Activation of Nitrogenase Function in *E. coli*

Christopher C. Parrish '21 and Michael J. Wolyniak

Department of Biology, Hampden-Sydney College, Hampden-Sydney, VA 23943

Nitrogen is one of the essential nutrients for life due to its role as a building block of amino acids. This fact is no different when it comes to plant growth; in fact, soil nitrogen levels are one of the most essential components to having a good crop harvest or plant growth in general. Often times, after years of growing crops on the same soil farmers deplete natural soil nutrients or have tracts of land with low natural bioavailability of these nutrients, and they must find ways to supplement soil nutrients to have good yields. The most common and easiest way to supplement soil nutrients tends to be using fertilizers: especially ones with high nitrogen, potassium, and phosphorus concentrations. While these do help crops grow more efficiently, a problem arises in their use when the impacts of runoff are considered. According to Townsend and Howarth, synthetic fertilizers can be compared to a "Jekyll and Hyde" type transformation because of how many harmful effects that it has on the environment[i]. Since these nutrients are essential to all life, they allow bacteria and algae in waterways to bloom.

The impact of these blooms is that there is less available oxygen in the water which fish need to breathe. Massive algae blooms have been part of the cause of the reduction of fish populations and habitats. Another issue with the use of fertilizers is that they do not allow tracts of land with depleted soil nutrients to regenerate what they have lost. Instead, farmers can use supplements and plant on the land again, but this is not a long-term solution: leading to more problems with no solution. If nothing is done about this arising problem, algae blooms will continue to grow larger, and fish populations will continue to dwindle. The smaller fish populations affect not only the vast fishing industry but also the casual fisherman. If precautions are not taken to prevent soil runoff, algae blooms could further threaten fish populations and eventually the commercial fishing industry if the blooms were allowed to grow large enough. Soil bacteria were examined to find a natural solution to the problem of runoff to try and combat fertilizer runoff into waterway; of the various species of soil bacteria, *Azotobacter chroococcum* was of interest due to its prevalence as the most common soil bacteria with the ability to fix nitrogen into the soil.

The function held by these bacteria is crucial to plant growth because they fix nitrogen from the atmosphere, N_2 gas, into usable nitrogen in the soil making ammonia or nitrite salts[ii]. Since the majority of available nitrogen in the atmosphere, and is not able to be used by plants, the majority of farmers have to resort to the use of fertilizers as a supplement. Luckily, there are soil bacteria that contain Nitrogenase proteins which fix nitrogen into the soil. There are specific genes that *A. chroococcum* has, and they are called nitrogen fixation genes, or *nif* genes[iii]. Two genes of interest can be found in Robson's research: the *nif*H gene and the Azotobacter Ferredoxin gene. The ferredoxin gene is on particular interest because ferredoxins are small proteins that donate electrons to several metabolic pathways making them function properly[iv]. Considering that nitrogen fixation is one of many different metabolic pathways, it was proposed that this gene could allow this pathway to function more efficiently. This experiment examined if these genes could be isolated and later enhanced to make a protein with a higher metabolic rate.

The specific genes that were targeted in this experiment were the *nifH* gene from *A. chroococcum* and the Azotobacter ferredoxin gene[v]. A *nifH* homolog that was found in *Pseudomonas aeruginosa* during protein BLAST test was also examined [vi]. As previously mentioned, the *nifH* and ferredoxin genes were selected for the hope of producing a more efficient protein to be tested in the future with plant growth trials in mind. The *P. aeruginosa* homolog gene was also selected for cloning purposes. If nothing else it had similar protein length to the *nifH* gene and allowed for the practice of protocols while waiting on the arrival of an *A. chroococcum* sample. The purpose of this experiment was to try and produce Estero coli samples that produced *nifH* proteins. The idea was that if a plasmid could be created to implant the possibly genetically enhanced *nifH* protein into E. coli, then this plasmid could be used to make Azotobacter samples that expressed an enhanced *nifH* protein as well as meaning it would allow the present metabolic pathway to work more efficiently. The desired product could have real-world applications if implemented in agriculture; it could mean less required fertilizers in agricultural crop growth in the long run.

MATERIALS AND METHODS

Bacterial Cultures

The first step in the experiment was to start liquid broth and agar plate cultures *of P. aeruginosa*. These cultures were later used for multiple purposes; for example, liquid cultures were used to isolate genomic DNA samples, and agar plate cultures were made as storage stock samples. Chemically competent cultures of E. coli also had to be prepared

to take up the generated plasmids. Liquid cultures of both bacterium were made in conical vials with 5mLs of LB broth, inoculated, then placed in the shaker incubator at 37ºC for 24-48 hours. Agar plate cultures were inoculated and placed in an incubator at 37ºC for 24-48 hours. Later, cultures of *A. chroococcum* were prepared, but difficulties arose in the culturing of the cells. Liquid cultures of Azotobacter had to be in 15mL conical vials with 5mLs of LB broth; the broth was then inoculated with *A. chroococcum* and incubated in a shaker incubator at 30ºC. The modifications had to be made to the culturing of each bacteria due to its inability to grow at standard growing temperature and time.

DNA Isolation and Quantification

The next essential step in the experiment was to extract, isolate, and quantify sampled DNA so that they could be used in further testing. Samples of DNA were extracted using the Qiagen Ultra Clean Microbial Kit and the protocol within that kit. This protocol required the contents of the kit along with a tabletop centrifuge and various Eppendorf tubes for sample collection. Liquid bacterial cultures were spun down over a series of steps leaving a pellet of cells; the contents of the kit were then utilized to break apart the cell wall and dispose of the intracellular organelles leaving the genomic DNA behind. Samples were then quantified on an EPOCH plate reader with a take three sample plate. These samples were later used in various PCR cycles.

PCR and Gene Isolation

The next, and most repeated, step in the experiment was to conduct various PCR cycles. Depending on various aspects of the cycle, sometimes the solutions had to be altered, but each followed a similar cocktail mixture. Each cocktail was different but had some things in common; each was set up to have approximately 100 ng/µl of genomic DNA, properly diluted primers, PCR Mean Green master mix, and DI water to a final volume of 20 µl. Primers were constructed using Gene Constructor software; some even were designed to include restriction sites for later experiments. The primers were ordered from IDT and were made for each target gene. Gene isolation was the next step of the project. This was accomplished using PCR cycles with site-specific primers. These primers allowed target genes to be copied and multiplied. The targeted genes were later used in various experiments; such as the *nifH* homolog gene being inserted into bacterial plasmid vectors such as the TOPO plasmid or pQE60.

Plasmid Selection and Insertion

Selection of a bacterial plasmid had to be considered many steps earlier when primers were being designed. It was decided to use the TOPO plasmid and pQE60 because samples could be found in the lab that had been used in previous experiments. Each plasmid then had to be examined to find the best restriction enzyme site with the least interference. After a suitable sample of DNA had been generated through PCR, it then had to be inserted into a plasmid. This was done in two different ways. Using the TOPO plasmid, the TOPO plasmid kit was used, and the general protocol in that kit was followed. When it came to making the pQE60 plasmid, the DNA segments first had to be digested with restriction enzymes and allowed to incubate for 37 degrees C overnight. The plasmids also had to be digested overnight to be prepared for their DNA inserts. The DNA inserts were then enveloped in the plasmid where the restriction enzymes had cut both the inserts and plasmids.

Plasmid insertion

After a plasmid had been prepared, it then had to be inserted into chemically competent E.coli cells. The E.coli cells had to be transformed so that they could produce the bacterial plasmid. In pQE60, this was accomplished through mixing the ligate from the plasmid preparation step with chemically competent cells and ice the cell mixture for 30 minutes. The cells were then heat shocked in a 42-degree water bath for 30 seconds then S.O.C. media was added to the solution. The cells were then placed in the shaker incubator at 37 degrees C and allowed to culture for 24 hours. The E.coli should now produce the plasmid within themselves if the plasmid were taken up properly. A similar procedure was followed for the TOPO plasmids, but as mentioned earlier the exact procedure was included in the TOPO plasmid kit.

Gel Electrophoresis

Another protocol that was repeated multiple times throughout the experiment was gel electrophoresis. Gel electrophoresis was essential to the project because it allowed for the analysis of PCR products and other DNA fragments that needed to be analyzed. Gels were made by putting 1 gram of LE agarose in 100mLs of TAE buffer. The mixture was then heated and then poured into the gel trays with the combs inserted to form the wells. The electrophoresis chambers were then filled with TAE buffer to allow current to pass through the gel. Ten µl of sample were then dyed with four µl of DNA dye, and the 14 µl of sample were loaded into each well. The chamber was then closed, and the electrodes were inserted into the power supply, and the current was applied at 100 volts.

The gels then ran for approximately 30 minutes before being analyzed with UV light. A standardized latter was also used so that DNA fragments could be sized within the gel.

SDS Page Gel Electrophoresis

The next and final step in the experiment was to conduct a sodium dodecyl sulfate page gel electrophoresis test. The purpose of this experiment was to determine the exact weight of the protein produced by the TOPO plasmid DNA. Knowing the weight of a protein would be helpful because it would confirm if the E.coli are producing the desired protein or not. A crude lysis procedure was used to determine the expression of proteins in the chemically transformed cells. Cells were crushed with a mortar and pestle in liquid nitrogen to remove all extracellular contents. The product was then placed in Eppendorf tubes and centrifuged for 30 minutes at a high RPM. The supernatant was then removed and placed in a new Eppendorf tube. Loading dye was made by mixing BME and lamella solution and boiling in an Eppendorf tube for 5 minutes. The dye was then mixed with the supernatant and the SDS page standard. The SDS page chamber was then filled with buffer before the gel was added and loaded with each sample. The chamber was then closed, and a current was applied, and it ran for 45 minutes or until the bands were reaching the bottom of the gel. Once the chamber was cleared of the current, the gel was removed and began to be washed in DI water. This wash step was repeated many times to make the gel more conclusive.

RESULTS

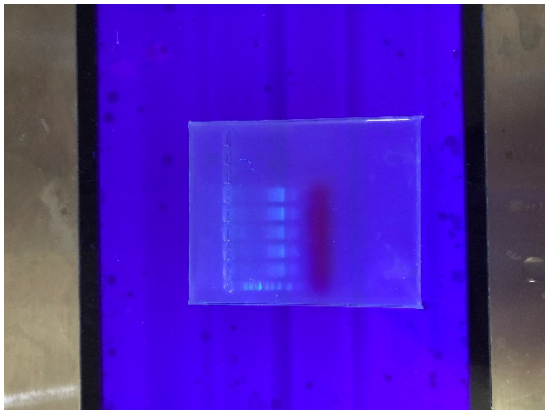

***Figure 1**: Gel Electrophoresis with possible nifH homolog*

The image in figure 1 shows the appearance of a single band around the 800 base pair region of the DNA latter. The template DNA used was that of *P. aeruginosa* with the suitable primers needed for the reaction. As desired, the band did appear in the 800 base pair region which is the approximate length of the *nifH* homolog protein. The presence of this band gives me confidence in saying the PCR cycle did produce the desired DNA strand.

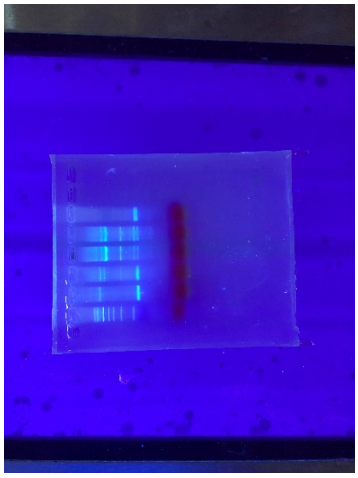

***Figure 2**: Gel electrophoresis of TOPO plasmid samples with P. aeruginosa gene*

Figure 2 shows another gel from electrophoresis, but this is slightly different from the first. The PCR cycle the sample came from tested the orientation of the TOPO plasmid vectors in colonies of chemically competent E. coli. Here It can be seen that in samples 2,3, and 4 that a band, however, faint it may be, does appear in the gel. This would mean that the TOPO plasmid in that sample of colonies would have the proper orientation and that they can be used for further experimentation.

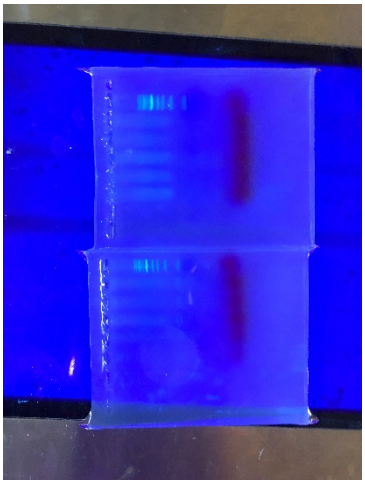

***Figure 3**: Gel electrophoresis of A. chroococcum*

Figure 3 shows another gel, but there are no bands present in the gel. This gel was one of the many attempts at running a PCR cycle for *A. chroococcum* samples. One of the most substantial issues with

conducting PCR cycles with the *A. chroococcum* genes was the inability to obtain a viable genomic DNA sample. This led to many issues throughout the project and inhibited the ability to obtain any real data on the *nifH* and ferredoxin genes.

Sample	260/280 ratio	ng/µl
1	1.892	60.544
1	1.846	59.503
2	1.849	177.781
2	1.83	170.138
3	1.844	34.699
3	1.754	39.497

***Table 1**: A. chroococcum Plate reader DNA sample data*

Table 1 shows a standard data output of the EPOCH plate reader, at least a streamlined version of that. The 260/280 ratio is a purity value and is desired to be at around 1.8. The concentration of the sample can also be found in ng/µl, which is desired to be around 50 or so. This data comes from one of the final *A. chroococcum* samples that were cultured and made into a genomic DNA sample, and it is also by far the most successful one of the project. If research were to be continued, these samples would jumpstart the process of PCR cycles.

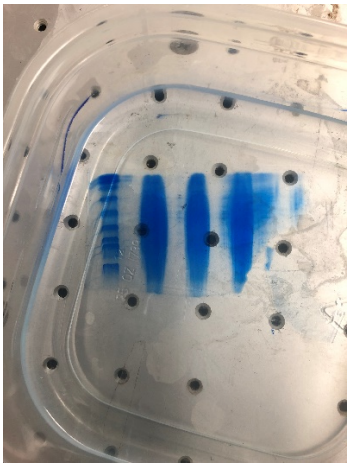

***Figure 4**: SDS Page Gel Analysis*

Figure 5 illustrates the output of an SDS page gel run. The experiment was the most daunting of the summer and had its own list of complications having no prior experience with SDS page gels. The cells were exposed to crude lysis, and proteins were not selected for so the gel is pretty inconclusive. A slight band can be seen in one of the rows, but I believe that nothing of importance can be taken from this experiment. This is due partially because of the crudeness of the lysing of the cells, or even possibly overloading the wells of the gel. Still, it cannot be determined if the transformed E. coli expressed the *nifH* homolog protein or not.

DISCUSSION

Overall a fair amount of progress was made on the main goals of the experiment, but it was not without its setbacks as well. Good progress was made, especially when it came to the *nifH* homolog gene from *P. aeruginosa*. The gene was isolated very early on in the summer, which allowed the gene to be implemented into the TOPO plasmid vector and pQE60 plasmids — working with the gene allowed for the practice of several protocols which allowed for familiarization with laboratory equipment. While the gene is not precisely like the *nifH* gene, it was a similar size and similar enough to work with. Even though the gene analysis went further with this gene, lacking was the ability to assess the proteins nitrogenase functions. Even in examining figure 4, there is no distinct protein to be seen, meaning no real evidence to support or deny the presence of the protein is available.

Working with *A. chroococcum* proved to be rather unsuccessful in more ways than one. Major issues were realized once an attempt was made at trying to culture the bacteria and obtain a genomic DNA sample. Initially, it was thought that the issue was with the media, which could still be the main issue; but after multiple attempts of producing a new bacterial media for the *A. chroococcum*, efforts were only met with failure. After a multitude of trial and error, a method was finally devised to culture the *A. chroococcum* cultures to the point where a viable genomic DNA sample could be obtained from it. The issue was, this discovery was made in the final days of research. In the future, these samples could be harnessed to expand the project to further goals in mind.

While goals were not met in this project, there is still a fair bit of knowledge that was learned about the culturing process of *A. chroococcum*. This newfound knowledge can be applied in the future to circumvent some of the significant issues that were encountered throughout the duration of this project.

Also, if nothing else, success was found in the cloning of the homologous gene found in *P. aeruginosa*. This allowed for the practice of critical laboratory protocols that can be applied in future research. The hypothesis of this experiment can neither be supported nor denied. The objectives of the experiment could not be realized, and proper evidence to confirm or deny the hypothesis could not be obtained. Further experimentation would be required to obtain this evidence.

ACKNOWLEDGEMENTS

I would like to thank the Hampden-Sydney undergraduate research program for letting me conduct this research project and for providing their facilities. My gratitude is also due to Dr. Wolyniak for sponsoring my research and helping me throughout the duration of the project. I would also like to thank Dr. Fischer for providing her assistance in the absence of Dr. Wolyniak and Dr. Hargadon for allowing me to use some of his equipment. Thank you to Mrs. Jenny Jenkins for her assistance with so many parts of my project and help with laboratory equipment. Lastly but not least, thank you to Alexander Washington for assisting throughout my experiment.

REFERENCES

- Townsend, Alan R., and Howarth, Robert W. "Fixing the Global Nitrogen Problem." *Scientific American*, vol. 302, no. 2, Feb. 2010, pp. 64–71., doi:10.1038/scientificamerican0210-64.
- Robson, Robert, et al. "Second Gene (NifH*) Coding for a Nitrogenase Iron Protein in Azotobacter Chroococcum Is Adjacent to a Gene Coding for a Ferredoxin-like Protein." *The EMBO Journal*, vol. 5, no. 6, 1986, pp. 1159–1163., doi:10.1002/j.1460-2075.1986.tb04341.x.
- Cassier-Chauvat, Corinne, and Franck Chauvat. "Function and Regulation of Ferredoxins in the Cyanobacterium, Synechocystis PCC6803: Recent Advances." *Life*, vol. 4, no. 4, 7 Dec. 2014, pp. 666–680., doi:10.3390/life4040666.
- "Nitrogenase Iron Protein [Azotobacter Chroococcum]." *National Center for Biotechnology Information*, U.S. National Library of Medicine, www.ncbi.nlm.nih.gov/protein/TBW04207.1.
- "MinD/ParA Family Protein [Pseudomonas Aeruginosa." *National Center for Biotechnology Information*, U.S. National Library of Medicine, www.ncbi.nlm.nih.gov/protein/WP_048303778.1?report=genbank&log%24=protalign&blast_rank=1&RID=EANHKBY7014.