in PTS-mediated regulation.

The basic composition of the PTS is in fact similar in all species studied so far (for reviews, see references 678 and 730). It is comprised of two "general" cytoplasmic components, EI and HPr, which are common to all PTS carbohydrates. Carbohydrate specificity resides in EII, and hence, bacteria usually contain many different EIIs. Each EII complex consists of one or two hydrophobic integral membrane domains (domains C and D) and two hydrophilic domains (domains A and B), which together are responsible for the transport of the carbohydrate across the bacterial membrane as well as its phosphorylation. In a sense, the EII complexes constitute parallel transport pathways connected to a common PEP/EI/HPr phosphoryl transfer pathway. *E. coli* contains at least 15 different EII complexes, and the existence and properties of these enzymes have been established by genetic, biochemical, and physiological studies. A similar number of PTSs is present in *B. subtilis* (178, 706). EII complexes are formed either by distinct proteins or by a single multidomain protein. Likewise, fusion proteins that contain EI and/or HPr domains exist. A prominent example of the latter is FPr, which consists of HPr and an EIIA domain and mediates the phosphotransfer in the uptake of fructose by *E. coli* and *Salmonella enterica* serovar Typhimurium (266). In *Rhodobacter capsulatus*, a similar fusion protein also contains an EI domain (958).

Genome sequencing projects have uncovered many proteins that are similar to either one of the two general PTS proteins or to parts of carbohydrate-specific EII complexes (345, 867, 935). For example, in *E. coli*, the protein DhaM is composed of an $EIIA^{Man}$-like regulatory domain followed by an HPr and a truncated EI domain (304). In other cases, the newly discovered proteins are formed by fusions between domains that originate from both PTS and non-PTS proteins, e.g., a *Clostridium acetobutylicum* response regulator (built from HPr and an NtrC-like protein) (721) and the *Streptococcus thermophilus* LacS protein (built from a melibiose carrier and $EIIA^{Glc}$) (673). The functions of most of these chimeric proteins remain unknown, but for some proteins, like LacS and DhaM, the function has been elucidated. Indeed, one of the present challenges is finding the function of the newly discovered PTS-like proteins, most of which have not been detected by any biochemical or mutant study. A very intriguing group consists of the paralogs of EI and HPr. In *E. coli*, at least five paralogs of each general PTS protein were uncovered (345, 720). We will discuss some of these proteins below (see "SOME UNUSUAL PTS PATHWAYS AND PROTEINS").

The general PTS proteins EI and HPr. EI is encoded by the *ptsI* gene. Sequence comparisons reveal that the EIs (about 570 residues; 63 kDa) from various gram-positive and gram-negative bacteria exhibit significant identity (345, 678). EI is autophosphorylated in the presence of Mg^{2+} (940) at the N-3 position of the imidazole ring of a conserved histidine (His-189 in *E. coli* EI) (16, 940), which is located on the N terminus of the protein. The C terminus contains the PEP binding site and is necessary for dimerization (114, 254, 798). The two-domain structure of the protein was discovered by high-sensitivity differential scanning calorimetry (478). Limited proteolysis of *E. coli* EI in vitro results in a specific split and provides a stable

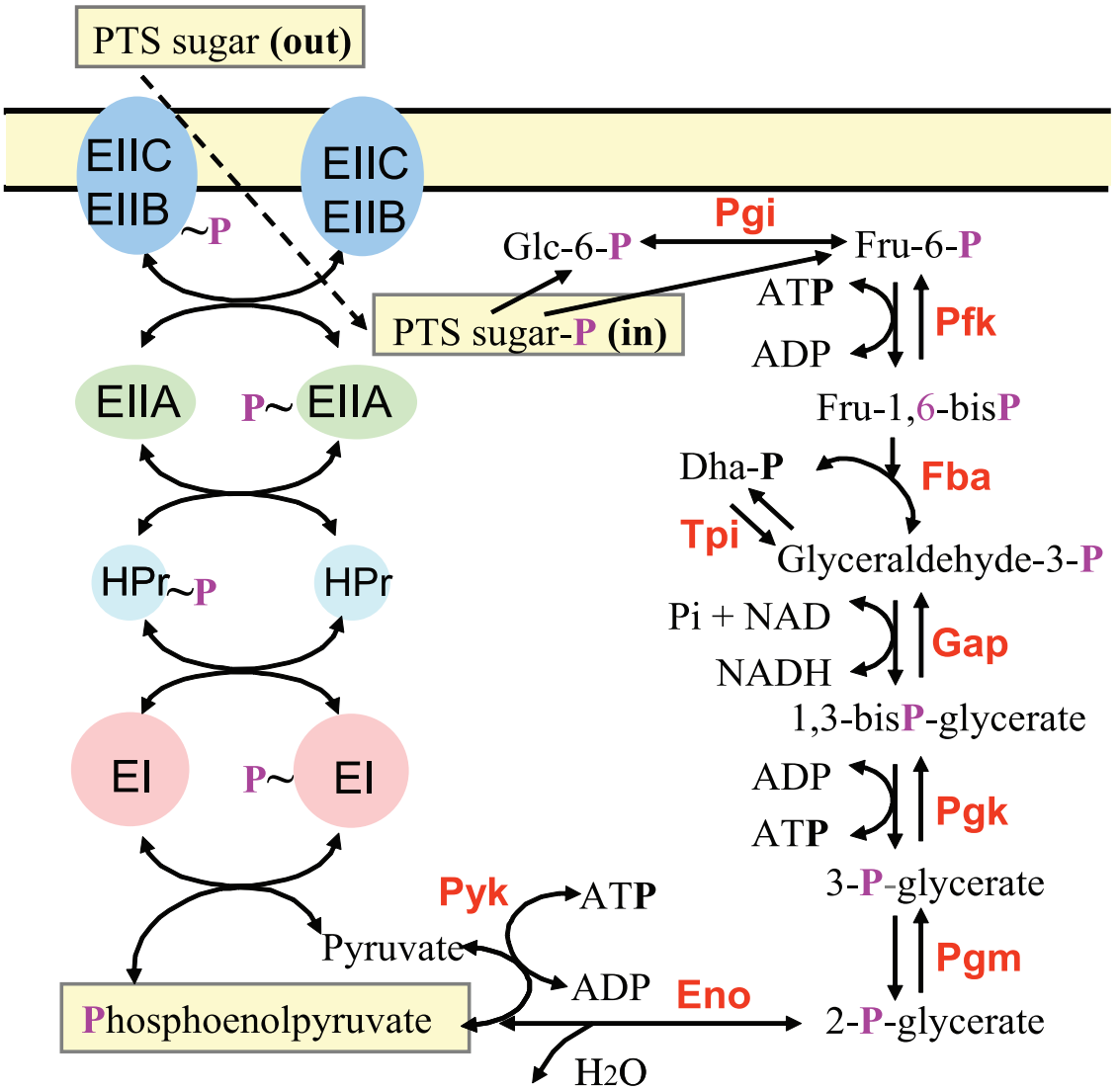

FIG. 1. Carbohydrate transport and phosphorylation by the PTS and their coupling to glycolysis. Carbohydrates are transported and concomitantly phosphorylated by the PTS. The phosphorylated carbohydrate feeds into glycolysis, normally at the glucose-6-P or fructose-6-P level. Two phosphoenolpyruvate molecules are usually formed in glycolysis, one of which is used to drive the transport and initial phosphorylation of the carbohydrate. As a result, the phosphorylation state of the PTS proteins depends on both the concentration of extracellular carbohydrates and the ratio of internal phosphoenolpyruvate and pyruvate. Abbreviations for enzymes (in boldface type) are as follows: Pgi, phosphoglucose isomerase; Pfk, phosphofructokinase; Fba, fructose-1,6-bisphosphate aldolase; Tpi, triose-phosphate isomerase; Gap, glyceraldehyde-3-phosphate dehydrogenase; Pgk, phosphoglycerate kinase; Pgm, phosphoglycerate mutase; Eno, enolase; Pyk, pyruvate kinase.

peptide representing the N-terminal domain (455). The N-terminal domain of EI (EI-N) (115) as well as the C-terminal domain (EI-C) (234) were cloned and characterized. EI-C was shown to complement EI-N both in vivo and in vitro; i.e., EI-N was phosphorylated in the presence of EI-C, PEP, and Mg^{2+} (234). A truncated protein composed of the first 259 amino acids has been synthesized and purified, and both the crystal (475) and solution (258) structures were resolved. It appears that phosphorylation does not drastically change the conformation of the N-terminal domain per se. Recently, the crystal structure of the C-terminal domain of EI of the thermophile *Thermoanaerobacter tengcongensis* was also elucidated (619). EI exhibits about 30% sequence similarity with pyruvate phosphate dikinase (PPDK) (328, 605, 670), and the various domains in both proteins have similar structures (619). PPDK, which converts ATP, P_i, and pyruvate into AMP, pyrophosphate (PP_i), and PEP; PEP synthase, an enzyme that catalyzes the conversion of pyruvate and ATP into PEP, AMP, and P_i and that is also phosphorylated on a histidyl residue (590); and EI have related functions. For PPDK, a structural model with three domains has been proposed: a C-terminal PEP/pyruvate binding domain, an N-terminal nucleotide binding domain, and a P~His domain linked to the other two domains via two flexible polypeptide segments (328). Although the nucleotide and PEP/pyruvate binding sites are approximately 45 Å apart, the P~His domain is thought to bridge the distance by swiveling between the two other domains to enable the phosphoryl transfer. Recent data obtained from a comparison of several

PPDK crystal structures are consistent with the proposed model (587). Similar to PPDK, a large distance (>20 Å) between the PEP/pyruvate binding site and the phosphorylatable histidine was observed in a model of EI in which the N-terminal and C-terminal EI structures were fused (619). Swiveling of the N-terminal domain by changing the position of the domains with respect to each other but retaining the structure of the individual domains reduces the distance significantly (to 7.4 Å). In the case of EI, the N-terminal domain contains the HPr binding site rather than the nucleotide binding site of PPDK and PEP synthase. This model was confirmed by recent studies in which the structure of full-length EI of *Staphylococcus carnosus* was determined (516). The phosphohistidine and HPr-binding domain are clearly separated from the C-terminal dimerization and PEP binding domain. The structure also revealed the extensive interaction surface related to the dimer. Thermodynamic analyses of the influence of several effectors on the conformational stability of EI of *Streptomyces coelicolor* showed that at a low pH, EI is partly unfolded and that the presence of PEP causes structural changes (356). Two other recent studies on the structure of EI (639) and its C-terminal domain (638) suggest a relatively large structural variability for monomeric EI, which progressively diminishes when EI dimerizes and binds Mg^{2+} and PEP. The observed effects are explained by a swiveling mechanism, as described for PPDK. The structure thus provides additional evidence for the proposed conformational change necessary to facilitate phosphotransfer.

HPr (about 90 residues; 9 to 10 kDa) is encoded by the *ptsH* gene and has been purified from a variety of organisms (678). Similarity is most pronounced around the active-site histidyl residue, His-15 in the HPr of most enteric bacteria and firmicutes (183, 681, 941). HPr is phosphorylated at the N-1 position of the imidazole ring of His-15. The crystal and solution structures of HPrs from *E. coli* and *B. subtilis* as well as a few other species are known (for reviews, see references 33, 123, 329, and 936). The molecule forms an open-faced β-sandwich consisting of a four-stranded antiparallel β-sheet that is covered at one side by one short and two long α-helices. The active-site His-15 is located in the N-terminal part of the first long α-helix and is exposed to the solvent.

The solution structures of the complex between HPr and the N-terminal domain of EI (260) and between HPr and $EIIA^{Glc}$ of *E. coli* (929) have been determined. The absence of large changes of the chemical shift values for the backbone atoms upon complexation indicates that the interactions of HPr with EI and $EIIA^{Glc}$ do not require considerable conformational changes in any of the domains of the PTS binding partners. The binding interface of HPr and glycogen phosphorylase (see "REGULATION OF CARBON METABOLISM IN GRAM-NEGATIVE ENTERIC BACTERIA") was also mapped, and the interaction surface of the three structures was compared (930). EI and $EIIA^{Glc}$ interact with the same narrow region of HPr, whereas the region of interaction with glycogen phosphorylase is somewhat wider (929, 930). This is consistent with the higher binding affinity reported for HPr with glycogen phosphorylase than for HPr with EI or $EIIA^{Glc}$ (259, 799). A similar interaction surface was reported for *E. coli* HPr binding either $EIIA^{Mtl}$ (139, 908) or $EIIA^{Man}$ (949) and *B. subtilis* HPr binding $EIIA^{Glc}$ (123). The key interacting residues are located in helices 1 and 2 and the loops preceding helix 1 and following helix 2. Studies with a large number of *ptsH* mutants, which show lowered affinity for binding to EI, suggest that the same residues are important for the interaction between EI and HPr (85).

In most low-G+C gram-positive bacteria and a few gram-negative organisms, HPr can also be phosphorylated by an ATP-dependent protein kinase on a seryl residue, e.g., Ser-46 in *B. subtilis.* We will discuss the importance of the second regulatory phosphorylation in detail below (see REGULATORY FUNCTIONS OF P-Ser-HPr). The regulatory phosphorylation is not part of the phosphoryl transfer to carbohydrates, but phosphorylation of the seryl residue slows the phosphoryl transfer from P~EI to HPr at least 100-fold. *E. coli* HPr contains a Ser-46 residue but lacks an HPr kinase. Nevertheless, replacement of Ser-46 in *E. coli* HPr by an aspartyl residue lowers the affinity of P~EI for HPr almost 1,000-fold (589). This effect is most likely caused by electrostatic repulsion, as the mutation caused by the replacement of Ser-46 with an aspartyl residue (Ser46Asp) leads to only weak structural changes (952).

Enzyme II complexes. The carbohydrate specificity of the PTS resides in the EIIs, which consist of an integral membrane domain facing both the periplasmic space and the cytoplasm and cytoplasmic domains or proteins. All EIIs are built basically in a similar modular manner (764) and can consist of up to four separate proteins (for reviews, see references 464, 678, 730, and 813). The PTSs were classified into four (super)families with distinct evolutionary origins on the basis of the phylogenies of the EIIs (759) as follows: (i) the glucose-fructose-lactose superfamily, comprised of the glucose family (e.g., *E. coli* $EIIA^{Glc}/EIICB^{Glc}$ or *B. subtilis* $EIICBA^{Glc}$ [CB, CBA, etc., indicate the domain order in multidomain EIIs]), the fructose-mannitol family (e.g., *E. coli* $EIICBA^{Mtl}$), and the lactose family (e.g., *L. casei* $EIIA^{Lac}/EIICB^{Lac}$); (ii) the ascorbate-galactitol superfamily, comprised of the ascorbate family (e.g., *E. coli* SgaA/SgaB/SgaT) (358, 989) and the galactitol family (e.g., *E. coli* $EIIA^{Gat}/EIIB^{Gat}/EIIC^{Gat}$) (610); (iii) the mannose family (e.g., *E. coli* $EIIAB^{Man}/EIIC^{Man}/EIID^{Man}$ or *B. subtilis* $EIIA^{Lev}/EIIB^{Lev}/EIIC^{Lev}/EIID^{Lev}$ [a mannose- and fructose-specific PTS]); and (iv) the dihydroxyacetone family (e.g., *E. coli* EIIA-HPr-EI^{Dha} [DhaM] [304] or $EIIA^{Dha}$ in firmicutes).

The presence of a common preserved domain in the latter two families (411) might indicate a similar evolutionary origin of the two families and thus the existence of a third superfamily. The sequence-based classification of the various PTSs is supported by X-ray crystallography and nuclear magnetic resonance (NMR) studies, which clearly show that the structures of the EIIA (474, 617, 818, 858, 906, 955) and EIIB (1, 2, 98, 459, 623, 778, 906) domains/proteins belonging to the various classes are quite different. Information on the structure of the integral membrane domain EIIC (and EIID) is limited. The membrane topologies of these proteins have been studied using Cys replacement mutagenesis and chemical modification by thiol reagents for $EIIBCA^{Bgl}$ (960) and $EIICBA^{Mtl}$ (918).

Extensive work on the intricate properties of the EII complexes and the detailed mechanisms by which they transport and phosphorylate carbohydrates has been performed in the laboratories of G. T. Robillard, B. Erni, and G. R. Jacobsen. The results that focus predominantly on $EIICBA^{Mtl}$, $EIIC^{Man}$/

$EIID^{Man}$, and $EIICB^{Glc}$ have been reviewed extensively (369, 730, 813), and we will therefore discuss only a few important aspects. Some more recent papers included work on the properties of a cyclized protein formed by the soluble $EIIA^{Glc}$ and the membrane-bound $EIICB^{Glc}$ (812), the dimerization of $EIICBA^{Mtl}$ (903, 904), sugar recognition by the $EIIC^{Man}$/$EIID^{Man}$ and $EIICB^{Glc}$ of *E. coli* (256), the membrane topologies of $EIIBCA^{Bgl}$ (960) and $EIICBA^{Mtl}$ (918), and transient-state kinetics of enzyme $EIICB^{Glc}$ (544).

The glucose-specific EII complex of enteric bacteria consists of two distinct proteins, the cytoplasmic protein $EIIA^{Glc}$ (gene *crr*) and the membrane-associated protein $EIICB^{Glc}$ (gene *ptsG*), in which the EIIB domain is hydrophilic and in contact with the cytoplasm and the EIIC domain is buried within the membrane. The EIIA and EIIB domains are defined as the domains that receive the phosphoryl group from P∼HPr and P∼EIIA, respectively. Whereas the phosphorylated residue in the EIIA domain/protein is a histidyl residue, that in the EIIB domain/protein can be either a histidyl residue (mannose family) or a cysteyl residue (all other families). The latter was first demonstrated in *E. coli* EII^{Mtl} (634), and the specific phosphocysteyl residue was thereafter identified in a number of cases by analytical methods (547, 633). The phosphoryl group of the EIIB domain is transferred to the carbohydrate after translocation of the carbohydrate by the EIIC domain across the membrane and delivery at the inside face of the cytoplasmic membrane. In the case of the mannitol-specific *E. coli* EII^{Mtl} or the glucose-specific *B. subtilis* EII^{Glc} complex, the EII is a single polypeptide that contains the two hydrophilic domains EIIA and EIIB as well as the transmembrane domain EIIC. Again, in other EII complexes, such as the mannose-specific *E. coli* EII^{Man}, phosphoryl groups are carried by two histidyl residues present in the A and B domains of the cytoplasmic $EIIAB^{Man}$, whereas translocation of the carbohydrate requires the two transmembrane subunits EIIC and EIID.

Organization of the PTS. Phosphoryl transfer between the PTS proteins proceeds by an in-line associative mechanism (48). The transfer is accommodated by the formation of heterodimeric transition complexes, and considering the kinetics of the transfer, the heterodimeric association should be transient. The solution structures of different transition complexes, including EI and HPr (260), HPr and $EIIA^{Glc}$ (124, 929), HPr and $EIIA^{Mtl}$ (139), HPr and $EIIA^{Man}$ (949), $EIIA^{Glc}$ and $EIIB^{Glc}$ (98, 270), and, finally, P∼$EIIA^{Chb}$ and Cys10Ser mutant $EIIB^{Chb}$ (398), could be determined. The formation of the heterodimeric complexes has a significant effect on the flux control properties of the individual components of the PTS (738, 896, 901).

Based on the observation that *E. coli* EI and HPr are attached to membrane fragments of *E. coli* (757), it was speculated that the PTS components associate into a metabolon (611), which would improve PTS function. However, it is not clear how phosphotransfer from one component to the next could take place in a fully associated system without linkers to provide room for the domains to move, and furthermore, as it was calculated that diffusion should not limit glucose influx (239, 240), there should be no functional driving force to fully associate into an EI-HPr-EII complex. Formation of larger complexes by EI linked to yellow fluorescent protein has been observed in cells grown on PTS as well as non-PTS carbohydrates when they reached stationary phase and high cell densities (637). The observed distribution of the fluorescence over the cells was punctate and sometimes even bipolar (after induction of yellow fluorescent protein-EI with IPTG [isopropyl-β-D-thiogalactopyranoside]). The aggregation appeared to be reversible, as it disappeared after the addition of a carbohydrate, which led to renewed growth of the cells and dispersion of the fluorescence. Thus, phosphotransfer activity within the PTS, as invoked by the addition of a carbohydrate, in fact seems to promote the overall dissociation of the PTS components rather than their association into a metabolon.

Notwithstanding, several PTS proteins were experimentally proved to associate to functional homodimers. The first PTS protein that was found to functionally dimerize was EI (432, 560; also reviewed in reference 114). Later, it was shown that the N-terminal domain does not dimerize (115) but that the C-terminal domain is responsible for dimerization (86). In fact, the association properties of the isolated C-terminal domain are similar to that of entire EI, although the binding constants are orders of magnitude larger (638). It was proposed that the monomer/dimer transition of EI could potentially regulate uptake flux (940), and as a result, the transition has been extensively studied. The idea was supported by the fact that PEP phosphorylates only dimeric EI, and the rate of association/dissociation is very slow, especially compared to sugar uptake (114, 539). The monomer/dimer transition has been studied by various analytical techniques, and many physiological conditions have been tested. It was observed that P∼EI has a 10-fold increased dimerization constant with respect to unphosphorylated EI and that the dimerization of EI is promoted by PEP and Mg^{2+} and inhibited by pyruvate (190–192). It was concluded that the stimulatory effect of Mg^{2+} and PEP on EI association results from a change in conformation. Association of both normal EI and the nonphosphorylatable mutants H189E, H189A, and H189Q was studied by carrying out sedimentation equilibrium experiments (639). The association constant appears to be very sensitive to pH, temperature, and ionic strength and may vary by as much as 700-fold. It was concluded that the ligands Mg^{2+} and PEP are the major determinants for dimerization and that their binding leads to conformational changes. A model that accommodates the known facts about the dimerization was presented. A potential regulatory function of EI dimerization with regard to the role of PEP is discussed below.

Several membrane-spanning EIIs have also been shown to dimerize, such as $EIICBA^{Mtl}$ (730) and $EIICB^{Glc}$ (213, 214, 548). Proteome analysis of stable oligomeric protein complexes confirmed the existence of $EIICBA^{Mtl}$ and $EIICB^{Glc}$ oligomers in the membrane of *E. coli* (826). In addition, oligomers of EII^{Nag} and EII^{Tre} were detected, which complies with a generalized picture in which the PTS permeases function as dimers (or oligomers). In contrast, it is generally assumed that HPr and EIIA do not form functional homooligomers. However, several experimental observations suggest the opposite. Biochemical evidence strongly suggested that $EIIA^{Lac}$ forms homotrimers (176, 319). This suggestion was supported by the crystal structure of $EIIA^{Lac}$ from *Lactococcus lactis*, which also revealed a homotrimer (818). In addition, it was reported that four molecules of $EIIA^{Glc}$ associate with one $EIICB^{Glc}$ homodimer (213). Moreover, multimeric $EIIA^{Glc}$ was observed

during gel filtration (786) and in crystals (224). Dimeric $EIIA^{Chb}$ and $P\sim EIIA^{Chb}$ were observed when ultracentrifugation was carried out (398). $EIIAB^{Man}$ was found in the dimeric form both in the cytoplasm and linked to the membrane (216, 826). Also, $EIIA^{Ntr}$, a protein homologous to EIIAs that are functional in sugar transport, crystallizes as a dimer (73), although it seems to be a monomer in solution (927). Crh, an HPr-like protein of *B. subtilis*, was shown to form homodimers in crystals (644), and F29W HPr from *Geobacillus stearothermophilus* (previously *Bacillus stearothermophilus*) forms similar domain-swapped dimers in crystallization experiments (827). On the other hand, several measurements performed with the protein in solution suggested that F29W HPr forms monomers. Recently, in vitro experiments provided kinetic evidence that all components of the *E. coli* glucose PTS form functional dimers (R. Bader, J. G. Blom, K. J. Hellingwerf, M. A. Pelletier, H. V. Westerhoff, and C. Francke, unpublished results).

Although the supposed primary function of the PTS is the transfer of the phosphoryl group from PEP to carbohydrates, all reactions up to and including the EIIs are reversible. The equilibrium constant, K_{eq}, for the reaction $PEP\Leftrightarrow P\sim EI\Leftrightarrow P\sim HPr$ is about 80 for the *E. coli* enzymes (539), whereas the K_{eq} for the phosphoryl group transfer between HPr and $EIIA^{Glc}$ from *E. coli* has a value of between 1 and 1.5 (540). Thus, the overall K_{eq} for the phosphoryl group transfer from PEP to $EIIA^{Glc}$ is in the order of 80 to 120. Furthermore, the K_{eq} for the reaction $P\sim EIIA^{Glc} + EIICB^{Glc}\Leftrightarrow EIIA^{Glc} + P\sim EIICB^{Glc}$ was determined to be 12 (539), very close to the estimated value of 7 (737). The number signifies that the phosphotransfer potential of the thiophospho bond in $P\sim Cys\text{-}EIICB^{Glc}$ is very close to that of the phosphoamidate bond in phosphohistidine of the other PTS proteins. Only the final step, $P\sim EIIB^{Glc}$ + glucose$\Leftrightarrow EIIB^{Glc}$ + glucose-6-phosphate (glucose-6-P), is virtually irreversible, with a measured forward rate of 3.2×10^6 $M^{-1}\ s^{-1}$ (539). These values indicate that under physiological conditions, the phosphoryl transfer reactions catalyzed by the PTS between PEP and $P\sim EIIB^{Glc}$ can run in both directions, from one high-energy P~enzyme intermediate to the next or back to the previous reactions. This differs from most eukaryotic signal transduction systems in which the seryl, tyrosyl, and threonyl residues that are often phosphorylated represent low-energy phosphoenzyme intermediates (837). The reversibility of the phosphoryl group transfer in the PTS has regulatory consequences because it allows the metabolic network to control the phosphorylation state of the PTS proteins in a number of ways, and we will discuss how numerous cellular processes are regulated by the phosphorylation states of certain PTS proteins below (see Table 1 for a summary).

REGULATION OF CARBON METABOLISM IN GRAM-NEGATIVE ENTERIC BACTERIA

In the following sections, we will discuss the numerous regulatory functions carried out by $EIIA^{Glc}$ in enteric bacteria. This protein not only is involved in the regulation of adenylate cyclase, and therefore in CCR, but also interacts with several non-PTS permeases and glycerol kinase to inhibit their activity. The latter phenomenon is described by the term inducer exclusion. We will present new insights into the complex regulation of the intracellular cyclic AMP (cAMP) level and the binding of $EIIA^{Glc}$ to adenylate cyclase and to non-PTS permeases.

$EIIA^{Glc}$ Is the Central Processing Unit of Carbon Metabolism in Enteric Bacteria

The integration of hundreds of enzymatic reactions into a metabolic network and the complex response of such a network to environmental changes require global regulation at the level of enzyme activity and gene transcription (89, 592, 917). A bacterium that is confronted with changes in the supply of nutrients can adapt its metabolic potential through the induction of specific catabolic operons (most often induced by the substrate) (90, 94). More drastic changes may require global adaptation and therefore the induction of complete regulons (89, 592, 917). Transcriptome data indicate that when *E. coli* cells that have been growing on a rich carbon source such as glucose are exposed to a carbon source allowing only slow growth, the number of induced genes increases progressively (485). Low growth rates result in the expression of many transport systems and catabolic genes for nutrients that might be absent but that would stimulate growth if present. Results obtained by high-throughput nutrient screening of glucose-grown cells and cells grown under carbon limitation confirmed the effects observed by transcriptome analysis: cells grown under carbon limitation do utilize a large variety of carbon sources, whereas cells growing on glucose do not (the result of CCR) (360). The large-scale transcriptional responses in *E. coli* are mediated via a limited number of global regulators (527). In enteric bacteria, global regulation of carbon metabolism involves several σ and transcription factors such as Crp (cyclic AMP receptor protein) (reviewed in references 76, 95, 96, 416, and 636), Mlc (666), FruR (145, 267, 268, 762), CsrA (carbon storage regulator) (451, 742, 743, 752, 861), and σ^{54} (25, 150, 843). The activities of some of these regulators are directly affected by the PTS.

The importance of the PTS in the regulation of carbon metabolism in enteric bacteria became apparent when *ptsHI* mutants containing defective EI and/or HPr were isolated. These mutants failed to grow not only on PTS carbohydrates but also on a large number of non-PTS carbon sources like lactose, melibiose, glycerol, and maltose (for reviews, see references 677, 678, and 763). Analysis of suppressor mutations, which restored growth of *ptsHI* mutants on the non-PTS carbon sources (765, 767), revealed that these mutations were in the *crr* gene (carbohydrate repression resistant), which was later shown to be the structural gene for $EIIA^{Glc}$ (542, 543, 786). While *ptsHI-crr* triple mutants grew on the non-PTS carbon sources, they did not resume growth on PTS carbohydrates. These results indicated that not only is $EIIA^{Glc}$ involved in glucose transport and phosphorylation via the glucose PTS, it also regulates the transport and/or metabolism of several non-PTS carbon sources.

The changes of structure or charge induced by phosphorylation or dephosphorylation determine the regulatory role of $EIIA^{Glc}$. $P\sim EIIA^{Glc}$ is required for the activation of adenylate cyclase. *S. enterica* serovar Typhimurium and *E. coli crr* mutants exhibit a low residual adenylate cyclase activity, generally 5 to 20% of the activity found in the parental strain (227, 469, 595). Unphosphorylated $EIIA^{Glc}$ can bind to and inhibit numerous non-PTS proteins, including the permeases for lactose (LacY) and melibiose (MelB), the ATP-hydrolyzing compo-

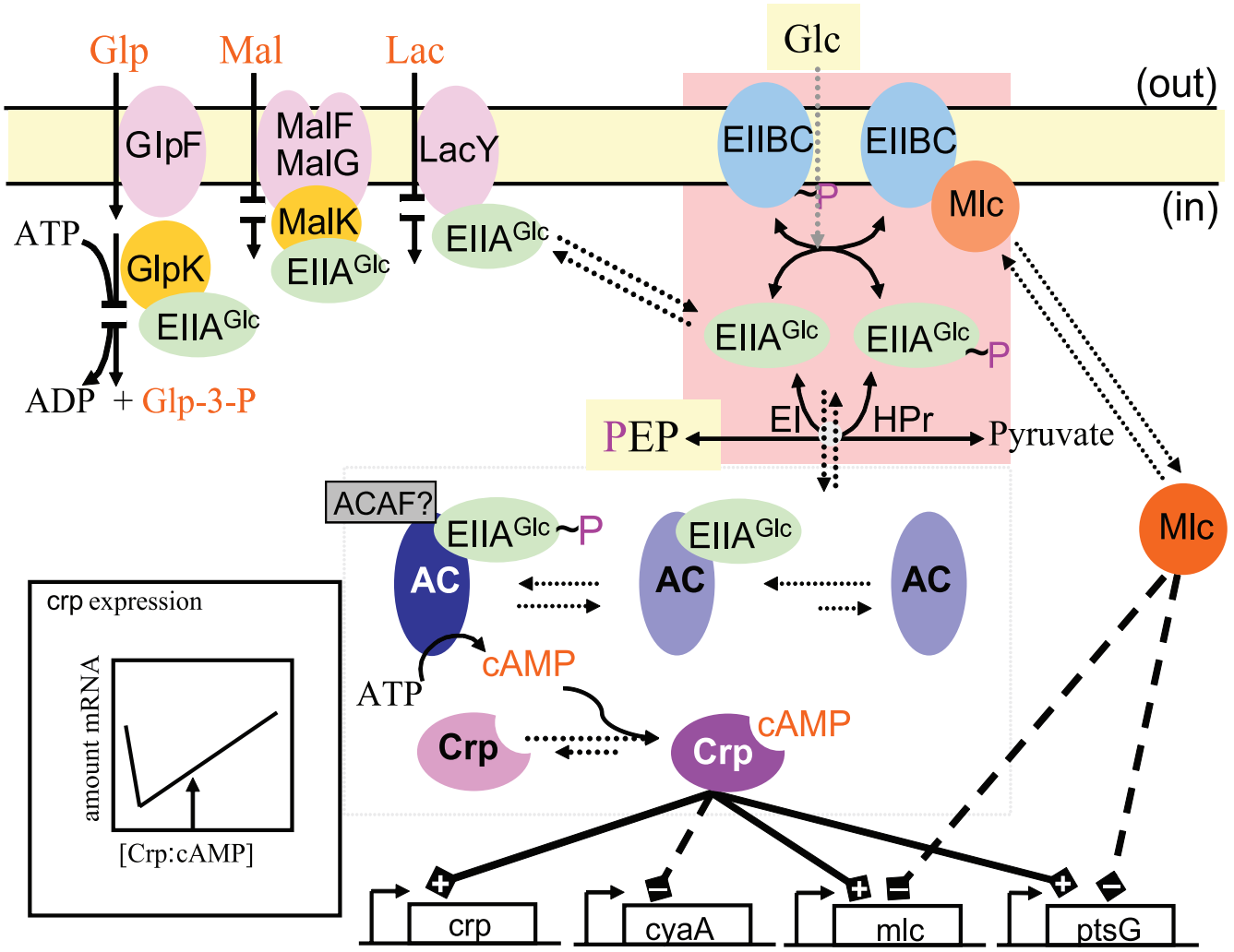

FIG. 2. Mechanisms underlying CCR and inducer exclusion in enteric bacteria. The import of glucose and other PTS carbohydrates leads to net dephosphorylation of the PTS proteins (including EIIA^{Glc} and the B domain of EIIBC^{Glc}) and thereby to inducer exclusion and recruitment of the transcription regulator Mlc to the membrane. The upper left part of the figure shows that unphosphorylated EIIA^{Glc} blocks the import of lactose, maltose, and melibiose and the phosphorylation of glycerol by binding to the respective transporter or kinase. The upper right part of the figure shows recruitment of Mlc by unphosphorylated EIIBC^{Glc}, which prevents the regulator from binding to its target sites on the DNA. In the absence of glucose and in the presence of phosphoenolpyruvate, the PTS proteins are found mainly in the phosphorylated state. The central part of the figure shows that phosphorylated EIIA^{Glc} activates adenylate cyclase (AC) but probably only in the presence of an unknown adenylate cyclase activation factor (ACAF). Adenylate cyclase binds phosphorylated as well as unphosphorylated EIIA^{Glc} (see reference 632). The bottom part of the figure shows the effect of the activated transcription factors (free Mlc and Crp:cAMP) on the transcription of the genes encoding Crp, adenylate cyclase, Mlc, and EIIBC^{Glc}, respectively. The inset shows the Crp:cAMP concentration dependence of *crp* transcription (deduced from data reported in references 310 and 311). The arrow indicates the physiological concentration of activated Crp in exponentially growing cells in the absence of PTS carbohydrates.

nent of the maltose transport system (MalK), and glycerol kinase (GlpK). As a result, induction of the genes involved in the uptake and metabolism of these substrates is prevented, and hence, the inhibitory process was called inducer exclusion.

Both control mechanisms, inducer exclusion and modulation of adenylate cyclase activity, have a physiological function and allow the cell to choose between different carbon sources as long as the uptake of glucose and other PTS carbohydrates leads to the dephosphorylation of EIIA^{Glc}. The connections between carbohydrate uptake, the phosphorylation state of EIIA^{Glc}, adenylate cyclase activity, and the intracellular concentration of metabolites such as PEP and pyruvate relate metabolic flux directly to the regulation of solute uptake and gene expression. In Fig. 2, a summary of the mechanisms underlying CCR in enteric bacteria is given. Although EIIA^{Glc}-mediated regulation has been found only in enteric bacteria so far, this signal transduction system has served and serves as a model for other regulatory processes that involve reversible phosphorylation, such as PTS-regulated transcription activation or antitermination.

Transcription Regulation by Crp/cAMP and Role of P~EIIA^{Glc}

In enteric bacteria, Crp is one of the few truly global regulators, as it controls the expression of a vast number of genes/operons (34, 291, 527, 990). Crp is activated by binding cAMP,

which is synthesized from ATP by adenylate cyclase (gene *cyaA*) (for recent papers on the structure of the Crp/cAMP regulator, see references 316, 471, 671, and 888). Different genes and operons require different levels of Crp/cAMP for full expression because the Crp/cAMP complex can adopt several conformationally active states and because the affinity for the recognition sites on the DNA varies (149, 316, 417, 484, 853, 888).

The concentration of Crp/cAMP is tightly regulated by the PTS. Soon after the discovery of cAMP in *E. coli*, it was observed, for example, that the addition of glucose to cells growing on lactate lowers the concentration of cAMP (508). Later results confirmed this finding (212, 384), and experiments with toluenized cells established that adenylate cyclase activity is inhibited by glucose (318, 648–652). Other PTS carbohydrates were also shown to inhibit cAMP synthesis, provided that the cognate EII complex was intact and induced (317, 767). Studies with *ptsHI* and *crr* mutants demonstrated that adenylate cyclase activity is low in both types of mutants (227, 469, 595). Based on these and other studies (see references 678 and 763), a regulatory mechanism for the activation of adenylate cyclase by P∼EIIA^{Glc} was proposed. In this scheme, transport and phosphorylation of glucose or other PTS carbohydrates decrease the extent of phosphorylation of the PTS proteins, including EIIA^{Glc}, and thus lower the activity of adenylate cyclase, whereas growth on non-PTS carbohydrates, particularly poor carbon sources like lactate and succinate, results in fully phosphorylated PTS proteins and the activation of adenylate cyclase. However, this model does not explain how growth on non-PTS carbon sources such as glucose-6-phosphate or gluconate can lead to intracellular cAMP levels similar to, or sometimes lower than, those observed in glucose-grown cells (212, 337). Moreover, the inhibitory effect of glucose-6-phosphate is found only in intact but not in toluenized cells (318), and we will discuss this important point later.

Regulation of *cyaA* and *crp* transcription. Most of our current knowledge regarding Crp/cAMP-mediated regulation is derived from the study of *cyaA*, *crp*, and/or *ptsHI-crr* mutants (76, 416, 636, 677, 678, 763, 890). The effect of different carbon sources on growth, gene expression levels, cAMP concentrations, and Crp levels has been tested with these mutants. The addition of exogenous cAMP was used to distinguish between the effects caused by concentration changes of either cAMP or Crp. One class of mutants, designated *crp** or *crp*(In), produced Crp, which is active without cAMP. Unfortunately, an interpretation of the mutant data is not easy, as the factors involved in cAMP-mediated regulation are interdependent, as depicted in Fig. 2.

Inactivation of the *cyaA* gene disables bacteria like *E. coli* and *S. enterica* serovar Typhimurium from growing on a large number of carbon sources (76), while *cyaA* overexpression appears to be detrimental (699). Inactivation of *crp* also affects the cAMP concentration by leading to a drastic increase in the rate of cAMP synthesis (75, 170, 241, 363, 679). The increase in the cAMP concentration depends on an intact *crr* gene. In *crp crr* double mutants, cAMP overproduction is markedly reduced (from about 60-fold of the wild-type level in the *crp* strain to only 4-fold of the wild-type level in the double mutant) (143, 170).

The phenomena described above are related to feedback regulation on the level of transcription. Expression of *cyaA* is negatively regulated by Crp/cAMP in both *E. coli* (10, 387, 395, 576) and *S. enterica* serovar Typhimurium (221), whereas expression of *crp* is both positively (310) and negatively (9, 140, 311) affected by Crp/cAMP. As a result, strong *crp* expression occurs at low and high Crp/cAMP concentrations (due to reduced inhibition and increased stimulation, respectively), whereas low *crp* expression is observed at intermediate Crp/cAMP concentrations (310). The concentration of Crp/cAMP in exponentially growing cells is an order of magnitude higher than that allowing minimal *crp* transcription (310, 365). Consequently, a decrease in the cAMP concentration caused by the addition of glucose to these cells should lower the transcription of *crp* and hence the concentration of Crp. Glucose-grown *E. coli* cells indeed contain less Crp, and this was shown not to be due to faster degradation of the *crp* mRNA (364, 365). The glucose effect on *crp* expression is absent in mutants lacking either adenylate cyclase or Crp, consistent with the postulated role of Crp/cAMP. The growth stage of *E. coli* cultures also has an influence on the amount of Crp (365). These variations might explain why some authors reported complete relief from glucose repression after the addition of cAMP (647), whereas others observe only partial relief (932).

Expression of the EIIA^{Glc}-encoding *crr* gene is almost completely independent of Crp/cAMP (857). In contrast, expression of the EIICB^{Glc}-encoding *ptsG* gene is almost fully repressed in *cyaA* or *crp* mutants (727). Owing to the low level of the glucose transporter EIICB^{Glc} in these mutants, the addition of glucose to *cyaA* or *crp* mutants does not lead to the same extent of dephosphorylation of P∼EIIA^{Glc} as in the wild-type strain. The regulation of *crp* transcription by factors other than Crp/cAMP adds further complexity to catabolite repression data. *crp* transcription is negatively regulated by both the DNA binding protein Fis (280), the transcription of which is in turn regulated by Crp/cAMP (591), and SpoT (378), the protein that mediates the stringent response and that catalyzes the synthesis of ppGpp (guanosine tetraphosphate).

Regulation of adenylate cyclase activity. Whether P∼EIIA^{Glc} exerts its regulatory effect on adenylate cyclase directly or indirectly has been an open question for a long time. Early experiments with permeabilized cells containing an intact PTS showed that adenylate cyclase activity is stimulated by potassium and phosphate and inhibited by α-methylglucoside (α-MG) and pyruvate (476, 477, 701), which is in line with a stimulatory effect by P∼EIIA^{Glc}. However, in vitro experiments with purified adenylate cyclase were not conclusive (963). The effect of EIIA^{Glc} phosphorylation on the activation of adenylate cyclase was studied in *crr* mutants producing EIIA^{Glc} in which the phosphorylatable His-90 (197) and the catalytically important His-75 had been changed. In comparison to wild-type EIIA^{Glc}, His75Gln EIIA^{Glc} synthesized in a *crr* mutant caused an approximately fourfold activation of adenylate cyclase. In contrast, neither His90Gln nor His90Glu EIIA^{Glc} activated adenylate cyclase (700, 852). The latter two mutant proteins are not phosphorylated by P∼His-HPr, while His75Gln EIIA^{Glc} is. Takahashi and coworkers also found that His75Glu EIIA^{Glc}, which is barely phosphorylated in vitro (700), can slightly activate adenylate cyclase (852). In wild-type

$EIIA^{Glc}$, His-75 and His-90 are in very close proximity, and the replacement of His-75 with a negatively charged Glu possibly mimics phosphorylated His-90. Although these results indicate that phosphorylation of His-90 of $EIIA^{Glc}$ is important for the activation of adenylate cyclase, they do not prove a direct interaction. Recent results with adenylate cyclase tethered to the membrane showed that although both $P\sim EIIA^{Glc}$ and unphosphorylated $EIIA^{Glc}$ bind with similar affinity to the protein, it is activated by $P\sim EIIA^{Glc}$ only when an additional, not-yet-identified factor from cell extracts is present (632) (Fig. 2).

From studies with truncated adenylate cyclase, it can be concluded that the catalytic activity resides in the N-terminal domain, with the C-terminal domain required for $EIIA^{Glc}$-mediated regulation (632, 699, 746). Starting from an *E. coli ptsHI-crr* deletion strain containing, in addition, an Asp414Asn mutation in *cyaA*, which prevents the large increase in adenylate cyclase activity in a *crp* mutant (143), suppressor mutants affected in the *cyaA* gene could be isolated. Most suppressor mutations were located in the region around Asp-414 and resulted in a truncated adenylate cyclase that was about half the size of the wild-type enzyme (144). The truncated molecules produce about 10 times more cAMP in a *crr* strain than the wild-type enzyme. These results suggest that the N-terminal domain of adenylate cyclase is active in the absence of $P\sim EIIA^{Glc}$ and that the C-terminal domain acts as an inhibitor that may be removed/inactivated by $P\sim EIIA^{Glc}$.

Mutation of *crp* leads to a drastic increase of extracellular cAMP. This increase is 50-fold diminished when the C-terminal 48 amino acids of adenylate cyclase are cut off. In contrast, the increase is diminished by only fourfold when the CRP/cAMP-mediated transcriptional regulation of the *cya* gene is prevented by replacing the *cya* promoter with the constitutive *bla* promoter (363). Therefore, it was concluded that the regulation of cAMP production occurs mainly posttranslationally (at the level of enzyme activity) and not at the level of transcription. However, because the regulation of *cyaA* transcription is affected by the Crp/cAMP concentration, which in turn is affected by CyaA activity, that conclusion does not seem to be justified. This can be illustrated by the changes in adenylate cyclase activity caused by a *crr* null mutation. Adenylate cyclase activity measured in *crr* mutants amounts to only 5% to 20% of the activity measured in wild-type strains (227, 469, 595), and the reintroduction of plasmid-borne *crr* increases it four- to fivefold, thus approaching levels of wild-type activity (477, 700). If $EIIA^{Glc}$ was the only factor interacting with and stimulating adenylate cyclase, *crr* mutants and strains producing adenylate cyclase missing the last 48 amino acids should exhibit the same phenotype. However, it is evident that the positive effect on adenylate cyclase caused by the expression of plasmid-borne *crr* in a *crr* mutant is much smaller than the negative effect (50-fold) caused by the deletion of the 48 C-terminal amino acids of adenylate cyclase in a *crp* mutant. This difference probably owes to the absence of the Crp-dependent effect of cAMP on transcription in a *crp* mutant.

Regulation by Crp/cAMP. Despite the enormous amount of experimental data, the control of cAMP and Crp levels in enteric bacteria is still not completely understood. Regulation occurs not only at the level of transcription but also at the level of enzyme activity. These two processes affect each other, making it difficult to quantify their individual contributions. Nevertheless, the general properties of the regulatory mechanism are clear. The immediate response of cells to the addition of a rapidly metabolizable carbohydrate involves regulation only at the level of enzyme activity. The addition of glucose or another PTS sugar causes the dephosphorylation of $P\sim EIIA^{Glc}$. This deactivates adenylate cyclase and hence lowers the concentration of cAMP and Crp/cAMP inside the cell. On a longer time scale, regulation on the transcriptional level becomes important. At normal physiological levels of cAMP and Crp, the *crp* gene is negatively regulated by Crp/cAMP. Thus, a reduced activity of adenylate cyclase leads to lower expression of *crp*. As a result, the concentration of Crp/cAMP drops even further. On the other hand, a lower Crp/cAMP concentration leads to an increased expression of *cyaA*, and more adenylate cyclase will accumulate, thereby counteracting the decrease in activity and raising the Crp/cAMP concentration. In principle, the elevated pool of adenylate cyclase provides a higher potential for cAMP production once the glucose is depleted, although the Crp concentration will be lower.

Some results cannot be explained by the proposed mechanism. For instance, in *S. enterica* serovar Typhimurium *crp** (595) and *E. coli crp* cya* (851) mutant strains, the addition of glucose lowers the concentration of Crp* and hence repression. However, because Crp* is constitutively active, i.e., it does not need cAMP for activity, its concentration should not be affected by the presence or absence of glucose. The occurrence of a component that is able to interact with Crp in the presence of glucose could explain the observed decrease in the Crp concentration. In fact, such a mechanism regulates the activity of the repressor Mlc (588): when glucose is present, $EIICB^{Glc}$ is dephosphorylated and binds the transcription factor Mlc, thus relieving repression. It is tempting to speculate that unphosphorylated $EIICB^{Glc}$ might also bind Crp, but as yet, there is no experimental proof to support this assumption.

Factors other than those mentioned above have been reported to influence the activity of adenylate cyclase, but their physiological relevance remains uncertain. A decrease in adenylate cyclase activity, for instance, was observed upon lowering the membrane electrochemical potential (649). Mutation of *fruB* (formerly *fruF*) can have a negative (272) or positive (225) effect on adenylate cyclase activity. Also, when GTP or the elongation factor Tu, a GTP-binding protein (702), was added to purified adenylate cyclase, it stimulated its activity almost twofold (829).

Growth rate effects on cAMP concentration. Although early studies reported hardly any effect of the growth rate on the cAMP concentration under glucose limitation (529, 957), more recent studies report a clear relationship, with elevated cAMP concentrations occurring at low growth rates (613, 614). During growth on glucose in continuous culture, expression of *lacZ* appears to be independent of ppGpp but directly related to the cAMP concentration and inversely related to the growth rate (438); i.e., growth rate and cAMP concentration are also inversely related. The effect of the growth rate on the cAMP concentration can be explained in terms of the stimulation of adenylate cyclase activity by $P\sim EIIA^{Glc}$. The phosphorylation state of the PTS components in these experiments should depend on the growth rate because the cells were grown in a chemostat under carbohydrate-limiting conditions, and the

growth rate was varied by changing the rate of carbon source supply. As a result, at high growth rates, less P~EIIA^{Glc} and hence less cAMP should be formed than that at slow growth rates.

Secretion and breakdown of cAMP. Makman and Sutherland (508) observed that following the addition of glucose, starved *E. coli* cells halt cAMP synthesis and excrete cAMP into the medium. In a later study, the presence of other metabolizable sugars was shown to elicit the same effect (758). Interestingly, *crp* mutants excrete much higher amounts of cAMP than wild-type cells (75, 241, 679). A putative low-affinity cAMP exporter (K_m of approximately 10 mM) was identified (275). However, as the internal cAMP concentration is generally in the micromolar range, excretion is not thought to be relevant to cAMP-mediated regulation.

The breakdown of cAMP by the endogenous cAMP phosphodiesterase (80) provides another possible way to affect the cAMP concentration. However, cAMP phosphodiesterase has a low affinity for cAMP (K_m of between 0.5 and 0.8 mM) (80, 604), making it unlikely that this enzyme exerts much, if any, control over cAMP levels. The findings that, in mutants lacking cAMP phosphodiesterase, glucose still lowers the cAMP level (508) and that CCR exerted by various carbon sources is as strong as that in wild-type cells (337) also argue against a physiological role of the cAMP phosphodiesterase.

Inducer Exclusion and Role of EIIA^{Glc}

Soon after the discovery of the PTS, it became clear that *ptsHI* mutants have a pleiotropic phenotype; i.e., they fail to grow not only on PTS carbohydrates but also on non-PTS carbon sources such as lactose, melibiose, maltose, and glycerol. Based on a number of growth and transport studies with several *ptsHI-crr* mutants, a model in which unphosphorylated EIIA^{Glc} inhibits systems catalyzing the transport and subsequent metabolism of these non-PTS sugars in enteric bacteria was proposed (765–767, 770; also see reference 678 for more references). An important observation strengthening the model was made in so-called "leaky" *ptsH* or *ptsI* mutants of *S. enterica* serovar Typhimurium (773), where "leaky" means that these mutants retain low EI or HPr activity. The "leaky" mutants do not grow on PTS sugars, but they do grow on the non-PTS sugars melibiose, maltose, and glycerol. Moreover, mutants growing on the latter sugars are hypersensitive to repression by the glucose analog α-MG. This phenotype can be explained by the fact that a low amount of EI or HPr strongly reduces the phosphorylation flux via the general PTS proteins EI and HPr, causing rapid and nearly complete dephosphorylation of EIIA^{Glc} in the presence of α-MG. The phenomenon was called inducer exclusion because the presence of unphosphorylated EIIA^{Glc} prevents either the import of these sugars or their subsequent metabolism, and as a consequence, bacterial cells are devoid of the inducer for the corresponding operons (see reference 502).

Inducer exclusion is mediated by unphosphorylated EIIA^{Glc}. During inducer exclusion, unphosphorylated EIIA^{Glc} binds directly to either the respective permease (lactose or melibiose), the ATP-binding subunit of the ATP binding cassette (ABC) transporter (maltose), or, in the case of glycerol, GlpK, the cytoplasmic enzyme catalyzing the formation of the inducer (inducer exclusion is depicted in Fig. 2). Initial evidence for a direct interaction between EIIA^{Glc} and the inhibited proteins came from studies showing that the lactose transport activity of LacY in *E. coli* vesicles is strongly reduced when the vesicles contain partially purified unphosphorylated EIIA^{Glc} (189, 559). Similar results were obtained with whole cells synthesizing either LacY or the melibiose transporter (MelB) (561). Direct binding of unphosphorylated EIIA^{Glc} to membrane preparations of an *E. coli* strain overproducing LacY was demonstrated by Osumi and Saier (624), who also found that the binding of EIIA^{Glc} correlates with the amount of transporter and is enhanced by the presence of lactose, while the phosphorylation of EIIA^{Glc} abolishes it. These results were confirmed and quantified by Nelson et al. (593, 597). Direct binding and inhibition by unphosphorylated EIIA^{Glc} were also established for the MalK component of the maltose transport system (163, 675) and for GlpK (166, 676).

The uptake systems for raffinose (876), galactose (559), and arabinose (338) are also subject to inducer exclusion. Raffinose uptake via the plasmid-encoded raffinose permease RafB is inhibited by α-MG, and the effect is enhanced in "leaky" *ptsI* mutants (876). It was suggested that galactose transport in *E. coli* would also be under the control of inducer exclusion (7, 385). Indeed, the uptake of galactose by membrane vesicles containing galactose permease was reported to be diminished by the addition of unphosphorylated EIIA^{Glc} (559). Nevertheless, other experiments from the same group (562, 767) did not reveal an effect of the PTS on galactose fermentation by intact cells. The authors of those reports argued that the absence of an effect probably results from the full induction of the galactose permease in intact cells, thereby reducing the effectiveness of inhibition by EIIA^{Glc}, as inducer exclusion is mediated by a 1:1 stoichiometric interaction (559). Nevertheless, *E. coli gal* operon expression is submitted to CCR, as was demonstrated by the early experiments of Stephenson and Gale (836).

Efficient binding of unphosphorylated EIIA^{Glc} to the target protein occurs only when the substrate of the target is present. For example, EIIA^{Glc} binds strongly to the lactose transporter LacY only in the presence of a β-galactoside (597, 624, 800, 825). This mechanism ensures that EIIA^{Glc}, the concentration of which is fairly constant in *E. coli* and *S. enterica* serovar Typhimurium cells, will not be wasted on useless binding, i.e., under conditions in which the substrate is not present in the environment. The necessity of substrate binding indicates that the target proteins probably have to undergo a conformational change before unphosphorylated EIIA^{Glc} can bind. Evidence for conformational changes associated with substrate binding has been presented for LacY (245, 390), MelB (586), MalK (71, 581), and GlpK (355, 654, 874).

Interactions of EIIA^{Glc}

Structure of EIIA^{Glc}. EIIA^{Glc} directly interacts with many different proteins as it serves in PTS phosphoryl transfer, inducer exclusion, and probably activation of adenylate cyclase (Table 1). Although most interaction partners are structurally unrelated, it appears that the binding surface of EIIA^{Glc} is always similar. The structure of EIIA^{Glc} is hardly affected by binding to its target proteins (98, 124, 270, 355, 929) or by phosphorylation (642). *E. coli* EIIA^{Glc} forms an antiparallel

TABLE 1. The PTS components and their various non-PTS interaction and/or phosphorylation partners

PTS component	Non-PTS phosphorylation/interaction partner(s)	Phosphorylation or interaction	Effect(s) of phosphorylation or interaction
EI (*E. coli*)	CheA, chemotaxis protein	Interaction	Stimulates CheA autophosphorylation
P∼EI (*B. subtilis*)	CheA, chemotaxis protein	Interaction	Inhibits CheA autophosphorylation
HPr (*E. coli*)	Glycogen phosphorylase	Interaction	Stimulates glycogen phosphorylase activity
P∼His-HPr (*E. coli*)	Glycogen phosphorylase	Interaction	Prevents binding of HPr to glycogen phosphorylase
P∼His-HPr	Antiterminators, BglG, SacY, LicT, etc.[a]	Phosphorylation in PRD2	Stimulates antitermination,[b] alternate CCR mechanism
	Transcription activators, LevR-like	Phosphorylation in the $EIIA^{Man}$ domain	Stimulates transcription, alternate CCR mechanism
	Transcription activators, MtlR-like	Phosphorylation in PRD2	Stimulates transcription, alternate CCR mechanism
	Transcription activators, LicR-like	Phosphorylation in PRD1 and PRD2	Stimulates transcription, alternate CCR mechanism
P∼His-HPr (firmicutes)	Glycerol kinase GlpK	Phosphorylation, His in the N-terminal half	Stimulates GlpK activity, inducer exclusion
	LacS, RafP; transporters for lactose, raffinose	Phosphorylation in the $EIIA^{Glc}$ domain	Stimulates substrate/galactose exchange
P-Ser-HPr (firmicutes)	CcpA	Interaction	CCR or CCA, catabolite corepressor
	Non-PTS transporters for maltose, ribose, etc.	Interaction	Inducer exclusion[c]
	RbsR	Interaction	Physiological role not yet established
P-Ser-Crh (bacilli)	CcpA	Interaction	CCR or CCA, catabolite corepressor
$EIIA^{Glc}$ (enterobacteria)	Non-PTS transporters LacY, MalK, MelB	Interaction	Inducer exclusion
	Glycerol kinase, GlpK	Interaction with the C-terminal domain	Inducer exclusion
	Fermentation respiration switch protein, FrsA	Interaction	Probably causes increased respiration
P∼$EIIA^{Glc}$ (enterobacteria)	Adenylate cyclase	Interaction	CCR, activation of adenylate cyclase[d]
P∼$EIIA^{Dha}$	DhaL, L subunit of dihydroxyacetone kinase	Phosphoryl transfer	ADP bound to DhaL is converted into ATP
$EIIB^{Glc}$ (enterobacteria)	Mlc	Interaction	Derepression of genes of the Mlc regulon
P∼EIIBs, Glc/Sac/Lac class	Antiterminators, BglG, SacY, LicT, etc.[a]	Phosphorylation in PRD1[e]	Inhibits antitermination, induction mechanism
P∼EIIBs, Man/Lac class	Transcription activators, LevR-like	Phosphorylation in PRD2[e]	Inhibits transcription, induction mechanism
P∼EIIBs, Mtl/Gut class	Transcription activators, MtlR-like	Phosphorylation in the $EIIA^{Mtl}$ domain[e]	Inhibits transcription, induction mechanism
P∼EIIBs, Lac/Cel class	Transcription activators, LicR-like	Phosphorylation in the $EIIA^{Mtl}$ domain[e]	Inhibits transcription, induction mechanism

[a] For a more detailed summary of well-studied antiterminators, see Table 4.
[b] Certain antiterminators (such as SacY and GlcT of *B. subtilis*) are phosphorylated in vitro by P∼His-HPr in PRD2, but their activity is not stimulated by this modification.
[c] An interaction of P-Ser-HPr with the maltose or ribose transport systems in *L. casei* and *L. lactis* has so far not been demonstrated but is suggested from genetic experiments.
[d] An additional cellular factor seems to be necessary for the activation of adenylate cyclase by P∼$EIIA^{Glc}$ (632).
[e] It is not clear whether phosphorylation of the antiterminators/transcription activators occurs via P∼His-HPr and P∼EIIBs stimulate only the phosphoryl transfer or whether the phosphoryl group is transferred from P∼EIIBs to the antiterminators/transcription activators. It is possible that both modes of regulation exist.

β-sandwich in which His-90 and His-75 of the active site are located off center on one face of the sandwich (224, 643, 955). Besides His-90, the residue that carries the phosphoryl group in *E. coli* P∼$EIIA^{Glc}$ (197), His-75, appears to be very important for the phosphocarrier activity. Replacement of His-75 with Gln results in strongly diminished phosphoryl group transfer (686). A structural study of His75Gln mutant $EIIA^{Glc}$ indicated no significant structural changes around the active site compared to the wild-type protein (643). Two possible explanations were proposed: (i) His-75 stabilizes the negative charge of P∼His-90, or (ii) the active site contains a proton relay network also involving Thr-73. The latter hypothesis was recently falsified by replacing Thr-73 with Ser, Ala, or Val and measuring the effect on the phosphotransfer rates to and from HPr (545). These rates were barely affected by the mutations.

The active site of *E. coli* $EIIA^{Glc}$ is surrounded by a ring of solvent-exposed hydrophobic residues flanked by some polar and charged residues. A similar arrangement was found for the $EIIA^{Glc}$ of *Mycobacterium capricolum* (349) and the EIIA domain of *B. subtilis* $EIICBA^{Glc}$ (122, 474). The NMR structure of *E. coli* P∼$EIIA^{Glc}$ shows that the phosphoryl group bound to His-90 (197) protrudes from the ring of hydrophobic residues that surround the active site (642). Supposedly, the dis-

TABLE 2. Residues involved in the interaction of $EIIA^{Glc}$ with partner proteins[a]

Partner protein	Interface residue(s) of:		Reference(s)
	Partner protein	$EIIA^{Glc}$	
HPr active site	**His-15**, Arg-17	**His-90**,[b] His-75[b]	330, 941 (HPr); 197, 686 ($EIIA^{Glc}$)
Interaction surface	Arg-17, Lys-24, Lys-27, Lys-49	Val-40, Phe-41, Val-46, Phe-71, Phe-88, Val-96, Lys-69 (aliphatic side chain), Asp-38,[c] Glu-72, Glu-80, Glu-86; Asp-94,[c] Glu-97, Glu-109, Asp-144, Ser-78, Ser-141	929
$EIICB^{Glc}$ active site	**Cys-421**, Arg-424, Arg-426		449, 547
Interaction surface	Arg-424,[d] Arg-426,[d] Lys-467, Asp-419; Asp-464	Val-40, Phe-41, Val-46, Phe-71,[e] Phe-88, Val-96, Lys-69,[e] Lys-99,[e] Asp-38, Glu-72; Asp-94, Glu-97; Ser-141	98
GlpK (*E. coli*) interaction surface	Residues 472-481	Val-40, Phe-41, Val-46, Phe-71, Phe-88, Val-96, Lys-69, Lys-99, Asp-38, Glu-72; Glu-92, Asp-94, Asp-97	355
Cofactor Zn^{2+}	Glu-478	His-75, His-90	223
LacY interaction surface	Val-132, Pro-192, Val-197, Ala-198, Arg-135, Arg-142, Ser-133, Thr-196, Asn-199, Ser-202, Asn-204, Ser-206	Gly-47, Phe-71, Ala-76, Lys-69, Glu-86; Asp-94, Ser-78	338, 823, 951 (LacY); 987 (EIIA)
MelB interaction surface	Ile-445, Asp-438, Asp-441; Asp-449, Arg-452		439, 440
MalK interaction surface	Ala-124, Phe-241, Gly-278, Gly-284, Gly-302, Arg-228, Glu-119, Ser-322		167, 431

[a] The active-site residues are indicated by boldface type.
[b] His-68 and His-83 in *B. subtilis* (124).
[c] Asp-31 and Asp-87 in *B. subtilis* (124).
[d] Arg-38 and Arg-40 of the *E. coli* $EIIB^{Glc}$ domain (270).
[e] Point mutations that block P transfer to HPr and/or $EIICB^{Glc}$ are Lys69Leu, Lys69Glu, Lys99Glu, Phe71Leu, and Phe71Ser (822).

turbance of the hydrophobic binding surface of $EIIA^{Glc}$ prevents the interaction between $P\sim EIIA^{Glc}$ and the target proteins. Furthermore, the introduction of a negative charge on the protein surface contributes to the diminished interaction.

Interaction with HPr and $EIICB^{Glc}$. The structures of *B. subtilis* HPr (330) and the EIIA domain of *B. subtilis* $EIICBA^{Glc}$ (474) were used to construct a model of the HPr:$EIIA^{Glc}$ transition complex (327). Herzberg concluded that (i) upon the formation of the transition complex, no major conformational change occurs and (ii) the central part of the binding surface consists mainly of hydrophobic residues. These assessments are sustained by the NMR solution structures of the HPr:$EIIA^{Glc}$ transition complexes of *B. subtilis* (124) and *E. coli* (929). The transition complex perfectly accommodates the binding of a phosphoryl group by both the active-site histidine of $EIIA^{Glc}$ and that of HPr in either an associative or dissociative mechanism. The active-site histidine of $EIIA^{Glc}$ (His-90 in *E. coli*) is located in a shallow depression of a slightly concave surface (224, 643, 955), whereas the active-site histidine of HPr is located at the end of the first α-helix on a convex protrusion of the protein (330). The binding of the phosphoryl group in *B. subtilis* $EIIA^{Glc}$ is stabilized by His-68 (His-75 in *E. coli*) (474) and by Arg-17 in HPr (330). In addition, two aspartyl residues are perfectly positioned to form alternate ion pairs with Arg-17 of HPr. The hydrophobic binding surface containing the active site of $EIIA^{Glc}$ includes several valine and phenylalanine residues (Phe-41, -71, and -88 and Val-40, -46, and -96 in *E. coli*) (929).

It appears that the binding surfaces of $EIIA^{Glc}$ for HPr and $EIIB^{Glc}$ are almost identical (Table 2). The soluble EIIB domain of *E. coli* $EIICB^{Glc}$ was isolated, and the solution structure of the $EIIA^{Glc}$:$EIIB^{Glc}$ transition complex was determined (98, 270). The interaction surface consists of a central hydrophobic patch composed of the phenylalanine and valine residues mentioned above, and the same two aspartyl residues form alternate ion pairs with arginine residues. $P\sim EIIA^{Glc}$ transfers its phosphoryl group to Cys-421 of the glucose permease $EIICB^{Glc}$ (92, 547, 618). This residue is located at the C terminus of β1 of the EIIB domain on a hydrophobic protrusion (203). Two arginine residues neutralize the negative charge added to Cys-421 upon phosphorylation of $EIIB^{Glc}$ (98). Like Cys-421 (92), the presence of these residues was reported to be compulsory for effective glucose transport and phosphorylation (449). Although the central interaction parts are very similar, the residues forming the edges of the binding surfaces are different between the transition complexes HPr:$EIIA^{Glc}$ and $EIIA^{Glc}$:$EIIB^{Glc}$ (98, 929). The edges of the binding surface of HPr contain only positively charged residues, whereas those of $EIICB^{Glc}$ include both positively and nega-

tively charged amino acids. Consequently, the edges of the binding surface of $EIIA^{Glc}$ for HPr include many negatively charged and some polar residues, while those of $EIIA^{Glc}$ for $EIIB^{Glc}$ include a polar residue and both negatively and positively charged amino acids.

Sequence comparisons of $EIIA^{Glc}$ and $EIIB^{Glc}$ from various bacteria reveal that the interfacial residues are strongly conserved (98). The few changes are mostly conservative, thus preserving the central network of intermolecular hydrophobic interactions. A higher variability is observed for the peripheral polar and charged residues. There are, for instance, several positively charged residues in *E. coli* $EIIA^{Glc}$ replaced with polar or negatively charged residues in the *B. subtilis* protein. Although these substitutions abolish some salt bridges, their contribution to the stability of the complex appears to be moderate, as illustrated by the similar apparent K_m values for heterologous (PTS proteins from different organisms) and homologous phosphoryl transfer (98, 723). Point mutations in several well-conserved residues of $EIIA^{Glc}$ also lower the phosphoryl transfer activity but only by between 20 and 70% (822). It was concluded from these observations (98) that multiple interactions contribute to the affinity between PTS protein partners.

The N terminus of $EIIA^{Glc}$ is disordered in the structures of both the HPr:$EIIA^{Glc}$ and $EIIA^{Glc}$:$EIIB^{Glc}$ transition complexes and is therefore probably not involved in the protein/protein interactions. Nevertheless, Meadow et al. (538, 541) established that although the naturally occurring N-terminal truncation (the first seven residues are absent, and the truncated $EIIA^{Glc}$ therefore migrates faster on nondenaturing gels and was called $EIIA^{Glc}_{fast}$) has no effect on phosphoryl transfer between HPr and $EIIA^{Glc}$, it reduces the phosphoryl transfer to $EIICB^{Glc}$ by about 97%. Recent measurements by the same group confirm these observations with genetically truncated $EIIA^{Glc}$ (deletion of the first 7 and 16 residues) (545). Simultaneously, they showed that phosphoryl group transfer to the soluble $EIIB^{Glc}$ domain remains unaffected. Misko et al. (559) found that compared to intact $EIIA^{Glc}$, $EIIA^{Glc}_{fast}$ has a lower affinity for LacY-containing membranes. These findings suggest that the N terminus plays a role in the recruitment of $EIIA^{Glc}$ to the *E. coli* inner membrane. Indeed, Wang et al. (926, 928) showed that the N terminus interacts with anionic lipids isolated from *E. coli*, suggesting that the N terminus serves as a kind of membrane anchor.

Interaction with GlpK. The crystal structure of the *E. coli* $EIIA^{Glc}$:GlpK complex has been determined (355). GlpK with bound glycerol and ADP forms tetramers in which each GlpK subunit interacts with one $EIIA^{Glc}$ molecule. Indeed, complete inhibition of glycerol kinase activity occurs only when at least four molecules of $EIIA^{Glc}$ are present per GlpK tetramer (900). The structure of $EIIA^{Glc}$ in the complex is very similar to that of $EIIA^{Glc}_{fast}$ (955), except that residues 1 to 11 are in contact with the surface of GlpK. Like $EIIA^{Glc}_{fast}$, the EIIA domain of *B. subtilis* $EIICBA^{Glc}$ lacks the seven N-terminal residues. Nevertheless, the protein complements an *E. coli ptsHI-crr* mutant defective in GlpK regulation (723). Therefore, the N-terminal residues cannot be very important for the inhibition of glycerol kinase (355). The inhibitory contact between the two proteins is mediated by a 3_{10} helix of GlpK (residues 472 to 481), which protrudes into the concave surface of the active site of $EIIA^{Glc}$. The contact surface of $EIIA^{Glc}$ is very similar to that in the $EIIA^{Glc}$:$EIIB^{Glc}$ complex (Table 2). The hydrophobic residues surrounding the active-site histidine are important, and binding involves negatively and positively charged residues.

The organization of the residues around the active site of $EIIA^{Glc}$ in the $EIIA^{Glc}$:GlpK complex suggests a possible role for Zn^{2+} in the association. Indeed, crystals soaked in a 20 mM $ZnCl_2$ solution adopted a Zn^{2+} ion at the expected position (223). Subsequent in vitro studies revealed that the presence of the metal ion lowers the K_i of $EIIA^{Glc}$ for GlpK by about 60-fold (223, 616). The Zn^{2+} ion is liganded by His-75, His-90, and a water molecule in $EIIA^{Glc}$ and by Glu-478 in GlpK. The stimulatory effect of Zn^{2+} on $EIIA^{Glc}$-mediated GlpK inhibition disappeared when Glu-478 was replaced with Cys, Asp, His, or Gln (657).

It is not known how the interaction with unphosphorylated $EIIA^{Glc}$ inhibits glycerol phosphorylation. The more than 30-Å distance between the sites of $EIIA^{Glc}$ binding and glycerol phosphorylation rules out a direct effect. A conformational change of glycerol kinase is part of the catalytic cycle, and so it was proposed that the binding of $EIIA^{Glc}$ could lock the protein in a closed state (355). The lower inhibition by $EIIA^{Glc}$ observed for some mutant GlpKs affected in the domain associated with "opening" and "closing" GlpK is consistent with this concept (655, 658).

Interaction with LacY. LacY is an H^+ symporter that uses electrochemical potential to drive the uptake of several galactosides such as lactose and melibiose (390, 391, 942). The recently resolved LacY structure confirms previous predictions that the protein contains 12 hydrophobic transmembrane domains connected by relatively hydrophilic loops (4). The major determinants of substrate binding are located at the interfaces of helices IV, V, and VIII (300, 444, 754). In contrast, the cytoplasmic loop between helices IV and V together with the flanking helical domains and some residues in the large cytoplasmic loop connecting helices VI and VII are important for the interaction of LacY with $EIIA^{Glc}$ (338, 800, 823). Based on the analysis of various *lacY* mutants, a model for LacY function in galactoside transport has been proposed (3, 391, 942). LacY is assumed to have a conformation in which the sugar binding site is facing the periplasm ("outward"). The binding of the sugar induces a structural change, which exposes the substrate binding site to the cytoplasm ("inward"). To explain the observation that $EIIA^{Glc}$ binds to LacY with higher affinity when its substrate is present (597, 624, 800, 825), the "outward"/"inward" transition is assumed to make the $EIIA^{Glc}$ interaction site more easily accessible (800, 823). Evidence supporting this concept comes from studies of a Cys154Gly mutant LacY. The mutation abolishes both lactose transport and binding of $EIIA^{Glc}$ and was originally thought to lock the protein in the "outward" conformation (819, 825, 902). However, the crystal structure of Cys154Gly LacY indicates that the protein is locked in an "inward" conformation (4). It was then proposed that $EIIA^{Glc}$ binds to the "outward" conformation of LacY, thus preventing the structural change to the "inward" conformation and, consequently, the entry of the bound sugar. Other observations support this interpretation. When galactosides are present at saturating amounts, ^{125}I-labeled unphosphorylated $EIIA^{Glc}$ binds in a 1:6 stoichiometry to wild-type LacY

(in inside-out vesicles) (824, 825). The replacement of residues Ile-129 and Lys-131 (these residues are not located in the $EIIA^{Glc}$ interaction site but are located on central helix IV, which is involved in substrate binding) with Cys allows more $EIIA^{Glc}$ molecules to bind to LacY but does not affect the apparent equilibrium dissociation constant, K_D, of the $EIIA^{Glc}$/LacY complex. In addition, different sugars lead to different binding stoichiometries, with the preferred substrates melibiose and lactose causing maximum binding of $EIIA^{Glc}$. The data were explained by assuming that LacY exists in two (or more) populations: one population that binds $EIIA^{Glc}$ ("outward") and one that doesn't ("inward"). The observed $EIIA^{Glc}$ binding stoichiometry was thought to reflect the ratio of these populations, which should be affected by the transported sugar (and in mutant LacYs by the conformational flexibility). According to the model, the binding stoichiometry should not affect the dissociation constant of the $EIIA^{Glc}$/LacY complex, which is consistent with the observations. It is noteworthy that in contrast to the data reported by Sondej et al. (824, 825), Nelson et al. (597) reported an $EIIA^{Glc}$/LacY binding stoichiometry of 1:1. The reason for the discrepancy is not clear.

The interaction surface of LacY and $EIIA^{Glc}$ has been probed by analyzing the effect of various mutations. Residues of the central loop of LacY were found to be involved in the interaction (338, 951). Changing Thr-7 or Met-11 to Ile prevents $EIIA^{Glc}$ from binding (597, 625). However, a deletion of residues 2 to 8 hardly affects the regulation of LacY by $EIIA^{Glc}$ (338). Other residues of LacY involved in binding $EIIA^{Glc}$ were identified by Cys-scanning mutagenesis (823). Although some of the LacY residues important for $EIIA^{Glc}$ binding are conserved in MalK, MelB, and RafB, no consensus $EIIA^{Glc}$ binding motif could be proposed (823). In contrast to the binding surfaces of other $EIIA^{Glc}$ target proteins (HPr, $EIIB^{Glc}$, and GlpK), the LacY binding surface seems to contain only a small number of hydrophobic residues. Several *E. coli* $EIIA^{Glc}$ (*crr*) mutants defective in inducer exclusion of LacY substrates have been identified (822, 987). Some of these residues are also important for the interaction of $EIIA^{Glc}$ with HPr, $EIIB^{Glc}$, and GlpK (Table 2).

Interaction with MelB. Similar to LacY, the melibiose permease MelB is an ion (Na^+/H^+) sugar symporter with 12 membrane-spanning segments (74, 586, 966). *S. enterica* serovar Typhimurium *melB* mutants synthesizing an inducer exclusion-resistant melibiose permease have been isolated (439) (Table 2). The mutated residues all lie on one side of an α-helical stretch situated in the cytoplasmic C-terminal region of MelB. Other substitutions located on the same α-helical stretch diminished inhibition of the permease by $EIIA^{Glc}$. Cutting off the last 22 amino acids of MelB reduces inducer exclusion by fourfold, and mutants lacking more residues exhibit low melibiose and methyl-α-thiogalactoside transport even in the absence of inducer exclusion.

Interaction with MalK. Maltose uptake in *E. coli* requires the *malE*-encoded periplasmic maltose binding protein and the multisubunit ABC transporter $MalFGK_2$ (72, 226, 447). The soluble MalK subunits are tightly associated with the two permease subunits MalF and MalG (354, 580). Binding of maltose-carrying maltose binding protein to $MalFGK_2$ stimulates ATP hydrolysis by MalK and therefore maltose transport (118, 161, 187). Stimulation of ATP hydrolysis is cooperative, suggesting that the MalK subunits interact with each other (159, 160, 226). MalK possesses an N-terminal ATPase domain (present in all ATP-binding proteins of ABC transporters) and, in certain bacteria including *E. coli* and *S. enterica* serovar Typhimurium, a specific C-terminal regulatory domain (66, 187, 782, 828). In the presence of maltose and glucose, $EIIA^{Glc}$ is assumed to bind to the regulatory domain, thus preventing maltose import. In addition, if no maltose is present, the C-terminal MalK domain is proposed to bind and inhibit the transcription activator MalT (380, 627, 828), which controls the expression of the *mal* regulon (65, 70).

Several *malK* mutants exhibiting weak inducer exclusion of maltose but normal inhibition of MalT by MalK and vice versa have been isolated (163, 431) (Table 2). In addition, the binding of monoclonal antibodies directed against linear epitopes extending from either amino acids 113 to 123 or 352 to 361 in the MalK sequence is diminished by the presence of $EIIA^{Glc}$ (828). The scattered location of the mutations (Table 2) and the $EIIA^{Glc}$/antibody competition sites made it difficult to propose a binding site for $EIIA^{Glc}$ (66, 828). In contrast to *Thermococcus litoralis* MalK (187), biochemical studies suggest that in the functional $MalFGK_2$ complex of *E. coli*, the C termini of the MalK subunits are in close contact (774). This assumption was confirmed by resolving the structures of three different forms of the MalK dimer (117). Several amino acids, the replacement of which leads to weak maltose exclusion, lie on the same face of the dimer, thus identifying a potential binding site for $EIIA^{Glc}$. The binding of $EIIA^{Glc}$ to the nucleotide binding domain of one subunit and the regulatory domain of the other could well prevent the "tweezers-like motion" necessary to accommodate the cycle of hydrolysis and subsequent transport (117).

Other interactions. Using ligand fishing, yet another interaction partner of unphosphorylated *E. coli* $EIIA^{Glc}$ was recovered (419). The protein was called fermentation/respiration switch protein (FrsA; formerly YafA) because *frsA* disruption causes increased respiration on several sugars including glucose, and overexpression results in increased fermentation. $EIIA^{Glc}$ forms a 1:1 complex with FrsA. Thus, in *E. coli*, the balance between fermentation and respiration seems to be regulated by the PTS. Furthermore, the *E. coli* genome has been screened for proteins containing a potential $EIIA^{Glc}$ binding site using sequence patterns deduced from mutagenesis studies of LacY (823). The search has yielded over 35 potential binding partners including HPr and MalK but excluding MelB and FsrA. Therefore, whether the recovered list of proteins is significant or not remains an open question.

Diauxic Growth

As mentioned in the introduction, diauxic growth was first described by Monod (572), who observed that when two carbon sources are supplied simultaneously, one (e.g., glucose or fructose) is often preferred over the other (e.g., glucan, maltose, lactose, or galactose). When the preferred carbon source is exhausted, growth is arrested. After a certain time (lag time), the genes involved in the catabolism of the second carbon source are expressed, and growth resumes. Inducer exclusion and/or poor expression of cata-

bolic genes due to low Crp/cAMP levels is responsible for this phenomenon (Fig. 2).

As outlined above, $EIIA^{Glc}$-mediated inducer exclusion prevents the uptake of carbohydrates such as lactose, melibiose, glycerol, and maltose and, consequently, the induction of the related catabolic genes. Expression of these genes is also regulated by Crp/cAMP, and growth on glucose reduces the cAMP levels by lowering the concentration of $P\sim EIIA^{Glc}$. Even when induced, non-PTS sugar transporters are present at relatively low concentrations compared to those of $EIIA^{Glc}$. The preferential uptake of glucose during the first part of diauxie is probably ensured by the inducer exclusion mechanism. In contrast, the induction of gene transcription during the lag phase should be caused by the entry/formation of the inducer as well as by rising concentrations of Crp/cAMP. This concept complies well with the available experimental data on glucose-lactose, glucose-maltose, and glucose-melibiose diauxie. An example is glucose-melibiose diauxie in *S. enterica* serovar Typhimurium, where inducer exclusion of melibiose is responsible for the preferential use of glucose (621). The addition of extracellular cAMP eliminates the lag period, an observation also reported for glucose-lactose diauxie (365, 502). However, in no case could the addition of extracellular cAMP prevent the preferred utilization of glucose over the second carbon source (242).

The concentrations of PEP and $P\sim EIIA^{Glc}$, which are low during growth on glucose, rise strongly when glucose is exhausted (335), which switches off the inducer exclusion mechanism and allows the second sugar to enter and eventually leads to the induction of the respective catabolic genes. In the case of glucose-lactose diauxie, the *lac* operon is initially repressed by LacI, as the presence of glucose does not allow the entry of lactose. When glucose is depleted, lactose is taken up via the small amount of LacY formed despite repression, the disaccharide is converted by β-galactosidase into allolactose, which binds to and inhibits LacI, and transcription commences (6). The effect of the entry of the inducer on transcription can be mimicked by the inactivation of *lacI* or by the addition of the nonmetabolizable inducer IPTG. Indeed, both abolish repression of the *lac* operon and the lag period in glucose-lactose diauxie but not the preferential uptake of glucose (362, 409).

While these results suggest that the repression of the catabolic *lac* genes is mainly a consequence of inducer exclusion, other data indicate that Crp/cAMP is also involved in the regulation of the *lac* operon. Four observations have lent support to the latter concept. First, the complete repression of the *lac* operon in *cyaA* and *crp* mutants shows that its expression requires a basal level of Crp/cAMP (6, 438). Second, the addition of extracellular cAMP abolishes the lag phase when switching from glucose to lactose utilization (362, 365, 621). Third, the addition of lactose to a *lacI* mutant or to IPTG-induced cells leads to a 1.5-fold increase in LacZ production, although inducer exclusion plays no role in this case (362, 409). An up to sixfold increase of LacZ is observed when the *E. coli* *lacI* strain KB53 switches from glucose to lactose utilization (425). Fourth, Crp/cAMP and LacI both bind in a cooperative manner to the *lac* promoter (40, 351, 470).

It is commonly assumed that the lag phase in diauxie is related to the time period required by the cells to synthesize the proteins necessary for the catabolism of the second sugar once glucose is exhausted. The addition of external cAMP abolishes the lag phase. It was therefore not surprising that a strong (sevenfold) transient increase in cAMP levels was observed during the switch from glucose to lactose metabolism (362). In addition, during the lag phase, Crp levels increased slightly before dropping again. We mentioned above that the low concentration of $P\sim EIIA^{Glc}$ present in cells growing on glucose stimulates the synthesis (but not activity) of adenylate cyclase due to a relief from repression by Crp/cAMP (763). At the onset of the lag phase, the rise in the $P\sim EIIA^{Glc}$ concentration together with the relatively high amount of adenylate cyclase should cause a transient surge in cAMP before it should drop again as a result of the down-regulation of the *cyaA* gene due to the increased concentration of Crp/cAMP and possibly by a decrease in $EIIA^{Glc}$ phosphorylation.

As described above, external cAMP is expected to increase the synthesis of the proteins necessary for lactose catabolism despite the presence of glucose, which can still prevent lactose catabolism via inducer exclusion. However, the addition of cAMP to *E. coli* cells growing on glucose leads to only a slight increase in β-galactosidase activity (362). This makes it difficult to explain why the addition of extracellular cAMP abolishes the lag phase but not the repression of β-galactosidase. It is possible that the slight increase of LacY and LacZ synthesis in the presence of high (extracellular) concentrations of cAMP might be sufficient to shorten the lag phase by allowing a more rapid induction of the relevant genes as soon as glucose is exhausted. However, this assumption does not explain why a lag phase is still observed in a *crp** mutant in which Crp activity is independent of cAMP (362). In this case, the activity of Crp* is possibly too low in the presence of glucose due to inhibition by an as-yet-unidentified mechanism (365, 851). This would be in line with the observed lowered Crp concentration in the presence of glucose. If glucose regulates the interaction of Crp with an effector and if the regulation would also depend on the concentration of cAMP, the addition of cAMP would, at least partly, relieve repression.

The experimental data discussed in this section concern mostly glucose-lactose diauxie. Although it is likely that the same concept holds for diauxie observed with other sugars that are sensitive to inducer exclusion, i.e., maltose and melibiose, there is little experimental evidence supporting this assumption. Indeed, experiments by Holtman et al. (340) indicate that inducer exclusion plays only a minor role in glucose-glycerol diauxie. The proteins involved in glycerol metabolism are encoded by the genes of the *glp* regulon. Similar to the *lac* operon, it is positively controlled by Crp/cAMP and negatively controlled by a repressor (GlpR) (479, 480, 944). The inducer *sn*-glycerol-3-phosphate is produced from glycerol by glycerol kinase (GlpK), the activity of which is regulated by the allosteric inhibitors fructose-1,6-bisphosphate (FBP) and $EIIA^{Glc}$ (487, 616, 676, 994). Reverse genetics has been used to distinguish between FBP- and $EIIA^{Glc}$-mediated allosteric regulation of GlpK (340). Surprisingly, *glpK* mutants that are insensitive to regulation by FBP exhibit no lag phase, consume glucose and glycerol simultaneously, and have only a transient induction of GlpK activity after the cells have depleted glucose. By contrast, *glpK* mutants that are insensitive to inducer exclusion exhibit diauxie similar to wild-type strains. Therefore, it was concluded that allosteric regulation by metabolic interme-

diates and poor expression due to low Crp/cAMP levels are probably the dominant mechanisms in glucose-glycerol diauxie. It was proposed that this unexpected behavior might be related to the fact that the inducer of the *glp* operon is also an intermediate of lipid metabolism.

The supposed direct link between the phosphorylation state of $EIIA^{Glc}$, glucose uptake, and adenylate cyclase activity has been questioned (614). In a glucose-limited chemostat, it was observed that the cAMP concentration rises sharply (8- to 10-fold) when the external glucose concentration drops below 300 μM, a concentration more than 20-fold higher than the K_m of glucose transport (5 to 15 μM). Notley-McRobb and Ferenci claimed that this result ruled out a direct role of the PTS in cAMP production and therefore that another signaling molecule would be required. However, Kremling et al. (425) reported that the *lac* operon is induced when the glucose concentration drops below 100 μM, which is much closer to the K_m. In addition, we found that in glucose-consuming *E. coli* cells, the cAMP concentration rises steeply between 120 and 30 μM glucose (M. Hoorneman, R. Bader, and P. W. Postma, unpublished results). At the same time, $EIIA^{Glc}$, which is predominantly unphosphorylated at higher glucose concentrations, becomes mostly phosphorylated. The latter observation complies with the concept that $P{\sim}EIIA^{Glc}$ stimulates adenylate cyclase activity.

Transcription Regulation by Mlc

When *E. coli* grows on PTS carbohydrates, the levels of both the general PTS proteins and the respective EIIs are often increased. The synthesis of EII complexes is usually controlled by sugar-related mechanisms. Operons encoding sugar-specific PTS components are often flanked by or contain a gene encoding either a specific repressor (operons encoding hexitol-specific PTS) (461, 465, 466), an antiterminator (the *E. coli bgl* operon [287] or the *L. casei lac* operon [288]), or a transcription activator (the *B. subtilis lev* operon) (518). These are examples of single operons controlled by their respective inducers. In enteric bacteria, the expression of several operons encoding either sugar-specific PTS components or the general PTS proteins is controlled by an additional mechanism responding to the phosphorylation state of a PTS protein (Fig. 2).

Repression by Mlc and its interaction with unphosphorylated $EIICB^{Glc}$. The utilization of a PTS substrate, such as glucose, by enteric bacteria increases the amount of not only $EIICB^{Glc}$ and the mannose-specific EIIs (215, 420, 769, 839) but also EI and HPr (172, 727, 771, 839). Unfortunately, a detailed analysis of transcription regulation of glucose-activated genes is complicated by the fact that most of them are also under the control of Crp/cAMP, i.e., partly repressed by the presence of glucose. For instance, *cyaA* or *crp* mutants have lower levels of EI, HPr, and several EIIs (727). On the other hand, *E. coli* wild-type cells grown on glucose exhibit increased levels of these proteins compared to cells grown on lactate. The relatively elevated synthesis in the presence of glucose appeared to require the activity of the repressor Mlc. Overexpression of the *E. coli mlc* gene leads to an increased colony size during growth on glucose-containing solid medium (hence the name *mlc*, for making large colonies), which is due to retarded glucose uptake and the reduced formation of acetate (343). This growth behavior led to the discovery of *mlc* in the first place.

Earlier studies of *E. coli* mutants that were affected in growth had already suggested that *ptsG* and *ptsM* expression might be controlled via a repressor. Suppressor mutants that were able to grow anaerobically on glucose, mannose, and glucosamine were obtained from *E. coli ptsG* strains, which cannot utilize the above-mentioned carbon sources in the absence of oxygen (732). The suppressor mutants were also 2-deoxy-D-glucose (2DG) sensitive under anaerobic conditions, and hence, the locus/gene was called *dgsA*. The toxic 2DG is transported mainly via $EIIC^{Man}/EIID^{Man}$, and indeed, after anaerobic growth, *dgsA* mutants exhibited elevated mannose and 2DG transport activities compared to the parental strain. DgsA was therefore assumed to be a repressor for the *man* operon. In fact, *mlc* was later found to be allelic with *dgsA* (662).

Strains overproducing Mlc (DgsA) were constructed, and the protein was purified (407, 857). It was found that the presence of a Zn^{2+} ion is essential for binding to the DNA (780). The amino acid sequence of the 44-kDa Mlc is about 40% identical to that of NagC (343, 579). NagC is a repressor/activator involved in *N*-acetylglucosamine uptake and metabolism (646, 667, 669, 922), and its activity is induced by *N*-acetylglucosamine-6-phosphate (668). Both Mlc and NagC belong to the ROK family, which contains repressors, open reading frames (ORFs), and kinases (314, 877). The DNA binding sequences of Mlc and NagC (663) and the putative sugar binding sites of Mlc and the structurally similar ROK family members glucokinase (GlcK) of *E. coli* and a putative fructokinase (FrcK) of *B. subtilis* (780) appear to be very similar. Nevertheless, in contrast to NagC, GlcK, and FrcK, Mlc does not seem to bind glucose or metabolites that are derived from it. Gel mobility shift assays showed that these molecules do not affect the interaction of Mlc with its various DNA target sites (167, 407, 408, 664). In addition, activation/deactivation of Mlc by phosphorylation seems unlikely, as no phosphorylation of Mlc has been detected (407).

While studying the expression of *ptsHI-crr* and *ptsG*, which are both regulated by Mlc, it became clear that the PTS controls Mlc activity (171, 172, 664, 665, 857). de Reuse and Danchin (171) concluded that transcriptional regulation of the *pts* operon is a consequence of an increase in the level of unphosphorylated $EIICB^{Glc}$. Plumbridge (665) then suggested that unphosphorylated $EIICB^{Glc}$ might interact with Mlc (Table 1). Affinity chromatography demonstrated that Mlc indeed binds to unphosphorylated, but not to phosphorylated, $EIICB^{Glc}$ (856). Mlc was found to be sequestered to the membrane when $P{\sim}EIICB^{Glc}$ was dephosphorylated during the uptake of glucose (458, 588). In addition, the overproduction of $EIICB^{Glc}$ resulted in the derepression of the Mlc-controlled operons *ptsG* and *ptsHI-crr* (588). Finally, surface plasmon resonance experiments and mobility shift assays confirmed that Mlc binds to $EIICB^{Glc}$ but not to EI, HPr, or $EIIA^{Glc}$ (588). The affinity of Mlc for unphosphorylated $EIICB^{Glc}$ ($K_D = 10^{-7}$ M) and for its various DNA target sites ($K_D = 10^{-8}$ to 10^{-7} M) is similar (588).

Initial studies with mutant $EIICB^{Glc}$ carrying various deletions suggested that Mlc binds to the EIIB domain only when

the EIIC-EIIB linker (residues 320 to 390) is present (458). Certain mutations in the EIIC domain interfere with Mlc-mediated regulation (615, 661, 988). In addition to *dgs* mutants, another class of mutants that also exhibited a phenotype resembling that of *mlc* strains was isolated. *E. coli manXYZ* strains grow only poorly on glucosamine, which is also slowly transported via $EIICB^{Glc}$. Starting from a *manXYZ* strain, several mutants that were able to grow efficiently on glucosamine were obtained. The mutants, which synthesized $EIICB^{Glc}$ constitutively, were called *umgC* (uptake of α-methylglucoside control), and the *umgC* mutations were mapped close to the *ptsG* gene (383). UmgC was hence originally postulated to be a repressor for *ptsG*. Investigation of the original mutants and some new *umgC* mutants revealed that these mutations actually map within *ptsG* (615, 661, 988). Interestingly, some of these mutations affect the putative cytoplasmic loops in the $EIIC^{Glc}$ domain and cause an up to fivefold increase in α-MG uptake rates compared to wild-type cells as well as elevated *ptsG* mRNA levels under noninducing conditions. The effects of *umgC* mutations resemble those found in *mlc* null mutants (615), and it was therefore assumed that they increase the affinity of $EIIB^{Glc}$ for Mlc. The binding of Mlc to EIIB is very sensitive to changes in the surface-exposed residues in the vicinity of the phosphorylatable Cys (795), and specific mutations in the EIIC domain possibly affect the arrangement of these residues in the EIIB domain, thereby affecting the binding of the transcription factor. However, the purified EIIB domain alone was found to be sufficient to bind Mlc, but anchoring to the membrane is necessary to prevent Mlc-mediated repression (795, 855). Nevertheless, other effects resulting from the membrane sequestration, like the induction of conformational changes or the masking of binding domains, might contribute to the regulation of Mlc (855).

Mlc has been crystallized, and its structure has been solved (780). The protein appears in two distinct homodimeric forms in the crystallographic unit, and the difference in the distances of the helix-turn-helix (HTH) DNA binding domains between the two forms suggests that only one should be able to bind to the DNA. The helix-turn-helix DNA binding domain of Mlc is stabilized by the C-terminal helix of the protein, and this structural feature seems to be crucial for Mlc function (780). Although deletion of the first 9 C-terminal amino acids does not affect Mlc activity, deletion of the first 18 C-terminal amino acids prevents repression and EII binding (795). The loss of function is accompanied by a transition from the tetrameric form, which is found in dilute solutions (588), to a dimeric form (795).

The Mlc regulon. DNase I footprinting experiments uncovered Mlc binding sites in the regulatory regions of five operons/genes in *E. coli*. They include *ptsHI* (407, 665, 857); *ptsG* (408, 664); *manXYZ* (662); *malT*, the positive regulator of the maltose regulon (167); and *mlc* itself (167). Transcription of these operons/genes is repressed by the binding of Mlc to a site overlapping the downstream promoter (except for the *pts* operon, where Mlc binds close to P_0) and is stimulated by Crp/cAMP, which binds upstream from Mlc. To find out to what extent Mlc and Crp/cAMP contribute to the regulation of the operons, expression of *lacZ* fusions in wild-type strains was compared to that in strains devoid of adenylate cyclase, Crp, Mlc, or $EIICB^{Glc}$.

A null mutation of the *mlc* gene caused a threefold increase in *manX* expression in glycerol-grown cells, and a similar increase was observed for *malT* (167). Synthesis of a ManX-LacZ fusion protein is very low in a *cyaA* mutant and is increased 14-fold by the addition of cAMP. The control exerted by Crp/cAMP appears to be stronger than that of Mlc, as a fivefold-higher amount of ManX-LacZ is present in cells grown on glycerol than in cells grown on glucose (662).

Glucose-grown cells also exhibit expression of *mlc* that is about twofold lower than that of cells grown on glycerol (167, 808). Whereas transcription regulators are often associated with one of the transcription units that they control, *mlc* is monocistronic. The coding sequence of the gene is preceded by a Crp/cAMP binding site, two promoters (579), and an Mlc binding site overlapping the second promoter (167). Although both promoters are recognized by RNA polymerase containing the housekeeping sigma factor σ^{70} ($E\sigma^{70}$), P_2 is also recognized by the heat shock factor σ^{32} complex ($E\sigma^{32}$) (808). In vitro transcription assays corroborated the finding that transcription is initiated from both promoters and that Crp/cAMP has a positive effect on P_2 in the presence of $E\sigma^{70}$ but a negligible effect in the presence of $E\sigma^{32}$. Crp/cAMP inhibits transcription from P_1, and Mlc inhibits transcription from P_2. The synthesis of σ^{32} in response to a heat shock leads to the overexpression of *mlc*. Nevertheless, the Mlc-to-$EIICB^{Glc}$ ratio remains fairly constant because *ptsG* transcription is also induced by $E\sigma^{32}$.

The $EIICB^{Glc}$-encoding *ptsG* gene is expressed from two promoters, which are under the control of Mlc (408, 664). One Mlc binding site overlaps P_1 and precedes a Crp/cAMP target site, and the other is upstream from P_2. Crp/cAMP-dependent expression from P_1 prevails, as P_2 transcripts amount to only 10% of the total *ptsG* mRNA (664). *crp* or *cyaA* null mutants do not express *ptsG* (408, 409, 727), while *ptsG* is up to 18-fold overexpressed in *mlc* null mutants (depending on the growth conditions) (373, 664). In wild-type cells, growth on glucose causes an eightfold induction of *ptsG* expression compared to growth on non-PTS carbon sources (215, 664). It follows that although affected by Crp/cAMP, the expression of *ptsG* is regulated mainly by Mlc. In addition, other factors have been implicated. It appears that the repression of *ptsG* transcription from promoters P_1 and P_2 by Mlc and the activation of transcription from promoter P_1 by Crp-cAMP are enhanced by Fis (807), one of the major histone-like proteins of *E. coli* (279). In addition, under conditions of nutrient limitation, *ptsG* expression is repressed by $E\sigma^{s}$ (RpoS), and this repression seems to act in synergy with that of Mlc (792). Furthermore, using ligand fishing with the promoter region of *E. coli ptsG*, it was found that the two-component response regulator ArcA, when phosphorylated, binds the promoter region at three positions: two between P_2 and P_1 overlapping the Crp/cAMP binding sites and one next to P_1 overlapping the Mlc binding site (373). P∼ArcA also binds to the promoter region of the *ptsHI-crr* operon but with lower affinity. The binding of P∼ArcA leads to a twofold reduction in *ptsG* transcription. The following link between the Arc two-component system and the glucose PTS might exist. An elevated glycolytic flux might cause a redox imbalance and hence lead to the activation of the response regulator ArcA, which is phosphorylated when reducing equivalents accumulate under aerobic conditions

(271). P~ArcA then binds to the *ptsG* promoter region and represses the transcription of *ptsG* (373), thus reducing the uptake of glucose and the glycolytic flux.

A different mode of regulation of the EIICB^{Glc} concentration is related to the stability of the RNA message. It was established that *ptsG* mRNA stability is reduced in an RNase E-dependent manner by metabolic blocks early in glycolysis (accumulation of glucose-6-P or fructose-6-P) (209, 410, 577, 578). Destabilization of the mRNA depends on enolase (578), a major component of the RNase E-containing degradosome, and the RNA chaperone Hfq (394). The chaperonin Hfq promotes the annealing of specific small RNA (sRNA) to target mRNA, thereby affecting the translation of the message (269, 842, 895). The *ptsG* mRNA is destabilized by an Hfq-binding sRNA called SgrS (898). Furthermore, expression of *sgrS* (formerly *ryaA*) is controlled by the transcription activator SgrR (formerly YabN) and becomes activated under conditions of α-MG-6-P (glucose-6-P) accumulation. However, Hfq-catalyzed annealing of the SgrS sRNA to *ptsG* mRNA alone is not enough to explain the observed instability of the *ptsG* mRNA. Kawamoto et al. (394) showed that the presence of the first two transmembrane helices of translated PtsG (i.e., localization near the membrane) is essential for an effective *ptsG* mRNA breakdown under conditions of hexose-6-P accumulation.

Transcription of the *ptsHI-crr* operon is initiated from three promoters (172, 173, 237, 753). P_0 and P_1 each yield two different transcripts: a short transcript coding for HPr only and a long transcript encompassing all three genes (172). The third promoter is located at the 3′ end of *ptsI* and allows *crr* transcription. Transcription from P_2 is independent of Crp/cAMP and Mlc (857) and accounts for about 80% of the total *crr*-containing mRNA (172). As a result, growth on glucose hardly affects the concentration of EIIA^{Glc}, whereas it increases the number of *ptsH* and *ptsI* transcripts three- to fourfold (171, 172, 727, 857). This induction is due to an increase in transcription from P_0. In the absence of glucose, Mlc binds to the P_0 promoter region and prevents the binding of the RNA polymerase (407, 665, 857). Although the presence of Crp/cAMP is essential for expression from P_0 (173, 665, 750, 857), this promoter is controlled mainly by Mlc (407, 857). The P_1 promoter is preceded by a second Crp/cAMP binding site, and expression from this promoter is weakly controlled by Crp/cAMP (173) but is not affected by Mlc (407, 857). Next to the Crp/cAMP is a FruR binding site (751), the inactivation of which has no effect on *ptsHI-crr* expression (665, 857). Recently, it was found that *crr* expression from P_2 is affected by the histone-like nucleoid-structuring protein (427), but the physiological relevance of this finding remains to be established.

Repression by Mlc and activation by Crp/cAMP are directly related to the phosphorylation state of EIICB^{Glc} and EIIA^{Glc}, respectively. As explained above, during growth on glucose, unphosphorylated EIIA^{Glc} and EIICB^{Glc} prevail, with the latter sequestering the repressor Mlc to the membrane. As a result, EIICB^{Glc} will be synthesized, whereas the amount of Mlc will decrease slightly due to the low amount of Crp/cAMP, which will further increase EIICB^{Glc} synthesis. Repression by Mlc is relieved not only by growth on glucose but also by growth on other rapidly metabolized PTS substrates such as *N*-acetylglucosamine and mannitol and to a lesser extent by growth on fructose and mannose (407, 661). Lactose, melibiose, and sucrose have no effect. Mlc repression is also affected by maltose, although maltose is not a PTS sugar. However, maltose degradation yields intracellular glucose and glucose-1-P. The import of maltose can therefore relieve Mlc repression inasmuch as intracellular glucose causes P~EIICB^{Glc} dephosphorylation (678).

Role of Phosphoenolpyruvate

The effects of PTS carbohydrates on the transport of non-PTS carbon sources and on cAMP synthesis seem to result mainly from changes in the phosphorylation state of EIIA^{Glc}. Early data on the phosphorylation state of PTS proteins showed that in galactose-grown *S. enterica* serovar Typhimurium cells, EIIA^{Glc} was mainly phosphorylated, whereas after the addition of α-MG, P~EIIA^{Glc} rapidly became dephosphorylated (596). In the meantime, the phosphorylation state of EIIA^{Glc} in *E. coli* cells growing on a number of PTS carbohydrates has been determined. When grown on glucose, more than 95% of the EIIA^{Glc} is unphosphorylated. A lower percentage of unphosphorylated EIIA^{Glc} was detected in cells grown on other PTS carbohydrates such as mannose, fructose, and mannitol (335). Interestingly, EIIA^{Glc} is also mainly dephosphorylated in wild-type cells grown only in rich medium, whereas in *cya* or *crp* mutants, EIIA^{Glc} is almost completely phosphorylated (852).

An unexpected finding was that EIIA^{Glc} is also partly unphosphorylated in cells grown on several non-PTS carbon sources, including lactose, melibiose, maltose, arabinose, and glucose-6-phosphate (334, 335). For instance, in cells growing on lactose or glucose-6-phosphate, approximately 70% and 60% of the EIIA^{Glc} is unphosphorylated, respectively. A correlation was found between the phosphorylation state of EIIA^{Glc} and the ratio of the intracellular concentrations of PEP and pyruvate, the substrate and product of EI phosphorylation, respectively. A decrease in this ratio leads to a decrease in EIIA^{Glc} phosphorylation. In harvested and washed *E. coli* cells, the intracellular PEP concentration ranges from 2 to 4 mM, and that of pyruvate ranges from 0.2 to 1.2 mM. The addition of glucose changes the concentration to about 0.3 mM PEP and 2.5 mM pyruvate within 15 s (335). For other carbohydrates, the changes are weaker and take slightly longer (30 to 60 s). The low PEP-to-pyruvate ratio observed during the metabolism of several carbon sources is expected to lead to poor phosphorylation of the PTS proteins, as the first phosphoryl transfer steps are reversible (540, 737, 940). An important corollary of these results is that carbon sources, whose transport/metabolism lowers the PEP-to-pyruvate ratio and is catalyzed by enzymes that are sensitive to inhibition by EIIA^{Glc}, should slow their own uptake. This has been confirmed for lactose. Cells lacking EIIA^{Glc} or producing a mutated LacY permease that is insensitive to inducer exclusion (inhibition by EIIA^{Glc}) exhibit elevated lactose influx (336).

It has been observed that when nonmetabolizable PTS substrates were added to starved cells of *S. enterica* serovar Typhimurium (839) or *L. lactis* (871), their uptake is initially fast and slopes off quickly. The phenomenon has been used as an argument for a possible regulatory role of the monomer/dimer transition of EI (639, 940). However, the observation

can be explained by the rapid drop of the PEP concentration upon the addition of sugar (335, 527, 871), which will cause a continuous decrease of the uptake rate until the PEP concentration reaches steady-state levels. In addition, model calculations (C. Francke, unpublished results) suggest that the initial uptake (first seconds) by starved cells might be extremely fast owing to a surplus of phosphoryl groups that are attached to PTS proteins in the nonfluxing state (equilibrium) compared to the state where phosphoryl group transfer towards the incoming sugar has commenced. The strongly diminished uptake rate that is sometimes observed can be explained by the accumulation of phosphorylated (nonmetabolizable) products and equilibrium, which will finally be reached under these conditions. In conclusion, although the extremely slow monomer/dimer transition rate of EI certainly shows regulatory potential, so far, uptake experiments can be perfectly explained by mechanistic models (239, 737) that do not take into account the dimerization of EI. In contrast, these models indicate that the PEP-to-pyruvate ratio is an important factor in regulation.

CCR Mediated by Non-PTS Sugars and Catabolic Intermediates

To determine the effect of Crp/cAMP on the expression of catabolic genes without the interference of inducer exclusion, *lacZ* expression was studied in the presence of the nonmetabolizable inducer IPTG (337), and *mal* operon expression was studied in the absence of its inducing sugar (210, 211). Lactose, gluconate, and glucose-6-P exert strong CCR on *lacZ* expression, even in a *crr* mutant, and a clear correlation between cAMP/Crp levels and *lacZ* expression was observed (337). Glycerol-3-P, glycerol, and other non-PTS sugars (i.e., arabinose, rhamnose, and especially xylose) cause strong repression of *malT* and *malK*, although phosphorylation of $EIIA^{Glc}$ is only slightly lowered (210, 211). Repression by glycerol is absent in a *glpK* mutant and enhanced in a *glpD* mutant (GlpD transforms glycerol-3-P into dihydroxyacetone-P), suggesting that repression is caused by elevated glycerol-3-P levels. Similar to glycerol-3-P, glucose-6-P and gluconate also lower *malT* expression (211). While growing a *pgi* mutant (Pgi, phosphoglucose isomerase, converts glucose-6-P into fructose-6-P) in the presence of glucose-6-P has only a weak effect on the phosphorylation state of $EIIA^{Glc}$ (334, 335), *malT* expression is still repressed under these conditions (211). If glucose-6-P is normally metabolized, mainly unphosphorylated $EIIA^{Glc}$ is present (334), and the repression of catabolic genes can be achieved via both inducer exclusion and low adenylate cyclase activity. However, if gluconate-6-P or glycerol-3-P accumulates, these metabolites seem to exert CCR even at high levels of $P\sim EIIA^{Glc}$. Although the phosphorylation state of $EIIA^{Glc}$ is barely altered, the cAMP concentration drops severalfold upon the addition of glucose-6-P or glycerol-3-P, whereas the concentration of Crp remains fairly constant (210, 337). A low cAMP level during growth on glucose-6-P has also been reported (201). It was therefore concluded that these metabolites interfere with the activation of adenylate cyclase by $P\sim EIIA^{Glc}$ (211).

2-Ketobutyrate, a precursor of leucine, is another metabolite involved in CCR. The addition of 2-ketobutyrate causes a strong transient decrease of the cAMP concentration in *E. coli* wild-type cells but not in *ptsI* or *crr* null mutants (152). The link between the PTS and the metabolism of 2-ketobutyrate is not clear. However, it has previously been reported that 2-ketobutyrate (which resembles pyruvate) accepts the phosphoryl group from P~EI (768). This could reduce the phosphorylation state of $EIIA^{Glc}$ and thereby reduce the activity of adenylate cyclase, especially when 2-ketobutyrate accumulates inside the cell. A transient accumulation of 2-ketobutyrate and a concomitant drop in cAMP concentration were indeed observed when cells growing on glucose were shifted from anaerobic to aerobic conditions (152).

Reverse Inducer Exclusion or Retroregulation

It is generally believed that PTS sugars, particularly glucose, are the preferred substrates in enteric bacteria and that they are on top of the hierarchy of carbohydrate utilization. However, one would expect that the overproduction of non-PTS target proteins of $EIIA^{Glc}$, such as GlpK or LacY, would lower the amount of free $EIIA^{Glc}$. Because the $EIIA^{Glc}$ phosphorylation site is usually part of the interface in the $EIIA^{Glc}$/non-PTS protein complexes, the phosphoryl group transfer through the glucose PTS will be diminished. This should lower the uptake of glucose via the PTS and at the same time diminish or even prevent inducer exclusion. The latter phenomenon has indeed been observed and was called escape from inducer exclusion (595) or desensitization (760). About 25% of the amount of $EIIA^{Glc}$ present in *S. enterica* serovar Typhimurium wild-type cells is sufficient for maximal uptake of α-MG (901). Lowering the amount of $EIIA^{Glc}$ below this level causes a decrease in the rate of α-MG transport. Interestingly, in cells producing high levels of GlpK, the rate of α-MG transport decreases by adding glycerol (734, 735), which reinforces the interaction between GlpK and $EIIA^{Glc}$. The competition of various target proteins for $EIIA^{Glc}$ and the resulting consequence for regulation in intact cells have also been studied. If different $EIIA^{Glc}$ target proteins are simultaneously present in the cell, they could compete for $EIIA^{Glc}$, especially when they outnumber the $EIIA^{Glc}$ molecules. Competition between the lactose carrier and GlpK for $EIIA^{Glc}$ was indeed observed in vitro (166). Similarly, inducer exclusion of glycerol or maltose in intact cells is relieved after the induction of the *lac* operon and the addition of thio-β-galactoside, which strengthens the interaction between LacY and $EIIA^{Glc}$ (761). In the same vein, inducer exclusion of maltose is relieved after the induction of GlpK synthesis and the addition of glycerol. Increasing the amount of $EIIA^{Glc}$ by introducing a *crr*-containing plasmid restores inducer exclusion (594).

Regulation by $EIIA^{Glc}$-Like Proteins

The *crr* mutation was isolated as a suppressor mutation in *ptsHI* strains, which restored growth on many non-PTS carbohydrates. No other mutations giving rise to this general suppressor phenotype have been reported, suggesting that $EIIA^{Glc}$ is unique in being able to regulate PTS-mediated inducer exclusion and to activate adenylate cyclase in enteric bacteria. Therefore, it came as a surprise that a residual level of inducer exclusion was observed in *S. enterica* serovar Typhimurium *crr* deletion strains (595). This effect is probably

mediated via EIIs containing an $EIIA^{Glc}$ domain such as $EIICBA^{Nag}$ and $EIIBCA^{Bgl}$. In bacteria grown on a carbohydrate that induces one of the EIIs, antibodies against $EIIA^{Glc}$ cross-react with membrane-associated proteins (786). In addition, *E. coli* $EIIBCA^{Bgl}$ and *Klebsiella pneumoniae* $EIICBA^{Nag}$ completely restore α-methyl-glucoside transport in a *crr* mutant (921), and $EIIA^{Glc}$ complements a C-terminal deletion in $EIICBA^{Nag}$ (923). The EIIA domain of $EIICBA^{Nag}$ restores inducer exclusion in a strain lacking both $EIIA^{Glc}$ and $EIICBA^{Nag}$ (899). In addition, several $EIIA^{Glc}$-like domains of proteins from gram-positive organisms, such as $EIIA^{Glc}$ of *B. subtilis* $EIICBA^{Glc}$ or *Streptococcus thermophilus* LacS, restore inducer exclusion in *E. coli* (302, 723).

Is $EIIA^{Glc}$-mediated regulation limited to enteric bacteria? Many bacteria contain an $EIIA^{Glc}$-like protein or domain, but whether $EIIA^{Glc}$ plays a regulatory role has been investigated for only a few organisms, such as *B. subtilis*, *M. capricolum*, *Haemophilus influenzae*, *S. thermophilus*, and *S. coelicolor*.

Similar to *E. coli ptsI* mutants, *B. subtilis ptsI* mutants are not able to grow on the non-PTS carbon source glycerol (265). However, in contrast to *E. coli*, inactivation of $EIIA^{Glc}$ does not restore growth of the *B. subtilis ptsI* strain on glycerol (282). It will be discussed below that in gram-positive bacteria, $EIIA^{Glc}$ does not interact with GlpK but that this enzyme is regulated via P∼His-HPr-mediated phosphorylation (158). In *M. capricolum*, glycerol kinase is also not inhibited by $EIIA^{Glc}$ (992).

Sequencing of the *H. influenzae* genome revealed genes encoding EI, HPr, and $EIIA^{Glc}$ (*ptsHI-crr* operon) as well as genes encoding an $EIIBC^{Fru}$-like protein (*fruA*) and a protein in which two FPr domains, each containing a phosphorylatable histidine (His-300 and His-424), are fused to $EIIA^{Fru}$ ($EIIA^{Fru}$-FPr-FPr; *fruB*) (500, 717). In *H. influenzae*, only fructose, but not glucose, is taken up via a PTS, and a *ptsI* mutant can therefore ferment glucose (499). Interestingly, the development of competence, which in *H. influenzae* requires cAMP and Crp (105, 196), was lowered about 70-fold in a *crr* mutant (305) and 250- to 500-fold in a *ptsI* mutant (305, 499). The addition of extracellular cAMP restored the development of competence. It is likely that *H. influenzae* P∼$EIIA^{Glc}$ stimulates adenylate cyclase activity in a manner similar to that observed in enteric bacteria. No $EIICB^{Glc}$ exists in *H. influenzae*, and thus, the presence of glucose does not lead to direct dephosphorylation of P∼$EIIA^{Glc}$. Nevertheless, the uptake of fructose will lower the amount of P∼EI and P∼HPr and consequently will also lower the amount of P∼$EIIA^{Glc}$.

Although the main uptake system for glucose in *S. coelicolor* is GlcP, a non-PTS permease of the major facilitator superfamily (911), CCR by glucose was observed (21, 217, 530, 912). Glucose kinase (443, 911) and the accumulation of glycolytic intermediates (694) are implicated in mediating the effect. Although *S. coelicolor* contains EI, HPr, $EIIA^{Glc}$ (designated $EIIA^{crr}$), and $EIIBC^{Glc}$ homologs as well as other sugar-specific EII complexes, PTS proteins do not seem to be involved in CRR in this high-G+C gram-positive organism (58, 629). In contrast to *E. coli*, the *crr* gene of *S. coelicolor* precedes *ptsI*, and *ptsH* is located somewhere else on the chromosome. $EIIA^{crr}$ restores growth of an *E. coli crr* strain on glucose (392). HPr from *S. coelicolor* is efficiently phosphorylated by *B. subtilis* EI and PEP but is barely modified by *B. subtilis* HprK/P and ATP (97, 630). In fact, *S. coelicolor* is missing HPrK/P, and the deletion of *ptsH* has no effect on the glucose repression of galactokinase (97) or glycerol kinase (612). The absence of fluctuations in the cAMP concentration (112) restricts the potential role of the PTS to inducer exclusion-mediated mechanisms. However, although expression of the *S. coelicolor crr* gene in an *E. coli ptsHI-crr* deletion strain significantly reduces maltose uptake and although a specific interaction between *E. coli* MalK and *S. coelicolor* $EIIA^{crr}$ was established by using surface plasmon resonance spectroscopy (392), no evidence showing that the PTS protein exerts a similar function in *S. coelicolor* has been obtained.

Mathematical Modeling of the PTS and Its Role in CCR

The study of complex metabolic and regulatory networks is facilitated by the availability of mathematical models (463). As a consequence of the vast amount of reliable experimental data on individual reaction rates, it has become possible to make such models for the PTS. A kinetic model for the glucose PTSs of *E. coli* and *S. enterica* serovar Typhimurium has been constructed (737) and converted spatiotemporally to unravel the possible effects of diffusion on PTS function (239, 240). The flux data obtained earlier in vivo (901) and in vitro (738) are described reasonably accurately by these models. When using the measured cellular protein concentrations, the computations indicate that most PTS components should be present as heterodimeric phosphoryl transfer complexes. Lowering the total $EIIA^{Glc}$ concentration in the calculation, for example, by binding to non-PTS components (735), has no significant effect on either the flux of phosphoryl groups or the concentration of unphosphorylated $EIIA^{Glc}$. Consequently, inducer exclusion will hardly have an effect on glucose influx, which was also observed experimentally (734). During the uptake of a rapidly metabolizable PTS sugar, the concentration of unphosphorylated $EIIA^{Glc}$ is predicted to be low. The low concentration of unphosphorylated $EIIA^{Glc}$ implies that the affinities between non-PTS transporters or GlpK and unphosphorylated $EIIA^{Glc}$ should be stronger than originally predicted. In fact, this prediction is in line with the interaction data for GlpK and $EIIA^{Glc}$. Initially, the K_i was reported to be 16.6 μM, but in the presence of 0.1 mM Zn^{2+}, it was found to be as low as 0.28 μM (223). When assuming a simple one-to-one association mechanism between GlpK and unphosphorylated $EIIA^{Glc}$ and a predicted concentration range for unphosphorylated $EIIA^{Glc}$ of 2.5 to 0.25 μM (239), the ratio of active/inactive GlpK can vary from 1.1 to 0.11. The prediction is also supported by the reported K_Ds for $EIIA^{Glc}$ from LacY (1.0 μM) (825) and FrsA (0.2 μM) (419).

A remarkable outcome of the spatiotemporal modeling is that although diffusion is not limiting for glucose uptake, it prevents an equal distribution of unphosphorylated $EIIA^{Glc}$ throughout the cell, with a significantly (30%) higher concentration near the cytoplasmic membrane (239, 240). LacY, MelB, MalK, and probably also GlpK (920) are associated with the cytoplasmic membrane, which implies that diffusion limitation enhances inducer exclusion. The calculations also clearly indicate that the PTS is a dual sensing system. In accordance with the experimental data, both the glucose and the PEP concentrations are predicted to affect the phosphorylation

state of $EIIA^{Glc}$ (335, 736). The same conclusion can be drawn from the response towards carbohydrate pulses of two other detailed kinetic models of the PTS (including glycolysis) (426, 777). The first model (426) was analyzed further with respect to the time hierarchy of the responses, and the second model (777) was analyzed further with respect to the regulatory roles of the various PTS components.

A mathematical model providing a different view of the PTS was presented by Thattai and Shraiman (868). Those authors attempted to describe the competition of different sugar-specific PTSs for the phosphoryl groups provided by the common PTS components EI and HPr and derived phase diagrams for the uptake rates of the "competing" PTS sugars. Their procedure revealed that there is only one "nontrivial switching" phenotype generated by the PTS. This phenotype corresponds to diauxic growth (winner-takes-it-all behavior). However, the calculations did not include the regulation of enzyme activity by catabolic intermediates and transcription regulation via Crp/cAMP and Mlc. In addition, the model is based on the assumption that the concentrations of EIIAs and EIIBCs are correlated and that the sugar uptake rate is maximized, while the phosphoryl group flux is limiting. In the case of glucose, these assumptions are problematic, as the expression of $EIIA^{Glc}$ and that of $EIICB^{Glc}$ are not correlated (see the section on the *mlc* regulon) and transport controls the flux of phosphoryl groups (240, 737, 901). Nevertheless, hierarchical utilization of PTS carbohydrates is a common feature of both gram-negative and gram-positive bacteria (see also reference 678).

Several mathematical models that describe the expression of the *lac* operon exist (626, 776, 802, 953, 978, 979). The model described by Wong et al. (953) contains regulation by inducer exclusion, cAMP, and LacI but lacks regulation of *cyaA* and *crp* expression. The expression of *lacI* is assumed to be constitutive, and the cAMP level is linearly proportional to the glucose transport rate. Intracellular phosphorylation of glucose by $EIICB^{Glc}$ is not considered. Nevertheless, for cells growing on glucose and lactose, the experimentally observed preferential utilization of glucose, the short lag phase when shifting to lactose utilization, the accompanying sharp rise in the cAMP concentration, and the induction of *lacZYA* transcription can be reproduced. However, in contrast to the experimental data (362), the concentration of cAMP remains high after the lag phase, and the time course predictions are incorrect. The models by the group of Mackey focus on the proposed bistable nature of the *lac* operon. A bistable system can convert a graded signal into a switch-like response; i.e., when the inducer concentration passes a certain threshold, the system is switched on. At the same time, to switch off and return to the initial state, the inducer concentration has to drop below a smaller threshold value (hysteresis). The initial models, which did not include the mechanisms responsible for CCR (978, 979), described the induction of the *lac* operon during growth on lactose well. By including experimental data on β-galactosidase expression, the models predicted bistability for the *lac* operon depending on the concentration of extracellular lactose and the growth rate. When activation/repression by Crp/cAMP and inducer exclusion were included (776), bistability was maintained. The potential for bistable behavior of the *lac* operon was further proved by the observed history dependence (i.e., a hysteretic dependence) of the induction of fluorescent reporter proteins synthesized from genes expressed from the *lac* or *gat* promoter (626). Based on these induction experiments, a mathematical model was constructed, which separates the effects of inducer exclusion (acting on LacI activity) and Crp/cAMP, as *E. coli* MG1655, which was used in these studies, lacks GatR. Its *gat* promoter is regulated solely by Crp/cAMP and can thus be used as a specific reporter of Crp/cAMP effects. It was found that repression by glucose is mediated by both inducer exclusion and Crp/cAMP and that the effect of the latter is stronger. Similarly, the calculations reported by Santillán and Mackey (776) indicate that the effect of activation/repression by Crp/cAMP and inducer exclusion on the induction of the *lac* operon are cumulative so that both the sensitivity of the system towards glucose and the concentration of lactose required to induce the system are elevated. However, in agreement with the conclusions drawn from "wet" experiments, the calculated effective β-galactosidase concentration appears to be more sensitive to inducer exclusion than to Crp/cAMP (see the section on diauxic growth).

A comprehensive model of diauxic growth was presented by Kremling et al. (425). Repression of *ptsG* by Mlc, inducer exclusion, and non-PTS transport of glucose were included, but the formation of heterodimeric phosphoryl group transfer complexes was not taken into account. Parameters were taken from the literature or otherwise estimated and optimized on the basis of growth experiments with isogenic mutants (*cyaA*, *lacI*, and *ptsG*) obtained from *E. coli* strain LJ110, a well-characterized derivative of the *E. coli* K-12 reference strain W3110. The model perfectly reproduces biomass yield, glucose and lactose consumption, the preferential use of glucose, and the induction of *lacZ* expression. In agreement with the experimental data, the model predicts high concentrations of unphosphorylated $EIIA^{Glc}$ during growth on glucose, followed by a rise in the $P{\sim}EIIA^{Glc}$ and cAMP concentrations when glucose is exhausted. When intracellular glucose is produced from lactose and phosphorylated by the PTS, $P{\sim}EIIA^{Glc}$ is predicted to drop again. A similar model describes the transport and metabolism of the non-PTS sugar glycerol and the PTS substrate sucrose as well as *glp* and *scr* gene expression (931). Again, the model reproduces the experimental data obtained with *E. coli* K-12 derivatives well. The models described by Wang et al. and Kremling et al. (425, 931) were used to construct a large-scale stochastic model of glucose, lactose, and glycerol metabolism (up to pyruvate), including transcriptional regulation (688). The model reveals potential effects of history and stochasticity, the latter especially in transcription, on reaction network behavior towards the changes in available nutrients.

Other Regulatory Mechanisms Involving the PTS in Enteric Bacteria

Phosphorylation of EI by ATP. PEP-dependent phosphorylation of EI was originally thought to be the only route to generate P~His-HPr (435, 436). However, two decades after the discovery of the PTS, PEP-independent EI phosphorylation was discovered (236). *E. coli* acetate kinase (AckA) and [γ-^{32}P]ATP phosphorylate EI in a reversible reaction (236). When catalyzing the formation of acetyl~P from acetate and ATP, AckA is transitorily phosphorylated on a glutamyl resi-

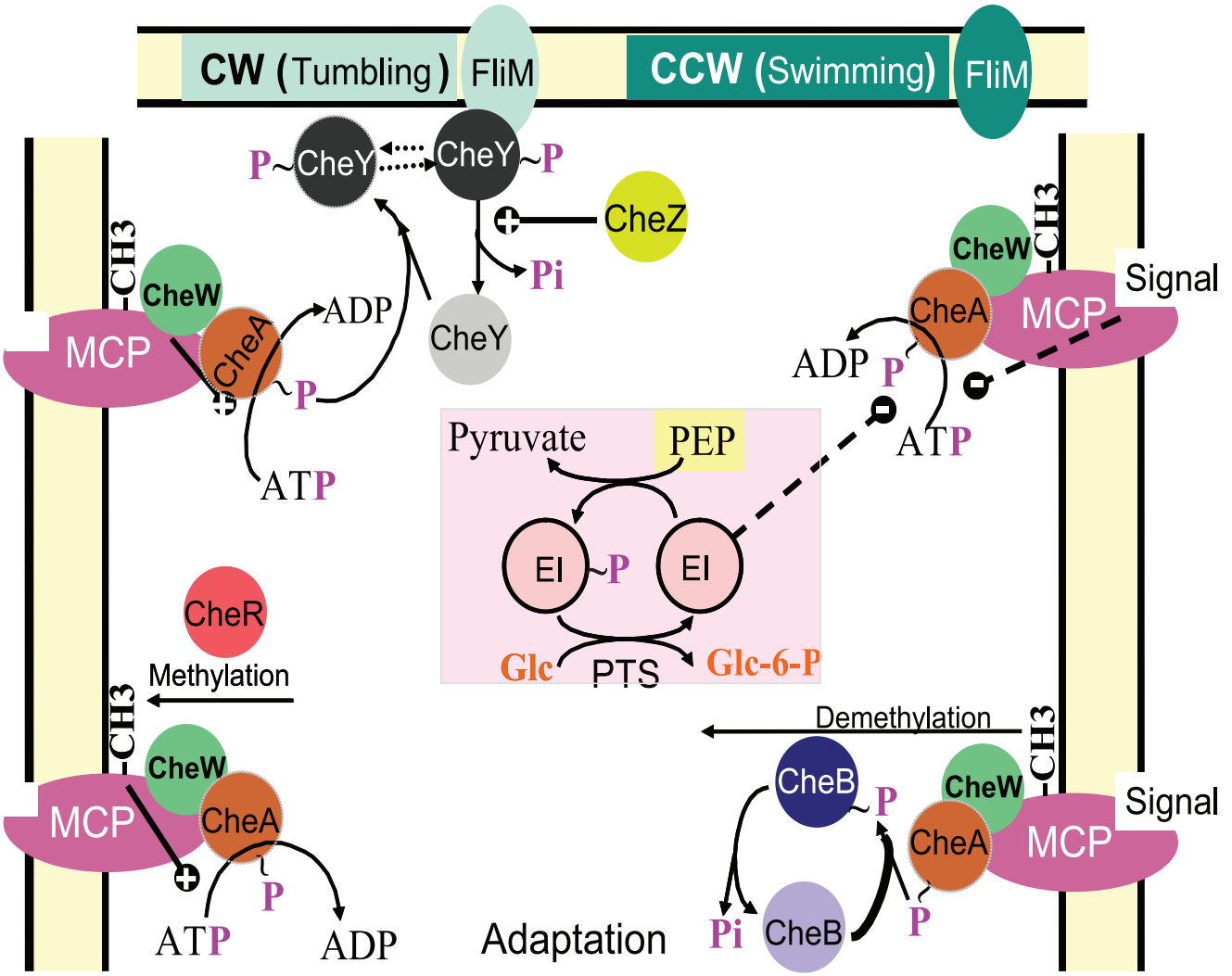

FIG. 3. Mechanism of chemotaxis in *E. coli* and role of the PTS in carbohydrate chemotaxis. In the absence of chemotactically active molecules (top left), CheA autophosphorylates at a histidyl residue. This reaction is stimulated by CheW, which recruits CheA to the MCP receptor protein. P~CheA transfers its phosphoryl group to CheY, and P~CheY binds the flagellar motor FliM, thereby evoking clockwise (CW) rotation of the flagella, which leads to tumbling of the bacterium. In the presence of chemotactically active molecules (top right), the autophosphorylation activity of CheA is inhibited, and as a result, the concentration of P~CheY drops. FliM molecules will no longer be complexed with CheY, which favors counterclockwise (CCW) rotation of the flagella. This results in smooth swimming towards increasing concentrations of the chemotactically active substance. The presence of an efficiently metabolizable PTS carbohydrate (or the absence of PEP) induces a similar response, as under these conditions, EI is present mainly in an unphosphorylated form. In fact, dephospho-EI inhibits the autophosphorylation of CheA (center) and therefore also favors smooth swimming. The sensitivity of the system towards the signal is regulated through methylation and demethylation of the MCPs (bottom), which are catalyzed by CheR and CheB, respectively. MCP methylation stimulates the autophosphorylation of CheA, and demethylation inhibits it.

due (238, 881), probably at Glu-387 in *E. coli* AckA (817). PEP and P~AckA most likely phosphorylate the same histidyl residue in EI (16), as the phosphoryl group of P~AckA can be passed to $EIIA^{Glc}$ via EI and HPr. A second protein kinase that also phosphorylates EI at the expense of ATP was found in *E. coli* (154, 155). It was partially purified, but its activity vanished during purification due to the loss of a cofactor, which was shown to be $NAD(P)^+$. The reaction catalyzed by this EI kinase is reversible; i.e., it can transfer the phosphoryl group of P~EI back to ADP, and the backward reaction requires the presence of NAD^+. In its absence, EI kinase acts as a phosphatase and converts P~EI to EI and P_i. It is not yet clear whether phosphorylated EI kinase is transitorily formed during P~EI dephosphorylation. Although AckA- and EI kinase-mediated phosphorylation of EI have been demonstrated in vitro, whether they have physiological importance remains to be shown.

Chemotactic response to carbohydrates. Motile bacteria such as *E. coli* and *B. subtilis* can be attracted to or repelled from various chemical and physical environmental signals. These signals can be processed via different pathways (see reference 11), but ultimately, they affect the frequency with which the rotation direction of the flagella changes. An increase in counterclockwise rotation results in smooth, straight

movement of the bacterium (e.g., towards the attractants), whereas an increase in clockwise rotation leads to "tumbling" and a change in direction (e.g., away from the attractant) (see reference 501). The most important chemotaxis signal processing pathway comprises chemoreception in the cytoplasmic membrane by methyl-accepting chemotaxis proteins (MCPs) (for reviews on chemotaxis, see references 77, 82, 838, and 850). Although the number of proteins involved in the chemotactic signaling pathway varies in different bacteria, the main mechanism follows similar principles (850). The chemotaxis pathway of enteric bacteria is displayed in Fig. 3. An incoming chemotactic signal is relayed to the flagellar motor via the CheA/CheY two-component system. The autophosphorylation activity of the ATP-dependent histidine kinase CheA is stimulated by its CheW-mediated recruitment to the MCPs. P~CheA transfers its phosphoryl group to an aspartyl residue of CheY, the response regulator. P~CheY binds to the flagellar motor protein FliM, and as a consequence, the tumbling frequency increases. To prevent the accumulation of P~CheY, the protein has autophosphatase activity, and in various bacteria (including *E. coli*), dephosphorylation is stimulated by a protein called CheZ. This signal transduction pathway is controlled by an adaptive feedback mechanism involving MCP methylation by the methyltransferase CheR, which stimulates CheA activity, and demethylation by the methylesterase CheB, which inhibits CheA activity. CheB is activated by phosphoryl transfer from P~CheA. Upon chemoreception, the MCP lowers the activity of CheA considerably, and as a result, the concentration of P~CheY decreases. The bacterium therefore tumbles less frequently and thus effectively moves up a concentration gradient of chemoattractant. Simultaneously, receptor demethylation diminishes due to lower levels of P~CheA/P~CheB, and CheA will be reactivated, leading finally to an adaptation of the system.

E. coli shows chemotaxis towards many nutrients, including several PTS sugars (8, 462). By using a variety of null mutants, it was established that the chemotrophic response towards PTS sugars was elicited by their transport and not by their binding or metabolism (8, 297, 462, 641, 948). It was established that chemotaxis towards PTS sugars at relatively high concentrations (>1 mM) is also mediated by the chemoreceptors Aer and Tsr (296), which sense redox state and proton motive force, respectively (865). In *E. coli*, chemotaxis by PTS sugars can be abolished by knocking out CheA, CheY, CheW, EI, HPr, or the corresponding sugar-specific EII complexes, whereas the removal of the MCPs, CheR, or CheB has no significant effect (8, 297, 460, 462, 498, 550, 609, 641, 745, 863, 864; see also reference 678). Despite the fact that PTS-mediated sugar uptake and Che protein-mediated chemotaxis involve similar phosphoryl transfer reactions, no phosphoryl transfer between PTS components and Che proteins has been demonstrated (379). Rather, in vitro experiments established that unphosphorylated EI interacts with CheA and thereby inhibits the autokinase activity of CheA by up to 10-fold (497). In addition, CheA activity drops about threefold upon shifts of the PEP concentration in the physiologically relevant range (from 2 to 0.3 mM). Further evidence for an effect of the phosphorylation state of PTS proteins on CheA activity was provided by kinetic analyses of the chemotactic response in *E. coli* wild-type cells and several *che* null mutants (498). The response, which was induced by flash photolysis of caged D-glucose and α-MG, shows rapid kinetics and high signal sensitivity (K_m for D-glucose is about 10 nM). Considering the rapidity and sensitivity of the response, those authors proposed that transport-induced dephosphorylation of P~EI affects CheA activity but has little influence on the PEP concentration (the intracellular concentration of PEP is about 200-fold higher than that of EI). This hypothesis holds only when phosphorylation of EI by PEP would be a slow process controlling glucose influx. However, because even at high glucose concentrations, nearly all control over the flux resides in the glucose transporter (901), it is highly unlikely that EI phosphorylation exerts control at low glucose concentrations. Rather, PTS model calculations predict that the extent of EI phosphorylation rapidly responds to changes in the PEP concentration (239, 240, 777). At high intracellular PEP concentrations, mainly P~EI and little EI have been calculated to be present. Thus, even a slight reduction in the PEP concentration (as anticipated for the chemotaxis experiments) (497) could cause a relatively significant increase of unphosphorylated EI, which would lower CheA activity. This could in turn slightly lower the amount of P~CheY and lead to a strong chemotactic response, as the binding of P~CheY to the flagellar motor is highly cooperative (133).

In contrast to enteric bacteria, chemotaxis towards PTS carbohydrates in *B. subtilis* is mediated by the methyl-accepting chemotaxis protein McpC, which is also a sensor for several amino acids (582). Deletion of *mcpC* abolishes chemotaxis towards the carbohydrates D-glucose, D-mannitol, D-fructose, trehalose, *N*-acetylglucosamine, and α-methyl-D-glucoside (261, 428). Nevertheless, chemotaxis in *B. subtilis* is affected by the PTS, and the cytoplasmic domain of McpC is thought to receive a transport-related signal from the PTS (428). As a consequence, EII^{Mtl}, EII^{Fru}, and EII^{Tre} null mutations resulted in a loss of chemotaxis towards the related carbohydrate. Deletion of *ptsH* also prevents *B. subtilis* from moving up a sugar gradient of mannitol and fructose (261). The response towards glucose is more complex and involves McpA (313). A *ptsH* or *mcpA* deletion strain shows chemotaxis towards glucose (which is taken up via a non-PTS transporter by the *ptsH* mutant), whereas a double mutant does not (261). Binding studies with purified CheA and EI indicate that P~EI, but not EI or PEP, binds CheA and thereby inhibits its autophosphorylation. In *B. subtilis*, P~CheY exerts an effect opposite that in *E. coli* (61, 62, 945) (i.e., P~CheY leads to smooth swimming), and therefore, the inhibition of CheA activity by phosphorylated EI in *B. subtilis* is consistent with CheA inhibition by unphosphorylated EI in *E. coli*. However, experimental findings with tethered cells raise doubts about the proposed mechanism. For instance, the addition and removal of mannitol had effects similar to those of the addition of glucose on the frequency of counterclockwise rotation in a *ptsH* mutant (261), although this mutant does not transport mannitol. It is not clear how the phosphorylation state of the PTS proteins could be affected by a sugar that is not transported and metabolized. In addition, conflicting results were obtained when the effect of glucose on the rotation of tethered cells was studied (261, 428).

Glycogen storage. Many bacteria, including *E. coli* and *S. enterica* serovar Typhimurium, store carbohydrates in the form of glycogen. They produce it from glucose-1-P by the sequen-

tial action of ADP-glucose pyrophosphorylase (GlgC) and glycogen synthase (GlcA) (see reference 683). When needed, glycogen is reconverted to glucose-1-phosphate by glycogen phosphorylase (GlgP) (962). The related *E. coli* genes are located in the *glgCAP* operon, which is positively regulated by Crp/cAMP (684) and negatively regulated by CsrA (38, 486, 962). *B. subtilis* possesses a *glgBCDAP* operon (*glgB* codes for a glycogen branching enzyme, and *glgD* codes for a second ADP glucose pyrophosphorylase), which is presumably negatively regulated by CcpA/P-Ser-HPr (178). Surface plasmon resonance-based ligand fishing revealed a strong interaction between *E. coli* glycogen phosphorylase (GlgP) and HPr; indeed, GlgP was the only protein "fished" out of a cell extract by immobilized HPr (799). The interaction between the two proteins was confirmed by mobility shift assays and sedimentation equilibrium centrifugation. Subsequent competition experiments established that the binding was highly specific. Neither *B. subtilis* and *Mycobacterium capricolum* HPrs nor *E. coli* NPr and diphosphoryl transfer proteins (FPr) bind to *E. coli* GlgP, and vice versa, *E. coli* maltodextrin phosphorylase, which is very similar to GlgP (352, 797), does not bind HPr (418, 799). The affinity of GlgP for P∼His-HPr is four times higher than that for unphosphorylated HPr. Binding of HPr stimulates the formation of GlgP dimers and tetramers. Only the binding of HPr, but not of P∼His-HPr, leads to a significant increase in GlgP activity (2.5-fold) (799). The concentration of HPr (531) is much higher than that of GlgP (116), and therefore, a major part of cellular GlgP will be complexed with HPr or P∼His-HPr. As a result, GlgP activity will be determined by the phosphorylation state of HPr. Conversely, overproduction of GlgP should lead to the sequestration of most HPr and thereby to the inhibition of the PTS, as was indeed observed (418).

Based on these results, the following model for the regulation of glycogen breakdown was proposed for *E. coli*. Cells growing on glucose start to accumulate glycogen during the late exponential growth phase and the onset of stationary phase (418, 683, 684). Under these conditions, the concentration of phosphorylated PTS proteins is relatively high (797). cAMP levels will therefore rise (653), and expression of the *glgCAP* operon will be stimulated. The activity of GlgP is low, because mainly P∼His-HPr is present, and glycogen will accumulate. Later, when growth of the cells has advanced far into stationary phase, the PEP concentration drops, PTS proteins will become dephosphorylated, and the cAMP concentration and expression of the *glgCAP* operon will decrease. However, GlgP is now activated by binding unphosphorylated HPr, and glycogen breakdown will therefore commence and dominate over glycogen synthesis.

REGULATION OF CARBON METABOLISM IN LOW-G+C GRAM-POSITIVE BACTERIA: REGULATORY FUNCTIONS OF P-Ser-HPr

HPr, the Central Processing Unit of Carbon Metabolism in Gram-Positive Bacteria

Most low-G+C gram-positive organisms do not possess adenylate cyclase, and the cAMP-dependent $EIIA^{Glc}$-controlled CCR mechanism of gram-negative bacteria can therefore not be operative in these organisms. In fact, the general PTS protein HPr turned out to be the master regulator of carbon metabolism in gram-positive bacteria. Similar to $EIIA^{Glc}$ in gram-negative bacteria, HPr carries out its diverse regulatory functions in response to changes in its phosphorylation state. In low-G+C gram-positive bacteria, HPr becomes phosphorylated not only by PEP at His-15 but also by ATP at Ser-46. As a consequence, four different forms of HPr exist in these organisms: dephospho-HPr, HPr phosphorylated at either His-15 (P∼His-HPr) or Ser-46 (P-Ser-HPr), and doubly phosphorylated HPr (571, 893). The rapid metabolism of various sugars affects the activities of a bifunctional protein kinase/P-protein phosphorylase, which responds to changes of the ATP, P_i, PP_i, and FBP concentrations, and of EI, which responds to alterations of the PTS phosphotransfer activity and the PEP-to-pyruvate ratio (335). These two enzymes control the concentration of the various forms of HPr, which regulate carbon metabolism via protein-protein interactions (HPr and P-Ser-HPr) or the phosphorylation of non-PTS proteins (P∼His-HPr) (Table 1). In gram-positive bacteria, HPr therefore functions as the "central processing unit" for carbon metabolism, as its phosphorylation state is determined by various signals (input), which in turn allow it to phosphorylate or to interact with numerous other proteins (output). In the following sections, we discuss the characteristics of the enzyme catalyzing the phosphorylation/dephosphorylation of HPr at Ser-46, the role of P-Ser-HPr in CCR and inducer exclusion, and the regulation of non-PTS proteins (antiterminators, transcription activators, carbohydrate transporters, and catabolic enzymes) via phosphorylation by P∼His-HPr and/or P∼EIIBs.

Characteristics of ATP-Dependent HPr Phosphorylation

In the following sections, we will describe the enzyme HPr kinase/phosphorylase (HprK/P), which catalyzes the ATP-dependent phosphorylation of HPr (and certain HPr paralogs) at Ser-46 as well as the dephosphorylation of P-Ser-HPr. We will include structural studies of HprK/P and the complexes with HPr and P-Ser-HPr and discuss the regulation of its two opposing activities by metabolites. The organization of *hprK* with specific genes, some of unknown function, in gram-positive bacteria will be discussed, and finally, results that suggest a function of P-Ser-HPr in the regulation of PTS activity by a feedback mechanism will be provided.

Phosphorylation of HPr at Ser-46. Isocitrate dehydrogenase from *E. coli* was the first bacterial protein shown to be phosphorylated by an ATP-dependent seryl protein kinase (257). In the early 1980s, when Deutscher, Reizer, and Saier investigated a possible implication of protein phosphorylation in inducer expulsion (185, 710, 712), a process where certain sugar phosphates that have accumulated in bacteria are dephosphorylated and expelled as soon as the cells are exposed to rapidly metabolizable carbon sources such as glucose or mannose, a second seryl-phosphorylated bacterial protein was detected in *Streptococcus pyogenes* and was identified as being HPr of the PTS (185). Unlike PEP-dependent EI-catalyzed phosphorylation at the catalytic His-15 of HPr, which occurs in both gram-negative and gram-positive organisms (Fig. 1) (262, 941), phosphorylation of HPr at a seryl residue, which requires a specific

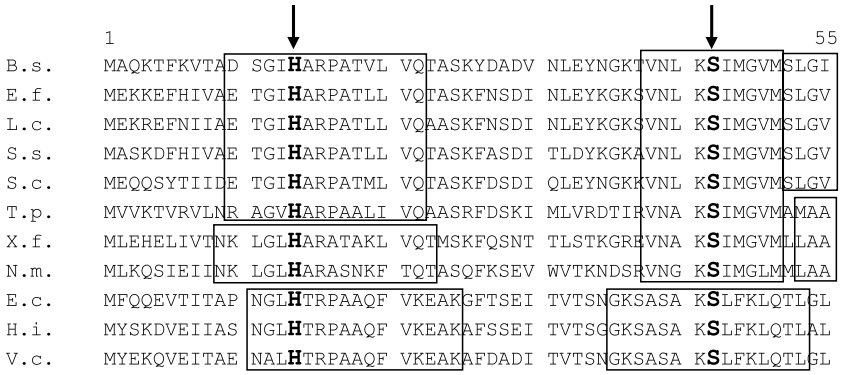

FIG. 4. Alignment of the first 55 amino acids of HPr proteins from firmicutes (B.s., *B. subtilis*; E.f., *E. faecalis*; L.c., *L. casei*; S.s., *S. salivarius*; S.c., *S. carnosus*), from a spirochete (T.p., *T. pallidum*), from proteobacteria with a Ser-46 region strongly resembling the corresponding sequence in HPr of firmicutes (X.f., *Xylella fastidiosa*; N.m., *N. meningitidis*), and from other gram-negative bacteria (E.c., *E. coli*; H.i., *H. influenzae*; V.c., *V. cholerae*) (*V. cholerae* possesses a second HPr in which the region around Ser-46 more strongly resembles the corresponding region in the HPr of firmicutes). The arrows indicate the amino acids His-15 and Ser-46. In HPrs from organisms, in which these residues are phosphorylated, they are shown in boldface type. Conserved regions around the phosphorylation sites are boxed.

protein kinase and ATP (185) or GTP (709), was originally believed to be restricted to gram-positive organisms with low G+C content.

The site of ATP-dependent phosphorylation in HPr of *Enterococcus faecalis* was identified as being Ser-46 (183). An equivalent of Ser-46 is present in HPr of all low-G+C gram-positive bacteria, and the surrounding sequence is usually well conserved (Fig. 4). However, in the HPr of some organisms, the phosphorylatable serine is in a slightly different position (position 45 in HPr of *Bacillus thuringiensis* subsp. *israelensis* [400] and position 47 in HPr of *Mycoplasma genitalium* [713]). Replacement of Ser-46 in *B. subtilis* HPr with an alanine, tyrosine, or aspartate prevents the FBP-stimulated ATP-dependent phosphorylation of HPr (206, 722), whereas HPr carrying a threonine at position 46 is still slowly phosphorylated (722). Mutations at Ser-46 also affect the PEP-dependent, EI-catalyzed phosphorylation at His-15. In a mutant complementation assay carried out with rate-limiting amounts of *B. subtilis* HPr (20 μM), the phosphoryl carrier activity was lowered to 35, 30, and 20% when Ser46Ala, Ser46Tyr, or Ser46Thr HPr, respectively, was used in place of wild-type HPr (206) and was lowered to 10% when Ser46Asp HPr was used (722). Phosphorylation of HPr at Ser-46 lowered the rate of PEP-dependent phosphorylation by 2 orders of magnitude (177). In addition, the apparent K_m value for the PEP-dependent phosphorylation of P-Ser-HPr was 15-fold higher than the apparent K_m value determined with HPr (723). In *E. coli* HPr, Ser-46 is located within a hydrophobic patch implicated in the interaction with EI (259) and EIIAs (139, 691, 949). The fact that Ser-46 of HPr faces the well-conserved Glu-84 of EI in the HPr:EI complex (260) probably explains why HPrs of gram-positive bacteria carrying a negative charge at Ser-46, due to either phosphorylation or mutation, interact poorly with EI. Vice versa, phosphorylation of HPr by EI at His-15 slowed its phosphorylation at Ser-46 by the ATP-dependent HPr kinase (177). In the HPr:HPr kinase complex, helix α4 of HPr kinase interacts with the region around His-15 (228, 533). Phosphorylation at His-15 probably diminishes the affinity between the kinase and its protein substrate. Indeed, in two-hybrid experiments, an interaction of *B. subtilis* HPr kinase with Ser46Ala mutant HPr could be detected. This interaction was prevented when EI was additionally produced in the yeast cells, suggesting that EI efficiently competes with HprK/P for binding to HPr and/or that EI phosphorylates HPr at His-15, which lowers its affinity for HprK/P (P. Noirot, S. Poncet, and J. Deutscher, unpublished results).

Gram-negative enteric bacteria do not possess HPr kinase, although their HPrs usually contain Ser-46 and exhibit about 45% overall sequence identity to HPrs from gram-positive organisms. Nevertheless, the region around Ser-46 is quite different. This difference is probably responsible for the failure of HPr kinase from gram-positive bacteria to phosphorylate *E. coli* HPr (177, 722). HPrs of *Mycoplasma capricolum*, in which amino acids next to Ser-46 were replaced with the corresponding amino acids of *E. coli* HPr, were poorly phosphorylated with ATP (991). Based on the solution and crystal structures of HPrs from *E. coli*, *E. faecalis*, and *B. subtilis*, the presence of a lysine in position 49 of *E. coli* HPr, which is a glycine in HPrs of most gram-positive bacteria (Fig. 4), was assumed to represent a major obstacle for the interaction of HPr kinase with HPr from *E. coli* and presumably with HPrs from other gram-negative bacteria (374).

Interestingly, several gram-negative nonenteric organisms possess an HPr kinase, and the sequence around Ser-46 of their HPrs resembles that of HPr from low-G+C gram-positive bacteria (64) (Fig. 4). In contrast, *Streptomyces coelicolor*, a gram-positive organism with high G+C content in its DNA, does not possess a protein exhibiting significant sequence similarity to HPr kinase (55), and the sequence around Ser-47 of its HPr barely resembles the sequence around Ser-46 in HPrs of low-G+C gram-positive bacteria. Nevertheless, HPr from *S. coelicolor* can be phosphorylated by the HPr kinase from *B. subtilis* (97, 630), suggesting that despite the sequence differences, the structure around Ser-47 sufficiently resembles the structure around Ser-46 in *B. subtilis* HPr to allow its phosphorylation.

HPr kinase also dephosphorylates P-Ser-HPr by producing PP_i. A soluble enzyme that specifically dephosphorylated P-Ser-HPr was purified to near homogeneity from *E. faecalis* (181). It had a molecular mass of 30 kDa (on sodium dodecyl sulfate-polyacrylamide gels), but it escaped the authors' atten-

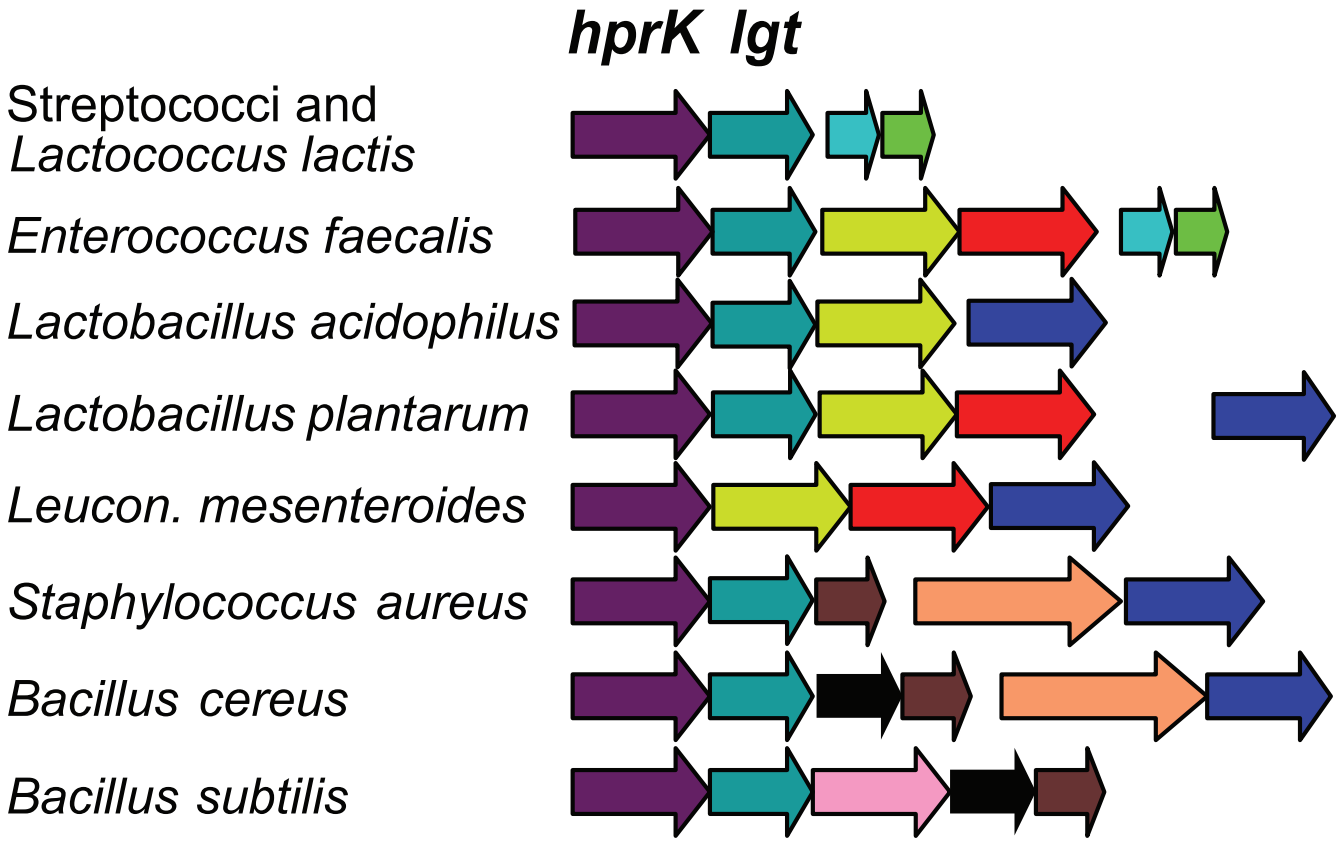

FIG. 5. The gene context of *hprK* in bacteria of the phylum *Firmicutes*. The *hprK* gene is followed by *lgt* in all sequenced genomes of the firmicutes except in *L. mesenteroides*, *Oenococcus oeni*, and some clostridiae. In addition, there are other genes associated with *hprK* that appear to be conserved. They include two genes without a known function (in *L. lactis*, *E. faecalis*, and several streptococci [*S. agalactiae*, *S. mutans*, *S. pneumoniae*, *S. pyogenes*, *S. suis*, *S. thermophilus*, and *S. uberis*]) and a glycerol-3-phosphate dehydrogenase gene and a UTP-glucose-1-phosphate uridylyltransferase gene (in *E. faecalis*) followed by a thioredoxin reductase gene (in *L. acidophilus*, *L. johnsonii*, *L. gasseri* [these species lack the uridylyltransferase], *L. brevis*, *L. plantarum*, *P. pentosaceus*, *L. mesenteroides*, and *O. oeni* [the latter two species lack the *lgt* gene]). Staphylococci contain YvoF, a protein with a hexapeptide transferase motif (*S. aureus*, *S. epidermidis*, *S. haemolyticus*, and *S. saprophyticus*). YvoF, together with YvoE (a pyrophosphatase), is also present in *B. anthracis*, *B. cereus*, *B. thuringiensis*, *Exiguobacterium* species, *L. innocua*, and *L. monocytogenes*, while YvoD, YvoE, and YvoF are found in the bacilli *B. clausii*, *B. halodurans*, *B. licheniformis*, *B. stearothermophilus*, *B. subtilis*, *G. kaustophilus*, and *O. iheyensis*.

tion that the purified enzyme must have also exhibited HPr kinase activity. In fact, it was later discovered that similar to the first described bacterial serine protein kinase, isocitrate dehydrogenase kinase/phosphatase (450), HPr kinase is also bifunctional and possesses P-Ser-HPr dephosphorylation activity (423). HPr kinase of *E. faecalis* has a molecular mass of 33 kDa, which is close to the molecular mass detected for the major protein in the P-Ser-HPr phosphatase preparation. Inorganic phosphate (P_i) was reported to stimulate P-Ser-HPr dephosphorylation (181). This was surprising, as P_i was assumed to be one of the products of P-Ser-HPr dephosphorylation and was therefore rather expected to inhibit this reaction. The paradox was resolved by demonstrating that P-Ser-HPr dephosphorylation is not a hydrolysis reaction. Dephosphorylation of either ^{32}P-Ser-HPr in the presence of P_i or P-Ser-HPr in the presence of $^{32}P_i$ leads to the formation of radioactive $^{32}PP_i$. P_i thus functions as a substrate, and lowering the P_i concentration below the μM range almost completely prevents the dephosphorylation of P-Ser-HPr (557). In analogy to the usual phosphohydrolysis reaction, this novel protein dephosphorylation mechanism was called phospho-phorolysis, and the responsible enzyme was termed HPr kinase/P-Ser-HPr phosphorylase (HprK/P). Because P-Ser-HPr dephosphorylation is reversible, PP_i can replace ATP as the phosphoryl donor for HPr phosphorylation. The kinetic parameters indicate that PP_i-dependent HPr phosphorylation is actually the energetically favored reaction (557). To allow the efficient dephosphorylation of P-Ser-HPr in the cell, the resulting PP_i therefore needs to be hydrolyzed. In *B. subtilis* and other bacilli, PP_i hydrolysis seems to be achieved by YvoE, which exhibits pyrophosphatase activity (557). The *yvoE* gene is located in the *hprK* operon of bacilli (Fig. 5), and since YvoE exhibits sequence similarity to P-glycolate phosphatases and stimulates P-Ser-HPr dephosphorylation, it was mistakenly assumed to be P-Ser-HPr phosphatase (251). It is likely that organisms missing a *yvoE* gene in the *hprK* operon use one of usually several YvoE homologs present in bacteria to hydrolyze PP_i formed during P-Ser-HPr dephosphorylation.

Cold-chase ATP labeling of HPr in *Listeria monocytogenes* crude extracts suggested that HprK/P of this organism cannot dephosphorylate P-Ser-HPr (129). However, later experiments

with purified HprK/P established that *L. monocytogenes* HprK/P functions as P-Ser-HPr phosphorylase, similar to the *B. subtilis* enzyme (326).

Interestingly, although *Mycoplasma pneumoniae hprK* mutant cells cannot form P-Ser-HPr, crude extracts prepared from this mutant were still able to dephosphorylate P-Ser-HPr. The dephosphorylation activity could be attributed to a PP2C-type P-protein phosphatase, which, in accordance with its *B. subtilis* homolog, was called PrpC (306). *M. pneumoniae* HprK/P is unusual, as it can phosphorylate HPr at relatively low ATP concentrations (831). In addition, in vivo P-Ser-HPr formation is stimulated by the presence of glycerol in the growth medium (307). Although the *M. pneumoniae* enzyme was reported to dephosphorylate P-Ser-HPr in vitro in a P_i-dependent manner, similar to HprK/Ps from other organisms (831), the P-Ser-HPr dephosphorylation activity exhibited by PrpC seems to be of physiological relevance. While an *M. pneumoniae* wild-type strain grown in the presence of glucose and glycerol contained only low amounts of P-Ser-HPr and doubly phosphorylated HPr, more than half of the HPr was found to be present as P-Ser-HPr and doubly phosphorylated HPr in the *prpC* mutant (306). Because a *B. subtilis* strain synthesizing Val267Phe HprK/P, which still functions as a kinase but has lost its phosphorylase activity (see "P-Ser-HPr Regulates PTS Transport Activity by a Feedback Mechanism"), contains more than 95% of its HPr as P-Ser-HPr (571), it is unlikely that PrpC of this organism plays a major role in P-Ser-HPr dephosphorylation. In *B. subtilis*, PrpC and the corresponding protein kinase, PrkC, were reported to play important roles in stationary-phase cells and to control the phosphorylation state of the translation elongation factor EF-G (248). It is interesting that HprK/Ps of certain gram-negative bacteria such as *Neisseria meningitidis* (S. Poncet, M.-K. Taha, M. Larribe, and J. Deutscher, unpublished results) and *Brucella melitensis* (S. Poncet, M. Dozot, X. de Bolle, J. J. Letesson, and J. Deutscher, unpublished results) exhibit no or very low P-Ser-HPr dephosphorylation activity, and although these organisms do not contain a homolog of PrpC, it is possible that they possess another P-Ser-HPr phosphatase.

Structure determination of HprK/P. The crystal structures of truncated HprK/P from *L. casei* (missing the N-terminal 120 amino acids) (229) and of full-length HprK/Ps from *Staphylococcus xylosus* (515) and *M. pneumoniae* (13) have been determined. The crystallized proteins form hexamers composed of two layers of trimers. Size exclusion chromatography and equilibrium sedimentation showed that *L. casei* HprK/P also forms hexamers in solution (229). *B. subtilis* HprK/P was reported to form hexamers at a neutral pH but was reported to form monomers and dimers at pH 9.5 (697). HprK/Ps do not exhibit similarity to eukaryotic protein kinases (312, 866) or P-protein phosphatases (43, 805) but resemble PEP carboxykinase and nucleosidediphosphate kinases (229, 252, 748). A P loop (or Walker motif A [GXXGXGKS]) that is usually located between amino acids 150 and 170 serves as a nucleotide binding site. The crystal structures of *L. casei* and *S. xylosus* HprK/Ps revealed that P_i binds to the same position in Walker motif A as the β-phosphate of the nucleotide. This explains the inhibitory effect of P_i on the kinase activity, as ATP and P_i compete for the same binding site. Vice versa, ATP inhibits the phosphorylase activity of HprK/P (181). The inhibitory effect of ATP was stronger at low Mg^{2+} concentrations. Mutant studies with *hprK* from various organisms suggested that Walker motif A is important not only for the kinase but also for the phosphorylase function (315, 571, 831).

The dual role of Walker motif A was confirmed by solving the crystal structures of HprK/Ps complexed with HPr or P-Ser-HPr, which also allowed the prediction of a detailed mechanism for HPr phosphorylation and P-Ser-HPr dephosphorylation (228). His-140 of *L. casei* HprK/P forms a hydrogen bond to Asp-179, which in turn acts as a base during HPr phosphorylation by forming a hydrogen bond to the hydroxyl group of Ser-46 in HPr. The structure of the HprK/P:P-Ser-HPr complex, which was obtained by cocrystallizing HPr, HprK/P, and PP_i, allowed the identification of the amino acids cooperating in the nucleophilic attack of P_i on the P-Ser bond and revealed that the binding of HPr and P-Ser-HPr to HprK/P involves nearly identical interfaces. Compared to the HprK/P:HPr complex, an additional contact is made between the phosphoryl group in P-Ser-HPr and Arg-245 of a neighboring HprK/P subunit (228). The loop containing Arg-245 is too flexible to be traced in the HprK/P and HprK/P:HPr structures, but it is stabilized in the HprK/P:P-Ser-HPr complex due to the interaction of Arg-245 with the phosphoryl group (228, 229). During P-Ser-HPr dephosphorylation, Asp-179 (present in protonated form) and His-140 (donates a proton to Ser-46 of HPr) (228) play an inverse role compared to the kinase reaction. The interaction site of *S. aureus* HPr with *S. xylosus* HprK/P was also determined by NMR, and the HPr interface appears to be similar in solution and in the crystal (533).

The crystal structure of HprK/P from *S. xylosus* revealed that a P_i ion binds not only to Walker motif A but also to the N-terminal domain (515). It was fixed by three conserved arginines located in two different subunits, one in the upper and one in the lower HprK/P trimer. The second P_i therefore bridges the two trimers (515), but the P_i bridge is not essential for hexamer formation, as truncated *L. casei* HprK/P (229) and *M. pneumoniae* HprK/P without bound P_i (13) also form hexamers. The function of the N-terminal domain, which protrudes from the central part like a propeller, is unknown. Truncated *L. casei* HprK/P that is missing this domain is active as a kinase and phosphorylase and responds to all known effectors (229). As will be discussed below, the N-terminal domain is naturally truncated in HprK/Ps of α-proteobacteria. Surprisingly, the N-terminal domain of HprK/P exhibits a fold similar to that of the N-terminal part of MurE (13), an enzyme implicated in the formation of cytoplasmic precursors for cell wall synthesis (284).

The structure of HprK/P with bound ATP or GTP has not yet been solved. If the nucleotide would occupy the same position as that in the closely related adenylate kinase, it would clash with the central K3 loop of a neighboring HprK/P subunit. Structural changes of ATP and/or HprK/P must therefore accompany nucleotide binding (13, 229, 515). Likewise, structural changes of HprK/P might be responsible for the observed cooperative binding of ATP (372).

Organization of the *hprK* operon. About 15 years after the discovery of the ATP-dependent phosphorylation of HPr at Ser-46 (185), at almost the same time, three laboratories succeeded in identifying the HprK/P-encoding *hprK* gene (named *ptsK* in one publication) from three different organisms. In the

first report, purification of *E. faecalis* HprK/P and sequencing of its N terminus and internal peptides allowed the amplification of the 5′ part of the *hprK* gene by PCR. This information was subsequently used to identify *hprK* of *B. subtilis* within the sequenced genome (251) and to obtain the complete *hprK* gene of *E. faecalis* (423). In the second report, purification and N-terminal sequencing of *B. subtilis* HprK/P was used to directly identify *hprK* of *B. subtilis* (709). Finally, purification of HprK/P and probing of a DNA library with a degenerate oligonucleotide derived from the N-terminal amino acid sequence allowed the identification of *hprK* from *Streptococcus salivarius* (84). More recently, the *hprK* genes from other low-G+C gram-positive bacteria including *S. xylosus* (357), *L. casei* (198), *M. pneumoniae* (830), and *G. stearothermophilus* (127) have been cloned and sequenced, and the corresponding HprK/Ps were purified and characterized. Genome sequencing revealed the presence of *hprK* in most low-G+C gram-positive organisms.

In the firmicutes, *hprK* is usually the first gene within an operon (Fig. 5), which is composed of five genes in *B. subtilis*. In most gram-positive bacteria, the second gene is the prolipoprotein diacylglyceryl transferase-encoding *lgt* gene, which carries out the diacylglyceryl lipidation of lipoproteins at a cysteyl residue. The frequent association of *hprK* with *lgt* might indicate that the functions of the two enzymes are somehow related, although no experimental evidence for this assumption has been obtained so far. A possible link between the PTS and Lgt is further suggested by the observation that in *Enterobacteriaceae* (*E. coli*, *S. enterica* serovar Typhimurium, *Yersinia pestis*, etc.), *lgt* is preceded by *ptsP* (T. Doan, personal communication), which encodes an EI with an N-terminal extension resembling the GAF domain in NifA. The *hprK* operon of *G. stearothermophilus*, *Oceanobacillus iheyensis*, *L. monocytogenes*, *Listeria innocua*, and the seven bacilli, whose genomes have been sequenced, contains the pyrophosphatase-encoding *yvoE* gene followed by *yvoF* downstream of *lgt*. *B. subtilis*, *Bacillus clausii*, *Bacillus halodurans*, *Bacillus licheniformis*, and *G. stearothermophilus* contain an additional gene, *yvoD*, inserted between *lgt* and *yvoE*. In *S. aureus* and *Staphylococcus epidermidis*, *yvoE* is missing, and *yvoF* directly follows *lgt*. The *yvoDEF* genes are absent from most other low-G+C gram-positive organisms. *O. iheyensis* possesses two genes encoding HprK/Ps, with one followed by *lgt*. It is not known whether the HprK/P ortholog encoded by the other *hprK* gene can phosphorylate HPr. In the genus *Clostridium*, *hprK* and *lgt* are located in different regions of the genome. In *Clostridium acetobutylicum*, *hprK* is preceded by a gene encoding a protein with significant sequence similarity to the glycerol-inducible *E. coli* GlpX, which resembles fructose-1,6-bisphosphatases (859).

Quite unique is a presumed *hprK* gene in *Fusobacterium nucleatum* ATCC 25586 and *F. nucleatum* subsp. *vincentii*, which encodes two complete HprK/P proteins fused together in tandem. The two HprK/P domains exhibit 22% sequence identity to each other and about 30% identity to HprK/Ps from gram-positive bacteria. However, a conserved Walker motif A is present only in the C-terminal domain. The corresponding sequence in the N-terminal domain is strongly altered and does not resemble Walker motif A. A prerequisite for HPr phosphorylation is the deprotonation of the hydroxyl group of Ser-46 of HPr by the concerted action of conserved histidyl and aspartyl residues (His-140 and Asp-179 in *L. casei* HprK/P). In *F. nucleatum* HprK/P, these residues are present in the C-terminal domain but are replaced with Glu and Lys, respectively, in the N-terminal domain. In contrast, Arg-245, which interacts with the phosphoryl group of P-Ser-HPr and probably plays a major role in P-Ser-HPr dephosphorylation, is present only in the N-terminal domain. It is therefore tempting to assume that in this duplicated enzyme, the two activities have been separated and that the C-terminal domain carries the kinase activity, whereas the N-terminal domain might function as P-Ser-HPr phosphorylase. The two sequenced *F. nucleatum* strains also contain HPr (FN1782) with conserved His-15 and Ser-46 regions and EI (FN1793), but although the genes are located close to each other on the chromosome, they do not seem to be organized in an operon.

The organization of the *hprK* gene in certain gram-negative bacteria will be discussed below in more detail.

Metabolites regulate the antagonistic activities of HprK/P. In vitro experiments suggested that the two antagonistic activities associated with HprK/P are regulated in the cell in response to changes in the concentrations of FBP, ATP, and P_i (177). FBP clearly stimulates the kinase activity of *B. subtilis* HprK/P (372), whereas its effect is less pronounced with the *L. casei* enzyme (198). The effect of FBP is usually stronger when low, nonphysiological concentrations of ATP are used. In addition, the stimulatory effect of FBP on HPr phosphorylation was more evident when the experiments were carried out in the presence of a few mM P_i (177, 198, 423), which lowers the HPr kinase activity (84, 181, 251). The main physiological function of FBP might therefore be to prevent the inhibition of HprK/P by P_i. For the *S. xylosus* enzyme, activation by FBP was observed only when HprK/P was present at low concentrations (357). Surprisingly, the *Streptococcus salivarius* enzyme was not at all stimulated by FBP (84). Similarly, the *M. pneumoniae* enzyme is fully functional at relatively low ATP concentrations, and its activity is also not stimulated by FBP (831). The intracellular concentrations of FBP, ATP, and P_i vary largely depending on whether the cells utilize a favored carbon source or not. During the uptake of a rapidly metabolizable carbon source such as glucose, fructose, or mannose by *L. lactis*, the concentrations of P_i, ATP, and FBP are about 5, 8, and 30 mM, respectively (528, 598, 873). Under these conditions, HprK/P functions as an HPr kinase and not as P-Ser-HPr phosphorylase. In contrast, in the absence of a rapidly metabolizable carbon source, the concentration of P_i increases to about 50 mM, whereas the concentrations of FBP and ATP drop to 1 to 3 mM (528, 598, 873). The effect of growth on a preferred or less favorable carbon source on P-Ser-HPr formation was confirmed with *S. salivarius* cells. By carrying out crossed immunoelectrophoresis experiments, the fractions of the various forms of intracellular HPr in *S. salivarius* cells grown in glucose-containing medium were found to be 45% for P-Ser-HPr and 50% for doubly phosphorylated HPr, whereas only little P~His-HPr (5%) and almost no dephospho-HPr could be detected (263). Cells grown on galactose, a less efficiently metabolized carbon source, also exhibited a high amount of doubly phosphorylated HPr (44%) and little P~His-HPr (3%), whereas P-Ser-HPr was lowered to 18%, and dephospho-HPr increased to 35%. A similar observation was made for *Strep-*

tococcus bovis, where an inverse correlation between P-Ser-HPr formation and the P_i concentration was observed. Cells growing on glucose contained more than half of the HPr as P-Ser-HPr, and the P_i concentration was in the low mM range. When glucose was exhausted, P-Ser-HPr disappeared, and the P_i concentration went up to 30 mM (26). In in vitro experiments, 20 mM P_i completely prevents the phosphorylation of HPr by 5 mM ATP, even when 25 mM FBP is present (177).

The observation that PP_i is formed during P-Ser-HPr dephosphorylation and used as a phosphoryl donor by HprK/P suggested that the PP_i concentration affects the intracellular amount of P-Ser-HPr. *B. subtilis* cells grown in the presence of glucose contain 10-fold more FBP than cells grown on succinate (from 1.4 to 14 mM). A similar increase was observed for PP_i (from 1.2 to 6 mM) (557). This metabolite distribution is probably the reason why about 65% of the HPr in glucose-grown *B. subtilis* cells is present as P-Ser-HPr, 35% is present as HPr, and little is present as P~His-HPr and doubly phosphorylated HPr (571). In contrast, in succinate-grown cells, mainly dephospho-HPr was present, together with a few percent of P~His-HPr. In in vitro experiments, HPr phosphorylation with *B. subtilis* HprK/P was maximal above 5 mM PP_i but negligible below 1 mM PP_i. The approximately 6 mM PP_i present in cells growing on glucose is therefore expected to be partly responsible for the increase of P-Ser-HPr, whereas in the absence of a rapidly metabolizable carbon source, the low PP_i and high P_i concentrations probably allow efficient P-Ser-HPr dephosphorylation. ATP-dependent HPr phosphorylation by *B. subtilis* HprK/P was maximal in the presence of 10 mM FBP, whereas it almost disappeared below 2 mM FBP (372). On the contrary, FBP was not required for efficient PP_i-dependent HPr phosphorylation (557).

An endogenous low-molecular-weight HprK/P inhibitor was detected when soluble extracts of *Lactobacillus brevis* were separated by size exclusion chromatography (715). However, no further details about the nature of the bacterial HPr kinase inhibitor were reported. Because the HPr kinase activity was detected by autoradiography after phosphorylation with [γ-^{32}P]ATP, it is possible that the reported HPr kinase inhibitor was PP_i. If the PP_i concentration in the assay mixtures exceeded the employed [γ-^{32}P]ATP concentration (10 μM) by severalfold, primarily nonradioactive (and therefore undetected) P-Ser-HPr would have been formed. Ironically, in the same publication, it was reported that the presence of PP_i can completely inhibit [γ-^{32}P]ATP-dependent HPr phosphorylation (715). Inhibition of [γ-^{32}P]ATP-dependent HPr phosphorylation by PP_i was also reported for HprK/P from *Treponema denticola* and was suggested to be probably due to the competition of the two phosphoryl donors for binding to HprK/P, as P-Ser-HPr was again detected by autoradiography (278). A synthetic inhibitor of HprK/P has also been identified. It is a heterocyclic bis-cationic compound, which was reported not to bind to the Walker motif (696).

In conclusion, the two opposing activities of HprK/P and consequently the ratio of the various HPr forms seem to be regulated mainly via metabolites. In agreement with this assumption, the amount of HprK/P present in *B. subtilis* cells was reported to have no influence on the ratio of the different HPr forms (59). The amount of HprK/P probably determines only how fast cells can adapt to changes in carbohydrate availability but apparently has no influence on the equilibrium reached under a certain condition.

Interestingly, in oral streptococci (glucose or lactose grown) (660, 893) and *S. thermophilus* (lactose grown) (134), a major part (up to 75%) of the HPr is converted into doubly phosphorylated HPr. A possible role of (P~His,P-Ser)-HPr in the regulation of the lactose transporter LacS of *S. thermophilus* will be discussed below (see "Regulation of EIIA-Containing Non-PTS Transporters by P~His-HPr-Mediated Phosphorylation").

P-Ser-HPr Regulates PTS Transport Activity by a Feedback Mechanism

P-Ser-HPr is a poor substrate for PEP-dependent phosphorylation by EI, although phosphorylation at Ser-46 causes only minor changes in the HPr structure (29, 952). One of these changes leads to an extension of the short β-helix, which is capped by Ser-46 at the N-terminal site. This alteration was assumed to affect the interaction of Met-48 with a hydrophobic pocket in EI, thus partly explaining why phosphorylation at Ser-46 drastically slows the EI-catalyzed phosphorylation at His-15. However, the main reason for the poor phosphorylation of His-15 in P-Ser-HPr is probably an electrostatic repulsion between the phosphoryl group at Ser-46 and residue Glu-84 of EI (260). As discussed in the preceding section, in glucose-grown cells, a major fraction of HPr is present as P-Ser-HPr or doubly phosphorylated HPr, which, compared to P~His-HPr, has a much lower affinity for EIIAs (723), but there is little P~His-HPr.

If P~His-HPr is present at low concentrations, it is expected to control the PTS sugar uptake rate. The efficient phosphorylation of HPr at Ser-46 during growth on glucose might thus reduce PTS-catalyzed sugar uptake. A *ptsH1* mutant (Ser46Ala replacement in HPr) was therefore expected to show deregulated PTS transport activity. However, a *B. subtilis ptsH1* mutant did not exhibit elevated uptake of glucose or other rapidly metabolizable PTS sugars (184), indicating either that additional regulatory mechanisms are operative or that in *B. subtilis*, the small amount of P~His-HPr detected in cells growing on glucose is sufficient to fully catalyze sugar transport via the PTS. In contrast, an *S. xylosus hprK* mutant, also unable to phosphorylate HPr at Ser-46, exhibited reduced growth in the presence of glucose, although glucose-grown *hprK* mutant cells showed a two- to fourfold-elevated initial glucose transport rate compared to the wild-type strain. It was therefore concluded that the growth deficiency of the *S. xylosus hprK* mutant is probably due to uncontrolled glucose uptake leading to the accumulation of deleterious amounts of metabolites (357).

In various gram-positive bacteria, the presence of glucose exerts an inhibitory effect on the uptake of other PTS sugars. This inhibitory effect could be due partly to the low amounts of P~His-HPr detected in glucose-grown cells, which probably aggravates the competition between the EIIAs for their common phosphoryl donor. Competition was expected to be less severe in a *ptsH1* mutant that is unable to form P-Ser-HPr. The effect of the *ptsH1* mutation on the simultaneous uptake of the two PTS substrates mannitol and glucose was measured with *B. subtilis* cells. If phosphorylation at Ser-46 of HPr and competition for the remaining P~His-HPr were involved in glucose-

mediated inhibition of mannitol uptake, elevated mannitol transport should occur in the *ptsH1* mutant. Indeed, mannitol uptake in the presence of glucose was two- to fivefold higher in the *ptsH1* mutant than in the wild-type strain (184, 974).

Evidence for a participation of P-Ser-HPr in the regulation of PTS transport activity was also provided by experiments with *L. lactis* vesicles. During the preparation of *L. lactis* vesicles, larger proteins such as HprK/P and EI remain entrapped in the vesicles, whereas almost all HPr is lost (970). An influence of the ATP-dependent phosphorylation of HPr at Ser-46 on PTS-catalyzed sugar transport could be demonstrated by 2DG uptake measurements using *L. lactis* vesicles into which *B. subtilis* HPr and various glycolytic intermediates had been electroporated (971). 2DG is transported via a PTS, and electroporation of HPr or coelectroporation of Ser46Ala mutant HPr with ATP and FBP into the vesicles stimulated 2DG uptake. In contrast, coelectroporation of wild-type HPr with ATP and FBP had no stimulatory effect. In addition, only slow 2DG uptake was detected with the P-Ser-HPr-resembling Ser46Asp mutant HPr (952), irrespective of whether it was electroporated alone or together with ATP and FBP. Similar results were obtained when the uptake of methyl-β-D-thiogalactoside (TMG) by *L. lactis* vesicles was studied (970). In addition to FBP, several other metabolites including gluconate-6-P, glycerate-2-P, and fructose-6-P were reported to inhibit TMG uptake when electroporated into *L. lactis* vesicles together with *B. subtilis* HPr. Moreover, when wild-type HPr was electroporated into *L. lactis* vesicles, the uptake of fructose was inhibited by the presence of glucose, while only slight inhibition occurred when Ser46Ala HPr was electroporated in place of HPr (974).

Although providing no direct proof, these in vitro results suggested that the formation of P-Ser-HPr during the utilization of a rapidly metabolizable carbon source can lower the amount of P~His-HPr to such an extent that it is insufficient to sustain the simultaneous uptake of two PTS carbohydrates. This hypothesis was supported by in vivo experiments using the *hprK*(*Val265Phe*) allele. The Val265Phe replacement causes an almost complete loss of the P-Ser-HPr phosphorylase activity but has little effect on the FBP-activated HPr kinase function (571). When grown in the absence of a rapidly metabolizable carbohydrate, a *B. subtilis* wild-type strain contains little P-Ser-HPr, whereas in strains carrying the Val265Phe *hprK* allele, almost all HPr is accumulated as P-Ser-HPr. The Val265Phe *hprK* strain grows barely or not at all on media containing the PTS sugar glucose, fructose, maltose, or mannitol as the sole carbon source. Similar results were obtained with a *B. subtilis* strain in which the *hprK* gene had been replaced with the *L. casei* Val267Phe *hprK* allele (571). A strain carrying the Val267Phe *hprK* allele transports glucose and mannitol at rate that is more than 10 fold lower than that of an integrant expressing wild-type *L. casei hprK* (571). The inability of *B. subtilis* strains expressing the *B. subtilis* Val265Phe or the *L. casei* Val267Phe *hprK* allele to grow on PTS sugars is due to strongly reduced PTS transport activity and, as will be explained below, to a kind of "permanent CCR." Expression of the $EIICBA^{Glc}$-encoding *ptsG*, which is not submitted to CCR, was not repressed by the presence of the *L. casei* Val267Phe *hprK* allele. The assumption that the accumulation of P-Ser-HPr in the Val267Phe *hprK* strain results in slow PTS phosphoryl group transfer, which in turn prevents PTS sugar uptake, was confirmed by introducing a *ptsH1* mutation in the Val267Phe *hprK* strain (no P-Ser-HPr formation). It restored growth on PTS sugars, whereas introducing a *ccpA* mutation, which prevents CCR, had only a slight effect on PTS transport (571).

As mentioned above, glucose-grown *S. salivarius* (263, 893) and *B. subtilis* (571) cells contain very little P~His-HPr. Nevertheless, the small fraction present as P~His-HPr (about 5%) seems to be sufficient to fully catalyze PTS-mediated uptake of glucose and probably other rapidly metabolizable PTS sugars in gram-positive bacteria. Similarly, less than 20% of the HPr present in wild-type *S. enterica* serovar Typhimurium cells can fully sustain PTS-catalyzed glucose uptake (901). However, P~His-HPr probably starts exerting flux control when two or more PTS sugars are simultaneously transported or when the natural balance of the different forms of HPr is disturbed by, for example, the conversion of an additional fraction of HPr to P-Ser-HPr, as is the case in *B. subtilis* strains expressing the Val265Phe *hprK* allele (571).

Role of P-Ser-HPr in CCR

The following sections will deal with the role of P-Ser-HPr and P-Ser-Crh in CCR/carbon catabolite activation (CCA). We will discuss some early results that suggested a connection between CCR and P-Ser-HPr formation and the first confirmation of this connection by construction of a *B. subtilis ptsH*(*Ser46Ala*) mutant. The *ptsH*(*Ser46Ala*) mutation has a pleiotropic effect and leads to a relief from CCR for numerous genes and operons. We will mention the interaction of P-Ser-HPr and P-Ser-Crh with the catabolite repressor/activator CcpA (Table 1) and how these interactions allow CcpA, a LacI/GalR-type DNA binding protein, to interact with the catabolite response elements (*cre*'s), operator sites which precede genes that are sensitive to CCR/CCA. The occurrence of Crh, an HPr paralog in which His-15 is replaced with a Gln, in bacilli, oceanobacilli, and geobacilli will also be discussed.

Loss of ATP-dependent HPr phosphorylation prevents CCR. Heterofermentative lactobacilli such as *L. brevis* and *Lactobacillus buchneri* were reported to contain HPr and HprK/P but to be devoid of EI and EIIs (715). Owing to the presumed absence of a functional PTS, there seemed to be no obvious regulatory role for the ATP-dependent phosphorylation of HPr at Ser-46 in these organisms. Although the report about the absence of EI and EIIs from heterofermentative lactobacilli later turned out to be wrong (an *L. brevis ptsHI* operon has been cloned and sequenced) (193) and the presence of a fructose-specific PTS induced under anaerobic conditions has been established (772), it initiated the search for additional functions of P-Ser-HPr, one of them being the regulation of CCR.

A connection between the phosphorylation of HPr at Ser-46 and CCR was suggested by the observation that CCR in *B. subtilis* requires the metabolism of the repressing sugar (490, 606). In gram-negative bacteria, the transport of a carbohydrate via the PTS is sufficient to elicit CCR. By studying CCR in mutants defective at various steps of glycolysis, the number of glycolytic intermediates potentially serving as a signal for CCR in *B. subtilis* could be narrowed to four candidates: dihydroxyacetone phosphate, glyceraldehyde-3-

phosphate, 1,3-diphosphoglycerate, and FBP (606). As FBP, the main activator of HPr kinase, was among these metabolites, ATP-dependent phosphorylation of HPr was suspected to play a role in CCR (184).

The *ptsH1* mutation has a pleiotropic effect on CCR and carbon catabolite activation. To test the proposed hypothesis that P-Ser-HPr plays a role in CCR, Deutscher and coworkers studied CCR in a *B. subtilis ptsH1* mutant (Ser46Ala replacement). Indeed, several enzymes, including gluconate kinase, mannitol-1-P dehydrogenase, inositol dehydrogenase, and glucitol dehydrogenase, which are all sensitive to CCR in a wild-type strain, were not or only partly repressed by glucose and other rapidly metabolizable sugars in the *ptsH1* mutant (184). Moreover, while the expression of a translational fusion (integrated at the *amyE* locus) of the promoter region and the 5′ part of *gntK* (encodes gluconate kinase) to *lacZ* was sensitive to CCR in a wild-type strain, it was not repressed by glucose in the *ptsH1* mutant (184). In addition, if a *B. subtilis* strain carrying a deletion of the *ptsGHI* genes, which encode $EIICBA^{Glc}$, HPr, and EI, respectively, was transformed with a plasmid that allowed the production of the P-Ser-HPr-resembling Ser46Asp HPr (952), partial repression of the *gnt* operon was observed even in the absence of a repressing sugar (184). These results clearly established the crucial role of the phosphorylation of HPr at Ser-46 in CCR of *B. subtilis*.

The *ptsH1* mutation turned out to be an important tool to study the implication of P-Ser-HPr in CCR in *B. subtilis* in more detail (184, 206). All *B. subtilis ptsH1* mutants used in various laboratories are derivatives of *ptsH1* strain SA003. They were obtained by either introducing additional mutations into SA003 or transforming specific mutants with chromosomal DNA of *ptsH1* strains carrying an antibiotic resistance cassette inserted about 6 kb downstream from *ptsH* (184). The *ptsH*-linked antibiotic resistance cassette was recovered from strain MO481 (P. Stragier, unpublished results). The use of the *ptsH1* mutant demonstrated that many other *B. subtilis* genes and operons are partly or completely relieved from CCR, such as the acetyl coenzyme A (acetyl-CoA) synthetase-encoding *acsA* gene (984) as well as the *lev* (523, 526), *xyl* (148), *xyn* (249, 250), and *glp* (158) operons (for a review, see reference 178). In a few cases, the effect of the *ptsH1* mutation on CCR depends on the growth conditions. CCR of the *B. subtilis hut* operon, for example, is not altered in a *ptsH1* mutant grown in minimal medium, whereas CCR in this strain completely disappears when it is grown in complex medium (985). A similar but less pronounced growth medium-dependent effect on CCR was observed with the *iol* operon (184). The mechanisms underlying the growth-dependent differences in CCR in *ptsH1* mutants are not understood but might be related to the different nitrogen sources present in the media (220).

Synthesis of the plasmid-encoded *B. thuringiensis* larvicidal protoxin Cry4A by stationary-phase cells is also sensitive to CCR. The repressive effect of glucose on Cry4A production disappeared in a *ptsH1* mutant (Ser45Ala replacement in *B. thuringiensis* HPr) (399). In *L. casei*, the *ptsH1* mutation causes a relief from CCR of 6-P-β-galactosidase and *N*-acetyl glucosaminidase and from diauxic growth in media containing glucose and either lactose, maltose, or ribose (919). An *L. lactis ptsH1* mutant is relieved from CCR of the *ptbA-bglH* operon (569). These results confirm that P-Ser-HPr plays an important role in CCR of not only bacilli but also other gram-positive organisms.

A chromosomal *B. subtilis ptsH2* mutant (Ser46Thr replacement) has also been constructed. Although the purified mutant HPr is slowly phosphorylated in ATP-dependent phosphorylation assays (722), the *B. subtilis ptsH2* mutant exhibits a relief from CCR of the *gnt* operon identical to that of the *ptsH1* mutant (M. Steinmetz and J. Deutscher, unpublished results). In contrast, an *L. casei ptsH2* mutant shows only partial relief from CCR and diauxic growth (919). It is therefore likely that Ser46Thr HPr from *L. casei* is more efficiently phosphorylated than the *B. subtilis* mutant protein.

Some genes and operons are more strongly expressed in the presence of glucose or other rapidly metabolizable carbohydrates. This phenomenon is known as CCA. In *B. subtilis*, the expression of several genes such as *alsS* (α-acetolactate synthase) (726, 983), *ackA* (acetate kinase) (886), and *pta* (phosphotransacetylase) (685) is stimulated by the presence of glucose. CCA of these *B. subtilis* genes is absent from *ptsH1* mutants. Expression of the *L. lactis las* operon containing the genes encoding the glycolytic enzymes phosphofructokinase, pyruvate kinase, and lactate dehydrogenase is also activated by the presence of glucose in the growth medium. The stimulatory effect of glucose on *las* operon expression disappeared in an *L. lactis ptsH*-disrupted strain transformed with a plasmid allowing the synthesis of *L. lactis* Ser46Ala mutant HPr (493), whereas transformants containing a plasmid carrying *L. lactis* wild-type *ptsH* exhibited normal glucose-mediated *las* activation.

The involvement of P-Ser-HPr in CCR and CCA was further supported by studying mutants that were unable to form P-Ser-HPr due to *hprK* inactivation. Such mutants have been obtained, for example, with *B. subtilis* (251, 315, 709), *S. xylosus* (357), and *L. casei* (198). In all three organisms, the inactivation of *hprK* causes a relief from CCR, but for several *B. subtilis* catabolic operons, the releasing effect in *hprK* mutants is markedly stronger than that in *ptsH1* mutants (249, 523, 685). This phenomenon was observed for many other genes when transcriptome analysis was carried out with *B. subtilis hprK* and *ptsH1* mutants (491). As will be explained below, the difference between *ptsH1* and *hprK* mutants is due to the presence of Crh, a *B. subtilis* HPr paralog, which is also phosphorylated by HprK/P (250) and which can partly substitute for P-Ser-HPr in CCR and CCA.

The Catabolite Response Element *cre*, an Operator Site for CCR and CCA

***A cis*-acting element regulating CCR and CCA.** Groundbreaking work in the laboratory of Glenn Chambliss led to the discovery of two other components involved in CCR in *B. subtilis*. Both were identified by isolating mutants in which the α-amylase-encoding *amyE* gene was relieved from CCR. Two independently isolated mutants were affected in a 14-bp imperfect palindromic sequence called *amyR1*, which is located just downstream from the *amyE* promoter and which exhibits similarity to the *lac* operator site of *E. coli* (601, 603). In both strains, replacement of the G:C base pair in position 8 of

amyR1 with an A:T base pair was responsible for the observed CCR resistance (603, 939). Expression of a plasmid-borne *cat* gene under the control of the *amyE* promoter followed by mutated *amyR1* was insensitive to CCR, while the simultaneous expression of the chromosomal *amyE* (promoter followed by wild-type *amyR1*) was repressed by glucose (602). These findings suggested that *amyR1* is a *cis*-acting element. A series of mutants in which one or two positions of *amyR1* were altered was constructed, and most of these mutants were partly or fully relieved from CCR of *amyE*. Based on mutational analysis and on the occurrence of similar imperfect palindromic sequences in the promoter regions of many other catabolite-repressed *B. subtilis* genes, the following consensus sequence was proposed for the catabolite response operator: TGWNANCGNTNWCA (939). Slightly different consensus sequences have been proposed by others (353, 563). The general term catabolite response element (*cre*) has been introduced for these operator sites (421).

Distribution of *cre*'s. Imperfect palindromic sequences resembling the proposed consensus *cre* sequence are located in the promoter region or at the 5′ ends of many catabolite-repressed transcription units. For some of these *cre*'s, their implication in CCR or CCA has been experimentally confirmed (178). By searching the *B. subtilis* genome with a degenerate *cre* sequence as a query, Miwa and coworkers detected a total of 126 tentative *cre*'s (565). Out of these 126 putative *cre* sites, 32 were inserted into a plasmid between the *spac* promoter and the *lacZ* gene, and the effect of these sequences on the glucose repression of β-galactosidase synthesis was tested. In 22 constructs, expression of the *lacZ* gene was repressed by glucose (565). By using a slightly different search method, 61 additional potential *B. subtilis cis* elements for CCR could be detected, including several *cre*'s preceding operons known to be submitted to CCR. A list of the about 160 identified or presumed *B. subtilis cre*'s was presented by Deutscher et al. (178). As the transcription units regulated by these *cre*'s contain, on average, two to three cistrons, about 400 genes, which represent 10% of the *B. subtilis* genome, are estimated to be regulated by CCR or CCA. A similar number was obtained when transcriptome analyses were carried out using a *ccpA* mutant (see below for *ccpA*) (575, 980). The number of genes presumed to be regulated by cAMP/Crp-mediated CCR in *E. coli* is in the same order (34).

A unique potential *cre* precedes the *B. subtilis uxaC* gene from the *yjm* operon, which codes for the enzymes necessary for glucuronide/galacturonate transport and metabolism (549, 729). While most *cre*'s are composed of an imperfect palindromic sequence, the putative *cre* of the *yjm* operon is a perfect palindrome. Moreover, the 14-bp-long putative *yjm cre* is part of a longer perfect palindrome composed of a total of 26 bp (TCAAAA**TGTTAACGTTAACA**TTTTGA [the 14-bp consensus *cre* is in boldface type]). To date, it is not known whether this extended palindrome plays a special role in CCR. The β-galactosidase-encoding *mbgA* gene of *Bacillus megaterium* contains two *cre*'s overlapping by 1 bp. Mutations in either one affect CCR of the *mbgA* gene (803). Two potential *cre*'s overlapping to a much larger extent (by 4 bp) begin at positions 278 and 287 within the *B. subtilis gudB* (former *ypcA*) gene, which encodes one of two glutamate dehydrogenases present in this organism and the expression of which is sensitive to the presence of glucose. The second *B. subtilis* glutamate dehydrogenase, RocG, is also submitted to CCR (53). The first 8 bp in the two presumed *gudB cre*'s are identical (**GTGAAGGCG*gtgaa*gg** *cgCTTTCA* [the first potential *cre* is in boldface type, the second is in italics, and the repeated sequences are underlined and in lowercase letters]). Interestingly, a *rocG* mutant is not able to utilize proline, ornithine, or arginine as the sole carbon source (growth on these carbon sources requires an active glutamate dehydrogenase), establishing that wild-type *B. subtilis* contains only low GudB activity. However, spontaneous revertants that were able to grow on these compounds appeared with high frequency. Surprisingly, in four independently isolated revertants, the GTGAAGGCG direct repeat was deleted, providing a *gudB1* allele with only a single *cre* (TGAAGGCGCTTTCA) (54). It is not clear whether the elevated GudB activity in the spontaneous mutants is due to the change in the two overlapping *cre*'s or to the deletion of the -V-K-A- repeat in the protein. The *B. subtilis iol* (563) and *ara* (361) operons also contain two functional *cre*'s, which, however, are not located next to each other. One *cre* precedes the first gene (*mmsA* and *araA*, respectively); the other is located within the second gene (*iolB* and *araB*). Several other catabolite-repressed or -activated genes or operons contain more than one potential *cre* in the promoter region or within the 5′ end (93, 290, 564, 886), but in several cases, only one of the *cre*'s is operative in CCR or CCA.

Two divergently transcribed genes or operons can potentially be regulated by a single *cre*. This seems to be the case for the putative *L. casei lev cre*, which overlaps the transcription start site of the *levABCDX* operon and the −10 box of the *levR* gene, which encodes the transcription activator for the *lev* operon. Simultaneous repression of *levABCDX* and the transcription activator gene *levR* presumably leads to very efficient CCR (534).

The *L. casei lev* operon is only one of many examples establishing the presence of functional *cre* sites in bacteria other than *B. subtilis* (Table 3). In *S. xylosus*, deletion of the *cre* located in front of the *malRA* operon (for maltose/maltotriose utilization) causes a complete relief from CCR for α-glucosidase (204). Similarly, deletion of a *cre* located right behind the transcription initiation site of the fructan hydrolase-encoding *fruA* gene of *Streptococcus mutans* causes a relief from glucose repression (93, 946). CCR of the β-amylase-encoding *bamM* gene from *B. megaterium* is mediated by a *cre* located in the DNA region encoding the signal peptide of this extracellular enzyme (457). A *cre* site is also present in front of the P-β-glucosidase-encoding *bglH* gene of *Lactobacillus plantarum* (511, 512) and the *pepQ* gene of *Lactobacillus delbrueckii* (779). These examples confirm that the *cre*-dependent CCR/CCA mechanism is widely distributed within gram-positive bacteria with low G+C content. Interestingly, the HPr-encoding *ptsH* gene of several streptococci is also preceded by a potential *cre* site, and expression of the *ptsHI* operon in *S. salivarius* and *Streptococcus bovis* is up-regulated in the presence of glucose (CCA) (894).

cre's can either overlap the promoter, overlap the transcription start site, or be located between the transcription start site and the initiation codon or within the 5′ part of catabolite-repressed genes (Table 3). Binding of a target protein to *cre* sites located within the promoter region is thought to interfere with the binding of the RNA polymerase holoenzyme, while *cre*'s situated downstream from the promoter are assumed to

TABLE 3. Genes and operons in gram-positive bacteria[a] containing functional *cre*'s or being regulated by the P-Ser-HPr:CcpA complex

Organism	Gene/operon	Function	*cre* sequence[b]	CcpA effect[c]	Reference(s)
B. megaterium	*bamM*	β-Amylase	+48TGTTAACGCTTTCA+61	−	457
B. megaterium	*mbgA*	β-Galactosidase	−34TGATAAGGTTTTCA−21	−	803
B. megaterium	*mbgA*	β-Galactosidase	−21AGAAAACGTTATCA−8	−	803
B. megaterium	*xyl*	Xylose metabolism	+124TGAAAGCGCAAACA+137	−	290
B. thuringiensis	*cry4A*	Cry4A toxin	NI	−	399
C. perfringens	*cpe*	Enterotoxin	NI	+	913
C. saccharobutylicum	*staA*	Starch degradation	NI	−	162
E. faecalis	*bkd*	β-Keto acid metabolism	NI	−	933
E. faecalis	*galKETR*	Galactose metabolism	NI	−	453
E. faecalis	*gdh dhaK*	Glycerol dehydrogenase/dihydroxyacetone kinase	NI	−	453
E. faecalis	?	Glucose starvation-induced proteins	NI	−	453
E. faecalis	*ldh*	Lactate dehydrogenase	NI	−	453
E. faecalis	*pfk*	Phosphofructo kinase	NI	−	453
E. faecalis	*pyk*	Pyruvate kinase	NI	−	453
E. faecalis	?	Serine dehydratase	NI	−	453
L. casei	*nag*	*N*-Acetylglucosaminidase	NI	−	568, 796
L. casei	*lac*	Lactose metabolism	NI	−	568
L. delbrueckii subsp. *bulgaricus*	*ccpA*	Catabolism repression	NI	−	574
L. delbrueckii subsp. *lactis*	*pepQ*	Peptidase	−61TGCAATCGCTTACA−48	+	799
L. lactis	*las*	Glycolysis	NI	+	494
L. lactis	*gal*	Galactose metabolism	NI	−	494
L. lactis	*ptbA bglH*	β-Glucoside metabolism	NI	−	569
L. lactis	*fruRCA*	Fructose metabolism	NI	−	45
L. monocytogenes	?	β-Glucosidase	NI	−	50
L. pentosus	*xpkA*	Xylulose-5-P phosphoketolase	NI	−	674
L. pentosus	*xylAB*	Xylose metabolism	NI	−	489
L. plantarum	*bglH*	P-β-glucosidase	−3TGTAAGGGCTATCA+11	−	511, 512
S. aureus	*pckA*	PEP carboxy kinase	NI	−	791
S. bovis	*ccpA*	CcpA	−68TGAAAAGGTTTTCA-54	+	27
S. bovis	*ldh*	Lactate dehydrogenase	NI	−	27
S. bovis	*pfl*	Pyruvate formate lyase	NI	+	27
S. gordonii	*abpA*	Amylase binding	NI	−	733
S. gordonii	*arcA*	Arginine deiminase	NI	−	195
S. mutans	*fruA*	Fructan hydrolase	−164AGATAGCGCTTACA−150	−	93, 946
S. mutans	*ftf*	Fructosyltransferase	NI	+	88
S. mutans	*gtfBC*	Glucosyltransferase	NI	+	88
S. pneumoniae	*cps*	Capsular polysaccharide	NI	+	273
S. rattus	*arcA*	Arginine deiminase	NI	−	298
S. thermophilus	*lacSZ*	Lactose metabolism	NI	−	897
S. thermophilus	*ldh*	Lactate dehydrogenase	NI	−	897
S. xylosus	?	β-Glucoronide	NI	−	204
S. xylosus	*ccpA*	Catabolic repressor	NI	−	204
S. xylosus	*lacPH*	Lactose metabolism	NI	−	46
S. xylosus	*mal*	Maltose metabolism	−58TGCAAACGCTTGCA−45	−	204
Consensus sequence			TGWNANCGNTNWCA		939

[a] Except *B. subtilis* (for this organism, see references 178, 575, and 980).
[b] The numbers indicate the position with respect to the transcription initiation site. NI means not experimentally identified.
[c] CcpA acts either as catabolite activator (+) or as catabolite repressor (−).

be regulated via a road block. As will be discussed below, most transcription units submitted to CCA contain a *cre* located in front of the promoter region.

Catabolite Control Protein A Functions as a Catabolite Repressor or Activator

A LacI/GalR-type *trans*-acting factor mediates CCR and CCA. Since *cre*'s exhibit similarity to the *E. coli lac* and *gal* operators, it was tempting to assume that a LacI/GalR-type repressor might be involved in CCR of *B. subtilis* and other gram-positive bacteria (603). By carrying out transposon mutagenesis, a *B. subtilis* strain in which *amyE* expression was resistant to catabolite repression could be isolated (322). Indeed, this strain carried the transposon inserted into a gene encoding a LacI/GalR-type repressor, which was called CcpA (catabolite control protein A) (938). Interestingly, genetic mapping revealed that the *ccpA* gene is located close to the previously described *B. subtilis alsA1* mutation, which pre-

vented the production of acetoin in glucose-grown cells (983). The failure of the *alsA1* mutant to produce acetoin during growth on glucose is due to the almost complete absence of the *alsS*-encoded acetolactate synthase. The *alsS* gene forms an operon together with the acetolactate decarboxylase-encoding *alsD* (726) and is induced by a CCA mechanism. Similar to the *alsA1* mutation, the *ccpA* mutation abolishes acetoin production. Introduction of an SPβ prophage carrying intact *ccpA* restored acetoin production in the *ccpA* and *alsA1* mutants, strongly suggesting that *ccpA* and *alsA* are identical loci (322). Indeed, the *alsA1* mutation was identified as a G-to-A exchange within the Shine-Dalgarno box of the *ccpA* gene (at position −14 with respect to the *ccpA* start codon) (566). This mutation lowers the synthesis of CcpA about 10-fold. The concentration of CcpA in wild-type *B. subtilis* was calculated to be 0.6 μM (566).

Disruption of *ccpA* not only prevented the inhibitory effect of glucose on α-amylase activity and the induction of acetolactate synthase but also affected CCR of many other *B. subtilis* enzymes (178, 184, 299, 566), thus establishing the pleiotropic character of the *ccpA* mutation. Whole-genome analysis turned out to be a powerful tool for detecting CcpA-regulated transcription units (575, 980). Interestingly, three *B. subtilis ccpA* mutants that specifically affected CCA of the *alsSD* operon, while CCA or CCR of other transcription units was not or only slightly altered, were isolated (887). Two of the mutations affect conserved regions in the DNA binding domain of CcpA (A18V and R48C replacements), whereas the third mutation was in a less conserved region in the core domain (Pro286Leu replacement).

The effect of *ccpA* inactivation on the synthesis of catabolic enzymes could also be demonstrated by comparing the protein patterns of two-dimensional gels obtained with crude extracts from *B. subtilis* (880) or *E. faecalis* (453) wild-type and *ccpA* strains. *E. faecalis* proteins isolated from a few spots exhibiting an altered intensity in the *ccpA* mutant compared to the wild-type strain were identified by microsequencing. They include a protein with strong similarity to the α subunit of L-serine dehydratase, an enzyme catalyzing the conversion of serine to pyruvate, as well as glycerol dehydrogenase and the two subunits of dihydroxyacetone kinase (DhaKL). The genes encoding the three latter proteins are present in the *dha* operon (453), which also contains the gene for an EIIA of the mannose class PTS (see "SOME UNUSUAL PTS PATHWAYS AND PROTEINS"). In addition, *galKETR* operon expression as well as the synthesis of 13 proteins identified as glucose starvation proteins were no longer sensitive to CCR in the *E. faecalis ccpA* mutant. Two-dimensional gel electrophoresis with *B. subtilis* crude extracts showed that in a *ccpA* mutant, seven tricarboxylic acid cycle enzymes (CitC, CitG, CitH, CitZ, OdhA, SdhA, and SucC/SucD) were relieved from CCR (880). The *citZ* (403) and *citG* genes and the *odhAB*, *sdhCAB*, and *sucCD* operons are preceded by potential *cre*'s (178, 565). Interestingly, *E. faecalis* and *B. subtilis ccpA* mutants contain a much larger amount of P-Ser-HPr than wild-type strains (453, 492). The increased percentage of P-Ser-HPr slows glucose uptake by the *B. subtilis ccpA* mutant when grown on synthetic medium (492), which in turn seems to prevent CCA of the *gapA* operon, which encodes the enzymes of the lower part of glycolysis (492, 880). When a *ptsH1* mutation, which prevents the formation of P-Ser-HPr, was introduced into the *ccpA* strain, the activating effect of glucose on *gapA* operon expression (230) was restored (492). Expression of *gapA* is controlled by the repressor CggR, which is inactivated by elevated amounts of FBP (194). A similar repressive effect of the elevated amount of P-Ser-HPr in a *ccpA* mutant was observed for the *B. subtilis* glutamate synthase-encoding *gltAB* operon. Introduction of a *ptsH1* mutation restored *gltAB* expression in a *ccpA* background (925). The mechanism leading to enhanced P-Ser-HPr formation in *ccpA* mutants is not known. In addition, when grown in complex medium, *ccpA* mutants seem to efficiently transport PTS sugars, because glucose utilization by a *B. subtilis ccpA* mutant represses the synthesis of several enzymes submitted to a second, CcpA-independent CCR mechanism. For example, CCR of the α-glucosidase- and glycerol kinase-encoding genes is not diminished in a *B. subtilis ccpA* mutant (184).

Genes regulated by CcpA include not only carbon catabolic genes but also genes related to carbon metabolism in a larger sense. The *L. delbrueckii* subsp. *lactis* CcpA ortholog PepR1 stimulates the expression of the prolidase-encoding *pepQ* gene and seems to affect the expression of *pepX*, *pepI*, and *brnQ*, which encode two other peptidases and a branched-chain amino acid transporter, respectively (779). A connection between CCR and amino acid metabolism also exists in *Streptococcus gordonii* and *Streptococcus rattus*, where the arginine deiminase operon is repressed by CcpA (195, 298), and in *B. subtilis*, where CcpA stimulates the expression of the *ilv-leu* operon, which encodes the enzymes for the synthesis of branched-chain amino acids (810, 882). A connection between CCR and the utilization of nitrogen sources was provided by the finding that the expression of *rocG*, which encodes the main glutamate dehydrogenase of *B. subtilis*, is subject to CcpA-mediated CCR (53). A connection between carbon and nitrogen metabolism in *B. subtilis* was further supported by the finding that the σ^{54}-encoding *sigL* gene is regulated by CcpA (128). Transcriptome analysis with *B. subtilis ccpA*, *hprK*, and *ptsH1* mutants revealed that numerous nitrogen and phosphorus metabolic genes are submitted to CCR or CCA (491). CcpA of *Lactobacillus pentosus* represses the activity of xylulose 5-phosphate phosphoketolase, the central enzyme of the phosphoketolase pathway (674). In *Clostridium perfringens*, inactivation of *ccpA* diminishes the expression of the enterotoxin-encoding *cpe* gene during entry into the stationary growth phase (913). *S. mutans ccpA* mutants exhibit diminished fructosyltransferase and glucosyltransferase gene expression (88) and biofilm formation (947).

CcpA-specific sequences. CcpA belongs to the family of LacI/GalR-type repressors but contains characteristic sequences located at the end of the DNA binding domain and within the core protein and can therefore easily be distinguished from other members of this family. These regions are important for CcpA function, because mutations affecting amino acids in the CcpA-specific sequences prevent or lower CCR of a *xylA-lacZ* fusion in *B. megaterium* (422). Interestingly, polyclonal antibodies directed against CcpA from *B. megaterium* do not recognize *E. coli* LacI (441). Phylogenetic analysis shows that all low-G+C gram-positive bacteria for which the genomes have been sequenced contain a *ccpA* ortholog (J. Deutscher and C. Francke, unpublished observa-

tion). The gene is probably always expressed, as a protein exhibiting the approximate size of CcpA and cross-reacting with the *B. megaterium* CcpA antibody was present in the crude extracts of all gram-positive bacteria tested (441). For several of these bacteria, including *E. faecalis*, *L. casei*, *Lactobacillus pentosus*, *L. lactis*, *L. monocytogenes*, *Clostridium saccharobutylicum* (formerly *C. acetobutylicum* P262) (162), and *S. xylosus*, a role of their CcpAs in CCR was demonstrated experimentally. Either their *ccpA* genes complement a *B. subtilis ccpA* mutant or *B. subtilis ccpA* complements *ccpA* mutants of these organisms. Genes affected by *ccpA* mutations in bacteria other than *B. subtilis* are listed in Table 3.

CcpA needs a corepressor. Interestingly, overexpression of the *C. saccharobutylicum ccpA* ortholog *regA* in an *E. coli* strain harboring the *C. saccharobutylicum staA* gene inhibited the expression of *staA*, which encodes a starch-degrading enzyme. As the corepressor of CcpA is absent from *E. coli* (see the next section), these results imply that, if overproduced, CcpA can bind to *cre*'s without a corepressor. Therefore, regulation of *ccpA* expression represents one way to control the binding of CcpA to *cre*'s. Indeed, in a few organisms (*S. xylosus*, *L. pentosus*, and *L. delbrueckii*), *ccpA* is preceded by a *cre*, and its expression is submitted to autoregulation (204, 507, 574). In *L. monocytogenes*, the synthesis of CcpA was elevated when the cells were exposed to prolonged salt stress (200). By contrast, expression of *B. subtilis ccpA* is constitutive and is not influenced by the presence or absence of glucose (566). CCR in *B. subtilis* is therefore not regulated via *ccpA* expression. Instead, additional factors must be involved in the CCR signal transduction pathway. Because CcpA at physiological concentrations was not able to bind to the *cre* of the catabolite-repressed *B. subtilis gnt* operon (566), it was likely that these additional factors would function as corepressors of CcpA.

P-Ser-HPr Functions as a Catabolite Corepressor

Interaction of P-Ser-HPr with CcpA. When the effects of the *B. subtilis ptsH1* mutation on CCR were compared to those of a *ccpA* mutation, it was found that all enzymes that are partly or completely relieved from CCR in the *ptsH1* mutant are also relieved from CCR in the *ccpA* mutant (184). In addition, two of the enzymes that remained repressed by glucose in the *ptsH1* mutant (GlpK and α-glucosidase) remained repressed in the *ccpA* mutant (184). The similar pleiotropic phenotypes observed for *ptsH1* and *ccpA* mutants suggested that P-Ser-HPr and CcpA participate in the same CCR mechanism and might interact. Binding of P-Ser-HPr to CcpA was indeed demonstrated by elution retardation experiments (182). In the presence of FBP, elution of P-Ser-HPr from a Ni-nitrilotriacetic acid column saturated with His-tagged CcpA was clearly retarded compared to the elution of HPr or P∼His-HPr, while doubly phosphorylated (P∼His,P-Ser)-HPr was only slightly retarded.

The specific interaction between P-Ser-HPr and CcpA was confirmed by NMR measurements (381). The formation of the P-Ser-HPr:CcpA complex could be observed in the absence of FBP, probably owing to the high protein concentrations used in the NMR studies. The NMR measurements also allowed the determination of the residues of P-Ser-HPr interacting with CcpA. In addition to the regions around the active-center His-15 and the regulatory Ser-46 (amino acids 43, 44, and 46 to 56), the interface includes amino acids 21 to 27. The regions at amino acids 21 to 27 and 46 to 56 are well conserved within the HPrs of gram-positive bacteria but are quite different in the HPrs of most gram-negative organisms (Fig. 4).

The size of CcpA (37 kDa) is too large to allow a determination of its interface in the P-Ser-HPr:CcpA complex by NMR measurements. However, based on the crystal structure of PurR (788), a LacI/GalR-type repressor missing the CcpA-specific sequences, and the failure of various mutant CcpAs to form a complex with P-Ser-HPr, a presumed surface-exposed area including Tyr-89, Tyr-295, Ala-299, and Arg-303 was proposed to be part of the interface in the P-Ser-HPr:CcpA complex (422). The four amino acids presumed to participate in P-Ser-HPr binding are located in CcpA-specific regions that are absent from other members of the LacI/GalR repressor family. The crystal structure of the *B. megaterium* P-Ser-HPr:CcpA complex revealed that residues Tyr-295, Ala-299, and Arg-303 are located on helix IX of the CcpA N subdomain (which directly follows the DNA binding domain) and indeed interact with P-Ser HPr (787). Val-300 and Leu-304 of CcpA are also part of this tight interface, which includes Ile-47, Met-48, and Met-51 of P-Ser-HPr. Replacement of Ile-47 with Thr in *S. salivarius* HPr leads to the disappearance of diauxic growth and the CCR observed with the wild-type strain during lactose and maltose utilization (263). However, crossed immunoelectrophoresis with HPr antibodies revealed that the amount of P-Ser-HPr in the *ptsH*(*Ile47Thr*) mutant was similar to that observed in the wild-type strain. The *ptsH*(*Ile47Thr*) mutation was therefore assumed to diminish the affinity of the mutant P-Ser-HPr for CcpA (263). The surface-exposed hydrophobic patch formed by Ile-47, Met-48, and Met-51 is also involved in the interaction of HPr with EI (260) and EIIAs (139, 691, 949).

NMR studies revealed an affinity of CcpA for P-Ser-HPr that was about 50-fold higher than that for HPr (381). This effect must be due mainly to electrostatic repulsion, as no significant structural changes accompany the phosphorylation of HPr at Ser-46 (29, 381). The electrostatic effects were assumed to involve the negatively charged phosphorylated Ser-46 of HPr and a positively charged counterpart in CcpA. According to the crystal structure, Arg-303 and Lys-307 indeed form hydrogen bonds with the phosphoryl group in P-Ser-HPr. Tyr-89, the fourth CcpA residue predicted to be involved in the interaction with P-Ser-HPr, is located on helix I of CcpA. Its close contact to helix IX probably explains why a mutation affecting Tyr-89 prevents the interaction with P-Ser-HPr (422). The P-Ser-HPr:CcpA example therefore proves that based on genetic and biochemical studies, reliable predictions about protein-protein interfaces can be made.

Similar to gram-negative bacteria, where PEP-dependent phosphorylation of $EIIA^{Glc}$ is important for CCR (see "REGULATION OF CARBON METABOLISM IN GRAM-NEGATIVE ENTERIC BACTERIA"), phosphorylation of HPr at His-15 affects CCR in gram-positive bacteria, probably by weakening the P-Ser-HPr:CcpA interaction. Indeed, the region around His-15 of P-Ser-HPr makes contacts to helix IX of subunit 1 and helices I and II of subunit 2 in the regulatory domains of the CcpA dimer (787). One P-Ser-HPr molecule therefore interacts with both subunits of the CcpA dimer.

Asp-69 and Asp-99 on helices I and II in the regulatory domain of subunit 2 of the CcpA dimer form hydrogen bonds with Arg-17 in P-Ser-HPr. The crystal structure provides an explanation as to why CcpA exhibits only a weak affinity for (P-Ser,P~His)-HPr (184) and why replacing His-15 with Glu or Ala caused an almost complete relief from CCR (707). When a *ptsH1* mutant strain was transformed with a plasmid carrying the His15Ala *ptsH* allele, the Ser46Ala mutant HPr synthesized from the chromosomal *ptsH1* allele allowed the uptake of glucose via the PTS, while the His15Ala mutant HPr synthesized from the plasmid-located *ptsH* allele could be phosphorylated at Ser-46 and was therefore expected to be active in CCR. However, only a weak repressive effect of glucose on gluconate kinase activity was observed in the *ptsH1* transformant (707), suggesting that seryl-phosphorylated His15Ala mutant HPr has only a low affinity for CcpA (707). Indeed, in the P-Ser-HPr:CcpA complex, Asp-296 forms hydrogen bonds with the Nδ1 of His-15 and the backbone amide nitrogen of Ala-16, which enhances the affinity between the two proteins. Phosphorylation at Nδ1 of His-15 in P-Ser-HPr by PEP and EI or replacing His-15 with Glu or Ala probably prevents the interaction of Asp-296 with HPr and, in the case of (P-Ser,P~His)-HPr and His15Glu mutant P-Ser-HPr, probably even leads to electrostatic repulsion. The participation of His-15 in the interaction with CcpA provides a link between CCR and PEP-dependent phosphorylation of PTS proteins (182).

Several mutations leading to "permanent" CCR have been described. Replacing Ser-46 of HPr with a negatively charged Asp provides a mutant protein resembling P-Ser-HPr (952), which was therefore able to bind to CcpA immobilized on a Ni-nitrilotriacetic acid column (707). In agreement with this finding, overexpression of the *ptsH*(*Ser46Asp*) allele from a plasmid led to partial CCR of the *B. subtilis gnt* operon even in the absence of a repressing sugar (184). Two other classes of mutations that caused similar "permanent" CCR have been described. Several *B. megaterium ccpA* mutants that exhibit partial CCR of the xylose utilization genes in the absence of a repressing sugar have been isolated (442). In contrast to wild-type CcpA, the mutant CcpAs are thought to interact with the *xyl cre* even in the absence of a corepressor (442). The mutations affected the DNA binding domain (Glu77Leu replacement) or the core domain of CcpA. Permanent CCR was also observed with a *B. subtilis* mutant producing Val265Phe mutant HprK/P, which exhibits normal kinase but strongly reduced P-Ser-HPr phosphorylase activity, which leads to the accumulation of P-Ser-HPr (571). Similar to the HPr(Ser46Asp)-producing strain, CCR in the *hprK* (*Val265Phe*) mutant occurs even in the absence of a repressing sugar. Expression of a *xynB′-lacZ* fusion in the *hprK* (*Val265Phe*) mutant grown in a medium containing xylose and succinate as the sole carbon sources is 100 times lower than that in a wild-type strain (571). This permanent repressive effect is responsible for the failure of the *hprK*(*Val265Phe*) mutant to grow on CCR-sensitive sugars such as gluconate, glucitol, and ribose. Inactivation of *ccpA* or the insertion of a *ptsH1* mutation in the *hprK*(*Val265Phe*) strain restored growth on the above-mentioned sugars (571). These results confirm that P-Ser-HPr formation represents the main signal for CCR in gram-positive bacteria. When present at elevated concentrations in the cell, P-Ser-HPr is sufficient to lead to strong CCR, while glycolytic intermediates do not seem to be essential.

Binding of the P-Ser-HPr:CcpA complex to *cre*'s. The above-described results indicate that in gram-positive bacteria with low G+C content, P-Ser-HPr functions as a corepressor by allowing the repressor CcpA to bind to *cre* sites. This concept was first tested with the *B. subtilis gnt* operon, which possesses a *cre* located within the 5′ end of *gntR*, the first gene of this operon (247). Footprint experiments revealed that CcpA or P-Ser-HPr alone does not bind to the *gnt cre*. By contrast, if P-Ser-HPr and CcpA were present together in the reaction mixture, a specific protection of a region corresponding to the *gnt cre* was observed, while no protection occurred with CcpA and dephospho-HPr (247). Mutations affecting the *gnt cre* and causing a relief from CCR prevented binding of the P-Ser-HPr:CcpA complex. Similar results were obtained with *cre*'s from many other catabolite-repressed or -activated transcription units. In addition, by isolating the DNA:protein complex and separating its components on a denaturing gel, it was demonstrated that P-Ser-HPr and CcpA bind together to *cre* sites (30).

The binding affinity of the P-Ser-HPr:CcpA complex for a *cre* site was first studied with circular dichroism spectroscopy. Based on association/dissociation-related changes in the spectrum, an apparent K_D of 4.5 μM was determined for the formation of the ternary complex P-Ser-HPr:CcpA:optimized *B. megaterium xyl cre* (A:T in position 10 replaced with T:A) (381). A 1:1:2 (*cre*:CcpA dimer:P-Ser-HPr) binding stoichiometry was proposed. Because no interaction with the *cre* could be observed when 0.5 mM HPr and CcpA was used, binding of P-Ser-HPr to CcpA was estimated to increase the affinity of the repressor for its DNA targets by more than 2 orders of magnitude (381). Besides, equilibrium constants for the binding of the P-Ser-HPr:CcpA complex to the *B. megaterium xyl cre* and the *B. subtilis amyE cre* were determined by electrophoretic mobility shift experiments and were in the orders of 5×10^6 and 3.5×10^8 M^{-1}, respectively (30, 404). A K_D (0.6 nM) that was much lower than the one determined by circular dichroism spectroscopy (4.5 μM) was reported for the formation of the ternary complex of *B. subtilis* CcpA, P-Ser-HPr, and the *xylA cre* (794).

The crystal structure of the triple complex composed of the CcpA dimer and two P-Ser-HPr molecules from *B. megaterium* and a *cre* has been solved (787). It revealed that the N-terminal HTH motifs and the hinge helices of the CcpA dimer make 32 phosphate contacts to the DNA. They interact with 10 bp out of the 16 bp forming the pseudopalindromic *cre*, an artificial operator site not present in *B. megaterium*. However, a reverse complementary sequence identical to bp 2 to 15 of the employed *cre* is present in *Streptococcus iniae* and probably controls expression of the lactose permease- and lactose oxidase-encoding *lctPO* operon (*cre* located 52 bp upstream from the start codon) (J. Deutscher, unpublished observation). In the ternary complex, important contacts are made with the central C:G and G:C base pairs via Leu-55, the "leucine lever." These contacts are responsible for DNA kinking and minor groove expansion. DNA bending by the P-Ser-HPr:CcpA complex (35°) is smaller than what was observed for LacI (40°) and PurR (50°). The HTH motif exhibits sufficient flexibility to allow CcpA binding to the variable half sites of

pseudopalindromic *cre*'s. The plasticity of the HTH motif also explains the capacity of CcpA to interact with the various *cre*'s of an organism, which generally exhibit significant sequence differences.

A comparison of the structure of the triple complex with that of unliganded CcpA (apoCcpA) revealed that the interaction of P-Ser-HPr with CcpA causes significant structural changes, primarily in the regulatory N subdomain, which allow CcpA to interact with *cre*'s (787). The N subdomain in both subunits of "activated" CcpA is rotated on average by 6° compared to apoCcpA. In addition, the position of helix IX (contains Arg-303) in "activated" CcpA is different from its location in apoCcpA. The interaction of Arg-303 of CcpA with the phosphoryl group in P-Ser-HPr requires the rotation of the side chain of Arg-303, which initiates a series of structural changes culminating at Thr-61, which is located at the core/DNA binding junction. The rotation of Tyr-91, which in apoCcpA interacts with Thr-306 via a hydrogen bond, towards the CcpA dimer interface plays an important role in this process, which ultimately allows high-affinity binding of CcpA to *cre* sites (787).

The P-Ser-HPr:CcpA interaction mode seems to be independent of the absence or presence of a *cre* site. The crystal structure of a complex formed between *B. subtilis* P-Ser-HPr and CcpA missing the first 57 amino acids was very similar to the corresponding structure in the *B. megaterium* P-Ser-HPr:CcpA complex (106). Interestingly, the *B. subtilis* protein complex contains two sulfate ions located close to each other. Both sulfate ions interact with Asp-276, and because an FBP molecule could easily be modeled into the structure with its two phosphoryl groups superimposed on the sulfate ions, it was predicted that the region around Asp-276 represents the FBP binding site.

Based on the above-described results, a general CCR and CCA mechanism, as outlined in Fig. 6 can be proposed for low-G+C gram-positive bacteria. The increase in FBP in cells growing on a rapidly metabolizable carbohydrate such as glucose stimulates the HprK/P-catalyzed formation of P-Ser-HPr, which forms a complex with CcpA and allows the LacI/GalR-type repressor to bind to the *cre*'s. For all genes of gram-positive organisms known to be submitted to CCA, the *cre* is located upstream from the promoter, and binding of the P-Ser-HPr:CcpA complex stimulates transcription, probably by a mechanism similar to that reported for Crp- or Cra-mediated activation of gene expression (142, 645). *cre*'s located upstream from the promoter are found in the catabolite-activated *B. subtilis ackA* (886), *glg* (178), *pta* (685), and *ilv-leu* (810, 882) transcription units and the *pepQ* and *las* genes of *L. delbrueckii* and *L. lactis*, respectively (494) (Table 3). CcpA-mediated CCA of the *B. subtilis als* operon occurs probably via an indirect mechanism, as no obvious *cre* is located in front of the *als* promoter (882). The *cre* of the CCR-sensitive *S. xylosus malRA* operon is also located in front of the promoter (205). However, the *mal cre* and the *mal* promoter are separated by only 8 bp. This might be close enough to enable the P-Ser-HPr:CcpA complex bound to the *cre* to prevent binding of the RNA polymerase holoenzyme. In carbon catabolite-activated genes (*ackA*, *pta*, *ilv-leu*, and *pepQ*), the *cre* and the corresponding promoter are separated by at least 12 bp.

Deviations from the general CCR mechanism in gram-positive bacteria. When Ramseier et al. studied the in vitro binding of *B. megaterium* CcpA to an "optimized" *B. subtilis xyl cre*, in which the G:C and A:T base pairs in positions 3 and 10 were replaced with T:A, an interaction was observed in the absence of P-Ser-HPr (695). Moreover, the presence of P-Ser-HPr or the structurally related Ser46Asp mutant HPr (952) inhibited the interaction of CcpA with the modified *xyl cre*. In addition to the two mutations introduced intentionally in the *B. subtilis xyl cre*, there is also an ambiguity for position 9, which was previously reported to be a T (421) but is a C in the published genome sequence (437). In the nearly identical *B. megaterium xyl cre* (only one base pair difference) (290), there is a C at position 9. As the experiments reported by Ramseier et al. (695) were carried out with the sequence carrying a T in position 9, the unexpected results obtained in the CcpA binding studies might be due to this sequence difference. Indeed, when footprint experiments were carried out with *B. megaterium* CcpA and an optimized *B. megaterium xyl cre*, which differs from the optimized *B. subtilis xyl cre* used previously (695) only at position 9 (C:G instead of T:A), results similar to those reported for the *B. subtilis gnt cre* were obtained; i.e., binding of *B. megaterium* CcpA to the *xyl cre* was observed only when P-Ser-HPr was present (290).

CcpA has been reported to bind in vitro to the *B. subtilis ccpC* (401) and *ackA cre*'s (886) in the absence of a corepressor. Nevertheless, the stimulating effect of glucose on *ackA* expression disappeared in a *ptsH1 crh* double mutant (for *crh*, see the next section) (886). The discrepancy between in vivo and in vitro results could not be explained.

Glucose-6-P allowed P-Ser-HPr-independent binding of CcpA to the *B. subtilis gnt cre* (564) and the optimized *B. megaterium xyl cre* (290), albeit only under nonphysiological conditions. For the *B. subtilis gnt cre*, an effect could be detected only when glucose-6-P was used at more than 30 mM, which is far above the 4 mM glucose-6-P detected in glucose-grown *B. subtilis* cells (233). For the *B. megaterium xyl cre*, an effect of glucose-6-P on the binding of CcpA could be observed only at pH values below 5.4 (290), i.e., far below the cytoplasmic pH of 7 to 8 reported for *B. megaterium* cells (503). Glucose-6-P was therefore not considered to play a role in regulating binding of CcpA to the *cre*'s but was suggested to mimic a possibly physiologically relevant unknown effector (290).

In footprint experiments, CcpA was found to bind weakly to the *B. subtilis amyE cre* in the absence of a corepressor (405). CcpA binding was stimulated by P-Ser-HPr and further enhanced by FBP (404). In in vitro transcription studies, CcpA in the 500 nM range was able to specifically inhibit *amyE* expression. In the presence of 2.7 μM P-Ser-HPr, inhibition of *amyE* expression occurred at slightly lower CcpA concentrations (around 100 nM) and was more efficient (90% inhibition). However, the P-Ser-HPr effect was considered to be nonspecific, as expression from control promoters was also inhibited. In addition, the *ptsH1* mutation had no influence on CCR of the *amyE* gene (924). Surprisingly, when NADP or NADPH was present, specific inhibition of in vitro transcription from the *amyE* promoter was observed at very low CcpA concentrations (in the 5 nM range), but NADP(H) was less efficient (70% inhibition) than P-Ser-HPr. Moreover, in footprint experiments, the addition of NADP(H) had only a weak effect on the protection of the *amyE cre* by CcpA. It was therefore concluded that NADP(H) does not function as a corepressor by enhancing the binding of CcpA to the *cre* but, rather, that it

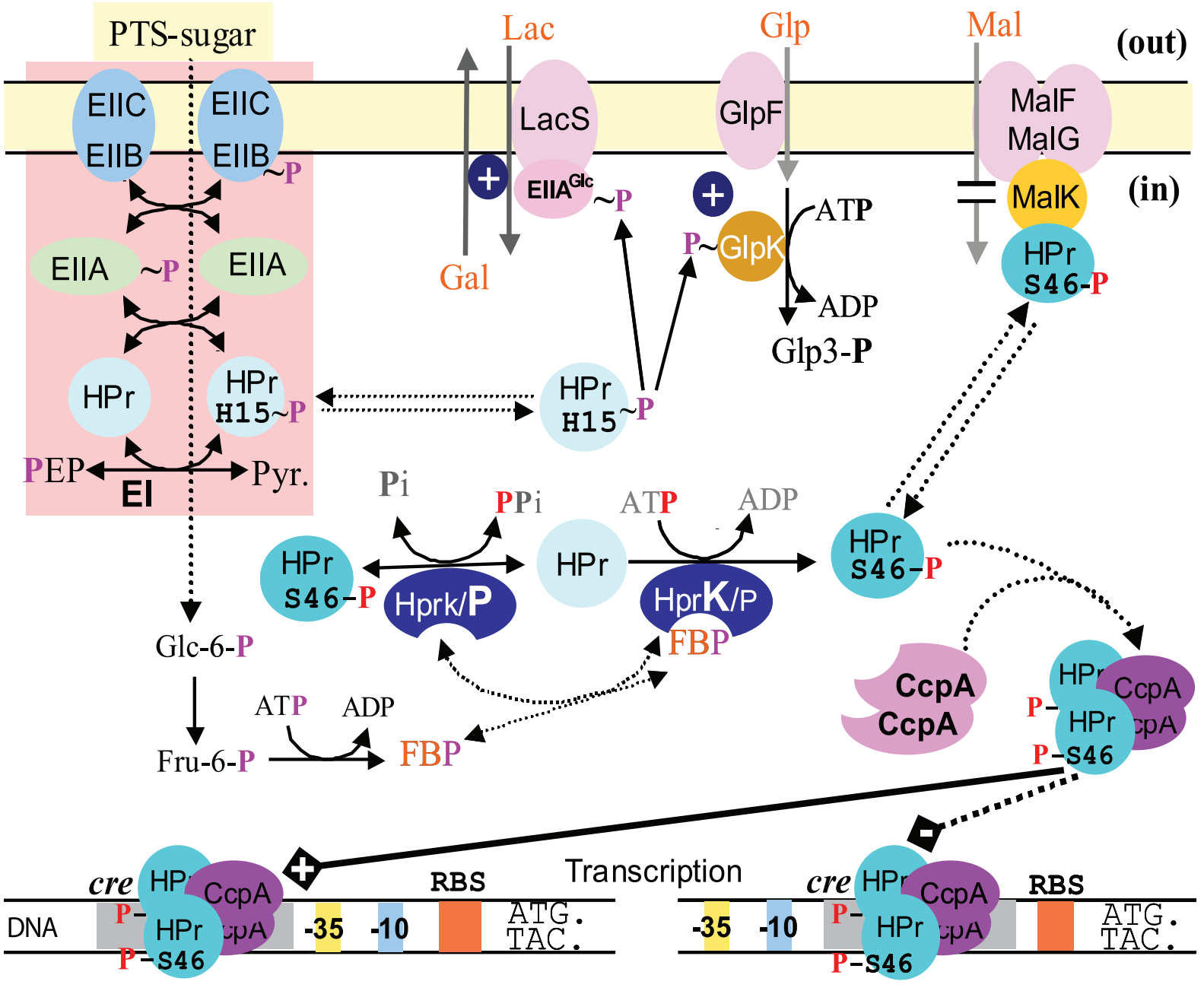
PTS-sugar
EIIC
EIIB
EIIC
EIIB ~P
EIIA ~P
EIIA
HPr
HPr H15~P
PEP
EI
Pyr.
Lac
LacS
Gal
EIIA^Glc ~P
Glp
GlpF
P~GlpK
ATP
ADP
Glp3-P
Mal
(out)
(in)
MalF
MalG
MalK
HPr S46-P
HPr H15~P
Pi
PPi
ATP
ADP
HPr S46-P
Hprk/P
HPr
HprK/P
FBP
HPr S46-P
Glc-6-P
ATP
ADP
Fru-6-P
FBP
CcpA
CcpA
HPr
CcpA
P-HPr
cpA
P-S46
Transcription
cre
HPr
CcpA
P-HPr
cpA
P-S46
-35
-10
RBS
ATG.
TAC.
DNA
cre
HPr
CcpA
P-HPr
cpA
P-S46
-35
-10
RBS
ATG.
TAC.

FIG. 6. Mechanisms underlying CCR in firmicutes. The uptake of glucose and other rapidly metabolizable PTS sugars (top left) leads to a net dephosphorylation of the PTS proteins. The center and bottom of the figure show that the high concentration of FBP present in cells growing on a rapidly metabolizable carbohydrate stimulates the HPr kinase activity of the bifunctional HprK/P and the formation of P-Ser-HPr. P-Ser-HPr interacts with CcpA, and the protein complex binds to the *cre* operator sites on the DNA. The promoter regions are indicated as −10 and −35. CCA occurs when the *cre* is located upstream from the promoter, while CCR requires a *cre* located within or downstream from the promoter. High concentrations of P_i present in resting cells favor the pyrophosphate-producing dephosphorylation of P-Ser-HPr by HprK/P. The top right part of the figure shows that P-Ser-HPr probably interacts with certain non-PTS carbohydrate transport systems, such as the maltose transporter from *L. casei*, and thereby inhibits their transport activity. Phosphorylation of LacS by P~His-HPr stimulates the lactose/galactose exchange reaction (in *S. thermophilus*). In the presence of rapidly metabolizable PTS substrates, the low level of P~His-HPr does not allow sufficient phosphorylation of GlpK, which leads to the inactivation of GlpK and to inducer exclusion. Pyr., pyruvate.

allows CcpA to inhibit the transcription machinery, thus leading to reduced *amyE* expression (404). CcpA has been proposed to directly interact with and to inhibit RNA polymerase, and NADP/NADPH was thought to stimulate the interaction of CcpA with RNA polymerase (406). The suggested role of NADP and NADPH cannot be related to the redox state of the cells, as both molecules were similarly efficient in the stimulation of CcpA-mediated inhibition of *amyE* expression, while NAD and NADH had no effect. With the exception of dormant spores, the concentration of the NADP(H) pool was reported to remain fairly constant in *B. megaterium* cells under various growth conditions, including growth in the absence and presence of glucose (801). It is therefore not clear to which signal the interaction of NADP(H) with CcpA responds.

In several instances, CCR of catabolic genes is mediated by an indirect mechanism. For example, *cre* sites can be located in front of genes encoding transcription regulators of catabolic genes. This is the case for *B. subtilis* CcpC, which controls the expression of *citZ* and *citB* (401) and for LevR of *L. casei*, which regulates the expression of the *levABCDX* operon (534). Similarly, the *acoABCL* operon of *B. subtilis* is controlled by the transcription activator AcoR, the gene of which is preceded by a *cre* recognized by CcpA (12). While a *cre* site also precedes *citZ* and the *L. casei levABCDX* operon, a *cre* seems to be absent from *acoABCL*, which is therefore only indirectly regulated by CcpA. A *cre* site has also been identified within the *sigL* gene of *B. subtilis*, which codes for σ^{54}, which is necessary for the transcription from several "−12, −24" promoters (128).

The *regM* gene of *S. mutans* encodes a homolog of CcpA. Surprisingly, the disruption of *regM* increased the glucose repression of α-galactosidase, mannitol-1-P dehydrogenase, and P-β-galactosidase, suggesting that RegM functions as a positive regulator for the expression of the corresponding genes. In addition, *regM* inactivation affected neither diauxic growth in media containing glucose and a less efficiently metabolizable sugar (816) nor expression of the fructanase-encoding *fruA* gene, although deletion or mutation of the *cre* preceding *fruA* led to a relief from CCR (946). Moreover, FBP, which stimulates HPr kinase activity in most bacteria, slightly inhibited HPr kinase from *S. mutans* (869). It therefore seems that *S. mutans* follows a CCR mechanism different from that operative in most other gram-positive organisms.

P-Ser-Crh, a Second Catabolite Corepressor in Bacilli, Geobacilli, and Oceanobacilli

A *B. subtilis* HPr-like protein without His-15. Although most catabolite-repressed genes and operons of *B. subtilis* are partly or completely relieved from CCR in both *ccpA* and *ptsH1* mutants, the degree of relief can be quite different for the two mutants. For example, CCR of the *amyE* gene and the *hut*, *cta*, *xyn*, and *iol* operons is only slightly affected by the *ptsH1* mutation (less than 20%), whereas these five transcription units are completely relieved from CCR in *ccpA* mutants (184, 250, 488, 924, 985). Similarly, CCA of the phosphotransacetylase-encoding *pta* gene is not affected in a *ptsH1* mutant but is completely lost in a *ccpA* strain (685). Less pronounced differences in CCR or CCA between *ptsH1* and *ccpA* mutants were observed for the glucitol dehydrogenase-encoding *gutB* (184),

the acetyl-CoA synthetase-encoding *acs* (984), and the acetate kinase-encoding *ackA* (886) genes as well as for the *lev* operon (250, 526). These results suggested that in *B. subtilis*, the binding of CcpA to certain *cre*'s is regulated not only by P-Ser-HPr but that a second corepressor might exist.

Evidence for a potential second corepressor came from the *B. subtilis* genome sequencing project, which led to the discovery of a gene encoding an HPr-like protein (219). Although this protein exhibits 45% sequence identity to HPr, there is a striking difference: His-15, the site of PEP-dependent phosphorylation in HPr, is replaced with a Gln in the HPr paralog. As a consequence, the HPr-like protein is not phosphorylated by PEP and EI (250). Nevertheless, the sequence around Ser-46 resembles that in HPr of gram-positive bacteria, and HprK/P-mediated, ATP-dependent, FBP-stimulated phosphorylation at Ser-46 in Crh could be demonstrated (250). These results suggested that the phosphorylated HPr paralog might play a role in CCR similar to that of P-Ser-HPr, and it was therefore called catabolite repression HPr (Crh).

However, when the *crh* gene is disrupted, no effect on CCR is observed. The participation of P-Ser-Crh in CCR can be detected only in a *ptsH1* background. The residual CCR of the *lev*, *iol*, *xyn* (250), and *hut* (985) operons as well as the remaining CCA of the *pta* (685) and *ackA* (886) genes observed in *ptsH1* mutants disappeared almost completely when *crh* was inactivated. Complete relief of the *acsA* (984), *xyn* (249), and *lev* (522) operons from CCR was also observed in a *ptsH1 crh1* double mutant in which Ser-46 of both HPr and Crh was replaced with Ala.

In vitro, Crh forms a mixture of monomers and dimers (644), which, similar to EI, are in slow equilibrium. The structure of the monomer and the interface of the dimer were determined by NMR spectroscopy (222). The NMR data suggested that domain swapping of the β1 strand is implicated in *B. subtilis* Crh dimer formation, and this was confirmed by the crystal structure (389). The Crh monomer strongly resembles HPr, except for a few specific differences. Their structural similarity explains why Gln15His mutant Crh can be phosphorylated by PEP and EI (524). When the Gln15His mutant Crh is overproduced in a *B. subtilis ptsH* deletion strain, it partly restores PTS transport activity and growth on PTS substrates or glycerol and allows the full activation of several transcription regulators possessing a PTS regulation domain (PRD) (156, 524). GlpK and PRD-containing transcription regulators (see "REGULATORY FUNCTIONS MEDIATED BY P~His-HPr AND P~EIIBs") are stimulated by PEP-dependent phosphorylation via EI and HPr (158, 844).

Binding of the P-Ser-Crh:CcpA complex to *cre*'s. To determine the binding characteristics of the P-Ser-Crh:CcpA complex, footprint experiments were carried out with *cre*'s from transcription units, which are not completely relieved from CCR in *ptsH1* mutants. The *xyn cre* site was indeed specifically protected by the P-Ser-Crh:CcpA complex, but the effect was slightly weaker than that with P-Ser-HPr:CcpA (249). The use of various mixtures of P-Ser-HPr and P-Ser-Crh did not increase the protective effect compared to the protection obtained with only a single corepressor. For a few catabolic genes, the binding of P-Ser-Crh:CcpA to the *cre*'s was stimulated by FBP, and in some *cre*'s, certain positions became hypersensitive towards DNase I digestion after binding the repressor:corepressor complex. P-Ser-HPr:CcpA and P-Ser-Crh:CcpA protected identical DNA regions, and FBP always had comparable effects on the DNA binding affinity of the two protein complexes. For example, FBP increased the protective effect of the P-Ser-HPr:CcpA and P-Ser-Crh:CcpA complexes against DNase I digestion of the *xyn cre* (249) and the *pta cre* (685). In contrast, FBP did not enhance the protective effect of both CcpA:corepressor complexes in footprint experiments with the *lev cre* (523). Both protein complexes rendered several nucleotides located outside the *lev cre* hypersensitive towards DNase I digestion. Expression of the *lev* operon is regulated by the transcription activator LevR, which binds to an upstream activating sequence (UAS) centered at about 130 bp upstream from the *lev* promoter. LevR was therefore assumed to exert its stimulating effect on *lev* operon expression by DNA bending (523). The *lev cre* is located between the UAS and the σ^{54}-dependent "−12, −24" promoter. Binding of the P-Ser-HPr:CcpA or P-Ser-Crh:CcpA complexes to the *lev cre* supposedly prevents LevR-induced DNA bending by altering the DNA structure, which might be responsible for the hypersensitivity towards cleavage during footprint experiments. Although in the case of the *pta cre*, only weak protection by P-Ser-HPr:CcpA or P-Ser-Crh:CcpA was observed, several nucleotides located outside the *cre* site became hypersensitive to DNase I digestion. When FBP was included in the reaction mixtures, the hypersensitive bands disappeared, and the protective effect on the *pta cre* was enhanced (685). The physiological significance of these in vitro observations remains to be determined.

In addition to the fact that certain *cre*'s are not recognized by the P-Ser-Crh:CcpA complex (when the *ptsH1* mutation leads to a complete relief from CCR or CCA), P-Ser-Crh was reported to have a 10-fold-lower affinity for CcpA than P-Ser-HPr. These differences are surprising, as, with the exception of two amino acids (His-15 and Thr-20), all P-Ser-HPr residues involved in the interaction with CcpA are conserved in Crh, and the crystal structures of the two proteins revealed a very similar fold. A possible explanation was that the dimerization of Crh (389) might have an influence on the formation of the P-Ser-Crh:CcpA complex and its interaction with *cre* sites. However, the crystal structure of the triple complex P-Ser-Crh(*B. subtilis*):CcpA(*B. megaterium*):*cre* (same *cre* as that used for the P-Ser-HPr:CcpA structure) (787) revealed that the two P-Ser-Crh molecules bound to the CcpA dimer are present exclusively in monomeric form (789). The binding of P-Ser-Crh:CcpA to the *cre* induces a kink (DNA bend angle of 31°) in the central conserved C-G that is almost identical to that observed in the triple complex with P-Ser-HPr (DNA bend angle of 35°). Nevertheless, specific differences between P-Ser-HPr:CcpA and P-Ser-Crh:CcpA occur in the contact region including His-15/Gln-15 and Ala-20/Thr-20 of HPr/Crh, respectively. In the complex with P-Ser-HPr, His-15 hydrogen bonds with the side-chain carboxyl of Asp-296 of CcpA. In contrast, Gln-15 of Crh makes only a weak contact to Asp-296 (distance of 4 Å), but instead, hydrogen bonds with the side chain of Arg-324, which, compared to the P-Ser-HPr:CcpA structure, needs to be rotated to come sufficiently close to the interface to allow the contact with Gln-15. Interestingly, an equivalent of Arg-324 seems to be present in CcpAs of only those organisms possessing Crh (789). If this assumption holds, *Bacillus pseudofirmus*, *Bacillus sphaericus*, and *Exiguobacterium*

sibiricum 255-15 should contain a Crh ortholog, as their CcpAs contain an equivalent of Arg-324, whereas *G. stearothermophilus*, in contrast to *Geobacillus kaustophilus*, should be devoid of Crh, as its CcpA is missing an equivalent of Arg-324 (replaced with a Glu). The presence of Crh in the above-mentioned four organisms is uncertain, as no or only incomplete genome sequences are presently available.

In conclusion, the structures of the complexes of P-Ser-HPr and P-Ser-Crh with CcpA and a *cre* exhibit extensive similarity, and the few differences that were observed do not explain why the *B. subtilis* P-Ser-HPr:CcpA complex binds to several *cre* sites, which, according to genetic data, are not or only barely targeted by the P-Ser-Crh:CcpA complex. The corresponding transcription units are partly or completely relieved from CCR in *ptsH1* and *hprK* mutants (*gnt*, *mtl*, etc.) (184). To better understand the different affinities of the two corepressor:CcpA complexes for certain *cre*'s, it might be useful to determine the structure of a triple complex of P-Ser-HPr, CcpA, and a *cre* that does not interact with the P-Ser-Crh:CcpA complex.

Distribution of Crh in gram-positive organisms. In addition to *B. subtilis* (250) and *B. megaterium* (794), Crh has been detected in most other bacilli (*Bacillus anthracis*, *B. clausii*, *B. cereus*, *B. halodurans*, *B. licheniformis*, *B. thuringiensis*, and *B. weihenstephanensis*) as well as in *G. kaustophilus* and *O. iheyensis*, which are both closely related to bacilli. The various Crhs exhibit a high degree of sequence identity (between 61 and 83%), whereas they show only 41 to 47% sequence identity to the HPrs of their respective organisms. Interestingly, *B. licheniformis* possesses two HPr-like proteins containing a Gln in place of His-15. One of them has a Ser-46 and therefore corresponds to Crh (83% sequence identity with Crh of *B. subtilis*). The other protein exhibits only 44% sequence identity to *B. subtilis* Crh, and its Ser-46 is replaced with an Asp, which probably mimics permanent phosphorylation at position 46 (Deutscher, unpublished). The function of the latter protein is unknown. *crh* is the last gene (or penultimate gene in *B. subtilis*) of the *yvc* operon. The last gene of the *B. subtilis yvc* operon (250), *yvcN*, is absent from the other organisms, and *O. iheyensis* also misses the first gene, *yvcI*.

The genome sequences of other gram-positive bacteria revealed that staphylococci, clostridia, lactobacilli, lactococci, enterococci, etc., also have a *yvc* operon, but none of them contains a *crh* gene. Although the in vivo and in vitro results unequivocally established that P-Ser-Crh can contribute to CCR in *B. subtilis*, the fact that *crh* is present only in bacilli and a few close relatives and that *crh* inactivation in wild-type *B. subtilis* has no effect on CCR raises serious doubts about whether CCR is the primary function of Crh. P-Ser-HPr seems to be sufficient to mediate CCR in gram-positive organisms, independently of whether they possess Crh or not. In addition, numerous *B. subtilis* operons are completely relieved from CCR in a *ptsH1* mutant, suggesting that their *cre* is not recognized by the P-Ser-Crh:CcpA complex. So far, only one P-Ser-Crh-specific function could be established. Expression of the Mg^{2+}-citrate transporter-encoding *B. subtilis citM* gene is strongly repressed during growth on glucose but is also slightly repressed when grown on glutamate/succinate as the sole carbon source. Whereas repression of *citM* by glucose is mediated via CcpA in complex with either P-Ser-HPr or P-Ser-Crh, repression by glutamate/succinate completely disappeared in a *crh*-disrupted strain (934). Interestingly, *crh* expression, which is much weaker than *ptsH* expression, is highest in cells grown on one of the two TCA cycle intermediates, citrate or succinate (286).

It is possible that additional functions of Crh are related to the genes cotranscribed with *crh*. It is noteworthy that the *yvcJ* gene organized in an operon with *crh* resembles *yhbJ* from the *E. coli rpoN* operon. YhbJ possesses Walker motifs A and B, but its function is not known. Because in many *hprK*-containing gram-negative bacteria, *yhbJ* and other genes of the *E. coli rpoN* region are organized in an operon with *hprK*, it is tempting to assume that Crh might somehow play a role in the regulation of SigL (σ^{54}), the RpoN ortholog of *B. subtilis*.

An HPr paralog containing a conserved Ser-46 but missing His-15 (His-15 is replaced with a Phe) is also present in the mollicute *Ureaplasma urealyticum* (also called *Ureaplasma parvum* serovar 1) (308). The sequence around Phe-15 (amino acids 12 to 22) completely differs from all known HPrs, while the other parts of the protein resemble HPr from gram-positive and gram-negative bacteria. In contrast to other mollicutes, *U. urealyticum* lacks EI as well as an HPr with His-15. Mollicutes generally lack CcpA but contain an *hprK* gene, as is also the case for *U. urealyticum* (308). The role of P-Ser-HPr formation in these organisms therefore remains unknown (307).

What Are the Functions of CcpB and CcpC?

A second *trans*-acting factor implicated in CCR in *B. subtilis* was identified. This protein, which was called catabolite control protein B (CcpB), exhibits 30% sequence identity to CcpA, and especially, the helix-turn-helix motifs of the two proteins are very similar. Indeed, *ccpB* disruption leads to a partial relief from CCR of the *gnt* and *xyl* operons in cells grown on solid medium (113). However, when the cells are grown in liquid media at a high agitation rate, no effect of the *ccpB* mutation is observed. In contrast, mutant cells grown at a low agitation rate exhibit partial relief from CCR. Based on these results, it was proposed that CcpB would affect CCR in response to changes in environmental conditions, such as oxygen supply, cell density, etc. However, the DNA targets and the factors controlling the binding of CcpB to these targets are not known. The structures of the P-Ser-HPr:CcpA and P-Ser-Crh:CcpA complexes (787, 789) revealed that those sequences that are specific for the CcpA repressor subfamily (422) are important for the interaction with P-Ser-HPr. The fact that these sequences are absent from CcpB argues against the assumption that it might use P-Ser-HPr as a corepressor (113).

A third *trans*-acting factor called CcpC is involved in CCR of the *B. subtilis citB* and *citZ* genes (which code for aconitase and citrate synthase, respectively) (386) and in CCR of the *L. monocytogenes* genes encoding a CitB ortholog and a presumed glutamine transporter (lmo0847) (402). However, CcpC is not a member of the LacI/GalR family but is a member of the LysR family of transcription regulators. In the absence of an effector, *B. subtilis* CcpC functions as a repressor by binding to both a dyad symmetry element centered at position −66 with respect to the transcription start site of *citB* and a repeat of only the downstream arm of the dyad symmetry element at position −27. In the presence of citrate, the putative inducer

for *citB* and *citZ*, CcpC, binds only to the complete dyad symmetry element (at position −66), which leads to transcription activation. Mutations affecting the CcpC binding sites in front of *citB* cause derepressed expression of only *citB*, whereas *ccpC* inactivation stimulates the expression of both *citB* and *citZ* (386). The dyad symmetry element shows no similarity to *cre*'s. Nevertheless, expression of the CcpC-regulated *citB* and *citZ* genes is elevated in a *ccpA* mutant (880), probably owing to two indirect effects. Firstly, a *cre* site is present in front of *citZ* (403). Inactivation of *ccpA* therefore leads to enhanced synthesis of the citrate synthase CitZ and probably to elevated amounts of citrate, the inducer of *citB* (403). Secondly, CcpA regulates *ccpC* expression by binding to a *cre* site located between the two promoters of the *ccpC* gene (401). Disruption of *ccpA* leads to elevated *ccpC* expression and, in the presence of high concentrations of citrate, to enhanced *citB* and *citZ* expression.

It seems contradictory that *B. subtilis* cells growing on rapidly metabolizable carbon sources such as glucose activate the expression of glycolytic enzymes but repress enzymes of the TCA cycle. However, during growth on glucose, this organism secretes large amounts of acetate into the medium (685) and also produces acetoin when approaching stationary phase (726). To divert the carbon flux towards acetate/acetoin production, the expression of the genes encoding the enzymes necessary for the synthesis of these metabolites (acetate kinase, phosphotransacetylase, acetolactate synthase, and acetolactate decarboxylase) is stimulated, whereas TCA cycle enzymes, cytochromes, and cytochrome oxidases are repressed. Once the rapidly metabolizable carbon source is exhausted, *B. subtilis* begins to utilize the previously secreted acetate and acetoin. Under these conditions, the synthesis of the enzymes for acetate and acetoin production is no longer activated, and TCA cycle enzymes, cytochromes, and cytochrome oxidases are derepressed. This coordinated regulation probably provides *B. subtilis* with a growth advantage, as not all bacteria are able to efficiently utilize acetate and acetoin.

CcpB and CcpC do not seem to be directly regulated by P-Ser-HPr. Nevertheless, the phosphoprotein has been shown to interact with *B. subtilis* RbsR (Table 1), a LacI/GalR-type repressor, which controls the expression of the ribose operon (583). When electrophoretic mobility shift assays were carried out, only P-Ser-HPr, but not HPr, allowed RbsR to interact with a DNA fragment containing the putative RbsR binding site, which also represents a perfect *cre* site. It was therefore proposed that this site might be the target for both RbsR and CcpA (731). The positively charged residues of RbsR that were predicted to interact with the phosphoryl group of P-Ser-HPr according to a structure model differ from the positively charged residues located in the interface of the P-Ser-HPr: CcpA complex (787). So far, no in vivo data that would attribute a physiological role to the observed in vitro P-Ser-HPr/RbsR interaction have been published.

Role of P-Ser-HPr in the Virulence of Certain Pathogens

P-Ser-HPr and the regulation of virulence genes in gram-positive pathogens. Surprisingly, in certain pathogens, P-Ser-HPr formation affects the activity of transcription regulators of virulence genes. The best-studied example is the Crp/Fnr-type transcription activator PrfA of *L. monocytogenes* (207). It regulates the expression of several virulence genes, including the hemolysin-encoding *hly* gene and the phospholipase-encoding *plcA* gene, which together with the *prfA* gene are clustered within a 10-kb region on the chromosome (129). Growth of *L. monocytogenes* on rapidly metabolizable carbon sources such as glucose and fructose leads not only to CCR of catabolic genes (50) but also to repression of the PrfA-controlled genes (558). While repression of catabolic genes requires CcpA, repression of the virulence genes is CcpA independent, since it persists in *ccpA* mutants (50). Nevertheless, P-Ser-HPr, the corepressor of CcpA, seems to play a role in the regulation of PrfA activity. A *B. subtilis* strain containing *L. monocytogenes prfA* under the control of the *spac* promoter and the *lacZ* reporter gene fused to the PrfA-activated *hly* promoter exhibits *lacZ* expression only in the presence of IPTG (804). Expression of *lacZ* and thus PrfA activity are inhibited by glucose and fructose, indicating that CCR of PrfA-controlled genes is also operative in *B. subtilis* (179, 326). Interestingly, introduction of the *hprK*(*Val267Phe*) mutation in the *B. subtilis* strain, which leads to the conversion of more than 95% of the HPr into P-Ser-HPr (571), strongly inhibits PrfA activity even in the absence of a repressing sugar (326). Replacement of Ser-46 in HPr with an alanine (*ptsH1* mutation) prevented the inhibitory effect of the *hprK*(*Val267Phe*) mutation, suggesting that P-Ser-HPr is directly or indirectly involved in PrfA regulation. In contrast, inactivation of *ccpA* or introduction of the *crh1* mutation in the *hprK*(*Val267Phe*) strain did not stimulate PrfA activity (326). The effect of P-Ser-HPr on PrfA activity seems to be indirect and based primarily on its poor phosphorylation by enzyme I, which probably leads to barely phosphorylated EIIAs and EIIBs. In agreement with this concept, deletion of *ptsH* or the enzyme I-encoding *ptsI* also led to strong inhibition of PrfA activity (326). PrfA therefore seems to require a fully functional PTS and might be either stimulated by P∼His-HPr, P∼EIIAs, or P∼EIIBs or inhibited by dephosphorylated EIIAs or EIIBs. A connection between the PTS components and PrfA was also suggested by the observation that the overproduction of PrfA inhibits glucose utilization via the PTS (517). In contrast, the utilization of glucose-6-P via the hexose phosphate transport protein (UhpT) was not affected by PrfA overproduction. In addition, in contrast to glucose, fructose, cellobiose, etc., glucose-1-P did not inhibit PrfA activity, although it is probably converted to glucose-6-P and metabolized via glycolysis (728). The failure of glucose-1-P, which is not transported via the PTS, to inhibit PrfA activity might therefore be related to its different mode of transport. In conclusion, these results suggest a direct or indirect effect of PTS components necessary for the uptake of glucose, fructose, cellobiose, etc., on PrfA activity.

In contrast to PrfA, the *S. pyogenes* virulence gene regulator Mga seems to be controlled by the classical CcpA-dependent CCR mechanism. Mga functions as a transcription activator for the expression of numerous genes encoding mainly surface-associated proteins involved in pathogenesis. Expression of the Mga regulon is growth phase dependent, and the presence of rapidly metabolizable carbon sources such as glucose stimulates the synthesis of members of the M-protein family (*emm*, *mrp*, *arp*, and *enn*) (424, 535). In addition, Mga controls genes encoding fibronectin-binding proteins (*sof* and *fbx*) and a collagen-like protein (*sclA*) (14, 15). Mga also controls its own

expression by interacting with two 59-bp binding motifs located within the *mga* promoter region (536). In addition, the −35 region of the first of the two *mga* promoters is preceded by a DNA sequence that almost perfectly matches the *cre* consensus sequence. Purified CcpA was found to bind to the presumed *cre* site (K. S. McIver, personal communication). Its location upstream from the −35 promoter region suggests that *mga* is submitted to CCA. This is in agreement with the previous observation that the presence of glucose stimulates the synthesis of the M protein.

The *Streptococcus pneumoniae* CcpA ortholog RegM represses α-glucosidase and β-galactosidase activities, but the inactivation of *ccpA* (*regM*) also attenuates the virulence of this organism. The mutation has no impact on the expression of several established pneumococcal virulence genes but, rather, affects the expression of genes from the capsular polysaccharide synthesis (*cps*) locus (273). Capsular polysaccharides are known to be important factors for host cell adhesion. Indeed, the *ccpA* mutant is severely attenuated for nasopharyngeal colonization and lung infection in mice (366). This effect might partly be related to the differences in surface protein composition observed between the wild type and the *ccpA* mutant.

P-Ser-HPr and the regulation of virulence genes in gram-negative pathogens. Genes encoding proteins with significant similarity to HprK/P are present in numerous gram-negative bacteria (α-, β-, γ-, and δ-proteobacteria), spirochetes, green sulfur bacteria, *Fibrobacteres*, and fusobacteria (41, 64). The existence of HprK/P homologs in gram-negative bacteria came as a surprise. Due to the absence of Ser-46 phosphorylation in the HPrs of *E. coli* and other *Enterobacteriaceae*, HprK/P was originally thought to exist only in gram-positive organisms. Interestingly, HprK/P-containing gram-negative bacteria possess an HPr in which the sequence around Ser-46 differs from the corresponding sequence in HPrs of *Enterobacteriaceae* and instead resembles those in HPrs from gram-positive organisms (64) (Fig. 4). For the gram-negative bacteria *Neisseria gonorrhoeae* and *N. meningitidis* (64), the spirochete *T. denticola* (278), and the α-proteobacteria *Agrobacterium tumefaciens* (S. Poncet, A. Khemiri, I. Mijakovic, A. Bourand, and J. Deutscher, unpublished results) and *B. melitensis* (S. Poncet, M. Dozot, A. Bourand, J. Deutscher, J. J. Letesson, and X. de Bolle, unpublished results), ATP-dependent phosphorylation of HPr by their HprK/Ps has been demonstrated in vitro. In several of the above-mentioned bacteria, *ptsH* and sometimes also *ptsI* are located in the *hprK* operon. Nevertheless, most of these bacteria seem to contain neither CcpA nor an EIIB or EIIC, excluding the possibility that P-Ser-HPr plays a role in the regulation of PTS transport activity or CcpA-mediated CCR in these bacteria.

In addition to *ptsH* and *ptsI*, the *hprK* operons of gram-negative bacteria frequently contain a gene encoding an EIIA of either the fructose/mannitol or the mannose class PTS (64, 345). The organization of the *hprK* operon in certain gram-negative pathogens and symbionts suggests a role of HprK/P in cell adhesion and virulence (64). In these proteobacteria, the *hprK* operon usually contains between 1 and 10 genes of the *E. coli rpoN* region or genes encoding a two-component system. Genes coding for an EIIA-like protein (PtsN) and an HPr-like protein (PtsO or NPr) are also present in the *rpoN* operon of *E. coli* and other enterobacteria (553) (for the regulatory functions of RpoN, PtsN, and PtsO, see "SOME UNUSUAL PTS PATHWAYS AND PROTEINS"). In *hprK*-containing β-, γ-, and δ-proteobacteria, *hprK* is usually located between *rpoN* and *yhbJ* (64). In a few α-proteobacteria, the *hprK* and *yhbJ* genes are also located next to each other. Interestingly, the *crh* gene of *B. subtilis* is also organized in an operon with a *yhbJ* homolog (64), while in *Ruminococcus albus*, *hprK* is followed by *murB* and a *yhbJ*-like gene (Deutscher, unpublished). These observations strongly suggest a functional connection between the PTS and the nucleotide binding protein YhbJ.

For β- and γ-proteobacteria containing a combined *hprK*/*rpoN* operon, HprK/P was proposed to control the phosphorylation state of the $EIIA^{Fru}$-like PtsN (64, 724), the gene of which often precedes *hprK*. In these organisms, signals causing enhanced HPr kinase and diminished P-Ser-HPr phosphorylase activity were assumed to lead to elevated amounts of P-Ser-HPr, which would slow the phosphorylation of the EIIA (180, 723). In *N. meningitidis*, such a phosphorelay system seems to somehow control the interaction with host cells, as the inactivation of *hprK* strongly reduces cell adhesion. The inactivation of *hprK* possibly leads to the enhanced formation of P∼PtsN. Dephospho-PtsN was therefore proposed to participate in a signal transduction pathway controlling cell adhesion (64).

Similarly, in several α-proteobacteria, the colocalization of *hprK* with genes encoding a two-component system suggests a role of HprK/P in the regulation of cell adhesion (64, 345). The *A. tumefaciens* EnvZ/OmpR-like two-component system ChvG/ChvI, which is encoded by the genes preceding *hprK*, controls the expression of the virulence genes *virB* and *virE* (472), which encode components of a type IV secretion system delivering oncogenic DNA to susceptible plant cells (100). Inactivation of *chvG* or *chvI* prevents tumor formation on the host plants (108). Similarly, inactivation of the *chvG*/*chvI*-like *bvrR* and *bvrS* genes of the pathogenic animal endosymbiont *Brucella abortus* affects cell invasion and virulence of this organism (821). Inactivation of the ChvG-like sensor kinase ExoS in the plant symbiont *Sinorhizobium meliloti* prevents the production of succinoglycan, the main exopolysaccharide necessary for the successful invasion of nodules on the host plant alfalfa (126). It has been proposed that HprK/P and the PTS proteins might somehow control the activity of the two-component systems (64). In α-proteobacteria, the *hprK* operon usually contains a gene encoding an EIIA of the mannose class PTS and sometimes also the genes for EI and HPr, whereas the gene for an EIIA of the fructose class PTS is located next to the *rpoN* gene (S. Poncet, unpublished observation). HprK/P was thought to control the phosphorylation state of the EIIAs. An EIIA or P∼EIIA was in turn suggested to interact with the two-component system (64), or alternatively, a P∼EIIA might phosphorylate one of the proteins of the two-component system. Interestingly, during a search for genes affecting the virulence of *Brucella melitensis*, inactivation of the $EIIA^{Fru}$-encoding gene was found to lower the pathogenicity of this organism (169).

α-Proteobacteria as well as *Coxiella burnetii* contain a truncated HprK/P missing the N-terminal domain, which resembles the N-terminal part of MurE (13), the enzyme catalyzing the formation of cytoplasmic precursors for cell wall synthesis (284). Similar to the artificially truncated *L. casei* HprK/P (229), the natural absence of the first 130 amino acids of *A. tumefaciens* HprK/P does not affect the known HprK/P func-

tions. Nevertheless, the *A. tumefaciens* enzyme is not able to efficiently use PP_i as a phosphoryl donor and to dephosphorylate P-Ser-HPr in the presence of P_i (I. Mijakovic, A. Khemiri, A. Bourand, S. Poncet, and J. Deutscher, unpublished results). However, the absence of these functions from *A. tumefaciens* HprK/P is likely due to sequence differences in the central loop compared to HprK/P from gram-positive organisms. Mutations affecting this loop in *L. casei* or *B. subtilis* HprK/P strongly diminish the phosphorylase activity (571).

Interestingly, all known bacteria with a truncated HprK/P possess an EI with an N-terminal extension resembling the N-terminal DNA binding domain of NifA, a transcription activator for certain σ^{54}-dependent promoters. EI^{Ntr} (PtsP) of *E. coli*, which, together with the HPr paralog PtsO, is assumed to catalyze the phosphorylation of PtsN, also contains this NifA domain (see "SOME UNUSUAL PTS PATHWAYS AND PROTEINS").

Is P-Ser-HPr Involved in Inducer Expulsion?

In the next few sections, we will deal with the regulatory phenomena inducer expulsion and inducer exclusion in low-G+C gram-positive bacteria, which were both suggested, based mainly on in vitro data, to be related to P-Ser-HPr formation. However, while in vivo experiments confirmed a participation of P-Ser-HPr in inducer exclusion, inducer expulsion also occurs when P-Ser-HPr cannot be formed due to the inactivation of *hprK* or the replacement of the Ser-46 of HPr with an alanine.

Simultaneous occurrence of inducer expulsion and P-Ser-HPr formation. Inducer expulsion is a regulatory phenomenon that has been known for at least 40 years. Halpern and Lupo observed that the presence of certain carbon sources accelerates the exit of α-MG from *E. coli* cells that had accumulated this nonmetabolizable PTS substrate (309). Glucose had the strongest effect and accelerated α-MG expulsion about sixfold compared to cells to which no carbon source was added. When discussing the expulsion of nonmetabolizable carbohydrates, at least two different forms need to be distinguished, as they follow different mechanisms: the expulsion of non-PTS sugars and PTS sugars. The latter are accumulated as phospho derivatives inside bacterial cells and are first dephosphorylated before they are expelled. We will discuss the expulsion of PTS sugars, because ATP-dependent HPr phosphorylation was discovered in connection with TMG expulsion from *S. pyogenes* cells (185, 710).

Streptococci and lactococci transport nonmetabolizable sugar derivatives such as TMG or 2DG via the PTS and accumulate these carbohydrates as P derivatives. Interestingly, these nonmetabolizable P-sugars are rapidly expelled when the cells are exposed to a well-metabolizable carbon source such as glucose, sucrose, or mannose (712, 872). Expulsion turned out to be a two-step process: the accumulated P-sugars are first intracellularly dephosphorylated before the dephosphorylated sugars are expelled (710). The expulsion could be prevented when the ATP synthesis was inhibited by poisoning the cells with arsenate, fluoride (712), or iodoacetate (872). It has subsequently been established that in poisoned cells, the dephosphorylation of the accumulated P-sugars is inhibited (710). Arsenate- or fluoride-poisoned *S. pyogenes* cells synthesize little ATP, but they can metabolize arginine via the deiminase pathway and thereby generate ATP. The presence of arginine indeed restored sugar-P dephosphorylation and inducer expulsion in arsenate- or fluoride-poisoned *S. pyogenes* cells (710). It was therefore concluded that the first step of inducer expulsion depends on the presence of intracellular ATP and that the sugar-P phosphatase catalyzing this step might be regulated directly or indirectly via ATP-dependent protein phosphorylation. The search for this presumed P-protein led to the discovery of P-Ser-HPr: under conditions provoking inducer expulsion, a low-molecular-weight protein became phosphorylated by ATP (185, 710). This protein was identified as P-Ser-HPr (185), and it was therefore assumed that P-Ser-HPr might play a role in inducer expulsion, probably by controlling the activity of the sugar-P phosphatase (708, 710).

Experiments aimed at studying in vitro TMG-6-P expulsion by *L. lactis* vesicles supported this concept. When we discussed the effect of P-Ser-HPr formation on PTS-catalyzed carbohydrate uptake, we mentioned that vesicles prepared from *L. lactis* cells contain EI, HPr kinase, and a sugar-P phosphatase but had lost most of the HPr (970). As a result, *L. lactis* vesicles exhibit only about half the TMG uptake rate measured with intact cells (970). The full TMG uptake rate was restored when 100 μM *B. subtilis* HPr was electroporated into the *L. lactis* vesicles. Coelectroporation of wild-type or mutant HPrs with various metabolites was used to test whether phosphorylation of HPr at Ser-46 affects expulsion of TMG from the vesicles. *L. lactis* vesicles were able to accumulate TMG-6-P, which, similar to in intact cells, was expelled when glucose was added. Interestingly, vesicles into which HPr had been electroporated exhibited glucose-induced TMG expulsion that was about three times faster than that of vesicles that had been electroporated in the absence of HPr or in the presence of Ser46Ala mutant HPr (970). In addition, a cytoplasmic sugar-P phosphatase was activated about sevenfold when glucose was added to toluenized vesicles into which HPr had been electroporated prior to toluenization. Coelectroporation of FBP and HPr similarly enhanced the sugar-P phosphatase activity, and stimulation was even stronger when ATP was included. Because glucose and FBP stimulate the ATP-dependent phosphorylation of HPr at Ser-46 in *L. lactis* vesicles, it was assumed that the sugar-P phosphatase catalyzes the first step in inducer expulsion and that it is allosterically regulated by P-Ser-HPr. In agreement with this concept, electroporation of the P-Ser-HPr-resembling Ser46Asp HPr stimulated the intravesicular sugar-P phosphatase even in the absence of FBP (970), whereas only a weak increase of the phosphatase activity was observed with FBP and Ser46Ala mutant HPr. In addition to this cytoplasmic phosphatase, a membrane-associated *L. lactis* sugar-P phosphatase was also reported to be activated by P-Ser-HPr (973). Similarly, *S. bovis* contains a cytoplasmic and a membrane-associated sugar-P phosphatase (138). However, the activity of only the latter enzyme was stimulated by Ser46Asp mutant HPr. Membrane-associated, Ser46Asp HPr-activated sugar-P phosphatases were also detected in *S. pyogenes* and *E. faecalis*, two other organisms exhibiting inducer expulsion (712, 967). Inducer expulsion in all these bacteria was therefore assumed to be triggered by P-Ser-HPr-mediated activation of the membrane-associated sugar-P phosphatase. However, as heterologous systems were used for the in vitro electroporation experiments (HPr from *B. subtilis* and vesicles

from streptococci, lactococci, or enterococci), these results needed to be confirmed by in vivo experiments.

Inducer expulsion in *L. casei* and *L. lactis* does not require P-Ser-HPr. Similar to *L. lactis* ML_3, *L. casei* strains 64H and BL23 transport TMG via a lactose-specific PTS, accumulate it as TMG-6-P, and expel it from the cells as TMG when glucose or another rapidly metabolizable carbon source is added (111, 198). An *L. casei* mutant synthesizing Ser46Ala HPr (*ptsH1*) was constructed from strain BL23. Although P-Ser-HPr cannot be formed in this strain, it expelled accumulated TMG-6-P similarly to the wild-type strain when it was exposed to glucose or mannose. Identical results were obtained with *ptsH* mutants in which Ser-46 or Ile-47 had been replaced with a threonine (198). These results established that the expulsion of TMG in *L. casei* does not depend on P-Ser-HPr. To exclude the possibility that an HPr-like protein capable of substituting for P-Ser-HPr in inducer expulsion might exist in *L. casei*, TMG expulsion was also studied in an *L. casei hprK* mutant (198). Compared to inducer expulsion by a wild-type strain, no effect of the *hprK* mutation on glucose- or mannose-triggered expulsion of accumulated TMG-6-P was observed.

Because most experiments suggesting a role for P-Ser-HPr in inducer expulsion were carried out with *L. lactis* vesicles, a *ptsH1* mutant of this organism was constructed and tested for the expulsion of preaccumulated TMG-6-P and 2DG-6-P. Expulsion of both nonmetabolizable sugar derivatives was observed in the *ptsH1* mutant. However, whereas 2DG expulsion was nearly identical in the wild type and the mutant, TMG expulsion was slowed by about 2.5-fold in the *ptsH1* strain (569). Because *L. lactis* possesses only HPr and no HPr-like proteins (68), these results unequivocally established that inducer expulsion in this organism does not depend on the presence of P-Ser-HPr. Nevertheless, HPr seems to play an indirect role in TMG expulsion in *L. lactis*, which might partly explain the vesicle results, which led to the conclusion that P-Ser-HPr participates in inducer expulsion. Differences in the phosphorylation state of the $EIIBC^{Lac}$, which was proposed to catalyze not only TMG uptake but also TMG expulsion (719), in the wild-type and the *ptsH1* strains were suggested to be responsible for the indirect effect of the *ptsH1* mutation on TMG expulsion (569).

Is P-Ser-HPr Involved in Inducer Exclusion?

In vitro interaction of Ser46Asp mutant HPr with non-PTS permeases. As explained above (see REGULATION OF CARBON METABOLISM IN GRAM-NEGATIVE ENTERIC BACTERIA), the inducer exclusion mechanism in enteric bacteria is mediated by dephospho-$EIIA^{Glc}$, which binds to several target proteins (permeases and catabolic enzymes), thereby inhibiting their activity and preventing the entry or the intracellular formation of the corresponding inducer. Several lines of evidence indicated that P-Ser-HPr might play a similar role in inducer exclusion in gram-positive bacteria (Fig. 6). In *L. brevis*, the galactose/H^+ symporter GalP also catalyzes the uptake of TMG (193, 739). The addition of glucose arrests TMG uptake and causes the rapid efflux of preaccumulated TMG from the cells. Both effects of glucose were ascribed to P-Ser-HPr, as its structural homolog, Ser46Asp HPr (from *B. subtilis*) (952), labeled with ^{125}I was able to bind to the galactose/H^+ symporter of *L. brevis* (972). Galactose as well as galactose derivatives stimulated the binding of ^{125}I-labeled Ser46Asp HPr to the galactose/H^+ symporter about 10-fold. Coelectroporated metabolites known to stimulate the kinase activity of HprK/P (FBP, gluconate-6-P, and glycerate-2-P) as well as extravesicular glucose inhibited TMG uptake by *L. brevis* vesicles preloaded with *B. subtilis* HPr by electroporation about 10-fold (969). No inhibitory effect of glucose or its metabolites on TMG uptake was observed when Ser46Ala mutant HPr was used in place of wild-type HPr. Moreover, Ser46Asp mutant HPr slowed TMG uptake even in the absence of extravesicular glucose or intravesicular metabolites. Studies of 2DG uptake by *L. brevis* vesicles electroporated with wild-type or mutant HPrs and with glycolytic intermediates provided results that were almost identical to those obtained for TMG uptake (968). In addition, by expressing the *L. brevis galP* gene (encodes the galactose/H^+ symporter) in a *B. subtilis* wild-type strain and an *hprK* mutant, both *B. subtilis* strains gained the capacity to efficiently take up TMG. When the *B. subtilis* strains also synthesized *L. brevis* wild-type or Ser46Ala or Ser46Asp mutant HPr, a strong inhibition of TMG uptake was observed with the strain producing Ser46Asp HPr (193), suggesting that the P-Ser-HPr-resembling Ser46Asp HPr causes inducer exclusion. However, when the strain producing *L. brevis* wild-type HPr was grown in the presence of glucose, which stimulates the formation of P-Ser-HPr, no inducer exclusion occurred. In addition, since the experiments with *L. brevis* vesicles were carried out in vitro with *B. subtilis* wild-type or mutant HPrs instead of the physiologically relevant *L. brevis* P-Ser-HPr, the proposed implication of P-Ser-HPr in inducer exclusion needed to be confirmed in vivo.

Mutations preventing P-Ser-HPr formation abolish inducer exclusion. The importance of ATP-dependent HPr phosphorylation for inducer exclusion could be established by studying this regulatory process in *L. casei* and *L. lactis* mutants that are unable to form P-Ser-HPr. To detect a potential influence of the *ptsH1* and *hprK* mutations on inducer exclusion in *L. casei*, transport studies with maltose and ribose were carried out (198, 919). In *L. casei*, maltose and ribose are taken up by ABC transport systems (570). When wild-type cells are grown in a medium containing glucose and either ribose or maltose, diauxic growth with a long-lasting lag phase is observed. In addition, maltose uptake is immediately arrested when glucose is added to maltose-transporting *L. casei* cells (198, 919), suggesting that glucose inhibits maltose uptake via an inducer exclusion mechanism. Interestingly, in *ptsH1* (919) and *hprK* (198) mutants, glucose no longer exerts its inhibitory effect on maltose transport. These results imply that the phosphorylation of HPr at Ser-46 plays a role in inducer exclusion in *L. casei*. Inactivation of the *ccpA* gene had no effect on inducer exclusion (919), confirming that the P-Ser-HPr-dependent inhibitory effect of glucose on maltose uptake is due exclusively to an inhibition of the transport step and not to CCR of the maltose operon mediated via the P-Ser-HPr:CcpA complex. Additional evidence for P-Ser-HPr-mediated maltose exclusion from *L. casei* cells came from direct measurements of sugar consumption in the growth medium. In wild-type cells, maltose consumption is instantaneously arrested when glucose is added to the incubation mixture and does not restart before glucose is exhausted. In contrast, in the *hprK* and *ptsH1* mu-

tants, the addition of glucose causes only a short transient stop of maltose consumption, and the two sugars are subsequently simultaneously utilized (198, 919).

Interestingly, ribose transport was stimulated by the presence of glucose. Glucose stimulation of ribose uptake has also been reported for *Lactobacillus sakei*. Dephosphorylation of the PTS proteins in the presence of glucose was suggested to be responsible for the stimulating effect, as the inactivation of *ptsI* also led to enhanced ribose uptake (834, 835).

The repressive effect of glucose on maltose uptake and maltose consumption had also disappeared in an *L. casei* strain producing Ser46Thr mutant HPr, which is a poor substrate for HprK/P. Unexpectedly, inducer exclusion in *L. casei* was also prevented by the *ptsH*(*Ile47Thr*) mutation, although the amount of P-Ser-HPr detected in the *S. salivarius ptsH* (*Ile47Thr*) mutant was similar to that in the parental strain (263). The hydrophobic patch on the surface of HPr, which is formed by Ile-47, Met-48, and Met-51 in gram-positive bacteria (375), is involved in the interaction of P-Ser-HPr with CcpA (381, 787) and in the interaction of HPr with EI (260) and various EIIAs (139, 691, 949). The finding that the *L. casei ptsH*(*Ile47Thr*) mutant does not exhibit inducer exclusion suggests that this hydrophobic patch might also be important for the interaction of P-Ser-HPr with non-PTS permeases regulated by inducer exclusion.

Inducer exclusion studies were also carried out with an *L. lactis ptsH1* mutant. Similar to *L. casei*, this organism takes up maltose and ribose via specific ABC transport systems (68). The inhibitory effect of glucose on the transport of the two non-PTS sugars in the wild-type strain had completely disappeared in the *ptsH1* mutant (569).

An approximately twofold decrease in the P-Ser-HPr level was reported to lead to an almost complete loss of inducer exclusion in *S. salivarius* cells (870). The lower amount of P-Ser-HPr was due to specific point mutations in the *ptsH* gene or the *ptsH* leader sequence, which did not affect the uptake rate of PTS substrates, although the metabolism of PTS sugars was slowed. Wild-type *S. salivarius* cells immediately stop the uptake of the non-PTS sugar lactose or galactose when glucose or fructose is added to the growth medium and resume the metabolism of the non-PTS sugars only when glucose or fructose is exhausted (660). In contrast, the uptake of lactose or galactose by the *ptsH* mutants containing twofold-lower amounts of P-Ser-HPr was not affected by the presence of the PTS sugar glucose or fructose (870). To exert inducer exclusion, gram-positive bacteria therefore seem to require high levels of P-Ser-HPr.

In summary, the above-described results support the concept that inducer exclusion in gram-positive bacteria is regulated via the metabolite-activated formation of P-Ser-HPr. Considering the similarities between the maltose uptake systems from *E. coli* and those from the lactic acid bacteria *L. casei* and *L. lactis*, it is tempting to assume that the exclusion of maltose follows a mechanism analogous to that in gram-negative organisms, except that P-Ser-HPr instead of dephospho-$EIIA^{Glc}$ binds to MalK (Fig. 6) (Table 1). This assumption is supported by a comparison of the sequences of the nucleotide binding and regulatory domains of MalKs from *E. coli* (117) and *L. casei* BL23 (570). The two proteins are composed of very similar N-terminal nucleotide binding domains (amino acids 1 to 241) (60% identity), while the C-terminal regulatory domains (amino acids 242 to 371) exhibit only 19% sequence identity (Deutscher, unpublished). The sequence difference in the regulatory domain might reflect the binding of different effectors: $EIIA^{Glc}$ in gram-negative bacteria and P-Ser-HPr in gram-positive bacteria.

REGULATION OF CARBON METABOLISM IN LOW-G+C GRAM-POSITIVE BACTERIA: REGULATORY FUNCTIONS MEDIATED BY P~His-HPr AND P~EIIBs

PEP-Dependent Phosphorylation of Non-PTS Proteins

Besides their role as phosphocarriers during PEP-dependent phosphorylation of carbohydrates transported via the PTS, P~His-HPr and several P~EIIBs also phosphorylate non-PTS proteins and regulate their activities. Based on the characteristics of the phosphorylation site, three different types of non-PTS proteins phosphorylated by P~His-HPr and/or P~EIIB can be distinguished. In the simplest case, an EIIA domain, the usual phosphoryl acceptor of P~His-HPr within the PTS phosphorylation cascade, or an HPr domain is fused to the target protein. These PTS protein domains are not active in sugar transport but, rather, regulate the activity of the fusion proteins in response to their phosphorylation state. For instance, an HPr domain is fused to the response regulator HprR of a *C. acetobutylicum* two-component system (721). EIIA domains are fused to non-PTS sugar transporters (673) and to transcription activators such as MtlR of *G. stearothermophilus* (324) or LevR of *B. subtilis* (178), which control the expression of operons encoding sugar-specific PTS components (Table 1). The transcription regulators also contain an EIIB domain (295) for which neither phosphorylation nor a regulatory function could be demonstrated so far.

The second class of proteins phosphorylated by P~His-HPr and/or P~EIIBs contains a PRD (844). The PRDs seem to have specifically evolved to control the RNA binding activity of transcription antiterminators and the DNA binding function of transcription activators in response to phosphorylation by PTS proteins (Table 1). PRDs exhibit no obvious similarity to PTS proteins. Nevertheless, they usually contain two histidyl residues, which are phosphorylated by P~His-HPr or a P~EIIB. The sequences around the two phosphorylatable histidyl residues are conserved. Most PRD-containing proteins possess two PRDs either organized in tandem or sometimes separated by EIIA and EIIB domains (178, 295) (Fig. 7). Depending on which of the usually four conserved histidyl residues in the two PRDs is phosphorylated, the regulatory domains stimulate or inhibit the activity of their antiterminator or transcription activator (520, 884).

The third class of non-PTS proteins phosphorylated by P~His-HPr contains GlpKs from low-G+C gram-positive bacteria (Table 1). These GlpKs contain neither a PTS protein domain nor a PRD but developed a novel P~His-HPr phosphorylation site, i.e., a histidine that is usually surrounded by three aromatic amino acids (Tyr and Phe) (109). The phosphorylation site is located on a surface-exposed loop.

Here, we will discuss the complex regulation of PRD-

containing antiterminators and transcription activators in both gram-positive and gram-negative bacteria. The activity of these transcription regulators is controlled by multiple (up to fivefold) PEP-dependent PTS-catalyzed phosphorylations. The phosphorylation events can have antagonistic effects on the antitermination and transcription activation function (Table 1). The observations that these transcription regulators occur mainly in low-G+C gram-positive bacteria and that there are only a few representatives in proteobacteria and high-G+C gram-positive organisms will also be discussed. We will subsequently describe the P~His-HPr-mediated phosphorylation of lactose and raffinose transporters with an $EIIA^{Glc}$ domain and of a conserved histidyl residue in GlpK, regulatory processes so far observed only in gram-positive organisms (Table 1).

Regulation of Transcription Antiterminators by PTS-Mediated Phosphorylation

Regulation of gene expression by transcription attenuation. In addition to the most common mechanisms regulating gene expression, i.e., transcription activation or repression, bacteria also developed a mechanism based on transcription termination/antitermination, i.e., transcription attenuation, which was first described for the *E. coli trp* operon (60). Two principal mechanisms of transcription termination have been reported for prokaryotes: factor-dependent termination and intrinsic termination (965). In the first case, the RNA polymerase is halted at a terminator located at the end of a transcription unit. Interaction with a ρ factor is necessary to dissociate the DNA:RNA polymerase complex and to terminate transcription. In contrast, transcription units submitted to intrinsic termination contain a ρ-independent terminator. When the ρ-independent terminator is located between the transcription start site and the start codon (Fig. 8), the expression of the corresponding gene or operon usually occurs from a constitutive promoter. However, under noninducing conditions, transcription stops when the terminator is formed on the nascent mRNA. Formation of the terminator leads to the disruption of the elongation complex and to the release of short, incomplete transcripts. Under inducing conditions, an antiterminator binds to the mRNA in front of the terminator or to a region overlapping the terminator, thereby preventing the formation of the disruptive stem-loop structure and allowing the RNA polymerase to complete transcription of the corresponding gene or operon (Fig. 8). Antiterminators can be either proteins or oligonucleotides. When tRNAs function as antiterminators, binding to their target site on the mRNA is usually prevented when they are charged with their corresponding amino acid (136). Polypeptide antiterminators frequently respond to changes in the concentration of intracellular metabolites, which serve as cofactors and allow the corresponding antiterminator to bind to its target on the mRNA. For example, glycerol-3-P binds to and activates the *B. subtilis* antiterminator GlpP (749). Another mode of regulation was reported for antiterminators controlling the expression of genes and operons encoding either sugar-specific PTS components or enzymes catalyzing the extracellular degradation of polysaccharides to mono- or oligosaccharides, which are subsequently taken up via the PTS. This group of polypeptide antiterminators is regulated by reversible PTS-catalyzed phosphorylation. They possess an N-terminal RNA binding domain (first 55 amino acids) usually followed by two regulatory domains, each containing two conserved histidyl residues (Fig. 7), which are phosphorylated or dephosphorylated depending on whether the corresponding carbohydrate is present in the growth medium.

ρ-Independent terminators. The *B. subtilis* levansucrase-encoding *sacB* gene (31, 806) and the *E. coli bgl* operon, which encodes a β-glucoside-specific EIIBCA (*bglF*), a 6-P-β-glucosidase (*bglB*) (504) (Table 4), and a carbohydrate-specific outer membrane porin (20), were the first two PTS-related transcription units for which experimental evidence for regulation by antitermination has been obtained. The *B. subtilis sacB* leader region contains an imperfect 28-base-long inverted repeat called *sacR* (833). When part of *sacR* was deleted or when a point mutation was introduced, *sacB* was expressed constitutively, and *sacR* was therefore proposed to function as a transcription terminator (31, 806). A sucrose-inducible *sacB′-′lacZ* fusion also became constitutive when *sacR* was mutated, which further supported the concept of transcription termination (806). Finally, S1 nuclease mapping revealed that in the absence of sucrose, mainly small transcripts terminating within *sacR* were formed. The presence of the inducer sucrose did not change the total number of transcripts but increased the number of long transcripts extending beyond the terminator sequence *sacR* (806).

An inverted repeat between the transcription initiation site and the start codon of the *bgl* operon of *E. coli* was also detected (505). In the noninduced state, more than 90% of the transcripts, which were initiated at the *bgl* promoter, were already terminated at the inverted repeat. Expression of a *bgl-lacZ* fusion containing the inverted repeat required an intact *bglG* (formerly *bglC*) gene, which is the first gene of the *bgl* operon, while *bgl-lacZ* fusions devoid of the inverted repeat exhibited constitutive, *bglG*-independent expression (505). Based on these results, it was proposed that the inverted repeat located between the transcription initiation site and the start codon of the *E. coli bgl* operon serves as a ρ-independent transcription terminator and that the protein encoded by *bglG* exerts a positive effect on *bgl* expression by functioning as an antiterminator. In agreement with this hypothesis, *bglG* disruption caused drastically lowered expression from the *bgl* promoter (504). However, a few *bglG* point mutations, including the one present in the *bglC9* strain described previously (682), can lead to the constitutive expression of the *bgl* operon.

Terminators similar to those preceding *B. subtilis sacB* and the *E. coli bgl* operon have been found in front of many other transcription units encoding sugar-specific PTS components of the glucose/sucrose class. Mutation or partial deletion of the terminator sequences preceding the *B. subtilis sacPA* (24), *bglPH* (430), or *ptsGHI* (448) operon also led to the constitutive expression of the corresponding transcription unit and thus confirmed their negative role in mRNA synthesis.

RAT sequences are the binding sites for PTS-regulated antiterminators. Sequencing of the *bgl* operon allowed the detection of a second terminator located in the intercistronic region between *bglG* and *bglF*. Interestingly, the sequences preceding the two *bgl* terminators are very similar. They were assumed to form weak stem-loops partly overlapping the downstream terminator (785). Insertion of 6 bp in the region

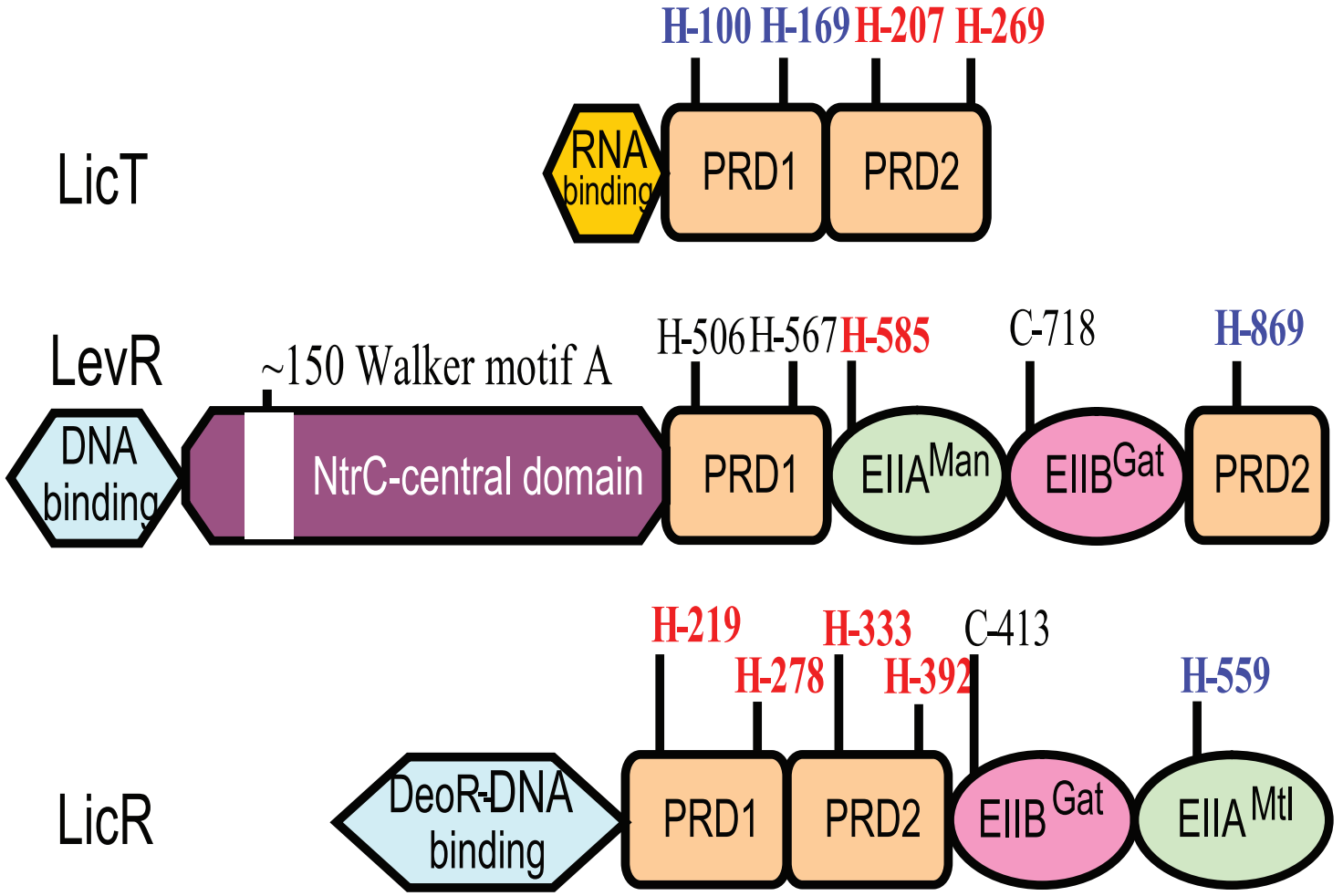

FIG. 7. Domain structure of the transcription antiterminator LicT and the transcription activators LicR and LevR of *B. subtilis*. The N-terminal RNA or DNA binding domain and the two regulatory domains PRD1 and PRD2 together with their conserved histidyl residues are indicated. Histidyl residues, which have been shown to become phosphorylated, are in boldface type. When the phosphorylation exerts a positive effect on the activity of the regulator, they are shown in red, whereas blue indicates the sites of negative regulation. For LicT, its four conserved histidyl residues become phosphorylated. Phosphorylation in PRD1 inhibits, while phosphorylation in PRD2 stimulates, LicT activity. Most other antiterminators of the BglG/SacY family also contain four conserved histidines, but sometimes, not all of them can be phosphorylated. LevR contains an NtrC-like central domain and $EIIA^{Man}$- and $EIIB^{Gat}$-like domains inserted between the complete PRD1 and a truncated PRD2. The positive site of phosphorylation is His-585, and the negative site is His-869. The conserved histidyl residues His-506 and His-567 in PRD1 and the $EIIB^{Gat}$ phosphorylation site (Cys-718) do not seem to become phosphorylated. An identical organization can be observed in most other NifA/NtrC-type PRD-containing transcription activators. However, a few LevR-like regulators contain a complete PRD2 with an additional potentially phosphorylatable histidyl residue. The DNA binding domain of LicR resembles that of DeoR, and PRD1 and PRD2 are followed by an $EIIB^{Gat}$-like and $EIIA^{Mtl}$-like domain. An identical domain organization can be observed in other DeoR-type PRD-containing transcription activators. All four conserved histidines in PRD1 and PRD2 are sites of positive regulation, while LicR activity is inhibited by phosphorylation at His-559 in the $EIIA^{Mtl}$-like domain.

upstream from the terminator preceding *bglG* indeed diminished the induction of a *bgl-lacZ* fusion (505), suggesting that the region upstream from this terminator is important for antitermination and might represent the binding site for BglG. The conserved sequences preceding the terminators were called ribonucleotidic antiterminator targets (RATs) (32). In in vitro experiments, BglG was indeed able to specifically bind to an RNA probe containing the entire *E. coli bgl* RAT sequence but only the 5′ half of the terminator (344). In contrast, if an RNA probe containing the entire RAT sequence and the complete terminator was used, very weak binding of BglG was observed. The formation of the terminator was therefore assumed to prevent the formation of the BglG binding site, i.e., the RAT stem-loop. In agreement with this concept, BglG binding to the longer RNA probe was improved when a DNA fragment, which was complementary to the 3′ part of the inverted repeat and which therefore prevented formation of the terminator, was included in the assay mixtures. These results suggested that there is competition between the formation of the terminator and the RAT stem-loop (344). Under noninducing conditions, BglG cannot bind to its mRNA, and the terminator will therefore be formed. Calculations revealed that the formation of the terminator releases much more free energy than the formation of the RAT stem-loop. Under inducing conditions, the binding of the "activated" BglG is assumed to favor the formation of the RAT stem-loop. BglG thereby prevents the formation of the terminator partly overlapping the RAT sequence and allows the synthesis of long transcripts (344).

In *B. subtilis*, sequences very similar to those of the *E. coli bgl* RATs precede the above-mentioned terminator *sacR* (833) and the terminators located in front of the *B. subtilis* 1,3–1,4-β-D-glucan endoglucanase-encoding *bglS* (formerly *licS*) gene (585, 785), the *sacPA* operon (164), which encodes a sucrose-specific EIIBC (*sacP*) and an endocellular sucrase (*sacA*) (32), and *ptsG* (847), which encodes the glucose-specific EIIBCA (Table 4). RAT sequences are also present in front of PRD-containing antiterminator-controlled genes from other organisms (964). An extensive genetic analysis of the RAT sequence preceding the *sacB* gene confirmed its importance for *sacB* induction. Expression of *sacB′-lacZ* fusions containing mutations at positions assumed to be important for the formation of the RAT stem-loop was barely inducible with sucrose (32).

The antiterminators regulating the expression of the four above-mentioned *B. subtilis* transcription units have been identified. SacY regulates *sacB* transcription (832), SacT controls the expression of *sacPA* (164), LicT is necessary for *bglS* but also for *bglPH* expression (430), and GlcT controls *ptsGHI* transcription (847) (Table 4). They all exhibit significant sequence similarity to BglG of *E. coli*. The new family of antiterminators was called the BglG/SacY family, as the *B. subtilis sacB* gene and the *E. coli bgl* operon, which are regulated by SacY and BglG, respectively, were the first transcription units for which this termination/antitermination mechanism was established.

The RAT sequences preceding *sacB* and *sacPA* differ in only three positions (Table 4), and consequently, cross talk has been observed: SacY also controls *sacPA* expression, and SacT regulates the transcription of *sacB* (32, 146). In gel shift experiments, not only SacT but also SacY could bind to RNA fragments containing the RAT sequence of *sacPA* (23). In vitro interactions of the RNA binding domains of LicT, SacY, and GlcT with their respective RAT sequences on *bglPA*, *sacB* (168), and *ptsGHI* (448) mRNA have been demonstrated by carrying out surface plasmon resonance experiments. The dissociation constants determined in these experiments varied from 3 μM (SacY) to 10 nM (LicT). The *S. carnosus* GlcT homolog regulates the expression of *glcA* and *glcB*, which encode a glucose- and a β-glucoside-specific EIICBA, respectively (131). The RAT sequence in the leader region of *glcAB* of *S. carnosus* strongly resembles the RAT sequence of *B. subtilis ptsGHI*. As a consequence, *S. carnosus* GlcT interacts with the RAT sequence of *B. subtilis ptsGHI*, as was shown by mutant complementation assays and surface plasmon resonance experiments (413). The high affinity between LicT and its *bglPA* RAT allowed the determination of the solution structure of the LicT catalytic domain:mRNA complex (964). The antiterminator interacts mainly with the minor groove of the double-stranded RNA formed by two internal loops and the stem in between. In summary, the above-described results make it clear that RAT sequences are the binding sites for BglG/SacY-type antiterminators.

In addition to regulating *sacB* expression, SacY controls its own synthesis by regulating the expression of the *sacS* locus containing the two genes *sacX* and *sacY* (993). The *sacX* gene encodes an EIIBC with strong similarity to sucrose-specific EIIs. It is preceded by a RAT sequence and a terminator, which are separated by about 100 bp, whereas in all other transcription units regulated by BglG/SacY-type antiterminators, the RAT sequence and the terminator are overlapping or located next to each other. Nevertheless, both regulatory elements are functional, as mutations within the RAT sequence prevent the induction of *sacXY* expression, while a partial or complete deletion of the terminator sequence causes constitutive expression (885). It was therefore proposed that the formation of the RAT stem-loop preceding *sacXY* is stabilized by binding SacY, which in turn prevents the formation of the distant terminator via not-further-specified long-range effects (885).

Phosphorylation of BglG/SacY-type antiterminators by PTS proteins. After RAT sequences were identified as target sites for proteins of the BglG/SacY family, the signal controlling the affinity of the antiterminators for the RAT elements remained to be determined. The early studies on SacY-regulated *sacB* and BglG-controlled *bgl* operon expression had already suggested that these antiterminators might be controlled by the PTS. In the late 1970s, it was discovered that the inactivation of *B. subtilis* EI causes "constitutive" expression of *sacB* (i.e., synthesis of full-length mRNA) (264, 635). In fact, it was this property of the PTS that was used to successfully clone the *B. subtilis ptsHI* operon (283). A promoterless kanamycin resistance gene put under the control of the *sacB* promoter conferred sucrose-inducible kanamycin resistance after integration into the *B. subtilis* genome. After transposon mutagenesis was carried out, a strain expressing the kanamycin resistance gene in the absence of sucrose was isolated. The mutant had the transposon inserted into the *ptsHI* operon (283), thus confirming that *ptsHI* inactivation leads to "constitutive" expression from the *sacB* promoter.

Another study from the same year showed that the inacti-

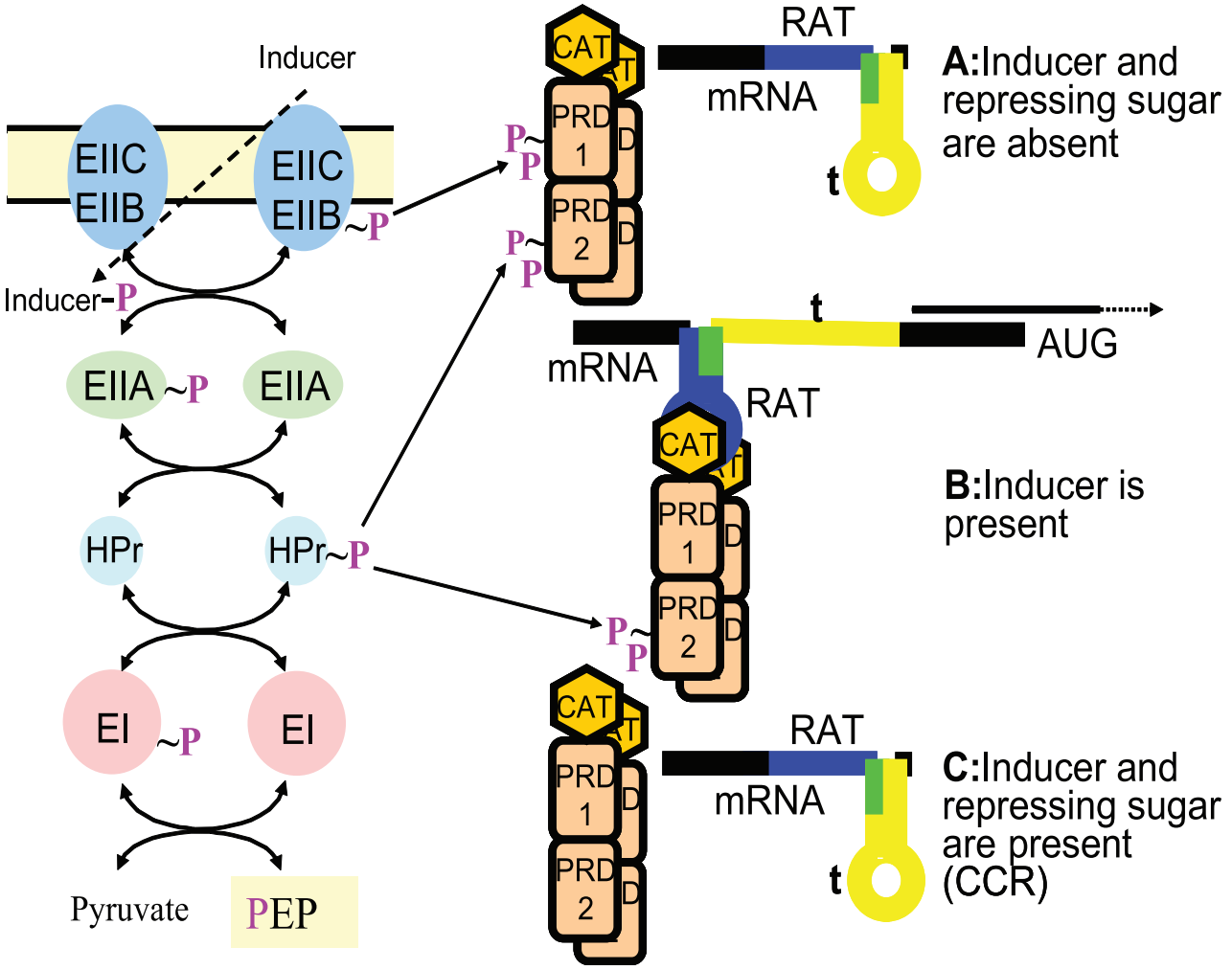

FIG. 8. Transcription regulation by the PTS via PRD-containing transcription antiterminators. (A) In the absence of the corresponding inducer, full-length transcription of several PTS-encoding genes/operons is inhibited owing to the formation of a terminator structure (t, yellow) on the nascent mRNA upstream from the start codon. Under these conditions, the corresponding antiterminator cannot bind to its RNA target, RAT (blue), because the EIIB is mainly phosphorylated and transfers its phosphoryl group to PRD1 of the antiterminator. The absence of a repressing sugar is expected to also allow phosphorylation at the activating domain (PRD2) by P~His-HPr. However, the negative effect of phosphorylation at PRD1 is dominant. The RAT sequence can also form a stem-loop, which, however, was calculated to free less energy than the terminator t. Interestingly, in most antiterminator-controlled PTS operons, the two sites RAT and t overlap (green), and the formation of the terminator therefore prevents the formation of the RAT stem-loop and vice versa. (B) If an inducer is present, the EIIB as well as PRD1 of the corresponding antiterminator will be present mainly in an unphosphorylated form. Because antiterminator-controlled PTSs are usually low-capacity sugar transporters, there will be sufficient P~His-HPr to guarantee activating phosphorylation in PRD2. The activated antiterminator binds to its RAT and thus favors the formation of the RAT stem-loop, thereby preventing the formation of the terminator stem-loop, as part of it (in green) is already used for the RAT stem-loop. (C) If, in addition to the inducing sugar, a repressing carbohydrate is present, the amount of P~His-HPr will be low in the cells. In firmicutes, P-Ser-HPr will also be formed, which further lowers the amount of P~His-HPr. These conditions prevent activating phosphorylation at PRD2, and most antiterminators are therefore inactive, although the presence of the inducer probably prevents the phosphorylation in PRD1. Dephosphorylation of PRD2 in the presence of a rapidly metabolizable PTS sugar therefore represents a CcpA-independent, P-Ser-HPr-dependent CCR mechanism. *ptsH1* as well as *licT*(Pia) but not *ccpA* mutants are relieved from this type of CCR.

vation of *E. coli bglF*, the gene located downstream from *bglG*, caused "constitutive" expression of the *bgl* operon (i.e., synthesis of full-length mRNA) (504). Because *bglF* encodes a β-glucoside-specific EIIBCA (79, 785), it was concluded that BglG/SacY-type antiterminators might be controlled by a phosphorylation cascade formed by the general PTS proteins EI and HPr and those sugar-specific EIIAs and EIIBs, the synthesis of which is regulated by the antiterminator. In vitro phosphorylation of BglG by P~EIIBCA^{Bgl} was indeed reported (18). However, the regulation of antiterminators by PTS-catalyzed phosphorylation turned out to be very complex. First, EI and HPr were later found to be able to phosphorylate most antiterminators in the absence of EIIA and EIIB. Second, the activity of some antiterminators depends on functional EI and HPr, and the inactivation of *ptsI* therefore does not lead to the "constitutive" expression of the genes controlled by these antiterminators but, on the contrary, lowered or prevented their expression. Third, BglG/SacY-type antiterminators are usually phosphorylated at more than one of the four conserved histidyl residues in their PRDs.

Extensive genetic, biochemical, and structural studies carried out mainly with the four *B. subtilis* antiterminators were necessary to understand in detail how BglG/SacY-type antiterminators are regulated.

In principle, two types of phosphorylation with antagonistic effects on the antiterminator activity have to be distinguished. P~EIIB-requiring phosphorylation in PRD1 inactivates the antiterminator (site of negative regulation). The absence of phosphorylation in PRD1 leads to full-length expression of the corresponding transcription units. By contrast, phosphorylation by EI and HPr in PRD2 stimulates the activity of antiterminators (site of positive regulation). The absence of phosphorylation in PRD2 leads to CCR of the corresponding genes and operons via a CcpA-independent mechanism (Fig. 8).

Antiterminator-dependent induction is regulated via P~EIIBs. "Constitutive" expression from the promoter of an antiterminator-controlled operon following inactivation of the related EII has been observed not only for *E. coli bglF* (504) but also for several *B. subtilis* transcription units as well as for the *lac* operon of *L. casei* (289). In *B. subtilis*, disruption of *sacX* (147), *bglP* (430, 454), or *ptsG* (847) leads to inducer-independent expression of *sacB*, *bglPH*, and *ptsGHI*, respectively. As mentioned previously, *B. subtilis ptsI* mutants exhibit "constitutive" *sacB* transcription (264, 283, 635), and the deletion of *ptsH* was reported to lead to the synthesis of full-length *ptsGHI* mRNA (847). It therefore appeared that a functional phosphorylation cascade composed of the general PTS proteins EI and HPr and sugar-specific EIIAs and EIIBs leads to the inactivation of the corresponding antiterminator.

Interestingly, SacX, the inactivation of which also results in "constitutive" *sacB* expression (147), contains EIIB and EIIC but no EIIA domain, and it was therefore concluded that EIIB, the last link in the PTS phosphorylation chain (Fig. 1), regulates the induction of antiterminator-controlled transcription units. Experiments with the *L. casei lac* operon confirmed the importance of P~EIIB for antiterminator-mediated induction, as the inactivation of *lacE* as well as *lacF* (encoding $EIICB^{Lac}$ and $EIIA^{Lac}$, respectively) led to the synthesis of full-length *lacTEGF* mRNA (289). A different mechanism had originally been proposed for the regulation of *E. coli* BglG, which was based on the erroneous assumption that His-306 of *E. coli* $EIIBCA^{Bgl}$ would be the phosphorylation site in the EIIB domain (784). The replacement of His-306 with nonphosphorylatable amino acids prevents β-glucoside transport but, in contrast to *bglF* disruption, does not lead to the synthesis of full-length *bgl* mRNA. It was therefore proposed that EIIA and not EIIB of $EIIBCA^{Bgl}$ would regulate BglG activity, and $EIIA^{Glc}$ was also suggested to phosphorylate and inhibit BglG (783). This concept proved to be wrong when the phosphorylation site in the EIIB domain of *E. coli* $EIICB^{Glc}$ was identified as being Cys-421 (547), which is equivalent to Cys-24 in $EIIBCA^{Bgl}$. A strain containing a *bglF*(*Cys24Ser*) mutation not only was unable to transport β-glucosides but also expressed a *bgl-lacZ* fusion in the absence of an inducer (β-glucoside) (120), confirming that *E. coli* BglG is also regulated via the EIIB domain of the corresponding PTS permease.

P~EIIBs are necessary for the phosphorylation of a histidine in PRD1. To determine which of the usually four conserved histidyl residues in the PRDs would be the target for P~EIIB-mediated regulation, extensive genetic analyses were carried out by replacing the histidyl residues in the *B. subtilis* antiterminators SacY and LicT with various amino acids and by measuring the effect of the mutations on reporter gene fusions in different genetic backgrounds. Replacement of His-99 in PRD1 of SacY with a Tyr (883) or Ala (884) causes constitutive expression of the *sacB* gene. In contrast, replacing the three other conserved histidyl residues in SacY with Tyr has little effect on the antitermination activity (883). Similarly, replacement of His-100 in *B. subtilis* LicT (884) or His-101 in *L. casei* LacT (288), the equivalents of His-99 of SacY, with alanine leads to the synthesis of full-length *bglPH* or *lacTEGF* mRNA, respectively. These results strongly suggest that phosphorylation of the first conserved histidyl residue in PRD1 by the corresponding P~EIIB prevents induction by PRD-containing antiterminators. Attempts to mimic phosphorylated antiterminators by replacing the first phosphorylatable histidine in PRD1 with negatively charged Asp or Glu were only partly successful. Putting a negative charge at position 99 by replacing His-99 with Glu or Asp indeed inactivates SacY and abolishes *sacB* induction (884). Similarly, the His101Asp replacement in *L. casei* LacT prevents *lac* operon expression (288). However, although His100Asp LicT exhibited significantly lower activity than His100Ala LicT, it was not completely inactive (884), indicating that in LicT, the His100Asp replacement is not completely phosphomimetic. Moreover, replacement of His-104 with an Asp in *B. subtilis* GlcT even led to strongly "constitutive" expression of a *ptsG'-'lacZ* fusion (35), suggesting that the mutant protein does not at all resemble GlcT phosphorylated at the first conserved histidine in its PRD1.

The original concept for the regulation of BglG/SacY-type antiterminators implied that P~EIIBs directly phosphorylate and thereby inactivate their corresponding antiterminator (18). The finding that the first conserved histidine in PRD1 of several antiterminators is phosphorylated in vitro by P~His-HPr therefore came as a surprise (413, 781, 883, 884). For instance, His-99, the site of negative regulation in *B. subtilis* SacY, is efficiently phosphorylated in vitro by EI and HPr in the absence of SacX (883). Because P~His-HPr is also necessary for the phosphorylation of EIIBs, it was not clear whether P~EIIBs directly phosphorylate their corresponding antiterminators or merely stimulate the P~His-HPr-mediated phosphorylation. It proved difficult to distinguish between the two possibilities, as it was not easy to obtain P~EIIB preparations that were free of HPr (EIIBs are often fused to their membrane-integrated EIICs, and HPr usually sticks to membranes). Because the inactivation of EI as well as SacX leads to constitutive expression of the SacY-controlled *sacB* gene, it was proposed that in the cells, efficient P~His-HPr-mediated phosphorylation of SacY at His-99 would require the presence of P~SacX. GlcT of *S. carnosus* also becomes phosphorylated in vitro by EI and HPr in the absence of an EIIB, and mass spectrometry with proteolytic fragments obtained from phosphorylated and dephospho-GlcT revealed that phosphorylation occurs primarily at His-105 (413), the equivalent of His-99 in SacY. In the case of *S. carnosus* GlcT, phosphorylation in PRD1 was assumed to stimulate the antitermination activity. In contrast, in experiments with GlcT from *B. subtilis*, P~His-HPr barely phosphorylated PRD1 but mainly transferred its phosphoryl group to His-210 in PRD2 (781) (for PRD2 phosphorylation, see the next section). In fact, the first conserved

TABLE 4. Description of some well-studied PRD-containing antiterminators

Antiterminator	Organism	Controlled gene(s)	Inducer(s)	RAT sequence
LicT	*B. subtilis*	*bglPH* *bglS*	β-Glucosides	GGATTGTTACTGCGAAAGCAGGCAAAACC (*bglP*) GGATTGTTACTGATAAAGCAGGCAAAACC (*bglS*)
SacT	*B. subtilis*	*sacPA* (*sacB*)[a]	Sucrose	GGATTGTGACTGGTAAAGCAGGCAAGACC (*sacP*)
SacY	*B. subtilis*	*sacB* (*sacPA*)[a] *sacXY*	Sucrose	GGTTTGTTACTGATAAAGCAGGCAAGACC (*sacB*) GGATTGTGACTGGGCAGGCAGGCAAGACC (*sacX*)
GlcT	*B. subtilis*	*ptsG*(*HI*)	Glucose	ACGTGTTACTGATTCGATCAGGCATCAGT (*glcT*)
GlcT	*S. carnosus*	*glcA*, *glcB*	Glucose, β-glucosides	ACGTGTAACTAATTCGATTAGGCATGAGT (*glcA*)
BglG	*E. coli*	*bglFB* *bglG*	β-Glucosides	GGATTGTTACCGCACTAAGCGGGCAAAACC (*bglF*) GGATTGTTACTGCATTCGCAGGCAAAACC (*bglG*)
LacT	*L. casei*	*lacTEGF*	Lactose	TGGATTGTGACTATTTAATTAGGCGACC (*lacT*)

[a] The similarity of the RAT sequences preceding *sacB* and *sacPA* probably explains the cross talk observed with the two antiterminators SacY and SacT.

histidine in PRD1 of *B. subtilis* GlcT has been shown to become phosphorylated by P∼EIIB^{Glc}. When the soluble *B. subtilis* EIIBA^{Glc} domains were synthesized without the membrane-integrated EIIC^{Glc} (to which they are normally fused), HPr-free P∼EIIBA^{Glc} could be prepared, which could phosphorylate wild-type GlcT but not GlcT(His104Ala). The antiterminator became phosphorylated in PRD2 only when HPr was added to the phosphorylation mixture containing P∼EIIBA^{Glc} and GlcT(His104Ala), thus confirming the nearly complete absence of HPr from the P∼EIIBA^{Glc} preparation (781). It therefore seems that phosphorylation of some antiterminators (*B. subtilis* SacY and *S. carnosus* GlcT) at PRD1 is catalyzed by P∼His-HPr, and this reaction is stimulated by the corresponding P∼EIIB, while phosphorylation in PRD1 of other antiterminators (GlcT of *B. subtilis*) occurs via the corresponding P∼EIIB.

Based on genetic studies with *licT*, it was suggested that P∼EIIB domains might also sequester their corresponding antiterminator (884). A similar mechanism is operative for the *E. coli* transcription regulator Mlc, which is sequestered by the unphosphorylated EIIB domain of EIICB^{Glc} (458, 588, 856) (see "REGULATION OF CARBON METABOLISM IN GRAM-NEGATIVE ENTERIC BACTERIA"). Preliminary evidence for the formation of a complex between an antiterminator and an EII was obtained for *S. carnosus* GlcT, which exists as an active dimer and an inactive monomer. Dimerization is favored by P∼His-HPr-mediated phosphorylation at His-105. Membrane fragments containing EIICBA^{Glc} (and possibly P∼EIICBA^{Glc}) specifically interacted only with the dimers in a GlcT monomer/dimer mixture (413). Calorimetric studies had established that the binding of sugar molecules to their respective EIIs, which leads to the dephosphorylation of the EIIB domain, causes extensive structural changes (546). In the case of *S. carnosus* EIICBA^{Glc}, the structural changes accompanying the binding of glucose were assumed to lead to a release of active P∼GlcT dimers, thus allowing the expression of the *glcAB* operon in response to the presence of a substrate.

Unlike antiterminators from *B. subtilis* and *L. casei*, the negative regulation site of BglG from *E. coli* was originally thought to be located in PRD2, and His-208 was reported to be phosphorylated by P∼EIIBCA^{Bgl} (121). However, the phosphorylation mixtures for BglG phosphorylation contained not only P∼EIIBCA^{Bgl} but also EI and HPr. Like most antiterminators, BglG is phosphorylated by EI and HPr in PRD2 (287) (see the next section), and Chen et al. (121) probably misinterpreted the P∼His-HPr-dependent modification as being P∼EIIBCA^{Bgl}-dependent phosphorylation. In fact, the negative regulation site of *E. coli* BglG has been identified as His-101, and phosphorylation at this amino acid requires the presence of P∼EIIBCA^{Bgl} (285).

In summary, it can be concluded that induction via most BglG/SacY-type antiterminators is regulated by P∼EIIB-controlled or -mediated phosphorylation of the first conserved histidyl residue in PRD1, which inhibits the activity of the antiterminator. In the presence of the corresponding inducer, i.e., the carbohydrate transported by (or, in the case of *B. subtilis*, SacX probably bound to) the EII regulating the antiterminator, the antiterminator will barely be phosphorylated at its first conserved histidine in PRD1, because phosphorylation of the inducer is probably faster than phosphorylation of the antiterminator (482) or rephosphorylation of the EIIB by its corresponding P∼EIIA (Fig. 8). For *B. subtilis* SacY, the presence of sucrose in the growth medium indeed significantly reduced the extent of its in vivo phosphorylation (359). Antiterminator molecules that are not phosphorylated at the first conserved histidine in PRD1 are able to bind to their RAT sequence, thus preventing the formation of the terminator and allowing the synthesis of full-length mRNA of their target gene or operon. If the inducer is absent, P∼EIIB will be formed, which leads to phosphorylation of the first histidine in PRD1 and/or sequestration of the corresponding antiterminator. Both effects prevent the binding of the antiterminator to its RAT sequence and therefore the induction of the corresponding gene or operon. According to genetic analyses with *licT* of *B. subtilis* (884) and *bglG* of *E. coli* (285), phosphorylation at the second conserved histidine in PRD1 plays a detectable but minor role in the induction process. By contrast, replacing either His-101 or His-159 in *L. casei* LacT with alanine leads to "constitutive" expression of the *lac* operon, suggesting that in

this antiterminator, the two conserved histidines in PRD1 contribute equally to the induction process (288).

Some antiterminators need to be activated by phosphorylation in PRD2. Based on the effect of *ptsI* or *ptsH* mutations on the activity of BglG/SacY-type antiterminators, two classes of transcription attenuators can be distinguished. The first class includes *B. subtilis* SacY and GlcT. Mutations in *ptsI* or *ptsH* result in the "constitutive" expression of their target genes *sacB* (147, 264, 635) and *ptsGHI* (847), respectively. In these mutants, neither P∼His-HPr nor P∼EIIB is formed, which prevents the inactivating phosphorylation at PRD1 of the antiterminator in the absence of an inducer. The second class of antiterminators includes *B. subtilis* SacT and LicT and *E. coli* BglG. The synthesis of the full-length mRNA of their target genes *sacPA* (24) and *bglPH* in *B. subtilis* (429, 430, 454, 482) and *bglGFB* in *E. coli* (287) is almost completely prevented when *ptsH* or *ptsI* is inactivated. These results were surprising, as we had learned in the previous section that the inactivation of, for example, the EIIBCA^{Bgl}-encoding *bglP* leads to the synthesis of full-length mRNA from the LicT-controlled *bglPH* promoter (430, 454). Because EI and HPr are essential for the phosphorylation of EIIBCA^{Bgl}, which in turn inactivates LicT by phosphorylating it at His-100 in PRD1, the disruption of *ptsH* or *ptsI* should also lead to the synthesis of full-length *bglPH* mRNA. The observed dependence of LicT, SacT, and BglG activity on functional EI and HPr therefore suggested that the second class of antiterminators is subject to dual control by PTS proteins: positive regulation by P∼His-HPr and negative control by their corresponding P∼EIIBs. Because the specific replacement of His-15, the site of EI-catalyzed phosphorylation in HPr, with an alanine also prevented LicT activity (429), it was likely that EI and HPr exert their positive effect by phosphorylating this class of antiterminators. Indeed, SacT (23), LicT (482), and BglG (287) are phosphorylated by PEP, EI, and HPr. In LicT, all four conserved histidines are phosphorylated by the general PTS proteins, with the main site of phosphorylation being His-207 in PRD2 (482, 884). Because the P∼EIIB-dependent negative effect is mediated via the first conserved His in PRD1 (see the previous section), it was likely that the positive effect of phosphorylation by EI and HPr is mediated via PRD2. Experiments with SacY/LicT hybrid proteins confirmed this assumption. When PRD2 of the EI/HPr-independent SacY was replaced with PRD2 of LicT, the resulting hybrid protein was EI and HPr dependent (884). This concept was further supported by an extensive genetic analysis, during which the conserved histidines in PRD2 of *B. subtilis* LicT were replaced with various amino acids and the effect of the mutations on *bglP′-lacZ* expression was measured in different *pts* genetic backgrounds. Replacement of His-207 or His-269 or both conserved histidines in PRD2 with Ala led to a complete loss of LicT antitermination activity, i.e., prevented the synthesis of full-length mRNA from the *bglPH* promoter even in the presence of its inducer salicin or in a *bglP* background. In contrast, in a *bglP* mutant, if either one of these two histidines or both were replaced with an Asp, which probably mimics a phosphorylated histidine, expression from the *bglPH* promoter remained "constitutive" irrespective of whether *ptsHI* were functional or deleted (884). These results demonstrate that PEP-, EI-, and HPr-mediated phosphorylation of PRD2 in antiterminators of the second class stimulates their activity. This is also true for *E. coli* BglG, where His-208, the only conserved phosphorylatable histidine in PRD2, was first claimed to be the site of negative regulation by P∼EIIBCA^{Bgl} (121) but was later identified as the site of positive regulation by P∼His-HPr (285, 287). Interestingly, *E. coli* FruB, a fusion protein composed of HPr- and EIIA^{Fru}-like domains (267), can replace HPr in BglG phosphorylation and activation (287).

CCR mediated by dephosphorylation of PRD2 of antiterminators. Surprisingly, although the activity of *B. subtilis* SacY and GlcT does not depend on EI and HPr, they are phosphorylated by PEP, EI, and HPr in their PRD2 (781, 883). It seems that during the course of evolution, SacY and GlcT developed a PTS-independent antitermination activity but retained the ability to become phosphorylated in PRD2. On the contrary, GlcT of *S. carnosus*, which is probably also EI/HPr independent, is not phosphorylated in its PRD2, as only one major P∼His-HPr-dependent phosphorylation site, His-105 in PRD1, could be detected (413). Interestingly, EI/HPr-independent antiterminators seem to regulate the expression of transcription units that are insensitive to CCR, whereas the EI/HPr-dependent LicT and SacT control operons that are preceded by *cre*'s recognized by the P-Ser-HPr:CcpA complex (429). It was therefore assumed that phosphorylation of LicT and SacT by PEP, EI, and HPr might serve as an additional, CcpA-independent CCR mechanism.

The first evidence for antiterminator-mediated CCR came from studies with a *B. subtilis ccpA* mutant. In this mutant, expression of a *bglP′-lacZ* fusion was not completely relieved from CCR. The residual CCR (about 20%) disappeared when the terminator preceding the *bglPH* operon was deleted, suggesting that *bglPH* is regulated by two CCR mechanisms: one that is CcpA dependent and one that is terminator dependent (429). Interestingly, the residual CCR also disappeared when a *ptsH1* mutation (which prevents the formation of P-Ser-HPr) was introduced into the *ccpA* mutant. It was therefore assumed that the CcpA-independent CCR mechanism operative for the *bglPH* operon was based on diminished phosphorylation in PRD2 of LicT due to the formation of P-Ser-HPr (a poor substrate for PEP-dependent phosphorylation by EI) during growth on a rapidly metabolizable carbon source. This assumption was supported by studies with *B. subtilis* mutants producing LicTs in which either one of the two conserved histidines in PRD2 was replaced with Asp. Expression of a *bglP′-lacZ* fusion remained inducible in both mutants, but β-galactosidase activity was less severely repressed by glucose than in a strain producing wild-type LicT (909). Unequivocal proof for the antiterminator-mediated CCR mechanism came from studies with strains producing EI/HPr-independent mutant LicTs, which were called LicT(Pia) (Pia stands for PTS-independent antitermination). Starting from a strain carrying a *bglPH′-aphA3* (*aphA3* confers kanamycin resistance to bacteria) and a *bglP′-lacZ* fusion as well as the LicT-inactivating *ptsGHI* deletion, several spontaneous mutants that had gained the ability to express the LicT-dependent *bgl* fusions despite the absence of functional EI and HPr were isolated (483). The isolated mutants were all affected in *licT*, and the various mutations apparently render LicT activity independent of functional EI and HPr. The *licT*(Pia) mutants can be divided into two classes: mutations affecting PRD1 (they lead to constitutive expression from the *bglPH* promoter) and mutations affecting PRD2 (they

remain inducible). The latter type of mutant LicTs resembles the naturally EI/HPr-independent SacY and GlcT. Interestingly, the residual CCR of the *bglPH* operon detectable in *ccpA* mutants disappeared when wild-type *licT* was replaced with one of the *licT*(Pia) alleles (483), thus confirming that it is the poor phosphorylation of PRD2 in LicT during the uptake of rapidly metabolizable carbon sources that triggers CcpA-independent CCR.

Based on the results obtained with the various LicT(Pia) proteins, distinct amino acids were proposed to be important for the transfer of the activating signal from PRD2 via PRD1 to the RNA binding domain (483), which was called CAT (coantiterminator) (964). When the crystal structures of the two PRDs of the constitutively active His207Asp, His269Asp mutant LicT (909) and of inactive wild-type LicT (292) were solved, a more detailed understanding of the structural changes involved in LicT activation was possible. In fact, the structures of the PRD2 of the two crystallized LicT forms are completely different, while the amino acid backbones of their PRD1s are almost identical. In inactive wild-type LicT, the two PRD2s of the LicT dimer make no contact to each other but are in contact with the two PRD1s. The four phosphorylatable histidines in the PRD2s are exposed to the surface and easily accessible for phosphorylation. In the activated His207Asp, His269Asp mutant LicT, a 180° swing movement of the C-terminal domain leads to extensive structural changes, placing the aspartyl residues (which replace the four histidines) in the center of PRD2, thus rendering them inaccessible from the outside. Two major rotations are responsible for the rearrangement of the PRD2s. Both take place in the interdomain region between PRD1 and PRD2, with one occurring at residues Leu-165 and Asn-166 and the other occurring at Met-169. As a consequence, the two PRD2s of His207Asp, His269Asp mutant LicT are in close contact, whereas most interactions with PRD1 are suspended. These alterations are probably responsible for the changes in the interdomain region connecting PRD1 and CAT, which in turn are thought to cause rearrangements of the CAT domain, ultimately leading to the activation of LicT (292). The monomer/dimer transition does not seem to be important for LicT activation, as the RNA binding domain CAT and active His207Asp, His269Asp mutant LicT as well as inactive dephospho-LicT form dimers (964). Nevertheless, the monomer/dimer transition might be important for the regulation of other antiterminators (56).

The structure of the RNA binding domain of the two antiterminators SacY and LicT has been intensively studied by NMR (510) and X-ray crystallography (910). The solution structure of the RNA binding domain of LicT (first 55 amino acids) attached to its mRNA has also been solved (964). However, the structure of an entire BglG/SacY-type antiterminator has so far not been determined.

Based on the above-described results, the following model for CCR mediated by EI/HPr-dependent antiterminators can be proposed. In the presence of a carbon source rapidly transported via the PTS, the antiterminators will have to compete with the corresponding sugar-specific EIIA for the common phosphoryl donor P~His-HPr. P~His-HPr probably transfers its phosphoryl group primarily to EIIAs, thus leaving the antiterminators in the unphosphorylated, less active state. In in vitro experiments, P~His-HPr indeed phosphorylates EIIAs much faster than antiterminators (482). In addition, the ATP-dependent phosphorylation of HPr at Ser-46 in low-G+C gram-positive bacteria, which is triggered by the presence of rapidly metabolizable carbon sources, drastically slows the PEP-dependent phosphorylation of HPr at His-15 and therefore further diminishes the activation of LicT by P~His-HPr-dependent phosphorylation. The proposed complex mechanism of induction and CCR of operons controlled by EI/HPr-dependent antiterminators is outlined in Fig. 8. The model requires that during PTS sugar uptake, the corresponding EIIB domain is present mainly in the dephospho form and therefore does not phosphorylate the first conserved His in PRD1 of LicT. On the other hand, P~His-HPr must be present in sufficient amounts to allow the phosphorylation in PRD2, which implies that PTS operons, the expression of which is regulated by an EI- and HPr-dependent antiterminator, encode low-capacity transport systems, as strong autorepression would otherwise occur. Probably in order to prevent autorepression, the operons for the *B. subtilis* high-capacity glucose and sucrose PTS are regulated by EI- and HPr-independent antiterminators.

Distribution of BglG/SacY-type antiterminators in bacteria. In a several-years-old review on PRD-containing transcription regulators, the BglG/SacY antiterminator family was reported to contain 13 members (844). In addition to the above-mentioned antiterminators, it included LicT of *Bacillus amyloliquefaciens*, AbgG of *Clostridium longisporum* (87), ArbG of *Pectobacterium chrysanthemi* (formerly *Erwinia chrysanthemi*) (208), BglR of *L. lactis* (42), CasR of *Klebsiella oxytoca* (446), and SurT of *G. stearothermophilus* (473). Owing to the many genome sequences completed in the meantime, a simple BLAST search yields numerous new potential family members. A few of these novel PRD-containing antiterminators have been studied in more detail. BvrA of *L. monocytogenes* controls the expression of the *bvrABC* operon encoding the antiterminator, a β-glucoside-specific EIIBCA, and a presumed ADP-ribosylglycohydrolase (81), respectively. In *L. monocytogenes*, repression of several PrfA-controlled virulence genes by the β-glucosides cellobiose and salicin requires an intact *bvr* locus, while repression by arbutin, glucose, or fructose persists in a *bvrAB* mutant. BglG of *L. plantarum* was reported to control the expression of the *bglGPT* operon encoding the antiterminator BglG, a β-glucoside-specific EII, and a 6-P-β-glucosidase (513), while LicT of *S. mutans* regulates an esculin-specific PTS (141). *C. acetobutylicum* ScrT was proposed to control the expression of the *scrAKB* operon encoding a sucrose-specific EII, a fructokinase, and a sucrose-6-P hydrolase, respectively (860). In the actinobacterium *Corynebacterium diphtheriae*, a BglG/SacY-type antiterminator-encoding gene is located downstream from the EIIBCA^{Glc}-encoding *ptsG* gene (631). BglT of *Pectobacterium carotovorum* probably controls the expression of an operon that encodes a β-glucoside-specific PTS and strongly resembles the *arb* operon of *P. chrysanthemi* (19). BglG/SacY-type antiterminators are especially abundant in clostridia. With nine BglG/SacY homologs, *Clostridium difficile* seems to be the organism that contains the highest number of PRD-containing antiterminators, followed by *L. plantarum*, which contains five such proteins. In fact, BglG/SacY-type antiterminators are present in most firmicutes including bacilli, clostridia, enterococci, lactobacilli, lactococci, listeriae, leu-

conostocs, staphylococci, streptococci, etc. BglG/SacY-type antiterminators occur less frequently in gram-negative bacteria. So far, in addition to *E. coli*, PRD-containing antiterminators have been detected in proteobacteria such as *P. carotovorum* (BglT) (19), *P. chrysanthemi* (ArbG) (208), *Klebsiella oxytoca* (CasR) (446), *Klebsiella aerogenes* (BglG) (690), *Shigella flexneri*, *Shigella sonnei*, *Shigella boydii*, *Photorhabdus luminescens*, and *Yersinia enterocolitica*. Interestingly, a gene coding for a BglG/SacY-type antiterminator is also present in the pathogenicity island of a uropathogenic *E. coli* strain (393). It is preceded by a gene encoding an EIICB resembling the glucose-specific EII (PtsG) of *E. coli*. However, whether this system is related to the virulence of this organism is not known. BglG/SacY homologs are also present in the actinobacteria *Bifidobacterium longum*, *Corynebacterium diphtheriae*, *Propionibacterium acnes*, and *Symbiobacterium thermophilum*. The antiterminator-controlled expression of a fructose PTS in *Bifidobacterium breve* has been studied in more detail (534a).

Regulation of Transcription Activators by PTS-Mediated Phosphorylation

PRDs in transcription activators. Transcription activators are DNA binding proteins usually containing a helix-turn-helix motif. They bind upstream from their target promoter, interact with a specific RNA polymerase holoenzyme, and thereby stimulate transcription. Based on the σ factor associated with the RNA polymerase, different classes of transcription activators can be distinguished. The first class comprises NifA/NtrC-type transcription activators. They usually interact with RNA polymerase containing σ^{54} (also called RpoN in *E. coli* and SigL in *B. subtilis*). They are composed of an N-terminal regulatory domain, which is usually a receiver domain of a two-component system, a central domain interacting with σ^{54}, and a C-terminal helix-turn-helix motif. A nucleotide binding site (Walker motifs A and B) is present in the central domain. In the case of NtrC, phosphorylation of its receiver domain by the sensor histidine kinase NtrB (396, 608) strongly stimulates its ATPase activity, which allows NtrC to enhance the formation of open complexes by the RNA polymerase/σ^{54} holoenzyme (943). NifA/NtrC-type transcription activators bind to the DNA at about 100 bp upstream from their target promoters, and DNA bending is necessary to allow an interaction with σ^{54} (705). In a few cases, the binding site for the transcription activator is located even more than 1 kb away from the target promoter (698, 809). Owing to their mode of action, these transcription activators are also called enhancer DNA binding proteins (EBP).

A second class of transcription activators interacts with the major σ^{70}-containing RNA polymerase holoenzyme. This class is very large and heterogenous and includes regulator proteins, which, depending on the location of their binding site, function as transcription activators (binding upstream from the promoter) and/or repressors (the binding site overlaps the promoter or is located downstream from it). This class includes the DeoR family of transcription regulators containing repressors such as *E. coli* GlpR (790) and SrlR (formerly GutR) (961), *B. subtilis* IolR (981), and *L. lactis* LacR (986) but also transcription activators such as *E. coli* FucR (125), *B. anthracis* AcpA (414), and *S. pyogenes* VirR (537). Often, DeoR-like transcription regulators do not contain a clearly identifiable nucleotide binding site. Recognition of their target sequence is controlled by low-molecular-weight effectors such as sugar phosphates, which interact with the C-terminal ligand-binding domain.

Several transcription activators belonging to one of the above-described classes are regulated neither by ligand-binding (DeoR type) nor via phosphorylation by a sensor kinase (NifA/NtrC type) but instead possess PRDs, suggesting that their activity is controlled by PEP-dependent, PTS-catalyzed phosphorylation. PRDs thus seem to be used not only to regulate the RNA binding activity of antiterminators but also to control the DNA binding function of some members of the above-described classes of transcription activators (Fig. 7). Interestingly, all PRD-containing transcription activators also possess an EIIA and an EIIB domain. The EIIB domain in transcription activators always exhibits similarity to EIIBs of the galactitol class PTS (295). In contrast, the EIIA domain belongs to the mannose class PTS in regulators with an NifA/NtrC-like σ^{54} binding domain (178) and to the mannitol/fructose class PTS in regulators with a DeoR-like DNA binding domain. The type of EIIA domain present in transcription regulators seems to be related to the specificity of the PTS that they control. Three cases can be distinguished: (i) transcription activators containing NifA/NtrC- and $EIIA^{Man}$-like domains control the expression of operons coding for PTSs belonging to the mannose or lactose class; (ii) transcription activators with DeoR- and $EIIA^{Mtl}$-like domains regulate the expression of operons coding for PTSs of the mannitol, lactose, or glucitol class; and (iii) PRD-containing antiterminators (see the previous section) are devoid of an EIIA domain and primarily regulate the expression of operons coding for PTSs belonging to the glucose/sucrose class but also several PTSs of the lactose class.

Domain organization in NifA/NtrC-type PRD-containing transcription activators. The best-studied PTS-controlled transcription activator is *B. subtilis* LevR, which regulates the expression of the *lev* operon (165). The *levR* gene is located just upstream from the *lev* operon, which encodes the EIIs for a low-capacity fructose/mannose-specific PTS (LevD, LevE, LevF, and LevG) and an extracellular levanase (SacC) capable of degrading fructose polymers such as levan (110, 519, 522). Expression of the *lev* operon occurs from a σ^{54} (σ^{L})-dependent −12, −24 promoter, is induced by low concentrations of fructose, and is repressed by rapidly metabolizable carbon sources. Although the Lev PTS transports fructose, high concentrations of this sugar repress *lev* operon expression, because *B. subtilis* also possesses a high-capacity fructose PTS encoded by the *fruA* gene (437).

LevR stimulates the activity of the RNA polymerase/σ^{54} holoenzyme after binding to a UAS, which was identified by footprint experiments (521) and is centered at about 110 bp upstream from the *lev* promoter. LevR contains an N-terminal helix-turn-helix motif (positions 34 to 54) (521) and thus differs from NifA/NtrC-type transcription activators with a receiver domain of two-component systems, which possess the DNA binding motif at the C terminus. The second domain of LevR resembles the central domain in NifA/NtrC-type transcription activators (165). This domain probably interacts with RNA polymerase associated with the alternate sigma factor σ^{54} and contains Walker motifs A (148-GPTGSGKS-155) and B (221-

GILFMDEI-228) for ATP binding and hydrolysis. In LevR, the NifA/NtrC-like central domain is followed by a complete PRD. A second, truncated PRD containing only one conserved histidyl residue is located at the C terminus of LevR (see Fig. 7 for the domain structure of LevR).

The region separating the complete and the truncated PRD of LevR (from positions 575 to 820) is much longer than that in BglG/SacY-type antiterminators. A BLAST search with the sequence separating the two PRDs in LevR revealed that the region from amino acids 578 to 707 exhibits significant similarity to EIIAs of the mannose class PTS (178) (Fig. 7). A histidine equivalent to the phosphorylatable histidine of *E. coli* $EIIA^{Man}$ (110, 840) is present in position 585. The strongest similarity was detected with $EIIA^{Man}$ of *Vibrio furnissii*. In addition, the sequence between the $EIIA^{Man}$-like domain and the truncated PRD from positions 708 to 807 was found to exhibit significant similarity to EIIBs from the galactitol class PTS (GatB, SgaB, and SgcB of *E. coli*) (295). These EIIBs contain a conserved cysteyl residue close to the N terminus, and an equivalent cysteyl residue is present in the EIIB-like domain of LevR at position 718. It therefore seems that LevR evolved by numerous domain fusions and insertions. It is tempting to assume that the two PRDs were added to the central domain of a NifA/NtrC-type regulator before the EIIA and EIIB domains were inserted between the PRDs. LevR contains a total of five potential PTS phosphorylation sites, two in PRD1, one in the EIIA and EIIB domains, and one in PRD2 (Fig. 7).

Interestingly, the *L. casei lev* operon (*levABCDX*) is not preceded by a −12, −24 promoter but is preceded by a −10, −35 promoter. It was therefore assumed that *L. casei* LevR, which controls the expression of the *lev* operon of this organism, does not interact with σ^{54}. Indeed, while the disruption of *levR* strongly diminished *lev* operon expression, inactivation of the σ^{54}-encoding *rpoN* gene had no effect (534). In addition, the amino acid sequence motif -GAFTGA-, known to be essential for the interaction of NifA/NtrC-type EBPs with σ^{54}, is absent from the central domain of *L. casei* LevR. Nevertheless, the domain organization of *L. casei* LevR is identical to that in *B. subtilis* LevR, and the two proteins exhibit 41% sequence similarity. So far, *L. casei* LevR is the only known protein of this family that does not interact with σ^{54} but that interacts with another σ factor (presumably σ^{70}).

Antagonistic effects of PTS-mediated phosphorylation reactions on LevR activity. The binding of *B. subtilis* LevR to its UAS and/or its interaction with σ^{54} was thought to be regulated by PTS-catalyzed phosphorylation (165). This assumption was based on the observation that mutations affecting LevD or LevE, the fructose-specific EIIA and EIIB of the Lev PTS, caused strong constitutive expression from the *lev* promoter (520, 522). Mutants carrying deletions of the EI- and HPr-encoding *ptsI* and *ptsH* genes or synthesizing His15Ala or Ser46Asp mutant HPr (the latter is a poor substrate for EI) also exhibit elevated LevR activity, which, however, is considerably lower than that in *levD* ($EIIA^{Lev}$) and *levE* ($EIIB^{Lev}$) mutants (526, 846). Deletion of *ptsHI* in a *levD* or *levE* strain diminished the constitutive LevR activity, indicating that EI and HPr exert a positive effect on LevR function (846). These results suggest that similar to the second, PTS-dependent class of BglG/SacY-type antiterminators, LevR might also be regulated by multiple PTS-catalyzed phosphorylations in its PRDs and possibly also in the EIIA and EIIB domains (Table 1).

Indeed, PEP-dependent, EI- and HPr-mediated phosphorylation of *B. subtilis* LevR occurs at His-585, which was originally thought to be part of PRD1 (520) but was later identified as the phosphorylatable His in the $EIIA^{Man}$ domain (178). His-488 of *L. casei* LevR is equivalent to His-585 of *B. subtilis* LevR and was accordingly identified as a phosphorylation site by P∼His-HPr (534). Replacement of this phosphorylatable His in LevR of *B. subtilis* or *L. casei* with an alanine diminishes *lev* operon expression, thus supporting the concept that EI/HPr-catalyzed phosphorylation exerts a positive effect on the transcription activation function of LevR (520, 534).

A second PEP-dependent phosphorylation requiring $EIIA^{Lev}$ and $EIIB^{Lev}$ (in addition to EI and HPr) occurs at the single conserved histidyl residue present in the C-terminally truncated PRD: His-869 in *B. subtilis* LevR (520) and His-776 in *L. casei* LevR (534). Experiments with *B. subtilis* $[^{32}P]$∼$EIIB^{Lev}$, which had been separated from EI, HPr, and $EIIA^{Lev}$ after its phosphorylation by these proteins, strongly suggested that P∼LevE directly phosphorylates LevR at His-869 (520). The fact that His869Ala mutant LevR was not phosphorylated by the $[^{32}P]$∼$EIIB^{Lev}$ preparation proved that it was almost free of $[^{32}P]$∼His-HPr. Inactivation of *levD* ($EIIA^{Lev}$) and *levE* ($EIIB^{Lev}$) as well as the replacement of His-869 of *B. subtilis* LevR (or His-776 of *L. casei* LevR) with an alanine cause constitutive expression from the *lev* promoter of both organisms. These results confirm that phosphorylation in PRD2 mediated by PEP, EI, HPr, $EIIA^{Lev}$, and $EIIB^{Lev}$ exerts an inhibitory effect on the transcription activation function of LevR.

Mutation of the first conserved histidine (His-506) in PRD1 also slightly affects *B. subtilis* LevR activity. However, *B. subtilis* and *L. casei* mutant LevRs in which both identified phosphorylatable histidines had been replaced with alanine were not phosphorylated in vitro either by EI and HPr or by EI, HPr, $EIIA^{Lev}$, and $EIIB^{Lev}$ (520, 534). These results suggest that in wild-type LevR, neither the two conserved histidyl residues in PRD1 nor the conserved cysteyl residue in the $EIIB^{Gat}$ domain is phosphorylated.

The results described above establish that the transcription activator LevR in *B. subtilis* and *L. casei* is regulated by at least two PTS-catalyzed phosphorylation reactions exerting antagonistic effects on LevR activity. Control of LevR therefore appears to be a complex kinetic phenomenon similar to LicT regulation. Although the kinetics of the phosphoryl group transfer reactions are barely understood, a model can be proposed on the basis of the above-mentioned biochemical and genetic results. In in vitro experiments, *B. subtilis* P∼$EIIB^{Lev}$ phosphorylates not only fructose (110) but also His-869 in PRD2 of LevR (520). According to genetic data, the latter reaction inhibits LevR activity, and it is therefore likely that the induction of the *lev* operon requires the dephosphorylation of His-869. In fact, the uptake of fructose via the Lev PTS will lead to the dephosphorylation of not only P∼$EIIB^{Lev}$ but, as a consequence of the reversibility of the phosphoryl transfer, probably also P∼His(869)-LevR. Because the phosphorylation of HPr by EI is very fast and because the Lev-PTS is a low-capacity fructose transport system, one can assume that cells

transporting fructose via the Lev-PTS probably contain relatively high concentrations of P~His-HPr, which are probably sufficient for the phosphorylation of LevR at His-585 in the $EIIA^{Man}$-like domain. As a consequence, during Lev-PTS-catalyzed fructose uptake, a major fraction of LevR will probably be present in the fully active form, i.e., phosphorylated at His-585 and dephosphorylated at His-869. Dephosphorylation of His-585 will occur when a rapidly metabolizable carbon source, such as glucose, is present in the growth medium. The uptake of glucose will lower the P~His-HPr level and, consequently, the activity of LevR, even when it is dephosphorylated at His-869. Indeed, LevR activity in *levD* Δ*ptsH* or *levE* Δ*ptsH* double mutants was reported to be about 15-fold lower than that in *levD* or *levE* single mutants (846). Poor phosphorylation of His-585 during growth on a rapidly metabolizable carbon source serves as a CcpA-independent CCR mechanism and accounts for the residual CCR, which was observed when a *ccpA* disruption was introduced into an inducer-independent mutant (*levE* or *levR8*). The residual CCR almost completely disappeared in a *ccpA ptsH1* double mutant (526), which, due to the inability to form P-Ser-HPr, probably contains a larger amount of P~His-HPr.

Because LevR contains a NifA/NtrC-like central domain, it is likely that LevR activates transcription by a mechanism similar to that operating in non-PRD-containing NifA/NtrC-type transcription activators. In the latter proteins, phosphorylation of the receiver domain by a sensor kinase stimulates the ATPase function located in the central domain and, consequently, the formation of open complexes by RNA polymerase (943). As mentioned above, the central domain of LevR also contains a nucleotide binding site. It is therefore tempting to assume that phosphorylation of the $EIIA^{Man}$-like domain by EI and HPr leads to increased LevR ATPase activity, while phosphorylation in PRD2 inhibits it. Nevertheless, how the inactivating/activating signals are transmitted from PRD2 and the $EIIA^{Man}$-like domain, respectively, to the NifA/NtrC-like central domain remains an open question. The structure of LevR or one of its homologs has so far not been solved.

Distribution of LevR-like transcription activators. For a long time, the only known homolog of LevR was CelR from *G. stearothermophilus* (445). CelR has the same regulatory domain organization as *B. subtilis* LevR (Fig. 7), to which it exhibits 47% sequence similarity. Histidyl residues equivalent to the two phosphorylation sites in *B. subtilis* LevR (His-585 and His-869) are also present in CelR. The *celR* gene precedes an operon encoding a cellobiose-specific PTS (lactose class) and a presumed 6-P-β-glucosidase (446). CelR was therefore assumed to control the expression of this operon by a mechanism similar to that used by LevR for the regulation of the *B. subtilis lev* operon.

The *L. monocytogenes* lmo1721 gene encodes a LevR-like regulator (called CsrA, but it is unrelated to *E. coli* CsrA), the inactivation of which prevents the repressive effect of cellobiose on the activity of PrfA, a transcription activator of numerous virulence genes in this organism, while the repressive effect of glucose on PrfA persisted in a *csrA* mutant (558a). The *csrA* gene precedes and probably regulates an operon containing a potential −12, −24 promoter and three genes, which code for an EIIA and an EIIB of a lactose/cellobiose class PTS and an outer surface protein. However, this operon is missing an EIIC, and it is therefore not clear whether the EIIA and EIIB are involved in cellobiose transport. Interestingly, *L. monocytogenes* contains an operon encoding a complete potential cellobiose-specific PTS (EIIA, EIIB, and EIIC, lmo2683 to lmo2685), but no regulator gene precedes or follows this operon. Nevertheless, this operon is preceded by a potential −12, −24 promoter identical to the one located in front of the *csr* operon [TGGCAC(N)5TTGCAT] (Deutscher, unpublished). It is possible that this operon is also regulated by CsrA and that *csrA* inactivation therefore prevents the expression of both potential cellobiose operons and, consequently, the synthesis of the cellobiose-specific PTS components and cellobiose transport. However, more specific effects of CsrA and/or the EIIA and EIIB encoded by the *csr* operon can presently not be excluded. It is noteworthy that cellobiose-specific inhibition of PrfA also disappeared in mutants affected in *bvrAB*, which code for a PRD-containing antiterminator and a β-glucoside-specific EIIBCA (81) (see the preceding section).

Homologs of *B. subtilis* LevR are present in many other low-G+C gram-positive organisms such as bacilli, clostridia, enterococci, lactobacilli, listeriae, *Pediococcus pentosaceus*, and *Thermoanaerobacter tengcongensis*, but they are also present in the actinobacterium *S. thermophilum* and in gram-negative bacteria such as *S. enterica* serovar Typhimurium, *Salmonella enterica*, *K. pneumoniae*, and *Salmonella enteritidis*, although they are much less frequent in the latter organisms. The highest number of LevR-like proteins (seven) was detected in *C. difficile*. The large number of LevR homologs in several bacteria suggests that these proteins are not used exclusively for the regulation of the expression of operons coding for fructose-, mannose-, or cellobiose-specific PTS. Indeed, the *L. casei esu* operon is regulated by a LevR-like protein and encodes a PTS that probably transports a sugar ester (976). Most of the approximately 100 LevR-like proteins detected have a size similar to that of *B. subtilis* LevR and exhibit between 40 and 60% sequence similarity to each other. They contain conserved equivalents of the two phosphorylatable histidyl residues, confirming the importance of these two amino acids in the regulation of the activity of LevR-like transcription activators. In contrast, an equivalent of His-506 of *B. subtilis* LevR, the first conserved histidine in PRD1, is absent from several LevR homologs, implying that this amino acid does not play an essential role in the regulation of LevR-like proteins. One *E. faecalis* and one *C. difficile* LevR homolog contain a complete second PRD, thus possessing an additional potentially phosphorylatable histidyl residue.

DeoR-type PTS-controlled transcription activators. A protein containing two PRDs and an N-terminal domain resembling the DNA binding domain in transcription activators of the *E. coli* DeoR family was first detected in *G. stearothermophilus* (324). The corresponding gene is located in the *mtlARFD* operon, in which *mtlA* codes for a mannitol-specific EIICB, *mtlR* codes for the PRD-containing regulator of the operon, *mtlF* codes for an $EIIA^{Mtl}$, and *mtlD* codes for a mannitol-1-P dehydrogenase. Soon afterwards, another protein with a DeoR-like N-terminal DNA binding domain followed by two PRDs was found in *B. subtilis* (878). This protein, LicR, controls the expression of the *licRBCAH* operon, which encodes the transcription activator LicR; the EIIB, EIIC, and EIIA components of a β-glucoside-specific PTS (belonging to the lactose class); and a presumed 6-P-β-glucosidase, respec-

tively. The genome sequence revealed that *B. subtilis* contains two other transcription activators possessing a DeoR-like N-terminal domain and PRDs: MtlR (YdaA) and ManR (YjdC). They were proposed to regulate the expression of a mannitol-specific and a mannose-specific PTS, respectively (844). Similar proteins in other bacteria have been described. They include MtlR (342) and SrlR (78) of *S. mutans* and MtlR of *C. acetobutylicum* (51). Extensive genetic and biochemical studies have been carried out only with MtlR of *G. stearothermophilus* and LicR from *B. subtilis*.

Domain organization in DeoR-type PRD-containing transcription activators. The transcription activators MtlR of *G. stearothermophilus* and LicR of *B. subtilis* possess an N-terminal DNA binding domain (Fig. 7), which exhibits significant similarity (around 50%) to the first approximately 180 amino acids of transcription activators of the DeoR family such as *E. coli* FucR (125), *B. anthracis* AcpA (414), and *S. pyogenes* VirR (537). In most DeoR-type transcription activators, a traditional helix-turn-helix motif is located at the beginning of the N-terminal domain. However, in a few PTS-controlled regulators, the helix-turn-helix motif is degenerated and difficult to detect (51). The suggested presence of a second helix-turn-helix motif in LicR (879) has so far not been confirmed. The DNA binding domain is followed by two PRDs, with PRD1 beginning at about amino acid 185 (Fig. 7). Each PRD usually contains two conserved histidyl residues. The PRDs are followed by an $EIIB^{Gat}$ domain containing a conserved and potentially phosphorylatable cysteyl residue (Cys-423 in *G. stearothermophilus* MtlR and Cys-413 in *B. subtilis* LicR) (295). The last domain of DeoR-type PRD-containing transcription activators is an EIIA domain belonging to the mannitol/fructose class PTS. In $EIIA^{Mtl}$ of *S. carnosus*, His-62 has been identified as a PEP-dependent phosphorylation site (703). An equivalent histidyl residue is present in the $EIIA^{Mtl/Fru}$-like domains of LicR of *B. subtilis* (His-559) (879) and MtlR of *G. stearothermophilus* (His-598) (323). In total, these regulators contain six potential PTS phosphorylation sites (Fig. 7).

PTS-mediated control of DeoR-type PRD-containing transcription activators. Phosphorylation of *G. stearothermophilus* MtlR was observed in the presence of PEP, EI, and HPr (323). The P~His-HPr-mediated phosphorylation was completely prevented when either one of the two conserved histidyl residues in PRD2 was replaced with an alanine. However, His348Ala, His405Ala, and His348Ala/His405Ala mutant MtlRs were phosphorylated when, in addition to EI and HPr, $EIIA^{Mtl}$ and $EIICB^{Mtl}$ were also present in the phosphorylation assay (323). The phosphorylation of MtlR in the presence of $EIIA^{Mtl}$ and $EIICB^{Mtl}$ was strongly diminished when the conserved His-598 in the C-terminal $EIIA^{Mtl/Fru}$-like domain was replaced with an alanine. The residual weak phosphorylation of His598Ala mutant MtlR was probably due to phosphorylation in PRD2 by the low amounts of P~His-HPr present in the reaction mixture. In conclusion, MtlR becomes phosphorylated by EI and HPr at one or both conserved histidyl residues in PRD2 and in an EI-, HPr-, $EIIA^{Mtl}$-, and $EIICB^{Mtl}$-requiring reaction at His-598 in the $EIIA^{Mtl/Fru}$-like domain. Phosphorylation of MtlR therefore clearly differs from *B. subtilis* LevR phosphorylation, where EI and HPr phosphorylate the $EIIA^{Man}$-like domain and phosphorylation in the presence of $EIIA^{Lev}$ and $EIIB^{Lev}$ occurs in PRD2 (520).

Similar to LevR, MtlR binds to a DNA region located upstream from the promoter, as was demonstrated in footprint experiments with *G. stearothermophilus* MtlR and a DNA fragment extending about 125 bp upstream and downstream from the transcription start site of the *mtl* operon. Apparently, MtlR does not interact with a continuous DNA stretch, as within a sequence of about 50 bp, five small regions were protected against DNase I digestion in the footprint experiments (325). The phosphorylation state of MtlR had a strong effect on its DNA binding activity. MtlR phosphorylated by EI and HPr in PRD2, but dephosphorylated in the $EIIA^{Mtl/Fru}$-like domain, exhibited an approximately 100-fold-higher affinity for its target DNA than did unphosphorylated MtlR (323). The DNA binding affinity of neither the His348Ala nor the His405Ala mutant MtlRs was enhanced by the presence of P~His-HPr (as mentioned above, mutation of either His prevents phosphorylation in PRD2). In contrast, phosphorylation in the presence of EI, HPr, $EIIA^{Mtl}$, and $EIICB^{Mtl}$ at His-598 in the $EIIA^{Mtl/Fru}$-like domain and in PRD2 lowered the affinity of MtlR for its DNA target about 10-fold compared to the unphosphorylated regulator. Surprisingly, not only phosphorylation of His-598 but also its replacement with an alanine had a negative effect on DNA binding, which could not be overcome by P~His-HPr-mediated phosphorylation (323). It is possible that the mutation causes structural changes leading to the inactivation of the transcription regulator. In conclusion, MtlR is controlled by at least two PTS-mediated phosphorylation events that exert antagonistic effects. Similar to LicT and LevR, the absence of EI- and HPr-mediated phosphorylation in PRD2 of MtlR serves as an alternate CCR mechanism. In contrast, phosphorylation in the $EIIA^{Mtl/Fru}$-like domain, which requires the additional presence of $EIIA^{Mtl}$ and $EIICB^{Mtl}$, exerts a negative effect on MtlR activity. This phosphorylation is probably prevented when mannitol is present in the growth medium, and it is therefore likely that dephosphorylation at His-598 serves as an induction mechanism for the *mtl* operon. The role of PRD1 in MtlR regulation is not clear. Although the replacement of either one of the conserved histidyl residues in PRD1 diminished the stimulatory effect of P~His-HPr on MtlR activity, phosphorylation of these histidines could not be detected. Similar to what was proposed for LevR, PRD1 in MtlR might be important only for the signal transfer from PRD2 to the DNA binding domain.

Expression of the *mtl* operon in *S. carnosus* (232) and *B. subtilis* seems to be regulated by a mechanism resembling that in *G. stearothermophilus*, as the *mtl* operon of both organisms contains a 50-bp-long upstream activating sequence similar to that recognized by MtlR of *G. stearothermophilus* (325). In addition, the *mtl* operon of *S. aureus* exhibits a gene order (*mtlARFD*) identical to those in *G. stearothermophilus* and *S. mutans* (341, 342). In contrast, *ydaA*, which encodes the *B. subtilis* MtlR homolog, is a monocistronic transcription unit located about 15 kb downstream from the *mtl* operon (437). YdaA was renamed MtlR (844), as it exhibits significant sequence identity (39%) to MtlR from *G. stearothermophilus*. MtlR of *S. mutans* exhibits only 28% sequence identity and 48% similarity to MtlR of *G. stearothermophilus*. Surprisingly, only a polar insertion into *mtlR* was reported to prevent mannitol utilization by *S. mutans*, whereas a nonpolar insertion had no effect (342). Therefore,

whether MtlR of *S. mutans* carries out functions similar to those of MtlR of *G. stearothermophilus* is questionable. Finally, an MtlR homolog of *C. acetobutylicum* was reported to control the expression of the *mtlARFD* operon of this organism (51).

The DNA binding site for LicR, the second PRD-containing DeoR-type transcription activator in *B. subtilis*, was determined by carrying out a deletion analysis with the promoter region of the *lic* operon. A sequence with a dyad symmetry precedes the *lic* promoter, which appears to be the target site for LicR (879). Similar to MtlR, the activity of LicR is negatively controlled by its corresponding sugar-specific PTS proteins, $EIIA^{Cel}$ (LicA) and $EIIB^{Cel}$ (LicB). Inactivation of $EIIA^{Cel}$ causes constitutive expression of a *licB′-lacZ* fusion. In contrast, deletion of the *ptsHI* genes prevents expression of the *licB′-lacZ* fusion, suggesting a positive role for the general PTS proteins in LicR regulation (878). The four conserved histidyl residues in the two PRDs of LicR were proposed to be the targets of positive regulation by P∼His-HPr, as the replacement of either one of them with a nonphosphorylatable amino acid results in inactive LicR (879). In contrast, replacement of His-559 in the EIIA-like domain of LicR with a glycine causes constitutive expression of the *licB′-lacZ* fusion. His-559 therefore appears to be the site of negative control of LicR activity. Based on the genetic results, it was proposed that P∼$EIIB^{Cel}$ transfers its phosphoryl group to His-559 in the $EIIA^{Mtl/Fru}$-like domain of LicR (879). Although $EIIB^{Cel}$ and $EIIA^{Mtl/Fru}$ belong to the same superfamily of PTS proteins (glucose-fructose-lactose), their phosphorylation sites do not exhibit significant sequence similarity (231, 703). In addition, the structures of EIIAs from these two PTS classes are completely different (730), and "cross"-phosphorylation between the EIIAs and EIIBs from the different PTS classes has not been reported. It therefore remains to be experimentally confirmed that the $EIIA^{Mtl/Fru}$-like domain in LicR can indeed be recognized and phosphorylated by P∼$EIIB^{Cel}$. Alternatively, His-559 in LicR could be phosphorylated by P∼His-HPr but only in the presence of P∼$EIIB^{Cel}$. This mechanism would resemble the regulation of the SacY antiterminator by P∼His-HPr-mediated phosphorylation at His-99 in the presence of P∼SacX (883).

Distribution of DeoR-type PRD-containing transcription activators. A third gene, *yjdC*, encoding a protein containing a DeoR-like DNA binding domain followed by two PRDs and $EIIB^{Gat}$- and $EIIA^{Mtl/Fru}$-like domains is present in *B. subtilis* (437). The sequence identities between YjdC and the other two related transcription activators in *B. subtilis* were 26% to LicR and 21% to MtlR. The *yjdC* gene is monocistronic and is located in front of the *yjdDEF* operon encoding an EIIBCA of a fructose/mannitol class PTS (*yjdD*), an enzyme exhibiting similarity to mannose-6-P isomerase (*yjdE*) and a protein of unknown function (*yjdF*). YjdC was called ManR (844), although the sugar specificity of the encoded PTS remains to be determined. Another DeoR-type transcription regulator, SrlR, containing PRDs and $EIIB^{Gat}$- and $EIIA^{Mtl/Fru}$-like domains has been described for *S. mutans*. SrlR controls the expression of the *srlDRMAEB* operon (78). Besides the putative transcription activator, the operon encodes a glucitol (sorbitol)-specific PTS (*srlAEB*) and a glucitol-6-P dehydrogenase (*srlD*). Surprisingly, the operon also contains a homolog of *E. coli* SrlM, a small protein that has been reported to function as an activator for the *srlR*-less *E. coli srl* operon (961).

Homologs of *G. stearothermophilus* MtlR and *B. subtilis* LicR are found in the genomes of numerous gram-positive bacteria, including other bacilli, clostridia, enterococci, lactobacilli, listeriae, streptococci, staphylococci, *Exiguobacterium* spp., *Geobacillus kaustophilus*, *Leuconostoc mesenteroides*, *L. lactis*, *Moorella thermoacetica*, *O. iheyensis*, *T. tengcongensis*, *Thermoanaerobacter ethanolicus*, etc. In total, more than 100 proteins were found to exhibit significant sequence similarity to *G. stearothermophilus* MtlR or *B. subtilis* LicR. In many of these proteins, including *S. mutans* SrlR, one or both conserved histidines in PRD1 are missing, whereas the phosphorylatable histidines in PRD2 and the $EIIA^{Mtl/Fru}$-like domain were always present, confirming the importance of the $EIIA^{Mtl/Fru}$ domain and PRD2 for the regulation of MtlR/LicR-type transcription activators. The organisms with the most DeoR-type PRD-containing transcription activators were *C. difficile* (13) and *L. innocua* (10). A few LicR/MtlR homologs are also present in gram-negative organisms such as *E. coli*, *P. carotovorum*, *S. enterica* serovar Typhimurium, *S. enterica*, *S. enteritidis*, *S. paratyphi*, *Vibrio fischeri*, and *Yersinia enterocolitica*.

The results obtained with the three best-characterized PRD-containing transcription activators LevR, MtlR, and LicR make it clear that the molecular mechanisms regulating their activity can vary largely. In LicR of *B. subtilis*, all four conserved histidyl residues in the two PRDs seem to be phosphorylated by P∼His-HPr, and their phosphorylation stimulates LicR activity. In MtlR of *G. stearothermophilus*, only the two histidyl residues present in PRD2 seem to have an equivalent function. Finally, *B. subtilis* LevR is activated by P∼His-HPr-mediated phosphorylation of a histidyl residue present in the $EIIA^{Man}$-like domain. For each transcription activator, the absence of the stimulatory phosphorylation(s) during the uptake of a rapidly metabolizable PTS substrate initiates a CcpA-independent CCR mechanism. The three PRD-containing transcription activators are inhibited by P∼EIIB-requiring phosphorylation in their C-terminal domain. The presence of the appropriate inducer (PTS substrate) prevents this phosphorylation and leads to the induction of the corresponding operon. Inhibition of LicR of *B. subtilis* is mediated by phosphorylation at His-559 in the $EIIA^{Mtl/Fru}$-like domain. Although the detailed mechanism of this phosphorylation reaction is not known, it is probably mediated by P∼His-HPr in the presence of P∼$EIIB^{Cel}$. Similarly, MtlR of *G. stearothermophilus* is inactivated by P∼His-HPr- or P∼$EIIB^{Mtl}$-mediated phosphorylation at His-598 in the $EIIA^{Mtl/Fru}$-like domain. In contrast, inactivation of LevR apparently occurs by P∼$EIIB^{Lev}$-mediated phosphorylation of a conserved histidyl residue in the second, truncated PRD.

It is tempting to assume that the four regulatory domains present in PRD-containing transcription activators are organized in a row, similar to the PRDs in antiterminators (909). The inactivating signal elicited by phosphorylation in the C-terminal domain therefore has to be transmitted through the other three domains to affect DNA binding. Structure determination of a transcription activator will hopefully allow a better understanding of the signal transmission.

Regulation of EIIA-Containing Non-PTS Transporters by P~His-HPr-Mediated Phosphorylation

Phosphorylation of an $EIIA^{Glc}$-like domain in certain non-PTS transporters. Lactose transport in *S. thermophilus* is catalyzed by LacS, an integral homodimeric membrane protein that exhibits similarities to the melibiose transporters of *E. coli* and other bacteria (673). Although LacS is an H^+/lactose symporter (235), it contains a C-terminal $EIIA^{Glc}$-like domain, which becomes phosphorylated by PEP, EI, and HPr (672). In *E. coli* and *S. enterica* serovar Typhimurium, the melibiose transporter is inhibited by an allosteric interaction with dephospho-$EIIA^{Glc}$, whereas P~$EIIA^{Glc}$ exhibits no affinity for the melibiose permease (439, 621) (also see the section on $EIIA^{Glc}$-mediated inducer exclusion above). Regulation of LacS-mediated lactose transport in *S. thermophilus* was hypothesized to follow a similar mechanism, except that the transporter is already fused to its regulatory $EIIA^{Glc}$ domain. EI/HPr-catalyzed phosphorylation of the $EIIA^{Glc}$ domain (302) was thought to prevent the inhibition of LacS. However, deletion of the $EIIA^{Glc}$ domain of LacS or mutation of its phosphorylatable histidyl residue (His-552) did not affect the H^+/lactose symport activity of LacS. In fact, when the lactose transport rate of LacS reconstituted into liposomes was measured, the presence of PEP, EI, and HPr even slightly inhibited proton motive force-driven lactose uptake.

Phosphorylation of LacS stimulates the lactose/galactose exchange reaction. More detailed studies revealed that monomeric LacS of *S. thermophilus* (244) catalyzes lactose uptake via a lactose/galactose exchange reaction (915). Galactose produced by the β-galactosidase-catalyzed hydrolysis of intracellular lactose cannot be metabolized by *S. thermophilus* and therefore needs to be expelled. Galactose export is also catalyzed by LacS and is coupled to the import of lactose (counterflow). The lactose/galactose exchange reaction has a higher V_{max} than LacS-catalyzed lactose/H^+ symport and should therefore represent the major entry route for lactose. The V_{max} of the counterflow reaction was two- to threefold higher when LacS was phosphorylated by PEP, EI, and HPr, and the K_m was twofold lower (301). Thus, PEP-dependent phosphorylation of LacS specifically stimulates lactose uptake via the lactose/galactose exchange reaction (Fig. 6).

A lactose transporter exhibiting 95% sequence identity to LacS from *S. thermophilus* is present in *S. salivarius*. In vitro studies revealed that LacS of *S. thermophilus* and *S. salivarius* becomes efficiently phosphorylated at His-552 not only by P~His-HPr but also by doubly phosphorylated (P~His,P-Ser)-HPr (134, 468), which is present in large amounts in oral streptococci grown on glucose or lactose (660, 893) and in lactose-grown *S. thermophilus* cells (134). However, in contrast to *S. thermophilus*, *S. salivarius* did not expel galactose during growth on lactose. In addition, when grown in medium containing lactose or lactose plus galactose, an *S. salivarius ptsI* mutant showed the same growth characteristics as the wild-type strain. The role of LacS phosphorylation in *S. salivarius* is presently not known.

Transporters of the galactoside-pentose-hexuronide family, to which LacS belongs, are abundant in bacteria. It appears that only a few of them (those that transport lactose or raffinose in gram-positive organisms) gained a regulatory domain by fusing $EIIA^{Glc}$ to their C-terminal end, similar to the PTS-regulated NifA/NtrC- and DeoR-type transcription activators discussed in the previous section. In addition to *S. thermophilus* and *S. salivarius*, transporters exhibiting similarity to the *E. coli* melibiose permease and containing an $EIIA^{Glc}$ domain fused to the C terminus have been described for a few other gram-positive organisms. They include the lactose transport protein LacS of *L. delbrueckii* subsp. *bulgaricus* (467) and *Leuconostoc lactis* (914). *L. plantarum* contains not only a LacS homolog but also a raffinose transporter (RafP) with an $EIIA^{Glc}$-like domain (815). Likewise, *Pediococcus pentosaceus* and *Lactobacillus johnsonii* possess both LacS and RafP homologs. *S. thermophilus* LacS-like proteins are also present in *L. mesenteroides*, *Lactobacillus acidophilus*, *Lactobacillus reuteri*, and *Lactobacillus helveticus* as well as in the proteobacterium *Mannheimia succiniciproducens* (MelB). The presence of an $EIIA^{Glc}$-like domain in these proteins indicates that P~His-HPr-mediated phosphorylation might represent a general mechanism for the stimulation of their lactose/galactose or raffinose/galactose exchange activities.

Regulation of Glycerol Kinase by P~His-HPr-Mediated Phosphorylation

Glycerol metabolism in gram-positive bacteria requires functional EI and HPr. Glycerol enters bacterial cells by facilitated diffusion, an energy-independent transport mechanism (321). The protein that catalyzes the equilibration of glycerol gradients across the bacterial cytoplasmic membrane is called the glycerol facilitator (GlpF) (481). GlpF, which has been studied extensively in *E. coli* (584, 849), is present in many other bacteria, archaea, and eukaryotes. It is a member of the major intrinsic protein family of transmembrane channels (39, 628). In addition to GlpF, the major intrinsic protein family includes other membrane-spanning bacterial proteins such as the water channel protein AqpZ from *E. coli* and several other bacteria (99) and PduF, a presumed propanediol diffusion facilitator of *S. enterica* serovar Typhimurium (119). A channel protein accepting glycerol and water as substrates was found in *L. lactis* (246). When the *E. coli glpF* gene was functionally expressed in *Xenopus laevis* oocytes, glycerol uptake by the oocytes exhibited the characteristics of diffusion by a pore-type mechanism (532). Because the glycerol facilitator catalyzes the reversible diffusion of glycerol across the cytoplasmic membrane, it is the subsequent metabolism of glycerol that allows a net (steady-state) flux of glycerol into the cell. The first metabolic step is catalyzed either by glycerol dehydrogenase, which converts glycerol into dihydroxyacetone, or by GlpK, which uses ATP to transform intracellular glycerol into glycerol-3-P (656). Glycerol-3-P is not a substrate for the glycerol facilitator and therefore remains entrapped in the cell. It functions as an inducer of the *glp* regulon by inactivating a repressor (GlpR) in *E. coli* (790) or by activating an antiterminator (GlpP) in *B. subtilis* (749). GlpP binds to the leader region in the mRNA of *glpD* but probably also of *glpFK* and *glpTQ* (274). The *glpD* gene encodes glycerol-3-P dehydrogenase (339), *glpT* encodes a glycerol-3-P transporter, and *glpQ* encodes a glycerophosphoryl diester phosphodiesterase.

Although glycerol is not transported by the PTS, mutants of gram-positive and gram-negative bacteria defective in one of

the general PTS proteins EI or HPr had lost the ability to grow on glycerol as the sole carbon source (52, 265, 282, 600, 711, 741, 766). The inhibition of *S. enterica* serovar Typhimurium and *E. coli* glycerol kinase by unphosphorylated $EIIA^{Glc}$ and the resulting dependence of glycerol metabolism on a functional PTS are described above (see "REGULATION OF CARBON METABOLISM IN GRAM-NEGATIVE ENTERIC BACTERIA") (166, 676, 899). A mechanism completely different from that operating in *E. coli* and *S. enterica* serovar Typhimurium seems to control glycerol metabolism in most low-G+C gram-positive bacteria.

EI- and HPr-catalyzed phosphorylation regulates GlpK activity in gram-positive bacteria. In contrast to the *Enterobacteriaceae E. coli* and *S. enterica* serovar Typhimurium, most gram-positive organisms do not contain free soluble $EIIA^{Glc}$ (130, 350). In these bacteria, either $EIIA^{Glc}$ is fused to $EIICB^{Glc}$, the membrane component of the glucose-specific PTS, or, when mannose and glucose are taken up by the same mannose class PTS, they possess no $EIIA^{Glc}$ at all. Even in *Mycoplasma capricolum*, which is one of the few gram-positive organisms possessing free $EIIA^{Glc}$, the PTS protein did not inhibit GlpK activity (992). Moreover, while the inactivation of the $EIIA^{Glc}$-encoding *E. coli* or *S. enterica* serovar Typhimurium *crr* gene enabled *ptsHI* mutants to grow on glycerol as the sole carbon source, deletion or disruption of the 3′ part of the *B. subtilis ptsG* gene encoding the $EIIA^{Glc}$ domain of $EIICBA^{Glc}$ had no such effect (282). Nevertheless, suppressor mutants that restored growth of a *B. subtilis ptsHI* mutant on glycerol have been obtained (52). They were not affected in *ptsG* but, rather, were affected in *glpK* (937), and glycerol uptake in gram-positive bacteria was therefore assumed to be regulated by an $EIIA^{Glc}$-independent mechanism.

The observation that GlpK from *E. faecalis* is phosphorylated by PEP, EI, and HPr (174, 186) also suggested that glycerol metabolism in gram-positive bacteria is regulated by a mechanism different from that operating in gram-negative bacteria. PEP-dependent phosphorylation occurred at the N-3 position of a histidyl residue and stimulated GlpK activity about 10-fold (186). A similar PTS phosphorylation-mediated stimulation was observed for GlpK from *Enterococcus casseliflavus* (109) and *B. subtilis* (158). No specific protein phosphatase dephosphorylating P∼GlpK could be detected. Instead, dephosphorylation of purified P∼GlpK was observed when it was incubated together with an excess of HPr, indicating that P∼GlpK can transfer its phosphoryl group back to HPr (186). The site of PEP-dependent, EI- and HPr-catalyzed phosphorylation was determined to be His-232 in GlpK of *E. casseliflavus* (109) and His-230 in GlpK of *B. subtilis* (158). Interestingly, in one of the *B. subtilis* suppressor mutants that were able to grow on glycerol despite the absence of active EI and HPr, His-230 of GlpK was replaced with an arginine, and in another mutant, the neighboring Phe-232 was replaced with a serine (937). These mutations were thought to cause structural changes similar to those triggered by phosphorylation, and the ability of these *glpK* mutants to grow on glycerol was assumed to be due to PTS-independent elevated activity of the mutant GlpKs. Indeed, GlpK activity in the four *B. subtilis ptsHI* suppressor mutants was about 10-fold higher than the activity measured in a *ptsHI* strain producing wild-type GlpK (158). In addition, replacing the phosphorylatable His-232 in *E. casseliflavus* GlpK or His-230 in *B. subtilis* GlpK with an arginine led to an increase in activity similar to that caused by phosphorylation (109, 158). In contrast, *E. casseliflavus* (or *B. subtilis*) mutant GlpKs, in which His-232 (or His-230) had been replaced with an alanine or glutamine, exhibited low catalytic activity, which could not be stimulated by phosphorylation with PEP, EI, and HPr (109, 158). Compared to EIIAs, the usual phosphoryl acceptors of P∼His-HPr within the PTS phosphorylation cascade, phosphorylation of enterococcal GlpKs was about 100 times slower. Based on these in vitro results, it was concluded that PEP-dependent, EI- and HPr-catalyzed phosphorylation of GlpK serves to regulate glycerol metabolism in response to the presence or absence of a PTS substrate. In the absence of a PTS sugar, GlpK was assumed to be present primarily in the active, phosphorylated form, allowing the rapid uptake and metabolism of glycerol. However, when bacteria grow in a medium containing a PTS substrate, the phosphoryl transfer flux through the PTS will be high, and the concentration of the common phosphoryl donor P∼His-HPr will be low. As a result, GlpK will be present mainly in the less active dephospho form (Fig. 6).

P∼GlpK dephosphorylation leads to CCR via inducer exclusion. *E. faecalis* GlpK and P∼GlpK migrate to slightly different positions on sodium dodecyl sulfate-polyacrylamide gels (175). By carrying out Western blot analyses with crude extracts from *E. faecalis* cells grown in the presence of various carbon sources, the influence of the growth conditions on the GlpK/P∼GlpK ratio could be determined. Growth in glycerol-containing medium induced the synthesis of GlpK, which was present primarily in the phosphorylated form under these conditions. If the growth medium contained glycerol plus glucose or mannitol, the synthesis of GlpK was strongly repressed, with the remaining GlpK being present mainly in the dephospho form. When cells were grown in glycerol-containing medium and glucose was added to one half of the culture 20 min before the cells were harvested, the amount of GlpK was similar to that in cells grown in the presence of glycerol only. However, while bacteria that continued to grow only on glycerol contained primarily P∼GlpK, the presence of glucose in the other half of the culture caused nearly complete dephosphorylation of P∼GlpK. These results support the above-described concept that the dephosphorylation of GlpK in response to the presence of a rapidly metabolizable PTS sugar inhibits glycerol uptake and metabolism. This regulatory mechanism can be considered a second form of "PTS-mediated inducer exclusion," as the presence of a PTS substrate prevents the GlpK-catalyzed formation of the inducer glycerol-3-P.

The P∼GlpK dephosphorylation-based inducer exclusion mechanism represents a CcpA-independent CCR mechanism and has been studied in detail in *B. subtilis*, where expression of the *glpFK* operon is also submitted to P-Ser-HPr:CcpA-mediated CCR (158). In fact, in *ptsH1*, *hprK* (both cannot form P-Ser-HPr), and *ccpA* mutants, expression of *glpK* or a *glpF′-lacZ* fusion was still strongly repressed by glucose (158, 184), confirming that a second, P-Ser-HPr:CcpA-independent CCR mechanism is operative for the *glp* operon. Interestingly, the repressive effect of glucose on *glpF′-lacZ* expression was absent in a *ccpA* mutant producing His230Arg mutant GlpK (158), which is highly active even without being phosphorylated by PEP, EI, and HPr. These results provide additional evidence

that the CcpA-independent CCR mechanism for the *B. subtilis glpFK* operon is based on poor phosphorylation of His-230 in GlpK. As discussed above, the prevalence of unphosphorylated, barely active GlpK during the rapid uptake of a PTS sugar is expected to lead to low concentrations of the inducer glycerol-3-P (Fig. 6). Growth of a *ccpA* mutant in the presence of glycerol-3-P, which is taken up by GlpT, should therefore also prevent CCR of *glpF′-lacZ* expression, and this has indeed been observed (158). In *B. subtilis*, the inducer glycerol-3-P activates the antiterminator GlpP, which prevents the formation of the ρ-independent terminator t_{glpFK} located in the *glpFK* leader region and consequently leads to the induction of the *glp* operon. Deletion of t_{glpFK} from the *glpF′-lacZ* fusion in a wild-type background therefore caused constitutive *lacZ* expression, which, however, was still strongly repressed by glucose (CcpA-mediated CCR). By contrast, when t_{glpFK} was deleted in a *ccpA* or *hprK* mutant, the repressive effect of glucose on *glpF′-lacZ* expression completely disappeared (158).

The phosphorylation loop of enterococcal GlpK binds FBP in the *E. coli* enzyme. The crystal structure of GlpK from *E. casseliflavus* (977) revealed that it forms dimers in which the phosphorylatable His-232 of each monomer is located about 25 Å from the active center on a loop that is part of the dimer interface. As a consequence, the two phosphorylatable histidyl residues of a GlpK dimer are located close to each other (about 5 Å apart). Because more than 80% of GlpK is present as P∼GlpK in cells grown in glycerol-containing medium (175), doubly phosphorylated GlpK dimers probably represent the activated form. Electrostatic repulsion due to the two neighboring negatively charged phosphoryl groups is likely to drive the structural changes leading to elevated activity of P∼GlpK. Owing to the instability of the P∼His bond, the structure of P∼GlpK could not be determined. Nevertheless, the *E. casseliflavus* His232Arg and Phe234Ser mutant GlpKs exhibit elevated activity even when they are not phosphorylated (109) and are therefore expected to possess a structure similar to that of P∼GlpK. It will therefore be interesting to determine the structure of one of the mutant proteins, work that is presently going on in the laboratories of J. Yeh, P. Briozzo, and J. Deutscher.

The overall structure of GlpK from *E. casseliflavus* is very similar to that of GlpK from *E. coli*. The loop containing the phosphorylatable histidine in *E. casseliflavus* GlpK protrudes less in *E. coli* GlpK. In addition, the corresponding loop in *E. coli* GlpK does not contain a histidine. While *E. coli* and *E. casseliflavus* GlpKs exhibit 60% overall sequence identity, the sequences corresponding to this loop are completely different in GlpKs from gram-positive and gram-negative bacteria. Interestingly, FBP, an allosteric inhibitor of GlpK from gram-negative bacteria, binds to this loop in *E. coli* GlpK (622), thereby connecting two GlpK dimers and leading to the formation of inactive GlpK tetramers. It therefore seems that GlpKs from gram-positive and gram-negative bacteria use the same regulatory loop but that the mechanisms controlling GlpK activity via this loop are completely different.

GlpKs from most other gram-positive bacteria with low G+C content, such as *E. faecalis*, *S. pyogenes* (109), *L. lactis* (67), *E. sibiricum*, *L. monocytogenes*, *L. sakei*, *O. iheyensis*, *S. pneumoniae*, *S. aureus*, etc., also contain a histidyl residue equivalent to His-232 of GlpK from *E. casseliflavus*, and the surrounding sequence, including the three aromatic amino acids, is well conserved. Interestingly, *L. plantarum* contains two GlpKs that exhibit 76% sequence identity. However, only GlpK1 possesses a regulatory loop with a phosphorylatable His (in boldface type) (T-K-D-Y-**H**-F-F-G-S), which is replaced with a Leu (in boldface type) in GlpK2 (T-K-D-Y-**L**-L-Y-G-S), implying that only GlpK1 is regulated by the PTS (Deutscher, unpublished). The gram-positive bacteria *M. pneumoniae*, *Mycoplasma genitalium*, and *Carboxydothermus hydrogenoformans* possess a GlpK that exhibits about 45% sequence identity to GlpK from *E. casseliflavus* and contains a histidine equivalent to His-232, but the surrounding amino acids are poorly conserved. Therefore, whether these three enzymes are phosphorylated and regulated by P∼His-HPr is questionable. Other gram-positive bacteria with low G+C content, such as clostridia, *Desulfitobacterium hafniense*, and *T. tengcongensis*, contain a GlpK exhibiting between 50% and 65% sequence identity to GlpK from *E. casseliflavus*, but they lack an equivalent of His-232, and the sequence corresponding to the regulatory loop is completely different. Surprisingly, the regulatory loop sequence of *C. acetobutylicum* and *Clostridium perfringens* GlpK is almost identical to that of *E. coli* GlpK, suggesting that these enzymes are regulated by FBP-stimulated tetramer formation and not by P∼His-HPr-mediated phosphorylation. In contrast, the site of interaction with $EIIA^{Glc}$ (Pro-472 to Tyr-481 in *E. coli* GlpK) (355) is not conserved in any known GlpK of gram-positive bacteria. These local sequence differences reflect the different modes of regulation of GlpK activity in gram-positive and gram-negative bacteria. Interestingly, amino acids Pro-472 to Tyr-481 are well conserved in GlpKs of only a few gram-negative bacteria including, in addition to *E. coli* and salmonellae, *P. carotovorum*, *Photorhabdus luminescens*, shigellae, and yersiniae, implying that the $EIIA^{Glc}$-mediated regulation of GlpK is not very widespread.

At first glance, the mechanisms regulating GlpK activity in gram-positive bacteria (stimulation by P∼His-HPr-dependent phosphorylation of GlpK) and certain gram-negative bacteria (inhibition by $EIIA^{Glc}$, which is prevented by P∼His-HPr-dependent phosphorylation at His-90 of $EIIA^{Glc}$) seem to be completely different. Nevertheless, the two distinct mechanisms of GlpK regulation, which apparently developed in gram-positive and gram-negative organisms after their separation during evolution, serve identical physiological functions; i.e., they slow glycerol uptake and metabolism when a rapidly metabolizable carbohydrate is transported via the PTS.

GlpK phosphorylation in bacteria of the *Thermus*/*Deinococcus* group. The thermostable GlpKs from *Thermus flavus* (347, 348) and *Thermus aquaticus* (346), which belong to the *Thermus*/*Deinococcus* group, exhibit more than 80% sequence identity to GlpK from *B. subtilis*. Other functionally related proteins from *T. flavus* or *T. aquaticus* and *B. subtilis* show a much lower degree of sequence identity (30 to 55%), implying that the GlpK-encoding gene present in *T. flavus* and *T. aquaticus* was gained by horizontal gene transfer from a gram-positive organism. Their GlpKs contain an equivalent of the phosphorylatable His-232, and the surrounding sequence is well conserved. It was therefore not surprising that EI and HPr from *B. subtilis* could phosphorylate *T. flavus* GlpK (157). In addition, *T. flavus* crude extracts were found to contain EI and HPr activity and were capable of phosphorylating *T. flavus*

GlpK (157). However, phosphorylation of *T. flavus* GlpK did not increase its activity. A similar observation was made with *G. stearothermophilus* GlpK (714), and it was therefore hypothesized that the modification of GlpK from these two thermotolerant organisms affects their thermostability (157).

Is GlpK the only carbohydrate kinase regulated by P~His-HPr? Glycerol kinase is a member of a large family of carbohydrate kinases including xylulose kinase, gluconate kinase, L-fuculose kinase, and many others (954). A data bank search revealed that none of the presently known eubacterial members of this family contains a histidyl residue equivalent to His-232 of GlpK from *E. casseliflavus*. Nevertheless, some bacteria such as *L. rhamnosus* (17) and *L. casei* BL23 (570) possess a *glpK*-like gene in addition to *glpK* in the *glpFK* operon (up to 80% identity at the protein level), which was called *gykA*. GykA of both organisms contains a P~His-HPr phosphorylation site in position 231 (His231, in boldface type; -Y-**H**-F-F-), which is identical to that in several other GlpKs. In *L. casei*, *gykA* is monocistronic, and its function is unknown (570). Because the *L. rhamnosus gykA* gene was not induced during growth on glycerol, it is possible that GykA phosphorylates a substrate other than glycerol. Nevertheless, the PTS-mediated activation mechanism based on P~His-HPr-dependent phosphorylation at a regulatory loop in the target enzyme seems to have almost exclusively evolved for GlpK of low-G+C gram-positive bacteria and certain members of the *Thermus*/*Deinococcus* group.

SOME UNUSUAL PTS PATHWAYS AND PROTEINS

Genome sequencing has unveiled many genes that encode PTS-like proteins (see http://www.membranetransport.org/ [725] and http://www.tcdb.org/ [755]) (345, 720, 759, 867). Most gram-positive bacteria as well as chlamydiae and the spirochetes have a single EI and HPr, whereas bacilli, geobacilli, and oceanobacilli possess an additional HPr paralog, Crh (see "REGULATORY FUNCTIONS OF P-Ser-HPr") (250). An HPr paralog is also present in *U. urealyticum* (308). In contrast, some proteobacteria carry several EI and HPr homologs. For instance, *E. coli* has at least five copies of each general PTS protein. Some of the paralogous genes encode multidomain proteins (e.g., diphosphoryl transfer proteins, triphosphoryl transfer proteins, or multiple-phosphoryl transfer proteins), which supposedly are the result of splicing and fusion as well as duplication of EI, HPr, EIIA, and EIIB domains (720, 867). EI, HPr, and EIIA homologs have even been identified in bacteria that lack any known EIIB and EIIC and therefore lack a PTS that is functional in sugar transport and phosphorylation (64). In the few cases studied in more detail, the paralogous proteins seem to constitute alternative phosphotransferase routes. We will discuss some of these alternative routes, which include (i) the unusual PTS-dependent utilization of dihydroxyacetone and (ii) the connection between carbon and nitrogen metabolism via the PTS^{Ntr}.

PEP-Dependent Dihydroxyacetone Phosphorylation

Dihydroxyacetone can be used by *E. coli* as the sole exogenous source of carbon. Although several bacteria metabolize dihydroxyacetone via ATP-dependent phosphorylation, a PEP-dependent pathway is operative in *E. coli* (376). Mutations in *ptsI* or the *dha* operon abolish phosphorylation of dihydroxyacetone and thereby growth on this carbon source. The *E. coli dha* operon comprises three cistrons (304, 640). *dhaK* (*ycgT*) and *dhaL* (*ycgS*) encode the dihydroxyacetone kinase subunits DhaK and DhaL (also called DhaK1 and DhaK2, respectively). Distinct DhaK and DhaL proteins are present in bacteria that phosphorylate dihydroxyacetone in a PEP-dependent reaction, whereas in bacteria that use ATP for dihydroxyacetone phosphorylation, such as *Citrobacter freundii*, DhaK and DhaL are fused to a single protein (153, 811). The third cistron encodes a tripartite PTS fusion protein, which was designated DhaH by Paulsen et al. (640) and DhaM by Gutknecht et al. (304). The corresponding gene is referred to as *dhaM* (*ycgC*) or *ptsD*. DhaM consists of an N-terminal $EIIA^{Man}$-like domain followed by an HPr-like domain and a C-terminal domain resembling the N-terminal part of EI. Each domain contains a phosphorylation site characteristic of the corresponding PTS protein (304). For a better understanding, we will refer to the *E. coli* DhaM protein as $EIIA\text{-}HPr\text{-}EI^{Dha}$.

The PEP-dependent phosphorylation of dihydroxyacetone by *E. coli* involves seven phosphotransfer steps (depicted in Fig. 9). $EIIA\text{-}HPr\text{-}EI^{Dha}$ was purified and shown to be phosphorylated by [^{32}P]PEP in the presence of both EI and HPr but was also shown to be slightly phosphorylated in the presence of EI only (304, 640). This is consistent with the absolute requirement for EI in dihydroxyacetone phosphorylation that was determined previously (376). Furthermore, it was found that the addition of equimolar amounts of purified DhaK and DhaL to purified $EIIA\text{-}HPr\text{-}EI^{Dha}$ allows PEP-dependent phosphorylation of dihydroxyacetone but that the phosphotransfer is blocked when either one of the phosphorylatable histidyl residues is replaced with an alanine (304). These observations imply that the phosphoryl group of PEP is sequentially transferred via EI and HPr to the EI domain of $EIIA\text{-}HPr\text{-}EI^{Dha}$ and, from there, to the HPr and the EIIA domain of the protein. Finally, it was established in vitro that DhaK binds the substrate dihydroxyacetone with high specificity (255, 814), even in the presence of 2 M glycerol, while DhaL carries a tightly bound ADP molecule (36). The final transfer steps thus involve phosphotransfer from the $EIIA^{Dha}$ domain to the ADP cofactor in DhaL and from the presumably intermediately formed DhaL/ATP to the dihydroxyacetone molecule bound to DhaK. It should be noted that, in contrast to the sugar PTS, the *dha* PTS is not involved in membrane transport. Transport of dihydroxyacetone occurs probably via GlpF-catalyzed facilitated diffusion or direct diffusion through the membrane (848).

Proteins homologous to DhaK, DhaL, and DhaM of *E. coli* are found among many bacterial orders including the *Enterobacteriales*, *Bacillales*, *Lactobacillales*, and *Clostridiales* (41). *K. pneumoniae* and several members of the *Clostridiales* contain not only a PEP-dependent *dha* system but also a "normal" ATP-dependent dihydroxyacetone kinase (848). The *dhaM* gene encodes either a tripartite protein (*E. coli*), a bipartite protein ($EIIA\text{-}HPr^{Dha}$) (*Corynebacterium diphtheriae* and *Leifsonia xyli*), or, as in most species, a single $EIIA^{Dha}$ protein (41). It is likely that in the latter group of organisms, the phosphoryl group is transferred from PEP to dihydroxyacetone in five consecutive steps.

For *E. coli*, it was observed that the *dha* operon was induc-

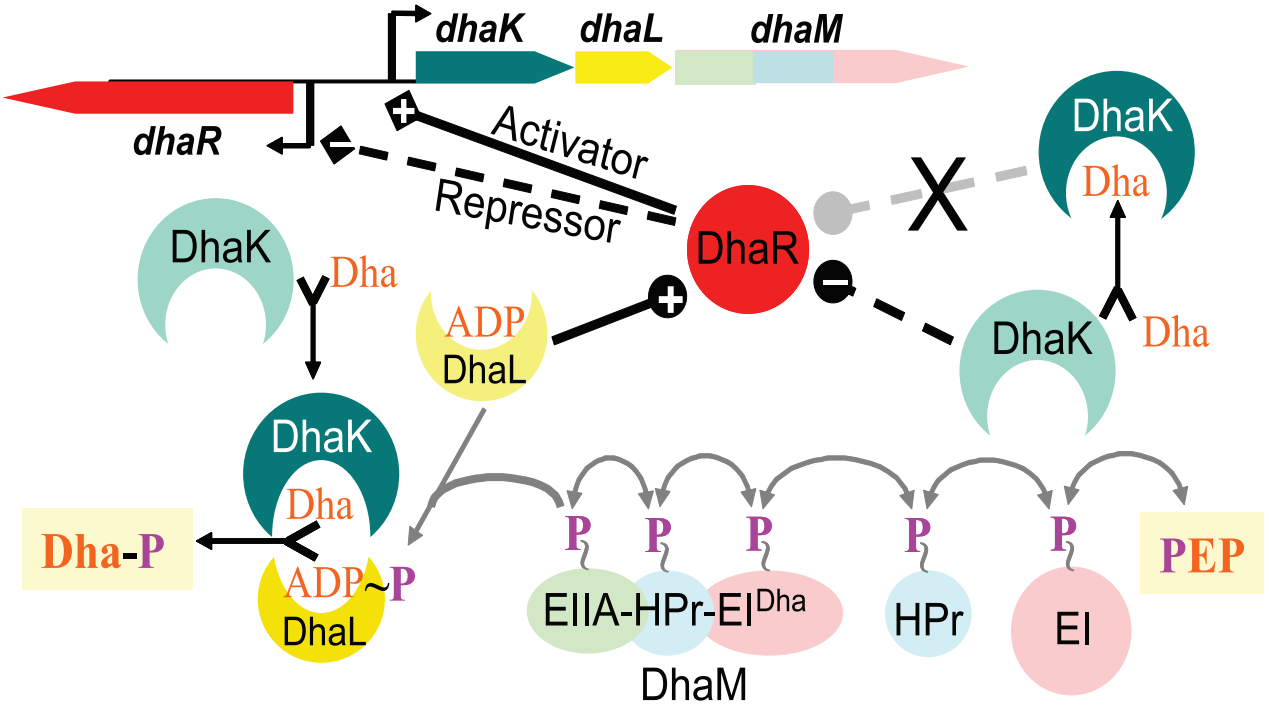

FIG. 9. The Dha PTS of *E. coli.* Phosphotransfer from PEP to Dha is mediated by five distinct proteins (EI, HPr, DhaM, DhaL, and DhaK). DhaM itself is composed of three PTS domains: a truncated EI, HPr, and an EIIA of the mannose class PTS. From the P~EIIA domain of DhaM, the phosphoryl group is transferred to an ADP molecule tightly bound to DhaL and from there is transferred to a Dha molecule bound to DhaK. Expression of the *E. coli dha* operon is regulated by the transcription activator DhaR. In addition, the gene encoding the activator DhaR is subject to negative autoregulation. When Dha is present in the cell, it is rapidly phosphorylated, and the ultimate phosphoryl donor, DhaL, therefore carries predominantly ADP. The DhaL:ADP complex binds to DhaR and stimulates its regulator functions. DhaK without Dha interacts with DhaR and down-regulates its activity, while the complex with its substrate, which is formed when Dha is present in the cell, does not interact with DhaR (37). It should be noted that many other organisms do not contain the three-domain DhaM but possess only $EIIA^{Dha}$ instead. In these bacteria, HPr probably transfers its phosphoryl group directly to $EIIA^{Dha}$.

ible; i.e., PEP-requiring dihydroxyacetone phosphorylation activity increased when cells were grown on dihydroxyacetone (376, 640). It appeared that *dha* operon induction is mediated by the dephosphorylation of the phosphotransfer proteins, as the inactivation of *ptsI* or *dhaM* as well as the replacement of any one of the three phosphorylatable histidyl residues in $EIIA\text{-}HPr\text{-}EI^{Dha}$ led to the constitutive expression of the *dhaKLM* operon (37). Transcription of the *dhaKLM* operon is subject to autoregulation by DhaK and DhaL, which interact with the N-terminal sensory domain of the transcriptional activator DhaR (Fig. 9). The protein is activated by DhaL/ADP and is inhibited by DhaK without bound dihydroxyacetone (37). This mode of autoregulation makes the expression of the *dhaKLM* operon sensitive to the availability of both substrate and sufficient phosphoryl donor molecules. In the presence of substrate, expression is expected to be high. The binding of dihydroxyacetone should prevent the inhibitory effect of DhaK on DhaR, and the formation of DhaL/ADP during dihydroxyacetone phosphorylation should stimulate DhaR (37). If, in addition to dihydroxyacetone, a rapidly metabolizable PTS sugar is present, PEP is probably used mainly for PTS-mediated sugar uptake and phosphorylation, and the metabolism of dihydroxyacetone should therefore be diminished. However, as a consequence of the expected decrease in phosphoryl group flux through DhaM, DhaL will primarily be present with bound ADP, and due to the presence of dihydroxyacetone, DhaK will primarily be present with bound substrate. Both signals favor *dhaKLM* expression, and this unusual regulation mode was therefore thought to ensure a constant dihydroxyacetone turnover rate and detoxification, which might be necessary if dihydroxyacetone accumulates owing to intracellular metabolic processes (567).

The PTS^{Ntr}

It has been known for quite some time that the extent of CCR depends not only on the nature of the carbon source but also on the available nitrogen source (137). Nevertheless, the underlying mechanism linking the regulation of carbon and nitrogen metabolism still remains obscure (107). Transcription of a number of genes involved in nitrogen assimilation, especially under nitrogen-limiting conditions, depends upon the sigma factor σ^{54}, encoded by the *rpoN* gene (previously designated *ntrA* or *glnF*) (for reviews, see references 91, 704, 705, and 843). Transcription initiation at σ^{54}-dependent promoters requires both the input of energy through nucleotide hydrolysis and the interaction with a specific EBP, such as NtrC or NifA, some of which can be activated by PTS-mediated phosphorylation (see "REGULATORY FUNCTIONS OF P-Ser-HPr"). Sequencing of the locus around the *K. pneumoniae rpoN* gene revealed two ORFs (ORF162 and ORF95) whose inactivation resulted in elevated transcription of σ^{54}-dependent genes

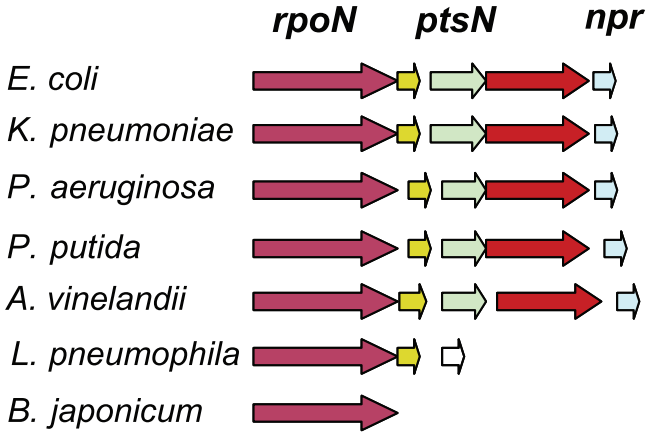

FIG. 10. Gene organization downstream from *rpoN* in several bacteria. The genes encode the sigma factor σ^{54} (*rpoN*), the ribosome-associated protein Y (*yhbH*, in yellow), $EIIA^{Ntr}$ (*ptsN*), an ATP-binding protein (*yhbJ*, in red), and the HPr paralog NPr (*npr*), respectively.

(553). ORF162 was shown to be homologous to EIIAs of the mannitol/fructose class PTS (718), which provided a putative link between nitrogen metabolism and the PTS for the first time. The gene was renamed *ptsN*, and the related protein was named $EIIA^{Ntr}$. In *K. pneumoniae*, $EIIA^{Ntr}$ is phosphorylated in vitro in the presence of [^{32}P]PEP, EI, and HPr (49). Overexpression of *ptsN* results in slightly slower growth on glucose minimal medium with NH_4^+ as the nitrogen source, which supports the assumption that $EIIA^{Ntr}$ negatively regulates transcription from σ^{54}-dependent promoters.

Sequencing of the equivalent locus in *E. coli* revealed four ORFs downstream from *rpoN*, with the second ORF being similar to *ptsN* and the fourth ORF encoding a protein strongly resembling HPr (Fig. 10) (382, 680). The latter ORF was designated *npr* (*ptsO*), and its gene product was named NPr. Like the *K. pneumoniae* protein, purified *E. coli* $EIIA^{Ntr}$ was reversibly phosphorylated by EI and HPr in vitro (680). However, although $EIIA^{Ntr}$ exhibits strong similarity to $EIIA^{Fru}$ and $EIIA^{Mtl}$, it does not complement mannitol or fructose phosphorylation in crude extracts prepared from mutants containing inactive $EIIA^{Fru}$ or $EIIA^{Mtl}$. Likewise, although NPr proved to be phosphorylated by P~$EIIA^{Ntr}$ and P~$EIIA^{Fru}$ of *E. coli* and $EIIA^{Glc}$ of *B. subtilis*, purified NPr does not replace HPr in the glucose or mannitol PTS assay (680), probably because phosphorylation of NPr by PEP/EI is a relatively slow process (689).

The PTS^{Ntr} was completed by the discovery of a third "alternative" PTS gene (*ptsP*) in the *E. coli* genome, which encodes EI^{Ntr}, a protein with considerable similarity to EI. Compared to EI, EI^{Ntr} contains an N-terminal extension of 170 amino acids, which resembles the N-terminal regulatory GAF domain of NifA, a protein controlling nitrogen fixation. EI^{Ntr} was therefore assumed to be involved in the regulation of nitrogen metabolism (716). PEP-dependent in vitro phosphorylation of NPr by EI^{Ntr} was observed. However, EI^{Ntr} does not replace EI in the overall PEP-dependent mannitol phosphorylation, which is at least 1,000-fold slower in the presence of EI^{Ntr} compared to assays with EI (689). This finding was not surprising, as *ptsI* mutants (lacking EI) do not grow on PTS carbohydrates, and no phosphoryl group transfer from PEP to HPr was observed in crude extracts of such mutants.

The localization of *ptsN* and *npr* in the *rpoN* operon and, as a consequence, their cotranscription with *rpoN* are conserved in several gram-negative bacteria (382, 552, 867). In *K. pneumoniae* (553) and *E. coli* (680), expression of the *rpoN* operon is constitutive, whereas in *Bradyrhizobium japonicum* (433), *Pseudomonas putida* (415), and *Rhizobium etli* (555), it is negatively autoregulated. EI^{Ntr}, NPr, and $EIIA^{Ntr}$ have so far not been detected in gram-positive bacteria. Attempts have been made to uncover the role of the PTS^{Ntr} in various species, mostly by studying growth and gene expression (via *lacZ* fusions) after inactivation of *ptsN*. The growth behavior was compared to those of a wild-type strain and mutants missing σ^{54} (RpoN). The results of these attempts are diverse and sometimes contradictory and will be described below.

Connection between carbon and nitrogen metabolism in *E. coli*. In *E. coli*, the regulation of nitrogen metabolism appears to be coupled to specific "classical" PTS components as well as to the PTS^{Ntr}. The key enzyme and key regulators for the utilization of nitrogen by *E. coli* are located in the *glnALG* operon, which encodes glutamine synthethase and the two-component system NtrB and NtrC, respectively (607, 704). Under conditions of nitrogen deprivation, P~NtrC blocks transcription from $glnAp_1$ and induces transcription from the σ^{54}-dependent $glnAp_2$ promoter (607, 704). Although the weak σ^{70}-dependent $glnAp_1$ promoter requires activation by Crp/cAMP, *glnA* expression was found to be much higher during growth on glucose or glucose-6-phosphate than during growth on fructose or glycerol. It was assumed that Crp/cAMP stimulates *glnALG* expression from $glnAp_1$ but represses expression from $glnAp_2$ and that the stimulatory effect is relatively stronger than the effect of repression (875). In line with this assumption, it was found that the addition of cAMP to glucose-grown cells lowered GlnA activity by about sevenfold (506). However, further analysis showed that the effect of glucose is probably not mediated via Crp/cAMP but that it is mediated indirectly via the uridylylation state of GlnB. Furthermore, under nitrogen-limiting conditions, growth of a *ptsN* mutant on glucose and glutamine had a small but significant negative effect on expression from $glnAp_2$ (680). The addition of cAMP under these conditions had no effect. *E. coli ptsN* mutants grow poorly on alanine, and this effect was accentuated by the presence of several sugars or TCA cycle intermediates (680). Repression is relieved by supplying the cells with ammonium salts or by expressing a *ptsN* gene in *trans*. Disruption of the gene preceding *ptsN* (probably also leading to the inactivation of *ptsN*) causes a slight reduction in transcription from $glnAp_2$ and p*nifL* when *K. pneumoniae* cells are grown on glucose (plus formate) under nitrogen limitation (serine) (553). In contrast, when serine is replaced with ammonium as the nitrogen source, expression from the $glnAp_2$, p*nifL*, and p*nifH* promoters is significantly higher than that in the wild type (553). A similarly elevated expression from $glnAp_2$ can be observed in a genuine *ptsN* mutant, whereas the inactivation of *npr* leads to a decrease in transcription from $glnAp_2$, p*nifL*, and p*nifH* (554). Overexpression of *K. pneumoniae ptsN* in an *E. coli* $rpoN^+$ strain inhibits growth on glucose minimal medium with either glutamine or ammonium as the nitrogen source but had no effect on an *rpoN* mutant (49). In *R. etli*, the inacti-

vation of *ptsN* slightly reduces growth on alanine or serine in the presence of mannitol or glucose, whereas growth is inhibited when the TCA cycle intermediates succinate and malate are present (555). In addition, a reduction in melanin production was observed. The activity of the p*nifH* promoter decreases, while the level of NifA, the activator controlling the expression of *nifH*, is not affected by the mutation. Mutation of *ptsA*, the gene that precedes the *frw* operon and codes for EI^{Frw} in *E. coli* (345, 720), has effects similar to those of the inactivation of *ptsN*. A functional link between EI^{Frw} and $EIIA^{Ntr}$ was therefore suggested (555).

A connection between $EIIA^{Ntr}$ and the regulation of branched-chain amino acid metabolism in *E. coli* was reported (456). The genes encoding the three heterodimeric *E. coli* acetohydroxy acid synthases, which catalyze the first step in leucine, isoleucine, and valine biosynthesis, are located in three different operons (891). One of the gene pairs (*ilvGM*) is normally not expressed due to a frameshift mutation. Among the other two gene pairs (*ilvBN* and *ilvIH*), the former is responsible for the majority of the enzyme activity (276, 277). The proteins encoded by *ilvBN* and *ilvIH* are sensitive to feedback inhibition by valine. As a consequence, wild-type cells exposed to valine as the sole amino acid or nitrogen source cannot make isoleucine and therefore cannot grow (452). Exposure to leucine-containing dipeptides also leads to an impairment of growth and a considerable increase in the intracellular leucine concentration (862). Besides abolishing the growth on leucine-containing dipeptides altogether, inactivation of *ptsN* completely prevents *E. coli* cells from growing on leucine and serine and reduces growth on isoleucine (456). Introduction of a plasmid encoding $EIIA^{Ntr}$ partly restores growth on these compounds, whereas transformants with a plasmid encoding His73Ala mutant $EIIA^{Ntr}$ (cannot be phosphorylated) grow even better than wild-type cells. Further experiments showed that unphosphorylated $EIIA^{Ntr}$ mediates derepression of the *ilvBN* operon and thereby enables the cells to synthesize the other branched-chain amino acids when grown on leucine, isoleucine, or serine.

Transcription regulation of the TOL plasmid of *P. putida*. In *P. putida*, $EIIA^{Ntr}$ affects the regulation of gene expression from the TOL plasmid. The TOL plasmid harbors the genes necessary for the metabolism of toluene and xylenes and contains four transcription units: a so-called upper pathway and a *meta* pathway operon flanked by the promoters *Pu* and P_M, respectively, and the regulator-encoding genes *xylS* and *xylR* connected to the promoters P_S and P_R, respectively (28, 388). Transcription from *Pu* and P_{S1} is σ^{54} dependent and is subject to CCR: it is low when cells grow exponentially on rich medium and is also down-regulated by glucose or gluconate but not by fructose (22, 101, 103). Repression of *Pu* (101, 103, 104) and P_{S1} (199, 281) transcription by rich medium or glucose is partially relieved (recovery of 50% of the activity) by the inactivation of *ptsN* or the catabolite repression control protein-encoding gene, *crc* (22). Relief of repression is achieved only in the presence of the inducer *o*-xylene (22), which increases the affinity of the activator XylR for *Pu* and P_{S1} (57, 514). Moreover, expression of *xylR* is repressed by rich medium, and this repression is absent in a *ptsN* mutant, whereas a *crc* mutation has no effect (22, 514). Thus, it seems that $EIIA^{Ntr}$ interferes with the activity of the transcription activator XylR, whereas repression via Crc should follow another mechanism (22). The involvement of Crc in the regulation of several sugar catabolic pathways (747) suggests a connection between the various metabolism-controlling regulatory networks (22). This complies with an observed down-regulation of *Pu* expression mediated via products of the Entner-Doudoroff pathway, where 6-phosphogluconate and/or 2-dehydro-3-deoxyphosphogluconate was identified as the intermediate that exerts repression (916).

Studies on active-site mutants suggest that the phosphorylated form of $EIIA^{Ntr}$ mediates repression of *Pu* and P_{S1}. It was found that a mutant producing His68Asp $EIIA^{Ntr}$ is permanently repressed, the aspartyl residue is thought to mimic a phosphorylated histidine, whereas a mutant producing His68Ala $EIIA^{Ntr}$ is not (103). Surprisingly, inactivation of *npr* does not relieve inhibition by glucose but leads to repression in the absence of glucose, a phenotype resembling that of the His68Asp *ptsN* mutant (104). A *ptsN npr* double mutant has a *ptsN*-like phenotype, and a mutant in which the conserved histidine of NPr has been replaced with an alanine exhibits the same phenotype as the *npr*-disrupted mutant. Although a *P. putida ptsP* gene has been identified, its inactivation had no effect. Taking all of these findings into account, it appears that $EIIA^{Ntr}$ of *P. putida* is partly phosphorylated during growth on glucose, contrary to what is seen for $EIIA^{Glc}$ in *E. coli*. The presumed partial phosphorylation of the PTS components in the presence of glucose might be explained by the fact that glucose is not a PTS sugar in *P. putida* (103). Because the uptake of glucose by *P. putida* does not use but yields PEP, it is possible that the PEP-to-pyruvate ratio is raised by the glucose catabolism, which in turn might increase the phosphorylation state of PTS proteins, including $EIIA^{Ntr}$. At the same time, the lack of repression by fructose can be explained by the fact that in *P. putida*, fructose uptake occurs via a PTS and thus lowers the concentration of $P\sim EIIA^{Ntr}$.

Other connections of the $EIIA^{Ntr}$. Inactivation of *ptsN* (and thereby also *npr*) in *Pseudomonas aeruginosa* impairs growth on minimal medium A (no carbon source mentioned) (377). Growth can be restored by the addition of glutamine, but not arginine, histidine, proline, or glutamate, and by *trans*-complementation (with only *npr*). There was no effect of *ptsN* overexpression or inactivation on the expression of the σ^{54}-regulated pilin or flagellin genes. Likewise, in *Caulobacter crescentus*, expression from several σ^{54}-dependent flagellar genes was unaffected by a *ptsN* mutation, although expression from the *fljK* promoter was slightly lower (371). Several bacteria, including numerous proteobacteria and the spirochetes *Treponema pallidum*, *T. denticola*, and *Leptospira interrogans*, the green photosynthetic bacterium *Chlorobium tepidum*, and the δ-proteobacterium *Geobacter metallireducens*, encode an EI, an HPr, an HPrK/P, and either one or two $EIIA^{Ntr}$ homologs within their genome but lack any known EIIB and EIIC (64, 345, 841). In *T. pallidum*, the EI-encoding gene seems to be an inactive pseudogene. Furthermore, it was shown in vitro that the *T. denticola* PTS^{Ntr} proteins are functional in phosphotransfer and that HPr is phosphorylated by HPrK/P requiring a much lower concentration of fructose-1,6-bisphosphate than the enzyme from *B. subtilis* for full activity (278).

Potential roles of EI^{Ntr}. EI^{Ntr}, which can easily be detected in numerous proteobacteria due to its typical N-terminal ex-

tension, exerts diverse functions. *Azotobacter vinelandii* does not transport carbohydrates via the PTS (44, 740), and a *ptsP* mutation hardly affects growth on carbon sources like fructose, mannitol, sucrose, acetate, pyruvate, or succinate (793). However, growth of a *ptsP* mutant on glucose or glycerol is drastically slowed, although glucose uptake and phosphorylation are not affected. The impeded growth was traced to the inactivation of the enzyme nitrogenase, and the suggestion was made that respiratory protection of nitrogenase under carbon-limiting conditions is absent in the *ptsP* mutant (793). At the same time, it was observed that the inactivation of the *ptsP* gene in *A. vinelandii* reduces the capacity of the organism to accumulate poly-β-hydroxybutyrate (793), a carbohydrate storage molecule made from acetyl-CoA building blocks (397). The signaling pathway might also include NPr and $EIIA^{Ntr}$, as genome sequencing has revealed the presence of both *ptsN* and *npr* in the *A. vinelandii rpoN* operon (551). Similarly, "leaky" *Ralstonia eutropha phbI* and *phbH* mutants show diminished accumulation of poly-β-hydroxybutyrate (687). A close relationship between *phbI* and *phbH* and the *ptsP* and *npr* genes of *E. coli* was established (680), although the PhbI protein of *R. eutropha* lacks the NifA-like extension typical of other EI^{Ntr}s.

Mutants of *P. aeruginosa* (854) or *Legionella pneumophila* (333) affected in *ptsP* do not show an obvious phenotype when grown in rich medium. However, the *ptsP* mutants have almost completely lost their virulence due to strongly reduced growth after infection of nematodes, plants, mice, guinea pigs, or human epithelial cells. *trans*-Complementation with *ptsP* restores pathogenicity, which proves that the loss of virulence is due to the loss of functional EI^{Ntr}. *L. pneumophila* stores poly-β-hydroxybutyrate in its cytoplasm as a nutrition source for long-term starvation survival (370). *ptsP* mutants showed normal accumulation of the carbon storage molecule when grown in rich medium, but unfortunately, poly-β-hydroxybutyrate levels were not monitored after infection of host cells (333).

In *B. japonicum*, *ptsP* is located next to *lysC* (encoding an aspartokinase), but the two genes do not form an operon (412). Nevertheless, inactivation of *lysC* or *ptsP* prevented the utilization of Pro-Gly-Gly and other oligopeptides. In vitro "pull-down" experiments revealed that EI^{Ntr} interacts with the aspartokinase. Purified EI^{Ntr} is phosphorylated by ATP in the presence of cell extracts, and the phosphorylation is prevented when aspartokinase is added (412).

What is the function of the PTS^{Ntr}? The discovery of paralogous PTS proteins associated with the transcriptional regulation of genes involved in nitrogen assimilation has provided candidates for a regulatory connection between nitrogen and carbon metabolism. In fact, a potential PTS^{Ntr} phosphorylation cascade is present in many bacteria, but a coherent concept for a signaling pathway is lacking. Growth of *ptsN* null mutants on poor nitrogen sources is impaired, especially when a TCA cycle intermediate is used as the sole carbon source, whereas growth on rich nitrogen media (glutamine and NH_4^+) is not affected. The PTS^{Ntr} therefore seems to enable bacteria to grow (faster) on poor nitrogen sources and TCA cycle intermediates. A similar role in adaptation to changes in growth conditions can be attributed to EI^{Ntr} of *P. aeruginosa* and *L. pneumophila*, considering that *ptsP* mutants of these pathogens grow normally in rich medium but grow very slowly after infection of a host cell. The effect of *ptsN*, *npr*, and *ptsP* mutations on gene expression is less clear. A connection to σ^{54}-directed transcription is conceivable, because $EIIA^{Ntr}$ and NPr are encoded within the *rpoN* operon and EI^{Ntr} has a putative regulatory domain present in other σ^{54}-interacting proteins, but no data showing exactly how σ^{54}-regulated genes are controlled by the PTS^{Ntr} are available. The amount of σ^{54} present in the cell does not seem to be controlled by PTS^{Ntr}, as it is always low and is affected neither by growth on glucose nor by *ptsN* or *npr* mutations (388). Many effects of the *ptsN*, *npr*, or *ptsP* mutations, such as those on melanin production, poly-β-hydroxybutyrate accumulation, *Pu* promoter expression, or *era* activity, seem to be unrelated to nitrogen metabolism. Two-dimensional gel electrophoresis with protein extracts prepared from *P. putida* wild-type or *rpoN* and *ptsN* mutant strains indicated that the role of $EIIA^{Ntr}$ in σ^{54}-mediated transcription can be only minor. On the other hand, in a *P. putida ptsN* mutant, the level of about 9% of the proteins is altered (102).

Also, whether the PTS^{Ntr} indeed forms a functional phosphorylation cascade remains uncertain. Some organisms contain only part of the system. In *R. etli*, the *rpoN* operon contains *ptsN* but seems to lack *npr*, while *ptsA* (EI^{Frw}) was identified based on sequence similarity (555). Inactivation of *ptsA* has effects similar to those of the inactivation of *ptsN*, suggesting a connection between EI^{Frw} and $EIIA^{Ntr}$. Moreover, genome analysis revealed that several proteobacteria, chlamydiae, and spirochetes lack any known EIIC permease but do contain EI, HPr, or both in combination with one or more components of the putative PTS^{Ntr} (41, 64). Although the PTS^{Ntr} is thought to require the phosphoryl group transfer $P\sim EI^{Ntr}\rightarrow P\sim NPr\rightarrow P\sim EIIA^{Ntr}$ in order to carry out its regulatory functions, the mixed appearance of the components in several bacteria suggests functional cross talk between the PTS^{Ntr} and "regular" PTS components. In fact, $EIIA^{Ntr}$ of the spirochete *T. denticola* (278) and $EIIA^{Ntr}$ and NPr of *E. coli* (680) can be phosphorylated in vitro by the regular PTS proteins. Interestingly, Powell et al. (680) found that in vitro NPr almost completely dephosphorylates $P\sim EIIA^{Ntr}$, implying that phosphoryl group transfer occurs in the opposite direction. In that case, $EIIA^{Ntr}$, which is phosphorylated via EI and HPr, could communicate the phosphorylation state of the PTS proteins to NPr and EI^{Ntr}. Nevertheless, as NPr was present in about an eightfold excess of both EI and $EIIA^{Ntr}$ in the phosphorylation assays, one might expect the dephosphorylation of $P\sim EI$ and $P\sim EIIA^{Ntr}$ independent of which phosphoryl transfer direction is favored. Moreover, the finding that unphosphorylated $EIIA^{Ntr}$ mediates derepression of the *ilvBN* operon in *E. coli* and that the inactivation of *ptsP* or *npr* renders the derepression constitutive suggests a "normal" direction of the group transfer (456).

A comparison of the crystal structures of *E. coli* $EIIA^{Ntr}$ and the $EIIA^{Mtl}$ domain shows that the fold of both proteins as well as the structure around the phosphorylation site, His-73 in $EIIA^{Ntr}$ and His-65 in $EIIA^{Mtl}$ (703), are nearly identical (73, 905). However, the second conserved histidine, His-120 in $EIIA^{Ntr}$ and His-111 in $EIIA^{Mtl}$, respectively, is differently oriented. His-111 of $EIIA^{Mtl}$ is part of the active site, whereas His-120 of $EIIA^{Ntr}$ is located in the vicinity but points away from the active site. Similar observations were made with the crystal structure of *N. meningitidis* $EIIA^{Ntr}$ (724). Interestingly,

$EIIA^{Ntr}$ of *H. influenzae* lacks equivalents of the two histidines of the phosphorylation site altogether (717). These findings are consistent with a regulatory role of $EIIA^{Ntr}$ through binding rather than through phosphoryl group transfer. Perhaps phosphorylation of the PTS^{Ntr} proteins serves only to alter binding affinities. On the other hand, the solution structure of the NPr:$EIIA^{Ntr}$ complex suggests that the protein interface resembles that in the $EIIA^{Glc}$:HPr complex (927).

CONCLUSIONS AND PERSPECTIVES

From an evolutionary point of view, it is likely that the PTS first served as a carbohydrate transport system before its numerous regulatory functions emerged. Although there is no direct evidence supporting this view, it is tempting to assume that during the course of evolution, the PTS grew in complexity by acquiring additional proteins/domains, which allowed additional regulatory functions. In this context, it is interesting that some PTS components resemble certain non-PTS enzymes, which, during catalysis, also become phosphorylated at histidyl or cysteyl residues. For example, EIIBs of the lactose/cellobiose class PTS exhibit structural similarity to low-molecular-weight P-tyrosine protein phosphatases (907) from either prokaryotes (such as YfkJ and YwlE of *B. subtilis*) (556) or eukaryotes. Proteins of both classes become phosphorylated at a cysteyl residue, which is located close to the N terminus and is surrounded by hydrophobic amino acids (132, 634). The sequence similarity between the lactose/cellobiose EIIBs and low-molecular-weight P-tyrosine protein phosphatases seems to be restricted to their N-terminal parts. Similarly, the active site of EIIAs of the mannose class PTS resembles the catalytic site in cofactor-dependent phosphoglycerate mutase and phosphatases dephosphorylating either low-molecular-weight substrates (such as eukaryotic fructose-2,6-bisphosphatase as well as *E. coli* glucose-1-P phosphatase and acid polyphosphatase AppA) or sensor kinases of two-component systems (such as *E. coli* SixA, which dephosphorylates P~ArcB) (Deutscher, unpublished). Common to these phosphatases is the R-H-G signature (620), which is preceded by four to five hydrophobic residues. The histidine probably functions as a phosphoryl acceptor during catalysis. For *E. coli* phosphoglycerate mutase 1 (GpmA), a partially phosphorylated histidine, which is part of the R-H-G motif and is located in position 10, was observed in the crystal structure (69). The enzyme becomes phosphorylated during the interconversion of 1,3- and 2,3-bisphosphoglycerate. The phosphorylatable histidine of *E. coli* $EIIA^{Man}$ is also located in position 10 and is part of the T-H-G signature, which, similar to the R-H-G motif in the above-mentioned phosphatases, is preceded by several hydrophobic amino acids. It is not clear whether the similarities between PTS components and certain enzymes, mainly transiently phosphorylated phosphatases, result from divergent or convergent evolution.

It is also surprising that the two groups of bacteria that are mainly discussed in this review, i.e., *Enterobacteriaceae* and *Firmicutes*, both use PTS components for the complex regulation of carbohydrate metabolism but that the mechanisms of PTS-mediated regulation are often completely different. It has been pointed out that while $EIIA^{Glc}$ is the master regulator in *Enterobacteriaceae*, HPr carries out this function in low-G+C gram-positive bacteria. For CCR, P~$EIIA^{Glc}$ stimulates the activity of adenylate cyclase and thereby activates Crp in *Enterobacteriaceae*, and P-Ser-HPr interacts with and stimulates CcpA in *Firmicutes*. For inducer exclusion, dephospo-$EIIA^{Glc}$ interacts with non-PTS permeases in *Enterobacteriaceae*, and P-Ser-HPr most likely interacts with non-PTS permeases in *Firmicutes*. For the regulation of GlpK, dephospo-$EIIA^{Glc}$ inhibits the catabolic enzyme by binding to its C-terminal part in *Enterobacteriaceae*, and P~His-HPr phosphorylates a histidyl residue in the N-terminal half of GlpK and thereby stimulates the glycerol kinase activity in *Firmicutes*. According to today's commonly accepted branching order, the *Firmicutes* branched off first, and the γ-*Proteobacteria*, to which the *Enterobacteriaceae* belong, branched off last (303). P-Ser-HPr formation in firmicutes probably developed after branching off, and the occurrence of HprK/P in several proteobacteria might be the result of horizontal gene transfer. These proteobacteria usually contain EI, two EIIAs, and an HPr, in which the region around Ser-46 resembles the corresponding region in HPr of low-G+C gram-positive bacteria, but lack CcpA (41, 64). The role of P-Ser-HPr formation, which is also regulated by metabolites in most proteobacteria, remains obscure. Similarly, proteins with PRDs are much more abundant in firmicutes, and their rare appearance in proteobacteria probably also results from horizontal gene transfer. The early branching off of the firmicutes might partly explain why completely different mechanisms for the regulation of carbon metabolism evolved in the two phyla. At the same time, it underlines the importance of the PTS, as in the two groups of bacteria, evolution favored the development of mechanisms for the regulation of carbon metabolism and other cellular functions, in which PTS components play a major role, but apparently came up with quite different solutions for *Enterobacteriaceae* and *Firmicutes*.

Whatever the course of evolution may have been, the vast number of physiological processes controlled by the PTS makes it difficult to decide whether the main function of the PTS lies in carbohydrate transport or in regulation of cellular processes. The majority of PTS-mediated control mechanisms relate to the phosphorylation state of the PTS components and, therefore, to the energy state of the cell. Among others, the PTS senses the PEP-to-pyruvate ratio and the intracellular concentrations of ATP, glycolytic intermediates, PP_i, etc., and its regulatory functions are therefore directly connected to the carbon and energy supply of the cell or, to use a more general term, to the fitness of the cell. The PTS-mediated regulatory functions can affect carbohydrate chemotaxis, the transport process, carbohydrate-specific metabolic steps, central metabolic pathways, and synthetic routes for secondary metabolites. In addition, specific mechanisms aimed at coordinating carbon metabolism and the metabolism of other essential elements, such as nitrogen and phosphorus, have developed. The scope of all these regulatory processes is to guarantee an optimal supply of carbon and energy without taking up too much of a carbon source. The latter might lead to futile cycles or might even have toxic effects. Although the general scope of the regulatory functions is very similar in gram-positive and gram-negative bacteria, the control mechanisms themselves can vary greatly.

Although a large number of PTS-mediated control mechanisms has been established, new PTS-related regulatory functions continue to emerge. An interesting new area is the rela-

tionship between the PTS and the pathogenicity of certain organisms. A connection between the PTS and the virulence of certain pathogens was suggested by the observation that some virulence genes underlie a kind of CCR. We extensively discussed this phenomenon for *L. monocytogenes*, where the diminished phosphorylation of PTS proteins during the uptake of glucose, fructose, cellobiose, etc., seems to be responsible for the inhibition of PrfA, a transcription activator of numerous virulence genes in this organism. In *C. difficile*, an important causative agent of nosocomial antibiotic-associated diarrhea and colitis, glucose and other rapidly metabolizable carbon sources inhibit the synthesis of toxin A and toxin B (202). It is not clear whether CCR of the *toxA* and *toxB* genes is mediated via P-Ser-HPr/CcpA or whether the repression of toxin synthesis occurs via another mechanism possibly involving the alternative σ factor TxeR, which is required for the expression of the *tox* genes (509). Synthesis of the catabolite-regulated transcription activator Mga, which is required for the expression of numerous surface-associated proteins involved in pathogenesis in *S. pyogenes*, seems to be controlled directly by CcpA. A connection between CcpA and virulence also exists in *S. pneumoniae*, where the inactivation of CcpA affects the synthesis of capsular polysaccharides and diminishes the virulence of this organism (273). Inactivation of the EI paralog PtsP in *P. aeruginosa* (854) or *L. pneumophila* (333) led to strongly reduced virulence, because the *ptsP* mutants exhibited reduced growth in their hosts. In addition, some EII components seem to play a role in the pathogenicity of certain bacteria. For example, inactivation of the *Streptococcus suis manN* gene, which codes for an EIID of the mannose class PTS, caused a hyperhemolytic phenotype due to the elevated synthesis of suilysin, the hemolysin produced by this organism. A mutant complementation test established that the presence of ManN lowers the synthesis of suilysin (496). Inactivation of an EIIABC of the glucose class PTS in *S. mutans*, the primary agent of dental caries in humans, led to the overproduction of the virulence factor fructan hydrolase. On the other hand, a mutant lacking the mannose-specific EIIAB exhibited an impaired capacity to form biofilms, which is probably due to the diminished synthesis of exopolysaccharide-forming glucosyltransferases (*gtfBC*) (5). EIIAs of the fructose and mannose class PTS were also proposed to play a role in the virulence of numerous gram-negative bacteria, which have in common that they possess an HprK/P and an incomplete PTS that is usually composed of an EI, an HPr, and one or two EIIAs. These bacteria therefore cannot transport sugars via the PTS, and P-Ser-HPr probably plays no role in CcpA-mediated CCR, as these organisms lack CcpA. Preliminary results suggesting a potential involvement of the incomplete PTS of these gram-negative bacteria in virulence regulation are discussed in this review.

A connection between the sensitivity/resistance of certain bacteria towards class IIa bacteriocins and PTS proteins has been reported. For example, the EIIAB component of a mannose class PTS (called Mpt) was found to be absent from several spontaneous leucocin A-resistant *L. monocytogenes* mutants (693), while overproduction of a β-glucoside-specific PTS in the same organism led to pediocin PA-1 resistance (294). The role of the Mpt PTS in class IIa bacteriocin resistance has been studied in more detail. The specificity of the Mpt PTS has not been determined, but it is likely to be the mannose/glucose-specific PTS, as its expression in *L. monocytogenes* is induced by these two sugars (151). In addition, the EIID domain of the Mpt PTS possesses a C-terminal extension characteristic of mannose/glucose-specific PTS, and the various sugar-specific EII^{Mpt} components exhibit the strongest similarity to those of the recently characterized mannose/glucose PTS of *L. casei* (975). Homologs of the Mpt PTS are present in *E. faecalis* and *Listeria innocua*, and the absence of expression of the *mpt* operon in these organisms also leads to resistance towards class IIa bacteriocins such as mesentericin Y105 for *E. faecalis* (320) and pediocin AcH for *L. innocua* (959). The expression of the *mpt* operon containing the *mptA*, *mptC*, and *mptD* genes, which encode EIIAB, EIIC, and EIID, respectively, depends on σ^{54} (RpoN) and on a LevR-like protein, which was called ManR. In addition, a novel activator for the *mpt* genes, which distantly resembles proteins of the Crp/Fnr family, has been identified in *L. innocua* (Lin0142) and is also present in *L. monocytogenes* (Lmo0095) (959). It contains a winged-helix DNA binding motif, and its gene precedes the *mpt* operon. Its inactivation prevents *mpt* expression and leads to pediocin AcH resistance. It seems that both membrane components of the mannose/glucose PTS play a role in bacteriocin sensitivity. An *L. monocytogenes* strain carrying a 28-amino-acid in-frame deletion in the C-terminal part of $EIID^{Mpt}$ was fully resistant to mesentericin Y105 (151). On the other hand, expression of *L. monocytogenes mptC* in *L. lactis* rendered the latter organism sensitive towards class IIa bacteriocins (692). It was therefore proposed that the membrane components of the mannose/glucose PTS are necessary for the docking of the bacteriocin. Interestingly, the *E. coli* membrane-spanning mannose-specific PTS components are also important for the infection by bacteriophage lambda (218, 950), and they can be replaced in lambda infection by the *B. subtilis* PTS components of the *lev* operon (525).

PTS proteins also seem to play a role in certain stress responses. Some PTS components are either overproduced or barely synthesized in response to a specific stress. For example, HPr of *L. lactis* is strongly diminished or almost vanishes in cells that had been exposed to a pH of 5.5 or 4.5, respectively (243). In contrast, *E. coli* cells exposed to low pH contain elevated amounts of the PTS components HPr and $EIIAB^{Man}$ (ManX) (63). Similarly, osmotic stress was found to increase the amount of CcpA and of an EIIA of a mannose class PTS in *L. monocytogenes* (200), while a cold shock led to the overproduction of HPr in *B. subtilis* (293) and *L. lactis* (956). Finally, the induction of the *E. coli ompC* gene in response to increasing temperatures was prevented by a specific mutation in the TD2 strain. A gene (*hrsA*, for heat response suppressor) that restored the thermoresponse in the TD2 mutant was identified (892). The *hrsA* gene codes for a fructose-like EIIABC, and it was renamed *mngA* when it was found to catalyze the transport of the osmolyte 2-*O*-α-mannosyl-D-glycerate (775). A connection between the cold shock response and the PTS was also inferred by the finding that an *L. casei ptsH*(*Ile47Thr*) mutant overproduced CspA (47), one of the small cold shock proteins found in most bacteria. Csps are normally among the first proteins overproduced in response to a cold shock. Interestingly, Csps exhibit sequence and structure similarities to the C-terminal part of EIIAs of the glucose class PTS. They con-

tain a histidine homologous to the phosphorylatable His-91 in *E. coli* EIIA^{Glc} (47), which, however, could not be phosphorylated by PEP, EI, and HPr. Nevertheless, this histidine is important for Csp function (659). In addition, various *L. casei* mutants affected in CCR exhibited reduced growth rates at low temperatures compared to that of the wild-type strain. Finally, the two mutants that were unable to form P-Ser-HPr (*ptsH1* and *hprK*) showed an almost 100-fold-reduced survival rate after repeated freezing and thawing compared to the wild-type strain and other *ptsH* and *ccpA* mutants (47). It is not yet understood how the ability to form P-Ser-HPr increases the resistance to freezing and thawing.

PTS components not only interact with other proteins and enzymes to regulate the activity of their targets but they are sometimes found as domains in non-PTS proteins, suggesting that the activity of these hybrid proteins is controlled by the PTS domain, most likely in a phosphorylation-dependent manner. The examples of the lactose and raffinose transporters, which contain a regulatory EIIA^{Glc}-like domain, and of transcription activators, which possess an EIIB^{Gat} and either an EIIA^{Man} or EIIA^{Mtl} domain, have been discussed in detail. Another example of a non-PTS protein containing a domain of a PTS component is the *C. acetobutylicum* multidomain HprR protein (for HPr response regulator), parts of which exhibit significant sequence identity (about 40%) to several response regulators of two-component systems (*B. subtilis* BkdR, RocR, and AcoR and *E. coli* NtrC). Interestingly, instead of the usual N-terminal receiver module, HprR possesses an HPr-like domain, which contains a phosphorylatable His-15 (721), while Ser-46 is missing. It is therefore tempting to assume that HprR activity is regulated by phosphorylation at its His-15 or via interactions of the HPr domain with EI or EIIAs.

Pyruvate kinase of *G. stearothermophilus* contains a C-terminal domain that strongly resembles the phosphorylation domain of EI (599). From the numerous available genome sequences, it became clear that, in fact, most gram-positive bacteria possess a pyruvate kinase with an EI-resembling C-terminal extension. The phosphorylatable histidine is nicely conserved (His-539 in *G. stearothermophilus* and *B. subtilis* pyruvate kinase), and it was therefore proposed that pyruvate kinase of gram-positive bacteria might be regulated via PTS-catalyzed phosphorylation. However, attempts to phosphorylate the EI domain of *B. subtilis* pyruvate kinase with PEP or PEP plus EI and HPr have not been successful so far (G. Boël and J. Deutscher, unpublished results). In fact, the EI domain of pyruvate kinase from gram-positive bacteria shows more extended sequence similarity to PEP synthases.

The above-mentioned examples make it clear that we are far from understanding all the regulatory roles of the PTS. It seems almost certain that future research will lead to the discovery of as-yet-unimagined regulatory functions of this complex sugar transport and global cellular control system.

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