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Tn*5*-Induced Mutants of *Azotobacter vinelandii* Affected in Nitrogen Fixation under Mo-Deficient and Mo-Sufficient Conditions†

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Mutants of *Azotobacter vinelandii* affected in N_2 fixation in the presence of 1 μM Na_2MoO_4 (conventional system), 50 nM V_2O_5, or under Mo deficiency (alternative system) have been isolated after Tn*5* mutagenesis with the suicide plasmid pSUP1011. These mutants can be grouped into at least four broad phenotypic classes. Mutants in the first class are Nif$^-$ under Mo sufficiency but Nif$^+$ under Mo deficiency or in the presence of V_2O_5. A *nifK* mutant and a mutant apparently affected in regulation of the conventional system belong to this class. Mutants in the second class are Nif$^-$ under all conditions. An FeMo-cofactor-negative mutant (NifB$^-$) belongs to this class, implying an involvement of *nifB* in both the conventional and the alternative N_2 fixation systems. The third mutant class consists of mutants incapable of N_2-dependent growth under Mo deficiency. Most of the mutants in this class are also affected in N_2 fixation in the presence of 1 μM Na_2MoO_4, with acetylene reduction rates ranging from 28 to 51% of the rates of the wild type. Strains constructed by genetic transfer of the Kan^r marker of mutants from this class into *nifHDK* or *nifK* deletion mutants showed N_2-dependent growth only in the presence of V_2O_5, suggesting that growth in the presence of V_2O_5 and growth under Mo deficiency are independent phenomena. The only mutant in the fourth class shows wild-type nitrogenase activity under Mo sufficiency, but only 10% of the acetylene reduction activity of the wild type in the presence of 50 nM V_2O_5. The acetylene reduction rates of whole cells of this mutant are identical in Mo-deficient medium and in medium containing V_2O_5. The conventional nitrogenase subunits are expressed in this mutant even under Mo deficiency or in the presence of V_2O_5; however, the NH_4^+- and Mo-repressible proteins normally seen under these conditions could not be detected on two-dimensional gels. The Tn*5* insertion carried by this mutant makes N_2 fixation dependent solely on the conventional system and consequently abolishes the vanadium effect.

Nitrogen fixation by *Azotobacter vinelandii* appears to include characteristics common to all known diazotrophic organisms as well as features that are novel (3, 5). The conventional nitrogenase system consisting of dinitrogenase and dinitrogenase reductase is responsible for the reduction of N_2 to NH_4^+ under Mo-sufficient conditions. Mutants unable to fix nitrogen under Mo-sufficient conditions (Nif$^-$) have been isolated after *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis (12, 36). Some of these mutants have been characterized biochemically (32). The *nif* mutations carried by these strains have also been genetically mapped by using recombination index values (2). Most of these mutants undergo phenotypic reversal to Nif$^+$ under Mo-deficient conditions. This led us to propose the existence of an alternative nitrogen fixation system in *A. vinelandii* that is expressed under Mo-deficient conditions (3, 4). Mutants carrying deletions in the structural genes for nitrogenase (*nifHDK*) are able to undergo phenotypic reversal under Mo-deficient conditions, thus providing definitive proof for the existence of an N_2-fixing system which operates without the participation of the conventional nitrogenase proteins (5; P. E. Bishop, M. E. Hawkins, and R. R. Eady, Biochem. J., in press). When cultured under Mo-deficient conditions both wild-type and Nif$^-$ strains produce NH_4^+-repressible proteins which are not observed in extracts from cells grown under Mo-sufficient conditions (3, 4). The significance of these NH_4^+-repressible proteins is not completely understood, although they may represent alternative nitrogenase proteins. Some of these proteins are also synthesized under N_2-fixing conditions in the presence of V_2O_5, and a connection between the stimulation of N_2 fixation by V (V effect) and the expression of the alternative N_2 fixation system has been suggested (4). Recently it has become clear that the alternative nitrogenase of *Azotobacter chroococcum* is a vanadium enzyme (28a).

Biochemical evidence for the presence of a dinitrogenase reductase which is expressed only under Mo-deficient conditions or in the presence of V_2O_5 has been reported for *A. vinelandii* (27). Furthermore, it has been proposed that, in addition to this dinitrogenase reductase, a third dinitrogenase reductase may exist in this organism (9). Multiple genomic regions homologous to *nifH* have been detected, and it has been shown that synthesis of *nifH*-homologous transcripts is regulated by Mo (15).

To advance our understanding of the genetics of N_2 fixation in *A. vinelandii* we have used Tn*5* mutagenesis to isolate mutants that are affected in N_2 fixation under Mo-deficient and Mo-sufficient conditions. In this study we describe the characterization of some of these Tn*5*-induced mutants.

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TABLE 1. Bacterial strains

Strain (plasmid)	Genotype	Phenotype	Reference
E. coli			
SM10(pSUP1011)	*thi thr leu recA* (::RP4-2-Tc-::Mu); pSUP1011	Tra^+ Kan^r Cm^r	(34)
A. vinelandii			
CA	Wild type	Nif^+ (+Mo, +V, −Mo)	(8)
CA11	Δ*nifHDK11*	Nif^- (+Mo), Nif^+ (+V, −Mo)	(5)
CA13	Δ*nifK13*	Nif^- (+Mo), Nif^+ (+V, −Mo)	(6)
UW1	*nif-1*	Nif^- (+Mo), Nif^+ (+V, −Mo)	(32)
UW6	*nif-6*	Nif^- (+Mo), Nif^+ (+V, −Mo)	(32)
UW45	*nif-45*	Nif^- (+Mo, +V, −Mo)	(32)

[a] Conditions used for the Nif phenotype observed: +Mo, 1 μM Na_2MoO_4 present in the medium; +V, 50 nM V_2O_5 present in the medium; −Mo, no added Mo and precautions taken to minimize contamination with Mo.

Oregon, 4 through 10 August 1985 [*in* H. J. Evans, P. J. Bottomley, and W. E. Newton eds., *Nitrogen Fixation Research Progress*, p. 525, Martinus Nijhoff Publishers, Dordrecht, The Netherlands 1985]).

MATERIALS AND METHODS

Bacterial strains and media. The *A. vinelandii* strains utilized in this investigation (Table 1) were cultured in modified Burk medium (38). When it was necessary to include fixed nitrogen in the medium, ammonium acetate (NH_4OAc) was added at 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 NH_4OAc contained 1.5% agar. Precautions were taken to minimize contamination by Mo when Mo-deficient medium was used. The same precautions were taken when V_2O_5 was added to the medium. All glassware was base and acid washed as described by Benemann et al. (1) and the medium was prepared with ultrapure chemicals as described previously (3).

Escherichia coli SM10 harboring plasmid pSUP1011 (34) (kindly provided by R. Simon) was grown in TYE medium (15 g of tryptone [Difco], 10 g of yeast extract, 5 g of NaCl per liter) containing kanamycin (40 μg/ml) and chloramphenicol (40 μg/ml).

Cultural procedures. The *A. vinelandii* strains were maintained on Mo-deficient agar medium containing NH_4OAc. For growth studies in liquid cultures, sidearm flasks (300 ml) containing 35 ml of culture were incubated at 30°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 4×10^6 cells per ml. Growth was monitored with a Klett-Summerson colorimeter (no. 64 red filter).

Tn*5* mutagenesis and mutant selection. Exponentially growing cells (5×10^8 cells) of *A. vinelandii* CA were mixed with an equal number of *E. coli* SM10(pSUP1011) cells growing exponentially in antibiotic-free medium. The cell mixture was filtered onto a Millipore type HA (0.45 μm) filter and incubated on TYE at 30°C for 6 h. The mating mixture was then suspended in Mo-deficient Burk medium containing NH_4OAc and kanamycin (10 μg/ml). This culture was subcultured six to eight times in this medium to ensure Mo starvation of the *A. vinelandii* cells and to eliminate the *E. coli* cells. (*E. coli* is unable to utilize sucrose as a carbon and energy source.) Dilutions of the final culture were plated on limited N medium (5 μg of N per ml) containing either 1 μM Na_2MoO_4, 50 nM V_2O_5, or no added Mo. Colonies which were noticeably smaller than others were isolated and tested several times for their Nif phenotypes.

Transformation. The transformation procedures for *A. vinelandii* employed were those of Page and von Tigerstrom (25). *A. vinelandii* was made competent as described by Page and von Tigerstrom (24). The crude DNA preparation was sheared to a size of approximately 30 kilobase pairs (kbp) by agitation on a Vortex apparatus for 30 s at maximum speed.

Acetylene reduction assays. Acetylene reduction assays on whole cells derepressed for nitrogenase were performed as described previously (3). Protein determinations were carried out by the method of Lowry et al. (18) with bovine serum albumin as a standard.

Two-dimensional gel electrophoresis. Two-dimensional gel electrophoresis of proteins in cell extracts was carried out by the procedures of O'Farrell (23), with modifications as described by Bishop et al. (3).

DNA preparation and restriction endonuclease reactions. Total *A. vinelandii* DNA was prepared from 200 ml of a late-log-phase culture by the procedure of Saito and Miura (29). The DNA was cleaved with *Eco*RI according to the specifications of the supplier.

Electrophoresis of DNA and hybridization reactions. Electrophoresis of DNA in 0.8% agarose gels and ethidium bromide staining were conducted as described previously (21, 26). The transfer of DNA to nitrocellulose filters (Schleicher & Shuell Co. Keene, N.H.) was a modification (35) of the technique of Southern (37). Filters were treated as described by Denhardt (10) and hybridized in 6 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate)–0.5% sodium dodecyl sulfate–0.02% bovine serum albumin–0.02% Ficoll–0.02% polyvinylpyrrolidone–50% formamide for 48 h at 42°C to DNA probes labeled with ^{32}P by nick translation (19). Sizes of fragments were determined by reference to known sizes of DNA fragments generated by *Hin*dIII digestion of phage lambda DNA.

Preparation of hybridization probes. Plasmid ColE1::Tn*5* (16) was digested with *Bgl*II. The restriction fragments were separated on an agarose gel, and the 2.7-kbp fragment containing the unique sequence of Tn*5* was isolated from the gel as described by Dretzen et al. (11) and used for nick translation. Plasmid pDB6 (kindly provided by D. Dean) was digested with restriction enzyme *Sma*I, and the 6.2-kbp insert spanning the *nifHDK* region (7) was isolated as described above.

Assay for FeMo-cofactor activity. Cells were grown in medium containing NH_4OAc. The cells were derepressed for nitrogenase during a 4-h incubation period in N-free Burk

medium containing 1 μM Na_2MoO_4. Extracts of the cells were prepared by osmotic shock treatment (31). Acid treatment of the extracts was performed as described by Nagatani et al. (22). Assays for nitrogenase activity were carried out as described by Premakumar et al. (27), except that bovine serum albumin and dithiothreitol were omitted from the buffer in making up the reaction volume. The incubation period was 15 min.

Complementation assays. Crude extracts from derepressed mutant strains were prepared and assayed as described by Premakumar et al. (27). Dinitrogenase was purified by the method of Shah and Brill (30), and dinitrogenase reductase purification followed the protocol of Shah et al. (33).

RESULTS

Isolation of Tn5-induced mutants. We isolated 46 independent Tn5-induced mutants that were affected in growth on Mo-sufficient or Mo-deficient N-free medium, but not in growth on medium supplemented with NH_4OAc. Backcross experiments with DNA-mediated transformation showed that linkage of the Kan^r marker and the observed Nif phenotype was always 100%. The mutants were stable with respect to their Nif phenotypes, and revertants, if observed at all, occurred with frequencies of less than 10^{-7}.

Based on growth characteristics in N-free Burk medium the mutants could be grouped into four broad phenotypic classes. The five mutants in the first class did not grow under N_2-fixing conditions in the presence of 1 μM Na_2MoO_4, but grew under Mo-deficient conditions or in the presence of 50 nM V_2O_5. The second class consists of 24 mutants that were unable to grow in the absence of combined N under any condition. The 16 mutants in the third class did not grow on N-free medium deficient in Mo. Most mutants in this class grew slower than the wild type in the presence of V_2O_5 or under Mo-sufficient conditions. The fourth class consists of only one mutant, which showed wild-type growth under Mo sufficiency but reduced growth in the presence of 50 nM V_2O_5 or under Mo-deficient conditions.

Characterization of representative mutant strains. (i) Class I mutants. Strains CA20 and CA25 did not grow in N-free Burk medium in the presence of 1 μM Na_2MoO_4. Both strains grew in the presence of 50 nM V_2O_5 or under Mo-deficient conditions (Table 2). Acetylene reduction activities for whole cells were about 1% of those for the wild type when derepressed in the presence of 1 μM Na_2MoO_4. However, both strains exhibited acetylene reduction activity in the presence of V_2O_5 or under Mo deficiency. These rates were lower than those seen for the wild type under the same conditions. This observation, which has been made previously with other Nif^- mutants (3, 5), could be explained by the inability of these mutant strains to utilize the conventional system which, if present, might operate by scavenging trace amounts of contaminating Mo. In complementation assays involving crude extracts of strain CA20, dinitrogenase activity was absent, but dinitrogenase reductase activity was detected (Table 3). Two-dimensional gel electrophoresis of proteins in cell extracts of strain CA20 derepressed for 3 h in medium containing 1 μM Na_2MoO_4 revealed the presence of the subunits of dinitrogenase reductase and the α-subunit of dinitrogenase but not the β-subunit of dinitrogenase. Thus Tn5 appears to be inserted in or near *nifK*. Furthermore, strain CA20 was transformed to Nif^+ by pLB1, a plasmid which contains a 2.6-kbp *Eco*RI insert carrying *nifK* (6). Hybridization data involving the 6.2-kbp *Sma*I insert (*nifHDK*) of pDB6 as a probe showed the absence of a hybridizing band at the 2.6-kbp position and the appearance of a new hybridizing band at a position corresponding to a fragment size that would have originated from the insertion of Tn5 (5.7 kbp) into the 2.6-kbp *Eco*RI fragment (data not shown).

Strain CA25 appears to carry a mutation that does not allow the expression of either the α- or β-subunit of dinitrogenase in the presence of 1 μM Na_2MoO_4. The spot corresponding to dinitrogenase reductase on two-dimensional gels was shifted from its usual position to a position where a putative alternative reductase protein subunit is found under Mo-deficient conditions (27). In complementation assays, very little dinitrogenase activity and virtually no

TABLE 2. Growth and acetylene reduction by representatives of each mutant class

Class	Strain	Liquid N-free Burk medium[a]	Whole cell acetylene reduction[b] (% of wild-type activity)	Generation time (h)
Wild type	CA	+Mo	28.97 (100.0)	2.5
		+V	7.47 (100.0)	3.3
		−Mo	1.79 (100.0)	4.0
I	CA20	+Mo	0.18 (0.6)	
		+V	6.22 (83.3)	4.5
		−Mo	1.21 (67.6)	6.0
I	CA25	+Mo	0.38 (1.3)	
		+V	5.71 (76.4)	5.0
		−Mo	1.13 (63.1)	8.0
II	CA30	+Mo	0.00 (0.0)	
		+V	0.00 (0.0)	
		−Mo	0.00 (0.0)	
II	CA31	+Mo	1.77 (6.1)	
		+V	0.26 (3.5)	
		−Mo	0.00 (0.0)	
IIIA	CA40	+Mo	8.25 (28.5)	9.5
		+V	1.21 (16.2)	12.5
		−Mo	0.05 (2.8)	
IIIA	CA41	+Mo	10.69 (36.9)	10
		+V	2.01 (26.9)	10
		−Mo	0.07 (3.9)	
IIIA	CA42	+Mo	15.06 (52.0)	10.5
		+V	1.43 (19.1)	11.0
		−Mo	0.09 (5.0)	
IIIA	CA45	+Mo	9.48 (32.7)	15.0
		+V	0.56 (7.5)	12.0
		−Mo	0.07 (3.9)	
IIIB	CA43	+Mo	11.35 (39.2)	4.0
		+V	0.51 (6.8)	6.0
		−Mo	0.17 (9.5)	
IIIC	CA44	+Mo	27.40 (94.6)	2.5
		+V	3.37 (45.1)	3.5
		−Mo	1.44 (80.4)	
IV	CA46	+Mo	27.50 (94.9)	2.5
		+V	0.74 (9.9)	5.5
		−Mo	0.74 (41.3)	5.5

[a] See footnote *a* of Table 1.
[b] Nanomoles of acetylene reduced per minute per milligram of protein.

TABLE 3. Complementation assays of *A. vinelandii* mutant strains CA20, CA25, and CA31

Mutant strain	Sp act (nmol of C_2H_4 formed per min per mg of protein)		
	Crude extract	Crude extract + dinitrogenase reductase[a]	Crude extract + dinitrogenase[b]
CA20	0.57	0.47	17.20
CA25	0.00	0.73	0.01
CA31	6.40	21.39	6.72

[a] Complementation assays were carried out with purified dinitrogenase reductase (specific activity, 950 nmol of C_2H_4 formed per min per mg of protein).

[b] The specific activity of purified dinitrogenase was 1,500 nmol of C_2H_4 formed per min per mg of protein.

dinitrogenase reductase activity could be detected (Table 3). Hybridization patterns obtained with the 6.2-kbp insert of pDB6 as a probe were identical for *Eco*RI digests of chromosomal DNA of wild-type strain CA and strain CA25. Therefore Tn*5* is not inserted in the genomic region harboring *nifHDK*.

Two-dimensional gels of proteins in cell extracts from strains CA25 and CA20 derepressed in Mo-deficient medium or in the presence of 50 nM V_2O_5 showed the protein patterns usually seen under these conditions in the wild type or in the deletion strain CA11 (4, 5).

(ii) Class II mutants. Strains CA30 and CA31 did not grow under N_2-fixing conditions regardless of the Na_2MoO_4 or V_2O_5 content of the medium. Whole cells of strain CA30 derepressed for 3 h did not reduce acetylene under any condition, whereas whole cells of strain CA31 reduced acetylene, albeit at very low rates (Table 2). Complementation assays showed that strain CA31 had high dinitrogenase activity; however, dinitrogenase reductase activity was not stimulated by the addition of purified dinitrogenase (Table 3). Strains CA30 and CA31 expressed the same NH_4^+-repressible proteins as the wild type. Crude extracts of strain CA30 were complemented by acid-treated extracts of wild-type strain CA, but not by acid-treated extracts of strain UW45, a FeMo-cofactor-negative mutant (33). Acid-treated extracts of strain CA30 did not complement crude extracts of strain UW45 (Table 4). Thus, strain CA30 was unable to synthesize active FeMo-cofactor.

The *Eco*RI fragments carrying Tn*5* in strains CA30 and CA31 were approximately 9.0 kbp in size (Fig. 1). These fragments were cloned into pUC9 (R. D. Joerger and P. E. Bishop, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, K157, p. 219; unpublished results). Digestions with *Kpn*I and *Sal*I showed that the *Eco*RI fragment from strain CA31 contained one *Kpn*I site, whereas the fragment from strain CA30 did not. The *Sal*I sites are located at different positions on these fragments (data not shown). Therefore, Tn*5* must be located on different *Eco*RI fragments in strains CA30 and CA31.

(iii) Class III mutants. The mutants in class III were unable to grow under Mo deficiency in N-free medium (Table 2). In the presence of 1 μM Na_2MoO_4 or 50 nM V_2O_5, the mutant strains CA40, CA41, CA42, and CA45 grew with generation times almost four times longer than those of the wild type. The generation times measured for strain CA43 were about twice as long under these conditions, whereas those for strain CA44 did not differ from the wild type. The addition of Na_2MoO_4 to concentrations of up to 1 mM did not increase growth rates in any of these mutants (data not shown). This indicates that these mutants are not equivalent to the $NifQ^-$ or Mol^- mutants of *Klebsiella pneumonia*, which have an elevated Mo requirement for N_2 fixation (13, 14). Acetylene reduction activity was detected in all of these mutants under all conditions (Table 2). The reduction rates were very low for the strains CA40, CA41, CA43, and CA45 when whole cells were derepressed under Mo deficiency. Strain CA44, on the other hand, exhibited acetylene reduction rates under Mo-deficient conditions that were comparable to those of the wild type. Strains CA40, CA41, CA42, CA43, and CA45 derepressed in the presence of 1 μM Na_2MoO_4 had 28 to 51% of the wild-type levels of nitrogenase activity. The activity for strain CA44 was essentially the same as that for the wild type.

To determine whether acetylene reduction activities under Mo deficiency in strains CA40, CA41, CA43, and CA44 were due to low levels of expression of the conventional nitrogenase system, mutant strains that also carried deletions in the structural genes for conventional nitrogenase were constructed. These double mutants still reduced acetylene under Mo deficiency despite the fact that they could not synthesize conventional nitrogenase (Table 5). This indicates that the acetylene reduction activities found under Mo-deficient conditions cannot be explained by activity originating from the conventional system. It should also be pointed out that these double mutants required V for N_2-dependent growth.

Two-dimensional gels of proteins in extracts of all of the mutants in this third class showed the same pattern as the wild type (Fig. 2A). When a *Bgl*II fragment of Tn*5* was hybridized to *Eco*RI-digested DNA from mutants in this class, a band corresponding to 9.9 kbp was observed for strains CA40 and CA45. For the strains CA41 and CA42 the hybridizing band was 9.6 kbp in size; however, the hybridizing bands for these two strains are probably same as those for strains CA40 and CA45, because the sizes were determined from two different gels (Fig. 1). The hybridizing fragment from strain CA43 was approximately 10.8 kbp in size, whereas that for strain CA44 was about 8.5 kbp in length (Fig. 1).

Two-factor crosses were conducted with five of these mutants by using the previously characterized Nif^- mutant strains UW6, UW45, and UW1 (Table 6). Tn*5* insertions carried by strains CA40, CA41, and CA42 showed a 14 to 23% linkage to the *nif-6* mutation (a *nifK* mutation [6]), whereas the Tn*5* insertions in strains CA43 and CA44 are not linked to *nif-6*. The mutation carried by strain CA44 is also unlinked to *nif-1* and *nif-45*. Both of these mutations (*nif-1* and *nif-45*) have previously been shown to map at some distance from mutations affecting the structural genes for nitrogenase (2). Both *nif-1* and *nif-45* appear to be unlinked to the Tn*5* insertions carried by strains CA40, CA41, and CA42. The Tn*5* insertion in strain CA43, however, is linked to *nif-45* by 42% and to *nif-1* by 53%.

TABLE 4. Characterization of *A. vinelandii* CA30 as a FeMo-cofactor-negative mutant

Source of extract (mg of protein)	C_2H_4 formed (nmol/min)
CA (5.9)	285.25
UW45 (5.9)	1.53
CA30 (5.5)	0.03
CA, acid treated (0.63)	0.02
CA30, acid treated (0.60)	0.01
UW45 (5.9) + CA, acid treated (0.63)	23.85
UW45 (5.9) + CA30, acid treated (0.60)	1.37
CA30 (5.5) + CA, acid treated (0.63)	18.88

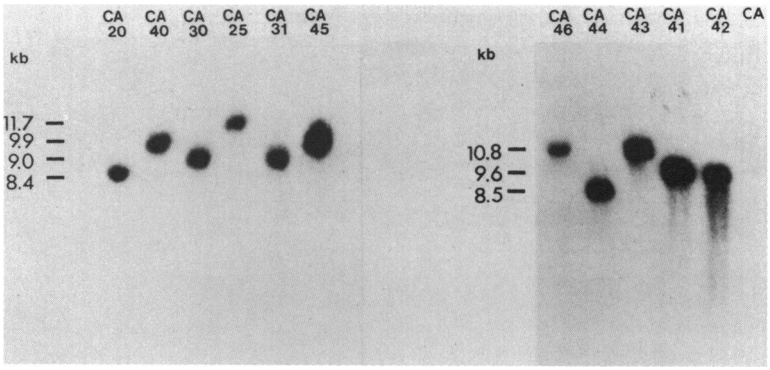


FIG. 1. Southern blots of chromosomal DNAs from Tn5-induced mutants and wild-type strain CA cleaved with *Eco*RI and probed with the *Bgl*II fragment of Tn5.

The third mutant class (Nif$^-$ under Mo deficiency) consists therefore of at least three phenotypic and genotypic subgroups. The strains CA40, CA41, CA42, and CA45 represent a group of mutants (class IIIA) with transposon insertions resulting in an Nif$^-$ phenotype under Mo deficiency and reduced N_2 fixation under Mo sufficiency. Strain CA43 exhibited a comparable phenotype; however, the mapping data placed this mutant into a different group (class IIIB). Strain CA44 (class IIIC) differed from the mutant strains of the previous two groups with respect to the position of its Tn5 insertion and its Nif phenotype, which was that of the wild type under Mo sufficiency and in the presence of V_2O_5 but not under Mo deficiency.

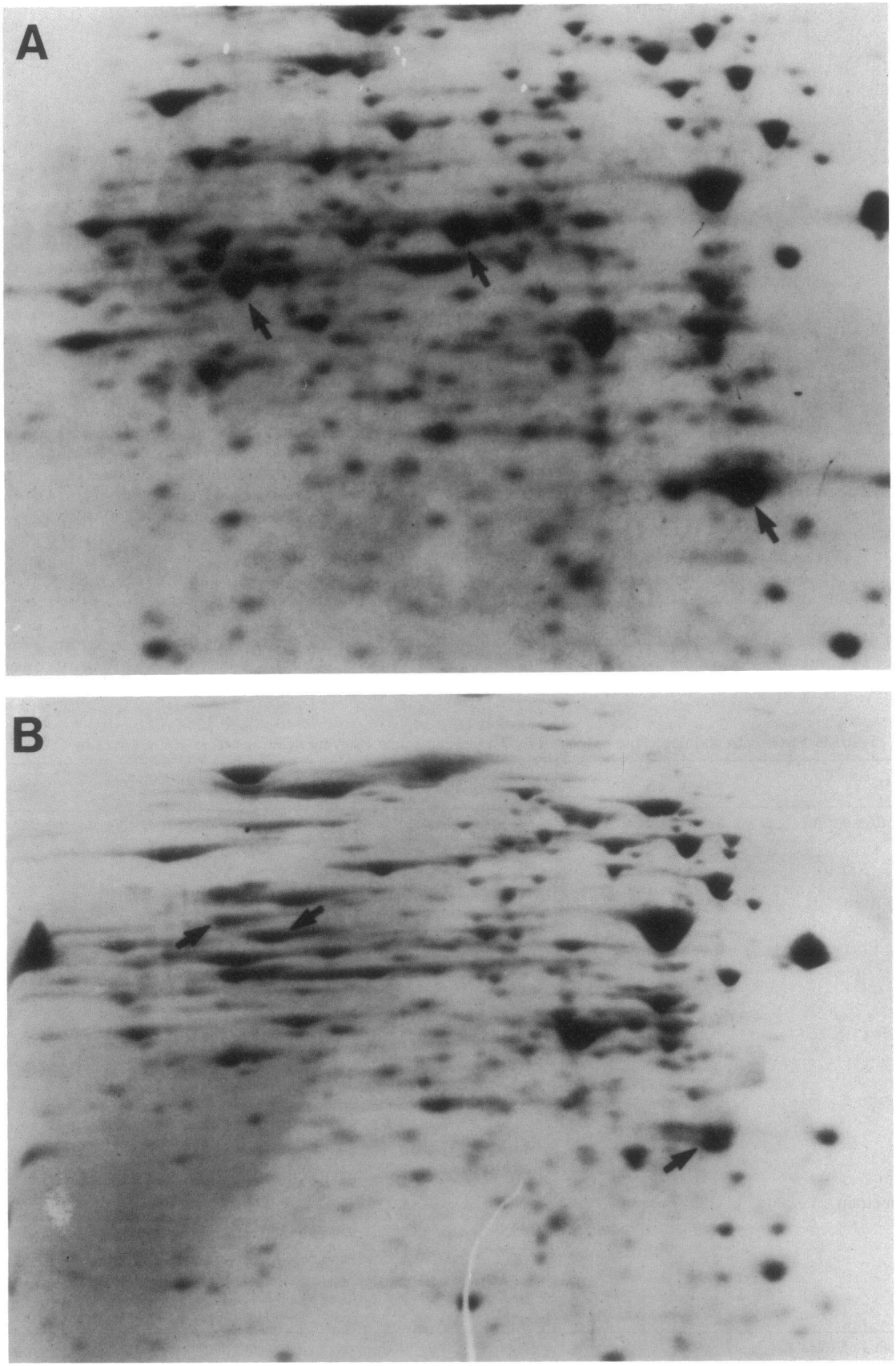
(iv) Class IV mutants. On solid N-free Burk medium containing 50 nM V_2O_5 or no added Na_2MoO_4, strain CA46 grew slightly slower than the wild type. Growth studies in liquid N-free medium showed that the rate of growth of this strain was identical in the presence of 50 nM V_2O_5 or under Mo-deficient conditions (Table 2). Vanadium also did not stimulate acetylene reduction by whole cells of this strain. Growth and acetylene reduction activity in the presence of 1 μM Na_2MoO_4 were virtually identical to those observed for the wild type.

Two-dimensional gel electrophoresis of proteins in extracts of cells derepressed in the presence of 50 nM V_2O_5 or under Mo deficiency revealed that the NH_4-repressible proteins previously seen in the wild type or Nif$^-$ mutants under these conditions were not present in strain CA46. Instead both the α- and β-subunits of dinitrogenase and dinitrogenase reductase were present (Fig. 2B). To obtain an

TABLE 5. Growth and acetylene reduction by Tn5 mutants also carrying deletions from strain CA11 or CA13

Class	Strain	Liquid N-free Burk medium[a]	Whole cell acetylene reduction[b] (% of wild-type activity)	Generation time (h)
nifHDK deletion strain	CA11	+Mo	0.00	
		+V	2.44 (100.0)	3.5
		−Mo	0.74 (100.0)	4.5
IIIA	CA41.11[c]	+Mo	0.00	
		+V	1.12 (45.9)	7
		−Mo	0.12 (16.2)	
IIIB	CA43.11	+Mo	0.00	
		+V	0.41 (16.8)	8
		−Mo	0.09 (12.2)	
IIIC	CA44.11	+Mo	0.00	
		+V	3.47 (142.2)	6
		−Mo	0.59 (79.7)	
IV	CA46.11	+Mo	0.00	
		+V	0.00	
		−Mo	0.00	
nifK deletion strain	CA13	+Mo	0.00	
		+V	5.42 (100.0)	3.6
		−Mo	0.44 (100.0)	5.0
IIIA	CA40.13[c]	+Mo	0.00	
		+V	1.61 (29.7)	9.5
		−Mo	0.26 (59.1)	

[a] See footnote *a* of Table 1.
[b] See footnote *b* of Table 2.
[c] Mutant strain containing both the deletion of strain CA11 or CA13 and the Tn5 insertion of the particular strain.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B

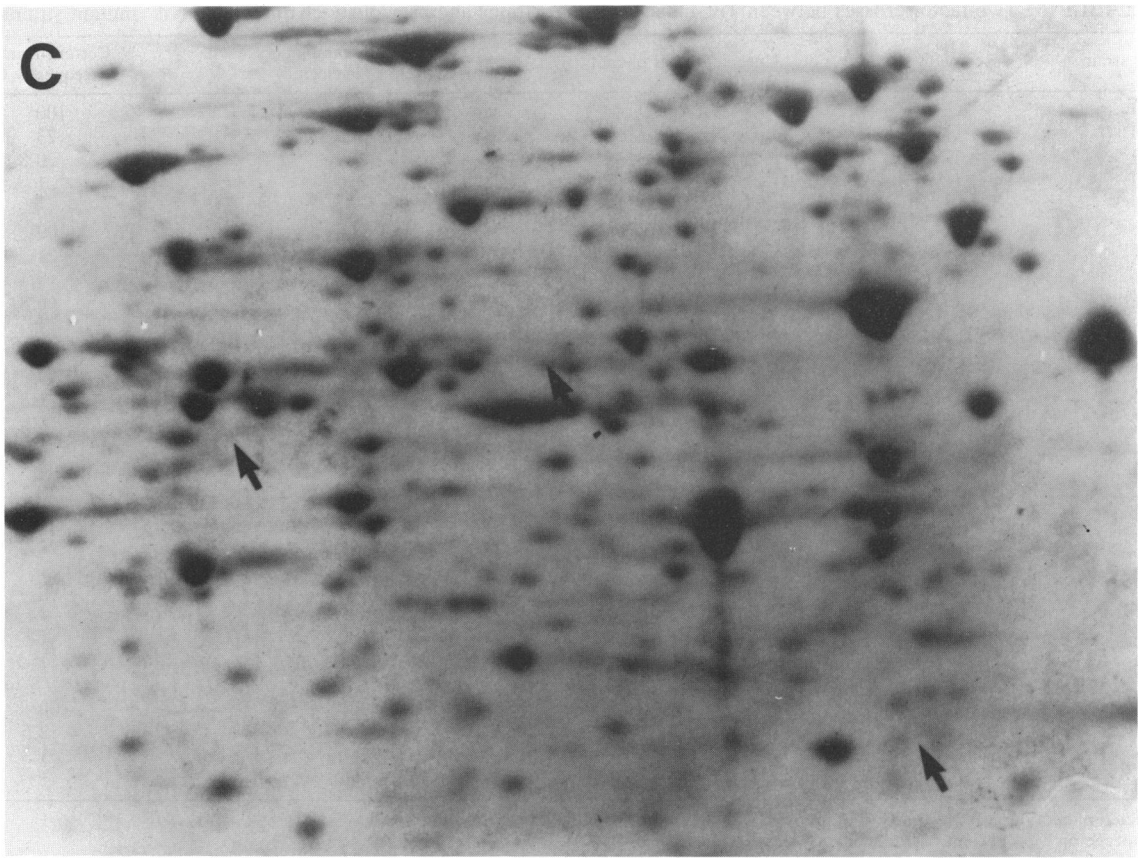

FIG. 2. Two-dimensional gel electrophoresis of proteins in cell extracts of mutant strains. (A) Strain CA44 derepressed under Mo deficiency. The arrows indicate spots corresponding to previously described (3, 4) NH_4^+-repressible proteins. (B) Strain CA46 derepressed under Mo-deficient conditions. The arrows in the upper left corner indicate the α- and β-subunits of dinitrogenase; the arrow at the bottom indicates dinitrogenase reductase. (C) Strain CA46.11 derepressed under Mo deficiency. The arrows indicate positions where the NH_4^+-repressible proteins are normally found.

indication as to whether growth and acetylene reduction activity measured for this strain in the presence of V_2O_5 or under Mo-deficient conditions were due to the residual expression of the conventional N_2 fixation system, we introduced the Tn*5* insertion mutation from strain CA46 into the deletion strain CA11. The resulting strain (CA46.11) did not grow or reduce acetylene in the presence of 1 μM Na_2MoO_4, in the presence of 50 nM V_2O_5, or under Mo deficiency (Table 5). As expected, strain CA46.11 did not express proteins for conventional dinitrogenase and dinitrogenase reductase (Fig. 2C). These results indicate that the Tn*5* insertion in strain CA46 prevents the expression of essential alternative nitrogen fixation system genes and allows the expression of conventional system genes even under Mo deficiency.

Two-factor crosses involving strain CA46 and strains UW1, UW6, and UW45 demonstrated that the Tn*5* insertion in strain CA46 is linked only distantly, if at all, to the other *nif* mutations (Table 6).

DISCUSSION

The use of Tn*5* to mutagenize *A. vinelandii* has enabled us to isolate a number of mutants with interesting Nif phenotypes. Although our primary goal was to isolate mutants affected in their capability to fix N_2 by the alternative nitrogen fixation system, we have also obtained mutants that are Nif^- for the conventional system. One of these mutants, strain CA20, appears to have Tn*5* inserted in *nifK*.

Another mutant, strain CA25, does not have Tn*5* inserted in its structural nitrogenase genes. Growth and acetylene reduction rates by these strains in the presence of 50 nM V_2O_5 and under Mo-deficient conditions are lower than those observed with the wild type under the same conditions. These differences between wild-type and mutant strains could be attributed to residual activity of the conventional system operating in the wild type with trace amounts of Mo scavenged from the environment. The deletion strains CA11, CA12, and CA13 and the mutant strains UW1, UW3, UW6, UW10, UW38, and UW91 also do not grow as fast as the wild type in the presence of V_2O_5 and under Mo deficiency (4, 5; unpublished results). All of these strains (which contain lesions in the conventional system), however, show a stimulation of nitrogenase activity by V_2O_5, indicating that the V effect is not tied to the conventional system. In strain CA25, the conventional N_2 fixation system is inactivated by a Tn*5* insertion which affects a gene with a possible regulatory role, since the subunits for conventional nitrogenase are absent. However, an apparently inactive dinitrogenase reductase was found on two-dimensional gels at a position where the putative alternative dinitrogenase reductase is normally seen (27). Strain UW1, which is thought to be a regulatory mutant (12, 32), also does not produce conventional nitrogenase subunits under Mo sufficiency. Determi-

TABLE 6. Two-factor crosses between Tn5-induced mutant strains and previously characterized Nif⁻ mutant strains

DNA donor strain	Recipient strain	Unselected Nif phenotype[a]	Fraction of recombinants (unselected/selected)	Cotransformation frequency (%)
CA40	CA	Nif⁻ (−Mo)	400/400	100
CA40	UW6	Nif⁺ (+Mo)	273/1,173	23
CA40	UW45	Nif⁺ (+Mo)	5/600	0.8
CA40	UW1	Nif⁺ (+Mo)	9/850	1
CA41	CA	Nif⁻ (−Mo)	400/400	100
CA41	UW6	Nif⁺ (+Mo)	128/600	21
CA41	UW45	Nif⁺ (+Mo)	1/250	0.4
CA41	UW1	Nif⁺ (+Mo)	6/750	0.8
CA42	CA	Nif⁻ (−Mo)	400/400	100
CA42	UW6	Nif⁺ (+Mo)	72/500	14
CA42	UW45	Nif⁺ (+Mo)	9/488	1.8
CA42	UW1	Nif⁺ (+Mo)	5/650	0.8
CA43	CA	Nif⁻ (−Mo)	400/400	100
CA43	UW6	Nif⁺ (+Mo)	1/300	0.3
CA43	UW45	Nif⁺ (+Mo)	341/800	42
CA43	UW1	Nif⁺ (+Mo)	404/750	53
CA44	CA	Nif⁻ (−Mo)	400/400	100
CA44	UW6	Nif⁺ (+Mo)	3/300	1
CA44	UW45	Nif⁺ (+Mo)	1/100	1
CA44	UW1	Nif⁺ (+Mo)	5/443	1
CA46	CA	Nif⁻ (−Mo)[b]	400/400	100
CA46	UW6	Nif⁺ (+Mo)	14/544	2.5
CA46	UW45	Nif⁺ (+Mo)	5/368	1.3
CA46	UW1	Nif⁺ (+Mo)	6/600	1

[a] The selected phenotype was Kan^r. See footnote *a* of Table 1 for growth conditions.
[b] Nif phenotype on Mo-deficient N-free medium was reduced growth.

nation of whether the phenotypic similarities between strains UW1 and CA25 are based on mutations at the same site will have to await further studies. Strain UW45, a mutant with a NifB⁻ phenotype (33), was shown to be unable to fix N_2 under any condition (unpublished results). A high percentage of the Tn5-induced mutants that we isolated were also Nif⁻ under any condition. Whether most of these mutants carry a defect in cofactor synthesis is not known. We demonstrated a FeMo-cofactor negative phenotype for strain CA30, and experiments by Shah et al. (33) showed that this mutant behaves identically to strain UW45 in FeMo-cofactor complementation assays. Kennedy et al. (17) described the isolation of a mutant of *A. vinelandii* (MV22) carrying Tn5 in or near a gene that is equivalent to *nifN* in *K. pneumoniae*. Strain MV22 is Nif⁻ under Mo-sufficient conditions but undergoes phenotypic reversal under Mo deficiency, indicating that not all of the genes necessary for the synthesis of FeMo-cofactor are required for a functional alternative nitrogenase.

Whole cells from strain CA31 reduce acetylene under Mo sufficiency, but fail to grow on N-free medium. Acetylene reduction by crude extracts from cells derepressed in Mo-sufficient medium was stimulated upon the addition of purified dinitrogenase reductase but not when purified dinitrogenase was added. The fact that Tn5 is not inserted in or near *nifH* in strain CA31 suggests that low dinitrogenase reductase activities are caused by a mutation affecting processing of dinitrogenase reductase. In *K. pneumoniae* it has been suggested that the products of *nifM* and *nifS* play a role in the processing of the *nifH* gene product (28). Kennedy et al. (17) have described a mutant of *A. vinelandii* (MV21) carrying a Tn5 insertion in a gene postulated to be *nifM*. Although this mutant reduces acetylene, it is unable to grow on Mo-sufficient or Mo-deficient N-free medium. Whether the phenotypic similarity between strain MV21 and strain CA31 is due to inactivation of the same gene is not known.

Whereas mutants affected in N_2 fixation under Mo-sufficient conditions could be readily isolated, the isolation of Tn5-induced mutants solely affected in their N_2 fixation capability in the presence of V_2O_5 or under Mo-deficient conditions proved far more difficult. The possibility that Tn5 does not insert randomly cannot be excluded, and difficulties in the detection of such mutants after mutagenesis might also be the cause. In this context it should be pointed out that data obtained through the analysis of two-dimensional gels of proteins in cell extracts indicate that the wild type expresses two distinct patterns of Mo− and NH_4^+-repressible proteins depending on the physiological age of the culture (4). Little is known about the regulatory processes involved here, except that in the presence of V_2O_5 only one pattern (nitrogenase B_1) appears to be expressed. This pattern is also seen in wild-type cells of early and mid-log cultures under Mo-deficient conditions. The other pattern (nitrogenase B_2) was detected in the Nif⁺ pseudorevertant strains CA2 and CA4 (3). In the wild type, the nitrogenase B_2 pattern is found in late-log-phase cells (4). Recent work of Chisnell and Bishop (9) suggests the existence of at least two different dinitrogenase reductases synthesized under Mo deficiency. The presence of more than one nitrogenase operating under Mo-deficient conditions may make it difficult to detect mutants with Tn5 insertions affecting only one of these nitrogenases. The mutant strains CA40, CA41, CA42, CA43, CA44, and CA45 exhibited the pattern of NH_4^+-repressible proteins previously described

for the wild type (4), but are unable to grow on N_2 under Mo deficiency. With the exception of strain CA44, which is comparable to the wild type with respect to Nif phenotype under Mo-sufficient conditions, the other five strains listed above show a marked decrease in growth and acetylene reduction activity in comparison to the wild type under Mo sufficiency. The site of the Tn*5* insertion in these mutants is therefore of importance to the conventional system but not essential. Under Mo-deficient conditions, however, these mutations cause a Nif^- phenotype. A defect in Mo uptake is an unlikely cause for the phenotype of these mutants, since growth stimulation could not be detected when the Na_2MoO_4 concentration in the medium reached 1 mM.

Transfer of the Tn*5* insertion from mutants of class III into *nifHDK* deletion strain CA11 or *nifK* deletion strain CA13 resulted in strains that were able to grow only on N-free medium containing V_2O_5. These mutant strains, with the exception of strain CA44.11, exhibited low acetylene reduction activities in the presence of 50 nM V_2O_5 and under Mo-deficient conditions.

The acetylene reduction rates by strains CA44 and CA44.11 were almost as high as those for strains CA and CA11 derepressed under Mo deficiency. The failure of strain CA44 to grow on N-free Mo-deficient medium is due to a defect that is specific for the alternative system and may be similar to that encountered in *nifV* mutants of *K. pneumoniae*, which are unable to grow under N-free conditions but exhibit near normal rates of acetylene reduction (20).

Mutant strain CA46 is interesting in two aspects. Not only has this strain lost the ability to respond with an increase in growth and acetylene reduction upon the addition of V_2O_5, but regulation of the conventional system by Mo has also been altered. The Nif^+ phenotype of this mutant under Mo-sufficient and Mo-deficient conditions is due solely to the expression of the conventional system, since the deletion of *nifHDK* causes a Nif^- phenotype under all conditions. The consequence of the mutation carried by strain CA46 therefore appears to be the inverse of mutations which confer tolerance to WO_4^{2-}, where only the alternative system is expressed, even in the presence of Mo (3, 4). The inability of this strain to respond to V_2O_5 suggests that V is specifically involved with the alternative N_2 fixation system (which is not expressed in strain CA46), as previously suggested (4). The ability of strain CA46 to grow in N-free medium and to reduce acetylene in the presence of V_2O_5 and under Mo deficiency while apparently only utilizing the conventional nitrogenase raises the question as to which cofactor is present in the dinitrogenase under these conditions. One might expect that some FeMo-cofactor could be synthesized with trace amounts of contaminating Mo, but it is also conceivable that a cofactor usually associated with the alternative N_2 fixation system can be incorporated into conventional dinitrogenase.

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