Activation of vanadium nitrogenase expression in *Azotobacter vinelandii* DJ54 revertant in the presence of molybdenum

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Abstract ***Azotobacter vinelandii*** **carries three different and genetically distinct nitrogenase systems on its chromosome. Expression of all three nitrogenases is repressed by high concentrations of fixed nitrogen. Expression of individual nitrogenase systems is under the control of specific metal availability. We have isolated a novel type of *A. vinelandii* DJ54 revertant, designated *A. vinelandii* BG54, which carries a defined deletion in the *nifH* gene and is capable of diazotrophic growth in the presence of molybdenum. Inactivation of *nifDK* has no effect on growth of this mutant strain in nitrogen-free medium suggesting that products of the *nif* system are not involved in supporting diazotrophic growth of *A. vinelandii* BG54. Similar to the wild type, *A. vinelandii* BG54 is also sensitive to 1 mM tungsten. Tn*5*-B21 mutagenesis to inactivate the genes specific to individual systems revealed that the structural genes for *vnf* nitrogenase are required for diazotrophic growth of *A. vinelandii* BG54. Analysis of promoter activity of different *nif* systems revealed that the *vnf* promoter is activated in *A. vinelandii* BG54 in the presence of molybdenum. Based on these data we conclude that *A. vinelandii* BG54 strain utilizes *vnf* nitrogenase proteins to support its diazotrophic growth.**

Key words: Nitrogenase; Tn*5*-B21 mutagenesis; Molybdenum; *nifHDK*; *Azotobacter*

1. Introduction

Azotobacter vinelandii harbors three genetically distinct nitrogenases [1–3], a well-characterized molybdenum nitrogenase, a vanadium-containing nitrogenase, and an iron-only nitrogenase. All three nitrogenase enzymes are oxygen-labile iron–sulfur proteins comprised of two separable components, the Fe protein and the dinitrogenase [1,2,4]. The Fe protein serves as the obligate electron donor to dinitrogenase during catalysis in a MgATP- and reductant-dependent process [5–8]; dinitrogenase contains the active site of the enzyme, the cofactor. The conventional Mo nitrogenase is synthesized in the absence of a fixed nitrogen source when Mo is available, whereas the V nitrogenase accumulates under Mo-deficient conditions in the presence of V. In the absence of both Mo and V, an alternative nitrogenase accumulates (Fe nitrogenase) that does not appear to contain either Mo or V [1,2]. The regulatory genes *nifA*, *vnfA*, and *anfA* are required for the expression of Mo, V and Fe nitrogenase, respectively [9–16]. NifA binds to an upstream activator sequence (5′-TGT-N10-ACA-3′) and activates the transcription of *nif* operons [17,18]. The VnfA binding site has been identified as 5′-GTAC-N6-GTAC-3′ [19,20]. The binding site for AnfA is unknown.

The structural genes encoding Mo nitrogenase and Fe nitrogenase are organized as single operons, *nifHDK* and *anfHDGK* respectively, while the genes encoding V nitrogenase are organized into two independently regulated operons, *vnfHFd* and *vnfDGK* [1,21,22]. The *nifH*, *vnfH*, and *anfH* genes encode the Fe protein for the three nitrogenases, respectively. The α and β subunits of Mo dinitrogenase, V dinitrogenase, and Fe dinitrogenase are encoded by *nifDK*, *vnfDK*, and *anfDK*, respectively. The δ subunits of V and Fe dinitrogenase are encoded by *vnfG* and *anfG*, respectively [1,6,8,21,23]. The *vnfHFd* and *vnfDGK* operons are expressed independently of each other. The Fe protein of V nitrogenase is expressed under Mo-deficient conditions in the presence or absence of V, whereas V dinitrogenase is expressed only in the presence of V [24–26]. It was shown that the Fe protein of V nitrogenase is required for the expression of Fe nitrogenase [27].

X-ray absorption spectroscopy studies [28,29] have shown that Mo and V in the MoFe and VFe proteins are present in analogous cofactor centers and have similar ligand geometry. A Fe- and V-containing cofactor (FeV cofactor), similar to the FeMo cofactor of MoFe proteins, has been extracted from *A. chroococcum* [1]. No comparable data are available for Fe nitrogenase. However, indirect evidence suggests that Fe nitrogenase contains a cofactor center similar to those in Mo and V nitrogenase. The genes *nifB* and *nifV*, which are involved in FeMo cofactor biosynthesis, are also required for V and Fe nitrogenase activity [21,30]. The sequence comparisons of the products of the structural genes show that amino acid residues that serve as ligands for FeMo cofactor in the MoFe protein are conserved in all three nitrogenases [22]. Moreover, a new species of V nitrogenase containing FeMo cofactor was reconstituted in vitro and found to be functional [31]. Here we report the isolation and characterization of an *A. vinelandii* mutant which supports diazotrophic growth due to the synthesis of V nitrogenase in the presence of Mo.

2. Material and methods

2.1. Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *A. vinelandii* strains were grown at 30°C in modified Burke nitrogen-free (BN^-) medium [32]. When it was necessary to include fixed nitrogen in the medium, ammonium acetate was added to a final concentration of 400 μg/ml. When antibiotics were needed, kanamycin and tetracycline were added to final concentrations of 5 μg/ml and 30 μg/ml, respectively. *Escherichia coli* strains were grown at 37°C in

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Luria broth or 2YT medium [33]. The antibiotics ampicillin and tetracycline were added to final concentrations of 50 μg/ml and 25 μg/ml, respectively.

2.2. Isolation of a revertant from ΔnifH A. vinelandii strain DJ54 and its growth characteristics

The Δ*nifH A. vinelandii* strain DJ54 (Table 1) was plated on BN^- plates containing 10 μmol Mo. The plates were incubated at 30°C to score for spontaneous revertants. The ability of these revertant colonies to grow on BN^- plates was re-assessed by plating on BN^- medium. The colony that showed consistent growth on BN^- medium was designated *A. vinelandii* BG54. To characterize *A. vinelandii* BG54 growth on nitrogen-free medium, overnight cultures were prepared by allowing them to grow in BN^+ medium at 30°C for about 15 h. These fully grown cultures were diluted into sterile BN^- medium at a ratio of 1:250 for overnight culture:fresh medium, and the growth was continued at 30°C while cell densities were recorded at various time intervals.

2.3. General molecular techniques

Restriction enzymes were purchased from Boehringer Mannheim (Indianapolis, IN, USA). Plasmid DNA isolations, DNA subcloning, restriction enzyme digestions, agarose gel electrophoresis, ligations and *E. coli* transformations were carried out as described in the laboratory manuals [33,34] or as suggested in the manufacturers' instructions. Oligonucleotides used for PCR amplification were purchased from Gibco BRL Life Technologies Inc. (Gaithersburg, MD). Tn*5*-B21 mutagenesis was carried out by following a modified method described by Joerger et al. [22]. PCR was performed using the reagents from the GeneAmp kit (Perkin-Elmer Corp.). 100 ng of the chromosomal DNA and about 50 nmol of the primers corresponding to sense and antisense DNA strands immediately upstream and downstream of the open reading frame for NifH were used in the reaction. The oligonucleotide primer corresponding to the *nifH* forward is 5′-TGGCTATGCGTCAATGCGCCATCTACGGC-3′ and the *nifH* reverse is 5′-TCAGACTTCTTCGGCGGTTTTGCCGACGATGGA-3′. PCR amplification was continued for 20 cycles under stringent conditions. The products were subjected to agarose gel electrophoresis to compare the size of the *nifH* genes from *A. vinelandii* strains OP, DJ54, and BG54.

3. Results and discussion

3.1. Isolation of A. vinelandii BG54 and verification of the genetic background

A. vinelandii DJ54, which has a defined deletion in the *nifH* gene, cannot support diazotrophic growth in a medium supplemented with Mo and Fe [35,36]. We were interested in testing whether Nif^+ revertants could be isolated from *A. vinelandii* DJ54 and how such revertants can gain nitrogen-fixing ability. As described in Section 2, *A. vinelandii* DJ54 cells were subjected to growth on nitrogen-free medium and spontaneous revertants that could grow diazotrophically were isolated. One of the colonies that showed consistent growth on BN^- medium supplemented with molybdenum, designated *A. vinelandii* BG54, was subjected to further analysis.

To verify that the *nifH* locus of *A. vinelandii* BG54 and *A. vinelandii* DJ54 are similar, and that the reversion to the Nif^+ phenotype in *A. vinelandii* BG54 is due to some other mutation, we performed PCR amplification of the *nifH* as described in Section 2. It was known that the *nifH* gene of *A. vinelandii* DJ54 is 340 bp since there was a 540 bp deletion in the coding sequence of the *nifH* in this strain. The PCR products obtained when chromosomes of *A. vinelandii* BG54 and *A. vinelandii* DJ54 were used as template were similar in size (about 340 bp) and much smaller than that of wild type which was 880 bp. This indicated that *A. vinelandii* BG54 retained the same Δ*nifH* originally present in the parental strain *A. vinelandii* DJ54. Therefore, the Nif^+ phenotype of *A. vinelandii* BG54 has presumably resulted, at least in part, from one of the Mo-independent nitrogenases.

3.2. Growth characteristics of A. vinelandii BG54

The growth rates of *A. vinelandii* strains OP, DJ54, and BG54 in BN^+ and BN^- liquid media were recorded at different time intervals. There was no apparent difference among the three strains when grown in BN^+ medium. On the other hand, *A. vinelandii* BJ54 could not grow in BN^- medium in the presence of Mo, indicating that Mo-independent nitrogenases were repressed by molybdenum in this strain. However, *A. vinelandii* BG54 was able to grow in BN^- medium in the presence of Mo and the growth rate of *A. vinelandii* BG54 was much slower when compared to that of *A. vinelandii* strain OP (Fig. 1). This is a typical growth phenotype of *A. vinelandii* using a Mo-independent nitrogenase to fix nitrogen [37] because V and Fe nitrogenases are not as efficient in substrate reduction as Mo nitrogenase [25].

It was shown previously that some strains of *A. vinelandii* that carried a deletion in the structural genes of Mo nitrogenase could grow in nitrogen-free medium in the presence of molybdenum and exhibited tungsten resistance [38–40]. The growth of these strains was not repressed by 1 mM of

Table 1
Bacterial strains and plasmids used in this study

Strain of plasmid	Relevant characteristics and description	Source or reference
E. coli TG1	K12, Δ(*lac-pro*), supE, *thi*-1, *lac*I^q, *lacZ*ΔM15, *hsd*D5 [F′ *traD36 proA*$^+$*B*$^+$]	Amersham Life Sciences Inc., IL
A. vinelandii OP	Wild type, Nif^+, soil bacterium	Laboratory stock
A. vinelandii DJ54	Nif^-; defined deletion in the *nifH* gene	[35]
A. vinelandii BG54	Nif^+; spontaneous revertant of *A. vinelandii* DJ54 with defined deletion in the *nifH* gene	This work
A. vinelandii DJ15	*nifH*::Tn*5*-B21; Nif^-; Tn*5*-B21 insertion in the *nifH*	[42]
A. vinelandii CA73	*anfH*::Tn*5*-B21: Nif^+; Tn*5*-B21 insertion in the *anfH*	[27]
A. vinelandii CA81	*vnfH*::Tn*5*-B21: Nif^+; Tn*5*-B21 insertion in the *vnfH*	[27]
A. vinelandii BG11K	nif^+; Δ*nifHDK*::Kan, in the genetic background of *A. vinelandii* DJ54 revertant BG54	This work
A. vinelandii BG73	Tet^r; *anfH*::Tn*5*-B21, in the genetic background of *A. vinelandii* DJ54 revertant BG54	This work
A. vinelandii BG15	Tet^r; *nifH*::Tn*5*-B21, in the genetic background of *A. vinelandii* DJ54 revertant BG54	This work
A. vinelandii BG81	Tet^r; *vnfH*::Tn*5*-B21, in the genetic background of *A. vinelandii* DJ54 revertant BG54	This work
pDB6	6-kb *Sma*I fragment spanning *A. vinelandii nifHDKY* genes cloned into the *Sma*I site of pUC8. Amp^r; ColE1 replicon	[42]
pBG506K	Derivative of pDB6 in which the *nifHDK* genes were deleted by digesting with *Bgl*II and inserting a 1.2-kb *Bam*HI fragment carrying a kanamycin resistance gene at this *Bgl*II site	This work

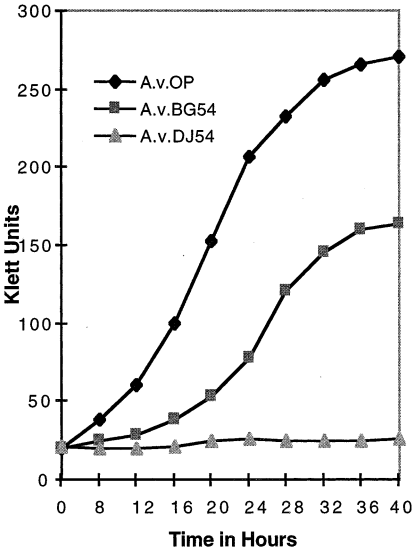

Fig. 1. Growth characteristics of *A. vinelandii* strains in BN^- liquid medium. Overnight cultures were prepared by inoculating freshly growing colonies from agar plates into Burke's medium supplemented with ammonium acetate. Cells from the overnight culture were collected and washed with BN^- and then transferred to sidearm flasks that contained sterile BN^-. Cell densities were recorded over 40-h time periods. This analysis showed that the *A. vinelandii* BG54 strain showed slower growth compared to the *A. vinelandii* OP and the parental strain *A. vinelandii* DJ54 did not show any growth.

Na_2WO_4. We characterized diazotrophic growth by *A. vinelandii* strain BG54 in the presence of various concentrations of Na_2WO_4. *A. vinelandii* strain BG54 showed resistance to tungsten in the nitrogen-free medium. The growth behavior of *A. vinelandii* strain BG54 was different from what has been reported on tungsten-resistant strains such as *A. vinelandii* strains CA6 [39], CARR [40], and WN101 [38], which could grow in nitrogen-free medium supplemented with 1 mM Na_2WO_4.

3.3. Deletion of nifDK from A. vinelandii BG54 has no effect on its diazotrophic growth

The Fe protein of any of the three nitrogenases can form a functional complex with the MoFe protein of Mo nitrogenase

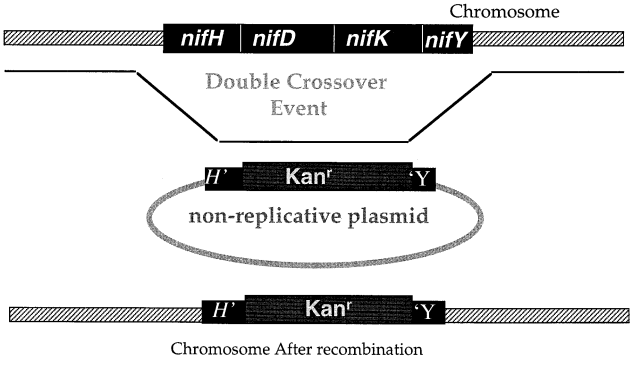

Fig. 2. General strategy of integration of kanamycin resistance gene cassette in the chromosome. The *nif* gene locus on the chromosome before and after the integration of deleted *nif* structural genes is shown. Using this genetic strategy the *A. vinelandii* strains in the wild type background and BG54(Δ*nifHDK*) in DJ54 revertant background designated *A. vinelandii* BG11K were constructed.

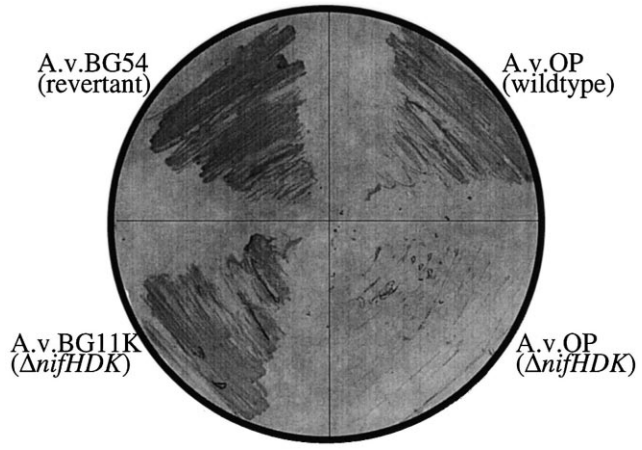

Fig. 3. The bacteria were tested for diazotrophic growth on BN^-. The strains used were *A. vinelandii* (wild type), *A. vinelandii* (Δ*nifHDK*), *A. vinelandii* BG54(Δ*nifH*) and *A. vinelandii* BG54(Δ*nifHDK*). Strain *A. vinelandii* (Δ*nifHDK*) was unable to grow on nitrogen-free medium and strain *A. vinelandii* BG54(Δ*nifHDK*) showed diazotrophic growth, indicating in the *A. vinelandii* BG54 background the *nif* structural genes are not needed for its diazotrophic growth.

to support substrate reduction and the diazotrophic growth of *A. vinelandii* [2,25,41]. Thus, it is possible that *A. vinelandii* BG54 is utilizing the MoFe protein of Mo nitrogenase and the Fe protein of either V or Fe nitrogenase to fix nitrogen. To test this possibility, we analyzed the role of *nifDK* genes in the diazotrophic growth of *A. vinelandii* BG54. Initially, we generated a derivative of *A. vinelandii* strain BG54 in which the *nifDK* genes were disrupted. To do this, we generated a derivative of pDB6 in which the *nifHDK* genes were deleted by digesting with *Bgl*II. Then we inserted a 1.2 kb *Bam*HI fragment carrying a kanamycin resistance gene at this *Bgl*II site. The resulting plasmid, which was designated pBG506K (Fig. 2), has a kanamycin resistance gene cassette in place of the *NifHDK*. The pBG506K was transformed into both *A. vinelandii* strains OP and BG54. The kanamycin-resistant colonies were selected from both transformations. The plasmid pBG506K is unable to replicate in *A. vinelandii* since it has a *ColE1* replicon. Therefore, the kanamycin-resistant colonies obtained after transformation carry the rescued kanamycin resistance gene in place of the *nifHDK* locus on the chromosome. It was observed that the transformants of *A. vinelandii* strain OP were unable to grow on nitrogen-free, Mo-containing medium suggesting that the products of *nifHDK* are essential for diazotrophic growth of this strain under these conditions (Fig. 3). In contrast, the transformants of *A. vinelandii* BG54 were able to grow on nitrogen-free, Mo-containing medium suggesting the products of *nifHDK* are not required for the diazotrophic growth of this strain (Fig. 3). The derivative of *A. vinelandii* BG54 in which the kanamycin resistance gene replaced the *nifHDK* locus on the chromosome was designated *A. vinelandii* BG11K. The growth characteristics of *A. vinelandii* BG11K and *A. vinelandii* BG54 showed that the deletion of *nifDK* did not affect the nitrogen fixation capability of *A. vinelandii* BG11K indicating that *A. vinelandii* BG54 was relying on either V or Fe nitrogenase to support nitrogen reduction in the presence of molybdenum (Fig. 3).

3.4. Expression of V nitrogenase is required for Nif$^+$ phenotype of A. vinelandii BG54

Since growth characteristics of *A. vinelandii* BG11K showed that the products of *nifDK* are not necessary for the growth of *A. vinelandii* BG54 on nitrogen-free medium supplemented with Mo, we analyzed whether V or Fe nitrogenase is used by this strain to support diazotrophic growth. Initially we constructed a series of strains which have *nifH*, *vnfH* or *anfH* mutated by Tn*5*-B21 in the genetic background of BG54. These strains were designated *A. vinelandii* BG15, BG81 and BG83, respectively. The numbering system corresponded to that of the *A. vinelandii* OP strains carrying similar mutations (CA15, CA81 and CA83, respectively, which were a gift from Dr. Bishop). The ability of these strains to grow on nitrogen-free medium containing Mo was analyzed and the results of these experiments are summarized in Table 2. It was found that disruption of *anfH* had no effect on the diazotrophic growth of *A. vinelandii* strains CA73 or BG73 (*anfH*::Tn*5*-B21) suggesting that the product of *anfH* is not involved in supporting the diazotrophic growth of these strains. Disrupting *vnfH* did not inhibit growth of *A. vinelandii* CA81 (*vnfH*::Tn*5*-B21 in the wild type genetic background) whereas disrupting *vnfH* inhibited growth of *A. vinelandii* BG81 (*vnfH*::Tn*5*-B21 in the BG54 genetic background) on nitrogen-free medium containing Mo (Table 2). These results clearly indicated that expression of V nitrogenase is essential for the growth of *A. vinelandii* BG54 on nitrogen-free medium containing Mo. *A. vinelandii* BG15 (*nifH*::Tn*5*-B21 in the BG54 genetic background) was able to grow on nitrogen-free medium containing Mo, further confirming the observation from the studies on *A. vinelandii* BG11K that the expression of *nifH* is not essential for the nitrogen-fixing ability of *A. vinelandii* BG54 (Table 2). In contrast, *A. vinelandii* DJ15 (*nifH*::Tn*5*-B21 in the wild type genetic background) was unable to grow on nitrogen-free medium containing Mo showing the dependence of this strain on the *nifH* product for diazotrophic growth under these conditions (Table 2). Taken together these data suggest that unlike wild type *A. vinelandii*, the *A. vinelandii* BG54 strain was synthesizing and assembling functional V nitrogenase in the presence of molybdenum.

3.5. Expression pattern of β-galactosidase from nifHp-lacZ, vnfHp-lacZ and anfHp-lacZ fusions in A. vinelandii BG54 genetic background revealed an activation of V nitrogenase in the presence of molybdenum

The growth phenotype of *A. vinelandii* BG54 was positive in nitrogen-free medium supplemented with molybdenum which was distinct from the negative phenotype of *A. vinelandii* DJ54, indicating that the expression profile of *A. vinelandii* BG54 nitrogenase genes was different from that of *A. vinelandii* DJ54. To further verify whether the transcription of the *vnf* system is activated in *A. vinelandii* BG54, cells were grown in nitrogen-free medium containing Mo and we examined whether the promoter of *vnfH* is activated in these cells. To do this, transcriptional fusions of the *vnfH* promoter and a promoterless *lacZ* gene in the *A. vinelandii* BG54 genetic background (*A. vinelandii* BG81) and the wild type genetic background (*A. vinelandii* CA81) were generated as described previously. The expression of *lacZ* by these strains when grown in the nitrogen-free medium supplemented with Mo was monitored. The results of these analyses are presented in Table 2. It was observed that the *A. vinelandii* CA81 that harbors the *vnfH*::*lacZ* fusion in the wild type genetic background expressed only the basal level of β-galactosidase. In contrast, *A. vinelandii* BG81, which harbors the *vnfH*::*lacZ* fusion in the BG54 genetic background, expressed elevated levels of β-galactosidase. This result clearly implies that in *A. vinelandii* BG54 cells grown in nitrogen-free medium supplemented with molybdenum, the *vnf* system is transcriptionally active. This observation, combined with the fact that impairing the expression of *nifHDK* genes does not alter the nitrogen fixation ability of *A. vinelandii* BG54, suggests that V nitrogenase is functional in these cells when grown in nitrogen-free medium supplemented with Mo. Therefore, *A. vinelandii* BG54 cells must have accumulated V nitrogenase with FeMo cofactor inserted in the presence of molybdenum. A hybrid *A. vinelandii* VFe protein containing FeMo cofactor has been reconstituted in vitro [31]. This VFe (FeMo co)

Table 2
β-Galactosidase activity measurements of *lacZ* reporter gene fusions to *nifH*, *vnfH* and *anfH* genes

Strain	Growth on BN$^-$ medium with molybdenum	Genotype/LacZ fusion	β-Galactosidase activity[a]	Percentage of activity
A. vinelandii DJ15	−	Wild type background and Tn*5*-B21 insertion in *nifH* gene; *nifH*::*lacZ*	129.15	100
A. vinelandii CA81	+	Wild type background and Tn*5*-B21 insertion in *vnfH* gene; *vnfH*::*lacZ*	14.28	11.05
A. vinelandii CA73	+	Wild type background and Tn*5*-B21 insertion in *anfH* gene; *anfH*::*lacZ*	ND	–
A. vinelandii BG15	+	*A. vinelandii* DJ54 revertant background and Tn*5*-B21 insertion in *nifH* gene; *nifH*::*lacZ*	122.85	95.12
A. vinelandii BG81	−	*A. vinelandii* DJ54 revertant background and Tn*5*-B21 insertion in *vnfH* gene; *vnfH*::*lacZ*	69.82	54.06
A. vinelandii BG73	+	*A. vinelandii* DJ54 revertant background and Tn*5*-B21 insertion in *anfH* gene; *anfH*::*lacZ*	ND	–

ND: No detectable activity was found.
[a]Cells from 1.5 ml of derepressed culture were collected by centrifugation and resuspended in 300 μl of Z buffer. A 100-μl aliquot of the resuspended cells was lysed using quick freeze-thaw (treatment with liquid nitrogen followed by thawing at 37°C). To measure the β-galactosidase activity in the cell lysate, 0.7 ml of the Z buffer/β-mercaptoethanol solution was added to each tube followed by 0.16 ml of Z buffer/ONPG (4 mg of ONPG per ml of Z buffer). The time of ONPG addition was recorded and the tubes were incubated at 30°C with shaking. When yellow color was visible, 400 μl of 1 M $NaCO_3$ was added to each tube to terminate the reaction and the time was recorded. The tubes were then centrifuged for 10 min at $10\,000\times g$ to remove cellular debris, and the OD_{420} was recorded. The β-galactosidase units were defined as the amount of enzyme which hydrolyzes 1 μmol of ONPG to *o*-nitrophenol and D-galactose per minute.

protein had a mixed EPR signal, which combined both MoFe protein and VFe protein signals and had similar electron allocation to the MoFe protein in nitrogen reduction and to the VFe protein in C_2H_2 reduction [31]. However, this V nitrogenase containing FeMo cofactor was not as efficient in nitrogen fixation as the Mo nitrogenase in vitro. Since the growth rate of *A. vinelandii* BG54 was much slower than that of *A. vinelandii* OP in ammonia-free medium in the presence of molybdenum, our results imply that the nitrogenase expressed by *A. vinelandii* BG54 is more similar to the V nitrogenase with the FeMo cofactor, rather than the Mo nitrogenase.

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References

[1] Eady, R.R. (1996) Chem. Rev. 96, 3013–3030.
[2] Bishop, P.E. and Premakumar, R. (1992) in: Biological Nitrogen Fixation (Stacey, G., Burris, R.H. and Evans, H.J., Eds.), pp. 736–762, Chapman and Hall, New York.
[3] Bishop, P.E. and Joerger, R.D. (1990) Annu. Rev. Plant Physiol. Plant Mol. Biol. 41, 109–125.
[4] Blanchard, C.Z. and Hales, B.J. (1996) Biochemistry 35, 472–478.
[5] Howard, J.B. and Rees, D.C. (1994) Annu. Rev. Biochem. 63, 235–264.
[6] Howard, J.B. and Rees, D.C. (1996) Chem. Rev. 96, 2965–2982.
[7] Dean, D.R. and Jacobson, M.R. (1992) in: Biological Nitrogen Fixation (Stacey, G., Burris, R.H. and Evans, H.J., Eds.), pp. 763–834, Chapman and Hall, New York.
[8] Burgess, B.K. and Lowe, D.J. (1996) Chem. Rev. 96, 2983–3011.
[9] Joerger, R.D., Jacobson, M.R. and Bishop, P.E. (1989) J. Bacteriol. 171, 3258–3267.
[10] Berger, D.K., Narberhaus, F., Lee, H.S. and Kustu, S. (1995) J. Bacteriol. 177, 191–199.
[11] Kennedy, C. and Robson, R.L. (1983) Nature 301, 626–628.
[12] Brito, B., Martinez, M., Fernandez, D., Rey, L., Cabrera, E., Palacios, J.M., Imperial, J. and Ruiz Argueso, T. (1997) Proc. Natl. Acad. Sci. USA 94, 6019–6024.
[13] Loroch, A.I., Nguyen, B.G. and Ludwig, R.A. (1995) J. Bacteriol. 177, 7210–7221.
[14] Santero, E., Toukdarian, A., Humphrey, R. and Kennedy, C. (1988) Mol. Microbiol. 2, 303–314.
[15] Walmsley, J., Toukdarian, A. and Kennedy, C. (1994) Arch. Microbiol. 162, 422–429.
[16] Santero, E., Hoover, T., Keener, J. and Kustu, S. (1989) Proc. Natl. Acad. Sci. USA 86, 7346–7350.
[17] Dixon, R. (1998) Arch. Microbiol. 169, 371–380.
[18] Hoover, T.R., Santero, E., Porter, S. and Kustu, S. (1990) Cell 63, 11–22.
[19] Woodley, P., Buck, M. and Kennedy, C. (1996) FEMS Microbiol. Lett. 135, 213–221.
[20] Lei, S., Pulakat, L. and Gavini, N. (1999) Biochem. Biophys. Res. Commun. 264, 186–190.
[21] Joerger, R.D. and Bishop, P.E. (1988) Crit. Rev. Microbiol. 16, 1–14.
[22] Joerger, R.D., Loveless, T.M., Pau, R.N., Mitchenall, L.A., Simon, B.H. and Bishop, P.E. (1990) J. Bacteriol. 172, 3400–3408.
[23] Georgiadis, M.M., Komiya, H., Chakrabarti, P., Woo, D., Kornuc, J.J. and Rees, D.C. (1992) Science 257, 1653–1659.
[24] Bishop, P.E., Jarlenski, D.M. and Hetherington, D.R. (1980) Proc. Natl. Acad. Sci. USA 77, 7342–7346.
[25] Chisnell, J.R., Premakumar, R. and Bishop, P.E. (1988) J. Bacteriol. 170, 27–33.
[26] Premakumar, R., Chisnell, J.R. and Bishop, P.E. (1989) Can. J. Microbiol. 35, 344–348.
[27] Joerger, R.D., Wolfinger, E.D. and Bishop, P.E. (1991) J. Bacteriol. 173, 4440–4446.
[28] Arber, J.M., Flood, A.C., Garner, C.D., Gormal, C.A., Hasnain, S.S. and Smith, B.E. (1988) Biochem. J. 252, 421–425.
[29] Harvey, I., Arber, J.M., Eady, R.R., Smith, B.E., Garner, C.D. and Hasnain, S.S. (1990) Biochem. J. 266, 929–931.
[30] Kennedy, C. and Dean, D. (1992) Mol. Gen. Genet. 231, 494–498.
[31] Moore, V.G., Tittsworth, R.C. and Hales, B.J. (1994) J. Am. Chem. Soc. 116, 12101–12102.
[32] Strandberg, G.W. and Wilson, P.W. (1968) Can. J. Microbiol. 14, 25–31.
[33] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[34] Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Strul, K. (1992) Greene/Wiley Interscience, New York.
[35] Robinson, A.C., Burgess, B.K. and Dean, D.R. (1986) J. Bacteriol. 166, 180–186.
[36] Robinson, A.C., Dean, D.R. and Burgess, B.K. (1987) J. Biol. Chem. 262, 14327–14332.
[37] Joerger, R.D., Premakumar, R. and Bishop, P.E. (1986) J. Bacteriol. 168, 673–682.
[38] Kajii, Y., Kobayashi, M., Takahashi, T. and Onodera, K. (1994) Biosci. Biotechnol. Biochem. 58, 1179–1180.
[39] Premakumar, R., Jacobitz, S., Ricke, S.C. and Bishop, P.E. (1996) J. Bacteriol. 178, 691–696.
[40] Umesh, K., Raina, R. and Das, H.K. (1998) FEMS Microbiol. Lett. 162, 161–167.
[41] Pau, R.N., Eldridge, M.E., Lowe, D.J., Mitchenall, L.A. and Eady, R.R. (1993) Biochem. J. 293 (Pt 1), 101–107.
[42] Jacobson, M.R. et al. (1989) J. Bacteriol. 171, 1017–1027.