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Biochemical and genetic analysis of the *nifUSVWZM* cluster from *Azotobacter vinelandii*

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Summary. *Azotobacter vinelandii* genes contained within the major *nif*-cluster and designated orf6, *nifU*, *nifS*, *nifV*, orf7, orf8, *nifW*, *nifZ*, *nifM*, and orf9 are organized into at least two overlapping transcriptional units. Nitrogenase derepressed crude extracts of *Azotobacter vinelandii* mutant strains having individual deletions located within *nifU*, *nifS*, *nifV*, *nifW*, *nifZ*, or *nifM* were examined for nitrogenase component protein activities. The results of these experiments indicated that, in *A. vinelandii*, the *nifU*, *nifS* and *nifM* gene products are required for the full activation or the catalytic stability of the nitrogenase Fe protein. Deletion of the *nifV* gene resulted in lower MoFe protein activity, probably resulting from the accumulation of an altered FeMo-cofactor. The *nifW* and *nifZ* gene products were required for the full activation or catalytic stability of the MoFe protein. Deletion of *nifZ* alone or *nifM* alone did not appear to affect FeMo-cofactor biosynthesis. However, deletion of both *nifZ* and *nifM* eleminated either FeMo-cofactor biosynthesis or the insertion of FeMo-cofactor into the apo-MoFe protein. Other genes contained within the *nifUSVWZM* gene cluster (orf6, orf7, orf8, and orf9) were not required for Mo-dependent diazotrophic growth.

Key words: *nifU – nifS – nifV – nifW – nifZ – nifM – Azotobacter vinelandii*

Introduction

The molybdenum (Mo)-dependent nitrogenase is a complex metalloenzyme composed of two component proteins called the Fe protein and the MoFe protein (for a recent review see Smith et al. 1988). The Fe protein acts as a specific, ATP-binding, one-electron reductant of the MoFe protein, which contains the active site for substrate binding and reduction. Native Fe protein is an approximately 60000 dalton dimer of identical subunits. A single 4Fe-4S cluster is believed to be symmetrically bridged between the Fe protein subunits. The MoFe protein is an $\alpha_2\beta_2$ protein with an M_r of about 220000 and it contains 2 Mo atoms and about 32 Fe and 32 S^{2-} atoms per molecule. About 16 of the Fe atoms can be extruded from each MoFe protein molecule in the form of 4Fe-4S clusters by treatment of the native protein with thiols in a denaturing organic solvent. All or most of the remaining Fe and both of the Mo atoms constitute the two identical iron-molybdenum cofactors (FeMo-cofactor). In addition to magnesium ATP, a source of reducing equivalents, protons, and an anaerobic environment are required for nitrogenase turnover.

The primary products of the genes encoding the nitrogenase structural components (*nifH*, Fe protein subunit; *nifD*, MoFe protein α-subunit; *nifK*, MoFe protein β-subunit) are not catalytically competent. Rather, immature nitrogenase components are processed to active forms through the action of the products of certain other *nif*-specific genes. For example, it is known that at least six *nif*-specific gene products (*nifH*, *nifE*, *nifN*, *nifB*, *nifQ*, and *nifV* gene products, see discussion in Jacobson et al. 1989) are required for the formation of the FeMo-cofactor and at least one *nif*-specific gene product (*nifM* gene product; Roberts et al. 1978) is required for the accumulation of active Fe protein. However, the individual roles of these *nif*-specific gene products in relation to the biosynthesis of the metalloclusters or insertion of metalloclusters into the immature nitrogenase component proteins remain unknown. Furthermore, the potential role of a number of the other identified *nif*-specific gene products (*nifT*, *nifY*, *nifX*, *nifU*, *nifS*, *nifW* and *nifZ* gene products) is entirely unknown.

One approach towards understanding the contribution that individual *nif*-encoded gene products make towards the formation of a catalytically competent nitrogenase involves elimination of *nif*-specific gene functions using gene-directed mutagenesis techniques. We recently determined the physical and genetic map of the major *nif* gene cluster in *Azotobacter vinelandii* by using DNA sequence analysis and directed mutagenesis of this genomic region (Jacobson et al. 1989). Here, the diazotrophic growth properties and nitrogenase component protein activities of mutant strains that have insertion or deletion mutations located within the *nifUSVWZM* region of the major *nif*-cluster of *A. vinelandii* are described.

Materials and methods

Materials. All of the restriction endonucleases, *Bal*31 nuclease, T4 DNA ligase and the large fragment of DNA polymerase (Klenow fragment) used in this study were purchased from either Bethesda Research Laboratories (Gaithersburg, MD), New England BioLabs (Beverly, MA), or Boehringer Mannheim Biochemicals (Indianapolis, IN).

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Deoxynucleotide triphosphates (dATP, dCTP, dGTP dTTP and 7-deazo-dGTP), dideoxynucleotide triphosphates (ddATP, ddCTP, ddGTP and ddTTP), and the plasmids pUC4-KAPPA and pUC4-KISS were purchased from Pharmacia (Piscataway, NJ). α-[^{35}S]dATP was purchased from DuPont, NEN Research Products (Boston, MA). Ultrapure acrylamide and DNA grade formamide were obtained from Bethesda Research Laboratories. Ultrapure urea was purchased from Boehringer Mannheim Biochemicals. Cesium chloride, ethidium bromide and all of the antibiotics used in this study were purchased from Sigma Chemical Company (St. Louis, MO). All other chemicals were reagent grade, available commercially.

Growth of A. vinelandii strains. The wild-type and mutant strains of *A. vinelandii* were grown at 30° C on modified Burk medium (Strandberg and Wilson 1968) containing 0.01 mM Na_2MoO_4. Thus, only diazotrophic growth using the molybdenum-containing nitrogen fixation system was examined in this study. When a fixed nitrogen source was required, either ammonium acetate or urea was added to a final concentration of 30 mM or 10 mM, respectively. Kanamycin was added to a final concentration of 0.5 μg/ml, and rifampin was added to a final concentration of 5.0 μg/ml, as required.

Growth rates for the wild-type and mutant strains of *A. vinelandii* were determined as follows. Cultures to be used as inoculum were grown to mid-log phase on ammonium acetate-supplemented Burk medium, and inoculated into 20 ml of nitrogen-free Burk medium in a 300 ml baffelled side-arm flask (Belco Glass, Vineland, NJ), to approximately 10 Klett units. Growth was followed using a Summerson-Klett meter, equipped with a no. 66 filter.

Derepression and assay of nitrogenase. Derepression and assay of nitrogenase were perforemd essentially as previously described (Brigle et al. 1987). Cultures of *A. vinelandii* were grown in 11 l of urea-supplemented Burk medium in a New Brunswick Microgen SF116 fermentor of 12 l working capacity. All cultures were stirred at 300 rpm at 30° C with an aeration rate of 12 l/min. Cultures were harvested at approximately 110 Klett units (no. 54 filter) and concentrated to 1 l with a Millipore tangential-flow ultrafiltration apparatus. Cultures were washed twice by adding 2 l of nitrogen-free Burk medium and concentrated again. The second wash was concentrated to 500 ml final volume. Eleven liters of sterile, nitrogen-free Burk medium was used to transfer cells back to the fermentor, followed by incubation as above for 3 h. Derepressed cells were harvested as above except that the cells were washed with chilled 0.05 M TRIS (pH 8.0), then centrifuged at $10000 \times g$ for 10 min and stored at −80° C until assayed. Frozen cells (approx. 13 g) were suspended in 3 volumes of cold 0.05 M TRIS (pH 8.0) containing 1 mM $Na_2S_2O_4$ and transferred into a pre-cooled rosette cell (Branson Ultrasonics, Danburg, CT) under an atmosphere of argon. The cell suspension was sonicated for 2 min using a Sonifier Cell Disruptor 350 (Branson) equipped with a microtip. The ruptured cell suspension was transferred into an anaerobic vial and degassed before being transferred into an anaerobic centrifuge tube and spun at $17000 \times g$ for 20 min. The crude extract was pelleted directly into liquid nitrogen. Nitrogenase component protein activities were examined in crude extracts as previously described (Brigle et al. 1987).

Extracts were examined for nitrogenase component protein accumulation by two-dimensional gel electrophoresis as previously described (O'Farrell 1975).

Escherichia coli *growth and DNA manipulations. E. coli* strain 71-18 [*Δ(lac-proAB) thi supE* (F′ *proAB lacI*^{q}*Z*M15)], which served for a host for all recombinant M13 bacteriophage used in this study and strain TB1 [*Δ(lac-proAB) strA ara thi* ∅80(d*lacZ*ΔM15) *hsdR*], which served as a host for all of the plasmids used in this study were grown and maintained as described by Messing (1983). When necessary, the medium was supplemented with ampicillin (100 μg/ml final concentration) or kanamycin (50 μg/ml final concentration). Recombinant M13 phage were grown and M13 single-stranded and replicative-form (RF) DNAs prepared as described in detail by Messing (1983). Preparation, restriction enzyme digestion, and ligation of hybrid DNAs were performed using previously described techniques (Beynon et al. 1987). All DNA sequence analyses were performed using the dideoxy chain-termination procedure (Sanger et al. 1977) with hybrids of M13 filamentous phage vectors described by Messing (1983). Deletion mutagenesis of recombinant M13 phage DNAs was accomplished as follows. The parent recombinant M13-RF DNA was first digested to completion with the appropriate restriction endonuclease, extracted with an equal volume of phenol saturated with 10 mM TRIS-HCl (pH 8.0) and ethanol precipitated. The restriction endonuclease digested RF DNA was then treated with 1 unit of nuclease *Bal*31 for varying periods of time (1–5 min) as described by the supplier, followed by another phenol extraction and ethanol precipitation. The *Bal*31 nuclease treated RF DNA was subsequently ligated using T4 DNA ligase, and transfected into *E. coli* strain 71-18 made competent by $CaCl_2$ treatment (Messing 1983). The extent of the deletion within each single-stranded DNA template was determined by dideoxy sequencing reactions using oligonucleotides synthesized on an Applied Biosystems 381A DNA synthesizer as primers.

All plasmids used in this study that were described previously are so indicated in Table 1. Plasmids pDB268 and pDB269 were constructed by inserting the 1.3 kb kanamycin resistance-encoding cartridge into the *Xho*I site of the parental plasmid, pDB50. Plasmid pDB271 was constructed by cloning a 1.0 kb *Bal*I-*Pst*I restriction enzyme fragment from pDB56 into the *Sma*I-*Pst*I restriction enzyme sites of pUC18. Plasmids pDB273 and pDB274 were constructed by inserting the 1.3 kb kanamycin resistance-encoding cartridge into the *Sal*I restriction enzyme site of the parental plasmid pDB271. Plasmid pDB270 was constructed by removing a 1.7 kb *Pst*I restriction enzyme fragment from the parental plasmid pDB133. Recombinant phage M13mp18/271 was constructed by cloning the 0.9 kb *Eco*RI-*Hin*dIII restriction enzyme fragment from pDB271 into the *Eco*RI-*Hin*dIII restriction sites of RF DNA from phage M13-mp18. Recombinant phage M13mp18/6-4 was constructed by digesting RF DNA from M13mp18/271 with the restriction enzyme *Sal*I, treating with *Bal*31 nuclease (see above), ligating with T4 DNA ligase and transfecting into *E. coli* strain 71-18. Single-stranded DNA templates were prepared and sequenced using the primer 5′-CGGCTCCAG-CATTCTTGG-3′ in order to determine the deletion endpoints within each template. Recombinant phage M13mp18/177 was constructed by cloning the 0.8 kb *Pst*I fragment from pDB177 (Jacobson et al. 1989) into the *Pst*I

Table 1. *Azotobacter vinelandii* strains used in this study

Strain	Mutation[a]	Gene(s) or orf[b] disrupted	Codons deleted	Doubling time (h)	Plasmid or M13 RF DNA used[c]	Parental plasmid or M13 RF DNA[d]
DJ236	::(<)	orf5	–	2.5	pDB268	pDB50[e]
DJ238	::(>)	orf5	–	2.5	pDB269	pDB50
DJ268	::(<)	orf6	–	N.G.[f]	pDB273	pDB271
DJ270	::(>)	orf6	–	N.G.	pDB274	pDB271
DJ293	Δ	orf6	36–68	2.5	M13mp18/6-4	M13mp18/271
DJ105[e]	Δ	*nifU*	116–231	N.G.	pDB114[e]	pDB111[e]
DJ116[e]	Δ	*nifS*	13–396	15.5	pDB136[e]	pDB133[e]
DJ262	Δ	*nifUS*	116–256[g]	N.G.	pDB270	pDB133[e]
DJ71[e]	Δ	*nifV*	91–288	9	pDB107[e]	pDB106[e]
DJ224	Δ	*nifW*	14–58	5.4	M13mp18/W-23	M13mp18/177
DJ194	Δ	*nifZ*	27–56	5.3	M13mp7/Z-3	M13mp7/124
DJ136[e]	Δ(fs)	*nifM*	105–207[h]	N.G.	pDB156[e]	pDB152[e]
DJ67[e]	Δ	*nifZM*	123–107[g]	N.G.	pDB98[e]	pDB97[e]
DJ77[e]	Δ	*nifH*	158–200	N.G.	pDB115[e]	pMJH5[i]

[a] Δ indicates a deletion, fs indicates a frame shift, :: indicates insertion of a kanamycin resistance-encoding gene cartridge and (>) or (<) indicates the direction of transcription of the kanamycin resistance gene cartridge relative to transcription of the *nif* genes
[b] orf, open reading frame
[c] Plasmid actually used to construct the mutant strain. RF, replicative form
[d] Parental plasmid from which the plasmid used to construct the mutant strain was derived
[e] Previously described (Jacobson et al. 1989)
[f] N.G., no growth
[g] The deletion results in a fusion of the indicated genes at the specified codons
[h] Results in a shift in the reading frame
[i] Previously described (Jacobson et al. 1986)

restriction enzyme site of RF DNA from phage M13mp18. Recombinant phage M13mp18/W-23 was constructed by digesting RF DNA from phage M13mp18/177 with the restriction enzyme *Xho*I, treating with *Bal*31 nuclease, ligating with T4 DNA ligase and transfecting into *E. coli* strain 71-18. Single-stranded DNA templates were prepared and sequenced using the primer, 5′-CCCACTGCGGCCAACGCG-3′ in order to determine the endpoints of the deletion within each template. Recombinant phage M13mp7/124 was constructed by cloning the 1.5 kb *Eco*RI fragment from pDB124 (Jacobson et al. 1989) into the *Eco*RI restriction enzyme site in the RF DNA from phage M13mp7. Recombinant phage M13mp7/Z-3 was constructed by digesting M13mp7/124 RF DNA with the restriction enzyme *Pst*I, treating with *Bal*31 nuclease, ligating with T4 DNA ligase and transfecting into *E. coli* 71-18. Single-stranded DNA templates were prepared and sequenced using the primer 5′-CGACCAACTGTT-GAGCTG−3′ in order to determine the deletion endpoints within each template.

Mutagenesis of A. vinelandii *genes.* The mutant strains of *A. vinelandii* used in this study are listed in Table 1. Strains DJ105, DJ116, DJ71, DJ136, DJ67 and DJ77 and the plasmids used for their construction have been previously described (Jacobson et al. 1989). *A. vinelandii* transformations were performed as described by Page and von Tigerstrom (1979). Specific *nif* deletions located within recombinant plasmids or M13 phage and which result in a Nif$^-$ phenotype when transferred to the chromosome, were recombined into the *A. vinelandii* chromosome by congression (coincident transfer of unlinked genetic markers), using the rifampin resistance locus *rpoB*113 (L.T. Bennett and D.R. Dean, unpublished results) as the selection marker. This procedure has been described in detail previously (Robinson et al. 1986). Methods for recombining recombinant clones having either the kanamycin resistance-encoding cartridge (isolated from pUC4-KISS or pUC4-KAPPA), cloned into the central portion of *A. vinelandii* DNA sequences or specific in-frame deletions of *A. vinelandii* genes were described previously (Jacobson et al. 1989).

Strain DJ236, DJ238, DJ268 and DJ270 were constructed by transformation of the wild-type strain to kanamycin resistance using the DNAs listed in Table 1. Strain DJ262 was constructed by recombination of the mutation in pDB270 into the *A. vinelandii* chromosome using congression as described above. After allowing time for phenotypic lag, the transformed cells were selected on Burk ammonium acetate-supplemented agar plates containing rifampin. Rif^r transformants were scored on Burk nitrogen-free and Burk ammonium acetate-supplemented agar plates to identify Nif$^-$ transformants. Strain DJ293 was constructed by transforming strain DJ268 with RF DNA from phage M13mp18/6–4. All resulting Nif$^+$ transformants were kanamycin sensitive. Strain DJ224 was constructed by transformation of strain DJ144 (Jacobson et al. 1989) with RF DNA from phage M13mp18/W-23. The transformed culture was plated on Burk nitrogen-free agar plates. After several days of growth of 30° C two different sized colonies were observed, the larger of the two being much less abundent. Isolated colonies of each type were scored on Burks ammonium acetate-supplemented agar plates and Burks ammonium acetate-supplemented agar plates containing kanamycin. Only the large colony type was Kan^s. Strain DJ194 was similarly constructed by transformation of strain DJ150 (Jacobson et al. 1989) with RF DNA from phage M13mp7/Z-3. Again the large colony transformants exhibited the Kan^s phenotype upon scoring

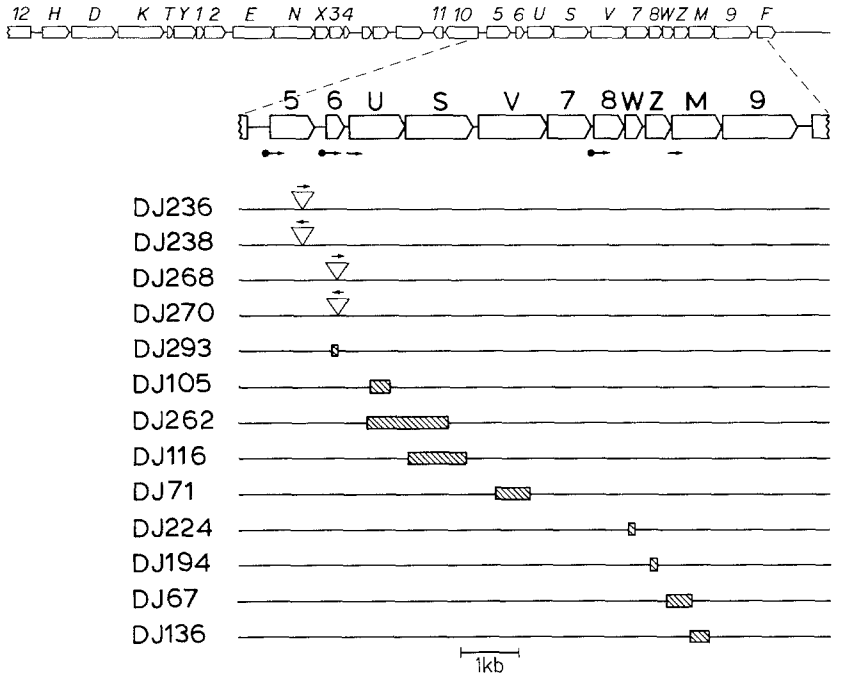

Fig. 1. Physical map of the *nifUSVWZM* region from *Azotobacter vinelandii*. The upper portion corresponds to the organization of the major *nif*-cluster from *A. vinelandii* (see Jacobson et al. 1989), with an expanded view of the *nifUSVWZM* region. *Arrows* under the *nif*-specific and *nif*-associated genes indicate consensus *nif* promoters and the direction of transcription. *Arrows with closed circles* indicate *nif* promoters with upstream activator sequences. The location of specific deletion or kanamycin-cartridge-insertion mutations within the *nifUSVWZM* cluster are indicated by hatched boxes or triangles, respectively. The orientation of the kanamycin resistance-encoded gene cartridge, relative to the kanamycin resistance promoter, is indicated

on Burk ammonium acetate-supplemented agar plates and Burk ammonium acetate-supplemented agar plates containing kanamycin.

Results

Transcriptional organization of the nifUSVWZM *region*

Identification of the *nifUSVWZM* region from *A. vinelandii* (Fig. 1) was previously determined by comparison with the corresponding region from *Klebsiella pneumoniae* (Beynon et al. 1987; Jacobson et al. 1989). There are five potential NtrA-dependent promoter sequences located within the *A. vinelandii nifUSVWZM* region (Fig. 1; Jacobson et al. 1989). Of these, a NifA-dependent upstream activator sequence, TGT-N_{10}-ACA (Buck et al. 1986), is also located upstream from the putative orf5, orf6 and orf8 promoters (Jacobson et al. 1989). There are no NifA-dependent activator sequences located immediately upstream from the putative *nifU* and *nifM* promoters. The apparent gene products encoded by orf7, orf8 and orf9 are not required for diazotrophic growth (Jacobson et al. 1989). However, polar mutations located within either orf7 or orf8 resulted in lower diazotrophic growth rates, indicating that transcription must occur through both orf7 and orf8 (Jacobson et al. 1989). The analysis of the diazotrophic growth rates of mutant strains having polar mutations located within orf7 or orf8 also indicated that transcription initiation must occur between orf7 and orf8 (Jacobson et al. 1989). This transcription probably originates at the proposed orf8 promoter. The absence of any obvious transcription termination signals located immediately after *nifM;* and the apparent translational coupling of *nifM* and orf9 deduced from the DNA sequence of this region, suggests that orf9 is cotranscribed with *nifM*.

Strains having polar insertion mutations located within orf5 were not affected in their diazotrophic growth capabilities (Table 1). Thus, neither transcription initiation from the putative orf5 promoter nor the presence of the gene product encoded by orf5 is essential for diazotrophic growth. Similarly, the gene product encoded by orf6 is not essential for diazotrophic growth because a strain having a deletion within orf6 was capable of normal diazotrophic growth. However, polar effects upon diazotrophic growth are observed in strains having insertion mutations within orf6, indicating that this region is transcribed and that transcription initiation must occur between orf5 and orf6.

These results show that the products of the *nif*-associated genes orf5, orf6, orf7, orf8 and orf9 are not essential for diazotrophic growth in *A. vinclandii*. However, homologs to orf6 have been identified in *Rhodobacter capsulatus* (B. Masepohl and W. Klipp, personal communication), *Bradyrhizobium japonicum* (H. Hennecke, personal communication) and *Anabaena* (D. Borthakur and R. Haselkorn, personal communication). A homolog to orf8 has also been identified in *R. capsulatus* (B. Masepohl and W. Klipp, personal communication). Thus, the conservation of these genes in such phylogenetically diverse organisms suggests that they are likely to have functions related to nitrogen fixation.

The *A. vinelandii nifUSVWZM* region is organized into a transcriptional unit that includes orf6-*nifU*-*nifS*-*nifV*-orf7-orf8-*nifW*-*nifZ*-*nifM*-orf9. Normal diazotrophic growth of *A. vinelandii* probably requires that transcription initiation occurs at the proposed orf6 and orf8 promoters. Thus, this gene cluster in *A. vinlandii* appears to be segmented into at least two overlapping transcriptional units as is the corresponding gene cluster in *K. pneumoniae* (Sibold 1982; Paul and Merrick 1987). The function, if any, of the putative *nifU* and *nifM* promoters is not apparent from these studies. One possibility is that the *nifU* and *nifM* promoters are involved in directing transcription of this region under conditions of molybdenum deficiency (see discussion in Kennedy et al. 1986).

Analysis of nifU *and* nifS

A collection of *A. vinelandii* mutant strains each having a deletion within one of the genes located within the

nifUSVWZM region is now available (Table 1; Fig. 1; Jacobson et al. 1989). With the exception of the deletion located in *nifM*, each of these deletions is in-frame with respect to the gene product coding sequence (Table 1). It is therefore unlikely that the deletions in these mutant strains cause any polar effects upon the transcription or translation of genes located downstream. Thus, phenotypes can be ascribed specifically to the elimination of the affected gene product. A large portion of the coding sequence is deleted in each mutant strain such that all functions of the respective products are expected to be eliminated.

Strain DJ105 is deleted for *nifU* codons 116–231 and strain DJ116 is deleted for *nifS* codons 13–396 (Table 1). Nitrogenase-derepressed crude extracts of strains deleted for *nifU* or *nifS* exhibited very low Fe protein activities and lowered levels of MoFe protein activities when compared to the wild type (Table 2). Thus, in *A. vinelandii*, both the *nifU* and the *nifS* gene products appear to be required for the activation or the stability of Fe protein. This conclusion, with regard to the *nifS* gene product is in agreement with similar data obtained for *K. pneumoniae nifS* mutants. Roberts et al. (1978) previously reported that *nifS* mutants of *K. pneumoniae* have low, but detectable, levels of MoFe protein activity and only a very low level of Fe protein activity. The data obtained for *K. pneumoniae* mutants is qualitatively the same as that reported here, the difference being that *A. vinelandii nifS* deletion strains have higher levels of both component protein activities. Roberts et al. (1978) suggested that the lower MoFe protein activities in *K. pneumoniae nifS* mutants could result indirectly from the accumulation of an inactive Fe protein. This hypothesis is reasonable because it is known that certain *K. pneumoniae* mutants impaired in the Fe protein structural gene also exhibit lowered accumulation of the MoFe protein subunits (Roberts et al. 1978). However, as shown in Fig. 2 and reported previously (Robinson et al. 1987), normal levels of both the α-subunit (*nifD* gene product) and the β-subunit (*nifK* gene product) of the MoFe protein accumulate in *A. vinelandii* mutant strains bearing deletions within the *nifH* coding sequence. Consequently a severe effect on MoFe protein activity in *A. vinelandii* is not expected if the primary consequence of deleting *nifU* or *nifS* is an alteration in the processing or stability of the Fe protein. Nevertheless, there could be an apparent decrease in MoFe protein activity in *nifU* or *nifS* deletion strains if the respective mutations alter the rate of component protein association and dissociation. Also, a lowered electron flux through nitrogenase in these and other mutants (see below) could result in a lowered in vivo catalytic stability of the complementary component protein.

Howard et al. (1986) used a binary plasmid system in *E. coli* to direct the synthesis of Fe protein in combination with other *nif*-specific gene products. Using a variety of hybrid plasmid constructions, they concluded that only the *nifM* gene product is required for the in vivo activation of Fe protein when expressed in *E. coli*. Using more specific plasmid constructs, Paul and Merrick (1987) confirmed this original conclusion. Nevertheless, the results of Howard

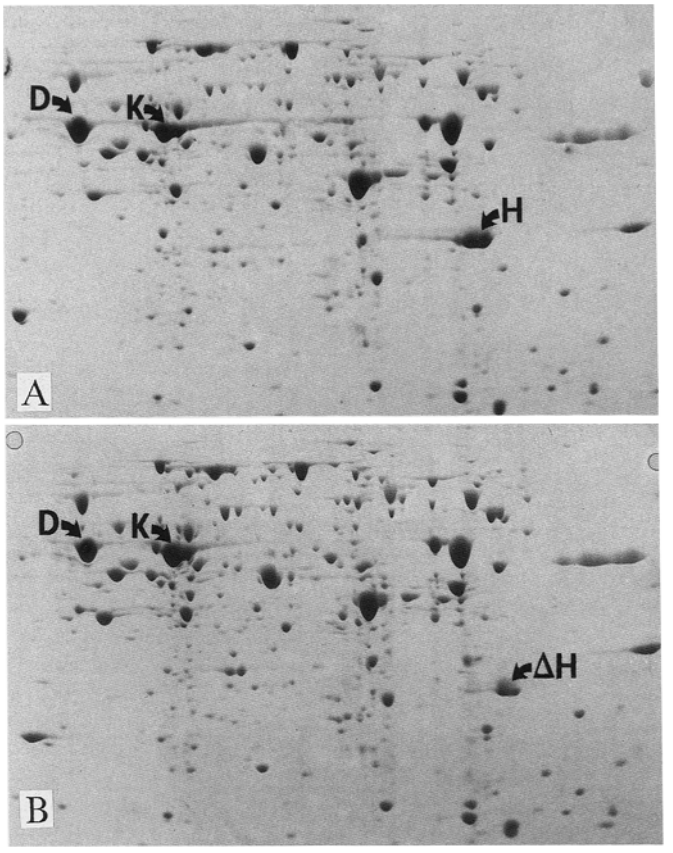

Fig. 2A and B. Two-dimensional gel analysis of extracts from nitrogenase derepressed wild-type *A. vinelandii* (**A**) and *A. vinelandii nifH* deletion strain DJ77 (**B**). The nitrogenase structural components; D (MoFe protein α-subunit), K (MoFe protein β-subunit), H (Fe protein) and ΔH (truncated Fe protein resulting from the in-frame deletion within the *nifH* coding sequence of strain DJ77) are indicated. The isoelectric focusing dimension is left to right (basic to acidic) and the size dimension is up to down (large to small)

Table 2. Nitrogenase component protein activities

Strain	Fe protein (Specific activity[a])	MoFe Protein (Specific activity[b])	Specific activity of FeMo-cofactor reconstitution[c]
Wild type	45.7	52.2	43.4
DJ105 (ΔU)[d]	2.5	15.4	14.8
DJ116 (ΔS)	3.5	13.1	13.3
DJ262 (ΔUS)	0.46	4.12	4.32
DJ71 (ΔV)	47.3	5.9	13.4
DJ224 (ΔW)	40.9	24.4	21.0
DJ194 (ΔZ)	48.4	17.3	16.0
DJ136 (ΔM)	<0.10	15.0	19.0
DJ67 (ΔZM)	<0.10	0.5	3.7

[a] Nanomoles of ethylene formed/min per mg of crude extract protein in the presence of saturation levels of purified *A. vinelandii* MoFe protein. All values represent the average of at least three independent determinations

[b] Nanomoles of ethylene formed/min per mg of crude extract protein in the presence of saturation levels of purified *A. vinelandii* Fe protein. All values represent the average of at least three independent determinations

[c] Nanomoles of ethylene formed/min per mg of crude extract protein in the presence of saturation levels of purified *A. vinelandii* Fe protein and FeMo-cofactor. All values represent the average of at least three independent determinations

[d] Genotypic designations are added only for clarity and do not strictly indicate the complete genotype of the organism. Δ, deletion

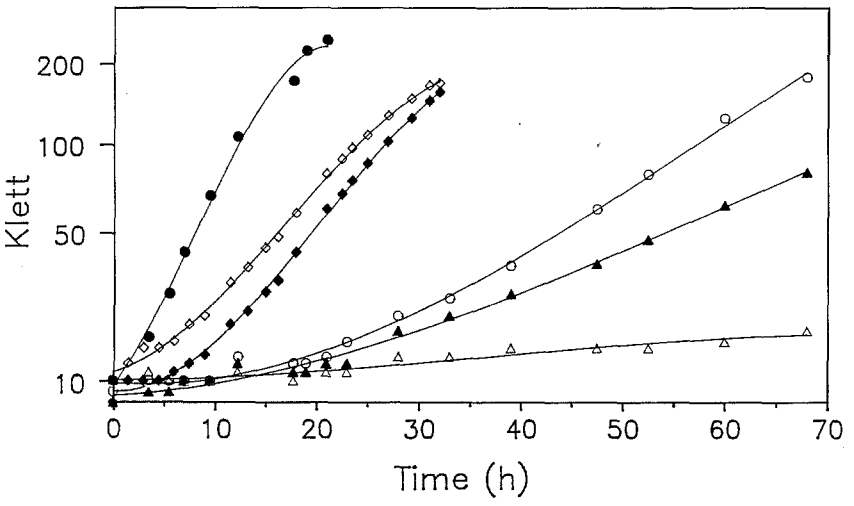

Fig. 3. Growth of the wild-type and mutant strains of *A. vinelandii* using N_2 as a sole source of nitrogen. Wild type (*closed circles*), DJ224 (*ΔW, open diamond*), DJ194 (*ΔZ, closed diamond*), DJ71 (*ΔV, open circle*), DJ116 (*ΔS, closed triangle*) and DJ105 (*ΔU, open triangle*)

et al. (1986) and Paul and Merrick (1987) did not eliminate the possibility that some *E. coli* encoded gene product can replace a requirement for the *nifU* or *nifS* gene product in Fe protein activation. Also, in the experiments of Howard et al. (1986) and Paul and Merrick (1989), the accumulation of Fe protein activity in *E. coli,* using the binary plasmid directed expression technique, was much lower than expected from the corresponding gene dosage.

The component protein activities in *A. vinelandii nifU* and *nifS* deletion strains show that the *nifU* and *nifS* gene products are required for the maturation or stability of the nitrogenase Fe protein. Because *nifU* and *nifS* are adjacent and cotranscribed, we wondered if their products might be involved in the same function. One possibility is that the *nifU* and *nifS* gene products could be subunits of the same enzyme. This notion is supported by the observation that nitrogenase derepressed crude extract component protein activities of strains deleted for *nifU* or deleted for *nifS* are very similar (Table 2). However, these mutant strains exhibited distinctly different diazotrophic growth phenotypes. Namely, strain DJ105 (*ΔnifU*) was incapable of diazotrophic growth while strain DJ116 (*ΔnifS*) exhibited slow diazotrophic growth (Fig. 3; Table 1). It was rather surprising that the strain deleted for *nifU* is not capable of at least weak diazotrophic growth, because readily detectable levels of both component protein activities are present in nitrogenase derepressed crude extracts of this strain (Table 2). This result suggests that one or both of the component proteins in the *nifU* deletion strain could have alterations which are not readily indicated by the acetylene reduction assay. If the *nifU* and *nifS* gene products are involved in catalysis of the same reaction, nitrogenase component protein activities in a strain deleted for both *nifU* and *nifS* should not differ significantly from component protein activities in those strains individually deleted for *nifU* or *nifS*. Strain DJ262 (*ΔnifUS*) has an in-frame deletion spanning *nifU* codon 116 through *nifS* codon 256 (Fig. 1). Polar effects upon transcription or translation of genes located downstream from *nifS* in this mutant are unlikely (see above). Deletion of both *nifU* and *nifS* resulted in almost complete loss of Fe protein activity and a marked reduction in MoFe protein activity (Table 2). Thus, although deletion of *nifU* alone or *nifS* alone resulted in similar reductions in nitrogenase component activities, their products are probably not participants in the same catalytic event.

Analysis of nifV

K. pneumoniae nifV mutants accumulate a MoFe protein having altered substrate reduction properties (McLean and Dixon 1981). This feature is the consequence of the synthesis of an altered FeMo-cofactor in *nifV* mutants (Hawkes et al. 1984). Hoover and co-workers have recently shown that homocitrate is an integral part of FeMo-cofactor and they have proposed that *nifV* encodes a homocitrate synthase (Hoover et al. 1987, 1989). Deletion of the *A. vinelandii nifV* gene resulted in a severe reduction in the diazotrophic growth rate (Table 1). Nitrogenase derepressed crude extracts of this strain exhibited lower levels of MoFe protein activity but normal levels of Fe protein activity (Table 2). The addition of purified FeMo-cofactor to nitrogenase derepressed crude extracts prepared from the *nifV* deletion strain stimulated MoFe protein activity (Table 2). This result suggests that either a portion of the MoFe protein in the *nifV* deletion strain is cofactorless or that native FeMo-cofactor can displace the altered form of FeMo-cofactor present in the *nifV* deletion strain. These results are consistent with the data of Hoover et al. (1986) who showed that *nifV* deficient extracts from *K. pneumoniae* are ineffective in in vitro FeMo-cofactor biosynthesis. Furthermore, Hoover et al. (1988a) have shown that the activity of apo-MoFe protein reconstituted with FeMo-cofactor synthesized in vitro in the presence of citrate, rather than homocitrate, is also stimulated by the addition of native FeMo-cofactor.

The addition of homocitrate to a nitrogenase derepressing culture of a *nifV*-specific *K. pneumoniae* mutant cures the $NifV^-$ phenotype (based on the catalytic properties of isolated MoFe protein; Hoover et al. 1988b). In contrast, addition of homocitrate (1 mM final concentration) to a similarly derepressed *A. vinelandii nifV* deletion strain did not stimulate the diazotrophic growth rate (data not shown). The failure of 1 mM homocitrate to cure the $NifV^-$ phenotype in *A. vinelandii* could reflect an ineffective homocitrate uptake ability in this organism. Nevertheless, comparison of *A. vinelandii* and *K. pneumoniae nifV* gene product primary sequences (deduced from nucleotide sequence data; Beynon et al. 1987), and the component protein activities in the *A. vinelandii nifV* deletion strain discussed above, indicate that the *nifV* gene product is likely to have the same function in *A. vinelandii* and *K. pneumoniae*.

Analysis of nifW *and* nifZ

Polar insertion mutations located within *nifW* or *nifZ* markedly reduce or abolish diazotrophic growth in *A. vinelandii* (Jacobson et al. 1989). Because these effects could be the result of a reduction in *nifM* gene expression, which is known to be required for diazotrophic growth (see below), strains of *A. vinelandii* having specific in-frame deletions within *nifW* or *nifZ* were isolated (Fig. 1). These mutant strains exhibited a moderate reduction in their diazotrophic growth capabilities (Fig. 3). Nitrogenase-derepressed crude extracts prepared from *nifW* or *nifZ* deletion strains contained normal levels of Fe protein activities but lowered MoFe protein activities (Table 2). Thus, the *nifW* and *nifZ* gene products are required for the full expression, stability, or processing of the MoFe protein. The similar diazotrophic growth rates and component protein activities in nitrogenase derepressed crude extracts of *nifW* and *nifZ* deletion mutants indicates that the *nifW* and *nifZ* gene products might be components of the same enzyme complex. This possibility is supported by the apparent translational coupling of the *nifW* and *nifZ* gene products as deduced from the nucleotide sequence of this region (Jacobson et al. 1989). However, the small differences in diazotrophic growth rates (Fig. 3) and the small differences in nitrogenase component activities (Table 2) in these respective mutants are reproducible and probably significant. Addition of purified FeMo-cofactor to nitrogenase derepressed crude extracts of these strains did not stimulate MoFe protein activity. This result does not, however, exclude the possibility that the *nifW* or *nifZ* gene product is involved in FeMo-cofactor biogenesis because strains deleted for *nifW* or *nifZ* could accumulate an altered FeMo-cofactor which is not displaced by addition of the purified native FeMo-cofactor.

The data reported here for *A. vinelandii nifW* and *nifZ* mutants is in general agreement with that of Paul and Merrick (1989) for *K. pneumoniae nifW* and *nifZ* mutants. They examined the accumulation of component protein activities in nitrogenase derepressing *K. pneumoniae nifW* and *nifZ* insertion mutants. In order to alleviate potential polar effects caused by the respective insertion mutations, these strains also carried plasmids encoding *nifM* or *nifZ* and *nifM*. Under the conditions examined, neither the *nifW* nor the *nifZ* gene product was found to be essential for nitrogen fixation in *K. pneumoniae*. However, reductions in MoFe protein and Fe protein activities were observed for both *nifW* and *nifZ* insertion mutants. The reduction in Fe protein activities in *K. pneumoniae nifW* and *nifZ* insertion mutants, which was not observed in *A. vinelandii nifW* and *nifZ* deletion mutants, could be attributed to polar effects upon *nifM* gene product accumulation resulting from the mutant constructs (Paul and Merrick 1989).

Recently, homologs to *nifW* and *nifZ* were identified in the photosynthetic bacterium *R. capsulatus* (B. Masepohl and W. Klipp, personal communication). The identification of these genes in three phylogenetically different diazotrophs, and the strong conservation in the deduced primary amino acid sequences of the respective gene products from each organism suggests similar functions in nitrogen fixation in all three organisms.

Analysis of nifM

An *A. vinelandii* mutant strain specifically deleted for *nifM* exhibited a strictly Nif$^-$ phenotype. Nitrogenase derepressed crude extracts of the *nifM* deletion strain contained a nearly negligible level of Fe protein activity and a reduced level of MoFe protein activity. In separate studies (Howard et al. 1989; Kennedy et al. 1986) polar insertion mutations located within the *A. vinelandii nifM* gene were found not to be significantly affected in MoFe protein activity. The basis for the difference in results could be related to the nature of the mutations in the respective mutants, i.e. insertion versus deletion mutation. Nevertheless, the *A. vinelandii nifM* gene product appears to function primarily in Fe protein activation. Roberts et al. (1978) also found that the *nifM* gene product is required for Fe protein activation in *K. pneumoniae*. On the basis of two-dimensional gel electrophoresis analyses (data not shown), no apparent difference in accumulation of Fe protein polypeptide was recognized in nitrogenase derepressed crude extracts of the *A. vinelandii nifM* deletion strain when compared to the wild type. Thus, over the 3 h nitrogenase derepression period used in this experiment, the *nifM* gene product function was not required for the chemical stability of Fe protein in *A. vinelandii*. In contrast, a role for *nifM* product in stabilizing *K. pneumoniae* Fe protein has been suggested (Howard et al. 1986).

Several possibilities for the function of the *nifM* gene product in activation of Fe protein have been suggested. These include: (a) biosynthesis or insertion of the Fe:S cluster into apo-Fe protein (Howard et al. 1986); (b) synthesis of the inorganic sulfide needed for Fe:S cluster assembly (Pagani et al. 1987); and (c) a conformational isomerization of the Fe:S cluster once it is assembled into an immature form of the Fe protein (Howard et al. 1989). If (c) is correct, it is predicted that inactive Fe protein accumulated in the *A. vinelandii nifM* deletion strain will contain Fe. This analysis is now in progress.

NifZ *and* NifM *are mutually required for FeMo-cofactor biosynthesis or insertion*

Nitrogenase derepressed crude extracts prepared from an *A. vinelandii* strain deleted for *nifZ* and *nifM* contained negligible Fe protein activity and only very low MoFe protein activity. This result was unexpected because individual loss of *nifZ* gene product function resulted only in a moderate reduction in MoFe protein activity and the *nifM* gene product appeared not to be required for MoFe protein activation (see above). Although the possibility has not been ruled out, neither the *nifM* nor the *nifZ* gene product has an apparent function in FeMo-cofactor biosynthesis or insertion. It was, therefore, even more surprising that addition of purified FeMo-cofactor to nitrogenase derepressed crude extract prepared from the *nifZM* deletion strain reconstituted MoFe protein activity (Table 2). This result shows that, in *A. vinelandii,* the *nifZ* gene product is required for FeMo-cofactor biosynthesis or insertion in the absence of the *nifM* gene product, or that the *nifM* gene product is required for FeMo-cofactor biosynthesis or insertion in the absence of the *nifZ* gene product. The basis for the cumulative effect of eliminating *nifZ* and *nifM* gene product functions upon FeMo-cofactor biosynthesis is not clear. It will be interesting to determine if extracts from *nifZ* deletion strains are competent in in vitro FeMo-cofactor biosynthesis.

There is a known physical requirement for Fe protein in FeMo-cofactor biosynthesis or insertion (Robinson et al.

1987; Filler et al. 1986). This function is somewhat different from Fe protein's role in nitrogenase catalysis because certain catalytically incompetent Fe protein species still function in FeMo-cofactor biosynthesis (see for example, Filler et al. 1986). It is not yet known whether a physical interaction between Fe protein and apo-MoFe protein or between Fe protein and one or more of the FeMo-cofactor biosynthetic gene products is required for FeMo-cofactor biosynthesis. However, if complexing of Fe protein with other proteins is required for MoFe protein maturation, it appears that the elimination of both *nifZ* and *nifM* gene products could result in the cumulative prevention or alteration of such interactions. These results also support the notion that the *nifZ* gene product has a function involving MoFe protein maturation and that this function is likely to be related to an event involving or affecting FeMo-cofactor.

Discussion

Twenty *nif*-specific genes whose products are either required for or associated with nitrogen fixation in *K. pneumoniae* have been identified and characterized. The *nif* genes identified in *K. pneumoniae,* however, do not represent the complete set of all genes associated with nitrogen fixation. Genes associated with nitrogen fixation and common to a wide variety of diazotrophic organisms, but apparently not present in *K. pneumoniae,* have been discovered. The *fixABC* gene cluster is one such example (Gubler and Hennecke 1986; Earl et al. 1987). Indeed, it is reasonable to expect that genes, which are unique to nitrogen fixation in a particular subgroup of organisms, are likely to exist. For example, the *nif*-specific pyruvate-flavodoxin-oxidoreductase is not expected to be present in aerobic diazotrophs. Conversely, the products of genes located within the *A. vinelandii nifUSVWZM* region that are designated as orf6, orf7, orf8, and orf9 could function in some aspect of nitrogen fixation operating under aerobic conditions.

Nevertheless, there is likely to be a minimum set of genes whose products are required for the maturation or stability of nitrogenase component proteins in all diazotrophic organisms. Based on comparative gene organization studies and nucleotide sequence data obtained from *K. pneumoniae, A. vinelandii* and *R. capsulatus,* this minimum set of common *nif*-specific genes is likely to include *nifE, nifN, nifU, nifS, nifV, nifW, nifZ, nifM, nifB,* and *nifQ.* The approach we have taken for the analysis of the functions of these genes in *A. vinelandii* involves the systematic deletion of each gene followed by examination of the nitrogenase component activities in nitrogenase derepressed crude extracts of such strains. Earlier studies of this nature demonstrated that *nifE* and *nifN* are required for FeMo-cofactor biosynthesis (Brigle et al. 1987; Kennedy et al. 1986). Similarly, it has been shown that *nifB* and probably *nifQ* are also involved in FeMo-cofactor synthesis (Joerger et al. 1986; Joerger and Bishop 1988). From the results of our study we conclude that in *A. vinelandii:* (a) *nifU, nifS* and *nifM* gene products are required for full activation or catalytic stability of Fe protein; (b) *nifV* gene product is involved in FeMo-cofactor biosynthesis; and (c) *nifW* and *nifZ* gene products are required for full activation or catalytic stability of the MoFe protein. In certain cases, there also appears to be indirect effects upon the full activation or enzymatic stability of one component when the complementary protein is altered. For example, *nifU* and *nifS* deletions, which appear primarily to affect Fe protein activity, also result in the accumulation of lower MoFe protein activity. A potential interplay among individual *nif*-specific gene products in the activation of the nitrogenase component proteins is indicated by the observation that deletion of *nifZ* alone or *nifM* alone does not have an apparent effect upon FeMo-cofactor biosynthesis, but deletion of *nifZ* and *nifM* results in the accumulation of FeMo-cofactorless MoFe protein. Thus, the isolation of mutant strains defective in combinations of *nif*-specific genes represents an approach for defining a phenotype and, potentially, a biochemical assay for the products of those genes whose functions are obscure.

Products of *nif*-specific genes from various diazotrophic organisms which have a high degree of primary amino acid sequence similarity are likely to have identical catalytic functions in their respective organisms. Nevertheless, the biochemical and physiological manifestations associated with the elimination of a particular *nif*-specific gene product is not necessarily expected to be the same for different organisms. For example, whereas the *nifU* gene product from *A. vinelandii* is absolutely required for diazotrophic growth, there is evidence that *nifU* is dispensable in *K. pneumoniae* (Roberts et al. 1978) and *R. capsulatus* (B. Masepohl and W. Klipp, personal communication). This feature might reflect the different physiologies of these respective organisms. These results point to the importance of comparative genetic and biochemical studies for the analysis of nitrogen fixation-related genes and their products and also indicate that a model organism for the overall study of biological nitrogen fixation does not exist.

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