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Identification of a DNA Polymerase Alpha Gene Sequence in *Thermomyces lanuginosus* Fungus

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IDENTIFICATION OF A DNA POLYMERASE ALPHA GENE SEQUENCE IN *THERMOMYCES LANUGINOSUS* FUNGUS

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Introduction

Studying growth control and expanding our knowledge on this topic would be helpful in understanding a number of current medical problems. For example, cancer is a problem of uncontrolled cell growth tumors, which are abnormal masses of tissue that may spread around the body and can prove to be fatal. Understanding cell growth control might suggest a successful approach to stopping cell growth that could be pivotal in some treatments. In contrast, spinal cord injuries are often accompanied by an inability to heal through reactivation of cell growth. Spinal cord regeneration would be helpful in potential treatments for injuries and the resulting paralysis, but no current methods promote sufficient cell growth in damaged areas of the spinal cord. This doesn't mean we are looking to cure cancer or reverse paralysis for an individual, as that is outside of our realm, but identifying a cell growth control point would be important in developing treatments for patients of both types.

With cell growth being the main focus of our research, we chose to study DNA polymerase alpha. DNA polymerase alpha is likely to be involved in cell growth as it is the enzyme that starts DNA replication. Control of this enzyme might lead to decreased cell growth in cancer or increased cell growth in damaged spinal cord tissue. We chose the thermophilic fungus *Thermomyces lanuginosus* for the study of the enzyme due to the ability to compare the enzyme's activity during fungal growth at different temperatures. Thermomyces exhibits rapid growth at high temperatures and slow growth at low temperatures. Studying this fungus allows us to study the enzyme in a system in which growth can be controlled.

DNA polymerase alpha has never been isolated from *Thermomyces lanuginosus*. This fungus is hard to grow in a culture and it is also difficult to extract its components, making it very hard to isolate and study its enzymes. Consequently, instead of getting DNA polymerase alpha directly from the fungus, our goal is to locate the gene sequence

from the enzyme and insert it into bacteria. The bacteria will then synthesize the enzyme that we wish to study. We are interested in this because of its presumed role in growth control. Also, it would be the first DNA polymerase from a thermophilic eukaryote.

Background Information

DNA synthesis plays a vital role in cell growth, which generally is achieved by mitosis. Mitosis is a multi-step process in which one cell gives rise to two identical daughter cells. An early step in mitosis is the S phase of interphase, when new DNA is being made so that when the cell divides into two, there will be enough DNA for both of the cells. DNA synthesis during this phase relies on DNA polymerases. DNA polymerases are heavily involved in DNA synthesis (Bailey). DNA polymerases are enzymes that are responsible for forming new copies of DNA. They are the only enzymes that have the capabilities of duplicating genetic information so they are responsible for duplicating the entire human genome before cell division. They create these new copies in the form of nucleic acid molecules, which are polymers. Specifically, DNA polymerase adds free nucleotides to the 3' end of a newly forming strand (Yokoyama). After DNA helicase, another enzyme, has unwound the tightly woven DNA molecule and opened it up to use as templates for replication. DNA polymerases are extremely accurate in copying the genetic information, in part because they proofread to assess for any misplaced base pairs that can then be corrected (Kornberg).

In total, there are 12 different eukaryotic DNA polymerases. We are focused on DNA polymerase alpha, because we know that it is involved in initiation of DNA replication. It has four subunits, two of which build small RNA sequences ("primers") that can be extended with DNA by a third "catalytic" subunit (Binas). Since DNA polymerase alpha functions primarily by providing primers for DNA replication, it is likely to be a control point in cell growth.

DNA polymerase alpha has been well-studied in systems before, but the systems

in which it was studied were chosen because of their ability to grow rapidly which is not ideal for understanding growth control. Other features make those studies less than ideal for examination of control mechanisms. For example, studies of the partially purified enzyme from embryonic chicken brain (Simet) were complicated because the brain had too many cell types. Looking for protein at one stage of replication was nearly impossible. In addition, embryonic chicken brains are very small so large numbers were needed to study the enzyme.

Due to lack of previous research on this topic there is very little known about the enzyme or its gene sequence. The only DNA polymerases from fungi that have been studied were from yeast (Sugino), and the DNA polymerase alpha enzyme is highly atypical, so that makes the enzyme that we will research and study unique. We chose the *Thermomyces* fungus in part because thermophilic systems are “on” when hot and “off” when cold, resulting in good studying conditions for growth control. If we want it to grow, we incubate it at 50^{o}C. When we want it to stop, we refrigerate it at 4^{o}C.

Identification of a DNA polymerase alpha gene sequence in *Thermomyces lanuginosus* fungus has never been done before. The overall fungal DNA sequence is known, but which specific part is the DNA polymerase alpha gene is still unknown. The purpose of this project is to identify this gene sequence so that it can further our understanding of cell growth control. Once we get the sequence, it can be inserted into bacteria which can then produce large quantities of the enzyme for further study.

Methods

Fungus Culture

Thermomyces lanuginosus fungus was cultured by Dr. Simet on 1% yeast extract/5% soluble starch/1% agar and can be seen below (Image 4). The white fungus is growing slowly, the gray fungus is growing more rapidly, and the black fungus is growing very quickly. All of

the fungus started off white, then changed color depending on how fast it grew. The white fungus is typically found on the outside, and the black fungus is found more towards the middle.

Image 4

DNA Extraction

DNA was extracted from frozen *Thermomyces lanuginosus* fungus using an Omega EZNA soil DNA kit. We took 150 mg of the fungus and 725 micro liters of SLX-Mlus Bugger and vortexed them for 4 minutes at maximum speed to lyse the sample After centrifuging at 500xg for five seconds, 72 microliters of DS Buffer was added and the sample was vortexed again. We incubated the sample at 70 degrees celsius for 30 minutes and centrifuged again at 10,000xg for 5 minutes. We then transferred 400 microliters into a new tube and added 135 microliters of chilled P2 lysis buffer solution and 200 microliters of cHTR reagent. The tube was vortexed followed by centrifuging at 10,000xg for 1 minute. We added an equal amount of XP1 buffer and vortexed the tube again. Next, we transferred 700 microliters of this sample to a hydroxyapatite mini column that was placed into a collection tube and centrifuged at 10,000xg for 1 minute. We repeated this process with the remaining volume until all of the

lysate had passed through. Following this step, 500 microliters of HBC buffer diluted with 100% isopropanol was added and then the column and collection tube were centrifuged at 10,000xg for one minute. We then took the minicolumn and placed it into a new tube and added 700 microliters of DNA wash buffer to eliminate any salts or contaminants.The tube was centrifuged again, and the step including the DNA wash buffer and centrifugation was repeated. ↯We centrifuged the minicolumn at 24000xg for two minutes in order to remove residual ethanol that could potentially interfere with any downstream applications. We then transferred the minicolumn into a new tube and added 50 microliters of Elution buffer that was heated to 70 degrees Celsius. After letting it sit for one minute at room temperature, we centrifuged it again at 24000xg for 1 minute. The eluate was returned to the same mini column and centrifuged one last time at 24000xg for 1 minute. The final eluate was stored at 4°C.

This procedure was repeated twice. Initial weights of fungus were increased to 250 mg, and vortex times were increased to five minutes to guarantee complete mixing. In the final run, the volume of the elution buffer used in the last step was reduced to 45 μL.

DNA Analysis

All three DNA preparations were screened for purity using a ThermoScientific NanoDrop 2000C microspectrophotometer. The results are shown in the images below.

Image 1

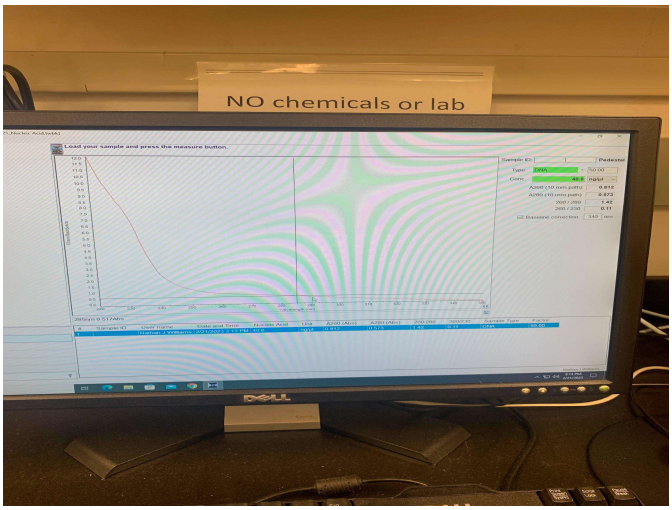
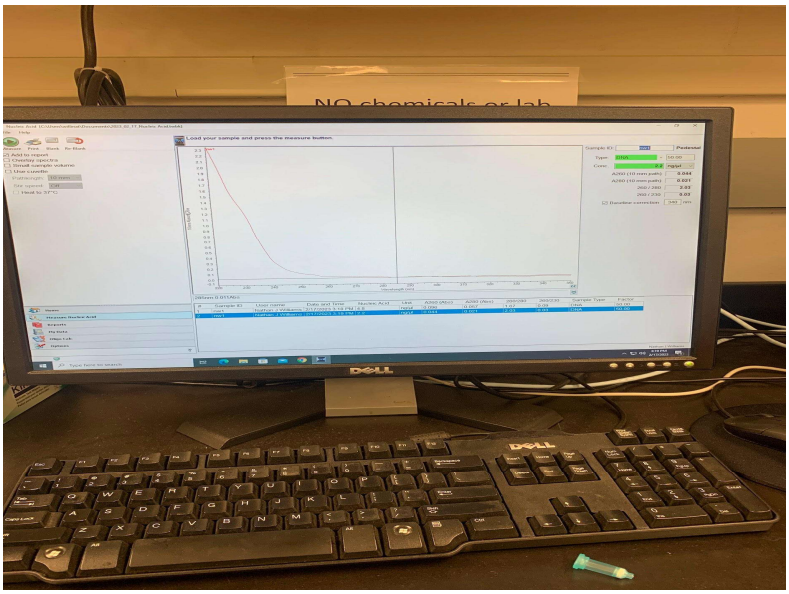


Image 2



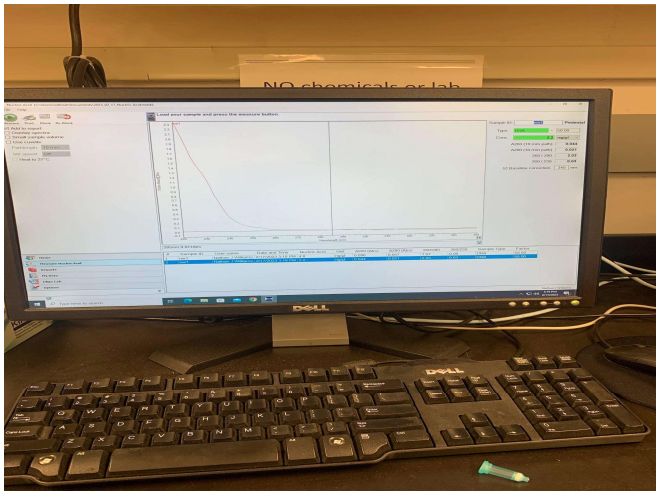


Image 3

All three spectra contain small peaks at 260 nm, the wavelength characteristic of DNA and RNA. No significant absorbance is seen at 280 nm, one of the absorbances associated with proteins. Also, the $A_{260\ nm}/A_{280\ nm}$ ratios for all three samples were below 2.0, results that confirm that no protein contamination is present. The first graph (Image 1) is from the first DNA extraction The DNA concentration was 2.2 ng/μL, which was far too low for a good sample.The results were better for the third extraction, where the concentration was 40.6 ng/μL (Image 2) and 78.4 ng/μL (Image 3). There were two graphs because I used two drops from the same DNA sample. respectively.

After confirming that what we had was truly DNA, we tested its size by using an agarose gel. The agarose gel was prepared by mixing 1 gram of agarose, 2 mL of 50x Tris Acetate EDTA buffer, and 100 mL distilled water. This mixture was heated in a microwave oven until it dissolved. After it cooled off enough to handle it, 5 microliters of 10 mg/mL ethidium bromide was added. The mixture was poured into a Liberty 1 agarose gel electrophoresis tray equipped with a nineteen-well comb and allowed to solidify. The DNA samples (10 μL) were mixed with 2 μL of tartrazine dye and placed into the wells. 2Log DNA size standards (5 μL) were added to 2 of the wells (Image 5). The gel was run for 30 minutes at 200 volts and 95 milliamps. The results were visualized in a UVP ultraviolet transilluminator equipped with a BioDoc-It imaging system (Image 6 pictured below). While the standards were visible, no other bands were visible. This is because the standards were small enough to move across the gel, while our DNA was too big to move across the gel. This is what we expected and wanted, as it proved our DNA was not too small or fragmented to be further used.

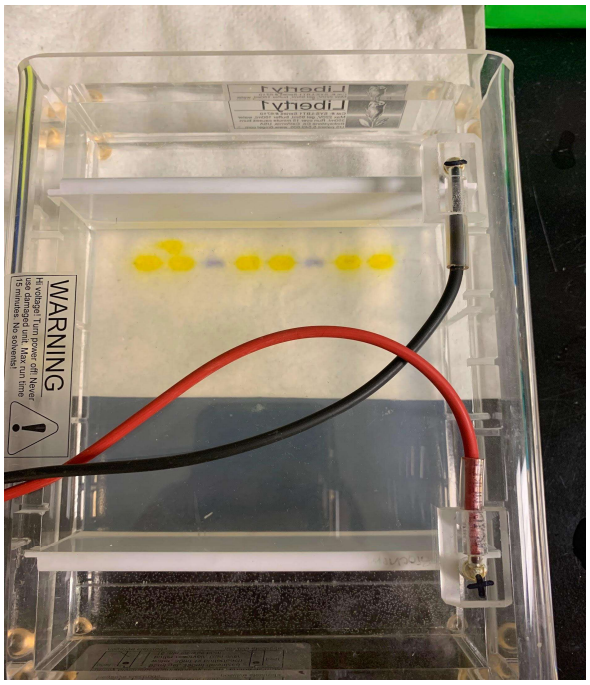
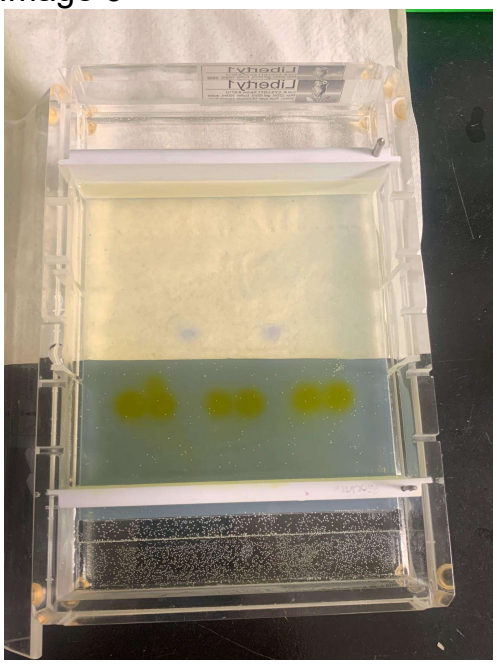


Image 6



Primer design

Using the Clustal Omega sequence alignment website (https://www.ebi.ac.uk/Tools/msa/clustalo/), we input the entire genome of our fungus in the FASTA format (Nokuthala). The FASTA format is a universally recognized format in which a gene sequence is identified by its unique sequence identifier. We compared our fungus's genome to other similar organisms' gene sequences. The first one we chose was *Ustilago maydis*, a corn smut similar to our fungus. The second organism we chose was *Aspergillus niger*, which is a mold. We were able to find these organism's sequences using the NCBI database. When searching for their sequences, we added "DNA polymerase alpha catalytic subunit" at the end of the search so we could compare the organism's DNA polymerase alpha gene sequence to our fungus's entire genome in order to narrow down our search to the specific sequence of our target gene. A search for matching base pairs showed an asterisk underneath the base pair if it was a match between the two organisms. We then attempted to find a range of 20 base pairs that had at least 14 matches, ideally in clusters. For example, every other base pair matching would not be ideal but 4 or 5 in a

row would be ideal. We were able to find a suitable sequence in *Ustilago maydis*. After finding that, we had to find another ideal region of the genome with the same criteria that was 400-800 base pairs either before or after the region that we picked. Both sequences of 20 that we picked had to end in a GC base pair so that binding would be secure. These sequences were needed to serve as “primers” for subsequent Polymerase Chain Reactions.

We entered our primers into the oligo analyzer tool on the Integrated DNA Technologies website. No undesirable hairpins or self-dimers (ΔG values lower than 8 kcal/mol) were found. However, the melting temperatures of the two primers were more than 5 degrees apart, so we wouldn’t be able to find an ideal temperature at which both could function. We changed one of them by substituting two A’s for two C’s. This made the melting temperatures compatible. The final sequences were: forward primer, 5’-TGG ACC GAC CAC CAG ATC-3'

(18 bases) and reverse primer, 5’-CAA CTG GCA CAG CCT GCT C-3' (19 bases).

Polymerase Chain Reaction

Polymerase chain reaction (PCR) uses primers to amplify a certain portion of a DNA genome, and it is a common and simple method to gather this needed information, The primers used in PCR are different from the primers referred to in previous sections. When talking about primers used for PCR, we are referring to short, single strands of DNA that flank the DNA sequence to be amplified. Primers that were referred to earlier when discussing DNA replication are short nucleic acid sequences used for initiation of DNA synthesis. The method of PCR includes denaturing a DNA sample by simply heating it. This will force the DNA to separate into two single strands instead of one double strand. The designed primers can then bind to the separated strands, and an enzyme will build

two new strands of DNA using the original strands as a template. This allows for each strand to have a new and an old/original strand of the DNA. From here, you can go on and on and create numerous copies.

For our PCR, we created three similar but different mixes. They all consisted of 2 microliters of our DNA sample, 4 microliters of each primer, 2 microliters of each dNTP, 70 microliters of water, and 2 microliters of. Taq DNA polymerase. The only difference between the three mixes were that their buffer components had either low (1 mM), medium (2 mM), or high (4 mM) magnesium concentrations. We did this because high magnesium encourages primers to bind even if they are mismatched, and low magnesium makes it hard for even good primers to bind to DNA. The optimal concentration can only be determined experimentally. Each of the three mixes was incubated at high (54^{o}C), medium (51^{o}C), and low (48^{o}C) temperature. We wanted different temperatures because if the temperature was too high, then the primers would fall off the DNA even if the base matches were almost perfect. If the temperature was too low, the primers would stick to the DNA even if there were many mismatched bases. Again, the optimal temperature can only be determined experimentally.

The PCR protocol, conducted in an Applied Biosystems Veriti termocycler, included an initial five minute denaturation at 94^{o}C, followed by thirty five cycles of 30 sec of primer binding at 54^{o}C, 51^{o}C, or 48^{o}C; one minute of synthesis at 72^{o}C; and 30 sec of denaturation at 94^{o}C, with a final five-minute extension interval at 72^{o}C.

The PCR products were analyzed using an agarose gel like the one described in the DNA Analysis section.

Results

Unfortunately, we were unsuccessful with the agarose gel and couldn’t get to the checkpoint of looking at our result under ultraviolet light. We know we have good DNA, as it is not fragmented, and we had a good plan that was just difficult to execute. We were

still optimizing all of the conditions as well, and we are unsure if it was a problem with the primer or a mistake in setting up the agarose gel. While it is frustrating that we didn't get the result that we desired, we went into this research knowing that it has never been identified before and would be a struggle.

Conclusion

The purpose of this study was to gain more knowledge about cell growth control. Through a series of experiments, we wanted to locate and identify an DNA polymerase alpha gene sequence in Thermomyces lanuginosus fungus. The identification of this sequence would ideally further our understanding regarding cell growth control points and potential ways to slow it down to help with cancer treatments, or to regenerate and grow new cells for spinal cord treatments. After this would be located, parallel experiments would need to find the other subunits and further tests along those lines. This can eventually lead to people to have a better understanding of DNA polymerase alpha and the role that it plays in cell growth control. All in all, someday down the road, once science understands and discovers this it can lead to better treatments of people with cell growth control diseases and can optimistically lead to better treatments for those people.

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