Role of N-terminal domain of Rns from Enterotoxigenic *Escherichia coli* in transcription activation

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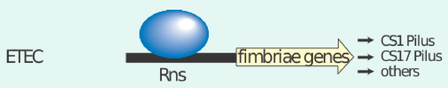
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Abstract

Enterotoxigenic *Escherichia coli* (ETEC) is a diarrhea causing pathogen which causes ~500,000 deaths/ year worldwide. Rns is a transcriptional activator of the AraC family and is required for virulence gene activation in ETEC. Rns has 2 domains: an amino terminal domain (NTD) and a DNA binding domain (DBD). Current evidence indicates that Rns functions as a monomer and may not require an effector to activate transcription. The main goal of this study is to identify the role of Rns-NTD in transcription activation. In previous work, two individual mutations I14T and N16D, near the amino terminus of Rns and far from known DNA-binding determinants, abolished Rns activation at its own promoter by hindering the binding of Rns to DNA. We have found that deleting the Rns-NTD (residues 1-156) leads to loss of Rns activity *in vivo*. Sequence analysis of Rns' closest homologs showed that a region of the NTD (residues 12- 30) shares high identity (74%), much higher than the 26% overall NTD identity. Further, this region aligns with a region in the ToxT structure that contacts the DBD. We hypothesize that residues in this region contact Rns DBD, causing structural rearrangements in the DBD that facilitate DNA binding. One test of our hypothesis is site-directed random mutagenesis to identify mutants in this region that are defective in transcription activation. To date, we have mutagenized residues 12 to16 and identified eight variants (at positions I12, I14, N15 and N16) that decreased Rns activity by > 2 fold, indicting that these residues are important for Rns activity. On the other hand none of the mutants of K13 had decreased Rns activity. We will perform Western blots to eliminate unstable variants from consideration. We have purified Rns DBD to near homogeneity by adding a solubility tag, GB1, at the C-terminus of the DBD. The purified Rns DBD will be used for antibody generation and structural studies. Our electrophoretic mobility shift analysis (EMSA) with purified Rns DBD-GB1 showed that Rns DBD can bind to DNA *in vitro* at sufficiently high protein concentrations.

Background and significance

- Rns is a AraC/XylS family transcriptional activator from enteric (Intestine) pathogen Enterotoxigenic *E. coli* (ETEC).
- AraC/XylS family proteins:
  - Have been identified in 70% of sequenced bacterial genomes.
  - Contain a conserved DNA-binding domain (DBD) with 2 HTH motifs

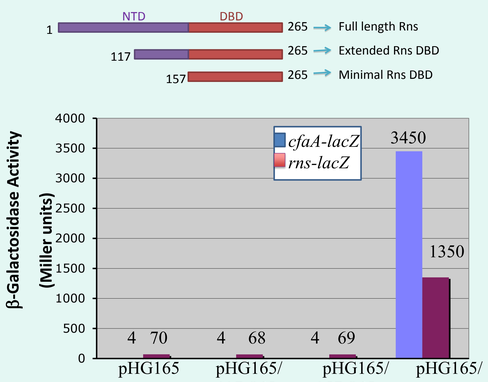


- ETEC is responsible for ~500,000 deaths annually in children under the age of five. (Fleckenstein *et al.,* 2010)
- Rns – activator of virulence genes in ETEC
- Since Rns is required for virulence gene expression in ETEC, it can be used as a potential anti-bacterial target

Objective

Identify the role of Rns-NTD in transcription activation

Deleting Rns-NTD led to loss of Rns activity *in vivo*



Transcription activation by Rns N-terminal deletion constructs: β-galactosidase activity of cfaA-lacZ and rns-lacZ fusions by plasmid encoded Rns or deletion variants

Rns-NTD residues 12-30

- Rns-NTD residues 12-30 share 74% identity with Rns' closest homologs which is higher than the 26% overall NTD identity, suggesting an important role for this region.

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HdaR_Ecoli        1 MKLKQNIEKEIIKINNIRIHQYTVLYTSNCTIDVYTKEGS 40
AggR_Ecoli        1 MKLKQNIEKEIIKINNIRIHQYTVLYTSNCTIDVYTKEGS 40
RNS_ECOLX         1 MDFKYTEEKETIKINNIMIHKYTVLYTSNCIMDIYSEEEK 40
Rns_EcoliX        1 MDFKYTEEKETIKINNIMIHKYTVLYTSNCIMDIYSEEEK 40
CFAD_ECOLX        1 MDFKYTEEKEMIKINNIMIHKYTVLYTSNCIMDIYSEEEK 40
CSVR_ECOLX        1 MDFKYTEEKELIKINNVMIHKYTILYTSNCILDISFGEDK 40
CS12actEcoli      1 MAFKYTEEKEIIKINNIRIHKYTLLYTSNCIMDIYSDEKK 40
Rns_Ecoli101_1    1 -MLIPHLESDQIKINNVRVHKYTILYTSNCIMDIDISGKT 39
Rns_Ealbertii     1 -MLIPHLESDQIKINNVRVHKYTILYTSNCIMDIDISGKT 39
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Alignment of Rns homologs showing a conserved region near the N terminus of Rns

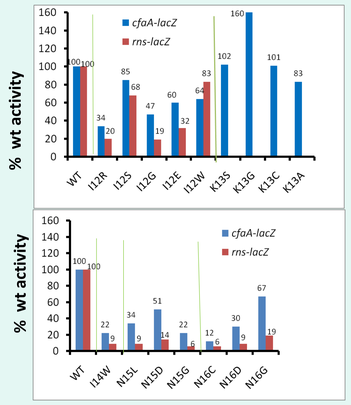
- This conserved region of Rns also aligns with a region in ToxT structure that contact DBD.
- Previous reports also indicated that two individual mutations I14T and N16D completely abolished Rns activity at its own promoter by hindering binding to DNA (Munson *et al*., 2010)

Hypothesis

- Residues (12-30) in the region conserved among Rns homologs contacts RnsDBD, causing structural rearrangements in the DBD that facilitate DNA binding

Site directed random mutagenesis of Rns residues 12-16

Among Rns homologs residues from 12-16 are 100% identical suggesting an important role for these residues. This led us to start our randomization experiments from this region



β-galactosidase assays of Rns variants at residues I12, K13 (Top), I14, N15 and N16(Bottom): Activity presented as the % activation of *cfaA-lacZ, rns-lacZ* fusion by Rns variants compared to WT Rns..

- We isolated variants at I12 (I12R, I12E), I14 (I14W), N15 (N15L, N15D, N15G) and N16 (N16C, N16D, N16G) that significantly decreased Rns activation (>2 fold) at both promoters. This indicates that these residues are important for transcription activation by Rns.
- Variants at K13 were not defective for Rns activation of the *cfaA-lacZ* fusion indicating that this residue is not key for Rns activation. Rns K13 variants have not yet been assayed at *rns-lacZ*.

Rns Purification

- We wished to purify Rns protein and its domains in order to:
  - Generate anti-Rns antibodies
  - Perform in vitro molecular assays of Rns funtion
  - Characterize Rns variants
  - Obtain structure information

•However, previous studies have found that Rns, like most AraC family proteins, was insoluble when overexpressed (Munson *et al.,* 1999).

•Relatively low concentrations of soluble Rns could be obtained as a fusion with the Maltose binding protein solubility tag. (Bodero *et al.,* 2007)

GB1 Solubility tag

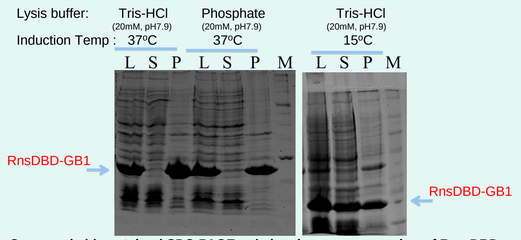


•GB1 is a small (6.2KDa), exceptionally soluble (>124mg/ml) protein that has been shown to increase solubility of many proteins. (Zhou and Wagner, 2010, J Biomol NMR, 46:23)

•Vectors are available to construct N- and C-terminal GB1-His_6 fusion proteins (De Guzman 2009 J Biol Chem, 284:8654-60)

- We constructed Rns, Rns-NTD, Rns-DBD fusions with GB1 at N- and C- termini of protein and tested their over expression. Among all the fusion proteins tested, only Rns-DBD-GB1 fusion protein showed a detectable level of overexpression

Rns-DBD-GB1 Overexpression

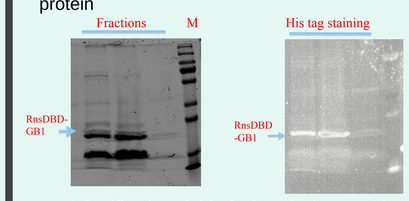


Coomassie blue stained SDS-PAGE gel showing overexpression of Rns-DBD-GB1. L, Total lysate; S, Supernatant; P, Pellet. Cells were grown to an A_{600} of 0.6, induced with 1mM IPTG. Cells were lysed by french press in Tris-HCl or phosphate buffer, run on SDS-PAGE gel and stained with coomassie stain.

- Up to 50% of Rns-DBD-GB1 was soluble in Tris-HCl lysis buffer when induced at 15ºC

Purification & EMSAs of Rns-DBD-GB1

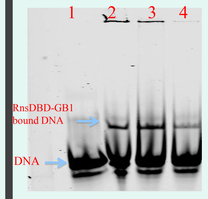
- Soluble RnsDBD-GB1-His_6 protein was purified using Ni^{2+} affinity chromatography and eluted with 0.5M imidazole. Elution fractions were run on SDS-PAGE and stained with coomassie blue and a fluorescent Ni^{2+} : nitraloacetic acid dye that stains His tag of fusion protein



Coomassie blue (Left) and His Stained (Right) SDS-PAGE gel showing purified Rns-DBD- GB1

- A Purification yield of ~2mg fusion protein per 100mL culture was obtained. The lower band in the purified protein, an apparent truncation product, was observed only in some purifications.

EMSA:



EMSA showing binding of purified Rns-DBD-GB1 protein to Rns DNA binding site : Undiluted (9μM) , 1:3 (3μM), 1:9 (1μM) dilutions of Rns-DBD-GB1 were mixed with double stranded Rns binding DNA and loaded into EMSA gel.
1: Free DNA
2: undiluted Rns-DBD-GB1 + DNA
3: 1:3 dilution of Rns-DBD-GB1 in EMSA buffer + DNA
4: 1:9 dilution of Rns-DBD-GB1 in EMSA buffer + DNA

- A concentration dependent change in binding of Rns-DBD-GB1 protein to DNA was observed

Summary & Conclusions

- Rns-NTD is required to activate transcription by Rns *in vivo.* Four out of the five residues tested in the conserved region (12-30) of Rns homologs are important for transcription activation .
- Have successfully purified Rns-DBD using GB1 solubility tag. This protein can be used to develop antibodies and potentially for structural studies.
- Demonstrated that Rns-DBD is capable of binding to DNA *in vitro* at high concentrations

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