Regulation of *crp* Gene Expression by the Catabolite Repressor/Activator, Cra, in *Escherichia coli*

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Key Words

Transcription factors · Genetic pleiotropy · Carbon catabolite repression

Abstract

Growth of *E. coli* on several carbon sources is dependent on the catabolite repressor/activator (Cra) protein although a Cra consensus DNA-binding site is not present in the control regions of the relevant catabolic operons. We show that Cra regulates growth by activating expression of the *crp* gene. It thereby mediates catabolite repression of catabolic operons by an indirect mechanism.

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Introduction

Several mechanisms of carbon catabolite repression are operative in large genome organisms such as *Escherichia coli* [Saier, 1996] and *Bacillus subtilis* [Stulke et al., 1998; Stulke and Hillen, 1998; Bruckner and Titgemeyer, 2002]. In *E. coli*, two dominant mechanisms involve transcriptional regulation by the cyclic AMP receptor protein (Crp) encoded by the *crp* gene, and by the catabolite repressor/activator (Cra) protein, encoded by the *fruR* gene [Ramseier et al., 1993]. Crp generally regulates the initia-

tion of carbon metabolism [Kolb et al., 1993] while Cra more frequently regulates carbon flux through the dominant metabolic pathways [Ramseier et al., 1995]. For example, the free (unliganded) form of Cra serves as an activator of catabolite repressible, oxidative Krebs cycle and gluconeogenic enzyme genes, but as a repressor of catabolite activatable, anaerobic glycolytic enzyme genes [Chin et al., 1987]. By contrast, the cyclic AMP-complexed form of Crp serves as an activator of almost all operons that initiate the metabolism of the many carbon sources that *E. coli* is capable of utilizing [Xu and Johnson, 1997; Busby and Ebright, 1999]. However, there is extensive overlap as Crp, for example, regulates transcription of most Krebs cycle enzymes while Cra directly regulates the initiation of the metabolism of some sugars (e.g. fructose, allose, etc.) [Ramseier et al., 1993, 1995].

We have recently found that Cra influences the expression of many cyclic AMP-Crp-activatable genes that initiate carbon metabolism even though no Cra-binding site could be identified in the control regions of the operons encoding the relevant transport systems and metabolic enzymes. We here document this phenomenon and provide compelling evidence that it is due to the fact that Cra positively controls expression of the *crp* gene. Although extensive evidence shows that Crp autoregulates expression of its own structural gene [Hanamura and Aiba, 1992; Ishizuka et al., 1993; Tagami et al., 1995], this is the

Table 1. Strains and oligonucleotides used in this study

Strain	Relevant genotype or characteristic	Source or reference
MG1655	Wild type	Blattner et al., 1997
$\Delta fruR$	$\Delta fruR$ in MG1655	this study
MG1655 $\Delta lacY$	$\Delta lacY$ in MG1655	Klumpp et al., 2009
MG1655 $\Delta lacY \Delta fruR$	$\Delta lacY \Delta fruR$ in MG1655	this study
MG1655 (<i>Pcrp-lacZ</i>)	MG1655 $\Delta lacY$ carrying <i>Pcrp-lacZ</i> at the <i>lac</i> locus	this study
MG1655 $\Delta fruR$ (<i>Pcrp-lacZ</i>)	$\Delta lacY \Delta fruR$ carrying <i>Pcrp-lacZ</i> at the <i>lac</i> locus	this study
MG1655 (<i>Pcrp</i> O _{Cra} - <i>lacZ</i>)	$\Delta lacY$ carrying <i>Pcrp</i> O _{Cra} - <i>lacZ</i> at the <i>lac</i> locus	this study
MG1655 $\Delta fruR$ (<i>Pcrp</i> O _{Cra} - <i>lacZ</i>)	$\Delta lacY \Delta fruR$ carrying <i>Pcrp</i> O _{Cra} - <i>lacZ</i> at the <i>lac</i> locus	this study
Oligonucleotide	Sequence	Use
fruR1-P1	gtgaaactggatgaaatcgctcggctggcgaggagtgctcgcgaccactg cgtgtaggctggagctgcttcg	<i>fruR</i> mutation
fruR2-P2	gctacggctgagcacgccggcgatagattacgtttaatgcgcgtta catatgaatatcctccttag	<i>fruR</i> mutation
Pcrp-Xho-F	atactgagcttgcatctttgtctactccactg	cloning <i>Pcrp</i> into pKDT
Pcrp-Bam-R	ttaggatccctggtgaataagcgtgctcttgatg	cloning <i>Pcrp</i> into pKDT
Pcrp-Z-P1	gcatttacgttgacaccatcgaaatggcgcaaaaccttgcggatgtgtgag gctggagctgcttc	integration of <i>Pcrp-lacZ</i> at the <i>lac</i> locus
Pcrp-Z-P2	gattaagtgggtaacgccagggtttccagtcacgacgttgtaaacgac ctggtgaataagcgtgctcttgatg	integration of <i>Pcrp-lacZ</i> at the <i>lac</i> locus
Pcrp-F	gttttagcatagcttgccttctgtctcctggtgcttaacaggagcatg	Cra operator mutation in <i>Pcrp</i> region
Pcrp-R	caccaggagacacaaagcgaaagc	Cra operator mutation in <i>Pcrp</i> region

first evidence that Cra cross controls *crp* gene expression. It therefore affects catabolite repression of these operons by an indirect secondary rather than a direct primary mechanism.

Materials and Methods

The strains used in this study are described in table 1. All strains were derived from *E. coli* K12 strain MG1655. The *fruR* gene was deleted using the method of Datsenko and Wanner [2000], yielding MG1655 $\Delta fruR$. To make the chromosomal construct in which the native *lacZ* gene is driven by the *crp* promoter (*Pcrp*), *Pcrp* was cloned into the *Sall* and *Bam*HI sites of pKDT [Klumpp et al., 2009], yielding pKDT-*Pcrp*. Using a site-directed mutagenesis approach, the Cra-binding operator in *Pcrp* was altered by changing GCTGAAGCGAGACACC to GCTCCTGTTAGACACC, yielding pKDT_*Pcrp*O_{Cra}. The *Pcrp* or *Pcrp*O_{Cra} promoter plus the upstream *rrnB* terminator and the *Km^r* (kanamycin-resistance) gene (*Km^r:rrnB*:*Pcrp* or *Km^r:rrnB*:*Pcrp*O_{Cra}) in pKDT was integrated into the chromosome of MG1655 $\Delta lacY$ [Klumpp et al., 2009] to replace the *lacI* gene and the native *lacZYA* promoter, yielding strain MG1655 *Pcrp-lacZ* or MG1655 *Pcrp*O_{Cra}-*lacZ*. In each of these chromosomal constructs there are a *Km^r* gene and an *rrnB*

terminator upstream of the promoter. The chromosomal *Pcrp-lacZ* and *Pcrp*O_{Cra}-*lacZ* in MG1655 were transferred to MG1655 $\Delta fruR$ by P1 transduction, yielding MG1655 $\Delta fruR$ *Pcrp-lacZ* and MG1655 $\Delta fruR$ *Pcrp*O_{Cra}-*lacZ*, respectively. The oligonucleotides used in this study are listed in table 1.

Miller's minimal medium 63 (M63) [Miller, 1972], supplemented with 0.5% (w/v) of various carbon sources, was used for measuring growth rates and promoter activities. Growth experiments were conducted following the method of Scott et al. [2000]. Seed cultures were grown in LB broth and used to inoculate appropriate pre-culture media. After overnight growth, pre-cultures were used to inoculate 5-ml of experimental media (at a 1,000 × dilution) contained in test tubes (20 mm × 25 cm). The tubes were shaken in a 37°C water bath at 250 rpm. Growth rate was monitored by measuring OD₆₀₀ in a Bio-Rad SmartSpec3000 spectrophotometer over time. At least 5 measurements were made during the exponential growth phase. The slope of the plot of logOD₆₀₀ versus time (min) was used to derive the growth rate in min/generation.

For *Pcrp* activity determination, 300 µl of culture samples were taken at least 4 times during exponential growth. β-Galactosidase assays were carried out using the method of Miller [1972]. The slope of the plot of activity (U/ml) versus the sample OD₆₀₀ yielded the activity in Miller units (U/ml/OD₆₀₀). To test if a similar activation effect occurred during the stationary growth phase, samples were also taken to determine *Pcrp* activity.

To determine cAMP-receptor protein (Crp) levels, a freshly isolated colony from an LB plate of each isolate (MG1655 and MG1655 Δ *fruR*) was used to inoculate 5 ml M63 supplemented with 0.5% (w/v) of xylose. The bacteria were incubated overnight in a shaking water bath at 37°C. The culture obtained was used for inoculation of 1 liter of the same medium in a 2-liter conical flask. The flasks were incubated on a rotary shaker at 37°C at 275 rpm until an OD₆₀₀ of about 0.7 was achieved. The cells were harvested by centrifugation in a Sorvall SS-34 rotor at 4°C at 6,000 rpm for 20 min, and washed twice with and resuspended in M63. The pellet suspension (15 ml) was lysed by passage 3× through a French Pressure cell at 0°C and 16,000 psi. The lysate produced was centrifuged at 40,000 rpm for 2 h at 4°C using a Beckman Ti70 rotor. The high-speed supernatant (HSS) produced was collected, and its total protein content was determined as described by Aboulwafa and Saier [2008] using the Bio-Rad colorimetric protein reagent (Cat. #500-0006) and bovine serum albumin as the standard protein.

SDS-PAGE was conducted as described by Aboulwafa and Saier [2008] using a stacking gel of 5% acrylamide, and a separating gel of 10% acrylamide. Equal volumes of protein solution and Laemmli sample buffer were placed in a boiling water bath for 3 min, cooled to room temperature, and applied in 30- μ l aliquots to a 10 × 7 cm gel. The samples were electrophoresed at 75 V. Proteins were transferred from the gel to nitrocellulose membranes (0.22 μ m) as described by Towbin et al. [1979] using Tris-glycine buffer/20% methanol, pH 8.3, no SDS, and at 100 V for 1 h. After electroblotting, the membrane was blocked in a Tris-buffered saline (TBS) solution containing 5% non-fat milk (Bio-Rad) and 0.1% Tween-20 for 4 h at 4°C with gentle shaking. The first antibody was rabbit polyclonal antibody raised against a peptide mapping within an internal region of *E. coli* Crp (Crp (T-14): sc-136636; Santa Cruz Biotechnology, Inc.) and used at a dilution of 1:50 in the same solution for 2 h at room temperature. The second antibody was mouse anti-rabbit IgG-HRP (sc-2357; Santa Cruz Biotechnology, Inc.) and used at a dilution of 1:500 in the same solution for 1 h at room temperature. Detection was performed using SuperSignal West Dura Chemiluminescent Substrate (Thermo Scientific Pierce Cat. #34075). Following the protocols described in Cheng et al. [2012], Chua et al. [2009], Sharma et al. [2008] and Sujobert et al. [2005], the Crp protein levels were determined by using the ImageJ software (National Institutes of Health, Bethesda, Md., USA) after scanning the film. The amount of Crp from MG1655 Δ *fruR* was normalized to 1.

In order to identify Cra-binding sites in the *E. coli* genome, experimentally determined 16-base Cra-binding site sequences from the following genes were used as input for the GRASP-DNA program; *fruB*, *icdA*, *aceB*, *epd*, *ppsA*, *mtlA*, *pykF*, *ptsH*, *nirB*, *fbp*, *pckA*, *adhE* and *edd* [Schilling et al., 2000]. The consensus-weighted matrix used to screen the genome was determined using this program.

Results

Table 2 shows the growth rates of a wild-type and an isogenic *fruR* mutant in M63 minimal medium containing various carbon sources (0.5%, w/v). As can be seen, the mutant grew at a much slower rate than the isogenic

Fig. 1. Control region preceding the *crp* gene in *E. coli*. The two Crp-binding sites (CrpI and CrpII) are indicated by lines above the sequence while the Cra-binding site is underlined. Mutations introduced in the Cra-binding site are labeled with letters above the sequence. For the primary promoter (*crpP1*), the -35 and -10 hexamers as well as the transcriptional start site (+1) are shaded. Two subsequent weak promoters (that are functional under specific conditions) are also indicated +1 [Gonzalez-Gil et al., 1998].

Table 2. Growth rates of *E. coli* wild type and the isogenic *fruR* deletion mutant in M63 minimal media with 0.5% (w/v) of various carbon sources

Carbon source	Wild type (min/ generation)	Δ <i>fruR</i> (min/ generation)
Xylose	80±3	95±4
Glycerol	89±2	130±6
Casamino acids (CAA)	84±5	129±5
Glycerol+CAA	58±3	63±3
Lactose	56±3	78±4
Sorbitol	88±3	>500
Succinate	119±5	468±12
Gluconate	67±3	72±3
Glucose	58±2	61±2
Fructose	85±2	63±4

wild-type strain on xylose. The same behavior was observed for several other carbon sources including glycerol, casamino acids (CAA) and lactose. When D-sorbitol or succinate was used as the carbon source, very little or no growth of the Δ *fruR* mutant was detected. The utilization of gluconate as well as glycerol + CAA was slightly decreased; glucose utilization was only slightly decreased, and fructose utilization was substantially increased by the loss of Cra (table 2).

Fig. 2. Activation of the *crp* promoter by Cra. The chromosomal *Pcrp-lacZ* fusion at the *lac* locus was made. MG1655Δ*lacY* and MG1655Δ*lacY*Δ*fruR* cells containing the *Pcrp-lacZ* fusion were cultured in M63 with 0.5% (w/v) of various carbon sources. For β-galactosidase assays, samples were collected during the exponential growth phase (first two columns) and the stationary phase (last two columns). For brevity, wt = MG1655Δ*lacY* (dark gray bars); Δ*fruR* = MG1655Δ*lacY*Δ*fruR* (light gray bars).

These last observations can be explained because growth of wild-type cells in the presence of fructose yields fructose-1-phosphate, the high-affinity ligand for Cra, while growth on glucose, the most rapidly utilized sugar in *E. coli*, and the primary substrate of both the glucose and mannose permeases, should produce the highest level of cytoplasmic fructose-1,6-bisphosphate, the primary low-affinity ligand for Cra [Ramseier et al., 1993, 1995].

The utilization of xylose, glycerol, lactose, succinate and sorbitol are subject to stringent Crp control as well as weak Cra control (table 2), but there is no Cra-binding site upstream of their catabolic enzyme-encoding operations. It appeared that these regulatory effects might be due to an indirect effect of the loss of Cra on an unidentified regulatory process.

A search for Cra-binding sites in the *E. coli* genome using the GRASP-DNA program [Schilling et al., 2000]

revealed genes that could potentially be regulated by this protein. The Cra-like sequence G C T G A A G C G A G A C A C C was found at positions –53 to –37 relative to the primary transcriptional start site of the *crp* gene. Figure 1 presents a schematic depiction of the control region of the *E. coli crp* gene. The Cra-binding site overlaps one of the two Crp-binding sites, the upstream CrpI site, by two nucleotides. While CrpI activates *crp* gene expression, CrpII represses expression [Hanamura and Aiba, 1992; Ishizuka et al., 1993; Tagami et al., 1995].

In order to test the possibility that Cra controls transcription of the *crp* gene, we constructed a chromosomal *Pcrp-lacZ* fusion in wild-type and *fruR* genetic backgrounds. The results are presented in figure 2. Cra activates expression of the *crp* gene when xylose, glycerol ± CAA or gluconate was used as the sole carbon source. Similar activation effects were observed in cells from the

Fig. 3. Western blot of Crp in HSS prepared from log phase cells of *E. coli* MG1655 wild-type and its *fruR* deletion mutant MG1655 Δ *fruR* grown in M63 containing 0.5% xylose (see Methods). Lanes 1–4 (MG1655 WT) and 5–8 (MG1655 Δ *fruR*) contained 34, 17, 8.5 and 4.25 μ g of total protein of the 2 h HSS of the corresponding strain. The amount of the Crp protein in each band was quantified using the ImageJ software (NIH) after scanning (see Methods). The three panels (top, middle and bottom) show the results of three independently conducted experiments.

Fig. 4. Effect of a Cra operator mutation on Cra activation of *Pcrp*. The Cra operator was mutated by changing GCTGAAGCGAGACACC to GCTCCTGTTAGACACC (see fig. 1). The effect of such an alteration on the *Pcrp* activity was determined using the altered promoter-*lacZ* fusion in the MG1655 Δ *lacY* and MG1655 Δ *lacY* Δ *fruR* strains in both the presence and absence of Cra.

Fig. 5. Expression of *lacZ* from the native *E. coli* *lac* operon promoter showing the effect of the loss of Cra. *lacZ* expression was measured in MG1655 Δ *lacY* and MG1655 Δ *lacY* Δ *fruR* cells grown in M63 medium plus xylose or glycerol.

stationary phase. Thus, the loss of this protein gives rise to reduced *crp* expression. As expected, the *Pcrp* activity was unaffected by the loss of Cra when cells were grown with fructose since fructose, when converted to fructose-1-P, releases Cra from its operator (fig. 2) [Ramseier et al., 1993].

These results were confirmed using Western blotting. As shown in figure 3, the wild-type strain contained higher levels of Crp (lanes 1–4) than did the isogenic *fruR* mutants (lanes 5–8). The figure shows the results for three independently conducted experiments all of which gave the same results. The relative amounts of the Crp protein in the Western blots were quantified using the ImageJ software (NIH). The ratio of the wild-type to the *fruR* mutant was 1.4 ± 0.1 .

Activation of the *crp* gene by Crp, but not by Cra, had been reported previously [Hanamura and Aiba, 1992; Ishizuka et al., 1993; Tagami et al., 1995]. Interestingly, these effects proved not to be additive as the *crp fruR* double mutant showed the same basal activity as the *crp* mutant in LB medium (data not shown). These results are in

accordance with expectation that gene expression is dependent on a complex of σ^{70} RNA polymerase with both Crp and Cra. Neither Crp nor Cra alone can fully activate gene expression. Because of the close proximity of the CrpI- and Cra-binding sites, these two proteins might form a complex without DNA looping. Alternatively, or in addition, Cra binding could antagonize the repressive effect of Crp bound to CrpII.

The Cra-binding site in the upstream *crp* operon regulatory region was altered by site-specific mutagenesis (see fig. 1). As shown in figure 4, this alteration resulted in decreased expression of the *Pcrp-lacZ* fusion construct. However, loss of Cra resulted in an even greater loss (fig. 4). These results are consistent with the conclusion that Cra mediates its effect, at least in part, by binding to the proposed Cra-binding site.

The wild-type chromosomal *lacZYA* operon is known to be positively controlled by Crp. This operon was similarly examined in wild-type and a $\Delta fruR$ mutant strain. As shown in figure 5, activity was decreased by the loss of Cra during growth in minimal medium containing 0.5% D-xylose (A) or 0.5% glycerol (B). All of the results presented in figures 2–5 as well as table 2 are consistent with the conclusion that Cra regulates *crp* gene expression and thereby indirectly controls expression of numerous operons involved in the initiation of carbon metabolism.

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Discussion

The results reported in this communication indicate a novel level of complexity in the regulation of carbon metabolism in *E. coli*. Cra and Crp both activate expression of the *crp* gene, apparently by a mechanism that requires the simultaneous presence of both proteins. The cytoplasmic concentration of Crp thus generated then determines the degree of Crp-regulon gene activation by the cyclic AMP-Crp complex. Those operons that bind the cyclic AMP-Crp complex with low affinity will be particularly sensitive to the Crp concentration. Regulation of *crp* gene expression by Cra and Crp is therefore suggested to be part of a single regulatory mechanism. The details of this proposed regulatory mechanism are currently under investigation. It is anticipated that interrelated networks of regulatory interactions such as those described here will provide bacteria with mechanisms for fine tuning and coordinating the different aspects of their metabolism and physiology [Amar et al., 2002; Babu and Teichmann, 2003].

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