

Expression of an Alternative Nitrogen Fixation System in *Azotobacter vinelandii*†

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Nitrogenase activities were determined from maximum acetylene reduction rates for mutant strains of *Azotobacter vinelandii* which are unable to fix N₂ in the presence of molybdenum (Nif⁻) but undergo phenotypic reversal to Nif⁺ under conditions of Mo deficiency. The system responsible for N₂ fixation under these conditions is thought to be an alternative N₂ fixation system (Bishop et al., Proc. Natl. Acad. Sci. U.S.A. 77:7342-7346, 1980). Phenotypic reversal of Nif⁻ strains to Nif⁺ strains was also observed in N-free medium without Mo but with either V or Re. Two protein patterns were found on two-dimensional gels of proteins from the extracts of wild-type cells cultured in N-free medium without Mo and with or without V or Re. The expression of each protein pattern in the wild-type strain of *A. vinelandii* seemed to depend upon the physiological state of the N₂-fixing culture. Electron paramagnetic resonance experiments were conducted on whole cells of *A. vinelandii* grown under conditions of Mo deprivation in the absence of fixed N. No $g = 3.65$ signal (an electron paramagnetic resonance signal characteristic of the Mo-containing component of nitrogenase) was detectable in these cells, regardless of whether V or Re was present during growth of these cells. These results are discussed from the perspective that the well-known effect of V on N₂ fixation by *A. vinelandii* may involve an alternative N₂ fixation system.

The conventional N₂ fixation system in *Azotobacter vinelandii* consists of dinitrogenase, a molybdenum- and iron-containing protein, and dinitrogenase reductase, an iron-containing protein. Dinitrogenase is a tetramer with a molecular weight of about 245,000 and is made up of two pairs of nonidentical subunits, each with a molecular weight of about 61,000 (16). Dinitrogenase reductase is a dimer (molecular weight, 60,500) containing two identical subunits, each with a molecular weight of 30,000 (15).

Mutant strains of *A. vinelandii* which are unable to fix N₂ in the presence of Mo (Nif⁻) have been isolated and characterized with respect to electron paramagnetic resonance (EPR) signals, their activities for dinitrogenase and dinitrogenase reductase, and antigenic cross-reactive material (13). The *nif* mutations carried by these strains have been genetically mapped with recombination index values (3).

We recently reported the existence of an alternative N₂ fixation system in *A. vinelandii* (4). Evidence for this alternative system is based primarily on the observation that Mo deprivation caused Nif⁻ mutant strains to undergo phenotypic reversal to Nif⁺. In this study, we

present further evidence for such an alternative N₂ fixation system in *A. vinelandii*.

MATERIALS AND METHODS

Bacterial strains and media. The *A. vinelandii* strains utilized in this investigation (Table 1) were cultured in modified Burk medium (14). When it was necessary to include fixed nitrogen (N) in the medium, ammonium acetate (NH₄OAc) was added to a concentration of 400 µg of N per ml. Solid N-free Burk medium contained 1.5% purified agar (Difco Laboratories), whereas solid medium containing NH₄OAc contained 1.5% agar (Difco). Precautions were taken to minimize contamination by Mo when Mo-deficient medium was used. The same precautions were employed when Re or V was added to the medium. All glassware was base and acid washed as described by Benemann et al. (2). Chemical components used to prepare the Mo-deficient medium were obtained from the following sources: KH₂PO₄ (ultra, ultrapure) and sucrose (ultra, ultrapure) were purchased from J. T. Baker Chemical Co.; CaCl₂ (puratronic), MgSO₄·7H₂O (ultrapure), and KOH (ultrapure) were purchased from Alfa-Ventron; and FeSO₄·7H₂O was purchased from Fisher Scientific Co. Both rhenium (VII) oxide (ultrapure) and vanadium pentoxide (ultrapure) were purchased from Alfa-Ventron. Mo contamination of the Mo-deficient medium was found to be 1.12 ppb (1.12 ng/ml) by the zinc dithiol method of Clark and Axley (8).

Cultural procedures. Strains were maintained on Mo-deficient agar medium containing NH₄OAc. For

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TABLE 1. *A. vinelandii* strains

Strain	Genotype	Phenotype	Reference
CA	Wild type	I ⁺ II ⁺ ^a	7
CA2	<i>rif-1 nif-10</i>	Rif ^r Nif ⁺	4
CA4	<i>rif-1 nif-6</i>	Rif ^r Nif ⁺ pseudorevertant of UW10	4
UW1	<i>nif-1</i>	I ⁻ II ⁻	13
UW3	<i>nif-3</i>	I ⁻ II ⁻	13
UW6	<i>nif-6</i>	I ⁻ II ⁺	13
UW10	<i>nif-10</i>	I ⁻ II ⁺	13
UW38	<i>nif-38</i>	I ⁻ II ⁺	13
UW91	<i>nif-91</i>	I ⁺ II ⁻	13

^a I and II represent dinitrogenase and dinitrogenase reductase, respectively. Superscripts + and - indicate active and inactive proteins, respectively, as determined by the acetylene reduction method.

liquid cultures, side-arm flasks (300 ml) containing 50 ml of culture were incubated at 28°C with vigorous shaking. Inoculum cultures for all experiments were subcultured at least twice in Mo-deficient medium. For each transfer in liquid culture, the medium was inoculated to a cell density of approximately 7×10^6 cells per ml. Growth was monitored with a Klett-Summerson colorimeter (no. 64 red filter) under conditions where 1 Klett unit equaled 7×10^6 viable cells per ml.

Biochemical assays. Acetylene reduction (a measure of nitrogenase activity) and two-dimensional gel electrophoresis were conducted as described previously (4). Low-temperature EPR spectroscopy experiments were conducted on whole cells at 5 to 12 K with a Varian E-9 EPR spectrometer as described by Davis et al. (9).

RESULTS

Growth and acetylene reduction under conditions of Mo deprivation. In a previous study (4) we observed that some Nif⁻ mutant strains of *A. vinelandii* underwent phenotypic reversal to Nif⁺ when starved for Mo. This led to the hypothesis that *A. vinelandii* possesses an alternative N₂ fixation system which permits it to grow under conditions of Mo starvation.

Growth of Nif⁻ strains UW3, UW6, and UW10 in Mo-deficient N-free medium is shown in Fig. 1. In this experiment the generation times ranged from 3 to 4 h. If growth of the Nif⁻ strains under these conditions is due to derepression of the alternative N₂ fixation system, then one might expect the addition of Mo to repress the alternative N₂ fixation system and cause derepression of the conventional molybdoenzyme system. Furthermore, since the conventional molybdoenzyme system of these strains is inoperative due to mutational lesions, these cells would therefore not be expected to grow under these conditions. This prediction is

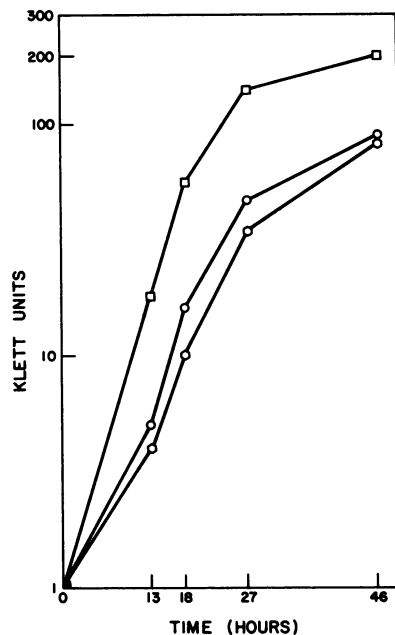


FIG. 1. Growth of Nif⁻ mutant strains in Mo-deficient N-free medium. Symbols: ○, strain UW3; ◯, strain UW6; □, strain UW10.

borne out as shown in Fig. 2, where growth of Nif⁻ strain UW3 is depicted after inoculation into N-free medium containing several concentrations of Na₂MoO₄·2H₂O. Similar results were obtained with strains UW6, UW10, and UW38.

Whole cell nitrogenase activities were determined throughout the growth cycle for several Nif⁻ strains and wild-type strain CA. The maximum values for each strain are shown in Table 2. From these data it is apparent that the wild-type strain had the highest maximum value, followed by Nif⁻ strain UW3. The nitrogenase activities, however, did not correlate with the generation times since there were no consistent differences between the strains with respect to generation times. The maximum acetylene reduction rates were always found during the early exponential growth phase at cell densities ranging from 1.4×10^7 to 4.9×10^7 cells per ml.

Growth and nitrogenase activity under conditions of Mo deprivation in the presence of Re and V. Several studies (5, 6, 12) suggested that V can substitute for Mo in dinitrogenase from *A. vinelandii* and *A. chroococcum*. Another report (10) indicated that Re might also replace the Mo requirement for N₂-fixing cultures of *A. vinelandii*. We speculated that the V or Re effects or both might be involved with the postulated alternative N₂ fixation system. If this were true, one might expect the Nif⁻ mutant strains to grow as well as the wild-type strain in Mo-deficient N-free medium containing these metals.

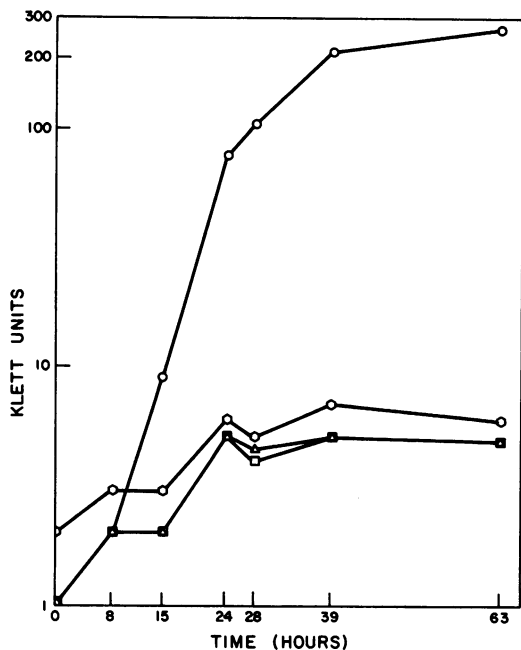


FIG. 2. Addition of $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ to cultures of strain UW3 growing in Mo-deficient N-free medium. Symbols: \circ , no addition; \square , $1 \mu\text{M}$ $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$; \triangle , $0.6 \mu\text{M}$ $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$; \square , $0.21 \mu\text{M}$ $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$.

Growth of strain CA in the presence of either $10 \mu\text{M}$ V_2O_5 or $10 \mu\text{M}$ Re_2O_7 is shown in Fig. 3. The generation times (ca. 3.5 h) for the V and Re cultures were essentially the same as those determined for strain CA growing in Mo-deficient medium in the absence of V and Re. Similar results were obtained with Nif^- strains UW10 and UW38 when grown in the presence of V or strain UW10 cultured in the presence of Re. Maximum nitrogenase values, as determined above for the Mo-deprived cultures, were 850 nmol of C_2H_4 per 10^8 cells per h and 313 nmol of C_2H_4 per 10^8 cells per h for strain CA growing in N-free medium in the presence of $10 \mu\text{M}$ V_2O_5 and $10 \mu\text{M}$ Re_2O_7 , respectively.

Two-dimensional gel electrophoresis. We used

TABLE 2. Maximum rates of acetylene reduction under conditions of Mo deprivation

Strain	nmol of C_2H_4 per 10^8 cells per h ^a (SEM)
CA	364 (7)
UW3	271 (3)
UW6	112 (1)
UW10	69 (5)
UW38	59 (2)

^a The acetylene reduction values represent the average of two determinations.

two-dimensional gel electrophoresis to demonstrate the presence of four new NH_4^+ -repressible proteins in extracts of cells grown under conditions of Mo deprivation in N-free medium (4). We hypothesized that the four-protein pattern might represent an alternative N_2 fixation system in *A. vinelandii* (4), even though these proteins cannot be assigned specific functions until they have been purified and characterized.

Figure 4A shows the nitrogenase proteins for the conventional molybdoenzyme system, and Fig. 4B shows the four new proteins found in extracts of strain CA grown in Mo-deficient N-free medium. When two-dimensional gel electrophoresis was conducted on proteins from extracts of cells cultured under N_2 -fixing conditions in Mo-deficient medium containing $5 \mu\text{M}$ V_2O_5 , two proteins were observed (Fig. 4C) which corresponded to neither the proteins found in the conventional nitrogenase system nor the four new proteins described above. The spots representing the two proteins were very faint on two-dimensional gels of proteins from extracts of cells grown in cultures containing V and NH_4^+ (data not shown). Thus, these proteins appear to be NH_4^+ -repressible, as might be

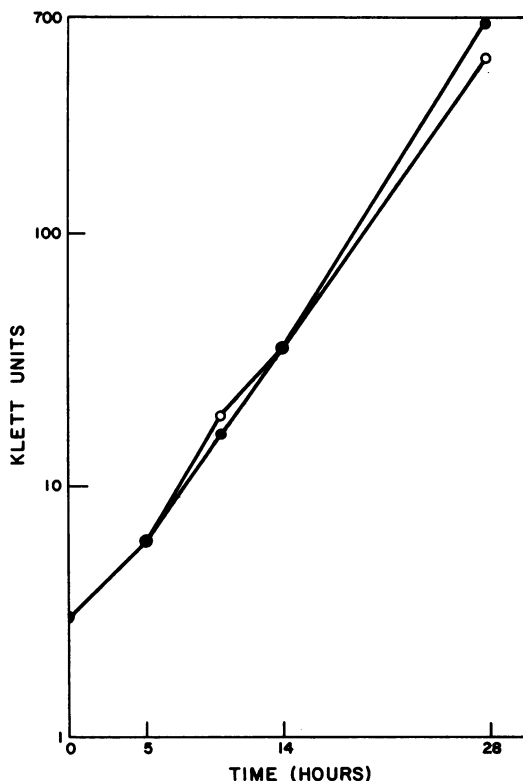


FIG. 3. Growth of strain CA (wild type) in Mo-deficient N-free medium containing V_2O_5 or Re_2O_7 . Symbols: \bullet , $10 \mu\text{M}$ V_2O_5 ; \circ , $10 \mu\text{M}$ Re_2O_7 .

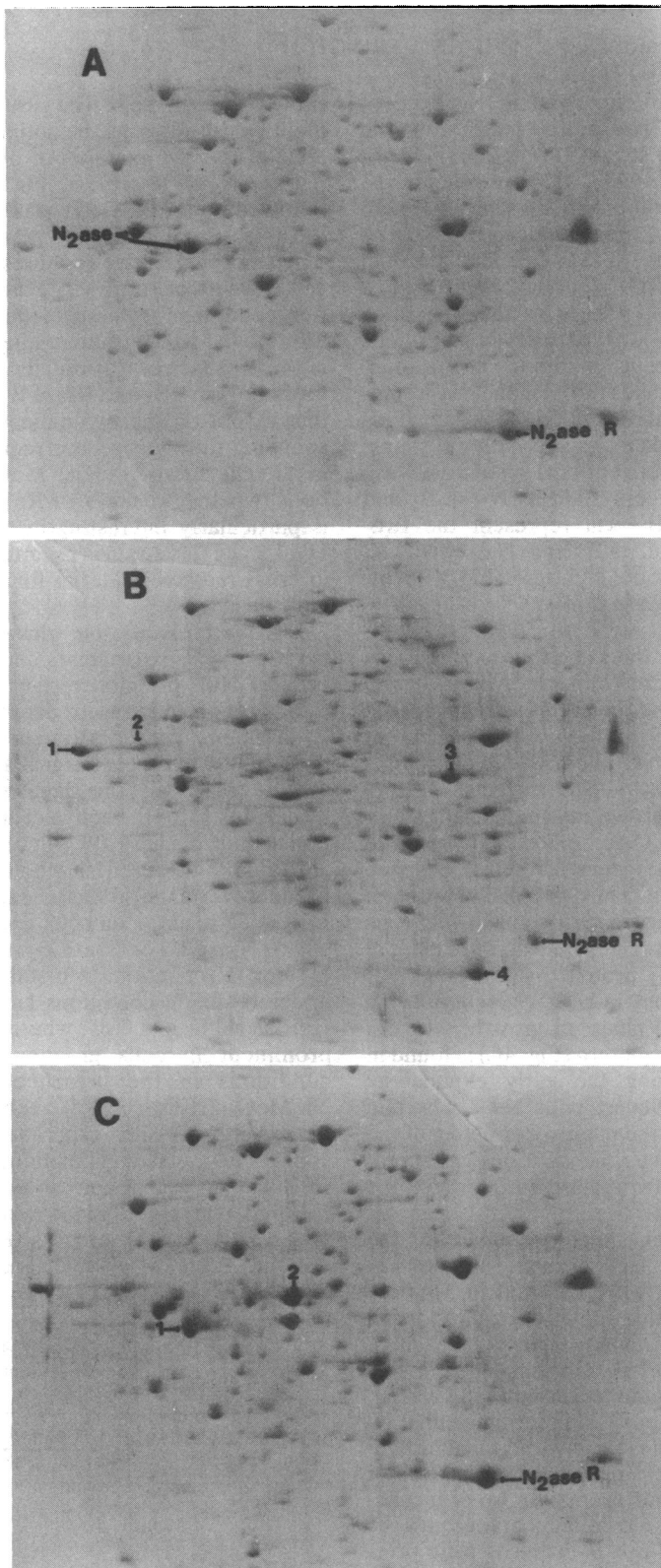


FIG. 4. Two-dimensional gel electrophoresis of proteins in extracts of strain CA (wild type). N₂ase, Dinitrogenase; N₂ase R, dinitrogenase reductase. (A) Strain CA grown under N₂-fixing conditions in the presence of 1 μ M Na₂MoO₄·2H₂O. (B) Strain CA grown under N₂-fixing conditions in Mo-deficient medium. Spots 1 through 4 represent new NH₄⁺-repressible proteins. (C) Strain CA grown under N₂-fixing conditions in Mo-deficient medium containing 5 μ M V₂O₅. Spots 1 and 2 represent new NH₄⁺-repressible proteins.

expected if they are involved in N_2 fixation. Furthermore, these two proteins were found in cell extracts of Nif^- strains UW1, UW3, UW10, UW38, and UW91 when they were cultured under N_2 -fixing conditions in Mo-deficient medium. Cell extracts of these strains grown under N_2 -fixing conditions in Mo-deficient medium containing $5 \mu\text{M}$ V_2O_5 also contained the two new proteins. Thus, it appears that the same proteins are observed in extracts of Nif^- cells grown under N_2 -fixing conditions in Mo-deficient medium regardless of whether V is present. In keeping with our previous designations (4) for the conventional and alternative systems, we will denote the conventional system as N_2 ase A and the alternative system as N_2 ase B₁ and N_2 ase B₂. N_2 ase B₁ will represent the two-protein pattern (Fig. 4C), and N_2 ase B₂ will designate the four-protein pattern (Fig. 4B; formerly designated N_2 ase B [4]).

The above results were surprising since we had expected to see the same four new proteins (N_2 ase B₂) that were found in wild-type cell extracts when these extracts were prepared from N_2 -fixing cells cultured under Mo-deficient conditions. Moreover, the two new proteins (N_2 ase B₁) were observed in extracts of strain CA cells that were grown in Mo-deficient medium containing NH_4OAc , followed by 3 h of derepression (for nitrogenase proteins) in N-free Mo-deficient medium. Thus, two distinct protein patterns can be observed in extracts of wild-type cells, depending on the physiological state of the cells. The N_2 ase B₂ protein pattern (Fig. 4B) appears to be present in cells collected during the late exponential phase of growth, whereas the N_2 ase B₁ protein pattern (Fig. 4C) is found in cells collected during the early exponential growth phase. However, with Nif^- cells and cells grown with V, the pattern consisting of the N_2 ase B₁ proteins was the only one observed, regardless of the growth phase during which the cells were harvested.

To further examine this phenomenon, the following derepression experiment was conducted. Strain CA cells were cultured in Mo-deficient medium containing NH_4OAc until a cell density of 4.1×10^8 cells per ml was reached. The cells were harvested and suspended in Mo-deficient N-free medium to a density of 3.9×10^8 cells per ml. At various times during incubation of this culture, samples were withdrawn for two-dimensional gel electrophoresis. The pattern of N_2 ase B₁ proteins was seen after 3 h of incubation; however, after 6 h of incubation, both the N_2 ase B₁ and the N_2 ase B₂ patterns were present. After 22 h of incubation, the N_2 ase B₂ pattern had become more prominent than the N_2 ase B₁ pattern as judged by the relative intensities of the spots representing each set of

proteins on the gels. The results of this experiment tend to support the notion that the appearance of the two patterns of proteins is sequential; the N_2 ase B₁ pattern (Fig. 4C) appears first, followed by the N_2 ase B₂ pattern (Fig. 4B).

Table 3 summarizes the results from the two-dimensional gel electrophoresis of proteins in cell extracts of strain CA grown under N_2 fixation conditions in the presence and absence of Mo, V, and Re. It is interesting that cells grown in the presence of Re contained protein patterns for both N_2 ase B₁ and N_2 ase B₂. It is noteworthy that a spot on the two-dimensional gels corresponding to dinitrogenase reductase was found in all cells cultured under Mo starvation conditions, whether or not V or Re was present. This is particularly interesting in the case of strain UW1 since this strain is reported to lack antigenic cross-reactive material for dinitrogenase reductase (13).

EPR spectroscopy on whole cells. Low-temperature EPR experiments conducted on whole cells of Nif^+ pseudorevertant strains CA2 and CA4 (strains which were derepressed for N_2 ase B₂ in the presence of Mo under N_2 -fixing conditions) failed to detect a signal at $g = 3.65$ (Table 4). The presence of this signal has been shown to be diagnostic for the active center of the conventional dinitrogenase (9). Since this EPR signal appeared to be absent in whole cells of the Nif^+ pseudorevertants, it was of interest to conduct EPR spectroscopy on cells under all conditions where N_2 ase B₁ or N_2 ase B₂ might be responsible for N_2 fixation. Mo-starved cells grown under N_2 -fixing conditions lacked a detectable EPR signal at $g = 3.65$, whereas this signal was prominent in cells growing under identical conditions in the presence of Mo ($1 \mu\text{M}$ $Na_2MoO_4 \cdot 2H_2O$) (Fig. 5). Table 4 summarizes the results of EPR experiments with several strain and metal combinations. No detectable EPR signal was found at $g = 3.65$ in cells cultured under conditions where N_2 ase B₁ or N_2 ase B₂ would be expressed; i.e., this signal was only found in whole cells of strains CA and

TABLE 3. Effect of Mo, V, and Re on the expression of nitrogenase systems in *A. vinelandii* CA

Metal added to the N-free medium	Expression of nitrogenase system ^a		
	N_2 ase A	N_2 ase B ₁	N_2 ase B ₂
None	-/+	-	+
Mo	+	-	-
V	-/+	+	-
Re	-	+	+

^a +, Presence of spots representing the nitrogenase system on two-dimensional gels; - absence of spots; -/+, faint trace of spots observed.

TABLE 4. Results of EPR spectroscopy on whole cells

Strain	Metal added to the N-free medium	Presence of a detectable EPR signal at $g = 3.65^a$
CA	Mo	+
UW91	Mo	+
CA2	Mo	-
CA4	Mo	-
CA	V	-
CA2	V	-
CA4	V	-
CA	Re	-
CA	None	-
CA2	None	-
CA4	None	-
UW1	None	-
UW10	None	-
UW91	None	-

^a +, Presence of an EPR signal at $g = 3.65$; -, absence of this signal.

UW91 that were grown in the presence of Mo, a condition which leads to the expression of N₂ase A.

DISCUSSION

Results have been presented in support of the hypothesis (4) that an alternative N₂ fixation system exists in *A. vinelandii* and is expressed during conditions of Mo starvation. This evidence centers principally on the ability of well-characterized Nif⁻ mutant strains to undergo phenotypic reversal during Mo deprivation under N₂-fixing conditions. We have demonstrated that Nif⁻ strains grow not only in a Mo-deficient

medium, but also in this medium with V or Re. Furthermore, growth of the Nif⁻ mutant strains is blocked upon transfer from Mo-deficient N-free medium to N-free medium containing Na₂MoO₄·2H₂O, as would be expected if Mo leads to derepression of the N₂ase A system while simultaneously repressing proteins involved in the N₂ase B₁ and N₂ase B₂ systems. Since some of the nitrogenase proteins in the N₂ase A system are either missing or inactive in these mutant strains, these strains will not grow in the absence of a N source in the presence of Mo.

Mutant cells cultured under conditions of Mo deprivation or in the presence of V in N-free medium lack dinitrogenase proteins for the N₂ase A system; however, two new NH₄⁺-repressible proteins were found. We have designated this protein pattern (as seen on two-dimensional gels) as N₂ase B₁. This protein pattern was the first discernible pattern in wild-type cells (strain CA) during derepression after transfer from Mo-deficient medium containing NH₄OAc to N-free Mo-deficient medium. The N₂ase B₁ protein pattern was followed by the appearance of the four NH₄⁺-repressible proteins observed previously in extracts of wild-type cells cultured in Mo-deficient medium under N₂-fixing conditions.

We previously designated this pattern as N₂ase B (4), but it is now designated as N₂ase B₂. The relationship of these two distinct protein patterns to each other (other than the sequence of appearance as observed on two-dimensional gels during derepression) is at present unknown. It is possible that the N₂ase B₁ proteins undergo

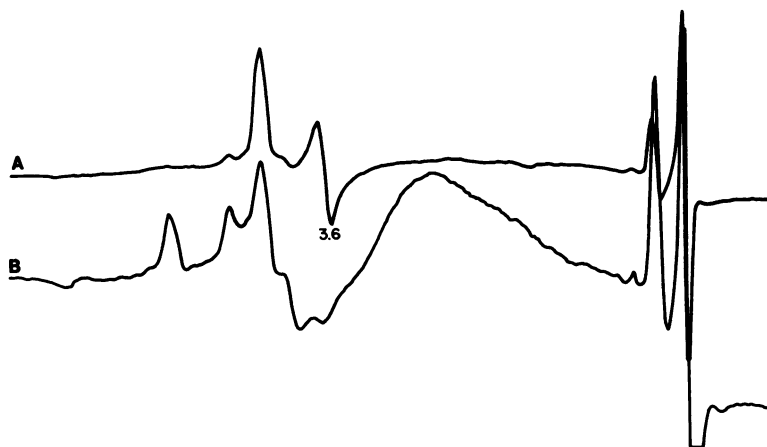


FIG. 5. EPR spectra of *A. vinelandii* strain CA cells grown under air (A) in the presence of 1.0 μ M Na₂MoO₄·2H₂O to a density of 57 Klett units and (B) in the absence of Na₂MoO₄·2H₂O to a density of 55 Klett units. Conditions of EPR spectroscopy were: temperature, 7 K; microwave power, 20 mW; instrument gain, 5×10^2 for (A) and 2.5×10^3 for (B); modulation amplitude, 16 G; time constant, 1.0 s; field sweep rate, 1,000 G/min; microwave frequency, 9 GHz.

post-translational modification that gives rise to the N_2 ase B_2 proteins or that these two sets of proteins may be coded by different sets of genes. Interestingly, only the N_2 ase B_1 proteins were observed in wild-type cells grown in N-free Mo-deficient medium containing V and in Nif^- mutant cells cultured in N-free Mo-deficient medium, even during the late exponential phase of growth. One can only speculate as to why expression of the N_2 ase B_2 proteins was not observed in Nif^- mutant strains; however, it is possible that expression of the N_2 ase B_2 proteins might require a very low level of expression of catalytically active N_2 ase A proteins, although it is uncertain as to how this type of control mechanism might operate.

An interesting observation is that a spot corresponding to the dinitrogenase reductase protein is found on all two-dimensional gels obtained with cells growing under conditions where either N_2 ase B_1 or N_2 ase B_2 proteins were expressed. This is intriguing in the case of strain UW1 because this strain is reported to lack antigenic cross-reactive material for dinitrogenase reductase (13). One possible interpretation of this finding is that there are two copies of the gene coding for the dinitrogenase protein and that each gene is under different regulation with respect to Mo; i.e., one gene codes for the dinitrogenase reductase which functions in the N_2 ase A system, and the other codes for the dinitrogenase reductase which functions in the N_2 ase B_1 and N_2 ase B_2 systems.

The lack of a detectable EPR signal in whole cells cultured under conditions where N_2 ase B_1 , N_2 ase B_2 , or both would be expressed suggests that the active center(s) in the dinitrogenase(s) is different from that found in the dinitrogenase belonging to the N_2 ase A system. The possibility that the lack of an EPR signal at $g = 3.65$ is due to the redox state of the enzyme rather than a different active center cannot be ruled out, however.

Our two-dimensional gel data suggest that V stimulation of N_2 fixation by *A. vinelandii* that is frequently observed by others (1, 2) may be due to derepression of at least some of the proteins involved in the alternative N_2 fixation system, since the protein pattern observed on two-dimensional gels of strain CA grown in the presence of V appears to be identical to the N_2 ase B_1 system (Fig. 4C). Burns et al. (6) reported that a purified preparation of V-Fe protein from *A. vinelandii* did not give any EPR signal at $g = 3.65$. This result agrees with our observation that no detectable EPR signal at $g = 3.65$ is found in whole cells cultured under N_2 -fixing conditions in the presence of V. Although Burns et al. (6) assumed that V replaced Mo in dinitrogenase isolated from V-grown cells, it is inter-

esting that their amino acid analyses for the Mo- and V-containing dinitrogenases were significantly different from each other with respect to several amino acids. Furthermore, the purified V-containing dinitrogenase lacked acid-labile sulfide and metals (including V) other than Fe. The explanation given for these results was that the dinitrogenase produced by cells grown on medium containing V might be unstable to fractionation on DEAE-cellulose, leading to a loss of metal and acid-labile sulfide. Our interpretation is that the V-Fe protein described by Burns and co-workers was composed of the nitrogenase proteins representing the alternative N_2 ase B_1 system. This interpretation is consistent with our observation that Nif^- mutant strains undergo phenotypic reversal when cultured in Mo-deficient medium containing V. This would not be expected if V simply replaced Mo at the active center of dinitrogenase since these strains do not contain an active dinitrogenase, and it is difficult to envision how the mutational lesion would be corrected by V. At this time we can only speculate on the role that V might play in expression of the postulated alternative N_2 fixation system; however, it is possible that V increases the maximum observed acetylene reduction values by excluding even trace amounts of Mo during starvation, which in turn could lead to maximal derepression of the alternative N_2 fixation system. This speculation is supported by a study of the effects of Mo and V on N_2 -fixing *Anabaena cylindrica*, where the addition of V to the growth medium amplified the symptoms characteristic of Mo deficiency (11).

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