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Journal

Microbial Physiology, 24(3)

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Publication Date

2014

DOI

10.1159/000362722

Peer reviewed



Published in final edited form as:

J Mol Microbiol Biotechnol. 2014 ; 24(3): 135–141. doi:10.1159/000362722.

Regulation of *crp* gene expression by the catabolite repressor/activator, Cra, in *Escherichia coli*

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Abstract

Growth 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.

Keywords

Transcription factors; genetic pleiotropy; carbon catabolite repression

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 initiation 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; Ramseier et al., 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 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 (Datsenko and Wanner, 2000), yielding MG1655 *fruR*. To make the chromosomal construct in which the native *lacZ* gene is driven by the *crp* promoter (*Pcrp*), *Pcrp* was cloned into the *SalI* and *BamHI* 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*_{Cra}. The *Pcrp* or *Pcrp*_{Cra} promoter plus the upstream *rrnB* terminator and the Km^r (kanamycin-resistance) gene (*Km^r:rrnBT:Pcrp* or *Km^r:rrnBT:Pcrp*_{Cra}) in pKDT was integrated into the chromosome of MG1655 *lacY* (Klumpp et al., 2009) to replace the *lacI* gene and the native *lacZYA* promoter, yielding strain MG1655 *Pcrp-lacZ* or MG1655 *Pcrp*_{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*_{Cra}-*lacZ* in MG1655 were transferred to MG1655 *lacY fruR* by P1 transduction, yielding MG1655 *fruR Pcrp-lacZ* and MG1655 *fruR Pcrp*_{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 (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 1000x 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 ul of culture samples were taken at least 4 times during exponential growth. β-Galactosidase assays were carried out using the method of Miller

(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 *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 L of the same medium in a 2 L 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 6000 rpm for 20 min, and washed twice with and resuspended in M63. The pellet suspension (15 ml) was lysed by passage 3X 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 µl 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 µM) as described by Towbin et al. (1979) using tris-glycine buffer/20% methanol, pH 8.3, no SDS, and a voltage of 100 V for 1 h. After electroblotting, the membrane was blocked in a tris buffered saline (TBS) solution containing 5% non fat milk (BioRad) 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 Institute of Health, Bethesda, MD) after scanning the film. The amount of Crp from MG1655 *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 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 *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).

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; Ramseier et al., 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 operons. 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. Fig. 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 Fig. 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 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 Fig. 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 Fig. 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 *fruR* mutant strain. As shown in Fig. 5, activity was decreased by the loss of Cra during growth in minimal medium containing 0.5% Dxylose (A) or 0.5% glycerol (B). All of the results presented in Figs. 2, 3, 4 and 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.

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).

Acknowledgments

We thank Carl Welliver and Maksim Shlykov for assistance in the preparation of this manuscript. This work was supported by NIH grants GM64368 and GM077402 from the National Institutes of General Medical Sciences.

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tgacagagtacgcgtactaaccaaatcgcg
                Crp I      CCTG
caacggaaggcgacctgggtcatgctgaag
                FruR
tt      -35
cgagacaccaggaacacaaagcgaaagct
-10      +1
atgctaaaacagtcagGatgctacagtaat
                Crp II
acattgatgtactgcatgtatgcaaaggac
                +1
gtcacattaccgtgcagtacagttgataGc
                +1
cctGcccaggtagcgggaagcatatttcg
gcaatccagagacagcggcgttatctggct
ctggagaaagcttataacagaggataaccg
                1st codon
cgcatg

```

Figure 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 (*crp*P1), 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).

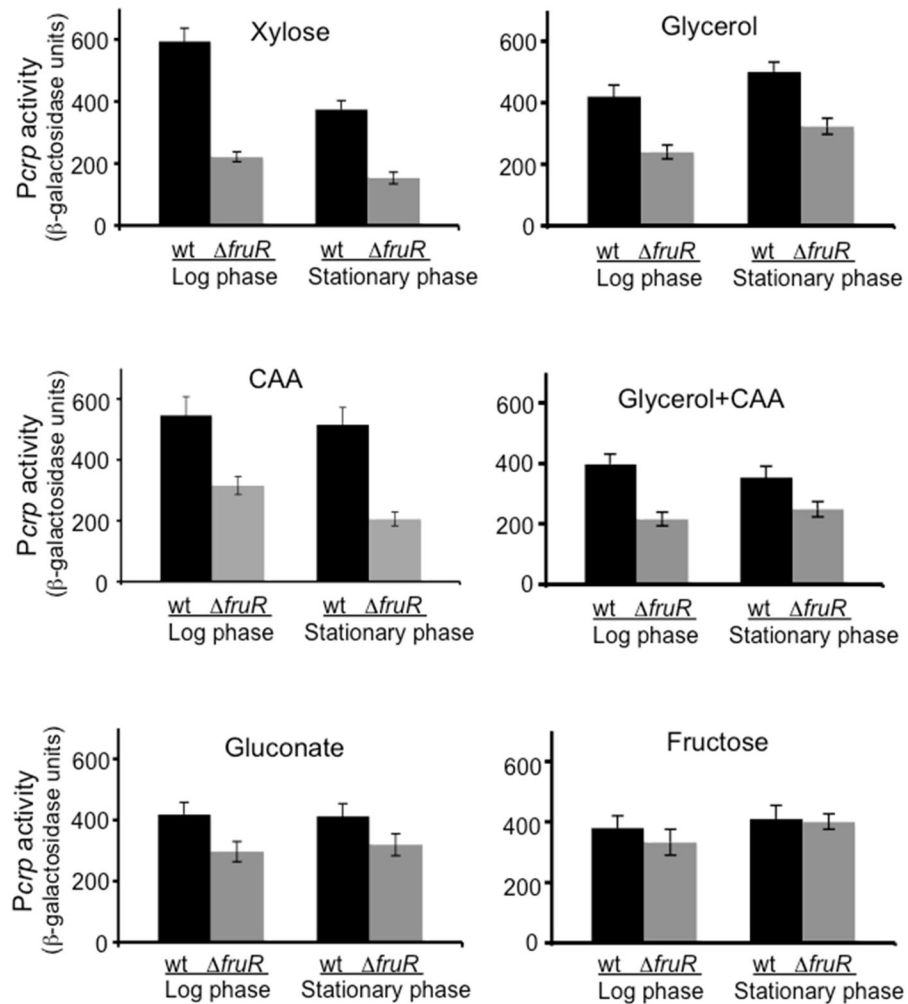


Figure 2.

The activation of the *crp* promoter by Cra. The chromosomal *Pcrp-lacZ* fusion at the *lac* locus was made. MG1655 *lacY* and MG1655 *lacY 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 = MG1655 *lacY*; *fruR* = MG1655 *lacY fruR*.

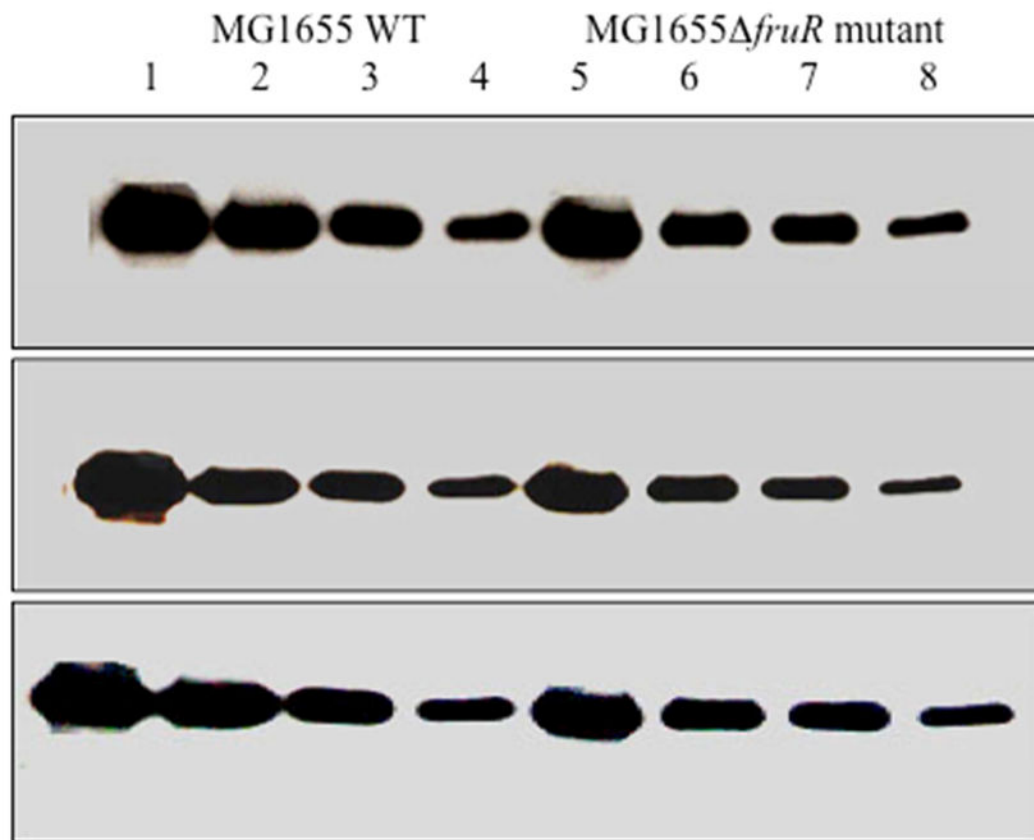


Figure 3.

Western blot of Crp in high-speed supernatants prepared from log phase cells of *E. coli* MG1655 wild type and its *fruR* deletion mutant MG1655 *fruR* grown in M63 containing 0.5% xylose (see Methods). Lanes 1 – 4 (MG1655 WT) and 5–8 (MG1655 *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).

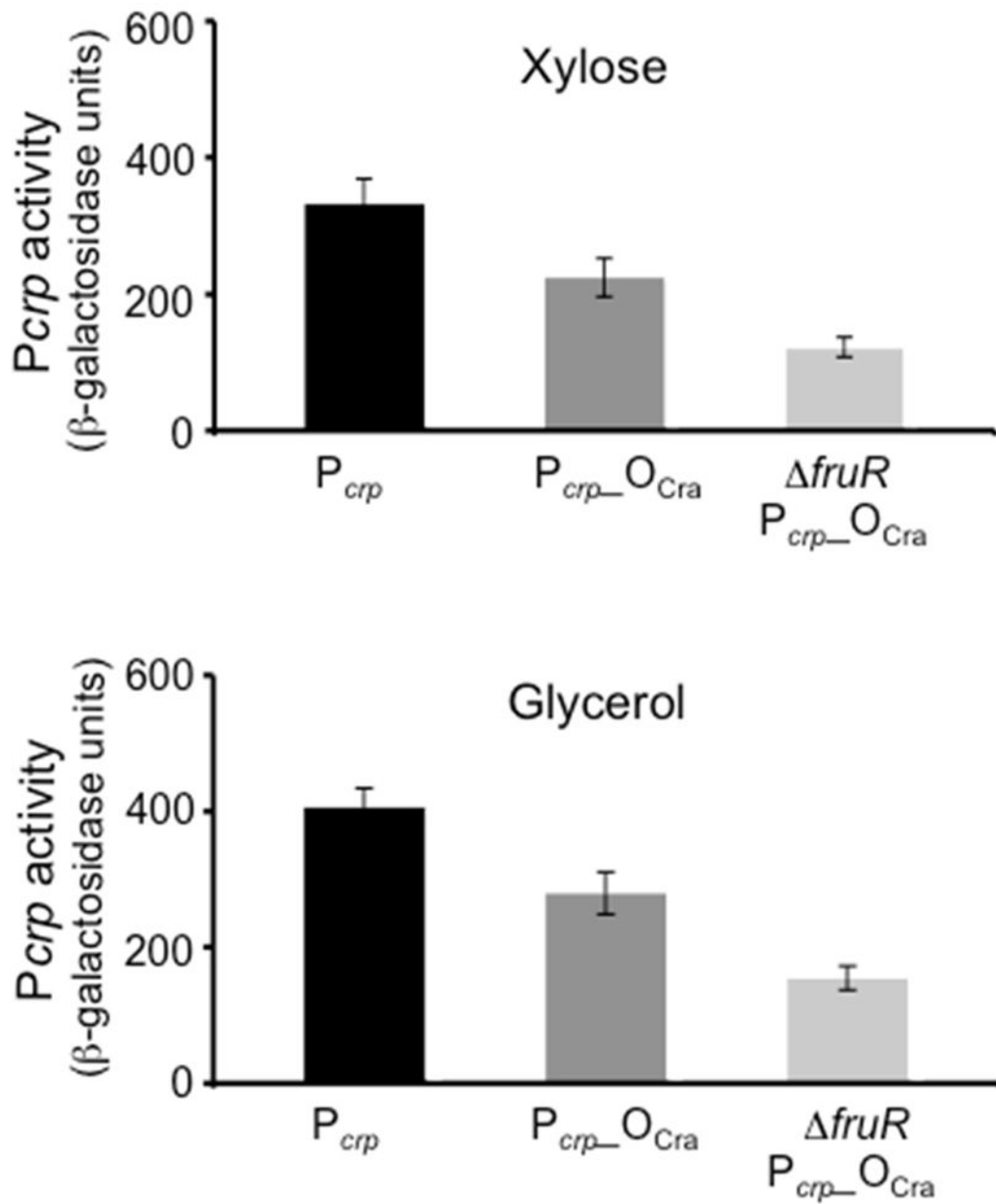


Figure 4. Effect of a Cra operator mutation on Cra activation of P_{crp} . The Cra operator was mutated by changing GCTGAAGCGAGACACC to GCTCCTGTTAGACACC (see Fig. 1). The effect of such an alteration on the P_{crp} activity was determined using the altered promoter-*lacZ* fusion in the MG1655 *lacY*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

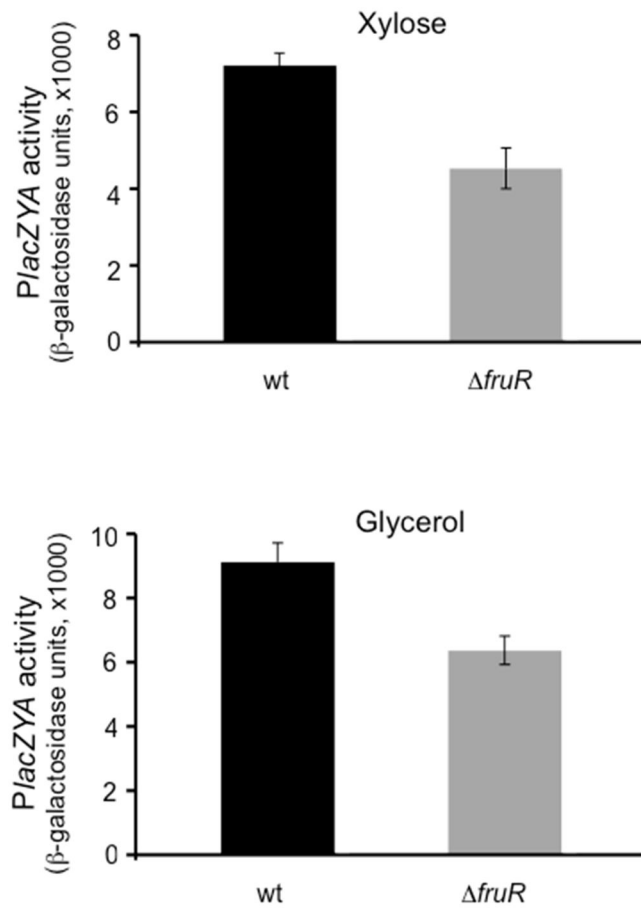


Figure 5. Expression of *lacZ* from the native *E. coli lac* operon promoter showing the effect of the loss of Cra. *lacZ* expression was measured in MG1655 *lacY* and MG1655 *lacY fruR* cells grown in M63 medium plus xylose or glycerol.

Table 1

Strains and oligonucleotides used in this study

Strain	Relevant genotype or characteristic	Source or reference
MG1655	Wild type	Blatner et al (3)
<i>fruR</i>	<i>fruR</i> in MG1655	This study
MG1655 <i>lacY</i>	<i>lacY</i> in MG1655	Klumpp et al (11)
MG1655 <i>lacY fruR</i>	<i>lacY fruR</i> in MG1655	This study
MG1655 (<i>Perp-lacZ</i>)	MG1655 <i>lacY</i> carrying <i>Perp-lacZ</i> at the <i>lac</i> locus	This study
MG1655 <i>fruR</i> (<i>Perp-lacZ</i>)	<i>lacY fruR</i> carrying <i>Perp-lacZ</i> at the <i>lac</i> locus	This study
MG1655 (<i>PerpO_{Cra}-lacZ</i>)	<i>lacY</i> carrying <i>PerpO_{Cra}-lacZ</i> at the <i>lac</i> locus	This study
MG1655 <i>fruR</i> (<i>PerpO_{Cra}-lacZ</i>)	<i>lacY fruR</i> carrying <i>PerpO_{Cra}-lacZ</i> at the <i>lac</i> locus	This study
Oligonucleotide	Sequence	Use
<i>fruR</i> 1-P1	gtgaaactggatgaaatcgctcggcggcgggaggtcgcggaccactgcgtgtaggcgtggagctgctcctcg	<i>fruR</i> mutation
<i>fruR</i> 2-P2	gctacggcgtgagcacgcggcggatagagattacgtttaatgcgcgttacatagaataatcctccttag	<i>fruR</i> mutation
<i>Perp</i> -Xho-F	atactgagcttgcttctctaccacg	Cloning <i>Perp</i> into pKDT
<i>Perp</i> -Bam-R	ttaggacctgggataaagcgtctcttggatg	Cloning <i>Perp</i> into pKDT
<i>Perp</i> -Z-P1	gcatttacgtgacaccatcgaatggcgaacaccttcgcggatggtgtaggscgtggagctgctc	Integration of <i>Perp-lacZ</i> at the <i>lac</i> locus
<i>Perp</i> -Z-P2	gattaagtgggiaacgccagggtttccagtcacagcttgtaaaacgacctgggataaagcgtgctcttggatg	Integration of <i>Perp-lacZ</i> at the <i>lac</i> locus
<i>Perp</i> -F	gitttagcatagcttcgtttgctctcctgggtctcaacaggagcatg	<i>Cra</i> operator mutation in <i>Perp</i> region
<i>Perp</i> -R	caccaggagacacaaagcgaagc	<i>Cra</i> operator mutation in <i>Perp</i> region

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)	<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