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A Crh-specific function in carbon catabolite repression in
Bacillus subtilis

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Abstract

Carbon catabolite repression in Bacillus subtilis is mediated by phosphorylation of the phosphoenolpyruvate:carbohydrate phosphotransferase system intermediate HPr at a serine residue catalyzed by HPr kinase. The orthologous protein Crh functions in a similar way, but, unlike HPr, it is not functional in carbohydrate uptake. A specific function for Crh is not known. The role of HPr and Crh in repressing the citM gene encoding the Mg²⁺-citrate transporter was investigated during growth of B. subtilis on different carbon sources. In glucose minimal medium, full repression was supported by both HPr and Crh. Strains deficient in Crh or the regulatory function of HPr revealed the same repression as the wild-type strain. In contrast, in a medium containing succinate and glutamate, repression was specifically mediated via Crh. Repression was relieved in the Crh-deficient strain, but still present in the HPr mutant strain. The data are the first demonstration of a Crh-specific function in B. subtilis and suggest a role for Crh in regulation of expression during growth on substrates other than carbohydrates.

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Keywords: Carbon catabolite repression; Crh; Gram-positive; HPr; CcpA

1. Introduction

In bacteria, the phosphoenolpyruvate-dependent phosphotransferase system (PTS) is the main carbohydrate uptake system and, in addition, plays an important role in the regulation of expression of catabolic genes and operons [1]. PTS-mediated uptake involves the transfer of the phosphoryl group of the high energy metabolite phosphoenolpyruvate to the carbohydrate through a cascade of phosphotransfer proteins including EI, HPr, and EIIABC. In PTS-mediated gene regulation, the phosphorylation state of the intermediate proteins is signaled to transcriptional regulators that repress the expression of genes encoding uptake systems and enzymes needed for the catabolism of less favored carbon sources (carbon catabolite repression; CCR). In Gram-positive bacteria, the signaling intermediate is HPr. HPr in Gram-positives is phosphorylated at two sites, a histidine and a serine (see Fig. 1). The histidine residue is phosphorylated by EI at the expense of phosphoenolpyruvate, the serine is phosphorylated by HPr kinase at the expense of ATP [2]. HPr(His-P) is involved in sugar transport, HPr(Ser-P) in CCR. The primary sensor in the regulatory pathway is HPr kinase, which is activated by glycolytic intermediates resulting in

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Fig. 1. Schematic representation of glucose-potentiated CCR in B. subtilis. See text for explanation.

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the phosphorylation of HPr at the serine residue. HPr(Ser-P) binds to the transcriptional regulator CcpA thereby inducing binding of the complex to so-called cre sites in the promoter region of the target genes, which prevents transcription [3]. During the genome sequencing project of Bacillus subtilis a second gene encoding a HPr-like protein was discovered [4]. The protein, Crh (catabolite repression HPr) shares 45% sequence identity with HPr and contains the regulatory site serine, but not the active site histidine. Accordingly, it was demonstrated that Crh was inactive in the PTS transport function but functional in CCR; Crh could be phosphorylated at the serine residue by HPrK, and then act as a corepressor of CcpA [5].

In B. subtilis the Crh- and the HPr-mediated regulatory pathways operate in parallel. Both are phosphorylated by the primary sensor HPr kinase at a serine residue and interact with CcpA to form a complex that binds to the cre sites on the DNA (Fig. 1). Glucose-induced CCR was not affected in knock-out strains of B. subtilis missing Crh [5,6]. Inactivation of the regulatory function of HPr by mutating the regulatory site serine to alanine resulted in partial or complete relief of repression depending on the target gene. Apparently, Crh is redundant in glucose- and maltose-mediated catabolite repression and can, at least in part, take over the regulatory function of HPr. No Crh-specific function is known.

The Mg$^{2+}$-citrate transporter CitM of B. subtilis is the major citrate uptake system during growth on citrate [7]. CitM is a secondary transporter that transports the complex of citrate or isocitrate and Mg$^{2+}$ into the cell [7,8]. The transporter also accepts the divalent metal ions Mn$^{2+}$, Zn$^{2+}$, Ni$^{2+}$, and Co$^{2+}$ in the complex [9,10]. Expression of CitM is strictly controlled by the medium composition. CitM is induced by citrate and isocitrate via a two-component system CitST [11] and repressed by glucose via the CCR route [12]. Growth substrates that potentiate CCR-mediated repression of the citM gene are more versatile than glucose alone. Repression was observed in media containing inositol and glycerol and the non-sugars succinate/gluatmate. CCR-mediated repression by succinate/glutamate is remarkable and the catabolites or signals that activate the pathway are unknown. Here we demonstrate that repression under these conditions is mediated specifically through Crh, and not through HPr.

2. Materials and methods

2.1. Strains and growth conditions

The B. subtilis strains used in this study are summarized in Table 1. All strains contain a fusion of the citM promoter region and the lacZ reporter gene encoding β-galactosidase, integrated in the amyE locus on the chromosome (PcitM-lacZ promoter fusion). The construction of the strains was described in [12]. Strain CM002 is referred to as the wild-type strain in this study. The strains were grown in C medium [13] and 10 mM of glucose (minimal glucose medium) or 6 g l$^{-1}$ sodium succinate and 8 g l$^{-1}$ potassium glutamate (CSE medium). Sodium citrate was added to a concentration of 10 mM. Auxotrophic requirements were added at 20 µg ml$^{-1}$ final concentration. When appropriate, antibiotics were added at the following concentrations: chloramphenicol, 5 µg ml$^{-1}$; kanamycin 5 µg ml$^{-1}$; spectinomycin 100 µg ml$^{-1}$; erythromycin 1 µg ml$^{-1}$. Overnight cultures were grown in media without citrate and used to inoculate 20 ml of medium with citrate. The cells were grown in 100-ml flasks at 37°C on a rotary shaker operated at 150 rpm. Growth was followed by measuring the optical density of the cultures at 660 nm (OD$_{660}$) using a Hitachi U-1100 spectrophotometer. The cells were harvested by centrifugation and washed once with 50 mM PIPES pH 6.5.

2.2. β-Galactosidase assay

β-Galactosidase activity was determined at 28°C by the method of Miller using o-nitrophenyl-β-D-galactopyranoside (ONPG) as the substrate [14]. Cells from 2 ml culture were harvested by centrifugation, frozen and stored at −20°C. The cell pellet was resuspended in a buffer containing 60 mM Na$_2$HPO$_4$, 40 mM NaH$_2$PO$_4$, 10 mM KCl, 1 mM MgSO$_4$, and 1 mM 1,4-dithiothreitol, pH 7.0 and the cells were lysed by treating with lysozyme in the presence of 10 µM DNase. The assay was started by the addition of 0.1 ml of ONPG (4.5 mg ml$^{-1}$) to the cell extract and stopped by the addition of 0.15 ml 1.2 M Na$_3$CO$_3$. After a brief spin to remove cell debris, the absorption of the sample was measured at 420 nm. Specific β-galactosidase activities were expressed as the o-nitrophenol released

<table>
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<tr>
<td>B. subtilis strains used in this study$^*$</td>
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<tr>
<td>Strain</td>
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<td>CM002</td>
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<td>CM010</td>
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$^*$Construction of the strains was described in [12].
$^+$Deficient in the CCR function of HPr, but not in PTS-mediated transport.
per minute per cell density at 28°C (Miller units). The values reported are averages of at least two independent measurements. Background activities were measured in *B. subtilis* strain 168 containing plasmid pJM116 integrated in the chromosome [12] and amounted to 0.4–2.5 Miller units.

3. Results and discussion

3.1. Functional equivalence of HPr and Crh

Transcription of the *citM* gene was studied in *B. subtilis* strains that contain a P*citM*-lacZ promoter fusion. Expression patterns of β-galactosidase (LacZ) were shown to correlate with Mg$^{2+}$-citrate uptake activity in these strains [12].

The *citM* promoter activity in the wild-type *B. subtilis* strain CM002 was completely inhibited during growth in a minimal medium containing glucose and citrate (Fig. 2, ●). In contrast, strain CM010, which is deficient in CcpA, the central component of CCR in Gram-positive bacteria, revealed high levels of expression when grown in the same medium, indicating that the repression in the wild-type cells was CCR-mediated (●). The increasing level of expression during exponential growth is caused by the fact that the expression level has not yet reached a steady-state value and represents a constant rate of transcription [15]. Strain CM006, which is deficient in Crh (Crh mutant), revealed the same expression pattern as the wild-type strain indicating that HPr was sufficient in mediating full repression of transcription of the *citM* gene under these conditions (●). *B. subtilis* CM004 expresses a HPr moiety that is mutated in the regulatory serine site (Ser46Ala). The mutant HPr is still functional in PTS-mediated uptake, but inactive in the signal transduction pathway [16]. Growth on glucose and citrate of strain CM004 resulted in complete repression during exponential growth followed by a slight relief of repression in later growth phases (●). It should be noted that the growth rates of the different mutant strains in the medium were of the same order of magnitude, ranging from 0.46 h$^{-1}$ for the CcpA-deficient mutant to 0.66 h$^{-1}$ for the Crh-deficient mutant. In conclusion, glucose-potentiated repression of *citM* transcription is equally well mediated by HPr(Ser-P) and Crh(Ser-P). A functional equivalence of HPr and Crh in the regulatory pathway was also observed for growth on inositol (not shown).

3.2. Functional discrimination between Crh and HPr

A similar set of experiments was done for growth on a minimal medium containing succinate, glutamate, and citrate (CSEC medium; Fig. 3). The growth rates of the different *B. subtilis* strains in CSEC medium were similar (Table 2). The β-galactosidase activity was significantly higher in strain CM008 than observed in the wild-type strain CM002 (Fig. 3, compare ● and ▲). Strain CM008 is deficient in both Crh and the regulatory function of HPr and, therefore, like the CcpA mutant, inhibited in CCR, but at a different level. The transcription rate of *citM* corrected for the growth rate was a factor 2.9 higher in the HPr(Ser)/Crh double mutant than in the wild-type strain (Table 2). Previously, we reported a 3.6-fold higher rate of transcription of *citM* in the CcpA-deficient mutant relative to the wild-type in the same growth medium [15]. It is concluded that *citM* expression is (incompletely) repressed in CSEC medium via the CCR mechanism. Surprisingly, a similar transcription rate as observed in the HPr(Ser)/Crh double mutant was observed in strain CM006, which is deficient in Crh, strongly suggesting that Crh was the essential component mediating repression in the wild-type (Fig. 3, ■ and Table 2). In
agreement, repression was only marginally relieved in the HPr mutant strain CM004 (Fig. 3, ▼ and Table 2).

The experiments suggest that HPr and Crh in *B. subtilis* may have specific functions in CCR depending on the growth factor(s) that potentiate repression. Glucose- and inositol-induced repression of transcription of the *citM* gene both follow the route via HPr(Ser-P) and Crh(Ser-P), with no apparent preference. It is likely that fructose-1,6-bisphosphate or some other glycolytic intermediate is the primary signal that activates HPr kinase under these conditions. Repression of *citM* in a medium containing succinate and glutamate seems to rely almost exclusively on Crh, while HPr does not seem to be functional. It is not clear what the primary signal is under these conditions, but it is unlikely that it would be a glycolytic intermediate. Both succinate and citrate are degraded in the citric acid cycle and it has been suggested that the redox state of the cells may affect CCR in *B. subtilis* [17]. In a recent report it was demonstrated that HPr of *B. subtilis* could be phosphorylated at the serine residue by pyrophosphatase [18]. The phosphorylation reaction that was catalyzed by HPr kinase was independent of fructose-1,6-bisphosphate. Possibly, this reaction plays a role in the initiation of the CCR route during growth in medium containing succinate and glutamate. At any rate, the data indicate that Crh may be specifically involved in regulatory pathways that are not linked to carbohydrate metabolism.

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### References


