Name: Taryn A. Bosquez

Title: Direct interactions between *Escherichia coli* transcription proteins Rho and NusA

Running title: Rho and NusA protein interactions

Abstract

Transcription termination is an important regulatory component within the *Escherichia coli (E. coli)* genome. Two proteins are known to influence transcriptional termination in *E. coli*: Rho and NusA. Rho only functions to induce transcription termination, while NusA is involved in both the enhancement and inhibition of termination. This study aimed to determine whether the two proteins influence each other's functions through direct or indirect interactions. Reciprocal yeast two hybrid (Y2H) analysis using vectors expressing Rho and NusA as fusion with the protein domain of the Gal4 transcriptional factor indicated direct interactions between the two proteins. These results will allow us to further study the mechanistic roles of each protein in transcription termination, as well as the process of gene expression throughout the *E.coli* genome.

***Keywords*:** Rho-dependent termination, pausing, yeast two hybrid system, RNA polymerase

The first step in prokaryotic gene expression, transcription, is one of the most highly regulated processes in the cell and consists of three stages: initiation, elongation, and termination. Prokaryotes use the same RNA polymerase (RNAP) to transcribe all of their genes. In *E. coli*, the RNAP is composed of five polypeptide subunits. Four of these subunits: alpha (*α)*, α, beta (*β)*, and β' comprise the polymerase core enzyme. Each subunit has a particular role; the two α-subunits are necessary to assemble the polymerase and are points of interaction with some transcription regulatory factors; the β-subunit binds to the ribonucleoside triphosphate that will become part of the nascent mRNA molecule; and the β' binds the DNA template strand. The fifth subunit, sigma factor (*σ)*, is involved in promoter recognition and aids the RNAP in binding to a promoter sequence (Finn et al. 2000).

In order for transcription initiation to begin, it is vital that the core enzyme and σ bind to form what is known as the holoenzyme. The holoenzyme then binds to the promoter region of the double stranded DNA, creating a closed promoter complex. DNA cannot be transcribed until it is unwound, but once this occurs the promoter complex is open and ready for the elongation phase. Once the RNAP moves along the DNA strand and synthesizes a few nucleotides, σ disassociates from the core enzyme and the elongation phase begins (Yang and Lewis 2010).

During transcription elongation RNAP continues along the template DNA strand synthesizing mRNA in the 5′ to 3′ direction. As elongation progresses, the DNA is repeatedly unwound ahead of the core enzyme and rewound behind it. Elongation is a highly stable process and will continue until a termination signal is encountered. Ultimately, transcription termination allows the RNAP to disassociate from the DNA and release the nascent transcript. There are two types of termination: Rho-dependent termination and Rho-independent intrinsic termination.

When RNAP encounters an intrinsic terminator, it releases the nascent RNA spontaneously, this is known as Rho-independent termination. Rho-dependent termination occurs when the RNAP encounters a protein factor called Rho (Artsimovitch and Landick 2000).

Rho, encoded by gene *rho,* is a hexameric helicase that uses the energy of hydrolysis of ATP to unwind the RNA/DNA duplex that is a required feature of the transcription elongation complex and thus cause transcription to terminate. The protein containing six identical subunits, is capable of binding nascent RNA chains containing C-rich segments of at least 60 nucleotides that are relatively low in secondary structure (Bogden et al. 1999, Schmidt and Chamberlin 1987). In vitro experiments showed that these specific sequences have an affinity for Rho and have been deemed Rho utilization (*rut)* sites (Richardson and Richardson 1996). These sites allow stable binding of mRNA to the primary RNA binding site of Rho. After the protein is bound to the nascent RNA, Rho's ATPase is activated and provides the energy to move along the transcribed RNA. When a transcription pause site is encountered by RNAP, Rho is able to catch up to RNAP and terminate transcription, via Rho's helicase activity (Richardson and Richardson 1996). When Rho acts on RNAP to terminate transcription, it does not act alone. There are multiple transcription factors that influence transcription (Sen et al. 2008), but for the sake of the study, we will be focusing on transcription factors NusG and NusA.

There have been a multitude of studies showing how NusG interacts with RNAP during the transcription process. In vitro studies have shown transcription factor NusG to influence transcription elongation and Rho-dependent termination through direct interactions with Rho and RNAP (Peters et al. 2012, Pasman and von Hippel 2000, Sen et al. 2008). One study showed how these direct interactions impact antisense transcription in *E. coli*. While analyzing antisense

transcripts, researchers found low C/G repeats are associated with a decreased probability of generating *rut* sites. Without a sufficient number of *rut* sites, the binding ability of Rho is compromised. With Rho-dependent termination down regulated, and the gene for the NusG protein being knocked out, the number of antisense transcripts significantly increased. Researchers compared these results with an *E. coli* genome containing healthy NusG proteins and found there was an upregulation Rho-dependent termination. This result endorsed the proposal of NusG aiding Rho in binding to nascent RNA at terminators with lower C/G ratios by tethering Rho near a RNAP exit channel (Peters et al. 2012).

Another study looked at the biological importance of Rho and why it is an indispensible protein for the *E.coli* genome. Researchers applied bicyclomycin (BCM), a Rho inhibitor, to determine its role within the genome. They found the addition of BCM inhibits Rho ATPase and blocks translocation. This resulted in RNAP continuing through important terminator sequences. This led to an increased expression of downstream toxic genes and genes derived from recent horizontal transfers from defective bacteriophages, which can be detrimental to the genome. These results led Cardinale et al. (2008) to postulate that one of Rho's jobs is to repress expression of foreign DNA. Increased sensitivity to BCM throughout the genome, can also lead to the deletion of transcription factor NusG which can delay or suspend of Rho function. When NusG is deleted, the growth rate of *E. coli* can be negatively impacted, with the doubling time significantly increasing. In turn, researchers concluded that Rho and its transcriptional factors act as gene regulators. When they are all present, Rho can easily bind to a *rut* site and terminate transcription of genes that are harmful to the genome (Cardinale et al. 2008). This study showed

transcription factor, NusG, aids Rho-dependent termination, but there are special circumstances in which it does the opposite.

An in vitro study by Linn and Greenblatt (1992) looked at how the presence of both NusA and NusG proteins upregulate the readthrough frequency of an attenuator site before the *rpoB* gene (gene for the *β* subunit of RNAP). Transcription of *rpoB* was decreased when Rho protein was added to the reaction and truncated transcripts, which is consistent with Rho function. Interestingly, addition of NusA and NusG proteins, which are both components of transcription antitermination systems (Sen et al. 2008, Linn and Greenblatt 1992), along with Rho resulted in decreased levels of truncated transcripts and increased levels of full length *rpoB* RNA. Apart from acting with NusG to impede upon Rho-dependent termination, protein NusA has individual capabilities to limit Rho function as well (Zheng and Friedman 1994).

NusA is a transcription factor that directly binds to RNAP and can influence transcription antitermination (Zheng and Friedman 1994). The protein binds to the β flap region of RNAP through its N-terminal domain and secures this position by wrapping its C-terminal domain back across the β` flap, where it interacts with the developing transcript (Traviglia et al. 1999). This specific interaction gives NusA the ability to induce transcriptional pausing, which in certain cases, can interfere with the functionality of Rho (Zheng and Friedman 1994).

Pausing during transcription elongation plays key regulatory roles for transcription in prokaryotes. Two classes of pausing have been identified and are classified as either a class I or class II pause (Artsimovitch and Landick 2000). Class II pauses, due to weak RNA:DNA interactions, are known to induce backtracking of RNAP to one or more states that occlude the enzyme's active site with nascent RNA . Class I pauses, which are enhanced by NusA

interactions, are characterized by the inhibition of nucleotide addition by stabilizing the RNA 3′ OH in a hyper-translocated position (Artsimovitch and Landick 2000). When class I pausing occurs, it supports the synchronization of transcription and translation. Though this function gives ribosomes the opportunity to bind to the nascent RNA to begin translation, it can prevent Rho from reaching RNAP. The presence of bound ribosomes interfere with Rho's ability access to nascent RNA and RNAP, which can delay Rho-dependent termination (Zheng and Friedman 1994).

Zheng and Friedman (1994) showed class I pauses ensure the close coupling of transcription and translation, which is essential for regulation at certain bacterial attenuators where premature termination is prevalent, in their in vitro study. Delaying the termination of essential genes is important for the viability of the *E.coli* genome. During Rho-dependent termination, Rho binds to the nascent RNA and at the expense of ATP hydrolysis moves toward the paused polymerase. During a class I pause, ribosomes bind to the RNA, and interfere with both the binding of Rho and the progress of Rho toward the paused polymerase. This lead to the conclusion that NusA, which induces the coupling of transcription and translation, impedes Rho-dependent termination when ribosomes are present. The presence of ribosomes are essential for this function, considering that in their absence a class I pause, induced by NusA, actually increases Rho-dependent termination at some sites (Zheng and Friedman 1994).

Previous analysis of potential interactions between NusA and Rho observed the different binding properties between the two. By specifically looking for contiguous sequences through high density tiling microarray profiles, Qayyum et al. (2016) revealed that specific operons within the *E.coli* genome contain overlapping *rut* and N-utilization (*nut)* binding sites. This

means that if the two proteins are present at the same time, they will directly compete with each other to bind to mRNA. For example, NusA can delay Rho-dependent termination if it were to bind to a *nut* site before Rho could bind to its respective *rut* site. When the binding of Rho is delayed, it leads to Rho-dependent termination being compromised. Conversely, if Rho were to bind to a *rut* site before NusA reaches a *nut* site, the occurrence of Rho-dependent termination would not be disrupted (Qayyum et al. 2016).

Past research indicates the two proteins' binding abilities put them in close proximity to one another, as Rho can bind directly with nascent RNA and transcription factor NusG (Bogden et al. 1999, Richardson and Richardson 1996, Peters et al. 2012, Pasman and von Hippel 2000, Cardinale et al. 2008, Sen et al. 2008) and NusA with nascent RNA and RNAP (Zheng and Friedman 1994, Artsimovitch and Landick 2000). Specific operons within the *E.coli* genome have also shown the two proteins to have overlapping binding sites, allowing them to influence the functionality of each other (Qayyum et al. 2016). The aim of this experiment was to identify whether Rho and NusA, due to their binding locations, directly interact with one another. By utilizing a yeast two-hybrid system, we were able to answer this question and establish whether Rho and NusA influence each other through either direct or indirect interactions.

Methods and Materials

Yeast two-hybrid system. In order to determine whether proteins NusA and Rho directly interact, a yeast two-hybrid approach (Y2H) was used. Physical protein interactions will be revealed by tagging our proteins with parts of a fragmented transcription factor (bait and prey vectors) and observing, through yeast growth, whether interaction of the two proteins constitutes a final transcription factor. A working transcription factor allows for the transcription of a gene product

which is necessary to support colony growth in nutrient-lacking media. Utilizing reporter gene *His3* in our Y2H system made it easier to determine whether our proteins, Rho and NusA, interacted. If *His3* was transcribed, due to a functioning transcription factor, we could deduce that our proteins were interacting, but if we could not detect any yeast growth it indicated no interaction. For this specific project we utilized a Y2H library as it aided in identifying the domain that plays a key role in the potential protein-protein interaction (Brückner et al. 2009, Vidal et al. 1996). Bait and prey vectors, as well as plasmids for both Rho and NusA were also constructed.

Preparation of pPC97 and pPC86 vectors. Y2H vector plasmids, pPC97 and pPC86 (Vidal et al. 1996), were prepared by digesting the plasmids with Sac 1 and Sma 1 restriction enzymes. The digested plasmids were electrophoresed through 1% agarose gels and an 8 kb vector (pPC97) and a 7 kb vector (pPC86) were isolated using a Wizard SV Gel and PCR cleanup kit (Promega).

Amplification and construction of Rho and NusA plasmids. The Rho and NusA open reading frames (ORFs) were amplified and isolated prior to the project by polymerase chain reaction (PCR) (Kainz, unpublished). The Rho and NusA PCR products were ligated into pGEM-T, a plasmid specifically designed for cloning PCR products, (Promega) following instructions provided by the manufacturer. Recombinant plasmids were transformed into *E. coli* strain DH5α and then isolated using an IBI rapid plasmid miniprep kit (MidSci). Candidate plasmids were characterized by a restriction digest with Sac 1 and Sma 1 and gel electrophoresis. Plasmids with digests that yielded an 3 kb plasmid vector and 1500 bp (Rho) or 1260 bp (NusA) restriction fragment were used as a source of the Rho and NusA ORF.

Construction of pPC97-Rho and pPC97-NusA plasmid. Digested fragments of Rho and NusA were ligated into Sac 1 and Sma 1 digested pPC97 using standard recombinant DNA methods. Ligations were transformed into DH5α. Plasmids were isolated from transformants using an IBI rapid plasmid miniprep kit (MidSci). Those that yielded a 1500 bp (Rho) or 1260 bp (NusA) and an 8 kb (pPC97) restriction fragment when analyzed by agarose gel electrophoresis, were prepared and submitted for DNA sequencing (Functional Biosciences).

Construction of pPC86-Rho and pPC86-NusA plasmid. Digested fragments of Rho and NusA were ligated into Sac 1 and Sma 1 digested pPC86 using standard recombinant DNA methods. Ligations were transformed into DH5α. Plasmids were isolated from transformants using an IBI rapid plasmid miniprep kit (MidSci). Those that yielded a 1500 bp (Rho) or 1260 bp (NusA) and an 7 kb (pPC86) restriction fragment when analyzed by agarose gel electrophoresis, were prepared and submitted for DNA sequencing (Functional Biosciences).

Yeast transformation. Actively growing cultures of yeast strain MaV203 (Vidal et al. 1996) were used to perform a high efficiency yeast transformation (Brückner et al. 2009, Vidal et al. 1996) with each of our newly constructed Y2H plasmids. Eight total transformations were completed: one containing experimental plasmids pPC86-Rho and pPC97-NusA, another with pPC97-Rho and pPC86-NusA, four containing different combinations of plasmids and empty vectors, one positive control, and one negative control. Y2H systems are known for producing false positive results; therefore, four different combinations containing plasmids and empty vectors acted as a control to test for self-activation. MaV203 cells transformed with both Y2H plasmids were selected by plating on media lacking leucine and tryptophan which are selectable markers for pPC86 (leucine) and pPC97 (tryptophan). If the fusion proteins from the Y2H plasmids interact,

it will result in the reconstitution of the Gal4 transcription factor and thereby cause expression of the *His3* reporter gene in the MaV203 genome (Fig. 1), which will enable cells to synthesize the amino acid histidine (Brückner et al. 2009).

Determining expression of His3. Viable yeast colonies from each transformation were then streaked on two 3-Amino-1, 2, 4-triazole (3AT) plates lacking leucine, tryptophan, and histidine where one plate had a 3AT concentration of 25 mM and another with 10mM. 3AT is a competitive inhibitor of *His3* gene product and thus growth on 3AT plates requires elevated levels of *His3* expression. The ability of yeast to grow on different [3AT] is an indirect measure of the strength of interaction between the two proteins involved in the reconstitution of the Gal4 transcription factor.

Results

We were able to isolate both pPC97 and pPC86 vector DNA from an agarose gel at the desired size of 8 kb and 7kb (Fig. 2).The Rho and NusA PCR products were transformed into *E. coli* strain DH5α, and the three transformants (Table 1) were used to create our pGEM-T+Rho and pGEM-T+NusA plasmids.

Candidate recombinant plasmids, pGEM-T+Rho and pGEM-T+NusA, were both isolated. The first containing an insert of the expected size of 3kb and 1500bp (Rho), and the second with inserts of sizes 3kb and 1260bp (NusA) (Fig. 3). These products were ligated pPC86 and pPC97 vectors and were used to transform DH5α. The transformants (Table 1) were used to create plasmids pPC97-Rho, pPC97-NusA, pPC86-Rho, and pPC86-NusA.

Each vector, along with our presumed Rho and NusA ORF plasmids, cut well in the Sac 1 and Sma 1 restriction digest and were isolated at 8kb (pPC97), 7kb (pPC86), 1500bp (Rho),

and 1260bp (NusA) (Fig. 4). Our gels indicated we had the correct ORF, but we have not verified our constructs by DNA sequencing.

After 48 hours, yeast strain MaV023 gave us a sufficient amount of yeast colonies to carry out yeast transformations. Viable colonies from all eight transformations grew on plates lacking tryptophan and leucine except for the transformation involving plasmid pPC86-Rho and empty vector pPC97- (Table 1). By successfully transforming seven different combinations of our Y2H plasmids into yeast, we could observe whether physical protein interactions were occurring between Rho and NusA.

Direct protein interactions were detected after analyzing colony growth on our 3AT plates lacking leucine, tryptophan, and histidine. Yeast colony growth from the two experimental yeast transformations were comparable to the growth of our positive control on both the 10mM (Fig. 5) and 25mM (Fig. 6) 3AT plates, indicating a physical interaction between proteins Rho and NusA. Our positive control contained a combination of each Y2H plasmid vector and viral N-proteins. These viral N-proteins are known to interact with each other, and provide a reliable model for positive protein-protein interactions. Both 3AT plates also showed signs of growth associated with our three controls testing for self-activation, but growth was not significant enough to indicate self-activation (Fig. 5, 6).

Discussion

Protein-protein interactions play important roles in almost every event that takes place in a cell. Since proteins are often assembled into large complexes to perform specific activities, the characterization of it's interaction patterns could provide some insight as to how that protein functions. With the availability of complete genome sequences we can study proteins on a large,

interactive scale. One way to study proteins, and their interactions, is the Y2H system (Brückner et al. 2009, Vidal et al. 1996). Rho is an essential protein that assists the *E.coli* genome in maintaining genomic integrity, by resolving transcription/replication conflicts through transcription termination (Artsimovitch and Landick 2000, Richardson and Richardson 1996, Cardinale et al. 2008). NusA is a protein associated with transcription antitermination within *E.coli*, but has also been shown to assist Rho in certain situations (Zheng and Friedman 1994, Qayyum et al. 2016). Identifying whether the two proteins interact, via the Y2H approach, gives us some understanding on how they influence one another.

By successfully constructing reciprocal Y2H vectors that express both Rho and NusA as a fusion with the Gal4 DNA binding domain (DB), suggests that NusA affects Rho function by direct interaction between the two proteins. The yeast growth associated with combinations of each plasmid-vector on both 10mM (Fig. 5) and 25mM (Fig. 6) 3AT plates indicated that sufficient amounts of reporter gene, *His3*, was being transcribed and detected to overcome the presence of 3AT. The presence of colonies on the 3AT plate with an increased concentration of 25 mM, indicated the interaction between Rho and NusA was fairly strong, which is consistent with our hypothesis.

While our yeast results suggest a direct protein interaction between Rho and NusA, our conclusion must be regarded as tentative until plasmid structure is verified by DNA sequence.

While analyzing yeast transformations grown on media lacking leucine and tryptophan, the lack of colony growth associated with Y2H plasmid construct pPC86-Rho and empty vector pPC97- was unexpected (Table 1). The unsuccessful transformation was not detrimental to the study, as it involved only one of the four controls being used to test for self-activation.

As mentioned in the discussion, minimal amounts of colony growth associated with our controls testing for self-activation were detected (Fig. 5, 6). To rule out the notion that self-activation occurred, we compared the growth of the three controls to the growth of our negative control on the same 3AT plates (Fig. 5, 6). After comparing each control, it was deduced that growth we observed is most likely background growth, and not significant.

The protein-protein interaction between Rho and NusA could have various outcomes within the *E.coli* genome, but in the future we would like to explore how the interaction affects Rho-dependent termination. Studies analyzing the inhibition of Rho-dependent termination indicate this can have either beneficial or adverse outcomes for the *E.coli* genome.

Rho's terminating function aids in halting the transcription of antisense products, which are known to inhibit translation of complementary mRNA by base pairing to it and obstructing the translation machinery. If Rho function is inhibited, important genes will not be optimally expressed, which can be detrimental to the *E.coli* genome (Peters et al. 2012). Inhibiting Rho would also make it easier for toxic activity of foreign DNA to cause cell death within *E.coli* cells (Cardinale et al. 2008, Pasman and von Hippel 2000, Sen et al. 2008).

Preventing Rho-dependent termination can also be beneficial for *E.coli*, as it can result in the upregulation of important genes. Rho has been known to prematurely terminate the transcription of genes associated with subunits of RNAP, which has negative effects on the genome (Sen et al. 2008, Linn and Greenblatt 1992). In order to avoid the suppression of essential genes, it is important that Rho function is inhibited.

It is important we continue this study because it is essential that we understand the implications of the interactions between Rho and NusA. Our results indicate the functionality of

Rho could be influenced by interaction with NusA. Though we do not know the outcomes of this interaction, expanding our knowledge on this specific protein interaction will allow us to understand the many aspects of Rho-dependent termination throughout the *E.coli* genome on a deeper level.

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Tables and Figures

Table 1. Data represents *E. coli* and *S. cerevisiae* transformed with either Rho or NusA to construct specific plasmids and the number of transformants represents the number of viable colonies.

Organism	Number of transformants	Plasmid
DH5α (Rho)	1	pGEM-T+Rho
DH5α (NusA)	2	pGEM-T+NusA
DH5α (Rho)	49	pPC97-Rho
DH5α (NusA)	63	pPC97-NusA
DH5α (Rho)	59	pPC86-Rho
DH5α (NusA)	52	pPC86-NusA
MaV203 (Rho)	7	pPC97-Rho+pPC86-
MaV203 (NusA)	9	pPC97-NusA+pPC86-
*MaV203 (Rho)	0	pPC86-Rho+pPC97-
MaV203 (NusA)	4	pPC86-NusA+pPC97-
MaV203 (Rho & NusA)	19	pPC86-Rho+pPC97-NusA
MaV203 (Rho & NusA)	8	pPC97-Rho+pPC86-NusA
MaV203 (negative control)	40	pPC97- + pPC86-
MaV203 (positive control)	12	pPC97-N+pPC86-N

*Indicates no growth

DH5α= *E.coli*

MaV203= *S. cerevisiae*

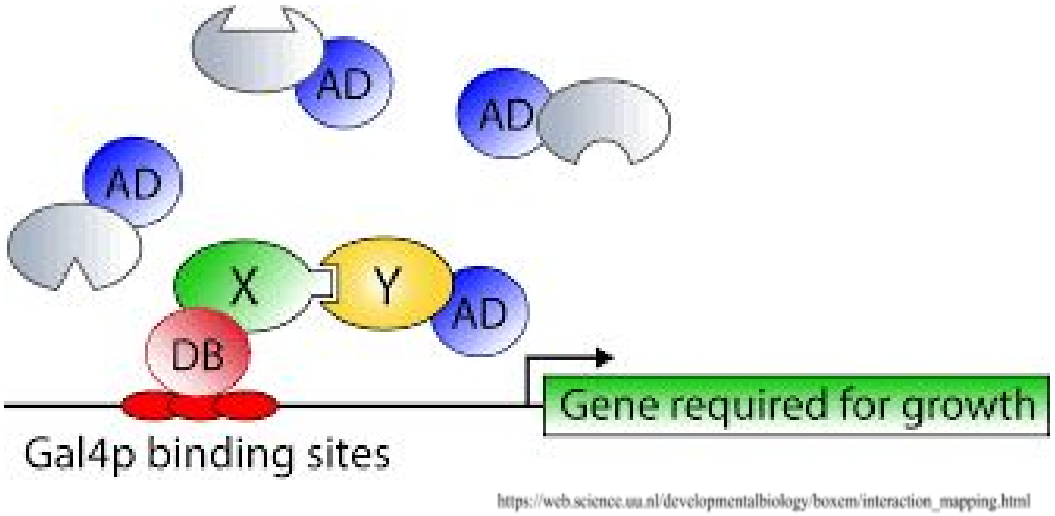

Figure 1. Y2H vectors fusing at the Gal4 binding site creating a viable transcription factor to enable the expression of an a important reporter gene.

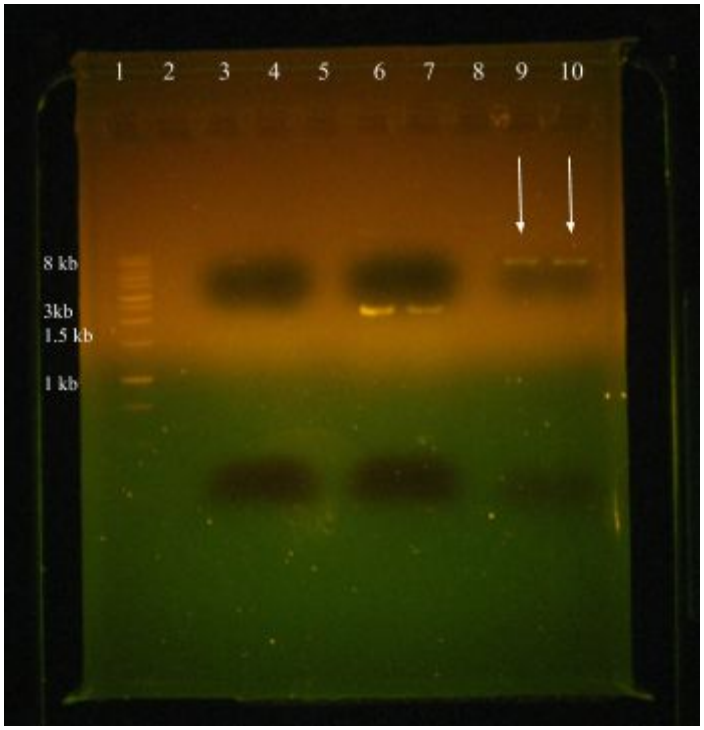

Figure 2. Yeast two-hybrid vector plasmid pPC97 and pPC86. Lanes 6 and 9 contain vector pPC97 and lanes 7 and 10 contains vector pPC86. Arrows indicate the vector product of the expected size. Lane 1 contains a kb DNA size marker.

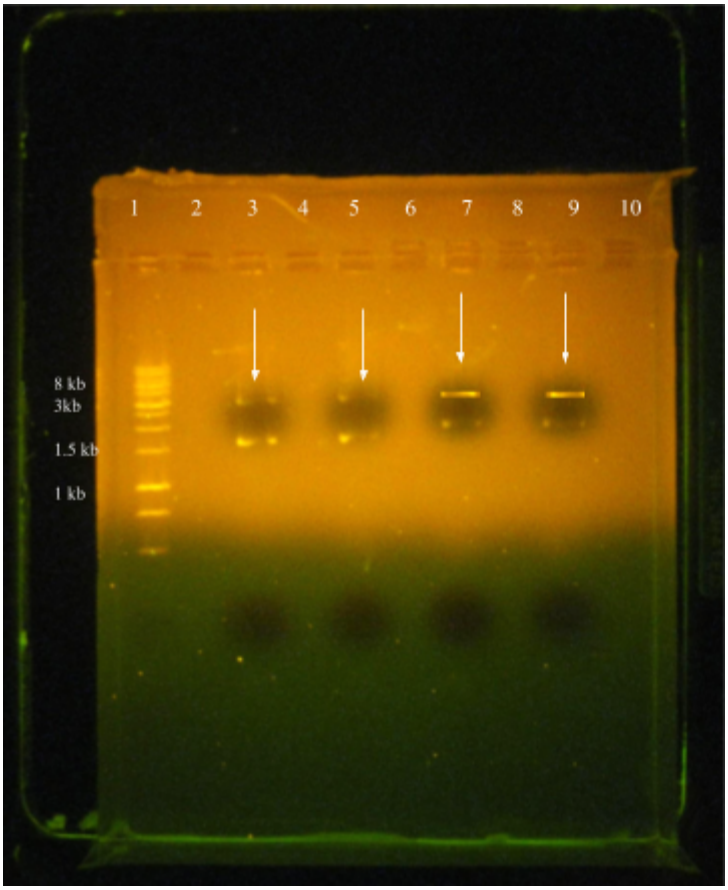

Figure 3. pGEM-T+Rho and pGEM-T+NusA plasmids containing Rho and NusA restriction digest fragments. Lanes 7 and 9 contains pGEM-T+Rho, and lanes 3 and 5 contain pGEM-T+NusA. Arrows indicate combined plasmid and PCR product of expected size. Lane 1 contains a 1 kb DNA size marker.

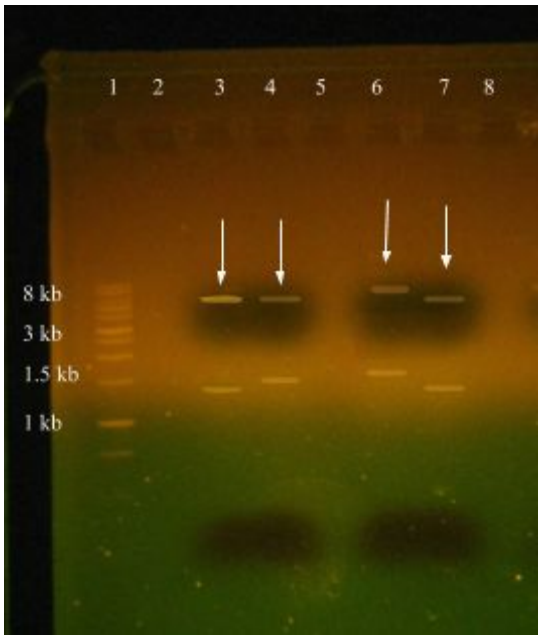

Figure 4. pPC97-Rho, pPC97-NusA, pPC86-Rho, and pPC86-NusA plasmids containing Rho and NusA PCR products. Lane 3 contains pPC97-NusA, lane 4 contains pPC86-Rho, lane 6 contains pPC97-Rho, and lane 7 contains pPC86-NusA. Arrows indicate plasmid and PCR product of expected size. Lane 1 contains a 1 kb DNA size marker.

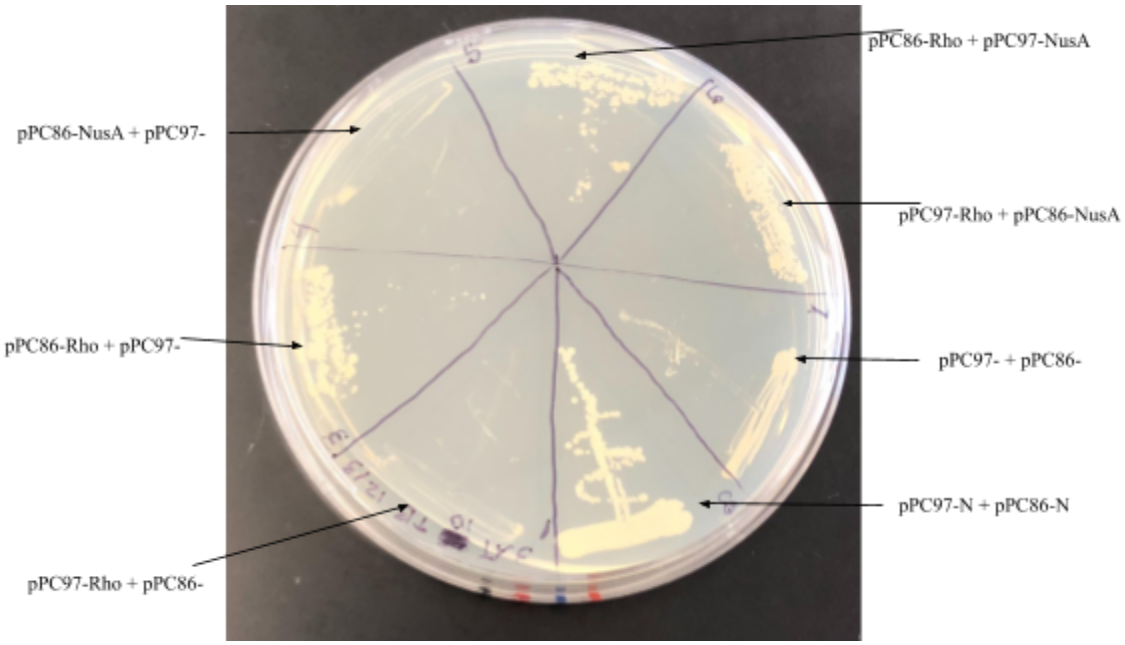

Figure 5. Media amended with 10mM of 3AT and lacking leucine, tryptophan, histidine contains colonies after constructed Y2H plasmids were transformed into MaV203 and streaked. Y2H vector-plasmid names indicate which plasmids were transformed into Mav203 and are present in the streaked colonies.

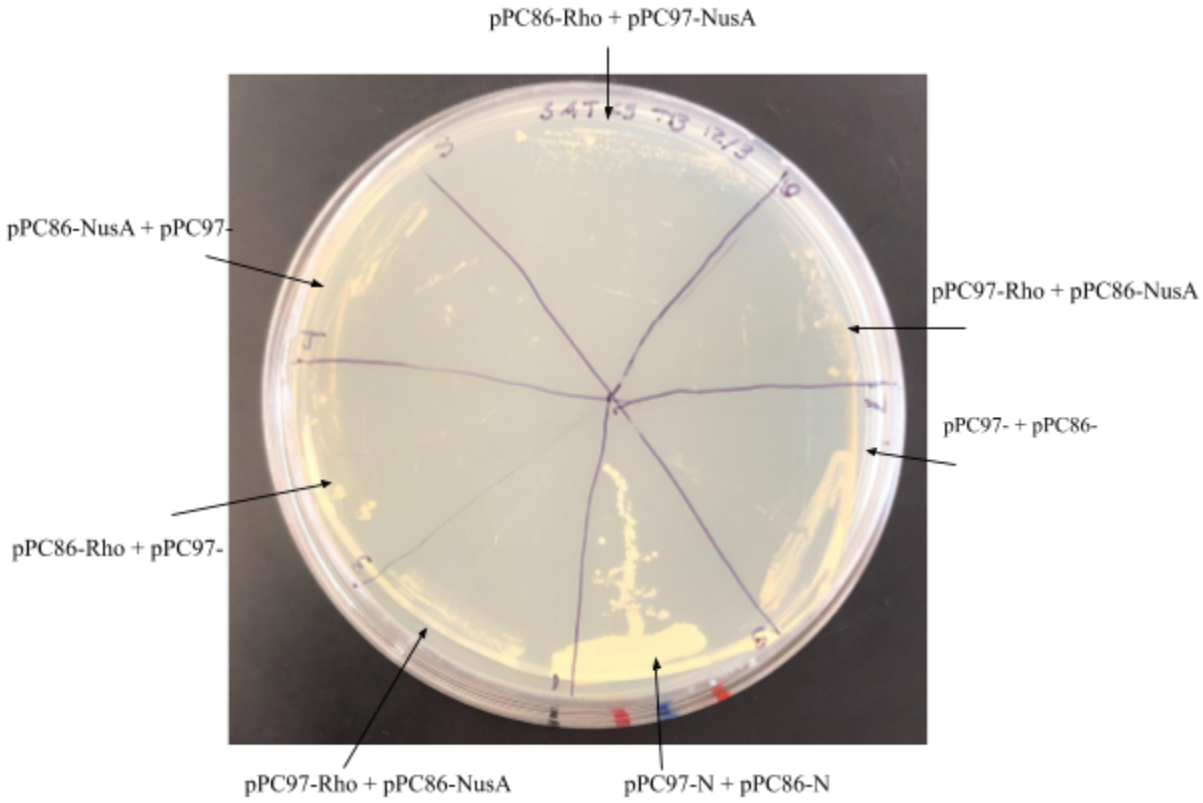

Figure 6. Media amended with 25mM of 3AT and lacking leucine, tryptophan, histidine contains colonies after constructed Y2H plasmids were transformed into MaV203 and streaked. Y2H vector-plasmid names indicate which plasmids were transformed into Mav203 and are present in the streaked colonies.