Assessing role of Cra in regulation of prpB and yahA genes of
Escherichia coli in vivo using lacZ transcriptional fusions

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This study presents in vivo role of Cra (Catabolite repressor activator) protein in regulation of two selected genes (prpB & yahA) of Escherichia coli. Gene prpB bears Cra binding site and yahA region binds to Cra in vitro. Therefore, in pBR322 background, pprpB-lacZ and pyahA-lacZ transcriptional fusions were generated and studied expression of b-galactosidase from both fusions in cra+ and cra− genetic backgrounds of E. coli. Results that both the promoters could possibly be up-regulated by Cra in vivo in E. coli were discussed in relation to transcription control by accessory transcription factor(s).

Keywords: Cra, lacZ, prpB, yahA, Transcriptional fusion

Introduction:
Core enzyme, RNA polymerase, transcribes specifically from promoters after assembly of sigma to the core enzyme. Even a simple bacterium, Escherichia coli harbours seven different sigma factors and the number rises to 17 distinct sigma factors in bacterium, Bacillus1-3. Besides sigma factors, a vast majority of accessory transcription factors (ATFs) help in fine tuning of transcription and help to achieve differential gene expression (GE). An average estimate gives an alarming picture that E. coli harbours ~300 ATFs1. Besides core RNA polymerase and sigma factors, ATFs further help in achieving selective GE. In toto GE modulation depends on interplay between sigma factors, ATFs, anti-sigma factors, small molecules/ligands and small regulatory RNAs. The very presence of ~300 ATFs in E. coli indicates gene regulation is being evolved as a very complicated process in E. coli1-2.

Studies pertaining to one such ATFs, Cra is gaining momentum these days. Cra (FruR), the Catabolite repressor / activator protein, a member of LacI-GalR family, regulates many genes involved in carbon-metabolism4-5. Some of the genes are up regulated and some are down regulated by Cra6-10. This transcription regulator recognizes particular palindromic sequence in chromosome and then binds to that to regulate the expression of downstream gene / operon. Activation / repression will be decided by the location of binding site of Cra in gene sequence. Cra-binding consensus sequence is asymmetric, with substantially greater conservation occurring in the left half-site than in the right half-site. DNA binding site of Cra, dual transcription regulator has been characterized well11. If Cra binding sequence precedes the binding site of RNAP in a gene, then gene will get activated. However, if the site overlaps or follows the binding site of RNAP in a gene, then transcription of gene will be repressed4. In a cra mutant, TCA cycle and glyoxylate shunt were down-regulated, while pentose phosphate (PP) pathway and Entner Doudoroff (ED) pathway were up-regulated6. Expression of icd gene of E. coli, which codes for isocitrate dehydrogenase, was positively regulated by Cra7. icsRSUA, Fe-S cluster assembly operon in Salmonella enterica8 was positively regulated by Cra. pfkB gene (encoding phospho fructose kinase-I) was negatively regulated by Cra9. Cra is also known for repression of adhE gene, codes for ethanol dehydrogenase under aerobic than anaerobic growth10.

This study presents two genes (prpB & yahA) to understand in vivo role of Cra in transcription regulation. BLAST analysis at MKU, Madurai indicated the presence of Cra binding site in prpB gene and Shimada et al12 had reported the binding of Cra in vitro to yahA sequence. Gene prpB codes for 2-methylisocitrate lyase and this enzyme catalyzes final step in propionate metabolism pathway in E. coli13-16. Therefore,
promoterless *lacZ* gene was fused with *prpB* promoter to generate transcriptional fusion (TF) in order to understand (Fig. 1) the effect of Cra in regulation of *prpB* expression. Gene yahA codes for phosphodiesterase and Cra down regulates *yahA* in *vitro*. Hence, to understand the role of Cra in expression of *yahA in vivo*, *yahA-lacZ* TF was also constructed. Both *prpB-lacZ* and *yahA-lacZ* TFs were analyzed for their β-galactosidase expression in *cra*+ and *cra¯* strains. In such TFs, *lacZ* expression is the indirect measure of expression of candidate genes.

**Experimental Section**

Conventional LB medium and M9 minimal medium were used according to Miller. Reagent grade materials used for preparation of various media, solutions and buffers were purchased mainly from Hi-media-India. Antibiotics and other fine chemicals were purchased from Sigma Company, USA; Streptomycin was purchased from Sarabhai Chemicals India. Restriction Enzymes, T4 DNA Ligase, dNTPs, *pfu* polymerase were purchased from MBI-Fermentas, Germany. *E. coli* strains are derivatives of K12. Strains JW0078-1 (F Δ(arad-araB)567 ΔfruR786::kan ΔlacZ4787::rrnB-3) LAM- rph-1 Δ(rhaDrhaB)568 hsdR514) and MC4100 (F araD139 Δ(argFlac)U169 rpsL150 relA1 flbB5301 fruA25 deoC1 ptsF25 e14) were obtained from Coli Genetic Stock Centre, USA. P1 *vir* was originally from Dr N Willetts, UK and maintained locally. Cultures were grown in 37°C unless stated otherwise. All molecular genetic techniques used were according to Miller. For other molecular biology experiments, protocols given in Sambrook *et al.* were used.

**Construction of *cra¯* Mutant Derivative of MC4100**

This was essentially done by P1 transduction. P1 made on ΔfruR786::kan was used as donor to transduce KanR (ΔfruR::kan) into MC4100 and *cra¯* (ΔfruR786::kan) transductants were selected as KanR transductants. P1 preparation and transduction experiments was done according to Miller. One of the selected KanR *cra¯* transductant was purified and named as MC4100 *cra¯*. MC4100 was used as wild type control (*cra*+). Both strains carry Δ(argF-lac) deletion and such Δlac strain is mandatory for using in the experiments pertaining to *lac* fusions.

**Vector Preparation for Generation of Transcriptional fusions (TFs)**

MKU, Madurai has already constructed a plasmid clone with *lacZ* TF for some other purpose with pBR322 vector (*prpsGL-lacZ*) and this pBR322 derived clone was used to clone *prpB* fragment to get *prpB-lacZ* TF. Initially, *rpsGL* fragment was cloned with its promoter in pBR322 (plasmid clone named as *prpsGL* with HindIII and *NheI* restriction sites and *lacZ* region was cloned in the same plasmid with *NheI* and *SalI* restriction sites to generate *rpsGL-lacZ* TF.

For cloning of *lacZ* in plasmid *prpsGL-lacZ*, genomic DNA was isolated from wild type *E. coli* MG1655. This DNA was used as template for PCR. *lacZ* was amplified without its own promoter but with its own ribosome binding site and ATG sequence with *lacZF* and *lacZR* primers (Table 1) and by using following cyclic conditions: initial denaturation, 94°C for 5 min; denaturation, 95°C for 45 s; primer annealing, 55°C for 45 s; extension, 72°C for 4 min; final extension, 72°C for 10 min with *pfu* polymerase. This *lacZ* fragment was cloned in *prpsGL* by using *NheI* and *SalI* restriction sites.

![Diagram of Cra binding Sequences](image-url)

**Fig. 1**—Putative Cra-binding site in *prpB* gene matched with Cra consensus sequence (R is G or A; S is C or G; W is A or T; H is A, T, or C; and N is A, C, G, or T)
Transformants were selected on ampicillin (100 μg/ml) plates with X-gal (30 μg/ml). Blue colonies were screened for plasmids with rpsGL-lacZ TF. Presence of lacZ insert was confirmed by NheI and SalI restriction. This clone was named as pprpB-lacZ and used to generate pprpB-lacZ and yahA-lacZ TFs.

β-Galactosidase Assay (GA)

GA was done especially as described by Miller but with minor modifications. Cells were grown in minimal medium with relevant carbon source (glucose, glycerol, arabinose and sodium lactate). Cells (0.5 ml) to be assayed were added to 2XZ-Buffer (0.5 ml). Two drops of chloroform was added to this and vortexed well. 0.2 ml of ONPG (Ortho nitro phenyl galactoside) (4 mg/ml) was then added to this cell suspension, mixed and incubated at 37°C for 30 min. Reaction was stopped by adding 1M sodium carbonate (0.5 ml). Prior to assay, density of cells was measured at 600 nm. Absorbance due to yellow colour during assay was measured at 420 nm. In order to avoid absorbance due to light scattering by cell debris, before measuring OD at 420 nm, cell suspension was spun down at 10000 rpm for 5 min. Miller Unit values were calculated using the formula given by Miller with minor modifications.

Results and Discussion

Rationale for Choosing prpB and yahA as Candidate Genes

One of the aspects of ongoing research in this laboratory pertains to transcription control, especially by ATFs in E. coli. Present work in this aspect had led to the elucidation of a second novel function (Fit-factors involved in transcription) associated with both α and β subunits of Phenylalanyl tRNA synthetase (PheRS) enzyme of E. coli. As a continuation of this aspect on transcription control in E. coli, in vivo role of Cra in regulation of two candidate genes (prpB & yahA) is studied. In case of prpB gene, putative Cra binding sequence CCTGAAACGTTAAC was found by BLAST analysis in its promoter (Fig. 1). In case of yahA, BLAST analysis did not show any previously defined putative Cra binding site in the promoter of yahA. However, purified Cra protein binds with the promoter region of yahA and down regulates expression of the same in vitro. Therefore, to validate in vitro results obtained with yahA and to understand involvement of Cra in prpB expression, these two candidate genes were chosen and analyzed the actual role of Cra in regulation of both genes in vivo.

Generation of prpB-lacZ Transcriptional Fusion (TF)

prpB fragment was amplified with prpBF and prpBR primers (Table 1), designed with HindIII and NheI restriction sites, using following cyclic conditions: initial denaturation, 94°C for 5 min; denaturation, 95°C for 45 s; primer annealing, 61°C for 45 s; extension, 72°C for 45 s; final extension, 72°C for 5 min using pfu polymerase. prpB fragment were cloned in pBR322 derived vector prpsGL –lacZ plasmid by replacing rpsGL with prpB using HindIII and NheI restriction sites. Transformants were selected on ampicillin (100 μg/ml) plates with X-gal (30 μg/ml). Blue colonies were screened for plasmids with prpB-lacZ TF. Presence of prpB insert was confirmed by HindIII and NheI restriction (Fig. 2a) and lacZ insert was also confirmed by NheI and SalI restriction (Fig. 2a). This clone was named as pprpB-lacZ, in which lacZ will get expressed only when prpB (promoter) gets expressed and this was used in further studies.

Generation of yahA-lacZ Transcriptional Fusion (TF)

yahA fragment (~800 bp) was amplified with yahAF and yahAR primers (Table 1), designed with HindIII and NheI restriction sites, using following conditions: initial denaturation, 94°C for 5 min; denaturation, 95°C for 45 s; primer annealing, 62°C for 45 s; extension, 72°C for 45 s; final extension, 72°C for 5 min by using pfu polymerase. To construct yahA-lacZ TF, rpsGL fragment of pBR322 derived vector prpsGL –lacZ plasmid was replaced with amplified yahA fragment with HindIII and NheI restriction sites. Presence of yahA fragment in the clone was confirmed by HindIII and NheI restriction digestion and lacZ gene was also confirmed by NheI and SalI restriction. This clone was named as pyahA-lacZ, in which lacZ will get expressed only if yahA (promoter) gets expressed (Fig. 2b), and this was used in further studies.
Evaluation of Extent of Expression of prpB and yahA Transcriptional Fusions (TFs) in isogenic Cra+ and Cra- Genetic Backgrounds and its Implications

pprpB-lacZ and pyahA-lacZ clones were transformed into isogenic cra+ and cra- derivatives of MC4100 (ΔargF-lac). GAs were done in strains with clones (bearing fusions) namely MC4100 cra- / ppprB-lacZ and MC4100 cra+ / ppprB-lacZ to check Cra effect on the expression of prpB (Fig. 3a). To check in vivo role of Cra in yahA expression, GAs were also done in MC4100 cra+ / pyahA-lacZ and MC4100 cra- / pyahA-lacZ strains (Fig. 3b). Actual values of β-galactosidase activity were given in Miller Units (Table 2). Different carbon sources were used to understand the effect of different carbon sources, if any, in transcriptional regulation of prpB and yahA by Cra. Carbon sources used in case of ppprB-lacZ were glucose, glycerol and arabinose, and for pyahA-lacZ were glucose, glycerol and sodium lactate. In case of cultures grown in minimal glucose, mid log phase cultures were used for GAs. But, in case of cultures grown with glycerol, arabinose and sodium lactate, GAs were done with 1.5-2 OD cultures, because activity of β-galactosidase was very less in those cultures when assayed with mid log phase cultures. Therefore, experiments were conducted with 1.5-2 OD cultures grown in other carbon sources except glucose.

In case of prpB-lacZ fusion, β-galactosidase expression (Table 2 & Fig. 3a) was found to be more in wild type cra+ strain and it is comparatively less in Cra- mutant, clearly indicating that prpB gene is up regulated by Cra. These results are in accordance with BLAST analysis, which revealed presence of Cra binding site preceding the binding site of RNAP in prpB promoter (Fig. 1). Up regulation of prpB by Cra in S. typhimurium LT2 had also been reported with single copy prp::MudI1734 fusion and this very much supports the result presented herein. In Fig. 3a (insert figures a & b), GA clearly indicated the up regulation of prpB in pprpB-lacZ fusion in minimal glycerol and arabinose.

In case of yahA gene, though putative Cra binding site was not identified previously, Shimada et al. reported down regulation of yahA by Cra in vitro. However, GA in yahA-lacZ fusion reported herein indicates that yahA-lacZ fusion gets expressed more in MC4100 cra+ genetic...
background. These results favor that yahA gene is also perhaps, up regulated by Cra in vivo. (Fig. 3b & Table 2). In Fig. 3b (insert figures a & b), GA clearly indicated up regulation of yahA by Cra in yahA-lacZ fusion in minimal glycerol and sodium lactate. Sequencing was done with relevant region of clone and BLAST analysis was also done with fusion sequences. Such analysis confirmed the absence of unexpected Cra binding sequence in the fusion region [data not shown].

Regulation of yahA gene by Cra may vary inside the cell, not only based on carbon source used but also based on phase of growth and also the involvement of other factors in the cell, which can not be considered in vitro.

Construction of prpB-lacZ and yahA-lacZ TF and analysis of GA in both the fusions in both cra+ and cra− backgrounds of MC4100 revealed that Cra perhaps up
regulates the expression of both prpB and yahA genes in vivo. This laboratory has been concentrating on genetics of fit loci, genes involved in transcription control in E. coli for many years (Vidhya et al.4 and references cited therein). In fact, only while studying in depth details of rpsG expression in fit mutants, the aspect reported in this paper were studied. Differential GE is the one, which is needed by any organism to survive in varied environmental conditions and it has been reviewed extensively.1,2,26. Although polynucleotide phosphorylase functions as an RNase in vivo, whereas it was extensively used for making RNA in vitro during initial studies pertaining to elucidation of genetic code.28,29. Similarly, E. coli RecA functions as a recombinase in a normal circumstance and becomes a protease during DNA damage and subsequent SOS induction.28,29. In PhoQ-PhoP two component system of E. coli and Salmonella, binding of PhoP differs in vivo and in vitro, which affect transcriptional regulation of set of genes by stimulus dependent manner30 and intracellular ionic environment also play a role in gene regulation.31 Not only the environmental status but also the medium of growth do play a role in gene regulation. Allelic status of strain also plays a role in differential GE. Early reports from this laboratory indicated that transcription defective temperature sensitive fitA76 mutant, which is virtually non-viable at 42°C (10-7), makes 50% RNA at 42°C, whereas fitA24 Ts mutant, which is partially viable at 42°C (10-3), makes only 25% RNA at 42°C.32 These results together with that selectivity of GE depends on interplay of conformational states of genome as well as RNAP complexes with various ATFs argue for the favor of the notion that ATFs are indeed vital to achieve selective GE.

Conclusions

In vivo regulation is presented of prpB and yahA genes of E. coli by Cra. Construction of prpB-lacZ and yahA-lacZ TF and analysis of GA from both fusions in both cra+ and cra− backgrounds of MC4100 revealed that Cra up regulates both prpB and yahA expression in vivo.

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