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Phosphorylation of HPr and Crh by HprK, Early Steps in the Catabolite Repression Signalling Pathway for the *Bacillus subtilis* Levanase Operon

ISABELLE MARTIN-VERSTRAETE,[1]* JOSEF DEUTSCHER,[2] AND ANNE GALINIER[3]

Unité de Biochimie Microbienne, CNRS URA 1300, Institut Pasteur, F-75724 Paris,[1] Laboratoire de Génétique des Microorganismes, INRA-CNRS ERS 567, F-78850 Thiverval-Grignon,[2] and Institut de Biologie et Chimie des Protéines, CNRS UPR 412, F-69367 Lyon Cedex 07,[3] France

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Carbon catabolite repression (CCR) of *Bacillus subtilis* catabolic genes is mediated by CcpA and in part by P-Ser–HPr. For certain operons, Crh, an HPr-like protein, is also implicated in CCR. In this study we demonstrated that in *ptsH1 crh1* and *hprK* mutants, expression of the *lev* operon was completely relieved from CCR and that both P-Ser–HPr and P-Ser–Crh stimulated the binding of CcpA to the *cre* sequence of the *lev* operon.

The main function of histidine-containing protein (HPr) is to participate in the phosphotransferase system (PTS)-catalyzed transport and phosphorylation of carbohydrates. Being part of a phosphoenolpyruvate-dependent protein phosphorylation chain, HPr is phosphorylated by enzyme I (EI) at His-15 (8) and transfers the phosphoryl group to the sugar-specific EIIAs. In gram-positive bacteria, the phosphoryl carrier protein HPr is also phosphorylated at a regulatory serine (Ser-46) by ATP and the HPr kinase (HprK) (2, 7, 22). This ATP-dependent phosphorylation regulates the induction and carbon catabolite repression (CCR) of several catabolic genes (23). Replacement of Ser-46 with alanine (*ptsH1* mutation) prevents the ATP-dependent phosphorylation of HPr and almost completely abolishes CCR of many operons (3). However, several operons such as the *xyn*, *iol*, and *lev* operons were not relieved or only partly relieved from CCR in the *ptsH1* mutant (3, 6a, 18). Growth conditions were also found to influence CCR in *ptsH1* mutants, and operons which were completely derepressed in minimal medium were only partly relieved from CCR in complex medium (3).

In addition to HPr, an HPr-like protein called Crh (for "catabolite repression HPr"), which was discovered during the *Bacillus subtilis* sequencing project and exhibits 45% sequence identity to HPr, was suggested to participate in CCR (6a). Since His-15 of HPr is replaced by a glutamine in Crh, no phosphoenolpyruvate-dependent, EI-catalyzed phosphorylation of Crh could be detected. However, Crh becomes phosphorylated by ATP and the metabolite-activated HprK at the conserved Ser-46 (6a, 7). If the *crh* gene of a *ptsH1* mutant was disrupted, almost complete relief from CCR was observed for β-xylosidase, inositol dehydrogenase, and levanase, indicating that both HPr and Crh are implicated in CCR of the corresponding operons (6a). In addition, HPr and Crh participate in glucose-induced activation of *ackA* expression (24).

Catabolite control protein A (CcpA), a member of the LacI-GalR family of repressors, acts as a pleiotropic regulator of CCR in *B. subtilis* and binds to the *cis*-active operator sequences (*cre*, for "catabolite response element") (11, 12, 25). An interaction of P-Ser–HPr with CcpA has been demonstrated in vitro (4, 13). The resulting complex binds specifically to the *cre* sequences of the *gnt*, *xyl*, and *xyn* operons and of the *amyE* gene (5, 6, 9, 14). P-Ser–Crh presumably also exerts its effect on CCR via CcpA, since those operons, which were only slightly relieved from CCR in a *ptsH1* mutant, were similarly relieved from CCR in *ccpA* and *ptsH1 crh* double mutants (6a).

The levanase operon of *B. subtilis* (*levDEFG-sacC*) encodes a fructose-specific PTS (*lev*-PTS) and the extracellular levanase, which hydrolyzes fructose polymers and sucrose (17). Expression of this operon is induced by fructose and repressed by glucose (16). CCR of the *B. subtilis* levanase operon seems to involve at least two mechanisms: one mediated by phosphorylation of the transcriptional activator LevR by P-H15–HPr observed in a constitutive background (*levR8*) (19) and the other mediated by the repressor CcpA (18). HPr and Crh are also involved in the CcpA-dependent CCR mechanism operative for the levanase operon (6a, 18). A potential CcpA binding site, *cre*, was identified between the −12, −24 promoter and its upstream activating sequence, the target site for LevR, the specific activator of the operon (18).

In this study, we have confirmed the role of P-Ser–HPr and P-Ser–Crh in CCR of the levanase operon by constructing a *ptsH1 crh1* double mutant and by testing the interaction of the CcpA/P-Ser–HPr and CcpA/P-Ser–Crh complexes with the *cre* sequence of the levanase operon. We have also tested the involvement of HPr kinase in the regulation of the levanase operon.

Ser-46 is the unique site of phosphorylation in Crh. The site of phosphorylation in Crh had been determined by mass spectroscopy to be Ser-46 (6a). However, mass spectroscopy can fail to detect minor phosphorylation sites. We therefore wanted to make sure that Ser-46 represents the only site of phosphorylation in Crh. For this purpose, a 250-bp DNA fragment containing either the *crh* or *crh1* allele was amplified by PCR with chromosomal DNA of the *ptsGHI* deletion strain GM273 (3) or plasmid pRC17 (6a) and appropriate primers containing an *Eco*RI site (3′ end) or a *Bam*HI site (5′ end). The *Eco*RI-*Bam*HI fragments were cloned into the expression vector pGEX-KT (10). Crh or CrhS46A fused to glutathione *S*-transferase (GST) was expressed from the resulting plasmids after transformation in *E. coli* NM522 and isopropyl-β-D-thiogalactopyranoside (IPTG) induction. Protein purification was carried out as described by Hakes and Dixon (10). Phosphorylation of GST-Crh and GST-CrhS46A was tested in the presence of HprK as described by Galinier et al. (6a) (Fig. 1).

* Corresponding author. Present address: Unité de Régulation de l'Expression Génétique, CNRS URA1129, Institut Pasteur, F-75724 Paris, France. Phone: 33-1-45688445. Fax: 31-1-45688948. E-mail: iverstra@pasteur.fr.

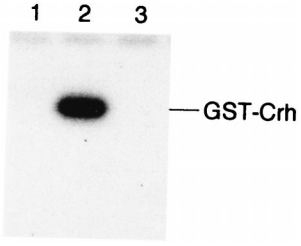

FIG. 1. ATP-dependent phosphorylation of GST-Crh and GST-CrhS46A. [γ-^{32}P]ATP and HprK were incubated together with GST-Crh (lane 2) or GST-CrhS46A (lane 3). Lane 1 is the control without GST-Crh or GST-CrhS46A. The phosphorylation reaction was stopped by adding sample buffer to the assay mixtures before loading them onto a sodium dodecyl sulfate-polyacrylamide gel. After electrophoresis, the gel was treated for 5 min with boiling 16% trichloroacetic acid before being dried and exposed to autoradiography (Biomax MR; Kodak). Coomassie blue-stained gels on which the same samples had been loaded revealed a single band for HPr and Crh (data not shown).

GST-Crh was phosphorylated by [γ-^{32}P]ATP (Fig. 1, lane 2), while GST-CrhS46A was not (lane 3), confirming that Ser-46 is the only site of HprK-catalyzed phosphorylation in Crh.

P-Ser–Crh participates in CCR of the levanase operon. Similar to the situation for a *ccpA* mutant, CCR of the levanase operon was abolished in a *ptsH1 crh*::*aphA3* double mutant (6a). Crh was therefore assumed to carry out its function in CCR via interaction of phosphorylated Crh with CcpA. To test this hypothesis, a chromosomal *crh1* mutant was constructed by using a pHT315 derivative (1) carrying the *crh1* allele and part of the downstream *yvcN* gene (pRC23, Fig. 2). A kanamycin resistance cassette was introduced into *yvcN*, giving plasmid pRC33 (Fig. 2). This plasmid was linearized with *Pst*I and used to transform QB5081 (*levD′-′lacZ*) and QB7148 (*ptsH1 levD′-′lacZ*). Cotransformation of the *crh1* allele with the kanamycin resistance cassette allowed us to isolate Km^r and Em^s clones containing the *crh1* allele (QB7158) or both the *ptsH1* and *crh1* mutations (QB7159). The presence of the mutations was confirmed by sequencing appropriate PCR products of these strains. Expression of the *levD′-′lacZ* fusion in the wild-type QB5081 and the *crh1* mutant QB7158 was decreased 13- and 10-fold, respectively, by the addition of glucose (Table 1). A 4.5-fold repression was observed in the *ptsH1* mutant, whereas the *ptsH1 crh1* double mutant was almost completely relieved from CCR (1.5-fold repression). These results suggest that CCR of the levanase operon is mediated via phosphorylation of HPr and Crh at Ser-46. However, under the experimental conditions used, HPr can completely replace Crh in CCR of the *lev* operon whereas Crh can only partly substitute for HPr, since a *ptsH1* mutant is partially relieved from CCR.

Both HPr and Crh are phosphorylated by ATP and HprK (6a, 7). The *B. subtilis hprK* (former *yvoB*) gene has recently been identified (7, 22), and HprK was found to be bifunctional, also catalyzing the dephosphorylation of P-Ser–HPr (15). To confirm the importance of ATP-dependent HPr and Crh phosphorylation for CCR of the levanase operon, we constructed an *hprK* mutant by using plasmid pRC37 containing *hprK* carrying a deletion from codons 26 to 232 and a kanamycin resistance cassette inserted at the newly created *Cla*I restriction site (Fig. 2). Plasmid pRC37 was cut with *Sca*I and used to replace *hprK* in QB5081 (*levD′-′lacZ*) with the modified *hprK*. A Km^r

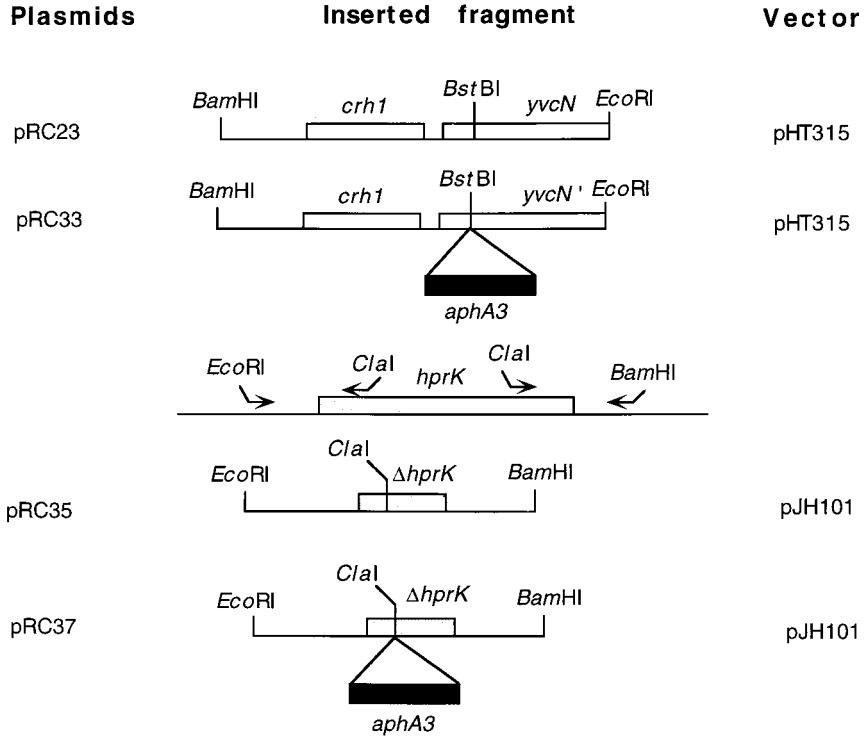

FIG. 2. Construction and restriction map of plasmids used in this study. A 1-kb DNA fragment containing the *crh1* allele and part of *yvcN* was cloned between the *Bam*HI and *Eco*RI sites of pHT315 (1) to give plasmid pRC23. A 1.5-kb *Cla*I DNA fragment containing the kanamycin resistance gene *aphA3* was inserted in *yvcN* at the unique *Bst*BI restriction site, providing plasmid pRC33. Plasmid pRC35 was constructed as follows. An *Eco*RI-*Cla*I and a *Cla*I-*Bam*HI fragment, corresponding to the 5′ and 3′ ends of *hprK*, respectively, were amplified by PCR. These two fragments were cloned into the integrative vector pJH101 digested with *Eco*RI and *Bam*HI, thus reconstituting an *hprK* gene deleted from codons 26 to 232. pRC37 was obtained by insertion of the 1.5-kb kanamycin cassette into the newly created *Cla*I site of *hprK*.

TABLE 1. Regulation of the expression of a *levD'-'lacZ* fusion by CcpA, P-Ser–HPr, and P-Ser–Crh

Strain[a]	Relevant genotype	β-Galactosidase activity (U/mg of protein)[b] in:		Repression factor
		CSK Fru	CSK Fru Glu	
QB5081	*crh*$^+$ *ptsH*$^+$	220	17	13
QB5224	*ptsH1*	405	87	4.5
QB7158	*crh1*	270	27	10
QB7159	*ptsH1 crh1*	390	220	1.5
QB7160	*hprK*::*aphA3*	370	282	1.3

[a] All strains contain a *levD'-'lacZ* translational fusion integrated in the *amyE* gene and a *trpC2* mutation.

[b] Specific activities of β-galactosidase were determined in extracts prepared from exponentially growing cells (absorbance at 600 nm, 0.7 to 1). The mean values of at least three independent experiments are presented. Cells were grown in CSK medium, which is C minimal medium supplemented with potassium succinate (6 g/liter) and potassium glutamate (8 g/liter) (17), or on CSK medium containing 0.2% fructose or 0.2% fructose plus 1% glucose. The method of Miller was used for the determination of β-galactosidase activity (21).

Cm^s clone (QB7160) was isolated, and the presence of the kanamycin cassette in *hprK* was confirmed by PCR. Similar to the situation for the *ptsH1 crh1* double mutant, expression of the *levD'-'lacZ* fusion in QB7160 was reduced only 1.3-fold by glucose (Table 1), confirming that ATP-dependent phosphorylation of HPr and Crh is part of the CCR signal transduction pathway operative for the levanase operon. However, we cannot exclude that in addition to the *hprK* mutation, alterations in the expression of the downstream genes due to the insertion of the kanamycin resistance cassette into *hprK* might influence CCR.

Binding of the CcpA/P-Ser–HPr and CcpA/P-Ser–Crh complexes to the *lev cre* sequence. To completely understand the signal transduction pathway in CCR of the levanase operon, we wanted to investigate whether both P-Ser-HPr and P-Ser-Crh influence the binding of CcpA to the *lev cre* sequence. P-Ser–HPr has been demonstrated to interact in vitro with CcpA (4, 13), and the resulting protein complex binds specifically to the *cre* sequences of the *gnt*, *xyn*, and *xyl* operons and of the *amyE* gene (5, 6, 9, 14). To test whether P-Ser–Crh can also interact with CcpA and allow specific binding of CcpA to the *lev cre* sequence, we performed DNase I footprinting experiments. A 178-bp fragment containing the *lev* promoter region (from positions −148 to +30) was 3′-end labelled with [α-^{32}P]dATP at the *Eco*RI site. The assay mixture contained 10 mM HEPES (pH 7.6), 1 mM $MgCl_2$, 200 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 50 μg of poly(dI-dC)-(dI-dC) per ml as the bulk carrier DNA, the radioactive DNA probe (100,000 cpm), 2.5 μM CcpA, and 10 μM HPr, P-Ser–HPr, Crh, or P-Ser–Crh. After a 15-min incubation at room temperature, 2 ng of bovine pancreatic DNase I (Worthington) was added to the assay mixture, which was incubated for a further 60 s at room temperature. DNase I digestion was terminated by adding stop solution (final concentration, 2.5 mM EDTA, 0.4 M sodium acetate, and 50 μg of calf thymus DNA) and subjecting the mixture to phenol extraction. All the proteins were purified on Ni-nitrilotriacetate-agarose columns, and HPr$(His)_6$ and Crh$(His)_6$ were phosphorylated by HprK$(His)_6$ in the presence of ATP as described by Galinier et al. (6a, 7). Under these conditions, HPr and Crh were about 90% phosphorylated. After phosphorylation, HprK was inactivated by keeping the assay mixture for 10 min at 80°C.

The results of the DNase I footprinting experiments are presented in Fig. 3. In the presence of either P-Ser–HPr or P-Ser–Crh, CcpA specifically recognized the *lev cre* sequence (Fig. 3, lanes 4 and 7, respectively). This interaction was not observed with HPr or Crh (data not shown) or when CcpA, CcpA and HPr, or CcpA and Crh were present in the assay mixture (lanes 2, 3, and 6, respectively). The slightly increased protection in the presence of CcpA and HPr or of CcpA and Crh appears to be nonspecific, since it affects the total DNA (lanes 3 and 6). By contrast, the region strongly protected in

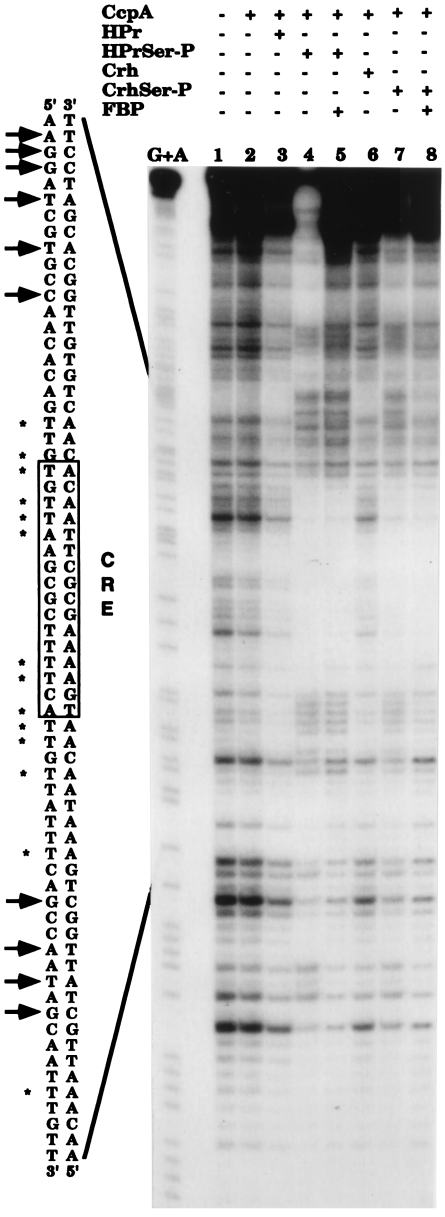

FIG. 3. DNase I footprinting experiments with the *lev cre* sequence in the presence of CcpA, P-Ser–HPr, and P-Ser–Crh. A 178-bp *Eco*RI-*Pst*I fragment (from positions −148 to +30) containing the *lev* promoter and the *cre* sequence (from position −50 to −36) was labeled at the 3′-end as described in the text. DNA was digested with DNase I in the absence of any protein (lane 1) or in the presence of 5 μM CcpA (lane 2); 2.5 μM CcpA and 10 μM HPr (lane 3), 2.5 μM CcpA and 10 μM P-Ser–HPr (lane 4), 2.5 μM CcpA, 10 μM P-Ser–HPr, and 20 mM FBP (lane 5), 2.5 μM CcpA and 10 μM Crh (lane 6), 2.5 μM CcpA and 10 μM P-Ser–Crh (lane 7), or 2.5 μM CcpA, 10 μM P-Ser–Crh, and 20 mM FBP (lane 8). The base-specific chemical cleavage reaction at guanine and adenine (20) is shown in lane G+A. The *cre* sequence is boxed, and the *cre* consensus sequence is indicated. The bases protected against digestion by DNase I are indicated by asterisks, while the sites of hyperdigestion by DNase I are indicated by arrows.

the presence of CcpA and P-Ser–HPr or of CcpA and P-Ser–Crh (AAATAACAACAAT**TGAAAACGCTTAACA**CAA) (lanes 4 and 7) contains the presumed *cre*-like sequence (in bold letters) located between positions −50 and −36 upstream of the promoter of the levanase operon (18). Moreover, sites of hypersensitivity to DNase I digestion were observed only when CcpA and either P-Ser–HPr or P-Ser–Crh were present in the reaction mixture. The addition of 20 mM fructose-1,6-bisphosphate (FBP) did not modify the binding of the CcpA protein in the presence of P-Ser–HPr or P-Ser–Crh (lanes 5 and 8).

Compared to the consensus *cre* sequence, the *lev cre* sequence contains additional base pairs close to the 3′ end. In addition, most of the *cre* sequences were found to be located either within the coding sequence of the corresponding gene or close to the transcription start site (12). In the levanase operon, the *cre* sequence (−50 to −36) is situated upstream from the −12, −24 promoter. Binding of the CcpA/P-Ser–HPr or CcpA/P-Ser–Crh complexes to the *cre* sequence located between the LevR binding site (upstream activating sequence) and the −12, −24 promoter may influence the formation of the complex between LevR and the RNA polymerase-σ^{54} which is necessary for melting the DNA and activating transcription. In this context, it is interesting that binding of the CcpA/P-Ser–HPr or CcpA/P-Ser–Crh complexes also caused significant alterations in the pattern of DNase I digestion outside the *cre* sequence (Fig. 3), suggesting that binding of CcpA to the *lev cre* sequence might induce changes in the DNA structure.

CCR of the *B. subtilis* levanase operon seems to involve at least two mechanisms: one mediated by the transcriptional activator LevR (19) and the other mediated by the repressor CcpA. The first CCR mechanism is based on activation of LevR by P-His–HPr-dependent phosphorylation at His-585. In the presence of a PTS sugar, the phosphoryl group of P-His–HPr is thought to be preferentially used for sugar phosphorylation, leading to poor phosphorylation of LevR. The reduced phosphorylation of LevR lowers its transcriptional activator function and leads to slowed expression of the levanase operon (19). The second CCR mechanism is based on the interaction of P-Ser–HPr or P-Ser–Crh with CcpA. Under the reaction conditions used, we observed similar binding affinities with both complexes. In the case of the *B. subtilis xyn* operon, a more detailed study had revealed that the CcpA/P-Ser–HPr complex is more effective in protecting the *xyn cre* sequence (6). The increased production of glycolytic intermediates accompanying the rapid metabolism of a carbon source is thought to activate HprK, which catalyzes the ATP-dependent phosphorylation of HPr and Crh. P-Ser–HPr and P-Ser–Crh function as corepressors by interacting with CcpA, the global regulator of CCR. They enable CcpA to bind to the *lev cre* sequence located between the binding site of the transcriptional activator LevR and the −12, −24 promoter, thus preventing expression of the *lev* operon.

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