The Q15H mutation enables Crh, a Bacillus subtilis HPr-like protein, to carry out some regulatory HPr functions, but does not make it an effective phosphocarrier for sugar transport

Isabelle Martin-Verstraete,1‡ Anne Galinier,2 Emmanuelle Darbon,3 Yves Quentin,4 Marie-Claude Kilhoffer,5 Véronique Charrier,2‡ Jacques Haiech,5 Georges Rapoport1 and Josef Deutscher3

Author for correspondence: Josef Deutscher. Tel: +33 1 30 81 54 47. Fax: +33 1 30 81 54 57.
e-mail: jdeu@platon.grignon.inra.fr

Keywords: PEP:sugar phosphotransferase system, HPr, Crh, catabolite repression

INTRODUCTION

The histidine-containing protein (HPr) is a phosphocarrier protein which plays a central role in the uptake of carbohydrates via the phosphoenolpyruvate (PEP):sugar phosphotransferase system (PTS). HPr can be phosphorylated by ATP at the regulatory Ser-46 and similar to P-Ser-HPr, P-Ser-Crh plays a role in carbon-catabolite repression. The sequence around the phosphorylatable Ser-46 in Crh exhibits strong similarity to the corresponding sequence of HPr of Gram-positive and a few Gram-negative bacteria. In contrast, the catalytic His-15, the site of PEP-dependent phosphorylation in HPr, is replaced with a glutamine in Crh. When Gln-15 was exchanged for a histidyl residue, in vitro PEP-dependent enzyme I-catalysed phosphorylation of the mutant Crh was observed. However, expression of the crhQ15H mutant allele did not restore growth of a ptsH deletion strain on the PTS sugars glucose, fructose or mannitol or on the non-PTS sugar glycerol. In contrast, Q15H mutant Crh could phosphorylate the transcriptional activator LevR as well as LevD, the enzyme IIA of the fructose-specific lev-PTS, which together with enzyme I, HPr and LevE forms the phosphorylation cascade regulating induction of the lev operon via LevR. As a consequence, the constitutive expression from the lev promoter observed in a ∆ptsH strain became inducible with fructose when the crhQ15H allele was expressed in this strain.

Crh of Bacillus subtilis exhibits 45% sequence identity when compared to histidine-containing protein (HPr), a phosphocarrier protein of the phosphoenolpyruvate (PEP):sugar phosphotransferase system (PTS). Crh can be phosphorylated by ATP at the regulatory Ser-46 and similar to P-Ser-HPr, P-Ser-Crh plays a role in carbon-catabolite repression. The sequence around the phosphorylatable Ser-46 in Crh exhibits strong similarity to the corresponding sequence of HPr of Gram-positive and a few Gram-negative bacteria. In contrast, the catalytic His-15, the site of PEP-dependent phosphorylation in HPr, is replaced with a glutamine in Crh. When Gln-15 was exchanged for a histidyl residue, in vitro PEP-dependent enzyme I-catalysed phosphorylation of the mutant Crh was observed. However, expression of the crhQ15H mutant allele did not restore growth of a ptsH deletion strain on the PTS sugars glucose, fructose or mannitol or on the non-PTS sugar glycerol. In contrast, Q15H mutant Crh could phosphorylate the transcriptional activator LevR as well as LevD, the enzyme IIA of the fructose-specific lev-PTS, which together with enzyme I, HPr and LevE forms the phosphorylation cascade regulating induction of the lev operon via LevR. As a consequence, the constitutive expression from the lev promoter observed in a ∆ptsH strain became inducible with fructose when the crhQ15H allele was expressed in this strain.

Keywords: PEP:sugar phosphotransferase system, HPr, Crh, catabolite repression

INTRODUCTION

The histidine-containing protein (HPr) is a phosphocarrier protein which plays a central role in the uptake of carbohydrates via the phosphoenolpyruvate (PEP):sugar phosphotransferase system (PTS) (for a review, see Postma et al., 1993). It is phosphorylated by the PEP-dependent protein kinase enzyme I at His-15 and transfers the phosphoryl group to several enzymes IIA, each specific for a certain carbohydrate. The phosphorylated enzymes IIA phosphorylate their corresponding enzyme IIB, which finally transfers the phosphoryl group to the carbohydrate bound to the membrane-spanning enzyme IIC. The enzymes II either can form a single protein with three domains (A, B and C) or can be split into two to four distinct proteins (Saier & Reizer, 1992). HPr of Gram-positive bacteria is also phosphorylated by the metabolite-activated ATP-dependent HPr kinase at Ser-46 (Deutscher & Saier, 1983; Galinier et al., 1991, 1998). HPr kinase is a bifunctional enzyme that also catalyses dephosphorylation of P-Ser-HPr (Kravanja et al., 1999). P-Ser-HPr functions as corepressor in carbon-catabolite repression by interacting with the catabolite control protein A (CcpA) (Deutscher et al., 1995), a member of the LacI/GalR family of repressors (Henkin et al., 1991).
The complex between P-Ser-HPr and CcpA binds to the catabolite response element (cre) (Fujita et al., 1995; Gössteringer et al., 1997; Kim et al., 1998; Galinier et al., 1999; Martin-Verstraete et al., 1999), an operator-like sequence present in most catabolite-repressed genes and operons (Hueck et al., 1994).

Most bacteria possess only one ptsI and one ptsH gene encoding the general PTS components enzyme I and HPr, respectively. In Bacillus subtilis, the general PTS components participate in sugar transport and phosphorylation via the approximately 15 different sugar-specific enzymes II detected within the genome sequencing programme (Kunst et al., 1997). However, a few Gram-negative bacteria possess more than one enzyme I or HPr. Escherichia coli and Salmonella typhimurium, for example, contain a fructose-inducible diphosphoryl transfer protein which is composed of an N-terminal enzyme IA<sup>Ptr</sup> and a C-terminal HPr-like domain (Waygood et al., 1984; Geerse et al., 1989) called FPr (fructose-specific HPr). The diphosphoryl transfer protein is phosphorylated by enzyme I at the HPr-like domain from where the phosphoryl group is subsequently transferred to the enzyme IIA<sup>Pfu</sup> domain. Interestingly, Haemophilus influenzae was found to possess a protein composed of a C-terminal enzyme IIA<sup>Pfu</sup> domain and two FPr domains fused in tandem to the C-terminus. This protein was also suggested to be active in fructose transport, but no specific function was attributed to the second FPr domain (Reizer et al., 1996).

Evidence for PTS-mediated regulation of nitrogen assimilation and fixation was provided by the finding that in several Gram-negative bacteria the two genes ptsN and ptsO, which encode an enzyme IIA<sup>Pfu</sup> and an HPr-like protein, respectively, were located in the vicinity of the rpoN gene (Reizer et al., 1992a; Jones et al., 1994). The rpoN gene encodes the alternate sigma factor σ<sup>II</sup>, which is necessary for transcription of genes required for nitrogen assimilation and fixation, and σ<sup>II</sup> was thought to be regulated by PtsN and PtsO. These two proteins were therefore renamed enzyme IIA<sup>Nr</sup> and NP<sup>r</sup> (for nitrogen-related HPr), respectively. Enzyme IIA<sup>Nr</sup> and NP<sup>r</sup> were found to exchange phosphate with enzyme I, HPr and enzyme IIA of the PTS (Powell et al., 1995). Loss of enzyme IIA<sup>Nr</sup> activity diminished carbon-catabolite repression of σ<sup>II</sup>-dependent transcription of the Pseudomonas putida xylS operon when expressed in E. coli (Du et al., 1996).

Neither FPr nor NP<sup>r</sup> were detected in B. subtilis (Kunst et al., 1997). Nevertheless, B. subtilis was found to possess a protein exhibiting 45% sequence identity to HPr. It contained the phosphorylatable Ser-46, but the catalytic His-15 was replaced with a glutamine (Galinier et al., 1997). As a consequence, no PEP-dependent, enzyme I-catalysed phosphorylation of this protein could be detected (Galinier et al., 1997). However, the HPr-like protein can be phosphorylated by the ATP-dependent HPr kinase at Ser-46 (Galinier et al., 1997). Similar to P-Ser-HPr, the seryl-phosphorylated HPr-like protein seems to interact with CcpA and to participate in catabolite activation of the ackA gene (Turinsky et al., 1998) and in catabolite repression of the B. subtilis lev, xyn and iol operons (Galinier et al., 1997, 1999; Martin-Verstraete et al., 1999) as well as the aceA gene (Zalieckas et al., 1998). It was therefore called Crh (for catabolite repression HPr). By replacing Gln-15 of Crh with a histidine we wanted to study to what extent this mutation will enable Crh to carry out the catalytic and regulatory functions of P-His-HPr.

**METHODS**

**Bacterial strains, growth conditions, transformation procedures and phenotypic characterization.** B. subtilis strain GM1341 carries an in-frame deletion of codons 7–42 of ptsI (Dral/HpaI excision, called ptsHADH) (Tortosa et al., 1997). Strains CRH168 and HC1341 were obtained by insertional inactivation of the crh gene of either B. subtilis wild-type strain 168 or the ptsH deletion strain GM1341 using plasmid pMTrch1. Transformation with this plasmid, derived from the vector pMUTIN1 (Vagner et al., 1998), leads to selective disruption of crh after a single recombination event with the bacterial chromosome. Chromosomal integration of a single copy of pMTrch1 and its insertion into crh was confirmed by PCR. Strain QB7111 was obtained by co-transformation of strain 168 with chromosomal DNA from strain QB5030 carrying a sacC::lacZ erm fusion (Martin-Verstraete et al., 1990) and from strain GM1341 containing the ptsH deletion. Erythromycin-resistant transformants were selected and subsequently tested for constitutive expression of the sacC::lacZ fusion. Constitutive expression from the lev promoter suggested the presence of the internal ptsH deletion ptsHADH, which was confirmed by PCR. Strain QB7114 was obtained by integrating the crhQ15H allele into amyE of strain QB7111 containing a sacC::lacZ fusion and the ptsH deletion ptsHADH. E. coli TG1 [K-12 Δlak::proE thi bsdF/F traD36 proA’ B’ lacI + lacZAM15] was used for cloning experiments, E. coli CJ236 [ΔgalU thi relA/pJCl05 (Cm<sup>4</sup>)] for site-directed mutagenesis of crh and E. coli BL21 (DE3) (Novagen) and M15[pREP4] (Qiagen) for overproduction of proteins. E. coli strains were grown in LB medium and B. subtilis in SP or C minimal medium. CSK medium is a minimal medium supplemented with potassium succinate (6 g l<sup>-1</sup>) and potassium glutamate (8 g l<sup>-1</sup>) (Martin-Verstraete et al., 1990).

Standard procedures were used to transform E. coli and B. subtilis strains (Sambrook et al., 1989; Kunst & Rapoport, 1995). Transformants were selected on agar plates containing ampicillin (100 μg ml<sup>-1</sup>), erythromycin (1 μg ml<sup>-1</sup>) plus lincomycin (25 μg ml<sup>-1</sup>) or chloramphenicol (5 μg ml<sup>-1</sup>). β-Galactosidase activities were measured after growth of B. subtilis strains in CSK medium with or without 0.2% fructose using the method of Miller (1972). One unit of β-galactosidase activity is defined as the amount of enzyme that produces 1 nmol of o-nitrophenol per min at 28 °C. Doubling times were determined by growing the bacteria in C minimal medium supplemented with glucose, fructose, mannitol, glycerol or glucitol (each at a concentration of 0.5%).

**Plasmid constructions.** Plasmid pMTrch1 was obtained by cloning a fragment of crh into the vector pMUTIN1 (Vagner et al., 1998) cut with HindIII and BamHI. The crh fragment was obtained by PCR using chromosomal DNA from B. subtilis strain 168 as template and the following two primers: 

CCCAAGGCTTGGATTAAAGGACAGGACTGCAAGCAGG

CCCAAGGCTTGGATTAAAGGACAGGACTGCAAGCAGG

CCCAAGGCTTGGATTAAAGGACAGGACTGCAAGCAGG

CCCAAGGCTTGGATTAAAGGACAGGACTGCAAGCAGG

CCCAAGGCTTGGATTAAAGGACAGGACTGCAAGCAGG

CCCAAGGCTTGGATTAAAGGACAGGACTGCAAGCAGG

CCCAAGGCTTGGATTAAAGGACAGGACTGCAAGCAGG

CCCAAGGCTTGGATTAAAGGACAGGACTGCAAGCAGG

CCCAAGGCTTGGATTAAAGGACAGGACTGCAAGCAGG

CCCAAGGCTTGGATTAAAGGACAGGACTGCAAGCAGG

CCCAAGGCTTGGATTAAAGGACAGGACTGCAAGCAGG

CCCAAGGCTTGGATTAAAGGACAGGACTGCAAGCAGG

CCCAAGGCTTGGATTAAAGGACAGGACTGCAAGCAGG
and CGCGGATCCGGAACGTAGCAGCAGCTTCTCC. The non-complementary HindIII and BamHI restriction sites are indicated in bold. A 1 kb DNA fragment of plasmid pRC12 containing the crb gene was cloned in M13mp19 to give M13mp19crb (Galinier et al., 1997). The Muta-Gene M13 in vitro megakinesis kit (Bio-Rad) was used to obtain the crbQ15H allele. A 434 bp PaeII-BstBI DNA fragment containing the promoterless crb gene or crbQ15H allele was inserted into pDR67 (Ireton et al., 1993) cut with Smal and Clal to give plasmid pRC16 or pRC18, respectively. Plasmid pDR67 carries the pH194 chloramphenicol acetyltransferase gene cat and a spac promoter between two fragments of the B. subtilis amyE gene. The crb and crbQ15H alleles were integrated downstream of the spac promoter allowing induction of their expression with 1 mM IPTG after single-copy integration at the amyE locus of the B. subtilis genome. Appropriate oligonucleotides and pRC16 or pRC18 were used to amplify a DNA fragment carrying the crb or crbQ15H alleles containing the spac promoter and a BamHI site introduced 85 bp downstream of the crb stop codon. These fragments were integrated into plasmid pHT315 cut with EcoRI/BamHI giving plasmids pRC19 (wild-type crb) and pRC20 (crbQ15H), respectively. A 285 bp fragment containing the crbQ15H gene was amplified by PCR using RF M13mp19crbQ15H as template and two oligonucleotides creating an Ndel and a PstI restriction site at the 5' and 3' end, respectively. The Ndel–PstI fragment was cloned into the expression vector pT7-7(6×His) (Cortay et al., 1994). The mutant protein CrbQ15H carrying a polyhistidine fused to the C-terminus was expressed from the resulting plasmid pAG6 after transformation in the E. coli strain BL21(DE3) (Novagen).

Plasmids pAG1, pAG2, pAG3, pQE30D and pQE30E, which were used for overproducing His-tagged Crh (His-tag attached to the C-terminus), HPr (His-tag added to the N-terminus), enzyme I, LevD and Leve, respectively, have been described previously (Galinier et al., 1997; Charrier et al., 1997a). Transformants carrying one of the above plasmids were grown at 37 °C in 2×TY medium until the culture had reached an OD₆₀₀ of 0.7. Expression of the genes encoding Crh, HPr, enzyme I, LevD or Leve was induced by adding IPTG to a final concentration of 1 mM, and incubation was continued for a further 4 h. Enzyme I(His)₄, Crh(His)₄ and HPr(His)₄ (Galinier et al., 1997) as well as LevD(His)₄ and Leve(His)₄ (Charrier et al., 1997a) were purified on Ni-NTA-agarose columns.

Two-dimensional gel electrophoresis. B. subtilis cells were disrupted by adding a solubilization solution containing 8 M urea, 4% CHAPS and 65 mM dithioerythritol to a cell suspension and by sonicating the cells three times for 20 s (Branson sonifier 430 equipped with a cuphorn). The volume of the added solubilization solution exceeded the volume of the cell suspension at least threefold. Aliquots of solubilized cells containing between 30 and 40 µg protein were used per silver-stained two-dimensional gel. Two-dimensional gel electrophoresis was performed using a commercially available immobilized pH gradient (IPG from Pharmacia Biotech) for the first dimension according to the method described on the web server (http://www.expasy.ch/ch2d/protocols.fln2.ht ml#998745). The voltage was increased stepwise from 100 V to 3900 V over a period of 4 h and then maintained at 3900 V until a total of 150 kVh was reached. The temperature was maintained at 20 °C. The IPG strips were then equilibrated and transferred to a slab gel containing a 12–20% polyacrylamide gradient, a 4–6% glycerol gradient and 0.1% SDS, and the electrophoresis was performed at 70 V overnight. Gels were silver stained using the Protein Silver Staining Kit from Pharmacia Biotech. The HPr migration position in the total cellular extract was first determined by comparison to the migration position of purified HPr (without His-tag). In the two-dimensional gels with the B. subtilis mutants GM1341 and CRH168, and HPr were also identified by mass spectrometry of tryptic fragments generated by digestion of the proteins present in the corresponding spots cut out of the gel.

Protein phosphorylation assays. Phosphorylation assays with [³²P]PEP, enzyme I(His)₄ and Crh(His)₄, CrbQ15H(His)₄, or HPr(His)₄ were carried out in a total volume of 20 µl. One-and-a-half micrograms of Crh(His)₄, CrbQ15H(His)₄, or HPr(His)₄ was incubated for 10 min at 37 °C with 2 µg enzyme I(His)₄ in 50 mM Tris/HCl, pH 7.4, 15 mM MgCl₂ and 10 mM [³²P]PEP (0.5 µCi; 18.5 kBq). [³²P]PEP was prepared from [³²P]ATP (Roossien et al., 1983). Leve was phosphorylated with [³²P]PEP, enzyme I(His)₄ and HPr(His)₄, Crh(His)₄, or CrbQ15H(His)₄ as described previously (Martin-Verstraete et al., 1998).

Phosphorylation of LevD and Leve was carried out by incubating a reaction mixture containing 0.2 µg enzyme I(His)₄, 0.5 µg LevD(His)₄, 5 µg Leve(His)₄, 12.5 mM MgCl₂, 50 mM Tris/HCl (pH 7.4), 10 µM [³²P]PEP (0.5 µCi) and 0.1 µg either HPr(His)₄ or Crh(His)₄ for 20 min at 37 °C in a total volume of 20 µl (Charrier et al., 1997a). All phosphorylation reactions were stopped by adding an equal volume of sample buffer (Laemmli, 1970) to the assay mixture. Proteins were separated by SDS-PAGE. Gels were dried for 2 h without prior fixation or coloration and exposed to X-ray film (Biomax MR, Kodak).

RESULTS

Sequence alignment of Crh with HPr from Gram-negative and Gram-positive bacteria

The amino acid sequence of Crh was compared to different HPr, FPr, NPr and multiphosphoryl transfer proteins, and to several putative HPrs, the sequences of which were derived from complete or incomplete microbial genome sequences (Fig. 1). The region around the PEP-dependent phosphorylation site His-15 (corresponds to Gln-15 in Crh) is similar in HPr from both Gram-positive and Gram-negative bacteria and only a few amino acids were found to be present exclusively or primarily in HPr of either Gram-positive (Thr-12, Leu-21 and Gln-24) or Gram-negative bacteria (Leu-14, Ala-20 and Phe-22) (see Fig. 1). Crh seems to contain the characteristics of the PEP-dependent HPr phosphorylation site from both phylogenetically distinct groups. In contrast, the region around the ATP-dependent phosphorylation site Ser-46 in Crh exhibited much stronger similarity to the corresponding region in HPrs from Gram-positive bacteria. The previously proposed consensus sequence for the ATP-dependent phosphorylation site in HPr of Gram-positive bacteria (V/G)(N/D)XKS(L/I)[M/L](G/N)(V/L)(M/L) (Galinier et al., 1998) is in full agreement with the corresponding sequence from position 42 to 51 in Crh. However, this consensus sequence has also been found in HPr of a few Gram-negative bacteria such as Alcaligenes eutrophus, Bordetella pertussis, Chlo-
Fig. 1. Alignment of proteins related to Crh. The multiple alignment was carried out by using the CLUSTAL W program included in the sequence editor SEA VIEW (Galtier et al., 1996). The shading of the multiple alignment has been done with BOX SHADE (K. Hofmann & M. D. Baron, unpublished). Residues in the HPr proteins that are identical to the corresponding residue in the Crh sequence are highlighted in black. The sequence KSIMGLMSL present in Crh from position 45 to 53, which is well-conserved in HPrs and has been shown, or assumed, to be phosphorylated at Ser-46, is boxed. The following protein sequences were extracted from SWISS-PROT (very similar sequences, such as those from E. coli and S. typhimurium, are represented by only one entry). HPr family: HPr from B. subtilis (PTHP J_BACSU), B. stearothermophilus (PTHP J_BACST), Lactobacillus sakei (PTHP J_LACSK), Listeria monocytogenes (PTHP J_LISMO), Staphylococcus carnosus, Sta. aureus (PTHP J_STACA), Streptococcus mutans, Str. salivarius (PTHP J_STRMU), Ent. faecalis (PTHP J_ENTFA), Mycoplasma capricolum (PTHP J_MYCCA), Mycoplasma pneumoniae, M. genitalium (PTHP J_MYPN), E. coli, S. typhimurium (PTFA J_ECOLI), H. influenzae (first and second HPr-like domain: PTFA J_HAEI1 and PTFA J_HAEI2); MTP family: HPr-like domain from Rhodobacter capsulatus (PTF1 J_RHOCA) and Xanthomonas campestris (PTF1 J_XANC). The other sequences were retrieved from complete or incomplete microbial genome sequences available on the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/index.html) by using the TBLASTN program (Altschul et al., 1997) and Crh as the query sequence. Preliminary genome sequence data were obtained from the website of The Institute for Genomic Research (http://www.tigr.org). Incomplete microbial genomes: Caulobacter crescentus (two occurrences), N. meningitidis, N. gonorrhoeae, Bor. pertussis, P. aeruginosa, Chl. tepidum, Clostridium acetobutylicum and complete genome: T. pallidum.

Pseudomonas aeruginosa, Neisseria gonorrhoeae, Neisseria meningitidis and Treponema pallidum. Interestingly, the HPrs of these organisms are most closely related to Crh (Fig. 2). Although belonging to the Gram-negative bacteria, several of these organisms including T. pallidum, C. tepidum, Bor. pertussis, N. meningitidis and N. gonorrhoeae were found to contain a homologue of the bifunctional enzyme HPr kinase/P-Ser-HPr phosphatase (Galilier et al., 1998; Reizer et al., 1998; Kravanja et al., 1999), suggesting that HPr of these Gram-negative organisms might also be phosphorylated at Ser-46. On the unrooted phylogenetic tree (Fig. 2), most Gram-positive HPrs are clustered and this is also true for several Gram-negative HPrs (E. coli, H. influenzae), whereas the other proteins including Crh and its closest relatives are all branched at the same node or very close to this node. This type of tree suggests that the proteins exhibiting the strongest sequence similarity to Crh do not constitute a homogeneous family but are intermediate between HPrs from Gram-positive and Gram-negative bacteria.

Mutation of ptsH and/or crh does not cause noticeable changes in the overall protein-synthesis pattern

Two-dimensional gel electrophoresis experiments were carried out with crude extracts prepared from the B. subtilis wild-type strain 168 (ptsH+/crb+), the two single mutants GM1341 (ptsH−) and CRH168 (crb−) as well as
from the \textit{ptsH crh} double mutant HC1341. Compared to the wild-type strain, no drastic changes of the protein synthesis pattern in the double or the two single mutants were observed (data not shown), suggesting that neither HPr nor Crh exerts a strong influence on gene expression when cells are grown in rich medium in the absence of carbohydrates. The migration position of HPr from \textit{B. subtilis} 168 has been determined as described under Methods. However, a spot migrating to the position of HPr was present not only in the \textit{crh} mutant CRH168, but also in the \textit{ptsH} mutant GM1341 carrying a 108 bp in-frame deletion from codon 7 to 42 in \textit{ptsH} (Tortosa \textit{et al}., 1997) (data not shown). This protein spot disappeared only in the \textit{ptsH crh} double mutant HC1341, suggesting that although Crh and HPr of \textit{B. subtilis} exhibit only 45\% sequence identity, the two proteins migrate to exactly the same position during two-dimensional gel electrophoresis. This assumption was confirmed by carrying out mass spectrometry with tryptic fragments derived from the corresponding protein spot obtained from strain CRH168, which was found to represent HPr, and from strain GM1341, which was identified as Crh. This finding is in agreement with the very similar molecular mass and pI values calculated for these two proteins (pI 4.86 and 4.9, and molecular mass 9058 Da and 9327 Da for HPr and Crh, respectively). The protein spots corresponding to Crh in strain GM1341 and to HPr in strain CRH168 were of similar intensity, suggesting that similar amounts of HPr and Crh are present in the cell.

**Q15H mutant Crh can be phosphorylated by enzyme I and PEP**

His-15 is the site of PEP-dependent enzyme I-catalysed phosphorylation of HPr. In Crh, His-15 was found to be replaced with a glutamine and no PEP-dependent, enzyme I-catalysed phosphorylation of wild-type Crh was detected (Galinier \textit{et al}., 1997). We wanted to test whether replacement of Gln-15 of Crh with a histidyl

**Fig. 2.** Unrooted tree showing the evolutionary relatedness of HPr and HPr-like proteins from various bacteria. The branch length is proportional to the relative evolutionary distance. The tree has been constructed with PUZZLE (Strimmer & von Haeseler, 1996), a program based on maximum likelihood, that gives a statistical estimation of each internal branch (numbers at nodes). We used the JTT models of substitution with a rate heterogeneity modelled by a discrete gamma distribution with 4 categories (abbreviations are as indicated in Fig. 1).
residue would allow PEP-dependent enzyme I-catalysed phosphorylation of the mutant protein. A mutation exchanging Gln-15 for histidine was introduced into \( crb \), the \( crbQ15H \) allele was inserted into a His-tag expression vector and His-tagged CrhQ15H was purified as described in Methods. PEP-dependent phosphorylation experiments were carried out with enzyme I and HPr, Crh or CrhQ15H. As previously reported (Galinier et al., 1997), no PEP-dependent phosphorylation was observed for wild-type Crh (Fig. 3a, lane 3). However, under the experimental conditions employed, the Q15H mutant Crh was found to be phosphorylated to a similar extent as HPr (Fig. 3a, lanes 2 and 4). In contrast to wild-type HPr and Crh, the two His-tagged proteins were found to migrate to slightly different positions during SDS-PAGE. This is probably due to the different composition and location of the His-tag in HPr and Crh (see Methods section).

**Q15H mutant Crh does not restore growth of the ptsHΔDH strain on PTS sugars and glycerol**

To find out whether CrhQ15H can replace HPr as phosphoryl carrier in PTS-catalysed sugar uptake, we tested whether a \( ptsH \) deletion strain producing CrhQ15H would be able to grow in minimal medium containing 1 mM IPTG (for induction of the \( crb \) allele under the control of the \( spac \) promoter) and 0.5 % of glucose, fructose, mannitol, glycerol or glucitol. Compared to the wild-type strain, the \( ptsH \) deletion strain QB7111 exhibited drastically increased doubling times when grown in the presence of the PTS sugars glucose, fructose or mannitol or the non-PTS sugar glycerol, whereas growth in the presence of glucitol was slowed only slightly (Table 1). Expression of the \( crbQ15H \) allele from the \( spac \) promoter in strain QB7114 had no detectable influence on its growth behaviour, indicating that, when integrated as a single copy into \( amyE \), the \( crbQ15H \) allele is not capable of restoring the uptake of the PTS sugars glucose, fructose or mannitol or the P-His-HPr-activated catabolism of glycerol (Deutscher et al., 1993; Charrier et al., 1997b). To test the effect of \( crb \) or \( crbQ15H \) expression from a multicopy plasmid on the growth behaviour of a \( ΔptsH \) strain, QB7111 was transformed with pRC19 or pRC20, \( crb- \) or \( crbQ15H- \) carrying derivatives, respectively, of the multicopy plasmid pHT315 (Arantes & Lereclus, 1991). Similar to strain QB7114, which contains the \( crbQ15H \) allele integrated into \( amyE \), the resulting transformants grew very slowly on minimal medium containing either glucose, fructose, mannitol or glycerol as the sole carbon source (data not shown), although the amount of Crh present in the cells transformed with plasmids pRC19 or pRC20 was found to be clearly elevated compared to the amount of Crh present in cells containing the \( crb \) allele in single copy. The amount of Crh in the various transformants and integrants was quantified by carrying out Western blots with crude extracts using rabbit polyclonal antibodies directed against \( B. subtilis \) Crh (data not shown).

**CrhQ15H can phosphorylate LevD and LevR**

To test whether CrhQ15H is capable of restoring other catalytic and regulatory functions carried out by P-His-HPr, we investigated whether it can phosphorylate the transcriptional activator LevR (Martin-Verstraete et al., 1998) and catalyse the LevD-mediated phosphorylation of LevE, a fructose-specific enzyme IIB (Charrier et al., 1997a). LevR and LevD have been demonstrated to be phosphorylated by P-His-HPr (Charrier et al., 1997a) (see also Fig. 3b, lanes 1 and 4). In the presence of wild-type Crh, no phosphorylation of LevR and of LevD/LevE was observed (Fig. 3b, lanes 2 and 5). The weak radioactive band migrating to the position of LevE in Fig. 3 (b), lane 5 is probably due to a contamination of LevD, since it was also observed when the phosphorylation mixture did not contain LevE (data not shown). However, LevR, LevD and LevE became clearly phosphorylated when Crh was replaced with CrhQ15H in the phosphorylation assay (Fig. 3b, lanes 3 and 6). Since the amount of LevE present in the phosphorylation assay was 10-fold higher than the amount of LevD, the phosphoryl group was mainly transferred to LevE (Fig. 3b, lane 6). The predominant phosphorylation of LevE suggests that, identical to the phosphorylation in the presence of HPr (Charrier et al., 1997a), LevD is probably simultaneously phosphorylated at His-9 in the presence of CrhQ15H and the phosphoryl group is subsequently transferred from phosphorylated LevD to His-15 in LevE. In contrast, although glycerol kinase from **Enterococcus casseliflavus** was found to be phosphorylated by HPr at a single histidyl residue (Charrier et al., 1997b), no phosphorylation of this protein was detected in the presence of CrhQ15H (data not shown).
**Table 1.** Doubling time of *B. subtilis* 168 and a *ptsHΔDH* strain with and without the *crhQ15H* allele integrated at the *amyE* locus

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant Genotype</th>
<th>Doubling time (min) in C medium supplemented with:</th>
</tr>
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<tr>
<td></td>
<td></td>
<td>Glucose</td>
</tr>
<tr>
<td>QB5030</td>
<td><em>ptsH</em></td>
<td>75</td>
</tr>
<tr>
<td>QB7111</td>
<td><em>ptsHΔDH</em></td>
<td>400</td>
</tr>
<tr>
<td>QB7114</td>
<td><em>ptsHΔDH</em></td>
<td>400</td>
</tr>
<tr>
<td></td>
<td><em>pspac crhQ15H</em></td>
<td></td>
</tr>
</tbody>
</table>

**Table 2.** Expression of a *sacC*::*lacZ* fusion in a *ptsHΔDH* strain in the presence and absence of *CrhQ15H*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant Genotype</th>
<th>β-Galactosidase activity [Units (mg protein)^-1]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CSK</td>
</tr>
<tr>
<td>QB7111</td>
<td><em>ptsHΔDH</em></td>
<td>470</td>
</tr>
<tr>
<td></td>
<td><em>sacC</em>::<em>lacZ</em></td>
<td></td>
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<tr>
<td>QB7114</td>
<td><em>ptsHΔDH</em></td>
<td>6</td>
</tr>
<tr>
<td></td>
<td><em>pspac crhQ15H</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>sacC</em>::<em>lacZ</em></td>
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Constitutive expression from the *lev* promoter in the *ptsHΔDH* strain becomes fructose-inducible when *CrhQ15H* is synthesized

In addition to phosphorylation of LevR at His-585 by enzyme I and HPr (Fig. 3b, lane 1), which stimulates its transcriptional activator activity, LevR was also found to be phosphorylated via enzyme I, HPr, LevD and LevE at His-869. This second phosphorylation inactivates LevR and occurs when fructose or mannose, the substrates of the lev-PTS, are absent from the growth medium. In the presence of one of its substrates, the components of the lev-PTS and consequently also His-869 of LevR are thought to become dephosphorylated leading to activation of LevR and to induction of the lev operon. In agreement with this concept, replacement of His-869 with alanine (Martin-Verstraete et al., 1998) or mutations inactivating enzyme I, HPr, LevD and LevE (Martin-Verstraete et al., 1990, 1995; Stülke et al., 1995) led to constitutive expression from the *lev* promoter. We wanted to test whether the synthesis of CrhQ15H would allow phosphorylation of LevR at His-869 and thus make the constitutive expression from the *lev* promoter in a *ptsH* deletion strain fructose-inducible. When grown in the absence of a sugar, the *ptsH* deletion strain QB7111 exhibited constitutive expression of a *sacC*::*lacZ* fusion (Table 2). The *sacC* gene encodes the enzyme levansucrase and is the fifth and last gene in the *lev* operon (Martin-Verstraete et al., 1990). A similarly high β-galactosidase activity was measured when the cells were grown in the presence of fructose. In contrast, the *ptsH* deletion strain QB7114 in which the *crhQ15H* mutant allele was integrated into *amyE* and expressed from the *pspac* promoter exhibited very low β-galactosidase activity in the absence of fructose. β-Galactosidase activity was more than 150-fold higher when strain QB7114 was grown in fructose-containing medium (Table 2), indicating that in the presence of CrhQ15H, LevR is inactivated by PEP-dependent phosphorylation when Gln-15 is replaced with a histidine, by PEP and enzyme I at His-15. Similar to that observed for a *ptsH* strain (Martin-Verstraete et al., 1990), expression from the *lev* promoter in QB7114 was fructose-inducible, as the uptake of fructose via the lev-PTS probably leads to dephosphorylation and activation of LevR.

**DISCUSSION**

Crh exhibits not only 45% sequence identity to HPr of *B. subtilis* and migrates to the same position during two-dimensional gel electrophoresis, it can also be phosphorylated by ATP and the HPr kinase at Ser-46 and, when Gln-15 is replaced with a histidine, by PEP and enzyme I at His-15. Similar to P-Ser-HPr, P-Ser-Crh was found to act as corepressor for CcpA during carbon-catabolite repression and catabolite activation (Galini et al., 1997; Turinsky et al., 1998; Zalieckas et al., 1998; Galinier et al., 1999; Martin-Verstraete et al., 1999). These results suggest that the structure of Crh strongly resembles that of HPr proteins. Separation of crude extracts from *B. subtilis* GM1341 (*ptsH* deletion) and CRH168 (*crh* disruption) on two-dimensional gels and identification of the spots representing HPr and Crh suggested that the two proteins HPr and Crh are...
synthesized in similar amounts. However, expression of the gene encoding CrhQ15H in a ptsH deletion strain did not restore growth on the PTS sugars glucose, fructose and mannitol, neither when expressed as single copy after integration into amyE, nor when expressed from a multicopy plasmid. Starting from a ptsH deletion strain transformed with a plasmid carrying crhQ15H (pRc20), we tried to obtain spontaneous mutations in crhQ15H allowing growth of the ptsHΔDH strain on mannitol as the sole carbon source. However, our attempts to isolate such crh alleles were not successful. The finding that the N-terminal part of Crh contains certain sequence characteristics found mainly in HPr from Gram-negative bacteria (Fig. 1) possibly provides an explanation for this failure. The $V_{\text{max}}$ for the PEP-dependent phosphorylation at His-15 in HPr from the Gram-negative bacterium E. coli by B. subtilis enzyme I was found to be 20-fold lower compared to the $V_{\text{max}}$ determined for the phosphorylation reaction with the two B. subtilis proteins (Reizer et al., 1992b). To make the PEP-dependent phosphorylation site of CrhQ15H more closely resemble the phosphorylation site in Gram-positive HPrs, more than one mutation in addition to the already present Q15H exchange (see Fig. 1) may be required.

Nevertheless, P-His-CrhQ15H was capable of efficiently phosphorylating the transcriptional regulator LevR. LevR has been shown to be phosphorylated by HPr at His-585 and this phosphorylation was suggested to play a role in a CcpA-independent CCR mechanism operative in the lev operon (Martin-Verstraete et al., 1998). In addition, P-His-CrhQ15H could phosphorylate LevD, a fructose-specific enzyme IIA, and the phosphoryl group was subsequently transferred from phosphorylated LevD to LevE, a fructose-specific enzyme IIB. This phosphorylation cascade fulfils two functions. First, it is required for the synthesis of CrhQ15H did not restore growth of a ptsH mutant on fructose (Table 1), although the mutant Crh was found to phosphorylate LevD (Fig. 3, lane 6). In addition, we cannot exclude that the mutant Crh phosphorlates LevD less effectively than HPr, which would further slow fructose uptake via the lev-PTS in the ptsH deletion mutant synthesizing CrhQ15H.

The second function of the enzyme I–HPr–LevD–LevE phosphorylation cascade is phosphorylation of LevR at His-869 (Martin-Verstraete et al., 1998). Phosphorylation of LevR by phosphorylated LevE regulates induction of the lev operon. A ptsH mutant strain, in which the phosphorylation cascade leading to the formation of P-His869-LevR is interrupted, exhibited constitutive expression from the lev promoter (Martin-Verstraete et al., 1998). Expression of the gene encoding CrhQ15H in a ΔptsH mutant allowed reconstitution of the phosphorylation cascade from enzyme I to LevD to LevE and finally LevR, which prevented the constitutive expression from the lev promoter observed in the ΔptsH mutant and restored its fructose-dependent induction.

Enterococcal glycerol kinases have been shown to be phosphorylated by PEP, enzyme I and HPr at a histidyl residue conserved in glycerol kinases of Gram-positive bacteria; phosphorylation increased glycerol kinase activity about 10-fold (Charrier et al., 1997b). Enterococcus faecalis or B. subtilis mutants defective in enzyme I or HPr therefore have low glycerol kinase activity and cannot grow on media containing glycerol as the sole carbon source (Reizer et al., 1984; Romano et al., 1990; Gonty-Treboul et al., 1991). Expression of crhQ15H was not able to restore growth of a B. subtilis ptsH deletion strain in the presence of glycerol. This is in agreement with the finding that although B. subtilis HPr could phosphorylate glycerol kinase from Ent. casseliflavus, no in vitro phosphorylation of this protein was observed in the presence of CrhQ15H. Again, the finding that Crh seems to contain certain characteristics of the PEP-dependent phosphorylation site of HPr from Gram-negative bacteria might explain that CrhQ15H is able to phosphorylate LevR, but not glycerol kinase. PEP-dependent phosphorylation of glycerol kinase has been observed only in Gram-positive bacteria (Charrier et al., 1997b). In contrast, proteins containing the PTS regulation domain, which carries the site of P-His-HPr dependent phosphorylation of LevR, have also been detected in Es. coli and other Gram-negative bacteria (Stülke et al., 1998).

In summary, the results presented here show that Crh in which Gln-15 has been replaced with a histidine is capable of carrying out some of the regulatory functions of HPr (phosphorylation of the transcriptional activator LevR at His-585 and His-869). The observed PEP-dependent enzyme I-catalysed phosphorylation of LevD/LevE in the presence of CrhQ15H suggests that CrhQ15H might be able to catalyse slow transport of fructose via the lev-PTS. However, the transport function for the most important PTS sugars glucose, fructose (via FruA) and mannitol as well as the P-His-HPr-dependent activation of glycerol catabolism could not be restored by the presence of CrhQ15H.

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REFERENCES


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