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Sarah Klass April 16, 2013

Characterization of Novel HydX Protein and its Involvement in the Formation of the Active Site in [Fe-Fe] Hydrogenases

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An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
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Emory College Chemistry Department

2013

Abstract

Characterization of Novel HydX Protein and its Involvement in the Formation of the Active Site in [Fe-Fe] Hydrogenases

By Sarah Harrington Klass

The novel HydX protein is expressed during anaerobic respiration in organisms containing [Fe-Fe] hydrogenase enzymes. In anaerobic conditions, protons are used as a final electron acceptor and are reduced at the active site of hydrogenases to produce H_2 (g). Although no prior research has been done on HydX, the structure and function of three accessory proteins (HydE, HydF, and HydG) known to be involved in the maturation of the active site in [Fe-Fe] hydrogenases have been identified, however, the complete maturation mechanism has yet to be elucidated. The gene sequences for the known accessory proteins directly flank the *hydx* sequence on either side and are also expressed during anaerobic along with the hydrogenase enzyme HydA. In this thesis the structure and function of HydX was explored to determine if it too had a role in the formation of the active site by acting as a scaffold or transport protein. First, a non-redundant BLAST search of the *hydx* sequence was performed, surprisingly, no homologs in structure or sequence were found. The CD spectrum of HydX indicated a primarily α-helical structure with a T_m of 48.5˚C. Size exclusion chromatography of the HydX protein indicated a trimeric oligomeric state. NMR spectroscopy revealed an apparently folded tertiary structure with some flexible regions. Pull down experiments with three known accessory proteins indicated possible protein-protein interactions between HydX and HydG.

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Abbreviations

SAM- radical S-adenosylmethionine

PCR- polymerase chain reaction

LB- Lysogeny Broth

Amp-Ampicillin

Sm-Streptomycin

IPTG- isopropyl β-D-1-thiogalactopyranoside

OD_{600}- Optical Density at a wavelength of 600nm

Ni-NTA column-nickel nitriloacetic acid column

FPLC- Fast protein liquid chromatography

DEAE- dimethylaminoethanol

SDS-PAGE- sodium dodecyl sulfate polyacrylamide gel electrophoresis

CD-spectroscopy- circular dichroism spectroscopy

NMR- nuclear magnetic resonance

M9-media- minimal media

DH5α cells- electro-competent E. *coli* strain used for plasmid replication

BL21(DE3) cells- electro-competent E. *coli* strain used for over-expression of genes

BLAST- basic local alignment search tool

NEB- New England BioLabs

Background

The use of hydrogen gas as an alternative energy source to natural gas and other fossil fuels as a source of energy would help to reduce the buildup of carbon dioxide in our atmosphere as well as reduce the need for importing foreign oil. Two of the most common methods for producing hydrogen gas are through steam methane reforming and through the electrolysis of water.[1] Steam methane reforming converts methane to hydrogen gas by reacting with steam at high temperatures. However, methane gas is not renewable and the byproduct of forming hydrogen gas in this way is carbon dioxide. Electrolysis can be an environmentally friendly way to produce hydrogen gas, but only if the energy used to power the process is from renewable sources such as wind or solar power.[2] Not only would the use of wind or solar power be costly, but it would also essentially just convert the product of one environmentally friendly energy source to another. The total global energy use is greater than $370 \cdot 10^{18}$ joules with less than 5% of that coming from sources other than fossil fuels.[3,4] With the growing environmental concerns as well as the rising cost associated with the use of fossil fuels, a demand for a renewable energy source that can be produced in a way that makes it financially competitive with fossil fuels has grown into a major world challenge. [4] Hydrogen gas is regarded as the most promising future candidate as a major energy source due to its high energy content (2.75 times greater than natural gas), and its capacity to be incorporated directly into a fuel cell to generate electricity.[5, 6] Possibly the most important reason why hydrogen gas should be considered as the next major energy source is that the only byproduct of combustion of hydrogen used to generate electricity is water. [7]

The utilization of metalloenzymes, known as hydrogenases, found in certain photosynthetic bacteria to catalyze the reduction of photons ($2H^+ + 2e^- \rightarrow H_2$) could provide a potentially cost effective renewable source of environmentally friendly energy that is currently in such high demand. There are many photosynthetic bacteria that contain these hydrogenase enzymes such as *Clostridium pasteurianum*, *Ignavibacterium album*, and *Thermotoga maritima*, that have homologous proteins used to facilitate their hydrogen metabolisms, utilizing protons as a final electron acceptor of photosystem I and II in anaerobic environments.[8,9,10] *S. oneidensis* is another anaerobic bacterium that produces hydrogen gas as a byproduct of its metabolic cycle. The genome of *S. oneidensis* also codes for the necessary accessory proteins used to create the active site in these hydrogenases. The active site of these hydrogenase enzymes is known as the H-cluster and it is where the redox chemistry occurs for molecular hydrogen production. In *S. oneidensis* there are two hydrogenases, HydA and HydB. HydA's active site contains only iron ions and is classified as an [Fe-Fe] hydrogenase, while the active site of HydB contains iron and nickel ions and is thus classified as an [Ni-Fe] hydrogenase. Typically [Fe-Fe] hydrogenases perform reductive chemistry ($2H^+ + 2e^- \rightarrow H_2$) while [Ni-Fe] hydrogenases usually perform oxidative chemistry ($H_2 \rightarrow 2H^+ + 2e^-$).[11,5] In this thesis only the chemistry involving HydA's active site or H-cluster is considered due to data suggesting a turnover rate 100 fold higher for [Fe-Fe] hydrogenases compared to [Ni-Fe] hydrogenases as well as the reductive nature of [Fe-Fe] hydrogenases.[12,13,11] The H-cluster in HydA is composed of two smaller clusters comprised of iron-sulfur components. One of the small clusters is a the standard cubane [4Fe-4S] iron sulfur cluster coordinated to HydA via cysteine thiol bonds, while the other cluster [2Fe-2S] is composed of two Fe ions coordinated to cyanide and carbon monoxide ligands (Figure 1).

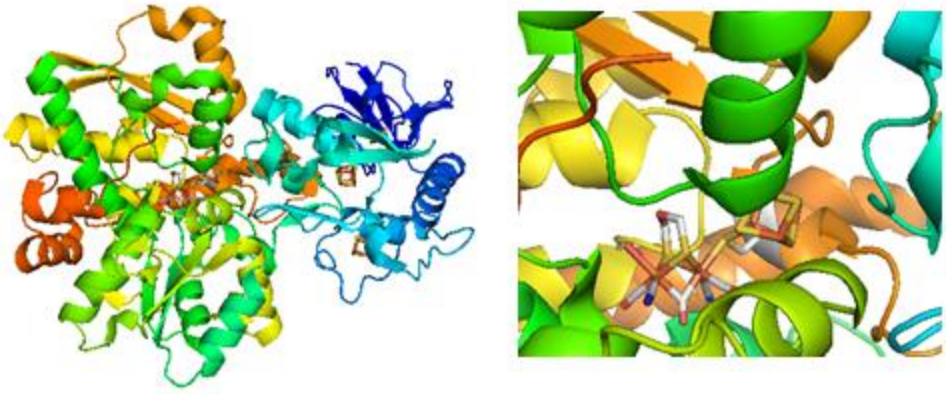

Figure 1: Structure of HydA and close-up of H-cluster: The HydA structure generated here was created in PyMOL using the atom assignments from the crystal structure of HydA determined to 1.39 Å by Peters et.al.[14]

The formation of this cluster is dependent upon three known accessory proteins, HydE, HydF, and HydG.[15,16] It has been determined by crystallographic structures that the HydF accessory protein serves as GTPase protein that acts as a scaffold for the [2Fe-2S] structure due to the presence of a GTP binding domain. HydE and HydG are both responsible for modifications to the [2Fe-2S].[16,17,18] Specifically, HydE forms the dithiolate ligand in the [2Fe-2S] sub-cluster by interacting with radical SAM proteins,[16,19,20] while HydG has two main functions; incorporating a previously synthesized [4Fe-4S] into the H-cluster and generating the CN^- and CO ligands on the [2Fe-2S] cluster (Figure 2).[16,21,22] The insertion of the two clusters has been determined to happen in a stepwise fashion where the insertion of [4Fe-4S] cluster into the active site precedes the insertion of the [2Fe-2S] cluster.[23,10]

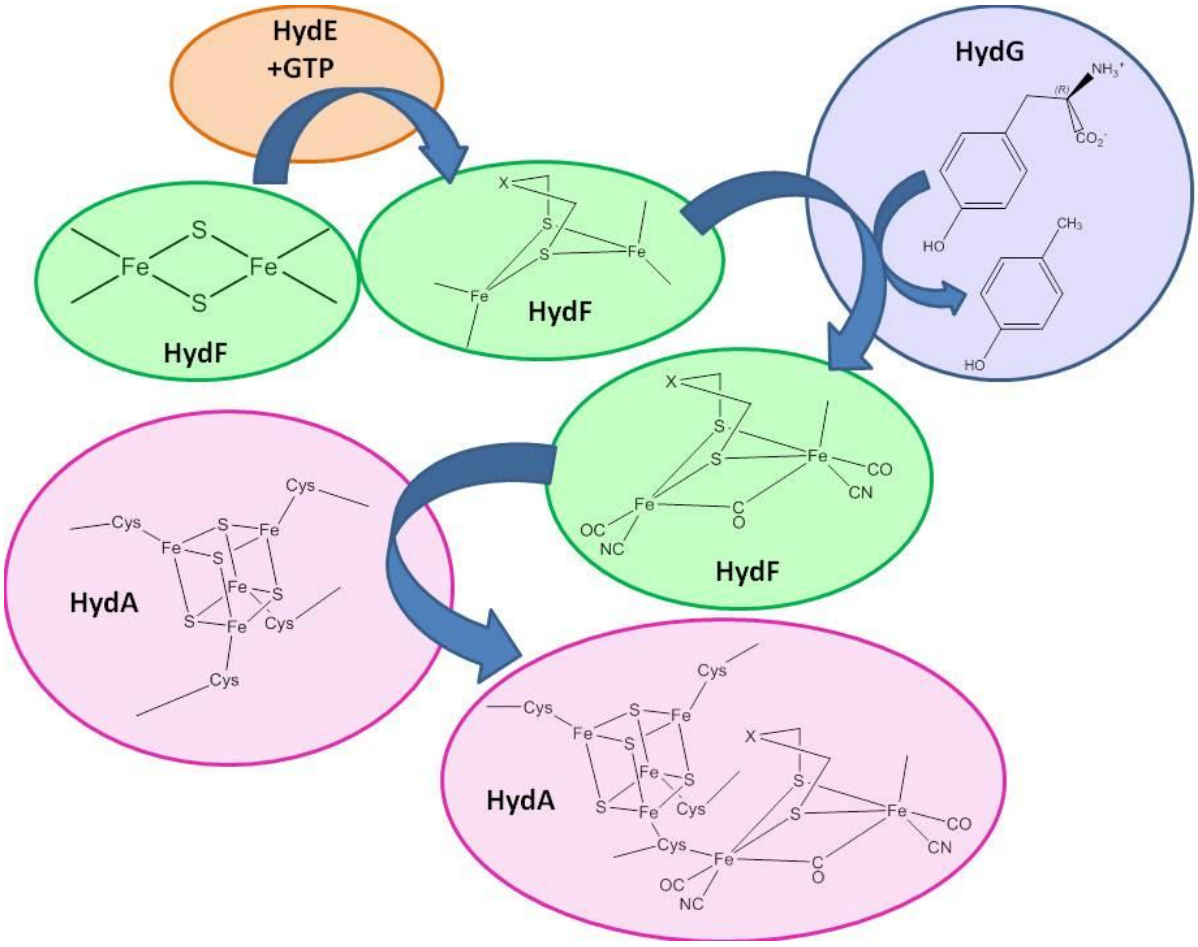

Figure 2: Assembly of The H-cluster active site in Hydrogenases: HydE forms dithiolate ligands on [2Fe-2S] cluster via interaction with SAM proteins. HydF serves as a scaffold for [2Fe-2S] cluster. HydG incorporates [4fe-4S] cluster into active site and generates CN^- and CO^- ligands on [2Fe-2S] cluster.[5]

The genes that code for the accessory proteins; HydE, HydF and HydG, all exist within the same gene cluster (Figure 3). Between the genes that code for HydG and HydE lies the coding region for a protein known as HydX. This HydX protein, along with the other accessory proteins is also expressed during H-cluster assembly. Though there has been extensive research

done on HydE HydF and HydG, there is little to nothing known about the structure or function of HydX. In comparison to the other three accessory proteins, HydX is relatively small with only 214 amino acids (HydE- 360aa, HydF- 395aa, HydG-480aa). Even more puzzling is that HydX is a novel protein in that it has no known structural or sequence homologs based on non-redundant BLAST searches. Due to hydx's proximity to the other accessory proteins necessary for the formation of the active site along with its relatively small size, my hypothesis is that HydX may serve as a scaffold or transport protein that helps to facilitate the production of the H-cluster.

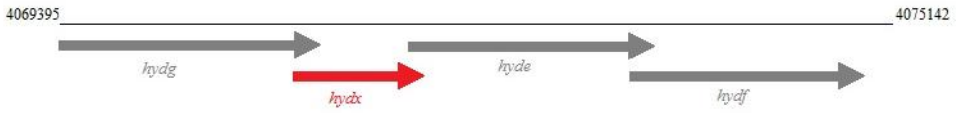

Figure 3: location of *hydx* gene relative to the *hyde, hydf,* and *hydg* genes that encodes for accessory protein responsible for the assembly of the H-cluster which are all contained in the [Fe-Fe] hydrogenase operon.

Further support for the HydX protein having a potentially important role in the maturation of the H-cluster comes from research done on the [Ni-Fe] cluster in hydrogenases that favors the oxidation reaction of hydrogen gas. It was determined that the hydrogenase maturation of the [Ni-Fe] cluster requires four accessory proteins HypC, HypD, HypE and HypF.[24,25] While HypD, HypE, and HypF are all involved in the biosynthesis of ligands on the [Ni-Fe] cluster much like HydE, HydF, and HydG do during the formation of the H-cluster. The protein HypC is

involved with the maturation process of the active site of [Ni-Fe] hydrogenases in a way that an analogous protein has not yet been identified in the maturation process for [Fe-Fe] active sites. HypC is in fact a small acidic protein that forms a complex with the precursor to the hydrogenase protein function as a chaperone to help metal ion acquisition.[25,26] However, after performing a BLAST search comparing the HydX sequence to the HypC sequence, no significant similarity between the two was discovered, and the possible hypothesis that HydX might be homologous to HypC was abandoned.

Heterologous expression of the accessory proteins, required for the formation of the active site of both [Fe-Fe] and [Ni-Fe] hydrogenases, followed by the *in vivo*[27,28] or *in vitro*[29] coupling to an electron donor such as photosynthetic machinery has been used to produce hydrogen gas from sunlight.[30] Although prior research has had success in producing H_2 from active hydrogenases, there are many obstacles associated with this process that prevent it from currently being used as major source of hydrogen gas. As previously mentioned, the hydrogenase enzyme produces hydrogen gas only when in anaerobic environments. When in the presence of O_2, the hydrogenase enzyme containing an active H-cluster exhibits irreversible inhibition by oxygen thus stopping the production of H_2.[31] The sensitivity to oxygen causes a major issue when attempting to produce hydrogen on a large scale.[32] Another problem encountered by producing hydrogen *in vitro* or *in vivo* is that the hydrogenase lacks a natural source of electrons. Under anaerobic conditions, these photosynthetic bacteria utilize protons to serve as a final electron acceptor for electrons produced by photosystem I and photosystem II. These electron reduce the protons thus producing H_2 as a byproduct of respiration. However, photosynthetic systems are highly complex and difficult to express heterologously for the *in vivo* or *in vitro* production of H_2 .[33,12,34] Though these issues do not directly impact the heterologous expression

and characterization of HydX, oxygen sensitivity and sources of electron donors will play a role when HydX is expressed with HydA and the other accessory proteins to form an active H-cluster and when the production of hydrogen gas from these active hydrogenases is attempted.

After characterizing and determining the role of the HydX protein, the ultimate goal of this experiment would be to perform heterologous expression of the HydA enzyme along with HydE, HydF, HydG, and HydX (assuming significance in H-cluster formation) in *E. coli* cells under anaerobic conditions to produce an active H-cluster. To avoid using photosystems I and II as the source of electrons to drive the reduction of protons, one possible alternative being explored is to attach a quantum dot close to the active site of the HydA protein. Quantum dots are semiconductor nanocrystals, when in the presence of light, absorb photons. This results in excited state electrons, followed by transfer to an electron acceptor such as HydA.[35] CdTe quantum dots have already been electrostatically bound to [Ni-Fe] hydrogenases from *Thiocapsa roseoperscina*, and have been shown to provide electrons for the reduction of protons to produce hydrogen gas when the quantum dot undergoes photoexcitation due to exposure to the sun.[36] The active enzyme could then be purified and immobilized on quantum dots for the in vitro reduction of protons in anaerobic conditions to form hydrogen gas. A schematic of the proposed production assembly is presented in Figure 4.

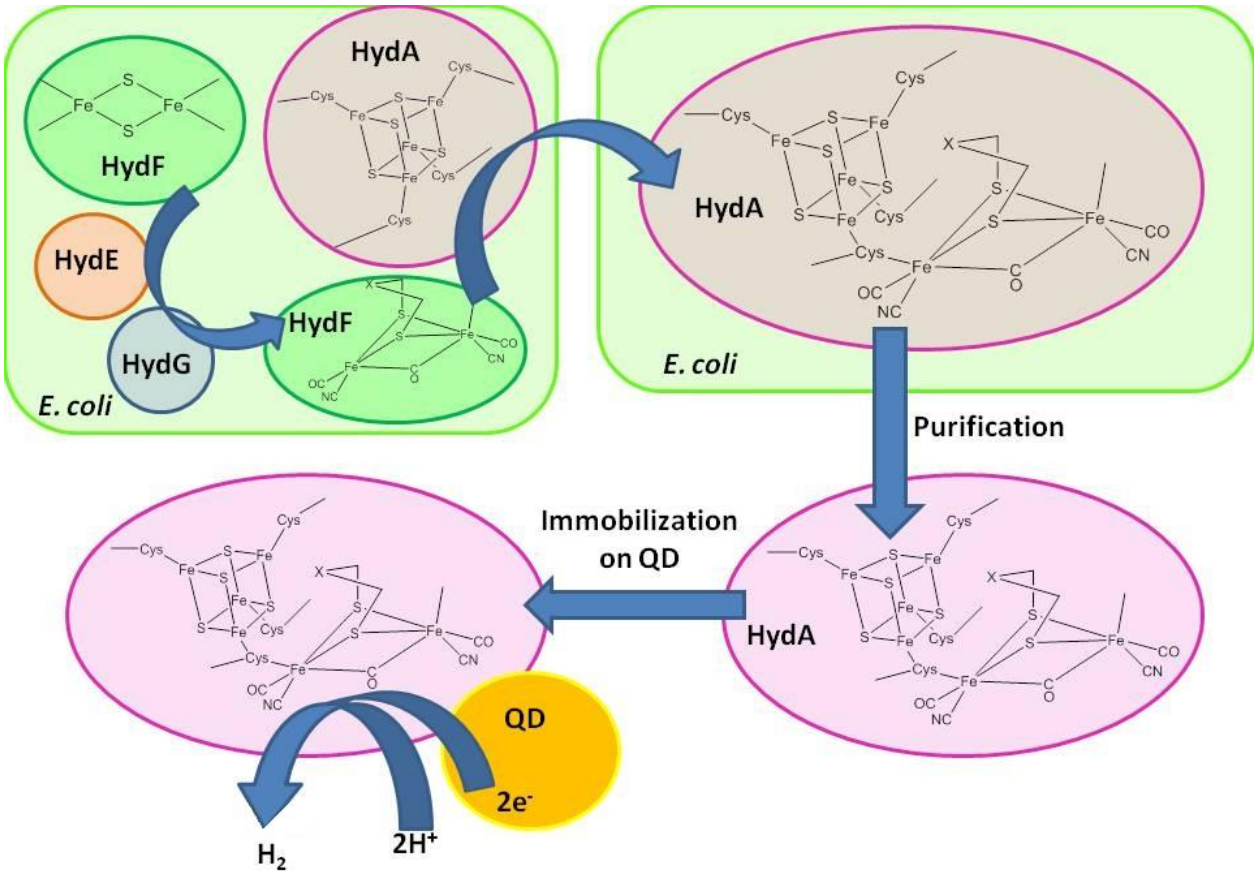

Figure 4: Schematic representing the construction of the active site (H-cluster) in HydA and the proposed method for producing hydrogen gas: due to the presence of the three known accessory proteins HydE, HydF, and HydG as well as the hypothesized accessory protein HydX, the H-cluster is assembled in the HydA protein in *E. coli* cells. The active hydrogenase enzyme is purified from E. coli cells and immobilized on quantum dots. When in a surrounding environment that is acidic, oxygen devoid, and exposed to light, the reduction of protons to hydrogen gas should occur.

Specific Aims

I. Obtain soluble HydX through heterologous expression of *Shewanella oneidensis* gene in *E. coli*

II. Study secondary, tertiary, and quaternary structure of HydX

III. Investigate the possible role of HydX as a scaffold or transport protein for HydE, HydF, and HydG by pull-down experiments

Experimental Methods

Materials

Taq DNA polymerase and 10x buffer used for PCR amplification was purchased from Promega (Madison, WI). dNTPs used in PCR amplification were purchased from Sigma-Aldrich. DNA samples were purified using the QIAquick Gel and PCR purification kits (Qiagen, Valencia, CA) using the provided buffers according to the manufacturer's protocol. Bacterial cells containing vectors were purified using QIAminiprep kit (Qiagen, Valencia, CA) using the provided buffers according to manufacturer's protocol.

Construction of Plasmid

To insert *hydx* into the pET21a vector (Figure 5) used for the over expression of the HydX protein, first *hydx* was isolated from the genomic DNA of *Shewanella oneidens*is by PCR amplification using gene specific primers (Appendix). The forward primer contained an NdeI restriction site while the reverse primer encoded for an XhoI restriction site. A histidine-tag was added onto the C-terminal primer to enable nickel column purification when the protein had been expressed. The amplified gene was then purified using a QIAquick purification column. Both the pET21a vector and amplified *hydx* gene were then digested (Appendix) with restriction enzymes, NdeI(NEB) and XhoI(NEB), to produce complementary sticky ends. The digested products were ligated together using T4 DNA ligase (Promega). The ligation product was purified the QIAquick PCR purification columns. The resulting ligation products were then transformed into electro-competent DH5α cells (Appendix). Taking advantage of the ampicillin (amp) resistance site on the pET21a vector, the transformed cells were grown on LB agar plates supplemented with 100 μg/ml amp (LB+amp plates) overnight at 37˚C. Colonies were selected

and then grown overnight in LB broth supplemented with 100 μg/ml amp (LB+amp media). Using colony PCR, the bacterial cultures containing the pET21a+hydx plasmids were determined and isolated by QIAminiprep. DNA sequencing was then used to confirm that the full-length *hydx* sequence was contained in the vector.

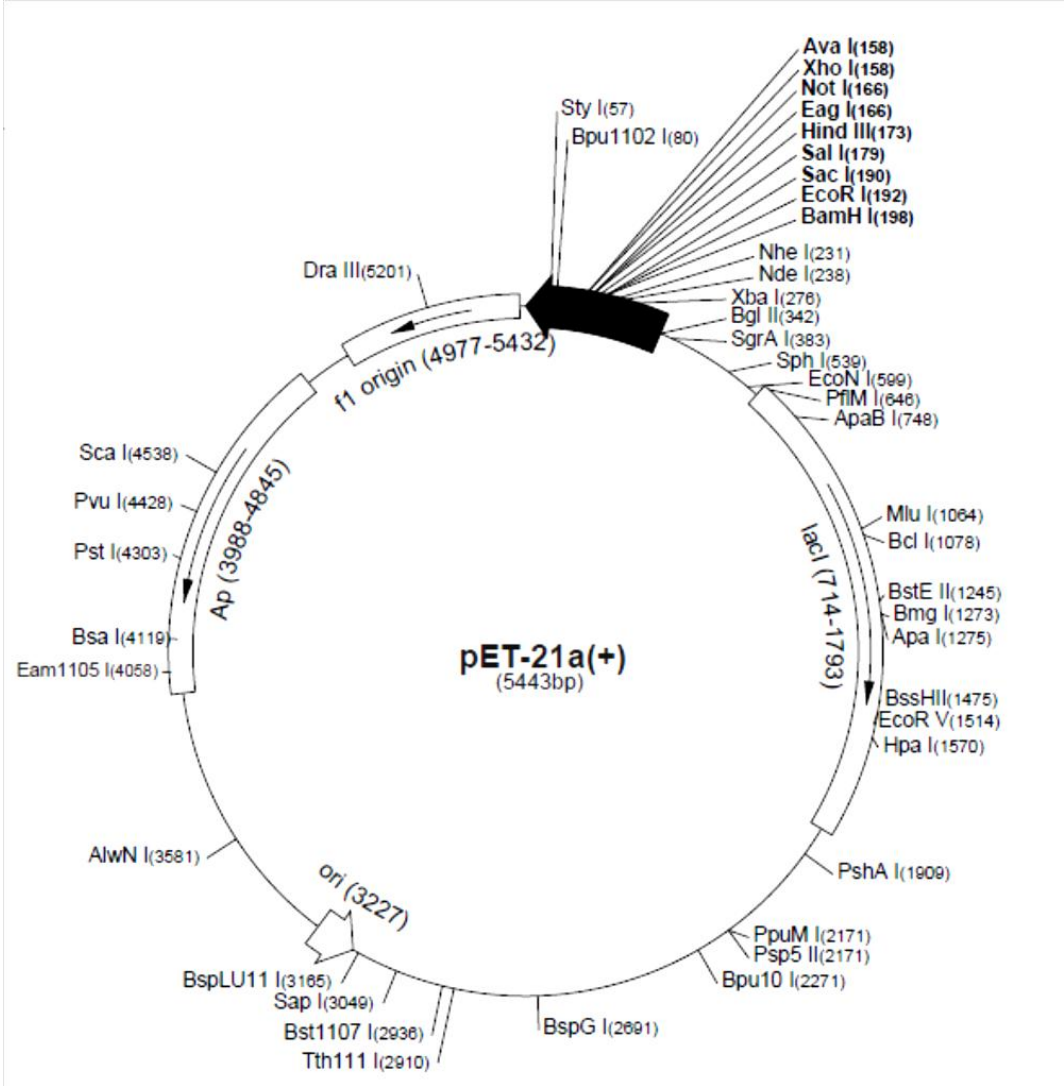

Figure 5: pET-21a vector: the vector used for expression of the *hydx* gene inserted between the NdeI and XhoI restriction sites. The vector also codes for ampicillin resistance, which can be used to select for bacteria containing vector. (Figure obtained from Novagen pET21a manual.)

Expression

Expression of HydX was carried out in *E. coli* BL21(DE3) cells transformed (Appendix) with pET21a-*hydx* vector. From a selected BL21(DE3) colony, a cell culture was then grown in 2 ml of LB+amp media shaken overnight at 37˚C. Using 500 µl of the overnight solution, a 500 ml solution of LB+amp media was incubated and grown at 37˚C until an OD_{600} of 0.4 at which point the temperature was dropped to 20˚C. The culture was then grown at 20˚C until it reached an OD_{600} of 0.6 when protein expression was induced with IPTG to a final concentration of 0.5 mM. The culture was grown for an additional 24 h at 37˚C with 500 µl samples taken at 1 h, 2 h, 6 h and 24 h time points to observe the time course expression of HydX. After 24 h the cells were centrifuged and the cell pellet was stored at -20˚C in 10% glycerol. The time course production of soluble HydX was analyzed on an SDS-PAGE gel (Appendix),

Purification

To purify the C-terminal His-tagged HydX protein, a Ni^{2+} affinity column was used to bind only the HydX protein. The cell pellet was resuspended in 10 ml of lysis buffer (Appendix) and suspension was incubated on ice for 30 min. The suspension was sonicated for 30 s on 30 s off for a total of 3 min and then centrifuged at 4˚C for 30 min. The supernatant was then loaded onto a 1 ml Ni-NTA column that had been washed with 10 column volumes of equilibrating buffer: 50 mM Tris-HCl (pH 8.0), 500 mM NaCl, and 5mM imidazole. The column was then washed with the equilibrating solution. The protein was eluted with the same equilibrating buffer, but with increasing concentrations of imidazole (up to 200 mM). Imidazole and Tris-HCl(pH 8.0) buffer were removed from the eluted HydX protein and exchanged with 20 mM phosphate buffer pH 7.0 and 50 mM NaCl using a dialysis bag. The protein concentration was

then analyzed using absorbance spectroscopy and a calculated molar extinction coefficient of 36440 $M^{-1}cm^{-1}$obtained from ExPASy online bioinformatics resource portal. Using 30 µl of the purified HydX protein solution was then loaded into a CD spectrophotometer to determine the secondary structure of the protein.

To make sure the histidine-tag on the protein was not altering the protein folding, HydX was also expressed without a histidine-tag following the same protocol as the tagged protein except during the purification step. To purify the untagged protein, anion exchange chromatography[37] and size exclusion chromatography were performed using an FPLC machine.

First anion exchange chromatography with a DEAE Sepharose column was used to separate proteins based on their charge. Based on the amino acid composition of HydX, the charge of the protein at pH 7 was determined to be 5.04 (ExPASy online bioinformatics resource portal). After lysing the cells to extract the HydX protein, the supernatant containing the HydX protein was loaded onto DEAE Sepharse column connected to the FPLC machine that had been equilibrated with a solution of 50 mM Tris-HCl(pH 7.0) and 20 mM NaCl. To elute HydX, the salt concentration of the eluent was slowly increased to a final concentration of 1 M NaCl. Fractions were collected when an increase in absorbance of the eluted product was detected and then run on an SDS-PAGE gel to determine which ones contained HydX.

Fractions containing HydX were concentrated down to 1 ml and then run on a 24 ml size exclusion column that have been washed with 1 column volume of 50 mM sodium-phosphate buffer(pH 7.0) and 50 mM NaCl. The contents were then eluted from the column with the same solution of 50 mM sodium-phosphate(pH 7.0) buffer and 50 mM NaCl. Fractions were collected when an increase in absorbance of the eluted product was detected. The elution time of the

fraction containing HydX could then be used to determine the molecular weight of the protein by comparing it to the elution time of the protein marker with known molecular weights. The molecular weight based on elution time was then compared to the molecular weight of the protein based on its amino acid composition to determine if the protein was being eluted as a monomer or a polymer. The fraction containing the protein was analyzed with CD-spectroscopy to determine the secondary structure of the protein.

NMR

The next step in determining the overall structure of HydX was to determine its 3D shape with ^{15}N NMR spectroscopy. To perform ^{15}N NMR spectroscopy the nitrogen atoms in the protein backbone were labeled with the ^{15}N isotope of nitrogen. To incorporate isotopic nitrogen into the backbone the HydX protein had to be grown in minimal media supplemented with ^{15}N-NH_4Cl. The same protocol as for the unlabeled protein was followed until protein expression. First one culture of *E. coli* BL21(DE3) cells that had been transformed with the pET21a-*hydx* vector was selected and grown for 3-5 h in 5 ml of LB+amp media at 37˚C. The culture was then spun down and the supernatant was decanted. The pellet was then resuspended in 25 ml of M9 media (Appendix) and grown at 37˚C in a 250 ml flask until it reached an OD_{600} of 0.5. The solution was and then spun down, and the supernatant was decanted. The pellet was resuspended in 500 ml of M9 media made with isotopic ammonium chloride ($^{15}NH_4Cl$) in a 1 L flask and grown until it too reached an OD_{600} of 0.5 at which point it was induced to a final concentration of 0.5 mM IPTG and grown at 20˚C overnight. Purification protocol of the labeled protein was the same as that for the untagged protein. The concentration of the protein was determined by absorbance spectroscopy at 280 nm. The labeled protein was partially deuterated by adding D_2O

to a final concentration of 10% and the entire sample was then analyzed with NMR spectroscopy.

Expression of Accessory Proteins

To overexpress the accessory proteins; HydE, HydF, and HydG, a streptomycin(sm) resistant vector pCDFDuet-*hydgx-ef* (Figure 6), constructed by Post Doc. Joel Panay, containing the entire operon of *hyde*, *hydf*, *hydg*, and *hydx* was used. Due to the genes' orientation, the continuous sequence of *hydg* and *hydx* was cloned into multiple cloning site 1 (MCS1) of the vector utilizing the BsphI and HindIII restriction sites. The continuous sequence of *hyde* and *hydf* was also cloned into multiple cloning site 2 (MCS2) utilizing the NdeI and the AvrII restriction sites.

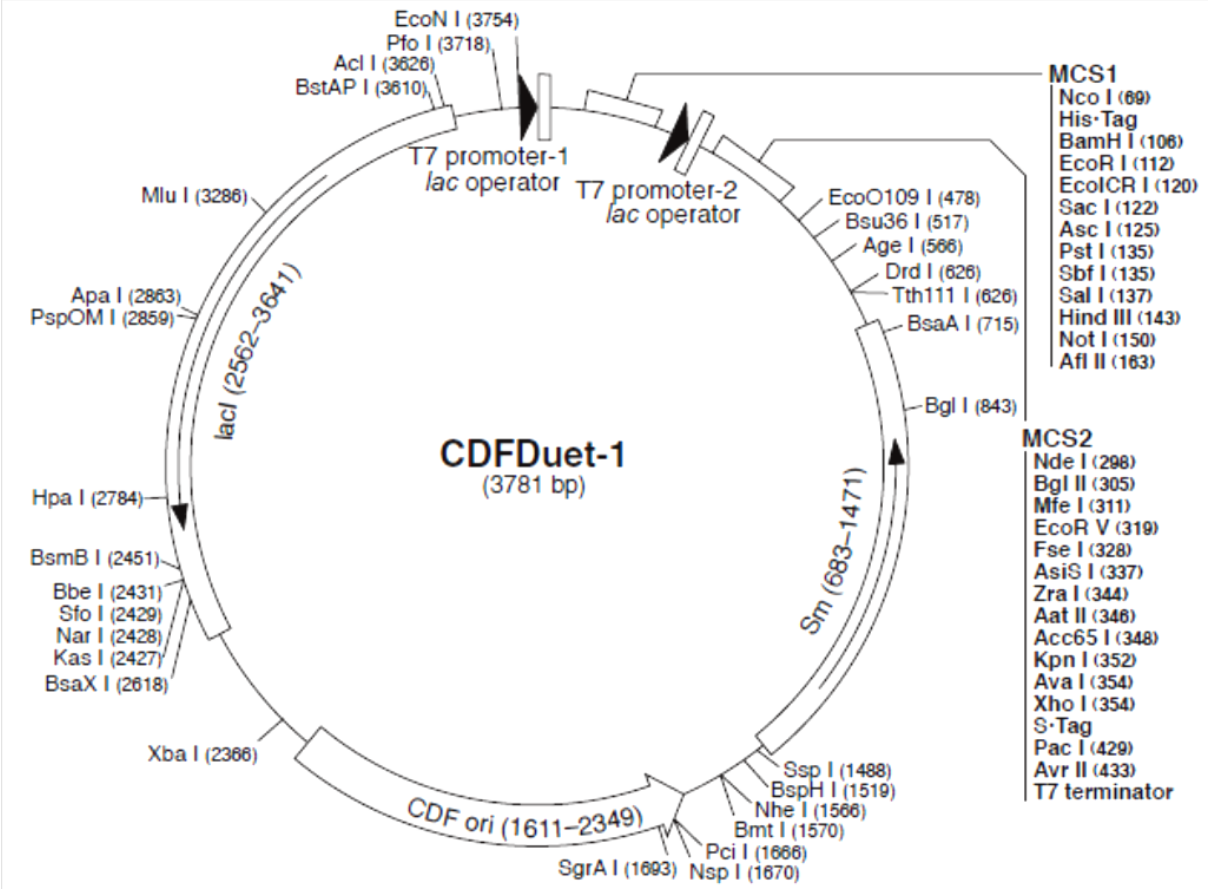

Figure 6: pCDFDuet-1 vector: the vector contains two cloning sites MCS1 and MCS2 and Streptomycin resistance. The *hydgx* sequence was cloned into MCS1 while the *hydef* sequence was subsequently cloned into MCS2 in a two-step digestion and ligation process. (Figure obtained from Novagen pCDFDuet manual.)

The pCDFDuet-1-*hydgx-ef* vector was transformed into *E. coli* BL21(DE3) cells by electroporation. One colony of the host cells was selected and grown in 2 ml of LB+sm media overnight. Using 500 µl of the overnight solution was used to incubate a 500 ml solution of LB+sm media and grown at 37˚C until an OD_{600} of 0.6 at which point the temperature was dropped to 22˚C. The culture was grown at 22˚C until it reached an OD_{600} of 0.9 at which point induced with IPTG to a total concentration of 0.5 mM. The culture was grown for an additional 20 h at 22˚C after which the entire culture was pelleted.

When determining soluble and total protein content on an SDS-PAGE gel, only bands corresponding to HydE, HydF, and HydG were present. The absence of the HydX protein was likely due to the presence of a TAA stop codon right after the *hydg* gene and immediately preceding the *hydx* gene. However, since purified HydX protein has already been made and the main purpose of this experiment was to obtain HydE, HydF and HydG, the fact that HydX expression levels in this system were low, was not an issue.

To purify HydE, HydF, and HydG accessory proteins, First the 22 h pellet of the containing the three proteins was resuspended in 10 ml of lysis buffer, incubated on ice for 30 min, and then sonicated for 30 sec on 30 sec off for 3 min. The suspension was then spun at 4˚C for 30 min and the supernatant was loaded into the FPLC machine to perform anion exchange chromatography with a DEAE Sepharose column. Based on the amino acid compositions of HydE, HydF, and HydG, the charge of the proteins at pH 7 was determined to be 4.35, 5.00, and 5.01 (calculated with the ExPASy pI/MW). Using the same procedure as for the untagged HydX protein, the FPLC machine was initially equilibrated with a solution of 50 mM Tris-HCl(pH 8.0) and 20 mM NaCl. The salt concentration of the eluent was gradually increased to a concentration of 1 M NaCl with fractions collected based on the elution absorbance at 280nm.

Pull down Experiment

To determine the interaction between known accessory proteins HydE, HydF, HydG and novel protein HydX, the C-terminally tagged HydX protein was run through the Ni-NTA column once again. However, this time after the HydX protein was bound to the column HydE, HydF, and HydG were also run on the column. The entire contents of the column were then eluted with 250 mM imidazole to remove the HydX protein and the proteins that may have attached to

HydX. By taking fractions after the addition of HydE, HydF, and HydG, and after elution with imidazole, the fractions were then analyzed on an SDS-PAGE gel to determine at what point in the experiment the proteins had come off the column. If the presence of HydE, HydF, or HydG was detected only in the fraction taken after elution along with HydX then the hypothesis that the four proteins have some sort of surface interaction would be supported.

Results and Discussion

Cloning and Expression of novel HydX protein

The *hydx* gene was successfully cloned from the genomic DNA of *Shewanella oneidensis* via a PCR reaction utilizing gene-specific forward and reverse primers (Appendix) containing NdeI and XhoI restriction sites, respectively. Figure 7 shows the amplified DNA band of a positive control and three bands corresponding to the amplified *hydx* gene. The resulting gene fragment was then cut with the restriction enzymes NdeI and XhoI to produce sticky ends for ligation with the pET-21a vector. The PCR of the *hydx* gene was performed twice, once where the reverse primer included a C-terminal tag and once without.

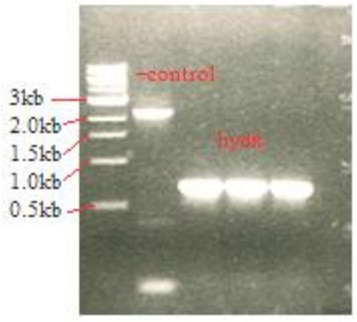

Figure 7: Determining successful PCR amplification of *hydx* gene: 1% Agarose gel run left to right with 1 kb molecular marker (NEB), gene of known length 2.3 kb and three samples of *hydx* gene, amplified from *Shewanella oneidensis* genomic DNA, mixed with 6X loading dye (NEB). After undergoing electrophoresis for 35 min at 80 mV, the PCR products of the expected size (*hydx*: 649 bp's) were detected.

The pET21-a vector was cut with the same restriction enzymes as the *hydx* gene to produce complementary sticky ends. The sticky ends of the *hydx* insert and the linearized pET-21a vector were ligated in insert: vector ratios of 0:1, 1:1, and 3:1. The 0:1 ligation served as a control to determine the vector's affinity for self re-ligation. Five of the colonies, resulting from

the transformation of the 3:1 ligation mixture into DH5α cells, were selected and screened by colony PCR to determine the presence of the *hydx* insert. Out of the five colonies selected, four had the vector containing the *hydx* insert. The positive hits were grown in LB culture, followed by plasmid isolation. Samples of the pET21a+*hydx* were submitted for DNA sequencing to confirm the correct gene sequence.

For heterologous overexpression of HydX, an aliquot of pET21a-*hydx* was transformed into *E. coli* BL21(DE3) cells. By taking samples over the course of expression, the increase in target protein from *hydx* could be observed. The optimal conditions for protein expression of HydX were determined to be overnight at 20˚C. In SDS-PAGE gel analysis for the culture samples, the resulting protein band showed up between the 17 kDa and 25 kDa marker bands as expected for the 24 kDa HydX protein carrying a C-terminal poly-His tag (Figure 8) and the untagged HydX protein (Figure 9).

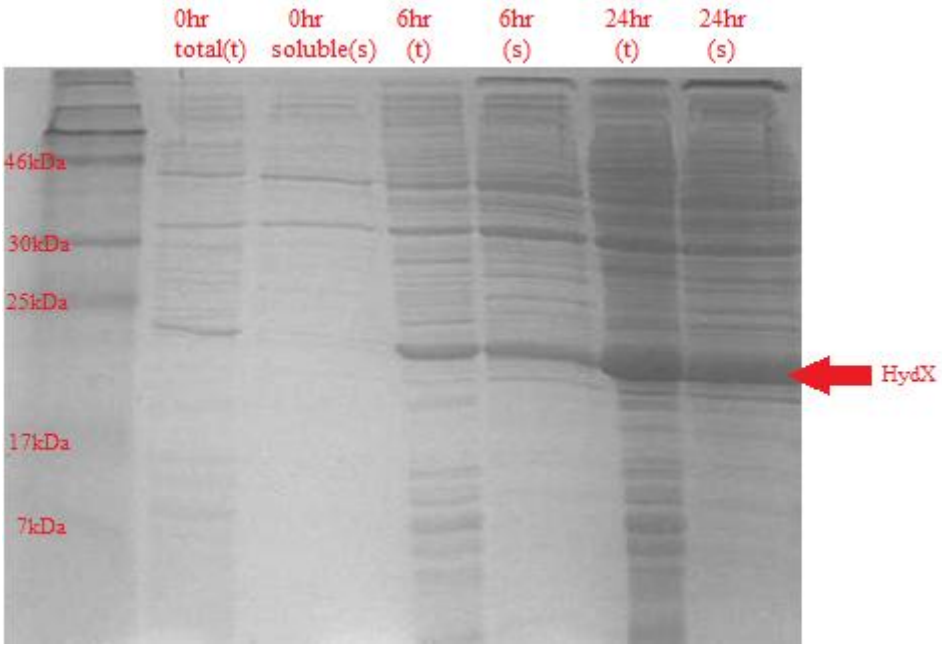

Figure 8: Determining the total and soluble protein expression of the C-Terminal his-tagged *hydx* gene: 18% SDS page gel run from left to right with 7-175 kDa protein ladder (NEB), the total and soluble protein content at 0 h, 6 h, and 24 h. The expressed band of protein appears just below the 25 kDa marker, which is where the HydX would be expected to be (24 kDa)

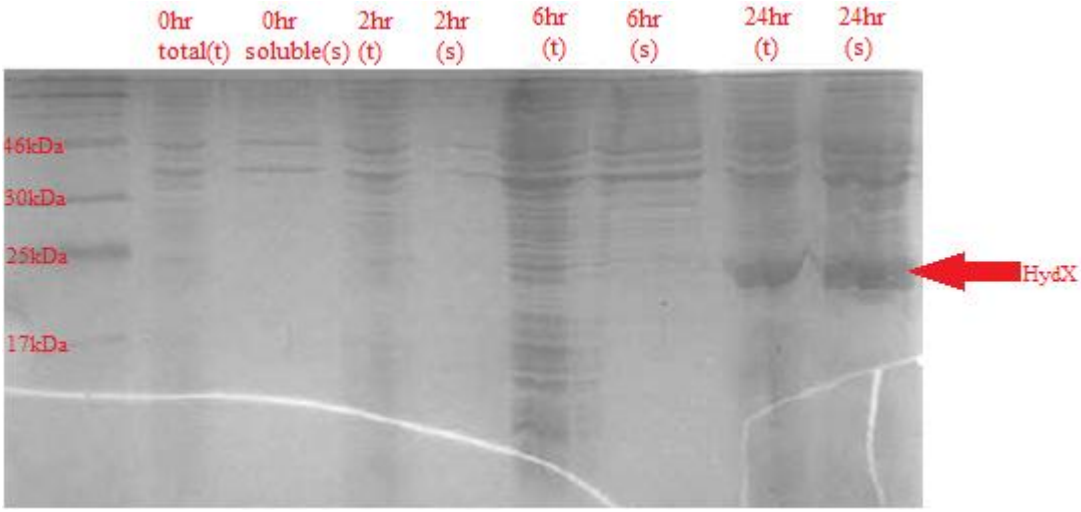

Figure 9: Determining the total and soluble protein expression of the untagged *hydx* gene 18% SDS page gel run from left to right with 7-175 kDa protein ladder (NEB), the total and soluble protein content at 0 h, 6 h, and 24 h. The expressed band of protein appears just below the 25 kDa marker, which is where the HydX would be expected to be (24 kDa)

The tagged protein was purified using a Ni-NTA column. The concentration of the HydX protein was determined by the absorbance at 280 nm and the molar extinction coefficient of 36440 $M^{-1}cm^{-1}$(ExPASy online bioinformatics resource portal). The untagged protein was purified in two steps using anion exchange and size exclusion chromatography. These purification methods resulted in a homogenous protein sample, supported by a singular distinct band when run on an 18% SDS-PAGE gel (Figure 10).

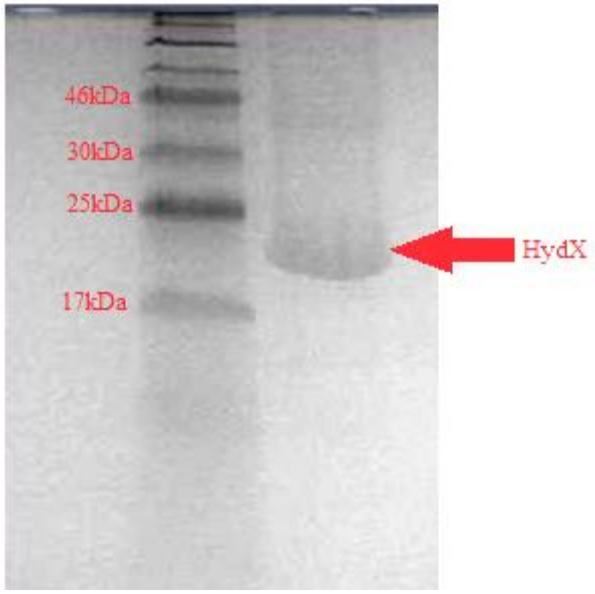

Figure 10: 18% SDS page gel of HydX after purification by anion exchange and size exclusion chromatography. The large band appearing at approximately 24 kDa corresponds to HydX protein. The sample is clean and devoid of other proteins of the host *E. coli* cells produced during the expression of HydX

The size exclusion chromatography was not only used as a purification method, but also as an analytical tool in determining the oligomeric state of HydX. By comparing the retention time of HydX against the molecular weights of the standards, the apparent molecular weight of

HydX was estimated to be 75.5 kDa. This indicated that HydX most likely exists in a trimeric oligomeric state (Figure 11).

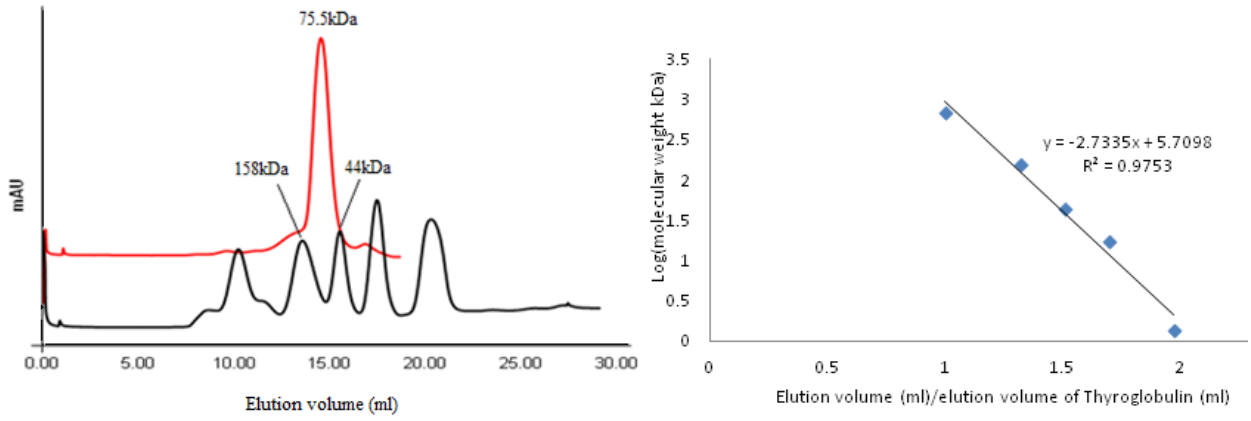

Figure 11: Determining oligomeric state of HydX with Size Exclusion Chromatography: A comparison of the elution time of HydX on a DEAE Sepharose 24ml column to the elution time of a molecular weight (mw) protein standard (Bio-Rad). The standard consisted of thyroglobulin-bovine (mw: 670 kDa), α-globulin-bovine (mw: 158 kDa), ovalbumin-chicken (mw: 44kDa), myoglobin-horse (mw: 17 kDa), and vitamin B_{12} (mw: 1.35 kDa) The HydX elution time is between that of the elution time for γ -globulin and Ovalbumin. By taking the logarithm of the standard proteins molecular weights and plotting against their respective elution's times in comparison of Thyroglobulin and the equation relating elution time and molecular weight was determined. The molecular weight of HydX, based on its elution time, was calculated to be 75.5 kDa. This molecular weight is approximately three times greater what is expected for HydX based on its amino acid composition (24 kDa) suggesting that HydX exists as a trimer.

The purified protein (tagged and untagged) were both analyzed using CD spectroscopy to determine the secondary structure of HydX and determine if the histidine tag altered the secondary structure of the protein. The results of the CD scan showed that the secondary structure of both the untagged protein and the histidine tagged protein showed primarily α-helical structure content, but the spectra of the two differed slightly when compared side by side (Figure 12).

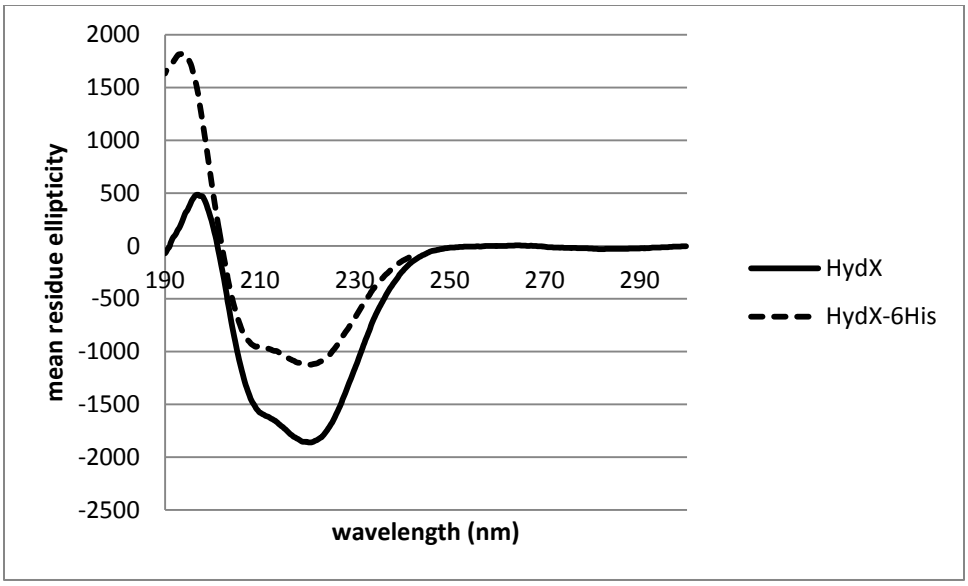

Figure 12: Determining Secondary Structure of HydX and HydX-6His with CD Spectroscopy: The CD spectrum taken from 190-260nm of HydX resembles that of an alpha helical curve. Differences in the spectrum of HydX-6His (dotted line) and the untagged HydX are observed however the general shape of both spectra correspond to primarily α-helical structure.

The stability of the HydX protein at room temperature was determined by a time course CD spectrum of the protein over the course of 72 h. A far-UV spectrum was recorded every 12 h (Figure 13). By the end of the third day the concentration of HydX had significantly decreased and visible precipitation was observed indicating that at room temperature the protein aggregated.

The

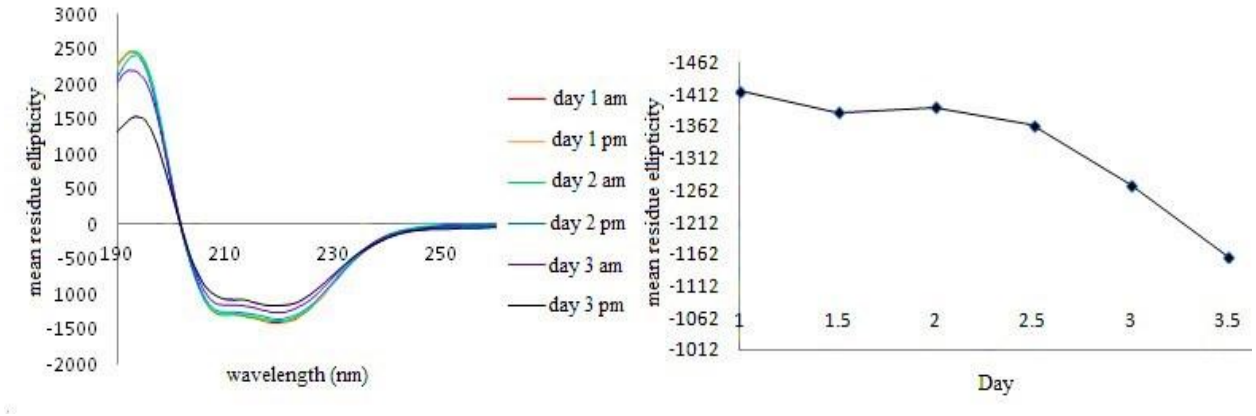

Figure 13: Determining aggregation of HydX protein at room temperature over 3 days: (Right figure)- By performing a time course CD analysis of a HydX protein sample that had been kept at room temperature conditions, the alpha helical secondary structure remained intact but the absorbance or amount of protein in the sample decreased. This is mostly likely due to aggregation of protein that appeared to be accumulating at the bottom of the sample vial as time went on. (Left figure)- The mean residue ellipticity was measured at 222nm to monitor the decrease in alpha helical composition of the protein at room temperature.

Another method to determine the stability of HydX was by thermodenaturation in the CD spectrophotometer. The sample was monitored at 222 nm as the temperature was gradually increased (Figure 14). The point at which 50% of the protein had unfolded (T_m) was determined by fitting the thermodenaturation curve to a Boltzmann equation (Origin software) and solving for the midpoint of the curve.

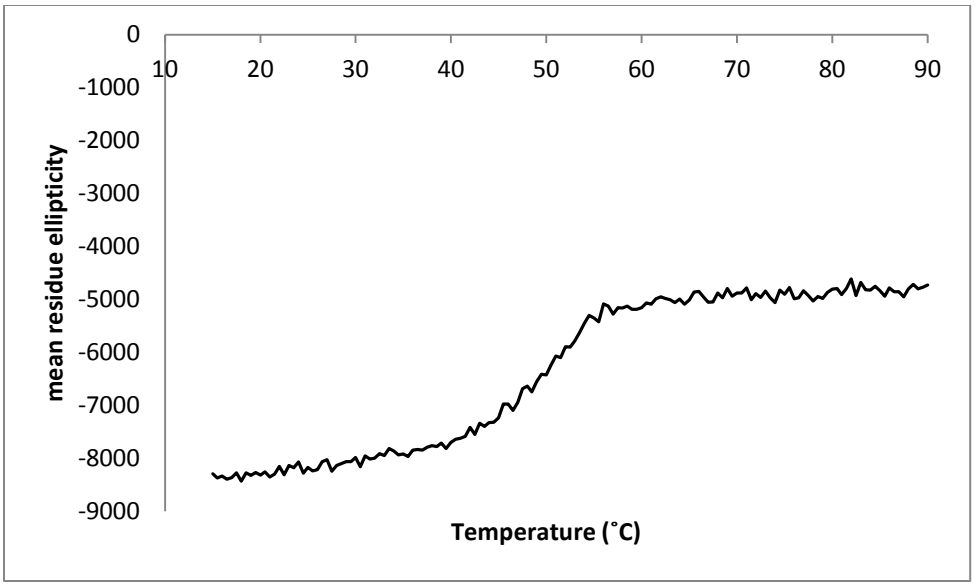

Figure 14: Determining T_m for HydX protein: The thermodenaturation curve of HydX from 15˚C to 90˚C was monitored at 222 nm. The data suggested a T_m of 48.5˚C.

Separately, the structure analysis of HydX by NMR spectroscopy was initiated. In collaboration with Professor Markus Germann of Georgia State University, a sample of HydX, expressed by *E. coli* host cells cultured on M9 minimal media in the presence of $^{15}NH_4Cl$, was analyzed by HSQC-NMR spectroscopy. Analysis of the resulting 2D spectrum indicated that the HydX protein is folded, but due to a possibly flexible structure the background noise ratio prevented the determination of specific amino acid assignments (Figure 15).

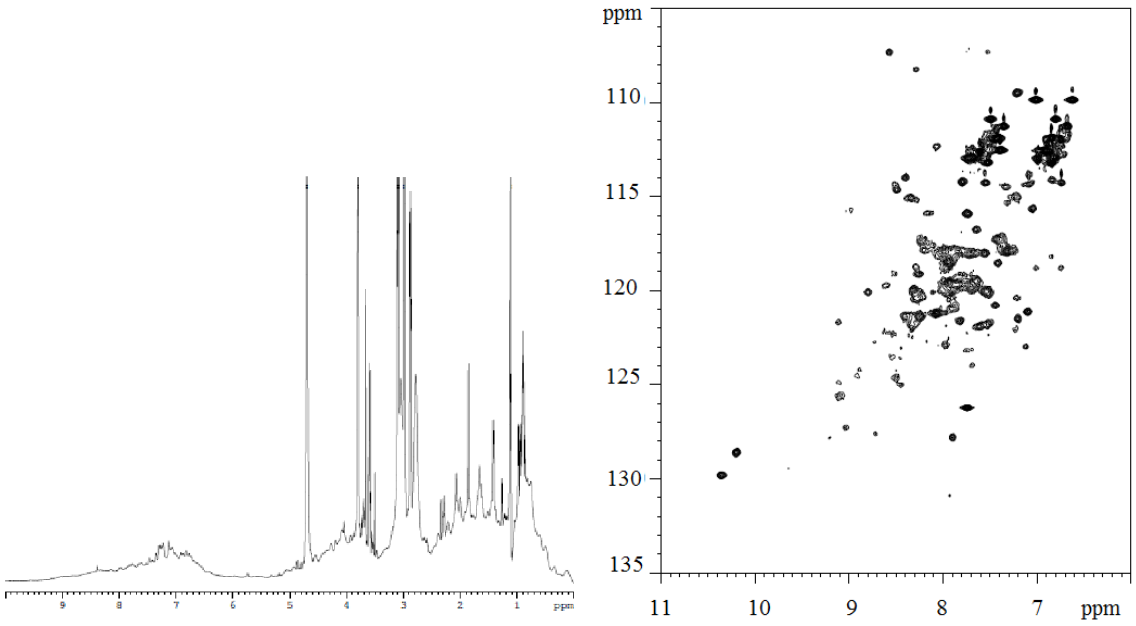

Figure 15: ^{1}H-^{15}N heteronuclear single quantum correlation (^{1}H-^{15}N-HSQC) of HydX: In the ^{1}H-^{15}N-HSQC spectrum one signal is expected to appear for each N-H bond in the amino acid sequence of HydX.

For the heterologous overexpression of HydE HydF HydG, an aliquot of pCDFDuet-*hydgx-ef* was transformed into *E. coli* BL21(DE3) cells. By taking samples over the course of expression, the increase in target protein from *hyde, hydf, and hydg* could be observed. The HydX protein did not express due to a stop codon after the *hydg* sequence. The optimal conditions for protein expression was determined to be overnight at 20˚C. From SDS-PAGE gel analysis for the culture samples, the resulting protein bands showed up between the 30 kDa and 58 kDa marker bands as expected for the 39 kDa HydE, 45 kDa HydF, and the 52 kDa HydG proteins (Figure 16).

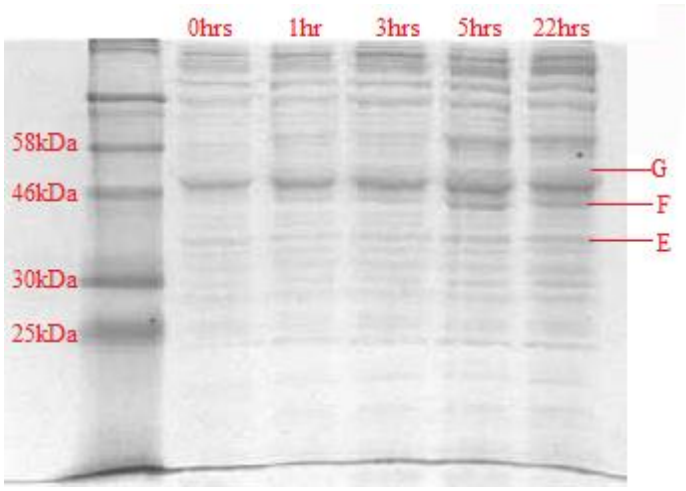

Figure 16: Expression of HydE, HydF and HydG: Soluble component of time course expression of HydE, HydF and HydG. Soluble protein bands appeared approximately 39 kDa, 45 kDa, and 52 kDa.

Subsequently, pull down experiments to determine HydX's interaction with HydE, HydF, and HydG were performed. Aliquots of the HydE, HydF, and HydG sample before it was loaded on the Ni-NTA column bound with HydX-6His, the flow-through after the sample had been loaded on the column, and the eluted sample was run on a 10% SDS-Page gel to determine at which stage the accessory proteins came off column (Figure 17). The gel indicated that HydG was the only accessory protein eluted with HydX that did not come off the column as flow-through as well. This suggests that HydG was retained by unspecific interactions with the column matrix or though protein-protein interactions with the HydX protein immobilized on the Ni-NTA column.

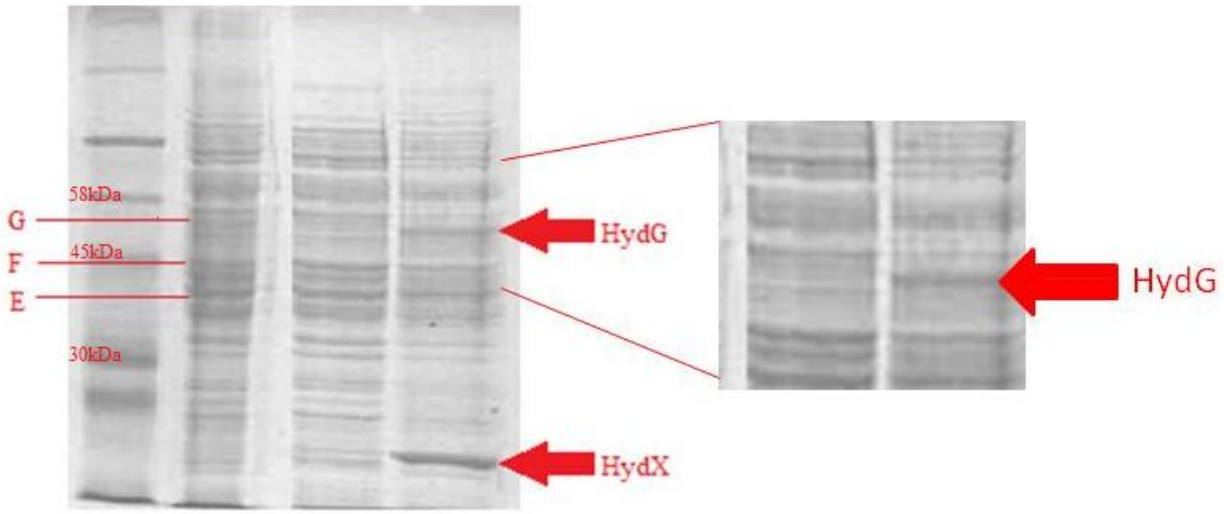

Figure 17: Pull down experiment to determine interaction between HydX and known accessory proteins: From left to right; Molecular weight marker, HydE HydF and HydG after purification by anion exchange, wash of Ni-NTA column with bound HydX, Elution of HydX and proteins bound to HydX or nickel column. The only accessory protein that appeared to only come off in the elution phase was HydG suggesting that HydG was retained due to unspecific interactions with Ni-NTA column or by protein-protein interactions with HydX.

Conclusions

The main findings of the experiments carried out in this thesis concerning the possible structure and function of HydX were the following:

I. Based on non-redundant BLAST searches, HydX is not homologous in structure or sequence to any known proteins.
II. Based on analysis by CD-spectroscopy, HydX's secondary structure is primarily α-helical, and the protein has a T_m of 48.5˚C.
III. Based on the elution time of HydX during size-exclusion chromatography, HydX exists in a trimeric state.
IV. Based on analysis by NMR-spectroscopy, HydX appears to be folded, but with a flexible structure.
V. Based on the pull-down experiment with HydE, HydF and HydG, protein-protein interactions between HydX and known accessory protein HydG are plausible.

From the findings in this thesis, the hypothesis that HydX is involved in the maturation of the active site in HydA is plausible, but not confirmed. However, the proposed protein folding along with the possible indication that protein-protein interactions occur between HydX and HydG provides substantial support that HydX is involved in the H-cluster assembly and that the characterization and function of HydX warrants further investigation.

Appendix

PCR-Protocol

Denaturation of 3 min at 95 ˚C and then cycled 20 times through a 30 sec at 95˚C denaturation step, a 30 sec at 50˚C annealing step, and a 1.5 min at 72˚C elongation step. Followed by 10 min at 72˚C and 10 min at 23˚C

Digestion-Protocol

500ng of both vector and amplified gene were digested with 0.5 µl of each restriction enzyme, NdeI(NEB) and XhoI(NEB) and 1 µl NEB- Buffer 4 in 10 µl reactions. The vector and gene were digested for 2 h at 37˚C followed by a 20 min kill stage at 65˚C.

Ligation-Protocol

The insert and vector containing sticky ends were ligated together with 1µl T4 DNA ligase (Promega) and 1µl T4 DNA ligase buffer in 10 µl reactions. The ligation reactions were done with ratios of insert to vector of 1:1 and 3:1 in 10 µl reactions. The reaction was set out overnight at 25˚C prior to transformation.

Transformation-Protocol

Ligation product was transformed into 50 µl DH5α /BL21(DE3) cells using an electroporation instrument set to 25 µF capacitance, 2.5 kV, and 200 Ω. After electroporation cells incubated with 500 µl LB medium for 30-60 min and then plated on LB plates with 100 µg/ml of the appropriate antibiotic.

Analysis of total and soluble protein expression (SDS-PAGE gel)-Protocol

Total protein content: the pellet was resuspended into 50 of 2x loading dye and 50 µl of water and boiled for 10 min. The suspension was then centrifuged for an additional 10 min and 10 µl from the supernatant was then loaded into an SDS page gel.

Soluble protein content: the pellets were resuspended in 100 µl of bug buster, 2% glycerol, 3 mM KCl, 0.1% tween, and 1 mM Tris-HCl(pH 8.0). The samples were rocked for 10min and then spun at 4˚C for 10 min. From the supernatant 10 µl were added to 10 µl of 2x loading dye and then heated for 3 min at 37˚C. After heating, the entire 20 µl solution was added to the SDS page gel was well. The samples were run a 220 mV for 45 min. To observe the gel bands, coomassie blue stain was added to the gel. After 1 h the stain was removed with 10% acetic acid destain.

Lysis Buffer-Recipe

To a total volume of 10 ml: 50 mM Tris-HCl(pH 8.0), 0.1% tween, 2% glycerol, 25 mM KCl, 1 µl/ml PMSF, 1 µl/ml Benzonase, and 20 mM NaCl.

M9 media Recipe[21]

First stock solutions of the following were made

- 10x M9 Salts: in 1L H_2O dissolve 67.8 g Na_2HPO_4, 30 g KH_2PO_4, 5 g NaCl. Autoclave.
- 10x NH_4Cl: in 1L H_2O dissolve 10 g NH_4Cl. Autoclave.
- 20% glucose: in 100 ml H_2O dissolve 20 g glucose. Autoclave.
- 0.1 M CaCl2: in 100 ml H_2O dissolve 1.11 g CaCl2. Autoclave.
- 1.0 M MgSO4: in 100 ml H_2O dissolve 24.6 g MgSO4. Autoclave.
- Thiamine solution: in 5ml H_2O dissolve 50 mg thiamine. Filter through 0.22 μm filter
- Biotin solution: in 5 ml H_2O dissolve 50 mg biotin. Filter through 0.22 μm filter
- $^{15}NH_4Cl$ solution: in 10 ml sterile and filtered H_2O dissolve 1 g of $^{15}NH_4Cl$
- Ampicillin (working concentration)

For 1 L M9 media (adjust concentrations for volume used): In a 2.8 L flask 100 ml 10x M9 salts, 100 ml of 10x NH_4Cl or 10 ml of $^{15}NH_4Cl$ solution, 15 ml 20% glucose, 1 ml of $CaCl_2$, 1ml $MgSO_4$, 1 ml thiamine, 1 ml biotin, 1 ml ampicillin, and Autoclaved H_2O to 1 L were combined.

Gene Sequences of Hydx, HydE, HydF, and HydG[24]

Hyd X: ATGGCATCTTATTTATTTTCTGAGCCCGGCGTGCAGTTGCATTTTGGCCTTAACACTAACGCCTTACTGA
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AGTAATTGCACCTCGGCTCAAGGACTTTGGCTGGAGTTTGATATCCTTGGTTTAGGCATTGCGCCAAACA
CCTTAATCGATTGGTTTGATACTGGCGTCCCCAGTGAGCAAAACTCAGCGGTGGCAATTGCCCTTGGCCG
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HydE: ATGATCACTCGCCCTAGCCCGCCGGCGCCAGTCACTCAGCCAACGTCCGTTAAACCGACGTTGCTTAACA
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HydF: ATGCGTTACCATATCGCCTTGGTCGGGCGGCGTAATTCGGGGAAATCCTCCTTACTCAATATGCTTGCAG
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HydG: ATGAGCACACACGAGCATCACTCCATTACACTTTCGGACTACAATCCCAACGTCAACTTTATCGACGATA
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GCAATGCGCTGATAGAGCGAGAGCTGGCTAAAATGAGCCCGTCACGGGCACGCAATGTGCGCGGCTGTTT
GCAAAAAACCGATGCGGGTGAACGGGATATCTATCTGTAA

hydx Primers

I. AGCTACATATGGCATGAATA
II. TCGATCTCGAGTCAGTGGTGGTGGTGGTGGTGTCATCTGTTAAA

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