UNIVERSITY OF FLORIDA
UFJUR
JOURNAL OF UNDERGRADUATE RESEARCH ISSN: 2683-0668

Expression and Characterization of YoaA, a Putative Helicase in Bacteria, Involved in Repairing Blocks to DNA Replication

Sneha Sathish[1], Leslie Morris[2], and Linda Bloom[2]
[1]*Department of Agriculture and Life Sciences, University of Florida*
[2]*Department of Biochemistry and Molecular Biology, University of Florida*

Faculty mentor: Leslie Morris, Department of Biochemistry and Molecular Biology

Abstract

While cells have efficient pathways for repairing damage to DNA, some DNA damage avoids repair and is found in DNA replication. Because the DNA polymerases that replicate the genome are high fidelity enzymes, DNA damage blocks DNA synthesis and ultimately progression of the replication fork. It is known that cells would not be able to survive without mechanisms to repair these replication blocks and to restart replication. With a genetic screen, our collaborators at Lovett laboratory, using 3'azidothymidine (AZT) as a tool to inhibit replication, identified a novel gene in *Escherichia coli*, *yoaA,* which was required along with *holC* to provide cells with tolerance to AZT. HolC is a protein subunit of the *E. coli* DNA polymerase III holoenzyme that does the bulk of synthesis during DNA replication, and yoaA binds HolC. Based on sequence, the *yoaA* gene encodes an iron-sulfur (Fe-S) helicases. *E. coli* contains a second Fe-S helicase, DinG, and human cells contain four Fe-S helicases, XPD, FANCJ, RTEL1, and ChlR1, that are involved in DNA repair. The overall goal of this project is to express YoaA protein in soluble form and characterize its biochemical activities to determine how YoaA aids in DNA replication fork repair. In our initial studies, we made fluorescent labeled DNA for helicase assays and tested them which allowed us to work towards our long-term goals of determining: 1) whether YoaA is DNA helicase and what are the best substrates for YoaA, and 2) how HolC affects YoaA activities. We have subcloned the *yoaA* gene into different expression vectors to express YoaA with and without affinity tags, and to co-express YoaA with HolC. We are developing strategies to purify YoaA alone and in a complex with HolC. We have subcloned the *yoaA* gene into different expression vectors to express yoaA with pCOLADuet. We are developing strategies to purify YoaA alone. The initial results working towards this are presented.

Introduction

It has been estimated that there are as many as 20,000 DNA damage events that occur in cells within a 24-hour period (Brown et al., 2015). Failure to fix these damages would result in an altered genetic code and possible chromosome breakage. Major DNA repair pathways in cells take advantage of the double-bonded nature of DNA. When one strand is damaged, it can be repaired based on the undamaged code in the complementary strand. While these strands

maintain the structure of DNA, some damage will ultimately not be repaired before cells undergo DNA replication. If DNA damage is encountered during replication it is a problem for two reasons: 1) the damaged site cannot be copied by a DNA polymerase so replication stops at this site, and 2) DNA damage at the replication fork is no longer in double-stranded DNA, so the normal repair pathways cannot repair the damage. Alternative strategies are needed to either get past DNA damage and repair it at a later time or repair the DNA damage at the fork. The Bloom research group studies DNA replication and repair mechanisms. They are interested in fundamental mechanisms by which cells duplicate their genomes and maintain the structure of DNA. We are investigating mechanisms by which cells cope with DNA damage that is encountered at the replication fork.

Background

The *E. coli yoaA* gene was identified in two different genetic screens for genes involved in DNA repair. In the first study **(**Brown et al., 2015), the *yoaA* gene was identified in a screen for high-copy suppressors that give *E. coli* tolerance to the DNA synthesis chain terminator, 3'azidothymidine (AZT). This screen also identified the *holC* gene, a subunit of DNA polymerase III holoenzyme, and the ground showed that YoaA and HolC interact both physically and functionally to give cells tolerance to AZT. A second study (Fan et al., 2008), identified the *yoaA* gene in a screen for synthetic lethality in a strain deleted for xthA and nfo, two AP endonucleases. This group showed that deletion of *yoaA* in combination with xthA increased sensitivity of cells to the DNA damaging agent, methyl methanesulfonate (MMS), and their data also indicated that yoaA functions with HolC. Thus, both of these genetic studies support a role for *yoaA* working with HolC in DNA repair. Known interactions between HolC (χ) and SSB and between HolC (χ) and HolD (γ) were also required for AZT tolerance (Brown et al., 2015).

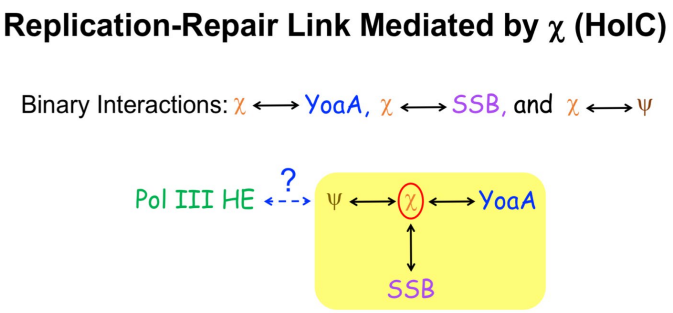

Figure 1. Genetic interactions between YoaA, SSB, and Pol III HE are established. The goal of our studies is to determine what the biochemical functions of YoaA are in DNA repair, and how YoaA works with HolC in repair. (Bloom Laboratory)

Lovett Laboratory

Our collaborators in the Lovett laboratory at Brandeis University use genetic approaches in *Escherichia coli*, a model organism, to discover processes that can repair DNA damage during replication. They recently discovered a pathway that allows cells to overcome DNA damage caused by 3'-azido-3'-thymidine (AZT). AZT is a chain terminator so that when it is incorporated into DNA by a DNA polymerase, the DNA polymer cannot be extended any further (Fan, et al 2008). Thus, DNA replication is blocked. The Lovett laboratory discovered a new gene, *yoaA*, which gives cells tolerance to AZT (Brown et al., 2015). Prior to this study, the function of the *yoaA* gene was not known. Based on its sequence, *yoaA* encodes a DNA helicase, and enzyme that unwinds DNA strands. Our hypothesis is that the YoaA DNA helicase unwinds the newly synthesized DNA strand so that a nuclease can remove the AZT from the end to repair the DNA. After AZT is removed, the DNA polymerase can return and continue synthesis.

DinG and YoaA

Based on the sequence of the *yoaA* gene, the YoaA protein is expected to be a DNA helicase that is related to DNA helicase, DinG, in *E.coli.*

DinG serves two functions in this project: 1) a positive control to show that our helicase assays are working properly. If DinG unwinds a DNA substrate, but YoaA does not, it can be said that our assay is working and that YoaA is not active in unwinding that particular DNA substrate. 2) DNA helicases are specialized. They have different preferences for the types of DNA structures that they unwind. By sequence analysis YoaA and DinG are related helicases, but by genetics, DinG cannot substitute for YoaA. This suggests that DinG and YoaA may have some differences

in the DNA structures they prefer to unwind. We will test YoaA and DinG on several DNA structures to determine what the similarities and differences in their DNA specificities are. It is expected that YoaA and DinG will unwind some of the same structures.

By purifying DNA molecules and the DinG and YoaA proteins and then performing helicase assays will allow us to compare DinG and YoaA activity. A fluorescence-based assay will measure DNA helicase activity. Labeling both strands of DNA with fluorescein will allow us to observe their characteristics and interaction during DNA unwinding.

When the DNA molecules are double-stranded the fluorescein molecules will be close enough to interact and become quenched. When the helicase unwinds DNA and separates the strands, the fluorescein molecules will no longer be able to interact and the fluorescence will increase. This is implemented by measuring the time it takes DinG and YoaA to unwind different DNA substrates. The faster the substrates can be unwound the better the substrates are. Our goal is to be able to answer:

1) What direction does the helicase move on DNA, 5'→3' or 3'→5'? 2) Does the helicase need to bind to a region of single-stranded DNA to unwind a double-stranded region? DinG moves 5'→3' and DNA substrates must have a region of single-stranded DNA at least 10 - 15 nucleotides long near the double-stranded region for DinG to bind DNA and unwind the double-stranded region (Raines, McCormick, Van Oosbree, & Mierendorf, 2008).

Methodology

The objective was to observe how *yoaA* interacts during DNA repair by using the S-tag to help purify the YoaA protein. It is believed that the peptide with its abundance of charged and polar residues could improve solubility of proteins it is attached to (Raines, McCormick, Van Oosbree, & Mierendorf, 2008). On DNA level the S-tag can be attached to the N- of C-terminus of any protein. After gene expression, the S-tag is used to purify the protein on the S- protein column. A tagged protein can be detected by commercially available antibodies. The amino acid sequence of the S-tag is: Lys-Glu-Arg-Gln-His-Met-Asp-Ser.

Growing Cells & Subcloning

E. coli strain DH5α bacterial cells, taken from frozen stocks in a -80°C freezer, were inoculated in 5mL Luria Broth (LB). The cells were incubated in LB at 37°C with shaking for 14-18 hours. The isolation of the pBluescript-yoaA plasmid from the DH5α cells was performed following the guidelines outlined in the Qiagen (QIAprep Spin Miniprep Kit) handbook. A DNA

concentration of 3791 ng/µL was obtained with the use of a microvolume spectrometer (nanodrop). The DH5α strain harbored the pBluescript-yoaA plasmid. By growing the strain, I could isolate the plasmid.

Plasmid Extraction

The intent was to cut the yoaA genes out of pBluescript and insert it into pCOLA. Inserting this will allow for the base pairs to do the same, due to being polydrones. A double digest in NEBuffer 3.1 at 37° C was performed with the NdeI and BamHI enzymes, as stated in NewEnglandBioLabs. A plasmid weighing of 15.164 µg was obtained.

Restriction Digest Preparation and Making Gel

With the intent of separating the plasmid from the bacteria strain, we created the gel using 0.3 g of Agarose, 30mL of 1xTAE, and 3µL of ethidium bromide. UV rays are used to determine if DNA is separated. We use ethidium bromide as molecular weight markers to determine the size of the fragments. We then cut the smaller *yoaA* vector fragment from and extract the DNA from the gel. A DNA concentration of 37.6 ng/µL was obtained.

Gel Extraction

Gel was extracted following the guidelines outlines in the Qiagen (QIAquick Gel Extracting Kit) handbook. A DNA concentration of 66.1 ng/µL was obtained.

Restriction Digest and Dephosphorylation of Vector

While referring to the S-tag, we see that the NdeI is on the 5' and there is no BAMHI on the 3'. DNA was digested with NdeI and BglII as restriction enzymes before purifying the vector using the Qiagen (QIAquick PCR purification Kit). A DNA concentration of 23.1 ng/µL was obtained.

Making Plates and Ligation Reaction

Kanamycin plates were made using 100mL solution of LB, which was then autoclaved for 1 hour and 3 minutes. Kanamycin was used because the pCOLA plasmid has a gene that provides cells with kanamycin resistance. Kanamycin serves as our control to see if our transformation worked. After allowing the stock to cool, 500µL of Kanamycin was inserted. I then dephosphorylated before ligating my DNA. DNA was ligated by measuring different ratios of DNA insert (*yoaA*) to the plasmid vector (pBluescript) and putting everything in 0.5mL tube. Another 0.5 mL tub was used, but filled with water in place of insert as a negative control and labeled 0:1. Both tubes were then put in an ice bath overnight.

Transforming Bacteria

With the intent of introducing the newly made plasmid to the bacteria, 2x50 µL DH5α was thawed on ice and 5 µL of the 0:1 I:V (ratio between Insert:Vector) was added to one tube and 5µL 3:1 I:V was added to the other tube. It was then incubated on ice for 30 min, heat shocked for 45 sec and 42°C, ice for 2 min, added 500µL LB to each tube and incubate with shaking at 37°C for 45 min, spread sample evenly across the plates. We wanted little to no colonies on the 0:1 ligation indicating that the vector was properly cut, making it a good control. We want colonies to be spread apart, so that we can physically remove a single clone colony. Since individual colonies could contain different plasmids (vector or vector with insert), we want a clone with vector plus insert and we do not want to contaminate it with a clone that only contains the vector. Doing this will ensure that the colonies we are using are genetically isolated and functioning on their own instead of depending on the adjacent colonies on the 50µL 3:1. We want the 500 uL plate to give us ten times more colonies. If our ligation did not work well and I only got one or two colonies on my 50 uL plate, I will still get some on my 500 uL plate. Incubate plates overnight at 37°C for 24 hours.

Isolating Plasmids

Created four overnight solutions from the isolated four colonies—1 from 0:1 plate, 2 from 50µL 3:1 plate, and 1 from 500µL 3:1 plate in 5mL LB solution with 25µL of Kanamycin to each tube. After 24 hours, glycerol stocks were created and the guidelines of the Qiagen (QIAprep Spin Miniprep Kit) were followed. DNA concentrations were measured with a nanodrop reader of 43.5 ng/µL, 37.5 ng/µL, 16.2 ng/µL, and 348 ng/µL.

Results

Test Digest

Use a molecular weight marker so we can see the size and weight of our DNA, the closer to the well= larger/heavier. The strand closest to the well indicated pCOLA vector and the yoaA insert (pCOLA=3.719bp, insert=2bp).

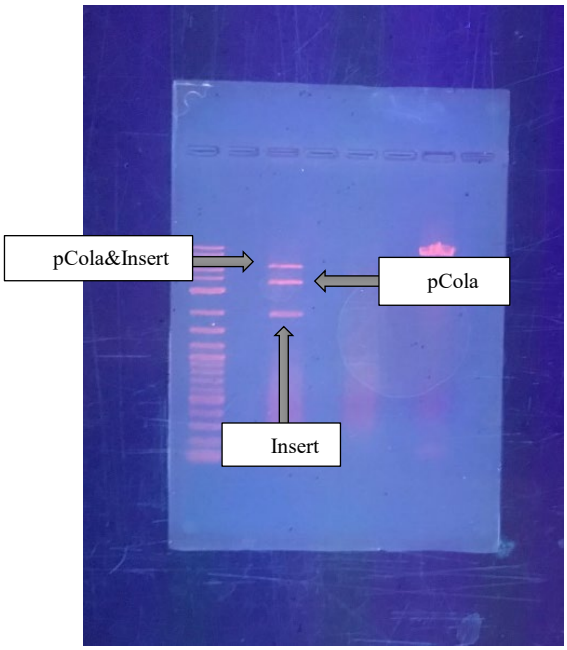

Figure 2. A successful gel stained with ethidium bromide. The strand closest to the well indicated pCOLA and Insert. The pCOLA vector is 3.719 bp and the insert is 2 bp. Insert is lighter because it's smaller, less base pairs run farthest from well due to smaller size. (Sathish, 2018)

Future Work & Applications

Future studies require us to test numerous variables to determine the optimization of the YoaA protein to work to express YoaA protein in a soluble form and characterize its biochemical activities. This will allow us to determine 1) whether YoaA is a DNA helicase and what are the best substrates for YoaA, and 2) how HolC affects YoaA activities. In the long run, future applications involve making clinical correlations between malfunctions in these biochemical pathways and diseases—e.g. colorectal cancer (HNPCC) and mismatch repair. We can also develop tools for biomedical research and diagnostics—PCR—DNA Sequencing, site-directed mutagenesis, cloning techniques. The work to express YoaA protein in a soluble form and characterize its biochemical activities.

Acknowledgments

Lovett Laboratory

References

Brown, L.T., Sutera, Jr., V.A., Shou, S, Weitzel, C.S., Cheng, Y., and Lovett, S.T. (2015) *Connecting replication and repair: YoaA, a helicase-related protein, promote azidothymidine tolerance through association with Chi, an accessory clamp loader Protein. PLoS Genet* 11, e1005651.

Fan, L., Fuss, J.O., Cheng, Q.J., Arvai, A.S., Hammel, M., Roberts, V.A., Cooper, P.K., Trainer, J.A. (2008) *XPD helicase structures and activities: insights into the cancer and aging phenotypes form XPD mutations. Cell* 133, 789.

Raines, R. T., McCormick, M., Van Oosbree, T. R., & Mierendorf, R. C. (2008). *[23] The S Tag Fusion System for Protein Purification*. doi:10.18411/a-2017-023.