GENETIC ANALYSIS OF NIFF AND NIFA AND SITE-DIRECTED MUTAGENESIS OF NIFE IN AZOTOBACTER VINELANDII

by

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(ABSTRACT)

Nitrogenase-catalyzed nitrogen fixation is a biochemically and genetically complex process requiring the participation of a number of different nif (nitrogen fixation) gene products. The nifF (electron transport), nifA (nif gene regulation) and nifE (FeMo-cofactor biosynthesis) genes from Azotobacter vinelandii were genetically analyzed. The nucleotide sequence of the nifF gene, which encodes a flavodoxin, was determined. Specific nifF mutation strains indicated that in A. vinelandii flavodoxin is not the unique physiological electron donor to nitrogenase. The nifF gene appears to be constitutively expressed but under nitrogen fixing conditions nifF gene expression is stimulated.

The nucleotide sequence of nifA was determined. This gene encodes a M_r = 58,100 polypeptide which shares significant sequence identity with the nifA genes from other organisms and which has a consensus ATP binding site and consensus DNA binding site. A potential regulatory gene precedes and is apparently co-transcribed with nifA. Specific nifA mutation strains were incapable of diazotrophic growth and failed to accumulate the nitrogenase structural gene products.

The nifE gene product is proposed to form a complex with the nifN gene product on which the FeMo-cofactor of the MoFe protein is synthesized. The FeMo-cofactor binding

regions in the MoFe protein and the NifEN complex are unknown but are expected to be similar. A potential FeMo-cofactor binding domain in NifE was altered, using a site-directed mutagenesis/gene replacement strategy, to more closely duplicate the corresponding region in the MoFe protein α-subunit. The diazotrophic growth capabilities of several of the nifE site-directed mutation strains were significantly reduced. The results of this study were discussed in terms of a model developed for the assignment of metallocluster binding ligands in the nitrogenase MoFe protein.

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Introduction

Nitrogen is a component of DNA and protein, and consequently it is essential for life. A continual cycling of nitrogen through the biosphere is maintained by the activity of bacteria. Nitrate (NO_3^-), nitrite (NO_2^-), ammonia (NH_3) and dinitrogen (N_2) are interconverted by the processes of nitrification ($NH_3 \rightarrow NO_2^- \rightarrow NO_3^-$), denitrification ($NO_3^- \rightarrow N_2$) and nitrogen fixation ($N_2 \rightarrow NH_3$). Nitrate and ammonia appear to be the preferred nitrogen sources for most plants and bacteria, respectively, while organic N-containing compounds from plants and animals are the most common nitrogen sources for animals. The process of denitrification is responsible for a loss of fixed nitrogen sources from the soil. In soils being continually cultivated nitrogen is usually the limiting nutrient. Nitrogen fixation is necessary to restore the fixed nitrogen levels in the soil by converting atmospheric dinitrogen to ammonia. Biological and industrial nitrogen fixation are the two most important means of obtaining fixed nitrogenous compounds for plant growth. The Haber Bosch process for the chemical synthesis of ammonia from dinitrogen and hydrogen is the most extensively used industrial process for this purpose. This process requires very high temperatures and pressures and consequently demands a large amount of energy. Approximately 50×10^6 metric tons of industrially synthesized ammonia are applied to the soil annually. Biological nitrogen fixation is performed exclusively by bacteria. This process converts dinitrogen to ammonia under physiological conditions

(25 °C, 1 atm) and accounts for approximately 150 x 10^6 metric tons of ammonia per year (ie. three times the amount of ammonia produced industrially).

Biological nitrogen fixation was first described in 1888 by H. Hellreigel and H. Wilfarth (114). They presented data demonstrating that atmospheric dinitrogen was the sole source of nitrogen for leguminous plants. They also stated that leguminous plants required a symbiotic association with a microorganism in order to use molecular nitrogen as a nitrogen source. In that same year, Beyerinck reported the isolation and cultivation of microorganisms capable of nitrogen fixation from the root nodules of many different leguminous plants. To date, a nitrogen fixing species of bacteria has been identified from all the major physiological groups including the following: 1) the free-living obligate anaerobe Clostridium pasteurianum, the free-living facultative anaerobe Klebsiella pneumoniae, the free-living obligate aerobes Azotobacter vinelandii and Azotobacter chroococcum, the fast and slow growing symbiotic organisms Rhizobium meliloti and Bradyrhizobium japonicum, respectively, the photosynthetic blue-green alga Anabaena, the photoautotrophic cyanobacterium Rhodobacter capsulatus, the non-leguminous symbiont Azospirillum brasilense, the chemolithotrophic H_2-utilizing organism Xanthobacter autotrophicus, the heterotrophic sulfur oxidizer Thiobacillus ferrooxidans and the methanogenic archaebacterium Methanococcus thermolithotrophicus.

Azotobacter vinelandii is the nitrogen fixing organism used in this study. It is a motile, Gram negative, ovoid bacterium isolated from alkaline soils and is one of only a few species of aerobic diazotrophic organisms known. A. vinelandii does not form a symbiotic relationship with leguminous plants but is instead a free-living nitrogen-fixer. This organism

provides an excellent system in which to study the genetics of biological nitrogen fixation because it possesses a natural transformation system; its physiology has been extensively studied; the DNA is easily extractable; growth rates are high; and handling is simple. A. vinelandii, and all other diazotrophic organisms, possess a nitrogen fixing system which catalyzes the reduction of dinitrogen to ammonia. A. vinelandii is unusual in that it possesses three nitrogen fixing systems; one is referred to as the conventional nitrogen fixation system and the other two are called the alternative nitrogen fixation systems. Conventional nitrogen fixation is catalyzed by the complex metallo-enzyme nitrogenase. The nitrogenase complexes isolated from many different nitrogen fixing organisms are remarkably similar in amino acid sequence, predicted secondary structure, protein composition and metal content, all of which is suggestive of a conserved catalytic mechanism.

Nitrogenase Structure and Mechanism of Action

Nitrogenase is a complex metallo-enzyme composed of two separately purifiable proteins, the Fe protein (dinitrogenase reductase or component-2) and the MoFe protein (dinitrogenase or component-1). The Fe protein is a dimeric protein of identical subunits with a native molecular weight of approximately 64,000. A single [4Fe-4S] cluster is believed to be symmetrically bridged between the two subunits (76). The Fe protein also harbors two ATP binding sites per dimer. A characteristic S = 3/2 electron paramagnetic resonance (EPR) signal is associated with isolated Fe protein. (70, 118, 145, 235) The MoFe protein is an $\alpha_2\beta_2$ tetrameric protein of two nonidentical subunits with a native

molecular weight of 220,000 (Mr_α = 50,000 and Mr_β = 60,000). The metal content of the MoFe protein consists of 2 Mo, 32 Fe, and $32S^{2-}$atoms arranged in at least two distinct types of metal clusters, designated the P clusters and the FeMo-cofactors (116, 171, 196). The four P clusters are proposed to be Fe-S centers of unknown structure and the two FeMo-cofactors are metal clusters also of unknown structure containing one Mo, 6-8 Fe, and 6-8 acid labile S^- atoms. The FeMo-cofactor is the species responsible for the characteristic electron paramagnetic resonance (EPR) signal of the MoFe protein and is the proposed site of substrate binding and reduction (77, 171).

The minimum requirements for the enzymatic reduction of dinitrogen to ammonia are the two component proteins, a low potential reductant, MgATP, a source of electrons, protons, and an anaerobic environment. The reaction catalyzed by nitrogenase is the following:

$$N_2 + 16MgATP + 8H^+ + 8e^- \rightarrow NH_3 + H_2 + 16MgADP + 16P_i.$$

The energetically wasteful ATP-dependent evolution of H_2 appears to be obligatorily coupled to the reduction of N_2 (30, 203, 208). However, the amount of H_2 evolved is dependent on the growth conditions. Under optimum conditions, a minimum of 25% of the electron flux is shuttled to proton reduction (203). In vitro H_2 production is a function of the ratio and concentration of the component proteins. In general, lower Fe protein/MoFe protein ratios favor H_2 evolution over NH_3 production (240). For optimal in vitro N_2 reduction, Fe protein/MoFe protein ratios of between three and five have been

TABLE 1. Substrates of nitrogenase.

Substrate		K_m(mM)*	Equation†
Dinitrogen	$N\equiv N$	0.1	$N_2 + 8H^+ + 8e^- \rightarrow 2NH_3 + H_2$
Azide	$[N\equiv N^+—N^-]^-$	1.0	$N_3^- + 3H^+ + 2e^- \rightarrow N_2 + NH_3$ $N_3^- + 7H^+ + 6e^- \rightarrow N_2H_4 + NH_3$ $N_3^- + 9H^+ + 8e^- \rightarrow 3NH_3$
Nitrous oxide	$N\equiv N^+-O^-$	1.0	$N_2O + 2H^+ + 2e^- \rightarrow N_2 + H_2O$
Cyanide	$[C\equiv N]^-$	0.4–1.0	$CN^- + 7H^+ + 6e^- \rightarrow CH_4 + NH_3$ $CN^- + 5H^+ + 4e^- \rightarrow CH_3NH_2$
Alkyl cyanides	$R-C\equiv N$	10–>500	$RCN + 6H^+ + 6e^- \rightarrow RCH_3 + NH_3$
Alkyl isocyanides	$R-N^+\equiv C^-$	0.2–25	$RNC + 6H^+ + 6e^- \rightarrow RNH_2 + CH_4$ $2RNC + 8H^+ + 8e^- \rightarrow 2RNH_2 + C_2H_4$ $2RNC + 10H^+ + 10e^- \rightarrow 2RNH_2 + C_2H_6$ $3RNC + 12H^+ + 12e^- \rightarrow 3RNH_2 + C_3H_6$ $3RNC + 14H^+ + 14e^- \rightarrow 3RNH_2 + C_3H_8$
Acetylene	$HC\equiv CH$	0.1–0.3	$C_2H_2 + 2H^+ + 2e^- \rightarrow C_2H_4$
Alkynes	$RC\equiv CH$		$RC\equiv CH + 2H^+ + 2e^- \rightarrow RCH=CH_2$
Allene	$H_2C=C=CH_2$	0.43	$H_2C=C=CH_2 + 2H^+ + 2e^- \rightarrow H_3C-CH=CH_2$
Cyclopropene		0.1–0.14	$+2H^+ + 2e^- \rightarrow$ $+2H^+ + 2e^- \rightarrow H_3C-CH=CH_2$
Diazirine		0.08	$+ 6H^+ + 6e^- \rightarrow CH_3NH_2 + NH_3$ $+ 8H^+ + 8e^- \rightarrow CH_4 + 2NH_3$ (H_2 also evolved)
Hydrogen ion (proton)	H^+	—	$2H^+ + 2e^- \rightarrow H_2$

*The Michaelis constant (K_m) is a measure of the interaction between substrate and enzyme. The lower the value of K_m, the more efficiently the substrate binds to the enzyme.
†e^- refers to an electron.

Taken from Postgate, Fundamentals of Nitrogen Fixation. 1982. Cambridge University Press, Cambridge, 39.

reported to be necessary. In addition to being a product of nitrogenase, H_2 is also an inhibitor of nitrogenase activity. Some organisms, including A. vinelandii, have developed hydrogen uptake systems coupled to the respiratory chain to recapture some of the energy lost by nitrogenase catalyzed H_2 evolution and to lower the concentration of H_2 available to function as an inhibitor of nitrogenase activity (166, 207).

The mechanism of nitrogenase action has been extensively studied in A. vinelandii and in the facultative anaerobe K. pneumoniae and shown to be extremely complex (121, 122, 219, 220, 221). A comprehensive understanding of the mechanism of nitrogenase must await the elucidation of the structures of the metal clusters contained within the Fe and MoFe proteins, particularly of the FeMo-cofactor. Some insight into how and where nitrogen fixation occurs on the nitrogenase complex comes from studying the reduction of substrates other than N_2. Besides N_2 and protons nitrogenase reduces a number of different triple bond-containing compounds including acetylene, azides, cyanides, cyclopropene, nitrous oxide, methylisocyanide, and cyanamide (141)(see Table 1). Carbon monoxide acts as an inhibitor of the nitrogenase catalyzed reduction of all of these substrates except protons. Reduction of acetylene to ethylene is the standard assay used for measuring in vivo and in vitro nitrogenase activity due to its ease and sensitivity.

The pathway of electron transport to nitrogenase is known for K. pneumoniae. The oxidation of pyruvate to acetyl-CoA and CO_2 is coupled to the reduction of flavodoxin through a pyruvate:flavodoxin oxidoreductase (200). Reduced flavodoxin (E_m < -400 mV) donates single electrons to the Fe protein. Reduced Fe protein binds two molecules of MgATP and associates with the MoFe protein where it functions as the unique reductant

$$N_2 + 8e^- + 8H^+ + 16ATP \longrightarrow 2NH_3 + H_2 + 16ADP + 16Pi$$

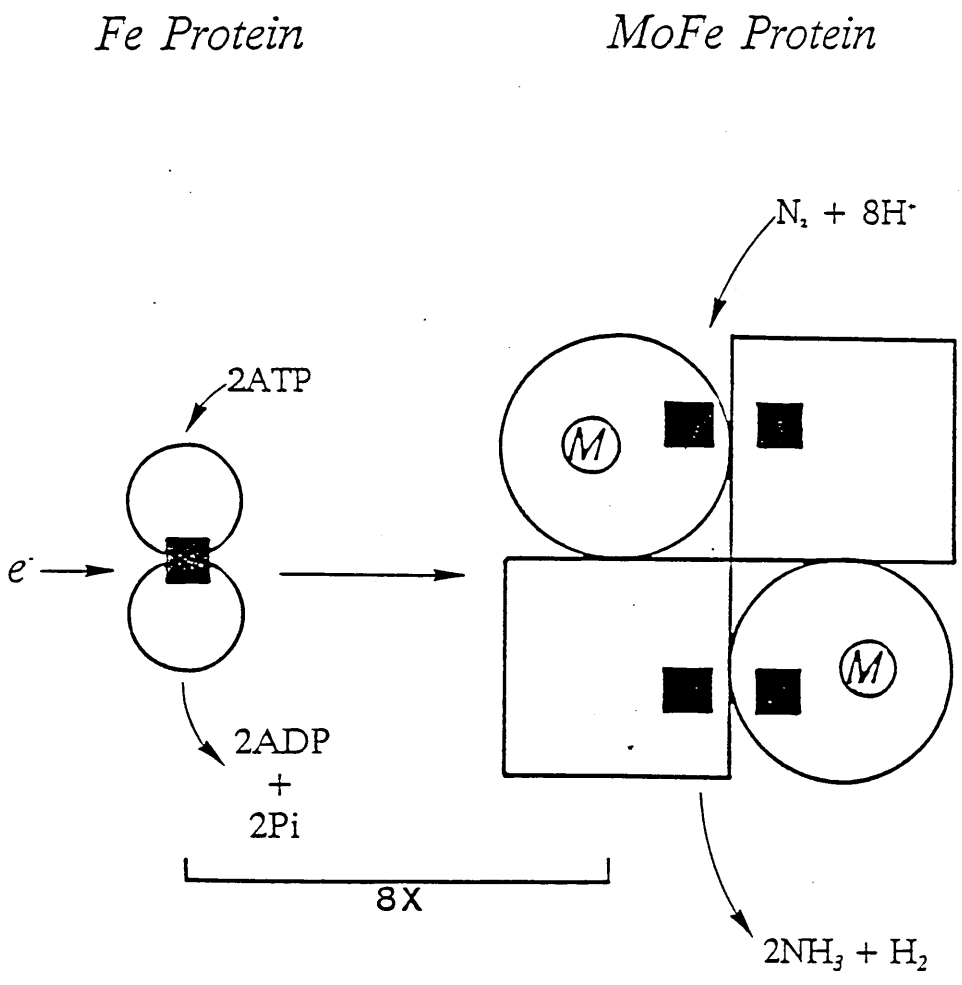

FIGURE 1. Diagrammatic representation of the pathway of electron flow through nitrogenase including the substrates and the products of the reaction catalyzed by nitrogenase.

of the MoFe protein (69). Transfer of a single electron from the Fe protein to the MoFe protein is coupled to the hydrolysis of two molecules of MgATP to two molecules of MgADP and two Pi (30, 69). Oxidized Fe protein dissociates from the MoFe protein and is reduced by flavodoxin to continue the reduction cycle. The cycle must be repeated at least eight times with the consequent consumption of at least 16 ATP molecules before dinitrogen can be reduced to two molecules of ammonia (69). The association and dissociation cycle of the Fe protein and the MoFe protein is the proposed rate-limiting step in the reaction (219). In the obligate anaerobe Clostridium pasteurianum, pyruvate metabolism is coupled to the reduction of a ferredoxin rather than to a flavodoxin which in turn donates electrons to nitrogenase (146). In A. vinelandii the physiological source of reducing equivalents, as well as the low potential reductant of the Fe protein, is unknown but is expected to be more complex, possibly involving the membrane generated proton motive force (67).

The structure and activity of all nitrogenases studied, despite the physiologically diverse organisms from which they come, are remarkably similar. In fact, in most cases the mixing of purified component proteins from different organisms results in nitrogenase activity as measured by acetylene reduction, N_2 reduction, H_2 evolution, and MgATP hydrolysis (55). One exception is with the component proteins from C. pasteurianum nitrogenase (54). The Fe protein from C. pasteurianum and the MoFe protein from A. vinelandii associate to form an inactive, tight binding complex which consists of two moles C. pasteurianum Fe protein bound to one mole A. vinelandii MoFe protein (56).

For turnover, nitrogenase requires an reducing environment. The MoFe protein and

particularly the Fe protein, as well as flavodoxin and ferredoxin, are irreversibly inactivated by oxygen (30). The obligate and facultative anaerobes meet the requirement for a reduced environment by only fixing nitrogen in the absence of oxygen. However, obligate aerobes like A. vinelandii and A. chroococcum are dependent upon oxygen for growth. How these organisms maintain both nitrogen fixation and respiration simultaneously without inactivating nitrogenase is not fully understood. Several means of protection against oxygen damage have been suggested to occur in Azotobacter. When the oxygen concentration reaches a certain level in the cell, nitrogenase assumes an inactive, yet oxygen tolerant conformation; this phenomenon is called conformational protection (80). An Fe-S protein isolated from A. vinelandii (known as Fe-S protein II, Shethna protein or ferredoxin II) is proposed to associate reversibly with the nitrogenase proteins to form an oxygen tolerant three component complex (176, 187, 188). The Fe-S protein II appears to contain two [2Fe-2S] clusters which are proposed to oxidize the metal clusters within the nitrogenase proteins, particularly the [4Fe-4S] center within the Fe proteins (176, 188). Azotobacter also possess a very high rate of respiration, shuttling the reducing power through the electron transport chain for the reduction of oxygen, and the subsequent generation of ATP by oxidative phosphorylation, before the oxygen can diffuse into the cell. This protection mechanism is known as "respiratory protection" (80). Some organisms utilize physical barriers to maintain a low intracellular oxygen concentration including slime production, thick cell walls and clumping of cells. The photosynthetic cyanobacteria, including Anabaena, compartmentalize the oxygen requiring and oxygen sensitive processes in two different cell types (74). Photosynthesis occurs in the vegetative cells, and nitrogen fixation occurs in

differentiated cells called heterocysts, which provide an anaerobic environment in which nitrogenase can function by minimizing the diffusion of oxygen into the cell and by eliminating the Photosystem II, the oxygen generating arm of the photosynthetic apparatus (74).

In addition to oxygen, ammonia has been implicated in the regulation of nitrogenase enzyme activity in a few organisms. Although ammonia repression of nitrogenase synthesis is established, the inhibition of nitrogenase activity by ammonia is not as well defined. Short-term nitrogenase "switch-off" by ammonia occurs in many species of Rhodospirillaceae, where this phenomenon has been most extensively studied (250). In the photosynthetic diazotroph Rhodospirillum rubrum, the Fe protein of the nitrogenase complex is covalently modified by ADP-ribosylation to an inactive form in response to the dark, as well as to ammonia (163). Specifically, a dinitrogenase reductase ADP-ribosyltransferase (DRAT) uses NAD as a ribosyl donor to modify arginine residue 101 of the Fe protein resulting in the inactive Fe protein (125). The reactivation of modified Fe protein is catalyzed by dinitrogenase reductase activating glycohydrolase (DRAG) which removes the covalently bound ribosyl moiety attached at arg101 (182). Purified R. rubrum DRAT catalyzes the ADP-ribosylation of the Fe proteins of A. vinelandii, K. pneumoniae, and C. pasteurianum at the analogous arg residues within these proteins (124, 126). However, this reaction does not appear to be physiologically significant in these organisms, in part due to the inability to isolate the DRAT and DRAG proteins or the genes which encode these proteins from these organisms (126).

Genetics of Nitrogenase

The biochemical complexity of nitrogenase is reflected in the genetic organization of the genes involved in the synthesis of a competent nitrogenase complex. In K. pneumoniae 20 nitrogenase (nif) specific genes are clustered in a single regulon and arranged in eight transcriptional units (2). The nucleotide sequence of the entire nif gene cluster in K. pneumoniae has been determined (2, 32, 191, 214, 94, 83, 13, 195, 14, 161, 46, 48, 29, 28, 109). In A. vinelandii 18 of these nif genes have been isolated and sequenced; nifJ and nifL have not been identified in A. vinelandii (96, 11, 12, 14, 20, 21 , 23, 39, 101,). These genes encode the nitrogenase structural proteins (nifH, nifD, and nifK), the nif-specific electron transport proteins (nifF and nifJ), the nif-specific regulatory elements (nifA and nifL), the proteins involved in processing and maturation of the structural proteins (nifB, nifE, nifN, nifH, nifM, nifV, nifQ, nifU, nifS, nifW, nifZ) and the gene products of unknown function (nifX, nifY, nifT). The identification and organization of the nif genes was accomplished by complementation analyses, cloning of the nif genes, identification of the nif encoded polypeptides, and DNA sequencing analyses. A comparison of the organization of the nif genes in K. pneumoniae and A. vinelandii demonstrates that although the sequential arrangement of the nif genes is identical in both organisms, the spatial arrangement is different (Fig. 2). Specifically, the nif genes of A. vinelandii are separated by a number of potential coding regions of unknown importance, which have not been identified in the K. pneumoniae nif cluster. Open reading frame (ORF) 2, ORF3, ORF8 and the fdxN (Fig. 2) gene appear to have counterparts in the photosynthetic diazotrophic organism Rhodobacter capulatus (130). Orf6 appears to have a counterpart

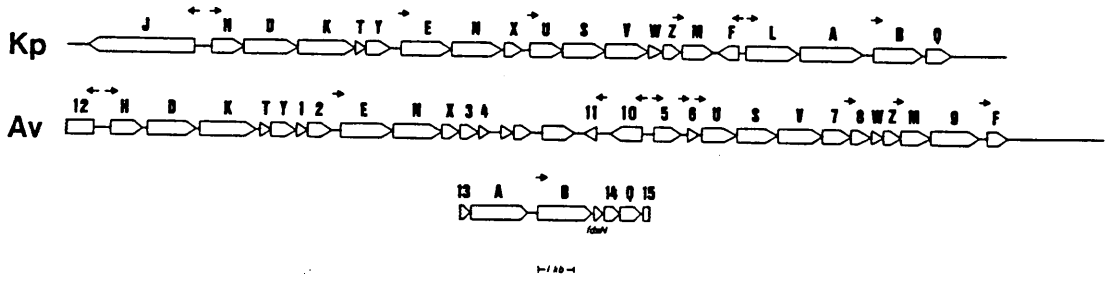

FIGURE 2. Comparison of the organization of the major nif clusters from A. vinelandii (lower portion) and K. pneumoniae (upper portion). Arrows indicate the approximate positions of identified or proposed nif-specific promoters and the direction of transcription. A. vinelandii genes whose deduced amino acid sequences exhibit identity with the nif genes products of K. pneumoniae are represented by the appropriate letter designation. Open reading frames (ORFs) which are potentially cotranscribed with the nif genes or are preceded by a consensus nif -specific promoter sequence are numbered. The numbering at the bottom refers to kilobases of DNA sequenced.

TABLE 2. Proposed functions of the nif gene products.

nif GENE	GENE PRODUCT FUNCTION
H	Fe protein subunit and FeMo-cofactor biosynthesis
D	α-subunit of the MoFe protein
K	β-subunit of the MoFe protein
M	Maturation of the Fe protein
E	FeMo-cofactor biosynthesis
N	FeMo-cofactor biosynthesis
B	FeMo-cofactor biosynthesis
Q	FeMo-cofactor biosynthesis
V	FeMo-cofactor biosynthesis (homocitrate synthase)
U	Maturation and/or stability of the Fe protein
S	Maturation and/or stability of the Fe protein
W	Maturation and/or stability of the MoFe protein
Z	Maturation and/or stability of the MoFe protein
T	Unknown
X	Unknown
Y	Unknown
F	Electron transport to nitrogenase (flavodoxin)
J	Electron transport to nitrogenase (pyruvate:flavodoxin oxidoreductase)
A	Regulation of nif gene expression (positive activator)
L	Regulation of nif gene expression (negative regulator of NifA)

in both R. capsulatus (130), Anabaena (personal communication) and B. japonicum (personal communication). The conservation of these sequences and the location on the chromosomes of A. vinelandii and R. capsulatus suggest that these ORFs encode gene products which function in nitrogen fixation. Furthermore, the nif genes of A. vinelandii are separated into two linkage groups; the major nif gene cluster contains nifHDKTYENXUSVWZMF (96) and another contains nifABQ (see this work and 101). Finally, in A. vinelandii, the nifF gene is transcribed in the same direction as the nifHDK operon while in K. pneumoniae nifF is transcribed in the opposite direction (2, 46). Fig. 2 compares the nif gene clusters of K. pneumoniae and A. vinelandii, and Table 2 lists the proposed products and functions of the known nif-specific genes. The remainder of this discussion will address the known functions of the nif gene products. The genetics and regulation of the alternative nitrogenases will also be discussed.

Fe protein: nifH

The nifH gene encodes the Fe protein component of the nitrogenase complex. The Fe protein functions as the unique ATP-dependent one electron donor to the MoFe protein component of nitrogenase (30, 91, 99, 119, 202, 224, 233, 236). In addition to its essential function in nitrogenase catalysis, the Fe protein is also necessary for the biosynthesis of the FeMo-cofactor (172, 175). This function of the Fe protein was identified in A. vinelandii by studying a specific in-frame nifH deletion strain (174,175). This strain accumulated the MoFe protein subunits but was devoid of in vitro MoFe protein activity (174, 175) . However, addition of purified FeMo-cofactor to a crude extract of this nifH

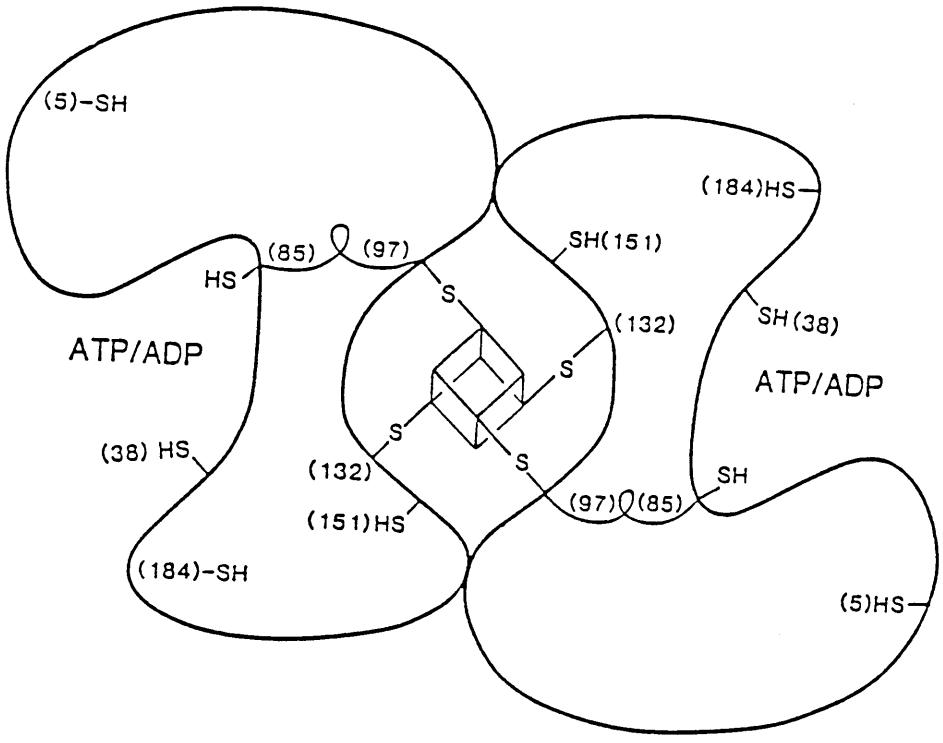

FIGURE 3. Proposed ligation of the [4Fe-4S] center in the Fe protein (taken from Hausinger and Howard, 1983).

deletion strain reconstituted MoFe protein activity (175).

The nifH gene has been isolated and sequenced in the following organisms: K. pneumoniae (191, 215), A. vinelandii (21), C. pasteurianum (34), Rhizobium trifolii (192), Parasponium Rhizobium (193), Rhizobium meliloti (222), Bradyrhizobium japonicum (60), Rhodobacter capsulatus (105), Rhizobium phaseoli (168), Rhizobium strain ANU240 (6), Anabaena 7120 (139), Thiobacillus ferrooxidans (165), Frankia strain HRN18a (154), Frankia strain Ar13 (155), Methanobacterium ivanovii (206), Methanococcus thermolithotrophicus (206), and Methanococcus voltae (207). The degree of amino acid sequence identity deduced from the nucleotide sequence of these nifH genes correlates with the biochemical relatedness observed by the Fe proteins isolated from different organisms. Sequence comparisons reveal significant overall identities with several regions exhibiting almost perfect identities. These regions include the following: 1) four invariantly conserved Cys residues (Cys39, Cys98, Cys133, Cys185). The conserved Cys residues are surrounded by other strongly conserved residues; 2) an Arg residue at position 101 (numbering represents that in A. vinelandii), and 3) a consensus ATP binding sequence (G-X-X-G-K-S). The N-terminal amino acids are also partially conserved while the C-terminal residues exhibit a significant but lower degree of sequence conservation.

Correlation of the sequence analyses with the biochemical and physical data has revealed some of the functional regions of the Fe protein. The Fe protein is homodimer containing a single [4Fe-4S] cluster which is thought to be symmetrically bridged between the two subunits and ligated to the protein subunits by cysteinyl ligands (76). The Cys residues functioning as ligands to the [4Fe-4S] cluster in A. vinelandii (Cys98 and Cys133) were

identified by chemical modification with iodoacetate (76). The results of recent site-directed mutagenesis studies are consistent with a potential role for these Cys residues as ligands to the Fe-S cluster (88). Analysis of the deduced amino acid sequence of nifH did not identify a typical bacterial ferredoxin type [4Fe-4S] cluster binding region (CysxxCysxxCysxxxxCys) (24). The region surrounding Cys98 and Cys133 exhibits limited sequence identity with other known Fe-S binding sites. In addition, the [4Fe-4S] center of the Fe protein is spectroscopically distinct from the [4Fe-4S] centers contained in other proteins as determined by EPR analysis (145, 158, 71). This difference in EPR spectra may be a result of the different environments shown to exist around the respective clusters to these different proteins.

To function as the specific electron donor to the MoFe protein, the Fe protein must bind and hydrolyze two molecules of MgATP (119, 99, 224, 233, 91, 202, 236). The details of MgATp hydrolysis, electron transport and component protein association in relation to nitrogenase catalysis are not well understood. However, the binding of MgATP to isolated Fe protein results in a number of biochemical and biophysical alterations which include 1) a reduction in the redox potential of the Fe-S center (145, 251), 2) a change in the lineshape of the EPR signal (158, 251), 3) an increase in the susceptibility of the Fe-S center to removal by chelation (120, 228) and 4) an alteration in the reactivities of certain thiol groups (76). A consensus ATP-binding site (G-X-X-G-K-S) was identified in the nifH coding sequence (75). Chemical modification studies have also identified at least one Cys residue as potentially being at or near the ATP-binding site (76).

As previously stated, the conservation of the Arg residue at position 101 (numbering as

in A vinelandii) of the predicted amino acid sequences of all Fe proteins sequenced is conserved. In the photosynthetic organism Rhodospirrilum rubrum, Arg101 is ADP-ribosylated to covalently modify the Fe protein to an inactive form in response to the dark and to ammonia (163). In K. pneumoniae, A. vinelandii and C. pasteurianum Arg101 of the Fe protein is a substrate for ADP-ribosylation in vitro resulting in the inactivation of their catalytic function, but in vivo this modification of the Fe protein has not been demonstrated (125, 163). Arg100 has been suggested to reside at a position in the tertiary structure of the Fe protein which interacts with the MoFe protein (126). When the Fe protein of C. pasteurianum is ADP-ribosylated, it can no longer form an irreversible complex with the A. vinelandii MoFe protein. There is also evidence that Arg101 may be important for electron transfer from the Fe protein to the MoFe protein. (33, 123) It was found that when Arg101 is substituted to His101, the Fe protein is no longer a substrate for ADP-ribosylation. In addition this mutant Fe protein cannot catalyze proton or acetylene reduction, but it can hydrolyze MgATP (123).

MoFe Protein: nifD and nifK

The MoFe protein is an $\alpha_2\beta_2$ tetrameric metalloprotein which functions in the terminal step of dinitrogen reduction to ammonia. The α- and ß-subunits of the MoFe protein are encoded by the nifD and nifK genes, respectively. The amino acid sequence of the α-subunit deduced from the nucleotide sequence of nifD has been determined from the following nine organisms: K. pneumoniae (2, 94), A. vinelandii (21), C. pasteurianum (231), B. japonicum (106), Parasponia Rhizobium (238), cowpea Rhizobium strain IRc78 (247),

Anabaena 7120 (61, 117), R. capsulatus (189) and Thiobacillus ferrooxidans (170). The amino acid sequence of the ß-subunit deduced from the DNA sequence of nifK has been determined from the following seven organisms: K. pneumoniae (2, 83, 209), A. vinelandii (21), C. pasteurianum (231), B. japonicum (218), Parasponia Rhizobium (238), Anabaena 7120 (131) and Thiobacillus ferrooxidans (170). C. pasteurianum MoFe protein subunits exhibit the least overall identity to the subunits of other organisms. Several features are strictly conserved in each of the subunits including the following: 1) five invariantly conserved Cys residues in the α-subunit (Cys62, Cys88, Cys154, Cys183, Cys275) and three invariantly conserved Cys residues in the ß-subunit (Cys70, Cys93, Cys153) all of which are contained within highly conserved regions, and 2) the presence of only one Cys residue (in the α-subunit) located within a region exhibiting some identity with a characteristic bacterial ferredoxin-like [4Fe-4S] cluster binding region (24).

Intersubunit comparisons between the α- and ß-subunits demonstrate sequence conservation around the first three invariant Cys residues. However, the importance of these identities has been questioned because of the different predicted secondary structures in the similar regions of the respective subunits (22, 40) Comparisons between the α-subunit of the MoFe protein and the nifE-encoded polypeptide and between the ß-subunit of the MoFe protein and the nifN-encoded polypeptide demonstrated significant sequence identities especially around the conserved Cys residues (23, 39). All of these observations have been useful in targeting specific amino acid residues as potential ligands to the metal clusters within the MoFe protein for further analyses.

The nature of the metal clusters within the MoFe protein has been studied using cluster

extrusion procedures (116, 150, 196), Electron Paramagnetic Resonance (EPR) (57, 71, 234, 248), Extended X-ray Absorption Fine Structure (EXAFS) (36, 52), Magnetic Circular Dichroism (MCD) (210), and Mossbauer spectroscopy (205, 90, 133, 248, 49), but little information is available concerning the structure and location of the metal clusters. Extrusion studies demonstrated at least two different types of metal clusters within the MoFe protein, Fe-S clusters (P centers) and FeMo-cofactor (M centers) (116, 150, 196). Sixteen Fe atoms are released from the MoFe protein as four [4Fe-4S] clusters after treatment with thiols in the presence of a denaturing organic solvent (116). Whether the 16 Fe atoms exist as four [4Fe-4S] centers in the native protein has not been definitively established (248). The spectroscopic properties of the Fe-S clusters within the MoFe protein are distinct from the properties of Fe-S clusters in other proteins. This physical difference correlates with the presence of only one characteristic bacterial ferredoxin type [4Fe-4S] binding site within the MoFe protein subunits (24). The remaining metal atoms, two Mo, 12-16 Fe, and 12-16 S^{-} atoms are extruded by anaerobic acid/base treatment of the native MoFe protein followed by extraction with N-methylformamide (NMF) (196). The structure of the FeMo-cofactor, which is unique among Mo containing cofactors, is unknown. Recent data suggests that homocitrate or a derivative of homocitrate is a component of FeMo-cofactor (85). Anaerobic extraction into NMF results in the isolation of a FeMo-cofactor which is capable of activating FeMo-cofactorless-MoFe protein (isolated from a nifB mutant strain) from any diazotroph, suggesting that the FeMo-cofactor is identical in all species (150, 196).

Although the location of the metal clusters within the MoFe protein is unknown,

correlation of the physical data with the sequence comparisons have revealed a number of residues which may function as potential ligands to either the Fe-S clusters or the FeMo-cofactor. The release of the Fe-S clusters by thiols strongly suggests at least partial ligation to these clusters by Cys thiol interactions (116). The conservation of Cys residues in both the α- and ß-subunits of the MoFe protein further supports this notion. However, the unusual spectroscopic features of the Fe-S clusters may be indicative of other types of ligands (230, 248). Cys thiol and amide ligation to the FeMo-cofactor have been proposed based partially on the thiol reactivity of purified FeMo-cofactor and the extraction of FeMo-cofactor by acid/base treatment in the presence of N-methylformamide or formamide (152, 196). In A. vinelandii, all conserved Cys residues in the α- and ß-subunits have seen changed to ser residues (19, 22). The majority of these changes resulted in strains exhibiting Nif$^-$ phenotypes and in the elimination or alteration of MoFe protein acetylene reducing activities. Changes of some conserved His, Asn and Gln residues resulted in the same drastic phenotypic alterations suggesting potential roles for some of these amino acids in metal binding or catalysis (19,22).

The functions of the metal clusters in the catalytic activity of the MoFe protein are also ill-defined. The [4Fe-4S] centers are speculated to capture electrons donated by the Fe protein and to some how transfer electrons to the active site (248). The FeMo-cofactor is the proposed site of substrate binding and reduction. Evidence for this is indirect and comes from studies on a nifV$^-$ mutant which has altered substrate reduction properties (132, 134). When the FeMo-cofactor isolated from the MoFe protein of the nifV mutant is used in reconstitution experiments with cofactor-less MoFe protein (isolated from a nifB

mutant), the same substrate reduction properties characteristic of the MoFe protein from the nifV mutant were observed (77).

FeMo-cofactor biosynthesis: nifE, nifN, nifB, nifQ, nifV, nifH

FeMo-cofactor is the unique prosthetic group contained within the MoFe protein and is thought to be the site of substrate binding and reduction. At least six polypeptides encoded by nifE, nifN, nifB, nifQ, nifV and nifH appear to be involved in the biosynthesis of FeMo-cofactor. The nifE-, nifN-, nifH- and nifB-gene products are absolutely required for nitrogen fixation, and mutations in the corresponding genes results in the accumulation of an inactive cofactor-less MoFe protein. The nifE and nifN genes encode polypeptides which are proposed to form a complex on which the FeMo-cofactor is proposed to be synthesized prior to insertion into the MoFe protein (23, 39). Considerable evidence has accumulated to support this concept. The nifE and nifN genes products have mutual stabilizing effects on each other as observed in two-dimensional electrophoretic gel analysis suggesting they form a complex (173). The MoFe protein is not required for the accumulation of FeMo-cofactor (199, 173). This was demonstrated in nifD and nifK mutant strains in both K. pneumoniae and A. vinelandii and suggests that the FeMo-cofactor associates with another protein complex. The amino acid sequence deduced from the nucleotide sequence of nifE and nifN exhibit significant identity with the deduced amino acid sequence of nifD and nifK, respectively (23, 39). Four of the five conserved Cys residues in nifD (Cys62, Cys88, Cys145, Cys275) are also conserved in nifE (Cys37, Cys62, Cys125, Cys250), and one of the three invariant Cys residues in nifK (Cys95) is present in

nifN (Cys44). The molecular weight and the ionic charge of the nifE and nifN encoded polypeptides closely resemble those of the nifD and nifK gene products, respectively (172, 173). Finally, the nifE and nifN gene products have been purified as a complex with M_r = 220,000 suggestive of an $\alpha_2\beta_2$ tetrameric arrangement (162). The presence of a single Fe-S center within the NifEN complex is suggested by the bleaching of a 425 nm absorption peak upon addition of dithionite.

The gene product encoded by nifB is essential for nitrogen fixation and MoFe protein activity, but its identity and specific function are unknown (101). The role of the nifB-encoded polypeptide in FeMo-cofactor biosynthesis was established when the MoFe protein purified from a K. pneumoniae NifB mutant strain was shown to be FeMo-cofactorless (78). In contrast to the nifB gene of K. pneumoniae, the nifB gene in A. vinelandii is contained in a gene cluster which in unlinked to the major nif gene cluster and which is co-transcribed with nifQ and two other open reading frames (101). The identity of one of these open reading frames appears to be a ferredoxin, fdxN, and transcription of both ORFs is ammonia repressible. The function of the polypeptides encoded by these ORFs is unknown.

An A. vinelandii NifQ mutant strain exhibits wild type nitrogen fixation capabilities in the presence of 1000 times the level of molybdate necessary to maintain diazotrophic growth by the conventional nitrogenase in the wild type strain (101). The exact function of the polypeptide encoded by nifQ is unknown but, in the presence of 50 nm molybdate, it is necessary for the synthesis of an active FeMo-cofactor. The nifQ gene product is not involved in Mo uptake since other Mo containing enzymes (ie. nitrate reductase) remain

functional in NifQ mutant strains (92, 93). The nucleotide sequence of the 5' end of nifQ shows some identity to a metal binding site and it has been suggested that this polypeptide may function to transport Mo to the site of FeMo-cofactor synthesis (101).

NifV mutant strains have reduced diazotrophic growth capabilities due to the synthesis of an FeMo-cofactor with an altered substrate affinity and inhibition specificity (84). In NifV mutants the rate of dinitrogen reduction is decreased and the evolution of H_2 is 70% inhibited by CO (in contrast to wild type in which protons are the only substrate not inhibited by CO) (132, 134). The in vitro acetylene reducing activity of the MoFe protein is affected by a mutation in nifV, and the FeMo-cofactor was identified as the site of the defect because apo-MoFe protein reconstituted with FeMo-cofactor isolated from a NifV mutant strain exhibited the characteristic NifV phenotype (134). Recently, homocitrate or a derivative of homocitrate has been shown to be a component of FeMo-cofactor, and nifV has been proposed to encode a homocitrate synthase (85, 86). Because FeMo-cofactor synthesized in vitro in the presence of citrate instead of homocitrate exhibits the same altered substrate reduction properties as the FeMo-cofactor isolated from a NifV mutant strain, the FeMo-cofactor synthesized in the absence of the nifV gene product is suggested to contain citrate (84).

Recent evidence has accumulated to implicate the Fe protein as a component in the biosynthesis of FeMo-cofactor. An A. vinelandii strain with a defined deletion in the nifH gene accumulated significant amounts of MoFe protein with a very low level of in vitro acetylene reducing activity which was restored upon addition of isolated FeMo-cofactor (175). In K. pneumoniae the Fe protein has also been suggested to play a role in FeMo-

cofactor synthesis or in the full expression of the FeMo-cofactor biosynthetic genes (58). The Fe protein apparently does not have to be active to function in FeMo-cofactor synthesis since only certain NifH mutant strains exhibit reduced MoFe protein activities (173). However, site-directed mutagenesis studies of the Fe protein indirectly demonstrated that the presence of the Fe-S cluster within the Fe protein is essential for both the reductase and FeMo-co biosynthetic activities (88).

Maturation of the Fe Protein: nifM, nifU, and nifS

The nifM gene encodes an essential nif polypeptide which activates the Fe protein in an undefined manner. Synthesis of an active K. pneumoniae Fe protein in E. coli requires only the proteins encoded by nifH and nifM (89). Strains of K. pneumoniae and A. vinelandii with a mutation in nifM are incapable of diazotrophic growth and exhibit nearly normal levels of in vitro MoFe protein activity, while exhibiting essentially no Fe protein activity (97, 161). Nitrogenase activity in NifM mutant strain extracts can be stimulated by the addition of purified wild type Fe protein. These results suggest that the nifM-encoded polypeptide modifies the Fe protein to a physiologically active form at a step after transcription. Originally the nifM gene product was thought to either stabilize the active form of the Fe protein or to function in the insertion of the Fe-S center (ie. as a rhodanese) (89). However, recent site-directed mutagenesis studies suggest that nifM may not necessary for the insertion of the Fe-S cluster into the Fe protein (88).

The nifU-gene product is essential for nitrogen fixation in A. vinelandii (97). NifS mutant strains of A. vinelandii are capable of only very slow growth under nitrogen fixing

conditions (97). In vitro component protein activities of nifU and nifS deletion strains suggest that both NifU and NifS are involved in Fe protein maturation or stability. Synthesis of an active K. pneumoniae Fe protein in E. coli requires only the nifH and nifM gene products (89). This suggests either that E. coli synthesizes proteins which can functionally replace NifU and NifS or that in K. pneumoniae NifU and NifS are not essential or have a different function (97).

Maturation of MoFe protein: nifW and nifZ

Deletion of nifW and nifZ results in moderate decreases in diazotrophic growth rates. Crude extracts of nifW and nifZ deletion strains of A. vinelandii and K. pneumoniae have normal Fe protein activity but reduced MoFe protein activity (97, 161). These results suggest that nifW and nifZ encode proteins which are necessary for the full activation, stability or processing of the MoFe protein, although they are not essential for nitrogen fixation. Because nifW and nifZ appear to be transcriptionally coupled and exhibit similar diazotrophic growth rates, the proteins may form a complex (97).

An interesting result was obtained in which deletion of nifZ and nifM in A. vinelandii produced a strain deficient in both Fe protein and MoFe protein activity (97). Furthermore, addition of purified FeMo-cofactor reconstituted MoFe protein activity in extracts of this mutant strain. This suggests that in A. vinelandii, nifZ is necessary for FeMo-cofactor biosynthesis or insertion in the absence of nifM, or nifM is necessary for FeMo-cofactor biosynthesis in the absence of nifZ (97).

Genes of Unknown Function: nifT, nifY, nifX.

A number of the nif genes contained within the major nif gene clusters of A. vinelandii, K. pneumoniae, and R. capsulatus encode polypeptides whose functions in nitrogen fixation are not yet established (96, 130, 142). The nifT and nifY genes appear to be contained in the same transcriptional unit as nifHDK, but transcripts encoding nifT and nifY are either at nondetectable levels or they are not synthesized (13, 19). Neither nifT nor nifY are essential for diazotrophic growth and strain with deletions within these genes exhibit normal nitrogen fixing growth rates as compared with wild type (96).

The nifX gene lies in the same transcriptional unit as nifE and nifN and encodes a polypeptide of unknown function (19, 96, 195). The nifX gene product is also non-essential for diazotrophic growth (96). The nifX genes of A. vinelandii and K. pneumoniae exhibit sequence identity, as do the nifY genes from these organisms (96, 167). In addition, nifX and nifY from A. vinelandii, and nifX and nifY from K. pneumoniae share sequence identities (96, 168). However, the regions of identities in the A. vinelandii nifX and nifY genes is different than in the K. pneumoniae nifX and nifY genes (19, 96).

Electron Transport: nifF and nifJ

The pathway of electron transport to nitrogenase is best understood in the facultative anaerobic organism K. pneumoniae. In this organism electron transfer to nitrogenase requires the polypeptides encoded by nifF and nifJ; both polypeptides have been purified and the genes have been sequenced (81, 151, 200). The nifF gene encodes an acidic polypeptide (M_r=22,000) belonging to the long chain class of flavodoxins (46). Flavodoxins

are low potential endogenous reductants (Em = -500mV) containing one mole of flavin mononucleotide (FMN) per molecule. In most organisms, flavodoxins are expressed under conditions of Fe deficiency to functionally replace ferredoxin, another low-potential reductant which requires Fe for activity (112). Flavodoxins can exist in three different redox forms: the fully oxidized quinone form, the semi-reduced semiquinone form and the fully reduced hydroquinone form (42, 226, 244). The flavodoxin semiquinone/hydroquinone redox couple is the only one of these species with a redox potential low enough to reduce the Fe protein. An unusual characteristic of flavodoxins is the stability of the flavodoxin semiquinone to oxidation (42, 226, 244). The K. pneumoniae flavodoxin is also unique in that to date it is the only flavodoxin having genetic evidence to support its in vivo physiological function which is as the specific reductant of nitrogenase.

The nifJ gene encodes the monomer of a dimeric pyruvate:flavodoxin oxidoreductase (M_r=240,000), which contains thiamine pyrophosphate and eight moles of Fe and acid-labile S^{2-} arranged as two typical [4Fe-4S] clusters (200, 227). The nifF-gene product of K. pneumoniae is functionally identical to the pyruvate oxidoreductase of Clostridium thermoaceticum (227). Both enzymes couple the phosphoroclastic oxidation of pyruvate (producing acetyl-CoA and CO_2) to the in vitro reduction of flavodoxin or ferredoxin. However, in vivo the oxidoreductase of K. pneumoniae is specific for the nifF-encoded flavodoxin (200). In C. pasteurianum, a pyruvate oxidoreductase is thought to mediate reduction of ferredoxin, which subsequently reduces the Fe protein (227, 229). In K. pneumoniae the flavodoxin encoded by nifF and the pyruvate:flavodoxin oxidoreductase encoded by nifJ function to specifically transfer the reducing power of organic metabolites

to the Fe protein of nitrogenase (227). In addition to pyruvate, formate, malate, and an NADPH-generating system all support nitrogenase catalyzed acetylene reducing activities.

Both nifF and nifJ are contained within the nif gene cluster of K. pneumoniae and are transcribed as ammonia repressible monocistronic transcriptional units (2, 32, 46). Strains with mutations within nifF or nifJ are incapable of diazotrophic growth and exhibit very low levels of in vivo nitrogenase activity (81, 151). However, nifF and nifJ mutant strains exhibit wild type levels of acetylene reducing activities in the presence of the artificial reductant dithionite (81, 151, 200). Acetylene reducing activities are restored by the mixing of nifF and nifJ mutant extracts and by the addition of purified A. chroococcum flavodoxin to nifF mutant extracts (151). Acetylene reducing activity in vitro requires the mixing of purified flavodoxin, purified pyruvate:flavodoxin oxidoreductase, purified Fe protein and MoFe protein, pyruvate, and an ATP regenerating system (200). Taken together these results demonstrate the essential nature of the nifF- and the nifJ-gene products for nitrogen fixation and identify them as the specific electron transport components linking the reducing power of the endogenous electron source to nitrogenase.

K. pneumoniae and C. pasteurianum fix nitrogen only when they are growing in an anaerobic environment, and pyruvate oxidation to acetyl-CoA and CO_2 mediated by a pyruvate oxidoreductase, is characteristic of a fermentative metabolism. Pyruvate oxidation in aerobic diazotrophic organisms like A. vinelandii is catalyzed by the pyruvate dehydrogenase complex which reduces NADH, rather than flavodoxin or ferredoxin, and shuttles the acetyl-CoA formed into the tricarboxylic acid cycle. The redox potential of the NAD(P)H/NAD(P)$^+$ couple (of about -330 mV at physiological pH) is in contrast to

the redox potential of flavodoxin and ferredoxin of about (-460 mV) and is not low enough to transfer electrons to flavodoxin, ferredoxin or the Fe protein of nitrogenase. Furthermore, although flavodoxins and ferredoxins from A. vinelandii have been purified, no enzyme activity has been detected which can couple the oxidation of pyruvate to the reduction of low potential electron carriers in this organism (8, 9, 10, 11, 201, 246). At the genetic level no gene with sequence identity to the nifJ gene of K. pneumoniae has been identified in A. vinelandii. Despite these results, the existence of an oxidoreductase-like gene cannot be completely discounted. However, the different physiologies of anaerobic and aerobic nitrogen fixers may demand different mechanisms of electron transport to nitrogenase.

Both flavodoxin and ferredoxin have been isolated and purified from A. vinelandii and implicated as components in electron transport to nitrogenase (8, 112, 201, 245, 246). The initial evidence to support this function include the following: 1) the ability of both flavodoxin and ferredoxin to couple the reducing power of illuminated spinach chloroplasts to nitrogenase activity (9, 201), 2) the observation that ferredoxin, flavodoxin, and a crude extract of nitrogenase could support low nitrogenase activities using NADPH as electron donor, and 3) the ability of dithionite reduced flavodoxin and ferredoxin, as well as photochemically reduced flavodoxin to reduce nitrogenase in vitro and to support high levels of acetylene reducing activity (10, 245). Flavodoxin was originally purified from A. vinelandii by Shethna in 1969 (201) and the amino acid sequence was determined from the purified protein (216). More recently, Veeger et al. identified three biochemically and immunologically distinct flavodoxin species (111). Although all are expressed constitutively,

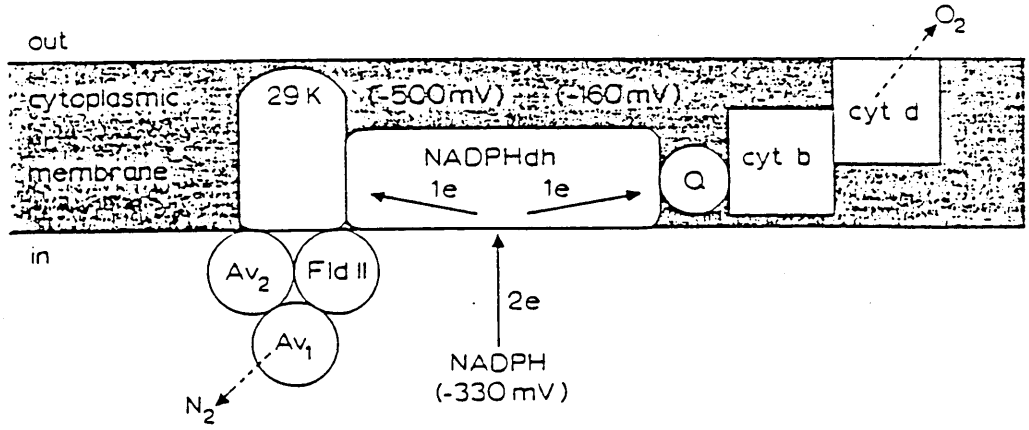

FIGURE 4. Model of the coupling of electron tranport to nitrogenase to the repiratory electron transport pathway in Azotobacter vinelandii. Av1, MoFe protein; Av2, Fe protein; Fld, flavodoxin; NADPHdh, NADPH dehydrogenase; cytb and cytd, cytochrome b and cytochrome d; 29K, 29 kD integral membrane protein. (taken from Hakker and Klugkist,1987)

the synthesis of one of these flavodoxins appears to be stimulated under nitrogen fixing conditions.

At least three genes within the A. vinelandii genome are proposed to encode ferredoxins, fdxA (144), fdxN (101), and the ferredoxin-like gene that is co-transcribed with the Fe protein of the V-nitrogenase (see **Alternative Nitrogen Fixing Systems**) (178), for which the physiological functions are unknown. Ferredoxin I was originally purified by Yoch et al. (245), and recently the structure has been examined by X-ray crystallography (212, 213). This ferredoxin is unusual in that its primary sequence is longer than other known ferredoxin, and it contains one [4Fe-4S] cluster and one [3Fe-4S] cluster. Ferredoxin I is encoded by the fdxA gene which has been cloned and sequenced (144). The gene is not contained within either of the nif gene clusters, is constitutively expressed and is not regulated by the presence of ammonia and is not essential for nitrogen fixation. Despite the ability of artificially reduced flavodoxin and ferredoxin to directly reduce nitrogenase, no physiological electron source has been identified which can support nitrogenase activity in the presence of oxidized flavodoxin and ferredoxin in A. vinelandii (8, 9, 10, 112, 201, 246). However, evidence has accumulated suggesting that the proton motive force may supply the energy necessary to transfer electrons from NADPH to flavodoxin, a thermodynamically unfavorable reaction, which can then reduce the Fe protein of nitrogenase (66, 110). An energized cytoplasmic membrane, particularly a high membrane potential, appears to be necessary for nitrogenase activity in A. vinelandii (68). A direct relationship between electron transport to nitrogenase and electron transport to oxygen through the respiratory chain has been proposed (66, 68, 110). Under nitrogen fixing

conditions two membrane-bound polypeptides are synthesized, a 29 kD and a 30 kD protein. An inner membrane-bound NADPH dehydrogenase activity is induced under derepressing conditions (110). These results, in addition to the characterization of an ammonia repressible flavodoxin, were used to formulate a model for electron transport to nitrogenase in aerobic nitrogen fixing organisms which is depicted in Fig. 4 (66, 67). However, there is no direct evidence to verify this model.

In several of the symbiotic microaerobic diazotrophic Rhizobium and Bradyrhizobium species another group of genes, fixA fixB and fixC, have been identified which may encode nitrogenase-specific electron transport components (51, 62, 63). The fixABC genes are essential for microaerobic nitrogen fixation in these organisms. They are not given a nif designation because the nucleotide sequence exhibits no identity with any of the identified nif genes of K. pneumoniae. The fixA, fixB, and fixC genes have been cloned and the nucleotide sequence determined in R. meliloti (51), cloned in B. japonicum (63), and cloned and partially sequenced in R. leguminosarum (62). In R. meliloti fixABC constitute a single operon which appears to be regulated by the product of nifA (51). In B. japonicum fixA is separated on the chromosme from fixB and fixC (63). Hybridization analysis using the fixABC genes of both R. meliloti (51) and B. japonicum (63) as a probe, identified similar sequences, not only in all tested species of Rhizobium and Bradyrhizobium, but also in A. vinelandii (51), A. chroococcum (51), and Azosprirrilum brasilense (53), and Azorhizobium caulinodans (107). In R. meliloti and B. japonicum another gene called fixX is located adjacent to fixC (51, 64). The suggestion that these genes may function in electron transport to nitrogenase is based on the strong amino acid sequence identity of the fixX

gene product with ferredoxin I from A. vinelandii (51, 64).

Transcriptional Regulation: nifA and nifL

K. pneumoniae has served as the model for studies on nif gene expression, and over the past decade significant progress has been made in understanding the details of the regulatory mechanisms involved. Comparisons of the nitrogen fixation regulatory systems operating in other diazotrophs including R. meliloti, B. japonicum, and A. vinelandii reveal many similarities, as well as a number of important differences, with the nif regulatory system in K. pneumoniae.

The nif genes are transcriptionally regulated in K. pneumoniae by the action of at least five proteins, NtrA (RpoN), NtrB, NtrC, NifA, and NifL, which function in a two-tiered cascade control mechanism (65). The first tier of regulation is mediated by the NtrA, NtrB and NtrC proteins which function to globally regulate transcription initiation from specific promoters, including pnifLA, in response to the cellular nitrogen status. The second tier involves the action of NifA and NifL in the transcriptional activation of the other nif genes in response to oxygen and nitrogen levels.

A consensus nif promoter sequence has been identified preceding nif transcriptional units in Rhizobium (222, 51), Bradyrhizobium (60, 106, 218), Azotobacter (96, 97, 12, 14, 21, 101), K. pneumoniae (15), Thiobacillus (166, 170), and Desulfovibrio (136) with the following sequence CTGG-N_8-TTGCA at the -24,-12 position relative to the transcription initiation site. This promoter sequence is specifically recognized by the sigma factor encoded by NtrA (σ54) (223, 243). In concert with core RNA polymerase, this sigma factor

directs transcription specifically from promoters with this sequence. The σ^{54} is distinct from σ^{70}, which is involved in transcription from the canonical E. coli -35, -10 promoter. The nucleotide sequence of ntrA was determined in K. pneumoniae, R. meliloti, and A. vinelandii and the predicted amino acid sequence is quite different from other known sigma factors (65, 223, 135, 137). NtrA is required for transcription of all genes under global nitrogen regulatory control, including the nif genes, and thus is required for diazotrophic growth.

In addition to NtrA, the polypeptides encoded by ntrB and ntrC, are also required for nif gene expression in K. pneumoniae (4, 243). NtrB and NtrC control transcription of genes involved in nitrogen metabolism and uptake and are proposed to function as a regulatory pair in which one protein acts as a sensory component and the other protein functions as a regulatory component (113). Other protein pairs with analogous regulatory schemes include E. coli PhoR/PhoB (phosphate limitation), EnvZ/OmpR (altered osmolarity), CpxA/SfrA (presence of F' cells), CheA/CheY + CheB (chemotaxis signalling and adaption), R. leguminosarum DctB/DctD (transport of C4-dicarboxylic acids), A. tumefaciens VirA/VirG (plant exudates), and R. meliloti FixL/FixJ (oxygen level) (1). All of the proteins in these pairs which function as the signaling component exhibit amino acid sequence identity in their C-terminal domains, while all of the proteins proposed to play the regulatory role have conserved N-terminal domains. According to the model, the N-terminal domain of the sensory component receives the environmental signal which causes a conformational change in the C-terminal domain of the protein. In this activated form, the sensory element interacts with the regulatory component at its N-terminal region, and

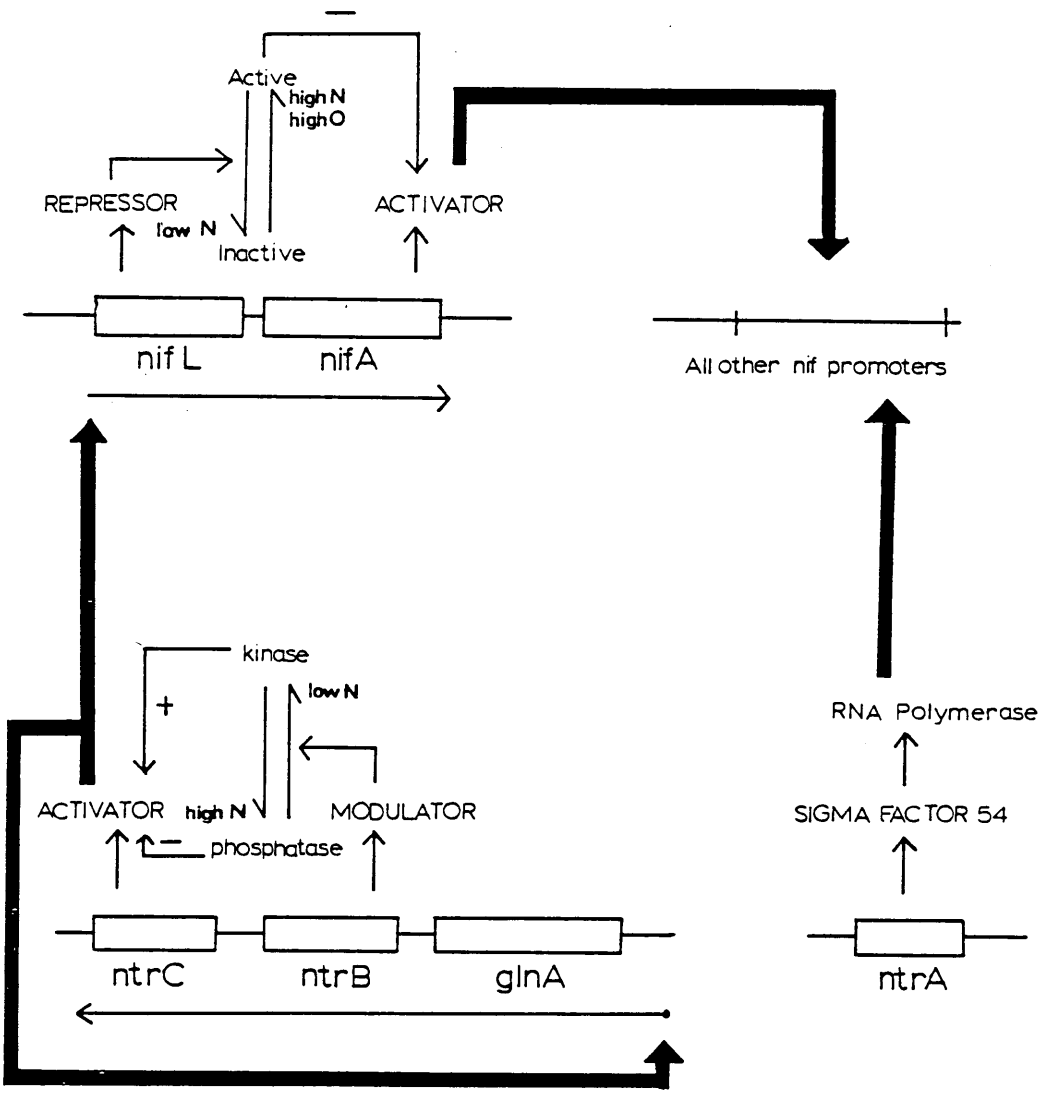

FIGURE 5. Model of the two-tiered cascade mechanism for the activation of nif gene expression in Klebsiella pneumoniae.

in this manner modulates the activity of the regulatory protein. The strongest support for this model comes from studies on the interaction between NtrB and NtrC in which NtrB regulates the activity of NtrC by covalent modification (127). NtrB phosphorylates NtrC in response to low cellular nitrogen levels (recognized as a high glutamate/glutamine ratio) and dephosphorylates NtrC in response to high nitrogen concentrations. In the phosphorylated form NtrC functions as an activator of transcription from NtrA-dependent promoters by cooperatively binding to specific sites near the promoter. The dephosphorylated NtrC is a much less efficient DNA binding protein and thus does not function as a transcriptional activator in this form. Several lines of evidence support NtrC mediated transcription activation from pnifLA. First, in K. pneumoniae both NtrB and NtrC are required for nif gene expression (4, 243). Two low affinity NtrC binding sites precede the nifLA promoter in K. pneumoniae (48, 44). Finally, purified NtrA, NtrB, NtrC and RNA polymerase from K. pneumoniae are all necessary for the in vitro transcription of nifLA (4). In E. coli, NtrC may mediate its activator function by catalyzing the formation of open complexes (44). In K. pneumoniae expression from pnifLA was reduced by aerobic growth and by addition of DNA gyrase inhibitor (45). These results suggest that expression of these genes may be a function of the DNA topology in this organism.

The deduced amino acid sequences of NifA and NifL exhibit significant identity to NtrC and NtrB, respectively, suggesting that NifA and NifL may function in an analogous manner to regulate expression of the nif genes (47, 48, 29). After expression of nifLA has been activated by the action of NtrC and NtrA, NifA acts as a positive activator of transcription

from the other nif-specific promoters in an NtrA-dependent manner (143, 159). In K. pneumoniae, NifL modulates the activity of NifA in response to the intracellular nitrogen and oxygen concentrations (3, 48). Although NifA and NifL can be overproduced in E. coli, purification of these proteins has proved unsuccessful, thus complicating the elucidation of how these regulators mediate their functions. The nucleotide sequences of nifA and nifL has been determined in K. pneumoniae and the predicted amino acid sequence has been compared to the amino acid sequences of other proteins to reveal several potential functional domains in each gene product (47, 48, 29). Within the N-terminal region of NifL is a sequence resembling the heme binding sites of other heme-containing proteins (48). However, heme biosynthesis is not required for oxygen regulation of the nif genes so the importance of a potential heme cofactor in nif regulation is questionable. NifL exhibits limited identity with the C-terminal end of NtrB suggesting NifL may physically interact with NifA in a manner analogous to NtrB and NtrC. However, deletion analysis of nifL demonstrated that in contrast to NtrB, NifL is required only for the inactivation of NifA in response to nitrogen and oxygen status (3).

Comparison of the predicted amino acid sequences of NifA and NtrC reveal extensive identity in all but the N-terminal domains. Both proteins have a conserved central domain containing a potential ATP binding site and a conserved C-terminal domain containing a helix-turn-helix motif characteristic of DNA binding regions in other known DNA binding proteins (29, 47). The importance of these conserved regions in functionality of both NifA and NtrC have been demonstrated using site-directed nucleotide and chemical mutagenesis approaches (143). All the nif transcriptional units are preceded by a consensus sequence,

TGT-N_{10}-ACA, between position -100 and -150 relative to the transcription start site (26). This sequence is proposed to function as an upstream activator sequence where NifA associates to enhance transcription from the consensus nif promoter (143). A DNA looping model in which NifA binds at the upstream activator sequence and then associates with sigma factor 54-core RNA polymerase at the -24,-12 region by a looping out of the DNA has been proposed to explain NifA mediated activation (181).

In A. vinelandii, nif gene regulation appears to be controlled by a two tiered cascade mechanism analogous to the system operating in K. pneumoniae. The ntrA, ntrB and ntrC genes have been isolated and mutagenized from A. vinelandii (136, 137, 223, 65). Mutations in any of these genes can be complemented by the addition of purified NtrA, NtrB or NtrC isolated from K. pneumoniae. Furthermore, expression of K. pneumoniae nifA in A. vinelandii activates expression of the nif structural proteins and supports nitrogenase activity (108). All identified nif transcriptional units are preceded by the consensus nif NtrA-dependent promoter at the -24,-12 position and by the consensus upstream activator sequence present in K. pneumoniae (2). These data suggest that in A. vinelandii NifA and NtrA may function together to initiate transcription from the nif promoters as occurs in K. pneumoniae. However, although NtrA is essential for nif gene expression, NtrB and NtrC are not required for nitrogen fixation suggesting that nif gene regulation is not coupled to the global nitrogen regulatory system (65, 223). Also, in contrast to K. pneumoniae, a NifL-like protein has not been identified by sequence or hybridization analyses. These features of A. vinelandii nif regulation more closely resemble the regulatory system operating in R. meliloti and B. japonicum nif gene expression. In R. meliloti transcription

of nifA is activated by FixJ, a recently identified gene which is proposed to function in a manner analogous to ntrC and nifA (79). NifA is necessary for expression of all nif operons except the operon containing nifA, but the upstream activator sequence appears to be non-essential for NifA mediated positive control of transcription (29, 65, 79). In these organisms the upstream activator sequence may be functioning as a transcriptional enhancer. In A. vinelandii a unique regulatory gene was identified by Tn5 mutagenesis, nfrX, which does not hybridize with the chromosomal DNAs of K. pneumoniae, R. meliloti, or B. japonicum (185). This gene appears to be essential for nitrogen fixation and may function to regulate the expression of nifA in A. vinelandii.

The nifA genes from R. meliloti and B. japonicum have been cloned and their nucleotide sequences determined (16, 29, 59). Comparison of the deduced amino acid sequences from these nifA genes and that of K. pneumoniae reveals the same conserved central and C-terminal regions as identified in K. pneumoniae nifA . However such comparisons also reveal the presence of an interlinker domain between the nonconserved N-terminal domain and the conserved central domain. Four conserved cys residues (two in the interdomain linker region and two in the central domain) are essential for NifA function and may specify a metal binding site (59). Binding of a metal cofactor is suggested to play a role in oxygen sensing. Deletion of the N-terminal domain of NifA from R. meliloti and B. japonicum, resulted in wild type levels of nif-gene expression, demonstrating the nonessential nature of this region for NifA mediated positive control of nif-gene expression (16, 59).

Alternative Nitrogen Fixing Systems

Genetic and biochemical studies in Azotobacter are complicated by the presence of the alternative nitrogen fixing systems which were discovered in 1980 by Bishop et al (18). In the absence of Mo and the presence of vanadium (V), A. chroococcum and A. vinelandii express a V-dependent nitrogen fixing system (nitrogenase-2, V-nitrogenase) (50, 72). A. vinelandii accumulates a third nitrogen fixing system (nitrogenase-3 or Fe-nitrogenase) in the absence of both Mo and V (35). The V-nitrogenase has been isolated and characterized from A. chroococcum and A. vinelandii nifHDK deletion mutation strains (50, 72). This enzyme is composed of two components, a dinitrogenase reductase (analogous to the Fe protein) and a dinitrogenase (analogous to the MoFe protein). The dinitrogenase reductase is a homodimer containing 4 Fe and 4 acid-labile S^{2-} atoms; the dinitrogenase is a tetrameric (M_r = 220,000) protein composed of two non-identical subunits of M_r = 50,000 and M_r = 55,000. Two V, 23 Fe and 20 acid-labile S^{2-} atoms are contained within the dinitrogenase component. An FeVa-cofactor species can be isolated from this component using acid/base treatment followed by extraction into N-methylformamide (204). A 13 kD polypeptide co-purifies with the dinitrogenase (177). Preliminary results suggest that this polypeptide is necessary for optimal nitrogenase activity by the V-dependent nitrogen fixing system. A large percentage of the electron flux through the V-nitrogenase is shuttled towards the reduction of protons. Acetylene is a poor substrate, but it is reduced not only to ethylene but also to ethane (50). Because ethane is not a product of acetylene reduction by the Mo-nitrogenase, this reaction is used to distinguish between the alternative and the conventional nitrogen fixing systems in vivo (102).

The genes encoding the structural components of the V-nitrogenase have been isolated in A. chroococcum, and the gene encoding the Fe protein of the V-nitrogenase has been isolated in A. vinelandii (169, 178). The dinitrogenase reductase is encoded by vnfH; the subunits of the dinitrogenase are encoded by vnfD and vnfK; and the 13 kD polypeptide may be encoded by vnfG. The vnfH gene is separated from vnfDGK, which comprise a single transcriptional unit. The vnfH gene appears to be co-transcribed with a ferredoxin-like gene which immediately follows vnfH (178).

In A. vinelandii, a third nitrogen fixing system (Fe-nitrogenase) has been identified which functions under conditions of Mo and V deficiency (35). This alternative nitrogenase has been purified and is composed of a dinitrogenase reductase and a dinitrogenase component similar in composition to the Mo- and V- nitrogenase components. The dinitrogenase component appears to contain only Fe and S^{2-} suggesting that in this nitrogenase complex, Fe may be functioning in a similar manner as Mo (in the conventional nitrogenase) and V (in the V-nitrogenase). Two active configurations of the dinitrogenase component of the Fe-nitrogenase can be isolated, an $\alpha_2\beta_2$ and an $\alpha_1\beta_2$ configuration. Protons are reduced with the greatest efficiency and at least 50% of the electrons are shuttled towards H_2 evolution. The complete nucleotide sequence of the structural genes for the Fe-nitrogenase was determined (104). The dinitrogenase reductase (anfH) and the dinitrogenase subunit (anfD and anfK) structural genes are contained in a single transcriptional unit. The anfD and anfK genes are separated by the anfG gene, which appears to encode a 13 kD protein similar to the 13 kD protein which co-purifies with the V-nitrogenase dinitrogenase component.

Jacobson et al. demonstrated that Mo regulated nitrogen fixation in A. vinelandii at the level of transcription, but the mechanism of this regulation is unknown (98). In contrast to the conventional nitrogenase, transcriptional activation of the alternative nitrogenase structural genes does not appear to be solely dependent on NifA. NifA is not required for the expression of the V-nitrogenase, but it may be involved in the expression of the Fe-nitrogenase (185). Two additional nifA-like genes, anfA and vnfA, were identified in A. vinelandii which appear to be required for the transcriptional activation of the Fe-nitrogenase and the V-nitrogenase genes, respectively (103). The conventional and the alternative nitrogen fixing systems appear to require several of the same gene products, including NtrA (sigma factor 54), NifB and NifM (102). Preliminary results suggest that several other gene products appear to be necessary for maximal diazotrophic growth by both the conventional and the alternative nitrogen fixation systems including nifU, nifS and nifV (Jacobson, 1989,unpublished).

RESEARCH GOALS

Biological nitrogen fixation is a biochemically and genetically complex process which is only partially understood. Besides the nitrogenase structural components, many other polypeptides encoded by nif-specific genes are necessary for the synthesis and accumulation of a competent nitrogenase complex. Some of the processes which these nif-specific polypeptides mediate include electron transport to nitrogenase, maturation of the nitrogenase structural proteins and the regulation of expression of nif-specific genes. In Azotobacter vinelandii the mechanism of electron transport to nitrogenase is unknown. In Klebsiella pneumoniae and Clostridium pasteurianum either a flavodoxin or a ferredoxin, respectively, function as low-potential electron donors to the Fe protein. In K. pneumoniae a nif-specific gene encodes the flavodoxin which functions as the unique electron donor to nitrogenase. Previous experiments using A. vinelandii suggested that a flavodoxin or a ferredoxin or both may provide electrons for nitrogenase. This study sought to determine whether A. vinelandii possessed a mechanism of electron transport analogous to the system operating in K. pneumoniae.

Expression of nif polypeptides is known to be transcriptionally activated under nitrogen-deficient conditions. In several diazotrophic organisms including K. pneumoniae a nif-specific activator protein regulates nif gene expression. A focus of this study was to isolate and genetically characterize the nif regulatory protein from A. vinelandii in order to provide a better understanding of nif gene regulation in this organism.

The MoFe protein is one of the structural components of nitrogenase. It is a metalloprotein containing two distinct types of metal clusters, P clusters and the FeMo-cofactors. The P clusters are Fe-S clusters of unknown structure and function. The FeMo-cofactor is a unique Mo-containing prosthetic group of unknown structure which has been implicated as the site on the nitrogenase complex where substrate reduction occurs. Evidence has accumulated to suggest that the FeMo-cofactor is synthesized on a protein complex composed of the nifE and nifN gene products. The NifEN complex appears to be structurally similar to the MoFe protein. Extensive research efforts have been applied towards understanding metallocluster binding in the MoFe protein of nitrogenase. The aim of this study was to provide additional information about the amino acid residues having potential ligating function to the FeMo-cofactor in both the catalytically active MoFe protein and the FeMo-cofactor biosynthetic protein complex.

MATERIALS AND METHODS

Growth of A. vinelandii strains

The wild type and mutant strains of A. vinelandii were grown on a modified Burk's medium (214) at 30°C. When a fixed nitrogen source was required, either urea or ammonium acetate was added to a final concentration of 10 or 29 mM, respectively. When required 0.5 μg/ml kanamycin, 5.0 μg/ml rifampicin and 20 μg/ml ampicillin were added to the medium.

Transformation of A. vinelandii

Transformations of A. vinelandii wild type cells and mutant strain cells with hybrid plasmids or M13 replicative form (RF) DNA were performed as described by Page and von Tigerstrom (160). The details of these experiments are described in the **Results** sections. When necessary cultures were outgrown at 30°C for approximately 18 hours and then plated on the appropriate medium. The recombinant plasmid DNA was recombined into the chromosome by a homologous recombination event.

Derepression of Nitrogenase and Whole Cell Acetylene Reducing Assay.

Cultures grown in a modified Burk's medium containing a fixed nitrogen source were harvested at mid- to late-log phase by centrifugation at 10,000 x g for 10 minutes. The cell pellet was washed once and immediately resuspended to the original volume in Burk's nitrogen-free medium and shaken vigorously for the required time. Whole cell acetylene

reduction assays were performed on cell cultures derepressed for three hours. One ml samples were added to stoppered 5.0 ml serum vials. After addition of 500 μl acetylene, the vials were shaken at 300 rpm at 30 °C for 20 minutes. The assays were terminated by addition of 100 μl 30% TCA. Ethylene production was measured on a Perkin-Elmer Sigma 2B gas chromatograph by injecting 50 μl of the gas phase onto a Porapak R column equipped with a flame ionization detector and a Varian 4290 integrator. The column was run at 82 °C with argon as the carrier gas.

Escherichia coli Growth, Phage DNA Preparation and DNA Fragment Purification, and DNA Sequencing.

The E. coli strain 71-18 ((lac-proAB), thi, supE, [F', proAB, lacI^{q}Z, M15]) which served as host for all plasmids and M13 phage used in this study was grown and maintained as described by Messing (138). Strain JM105 [thi, rpsL, endA, sbcB15 hsdR4 (lac proAB)F' traD36 proAB lacI^{q}Z M15] served as the host for the vector used to overproduce flavodoxin. When necessary the medium was supplemented with 100 μg/ml of ampicillin or 50 μg/ml of kanamycin. Recombinant M13 viruses and DNA were prepared as described in detail by Messing (138). DNA fragments used for cloning or as probes for Southern and northern hybridizations were purified by fractionation on agarose gels followed by isolation using the Elutrap (Schleicher & Schuell). All sequencing reactions were performed using the dideoxy sequencing method of Sanger et al. (184).

Plasmid Constructions

The plasmid pDB93 was constructed by cloning a 2.0 kilobase nifF-containing XhoI restriction fragment into the SalI restriction site of the plasmid pUC7 (138). This SalI-XhoI ligation results in the loss of the vector SalI site, as well as the terminal XhoI site of the insert DNA. Restriction endonuclease mapping of pDB93 and DNA sequence analysis revealed that three HincII restriction sites (one of which is also a SalI site) contained within the nifF sequence. The plasmid pDB94 was constructed by digesting pDB93 with the restriction endonuclease HincII and religating, resulting in the generation of a new SalI restriction site (see Fig.11) and a 318 base pair deletion of the HincII fragments contained entirely within the nifF gene. The plasmid pDB99 was constructed by ligation of the 1.3 kilobase SalI restriction enzyme fragment from pUC4-KAPA (Pharmacia), which encodes the kanamycin resistance cartridge from Tn903 (157), into the SalI site in pDB93. The plasmid pDB100 was similarly constructed by the insertion of the same kanamycin resistance cartridge into the SalI site of pDB94. Plasmid pDB89 was constructed by cloning the 2.0 kilobase nifF containing XhoI restriction enzyme fragment into the XhoI site of the broad host range vector pKT230 (5). A nifF-lacZ fusion plasmid, pDB154, was constructed by ligaton of a 6.2 kilobase lacZYA-containing SalI fragment from the plasmid pSKS104 (186) into the unique SalI site located within the nifF gene contained on pDB89.

The plasmid pDB150 was constructed by cloning a 1.5 kilobase SalI restriction enzyme nifA-containing fragment into the SalI site in pUC7. The plasmid pDB160 was constructed in a similar manner by ligating a 1.4 kilobase nifA and nifB-containing SalI restriction

fragment into pUC7, and pDB144 was made by inserting a 5.0 kilobase EcoRI fragment containing nifQ into the pUC7 EcoRI restriction site. The complete nifA coding sequence and flanking regions are contained on the hybrid plasmid pDB330 which was constructed by ligating the SalI restriction enzyme fragments from pDB150 and pDB160 in the presence of pUC7 digested with SalI. Recombinants were subsequently screened for the orientation which generated a complete nifA gene by restriction enzyme analysis. For construction of the nifA-specific deletion plasmid pDB155, the parental plasmid pDB150 was digested with the restriction enzyme PstI and then religated resulting in the deletion of 700 base pairs of nifA-coding sequence. The plasmid pDB167 was constructed by inserting the 1.3 kilobase kanamycin resistance-encoding cartridge from pUC4-KAPA (Pharmacia) into the PstI site in pDB155. Restriction digestion of pDB160 with SphI restriction enzyme resulted in the deletion of 700 base pairs of nifB-specific coding DNA; this was replaced by the 1.3 kanamycin resistance encoding cartridge from pUC4-KAPA to generate the nifB deletion and insertion recombinant plasmid pDB218. The plasmid pDB291 contains the 1.3 kilobase kanamycin resistance-encoding cartridge inserted into the unique SstI restriction enzyme site within pDB144 to produce a nifQ-Kan^r hybrid plasmid.

A. vinelandii Strain Construction

Attempts to detect mutant strains that had recombined the nifF-specific deletion contained on plasmid pDB94 into the A. vinelandii OP chromosome during transformation were not successful (see nifF **Discussion** section). A specific nif deletion within the nifA gene contained on the hybrid plasmid pDB155 was transferred to the A. vinelandii

chromosome by using congression. Congression of the nifA deletion was accomplished by adding equal amounts (1.0 μg each) of either genomic Rif^r DNA or pDB303, which contains the A. vinelandii rifampicin resistance determinant cloned into pUC7, and the hybrid deletion plasmid preparation to competent A. vinelandii cells. After allowing time for phenotypic lag (approximately 18 hours), the transformed cultures were spread on Burk's ammonium acetate-supplemented medium plates containing 5.0 μg of rifampicin per ml. Rif^r transformants were scored on Burk's nitrogen-free and Burk's ammonium acetate-supplemented medium plates to identify Nif$^-$ co-transformants. Transformation of competent A. vinelandii OP cells with hybrid plasmids results in two different recombination events. A single crossover event results in the incorporation of the entire hybrid plasmid into the host chromosome and the consequent interruption and duplication of the A. vinelandii nif region carried on the plasmid. Double reciprocal crossover events result in the transfer of the nif deletion contained within the plasmid to the homologous region on the host chromosome with the subsequent loss of plasmid vector. These different recombination events were easily distinguishable. Single crossover recombinants were endowed with the antibiotic resistance phenotype carried on the plasmid vector. The strains were also unstable reverting to the Nif$^+$ and antibiotic sensitive phenotype at a high frequency. Double crossover events carried the non-revertible Nif$^-$ phenotype and were used for further experiments.

The kanamycin resistance determinant carried on the nifF-hybrid plasmids pDB99 and pDB100, the nifA recombinant plasmid pDB167, the nifE deletion and insertion containing plasmid pDB259, the nifB hybrid plasmid pDB218 and the nifQ hybrid plasmid pDB291 was

recombined into the A. vinelandii OP chromosome by double reciprocal recombination events as described above. Transformed cells were outgrown for 18 hours to account for phenotypic lag. Following outgrowth, cells that had recombined the kanamycin resistance determinant into the chromosome were recovered by plating them on Burk's medium containing 29 mM ammonium acetate and 0.5 μg/ml kanamycin. DNAs prepared from recombinant plasmids pDB99, pDB100, pDB167, pDB259, pDB218, and pDB291 were used for the construction of strains DJ58, DJ60, DJ143, DJ228, DJ463, and DJ464, respectively. Strain DJ130 was isolated by transforming competent A. vinelandii OP cells with the nifF-lacZ fusion plasmid pDB154, and transformants containing the flavodoxin-β-galactosidase fusion protein were identified as blue colonies on Burk's medium supplemented with the β-galactosidase indicator, 5-bromo-4-chloro-3-indole β-D-galactoside.

Southern Blot Analysis.

Total genomic DNA was isolated from A. vinelandii using the procedure of Saito and Miura (183), digested with the appropriated restriction enzymes and fractionated by electrophoresis on a 0.6% agarose gel. The DNA was electrophoretically transferred to Genesreen™ hybridization transfer membrane (DuPont-New England Nuclear) following the procedure suggested by the manufacturer. Hybridizations were performed as described by the suppliers of GeneScreen™ in the presence of 50% formamide at 42°C. DNA fragments used as probes were labeled with [α-^{32}P] dCTP using the nick translation procedure of Maniatis et al. (128).

TABLE 3. A. vinelandii mutant strains constructed or used in this study.

Strain	Mutation[a]	nif gene(s) disrupted	DNA used[c]	Parental strain	Nif Phenotype[b]
DJ58	::	F	pDB99	WT	Nif+
DJ60	▲::	F	pDB100	WT	Nif+
DJ130	Z::	F-lacZ::	pDB154	WT	Nif+
DJ138	Z::,::	F-lacZ::-fdxA::	pDB217	DJ130	Nif+
DJ73	::	fdxA::	pDB217	WT	Nif+
DJ250		ORF12	pDB68	DJ249	Nif+
DJ140	▲	A	pDB155	WT	Nif-
DJ143	▲::	A	pDB167	WT	Nif-
DJ141	▲	B	pDB161	WT	Nif-
DJ315	::	Q	pDB291	WT	Nif+/-
DJ228	▲	E	pDB259	WT	Nif-
DJ448	sd	E	E-Mn1-RF	DJ228	Nif+
DJ449	sd	E	E-Mn2-RF	DJ228	Nif+
DJ450	sd	E	E-Mn3-RF	DJ228	Nif+
DJ451	sd	E	E-Mn1,2-RF	DJ228	Nif+
DJ452	sd	E	E-Mn1,3-RF	DJ228	Nif+
DJ453	sd	E	E-Mn2,3-RF	DJ228	Nif+
DJ454	sd	E	E-Mn1,2,3-RF	DJ228	Nif+
DJ463	sd,▲::	E:sd,▲B::	pDB218	DJ454	Nif-
DJ464	sd::	E:sd,Q::	pDB291	DJ454	Nif+/-
DJ466	sd,▲::	E:sd,▲B::	pDB218	DJ465	Nif-
DJ467	sd::	E:sd,Q::	pDB291	DJ465	Nif+/-

[a] ▲ indicates a deletion, :: denotes a kanamycin resistance-encoding cartridge insertion: z denotes the insertion of an in-frame translational fusion of the lacZ gene from E. coli; sd indicates an oligonucleotide-directed mutation.

[b] + indicates that the mutant strain was capable of normal diazotrophic growth, - indicates that the mutant strain was incapable of diazotrophic growth and +/- indicates that the mutant strain was capable of only very slow diazotrophic growth.

[c] pDBX refers to hybrid plasmid DNA. RF refers to the replicative form of the recombinant bacteriphage M13. E-Mnx-RF refers to the particular RF used in the respective strain construction. Mn indicates mutene; E indicates that nifE-coding sequence contains the mutation. A list of the mutenes (Mn) used for the site-directed mutagenesis of nifE is given in Table 4.

Northern Blot Analysis.

Total RNA was isolated as described by Krol et al. (115). Approximately 10-μg portions of RNA samples were glyoxylated and electrophoresed on a 1.1% agarose gel as described by McMaster and Carmichael (135). Electrophoretic transfer of RNA to GeneScreen™ and hybridization, in the presence of 50% formamide at 42 ° C, were performed as described by the manufacturer.

β-Galactosidase Activity Assays.

β-Galactosidase specific activities were determined as described by Miller (140) in both nitrogenase repressed and derepressed cultures of A. vinelandii OP. Cultures were grown in 100 ml of Burk's medium supplemented with 10 mM urea to a cell density of 60 klett. Cultures were derepressed for nitrogenase for three hours. One tenth ml of the culture was added to 0.9 ml of Z buffer (140) and cells were permeabilized by the addition of 50 μl of toluene and vortexing. The reaction was started by the addition of 0.2 ml of a 4 mg/ml solution of p-nitrophenyl-D-galactosidase. The reaction was terminated by the addition of 500 μl of 1.0 M sodium carbonate. The β-galactosidase activity was measured as the rate of cleavage of the chromatogenic substrate according to the absorbance reading at A_{420}. The units of activity used were calculated as described by Miller (140) and used the equation $U = OD_{420}/OD_{600}$ (t) (1000) where t = time of reaction in minutes.

Ouchterlony Double Diffusion Assay.

Anti-flavodoxin serum and purified flavodoxin OP were supplied by Dale Edmondson (Emory University). Approximately 50 μl of samples were added to an Ouchterlony plate (0.8% agarose in 0.1 M potassium phosphate buffer, pH 7.5) wells. Crude extracts were prepared using the French pressure cell in the presence of DNase and RNase. A. vinelandii wild type and strain DJ60 crude extracts (approximately 1.0 mg of protein), purified flavodoxin (10 μg) and purified MoFe protein (10 μg) from A. vinelandii OP were added to the appropriate wells and allowed to diffuse towards anti-MoFe sera or antiflavodoxin sera added to the central wells.

Oligonucleotide-Directed Mutagenesis.

Oligonucleotides (primers and mutenes) were synthesized on Beckman System 1 Plus or an Applied Biosystems 381A DNA synthesizer and purified by HPLC (95). Oligonucleotide-directed mutagenesis was performed using a modification of the procedure of Zoller and Smith (249) followed by a methylation step (87). The desired mutations were screened and confirmed using dideoxy sequencing. Replicative form DNAs were prepared from each mutant phage and used to transform competent A. vinelandii cells. A list of the mutagenic primers (mutenes) and sequencing primers are shown in Table 4.

Two-dimensional Gel Electrophoresis.

Preparation of extracts for two-dimensional electrophoresis were prepared as described by Bishop et al. (17). Two dimensional analysis was performed as described by 0'Farrell (156).

TABLE 4. Mutagenic primers (mutenes) and sequencing primer used in the construction of the nifE site-directed mutation strains.

	Mutene	Alteration
E-Mn-1	CCG TCG ACT GCG CCG GCT	153C→153S
E-Mn-2	ACG GCA CCC AGA ACC TCG	160K→160Q
E-Mn-3	ACC TCG GCC ACC GCA TCG	164N→164H

	Primer
EP10	ACG CAG TGT GCA AAG CC

ISOLATION, NUCLEOTIDE SEQUENCING, AND MUTAGENESIS OF THE NIFF GENE ENCODING A FLAVODOXIN AND DOWNSTREAM FLANKING REGION IN AZOTOBACTER VINELANDII

RESULTS

Introduction.

In the facultative anaerobe K. pneumoniae, 20 nif-specific genes have been identified (2, 32, 191, 214, 94, 83, 13, 195, 14, 161, 46, 48, 29, 28, 109). These genes are clustered on the chromosome and arranged into eight transcriptional units (Fig. 2). A. vinelandii possesses 18 of these nif-specific genes which exhibit structural and/or functional identity with the nif genes of K. pneumoniae (96, 11, 12, 14, 20, 21, 23, 39, 101). Both organisms share the same sequential arrangement with the nif genes. However, significant spatial differences exist between the nif gene clusters of these organisms. The most striking difference is the division of the A. vinelandii nif genes into two unlinked gene clusters (Fig. 2). The complete nucleotide sequence of the major nif gene cluster (encoding nifHDKTY nifENX nifUSVWZM nifF) and the minor gene cluster (nifABQ) in A. vinelandii has been determined. This section describes the restriction enzyme mapping, the nucleotide sequence analysis and the mutagenesis of the region including and downstream of the nifF gene in A. vinelandii. Analysis of this region resulted in the isolation of a flavodoxin encoding sequence and a loci which when deleted results in a strain requiring acetate for growth.

Isolation and DNA Sequence of the nifF Gene.

At the time of this study, only 17 nif-specific genes were identified in K. pneumoniae and 11 nif-specific genes were identified in A. vinelandii. Because of the conservation of nif gene organization between the two organisms, a nifF-like sequence was searched for in the region downstream of the proposed nifM gene. Random DNA sequence analysis of this region revealed a flavodoxin encoding sequence. Restriction enzyme mapping experiments demonstrated that the flavodoxin encoding gene was located approximately 1.6 kb downstream of the nifM gene and that it was located entirely within a 2.0 kb XhoI restriction enzyme fragment (Fig. 6). The complete nucleotide sequence of the flavodoxin gene was completed using the strategy depicted in Fig. 7. The DNA sequence and the restriction enzyme mapping showed that this gene was transcribed in the same direction relative to the other identified nif genes of A. vinelandii OP. In K. pneumoniae, the nifF gene is transcribed in an opposite direction when compared with the other nif genes. The flavodoxin peptide sequence deduced from the nucleotide sequence was compared with the peptide sequence previously determined from the purified protein (216). These sequences are in agreement with the exception that the DNA sequence indicated a glutamate residue at position 157 rather than the glutamine residue reported for the determined protein sequence. From this comparison the conclusion was reached that the flavodoxin gene isolated here encodes the previously isolated and characterized flavodoxin species. A potential nif promoter sequence and a nif promoter activator sequence precede the flavodoxin coding sequence (15,26). A potential rho-independent transcription termination

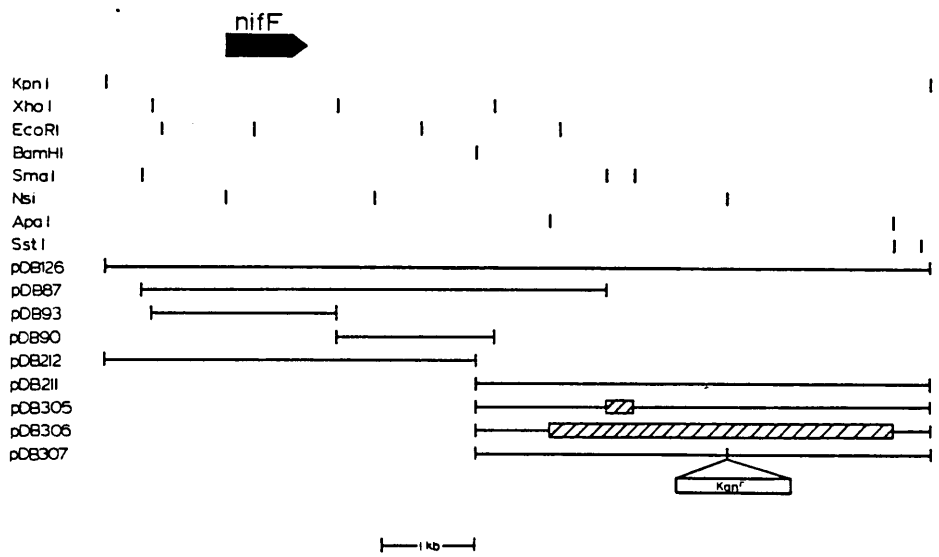

FIGURE 6. Restriction map of the nifF gene and the downstream flanking region. The region shown here includes approximately 9.0 kilobases of A. vinelandii genomic DNA. The direction of transcription is from left to right. The entire nifF coding region is contained on the 2.0 kilobase XhoI restriction enzyme fragment. The hybrid plasmid pDB93 containing this XhoI fragment was used to determine the nucleotide sequence nifF. The position of specific restriction enzyme sites are indicated by the vertical bars. The cloned regions within the hybrid plasmids are indicated as horizontal bars. Deletions of A. vinelandii sequences are indicated by cross hatched areas, and kanamycin resistance-encoding cartridge insertions are indicated by an arrowhead.

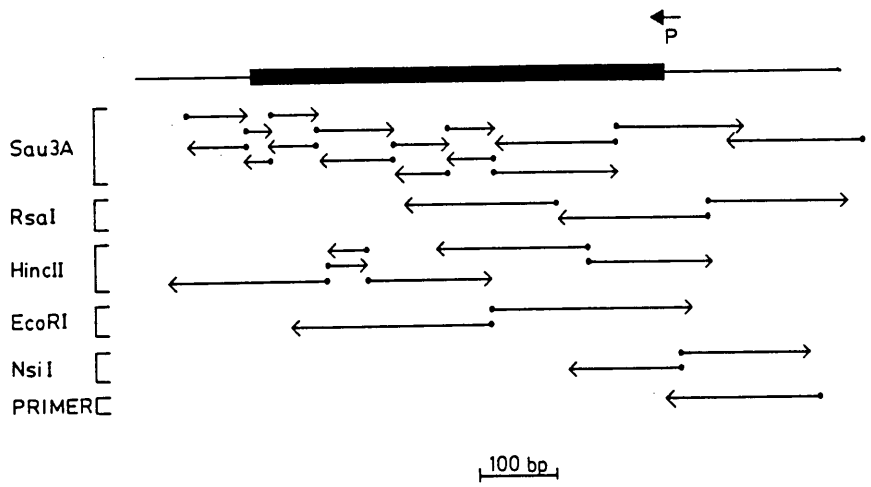

FIGURE 7. Strategy used in sequencing the nifF gene. The nifF gene coding region is indicated by a solid box and in the 3' to 5' direction. The direction of nifF transcription is indicated by the arrow above the solid box. The direction and extent of individual DNA sequencing experiments are indicated by horizontal arrows. Restriction enzyme sites used for subcloning experiments are indicated on the left.

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GTCGTAAAGAACTGTTGTAAAAACGACAAGGCCTGCCTGC

CCGCCCCATTCTTATCGTCGCATTTGCGACAGGATTTTTT

ACTGCCATAAAAAAATGCTTTTAAATCAATAAGTTGGTAAA

TGTGTTCGGGGTTTGGGGAGTGGTCTGCTTCTTGCTGTTA

CTCACCGGCCAGCCAGGTGTACAAGCCCGGAGACTGGCGC

TCCGACCACGAATCTCATGCATGCAGCCAGAGGTTAAGTT
                5                  10                  15                  20
ATG GCC AAG ATT GGA CTC TTC TTC GGT AGC AAC ACC GGT AAA ACC CGC AAG GTC GCC AAG
MET ALA LYS ILE GLY LEU PHE PHE GLY SER ASN THR GLY LYS THR ARG LYS VAL ALA LYS
                25                  30                  35                  40
TCG ATC AAG AAG CGT TTC GAC GAC GAA ACC ATG TCC GAT GCG CTG AAC GTC AAC CGT GTT
SER ILE LYS LYS ARG PHE ASP ASP GLU THR MET SER ASP ALA LEU ASN VAL ASN ARG VAL
                45                  50                  55                  60
TCC GCG GAA GAC TTC GCC CAG TAC CAG TTC CTG ATT CTG GGT ACT CCG ACC CTT GGC GAA
SER ALA GLU ASP PHE ALA GLN TYR GLN PHE LEU ILE LEU GLY THR PRO THR LEU GLY GLU
                65                  70                  75                  80
GGC GAA CTC CCC GGT CTG TCC TCC GAC TGC GAA AAC GAA AGC TGG GAA GAA TTC CTG CCG
GLY GLU LEU PRO GLY LEU SER SER ASP CYS GLU ASN GLU SER TRP GLU GLU PHE LEU PRO
                85                  90                  95                  100
AAG ATC GAG GGC CTG GAT TTC AGC GGC AAG ACC GTG GCG CTG TTC GGC CTG GGC GAT CAG
LYS ILE GLU GLY LEU ASP PHE SER GLY LYS THR VAL ALA LEU PHE GLY LEU GLY ASP GLN
                105                 110                 115                 120
GTT GGC TAT CCC GAG AAT TAC CTG GAT GCC CTG GGC GAA CTG TAT TCC TTC TTC AAG GAC
VAL GLY TYR PRO GLU ASN TYR LEU ASP ALA LEU GLY GLU LEU TYR SER PHE PHE LYS ASP
                125                 130                 135                 140
CGT GGC GCC AAG ATC GTA GGC TCC TGG TCG ACC GAC GGC TAC GAG TTC GAA AGC TCC GAG
ARG GLY ALA LYS ILE VAL GLY SER TRP SER THR ASP GLY TYR GLU PHE GLU SER SER GLU
                145                 150                 155                 160
GCC GTG GTT GAC GGC AAG TTC GTC GGC CTG GCG CTG GAT CTG GAC AAT CAG AGC GGC AAG
ALA VAL VAL ASP GLY LYS PHE VAL GLY LEU ALA LEU ASP LEU ASP ASN GLN SER GLY LYS
                165                 170                 175                 180
ACC GAC GAG CGC GTT GCT GCC TGG CTG GCA CAG ATC GCT CCC GAG TTC GGG CTG TCC CTG
THR ASP GLU ARG VAL ALA ALA TRP LEU ALA GLN ILE ALA PRO GLU PHE GLY LEU SER LEU

TAA AAGTCGATCCCCGGTCATGCGGTCTCCGTGCATGGCCG
END

GGTGACTTGGCATGTTTTCTTTTCGTTTCTCTGCGGATC
```

FIGURE 8. Nucleotide sequence and the predicted amino acid sequence of the nifF gene from A. vinelandii. The complete DNA sequence deduced from the DNA sequence is indicated below the nucleotide sequence. A consensus nif promoter sequence is boxed in the figure and a potential nif activator sequence preceding the promoter is underlined. A region of dyad symmetry located immediately distal to the nifF coding sequence is indicated by converging arrows. A pyrimidine-rich region immediately following the region of dyad symmetry is underlined.

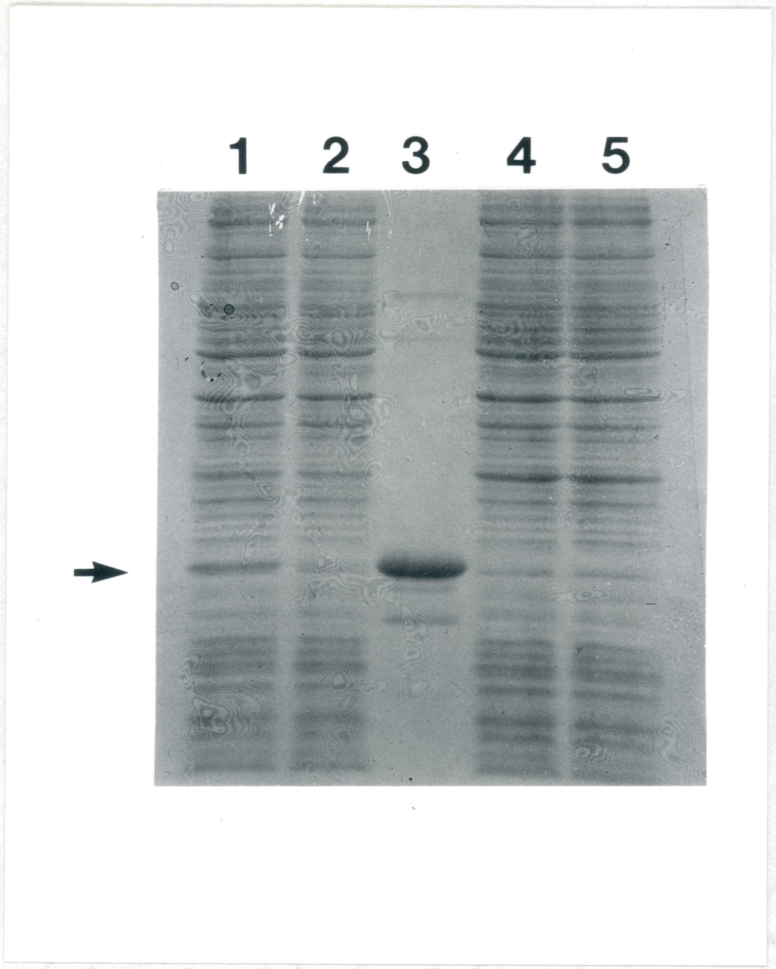
sequence (181) is apparently in the region just distal to the flavodoxin coding sequence (Fig. 8).

Overexpression of the nifF Gene Product in E. coli.

Additional support identifying the nifF gene product as flavodoxin was obtained by expressing the nifF gene product in E. coli. Overexpression of flavodoxin in E. coli would also simplify the purification of flavodoxin. Overproduction of the flavodoxin was achieved by cloning the entire proposed nifF coding region, minus the nif promoter sequence, behind the tac promoter contained in the expression vector pKK223-3. This construction allowed the expression of flavodoxin to be controlled by the IPTG inducible tac promoter in E. coli JM105. Induced and uninduced crude extracts prepared from the E. coli strains containing the recombinant plasmid, with nifF inserted in the correct orientation relative to the tac promoter (pDB343), and the control strain (pDB344) were electrophoresed on a denaturing 12% PAGE gel. The control strain contained a plasmid with the nifF gene inserted in the incorrect orientation relative to the tac promoter. An overproduced protein with the same approximate molecular weight as purified A. vinelandii flavodoxin (Mr = 20,000) accumulated only in the strain containing pDB343 (Fig. 9). This result demonstrated that the accumulation of the nifF gene product was dependent on expression from the tac promoter and that the nifF encoded polypeptide has the same electrophoretic properties as flavodoxin on SDS-PAGE gels.

Southern Hybridization Analysis of the nifF gene.

Multiple copies of nifH-like genes encoding nitrogenase Fe protein species were reported



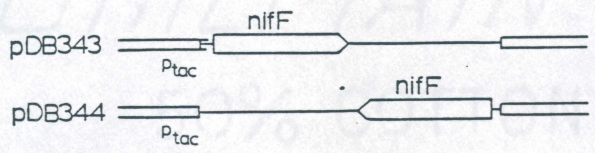


FIGURE 9. Overproduction of the nifF encoded flavodoxin in E. coli.
A) SDS-PAGE gel of IPTG induced and uninduced E. coli strain JM105 containing either plasmid pDB343 or plasmid pDB344. Lane 1: induced JM105 containing pDB343; Lane 2: uninduced JM105 containing pDB343; Lane 3: 1.0 ug purified flavodoxin; Lane 4: induced JM105 containing pDB344; Lane 5: uninduced JM105 containing pDB344.
B) Construction of plasmids pDB343 and pDB344 used for the overproduction of flavodoxin. The expression vector used for both plasmids is pKK233-3. The orientation of plasmid pDB343 places expression of nifF under the control of the tac promoter. The nifF coding sequence of plasmid pDB344 is in the opposite orientation as pDB343.

for A. vinelandii OP (98). In addition, Veeger et al. characterized three immunologically distinct flavodoxins from A. vinelandii OP (111). We therefore examined whether the nifF gene is also reiterated on the A. vinelandii OP chromosome. Southern analysis was performed using the 2.0 kb XhoI enzyme fragment containing the nifF gene as a probe, and A. vinelandii OP genomic DNA digested with the restriction enzymes XhoI, SmaI, or PstI (Fig. 10). Results of these experiments showed that the nifF probe hybridized to only a single XhoI, SmaI, and PstI restriction enzyme fragment in the respective Southern analyses. These results indicated that there is only a single nifF coding sequence on the A. vinelandii OP chromosome or that the reiterated nifF coding regions are contained on the same size XhoI, SmaI, and PstI restriction enzyme fragments. To eliminate this latter possibility, Southern analyses were also performed on XhoI and SmaI restriction enzyme digests of genomic DNAs prepared from two different mutant strains. One of these mutant strains (DJ60) contains a known deletion and insertion within the nifF gene and the other (DJ58) contains an insertion within the nifF gene (Fig. 11). The details for the construction of these strains are presented in **Materials and Methods** and are further discussed in the next section. Because the locations of the XhoI and SmaI restriction enzyme sites within the insert are known (157), the alteration in the pattern of restriction enzyme fragments hybridizing to the nifF probe in the respective Southern analyses could be predicted. The results of the Southern analyses of genomic DNAs from these mutant strains are shown in Fig. 10. From these results we can unambiguously conclude that there is only a single copy of the nifF coding sequence on the A. vinelandii OP chromosome.

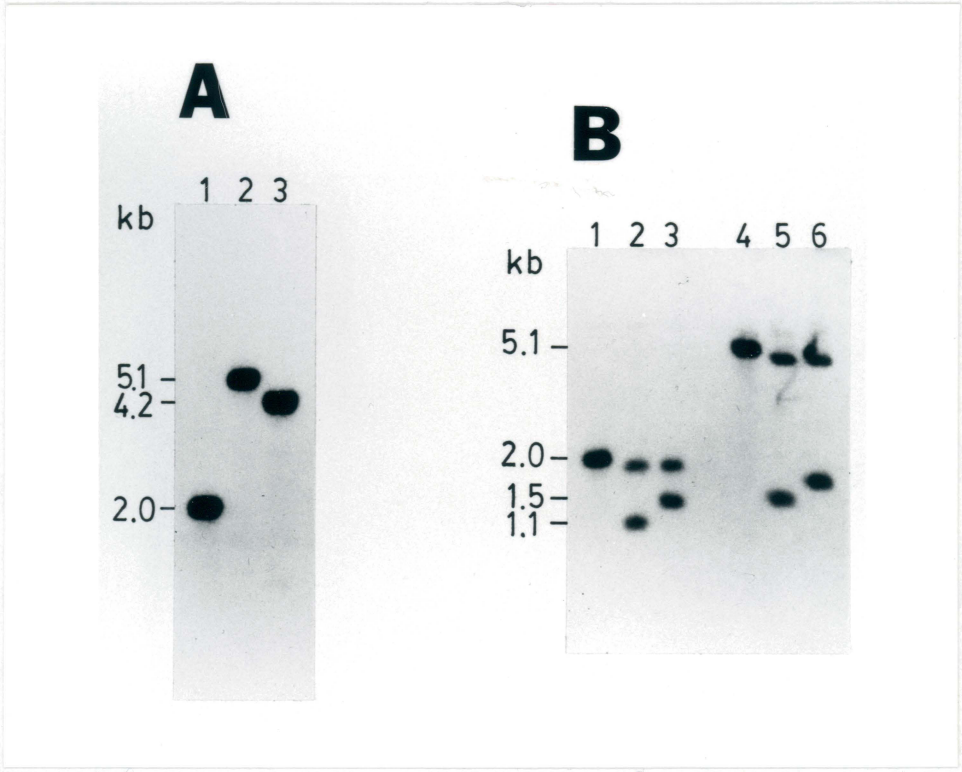


FIGURE 10. Southern hybridization analysis indicating the presence of only a single copy of the nifF gene on the A. vinelandii genome. For the experiments shown in both Panel A and Panel B the 2.0 kilobase nifF-containing XhoI restriction enzyme fragment was used as hybridization probe. Panel A, Southern analysis of genomic DNA prepared from wild type A. vinelandii digested with XhoI (lane 1), SmaI (lane 2), or PstI (lane 3). Panel B, Southern analysis of genomic DNA prepared from wild type A. vinelandii (lanes 1 and 4), strain DJ60 (lanes 2 and 5), and strain DJ58 (lanes 3 and 6). The respective DNAs were digested with XhoI (lanes 1-3) or SmaI (lanes 4-6). The approximate sizes of the hybridizing fragments (kb, kilobases) are indicated on the left of the panels. The hybridization patterns in Panel B should be compared with the physical maps of the wild type, strain DJ58 and strain DJ60 presented in Fig. 11.

Mutagenesis of the nifF Gene.

The details of the method used for the site-directed deletion of nif-specific gene fragments from the A. vinelandii chromosome is described in **Materials and Methods.** This procedure involves the deletion of the central portion of nif gene sequences from recombinant plasmids, followed by the recombination of such deletions into the A. vinelandii OP chromosome during reciprocal recombination events. Strains deleted for a nif-specific sequence generally exhibit a Nif⁻ phenotype, and consequently they are identified by their inability to grow in the absence of a fixed nitrogen source. Repeated efforts to isolate a mutant strain that has a deletion within nifF-coding sequence were unsuccessful. This result indicated that flavodoxin was either not essential for diazotrophic growth or that it was an essential cellular component required under all physiological conditions. To resolve these possibilities, a direct selection mutagenesis strategy was used. For this purpose two different hybrid plasmids were constructed, pDB99 and PDB100. A DNA fragment that encodes a kanamycin resistance determinant was inserted into cloned nifF coding sequences such that in the plasmid pDB100 it created an insertion mutation and in the plasmid pDB99 it created a deletion and an insertion mutation (Fig. 6). Purified preparations of these plasmids were added to competent A. vinelandii OP cells, and the transformed cells were plated in nitrogen-supplemented medium containing kanamycin. The inclusion of kanamycin in the medium provided a positive selection for transformants that integrated the kanamycin resistance determinant into the chromosome. Southern analysis of DNA prepared from the transformants confirmed the location and the orientation of the kanamycin resistance cartridge within the A. vinelandii OP chromosome in both mutant

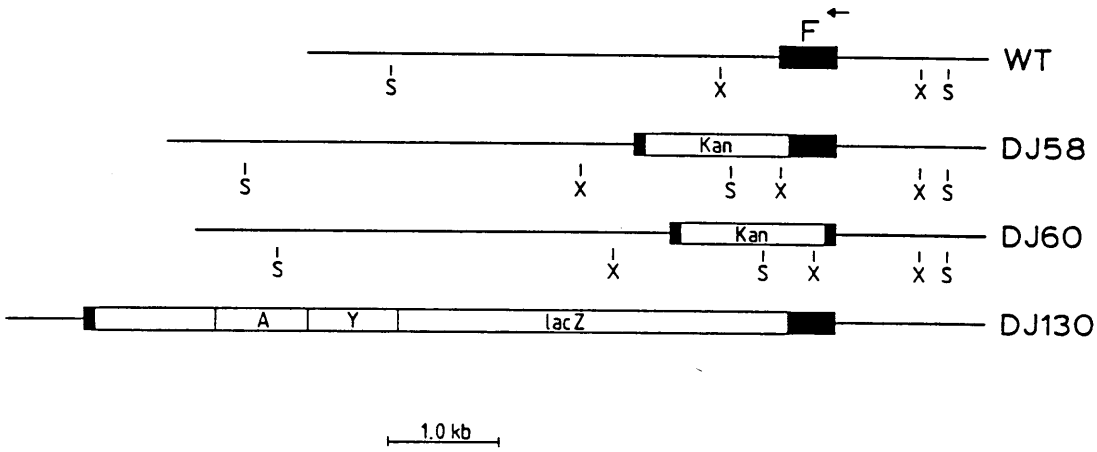


FIGURE 11. A. vinelandii strains used in this study. The construction of the mutant strains DJ58, DJ60 and DJ130 is described in **Materials and Methods** and their structures are shown in the figure. Strain DJ58 carries a 1.3 kilobase kanamycin resistance determinant inserted into the unique SalI restriction site located within nifF (the SalI site is located within codons 130 and 131 of nifF, see Fig. 8). Strain DJ60 carries a 1.3 kilobase kanamycin resistance determinant which replaces a 318 base pair deletion of the nifF coding region extending from the first to the third HincII restriction enzyme site within nifF. Strain DJ130 carries an in-frame fusion between the 130th codon of the nifF gene and the 12th codon of the lacZ gene (144) from E. coli. In the figure, dark shaded areas represent nifF coding sequences, thin lines represent A. vinelandii sequences flanking nifF, and open regions represent foreign DNA sequences integrated into the A. vinelandii genome. Restriction enzymes XhoI and SmaI are indicated by X and S in the figure, respectively.

strains (Fig. 10). The ability to obtain such transformants indicated that flavodoxin is not an essential cellular component. Both mutant strains were also tested for their ability to grow in the absence of a fixed nitrogen source. There was no difference in the mutant strain growth rates (0.62 generation per hour) compared with the wild type strain growth rate when they were cultured in a liquid Burk's medium containing no fixed nitrogen source. However, whole cell acetylene-reduction assays showed that the mutant strains had only 70% of the wild type acetylene-reducing activity. These results indicated that the reduced flavodoxin is not an essential physiological reductant of the nitrogenase in A. vinelandii OP. However, flavodoxin does appear to play a role in permitting the full in vivo activity of nitrogenase. Whether the role is as the direct reductant of the nitrogenase Fe protein or as a reductant of some other nif component is not known.

Proof that the mutant strains described above are actually devoid of the nifF gene product was obtained by performing one- and two-dimensional gel electrophoresis of wild type and mutant crude extracts (data not shown) and by Ouchterlony double diffusion using crude extracts and flavodoxin-specific antisera. In Fig. 12 the cross-reaction of flavodoxin specific antisera against wild type and mutant crude extracts are compared. The wild type crude extract shows a single precipitin band while the mutant strain shows no immunoprecipitin band. These results show that the mutant strain is indeed lacking the flavodoxin protein and that there are no other A. vinelandii OP proteins immunologically related to flavodoxin that can be detected by this method.

nifF Gene Expression.

Previous studies indicated that flavodoxin accumulated in A. vinelandii OP under fixing

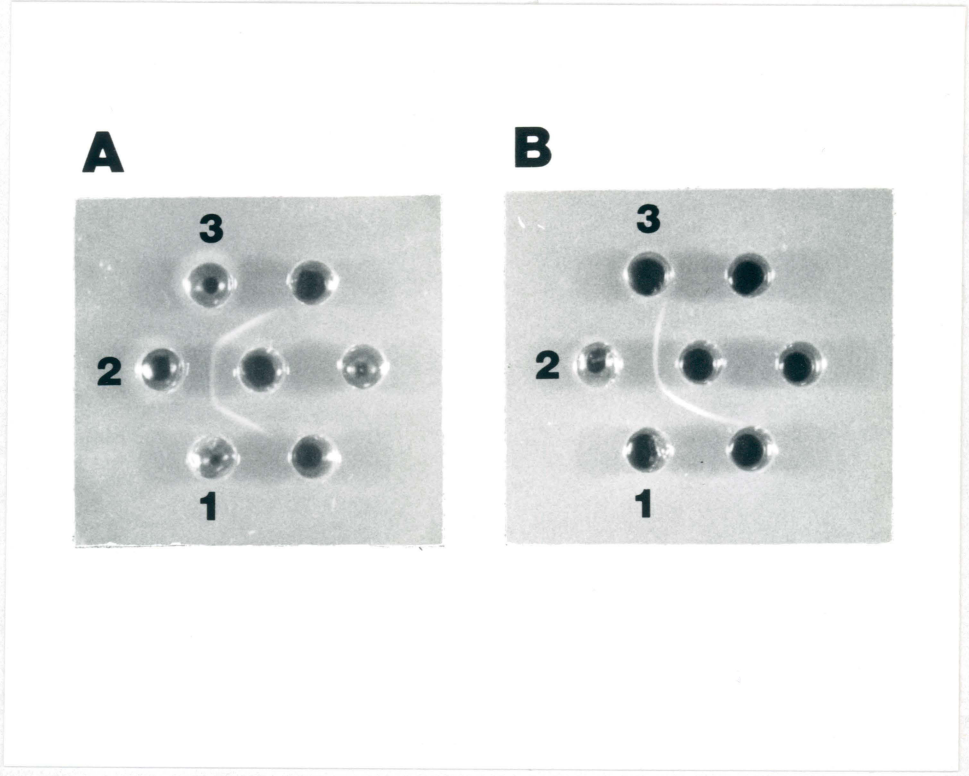


FIGURE 12. Ouchterlony double diffusion analysis. Panel A shows the results of a control experiment. The center well contains anti-MoFe protein. Outer wells contain purified MoFe protein (well 1), crude extract prepared from nitrogenase derepressed wild type (well 2) and crude extract prepared from nitrogenase derepressed DJ60 (well 3). Panel B has antiflavodoxin in the center well. Outer wells contain purified flavodoxin (well 1), crude extract prepared from nitrogenase derepressed wild type (well 2) and crude extract prepared from nitrogenase derepressed DJ60 (well 3). Note that there is no cross-reaction of DJ60 crude extract with antiflavodoxin.

nitrogen fixing and non-nitrogen fixing conditions (8). This observation raised the possibility that nifF expression does not respond to a physiological demand for a fixed nitrogen source. The characteristic nif-like promoter sequence preceding the nifF gene, however, indicated that nifF expression is likely to be regulated, at least in part, by a demand for fixed nitrogen. To examine the regulation of nifF expression, we constructed a recombinant plasmid that carries an in-phase translational gene fusion such that the lacZ gene (β-galactosidase) from E. coli is fused to the nifF gene. In this construction, the nifF promoter and the N-terminal coding portion of the nifF gene were fused close to the N-terminus of the lacZ gene (Fig. 11). Since this recombinant plasmid carries A. vinelandii OP nifF sequences flanking both sides of the lac gene cartridge, it was possible to use this gene fusion in a gene replacement experiment such that the fusion was integrated into the A. vinelandii OP chromosome (see Fig. 11). This strain, DJ130, thus produces a nifF-lacZ fusion protein that retains a significant amount of β-galactosidase activity and whose transcription and translation is under the control of the nifF regulatory elements.

The nifF-lacZ gene fusion strain was assayed for β-galactosidase activity under both nitrogenase repressing and derepressing conditions. A nitrogenase derepressed culture has nearly twice the β-galactosidase activity (specific activity, 355 Miller units (140)) as the repressed culture (specific activity, 187 Miller units (140)) indicating that synthesis of flavodoxin is elevated in response to a demand for a fixed nitrogen source. The high level of activity under nitrogenase repressing conditions indicated that either the proposed nifF promoter is partially active under non-nitrogen fixing conditions or that there is another promoter which directs the transcription of flavodoxin under non-nitrogen fixing conditions.

Northern Analysis of nifF Gene Expression.

Because of the results of gene fusion studies discussed above, northern analysis was used to determine the size and accumulation of the nifF-hybridizing mRNA. For these experiments, a nifF-specific probe was used to examine the accumulation of nifF mRNA under nitrogenase repressing and derepressing conditions (Fig. 13). Under nitrogenase repressing conditions two major nifF-hybridizing transcripts (approximately 1.6 and 3.0 kb) accumulated and a minor transcript (approximately 0.8 kb) accumulated as well. Under nitrogenase derepressing conditions the relative amounts of the two larger transcripts remained the same, but the smaller transcript accumulated to a much greater degree (Fig. 13). The 0.8 kb transcript is likely to originate from the proposed nif promoter and ending at the apparent rho-independent transcription termination signal (see Fig. 8)

Mutagenesis of other genes previously proposed to be involved in electron transport to nitrogenase in A. vinelandii.

Early studies on electron transport to nitrogenase suggested that both reduced flavodoxin and ferredoxin can donate electrons to the Fe protein of A. vinelandii in vitro. Because the flavodoxin encoded by nifF is not essential for diazotrophic growth in A. vinelandii, the possibility that a ferredoxin may be involved in electron transport to nitrogenase was further investigated. The fdxA gene had previously been cloned, sequenced and mutagenized (144). This study demonstrated that an A. vinelandii OP strain carrying the kanamycin resistance determinant from Tn903 inserted within the ferredoxin I coding sequence was capable of normal diazotrophic growth rates as compared to wild type growth rates. In

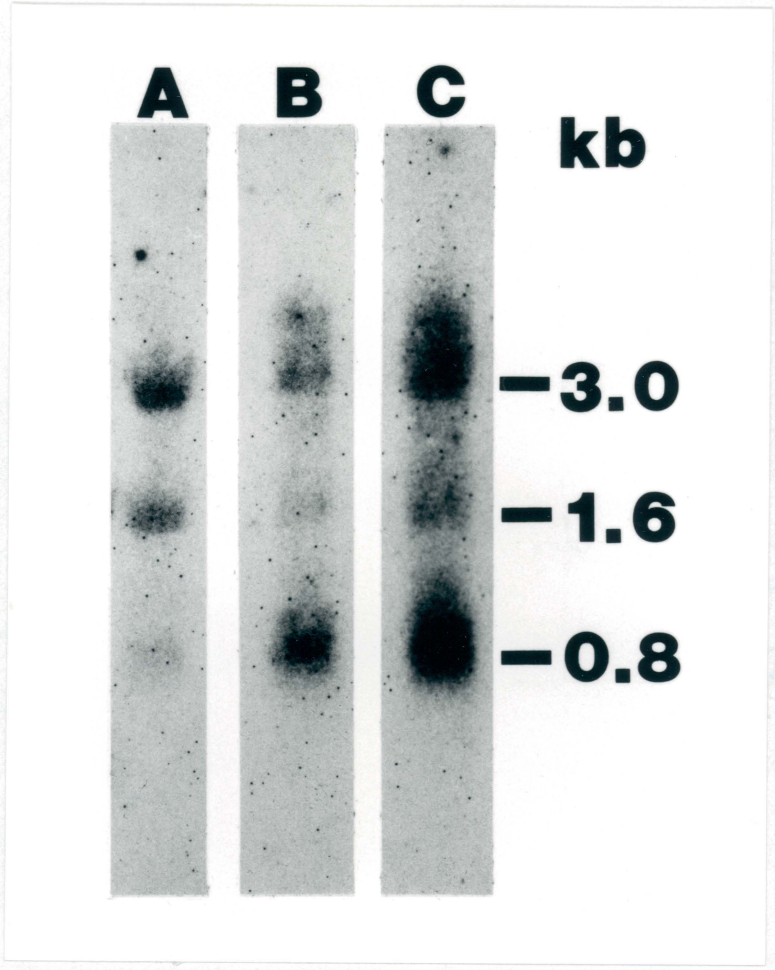


FIGURE 13. Northern hybridization analysis of nifF mRNA accumulation in A. vinelandii. Lane A, Northern analysis of total RNA isolated from a wild type culture grown in Burk's medium containing 29 mM ammonium acetate (nitrogenase repressed); lane B, Northern analysis of total RNA isolated from a wild type culture derepressed for nitrogenase for 30 min.; lane C, Northern analysis of total RNA isolated from the same culture derepressed for nitrogenase for 60 min. The 2.0 kilobase nifF-containing XhoI restriction enzyme fragment was used as a hybridization probe. Identical results, although less intense bands, were obtained when a 280 base pair nifF-specific HincII restriction enzyme fragment was used as the hybridization probe (data not shown). Note that the 0.8 kilobase band substantially accumulated only under nitrogenase derepressing conditions.

addition, fdxA was not preceded by a nif promoter sequence and its expression was not stimulated under nitrogen fixing conditions. These results suggested that ferredoxin I is also not the unique physiological reductant of the Fe protein of nitrogenase in A. vinelandii. However, flavodoxin and ferredoxin have been proposed to function together in electron transport to nitrogenase in A. vinelandii (9). The possibility that ferredoxin I could functionally replace flavodoxin in electron transport and visa versa was investigated by isolating a strain carrying a mutation in both nifF and fdxA. A recombinant plasmid containing the kanamycin resistance determinant from Tn903 inserted into the fdxA coding sequence was used to transform the A. vinelandii OP strain (DJ130) carrying a genomic nifF-lacZ fusion. The flavodoxin-ferredoxin double mutation strain was selected for kanamycin resistance and exhibited a slightly slower growth rate relative to the wild type strain, both in the presence and in the absence of a fixed nitrogen source on plated and liquid Burk's medium. These results indicated that neither flavodoxin nor ferredoxin I are essential for diazotrophic growth and that either another cellular component is capable of functioning as a non-specific low-potential reductant of nitrogenase or that a completely different electron transport mechanism to nitrogenase is operating in A. vinelandii OP.

In K. pneumoniae both nifF and nifJ are required for electron transport to the nitrogenase complex. Due to the conservation of nif gene organization in A. vinelandii and K. pneumoniae, the possibility that a nifJ coding sequence was present in the region immediately upstream of nifH was previously investigated (96). The 5' end of the open reading frame preceding nifH was sequenced and found to exhibit no significant identity with the nucleotide sequence of the nifJ gene sequenced from K. pneumoniae. Because

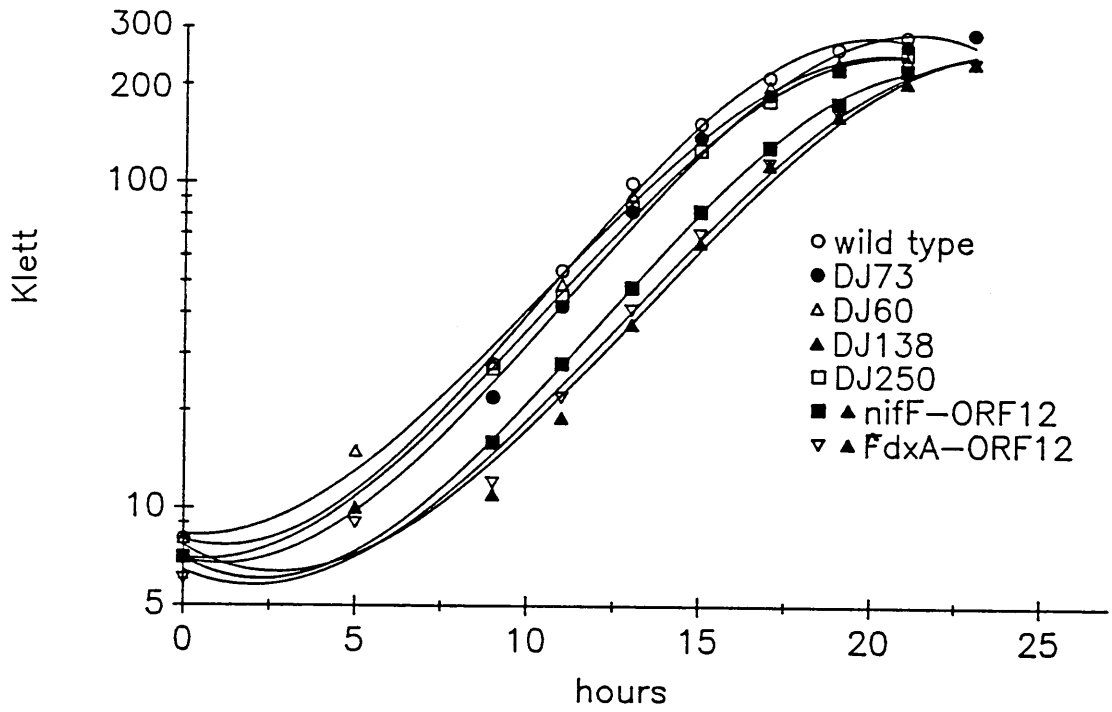


FIGURE 14. The diazotrophic growth rates of wild type A. vinelandii OP, DJ60 (▲nifF::kan), DJ73 (fdxA::kan), DJ154 (▲nifF::lacZ - fdxA::kan), DJ250 (▲ORF12), ▲nifF::kan - ▲ORF12, and fdxA::kan - ▲ORF12. All strains were grown in Burk's medium lacking a fixed nitrogen source.

the sequence identity could be restricted to the portion of the gene which had not been sequenced, the identity of ORF12 as a nifJ-like gene can not be eliminated. A specific deletion within ORF12 on the A. vinelandii OP chromosome was generated, and the resulting mutant strain was tested for diazotrophic growth capabilities. This strain was isolated by restoring a strain phenotypically Nif$^-$,due to a deletion extending from the 5' end of ORF12 through the nifH promoter, to Nif$^+$ by transforming with a recombinant plasmid carrying a specific deletion restricted to the ORF12 sequence. The ability to isolate the Nif$^+$ transformants demonstrated that ORF12 does not encoded a polypeptide essential for nitrogen fixation in A. vinelandii. Furthermore, a strain carrying a deletion within ORF12 and a kanamycin resistance determinant insertion and deletion within nifF, and a strain containing a deletion within ORF12 and a kanamycin resistance determinant insertion within fdxA, were also isolated and found to be capable of significant diazotrophic growth rates. A comparison of the growth rates of wild type A. vinelandii OP, a flavodoxin mutation strain (DJ60), a ferredoxin I mutation strain (DJ73), an ORF12 mutation strain (DJ250), a flavodoxin-ferredoxin I mutation strain (DJ130), a flavodoxin-ORF12 mutation strain, and a ferredoxin-ORF12 mutation strain is shown in Fig. 14. These results firmly show that the physiological electron transport mechanism to nitrogenase in A. vinelandii has not been discovered and is not dependent upon the presence of nifF, fdxA or ORF12.

Mutational Analysis of the Region Downstream of nifF.

The possibility that a gene required, or at least involved in electron transport to nitrogenase or some other aspect of nitrogen fixation might reside downstream of nifF, was

investigated. In K. pneumoniae, the nifL, nifA, nifB, and nifQ genes lie immediately downstream of nifF (see Fig. 2) However, in A. vinelandii the nifA, nifB and nifQ genes are located in another position on the chromosome which is unlinked to the major nif cluster (see nifA **Results**). A hybrid plasmid containing the nifF coding sequence and approximately five kilobases of downstream flanking sequence was constructed. The nucleotide sequence approximately two kilobases past the nifF coding sequence was determined and found to contain a potential open reading frame. Because nifL and nifJ genes are the only nif genes from K. pneumoniae which have not been identified in A. vinelandii and because of the sequential conservation of nif gene organization in K. pneumoniae and A. vinelandii, this sequenced region was analyzed for nucleotide and predicted amino acid sequence identity with the sequences of the K. pneumoniae nifL and nifJ genes and gene products, respectively. Using the Pustell Sequence Analysis Program, no significant nucleotide or amino acid sequence identity to nifL or to nifJ was revealed. This region was also analyzed for sequence identity to the Rhizobium meliloti fixABCX genes, which are proposed to function in electron transport to nitrogenase in this nitrogen fixing organism (51). Again no apparent sequence identity between this region in A. vinelandii and the fixABCX genes in R. meliloti was observed.

Evidence for the presence of a polypeptide coding region within the five kilobases downstream of nifF was obtained when a strain of A. vinelandii carrying a deletion from ORF5 to approximately eight kilobases past nifF (DJ40) (see Fig.2) was observed to be incapable of growth on Burk's medium supplemented with urea as the nitrogen source but capable of growth on Burk's medium containing ammonium acetate. The loci responsible

for generating this phenotype was isolated by the construction of a strain carrying either a deletion or an insertion mutation within the region downstream of nifF (Fig. 6). A strain containing a 3.5 kilobase deletion within this downstream region was isolated using the following strategy. First, wild type A. vinelandii OP was co-transformed with a recombinant plasmid containing the 3.5 kilobase deletion from the nifF upstream region (pDB306) and a hybrid plasmid carrying the rifampicin resistance determinant (pDB303, see Appendix I), and co-transformants selected for on Burk's medium supplemented with ammonium acetate and rifampicin. Then these transformants were screened for there ability to grow on Burk's medium with ammonium acetate but their inability to grow on Burk's medium containing urea. A strain carrying the kanamycin resistance determinant from Tn903 inserted within this region (see Fig. 6) was also isolated and found to be capable on growth Burk's supplemented with either ammonium acetate or urea. These results place the acetate requiring loci at least 4.5 kilobases downstream of nifF. The identity of this loci is unknown, as is the its involvement in nitrogen fixation.

DISCUSSION

It is now clear that the product of the nifF gene from A. vinelandii OP is not essential for diazotrophic growth. Consequently, this flavodoxin cannot be the unique source of reducing equivalents for nitrogen fixation in A. vinelandii OP. Furthermore, gene fusion experiments and Northern analyses of nifF mRNA demonstrate that the nifF gene is transcribed and translated under non-nitrogen fixing conditions. Based on these results it

could be argued that the flavodoxin-encoding gene from A. vinelandii OP should not be considered a nif gene. However, the nifF genotypic designation for this flavodoxin encoding gene is appropriate for the following reasons: 1) the gene encoding the flavodoxin is located adjacent to, or possibly within, the major nif gene cluster from A. vinelandii OP; 2) the nifF gene is preceded by a consensus nif-promoter sequences, as well as a nif-activator sequence; 3) one of the three hybridizing transcripts is substantially accumulated only under nitrogen fixing conditions; and 4) the A. vinelandii OP nifF gene product has a high level of sequence identity when compared to the nifF gene product from K. pneumoniae (46).

If the nifF gene product is not the unique source of reducing equivalents for nitrogenase turnover in A. vinelandii OP, what are other potential electron donors? There are several possibilities. Purified ferredoxin I has been shown to be an electron donor to nitrogenase in vitro (9). Thus, in the absence of flavodoxin it is possible that ferredoxin I acts as the direct electron donor to nitrogenase. Morgan et al. (144) have described the isolation, sequence analysis, and mutagenesis of the gene from A. vinelandii OP encoding ferredoxin I (fdxA). These studies show that ferredoxin I is not essential for diazotrophic growth. Unlike nifF a nif-specific promoter does not precede the fdxA gene and expression of fdxA appears to be constitutive. The isolation of a flavodoxin-ferredoxin double mutation strain of A. vinelandii OP demonstrated that in the absence of both low-potential reductants A. vinelandii OP is still capable of substantial levels of diazotrophic growth.

The presence of three different flavodoxins in A. vinelandii ATCC 478 had previously been reported (111). These flavodoxins have nearly identical molecular weights and isoelectric points, yet they are structurally and immunologically distinct. One of these

flavodoxins accumulated only in response to nitrogen fixing conditions (111). If there are also other forms of flavodoxin present in the strain used in this study, A. vinelandii OP, then perhaps a reduced form of one of these proteins might be used for the nitrogenase turnover. However, there is no evidence that more that a single flavodoxin species is present in A. vinelandii OP. Finally, it was recently shown that the fixABCX gene cluster from Rhizobium meliloti is capable of hybridizing to A. vinelandii OP genomic DNA (51), and it was suggested that the fixABCX gene cluster products could be involved in electron transport to nitrogenase.

In K. pneumoniae electron transport to nitrogenase is dependent on the action of the nifF encoded flavodoxin and the nifJ encoded flavodoxin:pyruvate oxidoreductase. The nifJ gene is located immediately upstream of nifH on the K. pneumoniae chromosome. The nucleotide sequence approximately two kilobases upstream of nifH in A. vinelandii was determined and an open reading frame (ORF12), was identified (96). However, a comparison of the sequences of ORF12 and nifJ did not reveal sequence identity at the DNA or the amino acid level. Furthermore, mutation of ORF12 did not effect diazotrophic growth capabilities. The possibility that the ORF12 encoded polypeptide functioned in concert with flavodoxin or was somehow involved in electron transport to nitrogenase was further investigated by the isolation of flavodoxin-ORF12 and ferredoxin-ORF12 double mutation strains. Because no substantial affect on nitrogen fixing growth rates was observed in the mutation strains in relation to the growth rates of the flavodoxin and ferredoxin mutation strains, respectively, ORF12 does not appear to function in the same physiological event as either flavodoxin or ferredoxin.

Northern analyses indicated that there are three overlapping nifF transcripts which accumulated in A. vinelandii OP. One of these transcripts substantially accumulated only in response to a demand for a fixed nitrogen source. The simplest explanation for these results is that a nif-specific promoter is located immediately adjacent to the flavodoxin coding region and that there may be an additional two promoters located approximately 0.8 and 2.2 kilobases preceding the nifF gene. Transcription from these latter two promoters is apparently insensitive to the nitrogen status of the cell. The nucleotide sequence two kilobases downstream of the nifF gene was determined and identified a potential open reading frame but did not identify an E. coli consensus promoter-like sequence or nif-specific promoter sequence. However, a consensus promoter sequence from constitutively expressed genes in A. vinelandii is not available and thus could be present and not recognized. Northern analyses using a series of probes from this upstream region are necessary to gain more insight into this transcriptional pattern.

Comparison of the sequence of the partial open reading frame identified downstream of nifF with the sequences of the K. pneumoniae nifJ and nifL genes and the R. meliloti fixABCX genes did not reveal any significant sequence identities at the DNA or the amino acid levels. However, mutational analysis of the four kilobases of DNA past the sequenced region identified a loci responsible for generating an acetate requiring strain of A. vinelandii. The identity of this loci is unknown but several possibilities exist. Absence of a functional pyruvate dehydrogenase complex would be phenotypically reflected by a strain requiring acetate for growth because the pyruvate generated during glycolysis could not be converted to acetyl-CoA. Acetyl-CoA is required for maintenance of the tricarboxylic acid

cycle which is a major energy generating pathway for the cell. The pyruvate dehydrogenase complex is composed of multiple copies of three enzymes, pyruvate dehydrogenase (E1), dihydrolipoate transacetylase (E2) and dihydrolipoate dehydrogenase (E3). The genes encoding each of these enzymes have been isolated and the nucleotide sequence determined in E. coli (211). The nucleotide sequences of the dihydrolipoate transacetylase and the dihydrolipoate dehydrogenase have also been determined in A. vinelandii (73, 239). Preliminary results (random sequencing of this nifF upstream region) suggest that the genes encoding any of the components of the pyruvate dehydrogenase complex are not located in this region. However, this result is far from conclusive. The pyruvate dehydrogenase enzyme of the complex contains thiamine pyrophosphate and dihydrolipotransacetylase contains lipoate as cofactors. The possibility that the acetate -requiring phenotype is due to a mutation is a gene encoding a polypeptide involved in the biosynthesis of lipoate is presently being investigated by Kok et al. (personal communication). Whether a relationship between the coding regions downstream of nifF and nitrogen fixation exists is unknown. The physiological role of flavodoxin in relationship to nitrogen fixation in A. vinelandii, as well as its function under non-nitrogen fixing conditions also remains an open question.

NUCLEOTIDE SEQUENCE AND MUTAGENESIS OF THE NIFA GENE FROM AZOTOBACTER VINELANDII

DNA Sequence of the A. vinelandii nifA Gene.

The nucleotide sequence of a 3.7 kilobase EcoRI restriction enzyme fragment which contains the A. vinelandii nifB and nifQ genes was previously reported (101). In K. pneumoniae the nifB and nifQ genes are contiguous (2), where as in A. vinelandii, nifB and nifQ are separated by two potential genes of unknown function (101). The deduced amino acid sequence of a partial open reading frame preceding the A. vinelandii nifB gene indicated that nifA was encoded in this region (101). Based on these results, we cloned two adjacent 1.7 kilobase SalI restriction enzyme fragments that encompass the entire nifA gene, as well as some flanking sequences (see Fig. 15). The entire sequence of the 1.7 kilobase fragment contained on pDB150 was determined, as was the nifA proximal portion on the 1.7 kilobase SalI restriction enzyme fragment contained on plasmid pDB160 (Fig. 15). These experiments confirmed that the A. vinelandii nifA and nifB genes are linked and completed the DNA sequence analysis of the nifA gene.

The deduced amino acid sequence for the nifA gene product has high sequence identity when compared with nifA-encoded gene products from several other diazotrophic organisms (29,32,109,216,236). The A. vinelandii nifA gene product is composed of 522 residues and has a calculated M_r = 58,100 if the N-terminal methionine residue is included in the calculation. In Fig. 17, the A. vinelandii and K. pneumoniae nifA gene products are compared and this comparison supports conclusions from previous interspecies comparisons

```
GTC GAC AAC GCC ATC GAG TCC ATG AGC CAG AAC CAG GTC AGC CGC CGC GAG CTG TTC ATC
VAL ASP ASN ALA ILE GLU SER MET SER GLN ASN GLN VAL SER ARG ARG GLU LEU PHE ILE

AGC ACC CGC GTG GAG AAC CAC CTG GTG CGC ATG GAG ATC ACC GAC AGC GGC CCG GGC ATT
SER THR ARG VAL GLU ASN HIS LEU VAL ARG MET GLU ILE THR ASP SER GLY PRO GLY ILE

CCG CCC GAC CTG GTG CTG AAG GTG TTC GAG CCG TTC TTC AGC ACC AAG CCG CCA CAC CGC
PRO PRO ASP LEU VAL LEU LYS VAL PHE GLU PRO PHE PHE SER THR LYS PRO PRO HIS ARG

GTC GGG CGC GGC ATG GGC CTG CCG GTG GTG CAG GAG ATC GTC GCC AAG CAC GCC GGC ATG
VAL GLY ARG GLY MET GLY LEU PRO VAL VAL GLN GLU ILE VAL ALA LYS HIS ALA GLY MET

GTG CAC GTA GAC ACC GAC TAT CGC GAA GGC TGC CGG ATC GTC GTC GAG CTG CCC TTC TCG
VAL HIS VAL ASP THR ASP TYR ARG GLU GLY CYS ARG ILE VAL VAL GLU LEU PRO PHE SER

GCC TCC ACC TGA A C A G C G A C A G G G A A T G C C C   ATG AAT GCA ACC ATC CCT
ALA SER THR END                                       MET ASN ALA THR ILE PRO
                10                                              20
CAG CGC TCG GCC AAA CAG AAC CCG GTC GAA CTC TAT GAC CTG CAA TTG CAG GCC CTG GCG
GLN ARG SER ALA LYS GLN ASN PRO VAL GLU LEU TYR ASP LEU GLN LEU GLN ALA LEU ALA
                30                                              40
AGC ATC GCC CGC ACG CTC AGC CGC GAA CAA CAG ATC GAC GAA CTG CTC GAA CAG GTC CTG
SER ILE ALA ARG THR LEU SER ARG GLU GLN GLN ILE ASP GLU LEU LEU GLU GLN VAL LEU
                50                                              60
GCC GTA CTG CAC AAT GAC CTC GGC CTG CTG CAT GGC CTG GTG ACC ATT TCC GAC CCG GAA
ALA VAL LEU HIS ASN ASP LEU GLY LEU LEU HIS GLY LEU VAL THR ILE SER ASP PRO GLU
                70                                              80
CAC GGC GCC CTG CAG ATC GGC GCC ATC CAC ACC GAC TCG GAA GCG GTG GCC CAG GCC TGC
HIS GLY ALA LEU GLN ILE GLY ALA ILE HIS THR ASP SER GLU ALA VAL ALA GLN ALA CYS
                90                                             100
GAA GGC GTG CGC TAC AGA AGC GGC GAA GGC GTG ATC GGC AAC GTG CTC AAG CAC GGC AAC
GLU GLY VAL ARG TYR ARG SER GLY GLU GLY VAL ILE GLY ASN VAL LEU LYS HIS GLY ASN
               110                                             120
AGC GTG GTG CTC GGG CGC ATC TCC GCC GAC CCG CGC TTT CTC GAC CGC CTG GCG CTG TAC
SER VAL VAL LEU GLY ARG ILE SER ALA ASP PRO ARG PHE LEU ASP ARG LEU ALA LEU TYR
               130                                             140
GAC CTG GAA ATG CCG TTC ATC GCC GTG CCG ATC AAG AAC CCC GAG GGC AAC ACC ATC GGC
ASP LEU GLU MET PRO PHE ILE ALA VAL PRO ILE LYS ASN PRO GLU GLY ASN THR ILE GLY
               150                                             160
GTG CTG GCG GCC CAG CCG GAC TGC CGC GCC GAC GAG CAC ATG CCC GCG CGC ACG CGC TTC
VAL LEU ALA ALA GLN PRO ASP CYS ARG ALA ASP GLU HIS MET PRO ALA ARG THR ARG PHE
               170                                             180
CTG GAG ATC GTC GCC AAC CTG CTG GCG CAG ACC GTG CGC CTG GTG GTG AAC ATC GAG GAC
LEU GLU ILE VAL ALA ASN LEU LEU ALA GLN THR VAL ARG LEU VAL VAL ASN ILE GLU ASP
               190                                             200
GGC CGC GAG GCG GCC GAC GAG CGC GAC GAA CTG CGT CGC GAG GTG CGC GGC AAG TAC GGC
GLY ARG GLU ALA ALA ASP GLU ARG ASP GLU LEU ARG ARG GLU VAL ARG GLY LYS TYR GLY
               210                                             220
TTC GAG AAC ATG GTG GTG GGC CAC ACC CCC ACC ATG CGC CGG GTG TTC GAT CAG ATC CGC
PHE GLU ASN MET VAL VAL GLY HIS THR PRO THR MET ARG ARG VAL PHE ASP GLN ILE ARG
               230                                             240
CGG GTC GCC AAG TGG AAC AGC ACC GTA CTG GTC CTC GGC GAG TCC GGT ACC GGC AAG GAA
ARG VAL ALA LYS TRP ASN SER THR VAL LEU VAL LEU GLY GLU SER GLY THR GLY LYS GLU
```

```
            250                                                 260
CTG ATC GCC AGC GCC ATC CAC TAC AAG TCG CCG CGC GCG CAC CGC CCC TTC GTC CGC CTG
LEU ILE ALA SER ALA ILE HIS TYR LYS SER PRO ARG ALA HIS ARG PRO PHE VAL ARG LEU
            270                                                 280
AAC TGC GCC GCG CTG CCG GAA ACC CTG CTC GAG TCC GAA CTC TTC GGC CAC GAG AAG GGC
ASN CYS ALA ALA LEU PRO GLU THR LEU LEU GLU SER GLU LEU PHE GLY HIS GLU LYS GLY
            290                                                 300
GCC TTC ACC GGC GCG GTG AAG CAG CGC AAG GGG CGT TTC GAG CAG GCC GAC GGC GGC ACC
ALA PHE THR GLY ALA VAL LYS GLN ARG LYS GLY ARG PHE GLU GLN ALA ASP GLY GLY THR
            310                                                 320
CTG TTC CTC GAC GAG ATC GGC GAG ATC TCG CCG ATG TTC CAG GCC AAG CTG CTG CGC GTG
LEU PHE LEU ASP GLU ILE GLY GLU ILE SER PRO MET PHE GLN ALA LYS LEU LEU ARG VAL
            330                                                 340
CTG CAG GAA GGC GAG TTC GAG CGG GTC GGC GGC AAC CAG ACG GTG CGG GTC AAC GTG CGC
LEU GLN GLU GLY GLU PHE GLU ARG VAL GLY GLY ASN GLN THR VAL ARG VAL ASN VAL ARG
            350                                                 360
ATC GTC GCC GCC ACC AAC CGC GAC CTG GAA AGC GAG GTG GAA AAG GGC AAG TTC CGC GAG
ILE VAL ALA ALA THR ASN ARG ASP LEU GLU SER GLU VAL GLU LYS GLY LYS PHE ARG GLU
            370                                                 380
GAC CTC TAC TAC CGC CTG AAC GTC ATG GCC ATC CGC ATT CCG CCG CTG CGC GAG CGT ACC
ASP LEU TYR TYR ARG LEU ASN VAL MET ALA ILE ARG ILE PRO PRO LEU ARG GLU ARG THR
            390                                                 400
GCC GAC ATT CCC GAA CTG GCG GAA TTC CTG CTC GGC AAG ATC GGC CGC CAG CAG GGC CGC
ALA ASP ILE PRO GLU LEU ALA GLU PHE LEU LEU GLY LYS ILE GLY ARG GLN GLN GLY ARG
            410                                                 420
CCG CTG ACC GTC ACC GAC AGC GCC ATC CGC CTG CTG ATG AGC CAC CGC TGG CCG GGC AAC
PRO LEU THR VAL THR ASP SER ALA ILE ARG LEU LEU MET SER HIS ARG TRP PRO GLY ASN
            430                                                 440
GTG CGC GAA CTG GAG AAC TGC CTG GAG CGC TCG GCG ATC ATG AGC GAG GAC GGC ACC ATC
VAL ARG GLU LEU GLU ASN CYS LEU GLU ARG SER ALA ILE MET SER GLU ASP GLY THR ILE
            450                                                 460
ACC CGC GAC GTG GTC TCG CTG ACC GGG GTC GAC AAC GAG AGC CCG CCG CTC GCC GCG CCG
THR ARG ASP VAL VAL SER LEU THR GLY VAL ASP ASN GLU SER PRO PRO LEU ALA ALA PRO
            470                                                 480
CTG CCC GAG GTC AAC CTG GCC GAC GAG ACC CTG GAC GAC CGC GAA CGG GTG ATC GCC GCC
LEU PRO GLU VAL ASN LEU ALA ASP GLU THR LEU ASP ASP ARG GLU ARG VAL ILE ALA ALA
            490                                                 500
CTC GAA CAG GCC GGC TGG GTG CAG GCC AAG GCC GCG CGG CTG CTG GGC ATG ACG CCG CGG
LEU GLU GLN ALA GLY TRP VAL GLN ALA LYS ALA ALA ARG LEU LEU GLY MET THR PRO ARG
            510                                                 520
CAG ATC GCC TAC CGC ATC CAG ACC CTC AAC ATC CAC ATG CGC AAG ATC TGA C G G G A C
GLN ILE ALA TYR ARG ILE GLN THR LEU ASN ILE HIS MET ARG LYS ILE END

C C T C C G G C A A T G G A T G G C C G G C T C C T C C C C C C T C C C C G T T

C T C C C C G C C C C C T C G T C C G C T A C G G G C G C C C G T C C A C G A C

G G A A C A T C G C C T C C G A C T C C C G C C A C A C T G C T T G T C G C T T
```

FIGURE 15. Nucleotide sequence of the A. vinelandii nifA gene. A potential open reading frame precedes the numbered nifA coding sequence. Numbers refer to the deduced nifA gene product amino acid sequence. The initiation codon for nifA is inferred by comparison with other nifA gene sequences.

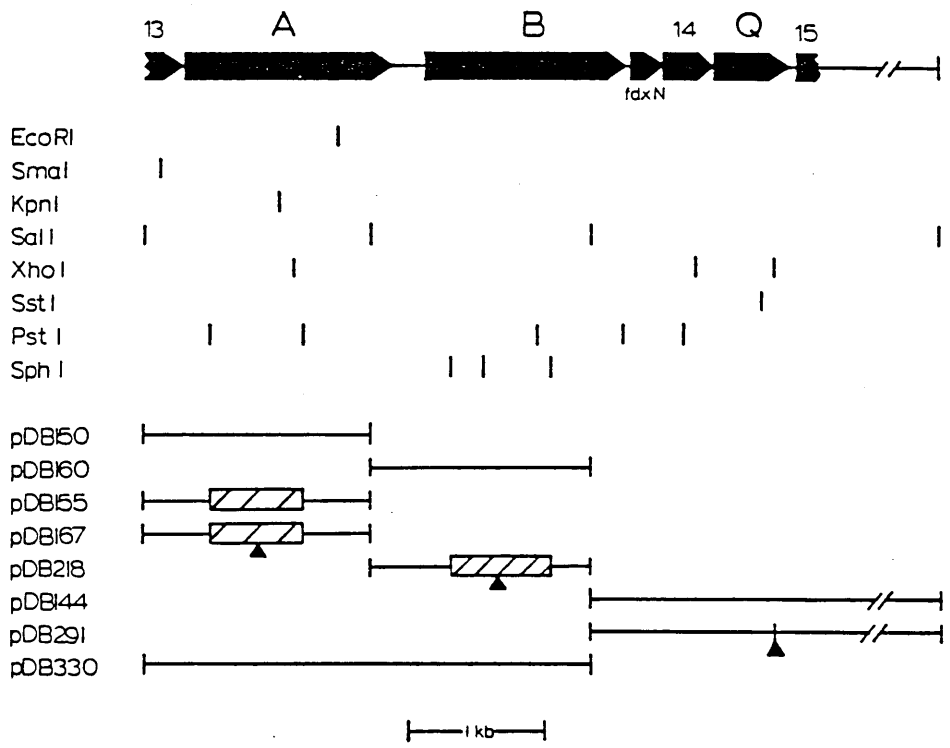


FIGURE 16. Physical organization of nifA-nifB-nifQ region from A. vinelandii. The direction of transcription is from left to right. The nifB and nifQ genes have been identified by their high level of sequence identity when compared with their K. pneumoniae counterparts. Two potential open reading frames one of which appears to encode a ferredoxin, fdxA, are located between nifB and nifQ. A potential nif-specific promoter has been proposed to be located within the nifA-nifB intergenic region (Joerger and Bishop, 1988) A potential open reading frame preceding nifA is indicated in the figure. The positions of specific restriction enzyme sites are indicated by bars. The cloned regions contained within hybrid plasmids pDB150 and pDB160 are indicated as horizontal bars. Both of the cloned fragments are approximately 1.7 kb in length and the fragment present in pDB330 is approximately 3.4 kb in length. The deletion in plasmid pDB155 is indicated by hatches. In pDB167, the deleted region was replaced by a 1.3 kb kanamycin resistance-encoding cartridge and this insertion is indicated by an arrowhead. The hybrid plasmid pDB218 contains an SphI deletion replaced by the 1.3 kb kanamycin resistance-encoding cartridge. The nifQ gene is contained entirely within the 5.0 kb EcoRI restriction fragment cloned in plasmid pDB144. The plasmid pDB291 carries the 1.3 kb kanamycin resistance-encoding cartridge cloned into the unique SstI restriction site within the nifQ coding region.

of nifA gene products (47,29). The A. vinelandii nifA gene is also related to the nifA gene of Rhizobium, though overall the similarity is less than that to the nifA gene of K. pneumoniae. The N-terminal portions of the respective nifA gene products have significant but relatively low interspecies sequence identity. This observation is in line with previous experiments which indicated that a significant portion of the N-terminal region of Bradyrhizobium japonicum (59) and the Rhizobium meliloti nifA gene products (16) is not required for activation of nif promoters. Beyond amino acid residue 210 and through the remainder of the A. vinelandii nifA product there is strong and continuous sequence identity in comparison with the K. pneumoniae nifA gene product. There are two conserved sequences present in all available nifA gene products that have been assigned potential structural significance (47, 29). These structures include a potential ATP-binding site located between amino acid residues 240 and 257 (using the A. vinelandii sequence as a reference; Fig. 16), and a potential DNA binding domain located near the C-terminus of the polypeptide (Fig. 17). The presence of a consensus DNA binding site within the nifA gene product is consistent with its proposed role as a transcriptional activator molecule. Whether or not ATP actually binds the nifA gene product is not yet known. However, such binding could have a regulatory function, since nitrogen fixation is an energy-intensive process.

Identification of an open reading frame preceding nifA

Examination of the available DNA sequence in the region preceding nifA did not reveal the presence of a promoter structure of any known type. However, the COOH terminal

```
MNATIPQRSAKQNPVELYDLQLQALASIARTLSREQQIDELLEQVLAVLHNDLGLLHGLVTISDPEHGAL 70
        *               *   *   ***       * **  ****    **     *     *
--MIHKSDSDTTVRRFDLSQQFTAMQRISVVLSRATEASKTLQEVLSVLHNDAFMQHGMICLYDSQQEIL

QIGAIHTDSEAVAQACEGVRYRSGEGVIGNVLKHGNSVVLGRISADPRFLDRLALYDLEMPFIAVPIKNP 140
 * *              *** ***  * **  * * ** *   * ****** ***   ******   *
SIEALQQTEDQTLPGSTQIRYRPGEGLVGTVLAQGQSLVLPRVADDQRFLDRLSLYDYDLPFIAVPLMGP

EGNTIGVLAAQPDCRADEHMPARTRFLEIVANLLAQTVRLVVNIEDGREAAD---ERDELRR-EVRGKYG 207
    ******    *  *  *  ***** **** *** **         *       *       *
HSRPIGVLAAHAMARQEERLPACTRFLETVANLIAQTIRLMILPTSAAQAPQQSPRIERPRACTPSRGFG

FENMVVGHTPTMRRVFDQIRRVAKWNSTVLVLGESGTGKELIASAIHYKSPRAHRPFVRLNCAALPETLL 277
 *** **  * **   * ** *  *  **** ********** ***  ****   **  ******  **
LENM-VGKSPAMRQIMDIIRQVSRWDTTVLVRGESGTGKELIANAIHHNSPRAAAAFVKFNCAALPDNLL
                                ATP Binding Site

ESELFGHEKGAFTGAVKQRKGRFEQADGGTLFLDEIGEISPMFQAKLLRVLQEGEFERVGGNQTVRVNVR 347
**************** ******* ************ *  ******* ***** *****  * *****
ESELFGHEKGAFTGAVRQRKGRFELADGGTLFLDEIGESSASFQAKLLRILQEGEMERVGGDETLRVNVR

IVAATNRDLESEVEKGKFREDLYYRLNVMAIRIPPLRERTADIPELAEFLLGKIGRQQGRPLTVTDSAIR 417
* ***** ** **  * ********** *  ******  ** *** **  **   *** *   * ***
IIAATNRHLEEEVRLGHFREDLYYRLNVMPIALPPLRERQEDIAELAHFLVRKIAHSQGRTLRISDGAIR

LLMSHRWPGNVRELENCLERSAIMSEDGTITRDVVSLTGVDNESPPLAAPLP-EVNLADETLDDRERVIA 486
***   ****************  ** * * ***       *    *  * *   *  ** * * **
LLMEYSWPGNVRELENCLERSAVLSESGLIDRDVILFNHRDNPPKALASSGPAEDGWLDNSLDERQRLIA

ALEQAGWVQAKAARLLGMTPRQIAYRIQTLNIHMRKI 486
*** ****************** *****   * *
ALEKAGWVQAKAARLLGMTPRQVAYRIQIMDITMPRL
        DNA Binding Site
```

FIGURE 17. Comparison of the A. vinelandii and the K. pneumoniae nifA gene products. The upper numbered sequence represents the A. vinelandii nifA-encoded product and the lower sequence corresponds to the K. pneumoniae nifA-encoded product. The entire nifA sequences for both organisms are shown. Dashes in the respective sequences represent packing characters used to allow optimum alignment. Sequence identities are indicated by astericks. A consensus ATP binding site and a consensus DNA binding site are underlined.

portion of a potential open reading frame was recognized in this region. The termination codon for this open reading frame precedes the proposed nifA initiation codon by 19 base pairs (Fig. 16). Since the gene encoding the negative regulatory element (nifL) precedes, and is cotranscribed with, nifA in K. pneumoniae (48), we compared the translation product of the identified open reading frame preceding nifA from A. vinelandii with the available K. pneumoniae product sequence. Some sequence identity was revealed by this comparison (Fig. 18). Drummond and Wooton (48) have suggested that the nifL gene product is structurally related to a class of regulatory proteins involved in transcription control, including the ntrB gene product. Ronson et al. (180) have reported that the C4-dicarboxylate regulatory gene product from Rhizobium leguminosarum (dctB gene product), as well as a number of other environmental sensory elements (including ntrB gene product from K. pneumoniae) share significant relationships in terms of primary sequence. Much of this conservation of sequence in confined to the COOH-end of the respective polypeptides. Because it is known that the ntrC product is not required for the activation of nif gene expression in A. vinelandii, it is possible that the product of the open reading frame preceding nifA might serve as an activator of nifA in a fashion analogous to the ntrB-ntrC and the dctB-dctD regulatory elements. If so primary sequence homology is predicted in the COOH-terminal portion of the open reading frame product when it is compared with the ntrB and dctB gene products. Such a comparison (shown in Fig.18) does reveal significant conserved identities among the COOH-ends of the R. leguminosarum dctB gene product, the K. pneumoniae ntrB gene product, and the identified A. vinelandii open reading frame. These conserved residues are the ones that have been recognized as

```
R1DctB VADNGPGIPTEIRKGLFTPFNTSKE---SGLGLGVISKDIVGDYGGRMDVASDS-GGTRFIVQLRKA
       * *******      ** *     *    * ***        *            * * * * *
KpNtrB VEDNGPGIPSHLQDTLFYPMVSGRE---GGTGLGLSIARSLIDQHSGKIEFTSWPGHTEFSVYLPIRK
         * *****  *    * *  *       * * **         * *              *
AV-ORF ITDSGPGIPPDLVLKVFEPFFSTKPPHRVGRGMGLPVVQEIVAKHAGMVHVDTDYREGCRIVVELPFSAST
                    *       *       * ** *   *  ** * *          *   *   *
KpNifL EGWLSLYLNDNVPLLQVRYAHSPDALNSPGKGMELRLIQTLVAYHRGAIELASRPQGGTSLVLRFPLFNTLTGGEQ
```

FIGURE 18. Comparison of the COOH-terminal regions of the R. leguminosarum dctB gene product (R1DctB), the K. pneumoniae ntrB gene product (KpNtrB), the potential open reading frame product which precedes the A. vinelandii nifA gene (AV-ORF) and the K. pneumoniae nifL gene product (KpNifL). Only adjacent sequences are directly compared and sequence identities are indicated by astericks. Dashes indicate packing characters used to achieve optimum alignment.

being generally conserved in environmental sensory elements (48,180). Conclusions regarding the function of the open reading frame product as a potential negative regulatory element (analogous to the nifL gene product) or as a potential environmental sensory element (analogous to the ntrB gene product), however, must await the genetic analysis of this region.

Mutagenesis of the nifA gene

A method for the site-directed deletion of nif-specific gene fragments from the A. vinelandii chromosome and a direct selection mutagenesis strategy for mutagenizing nif genes in A.vinelandii have been described in the **Material and Methods.** Using these procedures, two mutant strains deficient in nifA were isolated. One mutant strain has an in-frame deletion that removes the coding region for amino acid residues 71 through 327 (DJ140, nifA), and the other has the same region deleted and replaced with a kanamycin resistance encoding gene cartridge (DJ143, nifA::kan^r). Hybrid plasmids pDB155 and pDB167, respectively, were used for these mutant strain constructions (see Fig. 15). Neither mutant strain is capable of diazotrophic growth in Burk's nitrogen-free medium. Two-dimensional gel analysis of extracts from nitrogenase-derepressed DJ140 (nifA) revealed that the nitrogenase structural components do not accumulate in this strain (Fig. 19). This result demonstrates that the nifA gene product from A. vinelandii is required for the expression or the accumulation of the nitrogenase structural gene products.

An A. vinelandii mutant strain (UW1) pleiotrophically deficient in the accumulation of nitrogenase components was previously isolated and characterized (197). Results from two

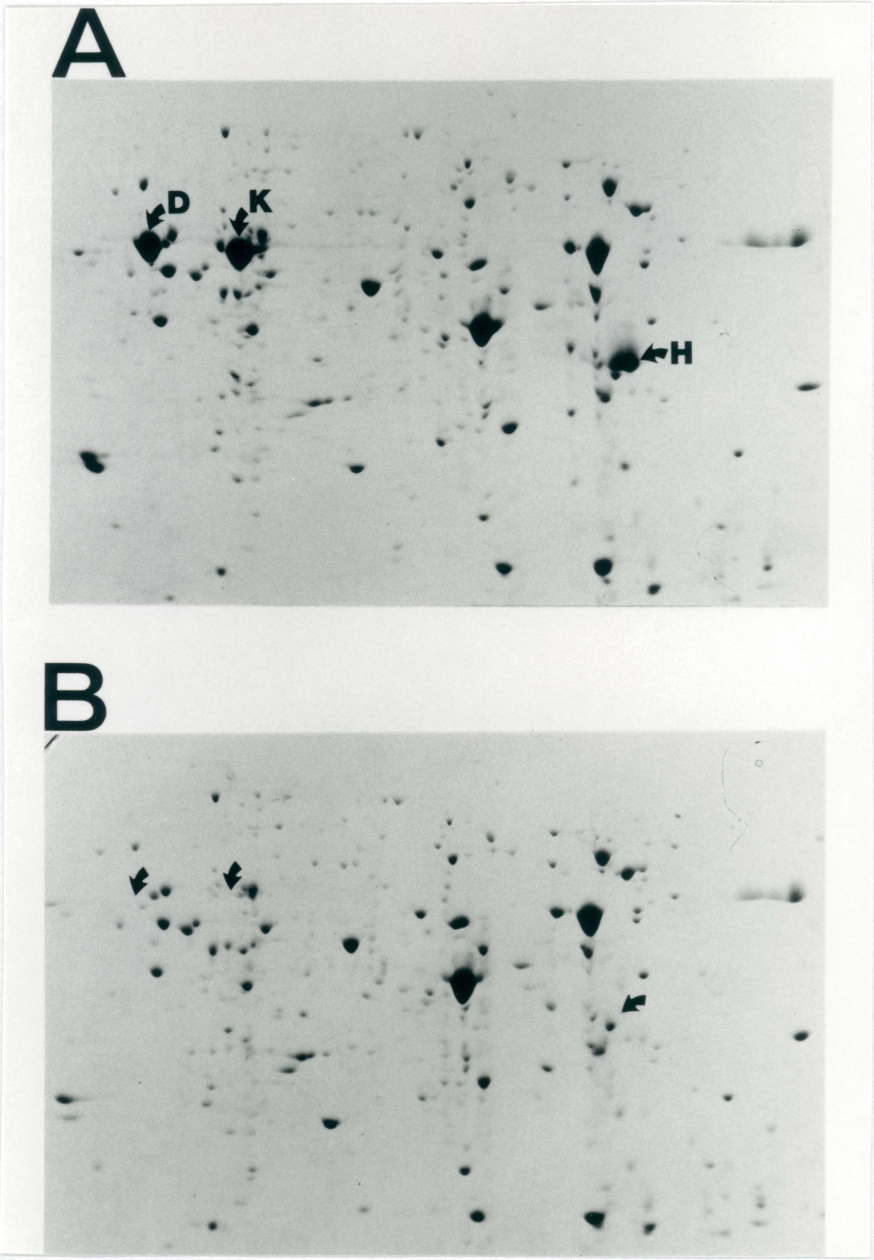


FIGURE 19. Two-dimensional gel analysis of extracts from nitrogenase-derepressed wild type A. vinelandii OP (Panel A) and mutant strain DJ140 (nifA). The nitrogenase structural conponents: H (Fe protein), D (MoFe protein α-subunit) and K (MoFe protein β-subunit) are indicated by arrows in Panel A. Note that spots corresponding to H,D and K are missing in the mutant strain extract shown in Panel B. The isoelectric focusing dimension is left to right (basic to acidic) and the size dimension is up to down (large to small).

factor crosses indicated that the mutation in UW1 is linked to another A. vinelandii nif-specific mutation that is carried in the mutant strain UW45 (17). It is known that the mutation in UW45 is located within the nifB gene (101). We therefore performed marker rescue experiments using purified DNA preparations of plasmid pDB150 and the deletion derivative of that plasmid (pDB155) to determine if the UW1 strain is altered within the nifA gene. It was possible to transform competent cells of UW1 to prototrophy using pDB150 DNA but not pDB155 DNA (see Fig. 15 for a comparison of pDB150 and pDB155). Thus, strain UWI is altered within the nifA gene and we propose to designate this mutation nifA1.

Summary

It is now established that a nifA gene product that is structurally and functionally related to the K. pneumoniae nifA gene product is encoded on the A. vinelandii genome. In a separate study, Santero et al. (185) have confirmed these results and also discuss the potential participation of the nifA gene product in regulation of the expression of alternative nitrogenases in A. vinelandii. Santero et al. (185) also present evidence for the existance of another nif-specific regulatory element that they propose to call nfrX. The specific roles of the products of nfrX, nifA and the open reading frame preceding nifA in regulating nitrogenase expression in A. vinelandii remain to be elucidated.

The available cloned nifA-specific DNA fragments, the nifA deletion strain described here, plus the previously described gene replacement technique for the genetic manipulation of A. vinelandii (23) now permit the site-directed mutagenesis of the A. vinelandii nifA

gene. Such experiments should help to test the various structural and functional predictions that have emerged from the interspecies comparisons of nifA gene products.

SITE-DIRECTED MUTAGENESIS OF THE NIFE POLYPEPTIDE

Construction of mutant strains.

The A. vinelandii mutant strain used in this study were constructed using a site-directed mutagenesis/gene replacement strategy described previously (22, 40). The basic steps of this strategy are depicted in Fig. 20. First, strain DJ228 was constructed which is deleted for part of the nifE-coding sequence (amino acids 15 through 344) including the amino acid residues targeted for substitution. In addition, this deletion is replaced by a kanamycin resistance-encoding cartridge. Isolation of this strain was performed as described previously for the isolation of the nifF and nifA deletion plus insertion mutation strains (DJ60 and DJ143, respectively). Competent cultures of wild type A. vinelandii OP were transformed with a hybrid plasmid carrying the nifE deletion and kanamycin resistance determinant insertion. Double reciprocal recombination events were directly selected for by plating on Burk's medium supplemented with ammonium acetate and kanamycin. Removal of a large portion of the nifE-coding sequence renders this mutation strain phenotypically Nif$^-$, which is essential for its use in the gene replacement procedure.

Site-directed mutation vectors were constructed in the bacteriophage cloning vector M13 using the procedure described in **Materials and Methods.** The recombinant M13 mutation vectors contained a 1.6 kilobase pair SphI fragment extending from 340 base pairs upstream of the nifE-coding sequence through 1292 base pairs into the nifE-coding region (which corresponds to 431 amino acids of the predicted amino acid sequence of NifE). Specific

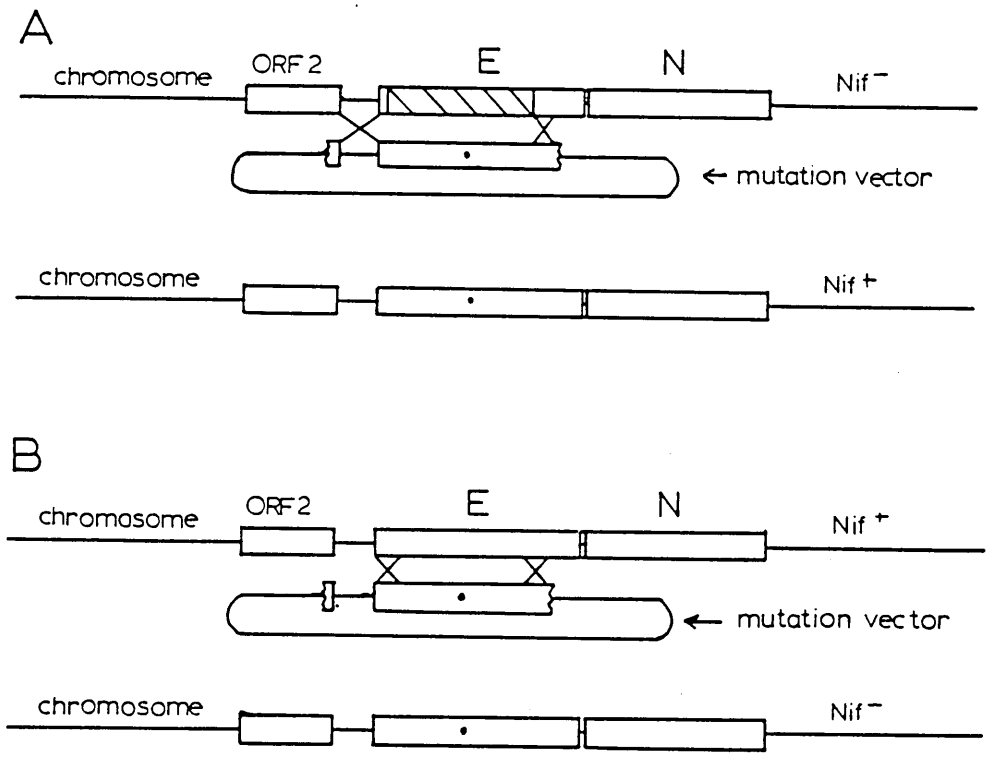


FIGURE 20. Strategy for the construction of site-directed mutantion strains. Introduction of a single point mutation, indicated by a dot on the mutation vector, into the A. vinelandii nifE coding sequence leads to either a Nif⁺ or a Nif⁻ phenotype in the resultant mutant strain. A) Mutations that do not cause a Nif⁻ phenotype are introduced into the A. vinelandii chromosome by transforming a Nif⁻ strain which carries a deletion within a nif structural gene (indicated by crosshatching). B) Mutations that result in a Nif⁻ phenotype are introduced into the A. vinelandii genome by transforming the wild type strain to Nif⁺

nucleotide changes were made in the recombinant vector by oligonucleotide-directed mutagenesis to generate specific amino acid substitutions within the nifE-coding sequence as described in **Materials and Methods.** Double stranded recombinant M13 mutation vectors containing single, double or triple amino acid substitutions were used to transform competent cultures of the nifE deletion plus insertion mutation strain. Double reciprocal recombination between the homologous regions of the recombinant nifE fragment within the mutation vector DNA and the nifE mutated chromosomal DNA from strain DJ228 results in the restoration of a complete nifE-coding sequence with an amino acid substitution(s). Thus, recombinants were screened for their diazotrophic growth capabilities on Burk's medium lacking a fixed nitrogen source.

If the amino acid substitutions at particular residue abolished nifE function, recombinants would remain Nif$^-$ and would be incapable of growth on Burk's medium. Mutant strains possessing the substitution of an essential amino acid residue can be isolated using the indirect selection scheme of congression which has been described in detail in **Materials and Methods** (see Panel B of Fig. 20). The recombinants carrying a substitution of an essential amino acid residue are identified by their ability to grow only on the media containing a fixed nitrogen source.

All site-directed mutant strains will be designated as in the following example: **'E153SC'** where 'E' represents the nif gene carrying the amino acid substitution, '153' represents the position of the substituted residue in the A. vinelandii predicted amino acid sequence, 'S' is the one letter abbreviation designating the amino acids residue in the wild type sequence and 'C' is the one letter abbreviation for the newly substituted amino acid residue.

Mutation strains containing two amino acid residue substitutions will be written in the same manner with each mutation being individually designated (for example E153SC-160KQ).

Rationale for Amino Acid Substitutions

A working model was developed in this laboratory for the assignment of potential metallocluster domains in the MoFe protein, one of the components of the nitrogenase complex. The basic criteria of this model have been previously described (22, 40). The MoFe protein is an $\alpha_2\beta_2$ tetrameric protein which contains at least two distinct types of metalloclusters, the P clusters and the FeMo-cofactors (M clusters) (116, 171, 196). The MoFe protein is believed to contain four P clusters, which may be [4Fe-4S] type clusters, and two FeMo-cofactors, which are complex clusters containing Fe, S and Mo in an unknown arrangement (116). The FeMo-cofactor is proposed to be located at the active site of the MoFe protein (77,116). Correlation of the quantitative metallocluster extrusion studies of the MoFe protein with the MoFe protein interspecies and intersubunit comparisons and with the MoFe protein subunits (NifD and NifK) and FeMo-cofactor biosynthetic gene product (NifE and NifN) comparisons were used for the formulation of this model. The spectroscopic properties of the metalloclusters also provided valuable information. In this study the nifE-gene product was altered by site-directed mutagenesis to more closely resemble the MoFe protein at a region in the amino acid sequence of the NifE product proposed to function in FeMo-cofactor binding.

a) MoFe protein metallocluster extrusion requirements.

Information regrading the identity of the metallocluster ligands can be obtained by considering that the solvents required for the extrusion of a particular metallocluster probably imitate the amino acid residues which function as ligands. Additional information about metallocluster ligation in the MoFe protein has come from the examination of the spectroscopic properties of the metalloclusters. Because the P clusters can be extruded from the MoFe protein by treatment with thiols in a denaturing solvent, cysteinyl mercaptide groups are proposed to function as P cluster ligands (116). The [4Fe-4S] centers of ferredoxins, which are known to be ligated by cysteinyl thiol linkages, can also be released from the protein with thiol treatment in a denaturing solvent, providing additional indirect support for the thiol ligation to the P clusters of the MoFe protein (24, 71). However, the unusual spectroscopic properties of the P clusters suggest that they exist in a different environment than the [4Fe-4S] centers in ferredoxin, and consequently the P clusters are not expected to be exclusively ligated by cysteinyl ligands (116).

The FeMo-cofactors are extruded from the MoFe protein by acid base treatment, which destroys the P clusters, followed by extraction into N-methylformamide (NMF) (196). That the amide containing compounds NMF and formamide facilitate FeMo-cofactor release suggests amide mediated ligation to the FeMo-cofactor. Spectroscopic studies also suggest that amide containing amino acid residues (His Asn, Gln) may function as FeMo-cofactor ligands (230). At least one cysteinyl mercaptide ligand to FeMo-cofactor has been proposed based on the reaction of a single thiolate per Mo atom (152).

b) MoFe protein interspecies and intersubunit comparisons

Spectroscopic studies, extrusion experiments and biochemical reconstitution studies suggest that the P clusters and the FeMo-cofactors are not physically associated in the MoFe protein (116, 196, 57, 71, 234, 248, 205, 133, 49, 210). This observation allows the assignment of specific regions in the primary sequence as either P cluster binding regions or FeMo-cofactor binding domains. Potential metallocluster binding regions have been targeted partially from the interspecies and intersubunit comparisons. Comparison of the amino acid sequences of the α- and β-subunits of the MoFe protein deduced from the nucleotide sequences of nifD and nifK from a number of different organisms (Fig. 21 and 22) revealed extensive sequence identities. Conservation of the secondary and tertiary structure is also suggested by computer assisted secondary structure predictions and by the conservation of residues (Pro and Gly) often located at reverse turns (22). Five Cys residues (Cys62, Cys88, Cys154, Cys183, Cys275) are invariantly conserved among all known α-subunits, and three Cys residues (Cys70, Cys95, Cys153) are conserved among all known β-subunits. Because of the extrusion data and the spectroscopic data implicating cysteinyl mercaptide as ligands to both the P clusters and the FeMo-cofactor, the conservation of Cys residues takes on greater significance. However, the conserved cys residues account for only 16 of the 18 cys residues necessary for total thiol ligation to the P clusters. This strongly suggests that other residues must function in both P cluster and FeMo-cofactor ligation.

Intersubunit comparisons of the amino acid sequences of the MoFe α- and β-subunits also revealed considerable sequence identities. The first three conserved cys residues in

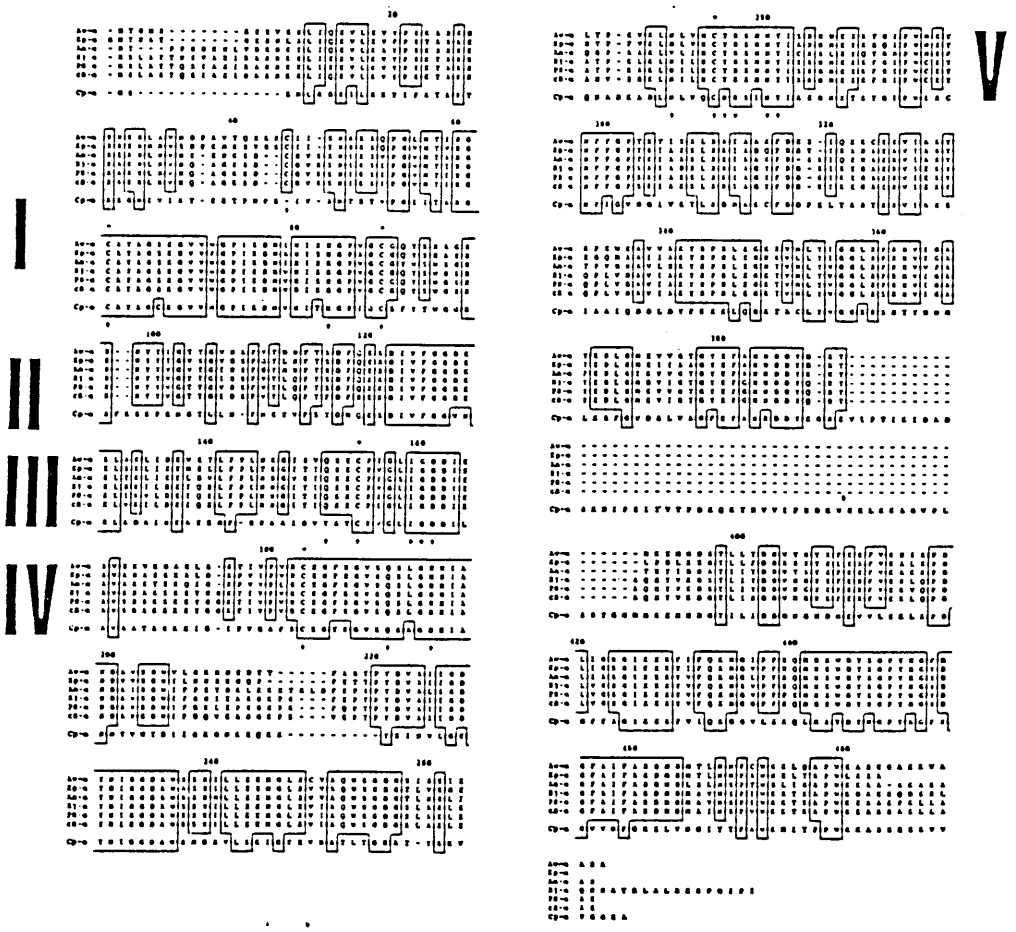


FIGURE 21. Alignment of the MoFe protein α-subunit sequences from A. vinelandii (Av), K. pneumoniae (Kp), Anabaena 7120 (An), B. japonicum (Rj), Rhizobium sp. Parosponia (PR), cowpea Rhizobium strain 1Rc78 (cR), and C. pasteurianum (Cp) (see text for references). Amino acid residues conserved in all seven organisms or in the six organisms excluding C. pasteurianum are boxed. Dashed lines represent alignment adjustments to provide best fit. The five conserved cysteine residues are indicated by triangles above sequence comparisons. Numbering refers to the A. vinelandii sequence. Region I refers to residues 60-100, Region II refers to residues 115-139, Region III refers to residues 148-163, Region IV refers to residues 180-206 and Region V refers to residues 270-288.

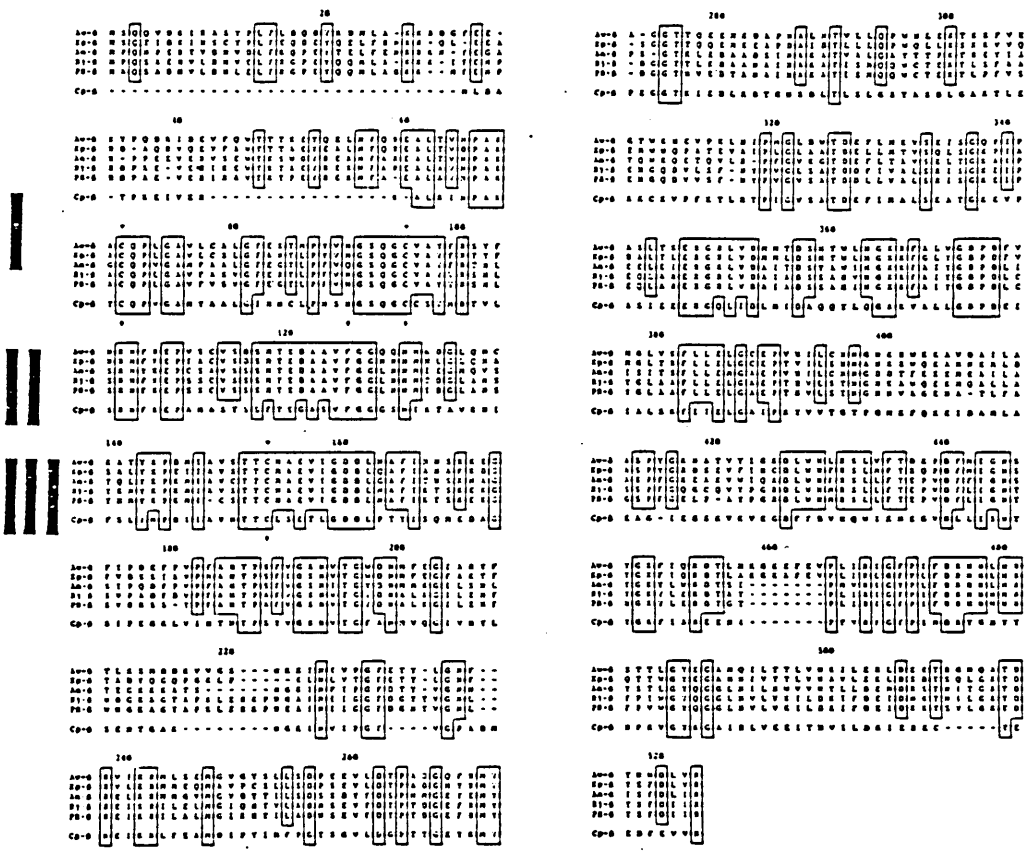


FIGURE 22. Alignment of the MoFe protein β-subunit sequences from A. vinelandii (Av), K. pneumoniae (Kp), Anabaena 7120 (An), B. japonicum (Rj), Rhizobium sp. Parasponia (PR), and C. pasteurianum (Cp). Amino acid residues conserved in all six organisms or in the five organisms excluding C. pasteurianum are boxed. Dashed lines represent alignment adjustments to provide best fit. The three conserved cysteine residues are indicated by triangles above the sequence comparison. Amino acid residues that were substituted in this study are indicated by arrows below the sequence comparison. Numbering refers to the A. vinelandii sequence. Regions I, II and III refer to residues 65-100, 110-135 and 147-162, respectively.

the α-subunit (Cys62, Cys88, Cys154) and the three conserved residues in the β-subunit (Cys70, Cys95, Cys153) are conserved sequentially and spatially.

c)Comparison of the MoFe protein subunits and the FeMo-cofactor biosynthetic gene products NifE and NifN

Comparison of the predicted amino acid sequence of the nifD-encoded α-subunit and the nifK-encoded β-subunit with the nifE-encoded product and the nifN-encoded product, respectively, revealed extensive regions of sequence identity (Fig.23) Four of the five conserved cys residues (Cys62, Cys88, Cys154, Cys275) in the MoFe protein α-subunit are also conserved in the NifE protein (Cys37, Cys62, Cys124, Cys250). One of the three conserved residues in the MoFe β-subunit (Cys95) is conserved in the NifN protein (Cys44). These amino acid conservations led to the proposal that the nifE- and nifN-gene products form a complex having a composition analogous to the MoFe protein, which functions as a scaffold for the biosynthesis of the FeMo-cofactor prior to insertion into the cofactorless-MoFe protein (23). The following evidence supports the formation of such a complex: 1) the MoFe protein is not required for the in vitro or in vivo biosynthesis of FeMo-cofactor (174), 2) the in vitro synthesis of the FeMo-cofactor is dependent on the presence of NifE and NifN (199), and 3) the NifEN protein complex was recently purified as an $\alpha_2\beta_2$ tetramer (162).

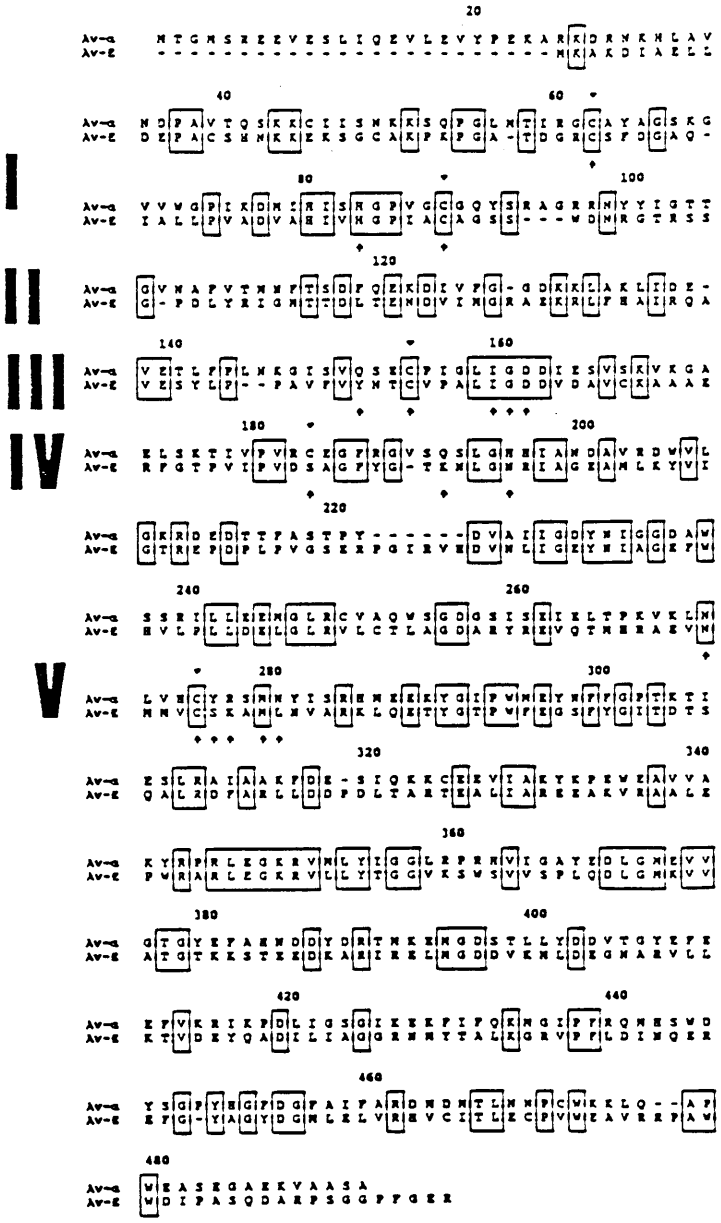


FIGURE 23. Comparison of the MoFe protein α-subunit and the nifE-encoded polypeptide. Dashed lines represent alignment adjustments to provide best fit. Numbering refers to the α-subunit sequence. The five conserved cysteine residues within the α-subunit are indicated by a triangle above the sequence comparison. Amino acids that were substituted in this study are indicated by arrows below the sequence comparison. Roman numerals refer to α-subunit regions as defined in Fig. 21.

d) Model for the assignment of potential MoFe protein metallocluster binding domains.

The P clusters and the FeMo-cofactors are proposed to be located in distinct regions of the MoFe protein (248, 205, 49, 210, 117, 116, 171, 133). Mossbauer spectroscopy indicates that the four P clusters have similar polypeptide environments, and therefore, four structurally similar regions are predicted to accommodate P cluster binding (248, 205, 133). Although no repeated sequences are present in either of the MoFe protein subunits, there are regions of the α-subunit which are similar to regions of the β-subunit, as previously discussed. Because the MoFe protein has an $\alpha_2\beta_2$ configuration, these conserved regions between the subunits provide four structurally similar environments and consequently are proposed to function as P cluster binding domains. The presence of a conserved Cys residues in each of these regions provides additional support for this model.

The two remaining conserved cys residues in the α-subunit of the MoFe protein (Cys 183 and Cys275) are proposed to function in FeMo-cofactor ligation. Evidence for this is that the regions around both of these cys residues is highly conserved in all known α-subunits, and there are significant sequence identity when these regions in the α-subunit are compared to the corresponding region in the nifE gene product. In addition. there are a number of potential N-donor ligands flanking Cys183 and Cys275 in the α-subunit (Gln191, His195, His196, Asn199, His274, Asn 281). This model also predicts that the FeMo-cofactor is contained mostly, or perhaps entirely, within the α-subunit of the MoFe protein (22).

The NifEN protein complex is proposed to form a scaffold on which the FeMo-cofactor is synthesized (23). Because the FeMo-cofactor must be able to escape the NifEN complex

during maturation of the MoFe protein, the regions of the MoFe protein and the NifEN protein which associate with the FeMo-cofactor are expected to be structurally similar but not identical so that they are functionally distinct. The FeMo-cofactor is predicted to bind more tightly to the MoFe protein than to the NifEN complex and the tightness of the association between the cofactor and the polypeptides is dependent upon the nature of the amino acid residues interacting with the FeMo-cofactor in each of these proteins. Furthermore, the model also predicts that changing the environment around the FeMo-cofactor in the NifE protein to more closely duplicate the environment around FeMo-cofactor in the MoFe protein could result in the trapping of the FeMo-cofactor on the NifEN complex.

Analysis of Mutant Strains

In this study, amino acid substitutions within the nifE gene product were made such that this polypeptide more closely resembled the corresponding MoFe protein α-subunit sequence in the region around Cys183. This is one of a number of different approaches which can provide information about potential FeMo-cofactor binding regions within the MoFe protein. If the amino acid residues which are important in ligation coordination or orientation of the FeMo-cofactor in the MoFe protein are substituted into the NifE protein in the corresponding locations, the environment around FeMo-cofactor in the NifEN complex might duplicate the MoFe protein closely enough to result in the trapping of FeMo-cofactor on the NifEN complex. The wild type NifEN complex and FeMo-cofactor are expected to form a weak association to allow FeMo-cofactor to escape the complex and

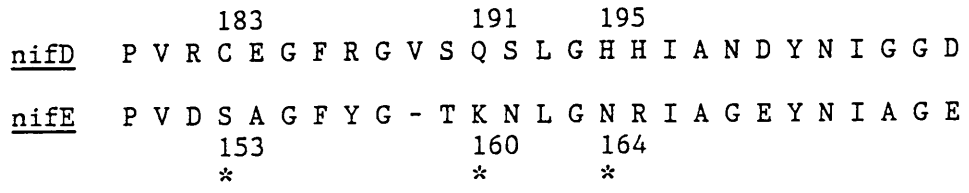


FIGURE 24. Comparison of the MoFe protein α-subunit and the nifE-encoded polypeptide in the Region IV where the site directed mutations were made in nifE. The stars indicate the amino acid residues which were changed in the NifE polypeptide. These residues were changed to the analagous residues in the MoFe protein α-subunit sequence indicated above.

associate with cofactorless MoFe protein. The following amino acid residues in NifE were systematically changed to the corresponding amino acid residues in the MoFe protein α-subunit: E-Ser-153 was changed to a Cys (corresponding to α-Cys-183), E-Lys-160 was changed to a Gln (corresponding to α-Gln-191) and E-Asn-164 was changed to a His residue (corresponding to α-His-195). The rationale behind targeting these amino acid substitutions was their identification as potential FeMo-cofactor coordinating ligands in the MoFe protein α-subunit (refer to previous section). The Cys residue at position 183 in the α-subunit is conserved among all known α-subunits (Fig. 21). Substitution of α-Cys-183 with either an Ala or a Ser residue results in severely reduced diazotrophic growth capabilities and MoFe protein activities suggesting that this Cys residue is important for function (19,22). Substitution of the α-Gln-191 with a glu residue completely abolishes diazotrophic growth, but nitrogenase-derepressed crude extracts of this strain (D191QE) exhibited significant levels of acetylene reduction activity (20% of wild type) (19). The difference in substrate utilization in D183CS and D191QE must be a result of their abilities to recognize or reduce different substrates. Because FeMo-cofactor is the proposed site of substrate binding and reduction, this feature is consistent with the hypothesis that the FeMo-cofactor environment was changed in the MoFe proteins of these mutant strains.

Additional experiments were performed to change residues α-Gln-191 and α-His-195 to the corresponding amino acid residues in NifE (19,22). Because NifE is predicted to have a similar structure as the α-subunit, particularly at the FeMo-cofactor binding region, and because FeMo-cofactor is predicted to bind less tightly to NifE that to the α-subunit, these amino acid substitutions were predicted to test the functionality of the α-Gln-191 and α-

His-195 in FeMo-cofactor binding without causing severe global structural perturbations in the MoFe protein. When α-Gln-191 was changed to a lys residue (corresponding to E-Lys-160) and α-His-195 was changed to an asn residue (corresponding to E-Asn-164), diazotrophic growth was abolished. However, nitrogenase-derepressed crude extracts from both of these mutant strains reduced acetylene to ethylene and ethane. Nitrogenase-derepressed crude extract of the wild type strain reduces acetylene exclusively to ethylene. In addition, both mutant strains D191QK and D195HN exhibited EPR spectra changed in both line shape and g value relative to that of the wild type strain (190). The interpretation of these results is that substitution of Lys191 and His195 into the α-subunit causes an alteration in the region providing ligands to FeMo-cofactor.

The above results suggest that the region around Cys183 (specifically, residues Gln191 and His195) of the MoFe protein α-subunit is involved in FeMo-cofactor binding in the MoFe protein. By performing the analogous experiments to alter the NifE polypeptide to more closely duplicate the MoFe protein at this region, additional information about the functions of these residues might be obtained. If the model is correct, as the MoFe protein FeMo-cofactor binding site is more closely duplicated in NifE, diazotrophic growth capabilities of the resultant mutant strains will progressively decrease. The nifE site-directed mutant strains constructed in this study (Fig. 24) and their Nif phenotypes are shown in Table 5. Fig. 25 shows a comparison of the diazotrophic growth rates of these mutant strains. The results appear to be consistent with what the model predicts. That is, in general, the strains containing multiple amino acid residue substitutions (E153SC-160KQ, E160KQ-164NH, and E153SC-160KQ-164NH) are more limited in their diazotrophic growth capabilities than are

TABLE 5. Growth characteristics of the nifE site-directed mutant and wild type strains.

Strain	Nif Phenotype	Doubling time (h)
wild type	+	2.5
E153SC	+	2.5
E160KQ	+	3.5
E164NH	+	2.5
E153SC-160KQ	+	3.5
E153SC-164NH	+	5.3
E160KQ-164NH	+	5.3
E153SC-160KQ-164NH	+	5.3

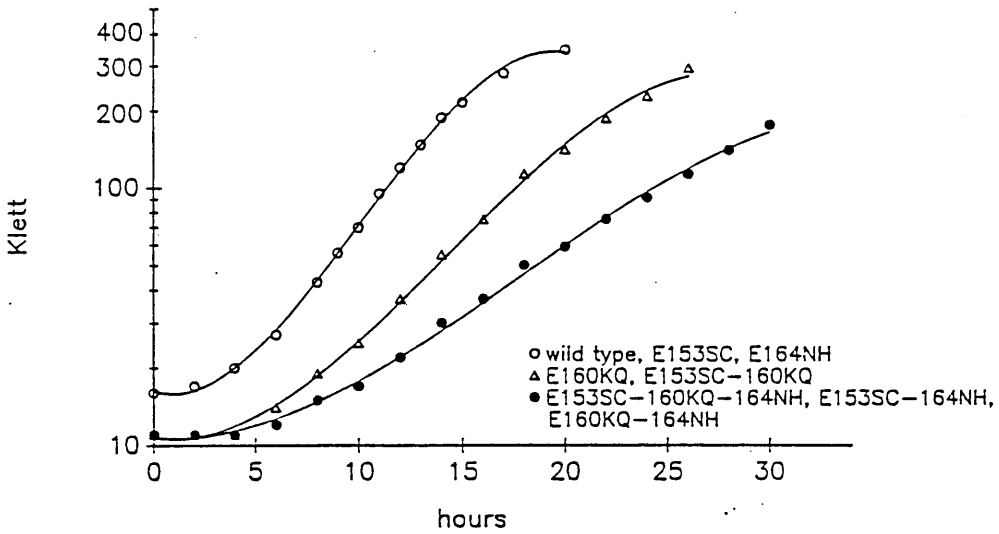


FIGURE 25. Diazotrophic growth of A. vinelandii wild type and nifE site-directed mutation strains.

the strains containing single amino acid residue substitutions (E153SC, 164NH, 160KQ). However, although the diazotrophic growth rates of some of these mutation strains is reduced all strains maintained the nitrogen fixing capabilities. One explanation for this result is that the binding region in the NifEN complex does not duplicate the FeMo-cofactor binding domain in the MoFe protein closely enough to completely trap the FeMo-cofactor on the NifEN complex. Another possibility is that the model is incorrect and the altered NifE protein is less capable of performing its function in the biosynthesis of the FeMo-cofactor.

Mutant strain E153SC and E164NH exhibit wild type diazotrophic growth rates. However, E153SC-E164NH exhibits significantly reduced diazotrophic growth relative to the wild type strain. A possible explanation for this result is that the Cys residue at position 153 and the His residue at position 160 in NifE are individually unable to bind FeMo-cofactor and cause a detectable change in binding. However, together these residues may provide an environment in the mutant NifE protein which associates more tightly with FeMo-cofactor than the wild type NifE protein. Substitution of an additional residue (E-Lys-160) with a Gln residue does not appear to generate an environment even more conducive to FeMo-cofactor binding. However, E-Gln-160 in the mutant strain E160KQ appears to associate with FeMo-cofactor in some fashion to retard its release from the NifEN complex.

In a previous experiment several of the amino acid residues around the conserved Cys residue at position 250 in the NifE protein (corresponding to α-Cys-275 in the MoFe protein) were changed to the corresponding residues in the MoFe protein (241). Cys275

in the MoFe protein α-subunit is the other Cys residue (in addition to α-Cys-183) proposed to provide a potential thiol ligand to the FeMo-cofactor. Substitution of Cys275 with seven different residues (Ser, Ala, Asp, Glu, Val, Thr and Gly) results in a strain which exhibits no diazotrophic growth or MoFe protein activity (19,194). These experiments further demonstrate the essential nature of this single Cys amino acid residue. When the corresponding Cys residue in NifE (E-Cys-250) was substituted to a Ser residue, the resulting nifE mutation strain is incapable of diazotrophic growth. This result strongly suggests that E-Cys-250 and α-Cys-275 are vital for NifE and MoFe protein functions, respectively.

Another nifE mutation strain was constructed in which the Val residue at position 249 was changed to a His (corresponding to α-His-274), the Ser residue at position 251 was changed to a Tyr (corresponding to α-Tyr-276) and the His residue at position 252 was changed to an Arg (corresponding to α-Arg-277) (241). By making these amino acid substitutions the environment around E-Cys-250 in NifE theoretically more closely duplicates the corresponding region (around α-Cys-275) in the MoFe protein α-subunit. This nifE mutation strain exhibited significantly reduced diazotrophic growth rates. The explanation for this result, as predicted by the model, is that the FeMo-cofactor is bound more tightly to the mutant NifEN protein in this strain than to the wild type NifEN complex because the FeMo-cofactor binding region more closely resembles the FeMo-cofactor binding region in the MoFe protein. Because this strain retains some diazotrophic growth capability, the FeMo-cofactor cannot be completely trapped on the mutant NifEN complex. In order to obtain a mutant strain which does accumulate a NifEN complex

which completely traps FeMo-cofactor, several other nifE mutant strains should be constructed. Regions around α-cys-183 and α-cys-275 in the MoFe protein α-subunit are proposed to bind to different regions of the FeMo-cofactor (see Fig. 26). Consequently, a NifEN complex which is altered in both of these corresponding regions (E-Ser-153 and E-Cys-250) to resemble the regions in the MoFe protein may bind FeMo-cofactor significantly tighter than the NifEN complexes altered at only one of these regions. Two additional nifE mutation strains, E153SC-160KQ-164NH-249VH and E153SC-160KQ-164NH-249VH-251SY, could be constructed to test this hypothesis from the nifE mutant DNAs made in this study.

To determine whether these altered NifEN complexes are binding FeMo-cofactor more tightly, the in vitro FeMo-cofactor biosynthesis procedure (recently developed by Shah and co-worker (199) could be used. In this assay cell-free extracts of mutants with complementary defects in FeMo-cofactor biosynthesis are mixed, and FeMo-cofactor biosynthesis is initiated by the addition of dithionite, molybdate and ATP. If the nifE mutant strains constructed in this study and the previous study (241) bind FeMo-cofactor more tightly than the wild type NifEN complex, then extracts from these strains should competitively inhibit in vitro FeMo-cofactor biosynthesis. These experiments have been initiated by placing a deletion and kanamycin resistance-encoding insertion into the nifB-coding region in combination with the nifE mutations in strains E153SC-160KQ-164NH and E248VH-251SY-252HR. NifB is one of the gene products essential for FeMo-cofactor biosynthesis. Its function is unknown, but it may be involved in a step preceding the involvement of the NifEN protein. NifB mutation strains accumulate a cofactorless-MoFe

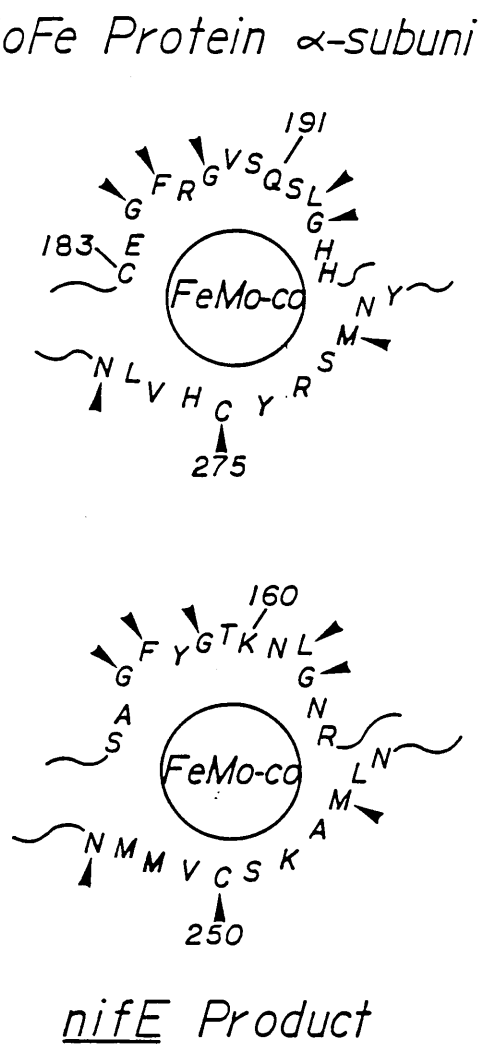


FIGURE 26. Model for FeMo-cofactor binding in the MoFe protein α-subunit. Letters indicate the one letter code for the respective amino acids. Residues α-cys-183 and α-gln-191 are located within Region IV of the α-subunit of the MoFe protein and α-cys-275 is located in Region V. Biochemical and spectroscopic data suggest that these residues interact with the FeMo-cofactor in the MoFe protein. E-lys-160 in NifE corresponds to α-gln-160 in the MoFe protein and E-cys-250 corresponds to α-cys-275. Preliminary data presented in this study suggest that E-lys-160 and E-cys-250 are involved in FeMo-cofactor binding in the NifEN protein complex. The NifEN complex is proposed to be the site of FeMo-cofactor biosynthesis. Arrows indicate amino acid residues which are conserved in the corresponding regions of the MoFe protein α-subunit and the NifE polypeptide.

protein. Inactivation of NifB is important because it will ensure that the altered NifEN product added to the experimental samples in the in vitro FeMo-cofactor biosynthetic assay will not be "pre-loaded" with FeMo-cofactor. If the altered NifEN protein donated from the nifE mutant strain extracts inhibits FeMo-cofactor biosynthesis in the assay, this will indicate that the available Mo is sequestered by the altered FeMo-cofactor biosynthetic complex.

Conclusions

The working model developed in this laboratory for the assignment of potential metallocluster ligands in the MoFe protein predicts that the regions around α-Cys-183 and α-Cys-275 of the MoFe protein α-subunit comprises the FeMo-cofactor binding domains. The NifEN protein complex appears to be structurally similar to the MoFe protein particularly around the proposed FeMo-cofactor binding domain. The NifEN protein complex is predicted to function as scaffold on which FeMo-cofactor is synthesized prior to insertion into immature MoFe protein. Because the FeMo-cofactor must escape the NifEN complex, the binding of FeMo-cofactor to the NifEN complex must be markedly weaker than the binding of FeMo-cofactor to the MoFe protein. This model predicts that if the NifEN complex is altered to more closely duplicate the MoFe protein at the FeMo-cofactor binding domain, FeMo-cofactor may be partially trapped on the altered NifEN protein complex. The results presented in this study are consistent with this prediction. However, the possibility exists that the model is incorrect and that the NifEN complex has another role in FeMo-cofactor biosynthesis. To verify the model several experiments should

be performed. Because the nifE mutation strains made in this study retained some level of diazotrophic growth, a strain in which diazotrophic growth is completely abolished should be isolated. Isolation of such a strain will provide additional indirect support for the identification of the region around α-Cys-183 and α-Cys-275 as the FeMo-cofactor binding domain. The altered NifEN complex should be tested for its ability to function in the in vitro FeMo-cofactor biosynthetic assay. The strains containing an inactive NifB in combination with the altered NifEN protein complex (DJ463 and DJ465) have been constructed and await analysis. The altered NifEN protein complex should be purified and the metal content analyzed. A purification scheme for the isolation of the wild type NifEN complex has been developed (162) and can be applied to the purification of the altered NifEN complexed to determine if Mo is bound tight enough to be co-purified. Determination of the EPR spectra of the altered NifEN products in whole cells and crude extracts of the respective mutant strains could provide information about the similarity of the environments around FeMo-cofactor in the NifEN protein and the MoFe protein. The analysis of the nifE mutant strains constructed in this study should help identify the amino acid residues which function as ligands to the FeMo-cofactor in the MoFe protein, may provide clues to the structure of the FeMo-cofactor, and may help determine the functions of some of the other FeMo-cofactor biosynthetic gene products.

LITERATURE CITED

1. Albright, L. M., E. Huala, Q. Gu, and F. E. Ausubel. 1988. Regulation of Rhizobium meliloti NifA function. In Nitrogen Fixation: hundred Years After. H. Bothe, F. J. de Bruijn and W. E. Newton (eds). 345-349.

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

3. Arnott, M. C. Sidoti, S. Hill, M. Merick. 1989 Deletion analysis of the nitrogen fixation regulatory gene nifL of Klebsiella pneumoniae. Arch. Microbiol. 151:180-182.

4. Austin, S. N. Henderson and R. Dixon. 1987 Requirements for transcriptional activation in vitro of the nitrogen-regulated glnA and nifLA promoters from Klebsiella pneumoniae: dependence on activator concentration. Mol. Microbiol. 1:92-100.

5. Bagdasarian, M., R. Lurz, B. Rucket, F. C. H. Franklin, M. M. Bagdarsarian, J. Frey and K. N. Timmis. 1981. Specific purpose plasmid cloning vectors. II Braod host range, high copy number, RSF1010 derived vectors and a host-vector system for gene cloning in Pseudomonas Gene (Amst) 16:237-249.

6. Badenoch-Jones, J., T. A. Holton, C. M. Morrison, K. E. Scott and J. Shine. 1989. Structural and functional analysis of nitrogenase genes from the broad host range Rhizobium strain ANU240. Gene 77: 141-153.

7. Bali, A., S. Hill, E. Santero, A. Toukdarian, J. Wamsley and C. Kennedy. 1988. NifA and NifC activate separate nitrogen fixation pathways in Azotobacter. In Nitrogen Fixation:Hundred Years After. H. Bothe, F. J. de Bruijn and W. E. Newton (eds). 316.

8. Bennemann, J. R., D. C. Yoch, R. C. Valentine and D. I. Arnon. 1969. The electron transport system in nitrogen fixation by AzotobacterI. Azotoflavin as an electron carrier. Proc. Natl. Acad. Sci. USA 64:1079-1086.

9. Bennemann, J. R., D. C. Yoch, R. C. Valentine and D. I. Arnon. 1971. The electron transport system in nitrogen fixation by Azotobacter. Requirements for NADPH-supported nitrogenase activity. Biochim. Biophys. Acta 226:205-212.

10. Bennemann, J. R. , D. C. Yoch, R. C. Valentine and D. I. Arnon. 1976. The electron transport system in nitrogen fixation by Azotobacter. Azotoflavin as an electron carrier. Proc. Natl. Acad. Sci. USA 64:1079-1086.

11. Bennett, L. T., F. C. Cannon and D. R. Dean. 1989. Nucleotide sequencing and mutagenesis of nifA from Azotobacter vinelandii. Molec. Micro. 2:315-321.

12. Bennett, L. T., M. R. Jacobson, D. R. Dean. 1988. Isolation, nucleotide sequencing and mutagenesis of the nifF gene encoding flavodoxin from Azotobatcer vinelandii. J. Biol. Chem. 263: 1364-1369.

13. Beynon, J., M. Cannon, A. Ally, V. Buchanan-Wollaston, R. Setterquist, D. R. Dean and F. Cannon. 1989. The nucleotide sequence of the nifT, nifY, nifX and nifW genes of Klebsiella pneumoniae Nucl. Acids Res. 16:9860

14. Beynon, J., A. Ally, M. Cannon, F. Cannon, M. Jacobson, V. Cash and D. Dean. 1987. Comparative organization of nitrogen fixation specific genes from Azotobacter vinelandii and Klebsiella pneumoniae: DNA sequence of the nifUSV genes. J. Bacteriol. 169:4042-4029.

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

16. Beynon, J., M. K Williams and F. Cannon. 1988. Expression and functional analysis of the Rhizobium meliloti gene. EMBO J. 7:7-14.

17. Bishop, P.E. and W. J. Brill. 1977. Genetic analysis of Azotobacter vinelandii mutant strains unable to fix nitrogen. J. Bacteriol. 130:954-956.

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

19. Brigle, K. E. 1989. Studies on the structure and function of various nif and nif-associated gene products encoded within the Azotobacter vinelandii nif gene cluster. (PhD thesis, VPI&SU, Dept. Anaerobic Microbiol.)

20. Brigle, K. E. and D. R. Dean. 1988. Revised nucleotide sequence of Azotobacter vinelandii nifE gene. Nucl. Acids. Res. 16:5214.

21. Brigle, K. E., W. E. Newton and D. R. Dean. 1985. Complete nucleotide sequence of the Azotobacter vinelandii structural gene cluster. Gene 37:37-44.

22. Brigle, K. E., R. A. Setterquist, D. R. Dean, J. S. Cantwell, M. C. Weiss and W. E. Newton. 1987. Site-directed mutagenesis of the nitrogenase MoFe protein of Azotobacter vinelandii. Proc. Natl. Acad. Sci. USA 84:7066-7069.

23. Brigle, K. E., M. C. Weiss, W. E. Newton and D. R. Dean. 1987. Products of the FeMo-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.

24. Bruschi, M. and F. Guerlesquin. 1988. Structure, function and evolution of bacterial ferredoxins. FEMS Microbiol. Rev. 54:155-176.

25. Buck, M. and W. Cannon. 1989. Mutations in RNA polymerase recognition sequence of Klebsiella pneumoniae nifH promoter permitting transcriptional activation in the absence of NifA binding to upstream activator sequences. Nucl. Acids Res. 7:2597-2612.

26. Buck, M., S. Miller, M. Drummond, and R. Dixon. 1986. Upstream activator sequences are present in the promoters of nitrogen fixation genes. Nature 320:370-378.

27. Bui, P. T. and L. E. Mortenson. 1986. Mechanism of the enzymatic reduction of nitrogen. The binding of adenosine 5'triphosphate and cyanide to the nitrogen reducing system. Proc. Natl. Acad. Sci. USA 61:1021-1027

28. Buikema, W. J., J. A. Klingensmith, S. L. Gibbons and F. M. Ausubel. 1987. Conservation of structure and location of Rhizobium meliloti and Klebsiella pneumoniae nifB genes. J. Bacteriol. 169:1120-1126.

29. Buikema, W. J., W. W. Szeto, P.V. Lemley, W. H. Orme-Johnson and F. E. Ausubel. 1895. Nitrogen fixation specific regulatory genes of Klebsiella pneumoniae and Rhizobium meliloti share homology with the general nitrogen regulatory gene ntrC of Klebsiella pneumoniae. Nucl. Acids Res. 13:4539-4555.

30. Bulen, W. A. and J. R. LeCompte. 1966. The nitrogenase system from Azotobacter: Two enzyme requirement for nitrogen reduction, ATP-dependent hydrogen evolution and ATP hydrolysis. Proc. Natl. Acad. Sci USA 56:979-986.

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

32. Cannon, M., F. Cannon, V. Buchanan-Wollaston, D. Ally, A. Ally and J. Beynon. 1989. Nucl. Acids. Res

33. Chang, C. L., L. C. Davis, M. Rider and D. J. Takemoto. 1988. Characterization of nifH mutations from Klebsiella pneumoniae. J. Bacteriol. 170:4015-4022.

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

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

36. Conradson, S. D., B. B. Burgess, W. E. Newton, L. E. Mortenson, K. O. Hodgson. 1987. Structural studies of the molybdenum site in the MoFe protein and its FeMo-cofactor by EXAFS. J. Am. Chem. Soc. 109:7507-7517.

37. Dalton, H. and J. R. Postgate. 1969a. Effect of oxygen on the growth of Azotoabcter chroococcum in batch and continuous cultures. J. Gen. Microbiol. 54:463-473.

38. Davis, L. C., V. K. Shah and W. J. Brill. 1975. Nitrogenase VII. Effect of component ratio, ATP and H_2 on distribution of electron to alternative substrates. Biochim. Biophys. Acta 403:67-78.

39. Dean, D. R. and K. E. Brigle. 1985. Azotobatcer vinelandii nifD- and nifE-encoded polypeptides share structural homology. Proc. Natl. Acad. Sci. USA 56: 5720-5723.

40. Dean, D. R., K. E. Brigle and W. E. Newton. 1988. Site-directed mutagenesis of the nitrogenase MoFe protein. In Nitrogen Fixation: Hundred Years After. H. Bothe, F. J. de Bruijn and W. E. Newton (eds) pp.107-114.

41. Deistung, J., F. C. Cannon, M. C. Cannon, S. Hill and R. N. F. Thorneley. 1985. Electron transfer to nitrogenase in Klebsiella pneumoniae. Biochem. J. 231:743-753.

42. Deistung, J. and R. N. F. Thorneley. 1986. Electron transfer to nitrogenase. Biochem. J. 239:69-75.

43. Dilworth, M. J., R. R. Eady, R. L. Robson and R. W. Miller. 1987. Ethane formation from acetylene as a potential test for vanadium nitrogenase in vivo. Nature 327:6118-6119.

44. Dixon, R. A., S. Austin, M. Buck, M. Drummond, S. Hill, A. Holtel, S. MacFarlane, M. Merrick, S. Minchin. 1987. Genetics and regulation of nif and related genes in Klebsiella pneumoniae. Phil. Trans. R. Soc. London B 317:147-158.

45. Dixon, R. A., N. C. Henderson and S. Austin. 1988. DNA supercoiling and aerobic regulation of transcription from the Klebsiella pneumoniae nifLA promoter. In Nitrogen Fixation: Hundred Years After. H. Bothe, F. J. de Bruijn and W.E. Newton (eds). p. 306.

46. Drummond, M. H. 1985. The base sequence of the nifF gene of Klebsiella pneumoniae and homology of the predicted amino acid sequence of its protein product to other flavodoxins. Biochem J. 232:891-896.

47. Drummond, M. H., P. Whitty and J. Wooton. 1986. Sequence and domain relationships of ntrC and nifA from Klebsiella pneumoniae. Homologies to other regulatory proteins. EMBO J. 5:441-447.

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

49. Dunham, W. R., W. R. Hagen, A. Braaksma, H. J. Grande and H. Haaker. 1985. The importance of quantitative Mossbauer spectroscopy of the MoFe protein from Azotobacter vinelandii. Eur. J. Biochem.146:497-501.

50. Eady, R. R., R. L. Robson, T. H. Richardson, R. W. Miller and M. Hawkins. 1987. The vanadium nitrogenase of Azotoabcter chroococcum. Purification and properties of the VFe protein. Biochem J. 244:197-207.

51. Earl, C. D. C. W. Ronson and F. E. Ausubel. 1987. Genetic and structural analysis of the Rhizobium meliloti fixA, fixB, fixC and fixX genes. J. Bacteriol. 169:1127-1136.

52. Eidsness, M. K., A. M. Flank, B. E. Smith, A. C. Flood, C. D. Garner, S. P. Cramer. 1986. EXAFS of Klebsiella pneumoniae nitrogenase MoFe protein from wild type and nifV mutant strains. J. Am. Chem. Soc. 108:2746-2747.

53. Elmerich, C. M. Calimand, C. Vielle, F. Delorne and M. de Zamaroczy. 1988. Nitrogen fixation genes in Azospirillum. In Nitrogen Fixation: Hundred Years After. H. Bothe, F. J. de Bruijn and W. E. Newton (eds). pp.327-331.

54. Emerich, D. W. and R. H. Burris. 1976. Interaction of heterologous nitrogenase components that generate catalytically inactive complexes. Proc. Natl. Acad. Sci.

USA 73:4369-4373.

55. Emerich, D. W. and R. H. Burris. 1978. Complementary functioning of the component proteins of nitrogenase from several bacteria. J. Bacteriol 134:936-943.

56. Emerich, D. W., T. Ljones and R. H. Burris. 1978. Nitogenase: properties of the catalytically inactive complex between the Azotobacter vinelandii MoFe protein and the Clostridium pasteurianum Fe protein. Biochim. Biophys. Acta. 527:359-396.

57. Euler, W. B., J. Martinsen, J. W. McDonald, G. D. Watt, Z. -C. Wang. 1984. Double integration and titration of the EPR signal in the MoFe protein of Azotobacter vinelandii. Biochem. 23:3021-3024.

58. Filler, W. A., R. M. Kemp, J. C. Ng, T. R. Hawkes, R. A. Dixon and B. E. Smith. 1986. The nifH gene product is required for the synthesis or stability of the iron molybdenum cofactor of nitrogenase from Klebsiella pneumoniae. Eur. J. Biochem. 160:371-377.

59. Fischer, H.-M., A. Alverez-Morales and H. Hennecke. 1986. The pleiotrophic nature of symbiotic regulatory mutants. Bradyrhizobium japonicum nifA gene in involved in control of nif gene expression and formation of the determinant of symbiosis EMBO J. 5:1165-1175.

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

61. Golden, J. W., S. J. Robinson and R. Haselkorn. 1985. Rearrangement of nitrogen fixation genes during heterocyst differentiation in the cyanobacterium Anabaena. Nature. 314:419-423.

62. Gronger, P., S. Manian, H. REilander, M. O'Connel, U. B. Priefer, A. Puhler. 1987. Organization and partial sequence of a DNA region of Rhizobium leguminosarum symbiotic plasmid pRL6J1 containing genes fixA, fixB, fixC, nifA, nifB and a novel open reading frame. Nucl. Acids. Res. 15:31-49.

63. Gubler, M. and H. Hennecke. 1986. fixABC genes are essential for symbiotic and free-living microaerobic nitrogen fixation. FEBS Lett. 200:186-192.

64. Gubler, M., T. Zurcher and H. Hennecke. 1989. The Bradyrhizobium meliloti fixABC operon: identification of fixX and of a 5' mRNA region affecting the level of the fixBCX transcript.

65. Gussin, G., C. W. Clive and F. M. Ausubel. 1986. Regulation of nitrogen fixation genes. Ann. Rev. Genet. 20:567-591.

66. Haaker, H. 1988. Biochemistry and physiology of nitrogen fixation. BioEssays 9:112-117.

67. Haaker, H. and J. Klugkist. 1987. The bioenergetics of electron transport to nitrogenase FEMS Microbiol. Rev. 46:57-71.

68. Haaker, H. and C. Veeger. 1977. Involvement of the cytoplasmic membrane in nitrogen fixation by Azotobacter vinelandii. Eur. J. Biochem. 77:1-10.

69. Hagemann, R. V. and R. H. Burris. 1978. Nitrogenase and nitrogenase reductase associate and dissociate with each catalytic cycle. Proc. Natl. Acad. Sci. USA 75:2699-2702.

70. Hagen, W. R., R. R. Eady, W. R. Dunham, H. Haaker. 1985. A novel S=3/2 EPR signal associated with native Fe proteins of nitrogenase FEBS Lett. 189:250-254.

71. Hagen, W. R., H. Wassink, R. R. Eady, B. E. Smith and H. Haaker. 1987. Quantitative EPR of an S=3/2 system in thionine-oxidzed MoFe proteins of nitrogenase: a redefinition of the P-cluster concept. Eur. J. Biochem. 169:457-465.

72. Hales, B. J., E. E. Case, J. E. Morningstar, M. J. Dzeda and L. A. Mauterer. 1986. Isolation of a new vanadium-containing nitrogenase from Azotobacter vinelandii. J. Biochem. 25:7751-7755.

73. Hanemaaijer, R., A. Janssen, A. de Kok and C. Veeger. 1988. The dihydrolipoyltransacetylase component of the pyruvate dehydrogenase complex from Azotobacter vinelandii. Molecular cloning and sequence analysis. Eur. J. Biochem. 174:593-599.

74. Haselkorn, R. 1978. Heterocysts. Annu Rev. Plant. Physiol. 29:319-344.

75. Hausinger, R. P. and J. B. Howard. 1982. The amino acid sequence of the nitogenase iron protein from Azotobatcer vinelandii. J. Biol. Chem. 257:2483-2490.

76. Hausinger, R. P. and J. B. Howard. 1983. Thiol reactivity of the nitrogenase iron protein from Azotobacter vinelandii. J. Biol. Chem. 258:13486-13492.

77. Hawkes, T. R., P. A. McLean and B. E. Smith. 1984. Nitrogenase from nifV mutants of Klebsiella pneumoniae contain an altered form of the iron molybdenum cofactor. Biochem J. 217:317-321.

78. Hawkes, T. R. and B. E. Smith. 1983. Purification and characterization of the inactive MoFe protein (NifB-Kp1) of the nitrogenase from nifB mutants of

Klebsiella pneumoniae. Biochem. J. 209:43-50.

79. Hertig, C., R. Y. Li, A.-M. Louarn, A.-M. Garnerone, M. David, J. Butut, D. Kahn and P. Boistard. 1989. Rhizobium meliloti regulator gene fixJ activates transcription of Rhizobium meliloti nifA and fixK in Escherichia coli. J. Bacteriol 171:1736-1738.

80. Hill, S. J. W. Drozd and J. R. Postgate. 1972. Environmental effects on the growth of nitrogen-fixing bacteria. J. Applies Chem. Biotechnol. 22:541-558.

81. Hill, S. and E. P. Kavanaugh. 1980. Roles of nifF and nifJ gene products in electron transport to nitrogenase in Klebsiella pneumoniae. J. Bacteriol. 141:470-475.

82. Hirschman, J., P. -K. Wong, K. Sei, J. Keener and S. Kustu. 1985. Products of nitrogen regulatory genes ntrA and ntrC of enteric bacteria activate glnA transcription in vitro. Evidence that the ntrA product is a sigma factor. Proc. Natl. Acad. Sci USA 82:7525-7529.

83. Holland, D., A. Zilberstein, A. Zamir and J. L. Sussman. 1987. A quantitative approach to sequence comparisons of nitrogenase MoFe protein α- and β-subunits including the newly sequenced nifK gene from Klebsiella pneumoniae. Biochem. J. 247:277-285.

84. Hoover, T. R., J. Imperial, J. Liang, P. W. Ludden and V. K. Shah. 1988. Dinitrogenase with altered substrate specificity results from the use of homocitrate analogues in the in vitro synthesis of the iron molybdenum cofactor. Biochem. 27: 3647-3652.

85. Hoover, T. R., J. Imperial, J. Liang, P. W. Ludden and V. K. Shah. 1989. Homocitrate is a component of the iron molybdenum cofactor of nitrogenase. Biochem. 28:2768-2771.

86. Hoover, T. R., A. D. Robertson, R. L. Cerny, R. N. Hayes, J. Imperial, V. H. Shah and P. W. Ludden. 1987. Identification of the V factor needed for synthesis for the iron molybdenum cofactor of nitrogenase as homocitrate. Nature 329:855-857.

87. Horton, R. D. and S. T. Lord. 1986. In vitro methylation doubles the efficiency of oligonucleotide-directed mutagenesis. Nucl. Acids Res. 14: 5112.

88. Howard, J. B., R. Davis, B. Moldenhauer, V. L. Cash, D. R. Dean. 1989. Fe-S cluster ligands are the only cysteines required for nitrogenase Fe-protein activites. J. Biol. Chem. 264:11270-11274.

89. Howard, K. S., P. A. McLean, F. B. Hansen, P. V. Lemley, K. S. Koblan and 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.

90. Huynh, B. H., M. T. Henzel, J. A. Christner, R. Zimmerman, W. H. Orme-Johnson and E. Munck. 1980. Nitrogenase XII. Mossbauer studies of the MoFe protein from Clostridium pasteurianum W5. Biochem. Biophys. Acta 623: 124-138.

91. Imam, S. and R. R. Eady. 1980. Nitrogenase of Klebsiella pneumoniae: reductant-independent AYP hydrolysis and the effect of pH on the efficiency of the coupling of ATP hydrolTP hydrolysis to substrate reduction. FEBS Lett. 110:35-38.

92. Imperial, J., R. A. Ugalde, V. K. Shah and W. J. Brill. 1984. Role of the nifQ gene product in the incorporation of molybdenum into nitrogenase in Klebsiella pneumoniae. J. Bacteriol. 158:187-194.

93. Imperial, J., R. A. Ugalde, V. K. Shah and W. J. Brill. 1985. Mol$^-$ mutants of Klebsiella pneumoniae requiring high levels of molybdate for nitrogenase activity. J. Bacteriol. 163:1285-1287.

94. Ioannidis, I. and M. Buck. 1987. Nucleoride sequence of Klebsiella pneumoniae nifD gene and the predicted animo acid sequence of nitrogenase MoFe protein. Biochem J. 247:287-291.

95. Itakura, K., J. J. Rossi and R. B. Wallace. 1984. Synthesis and use of synthetic oligonucleotides. Ann. Rev. Biochem. 53:323-356.

96. Jacobson, M. R., K. E. Brigle, L. T. Bennett, R. A. Setterquist, M. S. Wilson, V. L. Cash, J. Beynon, W. E. Newton and D. R. Dean. 1989. Physical and genetic map of the major nif gene cluster from Azotobacter vinelandii. J. Bacteriol 171:1017-1027.

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

98. Jacobson, M. R., R. Premakumar and P. E. Bishop. 1986. Transcriptional regulation of nitrogen fixation by molybdenum in Azotobacter vinelandii. J. Bacteriol.176:480-487.

99. Jeng, D. Y., J. A. Morris and L. E. Mortenson. 1970. The effect of reductant in inorganic phosphate release from adenosine 5'triphosphate by ourified nitrogenase of Clostridium pasteurianum.

100. Jensen, B. B. and R. H. Burris. 1985. Effect of High pN_2 and high pD_2 on NH_3 production, H_2 evolution and HD formation by nitrogenases. Biochem. 24:1141-1147.

101. Joerger, R. D. and P. E. Bishop. 1988. Nucleotide sequence and genetic analysis of the nifB-nifQ region from Azotobacter vinelandii. J. Bacteriol. 170:1475-1487.

102. Joerger, R. D. and P. E. Bishop. 1988a. Bacterial alternative nitrogen fixation systems. Crit. Rev. Microbiol. 16:1-14.

103. Joerger, R. D., M. R. Jacobson and P. E. Bishop. 1989. Two nifA-like genes required for the expression of alternative nitrogenases by Azotobacter vinelandii. J. Bacteriol. 171:3258-3267.

104. Joerger, R. D., M. R. Jacobson, R. Premakumar, E. D. Wolfinger and P. E. Bishop. 1989. Nucleotide sequence and mutational analysis of the structural genes (anfHDGK) for the second alternative nitrogenase from Azotobacter vinelandii. J. Bacteriol. 171:1075-1086.

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

106. Kaluza, K. and H. Hennecke. 1984. Fine structure analysis of the nifDK operon encoding the α- and β-subunits of dinitrogenase from Rhizobium japonicum. Mol. Gen. Genet. 196:35-42.

107. Kaminski, P.A., F. Norel, N. Desnoues, A. Kush, G. Salzano, C. Elmerich. 1988. Characterization of the fixABC gene region of Azorhizobium caulinodans ORS571 and identification of a new nitrogen fixation gene. Mol. Gen. Genet. 214:496-502.

108. Kennedy, C. and R. L. Robson. 1983. Activation of nif gene expression in Azotobacter by the nifA gene product of Klebsiella pneumoniae. Nature 301:626-628.

109. Kim, Y.-M, K.-J Ahn, T. Beppu and T. Uozumi. 1986. Nucleotide sequence of the nifLA operon of Klebsiella oxytoca NG13 and characterization of the gene products. Mol. Gen. Genet. 205:253-259.

110. Klugkist, J., H. Haaker and C. Veeger. 1986. Studies on the mechanism of electron transport to nitrogenase in Azotobacter vinelandii. Eur. J. Biochem. 155:41-46.

111. Klugkist, J., J. Voorberg, H. Haaker and C. Veeger. 1986. Characterization of three different flavodoxins from Azotobacter vinelandii. Eur. J. Biochem. 155:33-40.

112. Knight, E., Jr., A. J. D'Eustachio and R. W. F. Hardy. Flavodoxin: a flavoprotein with ferredoxin activity in Clostridium pasteurianum. Biochem. Biophys. Acta 113:626.

113. Kofoid, E. C. and J. S. Parkinson. 1988. Transmitter and receiver modules in bacterial signaling proteins. Proc. Natl. Acad. Sci. USA 85:4981-4985.

114. Kratzsch, G. 1987. Hellriegel's work at Bernburg. Phil. Trans. R. Soc. London B 317:107-109.

115. Krol, A. J. M., G. J. Holtelez, B. Roozendaal, and A. van Kammen. 1982. On the operon structure of the nitrogenase genes of Rhizobium leguminosarum and Azotobacter vinlenadii.Nucl. Acids Res. 10:4147-4157.

116. Kurtz, D. M., R. S. McMillan, B. K. Burgess, L. E. Mortenson and R. H. Holm. 1979. Identification of iron-sulfur centers in the iron molybdenum protein of nitrogenase. Proc. Natl. Acad. Sci. USA 76:4986-4989.

117. Lammers, P. J. and R. Haselkorn. 1983. Sequence of the nifD gene coding for the α-subunit of dinitrogenase from the cyanobacterium Anabaena. Proc. Natl. Acad. Sci. USA 80:4723-4727.

118. Lindahl, P. A., E. P. Day, T. A. Kent, W. H. Orme-Johnson and E. Munck. 1985. Mossbauer, EPR, and magnetization studies of the Azotobatcer vinelandii Fe protein. J. Biol. Chem. 260:11160-11173.

119. Ljones, T. and R. H. Burris. 1972. ATP hydrolysis and electron transfer in the nitrogenase reaction with different combinations of iron protein and molybdenum-iron protein. Biochem. Biophys. Acta 275:93-101.

120. Ljones, T. and R. H. Burris. 1978. Nitrogenase: the reaction between the Fe protein and bathophenanthrolinedisulfonate as probe for interaction with MgATP. Biochem. Biophys. Res. Commun. 80:22-25.

121. Lowe, D. J. and R. N. F. Thorneley. 1984a. The mechanism of Klebsiella pneumoniae nitrogenase action. Pre-steady state kinetics of H_2 formation. Biochem J. 224:877-886.

122. Lowe, D. J. and R. N. F. Thorneley. 1984c. The mechanism of Klebsiella pneumoniae nitrogenase action. The determination of rate constants required for the simulation of the kinetics of N_2 reduction and H_2 evolution. Biochem. J. 224:895-901.

123. Lowery, R. G., C. L. Chang, L. C. Davis, M.-C. McKenna, P. J. Stephens and P. W. Ludden. 1989. Substitution of his from arg101 of dinitrogenase reductase disrupts electron transfer to dinitrogenase. Biochem 28:1206-1212.

124. Lowery, R. G. and P. W. Ludden. 1988. Purification and properties of dinitrogenase reductase ADP-ribosyltransferase from the photosynthetic bacterium Rhodospirillum rubrum. J. Biol. Chem. 263:16714-16719.

125. Lowery, R. G, L. L. Saari and P. W. Ludden. 1986. Reversible regulation of the nitrogenase iron protein from Rhodospirillum rubrum by ADP-ribosylation in vitro. J. Bacteriol. 166:513-518.

126. Ludden, P. W., G. P. Roberts, R. G. Lowery, W. P. Fitzmaurice, L. L. Saari, L. Lehman, D. Lies, D. Woehle, H. Wirt, S. A. Murrell, M. R. Pope and 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 Bruijn and W. E. Newton (eds) 157-162.

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

128. Maniatis, T., A. Jeffrey and D. G. Kleid. 1975. Nucleotide sequence of the rightward operator of the phage Lambda. Proc. Natl. Acad. Sci. USA 72:1184-1188.

129. Martin, A. E., B. K. Burgess, S. E. Iismaa, C. T. Smartt, M. R. Jacobson and D. R. Dean. 1989. Construction and characterization of an Azotobacter vinelandii strain with mutations in the genes encoding flavodoxin anf ferredoxin I. J. Bacteriol. 171:3126-3132.

130. Masephohl, B., W. Klipp, A. Puhler. 1988. Genetic characterization and sequence analysis of the duplicated nifA/nifB gene region of Rhodobacter capsulatus. Mol. Gen. Genet. 212:27-37.

131. Mazur, B. J. and C.-F. Chui. 1982. Sequence of the gene encoding the β-subunit from the blue green alga Anabaena. Proc. Natl. Acad. Sci.USA 79:6782-6786.

132. McLean, P. A. and R. A. Dixon. 1981. Requirement of nifV gene for the production of wild type nitrogenase enzyme in Klebsiella pneumoniae. Nature 292:655-656.

133. McLean, P. A., V. Papefthymiou, W. H. Orme-Johnson and E. Munck. 1987. Isotopic hybrids of nitrogenase. Mossbauer study of the MoFe protein with selective ^{57}Fe enrichment of the P-cluster. J. Biol. Chem 262:12900-12903.

134. McLean, P.A., B. E. Smith and R. A. Dxion. 1983. Nitrogenase of Klebsiella pneumoniae nifV mutants. Biochem. J. 211:589-597.

135. McMaster, G. K. and G. G. Carmichael. 1977. Analysis of single- and double-stranded nucleic acids on polyacrylamide and agarose gels by use gyoxal and acridine orange. Proc. Natl. Acad. Sci. USA 74:4835-4838.

136. Merrick, M. 1988. Organization and regulation of nitrogen fixation genes in Klebsiella and Azotobacter. In Nitrogen Fixation: Hundred Years After. H. Bothe, F. J. de Bruijn and W. E. Newton. pp. 293-302.

137. Merrick, M., J. Gibbons, A. Toukdarian. 1987. Nucleotide sequence of the sigma factor gene ntrA (rpoN) of A. vinelandii: analysis of conserved sequences in NtrA protein Mol. Gen Genet. 210:323-330.

138. Messing, J. 1983. New M13 Vectors for cloning. Methods in Enzymology 101:20-78.

139. Mevarech, M., D. Rice and R. Haselkorn. 1980. Nucleotide sequence of the cyanobacterial nifH gene coding for nitrogenase reductase. Proc. Natl. Acad. Sci. USA 77:6476-6480.

140. Miller, J. H. 1972. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

141. Miller, R. W. and R. R. Eady. 1988. Cyanamide: a new substrate for nitrogenase. Biochim. Biophys. Acta 952:290-296.

142. Moreno-Vivian, C., M. Schehl, B. Masephohl, W. Arnold, W. Klipp. 1989. DNA sequence and genetic analysis of Rhodobacter capsulatus nifENX gene region: Homology between nifX and nifB suggests involvement of NifX in processing of iron molybdenum cofactor. Mol. Gen. Genet. 216:353-363.

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

144. Morgan, T. V.. D J. Lundell and B. K. Burgess. 1988. Azotobacter vinelandii ferredoxin I: cloning, sequencing and mutant analysis. J. Biol. Chem. 263:1370-1375.

145. Morgan, T. V., R. C. Prince and L. E. Mortenson. 1985. Electronchemical titration of the S=3/2 and S=1/2 state of the iron protein of nitrogenase. FEBS Lett. 206:4-8.

146. Mortenson, L. E. 1964. Biochim. Biophys. Acta. 81:413.

147. Mortenson, L. E. and R. N. F. Thorneley. 1979. Structure and function of nitrogenase. Ann. Rev. Biochem. 48:387-418.

148. Munck, E., H. Rhodes, W. H. Orme-Johnson, L. C. Davis, W. J. Brill and V. K. Shah. 1975. Nitrogenase. VIII. Mossbauer and EPR spectroscopy. The MoFe protein component from Azotobacter vinelandii OP. Biochim. Biophys. Acta. 400: 32-53.

149. Murrell, S. A., R. G. Lowery and P. W. Ludden. 1988. ADP-ribosylation of the dinitrogenase reductase from Clostridium pasteurianum prevents inhibition of nitrogenase from Azotobacter vinelandii. Biochem. J. 251:609-612.

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

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

152. Newton, W. E., S. F. Gheller, B. Hedman, K. O. Hodgson and S. M. Lough. 1986. Elicitation of thiomolybdates from the iron-molybdenum cofactor of nitrogenase. Comparison with synthetic FeMoS complexes Eur. J. Biochem. 159:111-115.

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

154. Normand, P. and J. Bousquet. 1989. Phylogeny of nitrogenase sequences in Frankia and other nitrogen-fixing organisms. J. Molec. Evol. 29:436.

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

156. O-Farrell, P. H. 1975. High resolution two-dimensional electrophoresis of protein. J. Biol. Chem. 250:4007-4021.

157. Oka, A, H. Sugisaka and M. Takanami. 1981. J. Mol. Biol. 147: 217-226.

158. Orme-Johnson, W. H., W. D. Hamilton, T. L. Jones, M. 0Y. Tso, R. H. Burris, V. K. Shah and W. J. Brill. 1972. EPR of nitrogenase and nitrogenase components from Clostridium pasteurianum W5 and Azotobatcer vinelandii OP. Proc. Natl. Acad. Sci USA 69:3142-3145.

159. Ow, D. W., S. Venkatesan, D. M. Rothstein, S. E. Brown and F. E. Ausubel. 1983. Promoters regulated by GlnG (NtrC) and NifA gene products share heptameric consensus sequence in -15 region. Proc. Natl. Acad. Sci USA 80:2524-2528.

160. Page, W. J. and von Tigerstom. 1979. Optimal conditions for transformation and Azotobacter vinelandii. J. Bacteriol. 139:1058-1061.

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

162. Paustian, T, D., V. K. Shah and G. P. Roberts. 1989. Purification and characterization of the nifE and nifN gene products from Azotobacter vinelandii mutant UW45. Proc. Natl. Acad. Sci USA 86:6082-6086.

163. Pope, M. R., S. A. Murrell and P. W. Ludden. 1985. Covalent modification of the iron protein of nitrogenase from Rhodospirrillum rubrum by adenosine diphosphorylation of a specific arginine residue. Proc. Natl. Acad. Sci USA 82:3173-3177.

164. Postgate, J. R. 1982. Fundamentals of Nitrogen Fixation. Cambridge Univerisity Press. Cambridge., pg 39.

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

166. Prosser, J., L. Graham and R. L. Maier. 1988. Hydrogen-mediated enhancement of hydrogenase expression in Azotobacter vinelandii. J. Bacteriol. 170:1990-1993.

167. Puhler, A. and W. Klipp. 1981. Fine structure analysis of the gene region for nitrogen fixation (nif) of Klebsiella pneumoniae. in Biology of Inorganic Nitrogen and Sulfur. H. Bothe and A. Trebst. (eds). 276-286.

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

169. Raina, R., M. A. Reddy, D. Ghosal and H. D. Das. 1988. Characterization of the gene for the Fe protein of the vanadium-dependent alternative nitrogenase of Azotobacter vinelandii and construction of a Tn5 mutant. Mol Gen. Genet. 214:121-127.

170. Rawlings, D. E. 1988. Sequence and structural analysis of the α- and β-dinitrogenase subunits of Thiobacillus ferrooxidans. Gene 69:337-343.

171. Rawlings, J., V. K. Shah, J. R. Chisnell, W. J. Brill, R. Zimmerman, E. Munck and W. H. Orme-Johnson. 1978. Novel metal cluster in the iron molybdenum protein of nitrogenase. J. Biol. Chem. 253:1001-1004.

172. Roberts, G. P. and W. J. Brill. 1980. Gene product relationships of the nif regulon of Klebsiella pneumoniae J. Bacteriol. 144:210-216.

173. Roberts, G. P., T. MacNeil, D. MacNeil and W. J. Brill. 1978. Regulation and characterization of protein products coded by the nif (nitrogen fixation) genes of Klebsiella pneumoniae. J. Bacteriol. 136: 267-279.

174. Robinson, A. C., B. K. Burgess and D. R. Dean. 1986, Activity, reconstitution and accumulation of nitrogenase components in Azotobacter vinelandii. Mutant strains containing defined deletions within the nitrogenase structural gene cluster. J. Bacteriol 166:180-186.

175. Robinson, A. C., D. R. Dean and B. K. Burgess. 1987. Iron-molybdenum cofactor biosynthesis in Azotobacter vinelandii requires the iron protein of nitrogenase. J. Biol. Chem. 262:14327-14332.

176. Robson, R. L. 1979. Characterization of an oxygen-stabile nitrogensae complex isolated from Azotobacter chroococcum. Biochem .J. 181:569-575.

177. Robson, R. L., R. R. Eady, T. H. Richardson, R. W. Miller, M. Hawkins and J. R. Postgate. 1986. The alternative nitrogenase of Azotobacter chroococcum is a vanadium enzyme. Nature 322:388-390.

178. Robson, R. L., P. Woodley and R. Jones. 1986. Second gene (nifH*) coding for a nitrogenase iron protein in Azotobatcer chroococcum is adjacent to a gene coding for a ferredoxin-like protein. EMBO J. 5:1159-1163.

179. Robson, R. L., P. Woodley, R. Pau and R. R. Eady. 1988. Cloning and analysis of the structural genes (vnfD,vnfG, and vnfK) for the VFe protein of the nitrogenase from Azotobacter chroococcum. In Nitrogen Fixation: Hundred Years After. H. Bothe, F. J. deBruijn and W. E. Newton (eds). p.232.

180. Ronson, C. W., P. M. Astwood, B. J. Nixon, F. E. Ausubel. 1987. Deduced products of C4-dicarboxylate transport regulatory genes of Rhizobium leguminosarum are homologous to the nitrogen regulatory gene products. Nucl. Acids. Res. 15: 7921-7934.

181. Rosenburg, M. and D. Court. 1979. Regulatory sequences involved in the promotion and termination of RNA transcription. Annu. Rev. Genet. 13:319-353.

182. Saari, L. L., E. W. Triplett and P. W. Ludden. 1984. Purification and properties of the activating enzyme for the iron protein of nitrogenase from the photosynthetic bacterium Rhodospirillum rubrum. J. Biol. Chem. 259:15502-15508.

183. Saito, H. and K. Miura. 1963. Preparation of the tranforming deoxyribonucleic acid by phenol treament. Biochim. Biophys. Acta 72:619-629.

184. Sanger, F., S. Nicklen and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74:5463-5467.

185. Santero, E., A. Toukdarian, R. Humprey and C. Kennedy. 1988. Identification and characterization of two nitrogen fixation regions nifA and nfrX in Azotobacter vinelandii and Azotobacter chroococcum. Mol. Microbiol. 2:303-314.

186. Shapira, S. K., J. Chou, F. C. Richard, M. J. Casadaban. 1983. New versatile plasmid vectors for the expression of hybrid proteins coded by a cloned gene fused to lacZ gene sequences encoding an enzymatically active carboxyl terminal portion of β-galactosidase. Gene (Amst) 25:71-82.

187. Scherings, G., H. Haaker and C. Veeger. 1977. Regulation of nitrogen fixation by Fe-S protein II in Azotobacter vinelandii. Eur. J. Biochem. 77:621-630.

188. Scherings, G., H. Haaker, H. Wassink and C. Veeger. 1983. On the formation of an oxygen tolerant three-component nitrogenase complex from Azotobacter vinelandii. Eur. J. Biochem. 135:591-599.

189. Schuman, J. P., G. M. Waitches and 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.

190. Scott, D. J., H. D. May. W. E. Newton, K. E. Brigle and D. R. Dean. 1989. Role of the nitrogenase MoFe protein α-subunit in iron molybdenum cofactor binding and catalysis. Nature. (submitted).

191. Scott, K. F., B. G. Rolfe and J. Shine. 1981. Biological nitrogen fixation. Primary structure of the Klebsiella pneumoniae nifH and nifD genes. J. Mol Appl. Genet. 1:17-81.

192. Scott, K. F., B. G. Rolfe and J. Shine. 1983. Biological nitrogen fixation: primary structure of the Rhizobium trifolii iron protein gene. DNA 2:149-155.

193. Scott, K. F., B. G. Rolfe and J. Shine. 1983. Nitrogenase structural genes are unlinked in the nonlegume symbiont Parasponia Rhizobium. DNA 2:141-148.

194. Setterquist, R. A. 1989. Site-directed mutagenesis of the nitrogenase MoFe protein from Azotobacter vinelandii. (MS Thesis, VPI&SU, Dept. Anaerobic Microbiol.)

195. Setterquist, R. A., K. E. Brigle, J. Beynon, M. Cannon, A. Ally, F. Cannon and D. R. Dean. 1988. Nucleotide sequence of the nifE and nifN genes from Klebsiella pneumoniae. Nucl. Acids Res. 11:5215.

196. Shah, V. K. and W. J. Brill. 1977. Isolation of an Iron-molybdenum cofactor from nitrogenase. Proc. Natl. Acad. Sci. USA 74:3249-3253.

197. Shah, V. K., L. C. Davis, J. K. Gordon, W. H. Orme-Johnson and W. J. Brill. 1973. III. Nitrogenaseless mutants of Azotobacter vinelandii: activities, cross-reactions and EPR spectra. Biochim. Biophys. Acta. 292:246-255.

198. Shah, V. K., T. R. Hoover, J. Imperial, T. D. Paustian, G. P. Roberts and P. W. Ludden. 1988. Role of nif gene products and homocitrate in the biosynthesis of the iron molybdenum cofactor. In Nitrogen Fixation: Hundred Years After. H. Bothe, F. J. de Bruijn and W. E. Newton (eds) pp.115-120.

199. Shah, V. H., J. Imperial, R. A. Ugalde, P. W. Ludden, W. J. Brill. 1986. In vitro synthesis of the FeMo-cofactor of nitrogenase. Proc. Natl. Acad. Sci. USA 83:1636-1640.

200. 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.

201. Shethna, Y. I., P. W. Wilson and H. Beinhert. 1966. Purification of non-heme iron protein and other electron transport components from Azotobacter vinelandii. Biochim. Biophys. Acta. 113:225-234.

202. Silverstein, R. and W. A. Bulen. 1970. Kinetic studies of nitrogenase-catalyzed hydrogen evolution and nitrogen reduction reactions. Biochem. 9:3809-3815.

203. Simpson,F. B. and R. H. Burris. 1984. A nitrogen pressure of 50 atmosphere does not prevent evolution of hydrogen by nitrogenase. Science 224:1095-1097.

204. Smith, B. E., R. R. Eady, D. J. Lowe and C. Gormal. 1988. The vanadium iron protein of V-nitrogenase from Azotobacter chroococcum contains an iron vanadium cofactor. Biochem. J. 250:299-302.

205. Smith, B. E. and G. Lang. 1974. Mossbauer spectroscopy of the nitrogenase proteins from Klebsiella pneumoniae. Biochem. J. 137:169-180.

206. Souillard, N., M. Magot, O. Possot and L. Sibold. 1988. Nucleotide sequence of regions homologous to nifH (nitrogenase Fe protein) from the nitrogen-fixing archaebacterium Methanococcus thermolithotrophicus and Methanobacterium ivanovii: Evolutionary implications. J. Mol Evol. 27:65-76.

207. Souillard, N. and 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.

208. Stam, H., A. H. Stouthamer and H. W. van Verseveld. 1987. Hydrogen metabolism and energy costs of nitrogen fixation. FEMS Microbiol. Rev. 46:73-92.

209. Steinbauer, J., W. Wenzel and D. Hess. 1988. Nucleotide and deduced amino acid sequences of the Klebsiella pneumoniae nifK gene coding for the β-subunit of the nitrogenase MoFe protein. Nucl. Acids. Res. 16:7199.

210. Stephens, P. J. C. E. McKenna, B. E. Smith, H. T. Nguyen, M. C. McKenna, A. J. Thompson, F. Devlin and J. B. Jones. 1979. Circular dichroism and magnetic circular dichroism of nitrogenase proteins. Proc. Natl. Acad. Sci. USA 76:2585-2589.

211. Stephens, P. E., H. M. Lewis, M. G. Darlson and J. R. Guest. 1983. Nucleotide sequnce of the lipoamide dehydrogenase gene of Escherichia coli K12. Euro. J. Biochem. 135:519-527.

212. Stout, C. D. 1988. 7-Iron ferredxoin revisited. J. Biol. Chem. 263: 9256-9260.

213. Stout, G. H., S. Turley, L. C. Sieker and L. H. Jensen. 1988. Structure of ferredoxin I from Azotobacter vinelandii. Proc. Natl. Acad. Sci. USA 85: 1020-1022.

214. Strandberg, G. W. and P. W. Ludden. 1968. Formation of the nitrogen-fixing enzyme system in Azotobacter vinelandii Can. J. Microbiol. 14: 25-31.

215. Sundaresan, V. and F. E. Ausubel. 1981. Nucleotide sequence of the gene coding for the nitrogenase iron protein from Klebsiella pneumoniae. J. Biol. Chem. 256:2808-2812.

216. Tanaka, M., M. Hanui, K. T. Yasunobu, D. C. Yoch. 1975. The amino acid sequence of the Azotobatcer vinelandii flavodoxin. Biochem. Biophys. Res. Commun. 66:639-644.

217. Thony, B., H.-M. Fischer, D. Anathamatten, T. Bruderer and H. Hennecke. 1987. The symbiotic nitrogen fixation regulatory operon (fixRnifA) of Bradyrhizobium japonicum is expressed aerboically and is subject to a novel NifA-dependent type of activation. Nucl. Acids Res. 15:8479-8499.

218. Thony, B., K. Kaluza and H. Hennecke. 1985. Structural and functional homology between the α- and β-subunits of the nitrogenase MoFe protein as revealed by sequencing the Rhizobium japonicum nifK gene. Mol. Gen. Genet. 198: 441-448.

219. Thorneley, R. N. F. and D. J. Lowe. 1983. Nitrogenase of Klebsiella pneumoniae kinetics of the dissociation of oxidized iron protein from the MoFe protein: identification of the rate-limiting step for substrate reduction. Biochem J. 215:393-403.

220. Thorneley, R. N. F. and D. J. Lowe. 1984b. The mechanism of Klebsiella pneumoniae nitrogenase action. Pre-steady state kinetics of an enzyme bound intermediate in nitrogen fixation of NH_3 formation. Biochem. J. 224:887-894.

221. Thorneley, R. N. F. and D. J. Lowe. 1984d. The mechanism of Klebsiella pneumoniae nitrogenase action. Simulation of the dependencies of H_2 evolution rate on component protein concentration and ratio and sodium dithionite concentration. Biochem. J. 224:903-909.

222. Torok, I. and A. Kondorosi. 1981. Nucleotide sequence of Rhizobium meliloti nitrogenase reductase (nifH) gene. Nucl. Acids Res. 9:5711-5723.

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

224. Tso, M. -Y. and W. J. Burris. 1973. The binding of ATP and ADP by the nitrogenase components from Clostridium pasteurianum. Biochim. Biophys. Acta 309:263-270.

225. Ugalde, R. A., J. Imperial, V. K. Shah and W. J. Brill. 1984. Biosynthesis of the iron molybdenum cofactor in the absence of nitrogenase. L. Bacteriol. 159:888-893.

226. van Lin, B. and H. Bothe. 1972. Flavodoxin from Azotobacter vinelandii Arch. Mikrobiol. 82:155-172.

227. Wahl, R. C. and W. H. Orme-Johnson. 1987. Clostridial pyruvate oxidoreductase and pyruvate-oxidizing enzyme specific to nitrogen fixation in Klebsiella pneumoniae are similar enzymes. J. Biol. Chem. 262:10489-10496.

228. Walker, G. A. and L. E. Mortenson. 1974. Effect of magnesium adenosine 5' triphosphate on the accessibility of the iron of Clostridial azoferredoxin a componetn of nitrogenase. Biochem. 13:2383-2388

229. Walker, G. A. and L. E. Mortenson. 1974. Evidence fot the existence of a fully reduced state of molybdoferredoxin during the functioning of nitrogenase and the order of electron transport from the reduced ferredoxin. J. Biol. Chem. 249:6356-6358.

230. Walter, M. A., S. K. Chapman and W. H. Orme-Johnson. 1986. The nature of amide ligation to the metal sites of FeMo-cofactor. Polyhedron 5:561-565.

231. Wang, S.-Z., J.-S. Chen and J. L. Johnson. 1987. Nucleotide and deduced amino acid sequence of nifD encoding the α-subunit of nitrogenase MoFe protein of Clostridium pasteurianum. Nucl. Acids Res. 15:3935.

232. Wang, S.-Z., J,-S. Chen and J. L. Johnson. 1988a. Distinct features of the α- and β-subunits of nitrogenase MoFe protein of Clostridium pasteurianum: an analysis of amino acid sequences. Biochem. J. 27:2800-2810.

233. Watt, G. D. and A. Burns. 1977. Kinetics of dithionite ion utilization and ATP hydrolysis for reactions catalyzed by the nitrogenase complex from Azotobacter vinelandii. Biochem. 16:264-270.

234. Watt, G. D., A. Burn, D. L. Tennent. 1981. Redox and spectral properties of oxidized MoFe protein from Azotobacter vinelandii. Biochem. 20:7272.

235. Watt, G. D. and J. W. MacDonald. 1985. Electron paramagnetic resonance spectrum of the iron protein of nitrogenase: existence of a g = 1.94 spectral component and its effect on spin quantization. Biochem 24: 7226-7231.

236. Watt, G. D., Wang, Z. -C. and R. R. Knotts. 1986. Redox, reactions of a nucleotide binding to the iron protein of Azotobacter vinelandii. Biochem. 25:8156-8162.

237. Weber, G., H. Reilander and A. Puhler. 1985. Mapping and expression of a regulatory nitrogen fixation gene (fixD) from Rhizobium meliloti. EMBO J. 4:2751-2756.

238. Weinman, J. J., F. F. Fellows, P. M. Greshoff, J. Shine and K. F. Scott. 1984. Structural analysis of the genes encoding the MoFe protein of nitrogenase in the Parasponia Rhizobium strain ANU289. Nucl. Acids Res. 12: 8329-8344.

239. Westphal, A. H. and A. de Kok. 1988. Lipoamide dehydrogenase from Azotobacter vinelandii: Molecular cloning, organization and sequence analysis of the gene. Eur. J. Biochem. 172:299-305.

240. Wherland, S., B. K. Burgess, E. I. Steifel and W. E. Newton. 1981. Nitrogenase reactivity: effect of the component ratio on nitrogenase flow and distribution during nitrogen fixation. Biochem. 20:5132-5140.

241. Wilson, M. S. 1988. Mutagenesis of nifE and nifN from Azotobacter vinelandii. (MS Thesis, VPI&SU, Dept. Anaerobic Microbiol.

242. Wilson, P. W., W. W. Umbreit and S. B. Lee. 1938. Mechanism of symbiotic nitrogen fixation IV. Specific inhibition by hydrogen. Biocem J. 32:2084-2095.

243. Wong, P. K. D. Papham, J. Kenner and S. Kustu. 1987. In vitro transcription of the nitrogen fixation regulatory operon nifLA of Klebsiella pneumoniae. J. Bacteriol. 169:2876-2880.

244. Yates, M. G. 1972. Electron TRansport to nitrogenase in Azotobacter chroococcum: Azotobacter flavodoxin hydroquinone as an electron donor. FEBS Lett. 27:63-67.

245. Yoch, D. C., Bennemann, J. R., R. C. Valentine and D. I. Arnon. 1969. Electron transport system in Azotobacter vinelandii. II Isolation and function of a new type of ferredoxin. Proc. Natl. Acad. Sci. USA 64:1404-4110.

246. Yoch, D. C. and D. I. Arnon. 1972. Two biologically active ferredoxins from the aerobic nitrogen-fixing bacterium Azotobacter vinelandii. J. Biol. Chem. 247: 4514-4520.

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

248. Zimmerman, R., E. Munck, W. J. Brill, V. K. Shah, M. T. Henzl, J. Rawlings and W. H. Orme-Johnson. Nitrogenase X: Mossbauer and EPR on reversibly oxidized MoFe protein from Azotobacter vinelandii OP. Nature of the iron clusters. Biochim. Biophys. Acta 537: 185-207.

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

250. Zumft, W. G. and F. Castillo. 1978. Regulatory properties of the nitrogenase from Rhodopseudomonas palustris. Arch. Microbiol. 117:53-60.

251. Zumft, W. G., L. E. Mortenson and G. Palmer. 1974. Electron paramagnetic resonance studies on nitrogenase. Eur. J. Biochem. 46:525-535.

APPENDIX I

The oligonucleotide mutagenic and sequencing primers used for the construction of the nifE site-directed mutagenesis project are located the draws labelled "Lisa" in the -80°C freezer. Mutagenic primers used in this study were the following:

E-Mn-1	E153SC	(CGG TCG ACT GCG CCG GCT)]
E-Mn-2	E160KQ	(TAC GGC ACC CAG AAC CTC)]
E-Mn-3	E164NH	(ACC TCG GCC ACC GCA TCG)

The sequencing primer used was the following:

EP10	(ACG CAG TGT GCA AAG CC)	used to screen E153SC, E160KQ, E164NH, E153SC-160KQ E153SC-164NH E160KQ-164NH E153SC-160KQ- 164NH

The mutagenic and sequencing primers used by Mark Wilson also located in the same box. The replicative form nifE site-directed mutant DNAs are also located in the -80°C freezer and labelled in the following manner:

E153SC RF,
E160KQ RF,
E164NH RF,
E153SC-160KQ RF,
E153SC-164NH RF,
E160KQ-164NH RF,

E135SC-160KQ-164NH RF

NifE site-directed mutant strains have been stocked and a description can be found in the Plasmid and Strain Log Book.

In addition to the nifE site-directed mutant DNAs described in this study, another site mutant template DNA was identified. This mutant template specifies four specific amino acid substitutions: E-Ser-153 to Cys, E-Lys-160 to Gln, E-Asn-164 to His and E-Val-249 to His. To construct this DNA, single stranded DNA from E153SC-160KQ-164NH RF was mutagenized using a mutagenic primer for position E249VH. One mutant template was identified after screening by single lane dideoxy sequencing (use **EP4**). The supernatant from this template was used to understreak, and four plaques were purified. Theses plaque purified template DNAs are located on Val Cash's shelf in the -20°C freezer. These plaques need to be sequenced and E153SC-160KQ-164NH-249VH RF DNA needs to be isolated. Single stranded DNA from E153SC-160KQ-164NH-249VH can subsequently be used to make a nifE sequence specifying five amino acid substitutions at E153SC-160KQ-164NH-249VH-251SY. The mutagenic primer needed for this site-directed mutagenesis experiment (**EP4**)is located in the box of nifE primers and mutenes.

The following is a list of Mark Wilson's nifE mutagenic and sequencing primers (see also Mark Wilson notebooks):

SEQUENCING PRIMERS

EP1	GAA AAG TCC GGC TGC GC
EP2	CGC ATC GGC ATG ACC AC

EP3	ACC AAG AAC CTC GGC AA
EP4	GAA CTG GGC CTG CGG CT
EP5	GAC ACC TCC CAG GCG CT
EP8	ATG AAA GCC AAG GAT AT
EP12	GAG CCC GCC TGC AGT CA

MUTAGENIC PRIMERS

E25SC	AAG TCC GGC TCC GCC AAG
E37CS	GAC GGT CGG TCC TCC TTC
E43Q-STOP	GAC GGC GCG TAG ATC GCC
E62CS	CCG ATC GCT TCC GCC GGC
E90EQ	CGA TCT CAC CCA GAA CGA
E92DE	CCG AGA ACG AGG TGA TCA
E283-STOP	GCC CGC ACC TAG GCG CTG
E401-STOP	GAC ATC AAC TAG GAG CGC
E42CS	CGT CAC GTC TCC ATC ACC

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