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UNDERSTANDING THE NIFM DEPENDENCE OF NIFH IN AZOTOBACTER VINELANDII: FUNCTIONAL SUBSTITUTION OF NIFH BY A NIFH-CHLL CHIMERIC CONSTRUCT IN A NIFM^{-} STRAIN

By

Kelvin Harris, Jr.

A Thesis
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Master of Science
in Biological Sciences
in the Department of Biological Sciences

Mississippi State, Mississippi

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Candidate for Degree of Master of Science

Nitrogenase catalyzes the reduction of dinitrogen to ammonia. No other mutual protein has shown to substitute the NifH protein, which is functional in the presence of the *nif*-accessory protein NifM. The ChlL protein of *Chlamydomonas reinhardtii* shows significant homology and structural similarity with NifH. Our laboratory has shown that the ChlL can substitute the Fe protein in the absence of NifM. We have also shown that the NifM is a PPIase and the Pro-258 within the C-terminus of NifH is a suggested substrate for NifM. We hypothesized that we could generate a NifM-independent NifH-ChlL chimeric protein by replacing the C-terminus of NifH (that spans the substrate of PPIase) with that of ChlL. The chimeric gene was then transformed into the *nifM*$^-$ *Azotobacter vinelandii strain* AV98. The chimera was able to impart a Nif$^+$ phenotype to this *nifM*$^-$ strain. This result demonstrated that the NifH-ChlL chimeric protein is NifM-independent.

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Key words: Nitrogenase, *Chlamydomonas*, *Azotobacter*

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DEDICATION

I would like to dedicate my research and thesis to the man whose true gift has yet to be encountered by a society that has need of it. His true gift is to be a foundation the society can stand on.

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ACKNOWLEDGEMENTS

I would like to express personal thanks and gratitude first and foremost to my Lord and Savior Jesus Christ and to the saints at Peter's Rock Temple COGIC. Truly there were some difficult times during my graduate tenure and I would like to thank all of those people that unselfishly assisted me in completing this feat. Thanks to an eminent educator and scientist for his unfailing support and concern, my advisor, Dr. Nara Gavini. In addition, appreciation and respect to his brilliant wife and committee member, Dr. Lakshmi Pulakat, Dr. Zhaohua Peng and all the members of the Gavini-Pulakat Lab. I wish to express gratitude to Mrs. Gloria Blakenship, the driving force of the entire Department of Biological Sciences.

My family has been such an integral part of my success. I would like for them to know that this was possible because they never stop believing in and encouraging me. My biggest supporter, my mom, has really been my backbone and I want her to know that I honor her for all that she is to me.

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CHAPTER I

INTRODUCTION

Nitrogen

French chemist Laurent Antoine Lavoisier named nitrogen *azote*, a word meaning without life (21) . However, nitrogen is an essential component of proteins, nucleic acids, and other cellular constituents. The element is abundant in our atmosphere, making up about 78% of the volume of the earth's atmosphere. To add, the volume of the atmosphere of Mars is comprised of less than 3% of nitrogen (21). The abundant amount of nitrogen however is in a metabolically useless form known as molecular nitrogen (N_2) or dinitrogen. Most plants and other nitrogen-dependent processes aren't benefited due to this abundant form being metabolically useless, mainly due to a triple bond present between the two nitrogen atoms. The feat of breaking the triple bond is only accomplishable with a large amount of energy (21, 22). In order for plants to utilize their nitrogen it must appear in a distinct fixed form. For this particular reason, animals secure their nitrogen compounds from plants directly or indirectly (22).

Nitrogen Fixation

Nitrogen fixation is the process by which inert atmospheric nitrogen gas is converted into ammonia. In the form of ammonia the former nitrogen gas is

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metabolically useful for amino acids, proteins, vitamins, and nucleic acids (24). In order for the fixing of nitrogen to be initiated, there must be a breaking of the very strong afore mentioned, triple bond between the two nitrogen atoms. This action is very energy expensive. Nitrogen fixation is either accomplished biologically or non-biologically (22, 29).

<u>Biological Nitrogen Fixation</u>

Biological nitrogen fixation is an energy expensive process in which atmospheric nitrogen is converted into ammonia by the metalloenzyme nitrogenase. The microorganisms that complete this particular process are referred to as diazotrophs. These nitrogen fixing organisms function to produce the metabolically useful ammonia in this form of nitrogen fixation. Higher plants and animals secure their nitrogen ultimately from nitrogen-fixing organisms or from nitrogen fertilizers that are composed of nitrogen compounds produced from lightning strikes (22). The ability to fix nitrogen is found only in certain bacteria. Some of the bacteria, *Rhizobium* for example, live in symbiotic relationship with plants of the legume family such as alfalfa and soybeans (28). Some nitrogen-fixing bacteria, like *Azotobacter*, live free in the soil, whereas some nitrogen-fixing cyanobacteria like *Anabena azollae* live in pores on the fronds of a water fern called *Azolla* (27). The symbiotic partnership of the latter bacterium is essential in enriching rice paddies with organic nitrogen (22, 27). Biological nitrogen fixation is a complex process requiring a complex set of enzymes and a huge expenditure of ATP. It is better expressed in the following equation, which depicts that minimal stoichiometry for the

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primary reaction, N_2 reduction to NH_3 (18, 22, 32).

$$N_2 + 8e + 8H^+ + 16ATP > 16ADP + 16\ Pi + 2NH_3 + H_2 \quad (10)$$

The reaction is accomplished by enzyme nitrogenase. In biological nitrogen fixation, the conversion of nitrogen into ammonia is catalyzed by the nitrogenase enzyme system. Nitrogen-fixing organisms have a nitrogenase system that occurs in Molybdenum (Mo) and Iron (Fe) and Vanadium (V) forms (30, 35, 38, 45). Essentially, there are systems that contain molybdenum only as their metal center like *Klebsiella pneumoniae* (45). In addition, there are some organisms that possess nitrogenase systems that are Mo- and vanadium(Vn)-containing, which is evident in *Anabaena variabilis* (45, 50). Iron-only nitrogenases, like *Rhodobacter capsulatus* and *Rhodospirillum rubrum,* are very unstable and aren't abundantly accessible (45, 50). In addition, *Azotobacter vinelandii* is capable of synthesizing any of the three distinct nitrogenase systems (22, 49).

Non-Biological Nitrogen Fixation

Non-biological nitrogen fixation is comprised in two different forms:

Atmospheric Nitrogen Fixation

The enormous energy of lightning fractures nitrogen molecules and enables their atoms to combine with oxygen in the air forming nitrogen oxides. The nitrogen oxides dissolve in rain and form nitrates that are carried to the earth. The dynamics in atmospheric nitrogen fixation contribute approximately 5-8% of the total nitrogen fixed (22).

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Industrial Nitrogen Fixation

The complex process of industrial nitrogen fixation is completed by the cooperation of atmospheric nitrogen, a specific catalyst, a high temperature of 600° C, great pressure, and hydrogen gas, usually derived from natural gas or petroleum, to form ammonia (18, 22, 43). This process in the industry is known as the Haber-Bosch process. The ammonia accumulated here is further processed as urea and ammonium nitrates to be used as fertilizers (20, 21, 24). Approximately 80 x 10^9 kg of ammonia is manufactured annually by the Haber-Bosch process. However, despite the commercial significance of the Haber-Bosch process, biological nitrogen fixation represents an even larger annual production at an estimated 170 x 10^9 kg of ammonia annually (18, 29). The lower reaction temperature of the nitrogenase catalyzed reaction suggests a more efficient activation of nitrogen as well as a thermodynamic advantage by favoring ammonia synthesis (18).

Nitrogen is an essential nutrient that is found to be limiting to the growth of green plants. This deficiency is the result of processes such as microbial denitrification, soil erosion, leaching, chemical volatilization and the removal of nitrogen-containing crop residues from the land, which deplete the nitrogen that would otherwise be present in the soil (29). Nitrogen is emitted into the atmosphere by industrial companies, which in turn increase the amount of nitrate and nitrite supplies in the soil and water (25, 29). The main cause of the nitrate and nitrite addition is the extensive use of fertilizers. These compounds are known to have health effects such as Vitamin A shortages, decrease in oxygen carrying capacity in

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blood, and can even lead to cause cancer (25). The reduction of nitrogen requires an enourmous amount of energy, whether chemical or biologically reduced. The process in producing fertilizers utilizes vast amounts of fossil fuels, which are irreplaceable and eventually exhaustible, as an energy source. Biological nitrogen fixation utilizes energy from the oxidation of carbohydrates formed by the photosynthetic activity of green plants. The energy for photosynthesis of course is supplied from sunlight; therefore, the energy for biological nitrogen fixation is indirectly supplied by an available and inexhaustible source (29). Because of the afore mentioned reasons, the chemical components of biological nitrogen fixation are preferred and researched for observation of the molecular basis of this enzyme catalyzed reaction's efficient environmental, health and economical costs.

Nitrogen-Fixing Organisms

The entire group of nitrogen-fixing bacteria are prokaryotic, and they have the ability to fix nitrogen across bacterial species. There are two classes of these nitrogen-fixing organisms, and they are referred to as free-living bacteria and symbiotic bacteria.

Symbiotic Bacteria

Symbiotic bacteria are protected from oxygen by inhabiting a plant host. Examples of this group of nitrogen-fixing organism include *Rhizobium* and *Bradyrhizobium*. *Rhizobium* is found free in the soil, but it only fixes nitrogen when inside the root nodules of its host plant, in a strictly controlled microaerophilic environment (22, 28). The bacteria infects the nodules of leguminous plants, leading

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to the formation of lumps or nodules where nitrogen fixation occurs. The nitrogenase enzyme complex within the Rhizobium supplies a constant source of reduced nitrogen to the host plant and the plant gives nutrients and energy for the activites of the bacterium (26). Although oxygen is needed to generate sufficient respiratory energy to drive nitrogen fixation, an excessive amount would inactivate nitrogenase. The oxygen level within the root nodules is regulated by a special hemoglobin protein called leghemoglobin (28). Plant genes encode for the globin protein and symbiotic bacteria produces the heme cofactor. However, this occurs only when the plant is infected with the *Rhizobium*.

Free-Living bacteria

Free-living bacteria are a particular group of microorganisms that independently function. The subcategories of this group includes anaerobic bacteria and aerobic bacteria.

Anaerobic Bacteria

Anaerobic bacteria are a specific set of bacteria that fix nitrogen in the absence of oxygen. There are several species in this subcategory such as *Clostridium, Klebsiella, Green sulfur bacteria, Purple sulfur bacteria,* and *Desulphovibrio.*

Aerobic Bacteria

Aerobic bacteria fix nitrogen under extreme microaerophilic conditions, meaning that there is limited exposure to oxygen. This group includes such genus as *Azotobacter, Bierjerinckia*, and some *Klebsiella* and *Cyanobacteria.* One of the most

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popular in nitrogen fixation research models, *Azotobacter vinelandii,* is a free-living, soil-dwelling, aerobic microorganism with a wide variety of metabolic properties. *Azotobacter* has evolved to allow it to fix nitrogen aerobically despite the inherent oxygen-sensitivity of nitrogenase. It has uniquely high rates of respiration coupled with specific cytochromes to ensure that nitrogenase experiences an essentially anoxic environment, despite the fact that energy is derived from aerobic metabolism (8,22,41). This particular characteristic of *Azotobacter* affords it the capability of synthesizing all three genetically distinct forms of nitrogenase (37).

Nitrogenase

In general, the nitrogenase enzyme is composed of two oxygen labile and seperable metalloproteins, dinitrogenase (component I) and dinitrogenase reductase (component II). Component I contains the active site for N_2 reduction, with a molecular weight of approximately 240kDa, and is composed of two heterodimers. Component II is a 60-70 kDa homodimer coupling ATP hydrolysis to interprotein electron transfer (8). There are three genetically distinct nitrogenase systems, respectively named according to the active-site central metal present (50). These systems are referred to as the Nif (Molybdenum), Anf (Iron), and Vnf (Vanadium) nitrogenase systems (50). The regulatory genes *nifA, anfA*, and *vnfA* are necessary for the expression of the respective Mo, V and Fe nitrogenase (37). In addition, a fourth nitrogenase system has been recently discovered which is not phylogenetically related to the afore mentioned systems. This particular nitrogenase, which exhibits a different protein composition, is found only in *Streptomyces thermoautotrophicus* and

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carries a Mo-molybdopterin cytosine dinucleotide cofactor (Mo-MCD) as the active site (45).

Mo-Nitrogenase

The molybdenum-containing nitrogenase system, the most universally distributed in nature and most studied form, consists of the two oxygen-labile metalloproteins, MoFe protein (dinitrogenase) and Fe protein (dinitrogenase reductase) (8, 33, 48). The MoFe protein of this system, the larger of the two, consists of a P-cluster and a FeMo cofactor, the site of substrate binding and reduction; whereas, the Fe protein consist of a single cluster that functions as the electron donor (8, 33, 45). This system is encoded by the *nifHDK* genes. This conventional system is synthesized in the absence of a fixed nitrogen source when molybdenum is available at the nitrogenase active metal site.

P Cluster

The P cluster consists of two [4Fe-4S] clusters that are bridged by both the thio side chains of the two cysteine residues (Cysα88 and Cysβ95) and a disulfide bond between two of the cluster sulfurs (Figure 1.1). The P cluster is believed to participate in electron transfer between the [4Fe-4S] cluster of the NifH and the FeMo cofactor of the NifDK.

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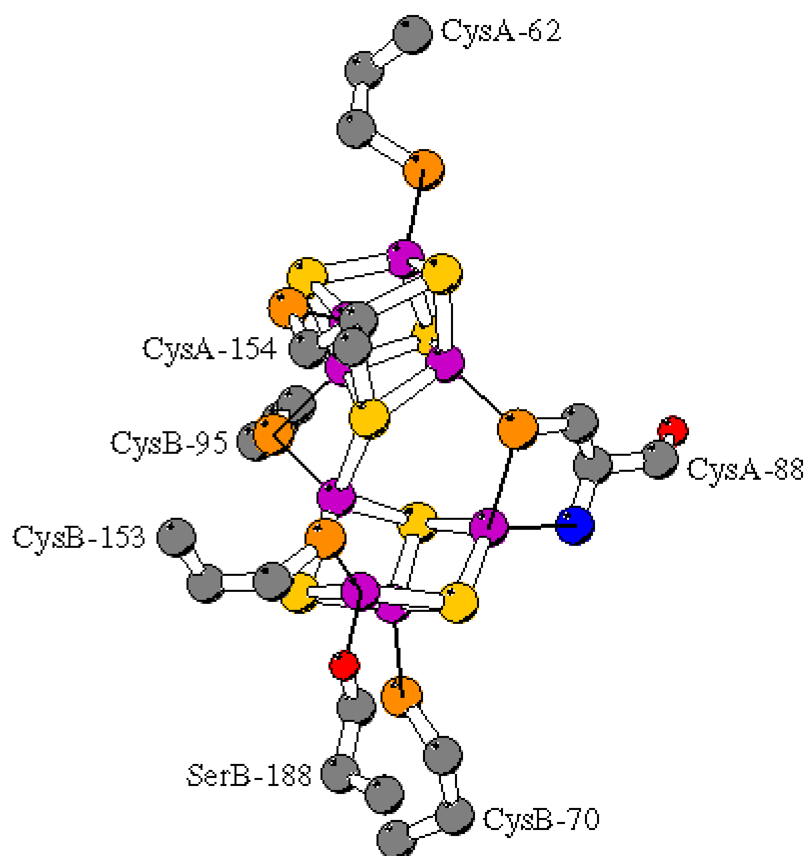

Figure 1.1 P cluster of the Nitrogenase MoFe Protein.

Structure is in its reduced state with environmental residues displayed as well (39).

FeMo Cofactor

The FeMo-cofactor, also referred to as the M-center or simply cofactor, was initially identified in 1977 as a stable metallocluster isolated from acid denatured MoFe protein. Much focus has been placed on the FeMo-cofactor since it contains molybdenum in a biologically unprecedented form and is believed to represent the site of substrate reduction (5, 8, 11, 12, 18, 30, 33, 35, 45, Figure 1.2).

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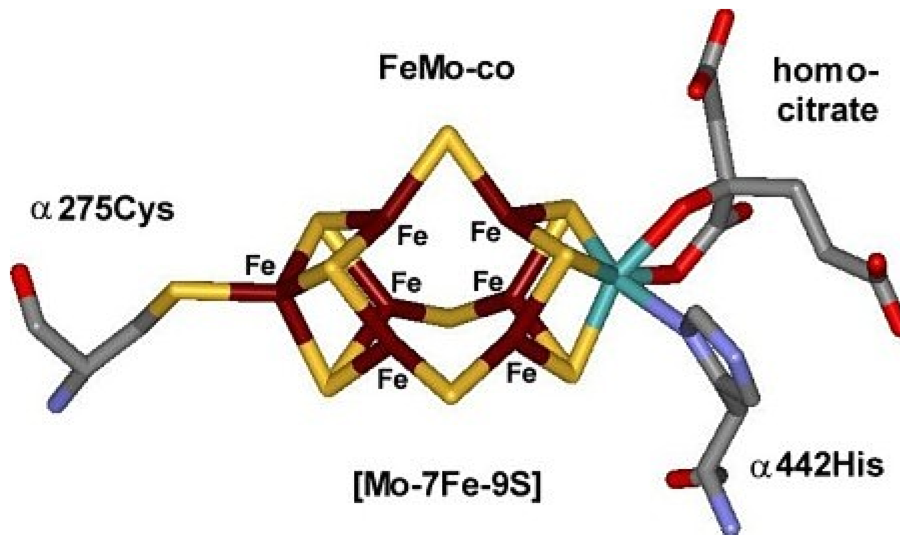

Figure 1.2 FeMo-Cofactor of the Nitrogenase MoFe Protein. (23)

Fe-Nitrogenase

The iron nitrogenase system contains two-segment proteins known as the FeFe protein or dinitrogenase and the Fe protein or dinitrogenase reductase (50). The FeFe component, encoded by the *anfD, anfG* and *anfK* genes, contains two metal clusters referred to as the M-cluster and the P-cluster. The M-cluster is representative of substrate reduction, whereas the P-cluster functions to transfer electrons to the M-cluster. The active Fe-nitrogenase complex was first purified from an *nifHDK* deletion strain of *A. vinelandii* that was grown in Mo- and V-deficient N-free medium (45, 50). Structural genes encoding Fe-nitrogenase and Mo-nitrogenase are organized as single operon, *vnfHDK* and *nifHDK* respectively (37, 50).

V-Nitrogenase

It was in 1986 that vanadium has been confirmed to play a role in biological nitrogen fixation after vanadium-containing nitrogenase was isolated from mutants of

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two species of *Azotobacter* (*A. chroococcum* and *A. vinelandii*) unable to synthesize Mo-containing nitrogenase (50). That particular study demonstrated how V-nitrogenase is accumulated under Mo deficient conditions in the presence of V (37, 50). The vanadium nitrogenase system consists of two proteins, the Fe protein and the vanadium iron (VFe) protein that alone consists of two polypeptides (50). The Fe protein is encoded by *vnfH.* The VFe protein is composed of two P-clusters and two FeV cofactors, and they are homologous to the P-clusters and FeMo cofactors of the MoFe protein. The V-nitrogenase system is encoded by the *vnfHDGK* genes and is detected in wild-type *A. vinelandii* in the presence of vanadium and the absence of molybdenum (37, 45, 50). In *A. vinelandii* and *A. chroococcum, vnfD* and *vnfK* genes are separated by the *vnfG* gene, which encodes an additional δ-subunit of the VFe protein that has no counterpart in the MoFe protein (50). However, the subunit is essential for V-nitrogenase function. It has been stated that the structural genes of V-nitrogenase are organized into two separate units allowing the two proteins to be expressed independently (50). Therefore, the Fe protein is expressed under Mo-deficient conditions in the presence or absence of V, where the VFe protein is expressed in the presence of V (50).

The Model Nitrogenase System

As mentioned, the most extensively characterized Mo-nitrogenase, consists of two metalloproteins, a smaller Fe protein and larger MoFe protein (15, 35, 50). The smaller Fe metalloprotein, is referred to as component II, or dinitrogenase reductase and is coded by the *nifH* gene. This protein will be referred to as NifH from now on.

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NifH is composed of two identical α subunits with a single [4Fe-4S] cluster bond between the subunits. It also contains two Mg-ATP binding sites, one on each subunit. The larger MoFe protein has been referred to as dinitrogenase, or component I. However, this protein will be referred to as NifDK from this point on. This protein is a heterotetramer that is composed of the gene products of two genes, the *nifD* and *nifK* genes.

NifDK

As mentioned, this is a 240kDa tetramer of the *nifD* and *nifK* gene products that contains two pairs of two complex metalloclusters known as the P-cluster and the iron molybdenum cofactor (FeMo-co). Each αβ pair of subunits of NifD and NifK contains one P-cluster and one molecule of FeMo-co. The FeMo-co is of interest because of its possession of molybdenum and its representation of the site for substrate reduction (30, 33, 44, 45). The FeMo-co is composed of homocitrate and a $MoFe_3$-S_3 cluster bridged to a Fe4-S3 cluster by three sulfur ligands (33, 45). The P-cluster is a [8Fe-7S] cluster with a structure similar to that of FeMo-co, which consists of two [4Fe-3S] cubanes connected by a central S atom (45). The P-clusters are located at the αβ subunit interface and are coordinated by cysteinyl residues from both subunits (33, 35). The P-cluster is believed to function in electron transfer between the [4Fe-4S] cluster of the Fe protein and the FeMo-co (45).

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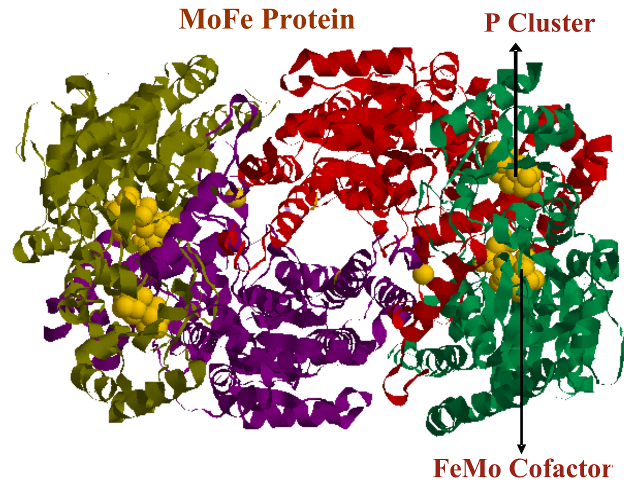

Figure 1.3 Ribbon Structure of the Nitrogenase NifDK Protein.

This structure, consisting of two P-clusters and two FeMo cofactors, is found in *A. vinelandii.*

<u>NifH</u>

The NifH protein is an important compound of nitrogenase, and no other structurally similar proteins or chemicals have been shown to be able to replace the role of NifH in nitrogenase thus far. The NifH is a 60kDa dimer of the product of the *nifH* gene, which contains a single [4Fe-4S] cluster at the subunit interface and two Mg-ATP-binding sites, one at each subunit. The NifH is the obligate electron donor to the NifDK. Electrons are transferred from the [4Fe-4S] cluster of the NifH protein to the P-cluster of the NifDK protein and in turn to the FeMo-co, the site of substrate reduction (14). In addition to the critical role the NifH protein plays in electron transfer to the NifDK, it has three other functions. First it is involved in the biosynthesis of the FeMo-cofactor, where there are at least seven *nif* genes known to

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be involved in this process (36, 37). Next function is the NifH's participation in the maturation of an inactive FeMo-cofactor-deficient NifDK protein (36, 41, 45). Finally, it has been suggested to be involved in regulating alternative nitrogenase systems.

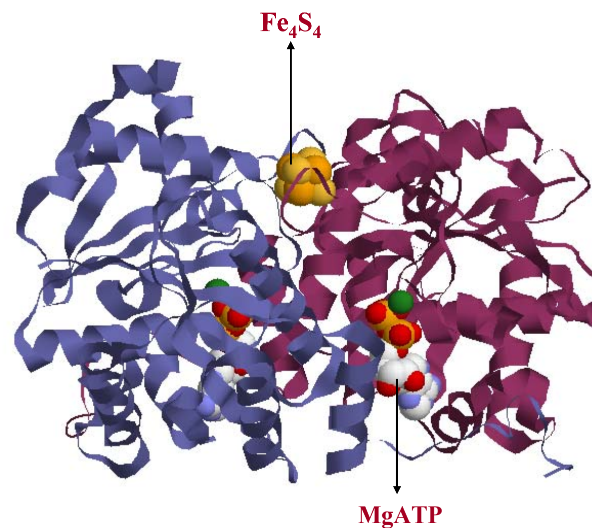

Figure 1.4 Ribbon Structure of the Nitrogenase NifH Protein.

This structure, which is present in *A. vinelandii*, contains a single [4Fe-4S] metal cluster and Mg-ATP binding site.

Although both nitrogenase components are irreversibly inactivated by the presence of oxygen, nitrogen-fixing organisms have developed different strategies to keep nitrogenase protected from oxygen. Some of the strategies include living in anaerobic environments, the depletion of intracellular oxygen by high respiratory activity when living in aerobic environments, or the development of specialized cells (heterocysts) that keep nitrogenase in a microaerobic environment (8, 24, 42).

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Mechanism of Nitrogenase

The mechanistic activity of the nitrogenase enzyme complex is at the protein level. It initially includes the formation of a complex between the reduced NifH with two bound ATP molecules and the NifDK. Secondly, electron transfer between the two proteins is coupled to the hydrolysis of ATP. Thirdly, there is a dissociation of the NifH accompanied by reduction and an exchange of ATP for ADP. Finally, there is a repetition of the previous cycle until sufficient numbers of electrons have been accumulated, in order for available substrates to be reduced (15, 20).

The dissociation between the NifH and NifDK plays an essential part in the mechanism of nitrogenase, being that it is the rate-determining step (18). The docking region of the two proteins is composed of specific residues of the metalloproteins (30). The docking region is the area for ATP hydrolysis coupled to electron transfer between the two proteins. The hydrolysis and electron transfer occurred through a series of conformational changes (36). At the initiation of the conformational changes, electrons transfer from the NifH to the P-cluster pair, followed by the electron transfer from the reduced P-cluster pair to the FeMo-co. After substrate binding at the FeMo-co, the oxidized NifH with MgADP bound, finally dissociates from the NifDK. Recent studies dealing with the biochemical and structural characterization of the cross-linked complex of nitrogenase have reported the specificity of the residues involved in the transient complex formation between the NifH and NifK proteins. It was shown that only Glu112 from one of the two NifH subunits and Lys400 of the NifK subunit were cross-linked, in spite of these

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residues being surrounded by numerous other charged residues that could potentially participate in this process (47). Other studies that substituted Ala in place of Phe125 of the α and β subunits of the NifDK protein, separately and in combination, showed that the doubly substituted NifDK was unable to form a tight complex with the MgADP-$A1F_4^-$ treated NifH or when using the altered 127ΔNifH (47). Thereby, suggesting that the Phe125 residues were involved in an early event(s) that occurred upon component protein docking and could be involved in eliciting MgATP hydrolysis (47).

Background on Nitrogenase Research

Nitrogen fixation has always been considered of fundamental importance, not only for its significance in global nutrition, but of the relevance of nitrogenase as a model system for examining processes such as multiple electron oxidation reduction reactions, complex biological metal assembly, and even nucleotide-dependent signal transduction. Over the past century, researchers have sought after an economically efficient avenue of fixing atmospheric nitrogen for use in fertilizers, explosives, and other essential chemicals. The development of an energy efficient, large scale process for nitrogen fixation was a significant chemical challenge until the introduction of the Haber-Bosch process. In recent years however, researchers worldwide have been aiming to engineer plants with the genes encoding the nitrogenase enzyme in order to generate plants that could fix nitrogen on their own. Accomplishing this feat would be monumental for plants, allowing them independence from nitrogen fixing bacteria and advancing their growth and yield.

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One complication that underlies this transformation would be the large size of the genes encoding the nitrogenase enzyme complex not being able to clone into the plant system. To add, Cheng *et al* states that the transformation appears problematic because nitrogen fixation is not compatible with the photosynthetic oxygen evolution (5, 12). Several experiments have thus been aimed at reducing the size of the *nif* complex in order to expedite this transformation.

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CHAPTER II

GENETICS OF NITROGENASE

Azotobacter vinelandii and the *nif* Gene Cluster

In order for biological nitrogen fixation to occur in *A. vinelandii*, there are about 15 *nif* (nitrogen fixing) genes required. The *nif* genes are clustered together in a genome and are referred to as the *nif* regulon. The *nif* genes include *nifH, nifD, nifK, nifT, nifY nifE, nifN, nifX, nifU, nifS, nifV, nifW, nifZ, nifM,* and *nifF* (31, 45). These *nif* genes have different functions and roles in the nitrogenase enzyme complex. Twelve other potential genes, or ORFs, whose expression are potentially subjected to nif-specific regulation are found interspersed among the identified nif-specific genes within the *nif* gene cluster. More details on *Azotobacter* were stated previously in introduction.

NifH, NifD, NifK

The *nifH, nifD* and *nifK* are the structural genes of the *nif* gene cluster. The *nifH* gene encodes for the NifH or dinitgrogenase reductase. The NifH participates in the transfer of electrons to the NifDK protein, biosynthesis of the FeMo-co, and the maturation of the apodinitrogenase (41). Apodinitrogenase is dinitrogenase lacking a FeMo-co, but containing the P-cluster. The *nifD* and *nifK* genes code for the α and

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the β subunits of the NifDK. Both the NifD and NifK proteins are associated with electron transfer and substrate reduction (8, 20, 41).

NifM

The *nifM* gene is an accessory gene located in the *nif* gene cluster that is transcriptionally regulated by *nifA* (18). The *nifM* gene of *A. vinelandii* is 879 bp and encodes 292 amino acids (40). The formation and maturation of an active and properly functioning NifH protein is reliant upon production of the *nifM* gene product NifM (13, 14, 19, 45). *nifM* is a nitrogen fixing gene that is identified by its requirement for the proper maturation of the NifH. However, the role of the *nifM* product in the maturation of the NifH component of nitrogenase is obscure. Jacobson et al compared the *nifM* sequences of *K. pneumoniae* and *A. vinelandii* and found an overall amino acid sequence identity of only 16% (31). This sequence identity was confined to the carboxyl terminal third of the polypeptides, and the sequences that made up the amino terminal two-thirds of the polypeptides shared very little sequence identity (31). The homology suggests that functional similarities are confined to the carboxyl terminal region of these gene products. After comparing the carboxyl regions of the *nifM* genes and generating a consensus sequence, the carboxyl terminal region of the NifM proteins were shown to have significant homology with a certain family of proteins, called the peptidyl-prolyl *cis/trans* isomerases (13, 14, Fig. 2.1).

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```
PpiC_in_Azotobacter_vineland      -MASERLADGDSRYYLLKVAHEQFGCAP-GELSEEQLQQADRIIGRQRHI
NifM_in_Azotobacter_vineland      -MASERLADGDSRYYLLKVAHEQFGCAP-GELSEDQLQQADRIIGRQRHI
NifM_of_Azotobacter_chroococ      -MSFEHPGDGDSRYYLLKIAHEQFGCAP-GELSEEQLQQAERIIGRQKHI
NifM_of_Klebsiella_pneumonia      -------MNPWQRFARQRLARSRWNRDP-AALDPADTPAFEQAWQRQCHM
PpiC_of_Arabidopsis_thaliana      ---MFRVTGTLSAASSPAVAAASFSAALRLSITPTLAIASPPHLRWFSKF
SurA_in_Escherichia_coli          --------------------------------------------------
PrtM_of_Lactococcus_lactis        MKKKMRLKVLLASTATALLLLSGCQSNQTDQTVATYSGGKVTESSLYKEL
Orf_of_Bacillus_subtillis         -MPLVSMTEMLNTAKEKGYAVGQFNLNNLEFTQAILQAAEEEKSPVILGV

PpiC_in_Azotobacter_vineland      EDAV--LR-SPDAIGVVIPPSQLEEAWAHIASRYESPEALQQALDAQALD
NifM_in_Azotobacter_vineland      EDAV--LR-SPDAIGVVIPPSQLEEAWAHIASRYESPEALQQALDAQALD
NifM_of_Azotobacter_chroococ      EDAV--LR-CPDAAGVVIPASQIEEAWTQIANRYESAEALQQALDAQGLE
NifM_of_Klebsiella_pneumonia      EQTI--VA-RVPEG--DIPAALLEN----------IAASLAIWLDEGDFA
PpiC_of_Arabidopsis_thaliana      SRQF--LGGRISSLRPRIPSPCPIRLSG------FPALKMRASFSSGSSG
SurA_in_Escherichia_coli          --------------------------------------------------
PrtM_of_Lactococcus_lactis        KQSP--TTKTMLANMLIYRALNHAYGKSVSTKTVNDAYDSYKQQYGENFD
Orf_of_Bacillus_subtillis         SEGAGRYMGGFKTVVAMVKALMEEYKVTVPVAIHLDHGSSFESCAKAIHA

PpiC_in_Azotobacter_vineland      AAGMRAMLARELRVEAVLDCVCAGLPEISDTDVSLYYFNHAEQFKVPAQH
NifM_in_Azotobacter_vineland      AAGMRAMLARELRVEAVLDCVCAGLPEISDTDVSLYYFNHAEQFKVPAQH
NifM_of_Azotobacter_chroococ      RVGMRAMLARELKVQAVLDCICAGLPEISDTDVSLYYFNHAEQFKVPARH
NifM_of_Klebsiella_pneumonia      PPERAAIVRHHARLELAFADIARQAPQPDLSTVQAWYLRHQTQFMRPEQR
PpiC_of_Arabidopsis_thaliana      SSASREILVQHLLVKNNDVELFAELQKKFLDGEEMSDLAAEYSICPSKKD
SurA_in_Escherichia_coli          -------------MKN-WKTLLLGIA-----------MIANTSFAAPQVV
PrtM_of_Lactococcus_lactis        AFLSQNGFSRSSFKESLRTNFLSEVALKKLKKVSESQLKAAWKTYQPKVT
Orf_of_Bacillus_subtillis         GFTSVMIDASHHPFEENVATTAKVVELAHFHGVSVEAELGTVGGQEDDVI
                                                 :
PpiC_in_Azotobacter_vineland      KARHILVTINEDFPENTREAARTRIETILKRLRGKPERFAEQAMKHSECP
NifM_in_Azotobacter_vineland      KA-HILVTINEDFPENTREAARTRIETILKRLRGKPERFAEQAMKHSECP
NifM_of_Azotobacter_chroococ      KARHILVTINEDFPENTREAARTRIEAILKRLRGKPERFAEQAAKHSECP
NifM_of_Klebsiella_pneumonia      LTRHLLLTVDND-----REAVHQRILGLYRQINASRDAFAPLAQRHSHCP
PpiC_of_Arabidopsis_thaliana      GGILGWVKLGQMVPEFEEAAFKAELNQVVR-CRTQFGLHLLQVLSEREPV
SurA_in_Escherichia_coli          DKVAAVVNNG--------VVLESDVDGLMQSVKLN----AAQARQQLPDD
PrtM_of_Lactococcus_lactis        VQHILTSDEDTAKQVISDLAAGKDFAMLAKTDSIDTATKDNGGKISFELN
Orf_of_Bacillus_subtillis         AEGVIYADP----KECQELVERTGIDCLAPALGSVHGPYKGEPNLGFKEM
                                                      .    .  :
PpiC_in_Azotobacter_vineland      TAMQGGL-LGEVVPGTLYPELDACLFQMARGELSPVLES-----PIGFHV
NifM_in_Azotobacter_vineland      TAMQGGL-LGEVVPGTLYPELDACLFQMARGELSPVLES-----PIGFHV
NifM_of_Azotobacter_chroococ      TAMQGGL-LGEVVPGTLYPELDACLFQMAQGQLSPVLES-----PIGFHV
NifM_of_Klebsiella_pneumonia      SALEEGR-LGWISRGLLYPQLETALFSLAENALSLPIAS-----ELGWHL
PpiC_of_Arabidopsis_thaliana      KDIQVEE-LHSKMQDPVFMDEAQLIDVREPNEIEIASLPGFKVFPLRQFG
SurA_in_Escherichia_coli          ATLRHQI-MERLIMDQIILQ------------------------------
PrtM_of_Lactococcus_lactis        NKTLDAT-FKDAAYKLKNGDYTQTPVKVTDGYEVIKMINHPAKGTFTSSK
Orf_of_Bacillus_subtillis         EEIGKSTGLPLVLHGGTGIPTADIKKSISLGTAKINVN------------
                                          :
PpiC_in_Azotobacter_vineland      LYCESVSPARQLTLEEILPRLRDRLQLRQRKAYQRKWLESLLQQNATLEN
NifM_in_Azotobacter_vineland      LYCESVSPARQLTLEEILPRLRDRLQLRQRKAYQRKWLVCLLQQNATLEN
NifM_of_Azotobacter_chroococ      LFCESVSTARQLTLEEILPRLRDRLQLRQRKAYQRKWLESLLQQNATLEN
NifM_of_Klebsiella_pneumonia      LWCEAIRPAAPMEPQQALESARDYLWQQSQQRHQRQWLEQMISRQPGLCG
PpiC_of_Arabidopsis_thaliana      TWAPDITSKLNPEKDTFVLCKVGGRSMQVANWLQSQGFKSVYNITGGIQA
SurA_in_Escherichia_coli          --------------------------------------------------
PrtM_of_Lactococcus_lactis        KALTASVYAKWSRDSSIMQRVISQVLKNQHVTIKDKDLADALDSYKKLAT
Orf_of_Bacillus_subtillis         --TENQISSAKAVRETLAAKPDEYDPRKYLGPAREAIKETVIGKMREFGS

PpiC_in_Azotobacter_vineland      LAHG--------
NifM_in_Azotobacter_vineland      LAHG--------
NifM_of_Azotobacter_chroococ      LAHG--------
NifM_of_Klebsiella_pneumonia      ------------
PpiC_of_Arabidopsis_thaliana      YSLKVDPSIPTY
SurA_in_Escherichia_coli          ------------
PrtM_of_Lactococcus_lactis        TN----------
Orf_of_Bacillus_subtillis         SNQA--------
```

Figure 2.1 Comparison of the Consensus Sequence of NifM to the Amino Acid Sequences of Peptidyl-Prolyl Isomerases.

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The homology is very evident throughout this entire sequence, which spans the carboxyl terminal regions of these proteins. The analysis was performed using the Clustal W Alignment using SDSC (San Diego Supercomputer Center) Biology Workbench. Peptidyl-prolyl *cis/trans* isomerases (PPIases) are said to be involved in assisting protein folding. These proteins catalyze the cis/trans isomerization of the peptidyl-prolyl peptide bond in oligopeptides and proteins, which is a rate-limiting step in the process of protein folding essential for generating functional proteins (13,14). Gavini *et al* demonstrated that comparison of NifM and predicted amino acid sequences showed three regions of significant amino acid similarity between the PpiC protein and the *Bacillus subtilis* lipoprotein PrsA, the *E. coli* protein SurA and the NifM proteins (Figure 5, 14). Based on these observations it was proposed that one function of the NifM protein could be to assist the proper folding of the NifH protein (14).

Peptidyl-Prolyl *cis/trans* Isomerases

Peptidyl-prolyl *cis/trans* isomerases (PPIases) enhance the rate of refolding of slowly folding forms of denatured proteins by playing a vital role in catalyzing the *cis/trans* isomerization of the peptidyl-prolyl peptide bond in oligopeptides and proteins (13, 14). Figure 2.2 demonstrates the functioning of the enzyme changing the conformation of a peptidyl-prolyl bond from *cis* to *trans.* The functioning of this enzyme is a rate-limiting step in the process of protein folding and is essential for generating functional proteins (14). PPIases are regulators of several biological functions, including, cell cycle progression, inhibition of apoptosis, carcinogenesis,

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association with hormone receptors and bacterial infection (14). The PPIase family consists of three subfamilies, the cyclophilins, the FK506 binding proteins and the parvulins. NifM, which is necessary for maturation, stability and activity of NifH, is a PPIase and is relative to the parvulin subfamily (14). The same study showed through a consensus peptide sequence of the NifH protein derived from comparing different NifH proteins that there exists seven fully conserved proline residues (14). Respectively, it is reasonable to assume that the stability and activity of the protein will largely depend upon the proper conformation of peptidyl-prolyl bonds in this protein.

For correct docking onto the NifDK protein, the NifH protein requires a stable conformation and orientation that is achievable only if it is folded and aligned properly (14, 36). Figure 2.1 displayed a consensus sequence comparison of NifM to other peptidyl-prolyl *cis-trans* isomerases, and clearly shows the significant homology of the related proteins.

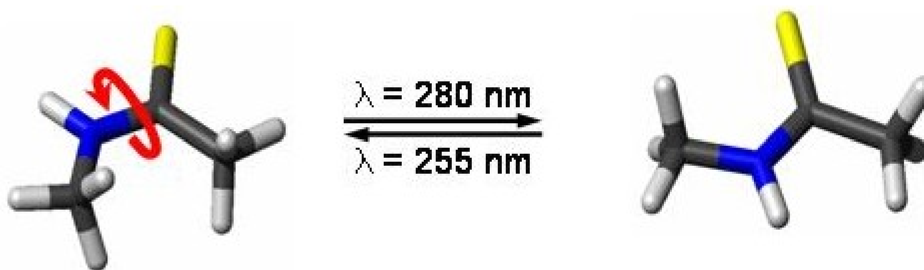

Figure 2.2 *cis*/*trans* Isomerization of the Peptidyl-Prolyl Bond.

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Proline Residue

Nineteen of the twenty amino acids form amide peptide bonds, whereas proline residues have an intrinsic probability to form imidic peptide bond. Due to the imidic structure, prolyl bonds have a particular electron distribution and steric structure. Proline restricts the conformational space for the peptides due to its geometry. Studies have shown that the folding intermediates have accumulated due to slow isomerisation of the prolyl peptide bonds. Therefore, it can be said that the amino acyl proline *cis-trans* isomerisation may become the rate-limiting step in protein folding. The amino acyl proline isomerisation not only limits protein folding and activity, but also determines the protein stability (47). For it's docking onto the NifDK protein, the NifH protein should have a stable conformation and orientation, which is possible if the NifH is folded and aligned properly (47). The NifH protein contains seven conserved proline residues. It is expected that the proline residues play a major role in the conformation and folding of the nascent NifH protein, due to the unique nature of the proline residue.

NifM-Mediated Activation of NifH

The activity of the *nifM* gene product is essential for the production of the NifH protein. The *nifM* gene product is required for the accumulation of the active NifH in *K. pneumoniae* (40). The carboxyl terminus of the NifM protein has been shown to have a significant homology with the PPIase family of proteins (13, 14, 40). The first discovered parvulin PPIase is a bacterial parvulin isolated from *E.coli*, known as PpiC (16). A multiple sequence alignment of the PpiC protein revealed that

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it shares significant homology with *Bacillus subtilis* lipoprotein PrsA, *E. coli* protein SurA and NifM protein from the nitrogen-fixing bacteria (16). Our laboratory has shown through a protease-coupled assay that NifM has a PPIase activity comparable to that of PpiC of *E.coli* (13, 14). Since NifM is a PPIase and has a beneficial effect on NifH, it's safe to predict that one or more of the conserved proline residues present in NifH could be a substrate for NifM. While constructing a PPIase-independent NifH mutant in *Azotobacter vinelandii,* Gavini *et al* were able to differentiate a proline-peptide bond (Pro 258) in NifH that serves as the substrate for the PPIase activity of NifM (14, 38). Considering this, we concluded that the NifM induces a conformational isomerization of the NifH protein and in doing so stabilizes and orients it in a way that docks it onto the NifDK protein and subsequently transfers the electrons. This conformational isomerization is associated with the PPIase domain of the NifM protein. Therefore, the presence of an intact and competent NifM is vital in maintaining the functional activity of the nitrogenase enzyme. Several studies have demonstrated, using structural analysis and consensus sequences, the importance of the NifM protein in relation with proper nitrogenase activity (13, 14, 19, 38, 40, 45). Studies on various accessory and functional proteins of nitrogenase have shown that deletion or mutation of the NifM protein results in a *nif-* phenotype (13, 14, 19, 40). It is certain that the NifM protein is a substrate for NifH, and that NifH is NifM-dependent in order for the nitrogenase to be functional and biologically fix nitrogen.

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ChlL Comparison to NifH

ChlL, a structurally similar protein that can function like NifH was observed as mutual protein expected to be capable of NifH characteristic. The *Chlamydomonas* species is a green algae, meaning that it is photosynthetic. Photosynthetic organisms use either of two different enzymes in order to carry out the formation of the reduced chlorophyllide from protochlorophyllide, the key intermediate in the biosynthesis of chlorophylls and bacteriochlorophylls (5). The one enzyme that we are interested in is the light-independent (dark) protochlorophyllide reductase (DPOR), of which photosynthetic bacteria, algae, and gymnosperms possess and thus are capable of synthesizing bacteriocholorophylls (Bchl) and chlorophylls (chl) (4, 5). The *Chlamydomonas reinhardtii* strain uses different genes to carry out the protochlorophyllide reduction referred to as *chlL*, *chlN*, and *chlB* (5). In an *in vitro* study at Indiana University, researchers showed that amino acid analysis demonstrated a significant sequence similarity BchL/ChlL, BchN/ChlN, and BchB/ChlB subunits of DPOR and the nitrogenase subunits NifH, NifD, and NifK (12). Of even more significance is the structural similarity of DPOR and nitrogenase that is apparent of the BchL/ChlL and NifH, of which there is 50% similarity and 33% overall identity (5, 12). The figure below shows the amino acid sequence homology between the ChlL protein of *C. reinhardtii* and the NifH of *A. vinelandii* (Figure 2.3).

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ChlL_of_Chlamydomonas_reinha     --MKLAVYGKGGIGKSTTSCNISIALAK-RGKKVLQIGCDPKHDST-FTL
NifH_in_Azotobacter_vineland     MTRKVAIYGKGGIGKSTTTQNTAAALAYFHDKKVFIHGCDPKADSTRLIL
                                    *:*:***********: * : ***  :.***:  ***** *** : *

ChlL_of_Chlamydomonas_reinha     TGFLIPTIIDTLSSKDYHYEDIWPEDVIYGGYGGVDCVEAGGP-PAGAGCG
NifH_in_Azotobacter_vineland     GGKPQETLMDMLRDK--GAEKITNDDVIKKGFLDIQCVESGGPEP-GVGCA
                                  *    *::* * .*    *.*  :***  *: .::***:*** * *.**.

ChlL_of_Chlamydomonas_reinha     GYVVGETVKLLKELNAFF-EYDVILFDVLGDVVCGGFAAPLN--YADYCI
NifH_in_Azotobacter_vineland     GRGVITAIDLMEENGAYTDDLDFVFFDVLGDVVCGGFAMPIRDGKAQEVY
                                 *  *  ::.*::* .*:  : *.::************* *:.   *:

ChlL_of_Chlamydomonas_reinha     IVTDNGFDALFAANRIAASVREKARTHPLRLAGLIGNR---TSKRDLIDK
NifH_in_Azotobacter_vineland     IVASGEMMAIYAANNICKGLVKYAKQSGVRLGGIICNSRKVDGEREFLEE
                                 **:.. : *::***.*. .: : *:   :**.*:* *     .:*:::::

ChlL_of_Chlamydomonas_reinha     YVEACPMPVLEVLPLIEEIRISRVKGKTLFEMSNKNNMTSAHMDGSKGDN
NifH_in_Azotobacter_vineland     FTAAIGTKMIHFVPRDNIVQKAEFNKKTVTEFAPEENQAKEYG-------
                                 :. *    ::..:*  : :: :..: **: *:: ::* :. :

ChlL_of_Chlamydomonas_reinha     STVGVSETPSEDYICNFYLNIADQLLTEPEGVI--PRELADKELFTLLSDFY
NifH_in_Azotobacter_vineland     -------------------ELARKIIENDEFVIPKP--LTMDQLEDMVVKYG
                                                    ::* ::: : * ***:*  *: .:*  :: .:

ChlL_of_Chlamydomonas_reinha     LKI
NifH_in_Azotobacter_vineland     IAD
                                          :
```

Figure 2.3 Sequence Homology of the ChlL of *Chlamydomonas reinhardtii* and the NifH of *Azotobacter vinelandii*.

As displayed in this figure, these two proteins show a 50% similarity and a 33% overall amino acid identity. However, they have the least homology in their C-terminal regions. This analysis was performed using the Clustal W Alignment using SDSC (San Diego Supercomputer Center) Biology Workbench (http://workbench.sdsc.edu/).

Another noteworthy case between NifH and ChlL would be the ATP-binding motif and two cysteine residues that conform the [4Fe-4S] cluster that are completely conserved among the NifH and are projected to be analogous with the BchL/ChlL proteins (3, 5, 12). This suggests that the BchL/ChlL protein catalyzes the transfer of electrons, dependent of ATP, from a certain compound to a reactionary group; therefore, its role is somewhat identical to that of the NifH. In studying the reconstitution of DPOR from purified Bchl and BchN-BchB subunits, scientists also

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found that the DPOR protein was dependent upon all three of its subunits, ATP and the reductant dithionite supporting the notation that the NifH and ChlL are similar in structure and function (5, 12).

Aim of Project

Several considerable facts about the two proteins include the amino acid sequence homology shown in Figure 2.3, which indicates that NifH and ChlL have similarities structurally and functionally, but have the least homology at their carboxyl terminal regions. It is also important to mention the fact that NifH is capable of partially functioning in the dark-dependent chlorophyll biosynthesis pathway. Ironically, the NifH protein is the most oxygen-sensitive component of nitrogenase and its ability to replace ChlL partially suggests that oxygen-sensitive enzymes may function in chloroplasts when *C. reinhardtii* is grown in the dark (5). Not surprisingly, hydrogenase, another oxygen-sensitive enzyme located in the chloroplast is active in dark grown *Chlamydomonas* or in anaerobically adapted cells grown in light (5). NifH of nitrogenase and the ChlL of protochlorophyllide reductase have somewhat of an extensive, comparative relationship. Since NifM is a PPIase and interacts with NifH, its assumable that the possible substate for NifM could be one of the seven fully conserved prolines of the NifH protein. This suggested notion is supported by a previous study in our lab that demonstrated that NifH can acquire NifM-independence when the conserved Pro258 located in the carboxyl terminal region of NifH is replaced by serine (14). Therefore, it can be proposed that the afore mentioned proline residue of NifH can serve as a substrate for

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the PPIase activity of NifM. Although beneficial to NifH, NifM was found to be detrimental towards the ChlL protein, which is structurally similar to the NifH. Interestingly enough, it has been a goal of our group to demonstrate chimeric proteins and *nifM⁻* mutants efficiency in nitrogenase. Ultimately, we proposed that it was possible to render a *nif⁺* phenotype, even through the development of a NifH-ChlL chimeric protein, independent of a NifM.

We predetermined to transform the chimeric gene construct of the *nifH* and *chlL* genes into a bacterial strain void of a functional NifM. The *Azotobacter vinelandii* strain AV98 has disrupted the *nifM* gene by the insertion of a kanamycin cassette (40). This choice was made after careful consideration of a previous study from our laboratory that indicated that *chlL* was incapable of producing a functional NifH after being transformed into a *nifH⁻* strain of *Azotobacter vinelandii*, DJ54. The experiment, which tested the hypothesis that *chlL* might complement *nifH⁻* strains of *A. vinelandii*, utilized *chlL* introducing it into *A. vinelandii* strains of different genetic backgrounds for complementation studies. In addition to complementation analysis confirming the ability of *chlL* to form a functional NifH in a *nifM⁻* strain, a series of growth curve analyses and protein interaction analysis were performed. The combined results of that study suggested ChlL was not functional in the *nifH⁻* strain of *A. vinelandii* DJ54, due to the presence of *nifM* in the strain. Protein analysis confirmed the existence of an interaction between NifM and ChlL. All prior supporting information lead us to employ the *A. vinelandii* strain AV98 to transform the chimeric gene construct.

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CHAPTER III

MATERIALS AND METHODS

General Molecular Biology Technique

Restriction enzymes were purchased from Promega (Madison, WI). DNA sub-cloning, plasmid DNA isolations, restriction enzyme digestions, agarose gel electrophoresis, ligations, and *E. coli* transformations were carried out as described in materials and methods. Oligonucleotides used for PCR amplification were purchased from Gibco-BRL Life Technologies (Gaithersburg, MD).

Growth Conditions

All bacterial strains and plasmids used in this particular study are described in Table 2. Specific bacterial strains were grown at 37ûC in 2YT media. Once transformed into AV98, the bacterial strain was grown at 30ûC in Burk's nitrogen and nitrogen-free media. Ampicillin, Chloramphenicol, and tetracycline were used to a final concentration of 50, 34, and 5 µg/ml, respectively, wherever the selection was necessary. All of the above growth media were dissolved in 1liter of distilled H_2O. In order to prepare agar plates, 20g of agar was added prior to autoclaving. Autoclaving was carried out at 121°C, 15 psi pressure, and 35 min/l.

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Table 1. Composition of Growth Media

Growth Media	Ingredients
2YT	Bacto-Tryptone-10g, Yeast Extract-16g, NaCl-10g
BN^+	KH_2PO_4-0.2g, K_2HPO_4-0.8g, $MgSO_4.7H_2O$-0.2g, $CaSO_4.7H_2O$-0.05g, $FeSO_4.7H_2O$-2ml, Na_2MoO_4-2ml, NaCl-0.2g, Sucrose-20g, NH_4Oac-2.31g
BN^-	KH_2PO_4-0.2g, K_2HPO_4-0.8g, $MgSO_4.7H_2O$-0.2g, $CaSO_4.7H_2O$-0.05g, $FeSO_4.7H_2O$-2ml, Na_2MoO_4-2ml, NaCl-0.2g, Sucrose-20g,
$BN^+Fe^-Mo^-$	KH_2PO_4-0.2g, K_2HPO_4-0.8g, $MgSO_4.7H_2O$-0.2g, $CaSO_4.7H_2O$-0.05g, NaCl-0.2g, Sucrose-20g, NH_4Oac-2.31g

Table 2. Bacterial Strains and Plasmids.

Strains/Plasmids	Relevant characteristics and description	Source/Reference
Escherichia coli TG1	K-12 *Δ(lac-pro) supE thi hsd-5/F' traD36 proA*$^+$*B*$^+$ *lacI*^{q} *lacZΔM15*	Amersham Life Sciences, Inc.
Escherichia coli XL-1 Blue	*MRF' K, Δ(mcrA) 183Δ[mcr CB-hsdSMR-mrr) 173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac (F' proAB lacI*^{q} *Z ΔM15 Tn5(Kan)*^{r}*]*	Stratagene Corp.
pCR 2.1 TOPO	*Amp*^{R}*, Kan*^{R}, 3908bp, used for direct cloning of PCR products, *lacZα fragment, MCS, M13*	Invitrogen Corp.
pBT	*Cm*^{r}, 53 bp *MCS*, 3.2 kb size, *MCS*, *p15A* origin of replication, *lac-UV5*, λ cl ORF	Stratagene Corp.
pTRG	*Tet*^{r}, 60bp *MCS*, 4.4kb size, *MCS*, *lac-UV5* promoter, ColE1 origin of replication, *RNAPα ORF*	Stratagene Corp.
Azotobacter vinelandii AV98	Nif-,defined deletion in the *nifM* gene, 309bp of *SmaI* fragment from *nifM* is replaced by kanamycin cassette	Laboratory stock
Azotobacter vinelandii DJ54	Nif-, defined deletion in the *nifH* gene	Laboratory stock
Azotobacter vinelandii wild type	Wild type, Nif+, soil bacterium	Laboratory stock
pBG1380	Expression vector (4500bp) regulates the expression of genes cloned downstream to the *nifH* promoter, *his tag (6XHis)*	ManHee Suh
pBG2433	Derivative of TOPO 2.1 pCR in which 280bp fragment of *chlL* (*chlL2*) interacting with *nifM* is cloned	Laboratory stock
pBG2434	Derivative of the expression vector, pBG1380 in which 873bp fragment of *nifH* is cloned. The fragment can be released by digestion with *EcoRV* and *HindIII*	Laboratory stock
pMH5405	Derivative of AV98, with pBG1380 transformed	Laboratory stock
pMH5403	Derivative of pBG2434 with partial *nifH*(1-248 a.a.) with *chlL*(249-302 a.a.) chimeric gene construct transformed into AV98	This work
pMH5404	Derivative of pBG2434, from which a 128bp fragment of *chlL* was digested at *SalI* and *HindIII* sites and cloned into TOPO 2.1 pCR	This work

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Plasmid Purification

All the plasmids used in this study were purified either by the phenol-chloroform extraction method or by the QIAprep Spin Miniprep kit by following the manufacturers protocol. The plasmid DNA sample was subjected to restriction digestion and electrophoresis on a 0.8% agarose gel to check for the fragment size. The restriction digestion mix was prepared in an eppendorf tube by adding distilled water, DNA solution, 10 X Restriction endonuclease buffer and Restriction endonuclease. They were mixed thoroughly. The tubes were then centrifuged for a few seconds and incubated at 37°C for 2-3 hrs. 6µl of 6X loading dye was added and the samples were loaded on the electrophoresis gel. To check the fragment size, the restriction digested sample was analyzed by gel electrophoresis.

PCR Amplification

PCR (Polymerase Chain Reaction) is a technique to exponentially amplify a small quantity of a specific nucleotide sequence in the presence of template sequence, two oligonucleotide primers that hybridize to opposite strands and flank the region of interest in the target DNA, a thermostable (*Taq*) DNA polymerase (Table 3). The reaction is cycled involving template denaturation at 95°C, primer annealing at 55°C, and the extension of the annealed primers by DNA polymerase at 72°C until enough copies are made for further analysis.

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Amplify the DNA Fragment Corresponding to the Region of *chlL* open reading frame spanning [a.a. 249-302]

In order to clone the correct DNA fragment of *C. reinhardtii*, the 280bp *chlL* fragment was PCR amplified from the pBG2433 template utilizing the 5’ ChlL(*SalI*) and 3’ (*HindIII*) primers (Table 2 and 5). The derivative of pCR 2.1 TOPO, in which 280bp fragment of *chlL* (*chlL2*) interacting with *nifM* is cloned, was taken from our laboratory’s frozen stock and grown on 2YT plates supplemented with 50µg/ml Ampicillin overnight, and the cells were transferred into 10ml 2YT media supplemented with 50µg/ml Ampicillin and the DNA was isolated by QIAprep Spin Miniprep (Table 1, Table 2). Following DNA isolation, the plasmid DNA was added into 75µl of dH_2O in a microcentrifuge tube and boiled for 5 minutes at 100° C for DNA PCR. 7µl of the now denatured DNA was added to a PCR tube with 18µl of the PCR mix (detailed in Table 3). The combined 25µl solution was added to new PCR tube. The tubes were then placed in PCR machine to undergo the afore mentioned number of cycles at the respective temperatures (Table 3, Table 4).

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Table 3. DNA PCR Mix.

Chemicals	Amount
$MgCl_2$ (25mM)	10µl
10XBuffer (Mg^{2+}) (15mM)	10µl
DNA template	1µl
dNTP (10µM)	1µl
Primer 5’	1µl
Primer 3’	1µl
Taq DNA Polymerase (5U)(Promega, Madison, WI)	1µl
dH_2O	75µl
Total	100 µl

Table 4. PCR Conditions.

95° C	1 min	Denaturing
72° C	2 min	Annealing
55° C	3 min	DNA synthesis

Table 5. List of DNA Primers.

Primers	Sequence
TG1 *chlL* (*SalI*)	5’GTC GAC AAT TCT ACA GTA GGA GTG TCA GAA ACT CC-3’
TG1 *chlL* (*HindIII*)	5’AAG CTT TTA AAT TTT AAG ATA GAA ATC TGA TAA AAG-3’

The 5’ *SalI* and 3’ *HindIII* primers used for the PCR were constructed to amplify the 128bp *chlL* fragment to correspond to the *SalI* and *HindIII* restriction sites of the pBG2434. These primers include specific built in *SalI* and *HindIII* restriction sites utilized in PCR amplification of *chlL* DNA fragment of *C.reinhardtii*.

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Insertion of PCR Amplified DNA into pCR 2.1 TOPO Vector

The PCR amplified 128bp *chlL* DNA fragment was inserted into pCR 2.1TOPO vector using TOPO TA Cloning kit (Invitrogen Corporation, Carlsbad, CA). The *Taq* DNA polymerase adds a single deoxyandenosine (A) to the 3' ends of the PCR products. The pCR 2.1 TOPO vector has single, overhanging 3' deoxythymidine (T) residue that allows the PCR product to ligate efficiently with the vector. All the instructions provided by the manufacturer were followed to perform the TOPO cloning reaction.

Transformation of *chl* fragment into *E. coli* Top10 cells

Top10 cells were grown overnight and 200µl of overnight culture diluted into 10ml of 2YT media and grown for 2 hrs. Cells were centrifuged for 5 minutes for 4000rpm, in order to harvest a pellet. Pellet was resuspended into 5ml of ice cold $CaCl_2$ and incubated in ice for 20 minutes. Cells were again centrifuged for 5 minutes at 4000rpm in order to harvest a pellet. Pellet was resuspended into 1ml of $CaCl_2$. 300µl of competent cells, along with 128bp *chlL* fragmaent DNA is added to a tube that will incubate in ice for 40 minutes. A heatshock followed at 42° C for 2 minutes and 45 seconds. Incubation in ice continued for another 10 minutes before adding 1ml of 2YT media to each tube, and incubating at 37° C for 1 hour. The cells were then centrifuged for 3 minutes at 3000rpm and 1ml of the supernatant discarded. The pellet was resuspended in the remaining media and plated on 2YT plates supplemented with 34µg/ml of chloramphenicol. Incubation at 37° C followed until the next day.

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Restriction Digestion

Before the chimera construction could begin, the 128bp *chlL* fragment was digested from pCR 2.1 TOPO vector, including the specific carboxyl terminal region of the pBG2433, spanning the region of the *nifM* substrate. In addition, the pBG2434, in which the *nifH* is cloned, was digested with the appropriate restriction endonucleases (Table 2). The 15µl of pBG2433 and 18µl of pBG2434 plasmid DNA was added to 67µl(pBG2433) and 70µl(pBG2434) of dH_2O, 10µl of buffer and 8µl of restriction enzyme. In the case of these two plasmids, we performed a double digestion using the respective *SalI* and *HindIII* restriction endonucleases. The digested plasmids were then purified by gene clean.

Purification of Amplified DNA Fragments

All PCR products and restriction digested DNA fragments were purified using the GENE CLEAN III kit. The DNA fragments were separated with a 0.8% agarose gel using TAE buffer (0.04M Tris-acetate, 0.0001M EDTA pH 8.0). During electrophoresis, the gel was protected from light in order to minimize DNA nicking in the presence of Ethidium Bromide. The DNA fragment of interest was cut from the gel and the fragment was transferred to a preciously weighed eppendorf tube. The eppendorf tube with the gel slice was weighed and three times the w/v sodium iodide was added. The tube was incubated in a 55-65° C water bath until the gel melted. 5µl of glass milk was added to the contents in the tube and was mixed by inverting several times allowing the DNA to bind to the glass milk. The tube was centrifuged at 14000rpm for 2-3 minutes. The supernatant was discarded and the pellet was

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resuspended in 250µl of New Wash solution. The sample was centrifuged for 2-3 minutes at 14000rpm and the supernatant was discarded. This washing step was repeated twice more. After the last washing step the supernatant was removed and the pellet was vacuum dried for 5 minutes in order to remove the residual ethanol. The pellet was resuspended in 20µl of dH_2O and incubated at 45-55° C for 2-3 minutes. The sample was then centrifuged for 2-3 minutes at 4000rpm and 30µl was carefully recovered in a new microcentrifuge tube. The sample was then stored at –20° C.

Ligation of pBG2434(with 745bp *nifH)* and the 128bp *chlL* Fragment

15µl of the pBG2434 plasmid with the partial 745bp *nifH* fragment and 11µl of the 128bp *chlL* fragment was added to a mixture of 1.5µl of 10X ligation buffer and 1.5µl of T4 DNA ligase. The mixture was incubated in an overnight water bath at 16°C. After the completion of the ligation experiment, a transformation was performed with the new pBG1380*nifH-chlL* chimeric construct, or pMH5403.

Transformation of *nifH-chlL* Chimera into *E. coli* Top10 cells

E. coli Top10 cells were grown overnight and 200µl of overnight culture diluted into 10ml of 2YT media. Cells were centrifuged for 5 minutes for 4000rpm in order to harvest a pellet. Pellet was resuspended into 5ml of ice cold $CaCl_2$ and incubated in ice for 20 minutes. Cells are again centrifuged for 5 minutes at 4000rpm in order to harvest a pellet. Pellet was resuspended into 1ml of $CaCl_2$. 300µl of competent cells, along with 1µl of plasmid DNA is added to a tube that will incubate

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in ice for 40 minutes. Following incubation will be a heatshock at 42° C for 2 minutes and 45 seconds. Incubation in ice will resume for another 10 minutes before adding 1ml of 2YT media to each tube, and incubating at 37° C for 1 hour. Centrifuge for 3 minutes at 3000rpm and take out 1ml of the supernatant and discard it. Resuspend the pellet in the remaining media and plate on 2YT plates supplemented with chloramphenicol. Incubate at 37° C overnight.

Screening for Presence of *nifH-chlL* Chimera

The transformants obtained were subcultured on 2YT plates supplemented with 34µg/ml of chloramphenicol and again grown overnight in 10ml of 2YT liquid media supplemented with 34µg/ml of chloramphenicol. The plasmid was isolated by the QIAprep Spin Miniprep protocol. Cell PCR was performed utilizing the 5'NifH (*EcoRV)* and 3'ChlL(*HindIII)* primers to check size for correct cloning. The 5' NifH (*EcoRV*) primer was utilized because the 873bp *nifH* fragment that was cloned into pBG1380 to create pBG2434 could be released by digestion with *EcoRV* and *HindIII* restriction endonucleases. The 3' ChlL (*HindIII*) primer was also utilized due to the built in *HindIII* restriction site of the 128bp *chlL* fragment; as well as, the *HindIII* restriction site of the *nifH* fragment.

Transformation into *Azotobacter vinelandii* strain AV98

Fresh *Azotobacter vinelandii* AV98 cells from a BN^{+} plate were transferred onto a $BN^{+}Fe^{-}Mo^{-}$ plate and incubated overnight at 30° C. A loop full of cells were transferred into 5ml of the *A. vinelandii* transformation media and grown in a 30° C shaker at 250rpm overnight. The 5ml of the cell suspension was then transferred into

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20ml of fresh transformation media and incubated overnight in 30° C shaker at 250rpm. 200µl of autoclaved MOPS buffer was added to sterile 15ml tubes along with 100µl of the prepared cells. 15-20µl of DNA added to the cells and this mixture was incubated at room temperature for 20 minutes. 3ml of BN^{+} media was added and the cells were incubated in 30°C shaker at 250rpm overnight. On the next day, the suspension was centrifuged at 3000rpm at 4°C for 5 minutes. The supernatant was discarded and the cells were resuspended into 3ml BN^{-} media. The suspension was centrifuged again at 3000rpm at 4°C for 5 minutes, the supernatant discarded and the pellet was resuspended in 300µl of BN^{-} media and spread on BN^{-} plates. The plates were incubated for 72 hours at 30°C.

Growth Curve Analysis

The transformants generated were subjected to growth curve analysis. The transformed strains were inoculated into 10ml of BN^{+} liquid media containing chloramphenicol and incubated in a 30°C shaker at 250rpm overnight. The cell suspension was then centrifuged at 3000rpm for 5 minutes. The supernatant was discarded and the pellet was resuspended in 5ml of BN^{-} media. The cell suspension was diluted using BN^{-} media until the O.D. (Optical Density) of 0.1 at λ=620nm. 3ml of the diluted cells were taken in culture tubes with caps and incubated in 30°C shaker at 250rpm. The O.D. was taken every twelve hours for 7 days.

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CHAPTER IV

RESULTS

Development of the Chimeric Construct

Our lab has previously shown that the *chlL* gene product protochlorophyllide reductase (ChlL) of *C. reinhardtii* was capable of substituting the NifH in the functions of nitrogenase, but only in the absence of NifM. However, the synthesis of a stable and functional NifH has demonstrated its dependence upon the availability of a *nif*-accessory protein, NifM (5, 13, 14, 19, 40). It has also been added that most likely, the NifM protein is involved in a catalytic event which results in activity and increased stability of the the NifH (19).

NifM Influence on NifH and ChlL Proteins

The comparison of *nifM* sequences of *K. pneumoniae* and *A. vinelandii* showed an overall amino acid sequence identity of only 16% (31). This sequence identity was confined to the carboxyl terminal third of the polypeptides, and the sequences that made up the amino terminal two-thirds of the polypeptides shared very little sequence identity (31). The findings suggested that the active portion of the *nifM* gene product is most likely located within the carboxyl terminal region of the protein. The carboxyl terminal region of NifM has sequence similarity to the peptidyl prolyl *cis/trans* isomerases (PPIases), particularly the PpiC. Gavini *et al* further

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showed that the NifM in *A. vinelandii* demonstrated PPIase activity with a specificity constant of 1.09 x 10^7/M/s, which is comparable to the reported E. coli PPIase, PPiC, being 1.69 x 10^7/M/s (14). Theoretically, the role of NifM could be the isomerization of a possible carboxyl terminally located substrate for the NifH (14). A consensus peptide sequence of NifH derived by comparing 60 different sequences shows the existence of seven conserved proline residues that are displayed in Figures 2.3 and 4.1 (13, 14, 40). Therefore, it was possible to assume that the stability and activity of NifH mostly depends upon the appropriate conformation of the peptidyl-prolyl bonds present in the protein. The proper folding and conformation of the NifH is necessary for it to be a stable and functional protein. Overall, this would be critical for further enzyme action and substrate reduction. One residue located in the carboxyl terminal region of NifH, Pro 258, was suggested to be a promising substrate for the NifM protein (13, 14, Figure 4.1). This discovery along with previous studies have suggested that the NifM-dependence of NifH could be confined to the carboxyl terminal region of NifH. Although the NifM protein has a beneficial effect upon the NifH, it has been found to confer a deleterious effect on the ChlL protein. According to Cheng *et al*, ChlL has been proposed to be the most promising substitute of the NifH (5).

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m-m---lrqiafYGKGGIgKSTtsqntlaalae-mgqkilivGCD**P**kaDsT

rlilhskaqdtvldaae-gsvedledledvlkeGyggikCvEsGG**P**e**P**GvG

CAGRGvItsinfleengay--ddldyvsyDvLGDVVCGGFAm**P**irenkAqei

YiVmsgemMAmYAANNIskGilkyansggvrLGGlicNsRtdrelelieala

klgtqlihfv**P**rdnivqhaElrrmTviey-pdskqadeyr-Larkihnn.g

*

kgvi**P**t**P**itmdelee.lmefgim..ede

Figure 4.1 Consensus Sequence of NifH Derived from Comparison of 60 Different NifH Proteins Showing Conservation of Seven Proline Residues.

The conserved prolines are shown in boldface. Uppercase letters indicate residues that are invariant. Pro258 is marked by an asterisk.

A Possible NifM-Independent Chimeric Construct?

The hypothesis adopted for this study includes constructing a protein that would be capable of biologically fixing nitrogen from the atmosphere, without the dependence of the *nif* accessory gene, *nifM*. A disruption of the active carboxyl terminal region of NifM, where its PPIase domain is located, would certainly render NifH unstable and inactive. Subsequently, we instituted the construction of a chimera gene consisting of a partial *nifH* gene [spanning amino acids 1-248] from *A. vinelandii,* and the *chlL* carboxyl region [a.a.248-302] from *C. reinhardtii*. The partial *nifH* excluded the carboxyl region [spanning amino acids 248-290] that comprises the suspected substrate for the NifM. Fujita and other researchers suggest

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the sequence homology between NifH and ChlL, shown in Figure 2.3, indicates that the ATP-binding motif and the two Cys residues that are involved in coordinating the [4Fe-4S] cluster are completely conserved among NifH and BchL/ChlL proteins (12). This proposes that the ChlL protein might catalyze an ATP-dependent transfer of electrons from a reductant to a catalytic protein complex via a Fe-S center (5). These suggestions imply mechanistic similarities between NifH and ChlL (3). Structural identity and similarity between the ChlL protein and NifH protein also suggest that an equivalent of *nifM* must be present in organisms which contain *chlL* (3, 5, 12). Cheng *et al* demonstrated that NifH has the ability to function partially in the dark-dependent chlorophyll biosynthesis pathway; of course raising concern and questioning the requirements for the biosynthesis and maintenance of activity of the Fe-protein within the chloroplast environment (5). The region of NifH spanning the Pro258 residue serves as the substrate for the NifM. The residue is located in the carboxyl terminal region of NifH, which wraps around the other subunit of the NifH dimmer. Therefore, it is possible that the *trans* conformation of the peptide-prolyl bond of Pro258 contributes significantly to the structure of this region and that NifM-mediated modification is essential to attain this structure. Therefore, it is proposed perhaps that the replacement of the region where NifM exerts its effect as a molecular chaperone of NifH with the protochlorophyllide reductase or ChlL will demonstrate an alternative, chaperone-independent mechanism by which the functional conformation is achieved as well.

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Cloning Strategy

Considering that ChlL is homologous with NifH and is able to function in the absence of NifM, it could be assumed that NifH might be capable of functioning in such an environment. The construction of the *nifH-chlL* chimeric gene is exhibited in Figure 4.5. The following list includes steps taken to construct the NifH-ChlL chimera.

1. Initially, both plasmids pBG2433 and pBG2434 were purified.
2. PCR pBG2433 with 280bp fragement of chlL spanning the carboxyl terminal region. Utilized the ChlL 5'*SalI* and 3'*HindIII* primers. The PCR product was purified by gene cleaning procedure explained in CHAPTER III.

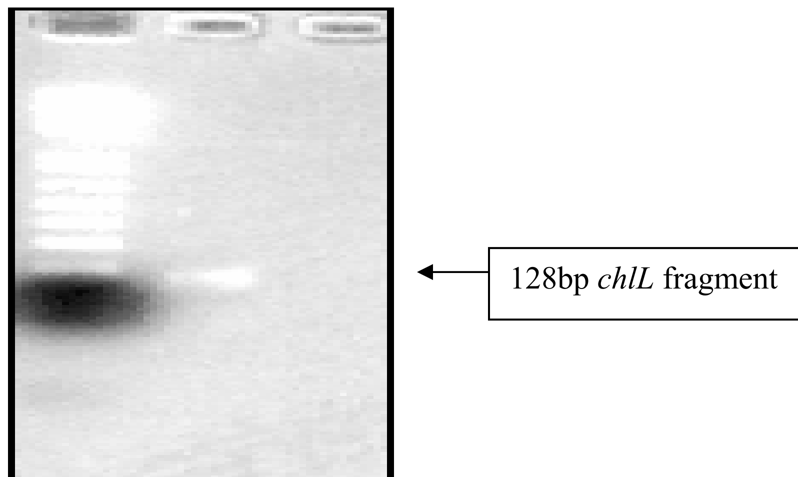

Figure 4.2 128bp *chlL* Fragment Purified by Gene Clean.

Procedure for gene cleanding is explained in CHAPTER III. DNA ladder used to show

size of fragment.

3. Insertion of the 128bp *chlL* fragment into the pCR 2.1 TOPO vector was achieved by ligation. The new TOPO*chlL* clone was identified as pMH5404. Restriction Digestion was performed with *EcoRI* to select for the 128bp *chlL* fragment cloned into the TOPO.

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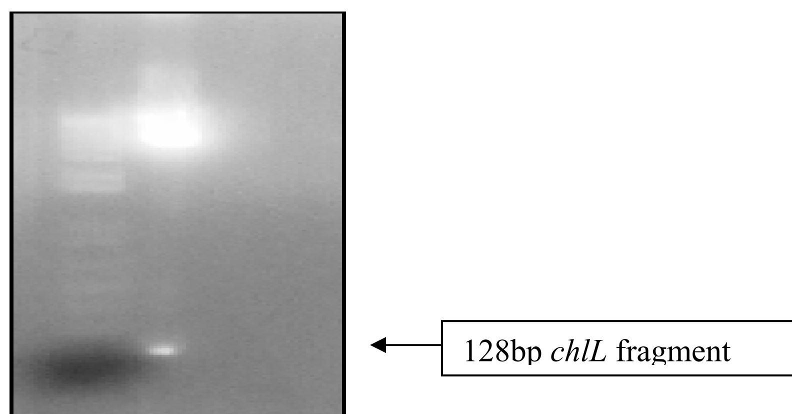

Figure 4.3 pMH5404 After *EcoRI* Restriction Digestion.

This step resulted in releasing the 128bp *chlL* fragment. DNA ladder used to show size of fragment.

4. Ligation into pCR 2.1 TOPO vector was followed by the transformation of the pMH5404 into *E. coli* Top10 cells. The selection for the plasmid's transfer into the Top10 cells was analyzed after plating cells on 2YT plates supplemented with 50µg/ml of ampicillin.

5. *SalI* and *HindIII* restriction digestion of pMH5404 followed and the 128bp *chlL* fragment was purified by gene cleaning procedure explained in CHAPTER III. .

6. Simultaneous restriction digestion of pBG2434 by *SalI* and *HindIII* restriction endonucleases was performed eliminating the proposed stretch of the gene including the suggested NifM substrate. This digestion produced a pBG1380 with a partial 745bp *nifH* fragment. pMH5404 was subjected simultaneously with *SalI* and *HindIII* restriction endonucleases.

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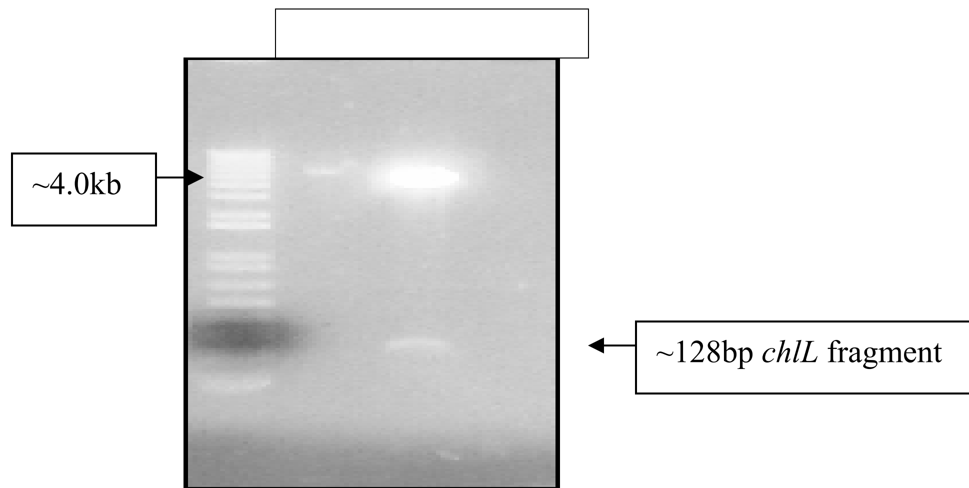

Figure 4.4 Double *SalI* and *HindIII* Restriction Digestion of pBG2434 and pMH5404.

7. The pBG1380 with 745bp *nifH,* and the 128bp *chlL* fragment were subjected to a DNA ligation. The ligation produced the *nifH-chlL* chimeric gene construct within the pBG1380, now referred to as pMH5403.

8. pMH5403 was transformed into E. coli Top10 cells and PCR amplified in order to the correct size of plasmid. Utilized the 5' NifH *EcoRV* and 3' ChlL *HindIII* primers for this procedure.

The pBG2434 is a derivative of the pBG1380 plasmid. The plasmid pBG1380 has been modified to express the protein of interest under the control of the *nifH* promoter, which is a strong promoter and utilizes the *A. vinelandii* transcriptional and translational regulation to over express specific target protein downstream of it (40). pBG1380 also contains a chloramphenicol resistance gene which allows for selection in *A. vinelandii* and *E. coli* (40). This vector also contains unique *EcoRV, BamH1* and *HindIII* cloning sites downstram of the *nifH* promoter, which allows the use of these sites for cloning of specific gene constructs into pBG1380 and express the proteins of interest in *A. vinelandii* under nitrogen fixing conditions (40). The purified 128bp *chlL* fragment was ligated to the partial 745bp *nifH* gene, in which was before cloned into the pBG1380 vector. Following a transformation, the

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chimeric construct was PCR amplified using the *5'*NifH *(EcoRV)* and *3'ChlL (HindIII)*.

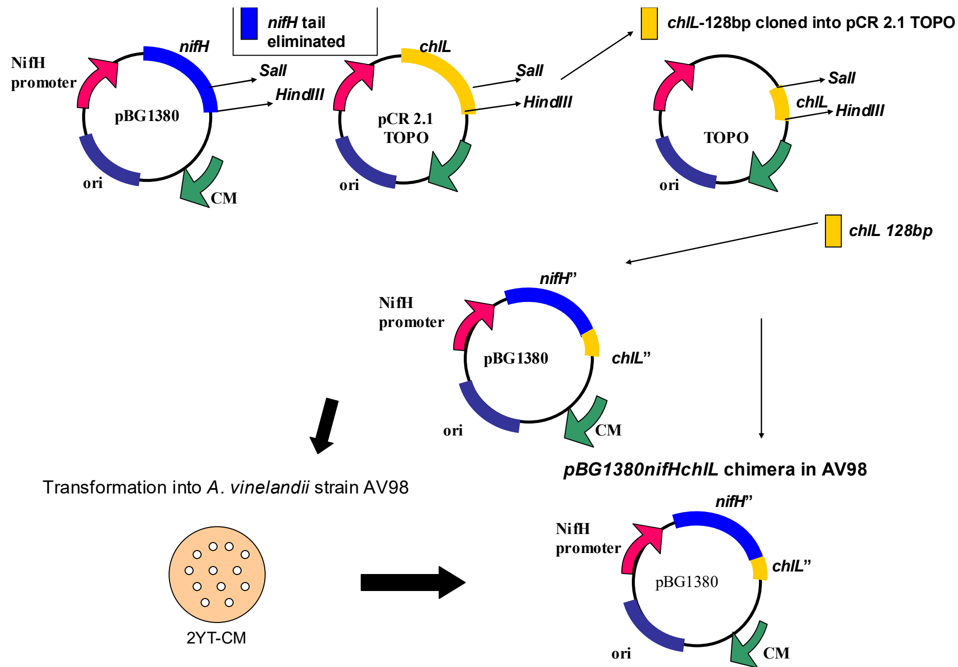

Figure 4.5 The Experimental Strategy to Construct the *nifH-chlL* Chimera into *Azotobacter vinelandii* Strain AV98.

The C-terminal of the *nifH* gene, including the substrate for the NifM protein, a Pro 258 residue, was eliminated at *SalI* and *HindIII* sites. It was replaced with the C terminal region of *chlL* gene, which was released from the pMH5404 at the exact restriction sites, and then ligated to the partial *nifH* gene to form the chimeric construct, pMH5403.

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Screening Transformants

Following transformation, the 7 colonies that grew on the 2YT plate supplemented with 34µg/ml chloramphenicol were all subcultured in 2YT media supplemented with 34µg/ml chloramphenicol and PCR amplified for verification of correct insertion. The chimera was subjected to cell PCR using the primers described in Table 6 below.

Table 6. PCR Primers Used to Amplify Chimera.

5'NifH (*EcoRV*)	5'GAT ^ATC ATG GCT ATG CGT CAA TGC GCC ATC TAC GGC-3'
3'ChlL(*HindIII*)	5'A^AG CTT TTA AAT TTT AAG ATA GAA ATC-3'.

As previously stated, these specific primers were utilized to amplify the *nifH-chlL* chimeric construct. The cells used for this PCR sample transferred into an eppendorf tube with 60µl of distilled water and boiled at 98°C for 20 minutes. The PCR contents are described in Table 7. 25µl of the boiled cells were added to the PCR mix described in Table 7.

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Table 7. Cell PCR Mix.

Chemicals	**Amount**
$MgCl_2$ (25mM)	10µl
10XBuffer (Mg^{2+}) (15mM)	10µl
Cells	25µl
dNTP (10µM)	1µl
Primer 5’	1µl
Primer 3’	1µl
Taq DNA Polymerase (5U)(Promega, Madison, WI)	1µl
dH_2O	51µl
Total	100 µl

The tubes were then placed in PCR machine to undergo the afore mentioned number of cycles at the respective temperatures (Table 3, Table 4). The PCR sample was subjected to 0.8% agarose gel electrophoresis to check size of the chimera construct (Figure 4.6).

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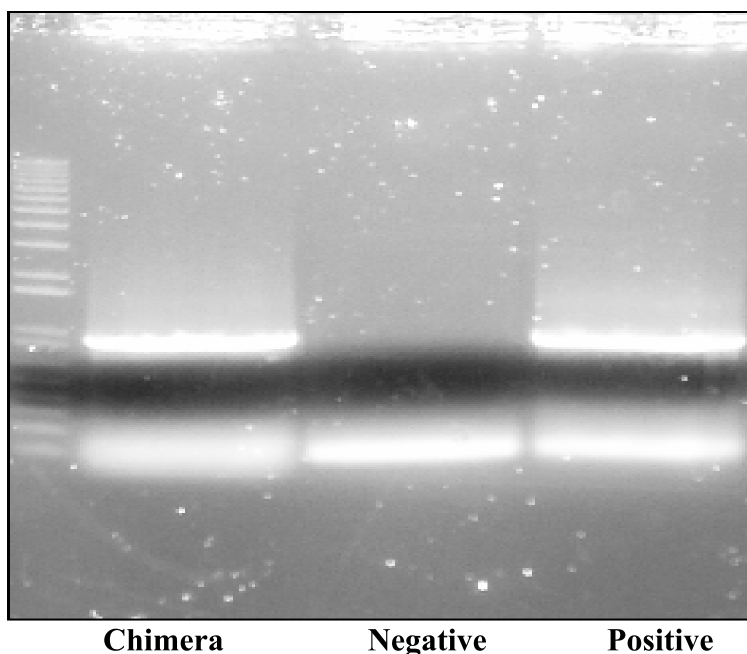

Figure 4.6 PCR Product of *nifH-chlL* Chimera.

Positive (TOPO*nifH)* and negative controls were amplified along with the chimera for verification of the primers and the insertion. The chimera was amplified using the primers displayed in Table 6.

Following the PCR amplification, the PCR products were restriction digested using the NifH *(EcoRV)* and ChlL *(HindIII)* restriction endonucleases, in order to release the chimeric construct.

Complementation Analysis of pMH5403 Transformants in AV98

The plasmid pBG1380, of which the pBG2434 was derived, has a chloramphenicol resistance marker. *A. vinelandii* AV98 carries a kanamycin resistance cassette which disrupts *nifM* open reading frame, thereby exhibiting a Nif^- phenotype. Therefore, the transformants were initially grown in BN^+ media supplemented with chloroamphenicol to show that the chimera construct was capable

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of rendering a Nif^{+} phenotype, and conferring antibiotic resistance in a *nifM*$^{-}$ strain (Figure 4.4). *Azotobacter vinelandii* exemplifies why it is the preeminent bacterial research model, being able to fix nitrogen even in the presence low concentrations of oxygen. The chimera construct exemplified that genetic engineering can generate a strain that is capable of growing in Burk's nitrogen-free media, indicating the presence of a functioning NifH protein. The construction and transformation of pMH5403 into *A. vinelandii* AV98 has resulted in an identified protein that was capable of fixing nitrogen in a *nifM*-independent environment. Figure 4.7 displays the growth yielded by pMH5403 when grown on BN^{+}, BN^{-}, BN^{-cm} and BN^{+cm} plates.

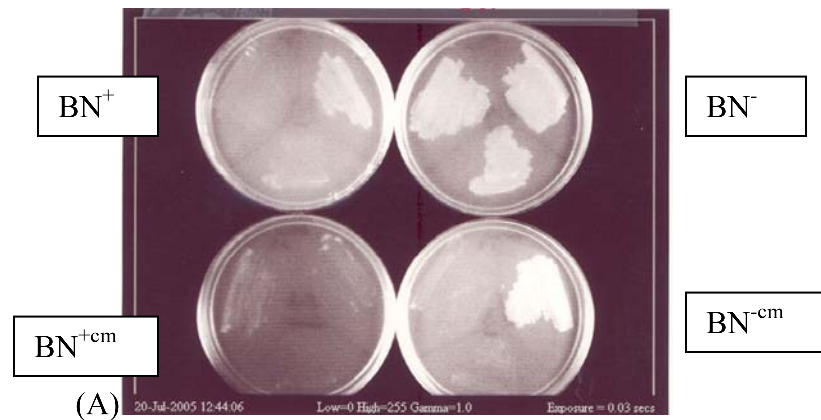

Figure 4.7 Complementation Analysis of NifH-ChlL Chimera in *Azotobacter vinelandii*

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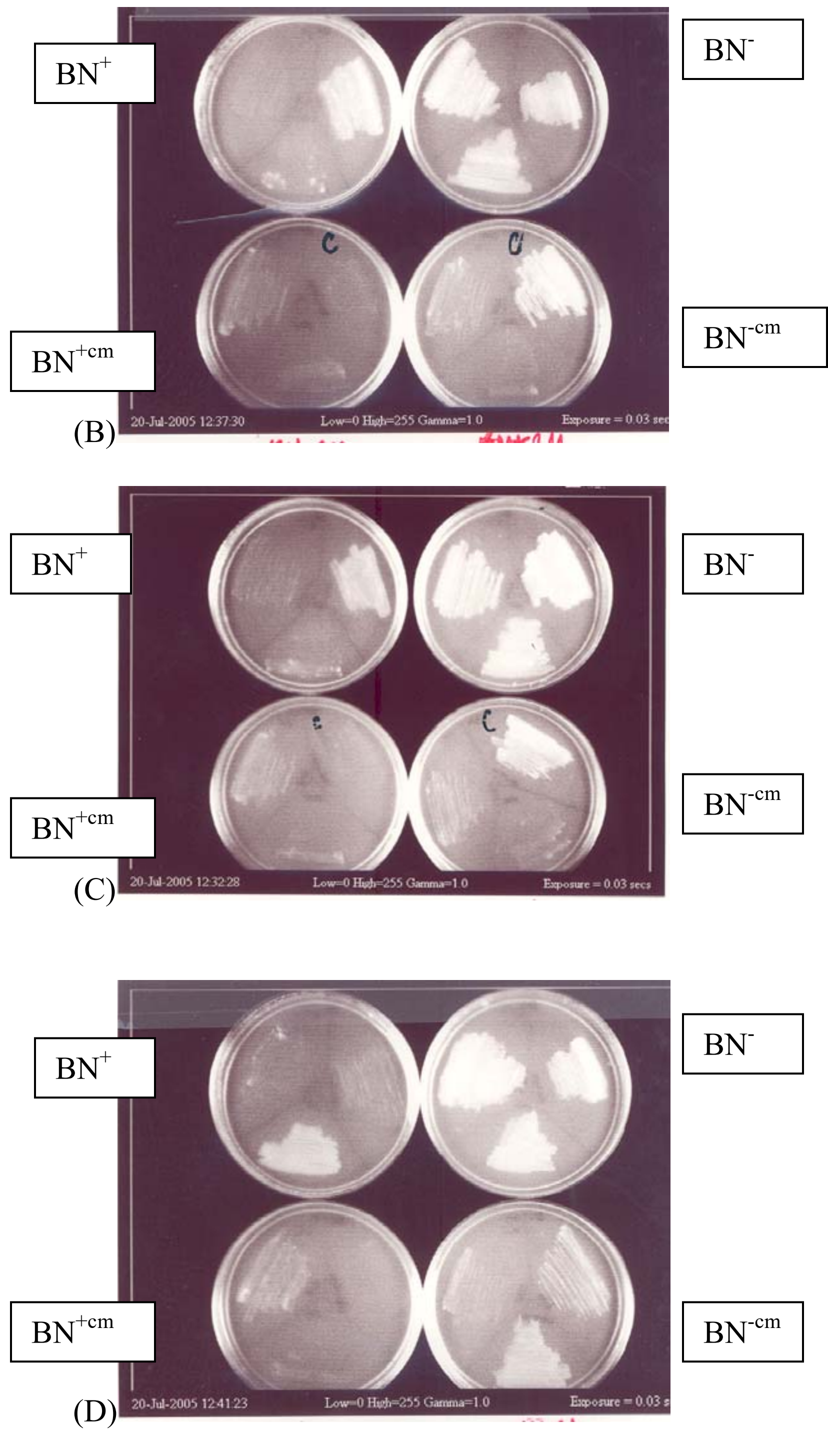

Figure 4.7 (continued)

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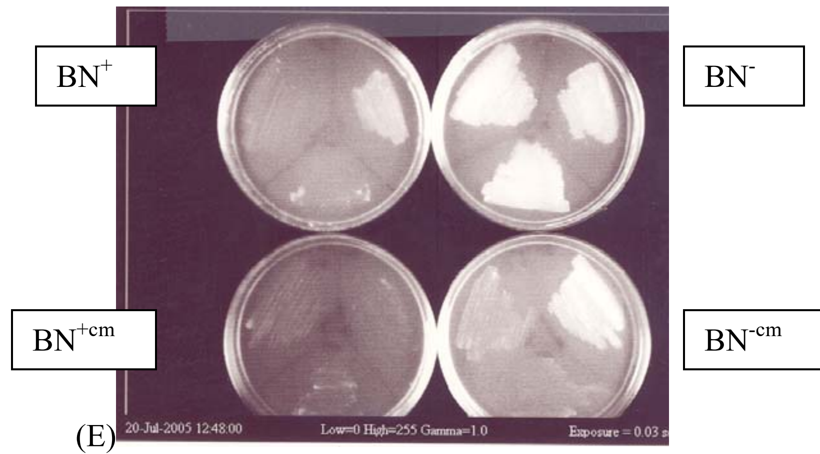

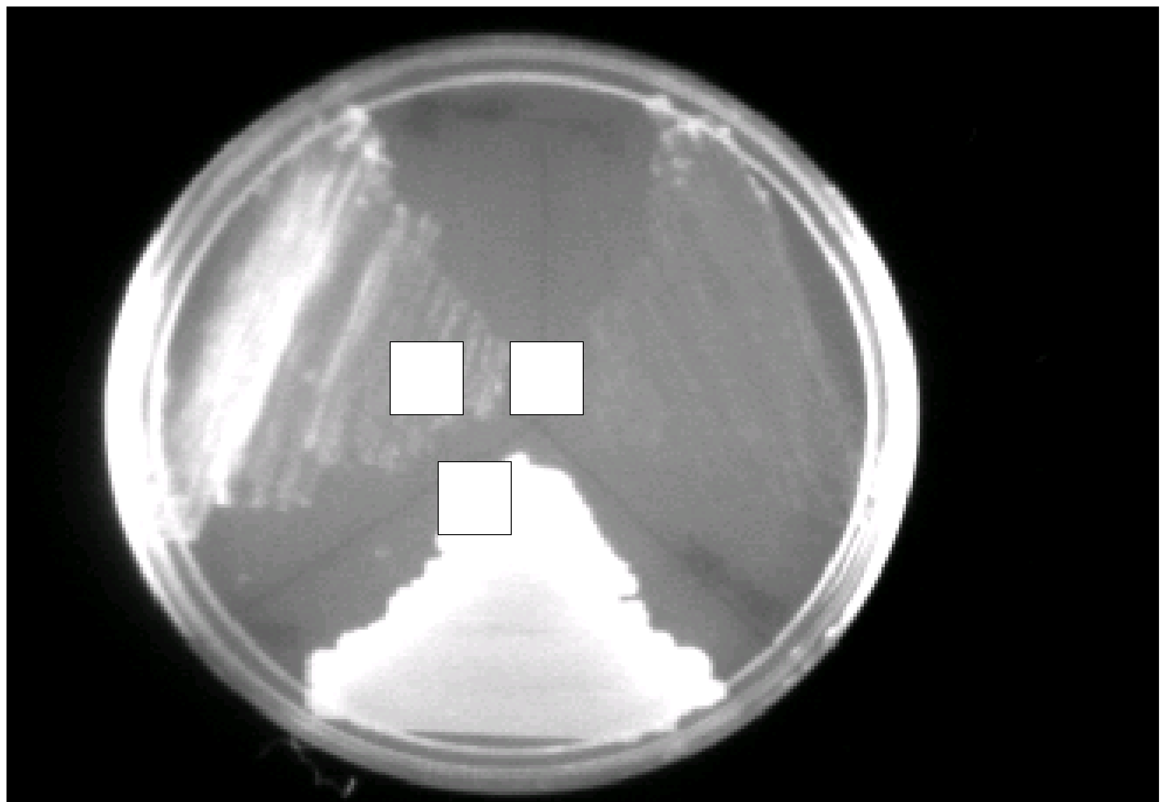

(F)

Figure 4.7 (continued)

AV98 on BN^{+}, BN^{-}, BN^{+cm} and BN^{-cm}. (A), (B), (C), (D), and (E) represent complementation analysis of 5 transformant carrying the *nifH-chlL* chimeric construct to yield growth in BN^{+}, BN^{+cm} and BN^{-cm} plates. The positive control, *Azotobacter*

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vinelandii wild type should yield a considerable amount of growth due to BN^- being its preferred optimal environment. The negative control, pBG1380 transformed into AV98 isn't expected to yield an adamant amount of growth due to the chloramphenicol resistance marker indigenous to the vextor. (F) represents the chimera, along with the same negative and positive controls on BN-.

Earlier, it was demonstrated that the ChlL was capable of substituting the NifH protein in the functioning nitrogenase, but only in the absence of NifM. The chimera was constructed in such a way that the *nifH* gene would be lacking one of the possible substrates for the NifM, Pro258 whose proposed location is in its carboxyl terminal region of NifH. Therefore, if there were any transformants generated on the BN^- plates that were supplemented with chloramphenicol, it suggests that the chimeric construct was capable of biologically fixing nitrogen in the absence of a nitrogen source in an *nifM*$^-$ *A. vinelandii* strain as well as conferring antibiotic resistance. The transformants obtained were PCR amplified with primers listed in Table 6. The PCR products were subjected to a 0.8% agarose gel electrophoresis to check the size of the construct (Figure 4.8) PCR products that displayed the correct band size were further studied through a BN^- growth curve analysis.

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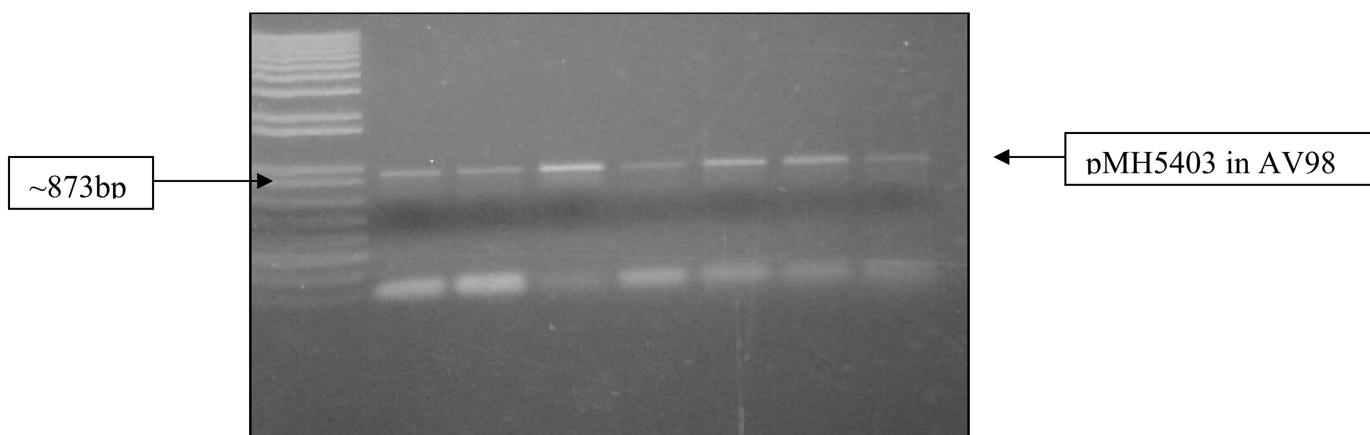

Figure 4.8 PCR Products of pMH5403 in *Azotobacter vinelandii* AV98.

Primers used in this procedure were given in Table 6.

Growth Curve Analysis

The growth curve was performed by using the procedure described in the materials and methods section of this paper. In order to monitor the growth rate of the pMH5403, a growth curve analysis was performed over 7 days in Burk's nitrogen-free media (Figure 4.9). The analysis compared the ability of the chimeric pMH5403, the *Azotobacter vinelandii* wild type (positive control) and the pBG1380 transformed into *Azotobacter vinelandii* AV98 to biologically fix nitrogen in an environment with removed antibiotics, that is nitrogen deficient and exposed to low amounts of oxygen. The difference in the presence and absence of *nifM* was shown to be a factor in the growth capabilities of biologically nitrogen-fixing organisms. This was seen in the growth yielded by the positive ($nifM^{+}$) and negative ($nifM^{-}$) controls. Figure 4.9 represents the Optical Density (nanometers) measurements of the chimera, positive and negative controls that were previously mentioned. The results

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demonstrate that the NifH-ChlL chimera protein, with sufficing proliferation, is capable of substituting the NifH protein in a functioning nitrogenase.

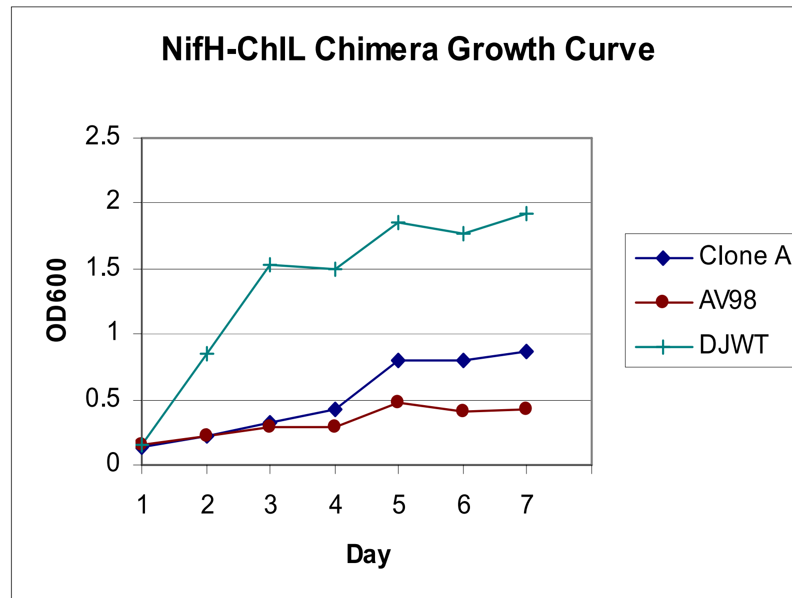

Figure 4.9 Growth Curve Analysis of NifH-ChlL Chimera.

The chimera(Clone A), with positive and negative controls, show a sufficing yield. The analysis was conducted in nitrogen deficient BN- liquid media for 7 days. The Optical Density was taken at 600(nm) every 12 hours.

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CHAPTER V

DISCUSSION

Proteins are the biochemical molecules that make up cells, organs and organisms. Proteins are linear polymers of amino acids that fold into conformations dictated by the physical and chemical properties of the amino acid chain. Making up most of the structure in the cell and also performing most of the work, proteins begin as long, thin molecules that resemble strings of beads. The folding of proteins is usually a spontaneous process, taking only a fraction of a second for a floppy chain of beads to fold into the shape it will keep for the rest of its working life. Proteins are synthesized in cells within ribosomes from the information encoded by the genes.

The products of the *nif* structural genes *nifHDK* are not catalytically competent and need further processing into their active forms by the action of *nif* accessory genes.The NifM protein is required for the accumulation of the active NifH protein in *K. pneumoniae* and in *E. coli*. Raja *et al* states that in A. vinelandii, the *nifU* and *nifS* together with the *nifM* gene product are required for full stability and activation of the NifH (14). A mutation or any disruption of the *nifM* gene results in the slower accumulation of non-functional NifH polypeptide (14, 31). The *nifH* is known to be responsible for four important functions within the nitrogenase complex. One very important and integral function of the NifH is its responsibility as obligate

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electron donor. As previously emphasized, the homology between the ChlL and NifH proteins is indisputable. Over the past two decades some attempts to introduce *nif* genes into the chloroplast has been proposed, but resulted in problems due to introduction of the NifH subunit into chloroplast via transit peptides. Mainly, the general concern remains that nitrogen fixation is not compatible with photosynthetic oxygen evolution. The structural similarities between the NifH proteins and the chlorophyll iron proteins suggest that an equivalent of *nifM* must be present in organisms which contain *chlL* (5). In addition, Cheng and coworkers used homologous recombination events to replace precisely the entire *chlL* coding region in the *C. reinhardtii* plastome with the eubacterial genes *uidA* and *nifH*. In accomplishing this replacement, they recommend that *nifH* can partially restore the capacity for chlorophyll biosynthesis in the dark; whereas, the *uidA* replacement inactivated the light-independent pathway of chlorophyll biosynthesis. The ability of the *nifH* of *K. pneumoniae* to replace the *chlL* function in *C. reinhardtii* suggested that it might function in ATP-coupled electron transfer to the other components of the light-independent protochlorophyllide reductase encoded by ChlN and ChlB (5, 12). The ability of NifH to function partially in the dark-dependent chlorophyll biosynthesis pathway raised a number of questions concerning the requirements for the biosynthesis and maintenance of activity of the NifH protein within the chloroplast environment. NifH-protein is the most oxygen-sensitive component of nitrogenase and its ability to replace ChlL partially suggestd that oxygen-sensitive enzymes may function in chloroplasts when *C. reinhardtii* is grown in the dark. This was not surprising since hydrogenase, another oxygen-sensitive enzyme located in the

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chloroplast, is active in dark-grown *Chlamydomonas* or in anaerobically adapted cells grown in light (5). In *Azotobacter*, a diazotroph possessing all three types of nitrogenase, the physiological mechanism of permitting nitrogen to be fixed despite the inherent oxygen-sensitivity of nitrogenase is uniquely advantageous to this species.

Although the NifM is found to be beneficial to the NifH, in a study in our laboratory it was shown to have a deleterious effect on the functions of ChlL. NifM, a PPIase, is required for the maturation of the NifH protein of nitrogenase (14). PPIases, as mentioned, are a class of enzymes that accelerate protein folding by catalyzing the *cis/trans* isomerization of proline imidic peptide bonds in oligonucleotides (13, 14, 47). Human Pin1, a PPIase that isomerized proline residues, showed similarity with the NifM of *A. vinelandii* in their respective PPIase domains (14). This sequence similarity, supported by Figure 2.1, is suggested to be confined to the carboxyl terminal region of NifM(14). A consensus sequence of NifH has revealed the existence of 7 fully conserved proline residues, suggesting that the stability and activity of NifH somewhat depends on the appropriate conformation of the peptidy-prolyl bonds present within (14, Figure 4.1). In a study by Gavini *et al*, it was conceived that the conformation of the peptidyl-prolyl bond of Pro258 contributed significantly to the structure of the carboxyl terminal region and that NifM-mediated modification is necessary to maintain the structure, because replacing this proline with serine renders partial NifM dependence of NifH (14). Therfore, we further proposed that Pro258 serves as a substrate for the PPIase activity of NifM

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(14). The Pro258Ser replacement suggested a specific site on NifH that NifM exerts its molecular chaperon effects, as well as generating an accessory-independent mechanism of accomplishing functional conformation. Interestingly, proline residues play a role in the structure of proteins.

Furthermore, we concluded to generate a chimeric construct gene that would not receive beneficial nor detrimental effect from PPIase activity and yet achieve functional conformation for the NifH protein in a *nifM-* environment. Our results show that the NifM-independence of NifH,

with the substitution of the *nifH-chlL* chimera, is capable in a functional nitrogenase (Figure 4.4).

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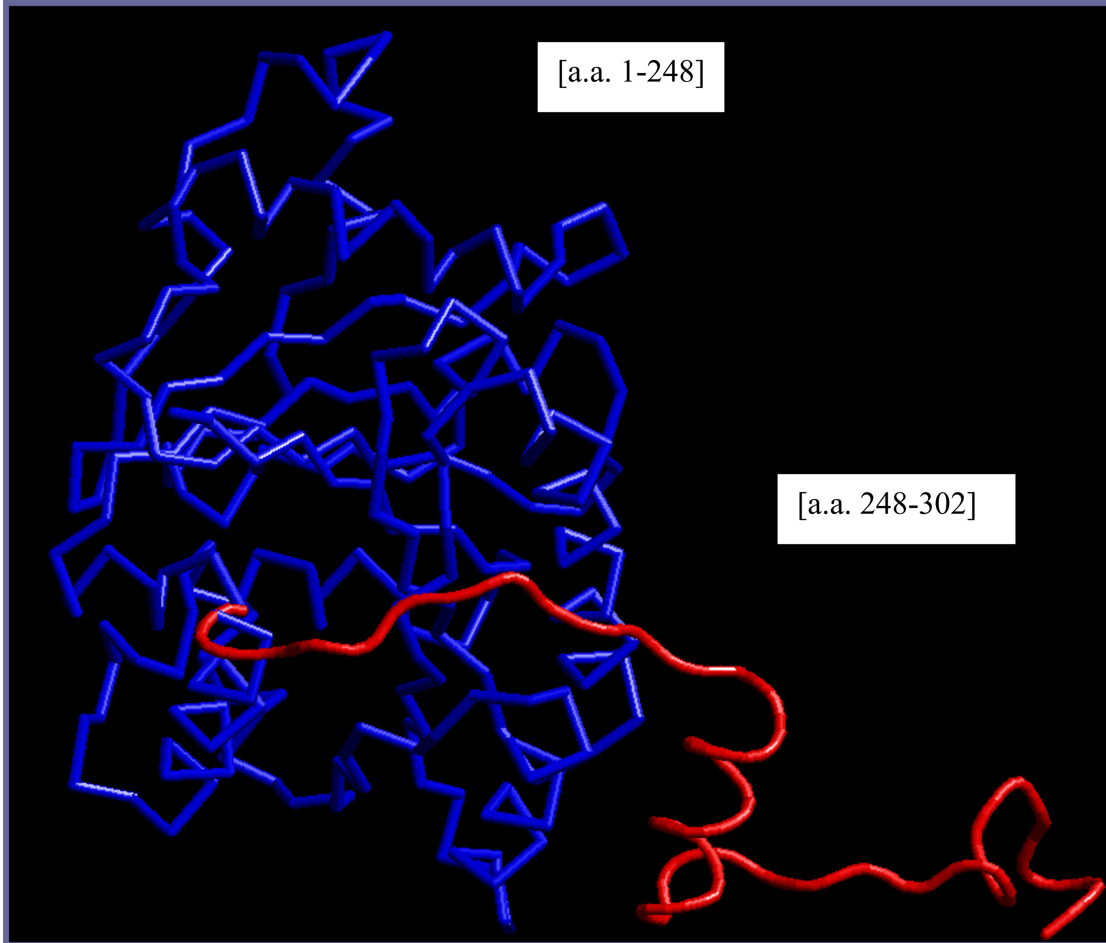

Figure 5.1 Swisspdb Model of the NifH-ChlL Protein.

This model of the chimeric protein was designed by Swiss Modeling. Displayed is only the e-chain of the protein. The original *nifH* spans from amino acid 1-248, and the *chlL* spans amino acids 249-302. The results from the complementation studies suggest that the pMH5403 is capable of conferring the antibiotic resistance of the vector (Figure 4.4). The growth, although partial, of the chimeric construct in AV98, suggest an equivalent of *nifM* being present within the *chlL* at the carboxyl terminal region. Our results have raised questions on the dependence of NifH on NifM in a functional nitrogenase.

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Future Analysis

Ultimately, this project has taken us a step closer to elucidating the collaboration between two life-giving processes, biological nitrogen fixation and photosynthesis. A previously mentioned, *chlL* is homologous to *nifH* and is able to function in the absence of *nifM*. Our plan is to continue experimentation with the chimera and transform it into *Azotobacter vinelandii* DJ54, which has a defined deletion in the *nifH* gene. Such a transformation, is expected to express the true flexibility of the synthesis of this coalescent construct.

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