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Constructing a Vector for Over Expressing Nitrobenzene Dioxygenase in E. coli

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Abstract:

Due to extensive use of nitroaromatic compounds in the production of dyes, pesticides, explosives, and their harmful effects on the environment, studying the enzyme mechanisms of bacteria capable of utilizing these compounds as nutrient sources have become a recent field of development. One example, the *Comamonas* sp. strain JS765, grows on nitrobenzene as its sole carbon, nitrogen and energy source. This bacterium contains a nitrobenzene dioxygenase enzyme system that incorporates O_2 into the nitrobenzene ring to form catechol and nitrite. In this project, the genes that code for the dioxygenase enzyme were cloned and then transformed into an expression vector for over-expression in *E. coli* NEB 5α and *E. coli* BL21 (DE3). This over-expression system will facilitate purification of the normal enzyme and of mutant forms for mechanistic studies.

Introduction:

Nitroaromatic compounds are commonly used as industrial feedstocks for the production of pesticides, dyes, and explosives.[1] When these compounds enter the environment through improper storage and disposal techniques, they present a hazard to living organisms. One solution for properly digesting these compounds before or after they have been introduced to the environment has been the use of aerobic bacteria that are capable of degrading these compounds. Due to the resistance of aromatic compounds,

such as nitrobenzene, the digestion of these compounds through the use of bacteria has been found to be specific to each bacterium rather than any one bacterium being able to digest a range of aromatic compounds. One such bacterium that is capable of digesting a nitroaromatic ring is the *Comamonas* sp. strain JS765. This bacterium digests nitrobenzene through a multicomponent dioxygense[1], and it is the physical mechanism of this molecular degradation that is of interest in this study.

The enzyme responsible for this degradation is nitrobenzene dioxygenase (NBDO), which is in the Rieske nonheme iron dioxygenase family. Rieske dioxygenases are a target of significant research for two main reasons; first, they incorporate two chiral centers adjacent to one another without producing stereochemical mixtures, and secondly they catalyze the first reaction in a pathway by which microbes break down aromatic compounds so that they can re-enter the global carbon cycle.[2] The reaction by which NBDO breaks down nitrobenzene can be seen in Figure 1, which shows that NBDO

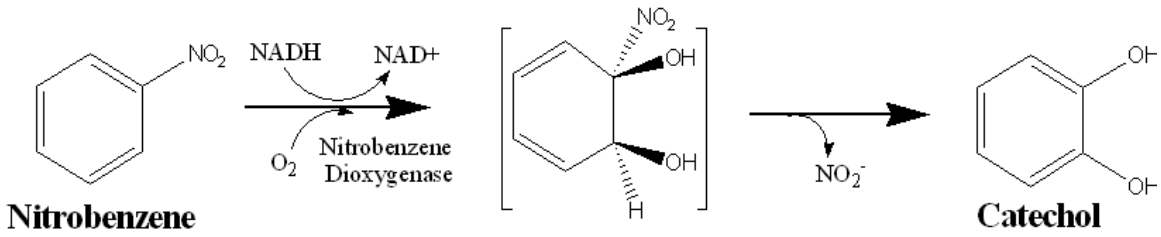

Figure 1: Enzymatic reaction of NBDO, depicting the characteristic cis-dihydroxilation reaction of a Rieske dioxygenase.[3]

utilizes an O_2 molecule from the atmosphere to oxidize the aromatic ring in a manner such that they are incorporated onto the same side of the ring. Also, an important environmental feature of this reaction is that it is converting nitrobenzene, an environmentally hazardous chemical, to catechol, which is activated by virtue of having

two adjacent hydroxyl groups on the ring. The benzene ring of catechol is then oxidatively cleaved by catechol dioxygenases, and the products are subsequently degraded to common metabolites, such as intermediates of the citric acid cycle.[2]

When looking at the gene sequence that codes for the overall nitrobenzene dioxygenase system (NBDOS), it is composed of five particular sequences; nbzAa, Orf2, nbzAb, nbzAc, and nbzAd[1]. However, NBDOS is only composed of three proteins; NBDR (for reductase), NBDF (ferredoxin), NBDO (for oxygenase). The nbzAa gene codes for NBDR, an iron- sulfur flavoprotein reductase (NBDR), and the nbzAb codes for ferredoxin$_{NBZ}$, a Reiske ferredoxin (NBDF). While both of these proteins are necessary for the NBDOS to function *in vivo*, it was the nbzAc and nbzAd genes that were of interest to this study. They code for oxygenase$_{NBZ\alpha}$, and oxygenase$_{NBZ\beta}$ respectively, which are the α and β subunits of the NBDO enzyme.

While it is unknown exactly how the NBDOS catalyzes the conversion of nitrobenzene to the corresponding *cis*-diol, a basic understanding has been established from other studies of Rieske dioxygenases. Two electrons are passed from the iron-sulfur flavoprotein reductase, which acts as an electron redox switch, to the Rieske ferredoxin which contains a iron-cluster capable of being reduced by the electrons.[4] These electrons are then passed to NBDO, where O_2 binds to the mononuclear iron center and becomes reduced to a (hydro)peroxo intermediate once nitrobenzene is introduced to the reaction. Two hypotheses have been put forward to describe how the oxygen-oxygen bond is cleaved so that the oxygen atoms can be inserted into nitrobenzene, as seen in Figure 2. One proposal is that the reaction proceeds through a concerted process where the

(hydro)peroxo intermediate reacts directly with nitro benzene[5], while the other is a two step process that forms a very reactive Fe(V) intermediate.[6,7]

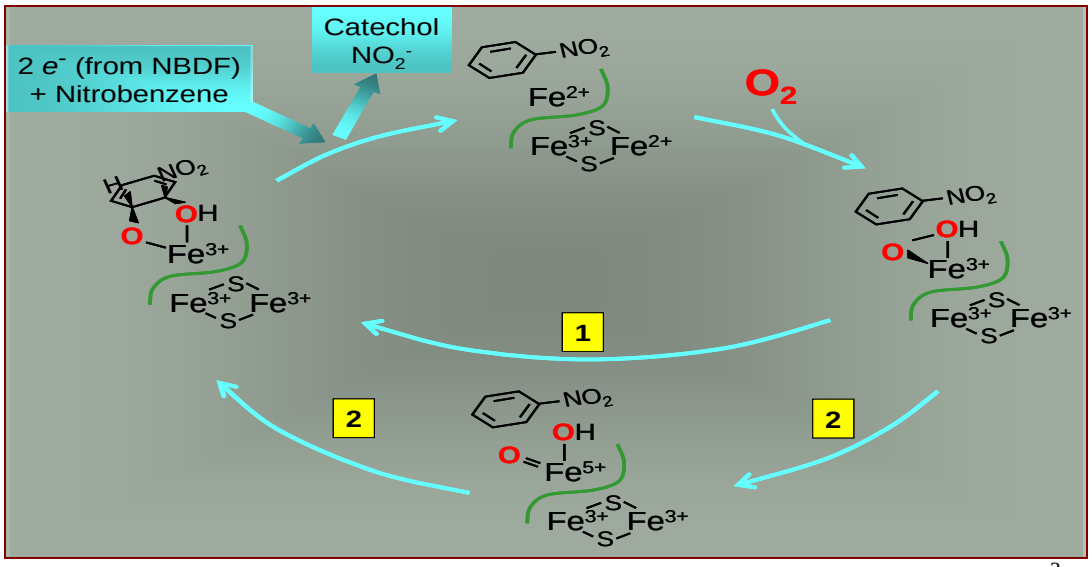

Figure 2: Proposed reaction mechanisms for *cis*-dihydroxylation of nitrobenzene.[3]

In order to determine which of the two proposed pathways is correct, it is necessary observe the intermediates that are being formed during the reaction. There is one report[5] of the detection of a peroxo-iron(III) intermediate by Mössbauer spectroscopy when a related Rieske dioxygenase was reacted with hydrogen peroxide and its substrate, but thus far neither this species nor the Fe(V) intermediate has not been detected during the reaction with O_2 and aromatic substrate. We hope to improve the likelihood of observing a reactive intermediate through the use of mutant enzymes and/or alternate substrates designed to maintain the normal rate of formation but slow the rate at which the intermediate decays. One way in which this might be done would be to mutate the asparagine 258 residue of the oxygenase subunit.[8] In a study by Parales et. al, a series of mutations were discussed, and it is the mutation from asparagine to valine at residue 258

that is of particular interest given our goal.[8] The group used a starting reactant of 4-nitrotoluene and monitored the product that was made. In this case, the untreated protein produced 4-methylcatechol 95% of the time, as opposed to the mutant which produced 4-nitrobenzylalcohol 99% of the time (Figure 3).[8] This difference in product distribution

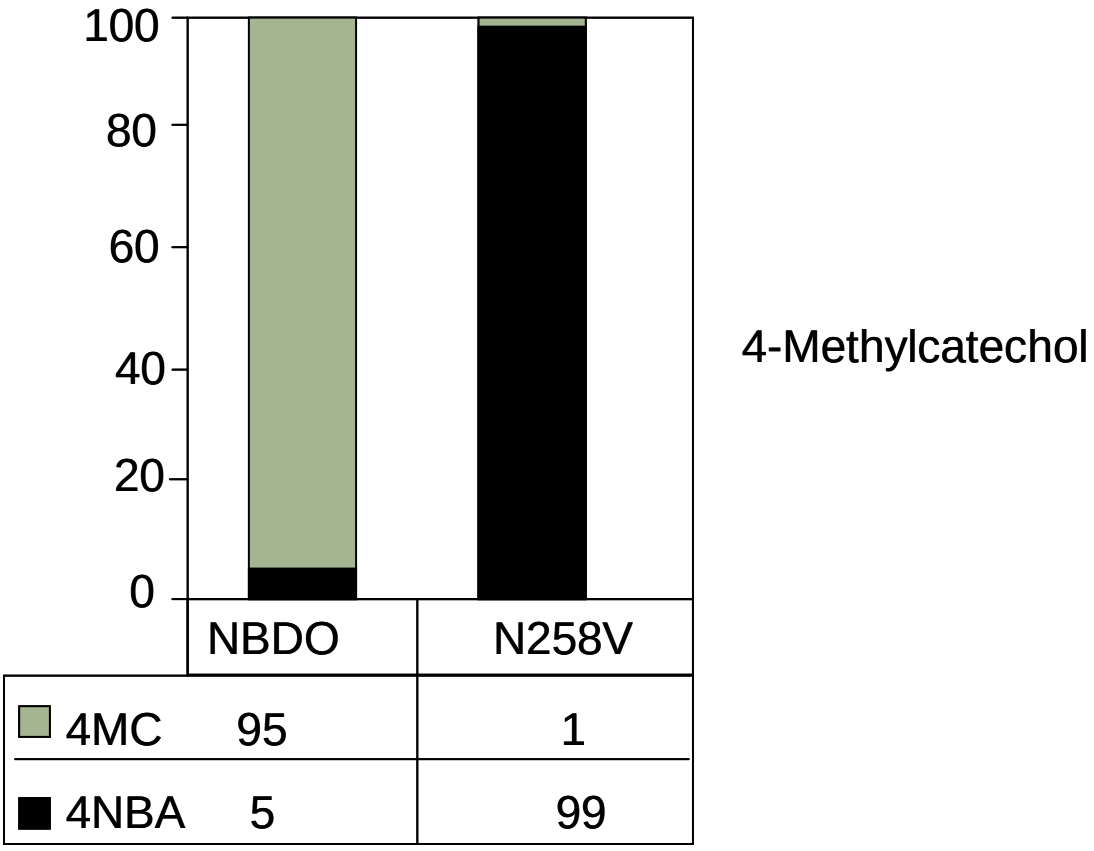

Figure3: Different products based on the 258 residue.[8]

indicates that the asparagine in the 258 position must have a significant role in the orientation of the incoming reactant, probably by way of hydrogen bonding to the nitro group of the substrate, because valine is incapable of such bonding (orientation of nitrobenzene in active site is displayed in Figure 4). It seems likely that the steps of the mechanism that generate the activated oxygen species in this mutant, such as the binding

of substrates and the reduction of O_2, would remain unchanged relative to the native enzyme. However, because the substrate is presented to the intermediate in a different orientation, it seems likely that the rate of reaction of the substrate with the activated oxygen intermediate will be affected, facilitating the observation of this intermediate through spectroscopic techniques.

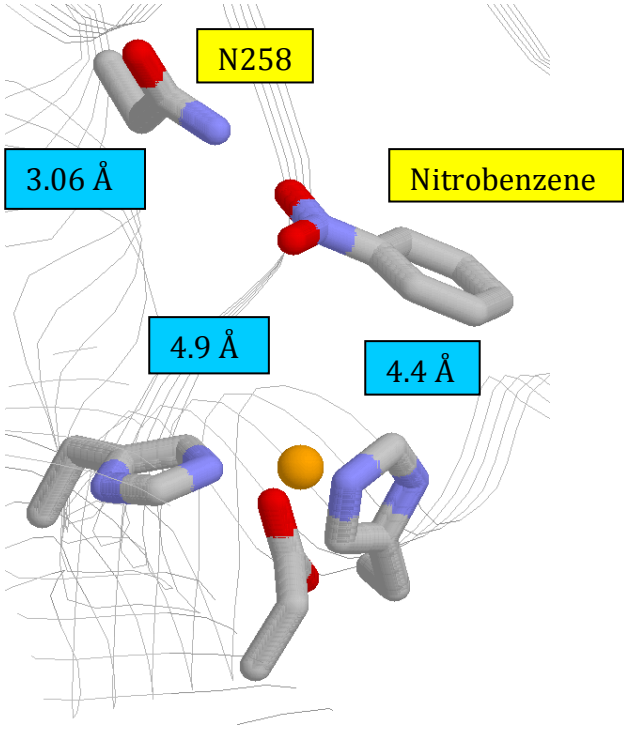

Figure 4: Orientation of nitrobenzene in the active site of NBDO.[4]

The goal of this project is to accomplish the initial preparations for carrying out large-scale productions of native or mutant proteins by cloning the nbzAc, and nbzAd genes into an expression vector and transforming them into *E. coli* (New England Biolabs, Inc. Cat. No. C2987I) NEB 5α and *E. coli* BL21 (DE3) for the over production of the α and β NBDO dioxygenase subunits. *E. coli* was selected because it is readily

available, inexpensive, easier to grow in the laboratory than *Comamonas* sp. strain JS765, commonly used for molecular biology applications, and does not express its own native NBDO system.

Materials and Methods:

The *Comamonas* sp. strain JS765 used was grown in either Difco Nutrient agar and Difco Nutrient broth, or BLK agar and BLK broth. However, the conditions used for the BLK agar and broths were determined by the concentration of the nitrogen and carbon source nitrobenzene. BLK is a minimal media that lacks a carbon and nitrogen source,[16] and in this study succinate was added to this media as the primary carbon source and nitrobenzene was used as the sole nitrogen source and secondary carbon source. The conditions used for bacterial growth was a nitrobenzene concentration of 0.750 mM for the broths, and the placement of a pipette tip filled with 0.750 mM nitrobenzene solution on the cap of a BLK agar plate. *Comamonas* was incubated at 30° C, and the liquid cultures were shaken at 250 rpm.

The genomic DNA was obtained by using the Gram-positive bacteria purification protocol from the DNeasy® Blood & Tissue Isolation Kit (Qiagen) because *Comamonas* sp. Strain JS765 is classified as a gram-positive bacterium in fresh cultures. The kit included a lysis buffer and proteinase K for cell lysis and protein degradation respectively, and it utilized a DNeasy Mini spin column and collection tubes for washing and collecting the desired DNA. Each sample was collected by eluting the DNA with buffer twice into the same collection tube to ensure maximal yield. Elution of the sample was then verified by gel electrophoresis using a 1% agarose gel with ethidium bromide as

the staining agent. Gels were visualized using a UV broad-spectrum light and DNA concentrations were determined by UV-spectroscopy prior to loading the gels.

Following genomic isolation, the Polymerase Chain Reaction (PCR) was used to amplify the desired α and β subunits of the NBDOS. PCR reactions contained1.1 µL of both forward and reverse primers (10 µM stock), 100 µg of sample DNA, 45 µL of Platinum PCR Supermix, and sufficient sterile deionzied water so that the total reaction volume was 55 µL. Forward and reverse primers for amplifying the nbzAc and nbzAd genes were designed according to the following criteria: each primer contains approximately 20 nt that are complimentary to the desired sections of the NDBO genes, their overall length was minimal to reduce the chances of binding to themselves but sufficient to be specific to only our area of interest, and that their T_m values were within 5°C of each other. As for the actual primers designed, the forward primer was a 38 bp long strand including the *Sgf I* restriction site with a total melt temperature of 64°C and a melt temperature of 53°C for the homologous region, and the reverse primer was a 33 bp long strand including the *Pme I* restriction site with a total melt temperature of 63°C and a melt temperature of 54°C for the homologous region. For the specific primer sequence see Table 3. The reaction conditions were then set to a hot start of 3.5 minutes at 94°C, 5

Table 3: Information on the forward and reverse primers used for PCR amplification and engineering of the *Sgf I* and *Pme I* restriction sites onto the NBDO genes.

	Length (bp)	Full Melt Temp	Homologous Region Melt Temp
Forward Primer	38	64°C	53°C
Reverse Primer	33	63°C	54°C
Forward Primer Sequence	5'- TTA TGC GAT CGC CAT GAG TTA CCA AAA CTT AGT GAG TG – 3'		
Reverse Primer Sequence	5 CGT AGT TTA AAC CAG GAA GAC CAA CAG GTT GTG 3		

Table 4: Color code guide for the primer sequence in Table 3.

Color	What it is
Yellow	Start codon of alpha subunit (2528 -30 bp)
Green	SgfI restriction sequence
Dark Blue	4 extra bp
Purple	Codon before/upstream of stop codon for the beta subunit (4465-67 bp)
Light Blue	PmeI restriction sequence
Red	4 extra bp

cycles of: 94°C for 30 seconds, 48°C for 30 seconds, and 72°C of 2 minutes, then 25 cycles of 94°C for 30 seconds, 54°C for 30 seconds, and 72°C or 2 minutes, and final cool period of 72°C for 10 minutes and 4°C until removed from the thermocycler. The PCR product was then analyzed by gel electrophoresis.

The PCR product was purified using the Promega Flexi® Vector Systems Kit, and then digested using the same kit. This kit utilized three key features; the rare restriction sites of *Pme I* and *Sgf I*, the lethal barnase gene, and the kanamycin resistance gene.[11] The restriction cite tagging was done by engineering one PCR primer with the reverse primer with the *Pme I* sequence and the forward primer with the *Sgf I*. The presence of a the lethal barnase gene was essential because not all of the plasmids had inserted genomic DNA, so this barnase gene was located between the two restriction cites and if one of these plasmids made it to a growth plate they would naturally die off. Finally, the kanamycin gene was utilized once the plasmids were inserted into the cells, because if a cell took up one of the transformed plasmids they were able to survive on kanamycin laced growth media. The digestion process then required the purified PCR product and the Promega Flexi® vector to be digested separately using 4 µL of a 5x

Flexi® Digest Buffer, 2 µL of the Flexi® enzyme blend (*Sgf I* and *Pme I*) and up to 200 ng of the purified PCR sample and the Promega Flexi® vector. The vector chosen for the transformations of both *E. coli* strands was the pF1K T7 Flexi® Vector (seen in Figure 5). This vector was chosen in combination with *E. coli* (New England Biolabs, Inc. Cat.

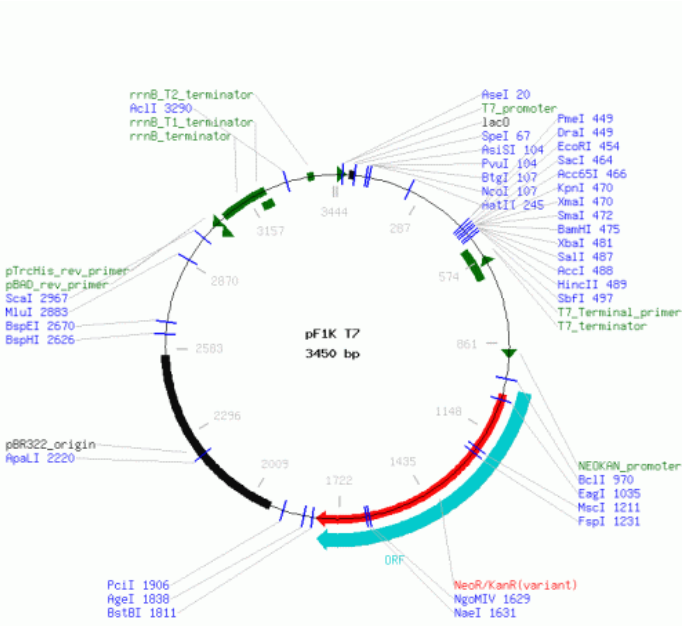

Figure 5: The restriction map of the pF1K T7 Flexi® Vector.[10] The *Sgf* 1 is located at100 bp, *Pme* 1 is located at 499, the T7 promoter is located at 21 bp and the *lacO* is located at 39.

No. C2987I) NEB 5α because it is a commonly used cell line for subcloning due to its lack of nucleases commonly found in other strains that would destroy the recombinant DNA the project is trying to generate, and *E. coli* BL21 (DE3) was chosen because it is able to produce the T7 RNA polymerase necessary for transcribing the NBDO gene located in the plasmid vector for later expression studies. The T7 RNA polymerase is a viral polymerase responsible for transcribing the inserted α and β NBDO genes, which will be inserted immediately downstream from a T7 promoter on the vector, and it is not found naturally in any prokaryotic cell lines.[9] What this means is that *E. coli* BL21

(DE3) is a line of *E. coli* that was previously genetically engineered to incorporate the gene responsible for the production of the T7 RNA polymerase. Also, this means that along with the α and β NBDO genes being present on the vector there must be a T7 promoter and transcription regulatory genes. Gene expression is regulated in the T7 Flexi® Vector by the lac operon. When the lactose analog IPTG is present, the *lac* operator is not repressed and transcription of the gene proceeds rapidly due to the abilities and tendencies of the viral T7 promoter and T7 RNA polymerase.[9] Since the maximum volume of the digest blend was 20 µL and the PCR product was not of high enough concentration to max out the 500 ng limit of the digest, all samples ran used a full 12 µL of purified PCR product to maximize the plasmid concentration (12 µL was the maximum volume aloud for in the reaction after the volumes required for the buffer and restriction enzymes were added). The digestion required a 30 minute incubation in a 37°C water bath for both samples and a second incubation of the vector in a 65°C water bath for 20 minutes. The PCR product and digested vector were then purified separately using a Wizard® SV Minicolumn and multiple rounds of washing by the according to the standard protocol described in the Promega Flexi® Vector technical manual. Finally, the purified, digested PCR product was ligated to the purified, digested vector. The ligation reaction consisted of 10 µL of 2x Flexi® Vector Buffer, 5 µL of digested vector, 4 µL of digested PCR product, and 1 µL of T4 DNA ligase followed by a hour long incubation at room temperature.

Competent *E. coli* (New England Biolabs, Inc. Cat. No. C2987I) NEB 5α cells were transformed with the products of the ligation reaction according to the High Efficiency Transformation protocol from New England Biolabs (protocol CC2987). In

this transformation procedure, 50 µL of cells were mixed with 5 µL (up to 100µg) of the ligated plasmid. The mixture was incubated for 30 minutes in an ice-water bath, 30 seconds at 42°C and then again on ice for 5 minutes. 950 µL of LB broth was then added and the mixture was incubated at 37°C for 1 hour in a shaker at 250 rpm. Cells were then plated onto Difco Nutrient agar plates containing the antibiotic kanamycin (50 µg/mL) to inhibit the growth of any *E. coli* cells that were not successfully transformed with the plasmid.

Plasmids from kanamycin-resistant transformants were isolated using the Quantum Prep Plasmid Mini Prep Kit (BioRad). Cells were grown in LB broth containing kanamycin (50 µg/mL) and harvested by centrifugation at 14,000xg. The cells were resuspended in the Cell Resuspension solution, then lysed using a lysis solution, and a Neutralization Solution to denature any proteins and stabilize the plasmid. The sample was then washed with buffer and collected by centrifugation and loaded onto a Wizard® SV Minicolumn. The sample bound to the column was washed with wash buffer and then eluted out in H_2O. The identification of proper transformation and isolation was then verified by gel electrophoresis of the product.

Following the isolation of the plasmid, the plasmid was transformed into *E. coli* BL21 (DE3). The transformation reaction contained 50 µL of cells and 100 ng of plasmid DNA. Incubations of 30 minutes on ice, a 10 second heat shock at 42°C, and then 5 minutes on ice again. 950 µL of SOC was then added and the solution was mixed at 37°C for 1 hour in a shaker at 250 rpm. After this incubation, the cells were plated out onto LB plates containing kanamycin and incubated overnight at 37°C.

Results:

The *Comamonas* sp. strain JS765 bacteria was found to grow best in a solution of BLK with a concentration of nitrobenzene at 0.750 mM. A series of tests were done in effort to find the most effective growing conditions, and the results of these tests can be seen in the Table 1.

Table 1: Nitrobenzene concentration dependence of *Comamonas* growth measured by UV-vis spectroscopy.

Nitrobenzene (mM)	Abs (600 nm)
0	0.26
0.250	0.44
0.500	0.67
0.750	0.80
1.000	0.67
1.250	0.59
1.500	0.21

The integrity of the genomic DNA isolated from *Comamonas* sp. strain JS765 using the Qiagen DNeasy® Blood and Tissue Isolation Kit was verified by agarose gel electrophoresis. The concentration of the genomic DNA was measured by UV-visible spectrometry. The spectrophotometric results showed a range on concentrations from 16.59 ng/µL to 45 ng/µL, and the results can be seen in Table 2. The band located in

Table 2: Absorbencies of collected genomic DNA of *Comanomas* sp. Strain JS765 as measured by UV spectroscopy.

Abs (260 nm)	Abs (280 nm)	ng/µL

0.3318	0.1652	16.59
0.4441	0.2122	22.21
0.6050	0.3125	30.25
0.3994	0.1732	19.97
0.90	0.55	45.
0.48	0.24	24.

Lane 4 of the gel indicates the genomic DNA due to its incredibly large size estimated at approximately 5 Mbp and can be see on Figure 6.

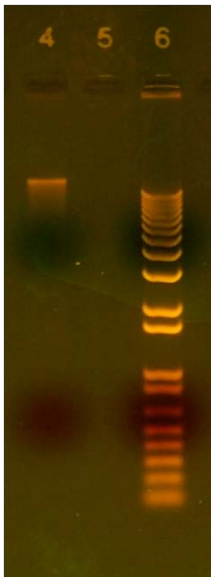

Figure 6: Gel of genomic DNA following the DNeasy Blood & Tissue Kit

After the genome was isolated, PCR was carried out under the conditions defined in the Materials and Methods section. The products of the PCR were analyzed by gel electrophoresis. The expected size of the PCR amplified product was a 1965 bp strand. However, as seen in Figure 7, the resulting strand was 2320 bp. This difference

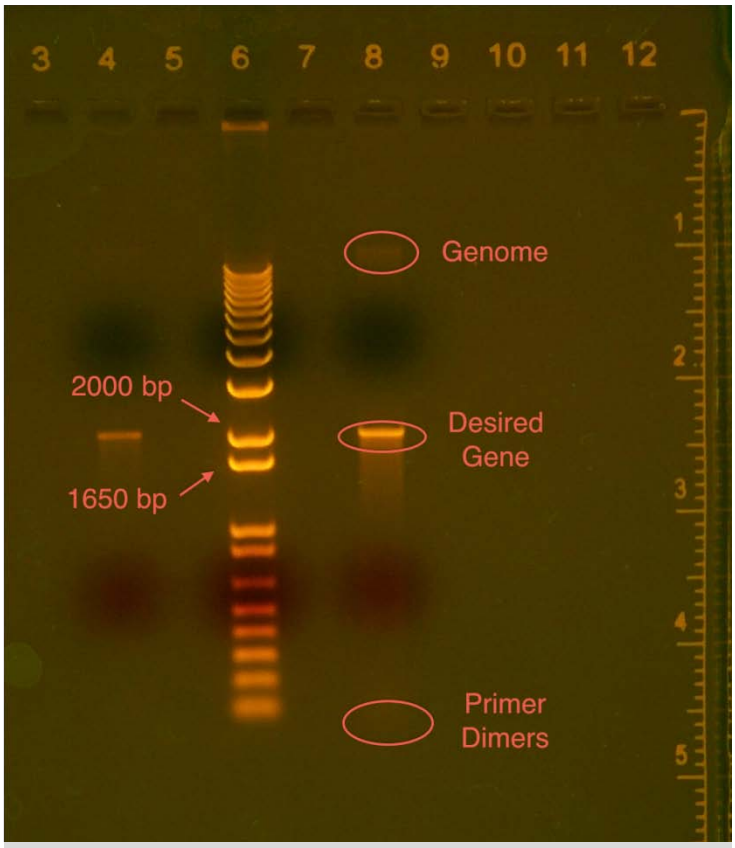

Figure 7: Gel of the amplified PCR product.

in size was deemed to be an acceptable deviation from the expected size, and the project was continued using these strands.

Following the PCR amplification, the product was digested using *Sgf I* and *Pme I* and ligated to the pF1K T7 Flexi Vector as described in the Materials and Methods section. This ligated product was then analyzed by gel electrophoresis, as seen by Figure 8. The expected size of the ligated plasmid was a circular plasmid of 5069 bp, which is the sum of the 1965 bp PCR product and the 3104 bp digested vector. Figure 8 shows that, when the plasmid construct was digested with Eco RI, a single band was observed at 6699 bp. And as seen in Figure 8, there is no such band at 5069 bp. However, the gel

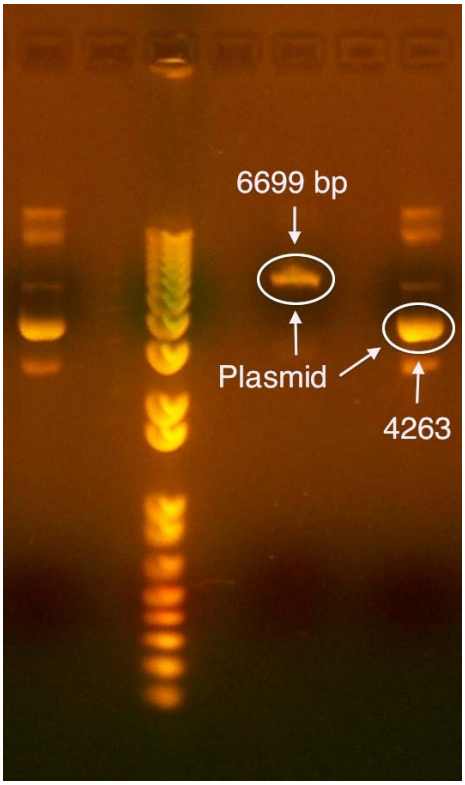

Figure 8: Gel of the ligated vector/NBDO gene plasmid. Lanes 1 and 4 are uncut plasmids, while lanes 3 and 5 are samples of the plasmid cut with *EcoR1* and are thus linear.

does suggest successful ligation because lanes 1 and Lane 4 contains the undigested circular ligated plasmid. Since the multiple bands seen in lane 4 all collapsed into one band in Lanes 3, it appeared as though the desired plasmid construct had been produced. As with the PCR results, even though the resulting product was larger than expected, the study proceeded to the next step, namely transformation of *E. coli* NEB 5α. This decision was made because the product would be sequenced later on in the study, and this later sequencing data is ultimately what is needed for confirming or rejecting that the gene was successfully cloned.

The transformation of the *E. coli* NEB 5α with the plasmid DNA construct generated kanamycin-resistant clones. Kanamycin resistance is conferred by the Flexi

Vector. In addition, since transformants carrying the original pF1K T7 Flexi Vector are not viable due to the lethal barnase gene, presumably all of the transformants carry foreign DNA. Figure 9 shows a negative control, in which untransformed NEB-5α cells were plated on nutrient agar plates containing kanamycin. As seen there is no growth evident on the plate indicating that the kanamycin successfully kills all bacteria that do not contain the kanamycin gene, such as the one found in the vector. In contrast, Figure 10 shows the results of the transformation experiment. Colonies indicate successful transformation because it must contain the kanamycin resistance gene found in the inserted plasmid. Also, it suggests that the plasmid contains the NBDO genes because the barnase gene, which would kill the bacteria, is absent from the inserted plasmid because the cells were able to grow and form colonies.

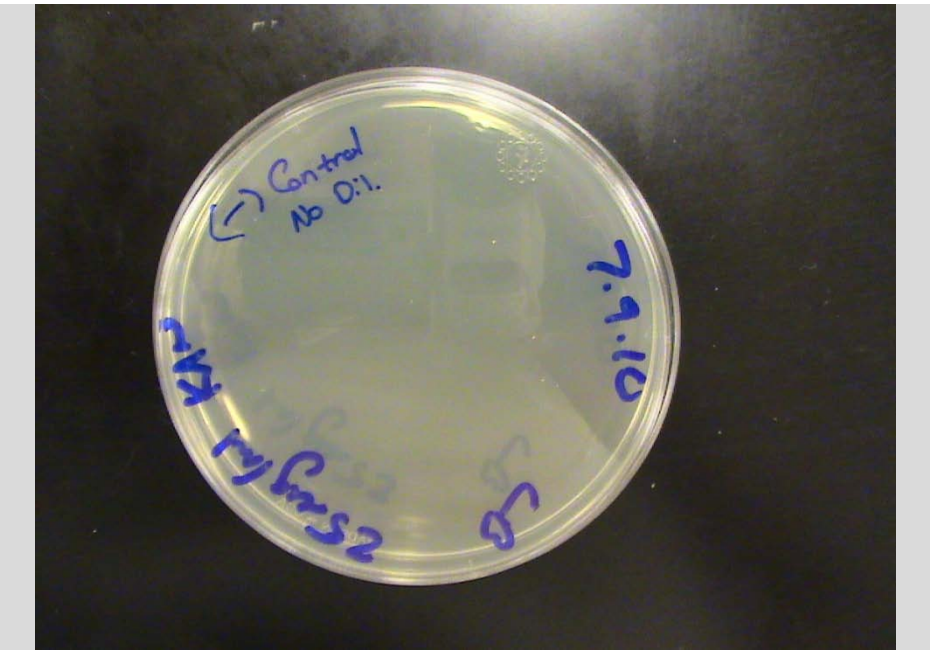

Figure 9: Picture of the negative control.

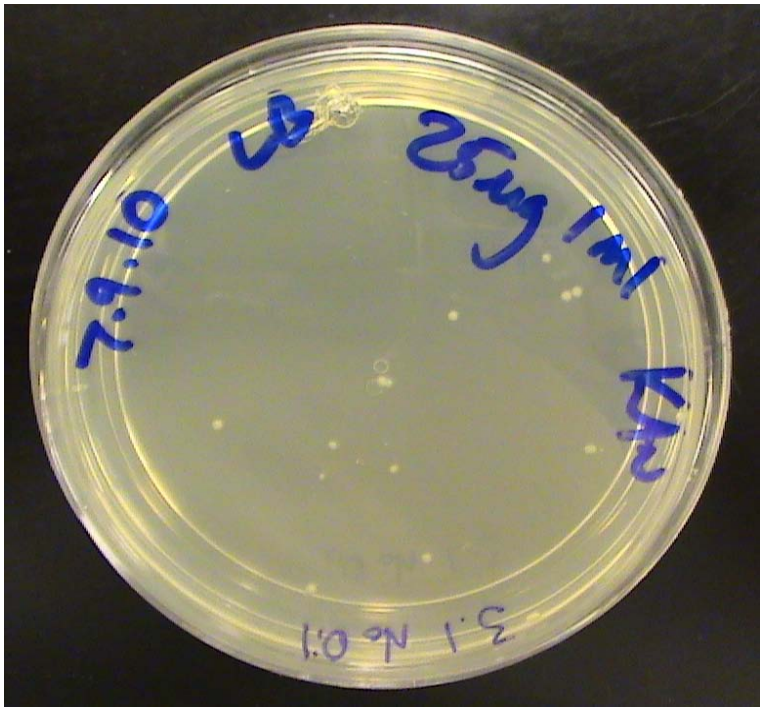

Figure 10: Picture of the transformed *E. coli* NEB 5α cells.

Sequencing was performed on the plasmids collected from three colonies of the transformed *E. coli* NEB 5α cells (Northwoods DNA Inc, Bemidji, MN). The resulting sequencing indicated that the NBDO gene was contained within the plasmid of more than one of the clones. Two of the clones showed improper copying of additional sequences, however, one clone showed proper sequencing except for a single amino acid, which is not found in or near the active site. So from these sequencing results, it was concluded that the NBDO was properly ligated into the vector, and that it was done so with the proper sequence.

After verifying that the ligated sequence was correct, the plasmid was isolated from the *E. coli* NEB 5α cell line and transformed into the *E. coli* BL21 (DE3) cell line. Again, Difco nutrient agar was used with kanamycin added. And as seen in Figure 11, transformants were successfully grown.

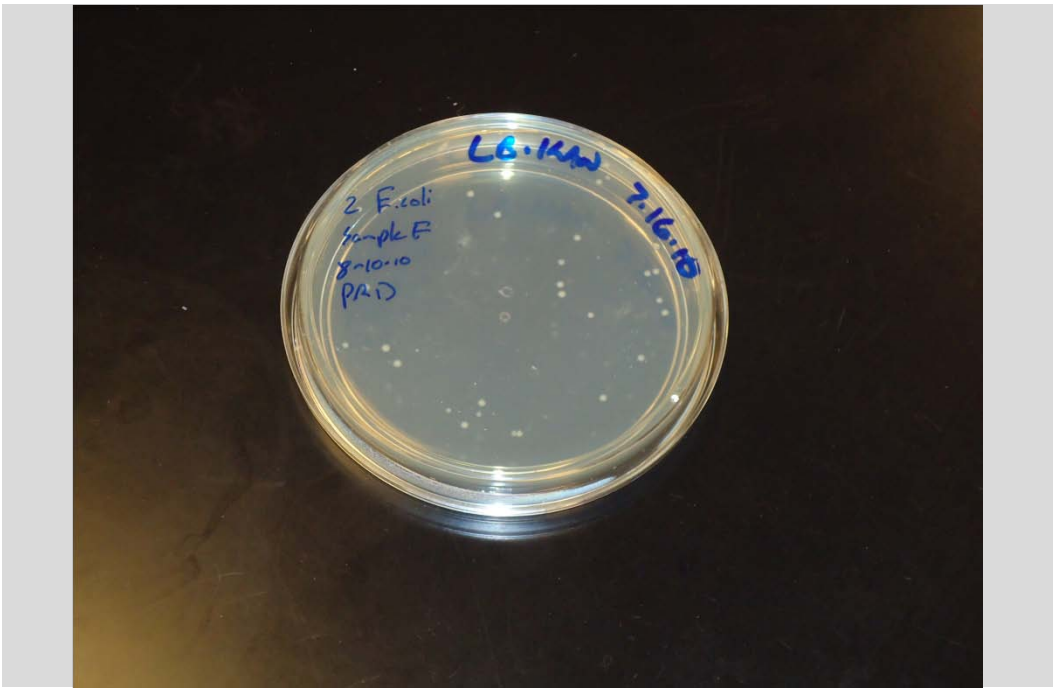

Figure 11: Picture of the transformed *E. coli* BL 21 (DE3) cells.

Conclusion

The overarching goal of this study is to determine the reaction mechanism of the Rieske dioxygenase enzyme found in the bacteria *Comamonas* sp. strain JS 765, and while this was not determined in this specific project, the first step necessary for this goal was successfully performed. As seen by the sequencing results and the successful growth of both the transformed *E. coli* (New England Biolabs, Inc. Cat. No. C2987I) NEB 5α and the *E. coli* BL21 (DE3) cell lines on kanamycin containing Difco Nutrient agar plates, it can said that the desired α and β subunits of the NBDOS were successfully cloned and are suitable for further use in the amplification and over production of the desired native Rieske enzyme and of the mutated Rieske enzyme. It is important to note that while the gene was cloned into these cell lines, it may be necessary to use a mutagenesis kit to change the cell line containing the single-nucleotide polymorphism

(SNP) back to the original nucleotide because this SNP changed the nucleotide from an adenosine to a guanine. Ultimately, it was not determined in this project if the change in amino acid substantially effected the function of the enzyme even though it was determined to be located away from the active site of the enzyme, but because the sequencing error was only a single change in nucleotide the cloning was determined to be a success and moving on towards mutating the enzyme for further study is going to be the next step for the project as a whole.

What this next step in the project would include after the mutation would be growing both the native protein and the mutated protein and comparing the rate at which O_2 is removed from the environment. This rate of O_2 loss would indicate the rate at which the enzyme was working because the enzyme uses atmospheric O_2 for the oxygen involved in the *cis*-dihydroxylation. And if it is found that the mutant is cable of utilizing the atmospheric O_2 and its enzymatic rate is slower than that of the native protein, then it may be possible to capture any intermediate reactants, possibly the postulated Fe(V) intermediate.

So while this study was unable to reach the point of studying the actual enzymatic mechanisms of the enzyme at hand, it was able to accomplish the first step of the process by successfully cloning the NBDO genes into both the *E. coli* (New England Biolabs, Inc. Cat. No. C2987I) NEB 5α and the *E. coli* BL21 (DE3) cell lines. Cloning the genes into these two cell lines accomplished two major advantages. The first being it is much easier to grow mass quantities of *E. coli* than *Comamonas*. The second being the *E. coli* BL21 (DE3) cell line is a line of *E. coli* designed for expressing proteins in higher than normal amounts, so when it is necessary for over-expressing the enzyme of interest

it will be possible to produce more protein per cell than if it were to be left in the initial *Comamonas* cell line.

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