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

¹ Unité de Biochimie Microbienne, CNRS URA 1300, Institut Pasteur, F-75724 Paris, France

² Institut de Biologie et Chimie des Protéines, CNRS UPR 412, F-69367 Lyon Cedex 07, France

³ Laboratoire de Génétique des Microorganismes, INRA-CNRS URA 1925, F-78850 Thiverval-Grignon, France

⁴ Laboratoire de Chimie Bactérienne, CNRS UPR 9043, F-13009 Marseille, France

⁵ Laboratoire de Biophysique, URA 491, Université Louis Pasteur, F-67401 Illkirch Cedex, France

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 $\Delta ptsH$ 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.*, 1998; Reizer *et al.*, 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).

[†] **Present address:** Unité de Régulation de l'Expression Génétique, CNRS URA 1129, Institut Pasteur, F-75724 Paris, France.

[‡] **Present address:** Dept of Biochemistry, The Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, NC 27157-1016, USA.

Abbreviations: FPr, fructose-specific HPr; HPr, histidine-containing protein; NPr, nitrogen-related HPr; PEP, phosphoenolpyruvate; PTS, PEP:sugar phosphotransferase system.

The complex between P-Ser-HPr and CcpA binds to the catabolite response element (*cre*) (Fujita *et al.*, 1995; Gösseinger *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 IIA^{Fru} 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^{Fru} domain. Interestingly, *Haemophilus influenzae* was found to possess a protein composed of an N-terminal enzyme IIA^{Fru} 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^{Fru}- 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^{54} , which is necessary for transcription of genes required for nitrogen assimilation and fixation, and σ^{54} was thought to be regulated by PtsN and PtsO. These two proteins were therefore renamed enzyme IIA^{Ntr} and NPr (for nitrogen-related HPr), respectively. Enzyme IIA^{Ntr} and NPr were found to exchange phosphate with enzyme I, HPr and enzyme IIA of the PTS (Powell *et al.*, 1995). Loss of enzyme IIA^{Ntr} activity diminished carbon-catabolite repression of σ^{54} -dependent transcription of the *Pseudomonas putida xylS* operon when expressed in *E. coli* (Du *et al.*, 1996).

Neither FPr nor NPr 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 *acsA* 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 *ptsH* (*DraI/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 pMTcrh1. 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 pMTcrh1 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 Δ (*lac-pro*) *supE thi hsdS/F' traD36 proA⁺ B⁺ lacI^q lacZAM15*] was used for cloning experiments, *E. coli* CJ236 [*dut ung thi relA/pJC105* (Cm^R)] 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 C minimal medium supplemented with potassium succinate (6 g l⁻¹) and potassium glutamate (8 g l⁻¹) (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 μ g ml⁻¹), erythromycin (1 μ g ml⁻¹) plus lincomycin (25 μ g ml⁻¹) or chloramphenicol (5 μ g ml⁻¹). 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 pMTcrh1 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: CCAAGCTTCGATTAAAGACAGGACTGCAAGCACC

and CGCGGATCCGAACGTAAGCAGCAGCTTCTCC. The non-complementary *Hind*III and *Bam*HI restriction sites are indicated in bold. A 1 kb DNA fragment of plasmid pRC12 containing the *crh* gene was cloned into M13mp19 to give M13mp19*crh* (Galinier *et al.*, 1997). The Muta-Gene M13 *in vitro* mutagenesis kit (Bio-Rad) was used to obtain the *crhQ15H* allele. A 434 bp *Pvu*II–*Bst*BI DNA fragment containing the promoterless *crh* gene or *crhQ15H* allele was inserted into pDR67 (Iretton *et al.*, 1993) cut with *Sma*I and *Clal* to give plasmid pRC16 or pRC18, respectively. Plasmid pDR67 carries the pC194 chloramphenicol acetyltransferase gene *cat* and a *spac* promoter between two fragments of the *B. subtilis amyE* gene. The *crh* and *crhQ15H* 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 *crh* or *crhQ15H* alleles containing the *spac* promoter and a *Bam*HI site introduced 85 bp downstream of the *crh* stop codon. These fragments were integrated into plasmid pHT315 cut with *Eco*RI/*Bam*HI giving plasmids pRC19 (wild-type *crh*) and pRC20 (*crhQ15H*), respectively. A 285 bp fragment containing the *crhQ15H* gene was amplified by PCR using RF M13mp19*crhQ15H* as template and two oligonucleotides creating an *Nde*I and a *Pst*I restriction site at the 5' and 3' end, respectively. The *Nde*I–*Pst*I fragment was cloned into the expression vector pT7-7(6×His) (Cortay *et al.*, 1994). The mutant protein CrhQ15H 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 OD₅₉₅ 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)₆, Crh(His)₆ and HPr(His)₆ (Galinier *et al.*, 1997) as well as LevD(His)₆ and LevE(His)₆ (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 450 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.fm2.html#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 kWh 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, Crh 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)₆ and Crh(His)₆, CrhQ15H(His)₆ or HPr(His)₆ were carried out in a total volume of 20 µl. One-and-a-half micrograms of Crh(His)₆, CrhQ15H(His)₆ or HPr(His)₆ was incubated for 10 min at 37 °C with 2 µg enzyme I(His)₆ in 50 mM Tris/HCl, pH 7.4, 15 mM MgCl₂ and 10 µM [³²P]PEP (0.5 µCi; 18.5 kBq). [³²P]PEP was prepared from [^γ-³²P]ATP (Roossien *et al.*, 1983). LevR was phosphorylated with [³²P]PEP, enzyme I(His)₆ and HPr(His)₆, Crh(His)₆ or CrhQ15H(His)₆ 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)₆, 0.5 µg LevD(His)₆, 5 µg LevE(His)₆, 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)₆ or Crh(His)₆ 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/I)(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*, *Chloro-*

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 SEAVIEW (Galtier *et al.*, 1996). The shading of the multiple alignment has been done with BOXSHADE (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 KSIIMGLMSL present in Crh from position 45 to 53, which is well-conserved in HPrs and has been shown, or is 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_BACSU), *B. stearothermophilus* (PTHP_BACST), *Lactobacillus sakei* (PTHP_LACSK), *Listeria monocytogenes* (PTHP_LISMO), *Staphylococcus carnosus*, *St. aureus* (PTHP_STACA), *Streptococcus mutans*, *Str. salivarius* (PTHP_STRMU), *Ent. faecalis* (PTHP_ENTFA), *Mycoplasma capricolum* (PTHP_MYCCA), *M. pneumoniae*, *M. genitalium* (PTHP_MYCPN), *Esc. coli*, *Sal. typhimurium* (PTHP_ECOLI), *H. influenzae* (PTHP_HAEIN), *Alcaligenes eutrophus* (PTHP_ALCEU); NPr family: NPr from *Esc. coli* and *Klebsiella pneumoniae* (PTSO_ECOLI); FPr family: FPr from *Esc. coli* and *Sal. typhimurium* (PTFA_ECOLI), *H. influenzae* (first and second HPr-like domain: PTFA_HAEI1 and PTFA_HAEI2); MTP family: HPr-like domain from *Rhodobacter capsulatus* (PTF1_RHOCA) and *Xanthomonas campestris* (PTF1_XANCP). 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*.

bium tepidum, *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 (Galinier *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*^{+*crh*⁺), the two single mutants GM1341 (*ptsH*⁻) and CRH168 (*crh*⁻) as well as}

from the *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 *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 *crh* mutant CRH168, but also in the *ptsH* mutant GM1341 carrying a 108 bp in-frame deletion from codon 7 to 42 in *ptsH* (Tortosa *et al.*, 1997) (data not shown). This protein spot disappeared only in the *ptsH crh* double mutant HC1341 suggesting that although Crh and HPr of *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 pro-

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 *et al.*, 1997). We wanted to test whether replacement of Gln-15 of Crh with a histidyl

Fig. 3. *In vitro* PEP-dependent enzyme I-catalysed phosphorylation of HPr, Crh and Q15H mutant Crh. Phosphorylation of the various proteins was carried out as described in Methods. (a) Autoradiogram obtained from a 15% SDS-PAGE gel on which samples containing the following proteins had been loaded: lane 1, enzyme I(His)₆; lane 2, enzyme I(His)₆ and HPr(His)₆; lane 3, enzyme I(His)₆ and wild-type Crh(His)₆; lane 4, enzyme I(His)₆ and CrhQ15H(His)₆. (b) Autoradiogram obtained from a 12.5% SDS-PAGE gel on which samples containing the following proteins had been loaded: lane 1, enzyme I(His)₆, HPr(His)₆ and LevR; lane 2, enzyme I(His)₆, wild-type Crh(His)₆ and LevR; lane 3, enzyme I(His)₆, CrhQ15H(His)₆ and LevR; lane 4, enzyme I(His)₆, HPr(His)₆, LevD(His)₆ and LevE(His)₆; lane 5, enzyme I(His)₆, wild-type Crh(His)₆, LevD(His)₆ and LevE(His)₆; lane 6, enzyme I(His)₆, CrhQ15H(His)₆, LevD(His)₆ and LevE(His)₆.

residue would allow PEP-dependent enzyme I-catalysed phosphorylation of the mutant protein. A mutation exchanging Gln-15 for histidine was introduced into *crh*, the *crhQ15H* 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 *ptsHADH* 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 *crh* 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 *crhQ15H* 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 *crhQ15H* 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 *crh* or *crhQ15H* expression from a multicopy plasmid on the growth behaviour of a $\Delta ptsH$ strain, QB7111 was transformed with pRC19 or pRC20, *crh*- or *crhQ15H*-carrying derivatives, respectively, of the multicopy plasmid pHT315 (Arantes & Lereclus, 1991). Similar to strain QB7114, which contains the *crhQ15H* 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 *crh* 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 intermediately 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 *ptsH*Δ*HDH* strain with and without the *crhQ15H* allele integrated at the *amyE* locus

Strain	Relevant Genotype	Doubling time (min) in C medium supplemented with:				
		Glucose	Fructose	Mannitol	Glycerol	Glucitol
QB5030	<i>ptsH</i> ⁺	75	90	95	96	100
QB7111	<i>ptsH</i> Δ <i>HDH</i>	400	> 600	> 600	> 600	132
QB7114	<i>ptsH</i> Δ <i>HDH</i> <i>pspac crhQ15H</i>	400	> 600	> 600	> 600	130

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

Strain	Relevant Genotype	β -Galactosidase activity [Units (mg protein) ⁻¹]	
		CSK	CSK + fructose
QB7111	<i>ptsH</i> Δ <i>HDH</i> <i>sacC</i> :: <i>lacZ</i> ⁺	470	415
QB7114	<i>ptsH</i> Δ <i>HDH</i> <i>pspac crhQ15H</i> <i>sacC</i> :: <i>lacZ</i> ⁺	6	953

Constitutive expression from the *lev* promoter in the *ptsH*Δ*HDH* 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 or 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 levanase 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 *spac* 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 catalysed by enzyme I, CrhQ15H, LevD and LevE. 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 (Galinier *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 *ptsH*ΔDH 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 $V_{\max}$ 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 $V_{\max}$ 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 HPr, 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 for 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 used for lev-PTS catalysed uptake of fructose (Charrier *et al.*, 1997a). However, fructose uptake via the lev-PTS is very slow (Gay & Delobbe, 1973; Martin-Verstraete *et al.*, 1990). Although the lev-PTS is fully active in *fruA* or *fruB* mutants, which are defective in enzyme IIA^{Fru} or phosphofructokinase 1, respectively, two components of the main fructose metabolic pathway in *B. subtilis*, doubling times exceeding 360 min have been reported for these mutants when they were grown on fructose as the sole carbon source (Gay & Delobbe, 1973). This low fructose-transport activity of the lev-PTS explains why 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 phosphorylates 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; Gonzy-Tréboul *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 *Esc. 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.

ACKNOWLEDGEMENTS

This research was supported by the Ministère de l'Éducation Nationale de la Recherche et de la Technologie, the Centre National de la Recherche Scientifique, the Institut National Agronomique, the Institut Pasteur, the Université Lyon I, the Université Paris 7 and the European Community BIOTECH programme contract number BIO4-CT96-0380. Sequence data from incomplete bacterial genomes were obtained from The Institute for Genomic Research through the website (<http://www.tigr.org>). Sequencing of the genome of *Caulobacter crescentus*, *Chlorobium tepidum* and *N. meningitidis*

was supported by DEO and Wellcome Trust. We are thankful to W. Hengstenberg for providing *B. subtilis* HPr without His-tag, to A. Guiseppe for constructing strains CRH168 and HC1341, to J.-P. Lecaer for mass spectrometry and to C. van Herrewwege and A. Bosch for their expert assistance during preparation of this manuscript.

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Received 19 April 1999; revised 2 July 1999; accepted 9 July 1999.