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Genetic Evidence for an *Azotobacter vinelandii* Nitrogenase Lacking Molybdenum and Vanadium

RICHARD N. PAU,* LESLEY A. MITCHENALL, AND ROBERT L. ROBSON†

Agricultural and Food Research Council, Institute of Plant Science Research, Nitrogen Fixation Laboratory, University of Sussex, Brighton BN1 9RQ, United Kingdom

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We have constructed a strain of *Azotobacter vinelandii* which has deletions in the genes for both the molybdenum (Mo) and vanadium (V) nitrogenases. This strain fixed nitrogen in medium that did not contain Mo or V. Growth and nitrogenase activity were inhibited by Mo and V. In highly purified medium, growth was limited by iron. Addition of other metals (Co, Cr, Cu, Mn, Ni, Re, Ti, W, and Zn) did not stimulate growth. Like the V-nitrogenase, the nitrogenase synthesized by the double deletion strain reduced acetylene to both ethylene and ethane (C_2H_6/C_2H_4 ratio, 0.046). There was an approximately 10-fold increase in ethane production when Mo was added to the deletion strain grown in medium lacking Mo and V. This change in reactivity may be due to the incorporation of an Mo-containing cofactor into the nitrogenase synthesized by the double-deletion strain. A strain synthesizing the V-nitrogenase did not show a similar increase in ethane production. The growth characteristics of the double-deletion strain, together with the metal composition reported for a nitrogenase isolated from a tungstate-tolerant strain lacking genes for the molydenum enzyme grown in the absence of Mo and V (J. R. Chisnell, R. Premakumar, and P. E. Bishop, J. Bacteriol. 170:27–33, 1988) show that *A. vinelandii* can synthesize a nitrogenase which lacks both Mo and V. Reduction of dinitrogen by nitrogenase can therefore occur at a center lacking both these metals.

Until recently, nitrogenases isolated from all nitrogen-fixing organisms consisted of an iron protein and a molybdenum (Mo)-iron protein. They contain an FeMo cofactor at the probable active site (21). The existence of Mo-independent nitrogenases was first proposed by Bishop et al. because Nif$^-$ mutants of *Azotobacter vinelandii* were able to grow and reduce nitrogen in the absence of Mo (1). An alternative nitrogenase, with a vanadium (V)-iron protein and an iron protein, was first isolated from the related species *Azotobacter chroococcum* (18). In this organism three contiguous structural genes (*nifHDK*) encode the polypeptides of the molybdenum-containing nitrogenase (4). Polypeptides of the V-containing nitrogenase are encoded by a distinct cluster of *nifHDK*-like genes (19). Mo- and V-nitrogenases have also been isolated from *A. vinelandii* (5, 11). However, *A. vinelandii* appears to differ from *A. chroococcum* in having three instead of two *nifH*-like genes (12). One is in the cluster of genes (*nifHDK*) which encodes the Mo-nitrogenase, and one is presumably associated with genes which encode the V-nitrogenase. The third *nifH*-like gene is in another *nifHDK*-like cluster which encodes a third nitrogenase (3). We show here that *A. vinelandii* has genes which are homologous to the genes for the V-nitrogenase of *A. chroococcum*.

A strain of *A. vinelandii* has been constructed with deletions in the genes encoding both the Mo- and V-nitrogenases. Characterization of nitrogen fixation by this double-deletion strain indicates that the third nitrogenase synthesized by *A. vinelandii* has novel properties.

(Preliminary results of this study were presented at the 7th International Congress on Nitrogen Fixation [15].)

* Corresponding author.

† Present address: Department of Biochemistry, Boyd Graduate Studies Research Center, The University of Georgia, Athens, GA 30602.

MATERIALS AND METHODS

Plasmids and bacterial strains. Plasmid pEF1 contains the genes encoding the VFe protein of *A. chroococcum* MCD1155 on an 8-kilobase (kb) *Eco*RI fragment cloned in pEMBL19$^+$ (7). It also contains the ferredoxinlike gene which is immediately downstream of the *nifH*-like gene encoding the Fe protein of the V-nitrogenase (20). pLAM3 contains sequences from *A. vinelandii* genomic DNA homologous to those of the *A. chroococcum* fragment in pEF1. They are on an 8-kb *Bam*HI fragment cloned in pTZ19R (Pharmacia Ltd).

A. chroococcum MCD1155 (17) and *A. vinelandii* CA11 (1) both contain deletions in *nifHDK*. They are derived from *A. chroococcum* MCD1 and *A. vinelandii* CA, respectively. *A. chroococcum* MCD50 is a tungstate-resistant strain derived from strain MCD1. *A. vinelandii* UW136 is a rifamycin-resistant strain derived from the wild-type *A. vinelandii* CA (1).

A. vinelandii mutant strain RP114 was constructed as follows. The 1.4-kb *Bgl*II fragment within the *nifDK*-like genes cloned in pEF1 (Fig. 1) was replaced in vitro by the 2-kb segment containing a spectinomycin resistance (Spc^r) gene (16). The fragment containing the inserted antibiotic resistance gene was cloned into the unique *Bam*HI site of the vector pCU101 (22), the resultant plasmid was introduced into *A. vinelandii* strain UW136 by conjugation (17), and Spc^r transconjugants were selected. The double-deletion strain RP206 was constructed by transforming (14) *A. vinelandii* CA11 with genomic DNA from strain RP114 and selecting transformants for Spc^r.

Media and growth conditions. *Azotobacter* strains were grown (in grams per liter of distilled water): sucrose, 20; $K_2PO_4 \cdot 3H_2O$, 0.64; KH_2PO_4, 0.16; Na_2SO_4, 0.142; $MgCl_2 \cdot 6H_2O$, 0.203; $CaCl_2 \cdot 2H_2O$, 0.074; $FeC_6H_5O_7$, 0.034. A 20-ml amount of the above medium or metal-supplemented medium in 50-ml sidearm flasks was inoculated with 1.3×10^7 cells per ml (12 Klett units) and

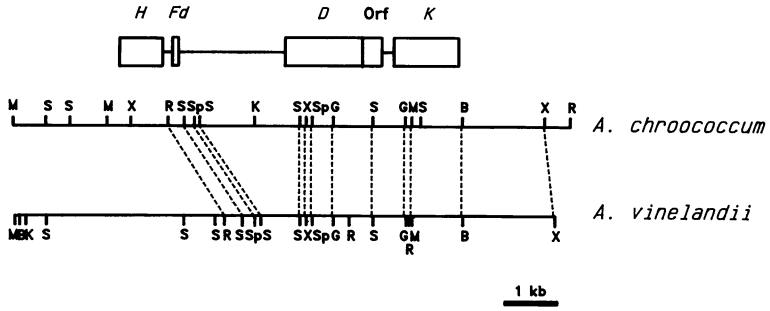

FIG. 1. Positions of the coding sequences of the structural genes for the vanadium nitrogenase in *A. chroococcum*, and comparison of the restriction maps of genomic DNA fragments containing the structural genes for the vanadium nitrogenases of *A. chroococcum* and *A. vinelandii*. H, D, and K indicate the *nifHDK*-like genes which encode the vanadium nitrogenase. The locations and identities of the nitrogenase genes were established by hybridization and DNA and amino acid sequencing (19). The *nifH*-like gene of *A. chroococcum* was previously called *nifH'* (20). The adjacent *nifDK*-like genes and the open reading frame (Orf) together encode the polypeptides of the VFe protein, which has three subunits. Fd, Ferredoxinlike gene. Common restriction sites are joined by dashed lines. Abbreviations: B, *Bam*HI; G, *Bgl*II; K, *Kpn*I; M, *Sma*I; R, *Eco*RI; S, *Sal*I; Sp, *Sph*I; X, *Xho*I.

incubated in air at 30°C with rotary shaking (200 rpm), and the growth was monitored with a Klett-Summerson photoelectric colorimeter with a no. 54 green filter. Medium containing Mo or V was supplemented with 10 μM Na_2MoO_4 or 10 μM VSO_4. The effects of $CoCl_3$, K_2CrO_4, $CuSO_4$, $MnCl_2$, $NiSO_4$, $NaReO_4$, $NaWO_4$, $TiCl_3$, and $ZnCl_2$ on growth were determined by the addition of 10 μM of the above salts (reagent grade) to the medium. The influence of iron was measured in iron-free medium chemically purified to remove traces of metals (9). This medium was supplemented with ultrapure $Fe_2(SO_4)_3$ (less than 10 ppm total metal impurities; Puratronic grade; Johnson Matthey Chemicals Ltd).

Southern hybridization and restriction analysis. *Azotobacter* genomic DNAs were isolated as previously described (17). DNAs (10 μg) were digested with restriction endonucleases, electrophoresed on 0.8% agarose gels in Tris-acetate-EDTA (13), transferred to nylon membranes (Hybond-N; Amersham International), and hybridized overnight at 42°C according to the membrane manufacturer's instructions. The hybridization solution consisted of 50% formamide, 6× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate), 0.5% sodium dodecyl sulfate, 0.1% bovine serum albumin, 0.1% Ficoll 400, 0.1% polyvinyl pyrrolidone. ^{32}P-labeled DNA probes were synthesized by random-primed labeling (10). The probe used to identify *nifDK*-like sequences of the V-nitrogenase genes in *A. vinelandii* genomic DNA was the 1.4-kb *Bgl*II fragment of pEF1 containing part of the *nifDK*-like genes and the open reading frame between these two genes. The *Sma*I site 5′ and the *Xho*I site 3′ to the *Bgl*II sites of pLAM3 (Fig. 1) were established from the sizes of genomic fragments which hybridized to the 1.4-kb *Bgl*II probe. The locations of the *nifH*-like and ferredoxinlike gene sequences in pLAM3 were confirmed by hybridization with fragments of *A. chroococcum* DNA which contained these genes (20). The probe for *nifD* was synthesized by using a 1.3-kb *Sac*I-*Bgl*II fragment of the *nifD* gene of *A. chroococcum*.

Enzyme activity analysis. Unlike the Mo-nitrogenase, the third nitrogenase isolated from *A. vinelandii* reduces protons more efficiently than acetylene (6). The nitrogenase activity of cells in iron-limited cultured was measured by hydrogen evolution because of the lower cell numbers in this experiment. Cultures (20 ml) in conical flasks were sealed, and incubation was continued at 30°C with shaking at 200 rpm for 2 h before the amount of hydrogen evolved was determined with a Pye Unicam 204 chromatograph fitted with a thermal conductivity meter and a 1-m column packed with an M5A molecular seive.

Repression of nitrogenase activity by Mo and V was measured by acetylene reduction as follows. Three 100-ml cultures were grown in 250-ml conical flasks at 30°C with rotary shaking at 180 rpm to a density of 3×10^8 cells per ml; 10 μM Na_2MoO_4 and 10 μM VSO_4 were each added to one culture, and incubation was continued. At the times indicated after the addition of these metals, 10 ml was transferred to a conical flask which was sealed (gas headspace, 27 ml), 3 ml of acetylene was added (0.1 atm, 10.13 kPa), and the cells were incubated as above for 2 h before ethylene and ethane formation was assayed by gas chromatography according to the method of Dilworth et al. (8). The effects of the addition of Mo and V on ethane production by *A. vinelandii* strains CA11 and RP206 was determined by growing 10-ml cultures to cell densities of 2×10^8 cells per ml in 25-ml flasks at 30°C with shaking at 180 rpm and adding 10 μM Na_2MoO_4 or 10 μM VSO_4 2 h before assaying for acetylene reduction as described above.

^{15}N enrichment. Cells were grown in 25-ml Mo- and V-free medium in sealed conical flasks at 22°C for 3 days. Air in the space (100 ml) above the experimental culture contained 10% $^{15}N_2$ and was replaced each day. The air above the control culture was changed each day. The atom% excess of $^{15}N_2$ derived from 4 mg of dried cells by oxidation with copper oxide at 700°C was measured with a VG Siara mass spectrometer.

RESULTS

V-nitrogenase structural genes. Comparison (Fig. 1) of the physical maps of the genomic DNA containing the *nifHDK*-like genes encoding the V-nitrogenase of *A. chroococcum* and the homologous fragment from *A. vinelandii* showed that the restriction sites in the DNA segments containing the genes were highly conserved in both species. In *A. chroococcum*, this cluster of genes was distinguished from the closely linked *nifHDK*, encoding the Mo-nitrogenase, in having a ferredoxinlike gene downstream of the *nifH*-like gene and an open reading frame located between the *nifD*- and *nifK*-like genes. Restriction sites present in the coding sequences for the Fe protein, ferredoxin, and VFe protein of *A. chroococcum* were also present in *A. vinelandii*. The principal difference between these gene clusters in the two

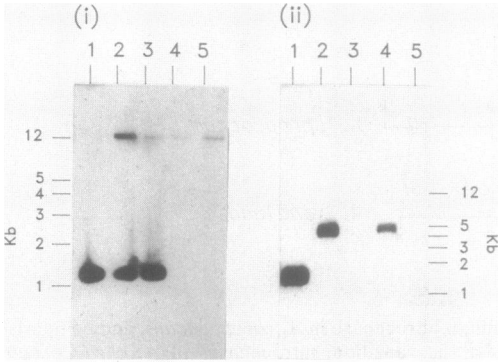

FIG. 2. Confirmation by Southern analysis of the construction of a strain (RP114) of *A. vinelandii* with a deletion in structural genes for the V-nitrogenase and a strain (RP206) with deletions in genes for both the V- and Mo-nitrogenases. Southern blots of *Bgl*II restriction endonuclease digests were probed with (i) the 1.4-kb *Bgl*II fragment from the *nifDK*-like genes for the vanadium nitrogenase of *A. chroococcum* and (ii) a 1.3-kb fragment of *nifD* for a subunit of the MoFe protein of *A. chroococcum*. Lanes: 1, *A. chroococcum* MCD50; 2, *A. vinelandii* UW136; 3, *A. vinelandii* CA11; 4, *A. vinelandii* RP114; 5, *A. vinelandii* RP206. (i) A 1.4-kb band indicates the presence of the *nifDK*-like genes encoding the VFe protein of the V-nitrogenase. (ii) The 1.8-kb band (lane 1, *A. chroococcum*) and 4.7-kb bands (lanes 2 and 4, *A. vinelandii*) indicate the presence of *nifD* (*A. chroococcum*) and *nifDK* (*A. vinelandii*) and hence the presence of genes encoding the MoFe protein of the Mo-nitrogenase.

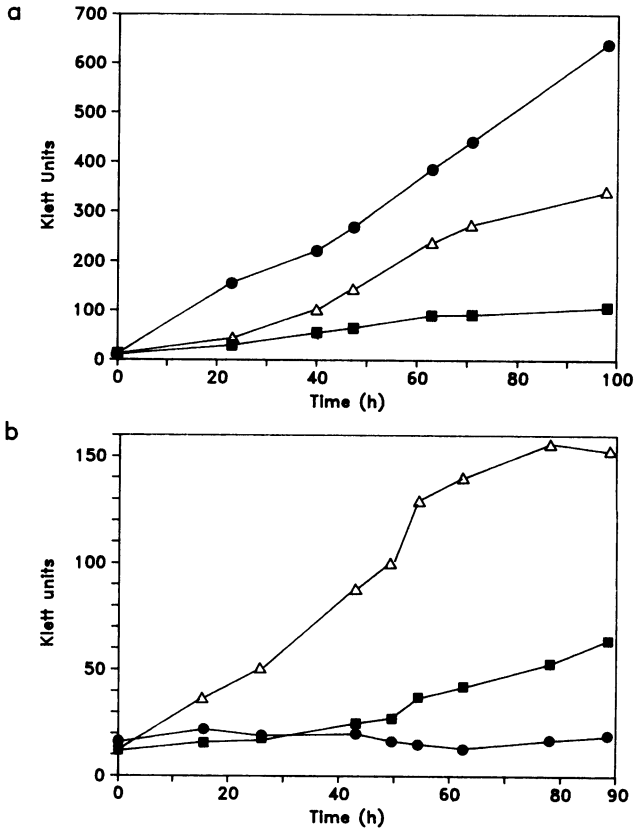

FIG. 3. Influence of Mo and V on (a) growth of a strain of *A. vinelandii* deleted for the vanadium nitrogenase genes (RP114) and (b) growth of a strain deleted for both molybdenum and vanadium nitrogenase genes (RP206). Metal added to medium: Mo (●), V (■), or none (△).

organisms was the presence in *A. chroococcum* of an additional region of approximately 1.2 kb between the ferredoxinlike gene and the *nifD*-like gene. A 1.4-kb *Bgl*II fragment from the *nifDK*-like genes, encoding the VFe protein of *A. chroococcum*, did not with hybridize with the 4.7-kb *Bgl*II fragment which contained *nifDK*, encoding the MoFe protein, of *A. vinelandii* (Fig. 2i, lane 2). Conversely, a fragment containing *nifD*, encoding the MoFe protein of *A. chroococcum*, did not hybridize with the 1.4-kb *Bgl*II fragment from the *nifDK*-like genes encoding the VFe protein of *A. vinelandii* (Fig. 2ii, lane 2). However, an additional weakly hybridizing band of about 12 kb was seen in digests of *A. vinelandii* DNA hybridized with the 1.4-kb *Bgl*II fragment of the genes for the VFe protein (Fig. 2i, lanes 2 to 5). This third *nifDK*-like region is linked to the third *nifH*-like gene to form a cluster distinct from those encoding the Mo- and V-nitrogenases (3). When a *Bgl*II digest of *A. chroococcum* DNA was hybridized with the 1.4-kb fragment of the *A. chroococcum nifDK*-like genes encoding the V-nitrogenase, no band was seen other than the strongly hybridizing 1.4-kb band derived from the V-nitrogenase gene cluster (Fig. 2i, lane 1). This suggests that *A. chroococcum* does not have a third *nifHDK*-like gene cluster.

Because of the close homology of the V-nitrogenase genes in *A. chroococcum* and *A. vinelandii*, we were able to use a fragment of *A. chroococcum* DNA with a 1.4-kb *Bgl*II deletion in the genes for the VFe protein replaced by an antibiotic resistance gene to create a corresponding mutation in the homologous genes of *A. vinelandii*. The absence of the deleted fragment in the resultant strain and the presence of the genes encoding the Mo-nitrogenase were confirmed by DNA hybridization (Fig. 2i, lane 4; Fig. 2ii, lane 4). DNA from this single-deletion strain (RP114) was then used to transform *A. vinelandii* strain CA11, which is deleted for the genes encoding the Mo-nitrogenase (*nifHDK*). The resulting strain (RP206) contained deletions in the genes for both the Mo- and V-nitrogenases (Fig. 2i, lane 5; Fig. 2ii, lane 5).

Growth of strains deleted for the Mo- and V-nitrogenases. As expected, Mo stimulated growth of the single-deletion strain RP114 in medium lacking a fixed nitrogen source (Fig. 3a). This strain grew less well in medium deficient in both Mo and V. Unexpectedly, very slow growth was observed in the presence of V, even though this strain contains a deletion in the genes for the V-nitrogenase. By contrast, growth of the double-deletion strain RP206 was completely inhibited by Mo and was greatest when both Mo and V were omitted from the medium (Fig. 3b). When V alone was added to the medium, the double-deletion strain also grew at a very slow rate, similar to that observed in the single-deletion strain. The ability of the double-deletion strain RP206 to fix nitrogen in medium lacking both Mo and V was confirmed by an increase in the $^{15}N/^{14}N$ ratio when it was grown in the presence of $^{15}N_2$. The atom% enrichment was 5.14% for the cells grown in the presence of $^{15}N_2$ and 0.02% for the control cells grown in air.

The metal dependence of diazotrophic growth of the double-deletion strain was tested in medium treated to remove transition metals and containing ultrapure iron. In this medium both growth and nitrogenase activity were found to be equally limited by iron (Fig. 4). The increased growth observed when a combined nitrogen source (1 mM urea) was added to iron-limited cultures indicated that limitation of growth by iron was principally due to the effect of

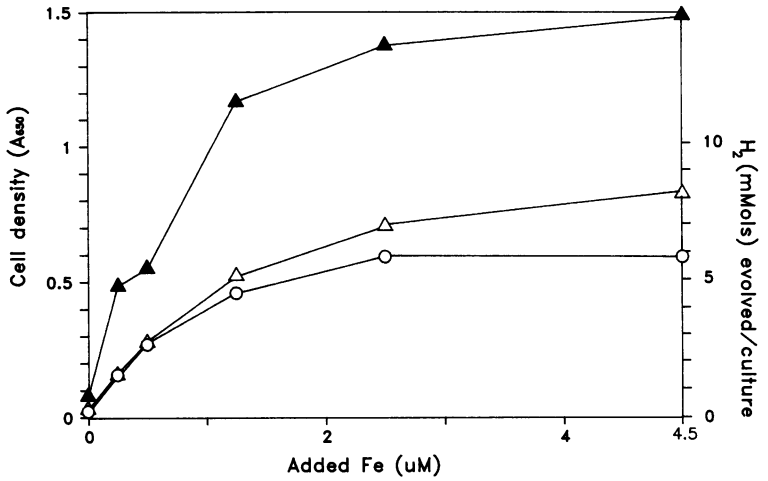

FIG. 4. Influence of iron on growth and nitrogenase activity. Symbols: ▲, medium containing 10 mM urea; △, medium with no fixed N source; ○, Hydrogen evolved per culture. Nitrogenase activity was determined by hydrogen evolution from cultures not containing urea.

iron limitation on nitrogen fixation and not on the function of other iron-containing enzymes. The ability of other metals to stimulate growth and nitrogenase activity under iron-limiting conditions (0.5 μM Fe) was tested by addition of the following (10 μM each): $CoCl_3$, K_2CrO_4, $CuSO_4$, $MnCl_2$, $NiSO_4$, $NaReO_4$, $NaWO_4$, $TiCl_3$, and $ZnCl_2$. None of these metals stimulated growth or nitrogenase activity. In contrast to this, similar amounts of V were essential for growth of *A. chroococcum* strain MCD1155, which contains only the structural genes for the V-nitrogenase (18).

Repression of nitrogenase activity by Mo and V. The nitrogenase activity of strain RP206, measured as acetylene reduction, was completely repressed by Mo (Fig. 5a). However, when V was added to the culture, there was a rapid decrease in activity which was not completely repressed. If the rate of nitrogen reduction is likewise not completely inhibited by V, it would explain the slow growth of the double-deletion strain in the presence of V.

Reduction of acetylene to ethane. The V-nitrogenase of *A. chroococcum* not only reduces acetylene to ethylene, but also produces a small amount of ethane (ethane/ethylene ratios from 0.015 to 0.040). It was proposed previously that ethane formation was a potential test of V-nitrogenase in vivo because comparable amounts of ethane were not observed when acetylene was reduced by the Mo-nitrogenase of *A. chroococcum* or *Klebsiella pneumoniae* (8). Strain RP206 also reduces acetylene to ethane (Table 1). While measuring the repression of nitrogenase activity of strain RP206 by Mo by using reduction of acetylene to ethylene (and ethane) as an assay, we found that addition of Mo to a culture of this strain 2 to 4 h prior to assay resulted in an approximately 10-fold increase in the ratio of ethane to ethylene (Table 1). By contrast, addition of V to strain RP206 did not result in an increase in ethane production. The increase in ethane production after the addition of Mo was transient, reaching a maximum in 2 to 4 h (Fig. 5b). It is therefore unlikely to have been caused by the induction of a new enzyme. We suggest that the change in reactivity may be due to the incorporation of the FeMo cofactor into the third nitrogenase to produce a hybrid enzyme. Initiation of synthesis of the FeMo cofactor by the addition of Mo would be accompanied by repression of synthesis of the polypeptides of the third nitrogenase. The lower enzyme activity observed when the double-deletion strain was grown in the

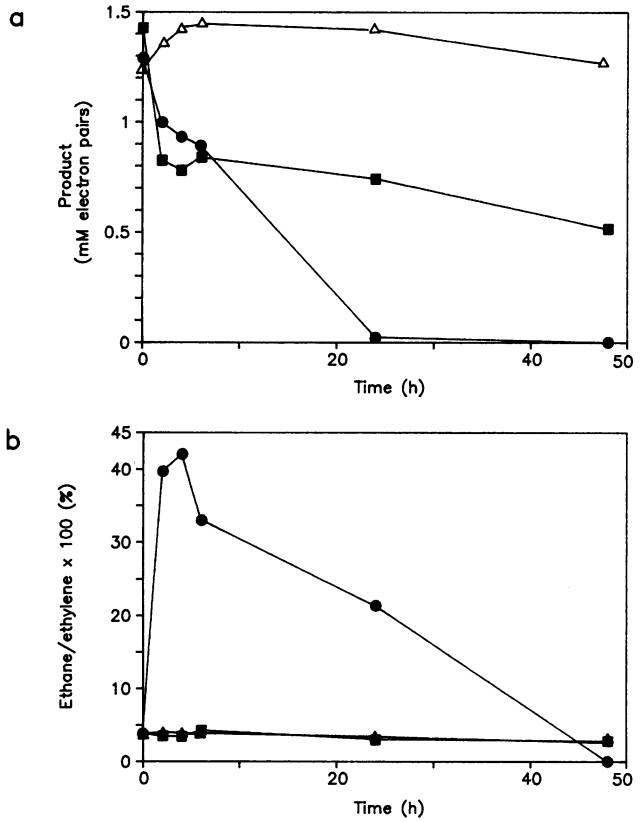

FIG. 5. (a) Repression of acetylene reduction of strain RP206 by addition of Mo (●) and V (■). △, No metal added. The total product formed, represented as millimoles of electron pairs transferred, was 2× millimoles of ethylene plus 4× millimoles of ethane. (b) Transient increase in reduction of acetylene to ethane after the addition of Mo to a culture of *A. vinelandii* RP206. The amounts of ethane produced after the addition of Mo and V to cultures is expressed as a percentage of the amount of ethylene produced. Metal added to medium: ●, Mo; ■, V; △, none.

TABLE 1. Effects of molybdenum and vanadium on ratios of ethane to ethylene produced from acetylene by *A. vinelandii* CA11 and RP206

Strain	Growth medium	Metal (10 μM) added 2 h prior to assay	C_2H_6/C_2H_4 ratio (10^{-2})
RP206	N-free	None	4.6
		Mo	48.1
		V	4.2
	N-free + 10 μM V	Mo	—[a]
		V	—
CA11	N-free	None	4.0
		Mo	19.8
		V	ND[b]
	N-free + 10 μM V	Mo	5.5
		V	1.9

[a] —, No growth.
[b] ND, Not determined.

presence of V may also be because the enzyme incorporated an inappropriate cofactor.

To test whether the addition of Mo to a strain synthesizing the V-nitrogenase resulted in a similar increase in ethane formation, we added Mo to strain CA11 grown in the presence of V. The ethane/ethylene ratio increased to 0.055. However, when this strain was grown in the absence of V or Mo, the ethane/ethylene ratio rose to 0.20 after the addition of Mo (Table 1). Therefore, although the basis for the increase in ethane production to a high level after the addition of Mo remains to be determined, it distinguishes the third nitrogenase of *A. vinelandii* from the V-nitrogenase.

DISCUSSION

Studies on the expression of NH_4^+-repressible polypeptides and on transcription of alternative *nifH* genes have pointed to the presence of a third nitrogenase in *A. vinelandii* (2, 12). This nitrogenase, which is synthesized when *A. vinelandii* is grown in the absence of both Mo and V, has recently been isolated from *A. vinelandii* strain CA11.6 grown under these conditions (6), and the third cluster of *nifHDK*-like genes has been isolated and sequenced (3). The present study provides definitive genetic evidence for the presence of a third nitrogenase which can be clearly distinguished from the Mo- and V-nitrogenases. Inhibition of diazotrophic growth of strain RP206 by Mo and V suggests that the nitrogenase in strain RP206 is unlikely to contain either of these two metals. The nitrogenase purified from strain CA11.6 grown in Mo- and V-deficient medium lacked significant amounts of Mo, V, tungsten, chromium, and rhenium (6). A low content of zinc (0.4 g atom/mol) was observed, but similar amounts were also found in preparations of the MoFe nitrogenase, where it has not been assigned a function. We showed that addition of $ZnCl_2$ did not enhance growth of strain RP206. These results, together with the iron-dependent diazotrophic growth of strain RP206 and the failure of other transition metals to stimulate growth, suggest that the third nitrogenase of *A. vinelandii* is an iron nitrogenase and so represents a third class of nitrogenase. The existence of such an enzyme implies that reduction of dinitrogen can occur in the absence of Mo and V.

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