Drastic Differences in Crh and HPr Synthesis Levels Reflect Their Different Impacts on Catabolite Repression in *Bacillus subtilis*

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In *Bacillus subtilis*, carbon catabolite repression (CCR) of catabolic genes is mediated by ATP-dependent phosphorylation of HPr and Crh. Here we show that the different efficiencies with which these two proteins contribute to CCR may be due to the drastic differences in their synthesis rates under conditions that cause CCR.

In *Bacillus subtilis*, carbon catabolite repression (CCR) of many catabolic genes is mediated by ATP-dependent phosphorylation of Ser-46 of HPr and of its homologue Crh (4). Although these two proteins exhibit high sequence identity (45%) and are both efficiently phosphorylated by the HPr kinase/phosphorylase, their contributions to CCR differ (4). P-Ser-Crh can only partly substitute for P-Ser-HPr in CCR, whereas P-Ser-HPr can completely substitute for P-Ser-Crh in this signal transduction pathway. In order to understand the different behaviors of these two proteins, we compared the expression levels of the corresponding genes, *crh* and *ptsH* (encoding HPr), in the presence of different carbon sources by using transcriptional and translational *lacZ* reporter gene fusions.

MATERIALS AND METHODS

Plasmids, bacterial strains, and growth conditions. The plasmids and the bacterial strains used in this study are listed in Table 1. *Escherichia coli* DH5 α was used as a general cloning host. Plasmid DNAs of the pMutin4 derivatives were prepared from *E. coli* BMH71-18 (*recA*⁺) prior to transformation into *B. subtilis*. *E. coli* and *B. subtilis* strains were routinely grown in Luria-Bertani broth supplemented with the appropriate antibiotics when necessary (ampicillin at 100 μ g/ml for *E. coli* and chloramphenicol at 5 μ g/ml and erythromycin at 0.3 μ g/ml for *B. subtilis*). Standard procedures were used to transform *E. coli* (15) and *B. subtilis* (7). Sequencing of PCR-derived DNA fragments in the final plasmid constructs was carried out by Genome Express (Meylan, France).

Construction of transcriptional and translational fusions of *crh* and *ptsH* to *lacZ*. To construct the fusion of *lacZ* to *crh*-5', the 5' region of *crh* (–120 to +90) was amplified by using the primers BG9 (*crh* [–120 to –103]) and BG10 (*crh* [+90 to +73]), digested at the HindIII and BamHI sites within the primers, and inserted between these sites in pMutin4, resulting in plasmid pBGM6. To construct the fusion of *lacZ* to *ptsH*-5', the 5' region of *ptsH* (–276 to +90) was amplified by using the primers LF1 (*ptsH* [–276 to –258]) and LF2 (*ptsH* [+90 to +73]), digested at the NotI and HindIII sites within the primers, and inserted between these sites in pMutin4, resulting in plasmid pLF2. Plasmid pBGM8 carrying a Φ (*crh-lacZ*)(Hyb) fusion gene was constructed by a three-fragment ligation. The entire *crh* gene with its ribosome binding site (RBS) was amplified by using the primers BG13 (*crh* [–20 to –3]) and BG14 (*crh* [+255 to +238]) and digested at the HindIII and XhoI sites within the primers. In parallel, the 5' part of *lacZ* was amplified from pMutin4 as a template by using the primers

BG15 (pMutin2 [382 to 399]) and BG16 (pMutin2 [698 to 681]) and digested with XhoI (located within primer BG15) and MstII (occurring naturally within *lacZ*). The two digested PCR fragments were ligated to the MstII-HindIII vector backbone of pMutin4 (lacking the RBS_{*spoVG*} and the *lacZ*-5' region). A CTC codon (Leu) was inserted together with the XhoI site between the *crh* and the *lacZ* parts of the fusion gene, and the Val initiation codon of *lacZ* had been removed. To obtain the isogenic construct pLF1, which carries a Φ (*ptsH-lacZ*)(Hyb) fusion gene instead of the Φ (*crh-lacZ*)(Hyb) fusion, the *ptsH* gene was amplified with primers LF3 (*ptsH* [–20 to –3]) and LF4 (*ptsH* [+264 to +247]), digested at the HindIII and XhoI sites within the primers, and used to replace the HindIII-XhoI fragment (encompassing *crh*) in pBGM8. The recombinant plasmids described above were used to transform *B. subtilis* 168 by a single crossover event by selection for erythromycin-resistant transformants. The resulting strains carried the fusions to *lacZ* described above in the respective chromosomal loci. For each strain, the correct structure of the plasmid insertion was verified by a set of seven different PCRs according to the strategy published by Pragai and Harwood (13).

Construction of the *glcT* Δ (–16 to +395) deletion mutants. First, the generalized deletion vector pBGM31 was constructed by a three-fragment ligation. The *cat* gene from plasmid pAC5 (9) was isolated as an EcoRI-EheI fragment (1,096 bp). In parallel, a fragment (311 bp) encompassing the three transcriptional terminators *rmBt₁₂- λ ₀* was isolated from pMutin2 by SmaI and PspI digestion. These fragments were ligated to the ClaI-EcoRI vector backbone of pBluescript SK[–]. The resulting plasmid, pBGM31, thus carries a *cat-rmBt₁₂- λ ₀* cassette encompassed upstream by a SacI-SacII-NotI-SpeI-SmaI-BamHI multiple cloning site and downstream by a SalI-XhoI-ApaI-KpnI multiple cloning site. Thereafter, the *glcT*-3' region (+396 to +889) was amplified with primers BG78 (*glcT* [+396 to +413]) and BG79 (*glcT* [+889 to +872]), digested at the BamHI and SacII sites within the primers, and inserted between these sites in plasmid pBGM31, resulting in the intermediate plasmid pBGM83. Next, the region upstream of *glcT* (–599 to –17) was amplified with the primers BG76 (*glcT* [–599 to –581]) and BG77 (*glcT* [–17 to –35]), digested at the ApaI and XhoI sites within the primers, and inserted between these sites in pBGM83. The resulting construct, pBGM84, was linearized by ScaI digestion and used to transform strains SG57 and SG58 by a double crossover with selection for chloramphenicol resistance. In the resulting strains, SG87 and SG88, the 5' part of *glcT* (–16 to +395) was deleted and replaced by the *cat-rmBt₁₂- λ ₀* cassette reading in the opposite direction of *glcT* transcription.

Construction of a control strain carrying a *Pspac-lacZ* cassette inserted in *amyE*. A control strain (SG68) carrying a constitutively expressed *Pspac-lacZ* cassette on the chromosome was constructed. The *Pspac* promoter was amplified with primers BG61 (pMutin2 [143 to 161]) and BG23 (pMutin2 [428 to 406]) from pMutin2 (19) as a template, digested with MunI (site located within primer BG61) and BamHI (site located downstream of the *Pspac* promoter in pMutin2), and inserted between the EcoRI and BamHI sites located in front of *lacZ* in pAC6. The resulting plasmid, pBGM55, was linearized by ScaI digestion and used to transform *B. subtilis* 168 by a double crossover with the *amyE* gene with selection for chloramphenicol resistance.

β -Galactosidase assays. Overnight cultures of the *B. subtilis* strains grown in C minimal medium (9) supplemented with L-tryptophan (100 μ g/ml), potassium glutamate (8 g/liter), and one of the various carbon sources (0.5%, wt/vol) as

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TABLE 1. Plasmids and bacterial strains

Plasmid or strain	Relevant structures or genotype ^a	Source or reference
Plasmids		
pAC6	Promoterless <i>lacZ</i> , <i>cat</i> encompassed by <i>amyE</i> -5' and <i>amyE</i> -3', <i>bla</i>	17
pMutin2 and -4	MCS <i>RBS_{spoVG}-lacZ lacI bla ery rrnBt₁₂-M₀ P_{spac}</i>	19
pBGM6	pMutin4 containing a 210-bp fragment of <i>crh</i> -5' region (−120 to +90) in MCS	This work
pBGM8	pMutin4 containing a Φ (<i>crh-lacZ</i>)(Hyb) fusion gene preceded by the RBS of <i>crh</i>	This work
pBGM31	<i>Bacillus</i> deletion vector; <i>cat</i> followed by <i>rrnBt₁₂-M₀</i> inserted in EcoRI/ClaI of pBluescript SK [−]	This work
pBGM55	<i>P_{spac}</i> in front of <i>lacZ</i> in pAC6	This work
pBGM84	<i>glcT</i> deletion vector; same as pBGM31 but <i>cat rrnBt₁₂-M₀</i> cassette encompassed by <i>glcT</i> 3' region (+889 to +396) and <i>glcT</i> upstream region (−17 to −599)	This work
pLF1	pMutin4 containing a Φ (<i>ptsH-lacZ</i>)(Hyb) fusion gene preceded by the RBS of <i>ptsH</i>	This work
pLF2	pMutin4 containing a 365-bp fragment of <i>ptsH</i> -5' region (−276 to +90) in MCS	This work
Strains		
<i>E. coli</i>		
BMH71-18	Δ (<i>lac-pro</i>) <i>thi supE F</i> [<i>lacI^a lacZ</i> Δ <i>M15 lacY⁺ pro⁺</i>]	6
DH5α	<i>gyrA96 recA1 relA1 endA1 thi-1 hsdR17 glnV44 deoR</i> Δ(<i>lacZYA-argF</i>) <i>U169</i> [ϕ 80dΔ(<i>lacZ</i>) <i>M15</i>]	22
<i>B. subtilis</i>		
168	<i>trpC2</i>	1
SG17	<i>trpC2 crh'::lacZ ery</i> (::pMutin4)	pBGM6→168
SG19	<i>trpC2</i> Φ (<i>crh-lacZ</i>)(Hyb) <i>ery</i> (::pMutin4)	pBGM8→168
SG57	<i>trpC2</i> Φ (<i>ptsH-lacZ</i>)(Hyb) <i>ery</i> (::pMutin4)	pLF1→168
SG58	<i>trpC2 ptsH'::lacZ ery</i> (::pMutin4)	pLF2→168
SG68	<i>trpC2 amyE::[P_{spac}-lacZ cat]</i>	pBGM55/ScaI→168
SG87	<i>trpC2</i> Φ (<i>ptsH-lacZ</i>)(Hyb) <i>ery</i> (::pMutin4) Δ <i>glcT</i> (−16 to +395):: <i>cat</i>	pBGM84/ScaI→SG57
SG88	<i>trpC2 ptsH'::lacZ ery</i> (::pMutin4) Δ <i>glcT</i> (−16 to +395):: <i>cat</i>	pBGM84/ScaI→SG58

^a MCS, multiple cloning site. Positions are relative to the initiation codon of the respective gene.

indicated in Tables 2 and 3 were inoculated at an initial optical density at 600 nm (OD₆₀₀) of 0.15 in the same medium. IPTG (isopropyl-β-D-thiogalactopyranoside) (1 mM) was added for induction of the *Pspac* promoter. The cultures were grown at 37°C with agitation (160 rpm), and cells were harvested when the cultures reached an OD₆₀₀ of 0.6 to 0.8. β-Galactosidase activities were deter-

mined as described by Miller (11), using lysozyme and Triton X-100 treatment (12). The untransformed strain (*B. subtilis* 168) yielded values of less than 1 U. Pilot experiments were performed in which the β-galactosidase activities were determined at different time points during growth on succinate (lowest growth rate) and glucose (highest growth rate). These experiments revealed that a

FIG. 1. Organization of the *crh*- and *ptsH*-containing operons. The gene *crh* is part of an operon composed of six ORFs which are cotranscribed from a promoter located in front of the gene *yvcI* (reference 4 and our unpublished data). The gene *ptsH* can be transcribed from two promoters, one directly in front of *ptsH* and another in front of the preceding *ptsG* gene. The hairpin structures correspond to transcriptional terminators. The promoters are indicated by arrows and the corresponding RNAs are indicated by dotted lines, according to previous data (4, 5, 17) and this study. The positions of the transcriptional and translational fusions of *lacZ* to *crh* and *ptsH*, respectively, and of the *glcT* deletion (−16 to +395) constructed in this study are indicated.

TABLE 2. β -Galactosidase activities of strains carrying transcriptional fusions of *crh* or *ptsH* to *lacZ*^a

Carbon source	β -Galactosidase activity ^b			β -Galactosidase activity ratio	
	<i>crh</i> :: <i>lacZ</i> (SG17)	<i>ptsH</i> :: <i>lacZ</i> (SG58)	<i>ptsH</i> :: <i>lacZ</i> Δ glcT (SG88)	<i>ptsH</i> :: <i>lacZ</i> <i>lacZ</i> / <i>crh</i> :: <i>lacZ</i>	<i>ptsH</i> :: <i>lacZ</i> <i>glcT</i> ⁺ / Δ glcT
Citrate	44	42	40	1.0	1.1
Succinate	40	43	39	1.1	1.1
<i>myo</i> -Inositol	29	51	52	1.8	1.0
Glycerol	33	52	51	1.6	1.0
Malate	36	53	53	1.5	1.0
Gluconate	42	66	61	1.6	1.1
Mannitol	36	91	59	2.5	1.5
Fructose	33	91	57	2.8	1.6
Sucrose	33	254	66	7.7	3.9
Glucose	29	268	44	9.2	6.1

^a Bacteria were grown in minimal medium supplemented with the indicated carbon source (0.5%, wt/vol) to an OD₆₀₀ of 0.6 to 0.8. For induction of expression of genes downstream of the plasmid insertion sites, 1 mM IPTG was added.

^b β -Galactosidase activities are expressed in Miller units, and each value is the average of three or four measurements of at least two independent cultures.

steady-state level of β -galactosidase activities was reached with all strains used when the cultures were in the OD₆₀₀ range of 0.6 to 0.8. In order to test whether growth conditions (i.e., utilization of different carbon sources) may directly affect β -galactosidase activities, we used the control strain SG68. The β -galactosidase activities (in Miller units) obtained for SG68 were as follows: 18 for citrate, succinate, and gluconate; 23 for *myo*-inositol; 25 for fructose and glucose; 26 for malate; 27 for sucrose; 28 for mannitol; 29 for glucitol; and 30 for glycerol. Thus, the various growth conditions had only a marginal effect on expression or activity of β -galactosidase in our assays.

RESULTS AND DISCUSSION

In order to compare the transcription rates, we constructed *crh*::*lacZ* and *ptsH*::*lacZ* transcriptional fusions. The resulting strains carry an RBS_{spoVG}-*lacZ* reporter cassette starting 90 bp downstream of the first base of the respective open reading frame (ORF) at their natural chromosomal loci (Fig. 1). In these constructs, the complete ORFs of *crh* and *ptsH* are restored downstream of the *lacZ* cassette insertion sites. Their expression is directed by the IPTG-inducible *Pspac* promoter present in the inserted cassette in order to overcome polar effects caused by the insertions. The bacteria were grown in minimal medium supplemented with different carbohydrates, and β -galactosidase activities were determined (Table 2). The

activities produced by the strain carrying the *crh*::*lacZ* fusion varied only slightly with the carbon source (29 to 44 U) (Table 2). In contrast, the strain carrying the *ptsH*::*lacZ* fusion showed remarkable differences in activities. High activities (91 to 268 U) were detectable when a substrate of the phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS) (mannitol, fructose, sucrose, or glucose) was the carbon source, whereas significantly lower activities (42 to 66 U) were obtained with the other carbohydrates, none of which is a substrate of the PTS (Table 2). In conclusion, transcription of *ptsH* relative to that of *crh* was found to be three- to ninefold stronger when a PTS substrate is utilized (Table 2). Control experiments (see Materials and Methods) excluded the possibility that the different growth rates may affect the β -galactosidase activities (21).

Thereafter, we aimed to compare HPr and Crh synthesis levels. We therefore constructed translational in-frame fusions of the entire *crh* and *ptsH* genes to *lacZ* and placed them at their natural chromosomal loci preceded by their natural RBSs. The strain which carries the chimeric Φ (*crh-lacZ*)(Hyb) fusion gene yielded β -galactosidase activities that varied only slightly with the carbon source, with the exceptions of citrate and succinate (Table 3). These substrates caused twofold-higher activities than the other tested substrates. The same effect, although less pronounced, was seen with the transcriptional *crh*::*lacZ* fusion (Table 2). The strain which carries the Φ (*ptsH-lacZ*)(Hyb) fusion gene produced very high activities (940 to 3,534 U) (Table 3). This indicates that *ptsH* is approximately 10- to 20-fold more efficiently translated than *crh*, which is in agreement with previous observations suggesting that HPr is synthesized to very large cellular amounts (18). Indeed, the RBS of *ptsH* is very similar to the optimal *B. subtilis* RBS (14), whereas the RBS of *crh* matches the consensus less well (Fig. 2). The activities produced by the Φ (*ptsH-lacZ*)(Hyb) fusion were significantly higher when the bacteria were grown on a PTS substrate (1,855 to 3,534 U) than when they were grown on a non-PTS carbohydrate (940 to 1,465 U). This confirms our observation that utilization of a PTS substrate stimulates *ptsH* transcription (Table 2). The combined effects of stimulated transcription and highly efficient translation of *ptsH* amplify the differences in HPr and Crh synthesis levels up to 100-fold (Table 3).

TABLE 3. β -Galactosidase activities of strains carrying translational fusions of *crh* or *ptsH* to *lacZ*^a

Carbon source	β -Galactosidase activity			β -Galactosidase activity ratio	
	Φ (<i>crh-lacZ</i>) (Hyb) (SG19)	Φ (<i>ptsH-lacZ</i>) (Hyb) (SG57)	Φ (<i>ptsH-lacZ</i>) (Hyb) Δ glcT (SG87)	Φ (<i>ptsH-lacZ</i>)/ Φ (<i>crh-lacZ</i>)	Φ (<i>ptsH-lacZ</i>) <i>glcT</i> ⁺ / Δ glcT
Citrate	103	940	872	9	1.1
Succinate	94	988	867	11	1.1
<i>myo</i> -Inositol	49	988	1,225	20	0.8
Glycerol	38	1,130	1,126	30	1.0
Malate	38	1,215	1,163	32	1.0
Gluconate	59	1,465	1,312	25	1.1
Mannitol	40	1,884	1,097	47	1.7
Fructose	46	1,855	1,191	40	1.6
Sucrose	39	3,150	1,307	81	2.4
Glucose	34	3,534	1,022	104	3.5

^a See Table 2.

optimal RBS **AAAGGAGG - 7-9 - ATG**
 RBS-*ptsH* **AAAGGAGAATGATAAAAATG**
 RBS-*crh* **AAAGGGGAGATCTTATG**

FIG. 2. Comparison of the RBSs of *ptsH* and *crh*. The optimal *B. subtilis* RBS is as described by Rocha et al. (14).

What may be the mechanism that accounts for the stimulatory effect of PTS substrates on *ptsH* transcription? The genes *ptsH* and *ptsI* are cotranscribed from a promoter located directly in front of *ptsH* (Fig. 1). This promoter is constitutively active and not subject to regulation by the carbon source (5). In the presence of glucose, *ptsHI* are, in addition, cotranscribed with the preceding gene *ptsG* (encoding the glucose-specific enzyme II [EII^{Glc}]) from a promoter located in front of *ptsG* (17). Induction of *ptsG* expression by glucose is mediated by antiterminator protein GlcT, which is in turn regulated by (de)phosphorylation via EII^{Glc} (2, 16). Upon transport of glucose, EII^{Glc} dephosphorylates and activates GlcT, which then prevents formation of a terminator present in the *ptsG* leader mRNA that otherwise blocks transcription elongation (8). This mechanism explains the high *ptsH* transcription rates that we observed in the presence of glucose (Table 2). Furthermore, it was shown that the expression of *ptsG* is also inducible by sucrose (17) and that this up-regulation is mediated by GlcT (8). To clarify whether cotranscription with *ptsG* also accounts for the higher *ptsH* transcription rates in the presence of various PTS substrates other than glucose, we deleted the left half of the *glcT* gene (−16 to +395) in the strains carrying the transcriptional and translational *ptsH-lacZ* fusions. This deletion abolishes GlcT antitermination activity but leaves the *ptsG* promoter-leader region unaffected (Fig. 1). As can be seen from the data (Tables 2 and 3), the stimulatory effect of the various PTS substrates was abolished in the Δ *glcT* strains. In contrast, the activities detected in the presence of the various non-PTS substrates were not affected by the Δ *glcT* deletion. These results establish (i) that in the absence of PTS substrates, *ptsH* is expressed exclusively from the promoter directly in front of it, (ii) that additional cotranscription with *ptsG* causes higher *ptsH* transcription rates in the presence of a PTS substrate in general, and (iii) that this cotranscription is mediated by antiterminator GlcT.

In conclusion, our data suggest that the highly efficient translation of *ptsH* and the increased transcription rate in the presence of PTS carbohydrates, i.e., under conditions of strong CCR, cause a higher cellular concentration of HPr than of Crh. These drastic differences in synthesis level, up to 100-fold, may explain why in previous studies (3, 4, 10) only a minor role of Crh in CCR was detected. Recently it was reported that *citM*, encoding a Mg²⁺-citrate transporter, is specifically repressed by Crh in a medium composed of glutamate, succinate, and citrate (20). Here we found that the differences in Crh and HPr synthesis levels are lowest (approximately 10-fold) when the bacteria utilize citrate or succinate (Table 3). This observation is in agreement with the proposal (20) for a specific role of Crh under these conditions.

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