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The Gene Encoding Dinitrogenase Reductase 2 Is Required for Expression of the Second Alternative Nitrogenase from *Azotobacter vinelandii*†

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Under diazotrophic conditions in the absence of molybdenum (Mo) and vanadium (V), *Azotobacter vinelandii* reduces N_2 to NH_4^+ by using nitrogenase 3 (encoded by *anfHDGK*). However, dinitrogenase reductase 2 (encoded by *vnfH*) is also expressed under these conditions even though this protein is a component of the V-containing alternative nitrogenase. Mutant strains that lack dinitrogenase reductase 2 ($VnfH^-$) grow slower than the wild-type strain in N-free, Mo-, and V-deficient medium. In this medium, these strains synthesize dinitrogenase reductase 1 (a component of the Mo-containing nitrogenase encoded by *nifH*), even though this component is not normally synthesized in the absence of Mo. Strains that lack both dinitrogenase reductases 1 and 2 ($NifH^-$ $VnfH^-$) are unable to grow diazotrophically in Mo- and V-deficient medium. In this medium, $NifH^-$ $VnfH^-$ strains containing an *anfH-lacZ* transcriptional fusion exhibited less than 3% of the β-galactosidase activity observed in the wild type with the same fusion. β-Galactosidase activity expressed by $VnfH^-$ mutants containing the *anfH-lacZ* fusion ranged between 57 and 78% of that expressed by the wild type containing the same fusion. Thus, expression of dinitrogenase reductase 2 seems to be required for transcription of the *anfHDGK* operon, although, in $VnfH^-$ mutants, dinitrogenase reductase 1 appears to serve this function. Active dinitrogenase reductase 1 or 2 is probably required for this function since a *nifM* deletion mutant containing the *anfH-lacZ* fusion was unable to synthesize β-galactosidase above background levels. An *anfA* deletion strain containing the *anfH-lacZ* fusion exhibited β-galactosidase activity at 16% of that of the wild type containing the same fusion. However, in the presence of NH_4^+, the β-galactosidase activity expressed by this strain more than doubled. This indicates that AnfA is required not only for normal levels of *anfHDGK* transcription but also for NH_4^+- and, to a lesser extent, Mo-mediated repression of this transcription.

The regulation of the expression of the three nitrogenases in *Azotobacter vinelandii* is responsive to the presence or absence of ammonium (NH_4^+), molybdenum (Mo), and vanadium (V) in the culture medium. The synthesis of all three nitrogenases is repressed by NH_4^+. Nitrogenase 1 is found in cells grown in the presence of Mo and nitrogenase 2 is expressed in the presence of V but in the absence of Mo, whereas nitrogenase 3 is synthesized only in the absence of both Mo and V (4, 9, 13, and references therein). Our knowledge of the molecular basis for nitrogen and metal regulation in *A. vinelandii* is still rudimentary. The regulatory genes *nifA*, *vnfA*, and *anfA* have been identified, and some of their functions have been described on the basis of the phenotypes of $NifA^-$, $VnfA^-$, and $AnfA^-$ mutants (1, 14). The *nifA* gene product is required for transcription of the structural genes for nitrogenase 1 (1). NifA binds to an upstream activator sequence (6) and activates transcription of *nif* operons preceded by this upstream activator sequence. The factors that influence this activation by NifA in *A. vinelandii* are presently not known.

VnfA is required for synthesis of nitrogenase 2 and directly or indirectly represses the synthesis of nitrogenase 1 in cells grown in Mo-deficient medium with or without V (14).

anfA deletion mutants were unable to grow diazotrophically in Mo- and V-deficient medium (14). The factors that regulate *vnfA* and *anfA* are also not known. Additional regulatory genes, designated *ntrC* and *nfrX* (28, 32), have been described. NtrC is required for diazotrophic growth in the presence of V but not in the presence of Mo or in the absence of Mo and V (32). NfrX, on the other hand, is required for growth in N-free, Mo-containing medium or in medium lacking Mo and V but not for diazotrophic growth in the presence of V (28). It is not understood how the products of these regulatory genes act within the regulatory circuits for expression of the three nitrogenases.

In contrast to the structural genes for nitrogenases 1 and 3 (10, 15), the structural genes for nitrogenase 2 are organized in two transcriptional units (16). This allows the independent expression of dinitrogenase reductase 2 (*vnfH* gene product) and dinitrogenase 2 (*vnfDGK* products). Unlike dinitrogenase 2, dinitrogenase reductase 2 is present not only under diazotrophic conditions in the presence of V but also in the absence of Mo and V, conditions where nitrogenase 3 is present (3, 8, 25). Dinitrogenase reductase 2 is unlikely to function in a catalytic role under Mo- and V-deficient conditions because purified dinitrogenase reductase 2 does not effectively complement dinitrogenase 3 in in vitro assays (8). Nevertheless, the *vnfH* gene product is important for diazotrophic growth under Mo and V deficiency because a $VnfH^-$ strain (*A. vinelandii* CA80) grew considerably slower than the wild-type strain CA in Mo- and V-deficient medium (16).

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics or genotype	Reference or source
E. coli		
HB101	F⁻ *hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44* lambda⁻	20
C600	F⁻ *thi-1 thr-1 leuB6 lacY1 tonA21 supE44* lambda⁻	20
S17-1	*thi pro* HsdR⁻ HsdM⁺ *recA* RP4 2-Tc::Mu-Km::Tn*7* (integrated plasmid)	29
JC5466	*trp his recA56 rplE*	C. Kennedy
A. vinelandii		
CA	Wild type	7
CA11	Δ*nifHDK*	5
CA11.66	Δ*nifHDK* Δ*anfA66*::*kan*	14
CA11.66.73	Δ*nifHDK* Δ*anfA66*::*kan anfH73*::Tn*5*-B21 Tc^r	This study
CA11.73	Δ*nifHDK anfH73*::Tn*5*-B21 Tc^r	This study
CA11.80	Δ*nifHDK vnfH707*::*kan*	16
CA11.80.73	Δ*nifHDK vnfH707*::*kan anfH73*::Tn*5*-B21 Tc^r	This study
CA30	*nifB30*::Tn*5*	12, 17
CA30.73	*nifB30*::Tn*5 anfH73*::Tn*5*-B21 Tc^r	This study
CA46	*vnfA46*::Tn*5*	14, 17
CA46.73	*vnfA46*::Tn*5 anfH73*::Tn*5*-B21 Tc^r	This study
CA66	Δ*anfA66*::*kan*	14
CA66.73	Δ*anfA66*::*kan anfH73*::Tn*5*-B21 Tc^r	This study
CA73	*anfH73*::Tn*5*-B21 Tc^r	This study
CA80	*vnfH707*::*kan*	16
CA80.73	*vnfH707*::*kan anfH73*::Tn*5*-B21 Tc^r	This study
DJ33	Δ*nifDK*	26
DJ33.73	Δ*nifDK anfH73*::Tn*5*-B21 Tc^r	This study
DJ33.80	Δ*nifDK vnfH707*::*kan*	This study
DJ33.80.73	Δ*nifDK vnfH707*::*kan anfH73*::Tn*5*-B21 Tc^r	This study
DJ54	Δ*nifH*	27
DJ54.73	Δ*nifH anfH73*::Tn*5*-B21 Tc^r	This study
DJ54.80	Δ*nifH vnfH707*::*kan*	This study
DJ54.80.73	Δ*nifH vnfH707*::*kan anfH73*::Tn*5*-B21 Tc^r	This study
DJ136	Δ*nifM*	11
DJ136.73	Δ*nifM anfH73*::Tn*5*-B21 Tc^r	This study
RP114	Δ*vnfDGK*::Sp^r	24
RP114.73	Δ*vnfDGK*::Sp^r *anfH73*::Tn*5*-B21 Tc^r	This study
RP114.80	Δ*vnfDGK*::Sp^r *vnfH707*::*kan*	This study
RP114.80.73	Δ*vnfDGK*::Sp^r *vnfH707*::*kan anfH73*::Tn*5*-B21 Tc^r	This study
RP206	Δ*nifHDK* Δ*vnfDGK*::Sp^r	24
RP206.73	Δ*nifHDK* Δ*vnfDGK*::Sp^r *anfH73*::Tn*5*-B21 Tc^r	This study
RP206.80	Δ*nifHDK* Δ*vnfDGK*::Sp^r *vnfH707*::*kan*	This study
R206.80.73	Δ*nifHDK* Δ*vnfDGK*::Sp^r *vnfH707*::*kan anfH73*::Tn*5*-B21 Tc^r	This study
Plasmids		
pMJH3	pUC9 containing *anfA* plus most of *anfH*	14
pWW1	pMJH3 containing Tn*5*-B21 insertion in *anfH*	This study

In this study, we investigated the effect of mutations in *vnfH* and *anfA* on the expression of the genes encoding nitrogenase 3 (*anfHDGK*).

MATERIALS AND METHODS

Maintenance and growth of bacteria. *Escherichia coli* and *A. vinelandii* strains used in this study are listed in Table 1. *E. coli* HB101 and S17-1 were maintained and cultured in TYE or LB medium. When required, kanamycin and ampicillin were added to final concentrations of 10 and 50 μg/ml, respectively. Lambda Tn*5*-B21 (30) was propagated in *E. coli* C600. This *E. coli* strain was grown in Y broth (Bacto-Tryptone [10 g/liter], NaCl [2.5 g/liter], yeast extract [0.1 g/liter]). Maltose was added to a final concentration of 0.2%. The *A. vinelandii* strains were grown in modified Burk medium (31) at 30°C. When required, antibiotics were added to the following final concentrations: kanamycin (5 μg/ml), ampicillin (50 μg/ml), tetracycline (20 μg/ml), and spectinomycin (20 μg/ml). For growth studies under Mo-deficient conditions and in the presence of V, precautions were taken to minimize contamination with metals as previously described (14). When required, Na_2MoO_4 and V_2O_5 were added to a final concentration of 1 μM. Fixed N was added as ammonium acetate (final concentration, 2.2 mg/ml).

Mutagenesis of pMJH3 with Tn*5*-B21. Plasmid pMJH3 (Table 1) was transformed into *E. coli* S17-1 (suppressor negative). The transformed strain was then grown to a cell density of approximately 10^9 cells per ml (A_{600} = 0.8). The cells were harvested and resuspended in 0.01 M $MgSO_4$ solution. A 400-μl volume of this cell suspension was mixed with 200 μl of lambda Tn*5*-B21 phage suspension (titer, 10^9 PFU/ml) followed by incubation for 2 h at 37°C. The infected cells were plated onto LB medium containing tetracycline (20 μg/ml). Tetracycline-resistant (Tc^r) cells were washed off the plates, and plasmid DNA was isolated from these cells. *E. coli* JC5466 was transformed with this plasmid DNA preparation, and Tc^r transformants were selected. Plasmid DNA was isolated from individual colonies, and the location and orientation of Tn*5*-B21 in the individual plasmids was determined by restriction enzyme analysis.

Construction of *A. vinelandii* mutant strains. *A. vinelandii* strains were made competent and transformed with genomic DNA or with plasmid DNA as described by Page and von Tigerstrom (23). When *A. vinelandii* strains were transformed with pMJH3 containing an insertion of Tn*5*-B21, transformants were selected initially on modified Burk medium containing tetracycline. These transformants were then tested on medium containing both tetracycline and ampicillin in order to detect transformants (Tc^r, Ap^s) in which a double crossover between homologous plasmid and chromosomal DNA had occurred. These transformants were subsequently tested for their ability to grow diazotrophically under different conditions. The locations of the desired genetic markers on the chromosomes of some of the transformed strains were verified by Southern hybridization analyses.

Two-dimensional gel electrophoresis. The *A. vinelandii* strains were derepressed for nitrogenases 1 and 2 for 3 to 5 h, and, for nitrogenase 3, the derepression time was 12 h. When possible, cells were grown to a cell density of 70 to 100 Klett units in N-free medium. Cell-free protein extracts were prepared as previously described (2). Isoelectric focusing and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of proteins in cell-free extracts were conducted by the method of O'Farrell (22) with modifications as described by Bishop et al. (2).

β-Galactosidase assays. The *A. vinelandii* strains containing Tn*5*-B21 were grown in Mo- and V-deficient Burk medium containing ammonium acetate (2.2 mg/ml) to a cell density of 70 to 100 Klett units. The cells were harvested and resuspended in four different media. These media were modified Burk medium containing ammonium acetate (2.2 mg/ml), N-free modified Burk medium containing 1 μM Na_2MoO_4 or 1 μM V_2O_5, and N-free, Mo-, and V-deficient modified Burk medium. Samples were taken from these cultures, and the accumulation of β-galactosidase was determined at various times after the onset of derepression. From these assays, a derepression time of 12 h was determined to be optimal for the comparative assays. Ten- to 50-μl volumes of these cultures were used for the β-galactosidase assays described by Miller (21). The cells were rendered permeable to proteins by the addition of 50 μl of 0.1% SDS and 30 μl of chloroform to the assay buffer.

RESULTS

Growth of mutant strains under diazotrophic conditions. The growth characteristics of the various mutant strains are summarized in Table 2. *A. vinelandii* CA (wild type) grows under all diazotrophic conditions tested. Mutants containing mutations in the structural genes for nitrogenase 1 (*nifHDK*) are unable to grow under N_2-fixing conditions in the presence of Mo but grow in Mo-deficient, N-free media. In agreement with previously reported results with a $NifM^-$ mutant (18), the *nifM* deletion strain DJ136 is unable to grow under any of the diazotrophic conditions listed in Table 2.

The growth characteristics of the *vnfDGK* deletion strain RP114 and the *nifHDK vnfDGK* deletion strain RP206 were reported previously (24). Both strains grow under Mo- and V-deficient conditions, but growth in the presence of V is considerably slower than that of strain CA. The $VnfH^-$ strain CA80 exhibits wild-type growth under Mo-sufficient conditions but grows slower than the wild-type strain in medium containing V or in Mo- and V-deficient medium. Strains CA11.80 and DJ54.80, which lack both dinitrogenase reductases 1 and 2, did not grow under any diazotrophic condition. Strain DJ33.80 does not grow under Mo-sufficient conditions, but growth in the presence of V and in the absence of Mo and V is similar to that of strain CA80. Growth characteristics of the $VnfA^-$ strain CA46 were reported previously (17). Because of the inactivation of *vnfA*, this strain is unable to synthesize nitrogenase 2. However, the lack of VnfA causes strain CA46 to synthesize both nitrogenase 1 and 3 under Mo-deficient conditions (in the presence or absence of V). Presumably, diazotrophic growth is due to nitrogenase 3 under these conditions. Growth under Mo sufficiency was identical to that of strain CA. Strain CA11.46, on the other hand, did not grow under any of the diazotrophic conditions mentioned above.

As described previously (14), the $AnfA^-$ strain CA66 grows as well as the wild-type strain CA under Mo- and V-sufficient conditions but is unable to grow in the absence of both metals.

TABLE 2. Summary of growth characteristics of *A. vinelandii* strains[a]

Strain	Genotype	Diazotrophic growth in the presence of:		
		1 μM Na_2MoO_4	1 μM V_2O_5	No Mo or V
CA	Wild type	+	+	+
CA11	Δ*nifHDK*	−	+	+
CA80	*vnfH707*::*kan*	+	Slow	Slow
CA11.80	Δ*nifHDK vnfH707*::*kan*	−	−	−
CA46	*vnfA46*::Tn*5*	+	+	+
CA11.46	Δ*nifHDK vnfA46*::Tn*5*	−	−	−
DJ33	Δ*nifDK*	−	+	+
DJ33.80	Δ*nifDK vnfH707*::*kan*	−	Slow	Slow
DJ54	Δ*nifH*	−	+	+
DJ54.80	Δ*nifH vnfH707*::*kan*	−	−	−
DJ136	Δ*nifM*	−	−	−
RP114	Δ*vnfDGK*::Sp^r	+	Slow	+
RP114.80	Δ*vnfDGK*::Sp^r *vnfH707*::*kan*	+	Slow	Slow
RP206	Δ*nifHDK* Δ*vnfDGK*::Sp^r	−	Slow	+
RP206.80	Δ*nifHDK* Δ*vnfDGK*::Sp^r *vnfH707*::*kan*	−	−	−
CA30	*nifB30*::Tn*5*	−	−	−
CA66	Δ*anfA*::*kan*	+	+	−

[a] Growth was monitored in liquid Burk medium as previously described (17).

Two-dimensional gel electrophoresis of protein extracts from mutant strains. The presence or absence of subunits for nitrogenase 3 or for dinitrogenase reductase 1 or 2 in protein extracts from cells derepressed or grown in Mo- and V-deficient, N-free medium is indicated in Table 3. Protein extracts from cells of strains CA, CA11, DJ33, and DJ54 contained the subunits of nitrogenase 3 (products of *anfHDGK*) as well as the dinitrogenase reductase 2 subunit (*vnfH* gene product). Extracts of cells of strains CA80 and DJ33.80 grown or derepressed in Mo- and V-deficient N-free medium contained lesser amounts of nitrogenase 3 subunits than those from wild-type cells. The *vnfH* gene product was not present; instead, a small amount of dinitrogenase reductase 1 (*nifH* gene product) was detected on two-dimensional gels. Protein extracts from cells of strains CA11.80 and DJ54.80 (derepressed for 4 to 24 h) did not contain detectable subunits for dinitrogenase reductases 1 and 2 or for nitrogenase 3.

We reported earlier that extracts from cells of the $VnfA^-$ strain CA46, which were grown or derepressed for nitrogenase under Mo- and V-deficient conditions, contained sub-

TABLE 3. Two-dimensional gel electrophoresis of protein extracts from cells derepressed in Mo- and V-deficient medium

Strain	Genotype	Presence or absence of proteins representing:				
		VnfH	NifH	AnfH	AnfD	AnfK
CA	Wild type	+	−	+	+	+
CA11	Δ*nifHDK*	+	−	+	+	+
CA80	*vnfH707*::*kan*	−	+[a]	+[a]	+[a]	+[a]
CA11.80	Δ*nifHDK vnfH707*::*kan*	−	−	−	−	−
CA46	*vnfA46*::Tn*5*	−	+	+	+	+
DJ33	Δ*nifDK*	+	−	+	+	+
DJ33.80	Δ*nifDK vnfH707*::*kan*	−	+[a]	+[a]	+[a]	+[a]
DJ54	Δ*nifH*	+	−	+	+	+
DJ54.80	Δ*nifH vnfH707*::*kan*	−	−	−	−	−

[a] Only small amounts of protein were present.

units for both nitrogenases 1 and 3 and that no nitrogenase subunits were found in extracts from CA11.46 cells (17). We also reported (14) that the AnfA$^-$ strain CA66 failed to synthesize nitrogenase 3 subunits. However, after reexamination of the published two-dimensional gels of protein extracts from strain CA66 cells derepressed under Mo- and V-deficient conditions, it appears that a faint spot that is present on two-dimensional gels of protein extracts of cells incubated under all nitrogen-fixing conditions may actually represent dinitrogenase reductase 3 (the product of *anfH*).

Construction of mutant strains containing *lacZ* under the control of *anfH*. In vivo mutagenesis of pMJH3 with Tn*5*-B21 resulted in a plasmid (pWW1) that contained Tn*5*-B21 in the correct orientation within the *anfH* gene, approximately 200 to 300 bp downstream from the predicted initiation codon. Transformation of the wild-type strain CA and the *nifHDK* deletion strain CA11 with pWW1 and selection for Tc^r Ap^s cells yielded strains CA73 (*anfH73*::Tn*5*-B21) and CA11.73 (Δ*nifHDK anfH73*::Tn*5*-B21). Total genomic DNA from strain CA73 or CA11.73 was used to transform the *anfH-lacZ* fusion into other *A. vinelandii* strains. The resulting mutant strains are listed in Table 1. Strains that contain a Tn*5*-B21 insertion in *anfH* are unable to grow diazotrophically under Mo- and V-deficient conditions (Anf$^-$). The presence of Tn*5*-B21 in *anfH* was also verified by Southern hybridization analyses.

Expression of the *anfH-lacZ* fusion in Nif$^-$, Vnf$^-$, and AnfA$^-$ mutants. The results of three independent experiments for each strain are shown in Table 4. Strains that are either NifH$^+$ or VnfH$^+$ accumulated β-galactosidase under Mo- and V-deficient conditions to levels that resulted in activities of 2,298 to 7,535 Miller units. NifH$^-$ VnfH$^-$ strains lacking both dinitrogenase reductases 1 and 2 accumulated between 176 and 196 Miller units of β-galactosidase activity. Previously, strains CA46 (17), RP114, and RP206 (24) were shown to grow under diazotrophic conditions where V was present in the medium. In agreement with these data, strains CA46.73, RP114.73, RP114.80.73, and RP206.73 accumulated β-galactosidase when derepressed in medium containing V. The NifB$^-$ strain CA30.73 also accumulated β-galactosidase when derepressed in V-containing medium. On the other hand, the *nifM* deletion strain DJ136.73, which synthesizes inactive dinitrogenase reductase 1 and presumably also dinitrogenase reductase 2, did not accumulate appreciable amounts of β-galactosidase when derepressed in Mo- and V-deficient medium or in medium containing V.

In contrast to all of the other mutant strains, the AnfA$^-$ mutant strains CA66.73 and CA11.66.73 synthesized about twice as much β-galactosidase in the presence of NH_4^+ as in its absence. When these strains were cultured in the presence of both Mo and NH_4^+, they had approximately 70% of the β-galactosidase activity that they had when cultured in Mo- and V-deficient NH_4^+-containing medium (data not shown).

DISCUSSION

This study was prompted by the observation that two mutant strains, CA11.46 (Δ*nifHDK vnfA46*::Tn*5*) and CA11.80 (Δ*nifHDK vnfH707*::*kan*), were unable to grow diazotrophically under Mo- and V-deficient conditions, whereas the *nifHDK*-containing strains CA46 (*vnfA46*::Tn*5*) and CA80 (*vnfH707*::*kan*) and the *nifHDK* deletion strain CA11 grew under the same conditions (5, 16, 17). The results of this investigation indicate that, under normal circumstances, dinitrogenase reductase 2 (*vnfH* gene product) is required for expression of nitrogenase 3; however, dinitrogenase reductase 1 can substitute for dinitrogenase reductase 2 in this role. Results of β-galactosidase assays with a NifM$^-$ mutant containing an *anfH-lacZ* fusion suggest that active dinitrogenase reductase 2 is required for transcription of the *anfHDGK* operon and, hence, for expression of nitrogenase 3. This interpretation, however, is based on the assumption that the *nifM* deletion results in the expression of an inactive dinitrogenase reductase 2 as well as inactive dinitrogenase reductase 1. Since the *nifM* deletion strain DJ136 is Vnf$^-$ and Anf$^-$ as well as Nif$^-$, it is highly probable that this assumption is correct. Nevertheless, a study of VnfH$^-$ mutants with point mutations in *vnfH* will need to be conducted before this conclusion can be substantiated.

The products of *vnfH* and *nifH* are very similar proteins (91% identical amino acid residues) (16), yet it appears that cells which synthesize dinitrogenase reductase 2 are better able to express nitrogenase 3 than are cells that contain only dinitrogenase reductase 1 (e.g., strain CA11 versus strain CA80; Table 3). This could be due to the minor differences in the amino acid sequences of the proteins, the lack of additional factors such as the ferredoxin-like protein predicted to be encoded by the open reading frame located 3′ to *vnfH* (assuming that polarity caused by the interposon insertion in strain CA80 prevents expression of the Fd-like gene) (16), or, more likely, the different levels to which the two dinitrogenase reductases accumulate under Mo- and V-deficient conditions. Differences in accumulation of these nitrogenase components could reflect differences in rates of peptide degradation or synthesis. The fact that dinitrogenase reductase 1 accumulates in mutant strains CA46 and CA80 (Table 3) under Mo-deficient conditions indicates that the usual regulatory constraints on its synthesis (i.e., expression only in the presence of Mo) are largely inoperative in strains that lack dinitrogenase reductase 2.

A regulatory role has been suggested for the products of *vnfDGK* in V-mediated repression of nitrogenase 3 on the basis of the incomplete repression of nitrogenase 3 by V in *vnfDGK* deletion strains of *A. vinelandii* (19). This proposal is corroborated by our observation that *anfH-lacZ* fusion strains (RP114.73 and RP206.73), which lack *vnfDGK*, accumulate moderate levels of β-galactosidase in the presence of V (Table 4). In this regard, it is interesting to note that, when the NifB$^-$ strain CA30.73 was derepressed in the presence of V, β-galactosidase was synthesized in amounts comparable to those found in *vnfDGK* deletion strains under the same conditions (Table 4). Therefore, it is possible that cofactors

TABLE 4. Expression of the *anfH-lacZ* fusion (*anfH73*::Tn*5*-B21) in Nif$^-$, Vnf$^-$, and AnfA$^-$ mutant strains

Strain	Phenotype	Medium[a]	β-Galactosidase	
			Activity[b]	%[c]
CA73	Nif$^+$ Vnf$^+$	−Mo, −V	7,535 ± 1,893	100.0
		+V	185 ± 78	2.4
		+Mo	50 ± 11	0.7
		+NH_4^+	36 ± 15	0.5
CA11.73	Nif(HDK)$^-$	−Mo, −V	7,293 ± 1,972	96.8
		+V	177 ± 100	2.3
		+Mo	24 ± 4	0.3
		+NH_4^+	43 ± 23	0.6
CA80.73	VnfH$^-$	−Mo, −V	4,324 ± 847	57.4
		+V	170 ± 40	2.3
		+Mo	37 ± 11	0.5
		+NH_4^+	56 ± 44	0.7
CA11.80.73	Nif(HDK)$^-$ VnfH$^-$	−Mo, −V	198 ± 111	2.6
		+V	79 ± 28	1.0
		+Mo	22 ± 5	0.3
		+NH_4^+	74 ± 27	1.0
DJ33.73	Nif(DK)$^-$	−Mo, −V	3,187 ± 1,452	42.3
		+V	49 ± 11	0.6
		+Mo	27 ± 23	0.4
		+NH_4^+	39 ± 32	0.5
DJ33.80.73	Nif(DK)$^-$ VnfH$^-$	−Mo, −V	2,298 ± 687	30.5
		+V	90 ± 33	1.2
		+Mo	35 ± 18	0.5
		+NH_4^+	59 ± 5	0.8
DJ54.73	NifH$^-$	−Mo, −V	2,666 ± 755	35.4
		+V	125 ± 66	1.6
		+Mo	24 ± 10	0.3
		+NH_4^+	27 ± 12	0.4
DJ54.80.73	NifH$^-$ VnfH$^-$	−Mo, −V	176 ± 147	2.3
		+V	93 ± 45	1.2
		+Mo	23 ± 9	0.3
		+NH_4^+	50 ± 14	0.7
DJ136.73	NifM$^-$	−Mo, −V	150 ± 61	1.4
		+V	64 ± 11	0.8
		+Mo	32 ± 4	0.4
		+NH_4^+	0	0.0
RP114.73	Vnf(DGK)$^-$	−Mo, −V	6,612 ± 1,932	87.7
		+V	1,726 ± 530	22.9
		+Mo	29 ± 7	0.4
		+NH_4^+	49 ± 7	0.6
RP114.80.73	Vnf(DGK)$^-$ VnfH$^-$	−Mo, −V	6,203 ± 433	82.3
		+V	1,673 ± 510	22.2
		+Mo	35 ± 4	0.5
		+NH_4+	87 ± 40	1.1
RP206.73	Nif(HDK)$^-$ Vnf(DGK)$^-$	−Mo, −V	6,800 ± 2,377	90.2
		+V	2,234 ± 1,199	29.6
		+Mo	22 ± 4	0.3
		+NH_4^+	52 ± 26	0.6
RP206.80.73	Nif(HDK)$^-$ Vnf(DGK)$^-$ VnfH$^-$	−Mo, −V	193 ± 76	2.6
		+V	192 ± 62	2.5
		+Mo	33 ± 10	0.4
		+NH_4^+	55 ± 30	0.7
CA46.73	VnfA$^-$	−Mo, −V	5,894 ± 209	78.2
		+V	5,880 ± 222	78.0
		+Mo	40 ± 22	0.5
		+NH_4^+	38 ± 6	0.5

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TABLE 4—*Continued*

Strain	Phenotype	Medium[a]	β-Galactosidase	
			Activity[b]	%[c]
CA30.73	NifB$^-$	−Mo, −V	5,657 ± 1,867	75.1
		+V	1,313 ± 369	17.4
		+Mo	40 ± 18	0.5
		+NH_4^+	136 ± 54	1.8
CA66.72	AnfA$^-$	−Mo, −V	1,245 ± 227	16.5
		+V	958 ± 9	12.7
		+Mo	970 ± 86	12.7
		+NH_4^+	2,551 ± 410	33.8
CA11.66.73	Nif(HDK)$^-$ AnfA$^-$	−Mo, −V	680 ± 72	9.0
		+V	524 ± 157	6.9
		+Mo	330 ± 94	4.4
		+NH_4^+	1,586 ± 244	21.0

[a] Cells were cultured in N-free modified Burk medium without added Mo and V (−Mo, −V), with 1 μM V_2O_5 (+V), with 1 μM Na_2MoO_4 (+Mo), or in modified Mo- and V-deficient Burk medium containing ammonium acetate (2.2 mg/ml) (+NH_4^+).

[b] β-Galactosidase activities are expressed in units described by Miller (21). Average and standard deviation of activity values from at least three independent experiments are listed.

[c] Percentage of average activity found in cells of strain CA73 derepressed for 12 h in modified N-free, Mo-, and V-deficient Burk medium.

are, in some way, involved in V-mediated repression of *anfHDGK*.

Although we do not yet understand the role played by dinitrogenase reductase 2 in the transcription of the *anfHDGK* operon, one can speculate that this nitrogenase component could interact with the transcription apparatus or with *anfHDGK*-containing transcripts. These functions might also be carried out by factors that are influenced by dinitrogenase reductase 2. On the basis of the known requirement of dinitrogenase reductase 1 for the synthesis of FeMo cofactor (27), it was considered possible that an alternative nitrogenase cofactor (e.g., the putative cofactor for dinitrogenase 3) was such a factor. However, *nifB*, a gene which has been postulated to be required for the synthesis of all three nitrogenase cofactors (12), is not required for transcription of *anfHDGK* as shown by the expression of the *anfH-lacZ* fusion in the NifB$^-$ mutant CA30.73 (Table 4). Accordingly, it is unlikely that dinitrogenase reductase 2 acts indirectly through an involvement in the synthesis of a nitrogenase cofactor which in turn is required for transcription of the *anfHDGK* operon.

When wild-type cells are grown in the presence of Mo or NH_4^+, the *anfHDGK* operon is completely repressed. However, in an *anfA* deletion background, regulation of this operon is altered, especially with respect to repression by NH_4^+. AnfA$^-$ cells (strains CA66.73 and CA11.66.73) accumulated twice as much β-galactosidase in the presence of NH_4^+ as in its absence. The levels of β-galactosidase in these cells, however, did not reach those in AnfA$^+$ cells derepressed under Mo- and V-deficient conditions (Table 4). RNA blot experiments also show a decreased accumulation of *anfH*-hybridizing transcripts in cells of strain CA11.66 as compared to those of strain CA11 under conditions where nitrogenase 3 would be expressed (24a). Finally, it should be mentioned that Mo repression of β-galactosidase is incomplete in Anf$^-$ cells. Thus, it is evident that AnfA not only functions as an activator of the *anfHDGK* operon but is also necessary for full repression by NH_4^+ and Mo.

In summary, dinitrogenase reductase 2 is essential for expression of the structural genes for nitrogenase 3, and AnfA is required for full expression and regulation of these genes.

Further study will be necessary to gain a better understanding of the complex interplay between the numerous components involved in the regulation of the three nitrogenase systems in *A. vinelandii*.

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