Construction of RhaS and RhaR overexpression plasmids and the effects of CRP mutants on *E. coli* L-rhamnose gene expression

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Abstract: The *rhaSR* operon in *Escherichia coli* encodes two proteins that act as transcription activators of the *rhaBAD* and *rhaSR* operons. The proteins RhaS and RhaR belong to the AraC family of activators. In this work, molecular techniques were used to clone the *rhaS* and *rhaR* genes into plasmid vectors which allowed for purification of the RhaS and RhaR fusion proteins. Transformations into *E. coli* strains with *rhaS* or *rhaR* deletions allowed for simple screening to identify the desired clones. The pTYB1 vector fused RhaS with an intein domain and a chitin-binding domain. The pET43a vector allowed for the production of NusA-His6 fusion protein aimed at increasing the solubility of the overexpressed RhaS and RhaR proteins. Immobilized Ni^{+2} affinity chromatography was used to purify the soluble NusA-His6-RhaS fusion protein, while the NusA-His6-RhaR fusion protein was not soluble. Experiments are underway to determine whether active RhaS protein can be released from the fusion. Isolation of the active protein is critical, as it will allow for biochemical analysis of RhaS and RhaR transcription activation.

In addition, characterization of the effects of mutations in CRP on *rhaBAD* and *rhaSR* activation is currently underway. This work involves transformations of strains carrying *lacZ* fusions with the *rhaBAD* or *rhaSR* promoters, a three-step growth regime, and assays to quantify the production of β-galactosidase in each mutant. To date we have found that mutation 165A greatly hinders activation of both *rhaB-lacZ* and *rhaS-lacZ* fusions.

BSU Faculty Advisor: Dr. Alice Lindgren

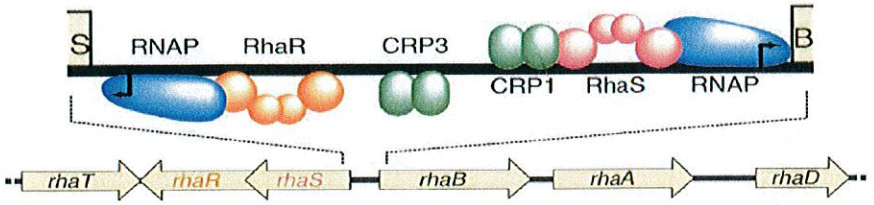
INTRODUCTION

RhaS and RhaR Overexpression Plasmids

The AraC family of transcription activators is a large family of regulators. Its members have been found in a variety of bacterial species and regulate the expression of genes involved in carbon metabolism, stress response, and pathogen virulence (1). In *Eschericia*

Fig. 1

E. coli L-rhamnose Regulon



coli RhaS and RhaR are proteins that act as transcription activators regulating metabolism of the sugar L-rhamnose.

RhaS activates L-rhamnose catabolism through expression of *rhaBAD* and *rhaT* operons, and RhaR regulates *rhaSR* expression. Both regulate the respective operons by binding as dimers to promoter DNA adjacent to RNA polymerase (Fig.1). At each promoter a second activator, CRP, is required (5).

Through the comprehensive research of the *rhaSR* operon, the details of transcription activation by AraC activators are better understood. As data is compiled, the applications of this knowledge may include the understanding of stress responses as well as pathogen virulence and sugar metabolism. Biochemical analysis of the system often requires purification of active proteins. A key component of this research is biochemical tests on the functions of the protein products of the *rhaBAD* and *rhaSR* operons.

To understand transcription activation in its entirety, the structure and function of all activators and inhibitors must be determined. The crystallized structure of the activator CRP is known (Fig. 9). The details of CRP folding and DNA binding is important to its role in activation. Altering the CRP protein in a known matter allows for specific analysis of amino acid effects on activity.

CRP Regulator Protein

CRP (cyclic AMP receptor protein) is a regulator protein that controls the expression of over 100 promoters in *E. coli*. As a prerequisite to activating transcription, CRP binds to a 22 bp twofold-symmetric recognition site on the bacterial DNA . The protein-protein binding between CRP and RNA polymerase seems to be the predominant means of regulation. Depending on the structure of the promoter region, one or two activation regions (AR1 and AR2) on the surface of CRP are involved in positive contacts with the α subunit of RNA polymerase (5).

In previous work (5) up to four CRP binding sites have been identified in the L-rhamnose regulon. (Fig 6). The effectiveness of CRP with known alanine substitution mutations for individual amino acids was assessed. These substitutions affect not only the

primary structure of the protein but potentially the secondary, tertiary, and quaternary structure of the regulatory protein. Most important to our research is the alteration of the CRP activity. This can be observed directly through β-galactosidase assays.

MATERIALS AND METHODS

Culture media. Cultures for β-galactosidase assay were grown in 1X MOPS [3-(N-morpholino) propanesulfonic acid]-buffered medium (9) (40 mM MOPS, 4 mM Tricine, 0.01 mM $FeSO_4$, 9.5 mM NH_4Cl, 0.267 mM K_2SO_4, 0.5μM $CaCl_2$, 0.528 mM $MgCl_2$, 50 mM NaCl, 3 X 10^{-9} M Na_2Mo_4, 4 X 10^{-7} M H_3BO_3, 3 X 10^{-8} M $CoCl_2$, 10^{-8} M $MnCl_2$, 10^{-8} M $ZnSO_4$, 1.32 mM K_2HPO_4, 10 mM $NaHCO_3$, 0.2% Casamino acids, 0.002% thiamine). Ampicillin and streptomycin were used at 125 μg/ml when necessary. For other experiments (cloning, etc.) cells were grown in tryptone-yeast extract medium (7) with or with our antibiotic or in TB-maltose (0.8% Bacto-Tryptone, 0.5% NaCl, 0.2% maltose).

General Methods. Standard methods were used for restriction endonuclease digestion, ligation, transformation, and purification of plasmid DNA. All PCRs done to generate DNA fragments for cloning were performed using the Expand High Fidelity PCR System from Roche (Indianapolis, Ind.) DNA sequences were verified by automated dideoxy sequencing on LI-COR 4000L sequencer. Sequencing reactions were performed using the Thermo Sequenase fluorescence-labled-primer cycle sequencing kit from Ambersham Pharmacia Biotech (Piscataway, N.J.) All DNA sequences were confirmed on both strands.

Plasmids and strains. Wild-type *rhaS* was amplified by PCR using primers 1170 and 898 and plamid pSE101as the template. Half of the PCR product was digested at the *XhoI* site in 1170 and at the *SmaI* site in 898 and the remainder was digested at the *NdeI* site in 1170 and the and the *SpaI* site in 898. Wild-type *rhaR* was amplified by PCR using primers **1170** and **898** and plamid pSE101as the template. The PCR product was digested at the *XhoI* site in **1170** and at the *PshAI* site in **898**

Cloning of *rhaS/rhaR* regulon

The *rhaS* and *rhaR* genes were amplified using polymerase chain reaction (PCR).

Two different vectors were used to produce fusion proteins of different natures. The pET43a vector (Fig. 2) allowed for the production of a NusA·His6 fusion protein aimed at increasing the solubility of the overexpressed RhaS and RhaR proteins. The *rhaS* gene was cloned into the *XhoI* and *SmaI* sites and *rhaR* was cloned into the *XhoI* and *PshAI* sites.

The pTYB1 vector (Fig. 3) (New England BioLabs) was used in the production of the fusion protein combining RhaS with an intein-chitin binding domain. The *rhaS* gene was cloned into the *NdeI* and *SpaI* sites of the pTYB1 vector.

Transformation of *E. coli* with Plasmid DNA

The transformation of *E. coli* strains SME2427 (Δ*rhaR*, Amp Resistant) and SME1087 (Δ*rhaS*, Streptomycin Resistant) was done in accordance to the revised techniques of D. Hanahan (4). SME2427 was transformed with the pET43a + rhaR plasmid and SME1087 was transformed with the pET43a + rhaS plasmid as well as the pTYB1 + rhaS plasmid. Transformed *E. coli* were grown on nutrient agar with antibiotic, L-rhamnose, and X-gal plates. The selection of a successful clone was identified by color change due to sugar metabolism

Clone analysis

The selected clones were cultured overnight in TY broth + antibiotic. Plasmid DNA was isolated and sequenced to ensure that rhaS and rhaR genes were incorporated in the correct location and without error in the gene or surrounding plasmid (Procedure: T. Cavileer, Univ. of Idaho).

Fig. 2

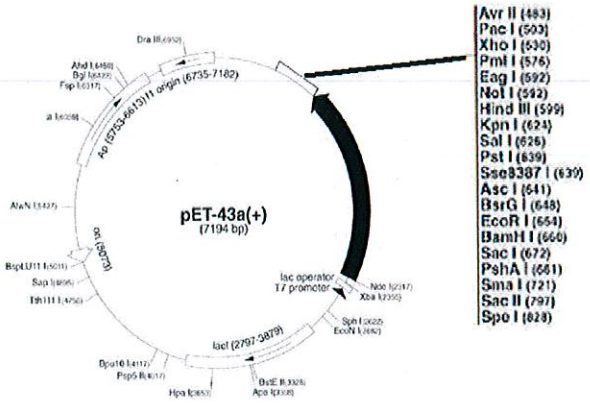
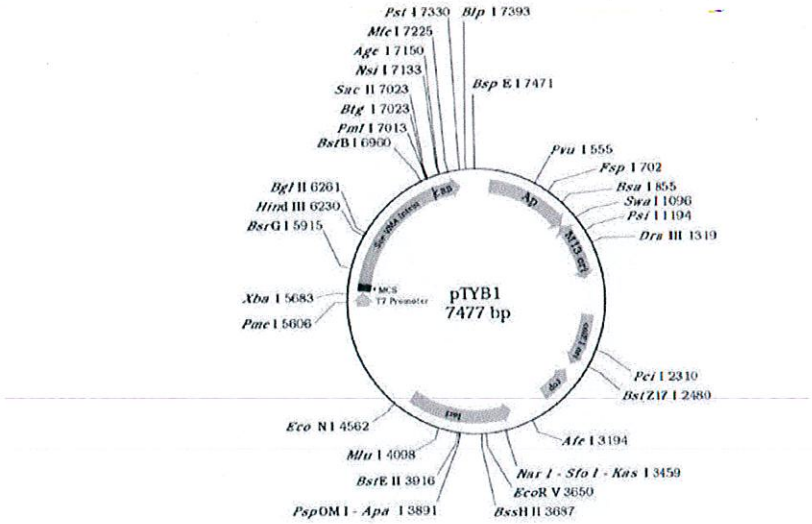


Fig. 3

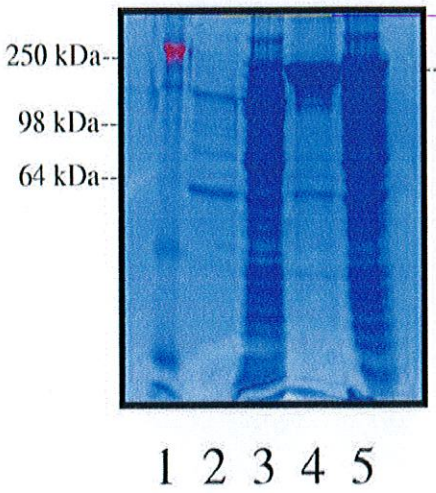


Test for protein solubility

The desired solubility of the RhaS and RhaR fusion proteins was tested. SME 2427 w/ pET43a + *rhaR*, SME1087 w/ pET43a + *rhaS*, and SME1087 w/ pTYB1 + *rhaS* clones were cultured overnight. Cells were collected by centrifugation and lysed by sonication.

Soluble and insoluble cell components were separated. The collected portions were run on a protein gel (Fig 4).

Fig. 4



Lane 1: Molecular Weight Marker

Lane 2: Insoluble cell components of SME 2427 w/ pET43a+*rhaR*

Lane 3: Soluble cell components of SME 2427 w/ pET43a+*rhaR*

Lane 4: Insoluble cell components of SME 1087 w/ pET43a+*rhaS*

Lane 5: Soluble cell components of SME 1087 w/ pET43a+*rhaS*

Protein Purification

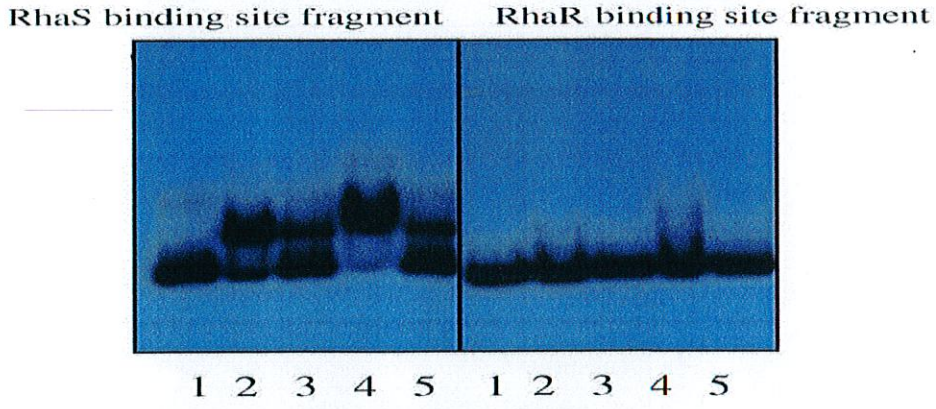
SME1087 w/ pET43a + *rhaS* was grown in a 4 L culture for 36 hrs. Cells were collected by centrifugation and lysed by sonication. Purification of the NusA·His6-RhaS fusion protein was completed by immobilized Ni^{+2} affinity chromatography. The His6 protein allowed for the binding of the fusion protein to a Ni^{+2} column.

Mobility Shift Assay

A mobility shift assay was used to test the DNA binding activity of the purified fusion protein NusA-RhaS (Fig 5).

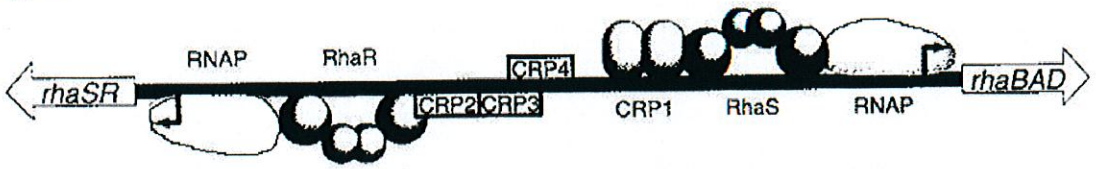
A *rhaS* DNA binding fragment was combined with the purified fusion protein. The combined sample, as well as a free DNA sample and purified NusA protein. The migrations of the respective samples were compared. A shift in the migration of the sample compared to the free RhaS binding DNA would indicate the binding of the active RhaS to the DNA binding site.

Fig. 5



Lane 1: Free DNA
Lane 2&3: NusA-RhaS
Lane 4&5: NusA

Fig. 6



The effects of CRP mutants on *E. coli* L-rhamnose gene expression.

β-galactosidase assays

Strains for β-galactosidase assay were grown as described by Bhende and Egan (1). Briefly, starter cultures were grown in tryptone-yeasy extract broth (with 125 μg ampicillin per ml added for strains containing plasmid) for approximately 8 hrs at 37 °C. The 40 μl of starter culture was used to inoculate 2.5 mls of 1X MOPS overnight medium (see recipe above). This culture was grown overnight. Approximately 200 μl were added to a growth flask to reach an optimum optical density at 600 nm of 0.01 to 0.02. This was cultured in 1X MOPS growth medium in 125 ml baffled flasks. Cultures were grown at 37 °C with vigorous shaking to an A_{600} of approximately 0.4. After resuspension of the cell pellets in Z buffer (8), β-galactosidase activity was determined as described by Miller (8) except that incubation with substrate (*o*-nitrophenyl-β-D -thiogalactopyranoside) was done at room temperature. Specific activities were averaged from at least three independent assays (unless otherwise noted), with two replicates in each assay.

RESULTS AND DISCUSSION

Overexpression Plasmids

Through DNA sequencing, successful cloning of the *rhaS* and *rhaR* operons into the pET43a vector (Fig. 2 was confirmed. However, the cloning of *rhaS* into pTYB1 was identified as an insufficient encorporation of the spliced DNA. The pET43a vector allowed for the production of a NusA·His6 fusion protein. The encorporation of the plasmid into E. coli (Δ*rhaS* or Δ*rhaR*) allowed for the isolation of a transformant. The test of protein solubility indicated that RhaS was roughly one-third soluble, however RhaR was shown to remain completely insoluble. The amplification and purification of RhaS yielded the desired fusion protein. The mobility shift assay showed the binding of this fusion protein to the *rhaS* DNA binding fragment. Initial results indicated the purification of an active *rhaS* protein, however further analysis indicated that NusA was actually binding to the *rhaS* DNA binding fragment. The possible activity of the purified RhaS fusion protein could not be determined.

β-galactosidase assays

Results of the β-galactosidase assay indicate that the alanine substitution at CRP residue Ile 165 results in a large defect in both *rhaSR* and *rhaBAD* expression (Fig. 7 & Fig. 8). However this residue is not very surface exposed and is unlikely to indicate a site of protein-protein interaction (Fig. 9).

Fig. 7

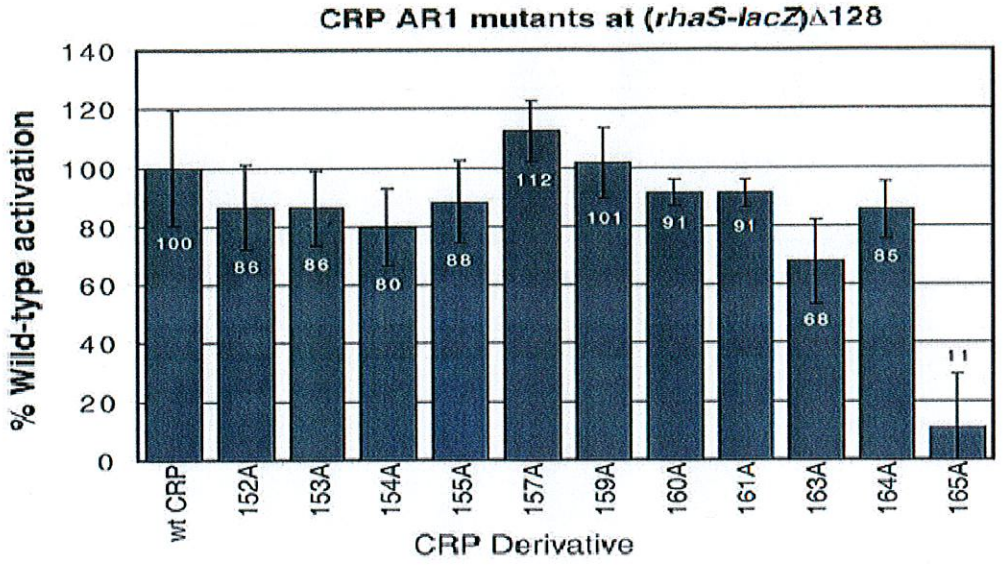

CRP AR1 mutants at (rhaS-lacZ)Δ128
% Wild-type activation
140
120
100
80
60
40
20
0
100
86
86
80
88
112
101
91
91
68
85
wt CRP
152A
153A
154A
155A
157A
159A
160A
161A
163A
164A
165A
CRP Derivative

Fig. 8

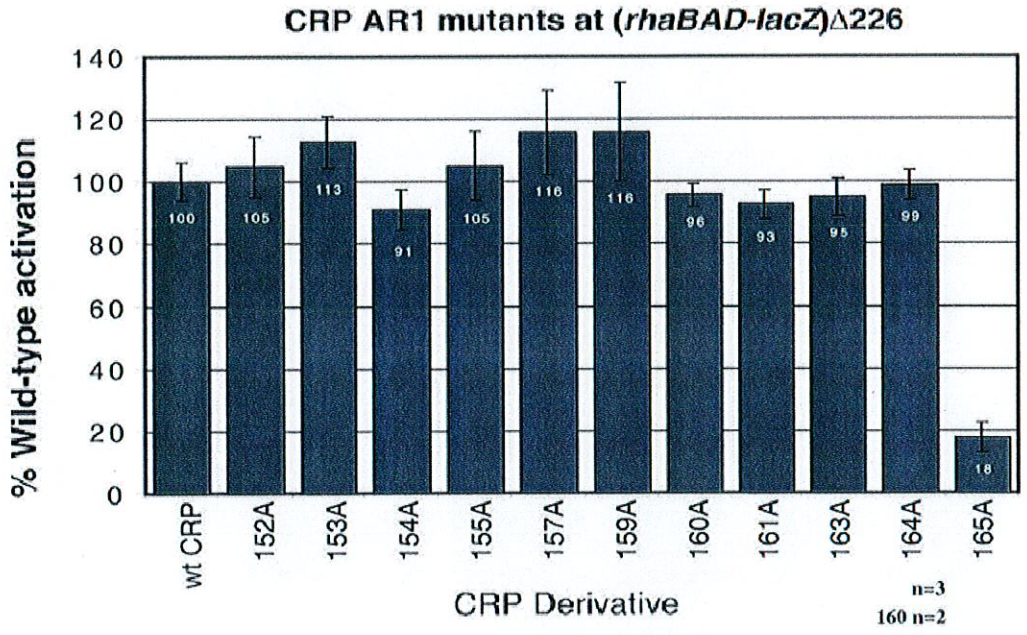
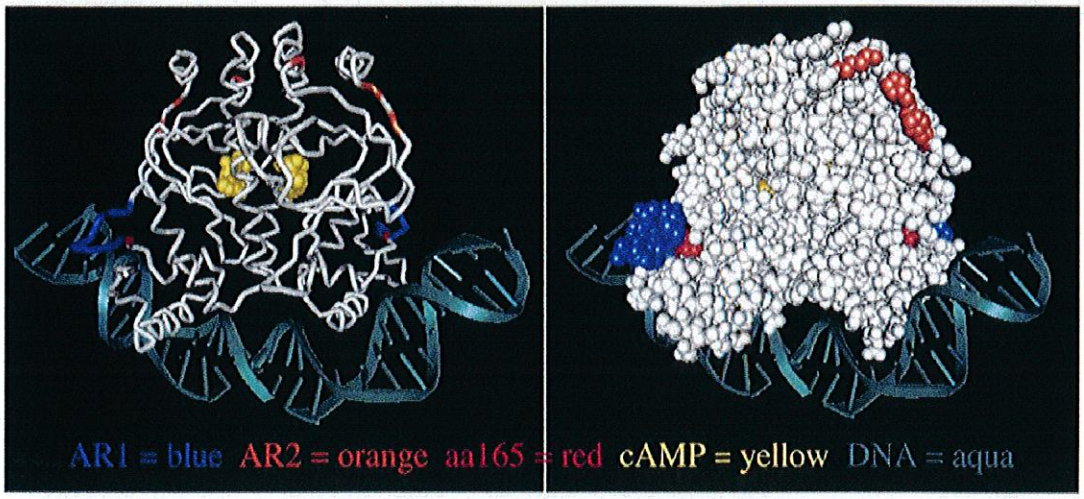

CRP AR1 mutants at (rhaBAD-lacZ)Δ226
% Wild-type activation
140
120
100
80
60
40
20
0
100
105
113
91
105
116
116
96
93
95
99
18
wt CRP
152A
153A
154A
155A
157A
159A
160A
161A
163A
164A
165A
CRP Derivative
n=3
160 n=2

Fig. 9

CRP 165 is not very surface exposed



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