# SITE-DIRECTED MUTAGENESIS OF THE NITROGENASE MoFe PROTEIN FROM *Azotobacter vinelandii*

by

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(ABSTRACT)

A model describing the potential amino acid ligands to the four 4Fe-4S centers (P-clusters) within the *Azotobacter vinelandii* nitrogenase MoFe protein is presented. Based on interspecies and intersubunit amino acid comparisons of the $\alpha$- and $\beta$-subunits of the MoFe protein, and the FeMoco biosynthetic proteins, NifE and NifN, four conserved residues (Cys62, His83, Cys88, Cys154 all proposed P-cluster ligands) within the $\alpha$- subunit were targeted for site-directed mutagenesis studies. In order to define a range of acceptable substitutions, 35 specific site-mutants have been constructed, each with a different amino acid replacement at one of the four targeted positions. Previous studies indicated that these residues were important for MoFe activity, and may act as metallocenter ligands. Unusual redox and spectroscopic properties of the Fe-S centers suggest the involvement of ligands other than the four typical cysteines, though extrusion requirements indicate that some thiol ligands are likely. Surprisingly, mutants with an Asp, Gly, Thr, or Ser substituted for Cys88 are still capable of diazotrophic growth (Nif+), though whole cell and crude extract acetylene reduction activity is lowered. Several substitutions (Cys, Asp, Phe, Asn, Met, Tyr, Leu) are tolerated at the His83

position, these Nif+ mutant strains also have varying acetylene reduction rates and growth rates. All mutants with substitutions at positions 62, 154, resulted in complete loss of diazotrophic growth. The results could be interpreted by the following explanations:

1) Our proposed model for the P-cluster ligation within the MoFe protein is incorrect.

2) Some substitutions permit P-cluster rearrangement to a semi-functional state.

3) Either, P-clusters are not absolutely essential for diazotrophic growth, or the enzyme can function with a reduced number of these metal centers.

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# LITERATURE REVIEW

## Introduction.

Nitrogen is an essential element for all living cells. It is a component in proteins, nucleic acids, amino acids and other important biomolecules. While the earth's atmosphere contains approximately 80% dinitrogen ($N_2$), most organisms can not utilize this biologically inert form, and must obtain a fixed source of nitrogen ($NH_3$, $NO_2^-$, $NO_3^-$, amino acids, etc) for growth and survival. Agriculturally, the demand for a fixed nitrogen source greatly exceeds its availability. Hence, fixed nitrogen is a limiting factor in the production of food for the earth's increasing population.

Nitrogen fixation, the conversion of atmospheric dinitrogen to a fixed nitrogen form, is one of three phases in the nitrogen cycle (Figure 1). Both biological and industrial processes are responsible for nitrogen fixation. Industrial nitrogen fixation converts $N_2$ and $H_2$ to ammonia using the Haber-Bosch process, which requires an enormous quantity of energy. The energy needed to create high temperatures and pressures, in combination with the cost of the catalyst and equipment, make the industrial production of ammonia very expensive. In contrast, the biological fixation of nitrogen occurs at ambient temperatures and pressures and is carried out solely by a few prokaryotic organisms known as diazotrophs. All these organisms synthesize nitrogenase, which catalyzes the reduction of $N_2$ to ammonia. In an effort to increase the world's food supply a great deal of research has focused on improving both the

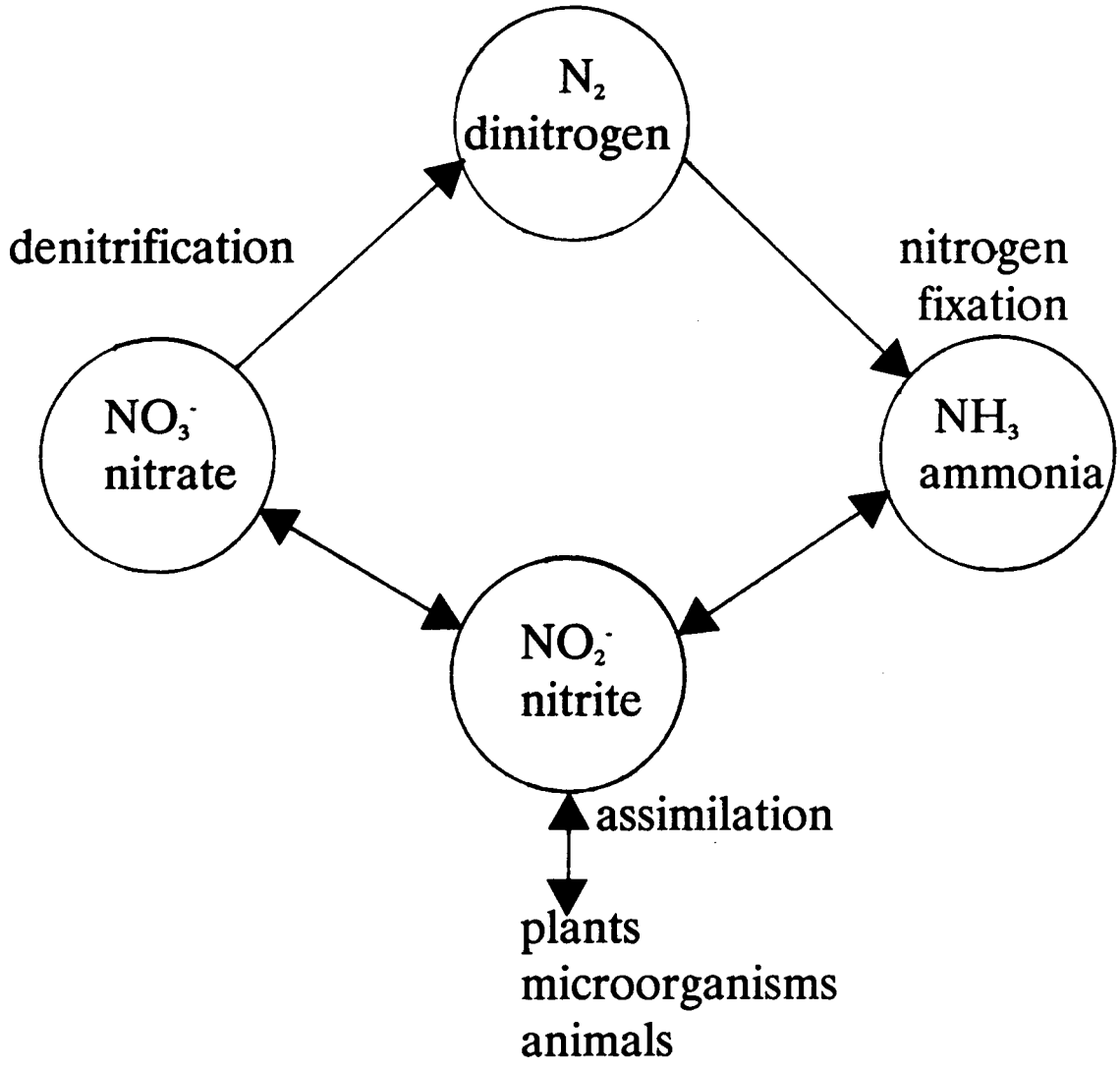


**Figure 1.** The Nitrogen cycle. The three phases are: Nitrogen fixation (conversion of atmospheric $N_2$ to $NH_3$), Assimilation of fixed nitrogen into the biosphere, and the Denitrification of organic and inorganic nitrogen forms.

biological and industrial processes of nitrogen fixation. A better understanding of diazotrophs and more specifically the structure and enzymatic mechanism of nitrogenase will inevitably lead to the development of better nitrogen fixing bacterial strains, and possibly, the improvement of catalysts for efficient production of fertilizers.

**Historical Perspective.**

The early Romans were the first to realize that rotating leguminous crops (ex. lentil beans) with non-legumes (ex. wheat) improved the overall crop production. However, it was not until 1838 that Boussingalt proved that legumes increase the nitrogen content of the soil by extracting nitrogen from the atmosphere. It was the replenishment of nitrogen in the soil that resulted in the increased productivity historically observed during crop rotation.

In 1888, the German researchers Hellriegel and Wilfarth discovered the precise source of nitrogen fixation associated with legumes (96). Their research conclusively proved that legumes contain bacteria that utilized atmospheric $N_2$ and these bacteria were found in the root nodules. Subsequent to Hellriegel and Wilfarth's discovery, several nitrogen fixing bacteria were isolated, including the root nodule bacterium *Rhizobium leguminosarum* (Beyerinck, 1888) and the free-living anaerobic bacterium *Clostridium pasteurianum* (Winogradsky, 1893) (96). At the present time, over one hundred different nitrogen fixing bacterial species have been described, including the free-living aerobe *Azotobacter vinelandii*. This organism was isolated from a Vineland, New Jersey soil sample in 1903. Table 1. has examples of some common diazotrophic species and their diverse habitats.

Table 1. Diazotrophic Bacteria.

| Habitat | Genus | Species |
|---|---|---|
| Obligate Aerobes | *Azotobacter* | *vinelandii, chroococcum* |
| | *Derxia* | *gummosa* |
| Strict Anaerobes | *Clostridium* | *pasteurianum, butyricum* |
| Facultative Anaerobes | *Klebsiella* | *pneumoniae* |
| | *Enterobacter* | *cloaciae, aerogenes* |
| | *Azospirillum* | *lipoferum* |
| Photosynthetic | *Rhodospirillum* | *rubrum* |
| | *Rhodobacter* | *capsulatus* |
| | *Anabaena* | |
| Symbiotic | *Rhizobium* | *leguminosarum, meliloti* |
| | *Bradyrhizobium* | *japonicum* |
| | *Frankia* | |
| Chemoautotrophs | *Thiobacillus* | *ferroxidans* |
| | *Methylosinus* | *trichosporum* |

Burris and Miller (15) made the first major technical advance in 1940 with the development of an assay using $^{15}N$ as a tracer for following nitrogen fixation in bacteria. Using the $^{15}N$ tracer, Carnahan et al (1960) showed nitrogen fixation in cell-free extracts of *C. pasteurianum* (16). In 1966 two independent groups observed the ability of the nitrogenase enzyme to reduce acetylene to ethylene (22,106). This observation, which made it possible to detect nitrogenase activity in a much simpler assay using gas chromatography, eventually led to the first description of nitrogenase. Purification of the two component enzyme quickly followed, as well as studies aimed at the identification of the nitrogen fixation genes (*nif* genes). In the last 20 years, there has been an explosion of genetic and biochemical research on the biological nitrogen fixation process.

**Enzymology of Nitrogenase**

Nitrogenase is a complex metalloenzyme composed of two separable components, the Fe protein, and the MoFe protein. The Fe protein, which contains Fe, is a specific ATP-binding, one electron reductant of the MoFe. The Fe protein is a dimer of approximate molecular weight of 63,000, composed of identical subunits with a single 4Fe-4S center thought to be symmetrically bridged between the subunits. The MoFe protein is a tetramer of two nonidentical subunits ($\alpha_2\beta_2$) with an approximate molecular weight of 220,000. The Fe and Mo is contained in two different types of metal centers, two FeMo cofactors (FeMoco) and 4 4Fe-4S centers (P-clusters). The FeMoco is thought to be, at least in part, the site of $N_2$ reduction. Both components are extremely oxygen labile. Biological $N_2$-fixation takes place according to the irreversible reaction:

$$N_2 + 8e^- + 8H^+ + 16MgATP \rightarrow 2NH_3 + 1H_2 + 16MgADP + 16Pi$$

This reaction requires an anaerobic environment, and both components of nitrogenase. Physiologically, the reaction is very costly, consuming $8e^-$ and 16ATP's for each molecule of $N_2$ reduced. The $H_2$ produced during catalysis is a wasteful side-reaction with no obvious apparent function. The energy lost ($2e^-/N_2$ reduced) in reducing $H^+$ to $H_2$ is regained in some organisms by hydrogen uptake systems (hydrogenases).

Because the Fe protein is an one electron donor to the MoFe protein eight cycles of electron transfer and ATP hydrolysis take place. The general reaction sequence is thought to proceed in this order:

1) Fe protein binds 2 MgATPs.

2) Fe protein is reduced by one electron.

3) Fe and MoFe proteins associate.

4) Single electron transfer from the Fe to the MoFe protein coupled to ATP hydrolysis.

5) Component proteins dissociate.

6) Cycle repeats until the MoFe protein (bound with substrate) is sequentially reduced.

The most comprehensive model describing the mechanism of nitrogenase was developed by Thorneley and Lowe (126). This model can accommodate all available chemical data on $N_2$ reduction. According to their model the rate limiting step is the dissociation of the component proteins (step 5). *In vivo* the electron donors to the Fe protein are probably a flavodoxin and ferredoxin. Specific electron donors to the Fe protein have been identified only for *K. pneumoniae* (83,112). These will be discussed latter in the

text. *In vitro* sodium dithionite is commonly used as a source of reducing equivalents. In either case the flow of electrons is from reductant → Fe protein → MoFe protein → substrate (see Figure 2). Although several studies have revealed important characteristics of MgATP-binding and hydrolysis, the exact function of this during catalysis is not known.

Nitrogenase is capable of reducing triple-bonded substrates other than $N_2$ (ex. acetylene, azide, nitrous oxide, cyanide, alkyl cyanides and alkyl isocyanides). All of the $N_2$ analogues inhibit $N_2$ reduction. Acetylene proves to bind to nitrogenase as well as $N_2$, (106) and the reduction product, ethylene, is easily detected by gas chromatography. Acetylene reduction is a standard method for both *in vitro* and *in vivo* measurements of nitrogen fixation. The reaction for acetylene reduction is:

$$C_2H_2 + 2H^+ + 2e^- + 4MgATP \rightarrow C_2H_4 + 4MgADP + 4Pi$$

Nitrogenase is the only enzyme known to date which can reduce acetylene. This has made the identification of new diazotrophs much less complicated. The triple bonded molecule, carbon monoxide (CO), is not reduced by nitrogenase but inhibits the reduction of all of the mentioned substrates including $N_2$. $H_2$ evolution is insensitive to CO inhibition suggesting separate active sites for $N_2$ and $H_2$ reduction.

It is important to emphasize that though the general mechanism for $N_2$ reduction is known, the exact function and structure of all the metal prosthetic groups in the MoFe protein, and the exact site for substrate binding is unknown. The molybdenum atom within the FeMoco was long thought to be an essential element for $N_2$-reduction, but the identification of Mo-free nitrogenase systems (alternative systems) (7) has

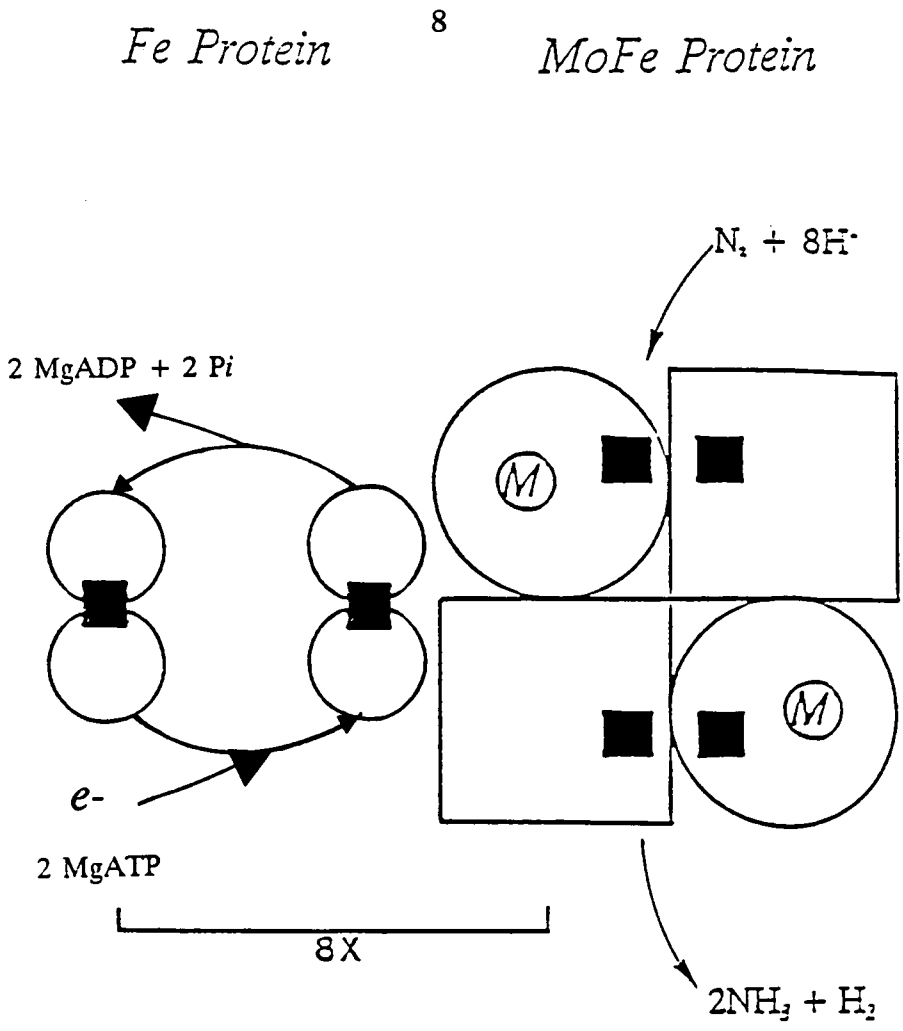


**Figure 2.** Electron transfer through nitrogenase. Eight cycles of protein association and disociation and ATP hydrolysis are required to fully reduce $N_2$ to ammonia. Circled M represents the FeMo cofactor and the black boxes represent Fe-S clusters.

raised questions concerning the catalytic importance of Mo. Alternative nitrogenases will be discussed in a separate section, and all reference to "nitrogenase" will imply the Mo-containing enzyme.

**The *nif* Genes: Products and Functions**

Most of the current knowledge of the nitrogen fixation genes (*nif*) has come from *K. pneumoniae* and *A. vinelandii* as both organisms are amenable to sophisticated genetic manipulation. The first important genetic studies of the *nif* genes were applied to *K. pneumoniae*. Thus, all *nif* genes, regardless of organism, are denominated on the basis of their homology to the twenty *nif* genes identified in *K. pneumoniae*.

The major *nif* gene cluster is comprised of eight transcriptional units containing 21 *nif* genes all arranged together on the chromosome. A comparison of the *nif* clusters from *K. pneumoniae* and *A. vinelandii* are presented in Figure 3. The entire nucleotide sequence for both the *K. pneumoniae* (1) and *A. vinelandii* (52) major *nif* clusters was recently completed. Some differences observed when comparing the two *nif* gene arrangements are: *A. vinelandii* has several open reading frames (ORFs) not present in *K. pneumoniae*, large spacer regions are found between the conventional *nif* genes in *A. vinelandii*, and neither a *nifJ* nor a *nifL* homologue has yet been found in *A. vinelandii*. One other genetic difference was revealed by mutational analysis of the *nifF* gene in *A. vinelandii* (3). Unlike *K. pneumoniae*, *NifF-* mutants in *A. vinelandii* are still capable of diazotrophic growth ($Nif^+$), indicating that the *nifF* gene product is not essential for nitrogen fixation in *A. vinelandii* (3). Overall, the organization of the nif genes in *A. vinelandii* and *K. pneumoniae* is conserved and many of the homologous proteins

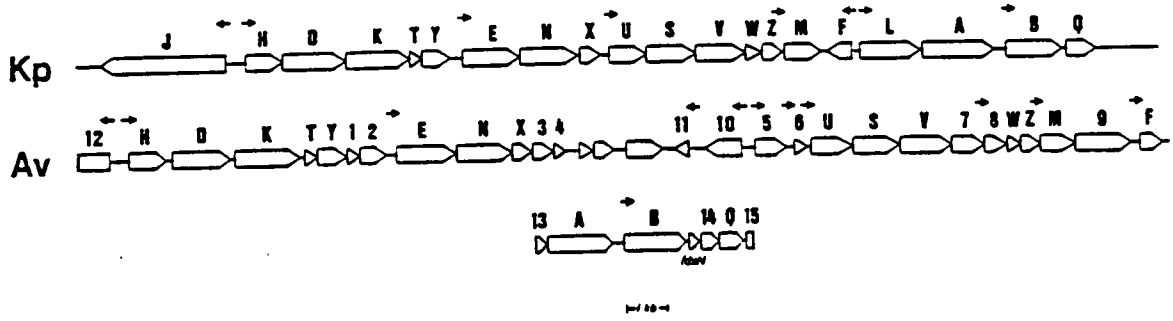


**Figure 3.** Comparison of the major *nif*-clusters from *K. pneumoniae* (top) and *A. vinelandii* (bottom). Arrows above genes represent the start and direction of transcription. Numbers corespond to open reading frames.

(especially the component proteins) have been shown to be functionally identical in genetic and biochemical complementation studies (30).

The structural proteins, Fe protein and the $\alpha$ and $\beta$ subunits of the MoFe protein, are encoded by the *nif H, D*, and *K* genes respectively. When synthesized, these polypeptides are not active until proper post-translational modifications are completed by other *nif*-specific gene products. The functional role(s) of each *nif* gene product is presented in Table 2. To date, the *nif* gene products are assigned to at least one of six functional groups: nitrogenase structural component, electron transport, FeMoco biosynthesis, Fe protein maturation, MoFe protein maturation, or regulatory element.

***nif H* gene product: The Fe protein**

Nif H encodes a 33 Kd monomer subunit of the Fe protein. The dimer contains a redox active 4Fe-4S center, two consensus ATP-binding sites and regions for MoFe protein interaction. Complete amino acid sequences are known for the *nifH* gene products from 16 different bacterial species (see Table 3.). Comparisons of the *nifH* gene products reveal strong sequence conservation (39,40). The common features observed are: presence of 5 conserved cysteines positioned in regions which are also conserved, a consensus ATP-binding site (101), and the conservation of an arginine at residue 100.

The importance of the conserved cysteinyl residues has been investigated by both chemical and genetic modification studies. Howard et al (41), proposed a model which places Cys97 and Cys132 as ligands to the Fe-S center within the Fe protein dimer.

Table 2. Proposed fuctions of the *nif* gene products

| *nif* gene | Function |
|---|---|
| H | Fe protein monomer, FeMoco biosynthesis |
| D | MoFe protein $\alpha$-subunit |
| K | MoFe protein $\beta$-subunit |
| E | FeMoco biosynthesis |
| N | FeMoco biosynthesis |
| B | FeMoco biosynthesis |
| Q | FeMoco biosynthesis |
| V | Homocitrate synthase, FeMoco biosynthesis |
| U | Maturation/stability of active Fe protein |
| S | Maturation/stability of active Fe protein |
| W | Maturation/stability of active MoFe protein |
| Z | Maturation/stability of active MoFe protein |
| M | Maturation of Fe protein |
| F | Flavodoxin, electron transport to nitrogenase |
| J | Pyruvate: flavodoxin oxidoreductase, electron transport |
| A | Positve activation of *nif* transcription |
| L | Negative regulator of NifA function |
| T | Unknown |
| Y | Unknown |
| X | Unknown |

Table 3. Available nitrogenase amino acid sequences

| Organism | NifH | NifD | NifK | Reference |
|---|---|---|---|---|
| *Anabaena* | x | x | x | 63,70 |
| *A. vinelandii* | x | x | x | 9 |
| *A. chroococum* | x | | | 103 |
| *Br. japonicum* | x | x | x | 33,58,125 |
| *C. pasturianium* | x | x | x | 17,130,134 |
| *K. pneumoniae* | x | x | x | 1,50,121 |
| *R. meliloti* | x | | | 127 |
| *R. phaseoli* | x | | | 95 |
| *R.* parasponia | x | x | x | 109,132 |
| *R.* IRc78 | | x | | 134 |
| *R. OR5571* | x | | | 85 |
| *R. trifolii* | x | | | 108 |
| *Rh. capsulatus* | x | x | x | 57,107 |
| *T. ferrooxidans* | x | | | 94 |
| *Mb. ivanovii* | x | | | 117 |
| *Mc. thermolithotrophius* | x | | | 117 |
| *Mc. voltae* | x | | | 116 |
| *Frankia* | x | | | 84 |

their studies show that residues 97 and 132 are selectively labeled with iodo [2-$^{14}$C] acetic acid (IAA) in the presence of nucleotide, and the labeling corresponds with the observable loss of the Fe-S center. In a separate experiment, Fe protein was incubated with IAA, nucleotide, and a chelator. Again, residues 97 and 132 showed enhanced labeling compared to the other cysteinyl residues in the protein. This suggested that as the Fe-S center is chelated, the exposed thiol ligands (Cys97,132) are readily labeled.

Genetic studies (47) further strengthened this model. Using site-directed mutagenesis it was possible to convert each individual conserved cysteine to a serine. Mutants with serines at either positions 97 or 132 were Nif - and had no detectable Fe protein activity, while alteration at the other three cysteines had moderate levels of enzyme activity. The absence of Fe protein activity for the 97 and 132 cys to ser mutants was attributed to the elimination of the Fe-S center as a result of removing the thiol ligands.

As stated before, the exact role of ATP-binding and hydrolysis is unclear, but some insight is revealed by these observations: binding of MgATP to the Fe protein lowers the redox potential, making it a more powerful reductant (137), EPR and CD spectra changes after MgATP binding, indicating a conformational change (118,138), and accessibility of various reagents to the Fe-S center is increased when MgATP is incubated with the Fe protein (41). One can envision, that when MgATP binds to the Fe protein it is readily reduced, and in a conformation with the reduced Fe-S center exposed, thus enabling easy association with the oxidized MoFe protein allowing efficient

electron transfer coupled to hydrolysis of the MgATP.

The mixing of purified component proteins from different bacterial species defined a range of functional complementary nitrogenases (30). Of particular interest is the inactive nitrogenase complex formed from mixing Fe protein from *C. pasteurianum* with the MoFe protein from *A. vinelandii* (29). The defective complementation is attributed to the tight Fe-MoFe protein complex formed, such that component dissociation is not possible. The Fe protein from *A. vinelandii* is elongated by 13 amino acids (including five negatively charged residues) when compared to *C. pasteurianum* (39). The C-terminal ends of these proteins have been suggested to provide proper MoFe protein recognition. An elaborate genetic manipulation has made possible the construction of an *A. vinelandii-C. pasteurianum* hybrid Fe protein. The last 18 residues of the *A. vinelandii* Fe protein are replaced by the last five residues from the *C. pasteurianum* Fe protein sequence. This hybrid protein strain is capable of diazotrophic growth, a result which demonstrates that the differences in the two Fe protein's C-terminal ends cannot fully account for the tight inactive complex seen *in vitro* (Dean, unpublished data).

The Fe protein from many photosynthetic bacteria is inactivated by the covalent attachment of ADP-ribose to one monomer at arginine 100 (38,93). The inactivation is catalyzed by an ADP-ribosyl transferase using NAD as a substrate, and reactivation (removal of the ADP-ribose) is catalyzed by another enzyme, glycohydrolase (38). Though this mode of regulation was demonstrated *in vitro* with Fe proteins from several sources, no physiological role has been shown *in vivo* for *A. vinelandii* (65).

Besides being the specific electron donor to the MoFe protein, the Fe protein also serves a function in the assembly of the FeMo cofactor (100). This will be discussed in a separate section describing all the known FeMoco biosynthetic genes and their products.

***nifD* and *nifK*: The MoFe protein**

The *nifD* gene encodes the 55-58 Kd $\alpha$-subunit, and *nifK* encodes the 56-59 Kd $\beta$-subunit, together in a $\alpha_2\beta_2$ conformation, they comprise the MoFe apo-protein. The active form contains 2 atoms of molybdenum and approximately 32 Fe and 32 $S^-$ atoms. The metals are arranged in six prosthetic groups (based on their Mossbauer and EPR properties) of at least two distinct types, four 4Fe-4S centers (P-clusters), and two FeMo cofactors (FeMocos) (115).

The complete amino acid sequences are known for the MoFe proteins of several diazotrophs (see Table 3). When primary sequences of the $\alpha$ and $\beta$-subunits are compared among different organisms as in Figures 4 and 5, strong sequence conservations are revealed. The high degree of conservation between nitrogenases from different organisms is usually attributed to the conformational constrains required for positioning the various metal centers. There are five conserved cysteines (positions 62,88,154,183,275) in the $\alpha$-subunit and three conserved cysteines (positions 70,95,153) in the $\beta$-subunit (position numbers correspond to the *A. vinelandii* sequence). These residues have received attention because cysteinyl-mercaptide ligands have been well characterized in Fe-S containing proteins, thus setting a precedence for possible cysteine metal ligands in the MoFe protein. The conservation of residues surrounding

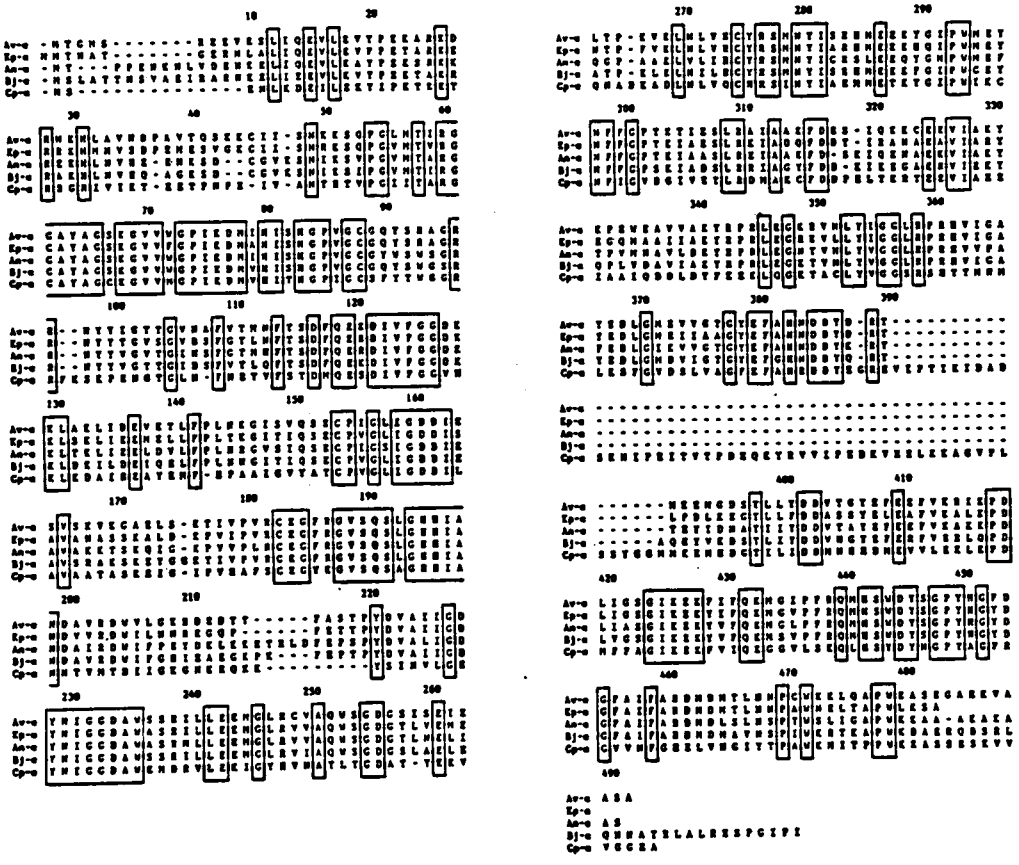

**Figure 4.** Alignment of MoFe protein α-subunit sequences from *A. vinelandii* (Av), *K. pneumoniae* (Kp), *Anabaena* 7120 (An), *Br. japonicum* (Bj), and *C. pastuerianum* (Cp). See table 3. for references. Amino acids conserved in all five organisms are boxed. Alignement adjustments were made to provide the best fit (dashed lines). Conserved cysteine residues discussed in the text are at positions 62, 88, 154, and 275 of the Av-α sequence (top line). Conserved histidine residues 80 and 83 are also discussed.

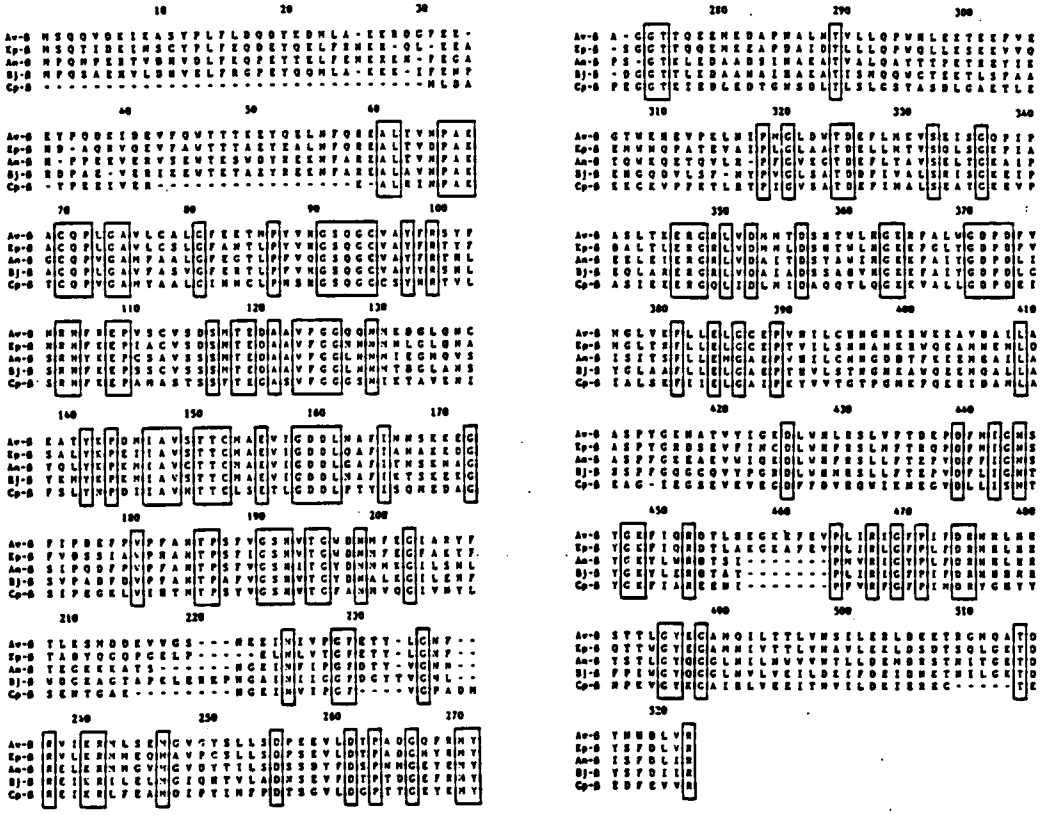

**Figure 5.** Alignment of MoFe protein $\beta$-subunit sequences from *A. vinelandii* (Av), *K. pneumoniae* (Kp), *Anabaena* 7120 (An), *Br. japonicum* (Bj), and *C. pastuerianum* (Cp). See table 3. for references. Amino acids conserved in all five organisms are boxed. Alignement adjustments were made to provide the best fit (dashed lines). Conserved cysteine residues discussed in the text are at positions 70, 95, and 154 of the Av-$\beta$ sequence (top line). Conserved histidine residue 90 is not boxed because the An sequence has a glutamine at the corresponding position.

each cysteine has important implication if these regions are indeed the metal-binding domains. As would be expected, the strong primary sequence conservations translates to a conservation at the secondary structural level, as predicted by computer analysis (50,63,130,132). Such analysis place each conserved cysteine in hydrophilic regions of the protein, usually a surface or near surface location. A surface location may be necessary for the acceptance of electrons from the Fe protein during catalysis.

Because only 16 cysteines are conserved, and 18 would be required if thiols were to supply the only ligands to the metal centers, other amino acids must be involved in metal binding. Genetic evidence and metal cluster extrusion requirements (which will be mentioned later) suggest that some thiol ligands are suspected in both the P-clusters and FeMocos. One residue, Cysteine 154 of the $\alpha$-subunit is considered a good candidate for P-cluster binding because it is contained in stretch of amino acids which show a remarkable identity with a ligand domain of some bacterial ferredoxins. The common sequence surrounding Cys 154 (Glu-Cys-Pro-Val-Gly-X-Ile) may provide an important structural pocket for a Fe-S center (64). A specific site-directed mutant of *A. vinelandii* which has the conserved Cys275 from the $\alpha$-subunit, replaced with a Ser, is incapable of diazotrophic growth, and acetylene reduction (11) and does not contain the $S=^3/_2$ EPR signal indicative of FeMoco (see below) (13). Three important observations which suggest that Cys275 supplies a thiol ligand to FeMoco are: 1) postulation of a single thiol ligand to FeMoco (14), 2) correlation between a conservation of amide containing residues near Cys275 and the nature of N-Methyforamide FeMoco extrusion (10), 3) increased availability of FeMoco in crude extracts of a Cys275 to Ala mutant of *K.*

*pneumoniae* (21).

Physical characteristics of the MoFe protein suggested the $\alpha$ and $\beta$-subunits may be similar (59,64,133). Besides their similar molecular weights and amino acid compositions, there are regions which appear very similar when the primary sequences of both subunits are compared. A diagrammatic comparison is shown and discussed in a later section (see Figure 11). The most striking observation is the nearly identical arrangement of the first three conserved cysteines in both the $\alpha$ and $\beta$- subunits. The predicted structural similarities surrounding conserved cysteines 62, 88, and 154 in the $\alpha$-subunit, and 70, 95, and 153 in the $\beta$-subunit, were found in two other gene products (located immediately downstream of the *nif*D and K genes), the NifE and NifN proteins. The relevance of this observation is examined in the FeMoco biosynthetic genes and products section. The subunit-subunit similarities may indicate functionally homologous regions within the MoFe tetramer.

The FeMo cofactor and P-clusters within the MoFe protein have been intensively studied because of their importance in the catalytic function of the enzyme. Arguably one of the most important advances in the study of nitrogenase was the identification and isolation of the FeMo cofactor (98,111). Brill and coworkers first demonstrated that a solution of HCl treated MoFe protein , which was completely devoid of activity, contained a factor which restored $N_2$-fixing activity to crude extracts of a mutant strain, *A. vinelandii* UW45 (defective in MoFe activity) (81). The activating factor (now known to be FeMoco) was demonstrated in preparations of *K. pneumoniae, C. pasteurianum*, and *R. rubrum*. UW45 has subsequently been shown (by DNA

sequencing) to be defective in a gene (*nifB*) required for FeMoco biosynthesis (55).

Shah and Brill isolated the low molecular weight activating factor which they named FeMoco. The extraction of FeMoco requires an anaerobic preparation of MoFe protein be partially denatured with citric acid, neutralized, and then extracted with alkaline N-methyforamide (NMF). Soon after the isolation procedure was worked out, FeMoco was examined by EPR and Mossbauer spectroscopy (98). The biologically unique $S = {}^{3}/_{2}$ EPR spectra detected in preparations of MoFe protein were nearly identical to the spectra obtained from purified FeMoco, therefore FeMoco was identified as the EPR active metal center in the MoFe protein. The EPR signal of isolated FeMoco could be reshaped to closer resemble that of native MoFe protein by the simple addition of thiolphenol, and the nature of NMF extraction was attributed to a N-deprotonated amide ligand (124,129), both indications of possible S and N-containing ligands to FeMoco in the native protein. Burgess et al. has shown that isolated FeMoco reacts with a single thilolate (one per Mo atom), further evidence of at least one S-ligand to FeMoco (14).

FeMoco within the protein exhibits three oxidation states: 1) FeMoco oxidized (EPR silent), 2) FeMoco semireduced (EPR active), 3) FeMoco reduced (EPR silent). The transition from FeMoco (ox) to FeMoco (semireduced) requires the addition of one electron (82) a second electron fully reduces it to the FeMoco (reduced) state.

The most informative FeMoco structural data has come from X-ray absorption near-edge spectra (XANES) comparisons between synthetic Mo-containing compounds and FeMoco, both bound and free of the MoFe protein (82). The comparison suggest

that FeMoco contains a $MoS_3$ core. A number of proposals for the structure of FeMoco have been presented, unfortunately none have been consistent with the observed spectroscopic data (32). Recently an organic compound (homocitrate) was found to be incorporated into FeMoco (remember the requirement for citric acid during FeMoco extrusion) (44).

Extrusion of the P-clusters involves an exchange of the protein ligands with an extracting thiol compound (62). This is suggestive of at least some thiol ligands supplied by cysteinyl residues within the protein. Characterization of the P-clusters is made difficult due to the complex mixture of metal groups (all containing Fe) which are seemingly close together. Mossbauer experiments detect four different Fe environments in the MoFe protein labeled, 'M', 'S', '$Fe^{2+}$', and 'D' (135). The 'M' Fe arises from the FeMoco, while the remaining Fe species are incorporated in two P-cluster (4Fe-4S) types, a $D_3Fe^{2+}{}_1$ cluster and a $D_2Fe^{2+}{}_1S_1$ cluster. The 'S' centers appear to be similar to the 'D' centers, and were once thought to be part of a separate metal center from both the P-clusters and FeMoco (88), but isotopic $^{57}Fe$ hybrid studies of the MoFe protein have proven their presence in the P-clusters (72,73). The four P-clusters undergo 4e- oxidation during thionine titration, and are oxidized before the FeMoco. The specific redox properties, protein ligands, subunit location, and catalytic function (if any) of the two P-cluster types is unknown. It is known that the P-clusters are novel Fe-S centers, and the environment in which the P-clusters reside is different than the well characterized bacterial ferredoxins Fe-S centers (135).

**Nif H, E, N, B, Q, and V gene products: FeMoco biosynthetic proteins**

The synthesis of FeMoco begins with the extracellular uptake of Mo and proceeds through a series of poorly understood steps where it is assembled and inserted in the MoFe-apoprotein. It is now known that at least six *nif*-specific gene products are required for the synthesis of the FeMo cofactor in *K. pneumoniae* and *A. vinelandii.* Lesions in the *nif*E, N, and B genes result in Nif- mutant strains which do not accumulate FeMoco. Addition of FeMoco to crude extracts of *nif*E, N, and B mutants restores nitrogen fixing activity. Recently an *in vitro* system for FeMoco biosynthesis was described (114). The system requires the mixing of crude extracts of *K. pneumoniae* (NifE- or N-) and *A. vinelandii* (NifB-) mutant strains, with $MoO_4^{2-}$ and MgATP. This has provided an assay for the purification and characterization of the *nif*EN gene products (92). As predicted by Dean and coworkers (10), the *nif*EN gene products appear to form an $\alpha_2\beta_2$ complex. Furthermore the complex has a UV-visible spectrum typical of an Fe-S protein suggesting the presence of Fe-S centers. The MoFe and *nif*EN proteins are similar in size, charge distribution, and oxygen sensitivity (92). The *nif*EN complex has been proposed to form a scaffold on to which the FeMo cofactor is assembled (10,19). The basis of this prediction relies on the striking amino acid sequence homology between the *nif*E and D, and *nif*N and K gene products respectively (see Figures 6 and 7). Comparison of the *nif*E protein sequence to the *nif*D protein sequence reveals four of the five cysteines conserved in MoFe $\alpha$-subunits are conserved in the *nif*E gene product. The four cysteines correspond to Cys62, 88, 154, and 275 of the *nif*D gene product. Comparison of the *nif*N protein sequence to the *nif*K protein sequence reveals that one of the three conserved cysteines of $\beta$-subunits is also

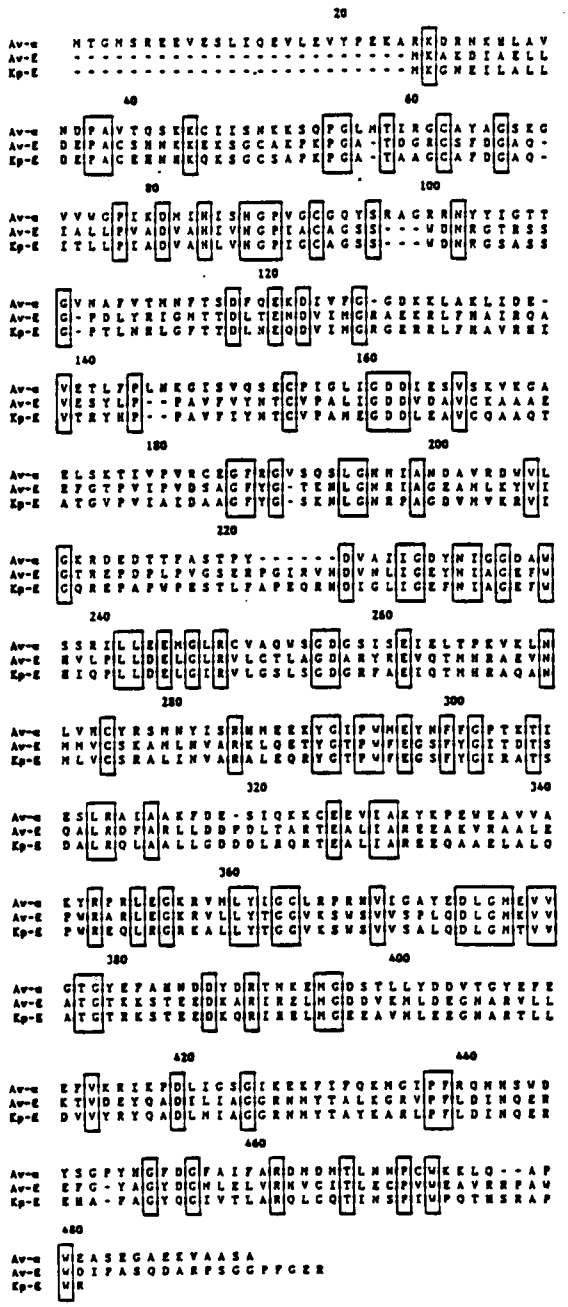

**Figure 6.** Comparison of the MoFe α-subunit and the *nif*E-encoded polypeptides from *A. vinelandii* (Av-E) and *K. pneumoniae* (Kp-E). Dashed lines represent alignment adjustments to provide the best fit. Note the conservation of cysteines 62, 88, 154 and the surrounding residues. Deduced amino acid seqence of Kp-E was accomplished in part of this study.

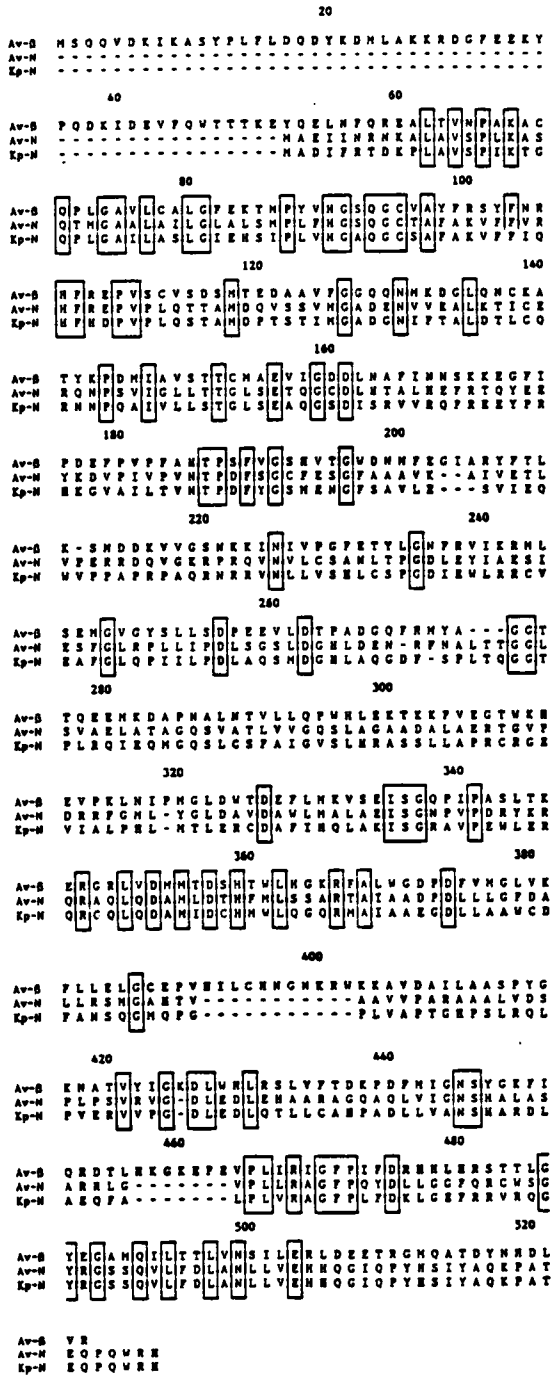

**Figure 7.** Comparison of the MoFe $\beta$-subunit and the *nif*N-encoded polypeptides from *A. vinelandii* (Av-N) and *K. pneumoniae* (Kp-N). Dashed lines represent alignment adjustments to provide the best fit. Note that only cysteine 95 ($\beta$-subunit) is conserved in both NifN proteins, this may reflect the different metal-binding properties of the NifEN complex. Deduced amino acid seqence of Kp-N was accomplished in part of this study.

conserved in the *nif*N gene product. The primary amino acid sequence of the of the *A. vinelandii* and *K. pneumoniae nif*EN gene products share significant identity, and the cysteinyl arrangement is identical (this study). The binding of FeMoco to the NifEN complex has not yet been proven, but it would be expected that the specific amino acid ligand arrangement would be different from that of the MoFe protein if the cofactor is to escape before entering the apo-MoFe protein (19).

Strains with deletions in the *nif*V gene produce a cofactor which exhibits substantial acetylene reduction activity but is incapable of efficiently reducing $N_2$ (53,71). The *nif*V gene product has been proposed to encode a homocitrate synthase, and as mentioned before, homocitrate has been shown to be an integral part of FeMoco (44). Addition of homocitrate to derepressing NifV- *K. pneumoniae* cells, cures the NifV- phenotype (43). Similar cultures of *A. vinelandii* are not cured by addition of homocitrate to 1mM. However, the amino acid comparisons of the *nif*V gene products from both organisms (6) suggests that the proteins have a similar if not identical function (ie. homocitrate synthase).

The *nif*H gene product (Fe protein), besides being the reductant to the MoFe protein during catalysis, is also physically required for the biosynthesis of FeMoco in *A. vinelandii* (100). Both *in vivo* and *in vitro* experiments support the participation of the Fe protein in FeMoco biosynthesis. *In vivo*, specific *nif*H deletion strains are defective in MoFe protein activity, and crude extracts of these strains assayed for MoFe activity (incapable of acetylene reduction) are reactivated by the addition of FeMoco. *In vitro*, FeMoco biosynthesis is carried out with *nif*H deletion strain extracts, only if intact Fe

protein is added. Further investigation has shown that the elimination of the Fe-S center within the Fe protein not only blocks substrate reduction, but also abolishes the FeMoco biosynthetic activity of the Fe protein (47). It has been proposed that the Fe protein serves as a electron donor to the *nif*EN complex during FeMoco biosynthesis, in a MgATP dependent reaction analogous to the redox step during substrate reduction (47,100).

NifQ- mutants of *K. pneumoniae* and *A. vinelandii* display a leaky phenotype (49,55). This mutation can be suppressed by the addition of molybdate (↑ 100$\mu$M) to the culture medium. Derepressed cultures of *K. pneumoniae nif*Q mutants grown in the presence of $^{99}$Mo prove that the uptake of Mo is not impaired, but at low concentrations of Mo, accumulation is decreased when compared to the wild type amount (49). Because a raised concentration of Mo can completely cure the NifQ- phenotype, the *nif*Q gene product is thought to be involved in an early step of Mo processing during FeMoco biosynthesis (49).

**nifF and nifJ: Electron transport to nitrogenase**

The transfer of electrons to nitrogenase appears to be different in *K. pneumoniae* and *A. vinelandii*. The biochemical and genetic analysis of electron transport to nitrogenase is well characterized in *K. pneumoniae* (23,35,42,83). The identification of *nif*F and *nif*J gene products as electron transport components came from the observation that extracts prepared from mutants with deletions in these genes could reduce acetylene only if dithionite was included as an electron donor (87,119). Dithionite directly reduces the Fe protein thus by-passing any specific physiological

donor (87). Both proteins have been purified and their redox properties have been studied extensively (112,83). The two electron oxidation of pyruvate is catalyzed by the *nif*J gene product, a pyruvate:flavodoxin oxidoreductase, this reduced enzyme then passes a single electron to the *nif*F gene product, a flavodoxin. The reduced flavodoxin is the specific one electron donor to the Fe protein (112).

The *nif*F gene product appears to have a different function(s) in *A. vinelandii*, for it is not absolutely required for nitrogen fixation (3). Earlier reports suggested that a ferredoxin could supply electrons to nitrogenase (4), as occurs in *C. pasteurianum* (80). An *A. vinelandii* ferredoxin (*fdx*A) has been sequenced and mutagenized (79). FdxA- mutants of *A. vinelandii* have normal diazotrophic growth rates. A double mutant, with defective NifF and FdxA proteins, has a slightly reduced growth rate under both nitrogen-fixing and non-nitrogen-fixing conditions. Therefore, NifF and FdxA may have other metabolic functions unrelated to nitrogen fixation (69), but it can not be ruled out that they can also participate in redox reactions of nitrogenase.

Recently the *fix*A, B, C, and X genes from *R. melilotii* have been cloned and sequenced (27). Preliminary DNA hybridization experiments have identified the presence of these genes in *A. vinelandii*. *Fix*A, B, C, and X gene products have been suggested to be electron transport components based on the amino acid sequence identity between the *fix*X gene product from *R. meliloti* and *fdx*A gene product (Ferredoxin I) from *A. vinelandii* (27). *R. meliloti* and *A. vinelandii* are microaerobic and obligately aerobic respectively, thus it is not inconceivable that these two organisms would have electron transfer systems quite different from the facultative anaerobe *K.*

*pneumoniae.*

**nifA and nifL: nif gene regulators**

*K. pneumoniae nif* gene expression is regulated in a two-tiered cascade (89). Gene products of *ntr*A, B, C control the first steps of global nitrogen regulation. Under nitrogen limiting conditions, *ntr*B (possible N-sensory element) activates the *ntr*C gene product (transcriptional activation element) via phosphorylation, which in turn stimulates *ntr*A-mediated transcription of the *ntr*-dependent promoters (66). NtrA, a RNA polymerase sigma-like factor, allows recognition of *ntr*-dependent promoters, specifically a -24 to -12 consensus sequence GG-$N_8$-TTGCA. An NtrA-NtrC (active) complex facilitates transcription of the *nif*LA operon. The second tier of regulation is controlled by the specific activities of the *nif*L and A gene products. The remaining *nif* operons have the -24 to -12 consensus sequence, as well as an upstream activator sequence (UAS) (78). Their transcription requires that NifA and NtrA recognize the UAS and *nif* promoter sequence respectively.

The *nif*A gene product itself is regulated by NifL in response to changes in environmental conditions. Under increased oxygen or nitrogen concentrations *nif*A is inactivated, thus shutting off all *nif* expression. NifL is believed to be responsible for the inactivation process (24,75). The *nif*A gene from *K. pneumoniae* has been sequenced, and the deduced polypeptide has been shown to contain an ATP-binding domain as well as a helix-turn-helix DNA-binding motif (a structure expected of a DNA-binding regulatory protein) (24).

*Nif* gene regulation in *A. vinelandii* is similar to the regulation discussed for *K.*

*pneumoniae*. Both organisms have consensus *nif* promoter sequences and UAS preceding most *nif* transcriptional units (1,52) as well as functionally homologous *ntr*A, B, and C gene products (76,128). The *nif*A gene product from *K. pneumoniae* has been shown to be functionally homologous in *A. vinelandii* (60). Indeed, the amino acid sequence derived from the *nif*A encoding region from *A. vinelandii* reveals the same structural domains (ATP-binding, DNA-binding domains) (2).

One fundamental difference between *A. vinelandii* and *K. pneumoniae nif* regulation is that the *ntr*C gene product is not required for *A. vinelandii nif* expression (128). Bennett et al. (2) has suggested that an open reading frame preceding the *A. vinelandii nif*A gene may act in a function analogous to the *ntr*B, C regulatory elements. To date, no *nif*L gene homolog has been found in *A. vinelandii.*

**_nif_U, S, W, Z, and M gene products: Nitrogenase maturation proteins**

NifM- mutant strains of *A. vinelandii* (53) and *K. pneumoniae* (99) have a strictly Nif$^-$ phenotype. *In vitro*, the addition of Fe protein stimulates acetylene reduction in crude extracts of NifM- mutant strains (99). Two functions of the *nif*M gene product which have been suggested both relate to the direct activation of the Fe protein. Howard et al. (46) proposed that the *nif*M gene product may be responsible for inserting the Fe-S center into the Fe protein. However, two separate observations lend evidence against this proposal. First, NifM- strains are fully capable of FeMoco biosynthesis and second, *nif*H site-mutants (discussed before) which do not contain the Fe-S center, are not capable of FeMoco biosynthesis. Considered together, this would indicated that the role of NifM is probably not the insertion of the Fe-S centers into

the Fe protein. The second suggestion for NifM's role in Fe protein activation is that is initiates a required conformational change upon the Fe-S center, after it has been inserted into the apo-Fe protein (47).

*A. vinelandii* mutant strains containing deletions in the *nif*U gene are incapable of diazotrophic growth and crude extracts of these strains are greatly reduced in Fe protein activity (52). Though the *nif*U gene products from *A. vinelandii* and *K. pneumoniae* share significant identity (6), the *nif*U gene product is not essential for diazotrophic growth in *K. pneumoniae* (74). Furthermore, an active *K. pneumoniae* Fe protein can be synthesized in *E. coli* (non-nitrogen fixer) in the absence of NifU (46).

The *nif*S gene product is not absolutely required for diazotrophic growth in *A. vinelandii* (52), *K. pneumoniae* (99), and *B. japonicum* (28), though NifS - strains from these organisms have a leaky Nif- phenotype. Nitrogenase derepressed crude extracts of *A. vinelandii* and *K. pneumoniae* NifS - strains have very low levels of Fe protein activity (53,99). *In vitro* fully active Fe protein requires some function supplied by NifS. Jacobson et al. has considered the possibility that NifU and NifS are subunits of a single enzyme (53).

*A. vinelandii* and *K. pneumoniae* strains defective in the *nif*W and *nif*Z genes are still capable of diazotrophic growth, but at a slower rate than the wild type strains due to decreased MoFe activities (53,91). Acetylene reduction assays of these strains were not enhanced by the addition of purified FeMoco (53,91).

**_nif_T, Y, and X: Functions unknown**

Deletions in *nif*T, Y, or X genes do not affect diazotrophic growth in *A.*

*vinelandii* and no function has yet been defined for these genes (52). The *nif*T, Y, and X genes have been sequenced from *A. vinelandii* (52) and *K. pneumoniae* (this study).

**Alternative Nitrogenase Systems**

The observation that certain Nif- strains of *A. vinelandii*, unable to grow on N-free medium containing Mo (10 uM), were able to grow on N-free medium when Mo was absent, led to the discovery of alternative Mo-free nitrogenases (7). The pseudorevertants were analyzed by two-dimensional gel electrophoresis, and were shown to synthesize at least four proteins not present in the $NH_4^+$ grown state, nor were these proteins present in normally derepressed wild type cultures (7). The first sound evidence for nitrogen fixation by an alternative system (not the Mo-nitrogenase) came from the labs of P. E. Bishop and D. R. Dean. They constructed *A. vinelandii* strains carrying specific deletions within the nitrogenase structural genes (▲*nif*HDK), these strains were able to grow on N-free medium in the absence of Mo (8).

Two different metal containing nitrogenases have been discovered, a vanadium nitrogenase called nitrogenase-2 (37,102), and a Fe nitrogenase, called nitrogenase-3 (18). Both alternative systems have been isolated from *A. vinelandii* and *A. chroococcum*. The expression of nitrogenase-2 requires the absence of Mo and the presence of V, while the expression of nitrogenase-3 requires the absence of both Mo and V. Regulation appears to be at the level of transcription, with the two alternative systems repressed by Mo (51).

Nitrogenase-2 Fe protein is very similar in structure to the conventional Fe protein. The Fe protein from nitrogenase-2 can effectively reduce the conventional

MoFe protein for enzymatic activity. The vanadium in nitrogenase-2 is contained within a VFe cofactor (FeVaco) which is extracted from an $\alpha_2\beta_2$ protein analogous to the MoFe protein (26). The genes encoding the nitrogenase-2 structural proteins (*vnf*HDK) have been sequenced from *A. chroococcum* (103), and the gene encoding the nitrogenase-2 Fe protein has been sequenced from *A. vinelandii* (97). Sequence analysis of the *vnf* genes reveals the presence of polypeptide subunits similar to the MoFe protein's subunits, with the conservation of all the cysteinyl residues.

Several genes from the conventional (*nif*) system are required for proper alternative nitrogen fixation. *Nif*M and *nif*B gene products are required for nitrogen fixation under all environmental conditions tested (54,61). The *ntr*A gene product is required for expression of all nitrogen fixation genes (conventional and alternative) (128). Preliminary reports suggest the *nif*U, S and V gene products may have some role in alternative nitrogen fixation, though these gene are not essential (Jacobson personal communication).

# MATERIALS AND METHODS

**_E. coli_ Strains and Growth.**

*E. coli* strain TB1 [▲*lac-pro strA ara thi @80dlacZ ▲M15 hsdR]* served as the host for all plasmids and *E. coli* strain 71-18 [▲*lac-pro thi strA supE endA sbcB15 hsdR4* (F'*pro*AB *lac*I$^q$Z M15)] served as the host for all M13 bacteriophage vectors. When necessary the medium was supplemented with 100ug/ml ampicillin. M13 viruses and DNA was prepared as described by Messing (77).

***A. vinelandii* Strains and Growth.**

A complete list of mutant *A. vinelandii* strains used in this study is found in Table 4. All strains were constructed from the wild type *A. vinelandii OP.*

Wild type and mutant strains were grown at 30° C on a modified Burk medium (120). When a fixed nitrogen source was required, filter sterilized ammonium acetate was added to a concentration of 30 mM for plates, and filter sterilized urea was added to a concentration of 10mM, for liquid media. When necessary the medium was supplemented with 20 ug/ml ampicillin, or 5 ug/ml rifampin. Diazotrophic growth rates were determined by inoculating 100 mls of N-free Burk medium in a 500 ml capacity Klett-Summerson flask with an actively growing culture, to a klett of 1-10. Klett measurements were taken every one to two hours until a stationary phase was observed.

**Site-directed Mutagenesis.**

Oligonucleotides with specific amino acid substitutions were synthesized on a Beckman System 1 Plus DNA synthesizer. At the nucleotide coding regions where

substitutions were desired an equal molar mixture of A, C, G, and T were programmed to be added to the growing oligonucleotide. Oligonucleotide-directed mutagenesis was performed by the primer extension method of Zoller and Smith (136) followed by a methylation step (45). Single stranded template DNA used for the primer extension was obtained by cloning the appropriate *nif*-specific region into M13mp18. Single lane dideoxy sequencing was used to screen for substitutions. Further DNA sequence analysis using all four bases was done to determine the exact substitution made. Replicative-form M13 mutant DNA was isolated for transformation of *A. vinelandii* cells. A list of oligonucleotides used to construct site-directed mutants is found in Table 5.

**Single Stranded Cassette Mutagenesis**

All mutations made at Histidine 83 (except D83HM which was made as described above) were made using a 60 base pair oligonucleotide with ends compatible for ligation into a Apa I and BssH II digested parent vector. The codon at position corresponding to His 83 was made degenerative in order to obtain a random sequence at this position (see Table 5.). Approximately 1-2 ug of the oligonucleotide was mixed with an equal amount short oligo adapters and annealed at 55°C for 30 minutes. This double/single stranded hybrid DNA has compatible sticky ends for ligation into the Apa I/BssH II digested vector (1-5 ug), pDB 252. The plasmid pDB 252 is a pUC 18 derivative containing a 1.5 Kb Eco RI fragment which codes for the first half of NifD. The ligation mixture was incubated overnight, then transformed into *E. coli* TB1, outgrown 2-4 hours, then plated on LB plates containing ampicillin. Plasmid DNA was

isolated from single colonies for screening by single base dideoxy DNA sequencing. Identification of plasmids containing inserts was simplified by the presence of a AAA sequence coding for Lys76 in *nif*D in all replacement positive plasmids, while all wild type plasmids contain an AAG-Lys codon. All recombinant plasmids containing a mutation at Histidine 83 were CsCl-ethidium bromide banded, resequenced, then transformed into *A. vinelandii*. A schematic representation of the cassette replacement strategy is shown in Figure 8.

**Transformation of A. vinelandii**

Transformations of *A. vinelandii* were performed in liquid cultures as described by Page and von Tigerstrom (90). Specific site-directed mutations contained within a *nif*D hybrid plasmids were recombined into the *A. vinelandii* chromosome by congression, with rifampin resistance as the selected marker. This procedure was described in detail previously (11). Also see Figure 9.

**PAGE of *A. vinelandii* proteins**

Preparation of *A. vinelandii* extracts for two dimensional gel electrophoresis was performed as described by Bishop et al (7). Two-dimensional gel analysis was performed as described by O'Farrell (86). Appendix II includes a detailed description of *A. vinelandii* nitrogenase derepression, sample preparation, and 2D-gel running procedures.

When single dimensional PAGE was performed, 1.5 mls of *A. vinelandii* (70-100 klett) cells were pelleted in microfuge tubes, boiled 5 minutes in 50 ul SDS-sample buffer, and 15-20 ul samples were loaded on a 10% gel. Gels were run at room temperature at 20 mA/gel constant amperage. Antibody precipitated crude extracts were

prepare as such: equal volumes of crude extract (50 ul) and polyclonal anti-MoFe antibody were mixed for 30 minutes at room temperature, then pelleted for 5 minutes at 14,000 rpms. Protein pellet was dissolved in 50 ul of SDS-sample buffer and boiled 5 minutes, 5-10 ul were loaded per lane.

**Dideoxy DNA sequencing**

Double stranded DNA sequencing of plasmid DNA was performed as stated in the manufactures instruction (Sequenase version 2.1, USB). Rapid mini-prep plasmid DNA (8 ul) was denatured in 2 ul of 2N NaOH for 5 minutes as room temperature, then 3 ul of primer (10 ng/ul) was added and the mixture neutralized with 3 ul of 3M Na-acetate pH 5.2, then precipitated with 75 ul of 95% ethanol. Precipitated DNA was pelleted for 15 minutes and resuspended in 10 ul of 1X Sequenase buffer for sequencing reactions.

Single stranded sequencing was performed by the Sanger dideoxy chain terminating procedure (104) using hybrids of filamentous phage vectors described by Messing (77). Specific plasmids used for sequencing the *nif*EN genes from *K. pneumoniae* were named pDB 339 and pDB 340. pDB 339 is a 2.7 Kb Sal I fragment cloned into pBR 322 and pDB 340 is a 2.0 Kb Eco RI fragment cloned into pSP 64 riboprobe vector (Stratagene). A physical map of these regions is shown in Figure 22. Seqences were obtained by digesting purified DNA fragments with the individual restriction enzymes Sau 3A, Eco RI, Sma I, Pst I, Sal I, Eco RV, HinP I or some combination of these enzymes, and ligated into the appropriately digested replicative form of the filamentous phage vector DNA. All enzymes, buffers and nucleotide mixes

were purchased from Bethesda Research Labs. (Bethesda, MD). All radiolabeled chemicals were purchased from New England Nuclear (DuPont, Boston, Mass).

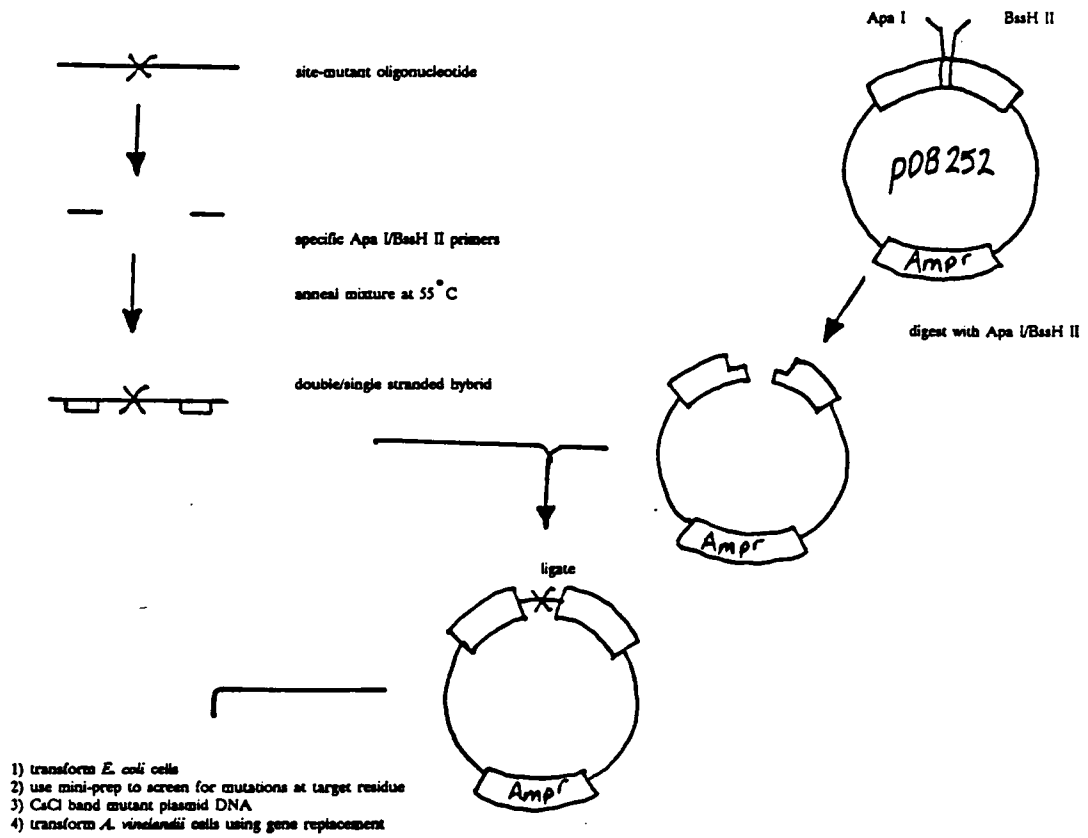


Figure 8. Method used for single stranded cassette mutagenesis of the *nif*D region surrounding His83. Site-directed oligonucleotide with variable coding sequence is designated by the single line with the X.

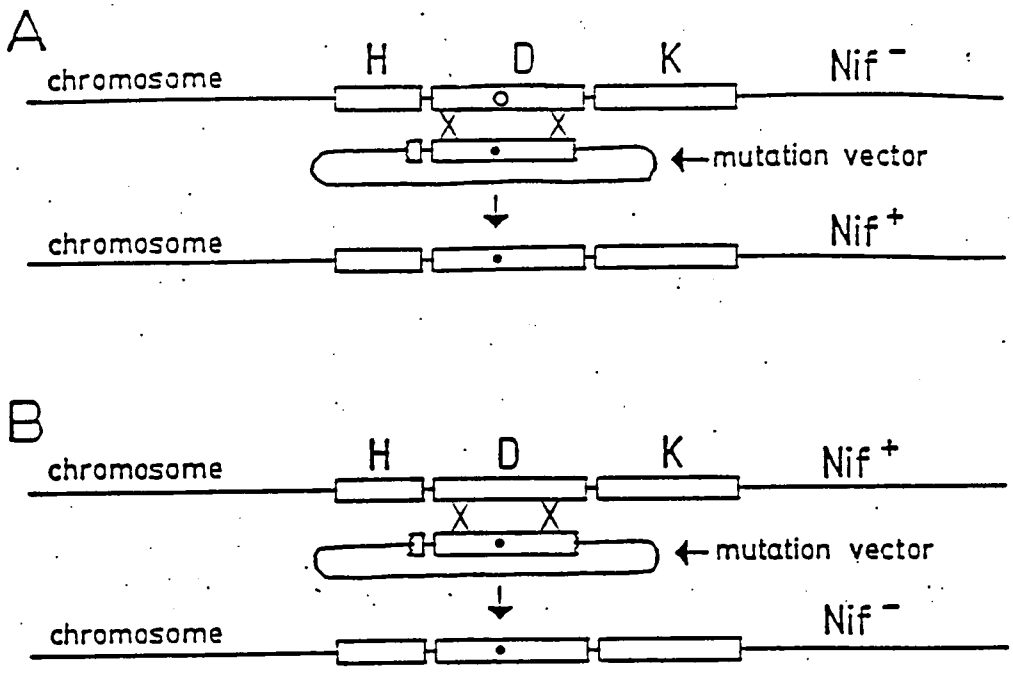


**Figure 9.** Gene replacement strategy for the construction of *A. vinelandii* site-directed mutant strains. Specific amino acid replacements are indicated by the black dot. Reciprocal recombination events between the mutation vector and the chromosome can lead to either a Nif+ or Nif- strain. Allowable Nif+ substitutions are introduced into the genome by transformation of the appropriate *nif*D (Nif-) site-directed mutant strain, indicated by the open circle (A). Nif- amino acid substitutions are introduced into the *A. vinelandii* chromosome by transforming the wild type strain to the Nif- character (B). The text refers to (A) as a genetic rescue.

Table 4. A. vinelandii strains used in this study

| Strain | nifD mutation | Nif phenotype | RF or plasmid |
|---|---|---|---|
| DJ273 | D62CA | - | Mn38 |
| DJ159 | D62CS | - | Mn27 |
| DJ436 | D62CV | - | RF2 |
| DJ438 | D62CL | - | RF4 |
| DJ440 | D62CK | - | RF1 |
| DJ442 | D62CG | - | RF5 |
| DJ445 | D62CE | - | RF6 |
| DJ447 | D62CD | - | RF3 |
| DJ326 | D83HC | + | PL12 |
| DJ328 | D83HF | + | PL15 |
| DJ330 | D83HL | +/- | PL9 |
| DJ333 | D83HG | - | PL7 |
| DJ337 | D83HP | - | PL18 |
| DJ341 | D83HR | - | PL16 |
| DJ343 | D83HI | - | PL22 |
| DJ345 | D83HD | + | PL21 |
| DJ392 | D83HY | + | PL19 |
| DJ393 | D83HM | + | RF14 |
| DJ181 | D83HN | + | Mn33 |
| DJ231 | D88CA | - | Mn39 |
| DJ162 | D88CS | +/- | Mn8 |
| DJ395 | D88CG | +/- | RF23 |
| DJ397 | D88CT | + | RF24 |
| DJ399 | D88CD | + | RF25 |
| DJ401 | D88CR | - | RF27 |
| DJ403 | D88CV | - | RF28 |
| DJ405 | D88CE | - | RF30 |
| DJ407 | D88CP | - | RF32 |
| DJ409 | D88CQ | - | RF33 |
| DJ253 | D154CA | - | Mn40 |
| DJ45 | D154CS | - | Mn3 |
| DJ411 | D154CE | - | RF35 |
| DJ413 | D154CQ | - | RF36 |
| DJ414 | D154CN | - | RF37 |
| DJ416 | D154CT | - | RF38 |
| DJ246 | D275CA | - | Mn42 |
| DJ56 | D275CS | - | Mn12 |
| DJ418 | D275CD | - | RF40 |
| DJ420 | D275CE | - | RF41 |
| DJ422 | D275CT | - | RF43 |
| DJ424 | D275CG | - | RF44 |
| DJ428 | D275CV | - | RF42 |
| DJ187 | nifD - | - | pDB243 |

Mn = mutene (RF) made in previous a study (Brigle,PhD thesis)
RF = Replicative form M13 DNA
PL = Plasmid DNA used in single stranded cassette mutagenesis
Nif+ capable of diazotrophic growth, Nif+/- very slow growth, Nif- no growth

Table 5. Amino acid replacement oligonucleotides used in this study

| Target amino acid | 5'-single stranded nucleotide sequence-3' |
|---|---|
| D62 CYS | CCATCCGCGGCCNNGCCTACG (N = A, G) |
| D88 CYS | GTCCGGTAGGCCNNGGCCAGT (N = A, G) |
| D154 CYS | TCCAGTCCGAGCNNCCGATCG (N = A, G) |
| D275 CYS | ACCTGGTTCACCNNTACCGCT (N = A, G) |
| D83 HIS | CGCGCGAATACTGGCCGCAGCCAACCGGACCCNNCCACATGTGGATCATGTCTTTGATGGGGCC (PRIMER 2; G; PRIMER 1) |
| PRIMER 1 | CCATCAAAGAC |
| PRIMER 2 | GGCCAGTATTCG |

N = Random addition of all four bases
Addition of A,C, and G at the first position of was to avoid synthesis of oligos with stop codons, and cys and ser codons (mutatants already been made).
PRIMER 1 and 2 are annealled to D83 HIS oligo to create sticky ends compatible for ligation with a Apa I and Bss HII restricted vector (see figure 8).

## RESEARCH GOALS

The nitrogenase MoFe protein contains six poorly understood metal-containing prosthetic groups of two distinct types. One type, the iron molybdenum cofactor (FeMoco) has received continuing attention because it is a likely participant in $N_2$-binding and reduction. The other type, the P-clusters, are thought to be biologically novel Fe-S centers. Their participation in the catalytic mechanism of nitrogenase is not known. Previous site-directed mutagenesis studies conducted in this lab have provided a rationale for assigning specific conserved amino acids as targets for potential metal center ligands. In a continuation of these studies, this study attempts to define a range of acceptable or non-acceptable amino acid substitutions at each targeted potential metal ligand contained in the MoFe protein $\alpha$-subunit from *A. vinelandii*. Site-specifically altered MoFe proteins, if not drastically destabilized, should exhibit changes in catalytic properties due to elimination or rearrangement of the metal centers, and aid in formulating a model for metal center binding within nitrogenase.

Amino acid sequence comparisons between the *Azotobacter vinelandii* $\alpha$ and $\beta$-subunits with the *nif*E and *nif*N gene products respectively, have been used as one criterion for targeting potential FeMoco-binding residues in site-directed mutagenesis studies. As part of this study, the nucleotide sequence of the FeMoco biosynthetic genes, *nif*E and *nif*N, from *Klebsiella pneumoniae* was determined by DNA sequencing. This information can increase the reliability of amino acid sequence comparisons by revealing conserved, as well as nonconserved residues from two physiologically different

species.

The results from the site-directed mutagenesis of the MoFe protein are presented and discussed in relation to the proposed metal binding models. The nucleotide sequence of the *K. pneumoniae nif*EN genes is presented in a publication found in Appendix I. Comparisons of these gene products with the *A. vinelandii nif*EN gene products are presented in the text.

# SITE-DIRECTED MUTAGENESIS OF THE NITROGENASE MOFE PROTEIN FROM *AZOTOBACTER VINELANDII*

## RESULTS

### Nomenclature

Specific site-directed mutants are named in order of gene, amino acid residue number, wild type residue, and then amino acid replacement. For example, a specific amino acid replacement in the *nif*D gene product at the amino acid Cysteine number 88, would be designated, D88CA, where A represents the single letter amino acid code of the new, substitute residue, Alanine.

D 88 C A

*nif* gene · residue # · WT-Cys · substitute amino acid Ala

All residue numbers will correspond to the *A. vinelandii* protein sequences, and in most cases amino acids will be designated by their single letter code. References to strains, proteins and plasmid DNA either containing or coding for single amino acid substitutions will follow this nomenclature. When the phrase "Nif + amino acid substitution" or "Nif - amino acid substitution" is used, it will refer to strains with single amino acid replacements within the MoFe protein which have the ability, or have lost the ability to grow diazotrophically. Other frequently used vocabulary is: allowable substitution (a Nif + amino acid replacement), and non allowable substitution (Nif - amino acid replacement).

### Construction of site-directed Mutants

Individual amino acid replacements in the MoFe protein was accomplished by a

combination of *in vitro* mutagenesis and *in vivo* gene replacement techniques. Two variations of *in vitro* mutagenesis were used in this study. Observations and comments concerning the two different techniques will be discussed later in this section. Both techniques result in DNA molecules randomized at two or three predetermined nucleotide positions, such that the amino acid coding information at this position can direct the synthesis of polypeptides containing different residues at the predetermined spot. After synthesis and construction of the mutagenic DNA molecules, both techniques utilize an *in vivo* gene replacement strategy, illustrated in figure 9. The benefits of gene replacement over the use of multicopy hybrid plasmids in the construction of site-directed mutants are: resultant properties of the mutated MoFe protein can be directly attributed to the known single amino acid replacement, ancillary *nif*-specific gene products are unaffected, Fe protein to MoFe protein ratios remain properly balanced, and mutations remain stable. Another advantage of the gene replacement strategy is the resultant phenotype of an amino acid replacement, either Nif + or Nif -, can be detected. Both phenotypes can be rapidly identified in genetic rescue experiments.

The basic concept of the rescue experiment is illustrated in figure 9A. Instead of using *nif*D specific deletion strains, this study made use of Nif - site directed mutants as the recipient strain to be rescued. In each case, the recipient strain to be rescued had a Nif -amino acid substitution at the particular NifD residue which was being phenotypically tested. For instance, a plasmid containing the mutation D88CG (unknown Nif phenotype), would be used to transform DJ231 (D88CA), a Nif - strain.

The transformed cells are plated on N-free medium and allowed to incubate several days. If the D88CG DNA recombined properly, and a Glycine at position 88 is an allowable substitution, this plate would show colonies with visible diazotrophic growth when compared to the untransformed DJ231 which can not grow diazotrophically. Alternatively, if D88CG is not an allowable substitution, the D88CG DNA would not rescue DJ231 and this experimental rescue plate would resemble the negative control (untransformed recipient DJ231).

All of the D83H and D88C Nif + substitutions were noticed to grow at a substantially slower rate than the wild type, this phenotype (very slow growth) allowed for a direct selection observable in the transformation of wild type cells by congression (see figure 10B). Congression (coincident transfer of genetic markers) with rifampin resistance was used to construct all strains, and rescue attempts were used to further confirm phenotypes, and for rapid screening.

The major differences in the two *in vitro* mutagenesis techniques is: cassette replacement relies on the presence of available, close by restriction sites flanking the desired amino acid of interest, while the oligo extension method can be used to introduce changes anywhere on the appropriate single stranded M13 hybrid clone. Both methods work well and can be used simultaneously with rescue experiments to rapidly determine the phenotypic result of a substitution.

Though the objective of single codon randomization was the construction of a set of mutants (each with a different amino acid at the same position), there appeared to be a bias toward the synthesis of a few mutagenic oligonucleotides. The reappearance

of particular codons limited the number of substitutions that could be made at each amino acid position. There did not appear to be any pattern in the bias and attempts to pull out different mutagenic oligos from the combined pool, by changing the sequence of the M13 template at the variable position, resulted in the isolation of the same reoccurring clones.

**Rationale for amino acid replacements**

Because only low resolution X-ray crystallographic data is available for the nitrogenase MoFe protein, an indirect approach has been used to design experiments which attempt to identify potentially important metal-binding domains. The rationale for targeting specific residues as potential metal ligands include: (1) consideration of extrusion requirements of the metal centers; (2) comparison of the MoFe sequences from different diazotrophic species (interspecies); (3) comparison of the MoFe protein $\alpha$- and $\beta$-subunit sequences (intersubunit); and (4) comparison of the MoFe $\alpha$- and $\beta$-subunit sequences with the FeMoco biosynthetic proteins NifE and NifN.

**(1) Extrusion requirements**

There are several indications which suggest that the FeMoco and P-clusters are not physically associated (62,73). Therefore, separate protein domains can be assigned to the two metal center types. The nature of extraction of FeMoco with NMF suggests that the protein functional group binding the FeMoco may resemble an amide (111). Further evidence suggesting N-ligands to FeMoco, comes from electron spin echo (124) and FT-IR analyses (129) of NMF extracted FeMoco. A S-ligand to FeMoco has also been suggested, since purified FeMoco reacts with a single thiolate (14). Considering

the above observations, protein bound FeMoco may have N-ligands and at least one S-ligand.

Fe-S centers of many ferredoxins are bound by thiol ligands (supplied by cysteinyl residues) and can be removed from the native protein by an organic solvent in the presence of excess thiols (o-xylyl-α-α'-dithiol). The process involves protein unfolding and ligand exchange between Cys-SH and the extracting thiol compound. The isolated Fe-S clusters can then be analyzed by various spectroscopic techniques to define the cluster type (2Fe-2S, 3Fe-3S, 4Fe-4S, etc.) Four P-cluster (4Fe-4S centers) can be removed from the MoFe protein (without interference from the FeMoco) by the thiol exchange method suggesting possible peptide thiol ligands. However, the P-clusters have unusual redox and spectroscopic properties, (when compared to other Cys-bound 4Fe-4S centers) suggesting other possible ligands besides cysteine, and there is still the possibility that the P-clusters undergo rearrangement during extrusion and really exist as two 8Fe-8S centers (36). Considering the thiol extrusion requirements and the unusual properties of the P-clusters, the four metal centers appear to be held in the MoFe protein mainly by cysteinyl thiol ligands and possibly one other non-cysteinyl residue.

**(2) Interspecies comparisons**

Complete amino acid sequences of the α- and β- subunits from five different bacterial species is presented in Figures 4 and 5. Comparison of the respective subunits reveal a high level of primary sequence identity. Wang et al. compiled the latest interspeices comparison between nitrogenases (130). Five cysteines are conserved in the α-subunit and three cysteines are conserved in the β-subunit (Cys 62,88,154,183,275 in

the α-subunit, and Cys 70,95,153 in the β-subunit). Because extrusion requirements of the P-clusters suggest thiol ligands, and FeMoco has been demonstrated to react with a single thiolate, it is highly probable that these conserved residues may supply a cysteinyl mercaptide ligand to one or the other metal center types.

Considering the total number of conserved cysteines (16) per tetramer, other ligands to the six metallocenters must be present, for 18 would be required to coordinate four P-clusters and two FeMocos. Other possible ligands would include conserved histidines. The α-subunit contains conserved His residues at positions 80, 83, 195, 196, and 275. Conserved Cys residues are located very close to three of these alternative amino acid ligands. Interspecies comparisons allow for the selection of eight potential cysteine and several potential histidine residues as targets for site-directed changes. Furthermore, assignment of both residue types as metal center ligands would be consistent with the observed extrusion properties.

**(3) Intersubunit comparisons**

There is significant sequence identity found surrounding the α-subunit Cys 62, 88, and 154, when they are respectively compared to β-subunit Cys 70, 95, and 153. The near identical spacial conservation and conservation of predicted secondary structure, has led to the suggestion that these regions may have similar structural and functional roles (binding of metal centers) in the MoFe protein tetramer (125). Five residues preceding the conserved α-subunit Cys 88, there is a conserved His residue present in the β-subunits of all sequenced MoFe proteins, with the exception of *Anabaena*, which has a Gln at this position. Histidine ligands to a 2Fe-2S cluster has

been suggested (122). The MoFe α-and β-subunit comparisons permit the assignment of four similar domains within the tetramer (two in the α-subunits and two in the β-subunits). Each domain contains four potential amino acid ligands (Cys 62, 88, 154, and His 83 corresponding to the α-subunit) which could accommodate the coordination of four 4Fe-4S clusters (P-clusters) (see Figures 10 and 11).

Mossbauer spectrum studies have shown that the Fe-S centers in the MoFe tetramer can be differentiated into two non-identical subsets, thought the specific coordinating ligands to each subset may be similar, if not identical (73). The differences observed by Mossbauer may reflect the two slightly dissimilar α- and β-domains surrounding the Fe-S centers.

**(4) Comparison of the MoFe α- and β-subunits with the FeMoco biosynthetic proteins, NifE and NifN**

DNA sequence analysis has revealed that the products of the *nif*E and *nif*N genes contain considerable identity when compared with the α- and β-subunits of the MoFe protein, respectively (10,19). Brigle et al. proposed that FeMoco is built upon a NifEN complex and then inserted into the apo-MoFe protein (10). This proposal is based upon two observations: *A. vinelandii* strains unable to make MoFe protein are still capable of synthesizing the cofactor (100), and the sequence identity observed when NifEN and NifDK proteins are compared. Recently, it has been shown that the *nif*E and *nif*N gene products do form a $\alpha_2\beta_2$ complex, which has been purified and shown to contain Fe and S, but FeMoco has not been demonstrated to be a part of the isolated complex (92). It seems likely that the FeMoco biosynthetic gene products, NifE and

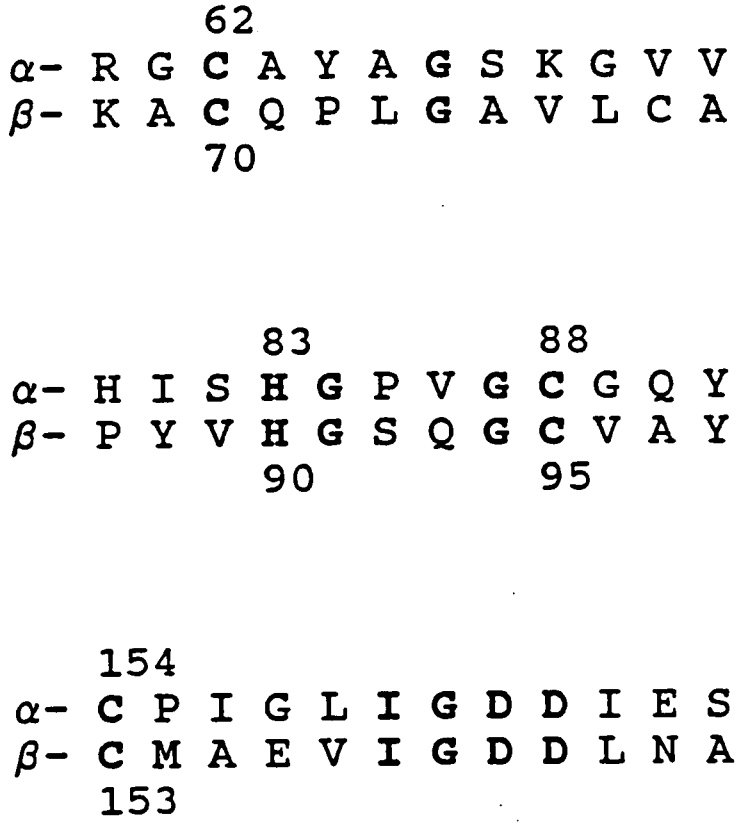


**Figure 10.** Alignment of amino acid sequences surrounding the proposed P-cluster ligands (numbered residues) in the MoFe α- and β-subunits. The numbered residues above the α-subunit were targeted for replacement in this study. The numbered residues below the β-subunit were targeted for site-directed replacements in a previous study (Brigle, PhD thesis).

```
      62                  83    88                              154
Avα- RxCxxxG-6aa-G-9aa-HGxxGC-7aa-RR-22aa-ExxxVFGG-26aa-CxxxxIGDDI
Kpα- RxCxxxG-6aa-G-9aa-HGxxGC-7aa-RR-22aa-ExxxVFGG-26aa-CxxxxIGDDI
Cpα- RxCxxxG-6aa-G-9aa-HGxxGC-7aa-RR-23aa-ExxxVFGG-25aa-CxxxxIGDDI
Avβ- KxCxxxG-6aa-G-8aa-HGxxGC-9aa-RH-13aa-ExxxVFGG-25aa-CxxxxIGDDI
Kpβ- KxCxxxG-6aa-G-8aa-HGxxGC-9aa-RH-13aa-ExxxVFGG-25aa-CxxxxIGDDI
Cpβ- KxCxxxG-7aa-G-8aa-HGxxGC-9aa-RH-13aa-ExxxVFGG-25aa-CxxxxIGDDI
```



**Figure 11.** Comparison of the spatially and structurally similar domains within the α- and β-subunits of the MoFe protein from *A. vinelandii* (Av), *K. pneumoniae* (Kp), and *C. pastueranium* (Cp). Numbered residues correspond to the conserved targeted amino acids which were replaced in the Av-α-subunit. Proposed structurally important regions for P-cluster binding are underlined, these regions correspond to the peptide arrangements shown in the proposed P-cluster model (figure 21).

NifN, would share some structurally and functionally related protein domains with the MoFe protein if the two tetramers bind the same metal centers. Indeed the regions conserved around the cysteines in the MoFe protein are also conserved in NifE and NifN (see Figures 12 and 13). FeMoco held to the NifEN complex would be expected to be more loosely bound if the cofactor is to escape from the complex and enter the apo-MoFe protein, therefore identical binding domains would not be suspected in the two tetramers. This could explain the failed attempts to isolate FeMoco from the NifEN complex. Comparison of the *nif*E and *nif*N gene products with the MoFe $\alpha$- and $\beta$-subunits has supplied additional information for site-directed mutagenesis studies aimed at determining the important metal-binding domains of the MoFe and NifEN complexes.

**Previous site-directed replacements in the nitrogenase component proteins**

Both the Fe protein and MoFe protein from *A. vinelandii* have been altered in this lab previously by the site-directed mutagenesis strategies discussed (11,47). The amino acid targets in these studies have been conserved, potential metal center ligands. The specific thiol ligands to the single Fe-S center in the Fe protein were indirectly identified by amino acid replacements (47). The MoFe protein has been subject to site specific alterations in both the $\alpha$- and $\beta$- subunits (11,13). Figure 14. illustrates the substitutions made previous to this study, which have aided in the targeting decision for multiple replacements.

In continuation of the study summarized in Figure 14, this study has concentrated on five conserved residues within the $\alpha$-subunit to further determine the result of, not just one substitution but several. Residues D83H and D88C were of

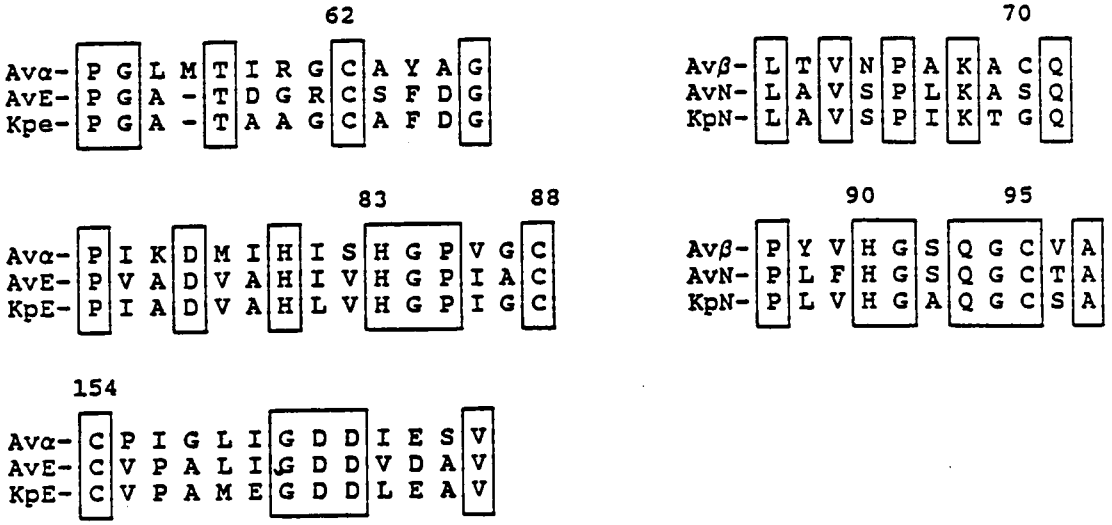


**Figure 12.** Alignment of amino acid sequences surrounding the conserved cysteines from the MoFe α- and β-subunits with the similar regions from the *A. vinelandii* (Av), and *K. pneumoniae* (Kp) NifE and NifN polypeptides. Boxed residues are conserved in all three proteins. Numbers correspond to the conserved cysteine and histidine residues of the *A. vinelandii* MoFe protein suspected to be involved in metal coordination.

| | | 83 | | | | | | | 88 | |
|---|---|---|---|---|---|---|---|---|---|---|
| Av | NifD | H | I | S | **H** | G | P | V | G | **C** | G |
| Kp | NifD | H | I | S | **H** | G | P | V | G | **C** | G |
| Cp | NifD | H | I | T | **H** | G | P | I | G | **C** | S |
| Av | NifK | P | Y | V | **H** | G | S | Q | G | **C** | V |
| Kp | NifK | P | Y | V | **H** | G | S | Q | G | **C** | V |
| Cp | NifK | P | H | S | **H** | G | S | Q | G | **C** | C |
| Av | NifE | H | I | V | **H** | G | P | I | A | **C** | A |
| Kp | NifE | H | L | V | **H** | G | P | I | G | **C** | A |
| Cp | NifE | H | L | V | **H** | G | P | I | G | **C** | A |
| Av | NifN | P | L | F | **H** | G | S | Q | G | **C** | T |
| Kp | NifN | P | L | V | **H** | G | A | Q | G | **C** | S |
| Cp | NifN | V | I | L | **H** | G | S | Q | G | **C** | S |

**Figure 13.** Comparison of the NifD, NifK, NifE, and NifN polypeptide regions at conserved residues histidine 83 and cysteine 88 (Av NifD sequence). Bolded residues are conserved in all four proteins. Note the similarities between the NifD and NifE, and NifK and NifN regions respectively. *A. vinelandii* (Av), *K. pneumoniae* (Kp), *C. pneumoniae* (Cp).

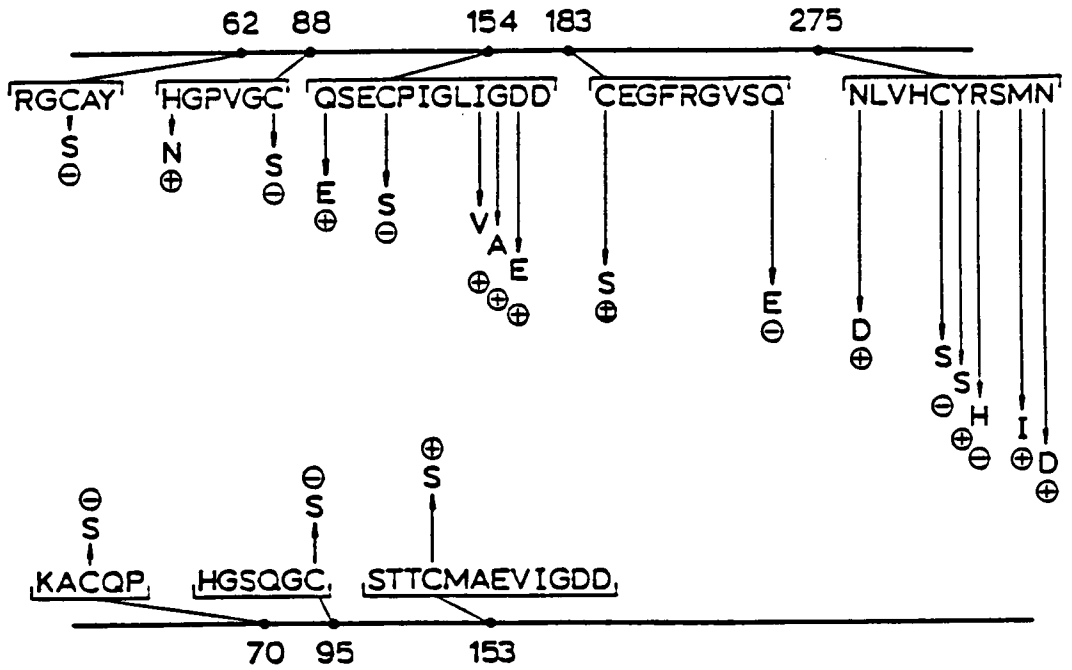


**Figure 14.** MoFe protein amino acid substitutions reported previously ( ). Top line represents the α-subunit polypeptide and the bottom line represents the β-subunit polypeptide. Specific substitutions are indicated by the arrows. The resulting Nif phenotype is shown for each substitution below the single letter amino acid code.

particular interest because of the observed allowable replacements D83HN and D88CS. While D88CS is shown to be Nif- in Figure 14, after approximately one week, signs of diazotrophic growth are apparent, and also this strain exhibits a very low acetylene reduction rate. The results of the replacements made at these and other amino acid positions are described next, followed by the discussion of these results in relation to the proposed metal-binding models.

**Characterization of amino acid replacement strains**

**D62C replacement strains**

There are eight different strains of *A. vinelandii* each containing a different amino acid replacement at D62C. All of these strains exhibit a strictly Nif - phenotype. The specific residue replacements are illustrated in Figure 15. Both positively and negatively charged amino acid replacements were constructed (D62CK, D62CD, and D62CE), as well as two hydrophobic replacements (D62CV and D62CL). The D62CA and D62CS mutant strains were used for genetic rescue attempts using newly constructed D62C mutant DNA. These experiments confirmed the Nif - character of all substitutions made at D62C. Protein samples taken at 0, 3, and 6 hours after resuspension in N-free medium were analyzed by PAGE, such analyses confirmed the presence of both MoFe subunits and the Fe protein. Rescue attempts of mutant strain D62CA with the combined *in vitro* mutagenized DNA failed to give rise to any Nif + colonies, this is considered indirect evidence that a cysteine at position 62 of the $\alpha$-subunit is required for nitrogenase activity. The strict requirement for D62C appears to reflect the thiol functional group of this residue, for a serine at this position (structurally

similar) is not allowable. Although a methionine substitution was not isolated for D62C, this would be an interesting replacement, for methionine is the only other S-containing amino acid and has been reported to supply a ligand to a heme Fe in cytochrome c (48). Another interesting replacement would be at the conserved D66G, for this residue obviously has no active functional group, but may have an important structural feature which helps form a active metal-binding domain at the D62C region. The $\beta$-subunit cysteine (K70C) substitutions also result in Nif - mutant strains, suggesting a similar required function of the two conserved residues.

| | 62 | | | | 66 |
|---|---|---|---|---|---|
| -arg -gly- | CYS- | ala- | tyr- | ala- | GLY |
| | ASP | | | | |
| | GLU | | | | |
| | ALA | | | | |
| | GLY | | | | |
| | VAL | | | | |
| | LEU | | | | |
| | SER | | | | |
| | LYS | | | | |
| Nif - | | | | | |

**Figure 15.** Site-directed replacements made at conserved D62C. All replacement strains with substitutions at D62C are strictly Nif -. Numbered and capitalized residues are conserved in both the $\alpha$- and $\beta$-subunits. The $\beta$-subunit contains a lysine at the position corresponding to arginine 60 (neutral substitution).

**D83H and D88C replacement strains**

The conserved possible N-ligand and thiol ligand residues D83H and D88C were replaced with several different amino acid residues (see Figure 16.). The D83HN mutant strain made previously, can grow diazotrophically, this was evidence that an allowable substitution at D83H was possible. Further identification of allowable substitutions at this position was attempted and indeed seven of the eleven substitutions made at this position were Nif + (allowable). Surprisingly, D83HY, D83HD, and D83HN are the "best" substitutions, these strains grow only slightly slower than the wild type strain when $N_2$ is the sole nitrogen source (see Figure 17). Component activities seem to reflect the observed diazotrophic growth rates of the Nif + D83H mutant strains (Table 6). Though the Nif - strains would not grow on N-free media, trace amounts of ethylene was detected during acetylene reduction assays (data not shown). Only D83HI and D83HG had specific activities above 0.3 nmoles ethylene produced/min/mg protein, a rate approximately 100 times slower than the wild type enzyme. The accuracy of measurements this low makes any conclusions about these substitutions unreliable. All D83H replacement strains produced visible Fe protein when derepressed cultures were analyzed by SDS PAGE, and Fe protein activities were comparable to the wild type strain. Explanations of the Nif + replacements are considered in the Discussion.

Like D83H, the available site-directed mutant data preceding this study aided in the targeting of D88C in an attempt to isolate a mutation(s) at this position which had obviously altered diazotrophic properties (not Nif -). D88CS mutant is a very slow

growing strain and has a low acetylene reduction rate. Three other amino acid substitutions were constructed at this position (D88CT, D88CD, and D88CG) which result in strains able to grow diazotrophically, and also capable of reducing acetylene. The nonallowabe substitutions at D88C were many (see Figure 16.). All derepressed mutant strains produced active Fe protein.

The possibility that serine, threonine, and aspartic acid, supply an oxygen ligand was considered, and may very well be the case, but obviously this is not the only possible replacement explanation, for D88CG has no functional group at this position. The growth of D88C replacement strains was compared with the wild type strain mainly on Burk's N-free agar plates (see figure 18). Attempts to accurately quantitate the growth rates were difficult under the conditions normally used (30°C, 300RPM, 100ml in 500ml flask). After four days the tested strains D88CD and D88CT had not reached a stationary phase. This appears to be a questionable result considering wild type doubling time of 2.5 hours. Perhaps this is a reflection of the mutant organisms response to changes in cellular oxygen concentration, and on plates these organisms can adjust the cellular $O_2$ concentrations to maximize their defective nitrogenase activity. An increase in $O_2$ concentration may be necessary for $N_2$ reduction by the mutant enzymes, if they require additional electrons for turnover (over the normal 8e-/$N_2$ reduced). Both D83H and D88C replacement strains gave rise to Nif + and Nif - phenotypes of varying magnitude, these strains help to conceive a P-cluster model, a model with possible cluster rearrangement qualities.

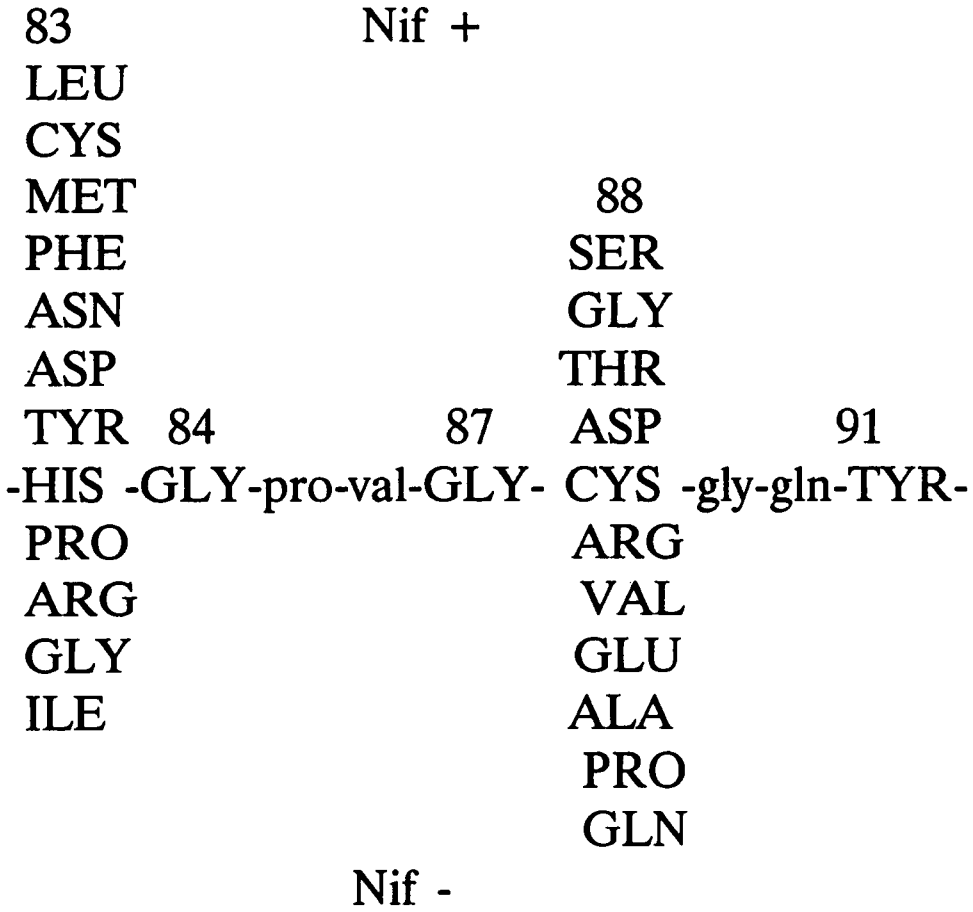


**Figure 16.** Site-directed replacements made at conserved D83H and D88C. Amino acid replacement strains which exhibit a Nif + phenotype (allowable substitution) have been placed above the polypeptide chain, and those below are strains which result in a Nif - phenotype (non-allowable substitution). The Nif + residue replacements are organized with the fasting growing strain being closest to the wild type residue (D83HL is slowest growing strain of all the D83H mutants). The numbered and capitalized residues are conserved in both the $\alpha$- and $\beta$-subunits.

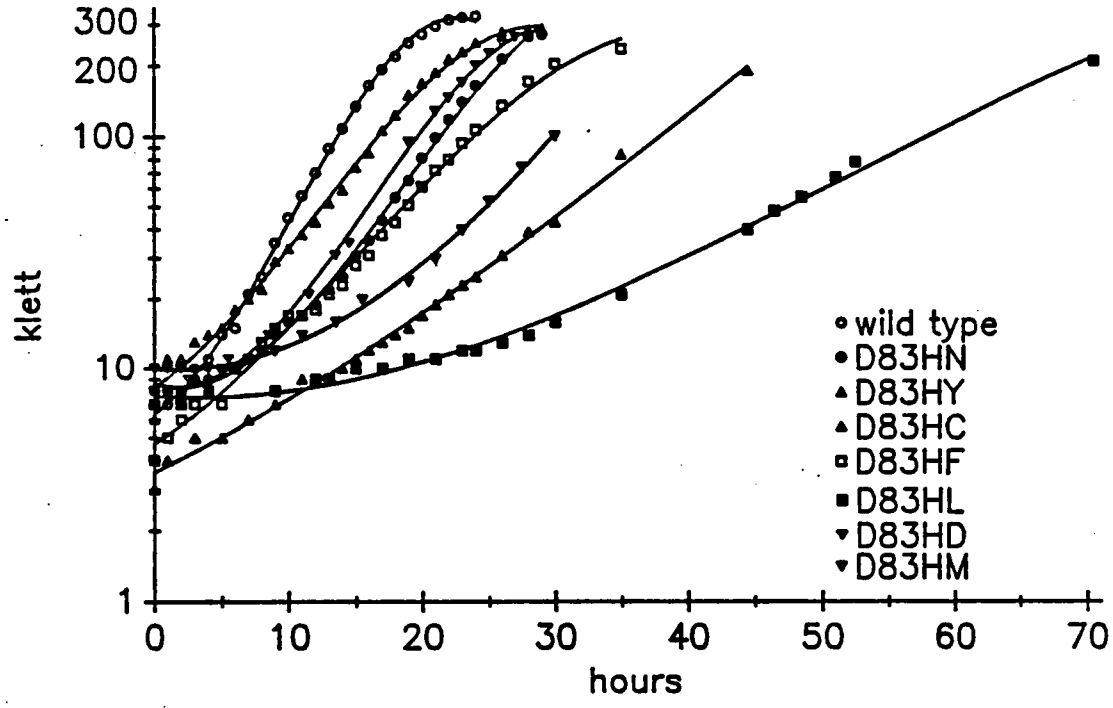


**Figure 17.** Diazotrophic growth of wild type *A. vinelandii* and Nif+ mutant strains containing amino acid substitutions at D83H. Growth of *A. vinelandii* is described in Materials and Methods.

**Table 6.** Growth rates and component protein activities of the wild type strain and Nif + replacement strains at D83H and D88C

| strain | mutation | % wt activity | doubling time (hrs) | MoFe specific activity[A]* | Fe specific activity[B]* |
|---|---|---|---|---|---|
| Wild type | none | 100 | 2.50 | 52.2 | 45.7 |
| DJ 181 | D83HN | - | 3.75 | 39.65, 39.05 | 16.52, 7.84 |
| DJ 332 | D83HY | - | 4.00 | 41.24, 39.09 | 29.22, 23.93 |
| DJ 328 | D83HF | - | 4.50 | 29.27 | 48.10 |
| DJ 326 | D83HC | - | 6.5-7.0 | 20.93 | 47.22 |
| DJ 330 | D83HL | - | 7.5 | 1.17, 1.02 | 29.38, 14.5 |
| DJ 345 | D83HD | - | 4.0 | 13.56, 4.46 | 33.22, 29.04 |
| DJ 393 | D83HM | - | 5.0 | - | - |
| DJ 399 | D88CD | 7.4 | >10 | 12.3 | 52.6 |
| DJ 397 | D88CT | 1.0 | >10 | 3.7 | 42.5 |
| DJ 395 | D88CG | 2.3 | >10 | 5.6 | 41.4 |
| DJ 162 | D88CS | - | >10 | 0.3 | 39.0 |

[A] Determined in the presence of saturating levels of purified *A. vinelandii* Fe protein
[B] Determined in the presence of saturating levels of purified *A. vinelandii* MoFe protein.
* nmoles of ethylene formed per min/mg crude extract protein

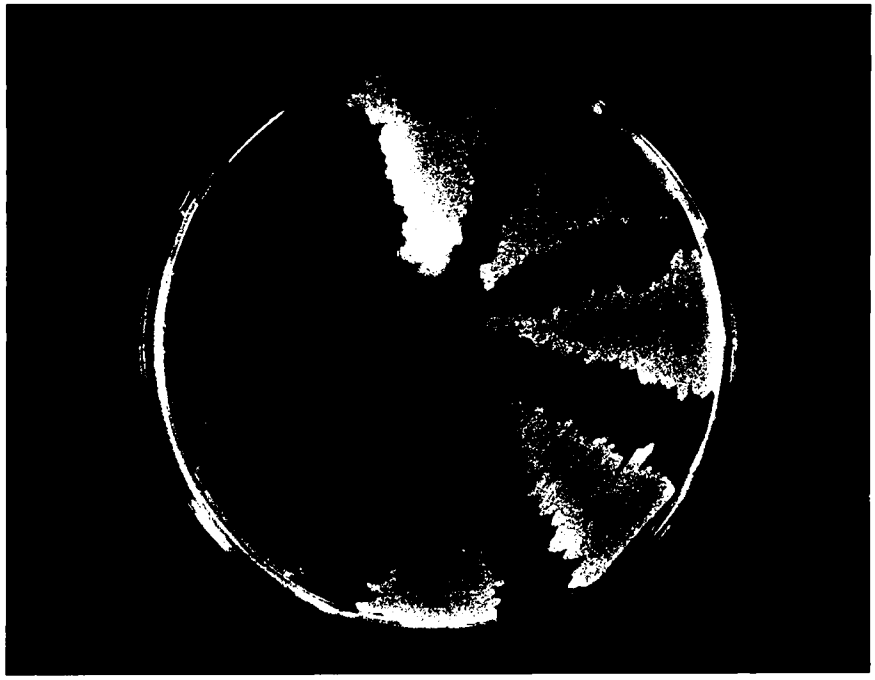

**Figure 18.** Diazotrophic growth characteristics of *A. vinelandii* mutant strains with specific amino acid substitutions at cysteine 88 of the MoFe α-subunit. Cells were streaked out on Burk's nitrogen-free medium and incubated for 6 days. A. wild type, B. D88CD, C. D88CT, D. D88CG, E. D88CS, F. D88CV, G. D88CE, H. DJ187▲nifD strain. Strains F-H are strictly Nif-.

**D154C replacement strains**

A variety of amino acid replacements at D154C resulted in mutant strains with a strictly Nif - phenotype (see Figure 19.). Both positive and negatively charged and hydrophobic amino acid replacements were identified. The replacement D154CP isolated would be expected to have gross structural changes because the immediate neighboring residue is also a proline (two $\alpha$-helix breakers). The possibility that D154C supplies an absolutely required thiol ligand to a Fe-S cluster is strengthened by these substitutions, but if the Fe-S cluster arrangement in the $\beta$-subunit is similar to the $\alpha$-subunit, the phenotypic result of K153CS would not be expected (this strain will grow diazotrophically). Two possible explanations (for the Nif+ K153CS mutant) places the neighboring methionine 163 as a functional replacement ligand, or alternatively the other amino acid neighbor, threonine 161 (if the D88CT mutant is used as an expmple).

Highly conserved $\alpha$-subunit residues , 159-162, are choice candidates for future site directed studies. Some alterations at these positions may change the D154C ligand environment in a manner not possible by directly altering the potentially essential thiol ligand (D154C), while it appears that K153C could be directly altered without complete loss of diazotrophy.

| | 154 | | | | 159 | 160 | 161 | 162 |
|---|---|---|---|---|---|---|---|---|
| | CYS | -pro- | ile- | gly- | leu- | ILE | GLY | ASP-ASP |
| | GLU | | | | | | | |
| | GLN | | | | | | | |
| | ARG | | | | | | | |
| | VAL | | | | | | | |
| | PRO | | | | | | | |
| | SER | | | | | | | |
| | ALA | | | | | | | |
| Nif | - | | | | | | | |

**Figure 19.** Site-directed replacements constructed at D154C. All strains with replacements at D154C are strictly Nif -. Numbered and capitalized residues are conserved in both the $\alpha$- and $\beta$-subuints. Substitution of the corresponding $\beta$-subunit Cys 153, with a Ser results in a mutant strain still capable of diazotrophic growth (see text for discussion).

**D275C replacement strains**

Several substitutions at D275C were made to provide further evidence that this cysteine provides and essential thiol ligand to the FeMo cofactor (11,115). This residue is not conserved in the $\beta$-subunit and is not expected to be involved in P-cluster coordination. As seen in figure 14 a D275CS and D275CA mutation is Nif -, but a mutation of D276YS is Nif +, and a combination of mutations which effectively moves D275C to position 276, results in a Nif - strain, thus suggesting that not only is the thiol group necessary, but the exact positioning of a thiol in the $\alpha$-polypeptide is required. All mutants constructed with replacements at D275C are Nif - (see figure 20). This is probably indicative of either elimination or perturbation of the FeMo cofactor. The component proteins appear to accumulate normally under derepressing conditions. A *K. pneumoniae* site-directed strain analogous to the *A. vinelandii* D275CA strain contains a loosely bound cofactor (21). Further biochemical characterizations are necessary for any conclusions pertaining to FeMoco binding in the recently constructed D275C replacement strains. It will be interesting to see if these mutants contain varying FeMoco binding properties. These strains can be used in current biochemical and genetic studies which are directed toward the elucidation of FeMoco structure and ligation in the MoFe protein.

| 271 | 275 | 279 |
|---|---|---|
| ASN-leu-val-his- | CYS | -tyr-arg-ser-MET |
| | ASP | |
| | GLU | |
| | VAL | |
| | ALA | |
| | GLY | |
| | SER | |
| | THR | |
| Nif - | | |

**Figure 20.** Site-directed replacements constructed at D275C. All strains with replacements at D275C are strictly Nif -. See figure 14 for other replacements made in this region. Numbered and capitalized residues are conserved in the NifE protein.

## Discussion

Amino acid sequence comparisons between the MoFe protein subunits, between the MoFe protein and NifEN complex,as well as extrusion requirements, have provided a rationale for indirectly probing the structure and function of Fe-S environments in nitrogenase. Four amino acid residues in the $\alpha$-subunit (D62C, D83H, D88C, and D154C) are predicted to provide ligands to a P-cluster, and the corresponding residues in the $\beta$-subunit (K70C, K90H, K95C, and K153C) are predicted to act in a similar manner for P-cluster coordination in this subunit. The functional equivalence of these four residues was predicted previously to be similar (20,50,125). Four P-clusters would be bound in this fashion in the $\alpha_2\beta_2$ nitrogenase complex. This proposal is shown schematically in Figure 21. The depicted model is compatible to the coordination of two 8Fe-8S centers, a structure of the P-clusters which cannot yet be ruled out (36). Coordination of an 8Fe-8S center could be accomplished by the four amino acid ligands proposed in the $\alpha$-subunit and the four proposed in the $\beta$-subunit, with the P-cluster bridged between.

Mutations described at each proposed Fe-S ligand in the $\alpha$-subunit result in altered MoFe proteins, both active and inactive. Alterations at D62C most likely abolish the function of the $\alpha$-P-cluster by disrupting a necessary thiol bond. Though not proven, it seems reasonable to describe D62C as indispensable to nitrogenase activity, and it would be very surprising if any of the remaining ten amino acids not used to replace D62C, would result in an active enzyme. Site-directed studies which are designed to alter this region (D62C) would have to target residues other than D62C; the

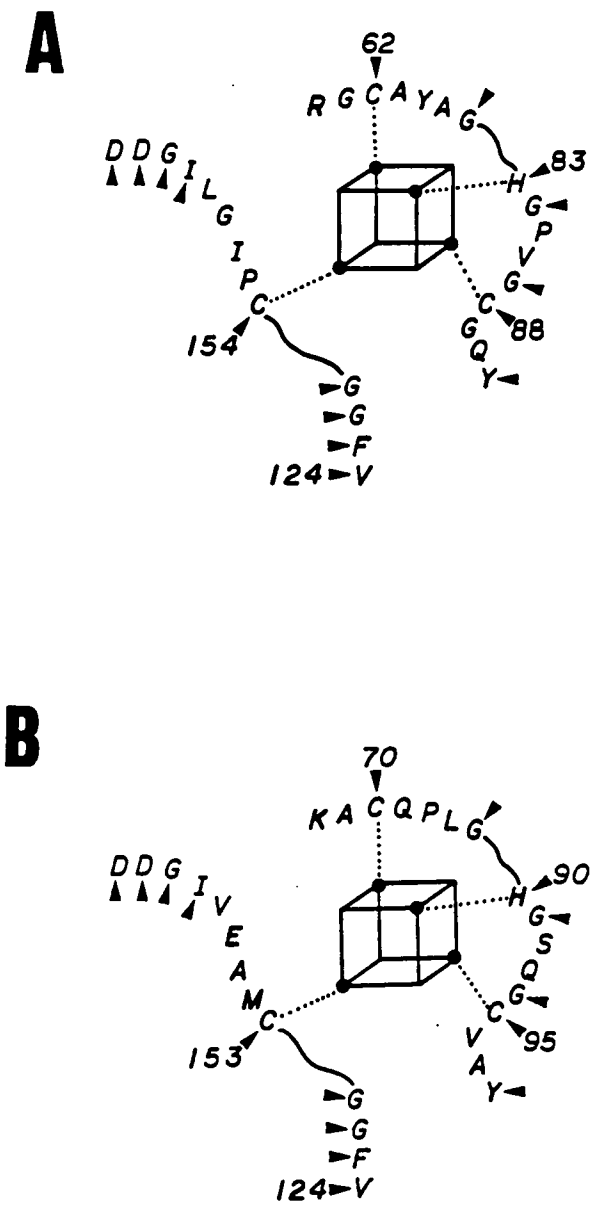


**Figure 21.** Proposed P-cluster coordination within the MoFe $\alpha$-subunit (A), and the $\beta$-subunit (B). The P-clusters (cubane 4Fe-4S centers) are coordinated by three conserved cysteines and one conserved histidine residues. Proposed structurally important residues conserved in both the $\alpha$- and $\beta$-subunits are indicated with arrows. The ligand arrangements are slightly dissimilar in the two subunits, this is consistent with the two different Fe environments ($D_3Fe^{2+}$ and $D_2Fe^{2+}{}_1S_1$) observed by Mossbauer spectroscopy.

residues of choice would have to be the conserved glycine at position 66 and the neutrally conserved arginine at position 60. Though only two substitutions have been made at the $\beta$-subunit cysteine (K70C) corresponding to D62C, this region would be expected to react the same to site-directed changes made in this study, for neither region has an available replacement ligand close-by.

The region containing D83H and D88C was particularly interesting. This peptide region seems quite "resistant" to amino acid replacements despite its highly conserved HGxxGC primary sequence. At least in the $\alpha$-subunit, the obvious explanation for the allowable substitutions at D83H is that another histidine residue (D80H) three positions away takes over the Fe-S binding role when D83H is altered by some replacements. This idea is not without precedence. When an *A. vinelandii* ferredoxin I cysteinyl residue (cys20) is replaced by alanine the available cysteine (cys24) four residues away takes over as a ligand and effectively binds the Fe-S center (68). In this case the rearrangement of the FdI Fe-S center actually changes the three-dimensional structure of the protein. In an analogous action, the substitutions at D83H may change the positioning of two Fe-S centers thus giving rise to various functioning mutant MoFe proteins. Only X-ray crystallographic data on these replacement enzymes can prove that possibility, but Mossbauer spectroscopy may reveal changes in the normally observed Fe environments, suggestive of different ligand arrangements.

At first sight, the D88C substitutions would seem to have a functional pattern (with the exception of D88CG). The Nif + replacements may be substituting a oxygen ligand for a thiol. *In vitro*, serine has been shown to complex with 4Fe-4S centers

(31). Though K95CS is a strict Nif- mutant, it would be interesting to see how threonine, glycine and an glutamic acid would alter nitrogenase activity if these residues replaced this cysteine. It cannot be ruled out that neither D83H and D88C are P-cluster ligands, but alternative conserved amino acids, which could provide a ligand known to bind Fe, are not obvious from the primary sequence. Clearly the $\beta$-subunit cysteine K95C responds differently to an identical substitution, for K95CS is strictly Nif- and D88CS is Nif+. This may reflect the slightly dissimilar peptide environments or may be characteristic of the serine substitution only (i.e. other substitutions may act in similar manners to the $\alpha$-subunit replacements).

All mutants with alterations at D154C are incapable of growing diazotrophically, a result which may indicates that a cysteine at this position is absolutely essential. There are two reports of site-directed mutations made at the conserved I-G-D-D region (see Figure 20) (20,34). A substitution of the *K. pneumoniae* $\alpha$-subunit glycine 161 (corresponding to D160G in the *A. vinelandii* sequence) with an aspartic acid, resulted in a Nif- mutant strain, while substitution of the *A. vinelandii* glycine with an alanine results in a Nif + strain. Because the region surrounding the glycine in both organisms is virtually identical, it would be expected that this region reacts similarly to specific substitutions, and furthermore multiple substitutions at this position may give rise to mutant strains with different characteristics, much like those observed with D83H and D88C mutants made in this study. The difference in this approach (targeting glycine), is that the potential ligand would be indirectly altered, through changes in potentially important conformations.

The dissimilar primary sequence of the K153C region, and the differences in phenotypes observed in the D154CS (Nif-) and K153CS (Nif +) mutants from both *A. vinelandii* and *K. pneumoniae* (21), raises the question of how functionally equivalent are the conserved cysteine residues. The multiple amino acid replacement strategy has the added feature over traditional single substitutions, of being able to compare several identical alterations between two different residues suspected to be functionally similar. Further studies which will give information concerning the structural equivalent regions of the MoFe protein would target the three conserved cysteines in the $\beta$-subunit for multiple replacements and compare the results with the identical changes in the $\alpha$-subunit.

The site-directed mutants made during this study will be valuable when detailed crystal structures of nitrogenase are made available. If the situation which occurs in the *A. vinelandii* Fd I is at all indicative of what may happen to the MoFe protein in response to site-directed changes at the P-clusters, then one could speculate that the P-clusters actually determine conformations of nitrogenase, and when normal ligands are denied, rearrangements are made to accommodate a slightly distorted, but functional Fe-S center. The only other Fe-S containing protein with an unknown structure which has been reported to have been studied by directly altering potential metal ligands, is glutamine phosphoribosylpyrophosphate amido transferase from *Bacillus subtilis* (67). Their results are also explained by potential forced Fe-S center rearrangements.

Due to the complexity of nitrogenase and the lack of structural data, only preliminary explanations can be made which account for the observed changes in

nitrogen-fixing activity of the replacement mutants. These are:

1) Amino acid substitutions permit protein rearrangements in order to accommodate a functional P-cluster with four coordinates.

2) P-clusters can function with three ligands, but D62C and D154C seem required.

3) Nitrogenase can function with a reduced number of P-clusters.

4) The predicted P-cluster coordinates are incorrect.

# NUCLEOTIDE SEQUENCE OF THE *Klebsiella pneumoniae* *nif*TYEN GENES

## Summary

The nucleotide sequence of the *nif*-specific region that is located between *nif*K and *nif*X in *K. pneumoniae* was determined (see Appendix I). This region (*nif*TYEN) spanning approximately 4.7 kb, was partially contained on two overlapping DNA fragments, a 2.7 kb Sal I , and a 2.0 kb Eco RI restriction fragment. These two fragments reside in pDB339 and pDB340, respectively. Specific DNA sequences were obtained from restriction fragments generated from these hybrid plasmids (see Material and Methods). A physical map of the sequenced region is found in Figure 22. The nucleotide sequence of *nif*T was determined at Biotechnica International, Cambridge, Mass., in the laboratory of F. Cannon.

The nucleotide sequence of the *nif*TYEN genes reported here, is in agreement with the sequence which was reported in a publication encompassing the nucleotide sequence of the entire *nif* cluster from *K. pneumoniae* (1). The deduced amino acid sequence of the FeMoco biosynthetic proteins (*nif*E and *nif*N) from *K. pneumoniae,* share significant identity with the *nif*E and *nif*N gene products from *A. vinelandii* (see Table 7). Alignment of the respective amino acid sequences of NifE and NifN proteins is shown in Figures 7 and 8. Specific sequences conserved include those regions proposed in FeMoco binding (11,20). The *nif*E and *nif*N genes are organized on one operon, and the proposed *nif*-specific promoter sequence, CTGG-$N_8$-TTGCA (5), precedes the *nif*E gene by 34 nucleotides. The *nif*T and *nif*Y gene products are

synthesized from a transcript directed by the *nif*H promoter. The function of these two genes remains unknown. The *nif*T and *nif*Y gene products from *A. vinelandii* are not essential for diazotrophic growth (13), and the physical characteristics of these gene products from the two diazotrophs appear to be quite different (see Table 7).

Comparison of the physical organization of the region between *nif*Y and *nif*E (Figure 23), reveals the presence of two open reading frames (ORFs) on the *A. vinelandii* chromosome not present in *K. pneumoniae* (110). The *nif*-related function of these coding regions is not known; perhaps their presence reflects the two organisms' physiological differences.

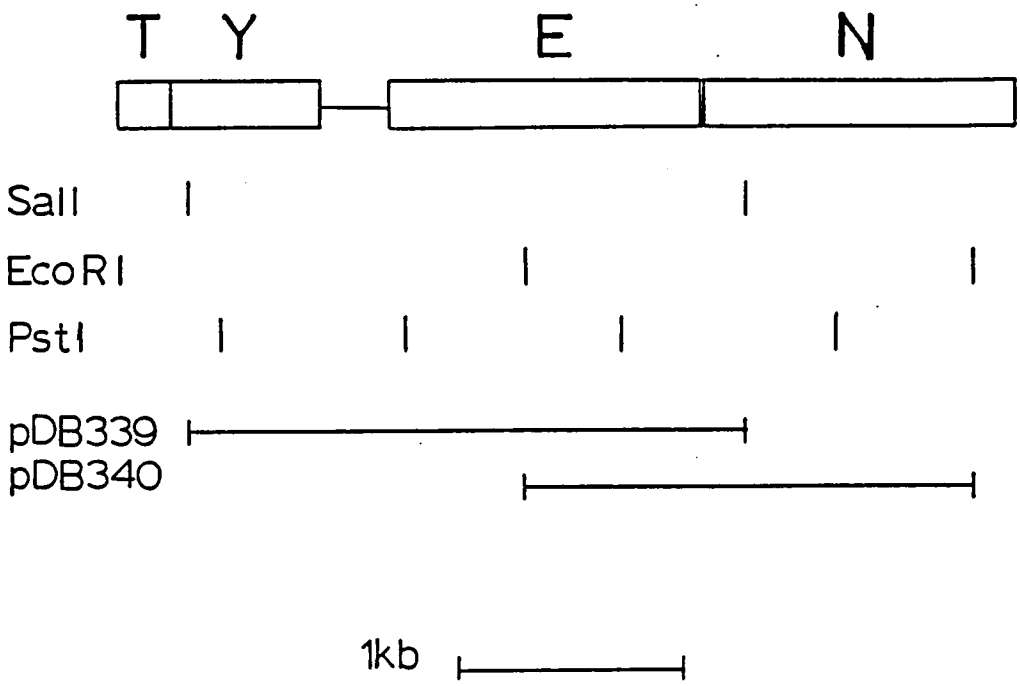


**Figure 22.** Physical Map of the *K. pneumoniae nif*TYEN region. Restriction sites and plasmids shown were used in part for DNA sequencing as described in the Materials and Methods section.

Table 7. Comparisons of the NifTYEN gene products of *A. vinelandii* and *K. pneumoniae*

| Gene | Bacterium | Product Mol.wt. | Product pI | %Identity amino acids |
|---|---|---|---|---|
| *nif*T | *A. vinelandii* | 8,307 | 5.51 | 41 |
| *nif*T | *K. pneumoniae* | 8,316 | 11.22 | |
| *nif*Y | *A. vinelandii* | 26,683 | 11.44 | 17 |
| nifY | *K. pneumoniae* | 25,498 | 10.58 | |
| ORFI | *A. vinelandii* | 9,552 | 4.93 | |
| ORFII | *A. vinelandii* | 27,963 | 5.99 | |
| *nif*E | *A. vinelandii* | 52,140 | 6.34 | 61 |
| *nif*E | *K. pneumoniae* | 50,145 | 6.47 | |
| *nif*N | *A. vinelandii* | 49,186 | 6.06 | 41 |
| *nif*N | *K. pneumoniae* | 50,621 | 6.47 | |

Product molecular weights and isoelectric points were calculated using the IBI Pustel DNA sequence analysis program.

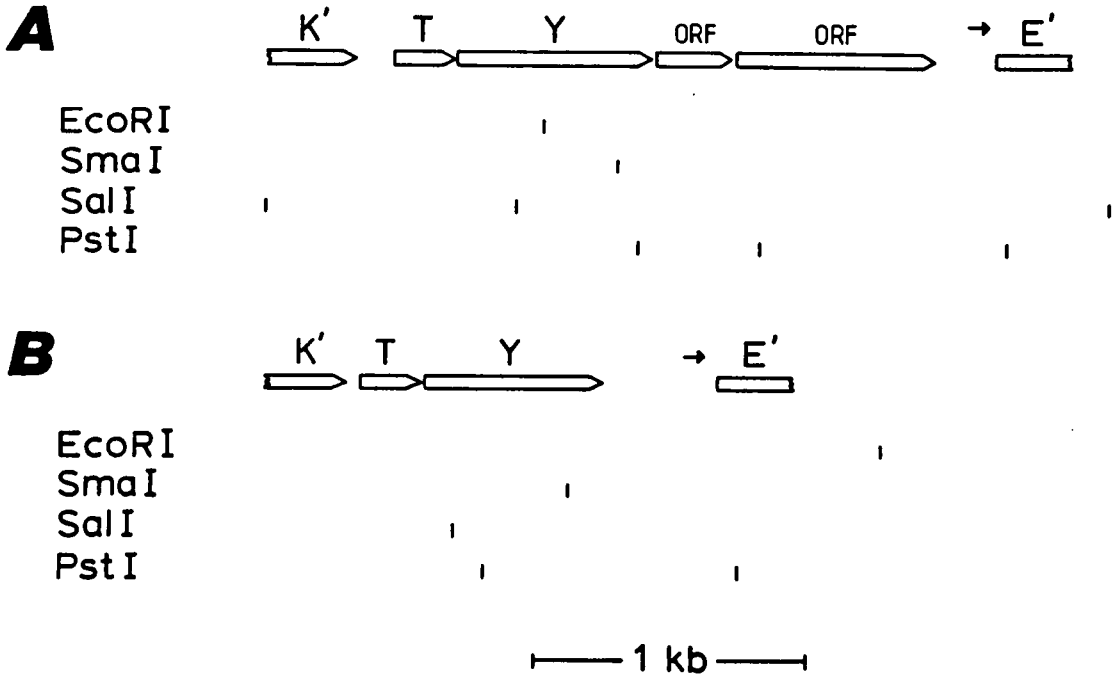


**Figure 23.** Comparison of the intergenic region between *nif*K and *nif*E from *A. vinelandii* (A) and *K. pneumoniae* (B).

## LITERATURE CITED


1. Arnold, W. A., W. Rump, U. B. Priefer, A. Puhler. 1988. Nucleotide sequence of a 24,206 base-pair DNA fragment carring the entire nitrogen fixation gene cluster of Klebsiella pneumoniae. J. Mol. Biol. 203:715-738.

2. Bennett, L. T., F. Cannon, D. R. Dean. 1988. Nucleotide Sequence and Mutagenesis of the nifA gene from Azotobacter vinelandii. Mol. Microbiol. 2:315-321.

3. Bennett, L. T., M. R. Jacobson, D. R. Dean. 1988. Isolation, sequencing, and Mutagenesis of the nifF Gene Encoding Flacodoxin from Azotobacter vinelandii. J. Biol. Chem. 263:1364-1369.

4. Beuemann, J.R., D. C. Yoch, R. C. Valentine, D. I. Arnon. 1979. The electron transport system in nitrogen gixation by Azotobacter vinelandii. Biochim. Biophys. Acta. 226:205-212.

5. Beynon, J., M. Cannon, V. Buchanan-Wollaston, F. Cannon. 1983. The nif Promoters of Klebsiella pneumoniae have a characteristic primary structure. Cell 34:665-671.

6. Beynon, J., A. Ally, M. Cannon, F. Cannon, M. Jacobson, V. Cash, D. Dean. 1987. Comparative organization of nitrogen fixation specific gene from Azotobacter vinelandii. and Klebsiella pneumoniae: DNA sequence of the nifUSV genes. J. Bacteriol. 169:4024-4029.

7. Bishop, P. E., D. M. L. Jarenski, D. R. Hetherington. 1980. Evidence for an alternative nitrogen fixation system in Azotobacter vinelandii. Proc. Natl. Acad. Sci. USA 77:7342-7346.

8. Bishop, P. E., R. Premakumar, D. R. Dean, M. R. Jacobson, T. Chisnell, M. Rizzo, J. Kpoczynski. 1986. Nitrogen fixation by Azotobacter vinelandii strains having deletions in structural genes for nitrogenase. Science 232:92-94.

9. Brigle, K. E., W. E. Newton, D. R. Dean. 1985. Complete Nucleotide Sequence of the Azotobacter vinelandii structural Nitrogenase gene cluster. Gene 37:37-44.

10. Brigle, K. E., M. C. Weiss, W. E. Newton, D. R. Dean. 1987. Products of the Iron Molybdenum Cofactor Biosynthetic genes, nifE and nifN, are Structurally Homologous to the Products of the nitrogenase Molybdenum-Iron Protein Genes nifH and nifK. J. Bacteriol. 169:1547-1553.

11. Brigle, K. E., R. A. Setterquist, D. R. Dean, J. S. Cantwell, M. C. Weiss, W. E.

Newton. 1987. Site-directed mutagenesis of the nitrogenase MoFe protein of Azotobacter vinelandii. Proc. Acad. Sci. USA 84:7066-7069.

12. Brigle, K. E., M. C. Weiss, W. E. Newton, D. R. Dean. 1987. Products of the Iron Molybdenum Cofactor-specific Biosynthetic Genes, nifE and nifN, are Structurally Homologous to the Products of the Nitrogenase Molybdenum-Iron Protein Genes, nifD and nifK. J. Bacteriol. 169:1547-1553.

13. Brigle, K. E. 1989. Studies on the structure and function of the various nif and nif associated gene products encoded within the Azotobacter vinelandii nif gene cluster. PhD. Thesis, Virginia Tech, Dept. Anaerobic Micro.

14. Burgess, B. K., E. I. Stiefel, W. E. Newton. 1980. Oxidation reduction properties and complexation reactions of the iron-molybdenum cofactor of nitrogenase. J. Biol. Chem. 255:353-356.

15. Burris, R. H. 1988. 100 years of discoveries in biological $N_2$ fixation. In Nitrogen Fixation: Hundred Years After Bothe, H., F. J. de Bruijn, W. E. Newton (eds). pp.21-30.

16. Carnahan, J. E., L. E. Mortenson, H. F. Mower, and J. E. Castle. 1960. Nitrogen fixation in cell-free extracts of Closdridium pasteurianum. Biochim. Biophys. Acta. 38:188-189.

17. Chen, K. C.-K., J. S. Chen. 1986. Structural features of multiple nifH-like sequences and very biased codon usage in nitrogenase genes of Clostridium pasteurianum. J. Bacteriol. 166:162-172.

18. Chisnell, J. R., R. Premakumar, P. E. Bishop. 1988. Purification of a second alternative nitrogenase from a nifHDK deletion stran of Azotobacter vinelandii. J. Bacteriol. 170:27-33.

19. Dean, D. R., K. E. Brigle. 1985. Azotobacter vinelandii nifD and nifE-encoded polypeptides share structural homology. Proc. Natl. Sci. USA 82:5720-5723.

20. Dean, D. R., K. E. Brigle, H. D. May, W. E. Newton. 1988. Site-directed Mutagenesis of the nitrogenase MoFe protein. In Nitrogen Fixation: Hundred Years After. H. Bothe, F. J. de Bruijn, W. E. Newton (eds). pp.107-113.

21. Dent H. M. I. Ioannidis, C. Gormal, B. E. Smith, M. Buck. 1989. Site-directed Mutagenesis of the Klebsiella pneumoniae nitrogenase-effects of modifiying conserved cysteine residues in the $\alpha$ and $\beta$ subunits. Biochem. J. (in press).

22. Dilworth, M. J. 1966. Acetylene reduction by nitrogen-fixing preparations from

Clostridium pasteurianum. Biochim. Biophys. Acta 127:285-294.

23. Drummond, M. H. 1985. The Base sequence of the nifF gene of Klebsiella pneumoniae and homology of the predicted amino acid sequence of it's protein product to other flavodoxins. Biochem J. 232:891-896.

24. Drummond,M., P. Whitty, J. Wooton. 1986. Sequence and Domain relationships of NtrC and NifA from Klebsiella pneumoniae: Homologies to other regulatory proteins. EMBO J. 5:441-447.

25. Drummond, M. H., J. C. Whooton. 1987. Sequence of the nifL gene from Klebsiella pneumoniae: mode of action and relationship to two families of regulatory proteins. Mol. Microbiol. 1:37-44.

26. Eady, R. R., R. L. Robson, T. H. Richardson, R. W. Miller, M. Hawkins. 1987. The vanadium nitrogenase of Azotobacter chroococcum. Biochem. J. 244:197-207.

27. Earl, C. D., C. W. Ronson, F. M. Ausubel. 1987. Genetic and Structural Analysis of the Rhizobium meliloti fixA, fixB, fixC and fixX Genes . J. Bacteriol. 169:1127-1136.

28. Ebling, S. M. Hahn, H. M. Fischer, H. Hennecke. 1987. Identification of nifE, nifN and nifS-like genes in Bradyrhizobium japonicum. Mol. Gen. Genet. 207:503-508.

29. Emerich D. W., R. H. Burris. 1976. Interaction of heterologous nitrogenase components that generate catalytically inactive complexes. Proc. Natl. Acad. Sci. USA 73:4369-4373.

30. Emerich, D. W. R. W. Burris. 1987. Complementary funtioning of Component Proteins of Nitrognease from Several Bacteria. J. Bacteriol. 134:936-943.

31. Evans, D. J., J. Leigh, 1988. Serinato , Tyrosinato, and Prolinato derivatives of $Fe_4$-$S_2$ clusters. J. Chem. Soc. Chem. Comm. 388-396.

32. Frank, A. M., M. Weininger, L. E. Mortenson, C. P. Cramer. 1986. Single-Cystal EXSAFS of nitrogenase. J. Amer Chem. Soc. 108:1049-1055.

33. Fuhrmann, M., H. Hennecke. 1984. Rhizobium japonicum nitrogenase Fe protein gene (nifH). J. Bacteriol. 158:1005-1011.

34. Govezensky, D., A. Zamir. 1989. Structure-function Relationships in the $\alpha$-subunit of Klebseilla pneumoniae Nitrogenase MoFe protein from analysis of nifD Mutants. J. Bact. 171:5729-5735.

35. Haaker, H., J. Klugkist. 1987. Bioenergetics of electron transport to nitrogenase. FEMS Microbiol. Rev. 46:57-71.

36. Hagen, W. R., H. Wassink, R. R. Eady, B. E. Smith, H. Haaker. 1987.Quantitative EPR of an S = $^7/_2$ systemin oxidized MoFe proteins of nitrogenase. A redefinition of P-cluster concept. Eur. J. Biochem. 169:456-465.

37. Hales, B.J., E. E. Case, J. E. Morningstar, M. F. Dzeda, L.A. Mauterer. 1986. Isolation of a new vanidium-containing nitrogenase from Azotobacter vinelandii. Biochem. 25:7251-7255.

38. Hallenback. P. C., M. C. Meyer, P. M. Vignais, 1982. Nitrogenase from the Photosynthetic Bacterium Rhodopseudomonas capsulata: Purification and Molecular properties. J. Bacteriol. 144:708-717.

39. Hausinger, R. P., J. B. Howard. 1980. Comparison of the iron proteins from the nitrogen fixation complexes of Azotobacter vinelandii, Clostridium pasteurianum, and Klebsiella pneumoniae. Proc. Natl. Acad. Sci. USA 77:3826-3830.

40. Hausinger, R. P., J. B. Howard. 1982. The amino acid sequence of the nitrogenase iron protein from Azotobacter vinelandii. J. Biol. Chem. 257:2483-2490.

41. Hausinger, R. P., J. B. Howard. 1983. Thiol Reactivity of the Nitrogenase Fe protein from Azotobacter vinelandii.J. Biol. Chem. 258:13486-13492.

42. Hill, S., E. P. Kavanagh. 1980. Roles of nifF and nifJ gene products is electron transport to nitrognease in Klebsiella pnuemoniae. J. Bacteriol. 141:470-475.

43. Hoover, T. R., J. Imperial, P. W. Ludden, V. K. Shah. 1988. Homocitrate cures the NifV - phenotype in Klebsiella pneumoniae. J. Bacteriol. 170:1978-1979.

44. Hoover, T. R., J. Imperial, P. W. Ludden, V. K. Shah. 1989. Homocitrate is a component of the Iron Molybdenum Cofactor of Nitrogenase. Bichem. 28:2768-2771.

45. Horton, R. D., S. T. Lord. 1986. In vitro methylation double the effeicency of oligonucleotide-directed mutagenesis. Nucl. Acids Res. 14:5112.

46. Howard, K. S., P. A. McLean, F. B. Hansen, P. V. Lemley, K. S. Koblan, W. H. Orme-Johnson. 1986. Klebsiella pneumoniae nifM gene product is required for stabilization and activation of nitrogenase iron protein in Escherichia coli. J. Biol. Chem. 261:772-778.

47. Howard, J. B., R. Davis, B. Moldenhauer, V. L. Cash, D. R. Dean. 1989. Fe-S cluster ligands are only Cysteines Required fro Nitrogenase Fe protein activities J. Biol. Chem. 264:11270-11274.

48. Ibers, J. A., R. H. Holm. 1980. Modeling Coodination Sites in Metallobiomolecules. Science 209:223-235.

49. Imperial, J., R. A. Ungalde, V. K. Shah, W. J. Brill. 1984. Role of nifQ gene product in the incorporation of molybdenum into nitrogenase in Klebsiella pneumoniae. J. Bacteriol. 158:759-765.

50. Ioannidis, I., M. Buck. 1987. Nucleotide Sequence of the Klebsiella pnuenomiae nifD gene and predicted amino acid sequence of the α-subunit of nitrogenase MoFe protein. Biochem. J. 247:287-291.

51. Jacobson, M. R., R. Premakumar, P. E. Bishop. 1986. Transcriptional Regulation of Nitrogen Fixation by Molybdenum in Azotobacter vinelandii. J. Bacteriol. 167:480-486.

52. Jacobson, M. R., K. E. Brigle, L. T.Bennett, R. A. Setterquist, M. W. Wilson, V. L. Cash, J. Beynon, W. E. Newton, D. R. Dean. 1989. Physical and Genetic Map of the Major nif Gene Cluster from Azotobacter vinelandii. J. Bacteriol. 171:1017-1027.

53. Jacobson, M. R., V. L. Cash, M. C. Weiss, N. F. Laird, W. E. Newton, D. R. Dean. 1989. Biochemical and genetic analysis of the nifUSVWM cluster from Azotobacter vinelandii. Mole. Gen. Genet (in press).

54. Joerger, R. D., R. Premakumar, P. E. Bishop. 1986. Tn-5 Induced Mutants of Azotobacter vinelandii affected in nitrogen fixation under Mo-deficent and under Mo-sufficent conditions. J. Bacteriol. 168:673-682.

55. Joerger, R. D., P. E. Bishop. 1988. Nucleotide Sequence and Genetic Analysis of the nifB-nifQ Region from Azotobacter vinelandii. J. Bacteriol. 170:1475-1487.

56. Joerger, R. D., M. R. Jacobson, R. Premakumar, E. D. Wolfinger, P. E. Bishop. 1989. Nucleotide sequence and Mutational Analysis of the Structural Genes (anfFDGK) for the second alternative Nitrogenase from Azotobacter vinelandii. J. Bacteriol. 171:1075-1086.

57. Jones, R., R. Haselkorn.1988. The DNA sequence of the Rhodobacter capsulatus nifH gene. Nucl. Acids Res. 16:8735.

58. Kaluza, K., H. Hennecke. 1984. Fine Structure analysis of nifHDK operon encoding the $\alpha$ and $\beta$ subunits of dinitrogenase for Rhizobium japonicum. Mol. Gen Genet. 196:35-42.

59. Kennedy, C., R. R. Eady, E. Kondorosi, D. K. Rekosh. 1976. The Molybdenum-iron protein of Klebsiella pneumoniae nitrogenase. Biochem. J. 155:383-389.

60. Kennedy, C., R. Robson. 1983. Activation of nif gene epression in Azotobacter vinelandii and Azotobacter chroococum by the nifA gene of Klebsiella pneumoniae. Nature 301:626-628.

61. Kennedy, C., R. Gamal, R. Humphrey, J. Ramos, K. D. Dean. 1986. The nif H, nifM and nifN genes of Azotobacter vinelandii characterization by Tn-5 mutagenesis and isolation from pLAFR1 gene banks. Mol. Gen. Genet. 205:318.

62. Kurtz, D. M. Jr., W. C. McMillan, B. K. Burgess, L. E. Mortenson, R. H. Holm. 1979. Identificaton of Fe-S centers in the MoFe protein of nitrogenase. 76:4986-4989.

63. Lammers, P. J., R. Haselkorn. 1983. Sequence of the nifD gene encoding for the $\alpha$-subunit of dinitrogenase from the cyanobacterium Anabaena. Proc. Natl. Acad. Sci. USA 80:4723-4727.

64. Lundell, D. J., J. B. Howard. 1981. Isolation and Sequence of the Tryptic Peptides of the MoFe protein of Azotobacter vinelandii. J. Biol. Chem. 256:6385-6391.

65. Ludden, P. W., G. P. Roberts, R. G. Lowery, W. P. Fitzmaurice, L. L. Aaare, L. L. Lehman, D. Lies, D. Woehle. H. Wirt, S. A. Murrell, M. R. Pope, R. H. Kanemoto. 1988. Regulation of nitrogenase activity by reversible ADP-ribosylation of dinitrogenase reductase. In Nitrogen Fixation: Hundred years after. H Bothe, F. J. de Bruijin, W. E. Newton (eds). 157-162.

66. Magasanik, B. 1988. Reversible phosphorylation of an enhancer binding protein regulates the transciption of bacterial nitrogen utilization genes. Trends in Biochem. Sci. 13:475-479.

67. Makaroff, C. A., J. L. Paluh, H. Zalkin. 1986. Mutagenesis of Ligands to the [4Fe-4S] Center of Bacillus subtilis Glutamine Phosphoribosylpryophasphate Amidotransferase. J. Biol. Chem 261:11416-11423.

68. Martin, A. B. K. Burgess, C. D. Stuout, V. Cash, D. R. Dean, G. M. Jensen, P. J. Stevens. 1989. Site-directed Mutagenesis of Azotobacter vinelandii Ferredoxin I: [FeS] cluster driven protein rearrangement. Proc. Natl. Acad Sci. USA (in

press).

69. Martin, A. E., B. K. Burgess, S. E. Iismaa, C. T. Smartt, M. R. Jacobson, D. R. Dean. 1989. Construction and Characterization of an Azotobacter vinelandii strain with mutations in Genes Encoding Flavodoxin and Ferredoxin I. J. Bacteriol. 171:3162-3167.

70. Mazur, B. J., C.-F. Chin. 1982. Sequence of the gene encoding for the $\beta$-subunit of dinitrogenase from the blue-green alga Anabaena. Proc Natl. Acad Sci. USA 79:6782-6786.

71. McLean, P. A., R. A. Dixion. 1981. Requirements of nifV gene for production of wild-type nitrogenase enzyme in Klebsiella pneumoniae. Nature 292:655-656.

72. McLean, P. A., V. Papaegthymiou, W. H. Orme-Johnson, E. Munck. 1987. Isotopic Hybrids of nitrogenase. Mossbauer study of MoFe protein with selective $^{57}$Fe enrichment of P-clusters. J. Biol. Chem. 262:12900-12903.

73. McLean, P. A., V. Papaefthymiou, E. Munck, W. H. Orme-Johnson. 1988. Use of Isotopic hybrids of the MoFe protein to study the Mechanism of nitrogenase catalysis. In Nitrogen Fixation: Hundred Years After. Bothe, H., F. J. de Bruijn, W. E. Newton. (eds.) p. 90.

74. Merrick, M.. M. Filser, R. Dixon, L. Elmerich, J. Houmand. 1980. The use of translocatable genetic elements to construct a fine-structure map of the Klebsiella pneumoniae nitrogen fixation (nif) gene cluster. J. Gen Microbiol. 117:509-520.

75. Merrick, M., S. Hill, H. Hennecke, M. Hahn, R. Dixon, C. Kennedy. 1982. Repressor properties of the nifL gene product in Klebsiella pneumoniae. Mol. Gen. Genet. 185:75-81.

76. Merrick, M., J. Gibbins, A. Toukarian. 1987. The nucleotide sequence of the sigma factor gene ntrA (rpoN) of Azotobacter vinelandii: analysis of conserved sequences in the ntrA proteins. Mol. Gen. Genet. 210:323-330.

77. Messing, J. 1983. New M13 vectors for cloning. Meth. Enzymol. 101:20-78.

78. Morett, E., M. Buck. 1988. NifA- dependent in vivo protection demonstrates that upstream activator sequence of nif-promoters is a protein binding site. Proc. Natl. Sci. USA 85:9401-9405.

79. Morgan, T. V., D. J. Lundell, B. K. Burgess. 1988. Azotobacter vinelandii Ferredoxin I: Cloning, sequencing and mutant analysis. J. Biol. Che,. 263:1370-1375.

80. Mortenson, L. E. 1964. Ferredoxin requirement for Nitrogen Fixation by extracts of Clostridium pasteurianum. Biochim. Biophys. Acta 81:473.

81. Nagatani, H. H., V. K. Shah, W. J. Brill. 1974. Activation of inactive nitrogenase by acid-treated Component I. J. Bacteriol. 120:697-701.

82. Newton, W. E., F. A. Schultz, S. F. Gheller, S. Lough, J. W. McDonald, S. S. Conradson, B. Hedman, K. O. Hodgoson. 1986. Iron-Molybdenum cofactor of Azotobacter vinelandii nitrogenase: Oxidation-Reduction Properties and structural insights. Polyhedron 5567-572.

83. Nieva-Gomez, D., G. P. Roberts, S. Klevickis, W. J. Brill. 1980. Electron transport to nitrogenase in Klebsiella pneumoniae. Proc. Natl. Acad. Sci. USA 77:2555-2558.

84. Norman, P., P. Simonet, R. Bardin. 1988. Conservation of nif sequences in Frankia. Mol. Gen. Genet. 213:238-246.

85. Norel, F, C. Elmerich. 1987. Nucleotide sequence and fuctional analysis of the two nifH copies of Rhizobium ORS571.J. Gen. Microbiol. 133:1563-1576.

86. O'Farrell, P. H. 1975. High resolution two-dimensional gel electrophoresis of proteins. J. Biol. Chem. 250:4004-4021.

87. Orme-Johnson, W. H., W. D. Hamilton, T. Ljoies, M.-Y. W. Tso, R. H. Burris, V. K. Shah, W. J. Brill. 1972. Electron Paramagnetic Resonance of Nitrogenase and Nitrogenase Components from Clostridium pasteurianum W5 and Azotobacter vinelandii OP. Proc. Natl. Acad. Sci. USA 69:3142-3145.

88. Orme-Johnson, W. H. 1985. Molecular Basis of Nitrogen Fixation. Ann. Rev. Biophys. Biophys. Chem. 14:419-459.

89. Ow, D. W., F. M. Ausubel. 1983. Regulation of Nitrogen Metabolism genes by nifA gene product in Klebsiella pneumoniae. Nature 301:307-313.

90. Page and von Tigerstrom. 1979. Optimal conditions for transformation of Azotobacter vinelandii. J. Bacteriol. 139:1058-1061.

91. Paul, W., M. Merrick. 1989. The roles of the nifW, nifZ and nifM genes of Klebsiella pneumoniae in nitrogenase biosynthesis. Eur. J. Biochem. 178:675-682.

92. Paustian, T. D., V. K. Shah, G. P. Roberts. 1989. Purification and Characterization of the nifN and nifE gene products from Azotobacter vinelandii.

Proc. Natl. Acad. Sci. 86:6082-6086.

93. Pope, M. R., S. A. Murrel, P. W. Ludden. 1985. Covalent Modification of Iron protein of nitrogenase from Rhodospirillum rubrum by adenosine diphosphoribosylation of a specific arginine residue. Proc. Natl. Acad. Sci. USA 82:3173-3177.

94. Pretorisu, I. M., D. E. Raulings, E. G. O'Neill, W. A. Jones, R. Kirby, D. R. Woods. 1987. Nucleotide sequence of the gene encoding the nitrogenase iron protein of Thiobacillus ferrooxidans. J. Bacteriol. 169:367-370.

95. Quinto, C., H. de la Vega, M. Flores, J. Leemans, M. A. Cevallos, M. A. Pardo, R. Azpiroz, M. de Lourdes Girard, E. Calva, R. Palacios. 1985. Nitrogenase reductase: a functional multigene family in Rizobium phaseoli. Proc. Natl. Acad. Sci. USA 82:1170-1174.

96. Quispel, A. 1988. Hellriegel and Wilfarth's discovery of (symbiotic) nitrogen fixation hundred years ago. In Nitrogen Fixation: Hundred Years After. Bothe, H., F. J. de Bruijn, W. E. Newton (eds.) ref. 5.

97. Raina, R., M. A. Reddy, D. Ghosal, H. K. Das. 1988. Characterization of the gene for the Fe-protein of the vandium dependent alternative nitrogenase of Azototbacter vinelandii and construction of a Tn5 muntant. Mol. Gen. Genet. 214:121-127.

98. Rawlings, J., V. K. Shah, J. R. Chisnell, W. J. Brill, R. Zimmerman, E. Munck, W. H. Orme-Johnson. 1978. Novel Metal Centers in the Iron Molybdenum Cofactor of nitrogenase. J. Biol. Chem. 253:100-1004.

99. Roberts, G. P., W. J. Brill. 1980. Gene Product Relationships of the nif Regulon of Klebsiella pneumoniae. J. Bacteriol. 144:210-216.

100. Robinson, A. C., D. R. Dean, B. K. Burgess. 1987. Iron- Molybdenum Cofactor Biosynthesis in Azotobacter vinelandii Requires the Iron Protein of Nitrogenase. J. Biol. Chem. 262:14327-14332.

101. Robson, R. L. 1984. Identification of possible adenine nucleotide-binding sites in nitrogenase Fe and MoFe proteins by amino acid sequence comparison. FEBS lett. 173:394-398.

102. Robson, R. L., R. R. Eady, T. H. Richardson, R. W. Miller, M Hawkins, P. J. Postgate. 1986. The alternative nitrogenase of Azotobacter chroococcum. Nature 322:388-390.

103. Robson, R. R., R. Woodley, R. Jones. 1986. Second gene (nifH*) coding for a nitrogenase iron protein in Azotobacter choococcum is adjacent to gene encoding for a ferredoxin-like protein. EMBO J. 5:1159-1163.

104. Sanger, F. S. Nicklen, A. R. Coulson. 1977. DNA Sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 77: 5463-5467.

105. Emerich, D. W., R. H. Burris. 1987. Complementary functioning of Component Proteins of Nitrogenase from Several Bacteria. J Bacteriol. 134:936-943.

106. Schollhorn, R., R.H. Burris. 1967. Acetylene as a competitive inhibitor of $N_2$ fixation. Proc. Natl. Acad. Sci. USA 58:213-216.

107. Schumann, J. P., G. M. Waitches, P. A. Scolnik. 1986 A DNA fragment hybridizing to a nif probe in Rhodobacter capsulatus is homologous to a 16S rRNA gene. Gene 48:81-92.

108. Scott, K. F., B. G. Rolfe, J. Shine. 1981. Biological nitrogen fixation: primary structure of the Rhizobium trifolii Iron protein gene. DNA 2:149-155.

109. Scott, K. F., B. G. Rolfe, J. Shine. 1983. Nitrogenase structural gene are unlinked in the non-legume symbiont Parasponia Rhizobium. DNA 2:141-148.

110. Setterquist, R. D. Brigle, J. Beynon, M Cannon, F. Cannon, D. Dean. 1988. Nucleotide Sequence of the nifTYEN region from Azotobacter vinelandii and Klebsiella pneumoniae. In Nitrogen Fixation: Hundred Years After. H. Bothe, F. J. de Bruijn, W. E. Newton (eds). p.324.

111. Shah, V. K., W.J. Brill. 1977. Isolation of an iron-molybdenum cofactor from Nitrogenase. Proc. Natl. Acad. Sci. USA. 74:3249-3253.

112. Shah, V. K., G. Stacey, W. J. Brill, 1983. Electron transport to nitrogenase: Purification and characterization of pyruvate flavodoxin oxidoreductase, the nifJ gene product. J. Biol. Chem. 258:12064-12068.

113. Shah, V. K., L. C. Davis, J. K. Gordon, W. H. Orme-Johnson, W. J. Brill. 1986. Nitrogenaseless mutants of Azotobacter vinelandii. Activities, cross reactions, and EPR spectra. Biochim. Biophysica Acta 292:246-255.

114. Shah, V. K., J. Imperial, R. A. Ungalde, P. W. Ludden, W. J. Brill, 1086. In vitro Synthesis of the Iron-Molymbdenum cofactor of nitrogenase. Proc. Natl. Sci. USA 83:1636-1640.

115. Smith, B. E., M. Buck, R. Eady, J. Lowe, N. F. Thorneley, G. Ashby, J.

Deistung, M. Eldridge, K Fisher, C Gormal, I. Ioannidis, H. Kent, J Arber, A. Flood, C. D. Garner, S. Hasnain, R. Miller. 1988. Recent studies on the structure and fuction of molybdenum nitrogenase. In Nitrogen Fixation: Hundred years after. H. Bothe, F. J de Bruijin, W. E. Newton (eds) pp.91-100.

116. Souillard, N., L. Sibold. 1986. Primary structure and expression of a gene homologous to nifH (nitrogenase Fe protein) from the Archaebacterium Methanococcus voltae. Mol. Gen. Genet. 203:21-28.

117. Souillard, N., M. Magot, O. Possot, L Sibold. 1988. Nucleotide sequence of regions homologous to nifH (nitrogenase Fe protein) from the nitrogen-fixing achcaeobacteria Methanococcus thermolithotropicus and Methanobacterium ivanovii: Evolutionary implications. J Mol. Evol. 2765-76.

118. Stevens, P. J., C. E. McKenna, B. E. Smith, H. T. Nguyen, M. C. McKenna, A. J. Thomson, F. Devlin, J. B. Jones. 1974. Circular dichroism and magnetic circular dichroism of nitrognease proteins. Proc. Natl. Acad. Sci. USA 76:2585-2589.

119. St. John, R. T., H. M. Johnston, C. Seidman, D. Carfinkel, J. K. Gordon, V. K. Shah, W. J. Brill. 1975. Biochemistry and genetics of Klebsiella pneumoniae mutant strains unable to fix $N_2$. J. Bacteriol. 121:759-765.

120. Strandberg, G. W., P. W. Wilson. 1969. Formation of the nitrogen-fixing enzyme systems in Azotobacter vinelandii. Can. J. Microbiol. 14:25-31.

121. Sundaresar, V., F. Ausubel. 1981. Nucleotide sequence of the gene coding for the nitrogenase iron protein from Klebsiella pneumoniae. J. Biol. Chem. 256:2808-2912.

122. Telser, J., B. M. Hoffman, R. LaBrutto, T. Ohnishi, A.-L. Tsai, S. Simpkins, G. Palmar. 1987. Evidence for N coordination to Fe in the [2Fe-2S] center in yeast mitochondrial complex II. FEBS lett. 214:117-121.

123. Teo, B.-K., R. G. Shulman. 1982. Low-Temperature Magnetic Circular Dichroism Studies of Iron-Sulfur Proteins. In Iron Sulfur Proteins. T. G. Spiro (ed) p.365.

124. Thomann, H., T. V. Morgan, H. Jin, S. J. N. Burgmayer,R. E. Bane, E. I. Stiefel. 1987. Protein Nitrogen Coordination of the FeMo Center of Nitrogenase from Clostridium pasteurianum. J. Am. Chem. Soc. 109:7913-7914.

125. Thony, B., K. Kaluza, H. Hennecke. 1985. Structural and functional homology between the $\alpha$ and $\beta$ subunits of the nitrogenase MoFe protein as revealed by seqencing the Rhizobium japonicum nifK gene. Mol. Gen. Genet. 198:441-448.

126. Thorneley, R. N. F., and Lowe, D. J. 1985. In Molybdenum enzymes. Spiro, T. G. (ed.) Wiley New York. pp. 221-284.

127. Toro, I., A. Kondorosi. 1981. Nucleotide sequence of the Rhizobium meliloti nitrogenase reductase (nifH) gene. Nucleic Acids Res. 9:5711-5723.

128. Toukdarian, A., C. Kennedy. 1986. Regulation of nitrogen matabolism in Azotobacter vinelandii: isolation of ntr and glnA genes and construction of ntr mutants. EMBO J. 5:399-407.

129. Walters, A. M., S. K. Chapman, W. H. Orme-Johnson. 1986. Nature of Amide ligation to the Metal sites of FeMoco. Polyhedron 5:561-565.

130. Wang, S.-Z., J. S. Chen, J. L. Johnson. 1987. Distinct Structural features of the $\alpha$- and $\beta$-subunits of Nitrogenase Molybdenum-Iron Protein of Clostridium pasteurianum: An Analysis of Amino Acid Sequences. Bichem. 27:2800-2810.

131. Wang, S.-Z., J. S. Chen, J. L. Johnson. 1988. Nucleotide and deduced amino acid sequence of the nifD encoding the $\alpha$-subunit of nitrogenase MoFe protein of Clostridium pateurianum. Nucl. Acid Res. 15:3959.

132. Wienman, J. J., F. F. Fellows, P. M. Gresstof, J. Shine, K. F. Scott. 1984. Structural analysis of the genes encoding the molybdenum-iron protein of nitrogenase in the Parasponia Rhizobium strain ANU298. Nucl. Acids Res. 12:8329-8344.

133. Yamane, T., M. W. Weiniger, L. E. Mortensen, M. G. Rossman. 1982. Molecular Symetry of the MoFe protein of nitrogenase. J. Biol. Chem. 257:1221-1223.

134. Yun, A. C., A. A. Szalay. 1984. Structural gene of dinitrogenase and dinitrogenase reductase are transcribed from two separte promoters in the range cowpea Rhizobium strain IRc78. Proc. Natl. Acad. Sci. USA 81: 7358-7362.

135. Zimmerman, R., E. Munck, W, J. Brill, V. K. Shah, M. T. Henzl, J. Rawlings, W. H. Orme-Johnson. 1978. Nitrogenase X: Mossbauer and EPR studies on reversibly oxidized MoFe Protein from Azotobacter vinelandii OP. Biochim. Biophysica Acta 537:185-207.

136. Zoller, M. J., M. Smith. 1983. Oligonucleotide-directed Mutagenesis of DNA fragments cloned into M13 vectors. Methods Enzymol. 100B:468-500.

137. Zumft, W. G., L. E. Mortenson, G. Palmer. 1974. Electron pramagnetic resonance studies on nitrogenase. Investigation of the oxidation reduction

behavior of azoferredozin and molybdferredoxin with potentrimetric and rapid-freeze techniques. Eur. J. Biochem. 46:525-523.

138. Zumft, W. G., G. Palmer, L. E. Mortensen, 1974. Electron Paramagnetic Resonance studies on Nitrogenase. II. Interaction of adenosine 5'-triphosphate with Azoferredoxin. Biochim. Biophys. Acta 292:413-421.

# APPENDIX I

Volume 16 Number 11 1988 Nucleic Acids Research

## Nucleotide sequence of the *nifE* and *nifN* genes from *Klebsiella pneumoniae*

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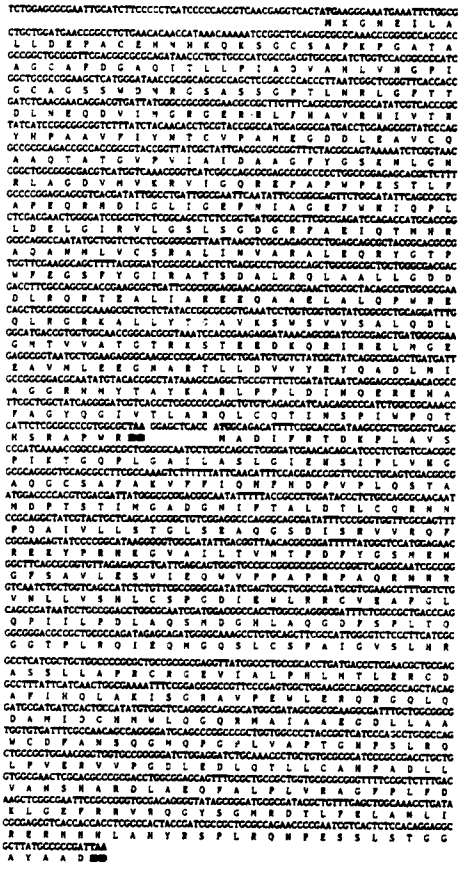
The nucleotide sequence of the K. pnuemoniae nifE and nifN genes has been determined. These genes share considerable sequence identity when compared to the previously published nifE and nifN genes from A. vinelandii (1,2). In the figure, the deduced amino acid sequence is given below the DNA sequence.



REFERENCES

1. Dean, D.R. and K.E. Brigle. (1985). Proc. Natl. Acad. Sci. USA 82, 5720-5723.
2. Brigle, K.E., Weiss, M.C., Newton, W.E., and Dean, D.R. (1987). J. Bacteriol. 169,1547-1553.

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# The nucleotide sequence of the *nifT*, *nifY*, *nifX* and *nifW* genes of *K.pneumoniae*

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The nucleotide sequence of the *K. pneumoniae nifX* and *nifY* genes has been determined. Also, we present two previously unreported *nif* genes: *nifT*, that lies between *nifK* and *nifY*, and *nifW*, that lies between *nifV* and *nifZ* in the *K. pneumoniae nif* cluster. *nifW* was previously partially sequenced and identified as a potential open-reading frame(1). The open reading frames which represent these genes can be defined as *nif* genes as they are within operons in the *K. pneumoniae nif* cluster and there are homologous genes in the same position in the *Azotobacter vinelandii nif* gene cluster(2). The function of none of these genes is known. However, since *nifD* and *nifK* share extensive homology with *nifE* and *nifN* respectively(3,4) and *nifY* and *nifX*, which follow *nifK* and *nifN* respectively, show significant homology to each other(2), this suggests comparable functions.

*nifTY* CTCGTTCGTTAACCATCAGGTGCCCCCGCGTCATGCGGGGCCAGGAGGGAGT ATG CCC ATC GTG ATT
TTC CGT GAG CGC GGC GCG GAC CTG TAC GCC TAT ATC GCG AAA CAG GAT CTG GAA GCG CGA
GTG ATC CAG ATT GAG CAT AAC GAC GCT GAA CGC TGG GGC GGC GCG ATT TCG CTG GAG
GGG GGA CGC CGC TAC TAC GTG CAT CCG CAG CCG GGG CGT CCC GTC TTT CCG ATA AGC CTG
CGC GCG ACG CGC AAT ACC TTG ATA TAA GGAGCTAGTG ATG TCC GAC AAC GAT ACC CTA TTC
TGG CGT ATG CTG GCG CTG TTT CAG TCT CTG CCG GAC CTA CAG CCG GCG CAA ATC GTC GAC
TGG CTG GCG CAG GAG AGC GGC GAG ACG CTG ACG CCA GAG CGT CTG GCG ACC CTG ACC
CAG CCG CAG CTG GCC GCC AGC TTT CCC TCC GCG ACG GCG GTG ATG TCC CCC GCT CGC TGG
TCG CGG GTG ATG GCG AGC CTG CAG GGC GCG CTG CCC GCC CAT TTA CGC ATC GTT CGC CCT
GCC CAG CGC ACG CCG CAG CTG CTG GCG GCA TTT TGC TCC CAG GAT GGG CTG GTG ATT AAC
GGC CAT TTC GGC CAG GGA CGA CTG TTT TTT ATC TAC GCG TTC GAT GAA CAA GGC GGC TGG
TTG TAC GAT CTG CGC CGC TAT CCC TCC GCC CCC CAC CAG CAG GAG GCC AAC GAA GTG CGC
GCC CGG CTT ATT GAG GAC TGT CAG CTG CTG TTT TGC CAG GAG ATA GGC GGG CCC GCC GCC
GCG CGG CTG ATC CGC CAT CGC ATC CAC CCG ATG AAA GCG CAG CCC GGG ACG ACG ATT
CAG GCA CAG TGC GAG GCG ATC AAT ACG CTG CTG GCC GGC CGT TTG CCG CCG TGG CTG
GCG AAG GGC TTA ACA GGG ATA ACC CTC TGG AAG AAC GCG TTT TTT AAT CCC TGT TTT GTG
CTT GTT GCC CGC TGA CCCCGCGGGCTTTTTTTCGCGTATGGACGCTCTTCCCCACGTTACGCTCAGGGGAATATTCCGTTCA
CGGTTGTTCCGGGCTTCTTGATGCGCCTAACCCCCTCGCTGCCAGCCTTTCATCAACAAATAGCCATCCCAGCGCGATAGGTCATAAAGC
ATCACATGCCGCCATCCCTTGTCCGATTGTTGGCTTTGTCGCAAAGCCAACAACCTCTTTTCTTTAAAAATCAAGGCTCCGCTTCTGGAGCG
CGAATTGCATCTTCCCCCTCA

*nifX* AGGAGGCGCTT ATG CCG CCG ATT AAC CGT CAG TTT GAT ATG GTC CAC TCC GAT GAG
TGG TCT ATG AAG GTC GCC TTC GCC AGC TCC GAC TAT CGT CAC GTC GAT CAG CAC TTC GGC
GCT ACC CCG CGG CTG GTG GTG TAC GGC GTC AAG GCG GAT CGG GTC ACT CTC ATC CGG GTG
GTT GAT TTC TCG GTC GAG AAC GGC CAC CAG ACG GAG AAG ATC GCC AGG CGG ATC CAC GCC
CTG GAG GAT TGC GTC ACG CTG TTC TGC GTG GCG ATT GGC GAC GCG GTT TTT CGC CAG CTG
TTG CAG GTG GGC GTG CGT GCC GAA CGC GTT CCC GCC GAC ACC ACC ATC GTC GGC TTA CTG
CAG GAG ATT CAG CTC TAC TGG TAC GAC AAA GGG CAG CGC AAA AAT ACG CGC CAG CGC
GAC CCG GAG CGC TTT ACC CGT CTG CTG CAG GAG CAG GAG TGG CAT GGG GAT CCG GAC
CCG CGC CGC TAG CCGTGTCGTTTCTGTGACAAAGCCCACAAAACATCGCGACACTGTAGGACGAACCTTGTCAGGACTAATACA
CAACCATTTGAAAAATATTAATTTTATTCTCTGGTATCGCAATTGCTAGTTCGTTATCGCC

*nifW* GGGCGAGGGGGTA ATG ATG GAG TGG TTT TAT CAA ATT CCC GGC GTG GAC GAA CTT CGC
TCC GCC GAA TCT TTT TTT CAG TTT TTC GCC GTC CCC TAT CAG CCC GAG CTG CTT GGC CGC
TGC AGC CTG CCG GTG CTG GCA ACG TTT CAT CGC AAA CTC CGC GCG GAG GTG CCG CTG CAA
AAC CGG CTC GAG GAT AAC GAC CGC GCG CCC TGG CTG CTG GCG CGA AGA CTG CTC GCG
GAG AGC TAT CAG CAA CAG TTT CAG GAG AGC GGA ACA TGA GACCGAAATTCACC

Flanking genes are underlined; some show translational coupling.

REFERENCES

1)Paul,W. and Merrick,M. (1987) Eur. J. Biochem. 170, 259-265. 2)Jacobsen,M.R. *et.al.* In preparation. 3)Dean,D.R. and Brigle,K.E.(1985) Proc.Natl. Acad. Sci. USA 82, 5720-5723. 4)Brigle,K.E.*et al* (1987) J. Bacteriol. 169, 1547-1553.

# APPENDIX II

## TWO-DIMENSIONAL POLYACRYLAMIDE GEL ELECTROPHORESIS OF AZOTOBACTER CRUDE EXTRACTS

A. Growth and Derepression of *Azotobacter vinelandii*

B. Sample Preparation

C. Isoelectric Focusing (1st dimension)

D. SDS PAGE (2nd dimension)

E. Reagents and Recipes

## A. Growth and Derepression of *Azotobacter vinelandii*

1. Streak a new plate for each strain to be derepressed. Use Burk's Urea media(BU). Remember to include wild type strain. Allow 2-3 days for growth.

2. Using fresh plates inoculate 20mls of BU media in a klett flask. Shake at 300rpm at 30 C°.

3. When culture is at mid-log phase (80-100 klett) inoculate 500mls of BU in a 2l flask. Using formula below calculate the volume of starter culture needed to make all 500ml cultures a density of 10 klett units.

$$\frac{\text{Desired Klett}}{\text{inoculum (klett of stater)}} \times 500\text{mls} = \text{mls of starter needed for desired klett}$$

4. When culture reaches a density of 100 klett harvest cells at 5000rpm 10 mins. in two plastic centrifuge bottles, wash with Burk's media (B) and combine pellets in one bottle, spin down again and resuspend in 500 mls of B media (NITROGEN-FREE). Do not waste any time during this step, make sure wash media and derepression media are at room temp. Also make sure derepression flasks are well rinsed if you are using same flask.

5. Replace flasks back in incubator, shake for 3-5 hours (derepression).

6. Note densities of cultures and harvest again. Wash once in 20mls sonication buffer and combine pellets into smaller glass centrifuge tube. Spin down, decant and place on ice or freeze in -80 C.

BEST TIME Streak plates on a thursday.
Start first culture on sunday evening.
Monday morning (early) start 500ml culture.
Monday afternoon derepress.

## B. Sample Preparation

1. Chill French pressure cell in refrigerator in advance.

2. If frozen, thaw cell pellets, and place buffers on ice.

3. Resuspend pellets in 2.0-2.5 mls of sonication buffer containing 50ug/ml RNAse (fresh). One way to determine approximate protein content of each sample at this stage is to mix 50ul of this cell concentrate with 5.0 mls of sonication buffer in a klett tube and measure density. Adjustments are easily made to samples before cell breakage.

4. Pipet 5ul DNAse stock per ml of cell resuspension in test tube. Weigh out 0.15g of urea and place in another test tube. Have theses two tubes on ice and one pasteur pipet for each sample.

5. Break cells at 20,000 psi (medium setting for small cell), let extract slowly drip from spout into tube containing DNAse. Run through twice if needed. WASH AFTER EACH BREAKAGE.

6. After extracts have mixed with DNAse for 10 mins., pipet 250ul of each extract into tube containing the urea (0.15g), mix in hands until urea has gone into solution, place back on ice until ready to load. Save remaining extract in Nunc tubes and place in -80 C freezer.

7. When ready to load samples on IEF tube gels, mix 250ul of lysis buffer with equal volume urea/extract (these volumes are adjustable). When urea is completely dissolved, load immediately.

BEST TIME Break cells right after derepression if not to late in day, (3:00), or break cells or break cells while IEF tube gels are polymerizing (after part C).
If extracts are frozen remove from refrigerator and place on ice before casting IEF tube gels.

## C. Isoelectric Focusing (1st dimension)

POURING GELS

1. Glass tubes should be cleaned with No-Chromix ($H_2SO_4$), rinsed well with water then acetone, and then air dried.

2. Make a mark ≈145mm from bottom of tube. Parafilm bottom, and place in casting stand. Level stand with bubble level.

3. In a small beaker mix the following:

5.50 g Ultra Pure Urea
1.33ml 30% IEF Acrylamide
2.00ml 10% NP-40
2.00ml $H_20$

Dissolve in 65° water for a few mins.

0.40ml 5-7 pH Ampholines (sterile)
0.10ml 3.5-10 Ampholines (sterile)
10 ul 10% Ammonium persulfate
7 ul TEMED

Fill tubes with long metal canulla starting at bottom. Avoid bubbles by keeping tip under surface of gel. Pour to mark, and overlay with 20ul of 8M urea. Allow 1 hour to polymerize.

RUNNING GELS

4. Prepare buffers for IEF.

Lower buffer- 0.01 M $H_3PO_4$ 0.68 ml/l 85% H3PO4 (need 4.5 l)
Upper buffer- 0.02 M NaOH 4 pellets/ 500ml (need 500ml)

5. Remove urea from top of gels and replace with 20ul of lysis buffer, let stand for 30 mins.

6. When ready to load, remove parafilm and place tubes into chamber, fill top with upper buffer leaving top of gels above level, then remove lysis buffer from top of gels and load sample (40-80 ul). Overlay with 20 ul sample overlay soln., then fill to top with buffer. When placing into tank do not mix upper and lower buffers.

7. Set power supply at constant Watts 0.5W/tube gel. Make sure Voltage and Amperage is not limiting. Run for 6-8 hours.

BEST TIME Pour gels first thing in the morning, then break cells.

C. Isoelectric Focusing continued

REMOVING GELS

8. Empty buffers into sink and set cooling core with tubes on bench in front of you. Will need:

5ml lure lock syringe with tygon tubing attached
2 1ml tuberculin syringes
Hamilton syringe
vial of india ink
beaker of water

9. Insert the Hamilton syringe into the top of the tube and remove the spent buffers. With the other syringe inject water around the the inside of the bottom of the gel to loosen it from the glass. Then carefully jab the acidic end a ink-coated needle.

10. To remove the gel, fill the 5ml syringe with water and attach the tygon tubing to the end of the tube, apply pressure until the gel has been forced out into a glass tube containing 5mls of sample equilabration buffer.

11. Freeze imediately in a dry ice ethanol bath. Then place in the -80 C for storage. May remain frozen for months. Do not freeze if you are going to run the second dimension that day, in which case you will need to equilibrate the tube gels for 10-30 mins. before placing on second dimension.

## D. Second dimension (SDS PAGE)

POURING GELS

1. Clean all plates with soap and water, then wipe off with ethanol. Clamp plates with spacers making sure that the bottom of the plates is flush with spacers. Place in the casting stand on two sheets of parafilm.

2. Heat in microwave 1% agarose in 1X lower buffer solution then seal the bottom edge of the plates to the parafilm. Close the clamps on the casting stand.

3. Recipes for lower gel:

10% PAGE separating gel

| | 1-gel | 2-gels |
|---|---|---|
| Acrylamide 30% | 10.0 | 20.0 |
| Lower Buf. | 7.5 | 15.0 |
| SDS 20% | 150 | 300 |
| $H_2O$ | 12.0 | 24.0 |
| Ammonium persulfate | 150 | 300 |
| TEMED | 15 | 30 |

Mix first four reagents and then degas for 3 mins. Add A.P. and Temed, swirl, and pour to a mark 1.5 cm from the top. Overlay top of gel with 300 ul saturated butanol-$H_20$.

Stacking gel

| | 1-gel | 2-gels |
|---|---|---|
| Acrylamide 30% | 1.34 | 2.68 |
| Upper Buf. | 2.50 | 5.00 |
| SDS 10% | 100 | 200 |
| $H_20$ | 6.0 | 12.0 |
| Ammonium persulfate 10% | 50 | 100 |
| TEMED | 8.0 | 16.0 |

When lower gel has polymerixed rinse off butanol and unpolymerized gel mix with water. Place back in the casting stand upside down until drained. Pour stacker to about 2 cm from top and carefully slide in comb avoiding bubbles. Rinse when polymerized and place in cooling core, add buffer and re-rinse wells with syringe.

4. Will need the following to place tube gels onto second dimension:
   - parafilm
   - blunt-ended pasteur pipet for tube gel manipulation
   - Hamilton syringe
   - boiling $H_20$ to melt tube gel agarose sealer
   - filter paper to blot gels

5. Thaw out frozen tube gels and gently pour out buffer through a ceramic funnel, let tube gel slip out of test tube into funnel. Then place the tube gel onto the parafilm and arrange it so it will easily slide into the notched side of the second dimension gel. With one hand lifting the gel with the Hamilton syringe, use the other hand to slowly inject melted agarose under the gel, avoid bubble. Large bubbles can be removed with syringe. Lable gels with resistant pen.

6. Fill top reservoir with SDS running buffer and watch for any obvious leaks.

SDS PAGE RUNNING BUFFER

| | 1 L | 2 L | 3 L |
|---|---|---|---|
| Tris HCl | 3.0 | 6.0 | 9.0 grams |
| Glycine | 14.41 | 28.82 | 43.23 grams |
| SDS | 1.0 | 2.0 | 3.0 grams |

7. Use remaining buffer for lower chamber. Place gels into lower chamber, rinse off bubbles which have collected on the underside of the gels, and run gels at 15-20 mA per gel (constant amperage). Running time approx. 4 hours.

8. When blue dye is near the bottom stop run, remove gel unit in sink, pry open with a plastic spacer (not metal spatula). Notch gels at corners for identification. Place in baking dishes with Coomassie blue stain.

COOMASSIE BLUE STAIN

| | |
|---|---|
| Coomassie blue | 1.0 gram |
| Methanol | 400 mls |
| Acetic acid | 100 mls |
| $H_20$ | 500 mls |

Dissolve dye in methanol first (5 mins), then add rest of ingredients.

9. Stain 1 hour then place in Destain I until backround is clear. Several changes will be necessary. When cleared place in Destain II to re-hydrate to normal size.

DESTAIN I

| | |
|---|---|
| Methanol | 800 mls |
| Acetic acid | 200 mls |
| $H_20$ | 1000 mls |

DESTAIN II

| | |
|---|---|
| Methanol | 100 mls |
| Acetic acid | 140 mls |
| $H_20$ | 1760 mls |

## E. Reagents and recipes

| | CONCENTRATIONS | RECIPES |
|---|---|---|
| 1. SAMPLE MIX | 9.5 M UREA | 5.705 G |
| | 2% NP-40 | 2.0 MLS |
| | 1.6% 5-7 AMPHOLYTES | 0.16 MLS |
| | 0.4% 3-10 AMPHOLYTES | 0.45 MLS |
| | 5% $\beta$-ME (W/V) | 4.6 MLS |
| | | 10.0 ML |
| 2. SONICATION BUFFER | 0.01 M TRIS PH=7.4 | 0.121 G |
| | 5 mM $MgCl_2$-$6H_20$ | 0.102 G |
| | | 100 MLS |
| 3. RNASE | 50 UG/ML RNASE IN SONICATION BUFFER | |
| 4. DNASE | 2.5 MG/ML DNASE IN: 0.01 M TRIS PH=7.4 1 mM $MgCl_2$-$6H_20$ | |
| 5. IEF ACRYLAMIDE | 30% ACRYLAMIDE | |
| | DO NOT USE SEQUENCING STOCK | |
| 6. NP-40 | 10% W/V NP-40 | 10 G/ 100 ML $H_20$ |
| 7. AMMONIUM PERSULFATE | 10% W/V IN $H_20$ | |
| 8. IEF OVERLAY | 8 M UREA | 4.8 G/ 10 ML $H_20$ |
| | STORE AT -20 C IN 500 UL ALIQUOTS | |
| 9. IEF ANODE BUFFER (LOWER) | 0.01 M $H_3PO_4$ | 2.04 MLS/ 3 L $H_20$ |
| 10. IEF CATHODE BUFFER (UPPER) | 0.02 M NaOH | 4 PELLETS/ 500 MLS $H_20$ |

11. SAMPLE OVERLAY SOLN.

| | |
|---|---|
| 9 M UREA | 5.4 G |
| 0.8% 5-7 AMPHOLYTES | 0.08 MLS |
| 0.2% 3.5-10 AMPHOLYTES | 0.02 MLS |
| | 10 MLS |

12. SDS EQUILIBRATION BUFFER

| | |
|---|---|
| 10% GLYCEROL | 10 G |
| 2.3% SDS | 2.3 G |
| 0.0625 M TRIS PH=6.2 | 0.757 G |
| 5% $\beta$-ME | 5 G |
| | 100 MLS |

13. TUBE GEL SEALER 1% AGAROSE IN SDS EQUIL. BUFFER, ADD VERY SMALL AMOUNT OF BROMPHENOL BLUE

14. CASTING STAND AGAROSE 1% AGAROSE IN 1X LOWER BUFFER

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